IOBC-WPRS

Working group "Insect Pathogens and Entomoparasitic Nematodes"

Preceedings of the Meeting

"Biological Control – its unique role in organic and integrated production"

at

Zagreb (Croatia)

16 – 20 June, 2013

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Johannes A. Jehle, Renata Bazok, Neil Crickmore, Miguel López-Ferber, Itamar Glazer, Enrique Quesada-Moraga, Michael Traugott

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Preface

BIOLOGICAL CONTROL OF INSECT AND SLUG PEST USING INSECT PATHOGENIC MICRO-O
VIRUSES AS WELL AS PARASITIC NEMATODES PLAYS AN IMPORTANT ROLE IN PEST CONTRO
FOOD PRODUCTION. THEREFORE THE SUBJECT OF THE 14TH MEETING OF THE IOBC-WPRS WORKING
GROUP **"Insect Pathogens and Entomoparasitic Nematodes"**, HELD ON **June 16 to 20, 2013**
IN **Zagreb**, IS: **"Biological control – its unique role in organic and integrated production".**

SINCE THE LAST MEETING OF THE WG IN INNSBRUCK 2011, SIGNIFICANT CHANGES CAN BE O
IN THE SOCIETY, IN POLITICS, AND IN BIOCONTROL INDUSTRY. MORE AND MORE CONSUM
ORGANICALLY PRODUCED FRUITS AND CROPS OR PRODUCTS GROWN WITH LOW INPU
PESTICIDES. THE DIRECTIVE 2009/128/EC ON THE SUSTAINABLE USE OF PESTICIDES CAME INT
THE EUROPEAN UNION AND AIMS TO REDUCE THE RISKS OF THE USE OF CHEMICAL PESTIC
CONSEQUENCE, NON-CHEMICAL PEST CONTROL MEASURES IS GIVEN PRIORITY. BIOLOGICAL C
THIS. THE MARKET OF BIOCONTROL INDUSTRY GROWS AND MULTI-NATIONAL COMPANIES (R
INTEREST IN BIOCONTROL PRODUCTS AS PROMISING CORNERSTONES IN THEIR PORTFOLIO.

IN 49 ORAL CONTRIBUTIONS, 41 POSTER PRESENTATIONS, A ROUND TABLE DISCUSSIO
WORKSHOPS, THE MOST RECENT PROGRESS AND NEW CHALLENGES IN THE USE OF INSECT P
ENTOMOPARASITIC NEMATODES WILL BE PRESENTED AND DISCUSSED. MORE THAN 110 DELE
COUNTRIES HAVE REGISTERED AND WILL EXCHANGE NEWS AND VIEWS FROM DIFFERENT P
WORLD. THEREFORE, WE HOPE THAT THIS MEETING WILL STIMULATE FURTHER INTERNATIO
AND EXCHANGE OF SCIENTISTS AND STUDENTS.

WE ARE PROUD THAT MORE THAN 20 STUDENTS ATTEND THIS MEETING. FOR SOME OF TH
THEIR FIRST INTERNATIONAL CONGRESS AND THE FIRST OPPORTUNITY TO PRESENT TH
INTERNATIONAL AUDIENCE. WE ARE GRATEFUL TO THE MINISTRY OF SCIENCE, EDUCATION A
REPUBLIC OF CROATIA, TO OUR SPONSORS, AND TO THE IOBC-WPRS FOR THEIR GENEROU
SUPPORT OF THE MEETING. YOUR CONTRIBUTIONS MADE IT POSSIBLE TO SUPPORT STUDENT
THIS MEETING.

ORGANIZING SUCH AN INTERNATIONAL MEETING IS A LOT OF WORK. WE CORDIALL
MEMBERS OF THE LOCAL ORGANIZATION TEAM FOR THEIR CORDIAL HOSPITALITY, TH
DEDICATION AND HARD WORK TO MAKE THIS MEETING A SUCCESS. WE ALSO THANK TO
AGRICULTURE OF THE UNIVERSITY OF ZAGREB FOR PROVIDING US SPACE FOR WORKSHO
SERVICES WE NEEDED FOR A SUCCESSFUL ORGANIZATION.

BECAUSE THIS BULLETIN WAS PREPARED TO BE HANDED OUT AT THE MEETING, THE C
WERE REVIEWED AND EDITED IN A VERY SHORT TIME. WE WISH TO THANK ALL THE ED
MEMBERS, IN PARTICULAR THE SUBGROUP CONVENORS AND THE TECHNICAL EDITOR UTE K
MANY HOURS OF WORKING TIME, EVENINGS AND WEEKENDS TO PREPARE THIS BULLETIN IN TI

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Authorisation of biological control agents - theory and practice

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Abstract: THE LEGISLATION OF THE EUROPEAN UNION REGARDING THE PLACEMENT OF PLANT PROTE ONTHE MARKET (REGULATION (EC) NO 1107/2009) AND ALSO THE DIRECTIVE 2009/128/EC ON THE SUST USE OF PESTICIDES PAVE THE GROUND FOR INCREASING USE OF BIOLOGICAL CONTROL AGENTS CLEARLY GIVE PRIORITY TO THE USE OF ALTERNATIVE, NON-CHEMICAL CONTROL MEASURES. HOWE PRACTICE IN MEMBER STATES IS DIFFERENT. WHEREAS MEMBER STATES LARGELY SEEM TO IGNOR BIOLOGICAL CONTROL, CHEMICAL CONTROL COMPANIES HAVE INCREASING INTEREST IN BIOLOGICAL ACQUIRED SEVERAL BIOCONTROL COMPANIES IN ORDER TO GET ACCESS TO BIOCONTROL BIODIVERS

Introduction

THEHISTORY OF PESTICIDE REGULATION HAS BEEN A PROCESS OF REPLACEMENT OF ONE C BY ANOTHER, WHICH OFTEN EXHIBITED ANOTHER SET OF PROBLEMS. THIS PROCESS WAS A THE DEVELOPMENT OF MORE AND MORE STRINGENT RULES TAKING INTO ACCOUNT SCIE DAMAGE CAUSED BY SYNTHETIC COMPOUNDS AND ANTICIPATED RISKS OF NEW GOVERNMENTS RESPONDED TO REPORTS OF DAMAGE WITH THE DEVELOPMENT OF NEW THAT SIMILAR IMPACTS WILL NOT OCCUR WITH NEW COMPOUNDS. CURRENTLY THE BAN OF COMPOUNDS IS DISCUSSED BECAUSE OF THEIR POTENTIAL INFLUENCE ON BEE HEALT INTRODUCTION OF REGULATION IN EUROPE, REGISTRATION REQUIREMENTS AND GUIDAN ALWAYS BEEN DEVELOPED IN CONSULTATION WITH MULTINATIONAL AGROCHEMICAL COM THAN REGULATION OF SYNTHETIC COMPOUNDS, REGULATIONS FOR BIOLOGICAL PLANT PRO HAVE NOT EVOLVED WITHIN SUCH A PROCESS:

- REGULATION OF BIOLOGICAL CONTROL AGENTS (BCAS) WAS NOT A GRADUAL EVOLUTI INDUSTRY
- REGULATION WAS NOT BASED ON SCIENTIFIC REPORTS OF DAMAGES, AS THERE A REPORTS ON DAMAGE OF BCAS
- BCAS HAVE NO EVOLUTION OF REGULATORY RULES; RULES FOR SYNTHETIC COMPO IMPLEMENTED ON BIOCONTROL WITHOUT CONSULTATION OF BIOCONTROL INDUSTRY

IN ORDER TO DISCUSS MORE ADAPTED APPROACHES TO REGULATION OF BIOLOGIC EXAGGERATING REGULATION REQUIREMENTS, THE EU SUPPORTED THE POLICY SUPPORT A (REGULATION OF BIOLOGICAL CONTROL AGENTS). REBECA MADE SEVERAL VALUABLE PROPO THE SYSTEM COULD BE IMPROVED IN ORDER TO ACCELERATE THE PLACEMENT OF BCAS C (EHLERS, 2011). THESE PROPOSALS HAVE BEEN DISCUSSED BUT, APART FROM MINOR IMP MAJOR CHANGES HAVE NOT BEEN MADE SINCE. NEW REGULATIONS ARE STILL IMPLEMENT SCIENTIFIC ANALYSIS OF POTENTIAL RISKS RELATED TO THE USE OF BCAS (CURRENTLY F BIOCIDE REGULATION 528/2012).

WAIVERS FOR DATA REQUIREMENTS ARE STILL LIMITED. ONE EXAMPLE *Bacillus* THE AGENT *thuringiensis israelensis* (BTI) IS USED TO CONTROL MOSQUITOES. BTI IS CONSIDERED THE INSECTICIDE BY THE WHO (WHO, 1999). IT MAY POSSIBLY BE THE NUMBER ONE PRODUCT IN

THE WORLD-WIDE BIOCONTROL MARKET AND NEGATIVE IMPACTS ON THE ENVIRONMEN
COMPLETELY ABSENT. A REGISTRATION FOR THE USED AGAINST TIPULIDS, A NEW INDICATION
DATA ON TOXICITY, CHEMICAL PROPERTIES, ANALYTICAL METHODS FOR QUALITY CONTROL A
TOXICITY AND NON-TARGET EFFECTS. COSTS HAVE BEEN ESTIMATED TO SURPASS 0.5 MILLI
(EC) NO 1107/2009 ANNEX1 LISTING. SUCH EXPENSES ARE NOT ACCEPTABLE FOR A PRODUC
HAS BEEN USED FOR DECADES WITHOUT CAUSING ANY DAMAGE. MANY OTHER PRODUCTS
TO THE MARKET BECAUSE THE COSTS FOR REGISTRATION ARE TOO HIGH.

ONE OF THE FUTURE TASKS OF THE EUROPEAN COMMUNITY (EC) COMMON AGRICU
(CAP) IS THE GREENING OF AGRICULTURAL PRACTICE. WHEN THE DIRECTIVE 91/414/EEC, REG
PLACEMENT OF PLANT PROTECTION PRODUCTS ON THE MARKET, WAS REPLACED BY REGU
1107/2009, SEVERAL GOOD STEPS TOWARDS GREENING OF CAP WERE TAKEN: THE NEW F
TOGETHER WITH THE DIRECTIVE 2009/128/EC ON THE SUSTAINABLE USE OF PESTICIDES (
GIVES PRIORITY TO NON-CHEMICAL PLANT PROTECTION METHODS. HOWEVER, THE ATTEMP
SUPPORT INTRODUCTION OF BIOCONTROL ARE MOST OFTEN FOILED BY MEMBER STATES (M
FOLLOW THESE RULES WHEN HANDLING AUTHORISATION OF PLANT PROTECTION PR
CONSEQUENCE, CHEMICAL PRODUCTS ARE GIVEN PRIORITY.

Articles 53 of Regulation (EC) No 1107/2009

ARTICLE 53 OF THE REGULATION (EC) NO 1107/2009 PROVIDES INFORMATION ON THE AUTHO
PLANT PROTECTION PRODUCTS (PPP) IN EMERGENCY SITUATIONS: “BY WAY OF DEROGATIO
28, IN SPECIAL CIRCUMSTANCES A MEMBER STATE MAY AUTHORISE, FOR A PERIOD NOT E
DAYS, THE PLACING ON THE MARKET OF PLANT PROTECTION PRODUCTS, FOR LIMITED ANI
WHERE SUCH A MEASURE APPEARS NECESSARY BECAUSE OF A DANGER WHICH CANNOT
ANY OTHER REASONABLE MEANS.” SEVERAL CASES OF AUTHORISATION OF CHEMICAL
ALTHOUGH BCAS WOULD HAVE BEEN AVAILABLE.

The SUD Directive

REGULATION (EC) NO 1107/2009 REFERENCES THE DIRECTIVE 2009/128/EC ON THE SUSTAINA
OF PESTICIDES (SUD) FOR SEVEN TIMES. IN THIS DIRECTIVE ARTICLE 14 INDICATES THAT “
TAKE ALL NECESSARY MEASURES TO PROMOTE LOW PESTICIDE-INPUT PEST MANAGE
WHEREVER POSSIBLE PRIORITY TO NON-CHEMICAL METHODS, SO THAT PROFESSIONAL USE
SWITCH TO PRACTICES AND PRODUCTS WITH THE LOWEST RISK TO HUMAN HEALTH AND
EU REGULATIONS ARE IMPLEMENTED IN EACH MS IN THE MOMENT THEY ARE ISSUED. DIREC
BE TRANSFERRED INTO MS LAWS. THE DEADLINE FOR DIRECTIVE 2009/128/EC IS JANUARY 2
MS CURRENTLY NEGOTIATE WITH THE COMMISSION TO PROLONG THIS PERIOD.

IT IS INTERESTING TO ANALYSE HOW MS WANT TO REACH THE GOAL OF PESTICIDE R
WHETHER THEY CONSIDER BCAS TO SUBSTITUTE FOR CHEMICAL COMPOUNDS. BIOCONTR
COULD CERTAINLY HELP MS TO PUT THIS DIRECTIVE INTO PRACTICE.

MS WERE ASKED TO DEVELOP NATIONAL ACTION PLANS (NAPS) TO PROVIDE STRAT
REDUCTION OF PESTICIDE USE. SOME MS HAVE PRODUCED THESE PLANS OTHERS ARE ST
THEIR PLANS. THE GERMAN ACTION PLAN WAS PUBLISHED 2005 AND IS A COLOURFUL DOCU
PAGES. WITHIN THIS DOCUMENT THE WORD “BIOLOGICAL CONTROL” OCCURS A SINGL
DOCUMENTS THE USE OF *Trichogramma* ON 3,000 HA OF CORN SEED PRODUCTION. BIOLOGICAL CONT
IS OF MUCH LARGER IMPORTANCE IN GERMANY BUT THIS IS NOT RECOGNISED BY THE
AGRICULTURE AND RELATED ORGANISATIONS, WHICH DEVELOP STRATEGIES TO REDUCE TH

EU SUPPORTED PROJECTS LIKE ENDURE OR PURE ALSO GIVE LITTLE ATTENTION TO BIOLOGIC
CONSIDERABLE AMOUNTS OF FUNDS ARE SPENT ON RE-DEFINING IPM INSTEAD OF SUP
INTRODUCTION OF BIOLOGICAL CONTROL STRATEGIES. ARE THE EU PLANS TO PRIORITIS
SERVICES WITH LITTLE POLITICAL CONSEQUENCES AND EVEN LESS PRACTICAL INFLUENCE FOR
AND THE ENVIRONMENT?

Example: Control of the invasive maize pest corn rootworm

THECORN ROOTWORM *Diabrotica virgifera virgifera* IS AN INVASIVE PEST IN EUROPE. IT CAUSES
MAJOR DAMAGE TO CORN AND MS AND THE EU TRY TO LIMIT SPREADING OF THE PES
ERADICATION PROGRAMMES HAVE FAILED AND IT HAS BEEN SPREADING FROM THE BALK
AUSTRIA, POLAND, GERMANY, ITALY AND FRANCE. THE PEST OVERWINTERS IN THE EGG
ROTATION IS A POSSIBILITY TO CONTROL THE PEST. HOWEVER, COSTS FOR ROTATION ARE
CONTROL MEASURES, EVEN BIOLOGICAL CONTROL.

NO CHEMICAL COMPOUND HAS AN OFFICIAL AUTHORISATION ACCORDING TO RE
1107/2009 FOR CONTROL OF THIS PEST. HOWEVER, MS HAVE GIVEN EMERGENCY AUTHORIS
120 DAYS (ACCORDING TO ART. 53 OF REG. (EC) NO 1107/2009) FOR NEONICOTINOIDS (IMIDA
THIAMETOXAM AND CLOTHIANIDIN) FOR SEED TREATMENT OF CORN AND/OR THE PYRETH
GRANULAR FORMULATION. SINCE 10.000 BEE HIVES IN GERMANY HAD SUFFERED FROM MA
DUE TO SEED TREATMENT WITH CLOTHIANIDIN IN 2008, GERMAN AUTHORITIES DID NOT P
NEONICOTINOIDS AGAIN. PLANT PROTECTION PRODUCTS CONTAINING NEONICOTINOID
IMIDACLOPRID, CLOTHIANIDIN) ARE UNDER SUSPECT TO CONTRIBUTE TO THE COLONY COLLA
BEES (E.G., HENRY *et al.*, 2012).

AS *Diabrotica* IS A QUARANTINE PEST, THE ARTICLE 53 OF REGULATION (EC) NO 1107/2
PROVIDES APPROPRIATE JUSTIFICATION FOR AUTHORISATION BECAUSE OF EMERGENCY. HO
53 THE WORDS “ANY OTHER REASONABLE MEANS” ARE IMPORTANT TO BE CONSIDERED
MEANS THAT THE ALTERNATIVES SHOULD BE AS EFFECTIVE AS CHEMICAL MEASURES
ECONOMICALLY VIABLE. CURRENT AUTHORISATION PRACTICE IN MEMBER STATES FOR
INVASIVE PEST *Diabrotica virgifera virgifera* NEVER TOOK INTO CONSIDERATION NON-CHEMIC
ALTERNATIVES.

SINCE 2011 A BIOLOGICAL CONTROL PRODUCT BASED ON THE ENTOMOPATHOGENI
Heterorhabditis bacteriophora IS ON THE MARKET. THE NEMATODES HAVE BEEN FIELD TEST
HUNGARY FOR 7 YEARS, IN AUSTRIA FOR 5 YEARS AND IN ITALY FOR 3 YEARS IN NUMEROUS
RESULTS INDICATE THAT THE *H. bacteriophora* ACHIEVED EQUALLY HIGH CONTROL RESULTS
COMPARED TO THE CHEMICAL SEED TREATMENT WITH A NEONICOTINOID OR APPLICATIO
PYRETHROID (E.G. TOEPFER *et al.*, 2010). NEMATODES ARE APPROXIMATELY 60 €/HA MORE EXPENSIV
THAN CHEMICAL CONTROL WITH TEFLUTHRIN IN GERMANY. HENCE, “OTHER REASONABLE M

THE GENERAL PRACTICE OF MS AUTHORITIES IS TO RENEW ARTICLE 53 AUTHORISATIO
EVEN FOR A PRODUCT WHICH HAS NOT EVEN AN AUTHORISATION FOR OTHER INDICATIO
BEEN REJECTED BY THE EC BECAUSE OF NEGATIVE SIDE-EFFECTS ON SOIL BIOTA AND IT W
IN 2008. IT WAS THEN AUTHORISED ONLY FOR USE AS INSECTICIDE FOR PELLETING OF SUG
BUT IT HAS NO ANNEX 1 LISTING TO BE USED AS GRANULAR FORMULATION WITH ADDITIO
AND IN HIGH CONCENTRATION IN THE SOIL.

IN 2011 AND 2012, ONLY TEFLUTHRIN WAS AUTHORISED IN GERMANY FOR CONTROL
BECAUSE OF THE NEGATIVE EFFECTS ON SOIL BIOTA, GERMAN AUTHORITIES ALLOW THE
THREE YEARS. HOWEVER, MANY GROWERS WHO COULD NOT ROTATE AFTER GROWING MA
TO CONTROL THE LARVAE BECAUSE OF ITS QUARANTINE STATUS (SEE ALSO EU REG
2006/565/EC AND DECISION 2003/766/EC). SO, THESE GROWERS EITHER MISSED TO CONTI

QUARANTINE PEST BREAKING THE EU LAWS OR THE GROWERS DID NOT COMPLY WITH REQU
USE OF TEFLUTHRIN ALLOWING THE USE ONLY EVERY THREE YEAR. IT WOULD HAVE BEEN POSS
THIS ILLEGAL SITUATION BY USE OF BIOLOGICAL CONTROL. HOWEVER, RESISTANCE AGA
BIOLOGICAL CONTROL METHOD WERE SO SEVERE FROM ALL DIFFERENT ORGANISATIONS
CONTROL WAS NOT USED. ONLY THIS YEAR 2013, FOR THE FIRST TIME, IT WILL BE USED ON
FINANCIAL SUPPORT BY THE STATE MINISTRY IN BADEN-WUERTTEMBERG, GERMANY. THE
THE END OF THE TUNNEL.

Example: Authorisation of antibiotic in fire blight control

MANY CASES OF IGNORANCE TOWARDS AVAILABLE BIOLOGICAL ALTERNATIVES CAN BE REP
CASE IS THE AUTHORISATION OF THE ANTIBIOTIC COMPOUND STREPTOMYCIN-SULPHATE
BLIGHT (*Erwinia amylovora*). OVER SEVERAL YEARS THIS ANTIBIOTIC WAS AUTHORISED IN GE
AGAIN, NO REGISTRATION ON ANNEX 1 EXISTS FOR CONTROL OF FIRE BLIGHT AND NOT EVE
INDICATION. IN GENERAL, ANTIBIOTICS ARE EXCLUDED FROM USE IN PLANT PROTECTI
CONCERNS REGARDING THEIR USE IN HUMAN CHEMOTHERAPY. RESISTANCE AGAINST ANT
ACHIEVED AFTER CONTINUOUS USE AND CAN OCCUR ALSO UNDER FIELD SITUATIONS. H
ORGANISATIONS URGENTLY REQUESTED AUTHORISATION TO GET THE EMERGENCE REGI
YEARS, ALTHOUGH BIOLOGICAL PRODUCTS WERE AVAILABLE, LIKE THE BIOLOGICAL CO
Aureobasidium pullulans. IN GERMANY FOR THE 2013 SEASON PROGRESS IS NOW MADE
BIOLOGICAL COMPOUNDS WERE ALSO GIVEN AN EMERGENCY AUTHORISATION.

The new EU Biocid Regulation (EU) No 528/2012

IN ARTICLE 3 OF THE REGULATION (EU) NO 528/2012 DEFINITIONS ARE PROVIDED FOR THE PU
THIS REGULATION. UNDER POINT 1 MICROORGANISMS ARE DEFINED: (B) "MICRO-ORGANIS
MICROBIOLOGICAL ENTITY, CELLULAR OR NON-CELLULAR, CAPABLE OF REPLICATION OR O
GENETIC MATERIAL, INCLUDING LOWER FUNGI, VIRUSES, BACTERIA, YEASTS, MOULDS, ALGA
MICROSCOPIC PARASITIC HELMINTHS. WITHOUT CONSULTATION OF BIOLOGICAL CONTRO
INDUSTRY, HELMINTHS HAVE BEEN INCLUDED UNDER “MICROORGANISMS”. SIMILAR AT
PREVENTED BEFORE REGULATION (EC) NO 1107/2009 WAS IMPLEMENTED. AFTER THE DRA
WAS MADE AVAILABLE BY THE COMMISSION, COST ACTION 850 “BIOCONTROL SYMBIOSIS” T
WITH SEVERAL STAKEHOLDERS FROM INDUSTRY AND THE ENVIRONMENTAL PROTECTION A
CHARGE OF PESTICIDE REGULATION IN THE USA, COULD PERSUADE THE EU OFFICIALS
NEMATODES FROM THE DEFINITION OF MICROORGANISMS. BUT SOMEONE HAS HAD THE I
THE PRACTICE FOR THE BIOCIDE DIRECTIVE WITHOUT A RISK-DAMAGE ANALYSIS OR A COS
OF THIS REGULATORY MEASURE. IS OUR SAFETY REGULATION MANAGED BY INCOMPETE
PERSONAL?

Member states disregard biocontrol, chemical companies discover their potential

WHEREAS MS AUTHORITIES AND OFTEN ALSO GOVERNMENTAL R&D ORGANISATIONS IGNOR
OF BIOLOGICAL CONTROL AGENTS, CHEMICAL INDUSTRY HAS DISCOVERED THE BENEFITS. IN
A SERIES OF TAKEOVERS OF COMPANIES SPECIALISING IN BIOCONTROL WERE REPORTED. IN
(28 NOVEMBER 2012) IT WAS ANNOUNCED THAT BASF HAS COMPLETED THE ACQUISITION
UNDERWOOD FROM NORWEST EQUITY PARTNERS, A US-BASED PRIVATE EQUITY INVESTME

FOR A PURCHASE PRICE OF US$ 1.02 BILLION (785 MILLION EURO). WITH THE ACQUISITION, I
NOWA LEADING GLOBAL PROVIDER OF TECHNOLOGIES FOR BIOLOGICAL SEED TREATMEN
PRODUCER OF ENTOMOPATHOGENIC NEMATODES AND *Steinernema*. ON 19
SEPTEMBER 2012, SYNGENTA ANNOUNCED THAT IT ACQUIRED PASTEURIA BIOSCIENCE INC
BIOTECHNOLOGY COMPANY DEVELOPING AND COMMERCIALISING BIOLOGICAL PRODUCTS TO
PARASITIC NEMATODES, USING THE NATURALLY OCCURRING SOIL BACTERIUM
SYNGENTA ACQUIRED THE COMPANY FOR US$ 86 MILLION, WITH ADDITIONAL DEFERRED PA
TO US$ 27 MILLION. ON 21 JANUARY 2013, BAYER CROPSCIENCE ANNOUNCED THE COMPLETI
PURCHASE OF PROPHYTA GMBH, GERMANY, A LEADING SUPPLIER OF MICROBIAL CROP
PRODUCTS. PROPHYTA, FOUNDED IN 1992, PROVIDES THE PRODUCT CONTAINING FOR CONTRO
SPP. AND BIOAPT, *Paecilomyces lilacinus*, FOR CONTROL OF *Meloidogyne* SPP. IN GREEN-HOUSE
VEGETABLES. APART FROM THESE ACQUISITIONS, CHEMICAL INDUSTRY IS HEAVILY INVESTING
BIOLOGICAL CONTROL AND REGISTRATION OF PRODUCTS BASED ON MICROBIAL CONTROL A
EXPECTED. OF COURSE, WE HAVE EXPERIENCED ENGAGEMENT INTO BIOLOGICAL CONTRO
UNSUCCESSFUL OUTCOME, WHY THESE ACTIVITIES ARE VERY CRITICALLY FOLLOWED. BUT TH
CHANGED: POLITICAL DIRECTIONS, LIKE GREENING OF CAP, PROBLEMS WITH RESIDUES
COMPOUNDS IN FOOD AND CHANGING CONSUMER BEHAVIOUR SET OTHER PRIORITIES. WE
MORE INPUT INTO BIOLOGICAL CONTROL, DESPITE THE FACT THAT SOME STAKEHOLDER AR
PARADIGMS OF THE LAST CENTURY.

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Dynamics of baculovirus as insect biocontrol agent

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Abstract: BACULOVIRUSES ARE PATHOGENS CAUSING DISEASE IN LEPIDOPTERAN, D
HYMENOPTERAN INSECTS. THEY ARE LARGELY SPECIFIC AND FORM A VIABLE BIOLOGIC
CHEMICAL INSECTICIDES OR TO GENETICALLY-MODIFIED CROPS IN THE CONTROL OF INSE
OVER SEVEN HUNDRED REPORTED BACULOVIRUSES, BUT ONLY A FRACTION OF THESE H
AND REGISTERED AS BIOCONTROL AGENT. BACULOVIRUSES ARE CONSIDERED SAFE FO
TARGET ANIMALS, EVEN WHEN USED IN FOOD CROPS. DRAWBACKS ARE THEIR SLOW SPE
UV-SENSITIVITY, BUT THESE CAN BE MITIGATED TO SOME EXTENT BY 'SMART-SPRAYING' T
BY USING PROPER FORMULATIONS.

BACULOVIRUSES WERE STRONGLY PROMOTED IN THE 1970S IN THE WAKE OF 'SILEN
MANY SMALL AND BIG SIZE COMPANIES STARTED TO DEVELOP BACULOVIRUSES COMM
RESEARCH CENTERS EMERGED AND STUDIED DIFFERENT ASPECTS OF BACULOVIRUS GENE
THE 1980S BOTH IN VIVO AND PROMISING IN VITRO PRODUCTION TECHNOLOGIES WERE S
INSIGHT IN THE MOLECULAR GENETICS OF BACULOVIRUSES ALLOWED ENGINEERING OF
IMPROVED INSECTICIDAL PROPERTIES. HOWEVER, *in vitro* PRODUCTION TECHNOLOGY NEVER MATURED
THE EXTENT THAT COULD COMPETE WITH *in vivo* PRODUCTION AND THE SOCIETAL DISCUSSION
GENETICALLY MODIFIED BACULOVIRUSES HALTED FURTHER DEVELOPMENT IN THAT DIRE

IN THE 1990S MAJOR INDUSTRIES REDUCED THEIR BACULOVIRUS ACTIVITIES OR WIT
SCENE ALTOGETHER AND ONLY A FEW BACULOVIRUS PRODUCTS AND COMPANIES SU
PRESENT DAY. ALSO MANY OF THE RESEARCH CENTERS ON BACULOVIRUSES HAVE DI
VARIETY OF REASONS AND ONE WONDERS WHETHER THERE IS ENOUGH VITALITY, OR NO
AND OPPORTUNITIES TO FORESEE ANOTHER WAVE OF INTEREST IN BACULOVIRUSES AS
THIS IS THE TOPIC OF THIS CONTRIBUTION AND AN EFFORT TO HIGHLIGHT RECEN
DEVELOPMENTS AND NOVEL OPPORTUNITIES IN THIS AREA. THE INTEREST IN BACULOVI
THE EXPRESSION OF FOREIGN GENES AND FOR GENE DELIVERY IN A HUMAN OR VET
(VACCINS, GENE THERAPY) NEVER CEASED TO EXIST AND IS EVEN ENHANCED WITH THE RE
HUMAN VACCINES (HUMAN PAPILLOMA VIRUS, INFLUENZA), THERAPEUTICS (PROSTATE C
THERAPY APPLICATIONS (LIPOPROTEIN LIPASE DEFICIENCY) PRODUCTS BASED ON THIS
RESEARCH IN THIS AREA ALSO HAS A POSITIVE FEEDBACK FOR THE FURTHER UN
BACULOVIRUSES AS INFECTIOUS AGENT.

THERE A NUMBER OF EMERGING AREAS IN BACULOVIRUS RESEARCH THAT ARE RE
BIOCONTROL AND WORTH DISCUSSING. ALTHOUGH THERE IS SUBSTANTIAL INFORMATIC
GENETICS AND FUNCTIONAL GENOMICS OF BACULOVIRUSES, MUCH LESS IS KNOWN ON
BEHAVIOR OF THESE VIRUSES AND THE GENES ASSOCIATED WITH THESE PROCESSES. BA
EVOLVED FROM A COMMON ANCESTOR, BUT HAVE DIVERGED AND ADAPTED TO THEIR R
OPTIMIZE THEIR OWN SURVIVAL, NOT NECESSARILY BY KILLING THEIR HOST FAST BUT
DISPERSAL. UNDERSTANDING THESE PROCESSES BETTER AND IDENTIFYING THE VIRUS
DRIVING THESE PROCESSES, CALLS FOR A DETAILED UNDERSTANDING OF THE HOSTS`
BIOLOGICAL AND BEHAVIORAL RESPONSE TO VIRUS INFECTION. THIS COULD MEAN
BACULOVIRUSES SHOW SPECIES SPECIFICITY BUT ALSO THE HOST RESPONSE MAY BE SI
THIS ASPECT WILL BE HIGHLIGHTED WITH A FEW EXAMPLES. A SECOND ASPECT TO DISCU

THAT BACULOVIRUSES SPECIES ARE IN FACT A CLOUD OF RELATED GENOTYPES (BACU
STRAINS) BUT ALSO MIXTURE OF GENOTYPES WITHIN EACH ISOLATE, THE RELATIVE PRO
MAY DETERMINE THE OUTCOME OF INFECTION. THE RECENTLY OBSERVED CASES OF RE
BACULOVIRUSES CAN BE OVERCOME BY USING OTHER STRAINS OF THE VIRUS, HIGHLIGHT
OF STRAIN SELECTION, IDENTIFICATION AND CHARACTERIZATION. THE INFERENCE OF TI
THE USE OF BACULOVIRUSES AS BIOCONTROL AGENT WILL BE DISCUSSED.

Key words: BACULOVIRUSES, BIOCONTROL, BIODIVERSITY, BEHAVIOR

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Insect pathogenic fungi: what was obtained and where to go?

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Abstract: SINCE ITS START IN 1991 THE IOBC WORKING GROUP 'INSECT PATHOGENS AND INSEC NEMATODES' HAS HELD 13 MEETING, PLUS SOME SUBGROUP MEETINGS. PAPERS ON FUNGAL EN HAVE BEEN PRESENTED AT ALL MEETINGS, AND LIKEWISE, FUNGI HAVE BEEN PART OF PRESENTA MEETINGS IN SOCIETY FOR INVERTEBRATE PATHOLOGY (SIP). BY LOOKING INTO ESPECIALLY TH PRESENTATIONS AND LITERATURE I WILL DISCUSS THE STATUS OF INSECT PATHOGENIC FUNGI WHAT WAS OBTAINED AND WHERE TO GO?

Key words INSECT PATHOGENIC FUNGI, HYPOCREALES, ENTOMOPHTHORALES

The fungal species: we have more species than we thought

THESPECIES CONCEPT OF INSECT PATHOGENIC FUNGI HAS MOVED SIGNIFICANTLY TOWAR SEVERAL WELL-KNOWN HYPOCREALES INTO CLADES, WHICH CAN BE CONSIDERED AS SP CRYPTIC SPECIES. THE SPECIES *Metarhizium anisopliae* IS NOT ANY MORE A SINGLE SPECIES, BUT SHOULD BE SPLIT INTO SEVERAL SPECIES, INCLUDING *M. anisopliae*, *M. brunneum* AND *M. robertsii*. IT IS A CHALLENGE FOR APPROVAL AUTHORITIES AND FOR SCIENTISTS TO KNOW WHEN A STRAIN OF *M. anisopliae* OR *M. brunneum* REFER TO THAT SPECIES OR MERELY REFERS ONE OF THE OTHER SPECIES. THE SAME DEVELOPMI FOR *Beauveria bassiana*. ALSO, THE APPLICATION OF NEW NOMENCLATURE RULES HAS SIG INFLUENCED OUR PERCEPTION OF THE IDENTITY OF CERTAIN HYPOCREALEAN FUNGI (HU EXAMPLE IF THE NAME OF THE TELEOMORPH SHOULD BE USED RATHER THAN THE NAME (VEGA *et al.*, 2012).

THE ENTOMOPHTHORALES HAVE BEEN LESS SUBJECTED TO SIGNIFICANT CHANGI PROBABLY DUE TO THE FACT THAT IS HAS FOR LONG BEEN KNOWN, THAT CORRECT IDE SPECIALIST FUNGI BESIDES MORPHOLOGICAL DATA ALSO NEED THE INCLUSION OF PA MOLECULAR DATA. FOR EXAMPLE, THE GENUS *Entomophthora* CONSISTS OF A NUMBER OF SPECIES WITH DIFFERENCES BOTH WITH RESPECT TO MORPHOLOGY, MOLECULAR BIOLOGY AND PA RANGE), AND BY THAT CAN BE DOCUMENTED AS BEING AN OLD LINEAGE (JENSEN *et al.*, 200

Field ecology: it is complicated

THEFIELD ECOLOGY OF ENTOMOPATHOGENIC FUNGI WAS OUTLINED BY ANDERSEN *et al.* (2009). A MISSING ELEMENT IS, HOW TRANSMISSION OF THE DISEASE TAKES PLACE IN NATURE, WHE CONCENTRATION WOULD NORMALLY BE FAR BELOW THE LEVELS USED FOR BIOCONTROL HAND, THERE WILL SURELY BE HOT-SPOTS WITH HIGHER SPORE CONCENTRATIONS AND A SEVERAL TYPES OF INTERACTIONS WITH HOSTS ALLOWING HOST AND FUNGUS TO MEET A STICK TO CUTICLE AND READILY INFECT. ON THE OTHER HAND IT APPEARS THAT FOR CLADES/SPECIES OF *Metarhizium* HAVE DIFFERENT NATURAL ECOLOGY (ST. LEGER AND WANG *et al.*, 2012).

BY THAT THE CONCENTRATION OF EACH OF THESE CLADES ARE EVEN LOWER THAN THE
GENUS IN TOTAL. A RECENT PROJECT IMBICONT (2012-2015), A BI-LATERAL COLLABORAT
UNIVERSITY OF SAO PAULO AND UNIVERSITY OF COPENHAGEN, HAS AS ONE AIM TO STUDY
THE FIELD ECOLOGY AND THE INTERACTION OF INSECT PATHOGENIC FUNGI AND THEIR
LEVEL.

Bio-assays: methods are well established

PERFORMING BIO-ASSAYS IS AN INDISPENSABLE WAY, FIRST TO SCREEN SEVERAL ISOL
SPECIFIC TARGET INSECTS, THEN TO DETAIL MORE THE CONDITIONS GOVERNING SUCCES
FINALLY TO TEST PERFORMANCE OF A SELECTED ISOLATE IN THE LABORATORY BEFORE
APPLICATION. THE IOBC PROCEEDINGS AND THE SIP ABSTRACTS AND PROCEEDINGS INCL
STUDIES, ESPECIALLY WITH HYPOCREALES. THE PRESENT KNOWLEDGE IS SUMMARIZED B
(2012) FOR HYPOCREALES AND BALA (2012) FOR ENTOMOPHTHORALES AND THESE CHAPT
CONTAIN SOME MAIN GUIDELINES. SOME PAPERS AND PRESENTATIONS ON BIO-ASSAYS F
YEARS (ESPECIALLY ON HYPOCREALES) APPEAR BE OF HIGH TECHNICAL VALUE FOR TH
FURTHER WORK ON SPECIFIC FUNGAL ISOLATES AGAINST SPECIFIC INSECT TARGE
CONTRIBUTIONS TO NEW GENERAL APPROACHES AND METHODOLOGIES ARE MORE RARE.

et al. *et al.*

Production and formulation: new approaches on their way

RECENTLY THE KNOWLEDGE ON MASS PRODUCTION OF HYPOCREALES WAS COMPILED
JACKSON, 2012). THEIR BOOK CHAPTER CONTAINS THE FULL INFORMATION PACKAGE NE
SCALE TO MEDIUM SCALE LABORATORY PRODUCTION AS WELL AS SIGNIFICANT CONSID
MASS PRODUCTION. FURTHER DEVELOPMENT OF INSECT PATHOGENIC FUNGI NEEDS
PRODUCTION AND FORMULATION AND NEW APPROACHES ARE NEEDED. A RECENT EU SU
INBIOSOIL (2012-2015) HAS AS ONE AIM TO STUDY EFFECTS ON TARGET AND NON-TARGE
FORMULATIONS OF HYPOCREALES.

Biocontrol strategies: Inundation, inoculation or conservation?

A BASIC QUESTION CONCERNING INSECT PATHOGENIC FUNGI: DO THEY FIT IN ALL THE M
STRATEGIES FOR BIOCONTROL. OBVIOUSLY YES CONCERNING INUNDATION AND INOCU
EXISTENCE OF HYPOCREALEAN FUNGI ON THE MARKET IN EUROPE AND ELSEWHERE
DOCUMENTS THAT THESE FUNGI CAN ACT IN BOTH STRATEGIES. THE PERSPECTIVES FOR
ALSO REALLY HIGH, ALTHOUGH THE POTENTIAL CANNOT BE EXPLORED MORE FULLY
KNOWLEDGE OF SPECIES COMPLEXES WITH DIFFERENT ECOLOGY AND PATHOLOGY ARE
CONSIDERATION.

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FOR RESEARCH (CENTRE FOR SOCIAL EVOLUTION).

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Fungi

Session 1:
Entomopathogenic fungi
in the control of soil-dwelling pests

Insect pathogens and entomoparasitic nematodes
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Biological control of wireworms with entomopathogenic fungi

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Abstract: THREE SPECIES OF WIREWORMS, *Agriotes lineatus, A. obscurus* AND *A. sputator*, ARE ECONOMICALLY IMPORTANT SOIL PESTS IN ARABLE AND VEGETABLE CROPS IN EUROPE. FUN *Metarhizium* ARE NATURAL PATHOGENS OF WIREWORMS. WE TESTED THE VIRULENCE OF TH *Metarhizium* STRAINS IN LABORATORY EXPERIMENTS AND FOUND A MAXIMUM MORTALITY OF UP WEEKS POST INOCULATION. WE FURTHER INVESTIGATED STABILITY OF THE VIRULENCE OF THE ART2825 AFTER TEN TIMES OF SUBCULTIVATION ON ARTIFICIAL MEDIA. THERE WAS NO DIFFERE COMPARED TO A TREATMENT OF LARVAE THAT WERE INFECTED WITH FRESHLY HOST-PASSED C THAT *Metarhizium* STRAIN ART2825 IS A POTENTIAL CANDIDATE FOR THE CONTROL OF WIREWORMS CONTINUE TO VALIDATE ITS EFFICACY UNDER FIELD CONDITIONS.

Key words: *Agriotes lineatus, A. obscurus, A. sputator*, ENTOMOPATHOGENIC FUNGI, POTATO, *Metarhizium*, VIRULENCE, HOST PASSAGE

Introduction

WIREWORMS, THE SOIL-DWELLING LARVAE OF CLICK BEETLES (*Agriotes* SPP.), CAUSE ECONOMICALLY SIGNIFICANT DAMAG PARTICULARLY ON POTATO TUBERS. THE SPECIES RESPONSIBLE FOR THIS DAMAGE ARE *A. lineatus*, *A. obscurus* AND *A. sputator* IN MANY EUROPEAN REGIONS (E.G. SURGHAUSE & SCHMITT, 2011, PARKER & HOWARD, 2011). CURRENTLY, WIREWORMS ARE CONTROLLED WITH CHEMICAL IN (KUHAR *et al.*, 2003). ALTERNATIVE CONTROL METHODS OF WIREWORMS FOR USE IN O INTEGRATED FARMING SYSTEMS ARE NOT YET AVAILABLE, ALTHOUGH HIGHLY ANTIC "SUSTAINABLE USE" DIRECTIVE 2009/128/EC.

SPECIES OF THE FUNGAL GENUS *Metarhizium* ARE NATURAL PATHOGENS OF A BROAD RANG INSECTS INCLUDING WIREWORMS (KABALUK *et al.*, 2005). HOWEVER, VIRULENCE OF *Metarhizium* STRAINS DIFFERS SIGNIFICANTLY AGAINST CERTAIN SPECIES OF WIREWORMS (ANSAR *et al.*, 2009). ADDITIONALLY, VIRULENCE MAY ATTENUATE AFTER SUCCESSIVE SUBCULTIVATION O (REVIEWED IN BUTT *et al.*, 2006). THE AIM OF OUR INVESTIGATIONS WAS TO IDENTIFY VIRULENT OF ENTOMOPATHOGENIC FUNGI (EPF) AGAINST DIFFERENT SPECIES OF WIREWORMS AN THEIR STABILITY AFTER REPEATED SUBCULTIVATION ON ARTIFICIAL MEDIA.

Material and methods

WIREWORM LARVAE AND FUNGAL STRAINS

LARVAE OF *A. obscurus*, *A. lineatus* AND *A. sputator* USED IN THESE EXPERIMENTS ORIGINATED FROM GREENHOUSE REARING, ESTABLISHED WITH FIELD COLLECTED ADULT CLICK BEETLES (K *et al.*, 2009). THREE *Metarhizium* STRAINS WERE TESTED: (1) BIPESCO 5 (= F52), ISOLATED FROM CODLING M *Cydia pomonella*, AUSTRIA, (2) ART2825 ISOLATED FROM *A. obscurus*, SWITZERLAND AND (3) V1002 ISOLATED FROM *A. lineatus*, UK. EACH FUNGUS STRAIN WAS PASSAGED THROUGH GREATE

MOTH LARVAE (*Galleria mellonella*), AND THEN RE-ISOLATED FROM SINGLE CONIDIA CO
MAINTAINED ON SABOURAUD DEXTROSE AGAR (MODIFIED, 1995) ACCORDING TO STRASSER *et al*

BIOASSAY WITH DIFFERENT METARHIZIUM STRAINS

TEN LARVAE OF EACH WIREWORM SPECIES WERE DIPPED INTO A SUSPENSION OF 10 ^{8} CONIDIA ml^{-1} WATER WITH 0.03% TWEEN® 80 FOR 20 S. EACH LARVA WAS INCUBATED SEPARATELY IN A SMALL CUP W 30 G NON-STERILE WET FIELD SOIL. A SLICE OF CARROT WAS PLACED IN EACH CUP AS FOOD S REPLACED WEEKLY. CUPS OF EACH TREATMENT WERE KEPT IN A PLASTIC BOX AND IN CONTROLLED CONDITIONS (AT 23 °C AND 65% RELATIVE HUMIDITY). THE NUMBER OF DE ASSESSED WEEKLY FOR EIGHT WEEKS. CADAVERS WERE INCUBATED UNTIL *Metarhizium* MYCOSIS BY WAS CLEARLY VISIBLE ON THE INSECT'S CUTICLE. A TREATMENT WITH WATER AND TWEEN® 80 AND AN INSECTICIDE TREATMENT WITH ETHOPROPHOS (9.6 MG PER CUP ACCORDING TO 60 G m^{-2}) SERVED AS CONTROLS. THE WHOLE EXPERIMENT WAS REPEATED THREE TIMES.

BIOASSAY WITH FRESHLY HOST-PASSED AND* in vitro *SUBCULTURED CONIDIA

THE STRAIN ART2825 WAS SELECTED FOR FURTHER INVESTIGATIONS BECAUSE OF ITS V PREVIOUS EXPERIMENT. CONIDIA FROM *in vivo* AND *in vitro* CULTIVATION WERE TESTED AGAINST WIREWORMS. CONIDIA FOR THE FIRST TREATMENT ORIGINATED FROM THE TENTH SUBSE THE FUNGUS ON SDA PLATES, WHILE CONIDIA FOR THE SECOND TREATMENT WERE DI FROM FRESH *Agriotes* CADAVERS. A TREATMENT WITH A WATER-0.05% TWEEN® 80 SOLUTION SERVED AS CONTROL. EIGHT LARVAE OF *A. lineatus* AND *A. obscurus* AND 12 LARVAE OF *A. sputator* WERE INFECTED PER TREATMENT AND EACH TREATMENT HAD FOUR REPLICATES. LARVAE WERE DIPPED I 10^{6} CONIDIA ml^{-1} FOR 5 S AND INCUBATED IN 10 G OF MOIST PEAT SUBSTRATE. INCUBATION AN WAS ASSESSED AS DESCRIBED ABOVE.

STATISTICAL ANALYSES

WE ANALYZED THE DATA FOR EFFECTS ON THE MORTALITY CAUSED BY THE TREATMENT W EFFECT MODEL BASED ON MAXIMUM LIKELIHOOD. THE STATISTICAL SOFTWARE R (VERS THE FUNCTION "LMER" WAS USED. THE STATUS OF THE LARVAE (ALIVE/KILLED BY CONSIDERED AS DEPENDENT VARIABLE WITH A BINOMIAL DISTRIBUTION. IN THE BIOASS VIRULENCE OF STRAINS, THE TREATMENT WAS USED AS THE INDEPENDENT VARIABLE. I BIOASSAY THE PRESENCE OF HOST PASSAGED CONIDIA (YES/NO) WAS USED AS THE INDEPE THE BLOCK REPRESENTED BY A BOX CONTAINING A TREATMENT OF WIREWORMS WAS RANDOM FACTOR.

Results and discussion

VIRULENCE OF DIFFERENT METARHIZIUM STRAINS

ART2825 WAS THE MOST EFFECTIVE STRAIN AGAINST *A. obscurus*. ON AVERAGE OF 80% OF THE LARVAE DIED OF MYCOSIS (FIGURE 1). THIS STRAIN KILLED SIGNIFICANTLY MORE *A. obscurus* LARVAE THAN BIPESCO 5 ($Z = 0.00014$) AND V1002 ($Z = 0.0063$). ADDITIONALLY, ART2825 WAS THE MOST EFFICIENT STRAIN BY KILLING MORE THAN HALF OF THE *A. obscurus* LARVAE WITHIN TWO TO THREE WEEKS RESULTS WERE SIMILAR FOR *A. lineatus*, WITH MORE THAN HALF OF THE LARVAE KILLED BY ART WITHIN FOUR WEEKS AND AN AVERAGE MORTALITY OF 70% AFTER EIGHT WEEKS. *A. sputator* WAS LESS SUSCEPTIBLE TO ART2825 (50% MORTALITY) THAN BIPESCO 5 (60%) AND V1002 (70%).

EFFECT OF A HOST PASSAGE ON THE VIRULENCE OF* Metarhizium *STRAIN ART2825

THE CULTIVATION BACKGROUND OF THE EPF INOCULUM HAD NO EFFECT ON THE VIRULE IN THE HOST PASSAGE TEST. AN AVERAGE OF 75% OF THE *A. obscurus* LARVAE DIED OF MYCOSIS WHEN TREATED WITH CONIDIA DIRECTLY HARVESTED FROM *A. obscurus* CADAVERS. A SIMILAR MORTALITY RATE O 70% WAS ACHIEVED WITH INOCULUM DERIVED FROM THE FIRST *in vitro* SUBCULTIVATION OF THE *Metarhizium* STRAIN. RESULTS WERE SIMILAR FOR *A. lineatus* AND *A. sputator* WITH GENERALLY LOWER MORTALITY RATES (DATA NOT SHOWN).

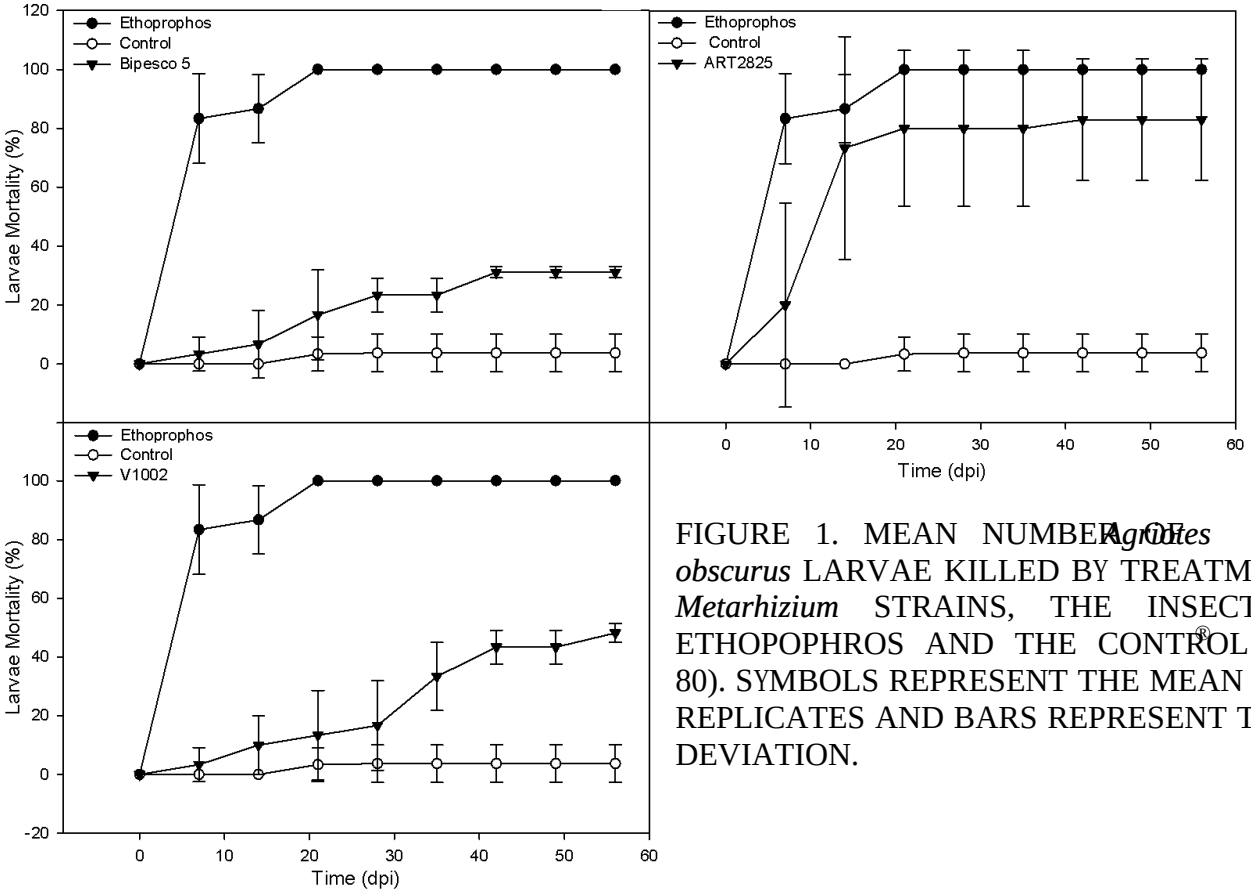

FIGURE 1. MEAN NUMBER OF *Agriotes obscurus* LARVAE KILLED BY TREATMENT WITH *Metarhizium* STRAINS, THE INSECTICIDE ETHOPOPHROS AND THE CONTROL (TWEEN® 80). SYMBOLS REPRESENT THE MEAN OF THREE REPLICATES AND BARS REPRESENT THE STANDARD DEVIATION.

BIOCONTROL AGENTS AND STRATEGIES AGAINST WIREWORMS HAVE BEEN RESEARCHED YEARS (E.G. ANSARI *et al.*, 2009; KABALUK, 2007). ART2825 IS A EUROPEAN *Metarhizium* STRAIN WHICH SHOWED HIGH VIRULENCE AGAINST TWO IMPORTANT WIREWORM SPECIES, *A. obscurus* AND *A. lineatus*. OUR RESULTS ARE IN ACCORDANCE WITH THOSE FROM KÖHLER *et al.*, 2011, WHO DEMONSTRATED SIGNIFICANTLY HIGHER MORTALITY OF WIREWORMS CAUSED BY ART2825 WITH A COMMERCIALLY AVAILABLE BIOCONTROL PRODUCT BASED ON *Beauveria bassiana*.

STABILITY OF VIRULENCE IS AN IMPORTANT CRITERION OF BIOCONTROL AGENTS IN THE ATTENUATION OF VIRULENCE DUE TO SUCCESSIVE SUBCULTIVATION ON ARTIFICIAL PHENOMENON AND MAY BE A PROBLEM FOR MASS PRODUCTION OF FUNGAL BIOCO (REVIEWED IN BUTT, 2006; ANSARI & BUTT, 2011). VIRULENCE MAY, HOWEVER, BE RESTO WITH HOST PASSAGES, AND IT HAS BEEN DEMONSTRATED THAT A SINGLE HOST PASSAGE (E. G. FARGUES & ROBERT, 1983). WE COULD NOT FIND SIGNS OF ATTENUATION IN ART282 SUBCULTIVATIONS ON ARTIFICIAL MEDIUM AND THEREFORE CONCLUDE THAT THE VIRU MAY BE RETAINED IN COMMERCIAL MASS PRODUCTION SYSTEMS.

IN CONCLUSION, ART2825 IS A PROMISING CANDIDATE FOR THE DEVELOPMENT OF A WIREWORM CONTROL PRODUCT. ADDITIONAL STUDIES UNDER SIMULATED AND ACTUAL CURRENTLY BEING CONDUCTED TO CONFIRM THE POTENTIAL OF THIS STRAIN AS A BIOCO

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Insect pathogens and entomoparasitic nematodes
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Monitoring of the entomopathogenic fungus *BEAUVERIA BRONGNIARTII* in cockchafer infested areas of the Euroregion Tyrol

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Abstract: THE PERSISTENCE OF THE ENTOMOPATHOGENIC FUNGUS *Beauveria brongniartii* IN SOILS IN THE EUROREGION TYROL HAS BEEN EVALUATED OVER A PERIOD OF TWO DECADES. THE FUNGAL PR® PILZGERSTE WAS SUCCESSFULLY APPLIED ON THE FIELDS IN DIFFERENT CONCENTRATIONS AN 1989 AND 2012. IN JULY AND AUGUST 2012 THE SOIL SAMPLES WERE DRAWN AND ANALYZED ON SE TO DETERMINE THE OCCURRENCE OF *Beauveria* SPP. PRELIMINARY RESULTS FROM MICROSATELLITE ANALYSIS THAT THE RE-ISOLATED *Beauveria* STRAINS FROM THE TEST SITES, WHICH HAD BEEN TREATED DURING T YEARS, WERE IDENTIFIED AS THE PRODUCTION STRAIN. NEW INSIGHTS INTO COLONISATION, MOBI OF *B. brongniartii* IN SOILS WILL BE DISCUSSED IN THE PRESENTATION.

Key words: *Beauveria brongniartii*, BIOLOGICAL CONTROL AGENT, PERSISTENCE, MONITORING, MICROS

Introduction

THE ENTOMOPATHOGENIC FUNGUS *Beauveria brongniartii* [(SACC.) PETCH ANAMORPHIC HYPOCREALES: CORDYCIPITACEAE] IS A COMMERCIALLY AVAILABLE BIOLOGICAL CONTRO OCCURS NATURALLY IN MANY HABITATS AROUND THE WORLD. IN EUROPE IT INFE COCKCHAFERS *Melolontha melolontha* AND *M. hippocastani* (COLEOPTERA: SCARABAEIDAE). THE SCARABS *Melolontha* SPP. ARE MAJOR PEST IN AGRO/FORST SYSTEMS AND ARE COMMONLY FO PROVINCES OF THE ALPINE REGION. TO CONTROL *Melolontha* SPP. IN THE PROVINCE OF TYROL *B. brongniartii* HAS BEEN SUCCESSFULLY APPLIED AND COMBINED WITH MECHANICAL TR MORE THAN TWO DECADES. MONITORING THE PERSISTENCE OF AN ENTOMOPATHONGEN IS AN INTEGRAL PART OF THE RISK ASSESSMENT OF MICROBIAL PESTICIDES. IN 2012 THE AND THE PANEL ON PLANT PROTECTION PRODUCTS AND THEIR RESIDUES (PPR) OF THE TENDER TO GENERATE A GUIDANCE DOCUMENT ON HOW TO CONDUCT RISK ASSESSM PESTICIDES. THIS GUIDANCE DOCUMENT SHOULD SUPPORT THE EU REGULATION (EC) NO TO FACILITATE THE ASSESSMENT OF RISKS POSED ON THE ENVIRONMENT BY BIOLOGICA LAENGLE AND STRASSER (2010) PROPOSED A RISK INDEX (RI). THE RI IS COMPOSED COMPONENTS INCLUDING PERSISTENCE. DATA ON THE SURVIVAL OF *B. brongniartii* AND OTHER ENTOMOPATHONGENIC FUNGI ARE NOT ONLY ESSENTIAL FOR RISK ASSESSMENT OF B AGENTS BUT ARE REQUIRED FOR REGISTRATION OF NEW MICROBIAL PESTICIDES AND BIOLOGICAL CONTROL STRATEGIES.

MOLECULAR METHODS ENABLE THE DISCRIMINATION BETWEEN DIFFERENT STRAI THEREFORE ADD AN IMPORTANT TOOL TO STUDY THE PERSISTENCE OF THE APPLIED BIOL VERSUS NATURALLY OCCURING STRAINS (ENKERLI *et al.* 2004). WE DEVELOPED A METHOD TO USE SIMPLE SEQUENCE REPEATS, ALSO CALLED MICROSATELLITES, TO DISCRIMINATE BETWEEN V *B. brongniartii*. THIS STUDY AIMED TO MONITOR *B. brongniartii* IN *Melolontha* INFESTED AREAS OF

Tyrol over a period of two decades. Additionally, the occurrence of indigenous *Beauveria* strains was compared to the density of the applied production strain of Melocont® Pilzgerste.

Material and methods

Selection of sampling sites

For the soil sampling, 20 sites (i.e. meadows and orchards) with a history of cockchafer infestation in East, North, and South Tyrol were selected. The sites were categorized according to the treatments with various concentrations of the product Melocont® Pilzgerste at different time periods between 1989 and 2012 (Table 1). The control sites had never been treated with Melocont® Pilzgerste. The fungal pesticide was applied according to the manufacture's guide lines (Agrifutur).

Table 1. Melocont® Pilzgerste treatments at sampling areas in the Euroregion Tyrol. With the exception of the control araea (C) all sites were treated with different quantities of Melocont® Pilzgerste and time frames.

Variations	Years of application	Number of treatments (T)	Rate of application (kg ha^{-1} and T)
C	0	0	0
1	1994-1997	1-2	25
2	2009-2012	1-2	25
3	1989-2012	1	20

Soil analysis

Soil samples from the test and control plots were taken with a split tube sampler. 40 samples ha^{-1} soil were drawn and combined in a plastic zip lock bag. Two horizons of each sample were analyzed: from 0 to 10 cm depth and from 10.5 to 20 cm depth. All soil plots were sampled in a Z-shape in July and August 2012. Soil samples were processed according to the standard protocol published by Laengle *et al.* (2005). To determine the number of fungal colony forming units (CFU) a selective medium was used (Strasser *et al.*, 1996). Three colonies per plate were selected and isolated. These isolates were grown in semi-synthetic complete medium (CM; Enkerli *et al.*, 2001) and fungal biomasses were filtered and washed with deionised water. Aliquots of 0.15 g of fresh biomass were frozen in liquid nitrogen and lyophilized.

Genetic analysis

After adding 0.15 g of glass beads (1 mm diameter) to the lyophilized fungal myzelia the samples were homogenized with a ball-mill (MM301, Retsch) at maximum speed for 15 to 45 seconds. For the DNA extraction the PL2 buffer of the DNA extraction kit Nucleo Spin Plant II (Machery & Nagel) was used. The following steps of the DNA extraction were performed according to the manufacturer's manual. The PCR reaction and the analysis of the *Beauveria* specific microsatellite markers were performed according to Enkerli *et al.* (2001). Amplified gene fragements were visualized with an Applied Biosystems 3130 Genetic Analyzer (Hitachi) and the output data were displayed with the software GeneMarker® (SoftGenetics).

EVALUATION OF THE INFESTATION WITH COCKCHAFERS

SPADE SAMPLING TECHNIQUES WERE USED TO EVALUATE THE INFESTATION RATE OF COC
TWELVE SQUARE HOLES 50 CM LONG AND UP TO 70 CM DEEP WERE DUG AND THE NUMBER
ASSESSED. SUPPLEMENTAL DATA ON THE INFESTATION OF THE SOIL PLOTS WITH LARVA
DAMAGES OF THE CROPS WERE COLLECTED IN TERMS OF A QUESTIONNAIRE PROVIDED BY

Results and discussion

PERSISTENCE OF BEAUVERIA BRONGNIARTII

THEAPPLICATION OF MELOCONT® PILZ CONTRA FOR FOUR YEARS IN *Melolontha* INFESTED AREA LEAD TO A CONTINUOUS INCREASE OF THE *B. brongniartii* DENSITY IN SOIL PLOTS COMPARED TO THE CONTRO FIELD. AFTER THE END OF THE TREATMENTS WITH THE *B. brongniartii* STRAIN THE DENSITY DECREASED FROM 1×10^5 TO 2×10^4 SPORES g^{-1} DRY WEIGHT OF SOIL WHICH IS THE RECOMMENDED FUNGAL D TOENSURE EPIDEMIC LEVELS IN GRASSLANDS (FERRON, 1967; FIGURE 1). FORNALLAZ (1992) 10- TO 50-FOLD REDUCTION OF *B. brongniartii* SPORES g^{-1} DRY WEIGHT OF SOIL PER YEAR IN THE ABSENC OFTHE HOST. FIFTEEN YEARS AFTER THE USE OF THE MICROBIAL PESTICIDE THE FUNGU LIMIT OF DETECTION WHICH IS DEFINED AS 2×10^1 SPORES g^{-1} DRY WEIGHT OF SOIL (LACEY, 2005). THE SUCCESSFUL TREATMENTS RESULTED IN LOW NUMBERS OF LARVAE, FOLLOWED BY A RED WHICH DECREASES RAPIDLY WITHOUT ITS HOST. THESE RESULTS ARE IN ACCORDANCE PROVIDED BY KESSEL *et al.* (2004).

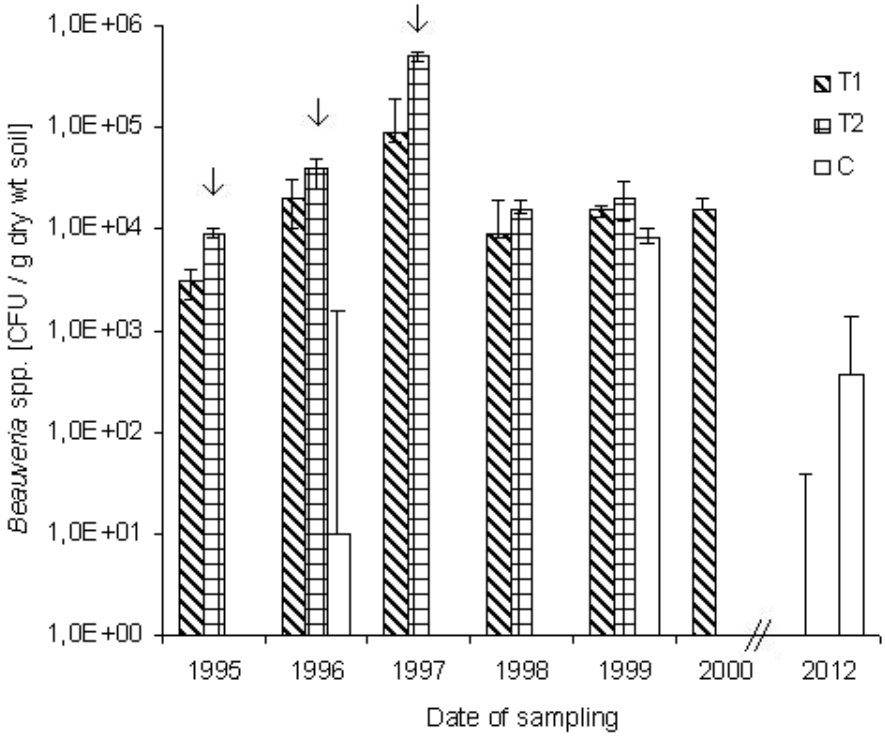

FIGURE 1. PRESENCE OF *Beauveria* SPP. (MEDIAN, UPPER AND LOWER QUARTILE) IN TEST FIEL AND THE CONTROL FIELD (C). ARROWS INDICATE APPLICATIONS OF THE MELOCONT®

SOILS WHICH HAD BEEN TREATED WITH THE FUNGAL PESTICIDE FOR A PERIOD OF FO
SAMPLING (VARIATION 2) CONTAINED APPROXIMATELY 10^5 SPORES OF *B. bongniartii* G^{-1} DRY
WEIGHT OF SOIL (TABLE 2). IN THESE SITES THE INFESTATION WITH *M. melolontha* WAS ESTIMATED
TO RANGE BETWEEN 75 AND 150 LARVAE M^{-2} ON AVERAGE BEFORE AND BELOW 5 LARVAE M^{-2} AFTER THE
PERIOD OF APPLICATION. FURTHERMORE, NO RELEVANT *M. melolontha* DAMAGES HAVE BEEN REPORTED
BY FARMERS AND EXPERT AUTHORITIES SINCE USING PILLER SERIE® ON CONTINUOUS
APPLICATION OF MELOCONT® PROFI GERSTE RESULTED IN A FUNGAL DENSITY UP TO 10^4 CFU G^{-1} DRY
WEIGHT OF SOIL (TABLE 2). THE *M. melolontha* LARVAE TESTIFIED THE EFFICACY OF THE FUN
PESTICIDE IN THOSE FIELDS. TWELVE YEARS AFTER OVERCOMING THE PLAGUE BY T
COCKCHAFER THE FUNGUS WAS NOT PRESENT IN THE SOIL AND NO DAMAGES OF THE
RECORDED. THE DETERMINATION OF THE NUMBER OF LARVAE IS IN PROGRESS.

TABLE 2. DENSITY OF *Beauveria* SPP. (MEDIAN) AND ITS HOST *M. melolontha larvae* (L2/3) IN SITE
(A, B, C) WHICH WERE TREATED WITH MELOCONT® 12 YEARS AGO (1) IN THE PREVIOUS 4 YEAR
(2) AND FOR TWO DECADES (3) AND THE CONTROL FIELD (C). * EVALUATION IN PROGRESS.

	***BEAUVERIA* spp. (CFU)**	**Number of larvae of *M. MELOLONTHA***
C	7.8 E + 01	*
1.a	0.0 E + 00	≤ 4*
1.b	0.0 E + 00	≤ 4*
1.c	0.0 E + 00	≤ 4*
2.a	9.0 E + 04	≤ 5
2.b	5.0 E + 05	≤ 5
2.c	1.5 E + 05	≤ 5
3.a	1.4 E + 04	≤ 1
3.b	3.0 E + 02	≤ 1

DISCRIMINATION BETWEEN THE APPLIED STRAIN AND INDIGENOUS STRAINS

THE GENETIC ANALYSIS OF THE MICROSATELLITE MARKERS IS STILL IN PROGRESS
PRELIMINARY STUDIES SHOWED THAT *Beauveria* STRAINS FROM THE TEST SITES VARIATION
WERE IDENTIFIED AS THE PRODUCTION STRAIN.

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Insect pathogens and entomoparasitic nematodes
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Susceptibility of *DIABROTICA VIRGIFERA VIRGIFERA* (Coleoptera: Chrysomelidae) to entomopathogenic fungi: Laboratory assays and field trials

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Abstract: SINCE THE INTRODUCTION OF *Diabrotica virgifera virgifera* TO EUROPE BETWEEN THE LATE 1980S AND THE EARLY 2000S, THE WESTERN CORN ROOTWORM SUBSEQUENTLY HAS EXTENDED ITS PRES PARTS OF EUROPE AND CAN CURRENTLY BE FOUND IN 20 EUROPEAN COUNTRIES. SEVERAL DI AIMING AT THE CONTROL OF *D. v. virgifera* HAVE AT LEAST PARTIALLY LIMITATIONS, MAKING THE BIOLOGIC PROBABLY THE MOST ENCOURAGING MANAGEMENT METHOD. BIOASSAYS REVEALED TWO *Metarhizium anisopliae* STRAINS AND ONE *Beauveria bassiana* STRAIN WITH THE HIGHEST PATHOGENICITY AGAINST 3RD INSTAR LARVAE OF *D. v. virgifera.* ALTHOUGH RESULTS OBTAINED FROM FUNGAL DENSITY MEASUREMENTS AFTER FUNG HUNGARIAN CROPLANDS REVEALED QUITE DISPLEASED PERSISTENCES, THE POTENTIAL OF CERTA THE CONTROL OF THE WESTERN CORN ROOTWORM CAN BE CONSIDERED INDISPUTABLE. HOWEVE AS WELL AS FIELD TRIALS ARE NEEDED TO CONFIRM THIS HIGH POTENTIAL.

Key words: ENTOMOPATHOGENIC FUNGI, *Metarhizium anisopliae*, WESTERN CORN ROOTWORM, BIOLOGICAL CONTROL AGENTS (BCA), EU FUNDED PROJECT INBIOSOIL

Introduction

SINCE THE REITERATED ACCIDENTAL INTRODUCTION OF *Diabrotica virgifera virgifera* TO EUROPE BETWEEN THE LATE 1980S AND THE EARLY 2000S (MILLER *et al.*, 2005), THE WESTERN CORN ROOTWORM (WCR) SUBSEQUENTLY HAS EXTENDED ITS PRESENCE ACROSS MANY PARTS OF EUROPE (E KISS, 2011). SEVERAL DIFFERENT NATURAL ENEMIES OF THE WESTERN CORN ROOTWORM WORLD, E. G. FUNGI, BACTERIA, PROTISTA, VIRUSES, NEMATODES, ARTHROPOD PREDATO ALL WITH DIFFERENT IMPACT ON WCR POPULATIONS (TOEPFER *et al.*, 2009). MANY OF THESE, PARTICULARLY SPECIALIZED PARASITOIDS, PREDATORS AND PATHOGENS, HAVE BEEN LEF OF ORIGIN, RESULTING IN A LACK OF OCCURRENCE IN MOST PARTS OF EUROPE (TOEPFE 2004). DIFFERENT MANAGEMENT PRACTICES AIMING AT THE CONTROL OF THE WCR, SU *i)* CROP ROTATION, *ii)* THE APPLICATION OF INSECTICIDES AND *iii)* THE USE OF ROOTWORM-RESISTANT TRANSGE MAIZE HYBRIDS PRODUCING *Bacillus thuringiensis* (*Bt*) TOXINS HAVE SHOWN TO EXHIBIT SEVERAL LIMITATIONS (GASSMANN *et al.*, 2011). THUS, THE USE OF BIOLOGICAL CONTROL AGENTS IN OR PROTECT PLANTS FROM WCR FEEDING IS CURRENTLY PROBABLY THE MOST ENCOURAGING

ENTOMOPATHOGENIC FUNGI, ESPECIALLY *Beauveria* SPP. AND *Metarhizium* SPP., HAVE ALREADY INDICATED TO BE EFFICIENT BIOLOGICAL AGENTS, SUPPRESSING WCR POPULATIONS ASSAYS, SEMI-FIELD- AND FIELD TRIALS (PILZ *et al.*, 2009). PRE-INVESTIGATIONS CARRIED OUT IN LABORATORY ASSAYS AND FIELD TRIALS AIMED AT THE EFFICAC ENTOMOPATHOGENIC FUNGAL STRAINS ON THE BASIS OF BIOASSAYS. FURTHERMORE CALCULATIONS OF *Beauveria* SP. AND *Metarhizium* SP. PRODUCTS IN *Diabrotica* INFESTED FIELDS IN HUNGARY WERE PERFORMED AND DAMAGE RATING ON MAIZE ROOTS WITH THE IOWA 1- AND PETERS, 1971) WERE EVALUATED.

Material and methods

BIOASSAYS OF BIOLOGICAL CONTROL AGENTS AGAINST **Diabrotica v. virgifera** *LARVAE*

THE VIRULENCE/EFFICACY OF 18 DIFFERENT FUNGAL STRAINS WERE CONSTANTLY BY EXPO
LARVAE TO DEFINED SPORE SUSPENSIONS OF BCAS. LARVAE WERE INFECTED BY EXPOSING
TO 30 THIRD-INSTAR LARVAE (*Zea mays*) IN THE SPORE SUSPENSIONS: EACH BATCH WA
TRANSFERRED TO A FILTER PAPER IN A 5-CM DIAMETER FUNNEL. FIFTY ML OF THE
SUSPENSION WERE GENTLY POURED OVER THE LARVAE. AFTER 5 S, THE SUSPENSION WAS
BY SUCTION. AFTER INOCULATION, FIVE TREATED LARVAE, EACH, WERE PLACED ON
INDIVIDUAL 10-DAY OLD CORN PLANT. ROOTS WERE PLACED ON A WET FILTER PAPER AN
PLASTIC FOIL BEFORE ROLLING. PLANTS WERE STORED UNDER HIGH HUMIDITY CONDITION
A PLASTIC BAG. CONTROL LARVAE WERE EXPOSED TO 0.1% (VOL/VOL) TWEEN 80 AND T
INOCULATED ONES. LARVAL DEVELOPMENT AT 25-28 °C WAS MONITORED FOR 10 DAYS.
DEAD INSECTS WERE RECORDED EVERY DAY. THE CAUSE OF DEATH WAS DETERMINE
DIFFERENTIATING BETWEEN LARVAE KILLED BY THE TESTED ENTOMOPATHOGENS AND D
OTHER CAUSES. DEAD LARVAE WERE TRANSFERRED TO SELECTIVE S4G AGAR MEDIUM
PROPORTION OF CADAVERS WITH RESULTING FUNGAL EMERGENCE AND SPORULATION.

BIOASSAY DATA WAS ANALYZED FOLLOWING THE PROBIT METHOD (FINNEY, 1971) BY USING
MATHEMATICA SOFTWARE 4.2. LT_{50}-VALUES WERE CALCULATED USING PROBIT TRANSFORMATI
PROPORTION OF INSECTS KILLED AND LOGARITHMIC TRANSFORMATION OF TIME.

FUNGAL DENSITIES IN SOIL AND EVALUATION OF DAMAGES ON MAIZE PLANTS

THE FUNGAL DENSITIES WERE ASSESSED BY DETERMINING THE NUMBER OF COLONY FORM
PER GRAM OF DRY SOIL ON A SELECTIVE MEDIUM (SZÉKÁCS *ET AL.*, 1998). THE NATURAL OCCURRENCE OF
THE ENTOMOPATHOGENIC FUNGI *Beauveria* AND *Metarhizium* WAS INITIALLY ASSESSED AT FIVE TRIA
SITES IN HUNGARY (ENYING, FONO, OZORA, GÖLLE AND BOYHAD) IN MAY 2005 AND MAY/JU
IN A SOIL DEPTH OF 0-10 CM AND 10.5-30 CM. TOTALLY TWELVE DIFFERENT *Beauveria*
SPP. AND *Metarhizium* SPP., E.G. AS GRANULES, WETTABLE POWDER OR IN COMBINATION
INSECTICIDE CARBOFURAN, WERE CARRIED OUT DURING SOWING IN 2005 AND 2006.

CFU MEASUREMENTS WERE ALSO CONDUCTED IN OCTOBER/NOVEMBER 2005 AS WELL
AUGUST AND NOVEMBER 2006. THE TRIAL SITES AT THE THREE LOCATIONS ENYING, FO
EXHIBITED HEAVY FEEDING DAMAGES THE YEAR BEFORE TREATMENTS. FEEDING DAMAGE
IN TREATED TRIAL SITES IN 2005 AND 2006 DEPENDING ON THE TIME OF HARVEST USING
SCALE (HILLS AND PETERS, 1971) AND COMPARED TO THE CONTROL FIELDS.

Results and discussion

BIOASSAYS OF BIOLOGICAL CONTROL AGENTS AGAINST **Diabrotica v. virgifera** *LARVAE*

FROM THE 18 DIFFERENT FUNGAL STRAINS TESTED, *Metarhizium anisopliae* (STRAIN V38E AND
BIPESCO 5) AND ONE *Beauveria bassiana* (STRAIN KVL 0433) WERE IDENTIFIED AS HIGH
PATHOGENIC AGAINST THIRD INSTAR LARVAE OF *Diabrotica v. virgifera*. PROBIT TRANSFORMATION GENERATE
LT_{50}-RATES OF 5.2 DAYS AND 4 DAYS FOR SPORE SUSPENSIONS OF 6×10^6 SPORES ML^{-1} OF *M. anisopliae*
BIPESCO 5 AND V38E, RESPECTIVELY.

SEVEN-DAY BIOASSAYS SHOWED LD_{50}-VALUES OF 1.76×10^5 AND 7.4×10^4 SPORES ML^{-1} FOR
M. anisopliae V38E AND BIPESCO 5, RESPECTIVELY. IT IS WORTH TO NOTICE THAT FROM *Diabrotica*
LARVAE RE-ISOLATED REUSED SPORES OF THE STRAIN V38E (V38E RI) EXHIBITED A HIGH
COMPARED TO THE PARENT STRAIN (FIGURE 1).

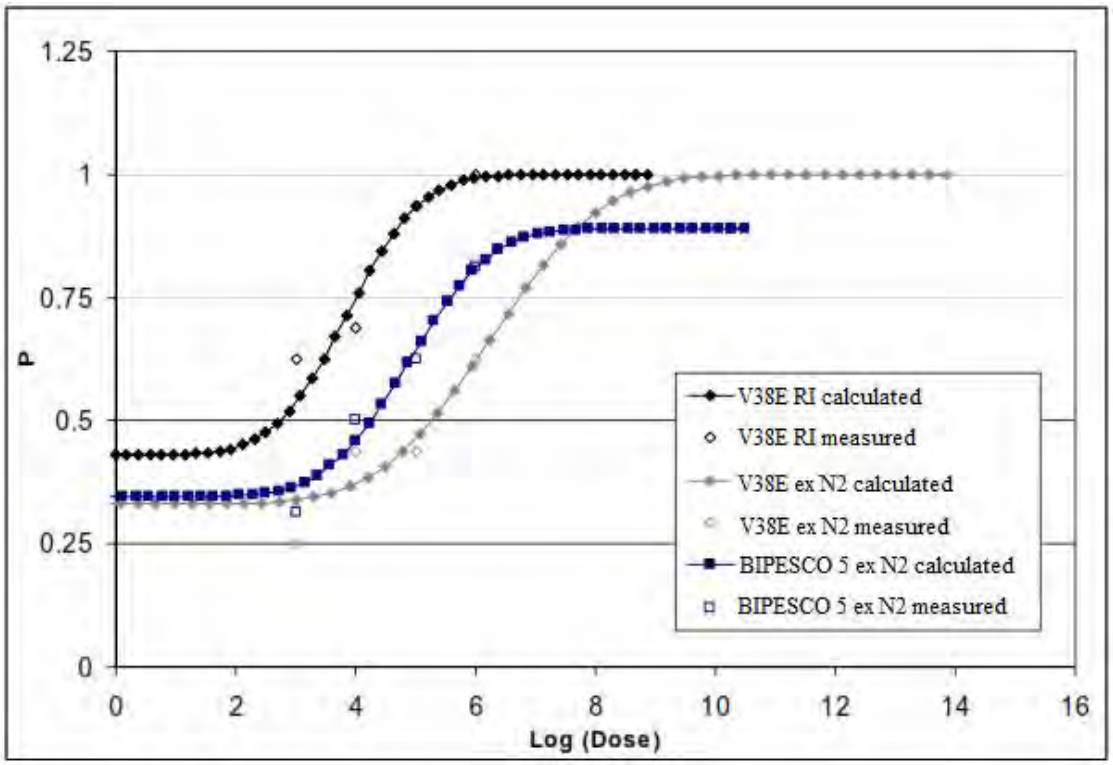

FIGURE 1: DOSE-RESPONSE-CURVES OF MEASURED AND WITH PROBIT CALCULATED DAT
BIOASSAYS ON 3RD INSTAR LARVAE OF *Diabrotica virgifera virgifera*. (EX N2 = PARENTAL STRAIN;
RI = FROM LARVAE RE-ISOLATED SPORES).

FUNGAL DENSITIES IN SOIL AND EVALUATION OF DAMAGES ON MAIZE PLANTS

EVALUATION OF NATURAL OCCURRENCE OF *Beauveria* SPP. REVEALED ONLY A LOW ABUNDANCE AT ALL
LOCATIONS. THE MAXIMAL FUNGUS DENSITY WAS MEASURED AT EXPERIMENTAL SITES IN
730 CFU PER GRAM DRY WEIGHT SOIL ($CFU\ g^{-1}\ DW$). *Metarhizium* SPP. DENSITIES WERE FOUND TO
BE HIGHER THAN 1000 $CFU\ g^{-1}\ DW$ UP TO A SOIL DEPTH OF 30 CM AT TRIAL SITES IN ENYING AND

IN SPITE OF THEIR FULLY VITALITY AND ABSENCE OF CONTAMINATION (PROVED IN QU
EXAMINATIONS), NEITHER *Beauveria* NOR *Metarhizium* WAS ABLE TO DECISIVELY ESTABLISH ITSELF
ANY OF THE EXPERIMENTAL SITES, REGARDLESS OF THE TREATMENT AND THEIR APPL
Metarhizium SPP. SHOWED A SLIGHT INCREASE OF DENSITIES (MAXIMAL INCREA
G^{-1} DW REACHED AT LOCATION ENYING), THE MINIMAL CONCENTRATION FOR A
SUSTAINABLE CONTROL OF *Diabrotica* LARVAE COULD, IF ANY, ONLY PARTIALLY REACHED.

ONLY IN ONE OF THREE CONTROL FIELDS THE DAMAGE RATE EXCEEDED THE ECON
LEVEL OF 3 ON THE IOWA SCALE (JOURNEY & OSTLIE, 2000). THUS, THE LARVAL FEEDING
CONTROL FIELDS CAN BE CONSIDERED TO BE LOW TO MODERATE, RESPECTIVELY, AND DI
DIFFER FROM THE TREATED SITES IN 2005. RELATIVE AMPLE PRECIPITATION DURING SU
MIGHT HAVE HELPED QUICKLY TO RESTORE ROOT LOSSES AND COULD BE A PLAUSIBLE E
GENERAL LOW LARVAL FEEDING DAMAGES OBSERVED.

PROSPECTS

ALTHOUGH THE RESULTS OBTAINED FROM THE ASSESSMENT OF FUNGAL DENSITIES I
CROPLANDS REVEALED QUITE DISPLEASED FUNGAL PERSISTENCES, BIOASSAYS HAVE
PATHOGENICITY OF *M. anisopliae* (STRAIN V38E AND BIPESCO 5) AND *B. bassiana* (STRAIN KVL
0433) AGAINST 3RD INSTAR LARVAE OF *D. v. virgifera*. THE FORMULATED GRANULAR PRODUC
GRANMET®, BASED ON BIPESCO 5 GROWN ON BARLEY KERNELS, IS ALREADY REGISTERED
FOR THE CONTROL OF THE GARDEN CHAFER *Phyllopertha horticola* (L.) AND COULD THUS POTENTIALLY BE
APPLIED IN THE FIELD AS SAFE BCA.

IN AUSTRIA, *D. v. virgifera* WAS FIRST DETECTED IN 2002 NEAR THE SLOVAKIAN BORDE
WHERE IT HAS CONTINUOUSLY SPREAD TO WESTERN PARTS OF THE COUNTRY. WELL E
POPULATIONS CAN CURRENTLY BE FOUND IN STYRIA, LOWER AUSTRIA AND BURGENLAND
STYRIA, FURTHER INVESTIGATIONS REGARDING THE GENERATION OF EFFICACY DATA OF
FUNGAL STRAINS (EPF) AND THE EVALUATION OF POSSIBLE SYNERGIES BETWEEN EPF
ENHANCING AGENTS (EEAS; I.E., SEMIOCHEMICALS, ENTOMOPATHOGENIC NEMATODES)
BEEN INITIATED IN THE FRAMEWORK OF THE EU FUNDED PROJECT INBIOSOIL (NO. 282767
SCHEDULED TO LAST THREE YEARS. THE FIELD TRIALS WILL BE COMPLEMENTED BY CON
TRIALS AND LABORATORY ASSAYS AIMING AT THE IMPROVEMENT OF THE APPLIED PRODU

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Insect pathogens and entomoparasitic nematodes
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Efficacy of biological control agents for the control of western corn rootworm

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Abstract: THE WESTERN CORN ROOTWORM, *Diabrotica virgifera virgifera* LECONTE, HAS BEEN INTRODUCED TO EUROPE MORE THAN 20 YEARS AGO, AND IT IS A WELL-ESTABLISHED MAIZE PEST 1995. THE LARVAE OF WCR CAUSE DAMAGE ON THE MAIZE ROOTS. THE EFFICACY OF VARIOUS BIOL AGENTS (BCAS), SUCH AS FERMENTED CULTURES OF VARIOUS ENTOMOPATHOGENIC TOXIN PRODUC OF *Bacillus thuringiensis*, AND SOME STRAINS OF THE ENTOMOPATHOGENIC FUNGUS *Metarhizium anisopliae*, WAS SCREENED AGAINST THE LARVAE OF WCR BUT THE PRACTICAL APPLICATION OF T ADDITIONAL RESEARCH AND DEVELOPMENT IS NEEDED. IN LABORATORY TESTS, WCR LARVAE WERE TREATED WITH MICROB PRODUCTS (FERMENTED CELL CULTURES OR SPORE SUSPENSIONS IN VARIOUS CONCENTRATIONS) A STAGE. LARVAE WERE FED WITH FRESHLY GERMINATED MAIZE ROOTS AND LARVAL MORTALIT PUPATION. IN GREENHOUSE EXPERIMENTS MAIZE PLANTS WERE GROWN IN POTS PLACED IN ISOLA (20 FOR EACH PLANT) WERE PUT DIRECTLY UNDER THE SEEDLINGS. IN THE FIELD EXPERIMENTS THE MICROBIAL PREPARATIONS WERE APPLIED AT THE TIME OF SOWING, IN THE SAME WAY AS THEY WERE APPL TRIALS. ONE MONTH AFTER THE PLANTING, THE ROOT MASS WAS MEASURED, AND THE DAMAG WAS DETERMINED BASED ON THE MODIFIED IOWA 1-6 SCALE. MOST OF THE BACTERIAL PREPARA STRAINS PROVED TO BE EFFECTIVE BOTH IN KILLING WCR LARVAE AND PREVENTING ROOT DAMA SOME MICROBIAL TREATMENTS ALMOST REACHED THE EFFICACY OF THE CONTROL TREATMENT 1.5 G) AND *Bacillus thuringiensis var. tenebrionis* (NOVODOR FC)) AND CAN BE CONSIDERED AS PROMISING CONTROL AGENTS OF WCR.

Key words: WESTERN CORN ROOTWORM, *Diabrotica*, ENTOMOPATHOGENIC FUNGI, MAIZE, *Metarhizium*

Introduction

THE WESTERN CORN ROOTWORM (WCR), *Diabrotica virgifera virgifera* LECONTE, (COLEOPTERA: CHRYSOMELIDAE) IS A WELL-ESTABLISHED MAIZE PEST IN HUNGARY. IT WAS FIRST DETE NEAR BELGRADE, SERBIA, IN 1992 (BERE, 1993). IN 1995 IT WAS FIRST RECORDED IN HUNGARY (PRINCZINGER, 1996) BUT THE MODELLING OF THE POPULATION DYNAMICS OF THE WCR SH COULD HAVE BEEN PRESENT WELL BEFORE THIS TIME (SZALAI *et al.*, 2011).

THE LARVAE OF WCR FEED ON THE MAIZE ROOTS CAUSING THE CHARACTERISTIC SYMPTOM AND SIGNIFICANT YIELD LOSS. THE ADULTS CAUSE DAMAGE ON ABOVE GROU FREQUENTLY ON THE GENERATIVE PARTS OF MAIZE, THUS REDUCING THE FERTILIZATI CHIANG, 1973).

ALTHOUGH VARIOUS CONTROL TOOLS (CROP ROTATION, CHEMICAL INSECTICIDE TR APPLIED BY FARMERS TO KEEP THE POPULATION BELOW ECONOMIC THRESHOLD LEVE CONSIDERED AS A MAJOR PROBLEM IN MAIZE PRODUCTION.

ENTOMOPATHOGENIC BACTERIAL AND FUNGAL SPECIES THRIVE IN THE SOILS IN HUNG *et al.*, 2007) AND THEY ARE PROMISING AGENTS FOR THE CONTROL OF WCR. THE ENTOMOPATH UNTIL NOW PROVED TO BE SUFFICIENTLY SELECTIVE, THEY IMPOSE NO RISK FOR NON-T AND THEY ARE CONSIDERED HARMLESS FOR HUMAN HEALTH. AMONG THESE ENTOMOPA

FUNGI (DEUTEROMYCOTA) ARE THE MOST PROMISING BECAUSE THEY HAVE WIDE HO
INOCULUM PRODUCTION AND FORMULATION IS COMPARATIVELY EASY (TURÓCZI, 2003).

Material and methods

IN VITRO TEST

WCR WAS BRED IN PETRI DISHES. TEN WCR EGGS WERE PUT IN THE PETRI DISH ON WET FI
COVERED BY 2 G STERILIZED SOIL AND INCUBATED AT 25 °C. THE HATCHED LARVAE WERE
GERMINATED CORN, PRESOAKED IN EDTA TO PREVENT THE GROWTH OF SAPROTROPHIC M

AT THE SECOND LARVAL STAGE THE LARVAE WERE TREATED WITH 2 ML OF THE MICR
WE APPLIED FERMENTED CELL CULTURES OF *B. thuringiensis var. tenebrionis*, (PATENT PENDING
STRAINS OF BIOFIL LTD.) IN TWO DIFFERENT CONCENTRATIONS (CONCENTRATION 10^8 CFU ML^{-1} AND 10^7 CFU ML^{-1}). ALSO,
SPOR SUSPENSIONS (ALSO OF 10^7 CFU ML^{-1}) OF 5 STRAINS OF *M. anisopliae* (MET-4, -16, -34, -43, -51)
WERE APPLIED. THROUGHOUT THE LARVAL DEVELOPMENT THE RATE OF MORTALITY WAS

GREENHOUSE EXPERIMENT

TWO MAIZE SEEDS WERE SOWN INTO POTS OF 15 CM DIAMETER AND THE POTS WERE GR
AND PLACED IN ISOLATORS. 20 WCR EGGS WERE PUT DIRECTLY UNDER EACH SEED.
M. anisopliae PREPARATIONS WERE APPLIED DIRECTLY ON THE SEEDS IN THE SAME DOSAGE
TRIALS. ALTOGETHER THERE WERE 21 TREATMENTS, EACH IN 6 REPLICATIONS. PHEROCON
TRAPS WERE PUT INTO THE UPPER PARTS OF THE ISOLATORS TO CAPTURE EMERGING ADULT

ONE MONTH FOLLOWING THE PLANTING, WE RECORDED THE PLANT HEIGHT, THE NU
THE ROOT MASS, THE NUMBER OF EMERGED ADULTS AND THE ROOT DAMAGE CAUSED BY L
LIMITED TERM OF EXPERIMENT (MAIZE IN THE ISOLATORS COULD REACH A MAXIMUM H
HALF A METER), THE MAXIMUM ROOT DAMAGE WAS MEASURED ON A MODIFIED IOWA 1-6
ALLOWS TO DETERMINE THE ROOT DAMAGE ALSO ON SMALL MAIZE PLANTS, SEE IN E
STANDARD, EPPO BULLETIN, 1999).

THE EFFICACY OF THE MICROBIAL TREATMENTS WAS COMPARED TO UNTREATED CO
TREATMENTS WITH INSECTICIDE TEFLUTHRIN (FORCE 1.5 G) AND *Bacillus thuringiensis var. tenebrionis* (NOVODOR FC).

Results and discussion

IN *in vitro* TEST, ALL OF THE MICROBIAL PREPARATIONS INCREASED THE MORTALITY OF V
RECORDED MORTALITY WAS LESS DEPENDENT ON THE CONCENTRATION OF THE APPLIED
VARIED SIGNIFICANTLY BETWEEN THE INDIVIDUAL MICROBIAL STRAINS.

IN *greenhouse* EXPERIMENTS THERE WAS NO DAMAGE IN CONTROL TREATMENT WITHOU
(CONTROL 0) AND THE MOST SERIOUS DAMAGE WAS RECORDED IN CONTROL TREATMENT
(CONTROL WCR). SOME OF THE MICROBIAL PREPARATIONS PROVED TO BE EFFECTIVE IN T
ROOT DAMAGE (FIGURE 1).

THE EFFICACY OF THE BACTERIAL PREPARATIONS AND *M. anisopliae* STRAINS WERE HIGHLY
VARIABLE, SOME OF THEM WERE SIGNIFICANTLY DIFFERENT FROM CONTROL TREATMENT
TREATMENTS ALMOST REACHED THE EFFICACY OF NOVODOR TREATMENT EITHER IN LA
IN THE REDUCTION OF ROOT DAMAGE.

THE EXAMINED *B. thuringiensis* PREPARATIONS AND *M. anisopliae* FUNGAL STRAINS ARE
PROMISING CONTROL AGENTS OF WCR. THEIR EFFICACY UNDER FIELD CONDITIONS WI
FURTHER EXPERIMENTS.

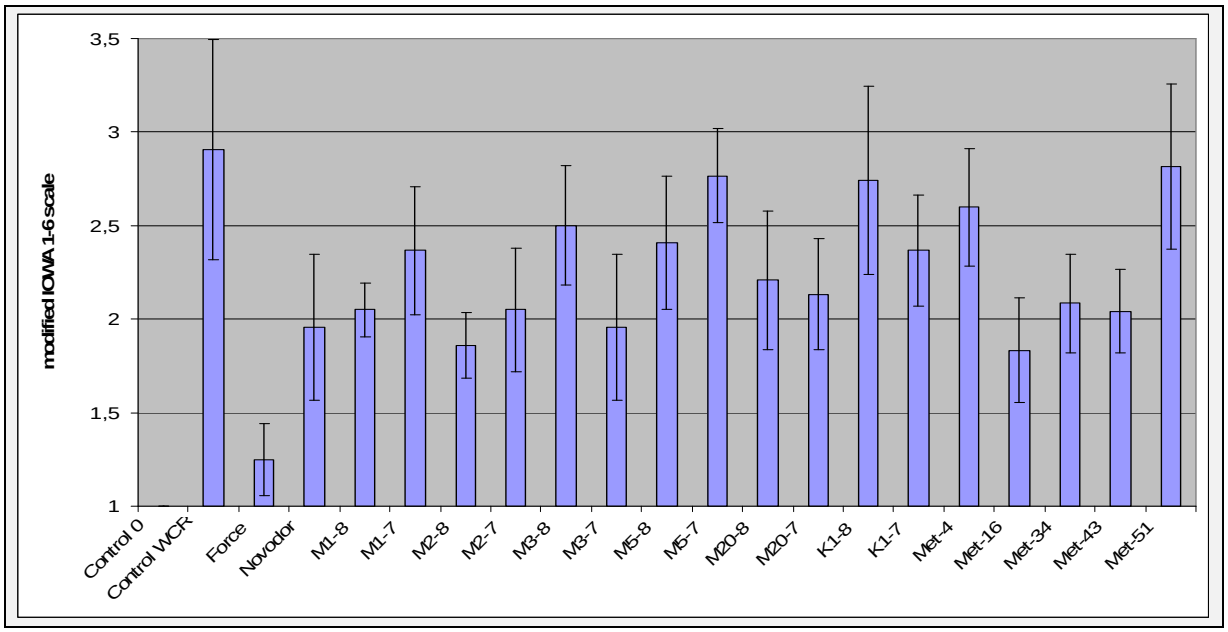

FIGURE 1. LARVAL DAMAGE ON MODIFIED IOWA 1-6 SCALE. BACTERIAL STRAINS (M AN
APPLIED AT CONCENTRATION OF 10 CFU ML (MARKED WITH 8 AND 7, RESPECTIVELY),
RESPECTIVELY. *Metarhizium* (MET) STRAINS WERE APPLIED AT A CONCENTRATION OF 10 CFU ML

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Exploring synergistic effects of semiochemicals, entomopathogenic fungi and nematodes against root-herbivores

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Abstract: ROOT-HERBIVORES MAY CAUSE IMPORTANT ECONOMIC YIELD LOSSES; FURT
SECONDARY STRESS FACTORS SUCH AS INCREASED WATER DEFICIENCIES OCCUR. SO FAR,
ABOUT THEIR ECOLOGY, ESPECIALLY WITH REGARD TO HOST FINDING STRATEGIES. SEVERAL
SHOWN THAT SEMIOCHEMICALS MAY SERVE AS HOST LOCATION CUES. RECENT STUDIES HAV
USE OF HOST SPECIFIC SIGNALS BY WESTERN CORN ROOTWORM LARVAE (*Diabrotica virgifera*
LECONTE) FOR LOCATING ITS HOST PLANT MAIZE (*Zea mays*). (TAKING INTO ACCOUNT THESE STUDIES AND THE
RECENTLY DISCOVERED SYNERGISTIC EFFECTS OF ENTOMOPATHOGENIC FUNGI AND NEMAT
POTENTIAL FOR REFINED BIOLOGICAL CONTROL STRATEGIES. FURTHERMORE, POLITICAL PR
REQUEST INNOVATIVE TECHNIQUES AND THE IMPLEMENTATION OF SUSTAINABLE STRATEGIES FO
ROOT-HERBIVORES, RESPECTIVELY.

WE AIM AT CONTROLLING LARVAE OF WESTERN CORN ROOTWORMS AND WIREWORMS (*Agriotes* SPP.)
IN MAIZE BY COMBINING SEMIOCHEMICALS, KNOWN AS COMPONENTS IN "ATTRACT" OR "CO
STRATEGIES, WITH *Metarhizium anisopliae* AND *Heterorhabditis bacteriophora* KNOWN AS KILL
COMPONENTS. THE CONCEPT USES BIOLOGICAL CONTROL AGENTS IN CO-FORMULATED C
PRESERVATION. FURTHERMORE, EITHER ATTRACTANT OR REPELLENT SEMIOCHEMICALS, W
TARGETED STRATEGY, ARE ADDED. WE FOCUS ON CO_2 AS ATTRACTIVE COMPONENT IN THE "ATTRACT
KILL" STRATEGY AND BOTANICALS AS THE REPELLENT COMPONENT IN THE "CONFUSE & KILL"
DATE, THREE DIFFERENT STRAINS OF *M. anisopliae* (BIPESCO5, ART2825 AND EAMA 01/58-SU) HAVE
BEEN TESTED IN BIOASSAYS AND IN THE GREENHOUSE. BIPESCO5 AND ART2825 SHOWED
PROMISING POTENTIAL, ALTHOUGH ALL STRAINS WERE NOT AS EFFICIENT AS A STANDAR
DIFFERENT CONIDIAL AND NEMATODE CONCENTRATIONS WILL BE TESTED IN THE FUTURE
MOST VIRULENT CONCENTRATIONS FOR SYNERGISTIC EFFECTS AND FOR CAPSULE FORMULATI

Key words: *Agriotes*, WIREWORM, *Diabrotica virgifera virgifera*, CONTROL STRATEGIES

Session 2:
Above-ground use of entomopathogenic fungi in protected and open field crops

Entomopathogenic fungi ecology and diversity from different Mediterranean ecosystems

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Abstract: THE OBJECTIVE OF THE PRESENT STUDY IS TO PROVIDE NEW INSIGHTS ON THE PRES DIVERSITY AND ECOLOGY OF ENTOMOPATHOGENIC FUNGI (EF) IN THE SOIL AND THE PHYLLOP EPHEMERAL (SUNFLOWER) AND PERMANENT (OLIVE AND HOLM OAK) MEDITERRANEAN AGR SYSTEMS WITH DIFFERENT MANAGEMENT STRATEGIES, ORGANIC OR CONVENTIONAL, D REFORESTATION. FOR THAT, SOIL AND PHYLLOPLANE SAMPLES FROM THE SAME GEOGRAPHICA POINT WERE GATHERED IN THE FOUR SEASONS AND IN THE FOUR CARDINAL DIRECTIONS USIN SAMPLING EFFORT. FROM 272 SOIL SAMPLES AND 840 PHYLLOPLANE SAMPLES, 693 EF ISOLAT OBTAINED. THEIR GENETIC DIVERSITY WERE CHARACTERISED BY THE MOLECULAR MARKER BA ELONGATION FACTOR 1-ALPHA (TEF1). NINE SPECIES WERE FOUND, INCLUDING THE GENERA *Beauveria*, *Metarhizium*, *Paecilomyces* AND *Purpureocillium*. *B. bassiana* WAS DETECTED MORE FREQUENTLY IN ALL ECOSYSTEMS AND EVEN IN THE PHYLLOPLANE (21.65% OF ISOLATES), WHEREAS *P. lilacinus* AND *P. marquandii* WERE RARELY DETECTED (0-0.29%, 0-0.43%, AND 0.14-0.43%, RESPECTIVELY). ALL ECOSYSTEMS SHOWED NO DIVERSITY OF EF ACCORDING TO SHANNON-WEAVE (H'), WHICH WAS LOWER THAN 1. LIKEWISE, THE FIVE ECOSYSTEMS PRESENTED A HIGHES DOMINANCE OF ONE SPECIES (*B. bassiana*) AS INDICATED BY SIMPSON DOMINANCE INDEX (D) AND PIELOU'S EVENNESS RATIO (J') VALUES LOWER THAN 1. FOR EACH AGROFORESTRY SYSTEM, DIFF BETWEEN HABITATS IN DIVERSITY OF EF WERE ALSO DETECTED WITH THE LOWER JACCARD'S I (J), AND THEREFORE HIGHER DIFFERENCES, OBSERVED FOR FUNGAL COMMUNITIES FROM PHYLLOPL SOIL.

IN GENERAL, THE PHYLLOPLANE HABITAT SHOWED MORE ISOLATES AND DIVERSITY THAI ECOSYSTEMS AND SPECIES. ECOSYSTEMS RANKING ACCORDING TO NUMBER OF FUNGAL ISOLATE HOLM OAK DEHESA > HOLM OAK REFORESTATION > ORGANIC OLIVE ORCHARD > TRADITIONAL OLIVE > SUNFLOWER PLANTATION, WHICH COULD INDICATE THAT THE HIGHER THE ECOSYSTEM MOD LOWER THE PRESENCE AND DIVERSITY OF EF.

Key words: PHYLLOPLANE, SOIL, DIVERSITY INDEX, *Beauveria*, *Metarhizium*, *Paecilomyces*, *Purpureocillium*

Efficacy of two strains of *BEAUVERIA BASSIANA* entomopathogenic fungus on the red palm weevil in France and in Spain

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Abstract: NPP/ARYSTA LIFESCIENCE, HELPED BY VEGETECH COMPANY, WORKS ON THE IMPLEMENTATION OF AN ALTERNATIVE BIOLOGICAL CONTROL METHOD AGAINST THE RED PA *Rhynchophorus ferrugineus*. SUCH A TOOL WOULD LIMIT THE ENVIRONMENTAL IMPACT OF TREATME A FIRST TRIAL IN OUTDOOR CAGES, SET UP AT THE END OF 2010 IN FRANCE, HAS SHOWN THE INTE TWO STRAINS OF THE ENTOMOPATHOGENIC FUNGUS *Beauveria bassiana*. DURING AUTUMN 2011 AND SPRING 2012, TWO NEW TRIALS WERE CARRIED OUT, IN SEMI-NATURAL CONDITIONS, IN FRANCE AN SPAIN, IN ORDER TO VALIDATE PREVIOUSLY OBTAINED RESULTS. THEY DEMONSTRATE THAT *Beauveria bassiana* STRAIN 147 (ACTIVE INGREDIENT OF BOTANIGARD®, ALREADY REGISTERED IN FRANCE FOR THE TREATMENT OF PALM TREES AGAINST *Paysandisia archon*, THE PALM BORER), IS AT LEAST AS EFFICIENT AS IMIDACLOPRID, THE CHEMICAL REFERENCE, AND THAT STRAIN NPP111B005 SHOWS AN INCREAS EFFICACY.

Key words: *Rhynchophorus ferrugineus*, PALM TREE, *Beauveria bassiana* STRAIN 147, *Beauveria bassiana* SOUCHE NPP111B005, BIOLOGICAL CONTROL

Insect pathogens and entomoparasitic nematodes
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BEAUVERIA BASSIANA strain ATCC 74040 interferes with oviposition behavior of Mediterranean fruit fly

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Abstract: THE ENTOMOPATHOGENIC FUNGUS *Beauveria bassiana* IS KNOWN TO INTERACT WITH INSECTS IN SEVERAL WAYS. THE PRESENT WORK REPORTS THE RESULTS OF OBSERVATIONS ON THE POTE *Beauveria bassiana* STRAIN ATCC 74040 AGAINST THE MEDITERRANEAN FRUIT FLY, *Ceratitis capitata*, WITH SPECIAL REGARD TO DISTURBANCE EFFECTS ON OVIPOSITION BEHAVIOUR. A COMMERCIAL FORMULAT DIFFERENT FUNGAL PREPARATIONS (PURE CONIDIA, HYPHAE, CULTURE SUPERNATANTS) WERE AP OFFERED TO OVIPOSITING MEDFLIES. A SIGNIFICANTLY LOWER NUMBER OF FLY VISITS AND OVI WERE RECORDED ON FRUITS TREATED WITH NATURALIS AND WITH PURE CONIDIA THAN ON OBSERVED EFFECTS ARE EXAMINED ON THE BASIS OF ADDITIONAL PROTEOMIC AND GENOMIC OB POTENTIAL MOLECULAR IMPLICATIONS OF THE RODLET LAYER OF AERIAL CONIDIA ARE DISCUSSE

Key words: *Ceratitis capitata*, OVIPOSITION, *Beauveria bassiana*, MICROBIAL CONTROL

Introduction

STUDIES WITH THE ENTOMOPATHOGENIC FUNGUS *Beauveria bassiana* CAN BE LISTED AMONG THE FIRST SIGNIFICANT EXPERIENCES WITH MICROBIAL CONTROL. SINCE THEN, THE INCREASING K ENTOMOPATHOGENIC SPECIES, INCLUDING ITS BIOLOGY, THE MECHANISM OF ACTION A TARGET INSECTS, AND THE IMPROVEMENT IN FORMULATION AND APPLICATION TECH COMMERCIALIZATION OF DIFFERENT PRODUCTS (WRAIGHT *et al.*, 2001). IN GENERAL, THE INSECTICIDAL ACTION RELATED TO CONIDIA GERMINATION AND HYPHAE PENETRATION INSIDE THE ASSOCIATED WITH VARIOUS MOLECULES INCLUDING DIFFERENT PROTEIN FAMILIES PROTEASES AND OTHER EXTRACELLULAR ENZYMES (ORTIZ-URQUIZA *et al.*, 2011). FURTHERMORE, RECENT INVESTIGATIONS BASED ON THE WHOLE GENOME SHOTGUN SEQUENCING OF *B. bassiana* STRAIN ARSEF 2860, EVIDENCED THE PRESENCE OF GENES ENCODING FOR BACTERIAL-LIKE TOXINS, S SHOWING SIMILARITIES TO *Bacillus thuringiensis* CRY TOXINS (XIAO *et al.*, 2012). HOWEVER, DIFFERENCES AMONG STRAINS IN TERMS OF INSECTICIDAL POTENTIAL HAVE BEEN DE DIFFERENT STRAINS MAY THEREFORE EXPRESS ENHANCED ACTION AGAINST SPECIFIC T *al.*, 2008). *Ceratitis capitata* WIEDEMANN (DIPTERA: TEPHRITIDAE), ALSO KNOWN AS MEDITERRANEAN FRUIT FLY, IS A MULTIVOLTINE AND POLYPHAGOUS PEST SPECIES AF HOST FRUITS. THE MANAGEMENT OF THIS PEST IS STILL MAINLY BASED ON REPEATED SYNTHETIC CHEMICALS. IN ORDER TO IMPLEMENT SUSTAINABLE AND INTEGRATED CR INTEGRATION OF THESE CONVENTIONAL CONTROL METHODS WITH BIOLOGICAL CONT HIGHLY DESIRABLE. PREVIOUS STUDIES (RUIU *et al.*, 2009) SHOWED THAT APPLICATIONS OF NATURALIS FRUITS COMPARED TO A BLANK (INERT CO-FORMULANTS OF THIS BIOINSECTICIDE) RESU REDUCTION OF OVIPOSITION PUNCTURES OF *C. capitata*.

IN THE STUDIES HEREIN REPORTED WE THUS DECIDED TO FURTHER INVESTIGATE INVOLVED IN THE DISTURBANCE EFFECTS OF *B. bassiana* STRAIN ATCC 74040 ON THE OVIPOSITION BEHAVIOUR OF *C. capitata*.

Material and methods

Fungal fractions

EXPERIMENTS WERE CONDUCTED WITH *B. bassiana* STRAIN ATCC 74040 WHICH WAS ISOLATED AND PURIFIED FROM THE COMMERCIAL FORMULATION BOTANIGARD® (CBC (EUROPE) S.R.L., NOVA MILANESE, ITALY). TO COLLECT PURE CONIDIA, THE MICROORGANISM WAS CULTURED ON SABOURAUD (SDA) PLATES AT 28 °C. CONIDIA WERE THEN COLLECTED BY SCRAPING FROM PLATES INTO SOLUTION FOLLOWED BY FILTRATION, WHEN NECESSARY. THE CONIDIA SUSPENSION PURITY UNDER A PHASE MICROSCOPE AND QUANTIFICATION WAS BASED ON THOMA CHAMBER. COLLECT HYPHAE AND CULTURE SUPERNATANTS 24-48 H AFTER CONIDIA GERMINATION, THE MICROORGANISM WERE GROWN ON SABOURAUD BROTH. THE EFFECTS OF THE FOR NATURALIS, OF PURE CONIDIA, HYPHAE AND OF CULTURE SUPERNATANTS ON THE OVIPOS *C. capitata* IN COMPARISON TO AN UNTREATED OR BLANK (CONTAINING ALL COMPO FORMULATION EXCEPT CONIDIA) CONTROL WAS THEN TESTED IN NO-CHOICE TESTS.

Insects and no-choice tests

C. capitata FEMALES WERE PROVIDED BY THE INSECT REARING FACILITY OF THE UNIVERS (SASSARI, ITALY). AFTER EMERGENCE, FEMALES WERE KEPT FOR 5 DAYS IN MATING CAGES WITH MALES, AND ALLOWED TO MATE AND TO GROW UP GONADS. THEN, GROUPS OF 5 EACH WERE TRANSFERRED INSIDE PLEXIGLAS CAGES (30X30X30 CM) WITH TWO LATERA WITH GAUZE TO ALLOW VENTILATION. IN DIFFERENT EXPERIMENTS, EITHER A TREATED F UNTREATED (CONTROL) FRUIT WAS OFFERED TO FEMALES IN EACH CAGE FOR OVIPOSITI TREATMENT). TO ESTIMATE THE NUMBER OF FEMALE VISITS/FRUIT, FRUITS WERE OBSERV HOUR, AND THE NUMBER OF FEMALES LANDING ON FRUITS WAS RECORDED. AFTER 4 REMOVED FROM THE CAGES, AND THE NUMBER OF OVIPOSITION PUNCTURES PER FRUIT W DIFFERENT FUNGAL FRACTIONS WERE APPLIED USING A SPRAY VOLUME OF 10 ML PER F SUFFICIENT TO ENSURE THOROUGH COVERAGE OF FRUITS. IN THE CASE OF NATURALIS A PRODUCT WAS APPLIED AT A CONCENTRATION OF 1.5 G L^{-1} FOLLOWING LABEL RECOMMENDATIONS. TH CULTURE SUPERNATANTS WERE APPLIED *ad libitum*, WHILE PURE CONIDIA WERE APPLIED AT A CONCENTRATION OF 10^5 CONIDIA ML^{-1}. HYPHAE WERE QUANTIFIED WITH OPTICAL MEASURES AND A AT A CONCENTRATION COMPARABLE TO CONIDIA BIOMASS. THE NUMBERS OF FEMALE OVIPOSITION PUNCTURES/FRUIT WERE COMPARED ACROSS TREATMENTS USING 1-WAY A BY LSD TEST FOR POST-HOC COMPARISONS OF MEANS.

Molecular studies

GENOMIC AND PROTEOMIC APPROACHES WERE FOLLOWED TO INVESTIGATE THE PROTE DIFFERENT FUNGAL FRACTIONS TESTED IN THE NO CHOICE TESTS. THE WHOLE PROTE DIFFERENT FRACTIONS WAS RESOLVED BY MONO- AND BI-DIMENSIONAL ELECTROPHORE FOLLOWED BY PEPTIDE MASS FINGERPRINTING USING TRYPSIN DIGESTION AND MALDI MASS SPECTROMETRY FOR MAIN COMPONENTS. FUNGAL DNA WAS EXTRACTED AND USED FOR T SEQUENCING OF GENES POSSIBLY CONNECTED WITH THE INSECTICIDAL MODE OF ACTIO PROTEINASES) AND THE INTERACTION WITH INSECTS (I.E. HYDROPHOBINS) (KUMAR *et al.*, 2010).

Results and discussion

BOTH THE NUMBER OF FEMALE VISITS/FRUIT AND THE NUMBER OF OVIPOSITION PUNCT SIGNIFICANTLY LOWER ON FRUITS TREATED WITH NATURALIS CONIDIA THAN ON BLANK C UNTREATED CONTROL FRUITS, RESPECTIVELY. ON FRUITS TREATED WITH HYPHAE AND CU

INSTEAD, NO SIGNIFICANT REDUCTION IN THE NUMBER OF VISITS/FRUIT AND IN THE NUM
PUNCTURES/FRUIT IN COMPARISON TO THE CONTROL WAS RECORDED (TABLE 1).

IN THE PROTEOMIC AND GENOMIC ANALYSIS OF *B. bassiana* STRAIN ATCC 74040, DIFFERENT
MOLECULES INVOLVED IN THE INTERACTION BETWEEN INSECTS AND THE FUNGUS WE
AMONG THESE A CHITINASE (CHIT1 HOMOLOGOUS), A CUTICLE-DEGRADING PROTE
HOMOLOGOUS) (KUMAR *et al.*, 2011) AND TWO HYDROPHOBINS (HYD1 AND HYD2 HOMOLOG
FORMING A RODLET LAYER, CONFERRING AERIAL CONIDIA HYDROPHOBIC FEATURES (BID

TABLE 1. MEAN NUMBER (M ± SE) OF FEMALE VISITS/FRUIT AND OF OVIPOSITION PUN
RECORDED IN THE DIFFERENT TREATMENTS.*

Treatment	Number of visits/fruit	Oviposition punctures/fruit
Experiment group 1		
CONTROL (BLANK)	17.4 ± 1.4 A	8.4 ± 0.5 A
NATURALIS®	5.0 ± 1.1 B	1.2 ± 0.4 B
Experiment group 2		
CONTROL (UNTREATED)	18.6 ± 1.4 A	10.4 ± 1.1 A
PURE CONIDIA (10^5 CONIDIA ML^{-1})	5.6 ± 0.6 B	3.4 ± 0.8 B
HYPHAE	18.4 ± 1.5 A	8.6 ± 1.0 A
CULTURE SUPERNATANTS	20.6 ± 1.8 A	10.6 ±1.5 A

*FOR EACH EXPERIMENTAL GROUP, MEANS IN THE SAME COLUMN FOLLOWED BY DIFFERENT LETTERS ARE SIGNIFICA
(LSD TEST, $P < 0.05$).

OVIPOSITION DETERRENT EFFECTS WERE OBSERVED FOR THE COMMERCIAL PRODUC
FOR PURE CONIDIA SUSPENSIONS, WHILE NO SUCH EFFECTS EMERGED FOR THE OTHER F
TESTED (HYPHAE AND CULTURE SUPERNATANTS). CONIDIA WERE THUS IDENTIFIED AS
RESPONSIBLE FOR THE OBSERVED EFFECTS. HOWEVER, THESE INHIBITORY EFFECTS WE
WHEN CONIDIA WERE APPLIED AT A CONCENTRATION EQUAL TO OR HIGHER THAN 10^5 CONIDIA ML^{-1} (DATA NOT
REPORTED). FURTHERMORE, ON FRUITS TREATED WITH PREPARATIONS OBTAINED BY
2-DAY-OLD CULTURES IN SABAURAUD BROTH, THUS IN THE IMMEDIATE POST-GERMIN
CONIDIA, NO SIGNIFICANT INHIBITING EFFECTS WERE DETECTED (DATA NOT REPORTED)
THE EFFECT OF THE CONIDIA ON MEDFLY OVIPOSITION BEHAVIOUR WAS OBSERVED IM
TREATMENT APPLICATION AND FOR THE FOLLOWING 48 H, AND CONSIDERING THAT CON
DAYS TO GERMINATE, ALL FURTHER INVESTIGATIONS WERE FOCUSED ON THE MAIN
STRUCTURE OF INTACT CONIDIA.

GIVEN THE RESULTS OF OUR STUDIES, WE ASSUMED THAT THE PHYSICAL AND BIOCHE
OF CONIDIA, IN PARTICULAR THE HYDROPHOBIC LAYER OF CONIDIA ON THE FRUIT SURFA
ABILITY OF MEDFLIES TO DETECT ORANGE-DERIVED STIMULI, SUCH AS ORANGE ODOURS
CONTENT (LEVINSON *et al.*, 2003), KNOWN TO AFFECT OVIPOSITION. ACCORDING TO THIS ASSU
THE HYDROPHOBINS OF THE EXTERNAL CONIDIA RODLET LAYER MAY BE OF PRIMAR
INHIBITING MEDFLY OVIPOSITION (BALOCH *et al.*, 1995). THE IMPLICATION OF HYDROPHOBINS IN THE
FUNGAL BIOCONTROL POTENTIAL HAS ALREADY BEEN SUGGESTED BY WANG AND LEGER (2007). IT HAS BEEN
SHOWN THAT INSECTS CAN BE REPELLED BY HYDROPHOBIC PARTICLE FILM BARRIERS (I
COATED PLANTS BECOME VISUALLY OR TACTUALLY UNRECOGNIZABLE AS A HOST AND I
BEHAVIOR CAN BE AFFECTED BY THE ATTACHMENT OF PARTICLES TO THE BODY (GLE *et al.*, 1999). IN

LINE WITH THIS, *B. bassiana* CONIDIA MIGHT WORK IN A SIMILAR WAY. HOWEVER, IN ADDITIO
BARRIER-EFFECT DETERMINED BY CONIDIA ON FRUITS, WE CANNOT EXCLUDE THAT PA
EFFECTS COULD BE DUE TO VOLATILE ORGANIC COMPOUNDS RELEASED BY THE FUNGUS
EFFECT ON *C. capitata* (CRESPO *et al.*, 2008).

AT PRESENT, FURTHER INVESTIGATIONS ARE BEING CONDUCTED TO CLARIFY THE
CONIDIA SURFACE COMPOUNDS. THESE STUDIES WOULD FURTHER SUPPORT THE POTENT *B. bassiana*
STRAIN ATCC 74040 IN PROTECTING FRUITS IN INTEGRATED MEDFLY MANAGEMENT PROGR

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Insect pathogens and entomoparasitic nematodes
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Pathogenicity of an indigenous strain of the entomopathogenic fungus *Beauveria bassiana* on larvae and adults of the sisal weevil, *Scyphophorus acupunctatus* Gyllenhal (Coleoptera: Curculionidae)

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Abstract: THE SISAL WEEVIL IS A SEVERE PEST OF BOTH ORNAMENTAL AND CULTIVATED AGAVE SP
OFSYNTHETIC INSECTICIDES CAUSES UNDESIRABLE EFFECTS, THE EVALUATION OF POTENTIAL
AGENTS IS NECESSARY. FIELD COLLECTED ADULTS AND LARVAE OF *Scyphophorus acupunctatus* WERE USED TO
EVALUATE THE PATHOGENICITY OF AN INDIGENOUS STRAIN OF THE *Beauveria bassiana*
(BALSAMO) VUILLEMIN (ASCOMYCOTA: HYPOCREALES). DIFFERENT CONCENTRATIONS OF SPORE S
TESTED. AS IN SOME CASES 100% MORTALITY WAS ACHIEVED IT IS INDICATING THAT THIS STRAI
POTENTIAL BIOLOGICAL CONTROL AGENT OF THE SISAL WEEVIL.

Key words: *Beauveria bassiana*, *Scyphophorus acupunctatus*, INDIGENOUS STRAIN, BIOLOGICAL CONTRO
AGENT, ENTOMOPATHOGENIC FUNGI

Introduction

THESISAL WEEVIL *Scyphophorus acupunctatus* GYLLENHAL IS ONE OF THE MOST IMPORTANT PEST
AGAVE SPECIES, WHICH ATTACKS BOTH CULTIVATED AND ORNAMENTAL PLANTS (GONZA
et al., 2011). IN ADDITION, OTHER ORNAMENTAL PLANTS (*Beaucarnea recurvata, Dasylirion longissimum, Dracaena draco*, *Furcraea foetida*, *Yucca* SPP. *Polianthes tuberosa*) HAVE BEEN REPORTED AS HOSTS OF THE SISAL WEEVIL (KONTODIMAS & KALLINIKOU, 2010). SUPPRESSION OF THE W
USE OF SYNTHETIC INSECTICIDES ON ORNAMENTAL PLANTS AS WELL AS IN CULTIVATE
SPIRIT (TEQUILA) PRODUCTION POSE A THREAT BOTH TO HUMAN HEALTH AND THE ENV
EFFECTIVE ALTERNATIVE CONTROL METHODS SUCH AS THE USE OF BIOINSECTICIDES OR T
OF CLASSICAL OR CONSERVATION BIOLOGICAL CONTROL ARE DESIRABLE FOR REPL
METHODS OF PEST MANAGEMENT.

THE WEEVIL LIVES AND DEVELOPS IN A PROTECTED HABITAT, ON THE BUTTON OF THE
THE PLANT'S HEAD (LOCK, 1962), WHERE IT IS PROTECTED BY ITS LIMITED NUMBER OF E
PARASITOIDS (VELÁZQUEZ *et al.*, 2006). THEREFORE, THE USE OF ENTOMOPATHOGENIC FUNGI THAT
THE ABILITY TO INFEST INDIVIDUALS INTO THEIR PROTECTED HABITAT COULD SERV
BIOLOGICAL CONTROL AGENTS AGAINST THE SISAL WEEVIL. IT HAS BEEN SHOWN THAT
EVEN DIFFERENT STRAINS WITHIN THE SAME SPECIES MAY EXHIBIT DIFFERENT BEHAVIOU
RANGE, PATHOGENICITY AND TEMPERATURE OPTIMUM LEVELS FOR DEVELOPMENT (SHAH

IN THE PRESENT STUDY AN INDIGENOUS STRAIN OF *Beauveria bassiana* (BALSAMO) VUILLEMIN
FROM GREECE OBTAINED FROM A NATURALLY INFECTED ADULT OF *Rhynchophorus ferrugineus* OLIVIER
(COLEOPTERA: CURCULIONIDAE) WAS EVALUATED FOR ITS PATHOGENICITY TO *R. ferrugineus* AND
S. acupunctatus BOTH BELONG TO THE SAME TRIBE (RHYNCHOPHORINI). THE FUNGUS WAS A
THREE DIFFERENT CONCENTRATIONS AND MORTALITY CAUSED TO DIFFERENT DEVELOP
INSECT WAS RECORDED.

Material and methods

INSECTS

INSECTS WERE COLLECTED FROM INFESTED ORNAMENTAL PLANTS OF *Agave americana*, LOCATED IN ARDITTOS HILL IN ATHENS (37° 58' 06", 23° 44' 18"). ADULTS WERE COLLECTED BY HAND WH INFESTED PLANTS WERE REMOVED AND TAKEN TO THE LABORATORY FOR THE ISOLATION ADULTS AND LARVAE WERE PLACED IN POLYESTER CAGES AND KEPT UNDER LABORAT 25 ± 1 °C, 50-65% RELATIVE HUMIDITY (R.H.) AND 12 H LIGHT:12 H DARK PHOTOPERIOD. W WERE PROVIDED WITH APPLE SLICES UNTIL USED IN TRIALS.

FUNGAL ISOLATES

THE *B. bassiana* STRAIN USED WAS OBTAINED FROM THE ENTOMOPATHOGENIC FUNGI CO BENAKI PHYTOPATHOLOGICAL INSTITUTE (ATTICA, GREECE). THE INITIAL STRAIN WAS NATURALLY INFECTED *R. ferrugineus* CADAVER FOUND IN ELLINIKON REGION (ATTICA, 37°53'15 23°43'42"E). AQUEOUS CONIDIAL SUSPENSIONS WERE PREPARED BY SCRAPING THE SURFACE OLD CULTURES, GROWN ON SABOURAUD DEXTROSE AGAR AT 25 °C IN DARK, INTO AQUEOU 0.2% TWEEN 80. CONIDIA CONCENTRATIONS WERE COUNTED WITH THE USE OF AN HAEMOC

BIOASSAYS

INFECTION OF ADULTS AND LARVAE WAS ACHIEVED BY CONTACT WITH THE INSECT TREATMENT WAS REPLICATED THREE TIMES, WITH EACH REPLICATE CONSISTING O INDIVIDUALS. APPLICATION WAS ACCOMPLISHED BY IMMERSING INDIVIDUALS IN GRO AQUEOUS CONIDIAL SUSPENSIONS FOR 60 S. ALL TREATMENTS CONTAINED A CONTROL GRO SOLUTIONS CONTAINING 0.2% TWEEN 80 WERE USED. CONCENTRATIONS OF 4×10^{7}, 4×10^{6} AND 4×10^{5} CONIDIA mL^{-1} WERE TESTED. MORTALITY WAS RECORDED DAILY FOR UP TO 11 AND 21 D FO ADULTS RESPECTIVELY. ADDITIONALLY, CADAVERS WERE KEPT INDIVIDUALLY IN STERILE WITH MOISTENED FILTER PAPER, IN A DARK ENVIRONMENT AT 25 °C AND CHECKED FOR F FUNGAL INFECTION.

STATISTICAL ANALYSIS

DATA ON PERCENTAGE MORTALITY WERE ARCSIN TRANSFORMED TO HOMOGENISE VAR ANOVA REQUIREMENTS. TRANSFORMED DATA WERE ANALYZED WITH ONE WAY ANOVA SPSS INC., CHICAGO, IL).

Results

MORTALITY OF ADULTS

ADULT WEEVILS EXHIBITED 100% MORTALITY IN THE HIGH CONCENTRATION TREATMI DIFFERED SIGNIFICANTLY ($F = 32.49$; $df = 3,8$; $P = 0.001$) BETWEEN CONCENTRATIONS OF 4×10^{6} AND 4×10^{7} CONIDIA mL^{-1}, WHILE CONTROL GROUP EXHIBITED ZERO MORTALITY (TABLE 1).

MORTALITY OF LARVAE

ALL TREATMENTS IN ALL CONCENTRATION LEVELS CAUSED HIGH MORTALITY TO LARVA *Sitophilus*, REACHING UP TO 100%. LOW LEVELS OF MORTALITY WERE OBSERVED IN CONTROL TREAT NOT EXCEED 6.7 ± 0.06%, DIFFERING SIGNIFICANTLY FROM THE TREATMENTS ($F = 41.41$, $df = 3,8$; $P = 0.001$) (TABLE 1).

MYCELIUM DEVELOPMENT

ALL ISOLATED CADAVERS PREVIOUSLY TREATED WITH THE FUNGUS DEVELOPED VISIBLE M
SURFACE WITHIN A WEEK. THE EXAMINATION OF THE CONIDIA UNDER MICROSCOPE AS
INVOLVEMENT OF THE ENTOMOPATHOGENIC FUNGUS *B. bassiana* IN THEIR DEATH. CADAVERS OF LARVAE
AND ADULTS DERIVING FROM CONTROL GROUPS DID NOT DEVELOP MYCELIUM OF *B. bassiana* OR ANY
OTHER ENTOMOPATHOGENIC FUNGUS.

TABLE 1. AVERAGE MORTALITY (%) (± STANDARD ERROR)[1] OF *S. acupunctatus* ADULTS AND LARVAE AFTER
AND 11 DAYS, RESPECTIVELY.

DEVELOPMENTAL STAGE OF *S. acupunctatus*	CONIDIA CONCENTATION			CONTROL
	4×10^7	2×10^7	4×10^6	
ADULTS	100% A	86.6 ± 0.21% AB	66.7 ± 0.09% B	0% C
LARVAE	100% A	100% A	93.3 ± 0.21% A	6.7 ± 0.06% B

[1] MEANS WITHIN ROWS FOLLOWED BY THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT (TUKEY-B, $\alpha = 0.05$).

Discussion

RESULTS INDICATE THAT THIS INDIGENOUS STRAIN OF *B. bassiana* HAS A HIGH VIRULENCE ON BOTH ADULTS
AND LARVAE OF *S. acupunctatus*. HENCE, IT COULD SERVE AS A POTENTIAL BIOLOGICAL CONTROL
IS OF HIGH IMPORTANCE THAT THE PEST ATTACKS THE PLANT FROM ITS BASE AND THAT I
MAINLY UNDER THE GROUND SURFACE HAVING DIRECT CONTACT WITH THE SOIL. *B. bassiana* IS A SOIL
BORNE FUNGUS, SO ENHANCEMENT OF THE GROUND WITH ITS CONIDIA COULD CONSTITUTE
APPROACH AGAINST THE SISAL WEEVIL. FURTHER RESEARCH IS REQUIRED FOR THE FIELD
PATHOGENIC CAPACITY OF THE CERTAIN STRAIN.

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Insect pathogens and entomoparasitic nematodes
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Microbial control of European red spider mite (*PANONYCHUS ULMI*) with *BEAUVERIA BASSIANA* strain ATCC 74040

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Abstract: THE EUROPEAN RED SPIDER MITE, *Panonychus ulmi*, CAN CAUSE SEVERE DAMAGE ON MANY FRUIT CROPS, ESPECIALLY ON APPLE. OUTBREAKS OF MITE POPULATIONS USUALLY OCCUR IN SUMME HUMID DAYS. NATURAL OCCURRING PREDATOR POPULATIONS MAY NOT ALWAYS BE ABLE TO K CONTROL, ESPECIALLY BECAUSE OF THE LIKELY OCCURRENCE OF A LAG IN TIME IN BUILD-UP OF POPULATIONS AND DUE TO THE USE OF NON-SELECTIVE CHEMICAL PESTICIDES. THE EFFICACY CONTROL AGENT *Beauveria bassiana* STRAIN ATCC 74040 (NATURALIS®) AGAINST *P. ulmi* WAS TESTED IN OPEN TRIALS ON APPLE. IN ONE OF THE TRIALS, ALSO OBSERVATIONS ON THE POTENTIAL SIDE EFFEC PRODUCT ON NATURAL OCCURRING PREDATOR POPULATIONS (*Phytoseiulus persimilis* AND *Stethorus punctillum*) WERE MADE. THE MICROBIAL CONTROL AGENT SHOWED HIGH EFFICACY AGAINST *P. ulmi* IN ALL TRIALS, AND DID NOT ADVERSELY AFFECT PREDATOR POPULATIONS. *Beauveria bassiana* STRAIN ATCC 74040 CAN BE CONSIDERED A VALUABLE TOOL TO BE INTEGRATED INTO *P. ulmi* CONTROL STRATEGIES.

Key words: *Panonychus ulmi*, *Beauveria bassiana* STRAIN ATCC 74040, MICROBIAL CONTROL, SELECTIVITY

Introduction

THE EUROPEAN RED SPIDER MITE, *Panonychus ulmi* (KOCH), IS CONSIDERED A SECONDARY PEST IN FRUIT CROPS. CONDITIONS FAVOURING *P. ulmi* OUTBREAKS ARE WARM AND HUMID SUMMER DAYS AN EXCESSIVE USE OF FERTILIZERS (CUTHBERTSON & MURCHIE, 2005). THE MITE IS USUALLY N IN ORCHARDS, WHERE PREDATOR POPULATIONS ARE WELL ESTABLISHED, BUT INJURY MA IMPORTANCE IN COMMERCIAL ORCHARDS DUE TO THE EFFECTS OF NON-SELECTIVE CHEM BENEFICIALS (COSTA-COMELLES *et al.*, 1990). HOWEVER, WHEN NO CHEMICAL SPRAYS ARE APPLIE NATURAL OCCURRING PREDATOR POPULATIONS ALONE MAY NOT BE ABLE TO KEEP THE BECAUSE OF THE LIKELY OCCURRENCE OF A LAG IN TIME IN BUILD-UP OF PREY AND PRED (CUTHBERTSON & MURCHIE, 2005). AN INTEGRATED CONTROL PROGRAMME BASED O ACARICIDES WHICH ARE SAFE TO PREDATORS IS THEREFORE DESIRABLE.

MICROBIAL CONTROL AGENTS, SUCH AS STRAINS OF THE ENTOMOPATHOGENIC FU *Beauveria bassiana* (BALSAMO) VUILLEMIN, CAN BE CONSIDERED INTERESTING CANDIDATES TO BE *P. ulmi* CONTROL STRATEGIES. THIS MICROBIAL CONTROL AGENT ACTS PRIMARILY BY ATTACHED TO THE HOST'S CUTICLE, THE CONIDIOSPORES GERMINATE PRODUCING PE WHICH ENTER AND PROLIFERATE INSIDE ITS BODY. THE PROLIFERATION OF THE FUNGU LEADS TO ITS DEATH. HOWEVER, PATHOGENICITY TOWARDS ARTHROPODS AND THUS TOWARDS BENEFICIALS IS VARIABLE AMONG DIFFERENT STRAINS (DE *et al.*, 1998). THE FUNGUS (MON OUR STUDIES WE INVESTIGATED THE *Beauveria bassiana* STRAIN ATCC 74040, KNOWN TO EFFECTIVELY CONTROL THE TETRANYCHID MITE *Tetranychus urticae* (CHANDLER *et al.*, 2005; DUSO *et al.*, 2008), AND TO SHOW LITTLE OR NO ADVERSE EFFECTS ON SEVERAL BENEFICIALS (DU SIMON *et al.*, 2010; LADURNER *et al.*, 2012). THE STRAIN HAS BEEN INCLUDED INTO THE EU LIST APPROVED ACTIVE SUBSTANCES (REGULATION EU 540/2011) IN 2009. THE FORMULATED PR

IN OUR STUDIES WAS NATURALIS® (BIOCAL (EUROPE) SRL – BIOGARD DIVISION, ITALY), AN OIL DISPERSION (OD) CONTAINING AT LEAST 2.3 × 10[7] VIABLE SPORES/ML OF *B. bassiana* STRAIN ATCC 74040.

THE RESULTS OF THREE FIELD TRIALS EVALUATING THE EFFICACY OF NATURALIS® AGAINST THE EUROPEAN RED SPIDER MITE ON APPLE ARE REPORTED. IN ADDITION, IN ONE OF THE TRIALS, OBSERVATIONS ON EFFECTS OF THE PRODUCT ON NATURAL OCCURRING PREDATOR POPULATIONS WERE CONDUCTED.

Material and methods

IN 2011-2012, THREE EFFICACY TRIALS WERE CARRIED OUT ON APPLE (*Malus domestica* BORKH.) IN COMPLIANCE WITH EPPO GUIDELINES AND PRINCIPLES OF GOOD EXPERIMENTAL PRACTICE. ONE TRIAL (TRIAL N. 1) WAS CONDUCTED IN SERBIA IN 2011 (45°09'N, 20°11'E), AND TWO OTHERS (TRIAL N. 2 IN 2011 AND TRIAL N. 3 IN 2012) WERE CONDUCTED IN ITALY (44°52'N, 11°40'E). IN ALL TRIALS, THE EFFICACY OF NATURALIS® AGAINST *P. ulmi* WAS COMPARED TO THAT OF A CHEMICAL REFERENCE TREATMENT AND AN UNTREATED CONTROL (TABLE 1). TO COMPARE THE DIFFERENT TREATMENTS A RANDOMIZED COMPLETE BLOCK DESIGN WITH 3 (TRIAL N. 1) OR 4 (TRIALS N. 2 AND N. 3) REPLICATES PER TREATMENT WAS USED (PLOT SIZE: 5 TREES), RESPECTIVELY. IN ALL TRIALS, TREATMENTS WERE APPLIED USING A SPRAY VOLUME OF 1000 L/HA. THE 1[ST] TREATMENT APPLICATION WAS CONDUCTED WHEN THE TARGET MITE WAS ALREADY PRESENT ON THE CROP.

TABLE 1. TESTED PRODUCTS, APPLICATION RATES AND TIMING OF APPLICATIONS IN THE TRIALS

N.	Active substance	Formulated product (conc. a.s.)	Applied rate	Timing (dd/mm)
Trial n. 1 (Serbia 2011)				
1	*Bb* ATCC 74040	NATURALIS (2.3 × 10[7] SPORES/ML)	1.5 L/HA	28/06, 04/07
2	ABAMECTIN	KRAFT 1.8 EW (18 G/L)	1.0 L/HA	28/06, 04/07
3	UNTREATED CONTROL			
Trial n. 2 (Italy 2011)				
1	*Bb* ATCC 74040	NATURALIS (2.3 × 10[7] SPORES/ML)	1.25 L/HA	03/08, 08/08
2	FENAZAQUIN	PRIDE 200 SC (13.8%)	0.75 L/HA	03/08
3	UNTREATED CONTROL			
Trial n. 3 (Italy 2012)				
1	*Bb* ATCC 74040	NATURALIS (2.3 × 10[7] SPORES/ML)	1.5 L/HA	17/07, 20/07
2	TEBUFENPYRAD	MASAI 20 WP (20.0%)	0.6 KG/HA	17/07
3	UNTREATED CONTROL			

IN ALL TRIALS, THE NUMBER OF LIVE MOBILE *P. ulmi* WAS COUNTED ON 25 RANDOMLY SELECTED LEAVES PER PLOT. THE 1[ST] (PRELIMINARY) ASSESSMENT WAS CONDUCTED JUST BEFORE 2[ND] APPLICATION, WHILE THE FINAL ASSESSMENT WAS CONDUCTED 7-10 D AFTER THE LAST APPLICATION (14 IN TRIAL N. 1, 9 IN TRIAL N. 2, AND 7 IN TRIAL N. 3), WHEN THE TARGET MITE POPULATION HAD REACHED A PEAK IN THE UNTREATED CONTROL. IN ADDITION, AN INTERMEDIATE ASSESSMENT WAS CONDUCTED JUST BEFORE THE 2[ND] APPLICATION IN TRIAL N. 1 AND JUST BEFORE THE 2[ND] IN TRIAL N. 2 AND 3.

FURTHERMORE, IN TRIAL N. 3, ALSO THE NUMBER OF LIVE MOBILE STAGES OF THE SPIDE
Phytoseiulus SPP. AND *Stethorus punctillum* PER 25 LEAVES WAS ASSESSED DURING THE STU
PERIOD. THE FINAL EFFICACY IN REDUCING THE NUMBER OF LIVE MOBILE STAGES OF *P. ulmi* PER 25
LEAVES OF THE DIFFERENT TREATMENTS WAS CALCULATED ACCORDING TO HENDERSON-

AT EACH ASSESSMENT, THE NUMBER OF LIVE MOBILE STAGES OF *P. ulmi* PER 25 LEAVES (TRIAL
N. 1-3) AND THE NUMBER OF LIVE MOBILE STAGES OF *Phytoseiulus* SPP. AND *S. punctillum* PER 25
LEAVES (TRIAL N. 3) WERE COMPARED ACROSS TREATMENTS USING ONE-WAY ANOVAS, FO
STUDENT-NEWMAN-KEULS TEST FOR POSTHOC COMPARISONS OF MEANS.

Results and discussion

SIGNIFICANT DIFFERENCES AMONG TREATMENTS IN THE NUMBER OF LIVE MOBILE STAG
25 LEAVES WERE NOT OBSERVED AT THE PRELIMINARY ASSESSMENT (TABLE 2). PEST DIS
BEGINNING OF THE TRIAL WAS THUS HOMOGENEOUS AMONG TREATMENTS. IN ALL TRIA
INCREASED CONSIDERABLY OVER TIME IN THE UNTREATED CONTROL. AT THE FINAL AS
INFESTATION WAS ALWAYS SIGNIFICANTLY LOWER IN *B. bassiana* TREATED WITH
74040 THAN IN UNTREATED CONTROL PLOTS, WITH MEAN EFFICACY VALUES OF THE M
AGENT ALWAYS EXCEEDING 70% (TABLE 2).

TABLE 2. NUMBER OF LIVE MOBILE STAGES OF *P. ulmi* PER 25 LEAVES (MEAN ± STANDARD DEVIATION) A
THE 3 ASSESSMENTS IN THE DIFFERENT TREATMENTS AND TRIALS, AND MEAN EFFICACY (
NUMBER OF LIVE MOBILE STAGES PER 25 LEAVES AT THE FINAL ASSESSMENT)*.

N.	Treatment	Preliminary assessment	Intermediate assessment	Final assessment	Efficacy (%)
Trial n. 1 (Serbia 2011)					
1	*Bb* ATCC 74040	34.3 ± 13.5 A	75.0 ± 49.3 A	121.5 ± 130.3 A	74.6
2	ABAMECTIN	29.8 ± 12.0 A	44.0 ± 36.5 A	67.8 ± 55.5 A	83.1
3	UNTREATED CONTROL	32.8 ± 11.0 A	362.3 ± 154 B	440.3 ± 181 B	-
Trial n. 2 (Italy 2011)					
1	*Bb* ATCC 74040	75.5 ± 42.8 A	52.5 ± 21.0 B	11.5 ± 9.0 A	93.4
2	FENAZAQUIN	54.0 ± 33.4 A	8.0 ± 1.6 A	1.5 ± 1.0 A	98.9
3	UNTREATED CONTROL	61.0 ± 20.6 A	72.5 ± 17.5 B	140.8 ± 74.4 B	-
Trial n. 3 (Italy 2012)					
1	*Bb* ATCC 74040	169.3 ± 27.6 A	67.3 ± 11.7 B	154.0 ± 28.3 B	71.9
2	TEBUFENPYRAD	158.5 ± 38.7 A	12.0 ± 10.5 A	41.0 ± 20.9 A	92.0
3	UNTREATED CONTROL	146.0 ± 39.2 A	163.5 ± 49.5 C	473.0 ± 79.9 C	

* DIFFERENT LETTERS WITHIN THE SAME COLUMN AND FOR THE SAME TRIAL INDICATE SIGNIFICANT DI
$P < 0.05$).

IN TWO TRIALS THE FINAL EFFICACY OF NATURALIS® WAS STATISTICALLY COMPARABLE TO THAT OF
CHEMICAL STANDARD, WHILE IN TRIAL N. 3 THE LATTER SHOWED SIGNIFICANTLY HIGHER
TESTED PRODUCT. HOWEVER, IN THIS TRIAL, THE INITIAL INFESTATION LEVEL WAS CONSI

IN THE OTHER TWO TRIALS (APPROX. 150 VERSUS LESS THAN 100 MITES PER 25
ENTOMOPATHOGENIC FUNGI HAVE A SLOW MODE OF ACTION COMPARED CHEMICAL PES
INFECTION BY *Beauveria bassiana* STRAIN ATCC 74040 CAN TAKE BETWEEN 24 AND 48 H DEPENDING ON
TEMPERATURE (BCPC, 2004). FURTHERMORE, BASED ON THE RESULTS OF DUSO *et al.* (2008) AND
SIMONI *et al.* (2010), IT CAN BE ASSUMED THAT AGAINST MITES THE STRAIN ACTS PRIMA
OVICIDE. STARTING WITH APPLICATIONS AT THE VERY FIRST APPEARANCE OF *P. ulmi*, BOTANIGARD®
THEREFORE BE ADVISABLE.

IN OUR TRIAL, THE MICROBIAL CONTROL AGENT DID NOT AFFECT THE NATURAL
POPULATIONS PRESENT IN THE FIELD. OUR FIELD OBSERVATIONS CONFIRM THE RESULTS
IN WHICH LITTLE OR NO SIDE EFFECTS ON NATURAL MITE SPECIES WERE OBSERVED
(DUSO *et al.*, 2008; SIMONI *et al.*, 2010). THE MICROBIAL CONTROL AGENT CAN THUS BE CONSID
VALUABLE TOOL TO BE INTEGRATED INTO SUSTAINABLE *P. ulmi* CONTROL PROGRAMMES.

TABLE 3. NUMBER OF LIVE MOBILE STAGES OF *Phytoseiulus* SPP. AND *S. punctillum* PER 25 LEAVES
(M ± S.D.) AT THE 3 ASSESSMENTS IN THE DIFFERENT TREATMENTS (*Bb* = *Beauveria bassiana* STRAIN)*.

N.	Treatment	Preliminary assessment	Intermediate assessment	Final assessment
	N. live mobile stages of *PHYTOSEIULUS* spp. per 25 leaves			
1	*Bb* ATCC 74040	0.5 ± 1.0 A	0.5 ± 1.0 A	3.5 ± 1.9 B
2	TEBUFENPYRAD	0.5 ± 1.0 A	0.5 ± 1.0 A	0.0 ± 0.0 A
3	UNTREATED CONTROL	1.0 ± 1.2 A	1.5 ± 1.9 A	5.5 ± 1.9 B
	N. live mobile stages of *S. PUNCTILLUM* per 25 leaves			
1	*Bb* ATCC 74040	N.A	6.5 ± 1.7 B	6.5 ± 1.3 B
2	TEBUFENPYRAD	N.A	2.8 ± 0.5 A	3.3 ± 0.5 A
3	UNTREATED CONTROL	N.A.	6.3 ± 2.1 B	6.8 ± 1.0 B

* DIFFERENT LETTERS WITHIN THE SAME COLUMN AND FOR THE SAME TRIAL INDICATE SIGNIFICANT DIFFERENCES (SNK
NA.= DATA NOT ASSESSED.

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Insect pathogens and entomoparasitic nematodes
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Mycopathogens of the corn leaf aphid, *RHOPALOSIPHUM MAIDIS* (Fitch.) infesting wheat plants at Assiut, Egypt

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Abstract: THE PRESENT STUDY WAS CARRIED OUT DURING 2008 AND 2010 WHEAT GROWING SEASONS. MYCOPATHOGENS OF THE CORN LEAF APHID, *Rhopalosiphum. maidis* WERE INVESTIGATED UNDER NATURAL CONDITIONS. SEVEN SPECIES OF ENTOMOPATHOGENIC FUNGI, INCLUDING FIVE ENTOMOPHTHORALES AND TWO HYPHOMYCETES WERE SURVEYED AND IDENTIFIED INFECTING THE COR LEAF APHID. ENTOMOPHTHORALES WERE REPRESENTED BY FIVE SPECIES BELONGING TO THREE FAMILI ANCYLISTACEAE WAS REPRESENTED BY ONE GENUS, *Conidiobolus* INCLUDING THREE SPECIES, NAMELY *C. coronatus, C. obscurus,* AND *C. thromboides.* ENTOMOPHTHORACEAE WAS REPRESENTED BY TWO GENERA, *Pandora* AND *Zoophthora* INCLUDING TWO SPECIES, *Pandora* (= *Erina*) *neoaphidis* AND *Zoophthora radicans*. THE IDENTIFIED SPECIES OF HYPHOMYCETES FUNGI BELONGING TO ORDER MONILIALES WERE REPRESENTED BY TWO MONILIACEAE SPECIES, NAMELY *Beauveria bassiana* AND *B. alba*. THE SPECIES *Beauvaria bassiana*, *B. alba* AND *Zoophthora radicans* REPRESENTED THE PREDOMINANT FUNGI SPECIES FOLLOWED BY *Pandora* AND *Conidiobolus obscurus.*

Key words: MYCOPATHOGENS, CORN LEAF APHIDS

Session 3:
New strategies for delivering and monitoring of entomopathogenic fungi

Insect pathogens and entomoparasitic nematodes
IOBC-WPRS Bulletin Vol. 90, 2013
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Exploiting vine weevil behaviour to disseminate an entomopathogenic fungus

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Abstract: CONTROL OF ADULT VINE WEEVIL (*Otiorhynchus sulcatus*) IS CURRENTLY RELIANT ON THE USE OF INSECTICIDES. HOWEVER, USING INSECTICIDE APPLICATIONS TARGETED AGAINST THIS PEST IS DIFFI TO BE APPLIED AT DUSK, AND ARE OFTEN INCOMPATIBLE WITH INTEGRATED PEST MANAGEMENT P STUDY INVESTIGATED THE POTENTIAL OF A NOVEL CONTROL STRATEGY THAT USES ARTIFICIAL REF OF AN ENTOMOPATHOGENIC FUNGUS AND EXPLOITS VINE WEEVIL BEHAVIOUR TO DISSEMINATE THROUGHOUT WEEVIL POPULATIONS.

PRELIMINARY EXPERIMENTS IDENTIFIED A SIMPLE PLASTIC CRAWLING INSECT TRAP AS A SUI VINE WEEVIL REFUGE. SUBSEQUENT SEMI-FIELD EXPERIMENTS USING FLUORESCENT POWDERS IN ENTOMOPATHOGENIC FUNGUS SPORE FORMULATION SHOWED THAT VINE WEEVIL AGGREGATIO MOVEMENT BETWEEN REFUGES EFFECTIVELY DISSEMINATED THE POWDERS THROUGHOUT WEEVIL

Key words: VINE WEEVIL, *Otiorhynchus sulcatus*, ENTOMOPATHOGENIC FUNGUS, REFUGE, AGGREGATION

Introduction

VINE WEEVIL (*Otiorhynchus sulcatus*) REMAINS ONE OF THE MOST SERIOUS PESTS OF SOFT FRUI NURSERY STOCK CROPS. DESPITE NON-CHEMICAL OPTIONS FOR THE CONTROL OF VINE WEEV AS USE OF ENTOMOPATHOGENIC NEMATODES AND THE ENTOMOPATHOGENIC FUNGUS (EPF), *Metarhizium anisopliae*, CONTROL OF ADULT WEEVILS IS CURRENTLY RELIANT ON INSECTICIDE APPLICATIO

THIS STUDY INVESTIGATED THE POTENTIAL OF EXPLOITING VINE WEEVIL BEHAVIOU CONTROL OF THIS PEST THROUGH THE USE OF AN EPF. THE APPROACH IS BASED ON THE WEEVILS ARE NOCTURNAL AND SEEK REFUGE DURING THE DAY, SHOW AGGREGATION BEHA INFECTED BY EPF (MOORHOUSE *et al.*, 1992). GIVEN THESE FEATURES OF VINE WEEVIL BIOLOGY IT M BE POSSIBLE TO USE ARTIFICIAL REFUGES TO DELIBERATELY INFECT WEEVILS WITH SPOR WEEVIL AGGREGATION BEHAVIOUR AND MOVEMENT BETWEEN REFUGES MAY THEN ALLOW OF THESE SPORES THROUGHOUT THE WEEVIL POPULATION.

Material and methods

INSECT REARING

ADULT VINE WEEVILS WERE COLLECTED FROM COMMERCIAL STRAWBERRY AND RASPBER WERE KEPT IN SMALL GROUPS IN VENTILATED PLASTIC CONTAINERS. EACH CONTAINER H MOISTURE (DAMP TISSUE PAPER), REFUGE (CORRUGATED CARDBOARD) AND FOOD SOURC LEAVES). THE WEEVILS WERE KEPT IN A CONTROLLED TEMPERATURE LABORATORY AT 21 °

ARTIFICIAL REFUGE TESTING

THREE SIMPLE ARTIFICIAL REFUGE DESIGNS WERE TESTED IN THESE EXPERIMENTS: (1) ROGUARD™ (RAS PLC, UK) – PLASTIC CRAWLING INSECT TRAP (80 MM DIAMETER X 15 MM) WITH FOUR SMALL (20 MM X 5 MM); (2) ROACHMASTER™ (RUSSELL IPM, UK) – PLASTIC CRAWLING INSECT TRAP (11 MM X 80 MM X 15 MM), HINGED ALONG ONE SIDE. EACH ROACHMASTER WAS MODIFIED FOR VINE WEEVIL REFUGE BY INSERTING A PIECE OF CORRUGATED CARDBOARD INSIDE THE TRA REFUGE WAS A NOVEL DESIGN BASED ON A BLOCK OF YEW WOOD (80 MM DIAMETER X 3 REFUGE WAS CREATED BY CUTTING A SERIES OF GROOVES (5 MM X 5 MM) IN THE WOOD.

ARTIFICIAL REFUGES WERE TESTED IN GAUZE CAGES (50 CM X 50 CM X 50 CM) PLA GLASSHOUSE COMPARTMENT AT ADAS BOXWORTH (CAMBRIDGE, UK) MAINTAINED AT 20 CAGE CONTAINED A DAMP COTTON WOOL PAD, YEW LEAVES AND ONE OR MORE ARTIF TWENTY VINE WEEVIL ADULTS WERE RELEASED INTO EACH CAGE DURING DAYLIGHT HOURS EACH WEEVIL WAS RECORDED 24 H AND 48 H AFTER RELEASE. DATA WERE SUBJECTED TO χ^2 TESTS. THE EXPERIMENTAL DESIGN WAS AS FOLLOWS:

A) SINGLE REFUGE (NO CHOICE) EXPERIMENT – A ROGUARD, ROACHMASTER OR 'WEEVI WAS PLACED INTO EACH CAGE. EACH REFUGE DESIGN WAS TESTED ON THE SAM EXPERIMENT WAS REPLICATED SIX TIMES.
B) TWO REFUGE (CHOICE) EXPERIMENTS – TWO REFUGES OF DIFFERENT DESIGNS WERE EACH CAGE. BASED ON THE RESULTS OF THE SINGLE REFUGE EXPERIMENT, TWO COM COMPLETED: ROGUARD + ROACHMASTER AND ROGUARD + 'WEEVILLE'. EACH COMP REPLICATED SIX TIMES.

DETERMINING POTENTIAL EFFICACY OF ARTIFICIAL REFUGES IN SPREADING SPORES OF AN EPF

ROGUARD REFUGES WERE TESTED IN LARGE GAUZE CAGES (145 CM X 145 CM X 152 CM) PL VENTILATED POLYTUNNEL AT ADAS BOXWORTH. TEMPERATURE DATA LOGGERS WERE P CAGES THROUGHOUT THE EXPERIMENTAL PERIOD. CAGES WERE PREPARED IN ONE OF TWO

A) TWO STRAWBERRY GROW-BAGS WERE PLACED INTO EACH CAGE. EACH BAG WAS C AND WAS PLANTED WITH FIVE STRAWBERRY PLANTS (CV. ELSANTA).
B) SIXTEEN *Euonymus fortunei* (CV. EMERALD GAIETY) PLANTS GROWN IN 1.5 L POTS.

FORTY ADULT WEEVILS WERE RELEASED INTO EACH CAGE AND THEN 24 H LATER 12 RO WERE SPREAD EVENLY THROUGHOUT THE CAGE (ON THE FLOOR AND CLOSE TO PLANT CONTAINED 0.2 G OF A HYDROPHOBIC FLUORESCENT POWDER (SWADA, STALYBRIDGE FLUORESCENT POWDER SERVED TO MARK WEEVILS ENTERING THE REFUGE AND WAS USE EPF SPORE FORMULATION. THE POWDERS USED WERE BRIGHTLY COLOURED AND FLUORES VIOLET LIGHT, ALLOWING EASY IDENTIFICATION OF WEEVILS THAT HAD ENTERED A REFUG CONTACT WITH A WEEVIL THAT HAD. WEEVILS WERE COLLECTED SEVEN DAYS AFTER PLA THE CAGES AND SCORED FOR THE PRESENCE OF FLUORESCENT POWDER. THE STRAWBER *Euonymus* EXPERIMENTAL DESIGNS WERE REPLICATED SEVEN AND EIGHT TIMES, RESPECTIVE

DETERMINING POTENTIAL EFFICACY OF WEEVIL TO WEEVIL CONTACT IN SPREADING SPORES OF AN EPF

LARGE GAUZE CAGES WERE PREPARED WITH *Euonymus* PLANTS AS PREVIOUSLY DESCRIBED. THIRTY FIV WEEVILS WERE RELEASED INTO EACH CAGE AND 24 H LATER 12 OF THE ROGUARD REFU INTO EACH CAGE AND ARRANGED AS PREVIOUSLY DESCRIBED. HOWEVER, IN THIS EXPERIME WAS CLEAN AND CONTAINED NO FLUORESCENT POWDER. FINALLY, FIVE ADULT VINE WEEVIL COATED IN FLUORESCENT POWDER WERE RELEASED INTO EACH CAGE. THESE WEEVILS W WITH WATER BASED PAINT. ALL WEEVILS WERE COLLECTED SEVEN DAYS AFTER RELEASI

POWDER COATED WEEVILS INTO THE CAGES. WEEVILS WERE SCORED FOR TT
POWDER, EXCLUDING THOSE THAT WERE COATED WITH POWDER AT THE ST.
EXPERIMENT WAS REPLICATED E

Results and discussion

ARTIFICIAL REFUGE TESTING

A) SINGLE REFUGE (NO CHOICE) EXPERI- REFUGE DESIGN SIGNIFICANTLY AFFECTED THE
OF ADULT VINE WEEVILS REFUGE DURING DAYLIGHT HOURS (^{2} = 17.82, P < 0.001)
AND 48 H (^{2} = 21.06, P < 0.001) AFTER THE EVILS WERE RELEASED IN (FIGURE 1).
INDIVIDUAL COMPARISONS BETWEEN THE TREATMENTS THAT A SIGNIFICANTLY HIGHER
OF WEEVILS WERE FOUND WITHIN THE ROGUARD OR REFUGE COMPARED TO THE
'WEEVILLE' REFUGES AFTER 24 AND 48 H. IN ADDITION, AFTER 48 H THE PROPORTION OF WEEVILS WITHIN
ROGUARD REFUGES WAS SIGNIFICANTLY HIGHER THAN IN ROACHMASTER REFUGES.

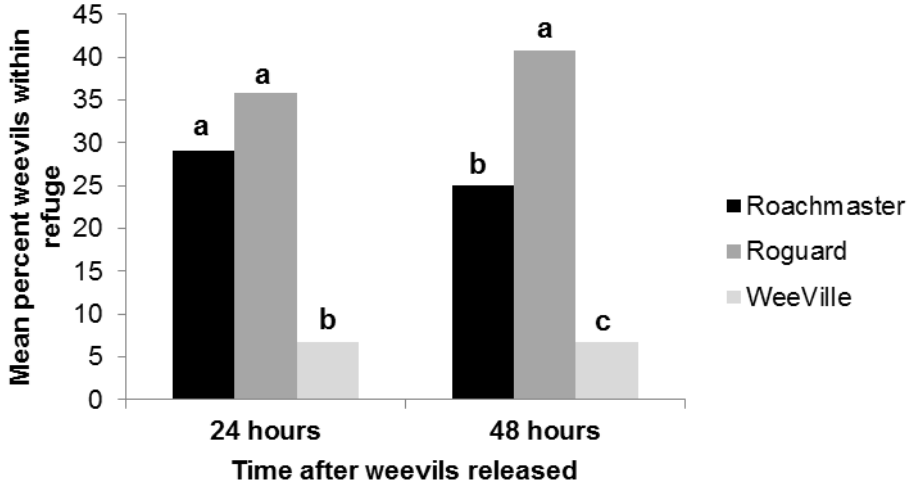

FIGURE 1. MEAN PERCENTAGE OF ADULT VINE WEEVILS WITHIN EACH DESIGN PRESENTED IN A NO-CHOICE ENVIRONMENT 24 AND 48 H AFTER WEEVILS WERE RELEASED INTO CAGES. DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES (P < 0.05).

B) TWO REFUGE (CHOICE) EXPERIMENT – A SIGNIFICANTLY HIGHER PROPORTION OF WEEVILS WERE FOUND WITHIN ROGUARD REFUGES THAN IN ROACHMASTER REFUGES WHEN PLACED ALTOGETHER WITHIN A CAGE (FIGURE 2A). THIS PREFERENCE WAS SEEN BOTH AT 24 H (^{2} = 7.48, P = 0.006) AND 48 H (^{2} = 15.38, P < 0.001). SIMILARLY, SIGNIFICANTLY MORE WEEVILS WERE FOUND WITHIN ROGUARD REFUGES WHEN PLACED ALTOGETHER WITH 'WEEVILLE' REFUGES WITHIN A CAGE (FIGURE 2B) AFTER 24 H (^{2} = 55.02, P < 0.001) AND 48 H (^{2} = 58.01, P < 0.001).

DETERMINING POTENTIAL EFFICACY OF ARTIFICIAL REFUGES IN SPREADING EPF FUNGUS

A) STRAWBERRY GROWER TRIAL – 92% OF ADULT VINE WEEVILS WERE RECOVERED FROM
DAYS AFTER ROGUARD REFUGES CONTAINING FLUORSCENT POWDER WERE PLACED
RECOVERED 94% HAD COME INTO CONTACT WITH FLUORESCENT POWDER IN CAGES
DURING THE EXPERIMENT WERE 21-24.1 °C (DAYTIME) AND 11.0-13.3 °C (NIGHT TIME

B) POTTED *Euonymus* – 94% OF ADULT VINE WEEVILS WERE RECOVERED FROM DAYS AFTER ROGUARD REFUGES CONTAINING FLUORESCENT POWDER WE OF THOSE RECOVERED HAD COME INTO CONTACT WITH FLUORESCENT POWDER. IN CAGES DURING THIS EXPERIMENT -26 °C (DAYTIME) AND 12ºC (NIGHT TIM

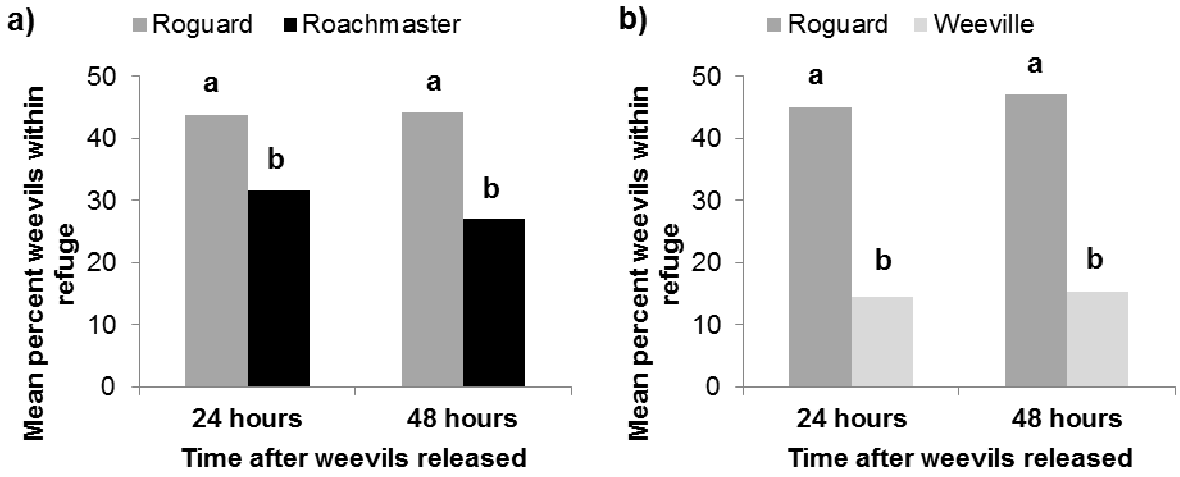

FIGURE 2. MEAN PERCENT ADULT VINE WE; A) ROGUARD + ROACHMASTE, AND B) ROGUARD + 'WEEVILLE' PRESENTED IN A CHOICE ENVIRO AND 48 H AFTER WE WERE RELEASED INTO CAGES. DIFFERENT LETTERS INDICATE A $P < 0.05$).

DETERMINING POTENTIAL EFFICACY OF WEEVIL TO WEEVIL SPREADING SPORES OF EPF

SEVEN DAYS AFTER INTRODUCING THE ADULT VINE WEEVILS COATED IN FLUOR ADULT VINE WEEVILS RECOVERED HAD COME INTO CONTACT WITH FLU TEMPERATURES DURING THE EXPERIMENT -28 °C (DAYTIME) AND 14ºC (NIGHT-TIME).

CONCLUSIONS

THESE RESULTS INDICATE THAT SIMPLE PLASTIC CRAWLING INSECT TRAPS ARTIFICIAL VINE WEEVIL REFUGES. FURTHERMORE, VINE WEEVIL AGGREGATION BEHAVIOUR AND REFUGES EFFECTIVELY ENABLES FLUORESCENT POWDERS PUT IN THESE REFUGES NUMBER OF WEEVILS. WORK IS CURRENTLY TO TEST THE EFFICACY OF A SUITABL FORMULATION IN THE REFUGES.

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Insect pathogens and entomoparasitic nematodes
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Field persistence of *METARHIZIUM* spp. strains applied as biocontrol agents against ticks (*IXODES RICINUS*)

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Abstract: IN TWO SEMI-FIELD TRIALS THE PERSISTENCE OF THREE *Metarhizium* STRAINS (BIPESCO 5, ARSEF 3297, ARSEF 4556) AFTER FOLIAR SPRAY APPLICATION WAS MONITORED AND *Tenebrio molitor* BIOASSAYS WITH LARVAE WERE MADE TO PRECLUDE A NEGATIVE EFFECT ON GERMINATION AND VITALITY OF C ADHESIVE AGENT NEO-WETT™ AND THE ANTIFOAMING AGENT ANTISCHIUMA SCHAUMSTOP™. OUTDOOR CONDITIONS (I.E. UNPROTECTED, RAINFALL PROTECTED, RAINFALL PROTECTED AND TESTED AND ALL STRAINS SHOWED AN ADEQUATE PERSISTENCE AFTER 25 DAYS (20-50%). BIPES WERE RE-ISOLATED FROM FOLIAGE EVEN AFTER HEAVY RAIN SHOWERS IN OPEN SITES AFTER SHOWED HIGH VIRULENCE IN BIOASSAY WITH AND WITHOUT NEO-WETT™ AND ANTISCHIUMA 90% OF ALL LARVAE WERE KILLED AFTER 5 TO 20 DAYS. NO NEGATIVE EFFECTS OF THE ADHESIVE AGENT ON THE GERMINATION ABILITY OF *Metarhizium* CONIDIA WERE OBSERVED.

Key words: *Metarhizium anisopliae*, *M. brunneum*, ADDITIVES, ABOVE GROUND APPLICATION

Introduction

Metarhizium IS ONE OF THE MOST IMPORTANT ENTOMOPATHOGENIC FUNGUS CURRENTL BIOINSECTICIDE AND BIOACARICIDE, RESPECTIVELY, FOR THE CONTROL OF A VARIETY O THE ABOVE GROUND APPLICATION OF THE FUNGUS IS A PROMISING NEW APPROACH FO TICKS REPORTED BY STAFFORD & ALLAN (2010). HOWEVER, LITTLE IS KNOWN ABOUT THE ENTOMOPATHOGENIC FUNGAL PROPAGULES ON PLANT SURFACES (INGLIS *et al.*, 2001). INGL AND JARONSKI (2010) PUBLISHED GENERAL INFORMATION REGARDING THE INFLUENCE BIOTIC FACTORS ON THE EFFICACY OF MYCOPESTICIDES IN FOLIAR APPLICATIONS. THE AU THAT THE MAIN ENVIRONMENTAL FACTORS ARE SOLAR RADIATION, TEMPERATURE, HUM SURFACE CHEMISTRY AND PHYLLOPLANE MICROBIOTA. THE USE OF STICKERS IN CONIDIA SPRAY APPLICATION IS ANNOUNCED TO ENHANCE HIGH CONIDIA CONCENTRATIONS ON CONSEQUENTLY A GOOD PERSISTENCE ON FOLIAGE (JENKINS *et al.*, 2001). PRELIMINARY STUDIES HAVE SHOWN THAT *Metarhizium* CONIDIA WITH A CONIDIA DENSITY OF 1×10^5 CONIDIA cm^{-2} LEAF DO NOT PERSIST LONGER THAN THREE WEEKS. TO ENSURE A SUCCESSFUL REDUCTION OF THE TIC STUDY WAS TO IMPROVE THE PERSISTENCE OF *Metarhizium* CONIDIA ON FOLIAGE BY USING THE ADHESIV AGENT NEO-WETT™ (STICKER) AND THE ANTIFOAMING AGENT ANTISCHIUMA SCHAUMSTO

Material and methods

FUNGAL ISOLATES

THREE DIFFERENT STRAINS OF *Metarhizium* SPP. WERE USED IN THIS STUDY: *Metarhizium anisopliae* VAR. *anisopliae* STRAIN BIPESCO 5 (MYCOLOGICAL COLLECTION, UNIVERSITY OF INNSBRUCK, ISOLATED FROM *Cydia pomonella*; *M. anisopliae* (ARSEF 4556) AND *M. brunneum* (ARSEF 3297), ISOLATED FROM *Boophilus* SP. (ACARI: IXODIDAE) IN FLORIDA/USA AND MEXICO (ANSARI

BUTT, 2011). BOTH STRAINS WERE PROVIDED BY DR. TARIQ M. BUTT, FROM SWANSEA UN
TECHNICAL SPORE POWDER PRODUCTS PRODUCED ON RICE.

QUALITY CONTROL TESTS

ALL TECHNICAL SPORE POWDER PRODUCTS WERE CHARACTERISED BASED ON THE ST
PUBLISHED BY LAEGDSGAARD (2005): (I) PURITY OF THE PRODUCTS, (II) VIABILITY OF CONIDIA AND
VIRULENCE OF THE TECHNICAL SPORE POWDER PROPAGULES.

INFLUENCE OF ADHESIVE- AND ANTIFOAMING AGENT

TESTING INFLUENCE OF THE ADHESIVE AGENT NEO-WETT™ (KWIZDA, 10640.001) AND THE A
AGENT ANTISCHIUMA SCHAUMSTOP™ (BASF ITALIA SRL) ON CONIDIA GERMINATION, BI
Tenebrio molitor LARVAE WERE CONDUCTED WITH SEVERAL VARIATIONS. SUSPENSIONS W
FOLLOWS: (A) CONIDIA WITH 0.1% (V/V) STEROL TWEEN® 80 (POSITIVE CONTROL), (B) CONIDIA
WITH 450 MG L^{-1} DODINE (1-DODECYLGUANIDIUM ACETATE) AND 70 MG L^{-1} CYCLOHEXIMID
(NEGATIVE CONTROL), (C) CONIDIA WITH H_2O, (D) CONIDIA WITH TAP H_2O AND 0.05% (V/V) NEO-
WETT™, (E) CONIDIA WITH TAP H_2O AND 0.0015% (V/V) ANTISCHIUMA SCHAUMSTOP™, (F) CONIDIA
WITH TAP H_2O, 0.05% (V/V) NEO-WETT™ AND 0.0015% (V/V) ANTISCHIUMA SCHAUMSTOP™.

SPRAY APPLICATION

Phaseolus vulgaris (BEAN PLANTS) AND *Malus domestica* (APPLE PLANTS) WERE USED IN THIS STUDY.
BEFORE SPRAY APPLICATION THE PLANTS WERE SEPARATED IN FOUR GROUPS DUE TO
DIFFERENT *Metarhizium* PRODUCTS AND ONE UNTREATED CONTROL. ALL BEAN PLANTS PER T
VARIATION WERE SPRAYED ONCE WITH A CONIDIAL SUSPENSION OF 1×10^8 CONIDIA mL^{-1} AND ALL APPLE
PLANTS WITH A CONIDIAL SUSPENSION OF 1×10^7 CONIDIA mL^{-1} (BIPESCO 5) AND 5×10^7 CONIDIA mL^{-1}
(ARSEF 3297),, RESPECTIVELY (TOTAL SPRAY VOLUME 400 ML PER PLANT). THE CONIDIAL SUSPENSION WAS
SUPPLEMENTED WITH A NEO-WETT™-SOLUTION [0.05% (V/V); KWIZDA, 10640.001]. T
SUSPENSIONS WERE SHAKEN FOR 2 MIN TO AVOID CLUMPING OF THE CONIDIA. A VARIATI
THE SPORE SUSPENSIONS WERE MADE IN THE SEMI FIELD TRIAL WITH APPLE TREES:
SUSPENSIONS WERE FIRST INCUBATED IN THE SONICATION BATH FOR 10 MIN, THEN FIL
COTTON CLOTH AND FINALLY SUPPLEMENTED WITH NEO-WETT™ [0.05% (V/V)]. ALL SUSP
APPLIED WITH A SPRAY-MATIC 1.25 P AEROSOL CAN (BIRCHMEIER) WITH A VOLUME CAPA
2 TO 3 S PER PLANT. AFTER A SHORT AIR DRYING PERIOD THIS PROCEDURE WAS REPEAT
DOSE OF 400 ML WAS APPLIED TO THE LEAVES. THIS APPLICATION TECHNIQUE ASSURED
AND BOTTOM SIDE OF THE BEAN AND APPLE LEAVES WERE COVERED WITH A FINE CON
RECOMMENDED CONCENTRATION OF MORE THAN 2×10^5 CONIDIA cm^{-2} LEAF.

MONITORING PERSISTENCE ON FOLIAGE

STANDARD PROTOCOL BY HUTWIMMER *et al.* (2007) WAS USED FOR MONITORING THE PERSISTENCE
CONIDIA ON LEAF SURFACES OVER TIME OF 25 AND 44 DAYS, RESPECTIVELY. THE TREAT
(N = 7; PER PRODUCT AND VARIATION) AND APPLE PLANTS (N = 6) WERE GROWN IN F
ENVIRONMENTS: ONE SET OF PLANTS WAS KEPT IN THE GREENHOUSE AND THE OTHER
PRESERVED OUTDOOR UNDER THREE DIFFERENT CONDITIONS: OPEN FIELD (UNPROTECTE
PROTECTED) AND ROOFED, FULLY COVERED WITH CANVAS COVER. AT THE SAMPLING D
TREATMENT AND STATION WAS CUT OFF THE BEAN SHRUBS (N = 7) AND THE APPLE TRE
STERILISED SCISSOR AND PUT INTO PLASTIC ZIP LOCK BAGS TO PROCESS THE SAMPLES
THE LEAVES WERE PUT INTO STERILISED 100 ML ERLENMEYER FLASKS CONTAINING 50 ML
(V/V) TWEEN® 80 SOLUTION. THE FLASKS WERE SHAKEN FOR 15 S TO WASH DOWN ALL CONI
LEAVES. THE HARVESTED AND DRIED LEAVES WERE USED TO DETERMINE THE SURFA
PROCESSED LEAVES. THE FORMER CONIDIA SUSPENSION WAS DILUTED TO OBTAIN A
CONIDIA μL^{-1}. THE SUSPENSION (50 µL) WAS PLATED ON *Metarhizium* SELECTIVE S4G AGAR PLATES
(N = 3). THE PLATES WERE INCUBATED AT 25 °C AND 60% RELATIVE HUMIDITY FOR UP TO
THE COLONY FORMING UNITS (CFUS) WERE COUNTED TO CALCULATE THE INITIAL SPORE DEN

Results and discussion

SONDERGEGGER (2012) REPORTED THAT ALL THREE STRAINS (BIPESCO 5, ARSEF 3297, ARSEF 4
SHOWED PERSISTENCE ON LEAVES BUT THE NUMBER OF DETECTABLE CONIDIA PER CM^2
DECREASED BETWEEN 4 AND 8 DAYS AFTER SPRAY APPLICATION, RESPECTIVELY. ALTHO
CONIDIA CONCENTRATION WAS TESTED, IN OUR STUDY ALL THREE STRAINS SHOWED AN A
EVEN AFTER 25 DAYS IN ALL OUTDOOR ENVIRONMENTS (FIGURE 1). BIPESCO 5 STRAIN W
OVER AN OBSERVATION PERIOD OF 44 DAYS. MORE THAN 9% OF VITAL BIPESCO 5 CON
DETERMINED. A MORE RAPIDLY DECREASE OF THE CONIDIA VIABILITY OF STRAIN ARS
BIPESCO 5 WAS ASSESSED IN THE UNPROTECTED OPEN FIELD SYSTEM. MONITORED MET
DATA LEAD TO THE CONCLUSION THAT HEAVY RAINFALL PERIODS HAD A SIGNIFICA
PERSISTENCE OF CONIDIA. DURING THE SEMI-FIELD TRIALS WITH *Phaseolus vulgaris* AND
Malus domestica SIX HEAVY RAIN SHOWERS OCCURRED (> 8 MM RAINFALL PER DAY) AND AI
PLANTS IN THE UNPROTECTED ENVIRONMENT. IN THE GREENHOUSE THE NUMBER OF C
FASTER AND ONLY BIPESCO 5 SHOWED AN ADEQUATE PERSISTENCE TILL THE END OF T
(FIGURE 1). THE DECLINED PERSISTENCE CAN BE TRACED BACK TO THE FACT, THAT T
CONDITIONS WERE SUBOPTIMAL, BECAUSE THE BEAN- AND APPLE PLANTS WERE EXPOSED
(> 45 °C) FOR MORE THAN 8 H A DAY BECAUSE OF THE MISSING AIR CONDITION IN THE BUIL

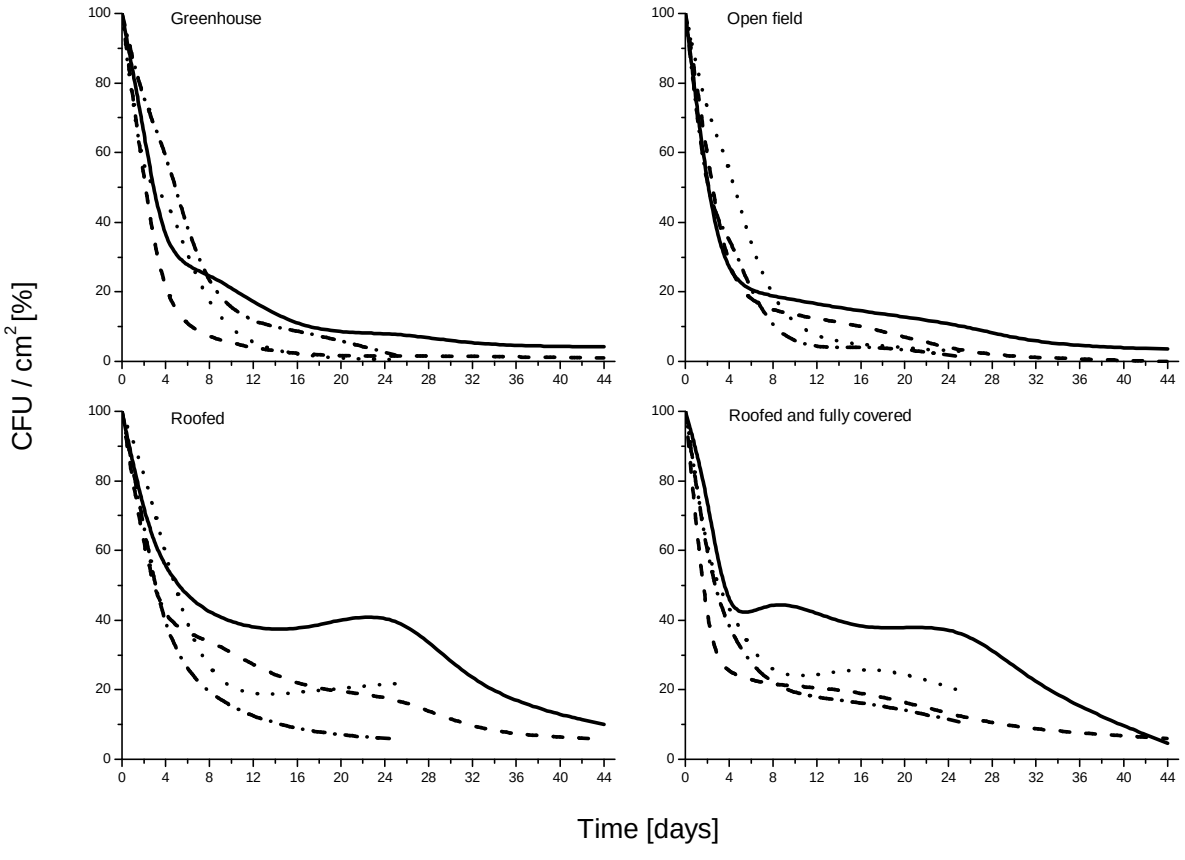

FIGURE 1: PERSISTENCE OF *Metarhizium* SPP. CONIDIA ON LEAVES OF *Phaseolus vulgaris* AND OF
Malus domestica DURING A TIME INTERVAL OF 25 AND 44 DAYS, RESPECTIVELY, IN FOUR
ENVIRONMENTS: GREENHOUSE, OPEN FIELD (UNPROTECTED), OPEN FIELD AND ROO
PROTECTED), OPEN FIELD AND FULLY COVERED WITH A CANVAS COVER. COMPARISON O
Metarhizium TECHNICAL SPORE POWDER PRODUCTS: BIPESCO 5 FROM JUNE 2012 (…), BIPESC
(—), ARSEF 4556 (–●–) AND ARSEF 3297 (– –) FROM JULY 2012.

AN ESSENTIAL PART OF A SUCCESSFUL SPRAY APPLICATION IS TO MAINTAIN THE V
BIOLOGICAL CONTROL AGENT. NO NEGATIVE EFFECTS ON THE VIRULENCE OF *Metarhizium* CONIDIA WERE
ESTIMATED BY ADDING THE ADHESIVE AGENT NEO-WETT™ AND THE ANTIFOAMING AGE
SCHAUMSTOP™. WITH THE EXCEPTION OF THE POSITIVE CONTROL SUBSTANCES
CYCLOHEXIMID (TWO POTENT FUNGICIDES) ALL THREE PRODUCTION STRAINS WERE HIGH
THE ADDITION OF THE ADDITIVES TO THE CONIDIA SUSPENSIONS. A FIFTY PERCENT M
Tenebrio LARVAE WAS ESTIMATED FOR ALL BIPESCO 5 AND ARSEF 4556 PRODUCTS WITHIN
DAYS (FIGURE 2). ARSEF 3297 CONIDIA SHOWED A DECREASED VIRULENCE, ESPECIALLY IN
NEO-WETT™- AND ANTISCHIUMA SCHAUMSTOP™ SUSPENSIONS. NEVERTHELESS, NINETY P
LARVAE WERE STILL KILLED BY ALL TESTED AGENTS AFTER AN INCUBATION TIME OF 5 TO

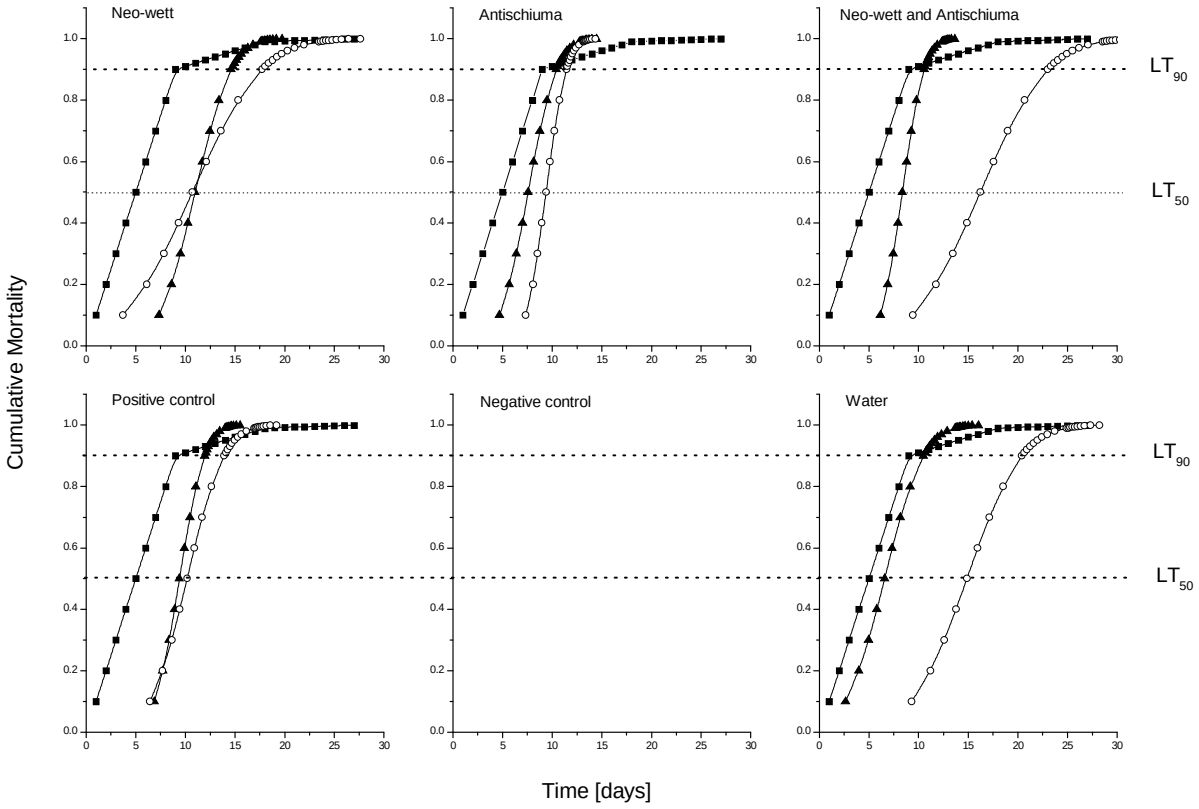

FIGURE 2: INFLUENCE OF THE ADHESIVE AGENT NEO-WETT™, THE ANTIFOAMING AGEN
SCHAUMSTOP™ AND TWEEN® 80 AND THE TWO FUNGICIDES DODINE AND CYCLOHEXIMID ON
VIRULENCE OF *Metarhizium* SPP. CONIDIA (LT_{50} AND LT_{90}). FOLLOWING VARIATIONS WERE TESTED
NEO-WETT™; ANTISCHIUMA SCHAUMSTOP™; NEO-WETT™ AND ANTISCHIUMA SCHAU
TWEEN® 80 [0.1% (V/V); POSITIVE CONTROL], 450 MG L^{-1} DODINE (1-DODECYLGUANIDIUM ACETATE)
AND 0.7 MG ML^{-1} CYCLOHEXIMID (NEGATIVE CONTROL) AND TAP WATER. BIPESCO 5 ( ), AR
4556 (▲) AND ARSEF 3297 ( ).

SUMMARIZING, THE PROPOSED CONIDIA DENSITY FOR FOLIAGE TREATMENT OF 2 X 10^5 CONIDIA CM^{-2}
LEAF (SONDEREGGER, 2012) ENSURES THAT THE CONIDIA PERSIST ON LEAF SURFACE FOR
WEEKS. EVEN AFTER HEAVY RAINFALLS CONIDIA WERE RE-ISOLATED IN THE OPEN FIELD
THE USE OF THE ADHESIVE AGENT NEO-WETT™ AND THE ANTIFOAMING AGENT
SCHAUMSTOP™ IS RECOMMENDED TO ENHANCE THE PERSISTENCE OF *Metarhizium* CONIDIA ON LEAF SURFACES.

Acknowledgements

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LAIMBURG (ITALY) FOR PROVIDING THE POTTED PLANT CULTURES.

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ricinus) DURCH DEN INSEKTENTÖTENDEN PILZ *Metarhizium anisopliae* (METCH.)
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Vertical transmission of an endophytic strain of *BEAUVERIA BASSIANA* (Ascomycota; Hypocreales) colonizing opium poppy *PAPAVER SOMNIFERUM*

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Abstract: *Beauveria bassiana* (BALSAMO) VUILL. STRAIN EABB 04/01-TIP OBTAINED FROM LARVAE OF THE OPIUM POPPY BORER *Eutreta luteipes* (HYMENOPTERA; CYNIPIDAE) ENDOPHYTICALLY COLONIZES OPIUM POPPY (*Papaver somniferum* L.) PLANTS. THE GOAL OF THIS STUDY HAS BEEN TO USE A SPECIES-SPECIFIC TWO-STEP NESTED PCR FOR IDENTIFYING A MONITORING THIS STRAIN IN PLANT TISSUES AND TO ASCERTAIN WHETHER THE FUNGUS IS TR VERTICALLY VIA SEEDS. SURFACE-STERILIZED SEEDS WERE TREATED (DRESSED) WITH A SUSPENSION AND ENDOPHYTIC COLONIZATION OF THE PLANT TISSUES BY THE FUNGUS W MONITORED AND ASCERTAINED THROUGHOUT DIFFERENT PLANT GROWTH STAGES INCLUDING SI ROSETTA, PRINCIPLE OF NOTCHING, END OF NOTCHING, CAPSULE FORMATION AND IN NEW SEEDS. U OF THE NESTED PCR PROTOCOL SHOWED THAT ALL PLANTS OBTAINED FROM SEEDS DRESSED W FUNGUS WERE ENDOPHYTICALLY COLONIZED AT THE DIFFERENT GROWTH STAGES, AND M IMPORTANTLY THE ENDOPHYTE WAS DETECTED IN 50% OF THE SEED SAMPLES FORMED IN THE CAPSULES. THREE SEED LOTS OBTAINED FROM THREE INDEPENDENT CAPSULES SHOWING *B. bas* COLONIZED SEEDS WERE SELECTED, AND THEIR SEEDS WERE SURFACE DISINFESTED AND SO ORDER TO MONITOR THE POSSIBLE PRESENCE OF THE FUNGUS IN THE TISSUES OF THE NEW PL THE SAME PHENOLOGICAL STAGES. IN TOTAL, 24 PLANTS WERE OBTAINED FROM THE A MENTIONED SEEDS, AND THE FUNGUS WAS DETECTED IN PLANT TISSUES AND EVEN SEEDS OF PLANTS, THEREFORE DEMONSTRATING THAT THE FUNGUS WAS TRANSMITTED VERTICALLY FR PLANTS VIA SEEDS, WHICH TO THE BEST OF OUR KNOWLEDGE IS REPORTED FOR THE FIRST TIM ENTOMOPATHOGENIC FUNGUS.

Key words: ENTOMOPATHOGENIC FUNGI, ENDOPHYTIC COLONIZATION, SYSTEMIC PROTECTION, SPECIES-SPECIFIC TWO-STEP NESTED PCR

Insect pathogens and entomopatrasitic nematodes
IOBC-WPRS Bulletin Vol. 90, 2013
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Development of a novel fermentation and formulation process for an endophytic *BEAUVERIA BASSIANA* STRAIN

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Abstract: THERE IS AN INCREASING DEMAND FOR ALTERNATIVE OR COMPLEMENTARY CROP PROT
A NOVEL APPROACH COULD BE THE USE OF THE ENTOMOPATHOGENIC AND ENDOPHYTIC FU
bassiana ISOLATE ATP-04. TO USE THE ENDOPHYTE AS A COMMERCIAL BIOCONTROL AGENT, THE FU
MASS-PRODUCED. *bassiana* WAS RAISED IN SHAKE FLASK CULTURES TO PRODUCE SUBMERGED CON
(SCS) WHICH ARE REPORTED TO SHOW A HIGHER SHELF LIFE THAN MYCELIUM AND BLASTOSPO
FOUND THAT IN MINERAL MEDIA WITH 5% SUGAR *B. bassiana* PRODUCED 0.1 X 1010 SCS G^{-1}
SUCROSE IN 192 H. BY ADDING 50 MM NACL 48 H AFTER INOCULATION THE SCS YIELD INCREASED TO 1.4 X
SCS G^{-1} SUCROSE. THE SCALE-UP TO A 2 L STIRRED TANK REACTOR WAS CARRIED OUT AT 25°C, 20
1 VVM AT PH 5.5. A TOTAL SPORE YIELD OF 5.2 X 1010 SPORES G^{-1} SUCROSE CORRESPONDING TO A SCS YIELD OF
0.2 X 1010 SCS G^{-1} SUCROSE WAS OBTAINED AFTER 216 H. ALSO THE YIELD OF SCS INCREASED TO
SCS G^{-1} SUCROSE BY THE ADDITION OF NACL. AFTER FERMENTATION *B. bassiana* WAS FORMULATED IN A NOVEL
SPRAY FORMULATION THAT DELIVERS THE FUNGUS ON OILSEED RAPE LEAVES, INCREASES PERS
AND GROWTH ON LEAVES AS WELL AS PENETRATION, COLONIZATION AND EFFICACY IN BIOA
xylostella.

Key words: ENDOPHYTE, *Beauveria bassiana*, SUBMERGED CONIDIOSPORES, FERMENTATION, SPR
FORMULATION

Introduction

B. bassiana IS AN ENTOMOPATHOGENIC FUNGUS THAT CAN COLONIZE A WIDE ARRAY OF
(OWNLEY, 2010) MANY OF THEM OF ECONOMIC INTEREST. THE *B. bassiana* ENDOPHYTIC
EFFICACY AGAINST A WIDE RANGE OF INSECT PESTS FROM WITHIN THE PLANTS AND HA
BECOMING A COST-EFFECTIVE BIOCONTROL AGENT (KHACHATOURIANS, 1986). FOR AP
COMMERCIAL BIOCONTROL AGENT THE *B. bassiana* HAS TO BE MASS-PRODUCED AND
FORMULATED IN SUCH A FASHION THAT IT COLONIZES PLANTS AND PROTECTS THEM FRO
WITHIN, JUST AS TRANSGENIC PLANTS DO.

MOST PUBLICATIONS ON CULTIVATION OF *B. bassiana* DEAL WITH SOLID-STATE FERMENTATION
EPIPHYTIC *B. bassiana* ISOLATES AND THEREFORE WITH THE MASS-PRODUCTION OF AERIAL
MYCELIUM (E.G. KANG *et al.*, 2005). HOWEVER THE PREFERRED METHOD FOR LARGE-SCALE PRO
OF MICROORGANISMS IS SUBMERGED CULTIVATION. THE OBVIOUS ADVANTAGES OF
CULTIVATION ARE THAT THE FUNGUS PRODUCES SPORES IN A RELATIVELY SHORT TIM
UNDER CONTROLLED STERILE CONDITIONS AS WELL AS A SIMPLER SCALE-UP IN CONT
FERMENTATION (FENG *et al.*, 1994, PATEL *et al.*, 2010). IN A SUBMERGED CULTIVATION *B. bassiana*
FORMS BS, SCS AND MYCELIUM. BS ARE RELATIVELY LARGE, THIN-WALLED AND SINGLE
BODIES (BIDOCHKA *et al.*, 1987). SCS, ON THE OTHER HAND, ARE SMALL, SPHERICAL, MORE UNIF
SIZE AND SHOW A HIGHER SHELF-LIFE THAN BS. THEY ARISE FROM FUNGAL MYCELIA OR
IN A PROCESS KNOWN AS MICROCYCLE CONIDIATION (THOMAS *et al.*, 1987).

WITH REGARD TO FORMULATION, THERE ARE NO SYSTEMATIC INVESTIGATIONS ON TH
NOVEL SPRAY FORMULATIONS FOR ENDOPHYTIC ENTOMOPATHOGENIC FUNGI WHICH LEA
COLONISATION OF OILSEED RAPE PLANTS (BURGES, 1998).

THE OBJECTIVES OF THE PRESENT WORK WERE TO PRODUCE SCS IN A COST-EFFE
MEDIUM ON LAB SCALE, TO INCREASE SCS YIELD BY ADDITION OF NACL AND SCALE-UP T
2 L STIRRED-TANK REACTOR. FURTHERMORE, WE WILL SHOW DATA ON DELIVERY OF FORM
RAPE LEAVES, PERSISTENCE OF FUNGUS, GERMINATION AND GROWTH ON LEAVE
COLONIZATION AND EFFICACY IN BIOASSAYS WITH *Plutella xylostella*.

Material and methods

STRAIN

Beauveria bassiana ISOLATE ATP-04 WAS PROVIDED BY THE GEORG-AUGUST-UNIVER
DEPARTMENT OF CROP SCIENCES/AGRICULTURAL ENTOMOLOGY, GOETTINGEN. THE STRA
25 °C ON SDA AGAR CONTAINING 1% CASEIN PEPTONE, 2% GLUCOSE AND 1.5% AGAR-AGAR
TEMPERATURE OPTIMUM WAS DETERMINED AT 25 °C AND PH OPTIMUM AT 5-6 (DATA NOT

CULTIVATION

B. bassiana WAS GROWN IN DIFFERENT LIQUID MEDIA IN SHAKE FLASKS WITH THREE BA
CONIDIA FROM AGAR PLATES (SEE ABOVE) WERE USED AS A STARTER INOCULUM. THE SH
WERE INOCULATED WITH THE SPORE SUSPENSION TO GIVE AN INITIAL SPORE DENSITY O
1×10^4 SPORES ML^{-1}. THE FLASKS WERE INCUBATED AT 25 °C, 150 RPM AND PH 5.5. AT SEVEN DIFFERENT T
INOCULATION DIFFERENT STERILE NACL STOCK SOLUTIONS WERE ADDED TO THE "OSMOTI
VARYING THE FINAL NACL CONCENTRATION IN THE MEDIA. BATCH FERMENTATION WAS
STIRRED TANK REACTOR (SARTORIUS STEDIM SYSTEM GMBH, GERMANY). FERMENTATION
INOCULATING 300 ML OF A CARBON SOURCE STOCK SOLUTION WITH A CONTENT OF 1.5×10^8 SPORES ML^{-1}). THE FERMENTATION WAS CARRIED OUT AT 25 °C, 1 VVM AND 200-600 RPM.

ANALYTICS

FOR THE DETERMINATION OF FUNGAL DRY BIOMASS 15 ML SAMPLES WERE CENTRIFUGE
20000 ×G, WASHED TWO TIMES WITH DD H_2O AND CENTRIFUGED AGAIN. THE CELL SUSPENSIONS W
DRIED AT 115 °C WITH A MOISTURE ANALYZER (SARTORIUS, GERMANY). THE COLONY FO
(CFU) OF BS AND SCS WERE DETERMINED BY SPREADING 100 µL OF DILUTED SAMPLES ON
(SEE ABOVE) AND INCUBATING AT 25 °C FOR 4-6 DAYS.

Results and discussion

IN TOTAL, 23 TECHNICAL CULTURE MEDIA BASED ON DIFFERENT CARBON SOURCES, MINE
YEAST EXTRACTS WERE SCREENED. THE MOST PROMISING CULTURE MEDIUM WAS A M
WITH 5% SUGAR BEET MOLASSES, WHICH CONSISTS OF 50% SUCROSE. IN THIS CULT
B. bassiana PRODUCED $5.32 \pm 0.24 \times 10^{10}$ TOTAL SPORES G^{-1} SUCROSE AT 192 H AFTER INOCULATION. BU
THE YIELD OF SCS WAS ONLY $0.12 \pm 0.04 \times 10^{10}$ SCS G^{-1} SUCROSE.

SUGAR BEET MOLASSES IS A RESIDUE OF THE AGRICULTURAL INDUSTRY AND CONSEQU
COST SOURCE. THEREFORE, THE COST OF 1 L CULTURE MEDIUM AMOUNTS TO ONLY 0.33
PROBLEM OF THIS CULTIVATION IS THE LOW CONCENTRATION OF SCS. ONE POTENTIAL
PROBLEM IS THE SELECTIVE PRODUCTION OF SCS BY OSMOTIC STRESS. TO THIS END TH

DIFFERENT TIMES OF ADDITION AND FINAL CONCENTRATIONS OF NACL ON THE PRODU
INVESTIGATED.

IN THE CONTROL, WHERE NO SALT WAS ADDED BUT THE SAME AMOUNT OF WATER AF
SPAN, A CONCENTRATION OF 0.02 ± 0.00 X 10^9 SCS ML^{-1} WAS OBTAINED. IN CONTRAST, 48 H AFTER
INOCULATION THE ADDITION OF 50 G L^{-1} NACL LED TO A CONCENTRATION OF 0.35 ± 0.03 X 10^9 SCS ML^{-1}
CORRESPONDING TO A YIELD OF 1.40 ± 0.12 X 10^{10} G L^{-1} SUCROSE AT THE END OF THE CULTIVATIO
(FIGURE 1).

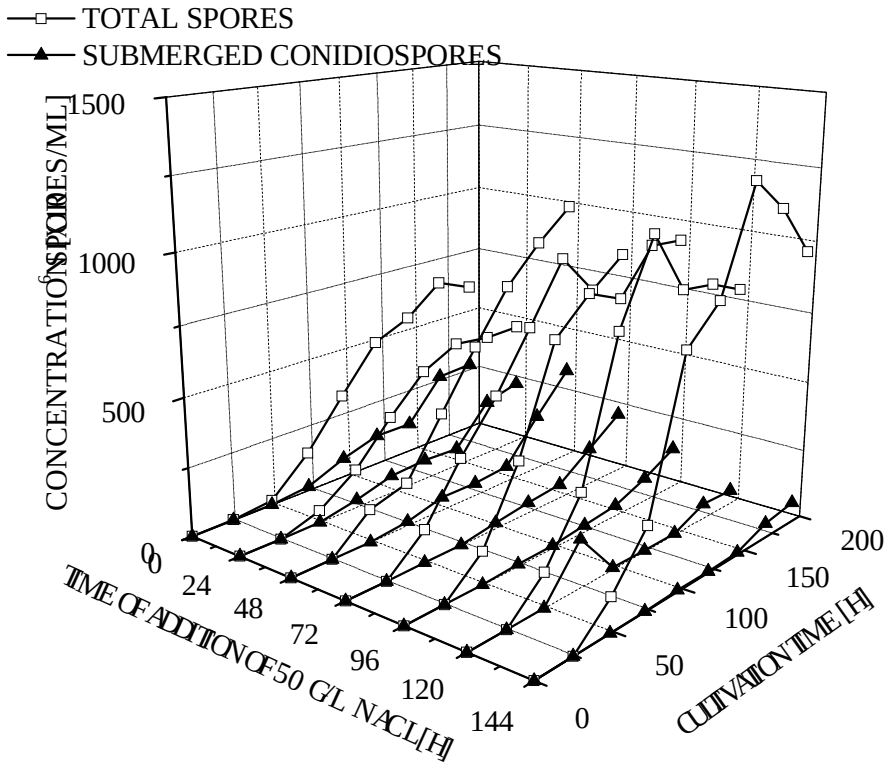

FIGURE 1. INFLUENCE OF DIFFERENT TIMES OF ADDITION OF 50 G L^{-1} NACL ON THE PRODUCTION OF SCS.

THUS, THE AMOUNT OF SCS WAS INCREASED 17.5-FOLD BY THE ADDITION OF NAC
APPROPRIATE TIME. IT WAS OBSERVED THAT THE HIGHEST YIELD OF TOTAL SPORES WAS
ANY ADDITION OF NACL. WHEN NACL WAS ADDED TO THE CULTIVATION BROTH, THE YIE
(TS) DECREASED AND A SHIFT FROM BS TO SCS WAS OBSERVED. THE EARLIER THE ADDITIO
HIGHER WAS THE CONCENTRATION OF SCS AND THE LOWER THE CONCENTRATION OF TOT

FIGURE 2A SHOWS A FERMENTATION OF *Trichoderma harzianum* IN A 2 L STIRRED-TANK REACTOR WITHOU
ADDITION OF NACL. IN THIS FERMENTATION *Trichoderma harzianum* PRODUCED 1.29 ± 0.04 X 10^9 TS ML^{-1}
CORRESPONDING TO A YIELD OF 5.16 ± 0.16 X 10^{10} G L^{-1} SUCROSE AT THE END OF THE FERMENTATIO
THEREFORE, THE COST OF TOTAL SPORES CAN BE ESTIMATED AT 0.26 €. BUT CONCENTRATION
WAS ONLY 0.06 ± 0.00 X 10^9 SCS ML^{-1}. THE AMOUNT OF DRY BIOMASS INCREASED AT THE BEGI
OF THE FERMENTATION BECAUSE THE FUNGUS PRODUCED MYCELIUM. AFTER 72 H, 21 G L^{-1} DRY BI
OBTAINED. THEN THE AMOUNT OF MYCELIUM DECREASED TO THE END OF THE FERMENTAT
BE DUE TO THE LIMITATION OF SUBSTRATES. 96 H AFTER INOCULATION THE CONCENTRAT
STARTED TO DECREASE TO A YIELD OF 3.12 X 10^{10} G L^{-1} SUCROSE AT THE END OF THE FERMENTATION.
THE SELECTIVE PRODUCTION OF SCS ALSO WAS ADDED TO THE CULTURE BROTH AFTER 48 H (

2B). IN CONTRAST TO A CULTIVATION WITHOUT NACL THE CONCENTRATION OF SCS COUL
$0.28 \pm 0.01 \times 10^9$ SCS ML^{-1} AT THE END OF THE FERMENTATION. THIS REPRESENTS A 5-FOLD IN
THE SCS YIELD. FURTHERMORE, *B. bassiana* DID NOT PRODUCE MYCELIUM DURING THE FERMENTAT
CONTRAST TO THE FERMENTATION WITHOUT NACL ADDITION.

IT COULD BE SHOWN THAT WETTERS BASED ON NON-IONIC SURFACTANTS COULD DE
ANGLE ON THE LEAF FROM 110 ° TO < 25 ° RESULTING IN AN INCREASE OF THE WETTED LEA
TO THE CONTROL BASED JUST ON WATER. HOWEVER, SOME WETTERS DECREASED VIAB
> 90%. BESIDES, WE WILL SHOW DATA ON DELIVERY OF FORMULATIONS ON OILSEED
PERSISTENCE OF FUNGUS, GERMINATION AND GROWTH ON LEAVES, PENETRATION, C
EFFICACY IN BIOASSAYS WITH *P. xylostella*.

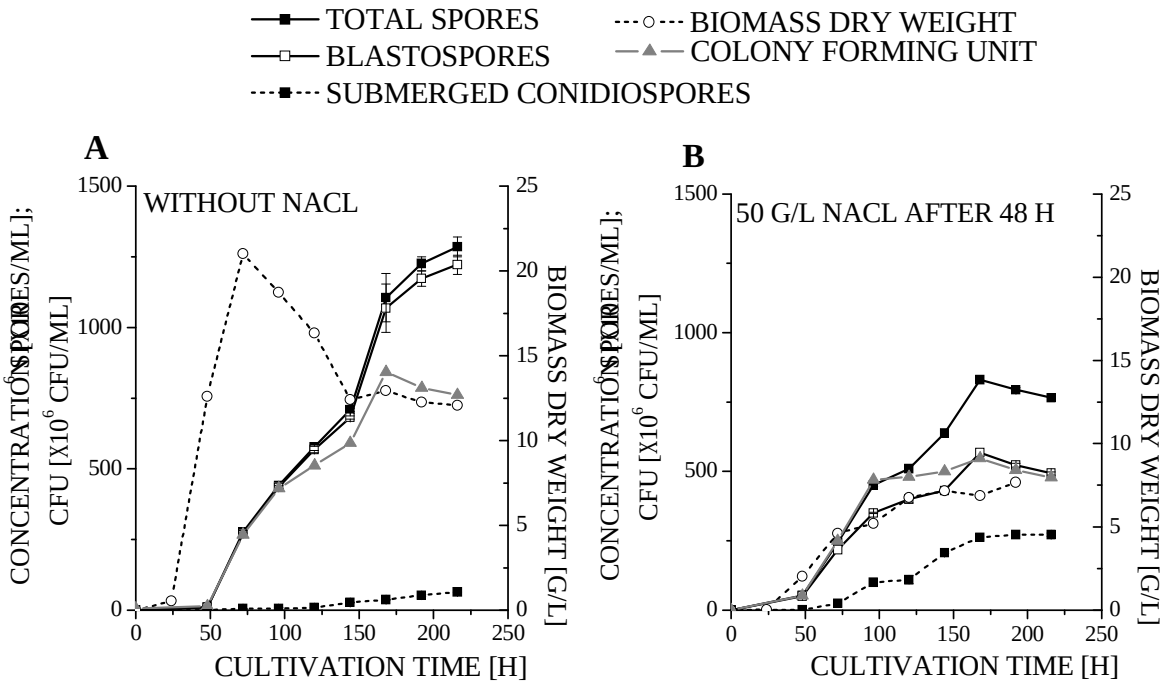

FIGURE 2. CULTIVATION OF *B. bassiana* WITHOUT NACL (A) AND IN PRESENCE OF NACL (B) IN A 2
STIRRED TANK REACTOR. THE CONCENTRATIONS OF TS, BS, SCS AND CORRELATION OF S
BIOMASS AND CFU ARE SHOWN.

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Insect pathogens and entomoparasitic nematodes
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Development of analytical tools to monitor the fate of *METARHIZIUM ANISOPLIAE* metabolites in the environment

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Abstract: DESTRUXINS (DTXS) ARE STRUCTURALLY CLOSELY RELATED CYCLIC HEXADEPSIPEPTI
RELEVANT METABOLITES BY THE ENTOMOPATHOGENIC FUNGUS *Metarhizium anisopliae*. TO MONITOR DTXS IN
FUNGAL CULTURE BROTH, PLANT DERIVED MATRICES AND CELL CULTURES, A FAST AND SE
UHPLC-DAD/MS METHOD WAS ESTABLISHED. SAMPLE PREPARATION WAS CARRIED OUT BY A S
EXTRACTION (SPE) ON A REVERSED PHASE MATERIAL. OPTIMAL PURIFICATION WAS ACHIEVED B
WITH 40% (V/V) METHANOL, REMOVING MOST OF THE POLAR COMPONENTS. THE HIGHEST AMOUN
OBTAINED BY USING 85% (V/V) METHANOL FOR ELUTION. AN UHPLC-DAD SYSTEM HYPHENATED TO
MASS SPECTROMETER WAS UTILIZED TO SEPARATE AND DETECT THE DTX CONGENERS. A SUB
COLUMN WAS USED AS STATIONARY PHASE, WITH A WATER/ACETONITRILE SOLVENT GRADIENT A
MIN^{-1} SERVING AS MOBILE PHASE. A TOTAL ANALYSIS TIME OF 12 MIN WAS ACHIEVED WITH THE
ASSAY WITH THE DTX CONGENERS ELUTING FROM 1 MIN TO 8 MIN WITH A HIGHER RESOLUTIO
COMPARED TO PREVIOUS HPLC-DAD ASSAYS. BESIDES THE AVAILABLE REFERENCE COMPOUNDS
DTXE, DTXE-DIOL 18 DTX DERIVATIVES WERE TENTATIVELY IDENTIFIED BY ANALYZING TOF-MS DA

Key words: *Metharhizium anisopliae*, ENTOMOPATHOGEN, METABOLITES, DESTRUXIN, HPLC-DAD/MS

Introduction

THE ENTOMOPATHOGENIC FUNGUS *Metarhizium anisopliae* PLAYS AN IMPORTANT ROLE AS A
BIOLOGICAL CONTROL AGENT (BCA), AND HAS BEEN USED FOR ABOUT 130 YEARS (ZIMME
IT IS KNOWN THAT THIS FUNGUS HAS AN EFFECT AGAINST MANY PEST INSECTS INCLUDI
WIREWORMS, WESTERN CORN ROOTWORM, BLACK VINE WEEVIL AND SCIARIDS. FUNGI
VARIETY OF BIOLOGICAL ACTIVE COMPOUNDS, SUCH AS SECONDARY METABOLITES OR T
ACT AS PATHOGENICITY DETERMINANTS BY IMPROVING THE INFECTION AND COLONISA
INSECT (STRASSER *et al.*, 2011).

THE MAIN METABOLITES PRODUCED BY THE FUNGUS *M. anisopliae* ARE DESTRUXINS (DTXS), CYCLIC
HEXADEPSIPEPTIDES COMPOSED OF 2-HYDROXY ACID AND FIVE AMINO ACID RESIDUES. THEY EXH
A WIDE VARIETY OF BIOLOGICAL ACTIVITIES, FOR EXAMPLE IMPORTANT CYTOTOXIC EFF
ARE BEST KNOWN FOR THEIR INSECTICIDAL AND PHYTOTOXIC ACTIVITIES (PEDRAS *et al.*, 2001).

HOWEVER, THERE ARE CONCERNS REGARDING WHETHER THESE FUNGI AND THEIR PR
METABOLITES ENTAIL RISKS TO HUMANS AND ENVIRONMENT, THE EU FUNDED PROJE
("INNOVATIVE BIOLOGICAL PRODUCTS FOR SOIL PEST CONTROL, NO. 282767") WILL IM
RAFBCA-REBECA DECISION SCHEME, WHICH HAS BEEN TESTED IN CASE STUDIES ON
METABOLITES AND CRUDE EXTRACTS (SCHAU *et al.*, 2014) CONCERNING THE QUESTION WHETHER
DESTRUXINS POSE A RISK TO HUMAN HEALTH THE AIM OF THIS STUDY IS TO ASSESS IF THE
ENTER THE FOOD CHAIN. CONSEQUENTLY, PROTOCOLS FOR ISOLATING, QUALITATIVE

QUANTITATIVE DETERMINATION OF SELECTED METABOLITES FROM *M. anisopliae* HAVE TO BE DEVELOPED.

AS PREREQUISITE A HPLC–DAD/MS ASSAY TO MONITOR DESTRUXINS IN FUNGAL CUL BASED ON A PREVIOUSLY REPORTED ASSAY (SEE *e.g.* [2]) WAS ESTABLISHED: THE NOVEL METHOD SHALL SERVE AS BASIS FOR FURTHER ASSAY DEVELOPMENT IN FOOD MATRICES.

Material and methods

CULTIVATION OF METARHIZIUM ANISOPLIAE

Metarhizium anisopliae var. anisopliae WAS CULTIVATED IN SABOURAUD DEXTROSE (SD) LIQ MEDIUM (S2G, MERCK 1.08339, VIENNA) SUPPLEMENTED WITH 2% (W/V) GLUCOSE (NEC 4445.5000 HEIDELBERG, GERMANY), AT 25 °C AND 65% RELATIVE HUMIDITY FOR 2 W INOCULATION WAS DONE BY PIPETTING 500 µL OF THE STOCK INOCULUM PER FLASK. TH CULTURE WAS STIRRED AT 250 RPM TO ENSURE A BIOMASS DRY WEIGHT OF 10 G L[1]. BEFORE HARVESTING THE LIQUID LOST DUE TO EVAPORATION WAS REPLACED WITH DEIONIZED W WERE UNIFIED TO ONE POOLED SAMPLE OF CULTURE BROTH. NON-INOCULATED MEDIUM CONTROL FOR ANALYTICS. THE POOLED CULTURE BROTH WAS CENTRIFUGED AND TH FILTRATED OVER A TARED FILTRATION GAUZE.

SAMPLE PREPARATION FROM CULTURE FILTRATE

SAMPLE PREPARATION WAS CARRIED OUT BY A SOLID PHASE EXTRACTION (SPE) ON A REVERSED PHASE MATERIAL (STRATA C18-E, PHENOMENEX, ASCHAFFENBURG, GERMA PURIFICATION WAS ACHIEVED BY A WASHING-STEP WITH 40% (V/V) METHANOL. FOR EL DESTRUXINS A 85% (V/V) METHANOLIC SOLUTION WAS USED.

HPLC-DAD/MS CONDITIONS

AN AGILENT 1200 UHPLC-DAD SYSTEM (AGILENT) WAS UTILIZED TO SEPARATE AND DE CONGENERS. A ZORBAX ECLIPSE XDB-C18 COLUMN (50 X 2.1 MM, 1.8 µM PARTICLE SIZE, AG WAS USED AS STATIONARY PHASE, WITH A WATER (A) / ACETONITRILE (B), EACH CONTAIN ACETIC ACID, GRADIENT AT A FLOW RATE OF 0.3 ML MIN[1] SERVING AS MOBILE PHASE. A BRUKER MICROTOF-QII MASS SPECTROMETER (TOF-MS; BRUKER DALTONICS, BREMEN, GERMANY) W TO DETECT AND IDENTIFY DTX CONGENERS. EXPERIMENTS WERE PERFORMED IN POSITIVE

ASSAY VALIDATION

FOR CALIBRATION FUNCTIONS METHANOLIC DILUTION SERIES OF DTXA, DTXB, DTXE RE WERE PREPARED. THE METHOD WAS FULLY VALIDATED INCLUDING THE LIMIT OF DETE LIMIT OF QUANTIFICATION (LOQ) VALUES, REPEATABILITY AND REPRODUCIBILITY.

Results and discussion

SAMPLE PREPARATION

A SOLID PHASE EXTRACTION (SPE) WAS DEVELOPED TO ISOLATE, CONCENTRATE, AND GENERS FROM *M. anisopliae* CULTURE BROTH PRIOR TO HPLC ANALYSIS. THE DEVELOPED S PREPARATION PROTOCOL MAKES IT POSSIBLE TO EXTRACT DTXS AND TO CLEAN UP SAM STEPS.

TO DETERMINE THE OPTIMUM RATIO OF METHANOL AND WATER FOR THE WASH STEP STEP SIX DIFFERENT METHANOLIC SOLUTIONS WERE PREPARED INCREASING IN 5% STEPS. CONDUCTED IN 5 REPLICATES FOR EACH CONCENTRATION AND SAMPLE PREPARATION ABOVE: FIRST USING THE DIFFERENT WASH SOLVENTS AT THE SAME ELUTION CONDITION

DIFFERENT ELUTION SOLVENTS AT THE SAME WASH CONDITIONS. FOR EVALUATION WHIC METHANOL CAN BE USED AS WASH SOLVENT, SO THAT POLAR COMPONENTS WERE REMO WERE RETAINED FROM THE SORBENT, THE ELUATE WAS MEASURED. FROM 25% (V/V) U METHANOL THE ANALYTE YIELDS WERE RATHER CONSTANT. UP TO 40% (V/V) METHANO WHICH INDICATES THAT ANALYTES STARTED TO ELUTE ALREADY IN THE WASH STEP (FIG 40% (V/V) METHANOLIC SOLUTION FOR THE WASH-STEP ALL UNDESIRED POLAR ANALYTE SO THAT DTXS CAN BE ELUTED WITH THE OPTIMIZED 85% (V/V) METHANOLIC SOLUTI WITHOUT DISTURBING COMPOUNDS.

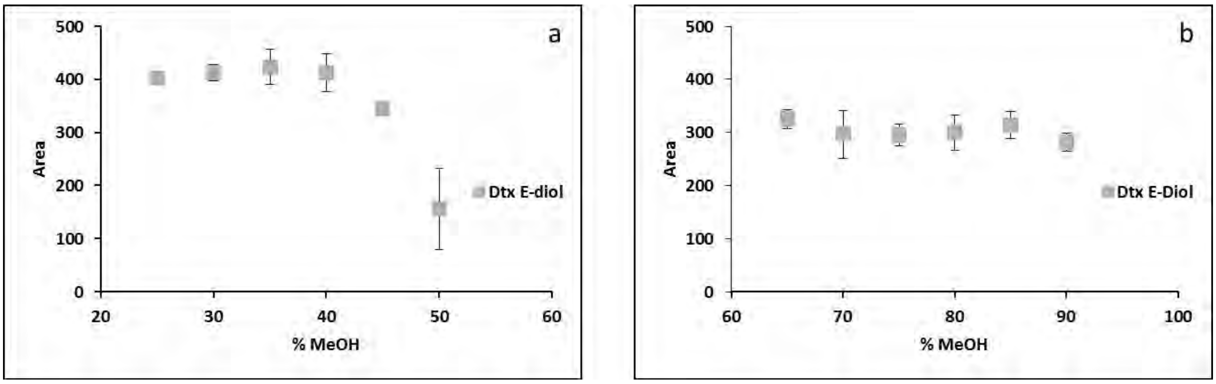

FIGURE 1: (A) COMPARISON OF ANALYTE YIELDS FOR DTX E-DIOL. MEAN RESULTS (N = 5) USING DIFFERENT METHANOLIC WASH SOLVENTS. (B) COMPARISON OF ANALYTE YIELDS MEAN RESULTS (N = 5) ACHIEVED BY USING DIFFERENT METHANOLIC ELUTION SOLVENT INDICATE THE STANDARD DEVIATION (SD).

HPLC-DAD METHOD DEVELOPMENT

THE METHOD DEVELOPMENT WAS CARRIED OUT USING *M. anisopliae* CULTURE BROTH SAMPLES. BEST RESULTS COULD BE ACHIEVED USING THE ZORBAX ECLIPSE XDB-C18 RAPID RESOLUTION ADDITION OF ACIDIC ADDITIVES SHOWED DIFFERENCES IN THE RESOLUTION OF PEAKS RE POLAR COMPOUNDS ELUTING IN THE FIRST MINUTES. AS FINAL SOLVENTS WATER (A) AND EACH CONTAINING 0.02% (V/V) ACETIC ACID, WERE USED. WITH THIS COMPOSITION OF THE THE OPTIMIZED GRADIENT PEAKS WERE BETTER SEPARATED, A HIGHER RESOLUTION CA BASELINE CAN BE STABILIZED.

HPLC-DAD METHOD VALIDATION

ASSAY VALIDATION WAS PERFORMED USING *M. anisopliae* CULTURE BROTH SAMPLES AND DILUTION SE OF REFERENCE MATERIAL IN METHANOL. FOR ALL DILUTION SERIES OF DTXA, DTXB CALIBRATION FUNCTIONS COULD BE REACHED. THE CALIBRATION RANGE OF DTXA WAS TO 600 MG L^{-1}, OF DTX B BETWEEN 0.5 TO 200 MG L^{-1} AND FOR DTXE BETWEEN 0.5 TO 400 MG L^{-1}. THE LODS OF DTX A, B AND E RANGED BETWEEN 0.05 AND 0.4 MG L^{-1} AND THE LOQS BETWEEN 0.14 MG L^{-1} AND 1.2 MG L^{-1}.

IDENTIFICATION OF DESTRUXINS FROM CULTURE FILTRATE

WITH THE OPTIMIZED METHOD IT WAS POSSIBLE TO SEPARATE 22 ANALYTES (FIGURE 2) MINUTES. COMPARED WITH THE METHOD OF HU *et al.* (2004) MORE PEAKS CAN BE BASELINE-SEPARATED, THE METHOD SHOWED A HIGHER RESOLUTION THAN THE PREVIOUS ASSA IDENTIFICATION WAS FACILITATED BY ANALYZING AND COMPARING TOF-MS DATA USIN AND SPECIFIC FRAGMENTATION PATTERN WITH DATA FROM LITERATURE (HU *et al.*, 2004; KRASNOFF *et al.*, 1998).

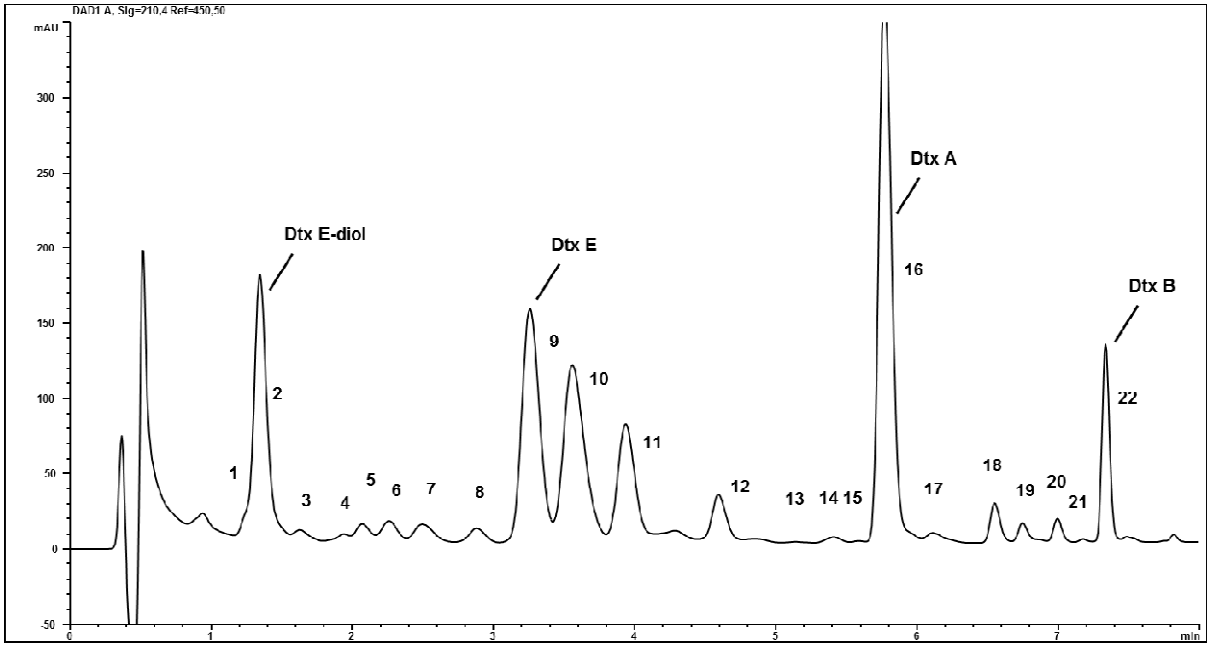

FIGURE 2: SEPARATION OF DESTRUXIN CONGENERS (E.G. DTX A, B, E, E-DIOL). REPRESENTATIVE – DAD CHROMATOGRAM OF *Metarhizium anisopliae* CULTURE BROTH, SAMPLE RECORD

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Cross-species transferability of 41 microsatellite markers for *METARHIZIUM* spp.

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Abstract: THE GENUS *Metarhizium* INCLUDES INSECT PATHOGENIC FUNGAL SPECIES, WHICH ARE U BIOLOGICAL CONTROL AGENTS (BCAS). GENETIC TOOLS FOR IDENTIFICATION AND MONITORING IMPORTANT. A GENOTYPING TOOL BASED ON 41 SIMPLE SEQUENCE REAPEAT (SSR) MARKERS HAS FOR *M. anisopliae s.l.* HOWEVER, DETAILED PHYLOGENETIC ANALYSES BASED ON A MULTILOCU REVEALED THAT *M. anisopliae s.l.* IS A CRYPTIC SPECIES COMPLEX OF NINE DIFFERENT *Metarhizium* SPE ACCORDING TO THIS NEW TAXONOMY, THE 41 SSR MARKERS WERE ISOLATED FROM *M. brunneum, M. robertsii* OR *M. anisopliae s.s.* THE GOAL OF THIS STUDY WAS TO ASSESS THE TRANSFERABILITY OF THE 41 SS INDIVIDUAL SPECIES OF THE *M. anisopliae* SPECIES COMPLEX. SUCCESSFUL PCR-AMPLIFICATION OF SS MARKERS WAS OBSERVED IN ALL SPECIES BUT THE NUMBER OF LOCI YIELDING PCR PRODUCT SPECIES. AMPLIFICATION OF INDIVIDUAL SSR LOCI DID NOT ALWAYS YIELD PRODUCTS FOR PARTICULAR SPECIES AND NOT ALL WERE POLYMORPHIC. THE STUDY REVEALED THAT SSR TRANSFERRED TO DIFFERENT SPECIES OF THE *M. anisopliae* SPECIES COMPLEX. HOWEVER, THE NUMBER OF AVAILABLE SSR MARKERS STRONGLY DEPENDS ON THE SPECIES TO BE ANALYZED. THE MARKE VALUABLE TOOL FOR IDENTIFICATION AND MONITORING OF *Metarhizium* BCAS AND THEY WILL ALLOW INVESTIGATION OF GENETIC DIVERSITY AND POPULATION STRUCTURE OF SEVERAL SPECIES OF THE FORMER *M. anisopliae* SPECIES COMPLEX.

Key words: SIMPLE SEQUENCE REPEATS, SSR MARKERS, *Metarhizium* SPP., GENOTYPING

Introduction

ENTOMOPATHOGENIC FUNGI OF THE GENUS *Metarhizium* CONSTITUTE AN IMPORTANT BIOTIC COMPONE IN THE NATURAL REGULATION OF ARTHROPOD POPULATIONS INCLUDING AGRONOMICA *Metarhizium* SPP. HAVE A HISTORY IN USE AS BIOCONTROL AGENTS (MEYLING & EILENBERG VARIOUS PRODUCTS ARE COMMERCIALLY AVAILABLE (JABA, 2009). IN A RECENT STUDY THE TAXONOMY OF *Metarhizium* SPP. AND IN PARTICULAR THE *Metarhizium anisopliae* SPECIES COMPLEX (*M. anisopliae s.l.*) HAS BEEN REVISED BASED ON A MULTILOCUS PHYLOGENETIC ANALYSIS (*Bischoff et al.*, 2009). WITHIN *M. anisopliae s.l.* NINE TERMINAL TAXA ARE NOW RECOGNIZED, I.E. *M. anisopliae s.s., M. brunneum, M. globosum, M. guizhouense, M. lepidiotae, M. majus, M. pingshaense* AND *M. robertsii* (FIGURE 1). CURRENTLY, SPECIES AFFILIATION OF *Metarhizium* ISOLATES IS PERFORMED BY SEQUENCING THE 5' END OF ELONGATION FACTOR 1-ALPHA ALIGNMENT OF OBTAINED SEQUENCES TO REFERENCE SEQUENCES AS DESCRIBED BY *Bischoff et al.* (2009).

AVAILABILITY OF EFFICIENT TOOLS THAT ALLOW GENOTYPING, AND DETECTION, OF CRUCIAL TO ALLOW MONITORING OF AN APPLIED BCA OR ASSESSMENT OF ITS HOST A DEPENDENT OCCURRENCE, POPULATION STRUCTURE, OR ITS POSSIBLE EFFECTS ON NON-T GENOTYPING TOOL, WHICH IS BASED ON 41 SINGLE SEQUENCE REPEAT (SSR) MARKERS (M HAS BEEN DEVELOPED FOR *M. anisopliae s.l.* (ENKERLI *et al.*, 2005, OULEVEY *et al.*, 2009).

MICROSATELLITES ARE SHORT DNA SEQUENCE MOTIVES (1 TO 6 BASES) THAT OCCUR AS
THE NUMBER OF REPEATS IN EACH PARTICULAR SSR LOCUS CAN BE HIGHLY VAR
POLYMORPHISM) BETWEEN INDIVIDUALS, WHICH MAKES THEM EFFECTIVE FOR IDENTIFI
AND POPULATION GENETIC ANALYSIS (QUELLER *et al.*, 1993). MICROSATELLITE MARKERS ARE AMPLIFIED BY
PCR AND THE SIZE OF THE RESULTING PRODUCTS (ALLELE SIZE) ARE DETERMINED BY C
ELECTROPHORESIS. ACCORDING TO THE NEW TAXONOMY, THE 41 SSR MARKERS HAVE BE
M. brunneum (27 SSR MARKERS), *M. robertsii* (6 SSR MARKERS) OR *M. anisopliae s.s.* (8 SSR
MARKERS). THE GOAL OF THIS STUDY WAS TO ASSESS THE TRANSFERABILITY OF THE 4
INDIVIDUAL SPECIES OF THE FORMER *M. anisopliae* SPECIES COMPLEX.

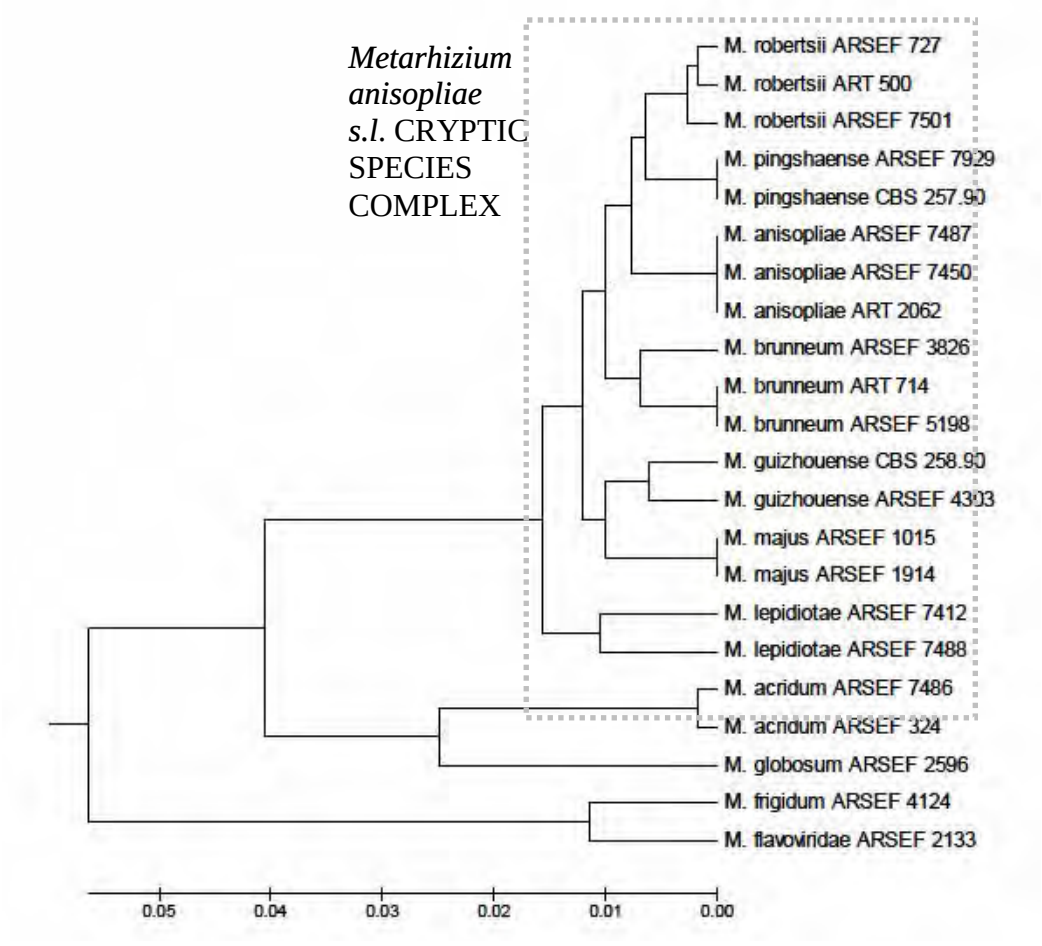

FIGURE 1. UPGMA PHYLOGENETIC TREE BASED ON AN ALIGNMENT OF 21 SEQUENCES OF T
ELONGATION FACTOR 1-ALPHA (~ 630 BP). 18 SEQUENCES WERE OBTAINED FROM
(HTTP://WWW.NCBI.NLM.NIH.GOV/GENBANK) AND 3 SEQUENCES WERE SEQUENCED IN THE
STUDY (ART 500, ART 714 AND ART 2062).

Material and methods

FUNGAL STRAINS AND DNA EXTRACTION

A COLLECTION OF 50 FUNGAL STRAINS INCLUDING THE *Metarhizium* SPECIES *M. acridum, M. anisopliae s.s., M. brunneum, M. guizhouense, M. lepidiotae, M. majus, M. pingshaense* AND *M. robertsii* WAS USED. FUNGAL STRAINS WERE OBTAINED FROM THREE DIFFERENT COLLE

ARS COLLECTION OF ENTOMOPATHOGENIC FUNGAL CULTURES (ARSEF, ITHACA, NY, U
COLLECTION (CENTRAALBUREAU VOOR SCHIMMELCULTURES, UTRECHT, NETHERLAND
RECKENHOLZ-TÄNIKON (ART) COLLECTION. *Metarhizium globosum* WAS NOT INCLUDED IN THIS STUDY AS ONL
ONE STRAIN IS AVAILABLE FROM ABOVE CULTURE COLLECTIONS. STRAINS WERE GROWN
SABOURAUD-DEXTROSE-AGAR (SDA, DIFCO BD, FRANKLIN LAKES, NJ, USA), MYCELIUM WA
AND HARVESTED AS DESCRIBED BY *et al.*, 2011). DNA OF *Metarhizium* STRAINS WAS
EXTRACTED FROM 20 MG LYOPHILIZED MYCELIUM USING PLANT NUCLEOSPIN® PLANT II KIT (MACHEREY-
NAGEL, EASTON, PA) ACCORDING TO MANUFACTURER'S INSTRUCTIONS. GENOMIC DNA C
WAS DETERMINED USING A NANODROP 1000 SPECTROPHOTOMETER (THERMO FISCHER
WALTHAM, MA).

SSR MARKER AMPLIFICATION AND DATA ANALYSIS

THE 41 SSR MARKERS PREVIOUSLY DEVELOPED (ENKERLI *et al.*, 2004; OULEVEY *et al.*, 2009) WERE
TESTED ON ALL STRAINS OF THE ISOLATE COLLECTION. PCR AMPLIFICATIONS WERE PERFORMED AS DESCRIE
BY OULEVEY *et al.* (2009). AMPLIFICATION PRODUCTS WERE ANALYSED ON AN ABI 3130XL SEQ
EQUIPPED WITH 36 CM CAPILLARIES FILLED WITH POP-7 POLYMER (APPLIED BIOSYSTEMS,
CA, USA) AND USING GENESCAN® 500 HD [ROX] SIZE STANDARD (APPLIED BIOSYSTEMS,
FOSTER CITY, CA, USA) AS INTERNAL SIZE STANDARD. ALLELE SIZES WERE SCORED WITH
GENMARKER V1.91 (SOFTGENETICS LLC, STATE COLLEGE, PA, USA).

Results and discussion

THE NUMBER OF SSR LOCI YIELDING SCORABLE PCR PRODUCTS VARIED STRONGLY AMON
Metarhizium SPP. (TABLE 1.). FOR *M. brunneum* PCR AMPLIFICATION OF 40 SSR LOCI, INCLUDING
THE 27 SSR LOCI ISOLATED FROM THIS SPECIES (ENKERLI *et al.*, 2005), REVEALED SCORABLE PRODUCTS.
FOR THE SPECIES *M. anisopliae s.s.*, *M. pingshaense*, *M. robertsii*, AND *M. guizhouense* 36, 34,
36, AND 33 SSR LOCI, RESPECTIVELY, REVEALED SCORABLE PCR PRODUCTS, WHEREAS FO
M. majus, *M. lepidiotae* AND *M. acridum* AMPLIFICATION PRODUCTS WERE OBTAINED FROM 27
AND 10 SSR LOCI, REPSECTIVELY. HOWEVER, SSR LOCI YIELDING SCORABLE PRODUCTS D
YIELD PRODUCTS FOR ALL STRAINS OF A PARTICULAR SPECIES. FOR EXAMPLE, FOR *M. brunneum*
40 SSR LOCI, INCLUDING 20 OF THE LOCI ISOLATED FROM THIS SPECIES, YIELDED PRODUCT
M. brunneum STRAINS ANALYZED. SEVEN SSR LOCI YIELDED PCR PRODUCTS FROM 6 TO 10
M. brunneum STRAINS ONLY. FOR *M. pingshaense*, *M. anisopliae s.s* *M. robertsii*, *M. majus*,
M. guizhouense AND *M. lepidiotae*, 30, 30, 28, 22, 21, AND 14 SSR MARKERS REVEALED PCR
PRODUCTS FROM ALL THE STRAINS, RESPECTIVELY. SEQUENCE DIFFERENCES IN THE PRIM
AMONG DIFFERENT SPECIES OR STRAINS ARE MOST LIKELY THE REASON FOR THE DIFFER
PCR AMPLIFICATION. POLYMORPHIC LOCI (I.E. DIFFERENT ALLELES ARE DETECTED AT A
SPECIES) WERE OBSERVED IN ALL SPECIES. THE LARGEST NUMBER OF LOCI DISPLAYING
POLYMORPHISM WAS DETECTED FOR *M. brunneum* (37 POLYMORPHIC LOCI) FOLLOWED BY
M. pingshaense AND *M. anisopliae s.s.* (27 POLYMORPHIC LOCI). LOCI DISPLAYING SPECIES SPECI
ALLELES ACROSS DIFFERENT SPECIES WERE ALSO OBSERVED.

THE PRESENT STUDY SHOWED THAT SSR MARKERS ISOLATED FROM *M. brunneum*, *M. robertsii*,
M. anisopliae s.s. CAN BE TRANSFERRED TO DIFFERENT SPECIES OF THE *M. anisopliae* SPECIES
COMPLEX. THE NUMBER OF SSR MARKERS THAT CAN BE APPLIED STRONGLY DEPENDS ON
BE ANALYZED AND AVAILABLE MARKERS FOR A GIVEN *Metarhizium* SPECIES DO NOT NECESSARILY
CORRESPOND TO THE MARKERS AVAILABLE FOR OTHER *Metarhizium* SPECIES. THIS FACT EXPLAINS
PREVIOUS OBSERVATIONS, WHERE SSR MARKERS WERE NOT CONSISTENTLY AMPLIFIED F
THE *M. anisopliae* SPECIES COMPLEX (ENKERLI, UNPUBLISHED). HOWEVER, THERE ARE MA

CAN BE USED TO ANALYZE MORE THAN ONE *Metarhizium* SPP. AT THE SAME TIME. FOR EXAMPLE, 26 SSR MARKERS ARE AVAILABLE THAT CAN BE APPLIED TO *M. brunneum* AND *M. robertsii* AND 14 OF THESE 26 MARKERS ARE POLYMORPHIC IN BOTH SPECIES. THE STUDY REVEALED THAT I NINE SPECIES OF THE *M. anisopliae* SPECIES COMPLEX 15 TO 37 POLYMORPHIC SSR MARKER ARE AVAILABLE. THESE MARKERS WILL PROVIDE A VALUABLE TOOL FOR IDENTIFICATIO *Metarhizium* BCAS AND THEY WILL ALLOW INVESTIGATION OF GENETIC DIVERSITY AND STRUCTURE OF SEVEN SPECIES OF THE FORMER *M. anisopliae* SPECIES COMPLEX.

TABLE 1. CROSS-SPECIES TRANSFERABILITY OF THE 42 *Metarhizium* SSR MARKERS. THE NUMBER OF STRAINS ANALYZED PER SPECIES, THE NUMBER OF SSR LOCI YIELDING PCR PRODUCTS, SSR LOCI SUCCESSFULLY AMPLIFIED FROM ALL STRAINS OF A SINGLE SPECIES AND POLYMORPHIC LOCI ARE SHOWN.

SPECIES	NR. OF STRAINS ANALYZED	SSR MARKERS YIELDING PCR PRODUCTS	AMPLIFICATION FROM ALL STRAINS	POLYMORPHIC LOCI
M. acridum	9	10	7	1
M. anisopliae s.s.	4	36	30	27
M. brunneum	11	40	33	37
M. guizhouense	5	33	21	21
M. lepidiotae	4	24	14	15
M. majus	6	27	22	18
M. pingshaense	4	34	30	27
M. robertsii	5	36	28	23

Acknowledgements

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Posters

Insect pathogens and entomoparasitic nematodes
IOBC-WPRS Bulletin Vol. 90, 2013
pp. 87-90

A review of the use of entomopathogenic fungi for the control of *BEMISIA TABACI* (Hemiptera: Aleyrodidae) in the UK

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Review

THE SWEETPOTATO WHITEFLY *Bemisia tabaci* GENNADIUS (HEMIPTERA: ALEYRODIDAE) IS A MAJO
PEST OF ECONOMICALLY IMPORTANT CROPS WORLDWIDE (Xie *et al.*, 2012). *Bemisia tabaci* DAMAGES
CROPS BY FEEDING ON PHLOEM SAP AND THE LARGE AMOUNTS OF STICKY HONEYDEW
LOWER THE RATE OF LEAF PHOTOSYNTHESIS. THIS SPECIES OF WHITEFLY IS ALSO A VEC
VIRUSES (POWELL, 2012). WITHIN THE UNITED KINGDOM *B. tabaci* REMAINS A NOTIFIABLE
PEST SUBJECT TO A POLICY OF ERADICATION IF FOUND ON PROPAGATORS PREMISES, P
TRADE, AND CONTAINMENT/ERADICATION IF OUTBREAKS OCCUR ATTANUBSE (CUTHBE
ENTOMOPATHOGENIC FUNGI CAN PENETRATE AND CAUSE THE DEATH OF MANY ECONOM
PESTS. THEY CAN FORM EFFECTIVE BIOLOGICAL ALTERNATIVES TO CHEMICAL PESTIC
CUTHBERTSON *et al.* (2005A, 2010, 2012) HAVE DEMONSTRATED THEIR POTENTIAL AND HAVE DET
THAT THE SECOND AND THIRD INSTARS OF *B. tabaci* ARE THE MOST SUSCEPTIBLE LIFE-STAGES TO B
Lecanicillium muscarium AND *Beauveria bassiana* (FIGURE 1)

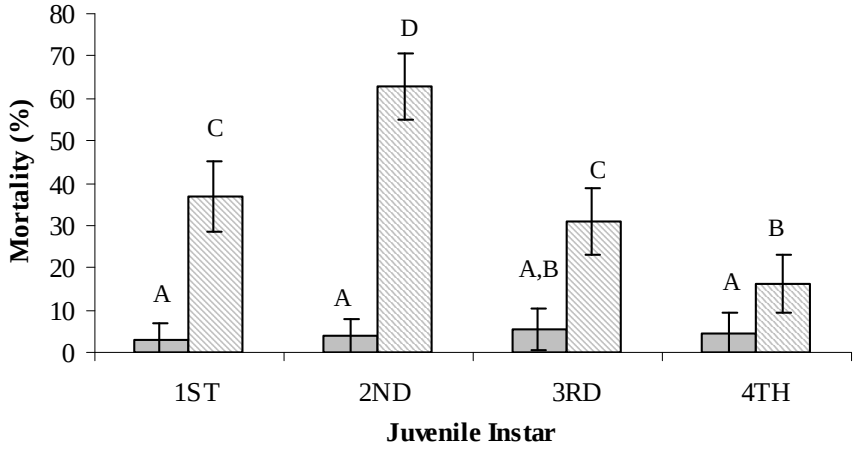

FIGURE 1. THE SUSCEPTIBILITY OF THE IMMATURE STAGES OF *Bemisia tabaci* TO ENTOMOPATHOGENIC FUNGI *Lecanicillium muscarium* ON VERBENA PLANTS. COLUMNS WITH THE SAME LETTER ARE NO SIGNIFICANTLY DIFFERENT. CONTROL, *Lecanicillium muscarium* + 0.02% AGRAL (CUTHBERTSON, *et al.*, 2005A).

DUE TO THE DIFFERENCES IN SENSITIVITY OF FUNGAL SPECIES TO DIFFERENT FORM
SAME INSECTICIDE, INFORMATION IS REQUIRED ON THE COMPATIBILITY OF EACH ENTOMO
AND CHEMICAL PRODUCT TO BE USED WITHIN A GIVEN IPM STRATEGY. FORMULATIO
INSECTICIDES MAY DIFFER IN TOXICITY TO FUNGI DUE TO THE USE OF DIFFERENT SUR
FUNGI SPECIES MAY ALSO DIFFER IN SENSITIVITY TO DIFFERENT FORMULATIONS OF THE

THEREFORE, INFORMATION REGARDING COMPATIBILITY BETWEEN ENTOMOPATHOGENI
CHEMICAL PRODUCT FOR AN IPM SYSTEM NEEDS TO BE TESTED INDIVIDUALLY WITHIN T
WHICH IT WILL BE APPLIED. THE OPTIMUM USE OF AN IPM SYSTEM FOR PEST CONTROL MA
SEQUENTIAL RATHER THAN SIMULTANEOUS APPLICATIONS OF INSECTICIDES AND ENTO
(CUTHBERTSON & WALTERS, 2005).

WITHIN THE UK, ONLY CUTHBERTSON *et al.* (2005B, 2010, 2012) HAVE INVESTIGATED THE COMBINATION OF CHEMICALS ROUTINELY USED FOR THE CONTROL OF WHITEFLY V PROMISING RESULTS HAVE BEEN OBTAINED. IN REGARDS TO MIXING CHEMICALS WITH *L. muscarium*, DIRECT EXPOSURE FOR 24 H TO IMIDACLOPRID, NICOTINE AND TEFLUBENZURON RESU SPORE GERMINATION, UNSUITABLE FOR COMMERCIAL USE. ONLY THE ACTIVE INGRE PROVIDED AN ACCEPTABLE LEVEL OF SPORE GERMINATION.

THE IMPLEMENTATION OF AN IPM SCHEME MAY REQUIRE SEQUENTIAL RATHER THA APPLICATIONS OF INSECTICIDES AND ENTOMOPATHOGENIC FUNGI BUT FEW PREVIOUS ST THE EFFECT OF DRY INSECTICIDE RESIDUES ON FUNGAL ACTIVITY. RECENT WORK (CU *et al.*, 2005B) HAS SHOWN THAT WHEN *L. muscarium* WAS APPLIED TO PLANTS SPRAYED 24 H EARLIER WI STANDARD COMMERCIAL APPLICATION OF ONE OF THREE CONTACT INSECTICIDES OR WI SYSTEMIC INSECTICIDE, NO SIGNIFICANT REDUCTION IN INFECTIVITY (MYCELIAL GROWT ANY CASES. THEREFORE, *L. muscarium* COULD BE APPLIED SEQUENTIALLY WITH IMIDACLO BUPROFEZIN, NICOTINE AND TEFLUBENZURON IN A COMMERCIAL IPM STRATEGY. IN *B. bassiana* PROVED SUITABLE FOR TANK MIXING WITH A RANGE OF PRODUCTS INCLUDING (CUTHBERTSON *et al.*, 2012). FOLLOWING SEQUENTIAL APPLICATIONS OF *L. muscarium* AND CHEMICALS, MORTALITIES OF UP TO 90% OF *B. tabaci* SECOND INSTARS WERE RECORDED (TABLE 1) (CUTHBERT *et al.*, 2005B). SEQUENTIAL TREATMENTS OFFER A GREATER FLEXIBILITY IN TIMING APPLI VARIOUS LIFE STAGES OF THE PEST.

TABLE 1. THE RESULTS OF EXPERIMENTS INVESTIGATING THE EFFECT OF CHEMICAL RES PLANTS ON THE INFECTIVITY OF *Lecanicillium muscarium* (CA 1.5 X 10^5 SPORES CM^{-2} OF LEAF AREA) AGAINST *Bemisia tabaci* SECOND INSTAR LARVAE. THE SECOND TREATMENT APPLICATION WAS HOURS FOLLOWING THE FIRST TREATMENT. DATA REPRESENT THE MODEL DERIVED PE (± 95% CONFIDENCE INTERVALS) OF *B. tabaci* LARVAE 3 DAYS AFTER FINAL TREATMENT APPLIC WITHIN BOTH ROWS AND COLUMNS MEANS WITH THE SAME LETTER EXHIBIT OVERLAP INTERVALS (CUTHBERTSON *et al.*, 2005B).

INSECTICIDE TESTED	NO. OF *B. tabaci*	1ST APPL. 2ND APPL.	MORTALITY OF *Bemisia tabaci* TREATMENT GROUPS A WATER WATER	B INSECTICIDE WATER	C INSECTICIDE *L. muscarium*	D INSECTICIDE *L. muscarium*
BUPROFEZIN (30ML/100L)	673		5.6 ± 5.2^{A}	67.0 ± 10.6^{BC}	68.5 ± 10.4^{BC}	77.4 ± 9.2^{B}
NICOTINE (500ML/5L)	521		7.7 ± 3.1^{A}	69.7 ± 4.9^{BC}	68.9 ± 4.9^{BC}	63.6 ± 5.3^{B}
IMIDACLOPRID (0.2G/L)	480		7.8 ± 11.3^{A}	89.1 ± 14.7^{BC}	89.2 ± 16.9^{BC}	91.4 ± 13.3^{B}
TEFLUBENZURON (500ML/1000L)	674		6.3 ± 10.0^{A}	52.1 ± 23.6^{BC}	52.8 ± 21.6^{BC}	75.6 ± 19.5^{B}

THE CHEMICAL GROUPS MOST TOXIC TO FUNGI ARE ORGANOPHOSPHATES AND CAR
COMMONLY USED INSECTICIDES, FOR EXAMPLE, BUPROFEZIN, HAVE NOW BEEN RENDERE
THE UK AGAINST *Trialeurodes vaporariorum* (GLASSHOUSE WHITEFLY) BY THE WIDESPREA
APPEARANCE OF RESISTANCE IN POPULATIONS. THIS PRODUCT HAS NOW ALSO JUST R
UNAVAILABLE FOR USE IN UK HORTICULTURE (CUTHBERTSON *et al.*, 2001). *Bemisia tabaci* HAVE ALSO
BEEN SHOWN TO OFFER A DEGREE OF RESISTANCE TO IMIDACLOPRID (HORTUS *et al.*, 2002) (SOUTHER
URGENCY TO THE DEVELOPMENT OF ALTERNATIVE IPM APPROACHES.

THE AMBIENT TEMPERATURE AND HUMIDITY ARE KNOWN TO BE IMPORTANT FACTO
FUNGI EFFICACY. TRIALS HAVE SHOWN THAT FOR OPTIMAL *Lecanicillium muscarium* FAVOURABLE CONDITIONS
FOR FUNGI SURVIVAL AND EFFICACY MUST BE MAINTAINED FOR UP TO 6-8 H FOLLOWIN
PLANT FOLIAGE. AS A RESULT, NO HOST PLANT EFFECTS ARE APPARENT (FIGURE 2) (
WALTERS, 2005).

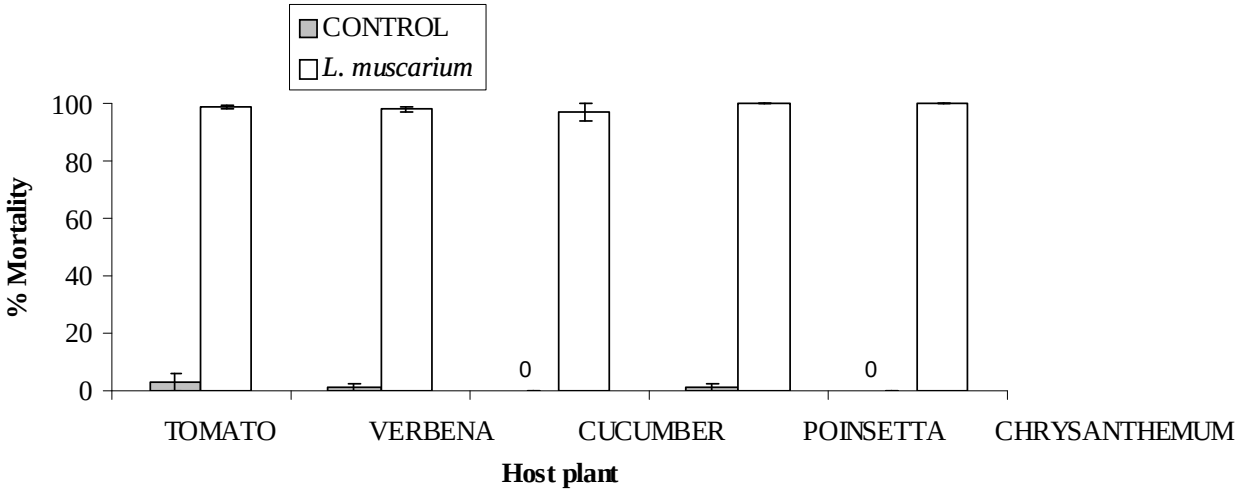

FIGURE 2. EFFICACY OF *Lecanicillium muscarium* (10^7 CONIDIA ML^{-1} 0.02% AGRAL) AGAINST
SECOND INSTAR *Bemisia tabaci* ON A RANGE OF HOST PLANTS APPLIED WITHIN A CONT
ENVIRONMENT CABINET, 20 °C, 85% RELATIVE HUMIDITY. MORTALITY RECORDED AFTER
STANDARD ERROR OF THE MEANS (± SEM) (CUTHBERTSON & WALTERS, 2005).

L. muscarium AND *B. bassiana* HAVE THE POTENTIAL TO BE IMPORTANT BIOLOGICAL C
AGENTS OF *B. tabaci*. INTEGRATED APPROACHES UTILIZING ENTOMOPATHOGENS ARE SHO
POTENTIAL. EARLY INSTARS OF *B. tabaci* ARE PROVING MOST SUSCEPTIBLE TO INFECTION, AN IMPO
FACTOR WHEN WANTING TO TARGET A QUARANTINE SPECIES AT AS EARLY A LIFE-STAGE
TO BREAK THE LIFECYCLE. THE LEVELS OF BOTH DIRECT AND INDIRECT COMPATIBILITY
CHEMICAL INSECTICIDES ALSO INCREASE THEIR POTENTIAL FOR INCORPORATING THEM IN
CONTROL OF *B. tabaci*. THEIR USE DEPENDS ON FURTHER WORK IN COMMERCIAL-SCALE GLASS
AND, IF SUCCESSFUL, THEY MAY CONTRIBUTE TO THE DEVELOPMENT OF SUSTAINABLE P
THROUGH A REDUCTION IN THE USE OF CHEMICAL INSECTICIDES AND, CONSEQUENTL
CHEMICAL RESIDUES ON PRODUCE AND INSECTICIDE RESISTANCE. FURTHER RESEARCH I
BOTH FINE TUNE THE APPLICATION TECHNIQUES AND OPTIMUM DOSE RATES REQUIRED F
THE GLASSHOUSE ENVIRONMENT.

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Effect of entomopathogic fungi against *TRIALEURODES VAPORARIORUM* and its parasitoid *ENCARSIA FORMOSA*: preliminary laboratory assays

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Abstract: *Trialeurodes vaporariorum* WESTWOOD, THE GREENHOUSE WHITEFLY, IS ONE OF T MAJOR PEST AFFECTING CROPS IN GREENHOUSE, PARTICULARLY TOMATO AND ITS BIOLOG POSSIBLE RELEASING ITS PARASITOID *Encarsia formosa* GAHAN OR USING MYCO-INSECTICIDES BASED ON THE ENTOMOPATHOGENIC FUNGI *Beauveria bassiana* VUILL. BALSAM. ENTOMOPATHOGENIC FUNGI ARE GENERALLY CONSIDERED NOT DETRIMENTAL AGAINST INSECTS NATURAL ENEMIES AND U FAUNA, DESPITE OF THE LACK OF DATA, PARTICULARLY ABOUT THE EFFECT OF THIS FUNG UNDER LABORATORY AND FIELD CONDITION.

THREE ISOLATES OF *B. bassiana* AND ONE OF *M. anisopliae* WERE TESTED FOR THEIR VIRULENCE AGAINST *Trialeurodes vaporariorum* WESTWOOD (HEMIPTERA: ALEURODIDAE) NYMPHS (SUB-PUPAE), PERFORMING 2.10^6 CONIDIA ml^{-1} SUSPENSIONS AND USING THE LEAF DISKS METHOD. TH COMMERCIAL MYCO-INSECTICIDE NATURALIS (INTRACHEM BIO ITALIA, ITALY), AND THE AT *B. bassiana* STRAIN, CONTAINED INTO THE COMMERCIAL PRODUCT, WERE INCLUDED IN TH COMPARISON. THE SAME ISOLATES WERE ALSO TESTED AGAINST WHITEFLIES SUB-PUPAE P *Encarsia formosa* GAHAN (HYMENOPTERA: APHELINIDAE) (ENCARSIA SYSTEM, BIOBEST) COMPLETE RANDOMIZED BLOCK DESIGN WITH FOUR REPLICATES WAS USED. THE WHITEFLIES THE EMERGENCE OF ADULT PARASITOIDS WAS RECORDED DAILY FOR 7 DAYS. FOR STATI CUMULATIVE MORTALITY AND CUMULATIVE NUMBER OF SURVIVING PARASITOIDS (%) WERE MEAN SURVIVAL TIME AND MEAN LETHAL TIME WERE DETERMINATED BY THE KAPLAN-MEI AND THE PROBIT ANALYSIS RESPECTIVELY. DATA WERE THEN ANALYZED PERFORMING ANALYSIS OF VARIANCE (ANOVA) AND THE HSD TUKEY TEST WAS USED TO COMPARE MEANS

RESULTS OF OUR PRELIMINARY ASSAY SHOWED A GOOD EFFICACY OF TESTED ENTOM ISOLATES AGAINST *T. vaporariorum*, WITH A FINAL CUMULATIVE MORTALITY (7 DAY AFTER TH INOCULATION) GREATER THAN THE 80% FOR ALL THE ISOLATES AND MEAN SURVIVAL TIMES 3.4 TO 4.5 DAYS. THE ATCC 74040 *B. bassiana* STRAIN AND THE COMMERCIAL PRODUCT NATURA RESULTED NOT SIGNIFICANTLY DIFFERENT FROM OUR ISOLATES. NOT SIGNIFICATIVE EFFEC THE *E. formosa* ADULTS EMERGENCE WERE DETECTED AMONG THE FUNGAL ISOLATES. THE EM PARASITOIDS IN THE UNTREATED CONTROL WAS SATISTICALLY NOT DIFFERENT FROM T TREATMENTS, EXCEPT THE CASE OF NATURALIS. THE MYCO-INSECTICIDE NATURALIS REDUC *E. formosa* EMERGENCE (20.3% 7 DAYS AFTER INOCULATION WHILE IN THE CONTROL WAS 57.6 NOT THE ATCC 74040 STRAIN ISOLATED FROM THIS PRODUCT. THIS EFFECT IS PROBABLY RE IMPROVING EFFECT OF CO-FORMULANTS, IN TERM OF ADHESION, PERSISTENCE AND PHY ACTION.

OUR RESULTS, EXCEPT THE CASE OF COMMERCIAL PRODUCT, ARE NOT IN CONTRAST WIT WHICH REVEALED THAT MATURE PARASITOID LARVAE ARE ABLE TO COMPLETE THEIR DE TREATED WITH ENTOMOPATHOGENIC FUNGI. SEVERAL AUTHORS SHOWED THAT THE TIME PARASITIZATION AND FUNGAL APPLICATION IS CRUCIAL FOR THE PARASITOID DEVELOPMENT WILL BE ANALYZED IN FUTURE TESTS UNDER LABORATORY AND FIELD CONDITIONS.

Key words: MICROBIAL PEST CONTROL, GREENHOUSE, NON TARGET INSECTS

Insect pathogens and entomoparasitic nematodes
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Laboratory *BEAUVERIA BASSIANA* (Bals.) Vuill. bioassays on spruce bark beetle (*IPS TYPOGRAPHUS* L.)

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Abstract: THE MASSIVE DAMAGES CAUSED BY *Ips typographus* IN SPRUCE FORESTS IN ROMANIA, THE SEVERE RESTRICTIONS ON THE USE OF CHEMICAL INSECTICIDES, THE IDENTIFICATION OF *Beauveria bassiana* NATURAL OUTBREAKS IN THE ROMANIAN FORESTS INFESTED WITH BARK BEETLE AND THE ISOLATION OF *B. bassiana* STRAIN, LED TO THE DEVELOPMENT OF SOME RESEARCHES ON THE POSSIBILITY TO USE THIS ENTO FOR REDUCING THE DAMAGE CAUSED BY BARK BEETLES. IN LABORATORY CONDITIONS, THE *I. typographus* TO INFECTION BY A NATURALLY OCCURRING *B. bassiana* STRAIN WAS TESTED. UTILIZATION OF A *B. bassiana* CONIDIAL SUSPENSION (3.31×10^{11} CONIDIA ml^{-1}) INDUCED BEETLE MORTALITY, LENGTH OF MOTH GALLERIES REDUCTION AND LARVAL GALLERIES NUMBER REDUCTION.

Key words: *Ips typographus*, *Beauveria bassiana*, BIOLOGICAL CONTROL

Introduction

EVERY YEAR, LARGE AREAS OF ROMANIAN SPRUCE FORESTS ARE AFFECTED BY THE ATT SPRUCE BARK BEETLE (*Ips typographus*). IN ROMANIA BETWEEN 2006-2010, THERE WERE STUDIES CONDUCTED CONCERNING THE AMOUNT OF LOSSES CAUSED BY *I. typographus* AND BY THE CONTROL MEASURES THAT HAVE BEEN APPLIED. THUS, IN 2006 THE *I. typographus* ATTACKS HAD A MODERATE INTENSITY AS RESULT OF CONTROL MEASURES, WHICH HAVE BEEN USED TO A RELATIVELY SMALL NUM PIECES); IN 2007 THE *I. typographus* INFESTATION LEVEL INCREASED DUE TO THE LARGE NU WINDFALL, ESPECIALLY IN THE NORTH-EASTERN FOREST DISTRICTS (CARPATII DE CURBENI BR CANDRILOR AND MOLDOVITA); IN 2008 THE *I. typographus* INFESTATION LEVEL CONTINUED TO GRO CREATING FAVORABLE CONDITIONS OF OUTBREAKS. BETWEEN 2009-2010, THERE WAS A W FORESTS HEALTH STATE, DESPITE THE CONTROL MEASURES APPLIED, MAINLY BECAUSE O OF FELLED TREES, WHICH WERE NOT EVACUATED ON TIME FROM THE FOREST.

CONSIDERING THE RESTRICTIONS, WHICH CURRENTLY APPLY IN CERTIFIED FORESTS AC STEWARDSHIP COUNCIL STANDARDS, IT IS INCREASINGLY IMPORTANT TO GIVE BIOLOGIC WHICH HAS MANY ADVANTAGES COMPARED TO CHEMICAL ONES. SOME STUDIES REGARD FLORA ASSOCIATED WITH *I. typographus* CONCLUDED THAT *Serratia liquefaciens* MAY HAVE POTENTIAL AS A BIOLOGICAL CONTROL AGENT AGAINST THE EURASIAN SPRUCE BARK BEETLE (MUR THE IDENTIFICATION OF *B. bassiana* NATURAL OUTBREAKS IN THE ROMANIAN FORESTS INFESTED WIT *duplicatus* (SAHLBERG) (DENUT *et al.*, 2012) AND THE ISOLATION OF A NEW *B. bassiana* STRAIN INFECTING *I. typographus* (ACCESSION NUMBER GIVEN BY THE INTERNATIONAL DEPOSITARY: (P) F 001,392), LED TO THE DEVELOPMENT OF RESEARCH ON THE POSSIBILITY TO ENTOMOPATHOGENIC MICROORGANISM TO REDUCE DAMAGE CAUSED BY BARK BEETLES.

Material and methods

B. bassiana EXPERIMENTAL BIOPRODUCT WAS OBTAINED USING SUBMERGED CULTIVATI
(ANDREI, 2004). LOGS REQUIRED FOR THE EXPERIMENT WERE OBTAINED FROM THE FOREST
AND WAS SUPPOSED NOT TO PROVIDE PREVIOUSLY EXPERIENCED INFESTATIONS. *Ips typographus* BEETLES
WERE CAPTURED USING PHEROMONE TRAPS. BEETLES WERE EXAMINED UNDER A MICRO
WERE BRANDED AND PLACED SEPARATELY ACCORDING TO MORPHOLOGICAL CHA
LABORATORY TESTS WERE PERFORMED IN SPECIAL CAGES (100×34×32 CM) WITH WOODE
SIDE WALLS MADE OF FINE WIRE MESH, WITH MESH SIZE SMALLER THAN *Ips typographus* BEETLE SIZE.
THE SPECIAL CAGES WERE SET OUT WITH A MOBILE SIDEWALL FOR AN EASY BIOLO
MANIPULATION (FIGURE 1). DEVICES, WHICH ALLOWED CONTROLLED INFESTATION OF
MADE USING EPPENDORF TUBES. TWO INDIVIDUALS, ONE MALE AND ONE FEMALE, WERE
DEVICE.

FIGURE 1. EXPERIMENTAL CAGES USED IN LABORATORY *B. bassiana* BIOASSAYS

Results and discussion

THE HOLES MADE BY MALES FOR PENETRATING THE BARK AND THE VENTILATION HOLES
THROUGHOUT MATERNAL GALLERY WERE USED AS PENETRATION ENTRANCES FOR *B. bassiana* INFECTIOUS
INOCULUM IN THE CAMBIAL ZONE (BETWEEN THE BARK AND THE WOOD). 300 ML FUNGAL
OF BARK WAS APPLIED IN THE FOLLOWING THREE EXPERIMENTAL VARIANTS: V1: 3.3×10^{11} CONIDIA ml^{-1};
V2: 9.9×10^{11} CONIDIA ml^{-1}; V3: 16.5×10^{11} CONIDIA ml^{-1}. A NATURAL DEGREE OF HYDRATION OF TH
SAMPLES FROM LABORATORY WAS MAINTAINED BY PERIODIC SPRAYING OF WATER ON THE
DAYS AFTER *B. bassiana* TREATMENT, IT WAS FOUND THAT APPROX. 60% BEETLES FROM GALL
DEAD, COVERED WITH *B. bassiana* WHITE MYCELIUM (FIGURE 2).

BY MEASURING THE MATERNAL GALLERIES IT WAS FOUND THAT THEIR LENGTH VARIE
AND 7.5 MM ON LOGS TREATED WITH *B. bassiana* CONIDIAL SUSPENSION AND BETWEEN 7.6 MM AN
9.9 MM ON CONTROL LOGS (FIGURE 3). AVERAGE NUMBER OF LARVAL GALLERIES CORRE
MATERNAL GALLERY WAS ALSO CONSIDERED. THE LARVAL GALLERIES NUMBER VARIED
PER CM MATERNAL GALLERY ON LOGS TREATED WITH *B. bassiana* CONIDIAL SUSPENSION AND BETWEEN 3.5
TO 4.4 GALLERIES PER CM MATERNAL GALLERY ON CONTROL LOGS (UNTREATED).
SIGNIFICANCE OF DIFFERENCES BETWEEN THE AVERAGE LENGTH OF MATERNAL GALLERI
IN DIFFERENT EXPERIMENTAL VARIANTS AND CONTROL SECTIONS, STUDENT T-TEST W
STATISTICAL PROCESSING OF THE EXPERIMENTAL DATA, THE RESULTS SHOWED THAT *B. bassiana* INFECTION
RESULTED IN A SIGNIFICANT REDUCTION IN THE MATERNAL GALLERY LENGTH (TABLE 1

TREATED TREES, A REDUCTION IN AVERAGE LENGTH OF MATERNAL GALLERIES FROM 8 T
COMPARED WITH CONTROL SECTIONS. THE NUMBER OF LARVAL GALLERIES CORRESPO
MATERNAL GALLERY WAS 19 TO 48% LOWER.

FIGURE 2. *I. typographus* ADULTS INFECTED WITH *B. bassiana* (ARTIFICIAL INFECTION)

FIGURE 3. GALLERIES OF *I. typographus* ON A *Picea abies* LOG

TABLE 1. AVERAGE LENGTH OF MATERNAL GALLERIES ON TREATED AND CONTROL SECTIO

VARIANTS	V1	V2	V3	CONTROL
AVERAGE	6.9	6.8	6.5	8.5
VARIANCE	0.263333	0.397037	0.169259	1.51
T-STATISTIC	-2.82431	-4.33674	-3.86184	-
SIGNIFICANCE OF DIFFERENCES	INSIGNIFICANT	HIGHLY SIGNIFIC	SIGNIFICANT	-

TABLE 2. NUMERICAL EVALUATION OF LARVAL GALLERIES CORRESPONDING TO TREATED

VARIANTS	V1	V2	V3	CONTROL
AVERAGE	2.8	2.8	2.6	4.0
VARIANCE	0.453848	0.074444	0.671481	0.203333
T-STATISTIC	-7.15335	-10.888	-4.35489	-
SIGNIFICANCE OF DIFFERENCES	HIGHLY SIGNIFIC	HIGHLY SIGNIFIC	HIGHLY SIGNIFICANT	-

Conclusions

B. bassiana IS A BIOLOGICAL CONTROL AGENT EFFECTIVE IN REDUCING THE *I. typographus* POPULATIONS BECAUSE OF THE BARK BEETLE SUSCEPTIBILITY TO FUNGAL INFECTION.

THE MAIN INDICATORS OF BARK BEETLE POPULATION REDUCTION BY *B. bassiana* FOLLOWING TREATMENTS WERE: MATERNAL GALLERY LENGTH, AVERAGE NUMBER OF LARVAL GALLE

GALLERY AND HIGH MORTALITY RATES RECORDED IN THE NUPTIAL CHAMBERS OF BARK
LOGS. THERE WAS A SIGNIFICANT REDUCTION OF THE NUMBER OF EGGS LAID BY INSE
GALLERIES OF TREATED LOGS AND A SIGNIFICANT REDUCTION OF LARVAL GALLERIES NU

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Insect pathogens and entomoparasitic nematodes
IOBC-WPRS Bulletin Vol. 90, 2013
pp. 97-101

Effect of local strains of *BEAUVERIA BASSIANA* (Bb024) and *METARHIZIUM ANISOPLIAE* (M7/2) against the fallweb worm *HYPHANTRIA CUNEA* (Lepidoptera: Arctiidae) in Georgia

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Abstract: THEINDIGENOUS ISOLATES OF THE ENTOMOPATHOGENIC FUNGI *Beauveria bassiana* (BB-024) AND *Metarhizium* ANISOPLIAE (M7/2) AGAINST FIFTH TO SEVENTH INSTAR LARVAE OF *Hyphantria cunea* IN TWO CONCENTRATIONS 1 X 10^7 AND 1 X 10^8 CONIDIA ml^{-1} WERE TESTED IN THE LABORATORY. MAXIMUM MORTALI LARVAE WAS OBSERVED 4-9 D AFTER TREATMENT. BOTH ISOLATES WERE HIGHLY PATHOGENIC TO *H. cunea* LARVAE. THE MORTALITY CAUSED BY *B. bassiana* RANGED FROM 59.8% TO 84.3% AND THAT OF *M. anisopliae* RANGED FROM 52% TO 68%. THE LIVING LARVAE HIDDEN UNDER LEAVES AND CORDON MADE COCOONS AND TR PUPAE. THE ADULT MOTHS APPEARED FROM THE OVERWINTERING PUPAE. THEY BEGAN TO EMERGE SIMULTANEOUSLY MATED. THE EMERGENCE OF *B. bassiana* WAS 69.6%, THAT OF *M. anisopliae* WAS 60%, AND THAT OF THE CONTROL WAS 55.7%. THE LARVAE HATCHED 7 TO 10 D LATER (THE HATCHING RATES WERE *B. bassiana* – 76.3%, *M. anisopliae* – 70%, CONTROL – 89.5%). IN CASE OF *B. bassiana* TREATMENT, DIMORPHIC MALES EMERGED FROM PUPAE OFTEN SHOWING UNDEVELOPED WINGS.

Key words: *Hyphantria cunea, Beauveria bassiana, Metarhizium anisoplia,* BIOCONTROL

Introduction

THE FALL WEBWORM (FWW), *Hyphantria cunea* DRURY (LEPIDOPTERA:ARCTIIDAE), IS A POLYPHAGOUS PEST HAVING A VERY WIDE RANGE OF HOST PLANTS. IT HAS BEEN ESTAB DAMAGE MORE THAN 400 PLANT SPECIES IN GEORGIA (EDILASHVILI, 2010). *H. cunea* WAS INTRODUCED IN1970, THE ABUNDANCE OF FEEDING PLANTS AND SUBTROPICAL CLIMATE APPEARED FAV WERE WELL ADAPTED AND SPREAD IN WESTERN GEORGIA AND THE BLACK SEA COAS PERIOD OF TIME

NOWADAYS, MECHANICAL AND CHEMICAL CONTROL METHODS ARE USED TO CONTR THE INSECT MOSTLY INHABITS THE POPULATED AND URBAN AREAS, WHERE THE APPLIC PESTICIDES IS PROHIBITED. *H. cunea*, THEREFORE, IT BECOMES NECESSARY TO USE ENVIRONMENTALLY FR SUCH AS BIOLOGICAL CONTROL. NUMEROUS EXPERIMENTS HAVE BEEN CARRIED OUT USING THE M AGENTS OF THIS INSECT IN LABORATORY (KENDERESHVILI *et al.*, 2012; CHKHUBIANISHVILI *et al.*, 2011; GORGADZE, 2000; LORTKIPANIDZE, 2010; SUPATASHVILI, BURJANADZE, 2008). THE STRATEGY FOR BIOLOGICAL CONTROL OF *H. cunea* INCLUDES THE USE OF ENTOMOPATHOGENIC FUNGI (EPF) AS W

IN THIS STUDY, LOCAL ISOLATES OF *Beauveria bassiana* (BB024) AND *Metarhizium anisopliae* AGG. (M7/2) WERE TESTED AGAINST *H. cunea* LARVAE AND THE EPF´S PATHOGENICITY WAS DETERMINED UNDER LABORATORY CONDITIONS.

Material and methods

FUNGAL CULTURE

THE INDIGENOUS STRAINS OF *Beauveria bassiana* BB024 (IMI#501797) AND *Metarhizium anisopliae* AGG. M7/2 (IMI #501805) WERE ISOLATED FROM SOIL SAMPLES USING THE BAIT METHOD' (ZIMMERMANN, 1986), THEN THEY WERE SUBJECTED TO MOLECULAR IDENTIFICATION AND UK GENETIC RECOURSE COLLECTION

INOCULUM PREPARATION

FUNGAL SUSPENSIONS OF THE ISOLATES WERE PREPARED FROM 2 WEEK-OLD CULTURES G 25 ± 2 °C, USING DISTILLED WATER CONTAINING 0.01% (W/V) TWEEN 80. THE CONCENTR SPORES IN THE SUSPENSIONS FROM EACH FUNGUS WAS DETERMINED USING A HAEMO ADJUSTED TO TWO CONCENTRATIONS OF 1×10^7 AND 1×10^8 CONIDIA ML^{-1} FOR BIOASSAYS.

BIOASSAYS

THE 5TH AND 7TH INSTARS (L5-L7) OF LARVAE OF *H. armigera* WERE COLLECTED MANUALLY FROM ORCHA AND FOREST TREES IN WEST GEORGIA. TARGET INSECTS USED FOR THE BIOASSAY WERE T CULTURAL SUSPENSIONS OF *B. bassiana* AND *M. anisopliae* (1×10^7 AND 1×10^8 CONIDIA ML^{-1}), AND PLACED IN GLASS JAR WITH MULBERRY TREE LEAVES. THEY WERE KEPT AT ROOM TEM (DAY) AND ~18 °C (NIGHT) AND WITH 14 H (LIGHT)/10 H (DARK) PHOTOPERIOD. DEAD OR INFECTED LARVAE WITH FUNGAL SYMPTOMS WERE REMOVED AND PLACED IN MOISTER ENVIRONMENT FOR CONIDIA. MORTALITY OF LARVAE WAS RECORDED ON 3-18 D AFTER TREATMENT.

DATA ANALYSIS

ALL MORTALITY DATA WERE CORRECTED FOR CONTROL MORTALITY USING THE FORMU THE PERCENTAGE OF LARVAL MORTALITY FOR EACH CONCENTRATION WAS ANALYZ ANOVA, MEANS WERE SEPARATED BY TUKEY'S MEAN SEPARATION TEST. MORTALITY W SIGNIFICANTLY DIFFERENT AT $P < 0.01$.

Results and discussion

BOTH FUNGAL STRAINS WERE PATHOGENIC TO *H. armigera* LARVAE. HOWEVER, VIRULENCE CONSIDERABLY VARIED. MYCOSIS BY *B. bassiana* BB-024 WAS OBSERVED IN L5-L6 LARVAE AND IN COCOONS. WI *M. anisopliae* M7/2 SYMPTOMS OF MYCOSIS WERE MOSTLY OBSERVED IN L5-L6 LARVAE. A RAPID DEVELOPMENT OF MYCOSIS WAS OBSERVED WITH BB-024. MAXIMUM MORTALITY OF MARKED 4-9 D AFTER TREATMENT, WHEREAS WITH M7/2 THE MORTALITY WAS OBSERVED AFTER TREATMENT (FIGURE 1).

BOTH ISOLATES WERE PATHOGENIC TO *H. armigera* LARVAE AND THE MEAN MORTALITY RANGED F 59.8% TO 84.3% FOR *B. bassiana* AND FROM 52% TO 68% FOR *M. anisopliae* (FIGURE 2).

MORTALITY CAUSED BY *B. bassiana* AND *M. anisopliae* WERE SIGNIFICANTLY DIFFERENT DEPENDING ON CONCENTRATIONS ($P < 0.05$). ONE-WAY ANOVA, USING FACTOR, $\alpha = 0.05$, SIGNIFICANT DIFFERENCES WERE FOUND BETWEEN THE PAIRS OF *B. bassiana* TREATMENTS FOR 10^7 CONIDIA ML^{-1} AND 10^8 CONIDIA ML^{-1}: $p = 0.0025$, $F = 18.7$; FOR *M. anisopliae* 10^7 CONIDIA ML^{-1} AND 10^8 CONIDIA ML^{-1}: $p = 0.000156$, $F = 44.6$; AND FOR *B. bassiana* 10^8 CONIDIA ML^{-1} AND *M. anisopliae* 10^8 CONIDIA ML^{-1}: $p = 0.0001$, $F = 46$. HENCE, AT THE HIGH CONCENTRATION (10^8 CONIDIA ML^{-1}) THE PROMISING ISOLATE OF *B. bassiana* SHOWED SIGNIFICANT DIFFERENCE COMPARED TO THE *M. anisopliae*.

THE LIVING LARVAE HIDDEN UNDER LEAVES AND CORDON MADE COCOONS AND TH PUPAE. THEY WERE LEFT TO OVERWINTER UNTIL SPRING AT 8 ± 5 °C IN ROOM CONDITIONS

ADULT MOTH APPEARED ON THE 4-5 D, THEY EMERGED MAINLY AND MATED. THE EMERGENCE OF ADULTS OF *H. cunea* TREATED WITH *B. bassiana* AND *M. anisopliae* IS GIVEN IN FIGURE 3. EGGS LAYING CONTINUED F-12 D.

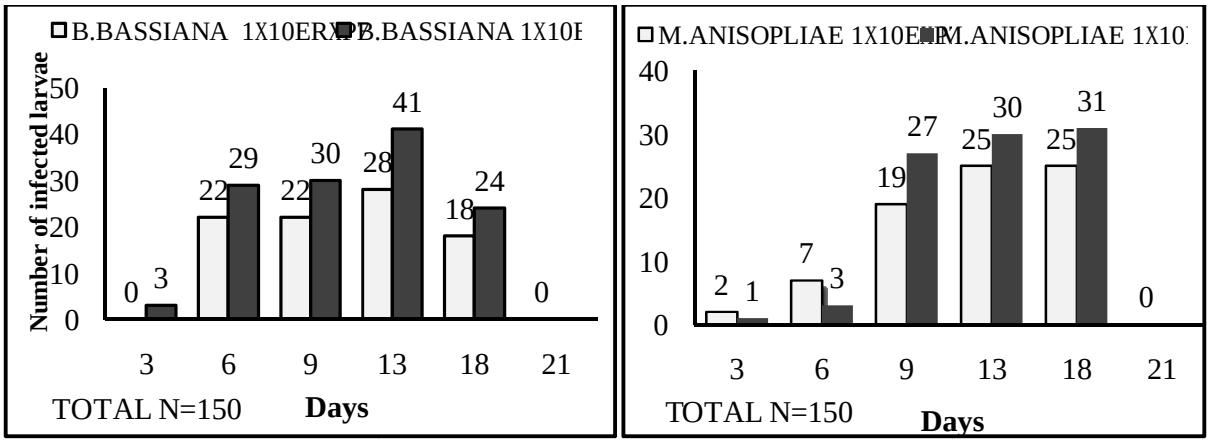

FIGURE 1. APPEARANCE OF MYCOSIS (MEASURED IN DAYS) OF LARVAE OF *Hyphantria cunea* TREATED WITH 10^{7} AND 10^{8} CONIDIA ML^{-1} OF THE *Beauveria bassiana* BB-024 AND *Metarhizium anisopliae* M7/2.

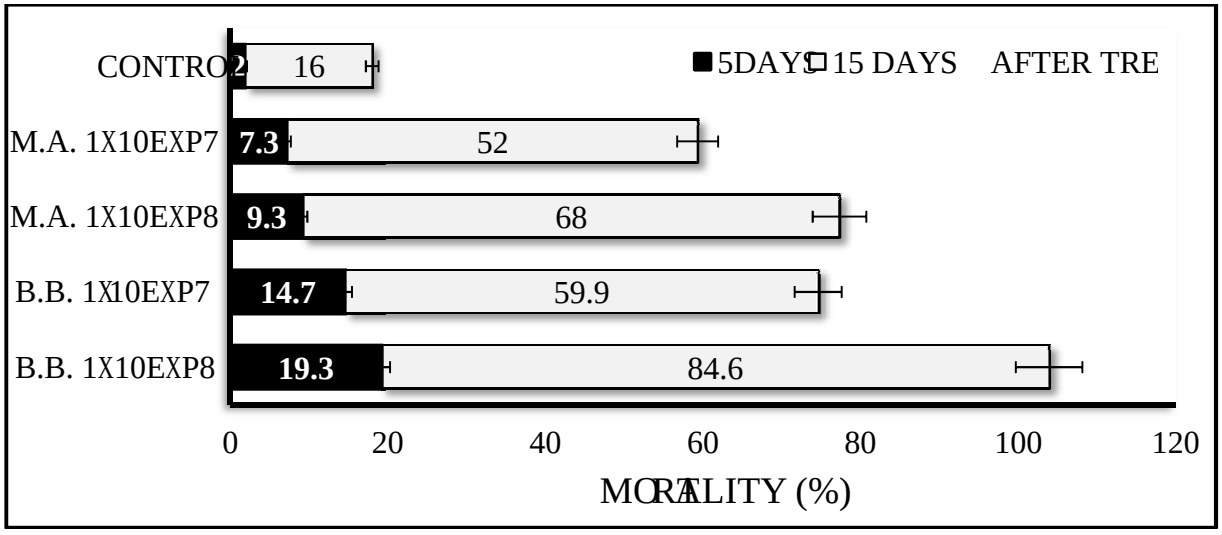

FIGURE 2. MORTALITY (%) OF LARVAE OF *Hyphantria cunea* AFTER TREATMENT WITH CONCENTRATIONS OF *Metarhizium anispiliae* AND BEAUVERIA BASSIANA (MD), SIGNIFICANT LEVEL=0.01

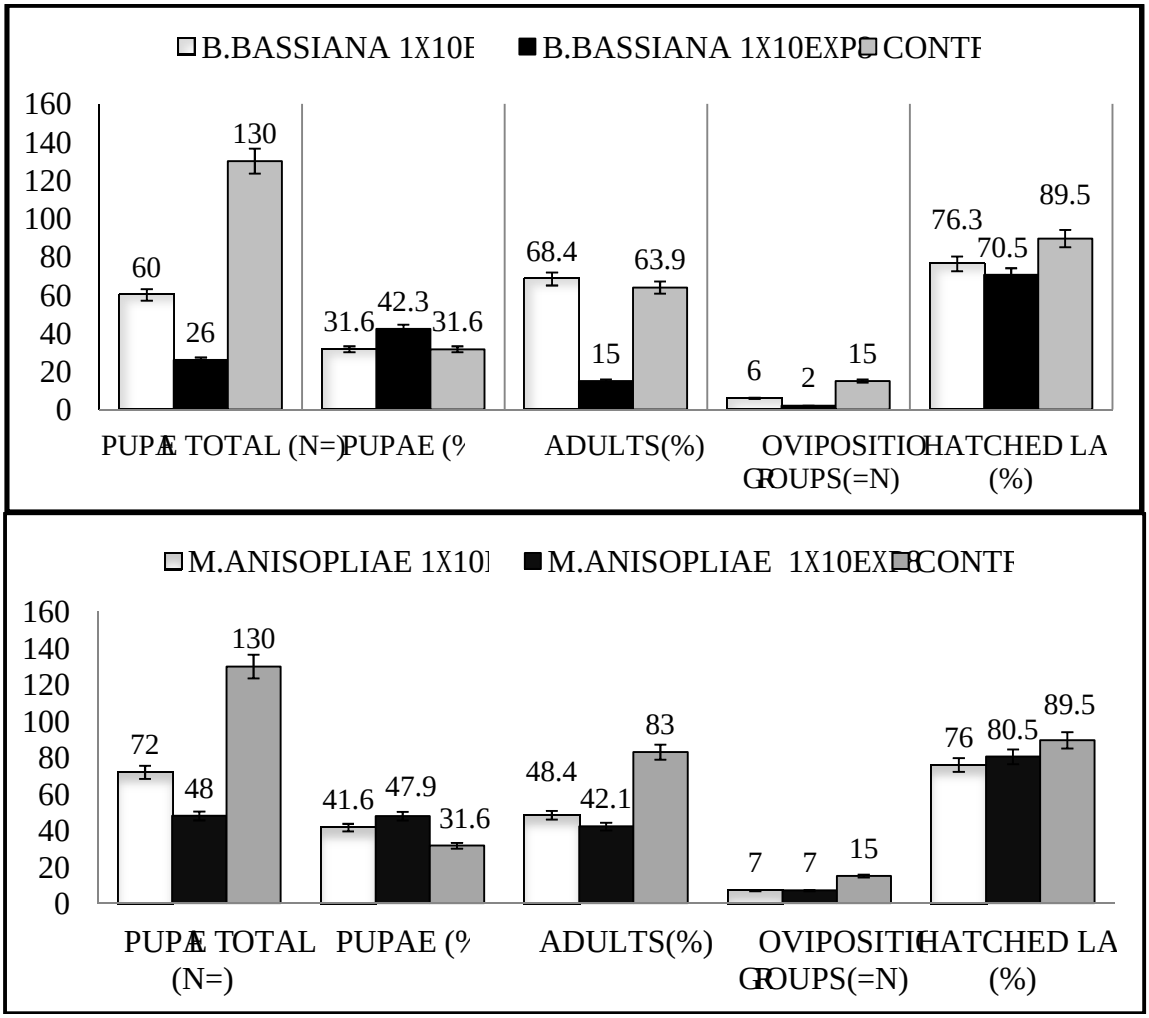

FIGURE 3. THE NUMBER OF EMERGED *Hyphantria cunea* ADULTS FROM THE OVERWINTERED PUPAE AND THEIR REPRODUCTIVE ABILITY (N – NUMBER)

AFTER 7-10 DAYS THE LARVAE HATCHED. THE HATCHING RATE WAS 76.3% AND 89.5% FOR *B. bassiana*, AND 76% AND 80.5% FOR *M. anisopliae*, THE HATCHING RATE OF THE CONTROL VARIANTS WAS 89.5% (FIGURE 3). IT SHOULD BE NOTED, THAT *B. bassiana* FROM DIMORPHIC MALE EMERGED SHOWING UNDEVELOPED FORE WINGS.

THE RESULTS SUGGEST *B. bassiana* (BB-024) AND *M. anisopliae* (M7/2) ISOLATES CAN BE USED TO CONTROL *H. cunea*.

Acknowledgements

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Highly effective *BEAUVERIA PSEUDOBASSIANA* strain (Dm-5) against the great spruce bark beetle, *DENDROCTONUS MICANS* (Kugelann) (Coleoptera: Scolytidae)

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Abstract: THE GREAT SPRUCE BARK BEETLE, *Dendroctonus micans* (KUGELANN) (COLEOPTERA SCOLYTIDAE), HAS BEEN A POTENTIAL THREAT FOR NOT ONLY TURKEY, BUT ALSO THE ENTIRE EURASIAN SPRUCE FORESTS FOR MANY YEARS. CONTROL STRATEGIES WHICH HAVE BEEN APPLIED SO FAR ARE STILL INSUFFICIENT PREVENT ITS DAMAGE. *Beauveria pseudobassiana* STRAIN (DM-5) WHICH WAS PREVIOUSLY ISOLATED FROM *D. micans* HAD 90% MORTALITY AFTER APPLICATION OF 1×10^{6} CONIDIA ML^{-1} SPORE SUSPENSION WITHIN 10 DAYS TOWARDS TO THE LARVAE AND ADULTS OF THIS PEST AND 90% MYCOSIS VALUE. IN THE DOSE-RESP EXPERIMENTS, A CONIDIAL SUSPENSION OF 1×10^{8} CONIDIA ML^{-1} CAUSED 100% MORTALITY ON BOTH LARVAE AND ADULT OF *D. micans* WITHIN 5 AND 6 DAYS, RESPECTIVELY. MORTALITY VALUES OF HORIZONTAL TRANSMISSION FROM LARVAE AND ADULTS WHICH WERE CONTAMINATED WITH SPORE SUSPENSIONS OF 1×10^{6} CONIDIA ML^{-1} OF *B. pseudobassiana* AT 25% WERE ALSO DETERMINED AS 100% AFTER 15 DAYS AT 20 °C UNDER THE LABORATORY CONDITIONS. WE ALSO DETERMINED THE DECREASE OF THE DAMAGE IN WOOD BLOCK (FROM SPRUCE 25 CM) EXPERIMENTS WHEN THE CONTAMINATION RATE OF THE LARVAE INCREASED. OUR RESULTS INDICAT *B. pseudobassiana* (DM-5) SEEMS TO BE A VERY PROMISING BIOCONTROL AGENT AGAINST *D. micans*, THIS STRAIN CAN SPREAD HORIZONTALLY AMONG BOTH LARVAE AND ADULT POPULATIONS OF *D. micans*, AND IT HAS ALSO A GOOD INSECTICIDAL EFFECT TOWARDS TO LARVAE IN THE WOOD BLOCK.

Key words: *Dendroctonus micans*, ENTOMOPATHOGENIC FUNGI, *Beauveria pseudobassiana*, MICROBIAL CONTROL

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Laboratory testing of insect associated fungi for the control of wireworms (*AGRIOTES* sp. L.)

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Abstract: THE AIM OF THE STUDY WAS TO ASSESS ENTOMOPATHOGENIC POTENTIAL OF 7 ISOL ENTOMOPATHOGENIC FUNGAL SPECIES (EPF) ISOLATED FROM VARIOUS SUBSTRATS IN SLOVENIA *Agriotes* SP. THE FUNGAL ISOLATES TESTED WERE *Beauveria bassiana, B. brongniartii, Metarhizium anisopliae* (2 ISOLATES), *M. robertsii, Purpureocillium lilacinum* AND *Clonostachys solani*. CONIDIA OF THESE SPECIES WERE INCORPORATED INTO THE TEST SUBSTRATE AS A WATER SUSPENSION CONCENTRATION OF 3.85 X 10^6 CONIDIA g^{-1} AIR-DRIED SOIL. THE LARVAL MORTALITY WAS OBSERVED ON A BASIS FOR A TOTAL OF 90 DAYS. THE MORTALITIES OBSERVED EXHIBITED A LINEAR TREND WITH S 0.20 TO 1.23 FOR THE FUNGAL TREATMENTS AND 0.08 TO 0.18 FOR THE CONTROL TREATMENTS. ABBOTT' MORTALITY AT DAY 90 RANGED FROM 20.7 TO 76.9%. THE MOST PROMISING CANDIDATE BIOLOGIC WAS *Metarhizium anisopliae* ISOLATE 1154.

Key words: *Agriotes* SP., BIOCONTROL, BIOLOGICAL CONTROL, BIOPESTICIDE, ENTOMOPATHOGENIC FUNGI, PESTS WIREWORMS

Introduction

WIREWORMS, SOIL-BURROWING LARVAL STAGES OF CLICK BEETLES (COLEOPTERA: ELAT PESTS OF CROPS INCLUDING POTATOES IN MANY PARTS OF THE WORLD (YANSARI *et al.*, 2009). THE ADULTS ATTACK A WIDE RANGE OF CROPS (FURLAN *et al.*, 2001). WIREWORM TUNNELING IN POTATO CREATES ENTRY POINT FOR OTHER PLANT PATHOGENS, WHICH CAN CAUSE TUBER ROT (ESTER & H AREAS HIGHLY INFESTED WITH WIREWORMS, ENTIRE BATCHES CAN BECOME UNMARKETA 2009). IN SLOVENIA, *Agriotes ustulatus* SCHALL., *A. lineatus* L., *A. obscurus* L., AND *A. sputator* L. LIVE IN GRASSLANDS AND FIELDS AND THUS HAVE THE POTENTIAL TO BE AGRICULTUR MILEVOJ, 2000).

SEVERAL ATTEMPTS HAVE BEEN MADE TO CONTROL WIREWORMS AND OTHER PEST BEETLE FAMILY WITH BIOLOGICAL AGENTS (TINLINE & ZACHARUK, 1960; ESTER & HU ANSARI *et al.*, 2009). THE EXPERIMENTAL METHODOLOGY IN MOST OF THESE ATTEMPTS WA ALSO, THE MORTALITY RATES AND LETHAL TIMES VARIED CONSIDERABLY. THEREF DISCOVERED EPF ISOLATE MUST UNDERGO RIGOROUS TESTING, IN ORDER TO DETERMINE BIOCONTROL AGENT. THE AIM OF THIS STUDY WAS TO ASSESS THE ENTOMOPATHOGE SEVERAL NEWLY DISCOVERED EPF IN SLOVENIA AGAINST WIREWORMS.

Material and methods

ENTOMOPATHOGENIC FUNGI (EPF) ISOLATION AND CULTURING

THEEPF WERE ISOLATED FROM VARIOUS SUBSTRATES IN SLOVENIA (TABLE 1). THE FUNGA
ROUTINELY CULTURED ON POTATO DEXTROSE AGAR MEDIA AT 24 °C IN DARKNESS.

TABLE 1: LIST OF ENTOMOPATHOGENIC FUNGAL ISOLATES TESTED IN THE STUDY.

Number	AIS ID* number	Genus	Species	Host organism / isolated from	Country of origin
1	1878	*Beauveria*	*bassiana*	*Melolontha melolontha*	SLO
2	1877	*Beauveria*	*brongniartii*	*Melolontha melolontha*	SLO
3	1154	*Metharhizium*	*anisopliae*	SOIL	SLO
4	1868	*Metarhizium*	*anisopliae*	*Agriotes* SP. ADULT	SLO
5	1880	*Metharhizium*	*robertsii*	UNKNOWN	SLO
6	1797	*Purpureocillium*	*lilacinum*	SOIL	SLO
7	1828	*Clonostachys*	*solani* F. *nigrovirens*	POTATO TUBER	SLO

* AGRICULTURAL INSTITUTE OF SLOVENIA MYCOLOGICAL COLLECTION IDENTIFICATION NUMBER

Agriotes *SP. LARVAE COLLECTION AND REARING*

Agriotes SP. LARVAE WERE COLLECTED IN MAIZE-WHEAT BAIT TRAPS ACCORDING TO THE I
BY KIRFMAN *et al.* (1986) AND CHABERT AND BLOT (1992). THE TRAPS WERE LAID OUT ON AP
2012 AND COLLECTED ON APRIL 28, 2012. THE CONTENTS WERE HAND-SORTED AND ALL L
SP. LARVAE TRANSFERRED TO A 15 L PLASTIC CONTAINER, CONTAINING CA. 8 KG OF DA
ORIGINAL LOCATION. THE CONTAINER WAS PLACED IN A GLASSHOUSE ON THE AIS PREMI
SLOVENIA. CARROT AND POTATO SLICES WERE ADDED REGULARLY AS FOOD AND THE CO
AS NEEDED.

SOIL EXPOSURE EXPERIMENT

CONIDIAL SUSPENSIONS WERE PREPARED BY TRANSFERRING CONIDIA TO 100 ML OF STER
80 SOLUTION. A HEMOCYTOMETER WAS USED TO ADJUST SPORE CONCENTRATION
CONCENTRATION OF EPF CONIDIA WAS 1.3×10^6 AS THE SUBSTRATE, WHICH WAS AIR-DRIED FOR 48 H BEFO
THECONIDIAL SUSPENSION WAS ADDED. THE TEST SUBSTRATE WAS A LIGHT COMMERCIAL
ORGANIC MATTER (BIO-PRESSTOPFERDE, FLORAGARD, OLDENBURG, GERMANY). THE
SUBSTRATE WAS MIXED THOROUGHLY IN A LARGE STERILE PLASTIC BAG TO INSURE HO
DISTRIBUTION. 30 ML OF SUBSTRATE CONTAINING CONIDIA WAS TRANSFERRED INTO
CENTRIFUGE TUBE. INTO EACH 50 ML CENTRIFUGE TUBE A SINGLE *Agriotes* SP. LARVA WAS PLACED. FINALLY,
A THIN SLICE (CA. 3 MM THICK) OF POTATO TUBER WAS PLACED ON TOP OF THE SUBSTR
THE TUBES WERE LOOSELY CAPPED, SO AIR COULD FREELY CIRCULATE. 15 TEST VESSELS
TREATMENT. 0.1% TWEEN WAS USED FOR NEGATIVE CONTROLS. THE POSITIVE CON
INSECTICIDE ‘MARSHALL 25 CS’, BASED ON CARBOSULFAN (24.5% ACTIVE INGREDIENT
RECOMMENDED CONCENTRATION OF 0.1%. THE LARVAL MORTALITY WAS OBSERVED ON A
A TOTAL DURATION OF 90 DAYS. DEAD OR IMMOBILE LARVAE LACKING A COAT OF SPOR
WERE REMOVED FROM THE TEST VESSELS AND PLACED IN STERILE 24-WELL PLATES TO
POTENTIALLY PRESENT FUNGI. THE EXPERIMENT WAS CARRIED OUT IN AN ENVIRONMENT

20 °C, 80% RELATIVE HUMIDITY AND TOTAL DARKNESS. POTATO SLICES AND WATER WAS
VESSELS AS NEEDED.

DATA CALCULATIONS AND STATISTICS

FROM THE NUMBER OF LIVING LARVAE AT EACH OBSERVATION POINT, RATE OF MORT
LIVING/INITIAL LARVAE) AND ABBOTT'S CORRECTED MORTALITY (ACM) WAS CALCULAT
((X-Y)/X), WHERE X REPRESENTS THE PERCENT OF LIVING LARVAE IN THE UNTREATED CO
Y THE PERCENT OF LIVING LARVAE IN THE TREATED SAMPLE. CALCULATION USING THIS
ERRORS DUE TO DEATHS IN THE CONTROL SAMPLES, WHICH WERE NOT DUE TO THE TR
SELECTED EPFS (ABBOTT, 1925). DATA PRESENTED ARE MEAN VALUES. THE EXPERI
PERFORMED TWICE INDEPENDENTLY. STATISTICAL ANALYSIS WAS PERFORMED BY CO
GRAPHPAD PRISM 5.00 AND MICROSOFT EXCEL 2007.

Results and discussion

THEMAJORITY OF MORTALITY CURVES OBSERVED IN THE SOIL EXPERIMENT EXHIBITED A
THE EXCEPTION OF THE POSITIVE CONTROL TREATMENT (MARSHALL 25 CS) (FIGURE 1, TA
CONFIDENCE INTERVALS OF THE EPF TREATMENTS' SLOPES DIFFERED SIGNIFICANTLY FR
CONTROL SAMPLES. THE SECOND EXPERIMENT GAVE SIMILAR RESULTS WITH TWO NOT
LOWER MORTALITY WAS OBSERVED IN THE TREATMENT WITH AIS 1154, AND HIGHER M
TREATMENT WITH AIS 1877 (NOT SHOWN).

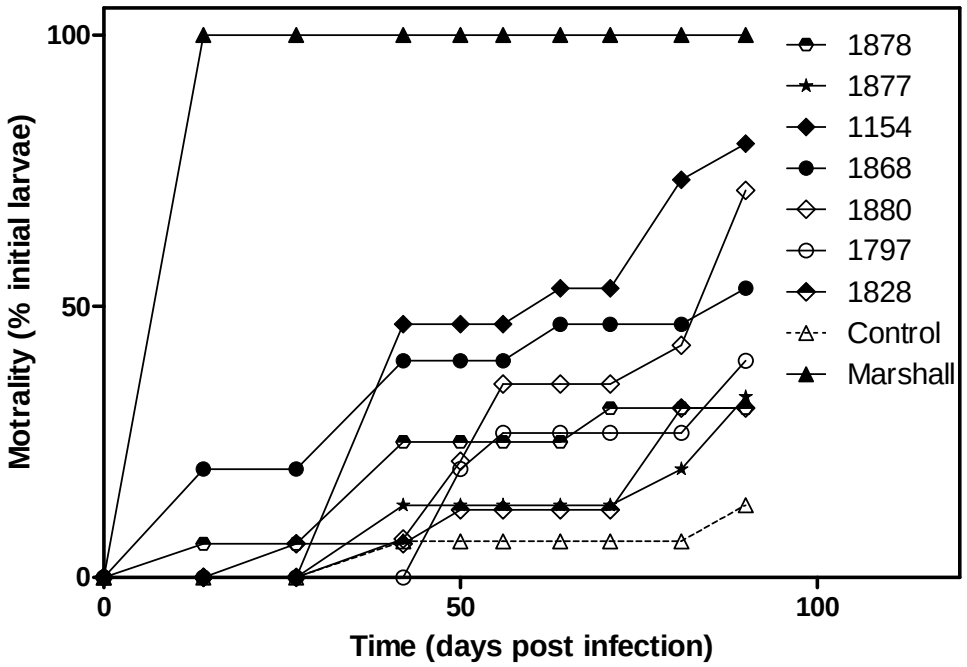

FIGURE 1. MORTALITY OF *Agriotes* SP. LARVAE DURING A TYPICAL EXPERIMENT. THE EXPERIMENT
FOLLOWED FOR 90 DAYS. THE SOIL WAS AMENDED WITH A CONCENTRATION OF 3.85 X 10^6 SPORES/G OF
DRIED SOIL AT THE START OF THE EXPERIMENT. MARSHALL – A CARBOSULFAN-BASED INS
POSITIVE CONTROL.

THE CALCULATED 95% CONFIDENCE INTERVAL OF TIME NEEDED TO REACH A 50% MO
WAS LOWEST IN THE TREATMENT WITH *M. anisopliae* STRAIN AIS 1154 (49.9 TO 64.7 DAYS) AND
HIGHEST IN THE CONTROL TREATMENT (118.8 TO 1426 DAYS). THE POSITIVE CONTROL (T
MARSHALL 25 CS) REACHED A LT_{50} OF LESS THAN A DAY (TABLE 2). THE HIGHEST ACM (AT DAY
WAS CALCULATED FOR THE TREATMENT WITH *M. anisopliae* STRAIN AIS 1154 (76.9%), FOLLOWED BY THE
TREATMENT WITH *M. anisopliae* STRAIN AIS 1880 (67.0%). THE LOWEST ACM WAS CALCULATED FOR TH
TREATMENT WITH *C. tenuani* STRAIN AIS 1828 AND *B. bassiana* STRAIN AIS 1878 (BOTH 20.7%)
(TABLE 2).

TABLE 2: STATISTICAL ANALYSIS OF THE MORTALITY CURVES AND ABBOTT'S CORRECTED
CALCULATED FOR DAY 90. SLOPE – 95% CONFIDENCE INTERVAL OF THE MORTALITY SL
LINEAR REGRESSION; r^2 – GOODNESS OF FIT OF LINEAR REGRESSION; LT_{50} – 95% CONFIDENCE INTERVAL OF
TIME NEEDED TO REACH A MORTALITY OF 50%; MARSHALL – A CARBOSULFAN-BASED INSE
POSITIVE CONTROL.

Treatment	1878	1877	1154	1868	1880	1797	1828	Control	Marshall
Slope	0.270-0.494	0.200-0.444	0.726-1.23	0.393-0.691	0.497-1.00	0.295-0.651	0.207-0.475	0.077-0.183	0-1.36
r^2	0.886	0.823	0.910	0.898	0.854	0.824	0.811	0.799	0.357
LT_{50} [days]	106.4-266.5	95.9-217.6	49.9-64.7	68.9-97.3	70.2-86.5	82.7-148.6	95.5-158.7	118.8-1426	0.12-0.15
ACM at day 90 [%]	20.7	23.1	76.9	46.2	67.0	30.8	20.7	0.0	100.0

THE RESULTS FROM THE TREATMENTS WITH *M. anisopliae* AIS 1154 AND *M. robertsii* AIS 1880
WERE COMPARABLE TO THE INSECTICIDAL ACTIVITY OF A *M. anisopliae* ISOLATE REPORTED BY KÖLLIKER
et al. (2011) FOR *A. lineatus*. THE AUTHORS OBTAINED LOWER LT_{50} VALUES AND HIGHER MORTALITY RATES AGAINST
A. lineatus AND HIGHER AGAINST *A. obscurus*. THEY HYPOTHESIZED THAT THE PATHOGENICITY OF THEIR ISO
SPECIES SPECIFIC. OUR STUDY DID NOT ALLOW FOR DIFFERENTIATION OF TOXICITY AS
DIFFERENT *Agriotes* SP. SPECIES AS WE PERFORMED OUR EXPERIMENTS WITH FIELD COLLECTED
DID NOT CLASSIFY THEM TO THE SPECIES LEVEL. THIS COULD BE OVERCOME BY REA
Agriotes SP. BY USING THE PROTOCOL OF KÖLLIKER *et al*. (2009) FOR IDENTIFYING AND EVALUATING INSECTICIDAL ACTIVIT
FOR INDIVIDUAL SPECIES. DESPITE THESE SHORTCOMINGS, THE *Metarhizium anisopliae* ISOLATES AIS
1154 AND *M. robertsii* AIS 1880 GAVE PROMISING RESULTS. AFTER SUCCESSFUL GLASSHOUSE
TESTING, THEY COULD BE CONSIDERED AS AN ENVIRONMENTALLY FRIENDLY ALTERNA
MANAGEMENT IN CONVENTIONAL OR ORGANIC FARMING SYSTEMS.

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Insect pathogens and entomoparasitic nematodes
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Laboratory and semi-field trials on the effects of *BEAUVERIA BASSIANA* (JW-1, ATCC 74040) against soil-dwelling stages of *FRANKLINIELLA OCCIDENTALIS* (Thysanoptera: Thripidae)

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Abstract: *Beauveria bassiana* (BALSAMO) VUILL. IS AN ENTOMOPATHOGENIC FUNGUS USED IN CON
VARIOUS PESTS. PREVIOUS RESEARCHES SHOWED THAT *B. bassiana*, APPLIED TO THE PLANT CANOPY, COULD EXERT
SIGNIFICANT CONTROL OF THRIPS POPULATIONS, *FRANKLINIELLA OCCIDENTALIS* PERGANDE.
HOWEVER, SOME STAGES (E.G., PREPUPAE AND PUPAE) OF THIS SPECIES DEVELOP IN THE SOIL BEIN
BY CONTROL TREATMENTS APPLIED TO THE CANOPY. THE IDENTIFICATION OF BIOLOGICAL CO
AGAINST SOIL-DWELLING STAGES OF *F. occidentalis* IS AN IMPORTANT ISSUE FOR THE IMPLEMENTATION OF I
HERE WE PRESENT LABORATORY AND GREENHOUSE EXPERIMENTS CARRIED OUT TO EVALUA
B. bassiana (JW-1 ATCC 74040) IN CONTROLLING SOIL-DWELLING STAGES OF *F. occidentalis*. IN LABORATORY
BIOASSAYS, *B. bassiana* REDUCED SIGNIFICANTLY THE EMERGENCE OF *F. occidentalis* ADULTS. IN THE GREENHOUSE
EXPERIMENT, A SIGNIFICANT CONTROL OF THRIPS POPULATION WAS OBTAINED ON CYCLAMEN PO

Key words: *Frankliniella occidentalis*, *Beauveria bassiana*, SOIL-DWELLING THRIPS STAGES, IPM, GREENHOUSE ORNAMENTALS

Introduction

THE WESTERN FLOWER THRIPS (WFT), *Frankliniella occidentalis* PERGANDE IS A WORLD-WIDE PEST OF
CULTIVATED PLANTS MAINLY OF GREENHOUSE ORNAMENTALS. THE PEST STATUS IS DUE
THE TRANSMISSION OF TOSPOVIRUSES, AND THE GREAT ABILITY TO DEVELOP RESISTANCE
PHENOMENON POSES MAJOR LIMITATION TO CHEMICALLY-BASED PEST CONTROL STRATE
LÓPEZ-SOLER *et al.*, 2008). OTHER PROBLEMS IN WFT CONTROL ARE RELATED TO ITS
DEVELOPMENTAL STAGES (PREPUPAE AND PUPAE). SINCE THE LATTER DEVELOP IN SOIL
THE EFFECTS OF CONTROL MEASURES (PESTICIDES OR BIOCONTROL AGENTS) APPLIE
(TOMMASINI & MAINI, 1995; CLOYD *et al.*, 2003). THEREFORE, SEVERAL STUDIES HAVE BEEN DEVO
TO THE DEVELOPMENT OF CONTROL STRATEGIES ALTERNATIVE TO PESTICIDES. MANY B
RESULTED EFFECTIVE IN CONTROLLING WFT POPULATIONS AND ENTOMOPATHOGENIC FU
POTENTIAL IN THIS FRAMEWORK (BROWNBRIDGE *et al.*, 1999; HEAVNER *et al.*, 2008).
AMONG ENTOMOPATHOGENIC FUNGI, *Beauveria bassiana* (BALSAMO) VUILL. IS A WELL-STUDIED
BIOCONTROL AGENT THAT HAS BEEN USED AGAINST VARIOUS PESTS INCLUDING WFT
NATURALLY OCCURRING IN THE SOIL (E.G., VÄNNINEN, 1995; QUESADA-MORAGA *et al.*, 2006; MEYLING
& EILENBERG, 2007). SOME STUDIES HAVE INVESTIGATED THE EFFECT OF SOIL APP
B. bassiana AGAINST WFT IN THE LABORATORY (ARAJ *et al.*, 2009), WHILE LESS WORK HAS BEEN
DONE ON CULTIVATED PLANTS. MOREOVER, THE EFFECTS OF *B. bassiana* IN CONTROLLING WFT AND OTHER
PESTS APPEAR TO BE STRAIN-DEPENDENT (SKINNER *et al.*, 2013). HERE WE TESTED THE EFFECT OF A
COMMERCIAL FORMULATION OF *B. bassiana* (JW-1, ATCC 74040) AGAINST SOIL-DWELLING STAGES OF
WFT IN LABORATORY AND GREENHOUSE CONDITIONS. PRELIMINARY RESULTS ARE REPOR

Material and methods

INSECT REARING

THRIPS USED IN THIS STUDY WERE OBTAINED FROM STOCK CULTURES WHERE INSECTS CUCUMBERS FOLLOWING A MODIFIED METHOD DESCRIBED BY DE KOGEL *et al.* (2000). REARING UNITS WERE KEPT AT ROOM TEMPERATURE [24 ± 1 °C; 60-70% RELATIVE HUMIDITY, (R.H.)] PHOTOPERIOD OF 16 H (LIGHT)/8 H (DARK).

EXPERIMENTAL PROCEDURES

IN ALL EXPERIMENTS A COMMERCIAL FORMULATION OF *B. bassiana* (STRAIN JW-1 ATCC 74040, NATURALIS®) WAS USED. LABORATORY BIOASSAYS WERE PERFORMED USING AN EXPERIM CONSTITUTED BY A 50 ML FALCON TUBE CONTAINING 30 ML OF PEAT. A PIECE OF TRANSPA MEMBRANE DIALYSIS TUBE, WAS PLACED ON THE TOP OF THE TUBE TO AVOID INSECT ES GAS-EXCHANGE. ALL THE MATERIAL INCLUDING PEAT WAS STERILIZED PRIOR TO THE EXP SECOND INSTAR LARVAE WERE TRANSFERRED FROM STOCK CULTURES TO EXPERIMENT CAMEL HAIR BRUSH. TWO TREATMENTS WERE COMPARED: *B. bassiana* WAS APPLIED TO THE SOIL BEFORE (2 H) LARVAE PENETRATION OR AFTER (24 H) LARVAE PENETRATION IN SOIL. STERILIZED WAS INCLUDED AS A CONTROL. THREE DOSES OF COMMERCIAL FORMULATION WE EXPERIMENT CORRESPONDING TO 3 L HA^{-1}, 9 L HA^{-1} AND 27 L HA^{-1}. EACH TREATMENT WAS REPLICATED 20 TIMES. EXPERIMENTAL UNITS WERE MAINTAINED AT 24 ± 1 °C AND 60% ± 5% RELATIVE (R.H.). ADULTS EMERGENCE WAS MONITORED DAILY FOR 11 DAYS FROM LARVAE INTRODU

A GREENHOUSE EXPERIMENT WAS PERFORMED TO EVALUATE THE EFFECT OF SOIL *B. bassiana* ON WFT INFESTATION ON CYCLAMEN POTTED PLANTS. TWO TREATMENTS WE SOIL APPLICATION OF *B. bassiana* (TWO APPLICATIONS IN 7 DAYS) VS. WATER TREATED CONTRO TREATMENT WAS REPLICATED 4 TIMES. EACH REPLICATION WAS PLACED IN INSECT-PROO THRIPS ESCAPING. THE DOSE OF *B. bassiana* FORMULATION CORRESPONDED TO 1 L HA^{-1}. PLANTS WERE INFESTED WITH ABOUT 10 ADULTS AND 50 JUVENILES TWO WEEKS BEFORE *B. bassiana* APPLICATION. WE EVALUATED THE PERSISTENCE OF *B. bassiana* IN SOIL SAMPLES COLLECTED FROM THE TWO TREATMENTS USING THE "GALLERIA BAIT METHOD" (ZIMMERMANN, 1986). SAMPLES OF FUNGA MYCELIUM PRESENT ON *Galleria mellonella* L. LARVAE WERE TRANSFERRED ON PETRI DISHES CONT A SELECTIVE MEDIUM AND HELD AT 25 °C FOR 5 DAYS TO OBTAIN NEW FUNGAL COLONIES IDENTIFIED UNDER MICROSCOPE USING DICHOTOMOUS KEYS (BARNETT & HUNTER, EVALUATION OF WFT POPULATION DENSITY AND STRUCTURE ON FLOWERS WAS PERFOR 35 D FROM THE *B. bassiana* APPLICATION. PLANTS WERE KEPT IN GREENHOUSE AT 18 ± 6 °C 63% ± 5% R.H. SOIL SAMPLING WAS PERFORMED WITH THE SAME TIMING.

Results and discussion

IN LABORATORY THE APPLICATIONS OF *B. bassiana* WERE ASSOCIATED TO A HIGHER MORTALITY COMP TO THE CONTROL TREATMENTS (*p* < 0.05) IN THE LATTER NATURAL MORTALITY WAS 3% FOR APPL BEFORE LARVAE PENETRATION IN THE SOIL AND 16% FOR APPLICATION AFTER LARVAE SOIL. *B. bassiana* APPLICATIONS EXHIBITED AN EFFECT THAT RANGED FROM 17% TO 57% IN CORRECTED MORTALITY. THE BEST RESULTS WERE OBTAINED WITH SOIL APPLICATION LARVAE PENETRATION IN SOIL. MORTALITY OF SOIL-DWELLING STAGES WAS DOSE-DE EFFECT WAS INFLUENCED BY THE TIMING OF APPLICATION. IN APPLICATION BEFORE LAR SOIL, MORTALITY WAS HIGHER FOR 27 L HA^{-1} (57% CORRECTED MORTALITY, C.M.) COMPARED T 9 LHA^{-1} (23% C. M.) AND 3 L HA^{-1} (17% C. M.) DOSES, WHILE IN APPLICATION MADE AFTER LAR

PENETRATION IN SOIL NO DIFFERENCES WERE OBSERVED BETWEEN 2.5 E 5 × 10[13] CONIDIA HA[-1] (49% C. M.) THAT INDUCED HIGHER MORTALITY COMPARED TO 1 × 10[13] CONIDIA HA[-1] DOSE (25% C. M.).

THE GREENHOUSE EXPERIMENT SHOWED THAT SOIL APPLICATIONS OF *B. bassiana* INDUCED SIGNIFICANTLY ($p < 0.05$) THE WFT INFESTATION ON CYCLAMEN POTTED PLANTS (TABLE 1) WITH TO THE WATER TREATED CONTROL. THE "APPLICATION METHOD" REVEALED THAT *B. bassiana* PERSISTED IN THE SOIL UNTIL THE END OF THE EXPERIMENT (35 DAYS FROM APPLICATION). NO SYMPTOMS AND SIGNS OF *B. bassiana* INFECTION WERE OBSERVED ON *G. melonella* LARVAE IN UNTREATED CONTROL. *B. bassiana* APPLICATION AFFECTED POPULATION STRUCTURE OF PLANTS RECEIVING SOIL APPLICATIONS WERE INFESTED ONLY BY LARVAE, WHILE CONTROL INFESTED BY LARVAE AND ADULTS. THESE RESULTS DEMONSTRATE *B. bassiana* SIGNIFICANT EFFICACY (JW-1, ATCC 74040) STRAIN AGAINST SOIL-DWELLING STAGES OF WFT.

TABLE 1. EFFECT (%) OF *B. bassiana* SOIL APPLICATIONS ON WFT POPULATION DENSITY DETECTED ON CYCLAMEN FLOWERS AND CALCULATED USING THE FORMULA OF HENDERSON AND TILTON

	TIME AFTER FIRST APPLICATION (DAYS)				
	7	14	21	28	35
Reduction of WFT infestation	37.04%	59.09%	40%	44.83%	66.67%

IN LABORATORY TRIALS WFT ADULTS EMERGENCE WAS REDUCED DEPENDING ON DOSE AND APPLICATION. BEST RESULTS WERE FOUND IN TREATMENTS AFTER LARVAE PENETRATION IN SOIL AT HIGHEST DOSE. WE CAN SUGGEST THAT *B. bassiana* ACTIVITY AFTER LARVAE PENETRATION IN SOIL CAN INCREASE THEIR EXPOSURE TO INFECTIOUS INOCULUM. IN TREATMENT MADE BEFORE LARVAE IN SOIL, THIGMOKINETIC BEHAVIOUR EXHIBITED BY WFT (JENSEN *et al.*, 2005) MIGHT BE RESPONSIBLE FOR LIMITED CONTROL EFFICIENCY AT LOWEST AND INTERMEDIATE DOSES. WE HERE CONFIRM THE POTENTIAL OF ENTOMOPATHOGENIC FUNGI APPLICATIONS AGAINST STAGES OF WFT EMERGED IN PREVIOUS INVESTIGATION (BROWNBRIDGE *et al.*, 1995). ANSARI FOUND THAT APPLICATIONS TO GROWING MEDIA OF *B. bassiana* STRAINS REDUCED FLOWER EMERGENCE OF ADULTS COMPARED TO A CHEMICAL INSECTICIDE (FIPRONIL). THE EMERGENCE OF ADULTS WAS COMPARABLE TO THAT FOUND HERE. GREENHOUSE EXPERIMENTS THE EFFECT OF SOIL APPLICATIONS OF *B. bassiana* ON WFT INFESTATIONS IN A REALISTIC CULTIVATION SCENARIO. THE FUNGUS PERSISTED IN SOIL FOR 35 DAYS AND THIS OBSERVATION IS USEFUL MANAGEMENT. NO ADULTS WERE FOUND ON PLANTS RECEIVING SOIL APPLICATIONS. THIS HAS IMPORTANT IMPLICATIONS FOR VIRUSES TRANSMISSION: WFT ACQUIRES TOSPOVIRUSES AS LARVAE AND TRANSMIT THEM AS ADULTS. IN PREVIOUS RESEARCH, SOIL APPLICATION OF AN EXPERIMENTAL *B. bassiana* (GRANULAR FORMULATION) WAS EFFECTIVE IN THE CONTROL OF WFT, WHILE BETTER RESULTS WERE OBTAINED WITH THE COMMERCIAL GHA STRAIN (BOTANIGARD ES). RESULTS OBTAINED HERE CONFIRM THE POTENTIAL OF *B. bassiana* IN IPM STRATEGIES AGAINST WFT. THIS STUDY STRESSES ON THE POTENTIAL OF THE COMMERCIAL *B. bassiana* STRAIN (JW-1, ATCC 74040) AS VALUABLE APPROACH FOR THE MANAGEMENT OF A CRITICAL POINT IN ACTUAL WFT CONTROL STRATEGIES.

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Insect pathogens and entomoparasitic nematodes
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Prevalence of the species *BEAUVERIA PSEUDOBASSIANA* among tick-associated fungal isolates from the Republic of Moldova

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Abstract: FUNGAL STRAINS ISOLATED FROM IXODID TICKS IN THE REPUBLIC OF MOLDOVA WE CHARACTERIZED USING THE RIBOSOMAL RNA OPERON INTERNAL TRANSCRIBED SPACER (ITS) REG THE 5.8S RRNA GENE AS WELL AS THE NUCLEAR CODING *ef1* GENE ENCODING THE ALPHA SUBUNIT OF EUKARYOT TRANSLATION ELONGATION FACTOR 1 AS FURTHER GENETIC MARKERS. ON THE BASIS OF THE SEQUENCE DATA EIGHT OUT OF TEN ISOLATES WERE ASSIGNED TO THE *Beauveria* SPECIES *B. pseudobassiana*. THE TWO REMAINING ISOLATES WERE CONSISTENTLY CHARACTERIZED AS *Isaria farinosa* AND AS *Aspergillus* SPECIES, RESPECTIVELY. FURTHER WORK TO ELUCIDATE IF THE PREVALENCE OF *B. pseudobassiana* IN TICKS IS OR NOT A REGIONAL PHENOMENON IS IN PROGRESS.

Key words: *Beauveria pseudobassiana*, *Isaria farinosa*, IXODID TICKS, INTERNAL TRANSCRIBED SPACER (IT ELONGATION FACTOR 1 ALPHA (*ef1*)

Introduction

AS VECTORS OF THE CAUSATIVE AGENTS OF SEVERAL SEVERE DISEASES OF HUMANS A LYME BORRELIOSIS, TICK-BORNE ENCEPHALITIS, COLORADO TICK FEVER, AND ROCKY M FEVER, TICKS POSE AN EMINENT THREAT TO PUBLIC HEALTH AND SET OFTEN IMPO LIMITATIONS TO STOCK-FARMING. TICK CONTROL AGENTS AND STRATEGIES ARE, THEREF

ONE KIND OF THE NATURALLY OCCURRING PATHOGENS OF TICKS ARE FILAMENTOUS THE HYPHOMYCETE GENERA *Metarhizium* OR *Beauveria* (KALSBEEK *et al.*, 1995; FERNANDES & BITTENCOURT, 2008; MEHTA *et al.*, 2011). THE FACT THAT INFECTION BY THESE FUNGI IS MORE FRE FOR ADULT FEMALE TICKS AS COMPARED TO MALES OR LARVAE (ZHIOUA *et al.*, 1999) AND THAT SUBLETHAL LEVELS CAUSES DECREASED FECUNDITY OF INFECTED FEMALES (MARKS & DE *et al.*, 2004) MAKES THEM PARTICULARLY INTERESTING CANDIDATES FOR BIOLOGICAL TICK CONTROL (SAMISH & CHANDLER, 2000; MANIANIA *et al.*, 2007; HARTE *et al.*, 2008). THEREFORE, AND AS SOUND TAXONOMIC CLASSIFICATION IS A PREREQUISITE OF THE REGISTRATION OF NEW BIOCON STRAINS ISOLATED FROM IXODID TICKS IN THE REPUBLIC OF MOLDOVA IN ORDER TO ASS POLYMORPHISM IN TICK-ASSOCIATED FUNGAL POPULATIONS WERE GENETICALLY CHA GENUS AND SPECIES LEVEL.

Material and methods

THE TEN INVESTIGATED FUNGAL STRAINS, TERMED TICK ISOLATE MDA#1 THROUGH MDA FROM TWO INDEPENDENT SAMPLINGS OF *Ixodes ricinus* TICKS AT DIFFERENT LOCATIONS OF THE REPUBL MOLDOVA WERE ISOLATED AS DESCRIBED BY ZIMIN *et al.* (2010). THE ITS AND *ef1* MARKERS WERE AMPLIFIED USING PRIMER PAIRS ITS4/ITS5 (WHITE *et al.*, 1990) AND 983F/1567R (REHNER & BUCKLEY, 2005), RESPECTIVELY. SEQUENCE ALIGNMENTS AND RECONSTRUCTION OF MAXIM (ML) PHYLOGENIES WERE PERFORMED WITH THE CLUSTALX AND PHYML SOFTWARE TOOL UNDER ASSUMPTION OF A GAMMA-DISTRIBUTION BASED MODEL OF RATE HETEROGENEITY RATE CATEGORIES. TREE TOPOLOGY CONFIDENCE LIMITS WERE EXPLORED IN NON-PAR ANALYSES OVER 1,000 PSEUDO-REPLICATES.

Results and discussion

THEITS1-5.8SRRNA-ITS2 SEQUENCES OBTAINED FROM ISOLATES MDA#1-10 WERE COMPAR ORTHOLOGOUS SEQUENCES FROM STANDARD STRAINS OF THE GENERA *Beauveria, Isaria, Lecanicillium, Metarhizium*, AND – AS AN OUTGROUP – *Aspergillus*. CONSISTENTLY WITH ITS PREVIOUS MORPHOLOGICALLY BASED CLASSIFICATION, MOST ISOLATES (MDA#2-9) CLUSTERED W STRAINS, WHEREAS ISOLATE MDA#1 APPEARED MOST CLOSELY RELATED TO *Isaria* SPP. AND ISOLATE MDA#10 CLUSTERED WITH THE OUTGROUP SEQUENCE (FIGURE 1).

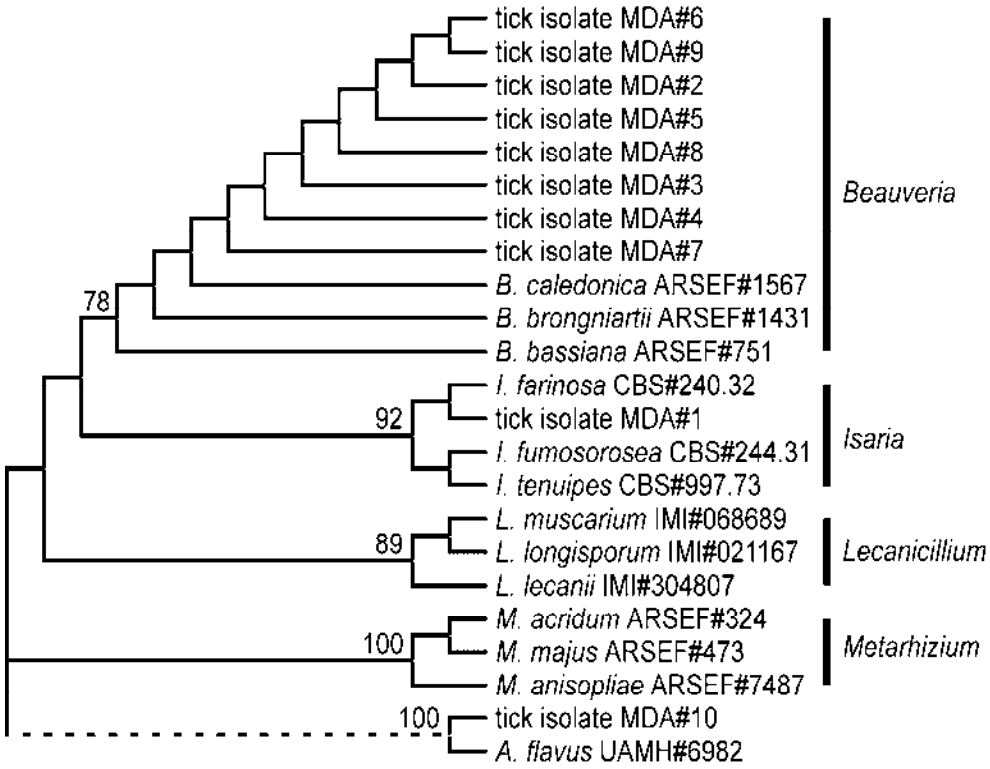

FIGURE 1. ITS SEQUENCE BASED ML CLADOGRAM FOR SEVERAL FUNGAL GENERA ROO *Aspergillus* BRANCH. NUMBERS ON BRANCHES DESIGNATE BOOTSTRAP SUPPORT PERCENTAG

IN ORDER TO OBTAIN A SPECIES CLASSIFICATION FOR TICK-DERIVED *Isaria* STRAIN MDA#1, ITS SEQUENCES REPRESENTING FURTHER *Isaria* SPECIES ACCORDING TO THE PHYLOGENY PRESENTED BY LUANGSA-*ard et al.* (2005) WERE INCLUDED IN THE ANALYSIS. THE TICK-DERIVED ISOLATE CLUSTERS WITH *Isaria farinosa* STRAINS IN A MAXIMALLY BOOTSTRAP SUPPORTED SUB-CLADE OF THE REFINED ITS PHYLOGENY AND SHOULD ON THIS BASIS BE ASSIGNED TO THIS SPECIES.

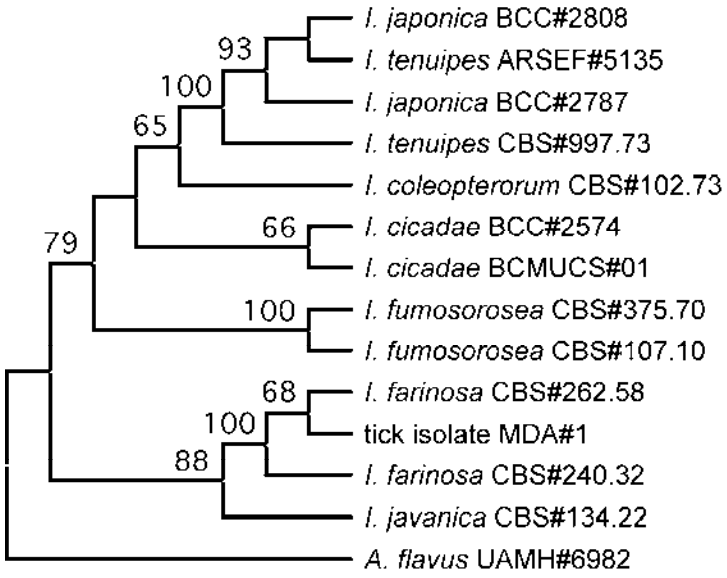

FIGURE 2. ITS SEQUENCE BASED ML CLADOGRAM FOR *Isaria* TICK ISOLATE MDA#1 WITH *Aspergillus flavus* SEQUENCE. NUMBERS ON BRANCHES DESIGNATE BOOTSTRAP SUPPORT PERCENTAGES

TICK-DERIVED *Beauveria* STRAINS MDA#2-9 WERE FURTHER ANALYZED WITHIN THE SYSTEMATIC FRAMEWORK CREATED BY REHNER & BUCKLEY (2005). BASED UPON A COMPARISON OF *tef1* PARTIAL GENE SEQUENCES (FIGURE 3), ALL EIGHT ISOLATES SHOULD BE ASSIGNED TO THE SPECIES *Beauveria pseudobassiana*. IMPORTANTLY, WITHIN THIS SPECIES, THE TWO ISOLATES FROM MOLDOVA DO NOT FORM A TIGHT (PRESUMABLY GEOGRAPHIC) CLUSTER ASSIGNABLE TO DIFFERENT INFRA-SPECIFIC SUB-CLADES, A FINDING VERY MUCH IN EVOLUTIONARY HISTORY OF THE FUNGUS-TICK RELATIONSHIP CHARACTERIZED BY MANY EVENTS.

Acknowledgements

WE GRATEFULLY ACKNOWLEDGE SUPPORT FROM BMBF GRANTS MDA 08/013 AND RUS 08/02

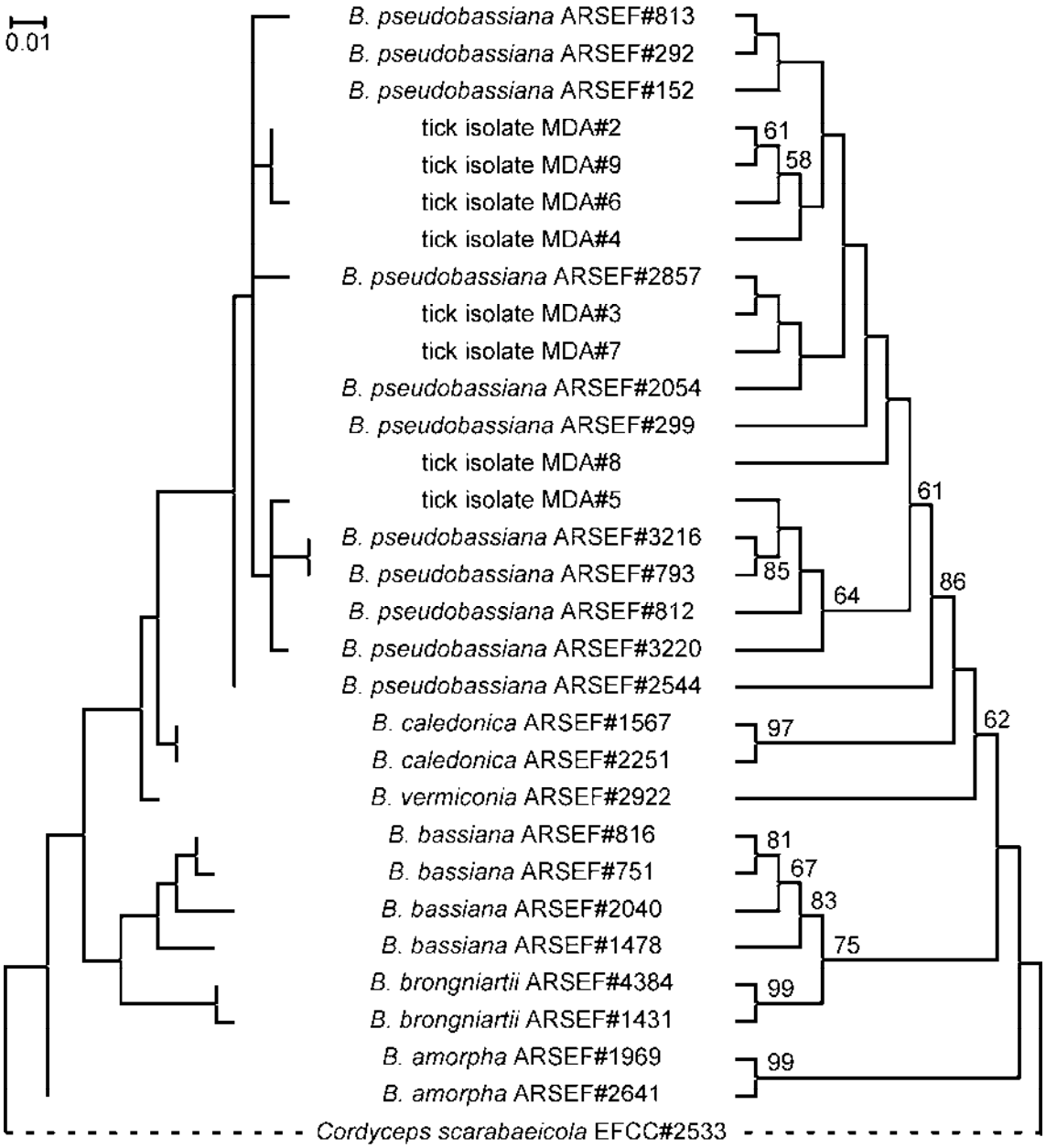

FIGURE 3. EF1PEPTIDE SEQUENCE BASED ML PHYLO- (LEFT) AND CLADOGRAM (RIGHT) FOR *Beauveria* ROOTED WITH A *Cordyceps* ORTHOLOG. NUMBERS ON BRANCHES DESIGNATE BOOTSTRAP SUPPORT PERCENTAGES. THE SIZE BAR DENOTES A 1% SEQUENCE DIVERGENCE.

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Evaluation of indigenous *BEAUVERIA* isolates as potential agents for emerald ash borer management and the development of a diagnostic marker to monitor a post-release isolate

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Abstract: TO SEARCH FOR EFFECTIVE AND SAFE INDIGENOUS BIOCONTROL AGENTS TO MANAGE E
(EAB), WE CONDUCTED A SURVEY IN 2008-2009 OF ENTOMOPATHOGENIC FUNGI (EPF) INFECTIN
OUTBREAK SITES IN SOUTHWESTERN ONTARIO, CANADA. *Beauveria* SPP. ISOLATES WERE RECOVERED FROM
DEAD AND MYCOSED EAB CADAVERS RESIDING IN THE PHLOEM TISSUES OF DEAD ASH BARI
EXTRACTED FROM FEEDING GALLERIES UNDER THE BARK OF DEAD TREES. MOLECULAR CHA
SEQUENCES OF THE ITS, 5' END OF ELONGATION FACTOR 1 ALPHA (*tef1*) AND THE GENOMIC BLOC REGION FRAGMENTS
REVEALED THAT *Beauveria bassiana* AND *B. pseudobassiana* WERE COMMONLY ASSOCIATED WITH EAB IN THE
SAMPLED SITES. INITIAL VIRULENCE SCREENING AGAINST EAB ADULTS OF 23 ISOLATES REPRESE
CLADES YIELDED 8 ISOLATES THAT PRODUCED MORE THAN 90% MORTALITY IN A SINGLE CONCEN
ISOLATES DIFFERED IN VIRULENCE BASED ON LT_{50} VALUES ESTIMATED FROM MULTIPLE CONCENTRATION BIOAS
BASED ON MEAN SURVIVAL TIMES AT A CONIDIA CONCENTRATION OF 1×10^6 CONIDIA ML^{-1}, *B. bassiana* ISOLATE
L49-1AA WAS SIGNIFICANTLY MORE VIRULENT AND PRODUCED MORE CONIDIA ON EAB CADAVE
THE OTHER INDIGENOUS ISOLATES AND THE COMMERCIAL STRAIN *B. bassiana* GHA, SUGGESTING THAT L49-1AA
MAY HAVE POTENTIAL AS A CONTROL AGENT AGAINST EAB. STUDIES HAVE BEEN DEVELOP
CONTAMINATION TRAPPING SYSTEM TO DISSEMINATE L49-1AA TO MANAGE EAB FIELD POPU
TARGETED THE *tef1* GENE SEQUENCE FROM L49-1AA TO DEVELOP AN ALLELE/STRAIN SPECIFIC PRIM
WILL BE USED TO MONITOR THE INTRODUCED L49-1AA IN TERMS OF ITS ESTABLISHMENT, P
VIRULENCE IN THE ENVIRONMENT.

Key words: EAB, *Beauveria* SPP. DIVERSITY, MOLECULAR CHARACTERIZATION, DIAGNOSTIC MARKER,
CONTROL

Introduction

THE EMERALD ASH BORER (*Agrilus planipennis* (COLEOPTERA: BUPRESTIDAE), IS AN INVASIVE
WOOD BORING BEETLE THAT IS DECIMATING NORTH AMERICAN ASH TREES (*Fraxinus* SPP.). SINCE ITS
ESTIMATED 30 MILLION ASH TREES HAVE SUCCUMBED TO EAB INFESTATION. THE C
EXPANSION OF EAB POSES A SUBSTANTIAL RISK TO THE REMAINING ASH RESOURCES OF
MULTI-TACTIC APPROACH THAT INCLUDE THE USE OF BIOLOGICAL CONTROL AGENTS HA
THE MOST SUITABLE LONG TERM PEST MANAGEMENT STRATEGY FOR INVASIVE SPECIES (

THE GOAL OF THIS STUDY WAS TO ISOLATE AND IDENTIFY *Beauveria* SPP. INFECTING
FIELD POPULATION OF EAB IN SOUTHERN ONTARIO, CANADA, AND ALSO EVALUATE TH
VIRULENCE AND CONIDIA PRODUCTION OF SELECTED *Beauveria* ISOLATES AGAINST EAB.
A POTENTIAL *B. bassiana* ISOLATE L49-1AA, WAS SELECTED FOR THE BIOCONTROL OF EMER
BORERS AND CHARACTERISTICS RELATING TO THE PRACTICAL APPLICATION IN AUTOCON
SINCE BEEN DISCUSSED AND DOCUMENTED (JOHNSONS *et al* 2012).

ONE SPECIFIC ASPECT THAT IS REQUIRED WHEN MICROBIAL CONTROL AGENTS ARE I
ENVIRONMENT IS BY MONITORING AND EVALUATING ITS ESTABLISHMENT, PERSISTENCE

MOLECULAR APPROACH UTILIZING SUITABLE GENETIC MARKERS WAS THEREFOR
IDENTIFICATION *B. bassiana* ISOLATE L49-1AA. A MOLECULAR MARKER DEVELOPMENT
UNDERTAKEN TO ALLOW FUTURE DISTINCTION BETWEEN L49-1AA AND NATURALLY OCC
STRAINS WITHIN RELEASED PLOTS.

Material and methods

FUNGAL-INFECTED EAB WERE COLLECTED FROM OLD OUTBREAK SITES IN SARNIA, WINDS
ONTARIO, CANADA DURING THE SUMMER OF 2008 AND 2009. FUNGAL ISOLATES WERE RE
DEAD AND MYCOSED INSECTS (LARVAE AND ADULTS) BASED ON STANDARD PROTOCOLS
1997). RECOVERED FUNGAL ISOLATES WERE IDENTIFIED BY CLASSICAL TAXONOMY F
GENERAL AND SPECIFIC IDENTIFICATION KEYS (HUMBER, 2009). ALL THE FUNGAL ISOL
IDENTIFIED BY MOLECULAR DIAGNOSTICS USING THE ITS 1 AND 2 SPACER REGIONS, *EF1-α*
SEQUENCES.

FOR PRELIMINARY VIRULENCE TEST, 23 ISOLATES WERE SELECTED FROM DIFFERENT
ITS PHYLOGENY (FIGURE 1) AND EVALUATED AGAINST ADULT EAB USING A SINGLE DOS
CONIDIA ML^{-1}. FROM THE RESULTS, EIGHT HIGHLY VIRULENT ISOLATES FROM DIFFERENT CLA
TESTED WITH FOUR DIFFERENT CONCENTRATIONS, 2.0×10^4, 2.0×10^5, 2.0×10^6, 2.0×10^7 CONIDIA
ML^{-1}. THE COMMERCIAL *B. bassiana* STRAIN, GHA WAS INCLUDED IN THE BIOASSAY AS A BASE
ISOLATE TO COMPARE VIRULENCE. CONIDIA PRODUCTION OF SELECTED *Beauveria* ISOLATES WERE
QUANTIFIED BY COUNTING THE CONIDIA FROM MYCOSED CADAVERS OBTAINED FROM TH
POST MORTALITY.

WE USED AN IMPROVED ALLELE-SPECIFIC POLYMERASE CHAIN REACTION (AS-PCR)
BASICALLY A CONCEPTUALLY SIMPLE SNP GENOTYPING STRATEGY. THE INHIBITION DISPL
OF AS-PCR REQUIRES ONLY TWO OUTER COMMON PRIMERS AND ONE INNER PRIMER WITH
3′ TERMINUS MISMATCH BUT WITH INCORPORATION OF A MISMATCH AT THE PENULTIMATE
BASE OF 3′ END OF ALLELE SPECIFIC INNER PRIMER. THE SEQUENCE DATA GENERATED
PRIMER SET FOR *B. bassiana* ISOLATE, L49-1AA, *Beauveria* SPP. EF1-α GENE SEQUENCES GENERATED
IN THIS STUDY AND THOSE ARCHIVED IN GENBANK WERE ALIGNED WITH BIOEDIT (HALL
SEGMENT OF THE ALIGNED SEQUENCES TARGETED TO DESIGN A STRAIN SPECIFIC PRIM
EFRO × EFF1 EXCLUSIVELY FOR L49-1AA. THE PCR TEMPERATURE PROFILE FOR THE
REACTION WAS 94 °C FOR 3 MIN INITIAL DENATURATION, FOLLOWED BY 35 CYCLES OF
58 °C FOR 30 S, 72 °C FOR 1 MIN AND A FINAL EXTENSION OF 72 °C FOR 10 MIN.

Results and discussion

A TOTAL OF 78 *Beauveria* ISOLATES WERE RETRIEVED FROM DEAD AND MYCOSED EAB CADA
GALLERY FRASS UNDERNEATH THE STRIPPED ASH BARK AT *viz.*, STERNEA, LAMBTON
AND WINDSOR IN SOUTHERN ONTARIO, CANADA. THE MAXIMUM LIKELIHOOD (ML)
INFERRED FROM THE ITS SEQUENCE ALIGNMENT USING MEGA 5.2.1 (TAMURA *et al.*, 2011) WAS
ON 573 CHARACTERS ALIGNMENT COMPRISING OF 112 SEQUENCES (FIGURE 1). SEVENT
CLUSTERED TOGETHER WITH *Beauveria bassiana* (CLADE A) WITH A STRONG BOOTSTRAP SUPPO
(> 95%), WHICH FURTHER SPLIT AND GROUPED INTO 3 DIFFERENT SUBCLADES (FIGURE 1).
ISOLATES CLUSTERED IN CLADE C OF *Beauveria* TAXONOMY TOGETHER WITH *Beauveria pseudobassiana*
(BLOC & EF1-α TREES NOT SHOWN).

A SINGLE DOSE BIOASSAY WAS CONDUCTED WITH A CONCENTRATION OF 2×10^7 CONIDIA ML^{-1} OF 2
FORMULATED FROM 23 DIFFERENT EAB DERIVED *Beauveria* ISOLATES AND THE COMMERCIAL ISOLAT

GHA. SIGNIFICANT DIFFERENCE IN TERMS OF BEETLE CUMULATIVE MORTALITY WAS NOT
DAYS FOLLOWING TREATMENT. BASED ON THESE RESULTS, EIGHT *Beauveria* VARIED
ISOLATES BELONGING TO DIFFERENT CLADES ON THE PHYLOGENETIC TREE AND GHA W
FURTHER VIRULENCE TESTING (TABLE 1). SIGNIFICANT DIFFERENCES WERE OBSERVED IN
MORTALITY BETWEEN 4 AND 14 DAYS AFTER TREATMENT WITH THE FOUR DIFFERENT CON
AMONG THE EIGHT ISOLATES DERIVED FROM EAB AND THE COMMERCIAL GHA ISOLATE.
OF LC_{50} VALUES OF THE *Beauveria* SPP. ISOLATES RANGED FROM 4.58 TO 5.87 (TABLE 1). ISOLA
L49-1AA HAD THE LOWEST LC_{50}, FOLLOWED BY COMMERCIAL ISOLATE, GHA. DOSE MORTA
REGRESSIONS HAD SIGNIFICANTLY DIFFERENT INTERCEPTS (χ^2 = 153.3, DF = 14, P < 0.001) BUT SHARED THE SAME SLOPE (TEST OF PARALLELISM: χ^2 = 8.94, DF = 7, P = 0.374). L49-1AA WAS ABOUT FIVE TIMES MORE VIRULENT THAN GHA IN ADDITION, ISOLATE L49-
EAB ADULTS FASTER THAN ALL OTHER ISOLATES (DATA NOT SHOWN).

TABLE 1. LOG (LC_{50}) VALUES OF DIFFERENT *Beauveria* SPP. AGAINST EAB ADULTS.

Beauveria ISOLATES	SLOPE ± SE	χ^2	LOG LC_{50} (95% CL)[A]	LETHAL CONCENTRATION RATIOS (95% CI)[B]
B. bassiana				
GHA	1.38 ± 0.23	0.35	5.27 (4.88-5.88)	--
L491-AA	1.23 ± 0.24	1.72	4.58 (4.02-4.94)	4.9 (1.4-16.9)*
L11A	0.98 ± 0.20	1.33	4.84 (4.20-5.25)	2.7 (0.7-10.3)
L19C	1.06 ± 0.20	0.77	4.91 (4.47-5.26)	2.3 (0.6-8.0)
B4B	1.55 ± 0.25	0.71	5.39 (5.05-5.68)	0.8 (0.3-2.1)
LHY48A	1.15 ± 0.21	1.85	5.54 (5.10-5.90)	0.5 (0.2-1.7)
LDY20A	1.76 ± 0.32	0.93	5.87 (5.52-6.14)	0.3 (0.1-0.7)
B. pseudobassiana				
L51D	1.39 ± 0.25	1.88	5.52 (5.12-5.84)	0.6 (0.2-1.7)
L25BC	1.29 ± 0.23	1.11	5.80 (5.41-6.13)	0.3 (0.1-0.9)

[A] EACH ASSAY INCLUDED 4 DIFFERENT CONCENTRATIONS (10^4 TO 10^7 CONIDIA/ML) AND A CONTROL; FIFTEEN INSECTS PER REPLICATE, THREE REPLICATION PER DOSE.
[B] LETHAL CONCENTRATION RATIO WERE ESTIMATED BY USING GHA AS STANDARD ISOLATE BAS ROBERTSON AND PREISLER (1992);
LC_{50} VALUES ARE SIGNIFICANTLY DIFFERENT IF THE 95% CI OF THEIR LETHAL CONCENTRATION RA 1.0 (ROBERTSON AND PREISLER 1992).
*SIGNIFICANTLY DIFFERENT FROM OTHER ISOLATES.

QUANTITATIVE SPORULATION OF THE *Beauveria* ISOLATES ON EAB CADAVERS OBTAINED WITH SINGLE CONCENTRATION (2×10^7 CONIDIA/ML) AFTER 14 DAYS OF INCUBATION WAS SIGNIFIC DIFFERENT. CONIDIA PRODUCED BY ALL EAB-DERIVED ISOLATES WERE SIGNIFICANTLY COMMERCIAL ISOLATE GHA ($P < 0.05$) (FIGURE 2).

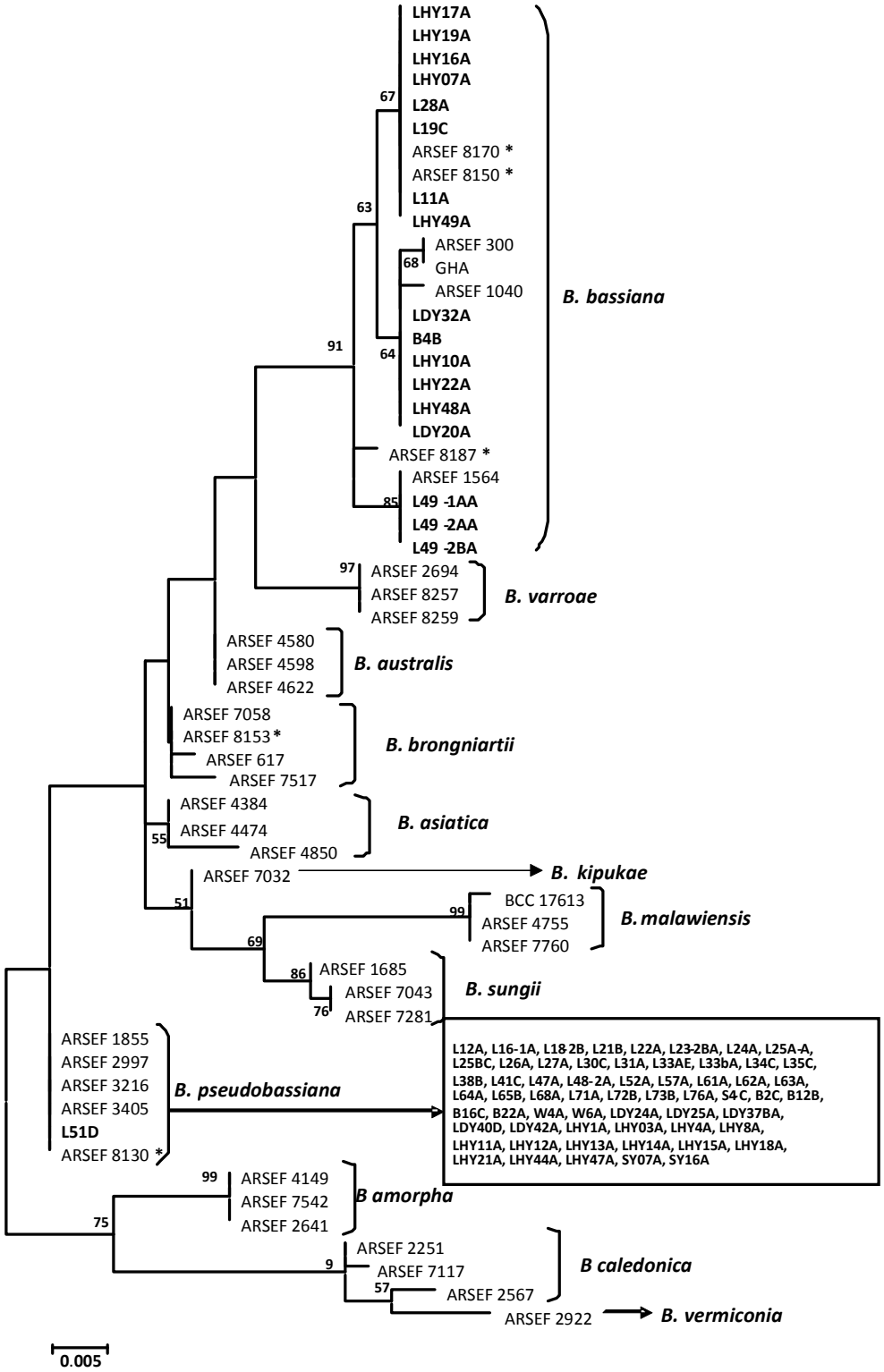

FIGURE 1. MAXIMUM-LIKELIHOOD TREE INFERRED FROM ITS1-5.8S-ITS2 RDNA GENE SEQU
Beauveria SPP. USING THE T92 + G MODEL OF SUBSTITUTIONS (117 TAXA AND 560 CHAR
BRANCH LENGTHS REPRESENT EVOLUTIONARY DISTANCE. NUMBERS AT THE NODES
BOOTSTRAP PERCENTAGES HIGHER THAN 50%. THE ISOLATES RECOVERED FROM EAB IN
THIS STUDY ARE IN BOLD LETTERING. ISOLATES INCLUDED IN THE BOX, CLUSTERED WITH
B. pseudobassiana.

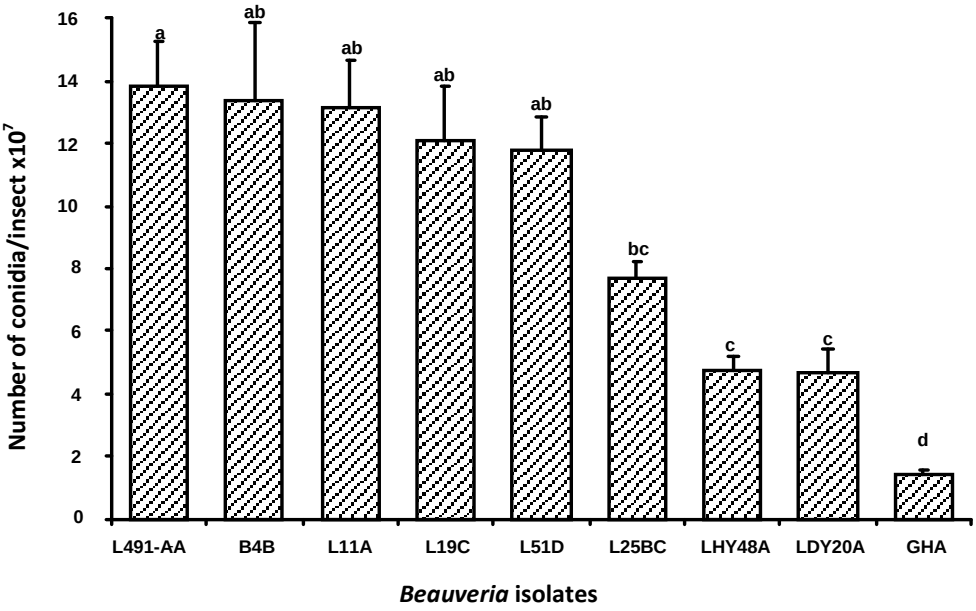

FIGURE 2. NUMBER OF CONIDIA RECOVERED FROM MYCOSED EAB 14 DAYS AFTER INCUBATION IN A HUMIDIFIED CHAMBER. VALUES PRESENTED ARE MEANS (± SE) FROM RANDOMLY SELECTED FROM THREE DIFFERENT BIOASSAYS. THE LETTERS ABOVE THE ER GROUPS OF SIGNIFICANCE (ANOVA PROTECTED TUKEY HSD TEST, α = 0.05).

PCR AMPLIFICATION USING THE AS-PCR PRIMER SET, EFFO × EFRO × EFF1 DESIGNED FR ALIGNMENT OF *Beauveria* SPECIES EF-1 GENE SEQUENCES ARCHIVED IN GENBANK AND THE TA L49-1AA PRODUCED A FRAGMENT 173 BP ONLY IN L49-1AA BUT NOT FROM ANY OTHE *Beauveria* SPECIES (FIGURE 3). THEREFORE THE DIAGNOSTIC TOOL DEVELOPED IN T DIFFERENTIALLY DETECTS AND RENDERS THE DISCRIMINATION OF L49-1AA FROM NAT *Beauveria* SPECIES AND STRAINS WITHIN OUR RELEASED PLOTS.

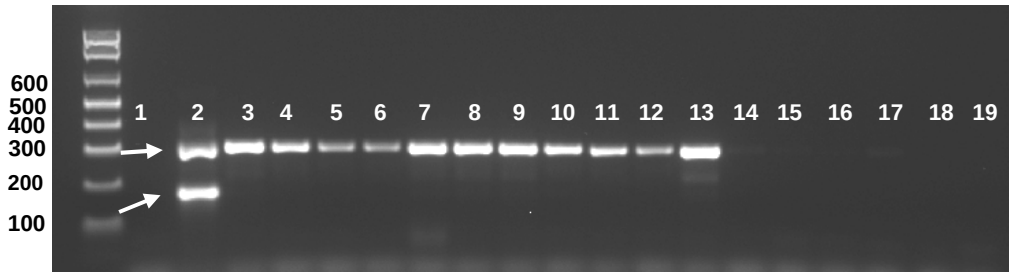

FIGURE 3. DIAGNOSTIC PCR USING THE AS-PCR PRIMER SET EFFO × EFRO × EFF1 AND DIFFE *Beauveria* SPECIES. LANE 1. 100 BP LADDER, LANES 2-13 *Beauveria bassiana*; 2) L49-1AA; 3) INRS CFL-A; 4) L11A; 5) L19C; 6) L28A; 7) B4B; 8) LHY48A; 9) LDY20A; 10) ARSEF 8187; 11) ARSEF 8170; 12) ARSEF 8150; 13) GHA, LANES 14-18 *Beauveria pseudobassiana*; 14) L51D; 15) L72B; 16) L25BC; 17) CAR1; 18) 8130; AND LANE 19 *Beauveria brongniartii*; ARSEF 8153. TOP ARROW-POSITIVE FOR *B. bassiana* AND BOTTOM ARROW-POSITIVE FOR L49-1AA ALONE.

Conclusions

- *Beauveria* SPP. WERE THE PREDOMINANT 'NATURAL' FUNGAL PATHOGENS RECOV MYCOSED EAB AND GALLERY FRASS.
- GREATER THAN 78% OF *Beauveria* ISOLATES RECOVERED FROM EAB CADAVERS WE *B. pseudobassiana;* WE SPECULATE THAT *B. pseudobassiana* MAY HAVE A POSSIBLE ENDOPHYTIC RELATIONSHIP WITH ASH TREES.
- INDIGENOUS *Beauveria* ISOLATES WERE COMPARATIVELY VIRULENT AS GHA AND INTE PRODUCED MORE CONIDIA THAN GHA
- THE MOST PROMISING *Beauveria* ISOLATE, IS CURRENTLY BEING EVALUATED IN THE FIEL AN AUTO-CONTAMINATION-DISSEMINATION APPROACH.
- SINCE RAPID DETECTION OF SINGLE-BASE CHANGES IS FUNDAMENTAL TO MODE GENOTYPING, A SIMPLE AND COST-EFFECTIVE METHOD LIKE AS-PCR WOULD IM ACCESSIBILITY TO SNP GENOTYPING FOR MINIMALLY EQUIPPED LABORATORIES WH A RELEASED ISOLATE. AN IMPORTANT PRACTICAL CONSIDERATION WITH THIS APP UNNECESSARY TO PREPARE A HIGH QUALITY DNA SUITABLE FOR RESTRICTION ENZY ANY OTHER DNA MANIPULATION PROCESS.

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Isolation and identification of endophytic entomopathogenic fungi from dent corn

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Abstract: IN THIS STUDY, WE SOUGHT ENDOPHYTIC ENTOMOPATHOGENIC FUNGI FROM DENT COR
POTENTIAL TO BE DEVELOPED AS ENDOPHYTIC BIOPESTICIDE WITH MULTIPLE ROLES. DENT CORN S
THIS STUDY WERE COLLECTED AT THREE LOCATIONS OF EAST HOKKAIDO ISLAND. EACH PLANT
INTO ROOT, STEM, LEAF AND KERNEL, AND THEN THESE WERE SURFACE-STERILIZED BY 70% E
SODIUM HYPOCHLORITE. PIECES OF EACH TISSUE WERE PLACED ON ENTOMOPATHOGENIC FUNGI S
ALL FUNGAL ISOLATES GROWING ON THIS PLATE WERE TRANSFERRED ONTO POTATO DEXTROSE
IDENTIFICATION TO GENUS LEVEL WAS CONDUCTED BY SLIDE CULTURE METHOD BY OBSER
MICROSCOPE (X100). IN TOTAL, 2252 FUNGAL ISOLATES (GREATER PART OF ISOLATES WERE *Penicillium* SPP. AND *Cladosporium* SPP.) WERE DETECTED ON SELECTIVE MEDIUM, AND AMONG THEM, 168 ISOLAT
ENTOMOGENOUS FUNGI. FIVE GENERA OF ENTOMOPATHOGENIC FUNGI, *Beauveria*, *Lecanicillium*, *Isaria*, *Metarhizium* AND *Simplicillium* WERE DETECTED IN THIS STUDY. IN THIS STUDY, ONLY FIVE F
SAMPLES WERE APPLIED, BUT ENTOMOPATHOGENIC FUNGI WERE DETECTED FROM ALL LOCATIO
PLANT TISSUE. MOREOVER, IT IS INDICATED THAT ENDOPHYTIC ENTOMOPATHOGENIC FUNGI MULT
PLANT BODY. ALTHOUGH *Beauveria, Lecanicillium, Isaria* AND *Metarhizium* SHOWED TENDENCY TO LOCALIZE TO SOME PLANT PARTS, *Simplicillium* TENDED TO BE UBIQUITOUS PRESENCE IN PLANT BODY. OUR RESU
INDICATE THAT ENTOMOPATHOGENIC FUNGI UNIVERSALLY COLONIZE INTO DENT CORN.

Key words: ENDOPHYTE, ENTOMOPATHOGENIC FUNGI, DENT CORN

Introduction

FUNGAL ENDOPHYTES HAVE BEEN DETECTED FROM MANY AGRICULTURAL CROPS, INCLUDI
CORN, COFFEE AND BANANA. SOME FUNGAL ENFOPHYTES BELONG TO ENTOMOPATHOGE
Beauveria bassiana VUILLEMIN (QUESADA-MORACA *et al.*, 2006), *Metarhizium robertsii* REHNER & HUMBER (RAMANPREET & BIDOCHKA, 2012), *Lecanicillium* SPP. (PETRINI, 1981) AND *Isaria farinosa* (HOLMSK.) FR. (BILLS & POLISHOOK, 1991). SEVERAL SPECIES OF ENDOPHYT
ENTOMOPATHOGENIC FUNGI HAVE BEEN SHOWN TO ACT AS PATHOGEN OF PEST INSECT
PLANT PATHOGENS AND PLANT-GROWTH-PROMOTING (QUESADA-MORACA *et al.*, 2008; TANG *et al.*, 2008; RAMANPREET & BIDOCHKA, 2012). FURTHERMORE, SOME FUNGAL ENTOMOPATHOGENS HAVE PO
FOR DUAL- OR MULTIPLE-CONTROL EFFECT AGAINST SEVERAL PLANT DISEASES, PEST
PARASITIC NEMATODES DUE TO ITS ANTAGONISTIC, PARASITIC AND DISEASE RESIS
CHARACTERISTICS (GOETTEL *et al.*, 2008). IN THIS STUDY, WE SEEKED ENDOPHYTIC ENTOMOPATHOG
FUNGI FROM DENT CORN WHICH HAVE POTAINTIAL TO BE DEVELOPED AS ENDOPHYTIC F
MULTIPLE ROLES.

Material and methods

PLANT SAMPLES

PLANT SAMPLES APPLIED TO THIS STUDY WERE COLLECTED AT THREE LOCATIONS OF EAS
TWO DENT CORN SAMPLES (UNKNOWN, TAKII & CO., LTD.) WERE FROM "SHIMIZU", TWO
SAMPLES (ASHILL, SNOW BRAND SEED CO., LTD.) WERE FROM "KAMI OBIHIRO" AND ONE
SAMPLE (P7631, HOKUREN) WAS FROM "ONBETSU" (FIGURE 1). DENT CORN SAMPLES FROM
AND KAMI OBIHIRO WERE WHOLE PLANT (INCLUDE ROOT, STEM, LEAVES AND EARS), BU
ONBETSU WAS ONLY ROOT AND SHORT STEM.

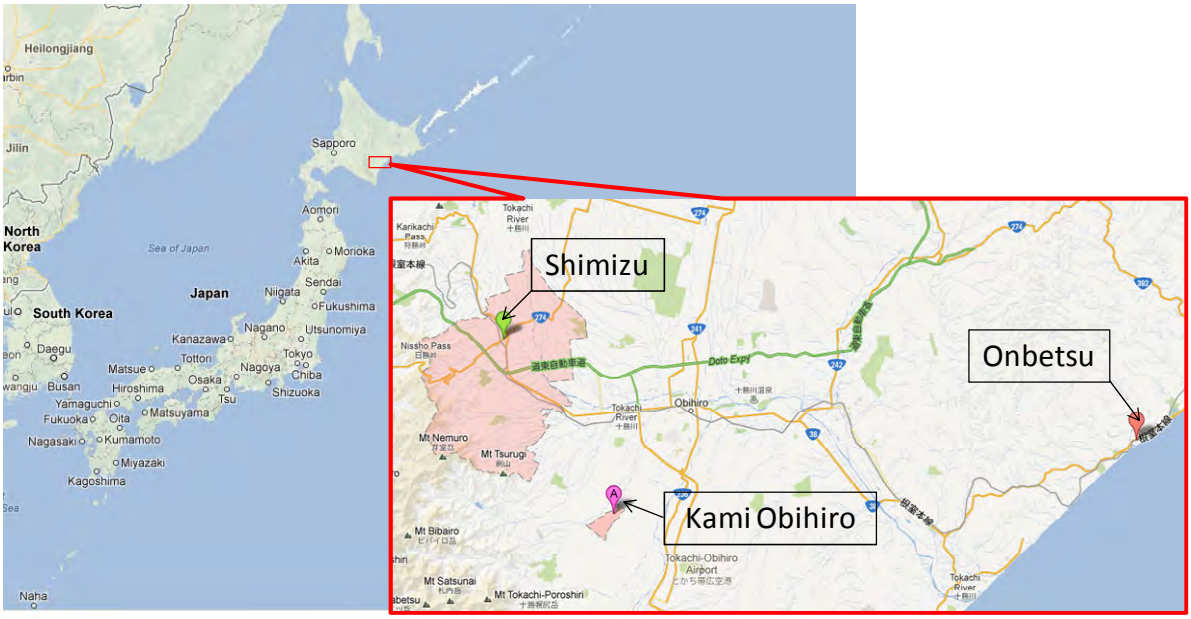

FIGURE 1. SAMPLING LOCATION OF DENT CORN.

ENDOPHYTE ISOLATION AND IDENTIFICATION

EACH PLANT SAMPLE WAS DIVIDED INTO ROOT, STEM, LEAF AND EAR. THEN, ALL STEM
INTO SMALL PIECES OF 10 CM LENGTH, LEAVES WERE CUT INTO 3 SEGMENTS (10 CM LON
CUT INTO 4 PIECES AND EARS WERE DIVIDED INTO KERNEL (27 KERNELS FOR EACH EAR
WERE SURFACE-DESINFECTED BY 70% ETHANOL AND 0.5% SODIUM HYPOCHLORITE (ARNOL
THESE WERE THEN FURTHER CUT INTO SMALLER SEGMENTS (25 MM^2; STEM, ROOT, ROOTLET,
KERNEL; HALF-CUT). PIECES OF EACH TISSUE WERE PLACED ON ENTOMOPATHOGENIC F
MEDIUM (GOETTEL & INGLIS, 1997). FUNGAL GROWTH WAS ASSESSED AFTER INCUBATING
24 °C FOR 1 WEEK. FUNGAL ISOLATES GROWN ON MEDIUM OR PLANT TISSUE WERE REPL
SELECTIVE MEDIUM AND INCUBATED FOR 1 WEEK. ALL FUNGAL ISOLATES GROWING ON
TRANSFERRED ONTO POTATO DEXTROSE AGAR. MORPHOLOGICAL IDENTIFICATION TO
CONDUCTED BY SLIDE CULTURE METHOD (GOETTEL & INGLIS, 1997) BY OBSERVING
MICROSCOPE (X100). MOLECULAR BASED IDENTIFICATION IS NOW ONGOING.

Results and discussion

FUNGAL ISOLATES OF ENDOPHYTIC ENTOMOPATHOGENIC FUNGI ISOLATED FROM DENT C
TABLE 1. IN TOTAL, 2252 FUNGAL ISOLATES (GREATER PART OF ISOLATES WERE *Penicillium* SPP. AND *Cladosporium* SPP.) WERE DETECTED ON SELECTIVE MEDIUM. AMONG THEM, 168 ISOLAT
ENTOMOGENOUS FUNGI. FIVE GENERA INCLUDING *Beauveria*, *Lecanicillium*, *Isaria*, *Metarhizium* AND *Simplicillium* WERE DETECTED IN THIS STUDY. FORMER 4 GERERA INCLUDE MAJOUR S
FUNGAL AGENTS OF BIOPESTICIDES (FARIA & WRAIGHT, 2007), AND *Simplicillium* SPECIES OF
KNOWN AS PARASITE OF MITE, PLANT PATHOGEN AND PLANT PARASTIC NEMATODE
BITTENCOURT, 2008; ZARE & GAMS, 2001). IN THIS STUDY, ONLY 5 PLANT SAMPLES WERE A
ENTOMOPATHOGENIC FUNGI WERE DETECTED FROM ALL LOCATIONS AND AT ALL PA
MOREOVER, IT IS INDICATED THAT ENDOPHYTIC ENTOMOPATHOGENIC FUNGI MULTIPLY
PLANT BODY. ALTHOUGH, *Beauveria*, *Lecanicillium*, *Isaria* AND *Metarhizium* SHOWED TENDENCY TO
LOCALIZE TO SOME PLANT PART, *Simplicillium* TENDED TO BE UBIQUITOUS PRESENCE IN PLANT BODY.
RESULT CAN INDICATE THAT ENTOMOPATHOGENIC FUNGI UNIVERSALLY COLONIZE INTO
RESEARCH WILL BE CONDUCTED TO REAFFIRM THE ENDOPYTIC ABILITY OF THESE FU
CONIDIAL INOCULATION TO DENT CORN, AND TO REVEAL CHARACTERISTICS OF THESE F
ON CONTROL EFFECT OF PEST INSECTS, PLANT DISEASES, AND PLANT PARASTIC NEMATO
BIOCONTROL AGENT.

TABLE 1. THE LIST OF ENDOPHYTIC ENTOMOPATHOGENIC FUNGI ISOLATED FROM 4 DIFFERE
CORN.

	SHIMIZU				KAMI OBIHIRO				ONBETSU		
FUNGAL GENERA	STEM	LEAF	ROOT	KERNEL	STEM	LEAF	ROOT	KERNEL	STEM		
Beauveria	-	1	-	-	4	1	1	-	-	-	
Lecanicillium	-	1	-	-	-	5	2	1	1	-	
Isaria	-	1	1	-	-	-	3	-	-	-	
Metarhizium	-	-	2	-	-	-	-	-	-	-	
Simplicillium	61	12	8	20	2	-	5	-	9	27	

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AUTHORS THANK TO TOKACHI FEDERATION OF AGRICULTURAL COOPERATIVE ASSOCI
SUPPLYING DENT CORN SAMPLES.

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Endophytic establishment of the entomopathogen *BEAUVERIA BASSIANA* in *VITIS VINIFERA* plants

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Abstract: FUNGAL ENTOMOPATHOGENS ARE IMPORTANT ANTAGONISTS OF ARTHROPOD PESTS AND H ATTRACTED INCREASED ATTENTION AS BIOCONTROL AGENTS IN INTEGRATED PEST MANAGEMENT PR ADDITION TO COLONIZING ARTHROPODS, EVIDENCE HAS ACCUMULATED THAT SOME ENTOMOPATH FUNGI LIKE *Beauveria bassiana* (BALS.) VUILL. (ASCOMYCOTA: HYPOCREALES) CAN ENDOPHYTICALLY COLONIZE A WIDE ARRAY OF PLANT SPECIES. FOR A COUPLE OF CROP PLANTS IT HAS BEEN PROVED ENDOPHYTIC *B. bassiana* CAN PROVIDE A SYSTEMIC PROTECTION AGAINST DAMAGE BY VARIOUS INSEC PESTS OR MIGHT TRIGGER INDUCED SYSTEMIC RESISTANCE MECHANISMS AGAINST PLANT PATH CURRENTLY, IT IS UNKNOWN WHETHER *B. bassiana* CAN EXIST AS AN ENDOPHYTE IN GRAPEVINE, *Vitis vinifera* (L.) PLANTS AND STILL MAINTAINS ITS ANTAGONISTIC POTENTIAL AGAINST INSECT PESTS.

IN THE PRESENT STUDY, GREENHOUSE EXPERIMENTS WERE CONDUCTED TO VERIFIY ENDOPHYT ESTABLISHMENT OF THE ENTOMOPATHOGENIC FUNGUS *B. bassiana* IN GRAPEVINE PLANTS AFTER INOCULATION. TWO DIFFERENT COMMERCIALIZED *B. bassiana* STRAINS (ATCC 74040 AND GHA) WERE USED AND APPLIED AS CONIDIAL SUSPENSIONS OR AS THE FORMULATED PRODUCT ON THE UPPER AND LO LEAF SURFACES OF POTTED GRAPEVINE PLANTS. TO DETERMINE IF ENDOPHYTIC COLONIZATION OF GR LEAVES BY *B. bassiana* WAS SUCCESSFUL, LEAF DISKS OF SURFACE STERILIZED CONTROL AND INOCULAT PLANTS WERE OBTAINED AND PLACED ON A SELECTIVE MEDIUM. VERIFICATION OF ENDOPH ESTABLISHMENT OF THE RESPECTIVE *B. bassiana* STRAIN WAS ACHIEVED BY THE AMPLIFICATION OF STRAIN-SPECIFIC MICROSATELLITE MARKERS. FURTHERMORE, THE ANTAGONISTIC ACTIVITY OF END *B. bassiana* AGAINST PUTATIVE TARGET PEST INSECTS LIKE THE VINE MEALYBUG *Planococcus ficus* WAS ASSESSED USING SURFACE STERILIZED LEAVES FOR A BIOASSAY. POSSIBLE EFFECTS OF ENDOPH *B. bassiana* ON THE FEEDING PREFERENCE OF BLACK VINE WEEVIL *Otiorhynchus sulcatus* CHOOSING BETWEEN CONTROL AND INOCULATED PLANTS WERE EXAMINED THROUGH BIOASSAYS.

ENDOPHYTIC SURVIVAL OF *B. bassiana* INSIDE LEAF TISSUES WAS EVIDENT AT LEAST 28 DAYS AFTER INOCULATION, IRRESPECTIVE OF THE INOCULUM USED. A SIGNIFICANT EFFECT OF ENDOPHYTIC *B. bassiana* ON GROWTH BUT NOT ON MORTALITY OF *P. ficus* WAS EVIDENT. ADULT *O. sulcatus* CHOSE SIGNIFICANTLY MORE OFTEN THE CONTROL PLANTS AS A HOST PLANT COMPARED TO GRAPEVINE PLANTS WITH EN *B. bassiana.*

Key words: ENDOPATHOGENIC FUNGI, *Beauveria bassiana*, ENDOPHYTIC GROWTH, GRAPEVINE, *Planococcus ficus, Otiorhynchus sulcatus*

Insect pathogens and entomoparasitic nematodes
IOBC-WPRS Bulletin Vol. 90, 2013
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Effect of temperature, water activity and UV-B radiation on conidia germination and colony growth of *BEAUVERIA BASSIANA* isolates from soil and phylloplane

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Abstract: THE EFFECT OF TEMPERATURE, WATER ACTIVITY (a_w) AND ULTRAVIOLET RADIATION (UV-B), KEY FACTORS DETERMINING THE ENVIRONMENTAL COMPETENCE OF ENTOMOPATHOGENIC FUNGI, HAV EVALUATED ON *Beauveria bassiana* ISOLATES FROM SOIL AND PHYLLOPLANE OF TWO HOLM OAK ECOSYSTEMS IN SOUTHERN SPAIN. THESE ISOLATES WERE MOLECULARLY CHARACTERIZED WITH BAS ELONGATION FACTOR 1-ALPHA ($tef1-\alpha$) GENE BELONGING TO 4 GENOTYPES.

EFFECT OF TEMPERATURE ON GERMINATION AND COLONY GROWTH RATE WAS MONITORED IN THE OF 15-35 °C, WITH OPTIMUM TEMPERATURE FOR GERMINATION AND GROWTH RANGING FROM 23.9 30.4 °C. NO SIGNIFICANT RELATIONSHIP WAS DETECTED BETWEEN OPTIMUM AND MAXI TEMPERATURES FOR GROWTH AND HABITAT, SOIL OR PHYLLOPLANE.

WATER ACTIVITY EFFECT ON THE ABOVE PARAMETERS WAS EVALUATED IN A RANGE OF POTENTIAL CONDITIONS (ψ = 0 TO 200 BARS) BY CHANGING THE GLYCEROL CONCENTRATION IN CULTURE MEDIA. AGAIN, NO SIGNIFICANT RELATIONSHIP WAS DETECTED BETWEEN HUMIDITY REQUIREMEN ISOLATES FROM SOIL AND PHYLLOPLANE, WITH MAXIMUM VALUES OF COLONY GROWTH AND GERMINA RATE BETWEEN 0 AND 5 BARS. NONE OF THE ISOLATES GREW ABOVE 100 BARS.

FINALLY, CONIDIA OF ALL ISOLATES WERE EXPOSED TO IRRADIANCES OF 920 AND 1200 MW M^{-2} 2, 4 AND 6 HOURS. IN GENERAL, THE DELAYING GERMINATION AND COLONY GROWTH WAS DIREC PROPORTIONAL TO UV-B RADIATION DOSE. THREE ISOLATES BELONGING TO A GENOTYPE INCLUDING PHYLLOPLANE ONES SHOWED A PARTICULAR RESPONSE TO UV IRRADIATION, WHICH MAY PROVIDE E ECOLOGICAL INSIGHTS ON THE ROLE OF THESE FUNGI IN THE PHYLLOPLANE.

Key words: ECOSYSTEM, HABITAT, ELONGATION FACTOR 1-ALPHA ($tef1-\alpha$), OSMOTIC-POTENTIAL

Viruses

Session 1

Insect pathogens and entomoparasitic nematodes
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pp. 133-136

Deletion genotypes influence occlusion body potency and production in insects infected by a *SPODOPTERA FRUGIPERDA* nucleopolyhedrovirus isolate from Colombia

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Abstract: THE COLOMBIAN FIELD ISOLATE (SFCOL-WT) OF *Spodoptera frugiperda* MULTIPLE NUCLEOPOLYHEDROVIRUS (SFMNPV) IS A MIXTURE OF DIFFERENT GENOTYPES. TO EVALUATE T PROPERTIES OF THE DIFFERENT VARIANTS IN SFCOL-WT A PLAQUE ASSAY WAS PERFORMED A GENOTYPES WERE IDENTIFIED. GENOTYPE SFCOL-A WAS THE MOST PREVALENT (71%) AND SHO RESTRICTION PROFILE IDENTICAL TO THAT OF SFCOL-WT. THE REMAINING NINE GENOTYPES PR DELETIONS OF 3.8-21.8 KB THAT AFFECTED THE REGION BETWEEN OPEN READING FRAMES (ORFS) THE POTENCY OF SFCOL-A OCCLUSION BODIES (OBS) WAS APPROXIMATELY 4-FOLD HIGHER THAN OBS, WHEREAS THE SPEED OF KILL OF SFCOL-A WAS SIMILAR TO THAT OF SFCOL-WT. DELETION G WERE SIMILARLY OR LESS POTENT THAN SFCOL-WT, BUT SIX DELETION GENOTYPES WERE FAS SFCOL-WT. THE POTENCY OF MIXTURES OF OBS AND CO-OCCLUDED MIXED GENOTYPE OBS WERE G REDUCED IN TWO-GENOTYPE MIXTURES INVOLVING EQUAL PROPORTIONS OF SFCOL-A AND ONE O GENOTYPES (SFCOL-C, -D OR -F). SPEED OF KILL AND OB PRODUCTION WERE IMPROVED ONLY WH GENOTYPE MIXTURES WERE CO-OCCLUDED, ALTHOUGH OB PRODUCTION WAS HIGHER IN THE SI THAN IN ANY OF THE GENOTYPES OR GENOTYPE MIXTURES THAT WE TESTED. THE SFCOL-WT POPUL BE STRUCTURED TO MAXIMIZE THE PRODUCTION OF OBS IN EACH INFECTED HOST SUGGESTIN PRINCIPAL LIMITATION TO TRANSMISSION.

Key words: SFMNPV, COLOMBIA,WILD-TYPE, GENOTYPES, MIXTURES OF OBS, PHENOTYPE

Introduction

PREVIOUS STUDIES ON *Spodoptera frugiperda* MULTIPLE NUCLEOPOLYEDROVIRUS (SFMNPV) AS A POTENTIAL BIOLOGICAL CONTROL AGENT IN COLOMBIA IDENTIFIED THE SFCOL ISOLA INSECTICIDAL OF A TOTAL OF 38 FIELD ISOLATES FROM COLOMBIA OR NICARAGUA (SFNIC 2011). SFMNPV POPULATIONS HAVE BEEN FOUND TO BE COMPOSED OF DIFFERENT G (HARRISON *et al.*, 2008; SIMÓN *et al.*, 2004). PREVIOUS STUDIES HAVE EXAMINED INTERACTIO BETWEEN GENOTYPES THAT DETERMINE THE TRANSMISSIBILITY OF THE WILD-TYPE POPUL *al.*, 1998; SIMÓN *et al.*, 2005). EVALUATING INTERACTIONS BETWEEN GENOTYPES CAN BE ADVANTAGEOUS DURING THE PROCESS OF SELECTING ACTIVE MATERIAL FOR THE DEV BASED BIOLOGICAL INSECTICIDES.

THE OBJECTIVES OF THE PRESENT STUDY WERE TO DETERMINE THE GENOTYPIC DIVE THE SFCOL ISOLATE AND EVALUATE THE CONTRIBUTION OF THE COMPONENT GEN INSECTICIDAL PROPERTIES OF THE NATURAL ISOLATE.

Material and methods

INDIVIDUAL GENOTYPES PRESENT WITHIN SFCOL-WT, 20 PLAQUES WERE ISOLATED BY PLAQUE ASSAY FOLLOWING THE PROTOCOL DESCRIBED BY SIMÓN *et al.* (2004). 10 PLAQUES WERE PICKED INDIVIDUALLY AND INJECTED INTO FOURTH INSTARS OF *Spodoptera frugiperda* FOR VIRAL AMPLIFICATION. OBS WERE PURIFIED AND DNA WAS EXTRACTED AND ANALYZED WITH THE RESTRICTION ENDONUCLEASE *Pst*I. PHYSICAL MAPS WERE CONSTRUCTED BY COMPARISON OF CO-MIGRATING AND GENOTYPE-SPECIFIC FRAGMENTS, AND CONFIRMED BY SEQUENCING THE POLYMORPHIC FRAGMENTS. RELATIVE QUANTITY OF THE COMPLETE GENOTYPE SFCOL-A WAS DETERMINED BY QPCR. *egt* GENE WAS USED AS AN INDICATOR GENE FOR THIS GENOTYPE, AS IT WAS THE ONLY GENE ABSENT IN ALL DELETION GENOTYPES AND PRESENT ONLY IN THE COMPLETE SFCOL-A GENOTYPE.

OB AND CO-OCCLUDED MIXTURES, INVOLVING EQUAL PROPORTIONS OF SFCOL-A AND DELETION GENOTYPES (SFCOL-C, -D OR –F), WERE PRODUCED AS DESCRIBED BY SIMÓN *et al.* (2005). THE INSECTICIDAL ACTIVITY OF THE SFCOL ISOLATE, INDIVIDUAL GENOTYPES AND OB AND OB MIXTURES WAS COMPARED WITH THAT OF SFCOL ISOLATE IN A CONTINUOUSLY RENEWED COLONY OBTAINED FROM LARVAE COLLECTED IN MAIZE FIELDS CLOSE TO BOGOTA, COLOMBIA. THE LC_{50} CONCENTRATION, (MEAN TIME TO DEATH (MTD) AND OB PRODUCTIVITY (OBS/LARVA) WERE DETERMINED USING POLOPLUS (LEORA-SOFTWARE, 1987), AND GLIM (CRAWLEY, 1993). OB PRODUCTION WAS DETERMINED BY COUNTING OB CONTENT IN COHORTS OF 24 OVERNIGHT FIFTH INSTARS INOCULATED WITH THE LC_{90}.

Results and discussion

THE COMPLETE SFCOL-A GENOTYPE ACCOUNTED THE MAJORITY OF GENOTYPES IN SFCOL-WT

TEN DIFFERENT GENOTYPES (NAMED SFCOL-A TO -J) WERE IDENTIFIED BY ANALYSIS OF PLAQUES WITH *Pst*I ENDONUCLEASE (FIGURE 1). SFCOL-A GENOTYPE WITH THE COMPLETE *Pst*I GENOME SHOWED A RESTRICTION PROFILE IDENTICAL TO THAT OF SFCOL-WT, AND WAS SHOWN TO BE PRESENT AT HIGH FREQUENCY (71%) IN THE POPULATION BY QPCR ANALYSIS

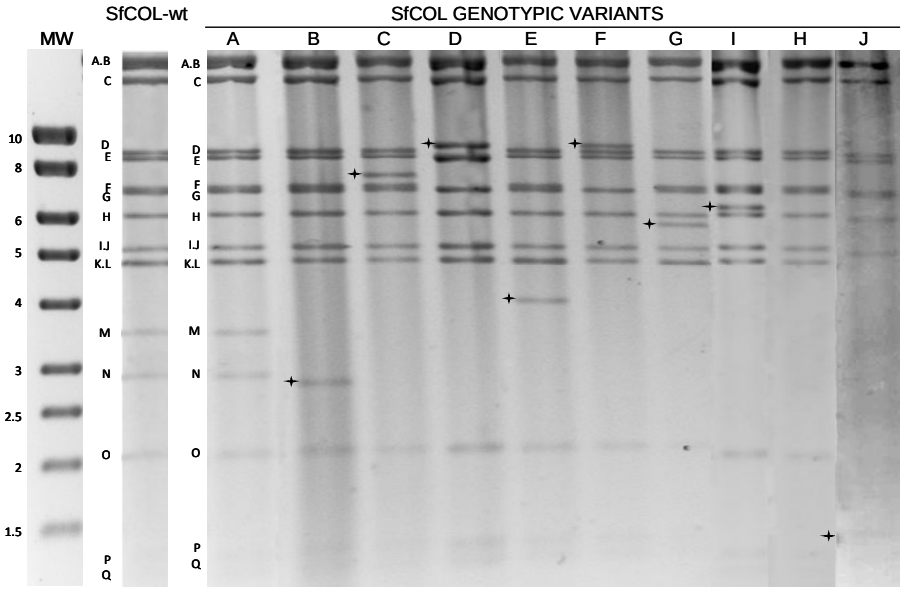

FIGURE 1. REN PATTERNS OF SFCOL-WT AND SFCOL VARIANTS DNA DIGESTED WITH *Pst*I. + INDICATES THE POLYMORPHIC FRAGMENTS OF EACH GENOTYPE.

ALL OTHER GENOTYPES DISPLAYED DELETIONS OF 3.8-15.1 KB TO THE LEFT OF HR1 (FIGURE 2). THIS REGION OF VARIABILITY AMONG THE GENOTYPES, WHICH INCLUDED ORFS S ESSENTIAL PROTEINS WITH AUXILIARY FUNCTIONS, WAS ALSO IDENTIFIED IN MISSOURI *et al.*, 2008) AND NICARAGUA (SIMÓN *et al.*, 2004) SFMNPV ISOLATES.

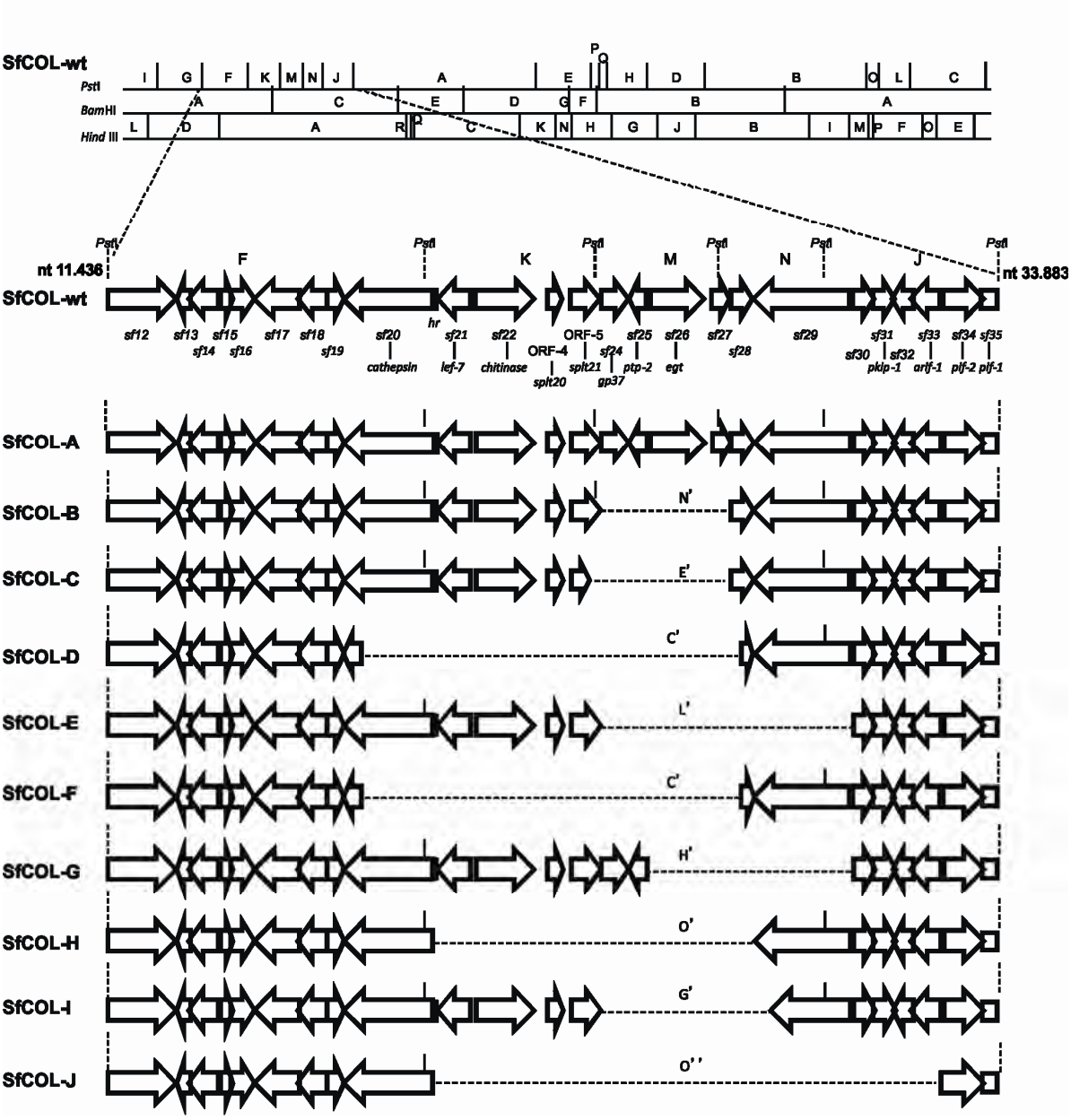

FIGURE 2. SCHEMATIC REPRESENTATION OF THE GENOMIC VARIABLE REGION AMONG SFC

DELETION GENOTYPES REDUCE PATHOGENICITY BUT INCREASE OB PRODUCTIVITY

SFCOL-A WAS APPROXIMATELY 4.4-FOLD MORE POTENT (IN TERMS OF CONCENTRATIO METRICS) THAN SFCOL-WT, INDICATING THAT THE OTHER GENOTYPES DIMINISH THE PATH POPULATION. SFCOL-WT AND PURE GENOTYPES SFCOL-A, -B, -D AND -G WERE THE LOWEST VIRUSES (IN TERMS OF MEAN TIME TO DEATH), WHICH IN THE CASE OF SFCOL-WT WAS R HIGHER PRODUCTIVITY. THE POTENCY OF OBS AND CO-OCCLUDED MIXTURES WERE CONSIS

IN TWO-GENOTYPE MIXTURES INVOLVING EQUAL PROPORTIONS OF SFCOL-A AND ONE OF T
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MIXTURES WERE CO-OCCLUDED, ALTHOUGH OB PRODUCTION WAS HIGHER IN LARVA
SFCOL-WT ISOLATE THAN IN LARVAE INFECTED WITH ANY OF THE COMPONENT GENOTY
THEREOF. CERTAIN DELETED GENOTYPES REDUCED OCCLUSION BODY POTENCY BUT INC
BODY PRODUCTION, SUGGESTING THAT SFCOL-WT IS STRUCTURED TO MAXIMIZE TRANSMIS

IN CONCLUSION, THE SFCOL-WT FIELD ISOLATE COMPRISES A HIGH GENOTYPIC DIVER
SFCOL-A WAS THE MOST PATHOGENIC AND WAS AS VIRULENT AS SFCOL-WT. GENOTYPI
REDUCED SPEED OF KILL BUT ALSO REDUCED OB PATHOGENICITY WHICH IS UNDESI
DEVELOPMENT OF A BIOLOGICAL INSECTICIDE. SFCOL-WT SEEMS TO BE STRUCTURED T
LIKELIHOOD OF TRANSMISSION BY MAXIMISING OB PRODUCTION. SFCOL-A, DUE TO ITS
PATHOGENICITY IS WELL SUITED TO BE DEVELOPED AS A BIOINSECTICIDE IN *S. frugiperda* CONTROL
COLOMBIA.

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On the role of baculovirus photolyases in DNA repair upon UV damage of occlusion bodies

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Abstract: THE USE OF BACULOVIRUSES IN INSECT BIOCONTROL IS HAMPERED BY THEIR SENSITIVI
(UV) LIGHT. THIS IRRADIATION INDUCES CYCLOBUTANE PYRIMIDINE DIMERS (CPDS) IN DNA. CPD-P
REPAIR CPDS USING VISIBLE LIGHT. PLUSIINE BACULOVIRUSES ENCODE PHOTOLYASES, WHICH
REPAIR UV-DAMAGE PRIOR TO INFECTION OF LARVAE. WHETHER THE PHOTOLYASES ENCODED
chalcites NUCLEOPLYHEDROVIRUS ARE INVOLVED IN UV DAMAGE REPAIR WAS TESTED BY INFECT
UV-IRRADIATED VIRAL OCCLUSION BODIES (OBS) THAT WERE SUBSEQUENTLY TREATED WITH VIS
THE DARK. THE OBSERVED MORTALITY WAS THE SAME FOR BOTH TREATMENTS. WE POSTULATE
NOT ACTIVE AS DNA REPAIR ENZYMES IN OBS, BUT MAY PLAY A ROLE IN OTHER ASPECTS O
PATHOGENESIS.

Key words: CPD PHOTOLYASE, *Chrysodeixis chalcites* NUCLEOPOLYHEDROVIRUS, DNA REPAIR, UV SENSITIVITY, BIOCONTROL, CIRCADIAN CLOCK

Introduction

SUNLIGHT IS (IN)DIRECTLY THE MAIN SOURCE OF ENERGY FOR ALL ORGANISMS. AT THE ULTRAVIOLET (UV) COMPONENT OF SUNLIGHT CAN HAVE DESTRUCTIVE EFFECTS BY C DNA: CIS-SYN-CYCLOBUTANE PYRIMIDINE DIMERS (CPDS) AND PYRIMIDINE-(6,4)-PYR PHOTOPRODUCTS (6-4PPS). CPDS ARE FORMED WHEN TWO ADJACENT PYRIMIDINES, USUA ARE LINKED BY TWO COVALENT BONDS. TO DEAL WITH THE HARMFUL EFFECTS OF UV- ORGANISMS (EXCEPT PLACENTAL MAMMALS) RELY ON LESION-SPECIFIC PHOTOLYASE INDUCED DAMAGE IN A LIGHT-DEPENDENT MANNER. CPD-PHOTOLYASES NEED BOTH FLAVINE ADENINE DINUCLEOTIDE (FAD) AND AN ANTENNA MOLECULE AS COFACTORS, B NUCLEOTIDES AND CAN FUNCTION IN AN EXTRACELLULAR ENVIRONMENT. THIS PI PHOTOREACTIVATION (BRETTEL & BYRDIN, 2010).

CPD-PHOTOLYASES ARE CONSERVED IN A SPECIFIC GROUP OF BACULOVIRUSES THAT INSECTS (XU *et al.*, 2008). BACULOVIRUSES ARE LARGE, ENVELOPED DOUBLE-STRANDED DN THAT INFECT INVERTEBRATES, PREDOMINANTLY INSECTS IN THE ORDERS LEPIDOPTERA DIPTERA (REVIEWED BY SLACK & ARIF, 2007) AND THAT CAUSE FATAL DISEASE. THE *Autographa californica* MULTIPLE NUCLEOPOLYHEDROVIRUS (AcMNPV) CPD-PHOTOLYASE (*phr*) GENES WERE REPORTED IN *Chrysodeixis chalcites* NUCLEOPOLYHEDROVIRUS (CHCHNPV) AND WERE NAMED CC-*phr*1 AND CC-*phr*2 (VAN OERS *et al.*, 2004; 2005). LATER ON, A *phr* GENE WAS ALSO IDENTIFIED IN THE GENOME OF *Trichoplusia ni* SINGLE NUCLEOPOLYHEDROVIRUS (TNSNPV) (WILLIS *et al.*, 2005). THE CHCHNPV-ENCODED PROTEINS SHARE 45% AMINO AC IDENTITY. CC-PHR2, IN CONTRAST TO CC-PHR1, POSSESSED CPD-PHOTOLYASE ACTIV HETEROLOGOUS (BACTERIAL) SYSTEM (*in vitro*) (VAN OERS *et al.*, 2008), BUT THERE IS NO EXPERIMENTAL EVIDENCE THAT THESE PHOTOLYASES REPAIR UV LIGHT-INDUCED DNA I FOR INSTANCE IN CHCHNPV OCCLUSION BODIES (OBS) PRIOR TO LARVAL INFECTION.

BACULOVIRUSES ARE APPLIED AS BIOCONTROL AGENTS SINCE THE 1950S AS A ALTERNATIVE TO CHEMICAL PESTICIDES (SZEWCZYK *et al.*, 2006). BUT QUICK INACTIVATION BY UV-LIGHT IN THE FIELD POSES A SEVERE CONSTRAINT ON THEIR USE (INGLIS *et al.*, 2001; SUN & PENG, 2007). TO LIMIT UV INACTIVATION EXPENSIVE UV PROTECTANTS ARE ADDED TO BACULOVIRU (BLACK *et al.*, 1997). THE DISCOVERY OF CPD-PHOTOLYASE GENES IN BACULOVIRUSES (VAN *et al.*, 2004; WILLIS *et al.*, 2005) POTENTIALLY PROVIDES A NOVEL TOOL TO REDUCE THE UV-SENS BACULOVIRUSES USED FOR BIOCONTROL. IN THIS PAPER, WE ANALYZED WHETHER CHCH BE PHOTO-REACTIVATED BY VISIBLE LIGHT AFTER INACTIVATION WITH UV LIGHT, THER INFECTIVITY.

Material and methods

A LABORATORY COLONY OF THE TOMATO LOOPER, *Chrysodeixis chalcites*, WAS REARED ON ARTIFICIAL DIET AT 28 ± 1 ºC AT A 16 H LIGHT/8 H DARK PHOTOPERIOD (MUELLER *et al.*, 2001). THE DUTCH ISOLATE OF CHCHNPV (CHCHNPV-NL) WAS USED IN THESE STUDIES AND HAS BEEN DESCRIBED BEFORE (ZUIDEMA *et al.*, 2004; 2005). AT FIRST THE 90% LETHAL CONCENTRATION (LC_{90}) OF NON-IRRADIATED, WILD TYPE CHCHNPV-NL WAS DETERMINED IN INSECT BIOASSAYS, WITH IN TOTAL 75 LARVAE PER T FIVE DIFFERENT CONCENTRATIONS OF OBS. THESE OBS WERE ISOLATED BY GRINDIN CADAVERS IN STERILE WATER, FILTERING THE HOMOGENATE THROUGH MUSLIN, FOLLOW AT 6000 RPM FOR 5 MIN. A SUSPENSION CONTAINING 10% SUCROSE, 0.001% FLUORELLA BL × 10^5, 1.7 × 10^4; 8.8 × 10^4, 3.5 × 10^3 OR 7 × 10^2 OBS ML^{-1} WAS GIVEN TO 2ND INSTARS, WHICH WERE STARVED FOR 24 H PRIOR TO DROPLET FEEDING (HUGHES & WOOD, 1981). AN INFECTION WI SERVED AS NEGATIVE CONTROL. SUBSEQUENTLY, LARVAE WERE TRANSFERRED TO INDI WELL PLATES CONTAINING DIET. MORTALITY WAS RECORDED DAILY UNTIL 8 DAYS POST THE DATA WERE ANALYZED USING POLO PLUS (LEORA SOFTWARE, 1987).

TO DETERMINE THE OPTIMUM IRRADIATION DOSE, 0.5 ML SUSPENSIONS OF 5 × 10^6 OBS ML^{-1} WERE IRRADIATED IN 35 MM PETRI DISHES (NUNC) WITH 250 NM UV-LIGHT AT TOTAL DOSES OF 150, 200 OR 300 J M^{-2} AS MEASURED BY A UVX RADIOMETER (UVP, LLC UPLAND, CA). TH IRRADIATED OB SUSPENSIONS WERE KEPT IN THE DARK FOR 6 H TO KEEP THE SAME SET PHOTO-REACTIVATION EXPERIMENTS DESCRIBED BELOW. SUBSEQUENTLY, 2ND INSTARS *C. cha* (~25 INSECTS PER TREATMENT, THREE-TIMES REPEATED) WERE DROPLET-FED WITH 5 × 10^6 OBS ML^{-1}. NEXT, CHCHNPV 5 × 10^6 OBS ML^{-1} OBS WERE IRRADIATED AT A UV DOSE OF 0 OR 200 J M^{-2}. THE IRRADIATED SAMPLES WERE EITHER INCUBATED IN COMPLETE DARKNESS OR EXPOSE WITH A REGULAR 13 W TL-TUBE (PHILIPS) AT 28 ± 1 ºC FOR 30 MIN, 1 H, 2 H OR 6 H. AN 8 MM PLATE WAS USED TO FILTER OUT SHORT WAVELENGTH UV-LIGHT. TWO INDEPENDENT PERFORMED AS DESCRIBED ABOVE.

Results and discussion

DOSE-RESPONSE RELATIONSHIP BETWEEN UV-DOSE AND MORTALITY

THE LC_{90} OF CHCHNPV FOR 2ND INSTAR *C. chalcites* LARVAE WAS DETERMINED AS 2.3 × 10^6 OBS ML^{-1} (χ^2=3.14; DEGREE OF FREEDOM: 3; HETEROGENEITY: 1.05). A VIRUS CONCENTRATION HIGH KIL APPROXIMATELY 100% OF THE LARVAE (5 × 10^6 OBS ML^{-1}) WAS SUBSEQUENTLY USED TO ESTABLISH THE DOSE OF UV LIGHT THAT WOULD REDUCE THE MORTALITY INDUCED BY THE CHCHNPV OBS NUMBER OF DEAD *C. chalcites* LARVAE DECREASED GRADUALLY WITH INCREASING UV DOSE AN ~12% AT 200 J M^{-2} (FIGURE 1). A UV DOSE OF 300 J M^{-2} COMPLETELY INACTIVATED THE OBS. THEREFORE A DOSE OF 200 J M^{-2} WAS USED IN EXPERIMENTS DESCRIBED BELOW.

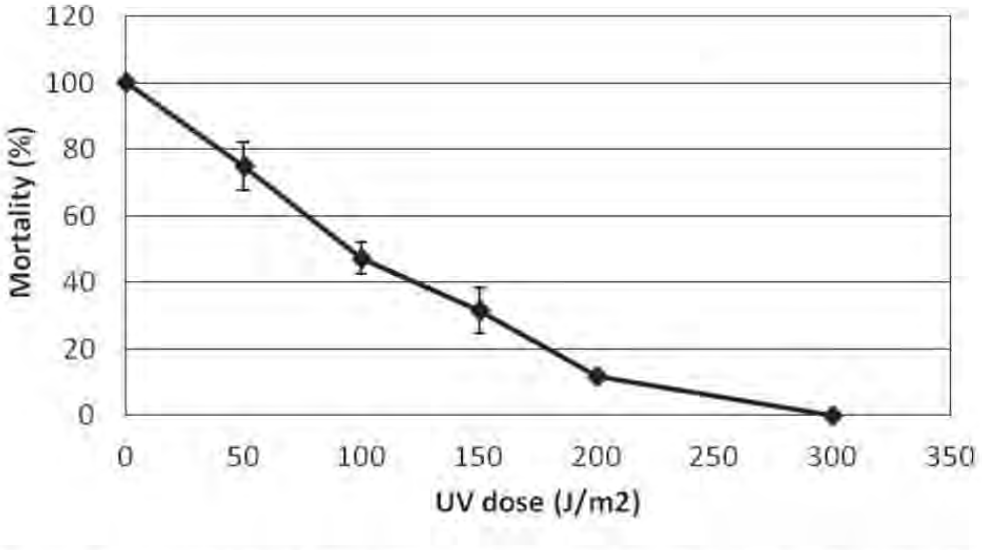

FIGURE 1. UV-SENSITIVITY OF CHCHNPV OBS. THE MORTALITY (%) OF *T. ni* LARVAE INFECTED WITH OBS FROM CHCHNPV TREATED WITH DIFFERENT UV-LIGHT DOSES (J M^{-2}) WAS MEASURED. MEAN AND STANDARD DEVIATION OF TRIPLICATE SAMPLES ARE SHOWN.

PHOTOREACTIVATION AS FUNCTION OF TIME

IN ORDER TO DETERMINE WHETHER CHCHNPV ODVS CARRY ACTIVE PHOTOLYASES THAT INACTIVATION BY UV-LIGHT, CHCHNPV OBS WERE THEN IRRADIATED AT A UV DOSE OF 200 J M^{-2}. THE IRRADIATED SAMPLES WERE INCUBATED IN COMPLETE DARKNESS OR EXPO LIGHT FOR VARIOUS LENGTH OF TIME. NON-IRRADIATED OBS (0 J M^{-2}) WERE USED AS A POSITIVE CONTROL AND RESULTED IN 100% MORTALITY OF *T. ni* LARVAE. NO SIGNIFICANT DIFFERENCE WAS FOUND FO CHCHNPV EXPOSED TO VISIBLE LIGHT (PHOTOREACTIVATION) OR KEPT IN THE DARK AFTE IRRESPECTIVE OF HOW LONG VISIBLE LIGHT WAS APPLIED (FIGURE 2). HENCE, THE BIOA THAT THE PHOTOLYASES ENCODED IN THE CHCHNPV GENOME ARE NOT PRESENT OR A CHCHNPV AND DO NOT PROTECT CHCHNPV AT THIS STAGE AGAINST UV-DAMAGE.

IN A RECENT PROTEOMIC STUDY BOTH PHOTOLYASES, PHR1 AND PHR2, WERE NOT DE CHCHNPV ODV PARTICLES (*Xu et al.*, 2011). THESE DATA COMBINED WITH THE DATA OF THE CU STUDY INDICATE THAT THE PHOTOLYASES ARE NOT PRESENT IN ODVS AND HENCE CA GENOMES PRIOR TO INFECTION OR IN THE VERY EARLY STAGES OF INFECTION BEFORE VI OCCURS. THE FACT THAT THESE PROTEINS WERE NOT FOUND IN ODVS IS IN LINE WITH BACULOVIRUS EARLY PUTATIVE PROMOTER IN THE MOTIFS FOR THE TWO PHR GENES (*phr1*, GATA FOR *phr2*) (VAN OERS *et al.*, 2004; 2005). THUS THE BACULOVIRUS PHOTOLYASES MAY EXPRESSED AT AN EARLY STAGE OF INFECTION, WHICH WOULD BE DIFFICULT TO CONCE CPDS ARE PRESENT IN THEIR GENE SEQUENCES. SINCE THE PHRS ARE ROUTED TO THE NU OUT THEIR FUNCTION (*Xu et al.*, 2010), THERE IS A POSSIBILITY THAT THESE PHOTOLYASES ARE S INVOLVED IN A REPAIR FUNCTION DURING BACULOVIRUS DNA REPLICATION (HUANG *et al.*, 2012). THE SITUATION FOR BACULOVIRUS PHOTOLYASES IS THEREFORE VERY DIFFERENT FROM WH FOWLPOX VIRUS, WHICH ENCODES A PHOTOLYASE THAT IS INCORPORATED INTO MAT WHERE THE ENZYME WAS ABLE TO REPAIR UV-INDUCED DNA DAMAGE IN EXTRACELLULA (SRINIVASAN *et al.*, 2001).

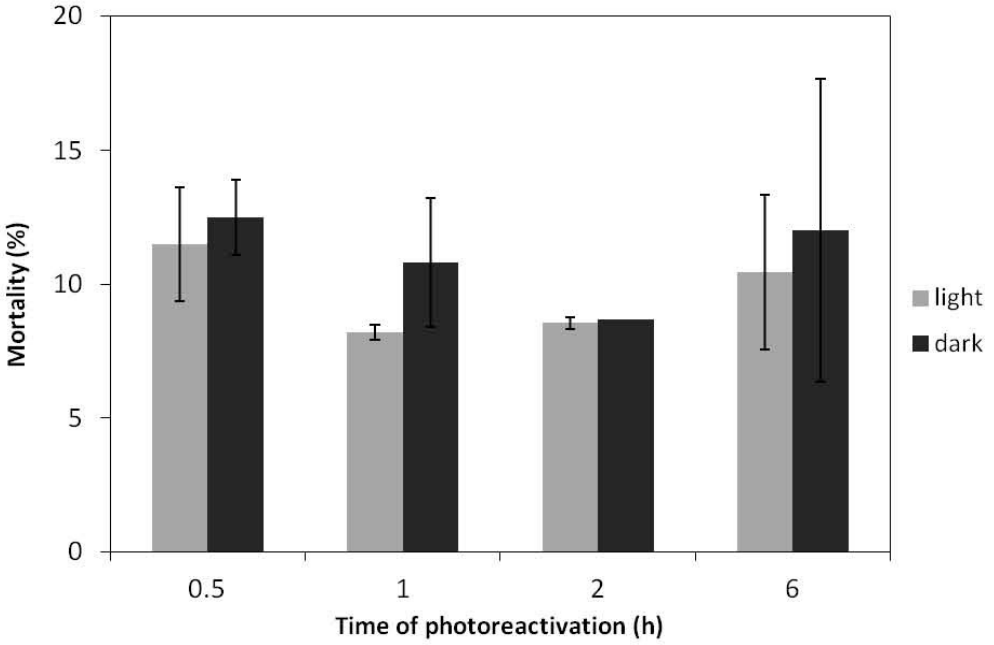

FIGURE 2. PHOTOREACTIVATION OF MORTALITY (%) OF *Chalcites* LARVAE WAS RECORDED AFTER INFECTION WITH OBS FROM CHCHNPV EXPOSED TO VISIBLE LIGHT OR KEPT IN VARIOUS PERIODS OF TIME AFTER UV-IRRADIATION AT A UV DOSE OF 2.0 J/M^2. MEAN AND STANDARD DEVIATION OF DUPLICATE SAMPLES ARE SHOWN.

THIS LEAVES OPEN THE QUESTION, WHAT THE FUNCTION OF BACULOVIRUS PHOTOLY IS KNOWN THAT PHOTOLYASES ARE HOMOLOGOUS TO CRYPTOCHROMES, PROTEINS TH CIRCADIAN CLOCK TO REGULATE OSCILLATION MECHANISMS, AND HENCE, PHYSIOLO METABOLISM OF ALMOST ALL ORGANISMS (VAN DER HORST *et al.*, 1999). RECENTLY, WE ILLUSTRATED THAT PHR2 CAN POTENTIALLY FUNCTION IN THE CIRCADIAN CLOCK BY MIMICKING THE ROLE O (BIERNAT *et al.*, 2012). WE POSTULATE THEREFORE THAT PHR2 COULD HAVE AN EFFECT ON TH CLOCK OF THE INSECT HOST. PHR2 MAY THUS PLAY A ROLE IN VIRUS-INDUCED BEHAVI INFECTED LARVAE BECOME HYPERMOBILE AND DIE AT ELEVATED POSITIONS (GOULSON, *et al.*, 2011; VAN HOUTE *et al.*, 2012) WITH A PUTATIVE BENEFIT FOR VIRUS TRANSMISSION AS OBS EASIER AND MORE EFFICIENTLY SPREAD OVER THE FOLIAGE. TO DETERMINE WHETHER PH BACULOVIRUS-INDUCED BEHAVIOR OR HAVE AN EFFECT ON VIRUS YIELD, E.G. BY CH PATTERNS, STUDIES IN *C. chalcites* LARVAE WITH *phr* KNOCKOUT BACULOVIRUSES ARE NEEDED.

IN CONCLUSION, WE HAVE SHOWN THAT THERE IS NO DIFFERENCE IN UV-SENSITIVITY OBS WHEN EXPOSED TO VISIBLE LIGHT (FOR PHOTOREACTIVATION) OR NOT, SUGGESTIN MAY PLAY A DIFFERENT ROLE IN THE PATHOLOGY OF BACULOVIRUS-INFECTED INSECT HAVE A DUAL FUNCTION, AT LEAST IN THE CASE OF CC-PHR2, AS AN ACTIVE CPD-PHOTO POINT AND AS A MODULATOR OF THE CIRCADIAN CLOCK, WITH POSSIBLE CONSEQUE BEHAVIOR AND ULTIMATELY FOR THE USE OF BACULOVIRUSES AS BIOCONTROL AGENT ONLY PLUSIINE BACULOVIRUSES, SUCH AS CHCHNPV AND TNSNPV HAVE SUCH GENES A EXTENT THIS RELATES TO THE BEHAVIORAL CONSEQUENCES OF THIS GROUP OF INSEC ELUCIDATED.

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Effect of top spray drying and freeze drying on the photostability and insecticidal activity of a *SPODOPTERA FRUGIPERDA* nucleopolyhedrovirus (SfMNPV 003) formulation

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Abstract: THE NUCLEOPOLYHEDROVIRUSES ARE CONSIDERED AS AN EFFECTIVE BIOPESTICIDE A ARMYWORM *Spodoptera frugiperda*. TOP SPRAY AND FREEZE DRYING METHODS WERE USED TO PREPA WETTABLE POWDER FORMULATION BASED ON NUCLEOPOLYHEDROVIRUS OF *Spodoptera frugiperda* (SFMNPV 003) AND ITS PHOTOSTABILITY AND VIRULENCE WERE ASSESSED. TOP SPRAY DRYING METHOD WAS MORE EFFIC MOISTURE CONTENT THAN FREEZE DRYING. NO OBVIOUS DIFFERENCES IN THE INSECTICIDA OBSERVED FOR BOTH DRYING METHODS ALTHOUGH A HIGHER PHOTOSTABILITY (88.54%) WAS FORMULATION PREPARED WITH TOP SPRAY DRYING METHOD COMPARED TO FREEZE DRYING UNFORMULATED VIRUS (15.62%) AFTER 6 HOURS OF UV RADIATION EXPOSURE. TOP SPRAY DRYIN SELECTED AS THE MOST FAVORABLE PROCESS FOR BEING IMPLEMENTED IN A MANUFACTURE PRO

Key words: BIOLOGICAL CONTROL, BIOPESTICIDE, ENTOMOPATHOGENIC, FALL ARMYWORM, DRYING

Introduction

THE FALL ARMYWORM *Spodoptera frugiperda* (JE SMITH, 1797) (LEPIDOPTERA: NOCTUIDAE), IS CONSIDERED AS THE MOST IMPORTANT PEST OF MAIZE CROP, REDUCING YIELDS UP TO 35 2002). USE OF ENTOMOPATHOGENIC VIRUS IS AN ALTERNATIVE TO CHEMICAL PESTICIDES CONSIDERED AS AN EFFECTIVE STRATEGY IN THE INTEGRATED PEST CONTROL MANAGEM CONCERNS CAN LIMIT ITS USE, SUCH AS INACTIVATION OF VIRUS CAUSED BY SOLAR RA AND SCALING UP PROBLEMS DURING VIRAL PROPAGATION AND FORMULATION PROCESS. FORMULATION BASED ON A COLOMBIAN *Spodoptera frugiperda* NUCLEOPOLYHEDROVIRUS (SFMNPV 003) WAS PREVIOUSLY DEVELOPED, WHICH SHOWED TO CONTROL THE PEST AT 100% UNI CONDITIONS. THE SAME FORMULATION REDUCED THE MAIZE PLANT DAMAGE UP TO 2.5 CONDITIONS (GOMEZ *et al.*, 2011). HOWEVER, THERE IS A LIMITED KNOWLEDGE ABOUT THE I METHOD NEEDED TO DRY THE FORMULATION, PARTICULARLY WHEN THE PROCESS HAS B THE COST OF PRODUCTION NEED TO BE REDUCED. THUS, THE EFFECT OF THE TWO DRYING AND TOP SPRAY DRYING) OVER PHOTOSTABILITY AND INSECTICIDE ACTIVITY OF FORMU SFMNPV 003 WAS EVALUATED.

Material and methods

VIRUS PRODUCTION

THE VIRAL INOCULUM WAS OBTAINED FROM INFECTED THIRD-INSTAR *S. frugiperda* LARVAE OF EXHIBITED SYMPTOMS OF VIRAL INFECTION. INFECTED LARVAE WERE HOMOGENIZED MORTAR WITH A STERILE SOLUTION OF SODIUM DODECYL SULFOXIDE (SDS) AT 0.1% CONCENTRATION OF VIRAL OCCLUSION BODIES (OBS). OBS WAS DETERMINED USING A NEUBAUER COUNTIN CHAMBER.

FORMULATION

A MIX OF 2300 ML OF VIRAL SUSPENSION CONTAINING 3.1×10^{9} OBS ML^{-1} AT PH OF 6.36 AND 3.10% SOLIDS CONTENT WAS USED TO PREPARE THE FORMULATION. ALL COMPONENTS INCLU BRIGHTENER AND OTHER SUNSCREENS WERE DILUTED IN PHOSPHATE BUFFER SOLUTION A WITH THE VIRAL SUSPENSION. THE FINAL MIXTURE WAS SUBJECTED TO TWO DRYING TECH DRYING AND FREEZE DRYING.

TOP SPRAY DRYING

A GLATT UNI GLATT 01277 FLUID BED DRYER WITH A NOZZLE OF 1 MM DIAMETER WAS BATCHES OF 250 G OF THE FORMULATED VIRUS WERE EVALUATED. EACH BATCH WA EQUIPMENT CHAMBER AT 10^{-1} $ML\ MIN^{-1}$. THE INLET AIR TEMPERATURE WAS MAINTAINED AT 90 ± 2 WHILE THE CHAMBER TEMPERATURE REMAINED BETWEEN 36 °C AND 55 °C. INTERNAL PR CHAMBER WAS 1 BAR. THE OPERATION EFFICIENCY WAS EXPRESSED BY THE AMOUNT OF V FROM THE INITIAL MIXTURE PER MINUTE ($G\ H_2O\ MIN^{-1}$).

FREEZE-DRYING

A VIRTIS GENESIS 25ES FREEZE DRYER WAS USED. THREE BATCHES WITH 1 KG OF THE FOR THE VIRUS WERE EVALUATED. TEMPERATURE IN THE CONDENSER WAS MAINTAINED UN 57 ± 5 MM TORR DURING 24 HOURS. PROCESS EFFICIENCY WAS DETERMINED ($G\ H_2O\ MIN^{-1}$) AS DESCRIBED ABOVE.

QUALITY CONTROL

THE PH IN SUSPENSION AND MOISTURE CONTENT WERE DETERMINED BY TRIPLICATE FOR E A POTENTIOMETER HANNA 8014 AND A KERN MLS 50 MOISTURE ANALYZER RESPECTIVEL ANALYZED BY ANOVA AND TUKEY TEST ($\alpha = 0.05$).

DETERMINATION OF VIRAL LC_{50}

NEONATE LARVAE (S1) OF *Spodoptera frugiperda* WERE USED TO DETERMINE THE MEAN LETHAL CONCENT (LC_{50}) FOR FORMULATED AND UNFORMULATED VIRUS. BRIEFLY, FIVE DIFFERENT CONCENT TO 1.0×10^{7} OBS ML^{-1}) DILUTED IN DISTILLED WATER AND A CONTROL WITHOUT TREATME FIFTEEN LARVAE FOR EACH TREATMENT WERE INFECTED WITH VIRUS USING THE DROP (HUGHES & WOOD, 1981). MORTALITY RESULTS WERE SUBJECTED TO PROBIT ANALYSIS (F USING POLO PC (POLO LEORA SOFTWARE, 1997).

EFFECT OF THE DRYING METHODS ON PHOTOSTABILITY

FORMULATED AND UNFORMULATED VIRUS WAS RECONSTITUTED IN DISTILLED W CONCENTRATION OF 2.0×10^{7} OBS ML^{-1}. SUBSEQUENTLY, 100 µL OF EACH FORMULATION WAS PLACE WELL OF A 96-WELL STANDARD MICROPLATE. THE MICROPLATE WAS EXPOSED TO M ULTRAVIOLET LAMP LIGHT (3VP-38) WITH A WAVELENGTH OF 375 NM (UV-B) AT 30 CM HEI (NO EXPOSURE), 2, 4 AND 6 HOURS OF EXPOSURE WERE EVALUATED. AFTER IRRADIATIO

were collected and the viral activity was assessed. Viral suspensions were used for a bioassay by the droplet feeding method (Hughes & Wood 1981). Mortality was corrected with the control (larvae without treatment) by the Schneider Orelli equation (Zar, 1999).

Results and discussion

Results of moisture content, pH and efficiency are described in Table 1. Significant difference ($p < 0.05$) were observed in final moisture content and suspension pH when drying techniques were compared (Table 1).

Table 1. Characteristics of the top spray dried and freeze dried formulations.

	Final moisture content (%)		**pH**		**Efficiency ($g\ H_2O\ min^{-1}$)**	
Batch	**Top spray drying**	**Freeze drying**	**Top spray drying**	**Freeze drying**	**Top spray drying**	**Freeze drying**
1	4.37a	1.54b	7.37a	6.06b	7.20	0.43
2	4.21a	1.73b	7.42a	6.06b	6.46	0.44
3	4.58a	1.75b	7.41a	6.05b	6.30	0.45
Mean					**6.65a**	**0.44b**

(Different letters indicate significant differences by Tukey test ($\alpha = 0.05$). Results of final moisture content (%) and pH were compared separately).

The efficiency was significantly higher in samples dried using top spray drying method compared with the freeze drying method (up to 16 times faster for removing the moisture). However, moisture contents obtained by freeze drying at the end of the process were significantly lower than spray drying (under 1.75%) (Table 2). The highest efficiency values obtained by top spray drying may be explained by the fact that heat and mass transfer phenomena are governed by convection, which generally have a higher dynamic compared to conductive phenomena which predominate in the freeze drying process (Al-Hakim & Stapley, 2004). Additionally, particles produced by top spray drying method have a superficial area larger than obtained by freeze drying method, this could increase transfer speeds. Significant differences found in the final pH could be attributed to the higher moisture content in the sprayed product, which is mainly buffer pH 7.0 remaining from formulation process.

The LC_{50} for products obtained by both drying methods were not significantly different, suggesting that drying methods did not affect viral insecticidal activity. In the other hand, significant differences between dried products an unformulated virus were not found, indicating that neither the formulation process nor the drying method affected the pathogenicity (Table 2). These results are similar to the obtained by Tamez *et al.* (2000) with 16 sprayed formulations, where bioassays demonstrated that viral occlusion bodies were unaffected.

TABLE 2. MEAN LETHAL CONCENTRATION (LC_{50}) OF UNFORMULATED AND FORMULATED VIRUS (TOP S
DRYING AND FREEZE DRYING).

	Fiducial limits (Obs ml^{-1}) 95%					
Treatment	**LC_{50} (OBs ml^{-1})**	**Lower (OBs ml^{-1})**	**Upper (OBs ml^{-1})**	**df**	**^{2}**	**P**
Unformulated Virus	1.64×10^5	5.17×10^4	4.68×10^5	3	4.738	0.57
Top spray drying	9.43×10^4	4.46×10^4	1.99×10^5	3	2.801	0.83
Freeze drying	6.26×10^4	1.89×10^4	1.91×10^6	3	3.166	0.57

REGARDING THE PHOTOSTABILITY TEST, THE RESULTS SHOWED THAT THE EFFICACY
VIRUS WAS SIGNIFICANTLY REDUCED ($P < 0.05$) BEING REDUCE TO 15.92% AFTER 6 HOURS C
DRIED FORMULATIONS SHOWED A SIGNIFICANT PROTECTION FROM UV RADIATION. HOWE
OF SPRAY-DRIED PRODUCT WAS SIGNIFICANTLY HIGHER (88.54%) THAN FREEZE-DRIED (77
HOURS OF EXPOSURE (FIGURE 1). SPRAY DRYING METHOD COULD FAVOR COATING OF O
MICROENCAPSULATION, PROTECTING THEM FROM THE DELETERIOUS EFFECTS OF UV RAD

THE FREEZE DRYING METHOD DEMAND HIGHER ENERGY CONSUMPTION AND IS LESS
TOP SPRAY DRYING TECHNIQUE. ADDITIONALLY, THE SPRAY DRYING SHOWED A SIGNIFICA
EFFECT ON THE PHOTOSTABILITY AND THIS TECHNIQUE COULD BE MORE FAVORABLE THA
A CONTINUOUS PRODUCTION.

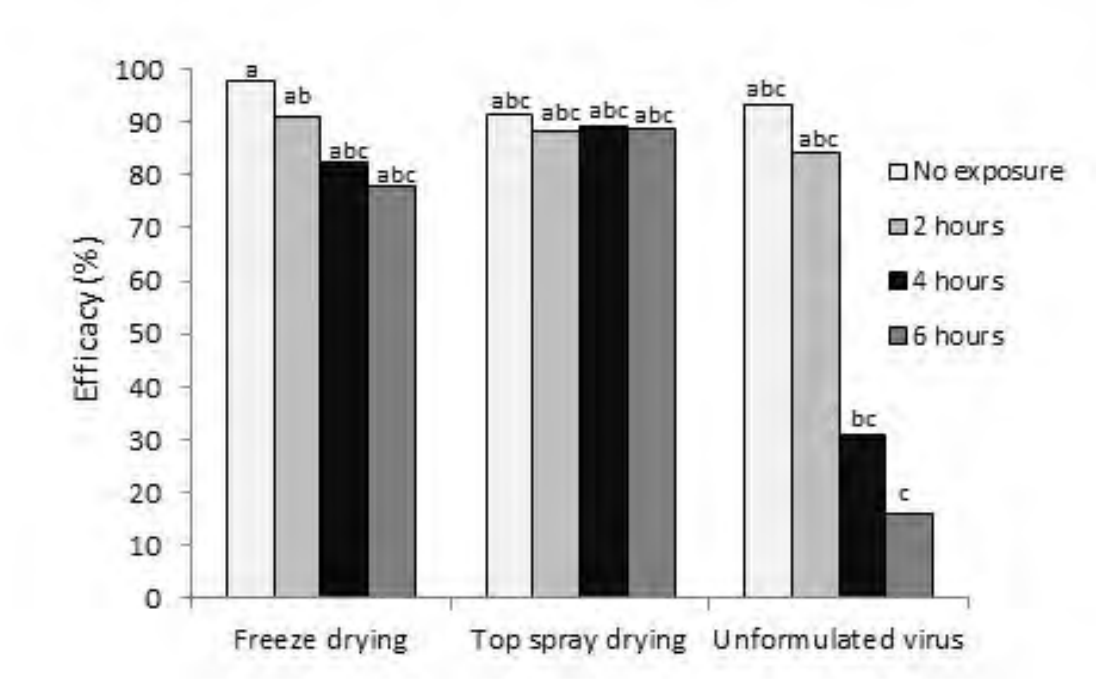

FIGURE 1. EFFECT OF UV-IRRADIATION TIME OVER EFFICACY OF UNFORMULATED AND F
DIFFERENT LETTERS MEAN SIGNIFICANT DIFFERENT ACCORDING TO DMS TEST ($\alpha = 0.05$).

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Variations in the susceptibility to CpGV in populations of the codling moth, *CYDIA POMONELLA*

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Abstract: FAILURE IN CODLING MOTH POPULATIONS CONTROL WITH CPGV IN APPLE ORCHAR ATTRIBUTED TO THE ACTION OF A SINGLE ALLELE LOCATED IN THE Z CHROMOSOME. HOWEVER MORTALITY PATTERNS BETWEEN GENETICALLY HOMOGENEOUS SUSCEPTIBLE AND RESISTANT IN STRONGLY SUGGEST THAT OTHER MECHANISMS ARE RESPONSIBLE OF VARIATION IN THE SUSCE CPGV ISOLATES.

Key words: *Cydia pomonella*, MULTIGENIC RESISTANCE

Introduction

THE *Cydia pomonella* GRANULOVIRUS (CpGV, *Betabaculovirus*) HAS BEEN EXTENSIVELY USED FOR THE CONTROL OF CODLING MOTH PROLIFERATION IN ORCHARDS SOON AFTER ITS FIRS 1964) AND CHARACTERISATION (CROSS *et al.*, 1998). IN 2005, A FAILURE OF CONTROL AND POSSIBL DEVELOPMENT OF RESISTANCE WAS REPORTED IN *C. pomonella* POPULATIONS FROM SOUTH (SAUPHANOR *et al.*, 2006) AND IN OTHER PARTS OF EUROPE (JEHLE & SCHMITT, 2009). FI INVESTIGATIONS INTO THE RESISTANCE OF *C. pomonella* TO CPGV-M CARRIED OUT IN GERMANY USING SINGLE PAIR CROSSES CLEARLY INDICATED MONOGENIC AND SEX-LINKED RESISTANCE (*ad hoc*, 2007). RECENTLY MORE DETAILED STUDIES SUGGESTED THAT OTHER MECHANISMS A EXPLAIN THE OBSERVED RESPONSE OF NATURAL AND LABORATORY POPULATIONS T (BERLING *et al.*, 2013; JEHLE & SCHMITT, 2009). IN THIS STUDY, THE RESPONSE OF LABOR COLONIES TO VIRUS CHALLENGE HAS BEEN ANALYSED FROM THIS PERSPECTIVE.

Material and methods

INSECT COLONIES

THREE *Cydia pomonella* COLONIES WERE USED IN THIS ANALYSIS: I) THE SUSCEPTIBLE LABORATORY IMBRED STRAIN, DERIVED FROM A FIELD POPULATION COLLECTED AT (VAUCLUSE, FRANCE) IN EARLY NINETIES, AND REARED WITHOUT SELECTION PRES (AVIGNON); II) THE V COLONY (BERLING *et al.*, 2009), DERIVED FROM THE INTROGRESSION OF THE MAJOR RESISTANT DETERMINANT FOUND IN A FRENCH NATURAL RESISTANT POPULAT (SAUPHANOR *et al.*, 2006), AND THE CPNPP COLONY, THAT IS THE SUSCEPTIBLE COLONY U INDUSTRIAL LEVEL FOR THE PRODUCTION OF CARPOVIRUSINE™. CPNPP HAS BEEN REAR FOR MORE THAN 20 YEARS AND ORIGINALLY COMES FROM NORTHERN FRANCE. ALL COL ON ARTIFICIAL DIET (GUENEL *et al*, 1983).

VIRUS ISOLATES

TWO *Cydia pomonella* GRANULOVIRUS (CPGV) (*Betabaculovirus, Baculoviridae*) ISOLATES WERE USED IN THIS STUDY, THE CPGV-M ISOLATE, FOUND IN MEXICO (TANADA, 1964), AND THE DERIVED FROM NPP-R1 BY SELECTION ON RGV INSECTS (BERLS *et al*., 2011) FOR 16 PASSAGES AS PREVIOUSLY DESCRIBED (BERLS *et al*., 2009).

BIOASSAYS

BIOASSAYS AGAINST NEONATE LARVAE (0 TO 12 H OLD) WERE CARRIED OUT USING CONTAMINATION METHOD IN 96-WELL PLATES CONTAINING ABOUT 200 µL OF A FORM ARTIFICIAL DIET (HELIOTHIS DIET; WARD'S NATURAL SCIENCE, USA). A 6 µL VOLUME SUSPENSION WAS DEPOSITED OVER THE DIET SURFACE OF EACH WELL (7.14 mm^2). THE SAME VOLUME OF DISTILLED WATER WAS USED IN CONTROL WELLS. BIOASSAYS WERE FIVE OR SIX CPGV CONCENTRATIONS, RANGING FROM 3 TO 729 OBS µL^{-1} FOR THE MOST EFFICIENT ISOLATES (CORRESPONDING TO 0.643 TO 156.2 OBS/mm^2 SURFACE) AND UP TO 3.125×10^6 OBS µL^{-1} FOR THE RGV COLONY FOR THE LEAST EFFICIENT ISOLATE (0.695 X 10^5 OBS/mm^2). ONE LARVA WAS PLACED IN EACH WELL. THE WELLS WERE SEALED WITH PARAFILM, AND THE MIC INCUBATED IN A GROWTH CHAMBER AT 25 °C WITH A 16 H LIGHT/8 H DARK PHOTOPERIOD DURING THE FIRST DAY POST INOCULATION WERE EXCLUDED FROM THE ANALYSIS. THR INDEPENDENT REPLICATE TESTS HAVE BEEN PERFORMED FOR EACH MODALITY REPRESE INFECTED INDIVIDUALS PER MODALITY. TESTS PRESENTING HIGH MORTALITY IN CONTRO OR HAVE BEEN REMOVED FROM THE ANALYSIS. MORTALITY WAS RECORDED AT 7 DA LARVAE THAT DID NOT REACT TO PHYSICAL STIMULI WERE CONSIDERED DEAD. DATA ANALYSIS USING THE SOFTWARE POLO+ (LEORA SOFTWARE 2012).

Results and discussion

ITHAS BEEN PROVEN THAT THE MAJOR DETERMINANT OF RESISTANCE TO CPGV-M IS L Z CHROMOSOME, AND THUS, FOLLOWS A SEXUAL TRANSMISSION PATTERN (ASSER-KAIS *et al*., 2007). HOWEVER, CAREFULL ANALYSIS OF THE AVAILABLE DATA FROM ISOGENIC STRAINS REVE WITH THIS PREDICTED MODEL (BERLS *et al*., 2013), IT HAS BEEN SUGGESTED THAT OTHER RESISTA MECAHNISMS COULD EXIST (JEHLE *et al*., 2011).

TABLE 1. BIOLOGICAL EFFICIENCY OF TWO CPGV ISOLATES ON THREE LABORATORY COLO

Virus	Insect	LD_{50}	95% Fiducial Limits	LD_{90}	95% Fiducial Limits	Slope	χ^2
CPGV-M	S_V	34	24-46	125	86-239	2.271 ± 0.387	3.782
	CPNPP	13	7-23	223	111-653	1.041 ± 0.087	5.9895
	R_{GV}	7122	1196-37429	$1.83X10^6$	$2.15X10^5$-$3.68X10^8$	0.531 ± 0.072	6.5308
CPGV-R5	S_V	32	4-106	438	127-19402	0.126 ± 0.175	8.4499
	CPNPP	7	3-12	60	28-279	1.355 ± 0.127	11.425
	R_{GV}	22	14-33	410	240-845	1.011 ± 0.102	3.622

Table 1 presents the results of bioassays on the three *C. pomonella* colonies. As expected, the CpGV-M isolate is efficient on susceptible insects (CpNPP and S_V) but not on resistant insects (R_{GV}). The CpGV-R5 isolate is efficient in all three colonies.

The comparisons of efficiency between each modality indicate that each virus displays a specific action on each insect colony excluding CpGV-R5 which shows the same effect on S_V and R_{GV} (Equality hypothesis: $P > 0.05$, $\chi^2 = 1.04$, df = 2, tail probability = 0.596; Parallelism hypothesis: $P > 0.05$, $\chi^2 = 0.34$, df = 1, tail probability = 0.559). This result could reflect the shared genetic background of S_V and R_{GV} colonies (Berling *et al.*, 2009).

When comparing the dose-reponse to the CpGV-M isolate of CpNPP and S_V, small but significatives differences are found (Equality hypothesis: $P < 0.05$, $\chi^2 = 24.80$, df = 2, tail probability = 0.00; Parallelism hypothesis: $P < 0.05$, $\chi^2 = 17.56$, df = 1, tail probability = 0.00), whereas CpGV-R5 virus impacts these colonies on a similar way (Parallelism hypothesis: $P > 0.05$, $\chi^2 = 1.02$, df = 1, tail probability = 0.314) but with different intensity (Equality hypothesis: $P < 0.05$, $\chi^2 = 30.40$, df = 2, tail probability = 0.00). CpNPP appears to be a laboratory colony "fully susceptible" to both CpGV isolates, while S_V and its derivate, R_{GV}, have a lower susceptibility level. As seen in Figure 1, although the global trends are similar for S_V and CpNPP, at low multiplicity of infection CpNPP is more susceptible than S_V to both virus isolates.

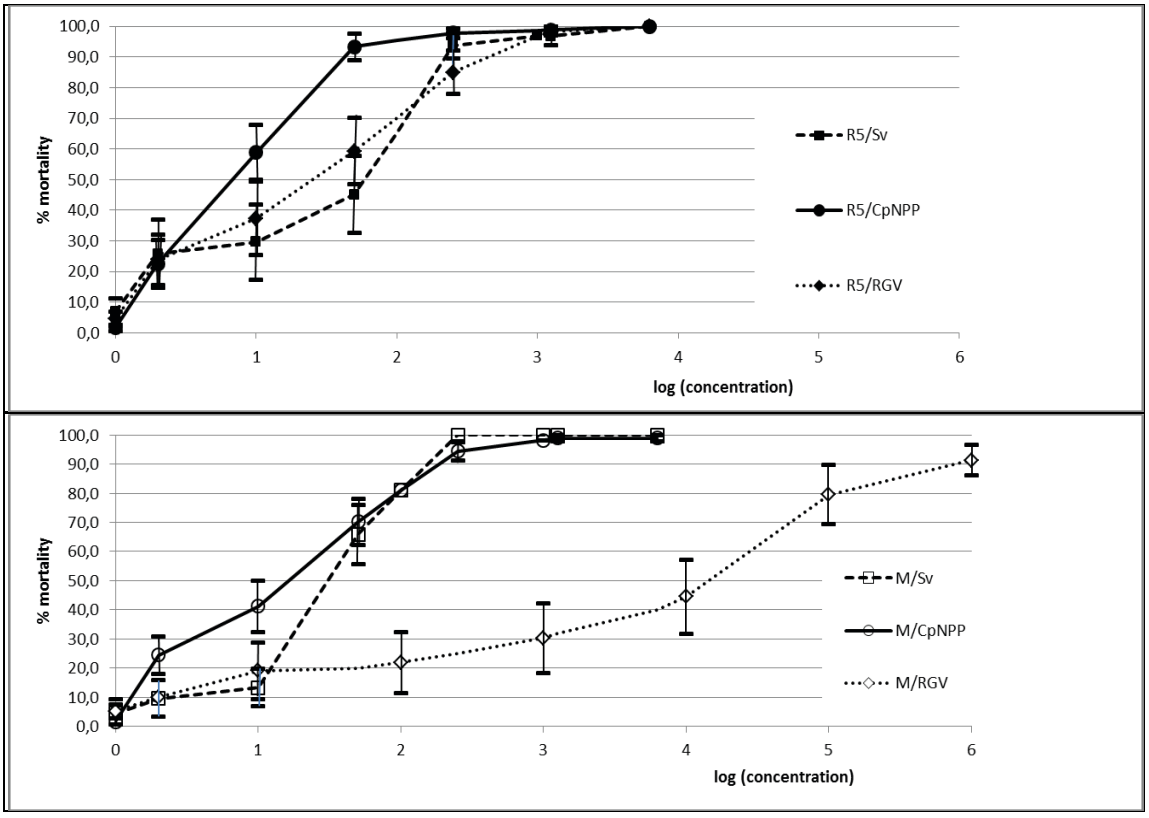

Figure 1. Dose/mortality plots for the two virus isolates CpGV-R5 (upper panel) and CpGV-M (lower panel) on three insect colonies (S_V, CpNPP, and R_{GV}).

THESE DIFFERENCES ARE PROBABLY THE REFLECT OF DIFFERENCES IN THE WAYS CONTROL THE INFECTION CYCLE IN THEIR HOSTS. IN CONDITION OF LOW VIRUS PREVALE BACKGROUND, IT SHOULD BE ENOUGH FOR BLOCKING AN OUTBREAK.

IN OTHER BACULOVIRUSES, VARIABILITY ON THE SUSCEPTIBILITY OF NATURAL PO INFECTION WITH A VIRUS ISOLATE HAS BEEN DESCRIBED (*e.g.*, BESSE, 1986). THE POSSIBILITY OF SELECTION OF A POPULATION WITH REDUCED SUSCEPTIBILITY TO CPGV SECOND MECHANISM REMAINS OPEN. THIS SECOND MECHANISM WOULD NOT NECESSARI ISOLATES IN A SIMILAR WAY AS THE RESISTANCE ACTUALLY PRESENT IN EUROPEAN ORCH

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Insect pathogens and entomoparasitic nematodes
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Characterisation of novel CrleGV isolates for false codling moth control - lessons learnt from codling moth resistance to CpGV

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Abstract: RECENTLY SOME CODLING MOTH, *Cydia pomonella*, POPULATIONS IN EUROPE DEVELOPED RESISTANC TO CPGV. IN ORDER TO PREPARE FOR THE POSSIBILITY OF A SIMILAR OCCURRENCE WITH THE FA *Thaumatotibia leucotreta*, IN SOUTH AFRICA, A SEARCH WAS CONDUCTED FOR NOVEL CRLEGV THROUGH OVERCROWDING, OUTBREAKS OF NOVEL ISOLATES WERE RECORDED FROM LABORATO GEOGRAPHICALLY DISTINCT HOST POPULATIONS. THE GENETIC NOVELTY OF THESE AND TWO CO ISOLATES WAS CONFIRMED THROUGH RESTRICTION ENZYME ANALYSIS AND SEQUENCE ANALYSIS *granulin* AND *egt* GENES. PHYLOGENETIC ANALYSIS SHOWED THE EXISTENCE OF TWO CRLEGV-SA GENOME TYP DIFFERENCES IN VIRULENCE WERE ALSO SHOWN BETWEEN CERTAIN ISOLATES AGAINST CERTAIN

Key words: *Cryptophlebia leucotreta* GRANULOVIRUS, *Thaumatotibia leucotreta*, NOVEL ISOLATES, DOSE-RESPONSE BIOASSAYS

Introduction

IN SOUTH AFRICA, TWO *Cryptophlebia leucotreta* GRANULOVIRUS (CRLEGV-SA) PRODUCTS ARE REGISTERED FOR THE CONTROL OF THE FALSE CODLING MOTH (FCM; *Thaumatotibia* (= *Cryptophlebia*) *leucotreta* (MEYRICK) (LEPIDOPTERA: TORTRICIDAE), AN IMPORTANT ECONOMIC PEST OF OTHER CROPS (NEWTON, 1998). THESE PRODUCTS, CRYPTOGRAN (RIVER BIOSCIENCE, SO (MOORE *et al*., 2011) AND CRYPTEX (ANDERMATT BIOCONTROL, SWITZERLAND) (KESSLER 2008), ARE ARGUABLY THE MOST WIDELY USED MODE OF CONTROL FOR THIS PEST IN SOUT

A RECENTLY NOTED RISK WITH THIS SORT OF USE OF BACULOVIRUSES IS HOST R POPULATIONS OF CODLING MOTH, *Cydia pomonella* (L.), IN EUROPE DEVELOPED RESISTANCE TO TH MEXICAN ISOLATE OF THE *Cydia pomonella* GRANULOVIRUS (CPGV-M) (ASSER-KAISER *et al*., 2007). THIS RESISTANCE WAS OVERCOME BY CHALLENGING RESISTANT INSECTS WITH DIFFERE THE SAME SPECIES (EBERLE *et al*., 2008; BERLINGER *et al*., 2009). THESE TRIALS LED TO COMMERCIAL REPLACEMENT OF PRODUCTS CONTAINING CPGV-M WITH THOSE CONTAINING GENETI CPGV ISOLATES (BESSE *et al*., 2011; ZINGG *et al*., 2011).

IN ORDER TO BE PREPARED IN CASE A SIMILAR SITUATION *T. leucotreta* OCCUR WITH SOUTH AFRICA, THIS STUDY AIMED AT BIOPROSPECTING FOR NEW CRLEGV ISOLATES AS THE EXISTING COMMERCIALLY USED ONES. ADDITIONALLY, VIRULENCE OF ISOLATE DIFFERENT HOST POPULATIONS WAS COMPARED.

Material and methods

HOST REARING

FIVE CULTURES OF INSECT POPULATIONS WERE ESTABLISHED AND MAINTAINED. THE (MIXC) CONSISTED OF A HETEROGENEOUS POPULATION WHICH HAD BEEN MAINTAINED C OVER 166 GENERATIONS. THE OTHER FOUR LABORATORY POPULATIONS (ADO, MBL, CIT ESTABLISHED FROM FIELD-COLLECTED LARVAE FROM FOUR DIFFERENT REGIONS IN SO (EASTERN CAPE) (ADO), MARBLE HALL (LIMPOPO) (MBL), CITRUSDAL (WESTERN CAPE) NELSPRUIT (MPUMALANGA) (NELS) (OPOKU-DEBRAH, 2008). THE CULTURES WERE REARED DIET AS DESCRIBED BY MOORE (2002).

VIRUS ACQUISITION AND PREPARATION

SYMPTOMATIC VIRUS INFECTION WAS INDUCED BY OVERCROWDING IN ALL GEOGRA POPULATIONS AS DESCRIBED BY OPOKU-DEBRAH *et al.* (2013). VIRUS WAS RECOVERED AND PURIFIED ACCORDING TO THE METHODS DESCRIBED BY MOORE *et al.* (2001) WITH MINOR MODIFICATIONS.

DNA CHARACTERISATION AND PHYLOGENETIC ANALYSIS OF TARGET GENE SEQUENCES

GENOMIC DNA WAS EXTRACTED USING A MODIFIED VERSION OF THE CTAB DNA EXTRACT DESCRIBED BY OPOKU-DEBRAH *et al.* (2013). SINGLE RESTRICTION ENDONUCLEASE (REN) DIGE REACTIONS WERE PERFORMED USING *Bam*HI, *Sal*I, *Xba*I, *Pst*I, *Xho*I, *Kpn*I, *Hin*DIII AND *Eco*R1. DIGESTS WERE ANALYSED BY 0.6% AGAROSE GEL ELECTROPHORESIS (AGE) IN 1 X TAE BU FOR 16 H FOLLOWED BY ETHIDIUM BROMIDE STAINING.

Granulin AND *egt* GENE SEQUENCES OF ALL ISOLATES WERE AMPLIFIED BY PCR USING SPECIFIC OLIGONUCLEOTIDES (LANGE & JEHLE, 2003). PHYLOGENETIC COMPARISONS BET WERE CONDUCTED USING THE NUCLEOTIDE SEQUENCES OF THE *gran* GENE OF ISOLATES (TAMURA *et al.*, 2011).

DOSE-RESPONSE BIOASSAYS

THE DROPLET FEEDING BIOASSAY TECHNIQUE DESCRIBED BY PEARCE *et al.* (2012) FOR THE BIOASSAY OF NEONATE LARVAE WAS USED. SEVEN-FOLD SERIAL DILUTIONS WERE U 6.07 X 10^2 TO 7.14 X 10^7 OBS ML^{-1}. THREE REPLICATES OF 48 LARVAE PER TREATMENT AND AN U CONTROL WERE CONDUCTED FOR EACH POPULATION; ASSAYS WERE EVALUATED FOR LARV POST INOCULATION.

DATA WERE ANALYSED BY PROBIT ANALYSIS USING PROBAN (VAN ARK, 1995). MEDI DOSE (LD_{50}) AND 90% LETHAL DOSE (LD_{90}) VALUES WERE DETERMINED AFTER POOLING DATA FR THREE REPLICATES. MULTIPLE COMPARISONS OF PROBIT REGRESSION LINES WERE ALSO CO BONFERRONI METHOD AND SIGNIFICANT DIFFERENCES BETWEEN SLOPES ($P<0.05$) WERE ESTABLISH

Results and discussion

DNA CHARACTERISATION

DNA PROFILES OBTAINED FOR *Bam*HI, *Sal*I, *Xba*I AND *Hin*DIII SHOWED SOME DIFFERENCES BETWEEN THE SEVEN CRLEGV-SA ISOLATES (DATA NOT SHOWN). THE CLEAREST DIFFERENCES BETW WERE EVIDENT WITH *Bam*HI (FIGURE 1). SEVERAL SUBMOLAR BANDS WERE OBSERVED IN TH PROFILES.

RESULTS FROM THIS ANALYSIS SHOWED THE PRESENCE OF TWO UNIQUE BANDING PA PLACEMENT OF ISOLATES INTO TWO GENOME GROUPS: CRYPTEX, CRLEGV-SA ADO, CRLE CRLEGV-SA CIT AND CRLEGV-SA MIXC (GROUP ONE) AS WELL AS CRYPTOGRAN AND CR NELS (GROUP TWO) (OPOKU-DEBRAH *et al.*, 2012).

COMPARATIVE ANALYSIS OF EGT AMINO ACID SEQU

SEQUENCE DATA FOR THE *granulin* GENE OF BOTH GROUP ONE AND GROUP TWO CRLEGV-SA ISOLATES SHOWED A FEW CHANGES IN THEIR NUCLEOTIDE SEQUENCE. THERE WAS NO ACID SEQUENCE, CONFIRMING THAT THIS GENE IS HIGHLY CONS

COMPARING EGT AMINO ACID SEQ GROUP ONE CRLEGV-SA SHOWED A 98% SIMIL (SIX SUBSTITUTIONS) TO-CV3 (GENBANK ID: AY229987; LANGE & JEHLE, 2003) AND GROUP TWO, A 99% SIMILARITY (SEVEN SUB OPOKU-DEBRAH *et al.*, 2013). THESE DIFFERENCES CONFIRMED THE ENESS OF THE SEVEN ISOLATES.

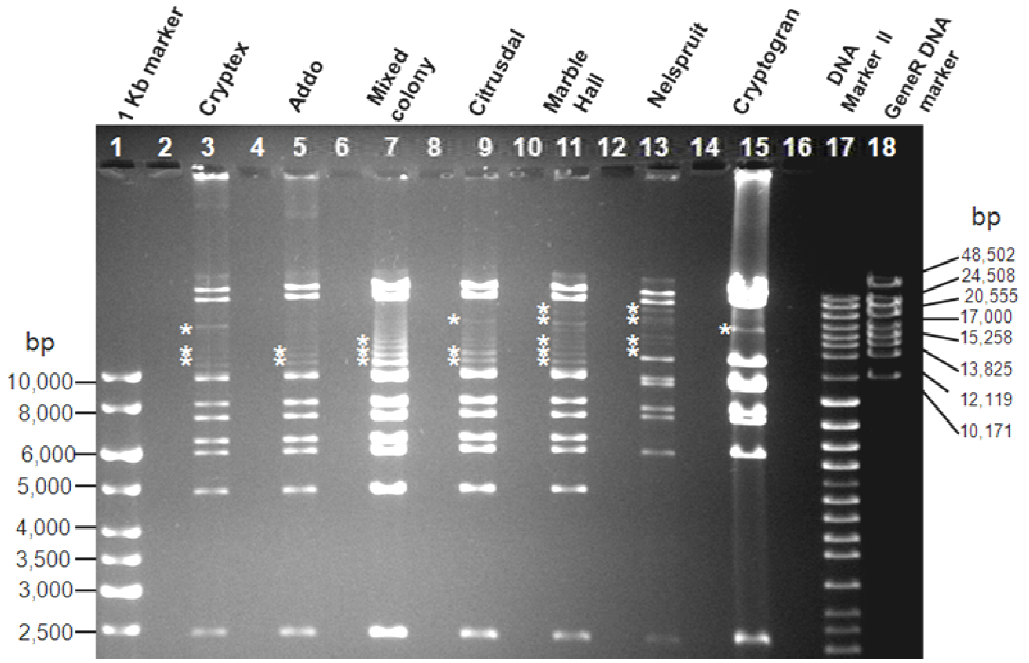

FIGURE 1. *Bam*HI RESTRICTION ENDONUCLEASE DIGEST PROFILES -SA ISOLATES, ANALYSED BY 0.6% AGE AT 30 V FOR 16 HOURS. ASTERISKS (*) INDICA

TABLE 1. LD_{50} AND LD_{90} (IN OBS PER LARVA) FOR NEONATE FCM LARVAE FROM TH AND THE MIXED POPULATIC-RESPONSE BIOASSAYS WITH SEVEN CRLEGV-SA ISOLATES

CrleGV-SA isolate	Addo population		Mixed population	
	LD_{50}*	LD_{90}	LD_{50}*	LD_{90}
CRYPTEX	2.58B	669.50	1.07A	324.42
CRYPTOGRAN	1.02A	272.86	1.06A	270.70
CRLEGV-SA ADO	1.14A	358.89	3.02B	754.57
CRLEGV-SA MIXC	3.12B	773.32	0.95A	331.02
CRLEGV-SA NELS	0.90A	250.11	0.79A	307.49
CRLEGV-SA CIT	0.97A	218.04	1.08A	332.67
CRLEGV-SA MBL	0.83A	289.25	0.99A	403.81

*VALUES IN THE SAME COLUMN FOLLOWED BY THE SAME LETTER DO NOT α=0.05)

DOSE-RESPONSE BIOASSAYS

DIFFERENCES IN VIRULENCE WERE OBSERVED IN BOTH THE ADDO AND MIXED POPU
EXAMPLE, IN ASSAYS WITH THE ADDO POPULATION BOTH CRLEGV-SA MIXC AND CRYPT
ALMOST 3 VIRUS PARTICLES PER LARVA TO ELICIT 50% MORTALITY (LD_{50}) IN THE ADDO POPULATION AS
OPPOSED TO 1 VIRUS PARTICLE REQUIRED FOR THE OTHER ISOLATES. THERE WERE NO SIGIF
IN VIRULENCE BETWEEN THE SEVEN ISOLATES AGAINST THE REMAINING HOST POPULATIO

CONCLUSIONS

THESE RESULTS PROVIDE US WITH SEVERAL POSSIBLE ALTERNATIVE CRLEGV ISOLATE
EVENT OF *C. leucotreta* DEVELOPING RESISTANCE TO THE COMMERCIAL ISOLATES. ADDITIONA
DIFFERENT ISOLATES AGAINST DIFFERENT REGIONALLY DISTINCT HOST POPULATIONS CO

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Elucidation of a novel mode of resistance of codling moth against *CYDIA POMONELLA* granulovirus by homogenization experiments

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Abstract: SINCE 2005, CODLING MOTH (*Cydia pomonella*) POPULATIONS WITH A REDUCED SUSCEPTIBILIT
TO *Cydia pomonella* GRANULOVIRUS (CpGV, *Baculoviridae*) PRODUCTS HAVE BEEN REPORTED FROM ABOUT
EUROPEAN ORCHARDS. THE RESISTANCE COULD BE TRACED BACK TO A SINGLE, DOMINANT,
CURRENTLY, RESISTANCE MANAGEMENT STRATEGIES ARE BASED ON THE APPLICATION OF IMPR
CONTAINING RESISTANCE-OVERCOMING CPGV ISOLATES. RECENTLY, TWO CM FIELD POPULATION
SA-GO) WITH A REDUCED SUSCEPTIBILITY TO EVEN THESE IMPROVED CPGV PRODUCTS WERE FOU
PAIR CROSSING EXPERIMENTS BETWEEN INDIVIDUALS OF THESE RESISTANT FIELD COLONIES
LABORATORY CM STRAIN (CPS) INDICATED THAT THE INHERITANCE OF RESISTANCE OF THESE
FOLLOW THE PREVIOUSLY DESCRIBED PATTERN OF Z-LINKED, DOMINANT RESISTANCE. IN SINGL
SUSCEPTIBILITY OF NEONATES OF THE RESISTANT CM COLONIES NRW-WE AND SA-GO TO DIF
ISOLATES (CPGV-M, -S, -V15 AND -E2) WAS ESTIMATED. THE AIM OF THE CURRENT STUDY WAS
HOMOGENIZATION OF THE GENETICALLY HETEROGENEOUS FIELD POPULATIONS NRW-WE AND
DIFFERENT METHODS: (I) REPEATED SINGLE PAIR CROSSINGS FOLLOWED BY FAMILY SELECTION
MASS CROSSING EXPERIMENTS UNDER VIRUS PRESSURE. THE RESULTING HOMOGENOUS STRAINS
SA-GO WITH FIXED RESISTANCE WILL BE USED FOR BACKCROSSING EXPERIMENTS WITH CPS T
MODE OF INHERITANCE OF THEIR RESISTANCE.

Key words: *Cydia pomonella*, CPGV RESISTANCE, BIOLOGICAL CONTROL, GENETICALLY HOMOGENIZA

Introduction

IN NEARLY ALL GROWING REGIONS OF APPLE AND PEAR WORLDWIDE, CODLING MOTH (*Cydia pomonella*) IS THE MOST DEVASTATING PEST; CM HAS DEVELOPED RESISTANCE TO MAN
INSECTICIDES (RODRIGUEZ *et al.*, 2011). AN ALTERNATIVE TO THE APPLICATION OF CHEMICAL INSE
IS THE USE OF *Cydia pomonella* GRANULOVIRUS (CPGV). CPGV PRODUCTS ARE APPLIED IN B
ORGANIC AND INTEGRATED PRODUCTION (HUBER, 1998).

FROM 2005 ON, THE FIRST CM POPULATIONS WITH REDUCED SUSCEPTIBILITY TO CPG
WERE REPORTED FROM SOUTH-WEST GERMANY (FRITSCH *et al.*, 2006) AND FRANCE (SAUPHANOR *et al.*, 2006). WHEN CM POPULATIONS OF 13 GERMAN APPLE PLANTATIONS WERE SYSTEMATICA
CPGV SUSCEPTIBILITY, A RESISTANCE RATIO UP TO 10.000 FOLD WAS DETERMINED (ASSER *et al.*, 2007). MEANWHILE, 38 CM POPULATIONS HAVE BEEN IDENTIFIED IN DIFFERENT EUROPEA
(SCHMITT *et al.*, 2013). SINGLE PAIR CROSSINGS WERE ACCOMPLISHED WITH A RESISTANT FI
(CPR) TO ACHIEVE A GENETICALLY HOMOGENOUS, RESISTANT CM INBRED STRAIN, REFERE
HYBRID CROSSING EXPERIMENTS BETWEEN CPRR1 AND THE SENSITIVE CM STRAIN (CPS
CLEAR EVIDENCE FOR A MONOGENIC, SEX-LINKED (CHROMOSOME Z) AND DOMINANT R
(ASSER-KAISER *et al.*, 2007; 2010; ZICHOVÀ *et al.*, 2013). CPGV-M, THE SO-CALLED MEXICAN
ISOLATE, WAS THE COMMON AGENT USED IN ALL COMMERCIAL CPGV PRODUCTS REGIST
TO PREVENT A FURTHER SELECTION FOR RESISTANCE TO CPGV-M, CURRENT RESISTAN
STRATEGIES ARE DERIVED FROM THE APPLICATION OF RESISTANCE-OVERCOMING CPGV IS

FIELD OBSERVATIONS OF SEVERAL CM FIELD POPULATIONS CONTROLLED WITH D
ISOLATES REVEALED TWO GERMAN CM POPULATIONS (NRW-WE AND SA-GO) WIT
SUSCEPTIBILITY TO BOTH CPGV-M AND THE NEW RESISTANCE OVERCOMING ISOLATES, SU
CROSSING EXPERIMENTS WITH INDIVIDUALS OF NRW-WE AND SA-GO, RESPECTIVELY,
SUSCEPTIBLE LABORATORY STRAIN CPS REVEALED A PATTERN OF RESISTANCE INHER
FOLLOW THE PREVIOUSLY DESCRIBED Z-LINKED, DOMINANT INHERITANCE (SCHULZE-
UNPUBLISHED).

IN ORDER TO PROVIDE A BASIC UNDERSTANDING OF THE COMPLEX BACULOVIRUS
UNDER FIELD CONDITIONS, IT IS NECESSARY TO GAIN A MORE DETAILED PICTURE ON T
COLONIES. THEREFORE, THE GENETICALLY HETEROGENEOUS FIELD POPULATIONS NRW-
NEED TO BE GENETICALLY HOMOGENIZED. TWO METHODS WERE APPLIED: LARVAE OF N
SELECTED FOR RESISTANCE BY FEEDING VIRUS CONTAMINATED DIET ACCORDING TO A M
et al. (2009) AND ZICHOVÁ *et al.* (2013). HOMOGENIZATION OF THE CM COLONY SA-GO WAS
ACCOMPLISHED BY SINGLE PAIR CROSSES AND EXPOSING LARVAE OF THE F1 GEN
DISCRIMINATION CONCENTRATION OF CPGV-M AND CPGV-S, ACCORDING TO THE METH
KAISER *et al.* (2007). FURTHERMORE, THE CONTROL POPULATION, MAINTAINED IN ABSENCE
WAS REARED TO ADULTHOOD. HENCE, THE SELECTED HOMOGENOUS COLONIES OF NRW-
WILL BE USED FOR BACKCROSSING EXPERIMENTS WITH CPS TO MONITOR AND COMPAR
THE TWO HOMOGENEOUS METHODS AND TO ELUCIDATE THE NOVEL MECHANISM OF RESIS

Material and methods

TEST INSECTS AND VIRUS

DIAPAUSING LARVAE OF NRW-WE AND SA-GO WERE FIRST COLLECTED IN 2009 FROM TW
PLANTATIONS IN GERMANY AND KEPT IN A LABORATORY REARING AT 26 °C, 60% RELATIV
AND AT 16 H PHOTOPERIOD BEFORE USING IN HOMOGENIZATION EXPERIMENTS. THE SUS
CPS DERIVED FROM ANDERMATT BIOCONTROL (SWITZERLAND) AND WAS FREQUENTL
SUSCEPTIBILITY TO CPGV-M. THE CPRR1 STRAIN IS A GENETICALLY HOMOGENIZED
DERIVING FROM A RESISTANT FIELD COLONY CPR, WHICH IS IDENTICAL TO THE RESISTAN
BY FRITSCH *et al.* (2005), CALLED ‘‘SUEDBADEN”.

THE ISOLATE CPGV-M USED IN THE BIOASSAY WAS A DESCENDENT FROM THE CPGV C
NORTHERN MEXICO (TANADA, 1964) AND THE ISOLATE CPGV-S ORIGINATED FROM THE CANA
PRODUCT VIROSOFT™ BIOTEPP INC. THE ISOLATE CPGV-V15 WAS DEVELOPED BY ANDERMA
BIOCONTROL AG (SWITZERLAND). CPGV-E2 DERIVED FROM THE SO-CALLED “ENGLISH ISO
E (CROOK *et al.*, 1985). VIRUS OCCLUSION BODIES (OBS) WERE COUNTED WITH PETROFF-
COUNTING CHAMBER (DEPTH 0.02 MM) USING DARK FIELD OPTICS.

GENETICALLY HOMOGENIZATION BY SINGLE PAIR CROSSING

LARVAE OF THE RESISTANT COLONY SA-GO WERE SEPARATED BY SEX IN THE FIFTH INSTA
OF GONADS VISIBLE UNDER THE SKIN OF MALE INDIVIDUALS. AFTER REARING TO PUPAE
MOTHS, 20 PAIRS WERE KEPT IN SMALL CLOSED PLASTIC BOXES AT 26 °C, 60% RH AN
PHOTOPERIOD. AFTER MATING, THE DEPOSITED EGGS WERE COLLECTED AND INCUBATED
LARVAE. THE OFFSPRING OF EACH PAIR WERE DIVIDED INTO THREE COHORTS. TWO COHO
SUSCEPTIBILITY TO CPGV-M AND CPGV-S USING BIOASSAYS WITH THE DISCRIMINATING C
OF THE VIRUS. LARVAE OF THE THIRD COHORT SERVED AS CONTROL AND WERE KEPT ON
UNTIL ADULTHOOD IN CASE THAT THE MORTALITY IN THE CORRESPONDING BIOASSAYS
AFTER 14 DAYS. ADULTS DERIVING FROM THE CONTROL COHORT WERE USED FOR A SECO
BY REPEATING THE PROCEDURE.

GENETIC HOMOGENIZATION BY MASS CROSSING UNDER VIRUSSELECTION

300 NEONATES OF THE F37 GENERATION OF THE RESISTANT LABORATORY COLONY
RANDOMLY SELECTED AND TRANSFERRED ON DIET CONTAINING BOTH CPGV-M AND CPGV
ML[1], RESPECTIVELY. THE SURVIVORS WERE REARED TO ADULTHOOD AND THEIR PRO
EXPOSED TO CPGV-M AND CPGV-S, RESPECTIVELY, AS DESCRIBED BEFORE. THE SELECTION
WAS REPEATED FOR FOUR GENERATIONS.

BIOASSAY

TO DETECT RESISTANT AND SUSCEPTIBLE INDIVIDUALS, LARVAE WERE SUBJECTE
DISCRIMINATING VIRUS CONCENTRATION (5.8 X 10[4] OB ML[1] DIET) (ASSER-KAISER *et al.*, 2007) CAUSING
> 95% MORTALITY FOR THE SENSITIVE STRAIN CPS WITHIN 7 DAYS POST EXPOSURE. AL
PREPARED VIRUS SUSPENSIONS WERE MIXED INTO ARTIFICIAL DIET (IVALDI-SENDER, 19
NEONATES (L1) OF THE DIFFERENT CM STRAINS. THE TEST INSECTS WERE KEPT AT 26 °C, 60
16 HR PHOTOPERIOD AND LARVAL MORTALITIES WERE RECORDED AFTER 7 AND 14 DAYS.

Results and discussion

MORTALITY DATA OBTAINED FROM 14-DAYS BIOASSAYS WITH THE CM COLONIES (SA-GO
CPRR1 AND CPS SUBJECTED TO CPGV-M, -S, -V15, AND -E2) ARE SHOWN IN FIGURE 1. T
LABORATORY STRAIN CPS WAS HIGHLY SUSCEPTIBLE FOR CPGV-M AND CPGV-S WITH A
100% AND 92%, RESPECTIVELY. THE RESISTANT STRAIN CPRR1 PRESENTED A HIGH MEA
(87.6%) WHEN CHALLENGED WITH CPGV-S BUT LOW MORTALITY WHEN TREATED WITH C
TWO FIELD POPULATIONS SA-GO AND NRW-WE SHOWED LOW MORTALITIES IN THE BIO
CPGV-M AND CPGV-S BUT MORTALITIES OF UP TO 100% IN BIOASSAYS WITH THE ISOLAT
V15 AND E2.

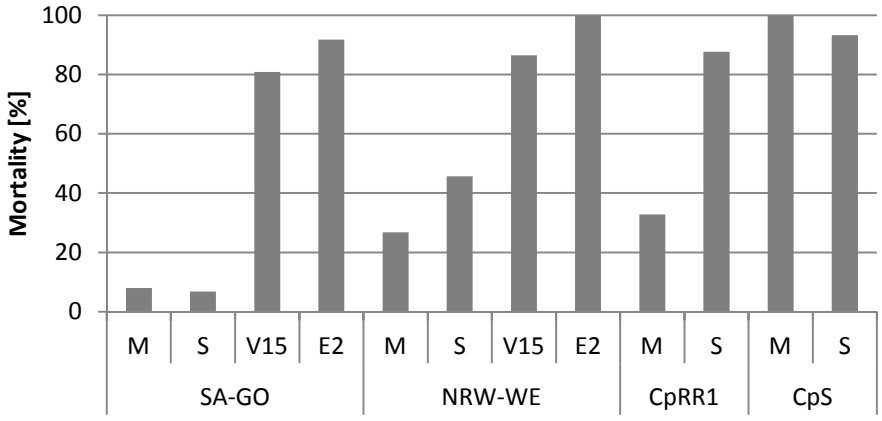

FIGURE 1. MORTALITY INDUCED IN NEONATE LARVAE OF DIFFERENT CM POPULATIONS
WE, CPRR1 AND CPS) AFTER 14 DAYS OF INCUBATION ON THE DISCRIMINATING VIRUS CO
OF 5.8 X 10[4] CPGV OB ML[1] WITH DIFFERENT CPGV ISOLATES (CPGV-M, -S, -V15, -E2). DATA
SHOW MEAN VALUES OF THREE INDEPENDENT REPLICATES.

BOTH OF THE NOVEL CM COLONIES NRW-WE AND SA-GO SHOWED RESISTANCE TO CPG
-M BUT THE ISOLATES CPGV-V15 AND CPGV-E2 OVERCAME THE RESISTANCE AND MORTA
100% WERE DETECTED.

PREVIOUS ANALYSIS OF THESE COLONIES DEMONSTRATED THAT THE INHERITANCE
DID NOT FOLLOW THE PREVIOUSLY DESCRIBED PATTERN OF Z-LINKED, DOMINANT RES
BOPP AND JEHLE, UNPUBLISHED). THE PURSUED HOMOGENIZATION OF THE FIELD COLON
AND SA-GO IS ESSENTIAL FOR BACKCROSSING EXPERIMENTS WITH CPS TO DETECT WHE
FURTHER MECHANISM OF RESISTANCE. FURTHERMORE, THE TWO DIFFERENT HOMOGENI
WELL AS THE TWO DIFFERENT RESISTANT FIELD COLONIES NRW-WE AND SA-GO WILL
BASED ON POTENTIAL DIFFERENCES IN THEIR MODE OF RESISTANCE. THE INTEND
BACKCROSSING EXPERIMENTS FOLLOWED BY BIOASSAYS CAN ALSO DEFINE SEX-LINKAGE

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Session 2

Insect pathogens and entomoparasitic nematodes
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Biological control of the box tree moth *CYDALIMA PERSPECTALIS* with *ANAGRAPHA FALCIFERA* nucleopolyhedrovirus (AnfaNPV)

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Abstract: THE BOX TREE MOTH *Cydalima perspectalis* ORIGINATED FROM EAST ASIA. SINCE SEVERAL YEARS IS A NOVEL INVASIVE INSECT PEST IN MANY EUROPEAN COUNTRIES, CAUSING WIDESPREAD DAM PLANTS. THE POTENTIAL OF THE *Anagrapha falcifera* NUCLEOPOLYHEDROVIRUS (ANFANPV) AS A POTENTIAL BIOLOGICAL CONTROL AGENT FOR *C. perspectalis* WAS INVESTIGATED IN THIS STUDY. TWO ANFANPV ISOLATES, TERMED DN10 AND BI-235, WERE USED. THE INFECTIVITY OF ANFANPV DN10 235 TO *C. perspectalis* WAS EVALUATED BY LEAF DISC BIOASSAYS AND THE MEDIAN LETHAL CONCEN LC_{50} WAS DETERMINED FOR BOTH ISOLATES. IN ADDITION, LIGHT AND ELECTRON MICROSCOPIC ANALY TO STUDY THE INFECTION PROCESS. IN CONCLUSION, *C. perspectalis* WERE SHOWN TO BE SUSCEPTIBLE TO BOTH ANFANPV ISOLATES.

Key words: *Cydalima perspectalis*, BIOLOGICAL CONTROL, BACULOVIRUSES, BIOASSAY, PATHOLOGY

Introduction

THE BOX TREE MOTH *Cydalima perspectalis* (WALKER 1859) (LEPIDOPTERA: CRAMBIDAE) IS ALSO KNOWN AS *Diaphania perspectalis* AND *Glyphodes perspectalis* (MALLY & NUSS, 2010). IT ORIGINATED FROM EAST ASIA BUT IN RECENT YEARS IT BECAME INVASIVE TO SOUTHW THE NETHERLANDS, UNITED KINGDOM, AUSTRIA, HUNGARY, SLOVENIA AND TURKEY (BIL DER STRATEN & MUUS, 2010; SÁFIÁN & HORVÁTH, 2011; SELJAK, 2012; HAYAL *et al*., 2012).

THIS INSECT IS SUSCEPTIBLE TO CHEMICAL INSECTICIDES, SUCH AS DELTAMETHRIN O AND TO *Bacillus thuringiensis* PREPARATIONS (KORYCINSKA & EYRE, 2011). RECENTLY, AN ISOL ANFANPV DN10 WAS IDENTIFIED TO INFECT LARVAE OF THE BOX TREE MOTH (*Diaphania nitidalis*) (LEPIDOPTERA: CRAMBIDAE) (JACKSON *et al*., 2009; KLEESPIES, UNPUBLISHED RESULTS). AS *D. nitidalis* IS RELATED TO *C. perspectalis* WE AIMED TO TEST, WHETHER ANFANPV IS ALSO ABLE INFECT BOX TREE LARVAE.

Material and methods

VIRUSES

THE ISOLATE ANFANPV DN10 ORIGINATED FROM DISEASED LARVAE OF THE PICKLEWORM (*Diaphania nitidalis*) (LEPIDOPTERA: PYRALIDAE) AND WAS OBTAINED FROM PROF. SAID EL-SALAMOUNY I (SOUTH CAROLINA, USA) (JACKSON *et al*., 2009). THE ISOLATE ANFANPV BI-235 HAD BEEN STORED IN THE JKI BACULOVIRUS COLLECTION. IT LIKELY ORIGINATED FROM HOSTETTER & PUTTL

BIOASSAYS

ANFANPV BI-235 AND DN10 WERE PROPAGATED IN SECOND TO FOURTH INSTAR L *C. perspectalis*. OCCLUSION BODIES (OBS) WERE ISOLATED ACCORDING TO STANDARD PROC DISC BIOASSAYS WERE PERFORMED TO DETERMINE THE MEDIAN LETHAL CONCENTRATIO LC_{50} (HEALY *et al*.

al., 2012). IN SHORT, PURIFIED VIRUS STOCKS WERE DILUTED WITH PBT BUFFER (PBS, 0.
BSA, 0.025% (V/V) TWEEN 20) AND SIX CONCENTRATIONS RANGING FROM 10^{8} TO 10^{4} OBS ML^{-1} WERE
PREPARED. SMALL PIECES (CA. 4 MM X 5 MM) OF BOX TREE LEAVES WITH THE UPPER DA
SURFACE WERE PLACED ONTO 3% AGAR IN SEPARATED WELLS OF AN AUTOCLAVABLE 50
BAD SALZUFLEN, GERMANY). AN ALIQUOT OF 1 µL OF THE OB DILUTION WAS PIPETTED ON
AND ALLOWED TO DRY FOR 1 TO 1.5 H. THIRTY TO FIFTY NEONATE LARVAE WERE
CONCENTRATION AND THE CONTROL (PBT BUFFER ONLY). ONE NEONATE LARVA WAS TH
WELL AND INCUBATED AT 26 °C AND A 16 H (LIGHT)/8 H (DARK) PHOTOPERIOD. LAR
CONSUMED MORE THAN 70% OF THE UPPER SURFACE OF LEAF DISCS WERE SUPPLEMENT
UNTREATED BOX TREE LEAF AFTER THREE DAYS. OTHER LARVAE WERE DISCARDED. LA
RECORDED SEVEN DAYS AFTER INITIAL VIRUS EXPOSURE. EACH TEST WAS REPLICATED T
VIRUS ISOLATE. MORTALITY DATA WERE CORRECTED BY ABBOTT'S FORMULA (ABBOT
MEDIAN LETHAL CONCENTRATION WAS DETERMINED USING PROBIT ANALYSIS WITH TH
(TOXRAT SOLUTIONS, ALSDORF, GERMANY).

MICROSCOPIC INVESTIGATIONS

FOR TRANSMISSION ELECTRON MICROSCOPIC (TEM) INVESTIGATIONS VIRUS INFECTED, M
OF SECOND TO FOURTH INSTAR WERE USED. LARVAE WERE DISSECTED, FIXED IN 3% GL
24 H, AND WASHED THREE TIMES IN VERONAL BUFFER. POST-FIXATION WAS PERFORMED W
TETROXIDE FOR 17 H. THEN, THE SAMPLES WERE WASHED THREE TIMES IN 2.5% SUCRO
FOLLOWED BY STAINING WITH URANYL ACETATE WOLFRAM PHOSPHORIC ACID FOR 5
DEHYDRATING USING ETHANOL THE SPECIMEN WERE EMBEDDED IN METHACRYLATE. U
WERE EXAMINED ON A ZEISS 902 TEM.

Results and discussion

THE MEDIAN LETHAL CONCENTRATION (LC_{50}) OF THE CLANFANPV ISOLATES DN10 AND BI-235 WERE
DETERMINED USING LEAF DISC BIOASSAYS AND PROBIT ANALYSIS (FIGURE 1). IN A SEVEN
THE LC_{50} VALUES WERE 7.8×10^{5} OBS ML^{-1} (95% FIDUCIAL LIMITS $5.5 - 11.6 \times 10^{5}$ OBS ML^{-1},
N = 685, SLOPE: 1.23, CHI^{2} = 29.0) FOR ISOLATE BI-235 AND 2.3×10^{6} OBS ML^{-1} (95% FIDUCIAL
LIMITS $1.4 - 3.9 \times 10^{6}$ OBS ML^{-1}, N = 680, SLOPE: 1.36, CHI^{2} = 17.70) FOR ISOLATE DN10. THE
DIFFERENCE BETWEEN BOTH ISOLATES WAS STATISTICALLY SIGNIFICANT ON THE BASIS O
FIDUCIAL LIMITS. THIS SUGGESTED THAT BI-235 WAS MORE VIRULENT THAN DN10, WITH
2.98.

INFECTION OF *C. perspectalis* LARVAE BY ANFANPV BI-235 AND DN10 WAS CONFIRMED BY LIG
MICROSCOPY (DATA NOT SHOWN) AND TRANSMISSION ELECTRON MICROSCOPIC STUD
INFECTION OF FAT BODY, TRACHEAL MATRIX AND EPIDERMIS CELLS OF BOX TREE MO
OBSERVED.

OUR RESULTS CLEARLY INDICATE THAT *C. perspectalis* IS SUSCEPTIBLE TO ANFANPV. THUS,
ANFANPV MIGHT BE A CANDIDATE FOR DEVELOPING A BIOCONTROL AGENT OF *C. perspectalis* ON THE
BASIS OF BACULOVIRUSES. HOWEVER EXPERIMENTS WILL BE NECESSARY TO DETERMINE ITS FIELD

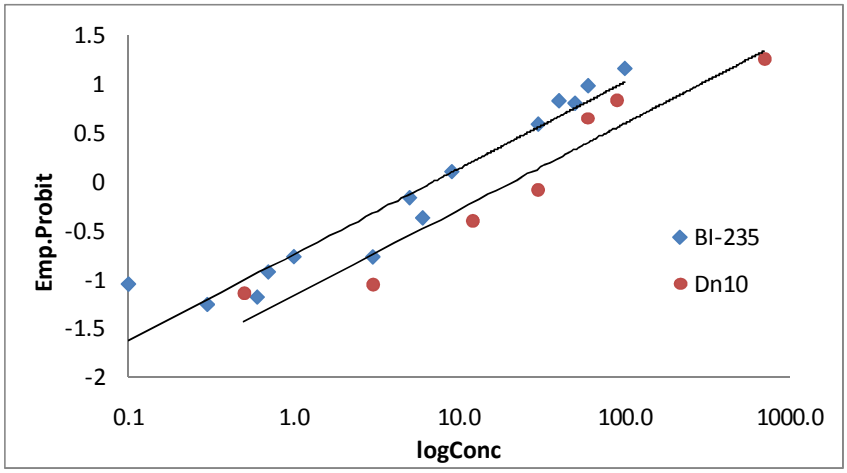

FIGURE 1. PROBIT ANALYSIS OF ANFANPV BI-235 AND DN10 ACTIVITY IN NEONATE LA
C. perspectalis.

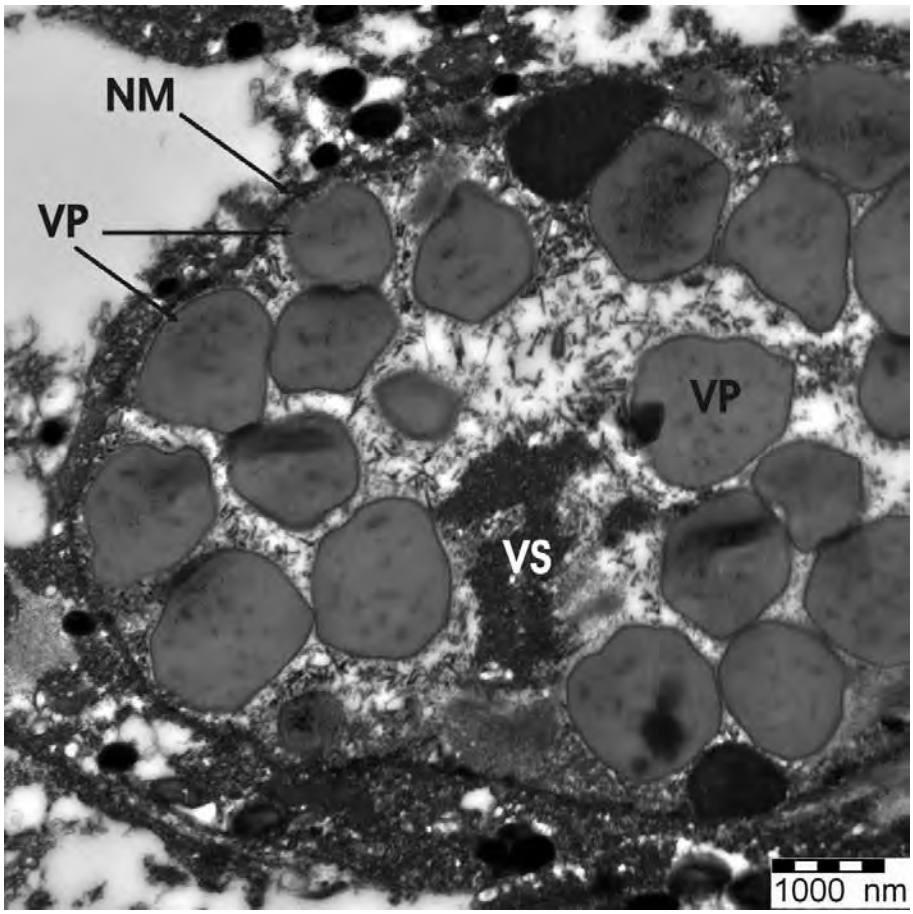

FIGURE 2. ULTRATHIN SECTION OF FAT BODY OF *C. perspectalis* INFECTED BY ANFANPV DN10. NOTE THE AREA OF VIROGENESIS (VS); NM = NUCLEAR MEMBRANE, VP = VIRUS POLYHEDRON.

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Interactions between structural proteins of *Chilo iridescent* virus

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Abstract: IRIDOVIRUSES INFECT A BROAD RANGE OF HOSTS INCLUDING INVERTEBRATE (ESP INSECTS), AMPHIBIANS, REPTILES AND FISH WHICH HAVE ECOLOGICAL AND ECONOMIC IMP KNOWLEDGE OF THE VIRAL INTERACTOME, PARTICULARLY AMONGST STRUCTURAL VIRION PRO AN EMERGING PICTURE OF THE PROTEIN–PROTEIN INTERACTIONS IMPORTANT FOR VIRAL ENTRY ASSEMBLY, AND EGRESS. PREVIOUS STUDIES INDICATED THAT *Chilo iridescent* VIRUS (CIV), THE TYPE MEMBER OF THE VIRUS FROM THE GENUS *Iridovirus*, CONTAINS 46 VIRION STRUCTURAL PROTEINS. IN THIS STUDY WE AIMED TO IDENTIFY THE PROTEIN-PROTEIN INTERACTIONS AMONG THESE PROTEINS BY TWO-HYBRID-SYSTEM. THE STRUCTURAL GENES WERE CLONED INTO BAIT AND PREY VEC *Saccharomyces cerevisiae* AH109 STRAIN WAS USED FOR TRANSFECTING THESE VECTORS. MINIM SYNTHETIC DEFINED (SD) MEDIA WERE USED FOR IDENTIFICATION OF PROTEIN-PROTEIN INTERACTI TO NOW, WE HAVE IDENTIFIED FIVE INTERACTIONS (118L-415R, 232R-142R, 337L-309L, 337L-117L, 337L-295L) AMONG CIV STRUCTURAL PROTEINS. WE HAVE CONFIRMED THE INTERAC BETWEEN 118L AND 415R BY A GST PULL-DOWN ASSAY. AFTER COMPLETING THESE INTERAC STUDIES, WE WILL HAVE A BETTER PICTURE OF THE STRUCTURE OF THE VIRUS AND MAY BE A INTERACTIONS BETWEEN VIRUS AND ITS RESPECTIVE HOSTS.

Key words: *Chilo iridescent* VIRUS, STRUCTURAL PROTEINS, YEAST-TWO-HYBRID, PROTEIN-PR INTERACTION

Insect pathogens and entomoparasitic nematodes
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Natural populations of *SPODOPTERA EXIGUA* are infected by multiple viruses: implications for the production and use of virus insecticides

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Abstract: COVERT INFECTIONS OF *Spodoptera exigua* MULTIPLE NUCLEOPOLIEDROVIRUS (SEMNPV) HAVE BEE
DETECTED IN LABORATORY OR FIELD POPULATIONS OF THE *S. exigua* HOST. TWO RNA VIRUSES
BELONGING TO *Iflaviridae* FAMILY (SEIV-1, SEIV-2) WERE IDENTIFIED IN TRANSCRIPTOME STUDIES
DIFFERENT LABORATORY COLONIES OF *S. exigua*. THE THREE VIRUSES ARE VERTICALLY TRANSMITED AND ES
PERSISTENT INFECTIONS. FOR THIS REASON, COINFECTION OF INDIVIDUAL INSECTS BY THESE VI
LIKELY. IN THIS STUDY, WE DETERMINED THE PREVALENCE OF COVERT INFECTIONS CAUSED
SEMNPV IN ORDER TO IDENTIFY VIRUS ASSOCIATIONS IN *S. exigua* POPULATIONS. SEMNPV WAS
DETECTED IN THE 54% OF FIELD-CAUGHT ADULTS, WHEREAS 13% AND 8% OF INSECTS WERE INFE
AND SEIV-2, RESPECTIVELY. THE PREVALENCE OF SEIV-1 AND SEIV-2 OBTAINED IN LABORATORY
SHOWED HIGHER LEVELS THAN IN THE PARENTAL GENERATION, WHEREAS THE PREVALENCE OF S
FROM PARENTS TO THEIR OFFSPRING. THESE FINDINGS HAVE IMPORTANT IMPLICATIONS FOR THE
OF VIRUS BASED INSECTICIDES USING MASS-REARED INSECTS AND THE EFFICACY OF THESE PRO
PEST POPULATIONS THAT MAY BE COINFECTED BY IFLAVIRUSES.

Key words: SEMNPV, IFLAVIRUS, COVERT INFECTION, FIELD POPULATION

Introduction

Spodoptera exigua IS A SERIOUS PEST OF SEVERAL CROPS GROWN IN THE GREENHOUSES O
SPAIN. THE LARVAE ARE SUSCEPTIBLE TO VIRAL PATHOGENS WHICH ARE CAPABLE OF PRO
SUBLETHAL INFECTIONS. THE *S. exigua* MULTIPLE NUCLEOPOLYHEDROVIRUS (SEMNPV) HAS BE
OBSERVED TO PRODUCE EPIZOOTICS IN LARVAL POPULATIONS AND A NUMBER OF BIOPES
THIS VIRUS HAVE BEEN DEVELOPED AND COMMERCIALIZED FOR USE AGAINST THIS PEST.
TRANSMITTED EITHER HORIZONTALLY, BETWEEN MEMBERS OF A COHORT, OR VERTICAL
OFFSPRING. THE LATTER TRANSMISSION ROUTE RESULTED IN COVERT INFECTIONS THAT
FIELD-CAUGHT ADULTS AND THEIR PROGENY (CABODEVILLA *et al.*, 2011A).

RECENTLY AN ANALYSIS OF THE TRANSCRIPTOME OF *S. exigua* LARVAE REVEALED NOVEL RNA VIRUSES
THAT HAVE BEEN IDENTIFIED AS BELONGING TO THE *Iflaviridae* FAMILY: *S. exigua* IFLAVIRUS-1 (SEIV-1;
S. exigua IFLAVIRUS-2: SEIV-2) (MILLÁN-LEIVA *et al.*, 2012; CHOI *et al.*, 2012). VERY LITTLE IS
KNOWN ABOUT IFLAVIRUSES, BUT THEY HAVE BEEN REPORTED IN ASSOCIATION WITH M
STUDIES; THESE VIRUSES APPARENTLY DO NOT CAUSE LETHAL INFECTION, BUT RESULT IN
GAIN (VAN *et al.*, 1983). THE AIM OF THIS STUDY WAS TO EVALUATE THE PREVALENCE OF BA
AND IFLAVIRUS COVERT INFECTIONS IN A FIELD POPULATION OF *S. exigua* AND TO DETERMINE THEIR
CAPACITY FOR VERTICAL TRANSMISSION TO THE OFFSPRING OF INFECTED PARENTS.

Material and methods

FIELD SAMPLING OF S. exigua *INSECTS*

FIELD ADULTS OF *Spodoptera exigua* WERE CAPTURED WITH UV LIGHT-TRAPS IN THE GREENHOU
SOUTHERN SPAIN. INSECTS WERE REARED INDIVIDUALLY IN 25 ML PLASTIC CUPS AND ALL
AFTER TWO DAYS ADULTS WERE FROZEN AT -80 ºC AND TWENTY FOUR NEONATES FROM
INDIVIDUALIZED IN CUPS CONTAINING DIET AND REARED THROUGHOUT LARVAL AND PUPAL STAG
CONDITIONS. ADULTS WERE FROZEN AT -80 ºC FOR THE SUBSEQUENT ANALYSIS.

TOTAL DNA AND RNA EXTRACTION AND RT-PCR AND QPCR ANALYSIS

FOR DETECTION OF COVERT INFECTIONS, TOTAL DNA AND RNA WAS OBTAINED FR
ABDOMENS OF BOTH FIELD-CAUGHT AND LABORATORY-REARED INSECTS, AFTER SEXING BY OBSERVATION
EXTERNAL GENITALIA. MULTIPLEX RT-PCR AND QPCR, BASED ON SYBR FLUORESCENCE, W
DETERMINE THE PREVALENCE OF INDIVIDUALS INFECTED BY *S. exigua* MULTIPLE NPV AND
NUCLEOPOLYHEDROVIRUS (SEMNPV), RESPECTIVELY.

Results and discussion

PREVALENCE OF COVERT INFECTIONS IN FIELD ADULTS

FIELD-CAUGHT ADULTS SHOWED HIGH LEVELS OF COVERT INFECTIONS FOR SEMNPV: 54%
BY QPCR. DETECTION OF IFLAVIRUSES WAS FAR LESS FREQUENT (19%). MALES AND F
INFECTED AT SIMILAR FREQUENCIES FOR BOTH SEMNPV ($P > 0.05$) AND IFLAVIRUSES
PREVIOUS STUDIES CARRIED OUT DURING 2006 AND 2007 DETECTED SEMNPV COVERT INF
16% OF FIELD-CAUGHT ADULTS BY RT-PCR (CABODEVILA *et al.*, 2011). HOWEVER, THE QPCR-BASED
TECHNIQUE USED IN THIS STUDY ALLOWED US TO INCREASE MARKEDLY THE SENSITIVITY

SEIV-1 SEEMS TO BE FREQUENT AND EASILY TRANSMITTED IN *S. exigua* LABORATORY COLONIES
(MILLÁN-LEIVA *et al.*, 2012), BUT THIS IS THE FIRST TIME THAT THIS VIRUS HAS BEEN DETECTE
CAUGHT INSECTS. CO-INFECTIONS OF BOTH VIRUS SPECIES WERE RELATIVELY RARE,
INDIVIDUALS HARBOUR BOTH SEMNPV AND ONE OR BOTH OF THE IFLAVIRUSES.

TRANS-GENERATIONAL TRANSMISSION

FIVE FEMALES EITHER INFECTED OR NON-INFECTED BY SEMNPV WERE RANDOMLY SELEC
CAUGHT ADULTS THAT HAD PRODUCED OFFSPRING. TEN ADULTS OF THE OFFSPRING P
ANALYZED TO DETERMINE TRANSMISSION RATES OF SEMNPV AND SEIVS. ALL THREE V
CAPABLE OF VERTICAL TRANSMISSION. OVERALL, SEMNPV VERTICAL TRANSMISSION
LEVELS OF COVERT INFECTION (10-20% DEPENDING ON MATING TREATMENT), WHERE
PREVALENCE INCREASED IN F1 RESPECT TO FIELD-CAUGHT ADULTS. THE REARING CONDI
PARTICULAR RELEVANCE, AS PREVIOUS STUDIES INDICATE THAT IFLAVIRUSES QUICKLY S
CULTURES IN LABORATORY CONDITIONS (MILLÁN-LEIVA *et al.*, 2012).

NO SIGNIFICANT DIFFERENCES WERE FOUND IN NUMBERS OF DESCENDENT POSITI
(36%) COMPARE TO THOSE FOR SEMNPV (20%) IN THE OFFSPRING OF COVERTLY INFECT
HOWEVER THE PROPORTION OF ADULTS DETECTED POSITIVE FOR SEIVS WAS SIGNIFICANTLY
(76%) THAN THAT FOR SEMNPV (10%) IN THE OFFSPRING FROM NON-INFECTED FEMALES
SEIV INFECTION WAS ALSO DETECTED IN THE OFFSPRING OF INFECTION NEGATIVE FEMA
DUE TO AN INFECTED MALE LINAGE THAT COULD CONTRIBUTE TO VIRUS TRANSMISSION
SEIVS IN THE OFFSPRING FROM SEMNPV COVERTLY INFECTED FEMALES (36%) WAS SI
LOWER THAN THAT REGISTERED FOR THE OFFSPRING FROM SEMNPV-FREE FEMALES (76

SUGGESTING THAT THE PRESENCE OF NPVS NEGATIVELY AFFECT THE SPREADING OF TH
INFECTION.

AS IFLAVIRUSES MAY AFFECT THE VIABILITY OF INSECT COLONIES USED FOR THE MA
NPV-BASED INSECTICIDES, PARTICULAR ATTENTION SHOULD BE PAID TO THE INTERACTI
VIRUSES DURING VIRUS PRODUCTION. FUTURE STUDIES SHOULD ALSO ADDRESS POTENTIA
SUSCEPTIBILITY TO NPV INFECTION IN INSECTS THAT ALREADY HARBOUR IFLAVIRUS INF
THE POTENTIAL TO AFFECT THE EFFICACY OF VIRUS BASED INSECTICIDAL PRODUCTS USE

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Estimating the importance of maternal and paternal contributions to the vertical transmission of *SPODOPTERA EXIGUA* multiple nucleopolyhedrovirus (SeMNPV)

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Abstract: VERTICAL TRANSMISSION OF *Spodoptera exigua* MULTIPLE NUCLEOPOLYHEDROVIRUS (SEMNPV) IS BELIEVED TO BE A COMMON FEATURE IN FIELD POPULATIONS OF *S. exigua*. TO ASSESS WHETHER GENDER AFFECTS TRANS-GENERATIONAL VIRUS TRANSMISSION, FOUR MATING GROUPS WERE PERFORMED U SUBLETHALLY INFECTED INSECTS: I) HEALTHY MALES (♂) × INFECTED FEMALES (♀), II) HEALTHY FEMALES (♀) × INFECTED MALES (♂), III) HEALTHY MALES (♂) × INFECTED FEMALES (♀) AND IV) INFECTED MALES (♂) × INFECTED FEMALES (♀). THESE ADULTS AND THEIR OFFSPRING WERE ANALYZED BY QPCR TO DETECT INFECTION. BOTH MALES AND FEMALES WERE ABLE TO TRANSMIT THE INFECTION TO THE NEXT G FEMALES INFECTED A HIGHER PERCENTAGE OF THE OFFSPRING AND FEMALE-MEDIATED TRAN CONSISTENT THAN THAT OF MALES. VENEREAL TRANSMISSION APPEARED TO BE HALF AS EFFE MEDIATED TRANSMISSION, AND THE MAIN ROUTE OF TRANSMISSION IS LIKELY TRANSOVARIAL R THE PREVALENCE OF THE INFECTION IN THE OFFSPRING DID NOT VARY ACCORDING TO GENDER, T AND FEMALES CAN BE INFECTED BY THEIR PARENTS IN SIMILAR PROPORTIONS. INCORPOR TRANSMITTED GENOTYPES IN BIOLOGICAL INSECTICIDES MIGHT HAVE THE POTENTIAL FOR RED AND EXTENDING PERIODS BETWEEN VIRUS APPLICATIONS.

Key words: SEMNPV, COVERT INFECTION, GENDER, TRANS-GENERATIONAL TRANSMISSION

Introduction

BACULOVIRUSES ARE THE MOST EXTENSIVELY STUDIED ARTHROPOD-SPECIFIC VIRUSE EXTREMELY HIGH VIRULENCE TO CERTAIN INSECT PESTS AND FOR THEIR BIOSAFETY CHA PRODUCTION OF RECOMBINANT PROTEINS (MOSCARDI, 1999). BACULOVIRUS SURVIVAL TRANSMISSION PATHWAYS. VIRAL OCCLUSION BODIES (OBS) ARE RESPONSIBLE FO TRANSMISSION BETWEEN LARVAE THAT IS THOUGHT TO BE THE MAJOR PATHWAY TRANSMISSION. LITTLE IS KNOWN ABOUT VERTICAL TRANSMISSION OF VIRUSES IN INS BEEN PROPOSED AS A SURVIVAL STRATEGY TO OVERCOME PERIODS OF HOST POPULATIO FACILITATE THE VIRUS DISPERSAL TO GEOGRAPHICALLY DISTANT NICHES. TRANS-GENER INVOLVES COVERTLY INFECTED ADULTS THAT PASS VIRUS TO THEIR PROGENY VIA TRANSOVARIAL PATHWAY (CORY & MYERS, 2003).

A PREVIOUS STUDY SHOWED THAT *Spodoptera exigua* FEMALES WITH NO EVIDENCE OF A NUCLEOPOLYHEDROVIRUS (NPV) COVERT INFECTION PRODUCED VIRUS-INFECTED OFFSP WITH FIELD-CAUGHT MALES. THIS LED US TO SUSPECT THAT BOTH MALES AND FEMALES VERTICAL TRANSMISSION OF THE PATHOGEN. HOWEVER, THE DIFFERENTIAL PREVALENCE BETWEEN MALES AND FEMALES (CABODEVILLA *et al.*, 2011) SUGGESTS A POSSIBLE GENDER EFFECT ON THE TRANSMISSION PROCESS. IN THIS STUDY WE ANALYZED THE EFFECT OF GENDER ON EFFICIENCY OF COVERTLY INFECTED *S. exigua* TO THEIR PROGENY.

Material and methods

INSECT AND VIRUS

THE EXPERIMENT WAS PERFORMED WITH A VIRUS-FREE LABORATORY STOCK OF *Spodoptera exigua* (HÜBNER). A SINGLE GENOTYPE OF SEMNPV, NAMED VT-SEAL1, WAS USED IN THE EXPERIMENT. THIS GENOTYPE WAS PREVIOUSLY ISOLATED FROM SUBLETHALLY INFECTED INSECTS COLLECTED IN THE GREENHOUSES (SPAIN) AND WAS TRANSMITTED FROM PARENTS TO OFFSPRING.

DNA EXTRACTION AND DETECTION OF COVERT INFECTIONS

TOTAL DNA WAS EXTRACTED FROM THE ABDOMENS OF ADULTS. QUANTITATIVE PCR BASED ON SYBR GREEN FLUORESCENCE WAS PERFORMED TO DETECT SEMNPV INFECTION. SPECIFIC PRIMERS WERE DESIGNED TO AMPLIFY A 149-BP REGION OF THE *DNA polymerase* GENE BASED ON THE COMPLETE GENOME SEQUENCE OF THE SEMNPV STRAIN VT-SEAL1 (UNPUBLISHED DATA). FOR THE STANDARD CURVE, VT-SEAL1 DNA WAS EXTRACTED FROM OBS, PURIFIED THOROUGH CSCL GRADIENTS, QUANTIFIED BY SPECTROPHOTOMETER AND THEN SERIALLY DILUTED TO THE FOLLOWING CONCENTRATIONS (5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, AND 0.001 NG μL^{-1}). QUANTIFIED VIRAL DNA WAS NORMALIZED BASED ON THE INSECT DNA CONCENTRATION FOR EACH SAMPLE AND MEASURED USING NANODROP 2000.

BIOASSAYS

TO DETERMINE THE INFLUENCE OF GENDER ON VERTICAL TRANSMISSION, GROUPS OF SUBLETHALLY INFECTED (INFECTED MALES: ♂ AND INFECTED FEMALES: ♀) OR VIRUS-FREE ADULTS (HEALTHY MALES: H♂ AND HEALTHY FEMALES: H♀) WERE REQUIRED. SUBLETHALLY INFECTED INSECTS WERE PRODUCED FROM A VIRUS-FREE INSECT CULTURE USING FOURTH INSTARS TREATED WITH 9 × 10^3 OB ML^{-1} SUSPENSION. A GROUP OF 100 LARVAE WERE TREATED IN THE SAME CONDITIONS WITH SOLUTION WITHOUT OBS. SURVIVING INSECTS WERE REARED, SEXED AND THEN CLASSIFIED IN GROUPS ACCORDING TO THEIR SEX AND VIRAL TREATMENT. ONCE THE ADULTS EMERGED, FOUR MATING TREATMENTS WERE PERFORMED: I) HEALTHY MALES (H♂) × HEALTHY FEMALES (H♀), II) INFECTED MALES (I♂) × HEALTHY FEMALES (H♀), III) HEALTHY MALES (H♂) × INFECTED FEMALES (I♀), AND IV) INFECTED MALES (I♂) × INFECTED FEMALES (I♀). FIVE ADULT PAIRS WERE CONFINED IN PAPER BAGS FOR OVIPOSITION. EGGS BATCHES FROM EACH TREATMENT GROUP WERE HARVESTED AND THEN USED IN SUBSEQUENT ANALYSES (INCUBATION). EGG MASSES FROM EACH PAPER BAG WERE DIVIDED INTO TWO PARTS, AND EITHER SOAKED IN A 0.25 PPM HYPOCHLORITE SOLUTION (SURFACE DECONTAMINATION) OR DISTILLED WATER (NO DECONTAMINATION) FOR FIVE MINUTES. TWENTY-FIVE NEONATES WERE REARED ON SEMI-ARTIFICIAL DIET THROUGH TO ADULTHOOD AND THEN FROZEN FOR SUBSEQUENT DNA ANALYSIS. THE WHOLE PROCEDURE WAS PERFORMED FOUR TIMES.

Results and discussion

OF THE LARVAE INITIALLY TREATED WITH VT-SEAL1 OBS, 58% SUCCUMBED TO VIRUS INFECTION, WHEREAS NO MORTALITY WAS REGISTERED IN MOCK-INFECTED CONTROL LARVAE. THE DETECTION LIMIT OF QPCR REACTION WAS ESTIMATED AT 1×10^{-3} NG OF GENOMIC DNA, WHICH EQUATES THEORETICALLY TO A VALUE BETWEEN 6 AND 7 VIRAL GENOME COPIES.

THE FREQUENCIES OF QPCR POSITIVE SURVIVORS TO A VIRUS CHALLENGED WERE SIGNIFICANTLY HIGHER THAN THOSE MEASURED IN CONTROL INSECTS ($P < 0.05$; CHI-SQUARE TEST). VIRUS (1.0–5.4) IN F_0 PARENTAL ADULTS AVERAGED 10.3 ± 2.0 GENOME COPIES PER ADULT (N = 72, POSITIVES = 1

SUBLETHALLY INFECTED MALES THAT MATED HEALTHY FEMALES PRODUCED OFFSPRING WITH INFECTED INDIVIDUALS ON AVERAGE, COMPARED TO 8% IN THE OFFSPRING OF THE CONTROL. IN CONTRAST, IN THE MATING GROUPS IN WHICH THE FEMALES WERE SUBLETHALLY INFECTED

OF COVERT INFECTION IN OFFSPRING VARIED BETWEEN 44% AND 49%. THEREFORE, FEM
VERTICAL TRANSMISSION WAS APPROXIMATELY TWICE AS EFFICIENT AS MALE-MEDIATED

THE PREVALENCE OF INFECTION IN ADULTS DID NOT DIFFER SIGNIFICANTLY ACCORDING
SURFACE DECONTAMINATION TREATMENT ($P > 0.05$). THIS RESULT IS IN AGREEMENT WITH RECENT STU
ON *Spodoptera exempta* NUCLEOPOLYHEDROVIRUS IN WHICH SURFACE DECONTAMINATION
NOT AFFECT THE DETECTION OF THE VIRUS IN THE OFFSPRING OF INFECTED INSECTS (VILA
SUGGESTING THAT TRANSOVARIAL, RATHER THAN TRANSOVUM TRANSMISSION REPRES
PATHWAY FOR TRANSMISSION.

STUDIES WITH *Drosophila* SIGMA VIRUS, HAVE INDICATED THAT TRANSMISSION RATES ARE
FEMALES THAN MALES (LONGDON *et al*, 2011), ALTHOUGH TRANSMISSION HAS BEEN OBSERVED TO
THROUGH BOTH EGGS AND SPERM. IN CONTRAST, STUDIES ON *Baculoviruses* (GENUS
DEMONSTRATED THAT BOTH SEXES WERE INVOLVED IN VERTICAL TRANSMISSION FOR *Plodia interpunctella*
GRANULOVIRUS, WITH VIRAL PARTICLES PRESENT IN BOTH TESTIS AND OVARIES OF S
INDIVIDUALS BY VIRAL TRANSCRIPT DETECTION (BURDEN *et al*, 2002).

MEAN VALUES OF VIRAL LOAD IN ADULTS DID NOT DIFFER SIGNIFICANTLY BETWEEN MATING
($P > 0.05$) I.E., THE QUANTITY OF VIRAL DNA PER SUBLETHALLY INFECTED INSECT WAS IN
THE PARENTAL LINEAGE PASSING ON THE VIRUS (MALE, FEMALE OR BOTH). IN CONTRAS
(2011) DETECTED LOWER TITRES OF SIGMA VIRUS IN THE EMBRYOS OF *Drosophila* SPECIES WHEN
THE VIRUS WAS PATERNALLY TRANSMITTED.

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Baculoviruses for the biological control of cutworms (*AGROTIS* spp)

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Abstract: CATERPILLARS OF THE COMMON CUTWORM *Agrotis segetum* AND BLACK CUTWORM *A. ipsilon* (LEPIDOPTERA: NOCTUIDAE) ARE WASTEFUL FEEDERS OF VARIOUS CROPS IN AGRICULTURE. THES MAINLY CONTROLLED BY CHEMICAL PESTICIDES BUT RECENT ATTEMPTS ARE AIMED TO CONTRO THE APPLICATION OF BACULOVIRUSES. FOUR DIFFERENT BACULOVIRUSES OF *Agrotis segetum*, NAMELY NUCLEOPOLYHEDROVIRUS A (AGSENPV-A), *Agrotis segetum* NUCLEOPOLYHEDROVIRUS B (AGSENPV-B), *Agrotis ipsilon* MULTIPLE NUCLEOPOLYHEDROVIRUS (AGIPMNPV) AND *Agrotis segetum* GRANULOVIRUS (AGSEGV), WERE ISOLATED FROM LARVAE OF *A. segetum* AND *A. ipsilon* AND ARE CONSIDERED AS POTENTIAL BIOCONTROL AGENTS. IN NATURAL INFECTIONS, LARVAE OF BOTH HOSTS ARE SUSCEPTIBLE TO INDIVIDUAL CATERPILLARS OF THE COMMON CUTWORM WERE OBSERVED TO BECOME IN INFECT BY AGSENPV-B AND AGSEGV. CO-INFECTIONS MAY BE ADVANTAGEOUS IN TERMS OF VIRULENCE AN MANAGEMENT, ALTHOUGH THE LEVEL OF INTERACTION IS CRITICAL. TO TEST FOR A MUTUA ANTAGONISTIC INTERACTION AND TO EVALUATE A COMBINED APPLICATION OF *Agrotis*-SPECIFIC BACULOVIRUSES, WE EXEMPLARY PERFORMED MIXED INFECTION EXPERIMENTS WITH *A. segetum* LARVAE THAT WERE EXPOSED TO AGSENPV-B AND AGSEGV AT DIFFERENT CONCENTRATION. FOR QUANTITATIVE ANALYSIS OF THE C INFECTIONS AS WELL AS FOR QUALITY CONTROL IN VIRUS PRODUCTION A RELIABLE METHOD DISCRIMINATIVE QUANTIFICATION OF *Agrotis*-SPECIFIC BACULOVIRUSES IS REQUIRED. WE ESTABLISHED A MULT PCR ANALYSIS BASED ON HIGHLY SPECIFIC OLIGONUCLEOTIDES WHICH ALSO PERMIT QUAN QUANTITATIVE PCR. AS A PREREQUISITE OF THESE STUDIES THE GENOME OF AGSENPV-B WAS SEQUENCED BY 454 SEQUENCING TECHNIQUE. COMPARATIVE GENOME SEQUENCE ANALYSES GAV INSIGHT INTO THE MOLECULAR SETUP OF THE THREE *Agrotis*-SPECIFIC NPVS AND CONFIRMED THAT THEY CAN BE REGARDED AS THREE DIFFERENT BUT CLOSE RELATED SPECIES. OUR RESULTS WILL HELP TO DE *Agrotis*-SPECIFIC BACULOVIRUSES AS BIOCONTROL AGENTS AND TO UNDERSTAND THE EVOLUTIONA VIRUSES THAT ARE HIGHLY ADAPTED TO THE SAME HOSTS.

Key words: CUTWORMS, *Agrotis*, BACULOVIRUSES, PEST CONTROL, CO-INFECTION

Introduction

CATERPILLARS OF SEVERAL NOCTUID MOTHS THAT LIVE IN SOIL AND FEED ON ROOTS OF P AS CUTWORMS. THE TERM CUTWORMS INCLUDES MOTHS OF THE GENERA *Agrotis*, *Euxoa*, *Feltia*, *Peridroma* AND *Xestia* (BOURNER & CORY, 2004). THEY ARE WORLDWIDE DISTRIBUTED AND KNO WASTEFUL FEEDERS ON VARIOUS AGRICULTURAL PLANTS. TWO HIGHLY HARMFUL CUTW FROM THE GENUS *Agrotis* (NOCTUIDAE): THE COMMON CUTWORM *A. segetum* (DENIS & SCHIFFERMÜLLER) AND THE BLACK CUTWORM *A. ipsilon* (HUFNAGEL). TO DATE, CUTWORMS (*Agrotis* SPP.) ARE MAINLY CONTROLLED BY CHEMICAL PESTICIDES (E. G. PYRETHROIDS). BA ARE CONSIDERED AS BIOLOGICAL CONTROL AGENTS FOR A SUSTAINABLE CONTROL OF TH CUTWORM IN AGRICULTURE AND HAVE BEEN ALREADY SUCCESSFULLY TESTED FOR THE WASTEFUL SOIL PESTS (*e.g.* BOURNER *et al.*, 1992). IN THE PAST, THREE DIFFERENT NUCLEOPOLYHEDROVI (NPV) AND ONE GRANULOVIRUS (GV) WERE ISOLATED AND CHARACTERIZED FROM *A. segetum* AND *A. ipsilon* (AGIP) LARVAE: AGSENPV-A (JAKUBOWSKA *et al.*, 2005, 2006), AGSENPV-B (ALAWAY & PAYNE, 1983), AGIPMNPV (BOUGHTON *et al.*, 1999; HARRISON, 2009) AND AGSEGV

(LIPA & ZIEMNICKA, 1971). AS ONLY LIMITED GENETIC INFORMATION OF AGSENPV-B WAS A
ITS GENOME WAS COMPLETELY SEQUENCED AND WHOLE GENOME COMPARISONS OF *Agrotis*-SPECIFIC
NPVS WERE PERFORMED.

BIOASSAYS REVEALED THAT *A. ipsilon* AND *A. segetum* ARE SUSCEPTIBLE TO MORE THAN ONE
Agrotis BACULOVIRUSES (EL-SALAMOUNY, 2003; BOURNER & CORY, 2004). CO-INFECTIONS
BETWEEN AGSEGV AND AGSENPV-B ARE FREQUENTLY OBSERVED, ALTHOUGH THEIR TYPE
IS NOT YET WELL UNDERSTOOD. HOWEVER, IN TERMS OF RESISTANCE MANAGEMEN
APPLICATION OF TWO BACULOVIRUSES IS CONSIDERED AS BENEFICIAL AND OCCURRING IN
CRITICAL FOR A SUCCESSFUL APPLICATION. MAINLY THREE DIFFERENT TYPES OF I
CONCEIVABLE: MUTUALISM, NEUTRALISM AND ANTAGONISM. TO INVESTIGATE AND OPTIM
APPLICATION OF THE FOUR KNOWN *Agrotis* BACULOVIRUSES FOR CUTWORM CONTROL, THE SUSCEPTIBI
A. segetum AND *A. ipsilon* LARVAE TO THESE VIRUSES NEEDS TO BE DETERMINED NOT ONLY IN
ALSO IN MIXED INFECTIONS. IN THIS STUDY, THE LEVEL OF INTERACTION WAS INVESTIGAT
B AND AGSEGV IN SIMULTANEOUSLY INFECTED *A. segetum* LARVAE. THE PRESENT RESULTS HELP TO
UNDERSTAND THE *Agrotis* BACULOVIRUS COMPLEX, HOW CLOSELY RELATED BACULOVIRUSES EVO
SAME HOST GENERA, HOW THEY DIFFER ON THE MOLECULAR LEVEL AND HOW THEY
INFECTIONS.

Material and methods

WHOLE GENOME SEQUENCING

PURIFIED GENOMIC DNA OF AGSENPV-B WAS COMPLETELY SEQUENCED BY 454 WHOLE G
SEQUENCING TECHNIQUE. READS WERE ASSEMBLED TO A CONSENSUS SEQUENCE AND
FRAMES AND HOMOLOGOUS REPEAT (HR) REGIONS WERE ANNOTATED BY GENEQU
(DNASTAR LASERGENE V8.1.4). BACULOVIRUSES SHARE 30 CORE GENES THAT WERE FOU
COMPLETELY SEQUENCED AND PUBLISHED VIRUS GENOMES. THE CONCATENATED SEQUE
CORE GENES OF AGSENPV-B AND OTHER SELECTED BACULOVIRUSES WERE USED TO
PHYLOGENETIC RELATIONSHIP BY MAXIMUM PARSIMONY ANALYSIS.

PCR BASED DETECTION, DISCRIMINATION OF AGROTIS BACULOVIRUSES

BASED ON THE COMPLETE POLYHEDRIN AND GRANULIN SEQUENCES OF AGSENPV-A, A
AGIPMNPV AND AGSEGV, FOUR HIGHLY SPECIFIC DIFFERENT PAIRS OF OLIGONUCLEOTIDE
PAIR FOR EACH VIRUS, WERE DESIGNED. TO DISCRIMINATE BETWEEN ALL FOUR VIRUSE
PCR PRODUCTS DIFFERED IN SIZE. THE PRIMERS ALSO ALLOWED A MULTIPLEX PCR AM
WHICH ALLOWED DETECTING ALL FOUR BACULOVIRUSES WITHIN A SINGLE PCR REACTION.

BIOASSAYS

BIOASSAYS FOR AGSENPV-B, AGIPMNPV AND AGSEGV WERE PERFORMED FOR *A. segetum* NEONATE
LARVAE. FOR EACH VIRUS, LARVAE WERE FED ON SEMI-ARTIFICIAL DIET CONTA
CONCENTRATIONS OF VIRUS OCCLUSION BODIES (OB). FIFTY *A. segetum* LARVAE WERE USED FOR
EACH CONCENTRATION AND MORTALITY WAS SCORED AFTER 14 D POST INFECTION (P.I
WERE REPEATED IN TRIPLICATE. MEDIAN LETHAL CONCENTRATIONS (LC$_{50}$) AND 10% MORTALITY (LC$_{10}$)
WERE CALCULATED BY PROBIT ANALYSES.

MIXED INFECTION STUDIES

THE LC$_{50}$ AND LC$_{10}$ (14 D P.I.) OF AGSENPV-B AND AGSEGV WERE USED FOR CO-INFECTIO
EXPERIMENTS. NEONATE *A. segetum* LARVAE WERE EXPOSED TO COMBINED LETHAL CONCENTRAT
WERE PREVIOUSLY DETERMINED BY BIOASSAYS (14 D P.I.): LC$_{50}$:LC$_{50}$, LC$_{10}$:LC$_{10}$, LC$_{10}$:LC$_{50}$,

LC_{10}:LC_{10}. EACH TREATMENT COMPRISED 25 NEONATE LARVAE AND WAS REPEATED SIX TIM
WAS SCORED AFTER 14 DAYS AND CADAVERS WERE INDIVIDUALLY COLLECTED. VIRAL GE
ISOLATED FROM DEAD LARVAE AND THE PRODUCTION OF AGSENPV-B AND AGSEGV PE
DETERMINED BY QUANTITATIVE (Q) PCR USING THE DESIGNED, HIGHLY SPECIFIC PCR P
AGSEGV AND AGSENPV-B.

Results and discussion

THECOMPLETELY SEQUENCED AGSENPV-B GENOME SHOWED A HIGH SIMILARITY IN GC
NUMBER OF DETECTED ORFS AND GENOMIC LENGTH TO AGSENPV-A (TABLE 1). DESPITE TH
SIMILARITIES, THE DNA SEQUENCE OF AGSENPV-B APPEARED TO ME MORE SIMILAR T
AGIPMNPV THAN TO THAT OF AGSENPV-A. THIS WAS PROVEN BY MAXIMUM PARSI
PHYLOGENETIC ANALYSIS BASED ON THE CONCATENATED 30 BACULOVIRUS CORE GENES. A
ANALYSIS AGSENPV-B IS MORE CLOSE RELATED TO AGIPMNPV THAN TO AGSENPV-A.
GENOME COMPARISONS OF AGSENPV-A, AGSENPV-B AND AGIPMNPV ALSO REVEALED T
ARRANGEMENT OF ORFS AND HOMOLOGOUS REPEAT REGIONS (HR) WERE HIGHLY SIMILAR

TABLE 1. CHARACTERISTICS OF THE GENOMES OF AGSENPV-B, AGSENPV-A, AGIPNPV
AGSEGV.

	LENGTH (BP)	ORF	% GC	REFERENCE
AGSENPV-B	148,986	154	45.69	THIS STUDY
AGSENPV-A	147,544	153	45.71	JAKUBOWSKA *et al.* (2006)
AGIPMNPV	155,122	163	48.57	HARRISON (2009)
AGSEGV	131,680	132	37.31	GENBANK (NC_005839)

IN MIXED INFECTIONS USING DIFFERENT CONCENTRATIONS OF AGSENPV-B AND A
INCREASE OF MORTALITY WAS OBSERVED COMPARED TO SINGLE INFECTIONS (DATA NOT S
OBSERVED THAT THE AMOUNT OF CO-INFECTED LARVAE WAS DEPENDENT ON THE APP
CONCENTRATION AND THAT A HIGHER AGSENPV-B CONCENTRATION REDUCED THE PRODU
PER LARVA. HOWEVER, THE AGSEGV CONCENTRATION IN MIXED VIRUS TREATMENTS DI
AFFECT THE AMOUNT OF PRODUCED AGSENPV-B PER LARVA. IT COULD BE CONCLUDED F
THAT NO MUTUALISM WAS FOUND, RATHER A COMPETITION FOR RESOURCES.

BASED ON THE POLYHDERIN AND GRANULIN GENE SEQUENCES OF THE VIRUSES *Agrotis segetum* NUCLEOPOLYHEDROVIRUS
OLIGONUCLEOTIDES TO BE USED IN PCR WERE DESIGNED. THE OLIGONUCLEOTIDES DID N
UNDESIRED BINDING IN MULTIPLEX PCR CONTROL REACTIONS AND WERE ALSO FULLY FUNC
ANALYSES. BIOASSAYS SHOWED THAT *A. segetum* LARVAE ARE LESS SUSCEPTIBLE TO AGIPMNPV
(LC_{50} = 7.3 X 10^3 OB ML^{-1}) AND AGSEGV (LC_{50} = 27.0 X 10^3 OB ML^{-1}) THAN TO AGSENPV-B
(LC_{50} = 3.3 X 10^3 OB ML^{-1}) (TABLE 2). FURTHERMORE, THE SPEED OF KILLING OF AGSEGV WAS
LOW AND RESULTED IN A HIGH LC_{50} VALUE AFTER 7 AND 14 D P.I.

TABLE 2. MEDIAN LETHAL CONCENTRATIONS (LC_{50}) OF AGSEGV, AGSENPV-B AND AGIPMNPV IN 7-DAY BIOASSAYS IN NEONATE *A. segetum* LARVAE.

VIRUS	LC_{50} (95% CL) [OB ML^{-1}] ($X10^3$)	LC_{10} (95% CL) [OB ML^{-1}] ($X10^3$)	SLOPE	DF	χ^2
AGSEGV	27 (5-131)	0.1 (0.001-0.7)	0.53	3	24.53
AGSENPV-B	3.28 (2.62-4.00)	0.34 (0.21-0.50)	1.30	3	9.56
AGIPNPV	7.29 (5.90-8.90)	0.55 (0.36-0.79)	1.15	3	8.94

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Posters

Insect pathogens and entomoparasitic nematodes
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Insecticidal activity of a spray dried formulation based on a Colombian *SPODOPTERA FRUGIPERDA* nucleopolyhedrovirus

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Abstract: A COLOMBIAN *Spodoptera frugiperda* NUCLEOPOLYHEDROVIRUS (SFMNPV 003) WITH HIGH POTENTIAL FOR THE DEVELOPMENT OF AN EFFICIENT BIOPESTICIDE WAS MICROENCAPSULATED WITH A PH DEPENDENT POLYMER® (EUDRAGIT) AND ITS INSECTICIDAL ACTIVITY WAS EVALUATED U LABORATORY AND GREENHOUSE CONDITIONS. SIGNIFICANT DIFFERENCES BETWEEN LC_{50} VALUES MICROENCAPSULATED VIRUS, THE DRIED VIRUS AND THE VIRUS WITHOUT ANY TREATMENT WER LABORATORY CONDITIONS, SUGGESTING THAT MICROENCAPSULATION BY TOP SPRAY DRYING D INSECTICIDAL ACTIVITY. THREE DIFFERENT MICROENCAPSULATED BATCHES SHOWED THE SA GREENHOUSE CONDITIONS AND SIGNIFICANT DIFFERENCES BETWEEN FORMULATED AND UNFO NOT DETECTED (P > 0.05). IN CONCLUSION, SFMNPV003 INSECTICIDAL ACTIVITY WAS NOT A FORMULATION PROCESS AND DEVELOPED BIOPESTICIDE DEMONSTRATED POTENTIAL FOR *S. frugiperda* CONTROL AND COULD BE INCLUDED IN PROGRAMS OF INTEGRATED PEST MANAGEMENT (IPM).

Key words: MICROENCAPSULATION, INSECTICIDAL ACTIVITY, BACULOVIRUS, ARMYWORM

Introduction

BACULOVIRUSES HAVE BEEN WIDELY STUDIED DUE TO THEIR HIGH PATHOGENICITY FOR SPECIES OF INSECTS CONSIDERED AS PESTS (MILLER, 1997; MOSCARDI *et al.*, 1999; CABALLERO HOWEVER, ONE LIMITATION FOR ITS USE IS THE ACTIVITY LOSSES OBSERVED UNDER (CABALLERO *et al.*, 2001), BEING NECESSARY TO DEVELOP FORMULATIONS ABLE TO RED INACTIVATION. THE MICROENCAPSULATION CONSTITUTES A PROMISING TECHNIQUE T BACULOVIRUS FORMULATIONS (VILLAMIZAR *et al.*, 2012). MICROENCAPSULATION WITH POLYMERIC MATERIALS IS VERY USEFUL FOR BIOPESTICIDES DEVELOPMENT BECAUSE THIS PROCESS PARTICLES FROM ENVIRONMENTAL CONDITIONS AS UV LIGHT OR TEMPERATURE. MOR RESISTANT TO RAIN AND DEW AND CAN BE EASILY DISPERSED IN THE AIR TO BE CONSUME (WINDER *et al.*, 2003).

S. frugiperda JE SMITH (1797) (LEPIDOPTERA: NOCTUIDADE) KNOWN AS ARMYWORM CA IMPORTANT ECONOMIC LOSSES IN DIFFERENT CROPS AS SORGHUM, RICE AND MAIZE (GAR 1999). A NATIVE *S. frugiperda* NUCLEOPOLYHEDROVIRUS (SFMNPV) WITH HIGH POTENTIA BIOPESTICIDE (GOMEZ *et al.*, 2010) WAS MICROENCAPSULATED BY OIL-IN-OIL EMULSION (O/O) SO EVAPORATION METHOD AND IMPROVE VIRUS PHOTOSTABILITY (VILLAMIZAR *et al.*, 2012). CONSIDERING THE RISKS OF WORKING WITH ORGANIC SOLVENTS FOR MICROENCAPS DEVELOPED METHOD A NEW FORMULATION PROCESS BY USING TOP SPRAY DRYING WA SPRAY DRYING ALLOWS TO PROCESS EXTREMELY HEAT-SENSITIVE MATERIALS AS BACUL SHORT DRYING TIMES AND THE LOW PRODUCT TEMPERATURES. MOREOVER, THIS TE ATTRACTIVE ADVANTAGES FOR PRODUCING MICROCAPSULES IN A RELATIVELY SIMPL CONTINUOUS OPERATION. (RE & RAZNSTEIN, 2004). THE PRESENT STUDY WAS CONDUCTED

DETERMINE THE EFFECT OF THE NEW FORMULATION PROCESS BY SPRAY DRYING OVER
INSECTICIDAL ACTIVITY.

Material and methods

VIRUS PRODUCTION

VIRUS OCCLUSION BODIES (OBS) PRODUCTION WAS CONDUCTED BY INOCULATING THIRD
S. frugiperda WITH A SFMNPV 003 SUSPENSION USING THE DROPLET FEEDING METHOD (HU
WOOD, 1981). OBS WERE RECOVERED FROM DEAD LARVAE BY MIXING AND FILTERING A
THEN DRIED IN A FLUID BED WITH AN INTERNAL PRESSURE OF 1 BAR, A FLOW RATE OF $1 L \cdot min^{-1}$,
INLET TEMPERATURE OF 92 ± 5 °C AND AN OPENING GATE INLET AIR ANGLE OF 25° AT THE S
AND 35° AT THE END.

MICROENCAPSULATION BY TOP SPRAY DRYING

MICROENCAPSULATED PRODUCT WAS PREPARED BY SPRAYING AN AQUEOUS SUSPENSIO
(1.35% W/V) AND EUDRAGIT® L100 (6.0% W/V) IN A GLATT UNI GLATT 01277 FLUID BED DRYE
EQUIPPED WITH A NOZZLE TO 1.0 MM TO ADJUST THE AIRFLOW. THE OPERATION CONDITION
TEMPERATURE OF 80 ± 5 °C, AN INTERNAL PRESSURE OF 2.23 BARS, A FLOW RATE OF 4.12 $ML \cdot min^{-1}$ AND
OPENING GATE INLET AIR ANGLE OF 25°.

INSECTICIDAL ACTIVITY UNDER LABORATORY CONDITIONS

THE BIOASSAY WAS CARRIED OUT FOLLOWING THE METHODOLOGY DESCRIBED BY H
(1981). SUSPENSIONS WERE PREPARED AND ADJUSTED TO FIVE CONCENTRATIONS BETWE
2.0×10^{8} OBS ML^{-1} FOR THE THREE MICROENCAPSULATED PRODUCT BATCHES. CONTRO
CONSISTED IN NON-TREATED LARVAE. EXPERIMENTAL DESIGN WAS COMPLETELY RANDOM
FACTORIAL ARRANGEMENT AND THREE REPLICATIONS PER TREATMENT, EACH ONE WITH
WAS DETERMINED SEVEN DAYS AFTER INOCULATION AND RESULTS WERE ANALYZED
(FINNEY, 1952) IN ORDER TO DETERMINE LC_{50} VALUES (BIOSTAT, 2007).

INSECTICIDAL ACTIVITY UNDER GREENHOUSE CONDITIONS

PLANTS OF MAIZE (*Zea mays* L.) ICA 508 VARIETY (SPECIAL FOR COLD WEATHER) WERE GROWN
UNDER GREENHOUSE CONDITIONS. RANDOMIZED COMPLETE BLOCK DESIGN (RCBD)
REPLICATES WAS USED. TREATMENTS WERE THREE BATCHES OF MICROENCAPSULAT
UNFORMULATED DRIED VIRUS BOTH ADJUSTED TO 1×10^{7} OBS ML^{-1} AND A CONTROL WITHOUT ANY
APPLICATION. THE EXPERIMENTAL UNIT CONSISTED IN A ROW OF 1.5 M LONG WITH 10 PLAN
OF PLANTING DISTANCE. THE CROP WAS SUBJECTED TO USUAL IRRIGATION, FERTILIZATIO
CONDITIONS. THIRTY DAYS AFTER SOWING, PLANTS WERE SPRAYED WITH 2 ML OF TREAT
HANDHELD SPRAYER. ONE HOUR AFTER APPLICATION, TWO SECOND INSTAR *S. frugiperda* LARVAE OF
PLACED PER PLANT. AFTER TWO DAYS, 10 LARVAE FROM EACH REPLICATE PER TREATME
LARVAE WERE PLACED IN SEPARATE PLASTIC CUPS CONTAINING ARTIFICIAL DIET AND
LABORATORY AT 28 ± 2 °C AND AT 60% OF RELATIVE HUMIDITY. MORTALITY RATE WAS
SEVEN DAYS AND EFFICACY WAS DETERMINED USING THE SCHNEIDER-ORELLI'S FORMU
THE NORMALITY OF THE DATA WAS ESTIMATED BY SHAPIRO-WILK TEST AND HOMOGEN
USING BARTLETT'S TEST. DIFFERENCES BETWEEN TREATMENTS WERE EVIDENCED BY AN
TEST ($\alpha = 0.05$) WITH THE PROGRAM STATISTIC 8.1.

Results and discussion

MICROENCAPSULATION BY TOP SPRAY DRYING

THREE MICROENCAPSULATED PRODUCT BATCHES PRESENTED A MEAN VIRAL CONCENTR
OBS G^{-1}, A PARTICLE SIZE OF 18.84 µ AND A MOISTURE CONTENT OF 10.38%.

INSECTICIDAL ACTIVITY UNDER LABORATORY CONDITIONS

TREATED LARVAE WITH MICROENCAPSULATED PRODUCT AND UNFORMULATED DRIED VI
SIGNS OF INFECTION AS CHANGE OF COLOR FROM PINK TO DARK BROWN, DEVELOPM
REDUCTIONS IN FEEDING AND MOBILITY (MOSCARDI, 1999). DISEASED LARVAE PRESI
TEGUMENT WHICH IS EASILY BROKEN DELIVERING A BROWN FLUID MAINLY CORRESP
(CABALLERO *et al*, 2001).

THE LC_{50} VALUES FOR THREE DIFFERENT BATCHES OF MICROENCAPSULATED PRODUCT AR
TABLE 1. THE COMPARISON OF THE CONFIDENCE LIMITS (95%) DID NOT REVEAL SIGNIFICA
BETWEEN THE LC_{50} OF THREE BATCHES SUGGESTING REPEATABILITY DURING THE MANUFAC
THESE RESULTS WERE COMPARED WITH THE LC_{50} DETERMINED PREVIOUSLY FOR UNFORMULATED
PURIFIED VIRUS AND EVEN THE LETHAL CONCENTRATION FOR MICOENCAPSULATED PRO
OBTAINED FOR UNFORMULATED VIRUS, FIDUCIAL LIMITS COMPARISON SUGGEST THAT E
THE SAME PATHOGENICITY AND MICROENCAPSULATION PROCESS BY TOP SPRAY DRYING
S100 DID NOT AFFECT THE INSECTICIDAL ACTIVITY OF VIRAL ISOLATE SFMNPV 003, E
TEMPERATURE DURING SPRAYING DRYING PROCESSES WAS 42.15 ± 5 °C, WHICH DID NOT
INACTIVATED THE VIRUS.

TABLE 1. MEAN LETHAL CONCENTRATIONS OF MICROENCAPSULATED AND UNFORMULATE

Batch	LC_{50} (OBs ml^{-1})	95% fiducial limits (OBs ml^{-1})		p	χ^2
		Lower	Upper		
1	1.3 X 10^4	2.8 X 10^2	6.4 X 10^5	0.53	2.19
2	3.1 X 10^4	9.1 X 10^2	1.0 X 10^6	0.63	1.70
3	3.1 X 10^4	4.0 X 10^3	1.4 X 10^5	0.94	0.39
AVERAGE	2.5 X 10^4	1.7 X 10^3	5.9 X 10^5	0.70	1.42
UNFORMULATED VIRUS (GÓMEZ *et al*, 2010)	2.3 X 10^5	5.4 X 10^4	4.7 X 10^6	0.25	4.72

INSECTICIDAL ACTIVITY UNDER GREENHOUSE CONDITIONS

TWO DAYS AFTER TREATMENTS APPLICATIONS THE DAMAGE CAUSED BY THE LARVAE W
APPLIED WITH THE VIRAL TREATMENTS COMPARED WITH THE CONTROL (NO APPLICA
DAMAGE CAUSED BY *S. frugiperda* OCCURS ON THE WHORL LEAVES, BEING HIGHER IN NOT TREAT
(CONTROL) (FIGURE 1).

YOUNG LARVAE OF *S. frugiperda* MAKE SCRATCHES ON THE SOFT PARTS OF THE LEAVES, WHI
APPEAR AS SMALL TRANSLUCENT AREAS. WHEN LARVAE ARE IN ADVANCED INSTAR
PERFORATIONS OR AREAS FEED WHEN ARE OPENED THE LEAVES. IN THIS PHASE IS CHAR
LARVAL WASTES (NEGRETE GONZALES, 2003). SYMPTOMATIC LARVAE WERE ONLY OBSERVE
FROM VIRUS APPLIED TREATMENTS. EFFICACIES OF THREE BATCHES OF MICROENCAP
APPLIED AT 1 X 10^7 OBS ML^{-1} WERE 82.36%, 87.40% AND 62.22% RESPECTIVELY WITH AN AVERAG

VALUE OF 77.32% AND UNFORMULATED VIRUS REACHED AN EFFICACY OF 77.33%. DIFFERE
MEANS WERE DETECTED USING TUKEY'S TEST (95%), WHICH DID NOT DETECT SIGNIFICA
BETWEEN ALL VIRAL TREATMENTS (P>0.05) CONFIRMING THE REPEATABILITY BETWEEN
AND SUGGESTING THAT THE DEVELOPED FORMULATION AND TOP SPRAY DRYING PROC
VIRAL ACTIVITY.

THE MICROCAPSULES PRODUCED BY THE METHOD OF MICROENCAPSULATION BY OIL-
(O/O) SOLVENT EVAPORATION IMPROVED VIRUS PHOTOSTABILITY, BUT PRESENTED RESIDUES O
SOLVENT IN THE FORMULATION (*Vital*, 2012), WHILE MICROCAPSULATION BY TOP SPRAY
DRYING AVOIDED THIS RESIDUES AND MICROCAPSULES SHOWED A SMALLER PARTICLE
THAN OBTAINED WITH THE SOLVENT EVAPORATION (18 µM). IN CONCLUSION, TOP SPRAY DRYING
METHOD DEMONSTRATED HIGH POTENTIAL FOR BEING USED AS FORMULATION PROCESS
MICROCAPSULES BASED ON SFMNPV 003, BIOPESTICIDE THAT COULD BE INCLUDED IN IN
MANAGEMENT PROGRAMS.

FIGURE 1. RECENT DAMAGE PRODUCED BY *S. frugiperda* IN MAIZE PLANTS, (A) UNTREATED PLANT (B
TREATED PLANT.

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CYDIA POMONELLA granulovirus knockout mutants: The potential role of *PE38* in overcoming codling moth resistance

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Abstract: THE *Cydia pomonella* GRANULOVIRUS (CPGV) BELONGS TO THE GENUS *Betabaculovirus* OF THE FAMILY BACULOVIRIDAE. CPGV IS A WORLDWIDE USED BIOLOGICAL AGENT TO CONTR INFESTATION OF APPLES, PEARS AND WALNUTS BY CODLING MOTH (*Cydia pomonella* L.). IN 2005, THE FIRST RESISTANCE OF FIELD POPULATIONS OF *Cydia pomonella* (CM), WITH UP TO 1000-FOLD REDUCED SUSCEPTIBILITY TO CPGV PRODUCTS CONTAINING THE ISOLATE CPGV-M (FOUND 1964 IN MEXICO), W DISCOVERED IN EUROPE. SINCE THEN, SEVERAL CPGV ISOLATES (E.G. CPGV-I12, -S) HAVE BEEN FO THAT WERE ABLE TO OVERCOME THE RESISTANCE OF CM UNDER LABORATORY CONDITIONS. I ANALYSIS OF DIFFERENT CPGV ISOLATES HAVE SHOWN THAT THE ONLY GENOMIC DIFFERENCES, WH RESISTANCE OVERCOMING ISOLATES HAVE IN COMMON, ARE AN INSERTION OF 24 NUCLEOTIDES IN GENE *pe38* (EBERLE & JEHLE, UNPUBLISHED). PRELIMINARY RESULTS SUGGEST THAT RECOMBINA WITH A KNOCKOUT *pe38* LOSES THEIR ABILITY TO INFECT SUSCEPTIBLE OR RESISTANT CM LARV AIM OF THIS WORK IS TO CONFIRM THE ROLE OF *PE38* IN OVERCOMING THE RESISTANCE OF CM BY CREATING KNOCKOUT AND RESCUE MUTANTS BASED ON AN ALREADY EXISTING CPGV-M BA ACCORDING TO THE SOURCE OF *pe38* OF EITHER RESISTANCE OVERCOMING ISOLATE (E.G. CPGV-S) OR NO RESISTANCE OVERCOMING ISOLATE (E.G. CPGV-M), WE ASSUME THAT THE RECOMBINANT VIRUS BE INFECTIVE AGAINST SUSCEPTIBLE LARVAE ONLY IF *pe38* FROM CPGV-M - OR AGAINST BOTH SUSCEPTIBLE AND RESISTANT LARVAE - IF *pe38* ORIGINATES FROM CPGV-S. RESULTS OF THE STRATEGY OF ELUCIDATING THE VIRAL MECHANISM OF OVERCOMING CPGV RESISTANCE PRESENTED.

Key words: RESISTANCE, BACULOVIRUS, CODLING MOTH

Insect pathogens and entomoparasitic nematodes
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Sequence analysis of CpGV-R5 isolate, able to efficiently control CpGV-M resistant insects: relation between biological activity and genome

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Abstract: THE CPGV-R5 ISOLATE IS ABLE TO OVERCOME RESISTANT POPULATIONS OF CODLING CPGV-M ISOLATE. THE COMPLETE SEQUENCES OF CPGV-R5 AND THE CPGV-M USED FOR INI PRODUCTION AT NATURAL PLANT PROTECTION HAVE BEEN DETERMINED. AMONG THE DIFFEREN SPECIFIC TO THE R5 ISOLATE, OTHERS ARE COMMON TO VARIOUS ISOLATES ABLE TO OVERCOME MODIFICATION ON THE P38 GENE PRODUCT.

Key words: GRANULOVIRUS, CPGV, RESISTANCE, SEQUENCE ANALYSIS

Introduction

THE FIRST ISOLATE OF *Cydia pomonella* GRANULOVIRUS, CPGV WAS FOUND IN MEXICO (CPGV-M (TANADA, 1964). CPGV IS USED IN BIOLOGICAL CONTROL AGAINST CODLING MOTH. IN COMMERCIAL FORMULATIONS OF CPGV ARE DERIVED FROM THE SAME CPGV-M ISOLATE (*et al.*, 2008). IN 2005, FIRSTS CASES OF RESISTANCE WERE DETECTED IN GERMANY (FRITSCH *et al.*, 2006) AND FRANCE (SAUPHANOR *et al.*, 2006). RESEARCHES CONDUCTED IN VARIOUS LABORATORIES AND CO RESULTED IN THE CHARACTERIZATION OF VARIOUS VIRUS ISOLATES THAT COULD CO RESISTANT INSECTS (BERLING *et al.*, 2009; EBERLE *et al.*, 2009; REZAPANAH *et al.*, 2008; ZINGG, 2011). AMONG THEM, THE CPGV-R5 HAS BEEN SELECTED FOR COMMERCIALIZATION BY NA PROTECTION (ARYSTA LIFESCIENCE) (NPP) (BESSE *et al.*, 2013). THE COMPLETE SEQUENCE OF CPGV-M IS KNOWN (NC_002816.1)(LUQUE *et al.*, 2001). COMPARISON BETWEEN THE SEQUENCES OF THE NEW ISOLATES CAN ALLOW THE IDENTIFICATION OF THE VIRUS GENES INVOLVED IN RES IN A FIST STEP TO UNVEIL THE MODIFICATION IN THE HOST-VIRUS RELATIONSHIPS IN CI INSECTS. THIS APPROACH HAS BEEN USED WITH VARIOUS CPGV ISOLATES (EBERLE *et al.*, 2011).

IN THIS STUDY THE SEQUENCES OF THE CPGV-M ISOLATE PRESENTLY USED BY NPP (C AND THE CPGV-R5 ISOLATE HAVE BEEN DETERMINED AND COMPARED TO THE REFERENCI CPGV-M1 (LUQUE *et al.*, 2001).

Material and methods

VIRUS AMPLIFICATION AND DNA EXTRACTION

ALL VIRUSES USED WERE PROVIDED BY NATURAL PLANT PROTECTION (ARYSTA LIFESCIEN R1 WAS PREVIOUSLY DESCRIBED BY BERLING AND COWORKERS (BERLING *et al.*, 2009). NPP-R5 WAS DERIVED FROM NPP-R1 THROUGH SELECTION BY PASSAGING ON RGV RESISTANT INSECTS IS THE STOCK USED FOR CARPOVIROUSINE™ PRODUCTION. IT COMES FROM THE ORIGINAL MEXICAN IS

(KLINGAUF, 2006). ALL VIRUSES WERE AMPLIFIED ON SV LARVAE AS PREVIOUSLY DESCRIBED (ZOUBA *et al*. 2008). BODIES WERE PURIFIED AND VIRAL DNA EXTRACTED AS DESCRIBED IN BERLING *et al* (2009).

SEQUENCING

SEQUENCING WAS CARRIED OUT BY THE GENTYANE PLATFORM OF GENOTYPING (INRA U CLERMOND-FERRAND, FRANCE) USING A SHOTGUN APPROACH. FOR CLOSING THE REMAI GAPS, A PRIMER WALKING STRATEGY WAS USED. PURIFIED PCR AMPLICONS WERE S EUROFINS MWG (EBERSBERG, GERMANY).

PCR AMPLIFICATION AND GEL PURIFICATION OF AMPLICONS

SPECIFIC PRIMERS WERE DESIGNED CLOSE TO THE BORDERS OF THE GAPS IN THE DNA SE THE SEQUENCE OF CPGV-M PREVIOUSLY PUBLISHED (SHELL *et al*., 2011) WAS A REFERENCE. PCR REACTIONS WERE CARRIED OUT USING STANDARD PROTOCOLS. THE PRESENCE OF T CONTROLLED ON A 1% AGAROSE GEL STAINED WITH ETHYDIUM BROMIDE. THE AMPL PURIFIED USING THE QIAQUICK GEL EXTRACTION KIT (QUIAGEN) FOLLOWING MANUFACTU

GENOME ASSEMBLY AND SEQUENCE ANALYSIS

THE SEQUENCES WERE ASSEMBLED AND ANALYSED USING CLONE MANAGER V9 (SCI-E WEB AVAILABLE PROGRAMS WERE USED FOR SEQUENCE COMPARISON.

Results and discussion

TABLE 1. MAIN DIFFERENCES OBSERVED BETWEEN CPGV-M1 AND CPGV-R5. Δ AND ▼ INDICATE DELETIONS AND ▼ INDICATES INSERTIONS.

CPGV-R5 ORF	CPGV-M1 ORF	NAME	POSITION IN CPGV-M1	LENGTH (NT)	LENGTH (AA)	POSITION AND TYPE OF AA DIFFERENCES
7	7	*ie-1*	3392 < 4858	1479	493	E367D, ▼VPHW463, R466I
24	24	*pe38*	18574 < 19722	1125	374	Δ312-319, K355Q, Q355K
32	32		27331 > 28671	1341	447	Δ14-21, L71M, ▼TEEEIQQNT120, V250A, V264A, M429T
46	46	*mp-nase*	36835 < 38472	1641	546	V85L, E177D, ▼G472
62	62		51047 < 51616	507	169	D38Y, Δ122-136, Δ157, D158E, Δ159-162
70	70		56506 < 57060	876	292	L20I, ▼S23, ▼D34, ▼EYQ37, V38S, D39E, ▼Y47, P48Q, S51D, ▼H52, P53Y, S54E, V55P, D57E, V59E, S60P, E61S, Y62S, ▼S64, ΔS81
87	87	*lef-5*	68491 < 69219	726	242	A87L, E88R, N89T, P90T

SEQUENCE COMPARISON BETWEEN CPGV-M1 AND CPGV-M$_{NPP}$

THE CPGV-M$_{NPP}$ SEQUENCE IS SIMILAR TO THE CPGV-M1 SEQUENCE (*ET AL.*, 2001). THIRTY ONE CHANGES AFFECTING ORFS WERE FOUND, SOME CONTRIBUTING TO FUSE TWO CO THE MOST IMPORTANT CHANGE IS LOCATED IN ORF32, WITH A SERIES OF SUBSTITUTION *polh* AND *lef8* OF CPGV-M$_{NPP}$ ARE CONSERVED (EBERL *ET AL.*, 2009). FOURTEEN OTHER DIFFERENCES ARE DETECTED IN NON-CODING REGIONS. A 16 NT INSERTION IS FOUND BETWEEN ORFS 15 VARIABILITY REGION IS LOCATED BETWEEN ORFS 50 AND 51.

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IE-1 CpGV-M1        351 KELQNLKNEYGTEADVEEFMRLSVAHPRGDVVFNMKVRDTNTQRYRINCF
IE-1 CpGV-MNPP      351 KELQNLKNEYGTEADVEEFMRLSVAHPRGDVVFNMKVRDTNTQRYRINCF
IE-1 CpGV-I01       351 KELQNLKNEYGTEADVDEFMRLSVAHPRGDVVFNMKVRDTNTQRYRINCF
IE-1 CpGV-R1.8      351 KELQNLKNEYGTEADVDEFMRLSVAHPRGDVVFNMKVRDTNTQRYRINCF
IE-1 CpGV-R5        351 KELQNLKNEYGTEADVDEFMRLSVAHPRGDVVFNMKVRDTNTQRYRINCF

IE-1 CpGV-M1        401 RMDSVHVWVNSMVYSDVQQFNLKKMIQRHRWGTHHILQFDYMYNSMMSKL
IE-1 CpGV-MNPP      401 RMDSVHVWVNSMVYSDVQQFNLKKMIQRHRWGTHHILQFDYMYNSMMSKL
IE-1 CpGV-I01       401 RMDSVHVWVNSMVYSDVQQFNLKKMIQRHRWGTHHILQFDYMYNSMMSKL
IE-1 CpGV-R1.8      401 RMDSVHVWVNSMVYSDVQQFNLKKMIQRHRWGTHHILQFDYMYNSMMSKL
IE-1 CpGV-R5        401 RMDSVHVWVNSMVYSDVQQFNLKKMIQRHRWGTHHILQFDYMYNSMMSKL

IE-1 CpGV-M1        451 HAEVSKLVIRYV----LSRRSFDLLQNDCSKLKLSYKKIVYE
IE-1 CpGV-MNPP      451 HAEVSKLVIRYV----LSRRSFDLLQNDCSKLKLSYKKIVYE
IE-1 CpGV-I01       451 HAEVSKLVIRYV----LSRRSFDLLQNDCSKLKLSYKKIVYE
IE-1 CpGV-R1.8      451 HAEVSKLVIRYV----LSRRSFDLLQNDCSKLKLSYKKIVYE
IE-1 CpGV-R5        451 HAEVSKLVIRYVVPHWLSIRSFDLLQNDCSKLKLSYKKIVYE

PE38 CpGV-M1        101 PRVQTAERNYNEFVGAIRNAAGEPMEAEQESPANEPAADYNSMMDDMINN
PE38 CpGV-NPP       101 PRVQTAERNYNEFVGAIRNAAGEPMEAEQESPANEPAADYNSMMDDMINN
PE38 CPGV-I01       101 PRVQTAERNYNEFVGAIRNAAGEPMEAEQESPANEPAADYSSMMDDMINN
PE38 CpGV-R1.8      101 PRVQTAERNYNEFVGAIRNAAGEPMEAEQESPANEPAADYNSMMDDMINN
PE38 CpGV-R5        101 PRVQTAERNYNEFVGAIRNAAGEPMEAEQESPANEPAADYNSMMDDMINN

          Perfect match between all five sequences from 150 to 300

PE38 CpGV-M1        301 TEDDITKSVANDTVDDTVDDTVDDTIMRDDSLMVANDTPSRKSYKILKRR
PE38 CpGV-MNPP      301 TEDDITKSVANDTVDDTVDDTVDDTIMRDDSLMVANDTPSRKSYKILKRR
PE38 CpGV-I01       301 TEDDITKSVAN--------DTVDDTIMRDDSLMVANDTPSRKSYKNLKKR
PE38 CpGV-R1.8      301 TEDDITKSVAN--------DTVDDTIMRDDSLMVANDTPSRKSYKILKRR
PE38 CpGV-R5        301 TEDDITKSVAN--------DTVDDTIMRDDSLMVANDTPSRKSYKILKRR

PE38 CpGV-M1        351 YLNLKQKFISHQYIVKSLTDSLRRATKKPIKY
PE38 CpGV-MNPP      351 YLNLKQKFISHQYIVKSLTDSLRRATKKPIKY
PE38 CpGV-I01       343 YLNLKQKFISHQYIVKSLTDSLRRATKKPIKY
PE38 CpGV-R1.8      343 YLNLQQKFISHKYIVKSLTDSLRRATKKPIKY
PE38 CpGV-R5        343 YLNLQQKFISHKYIVKSLTDSLRRATKKPIKY
```

FIGURE 1. PARTIAL ALIGNMENT OF THE PREDICTED AMINO ACID SEQUENCES OF VARIOUS A THE ORF7 (IE-1) AND ORF24 (PE38).

SEQUENCE COMPARISON BETWEEN CPGV-M-1 AND CPGV-R5

CPGV-R5 APPEARS TO BE DIFFERENT FROM THE 4 OTHER CLASSES OF CPGV ISOLATES FUNCTION OF THE NUCLEOTIDE VARIABILITY AT THE *polh* AND *lef-8* GENES (EBERL *ET AL.*, 2009). CPGV-R5 IS SIMILAR TO B CLASS (AS CPGV-E2) ON GRANULIN AND TO A CLASS WHEN CO *lef-8*.

THE ISOLATE CPGV-R5 HAS 124 DIFFERENCES IN RESPECT TO CPGV-M1 (LUQUE
EBERLE (2010) HAS COMPARED CPGV-M, CPGV-I12 AND CPGV-S. TWO THIRDS OF THE 66 OF
CONSERVED BETWEEN CPGV-M1 AND CPGV-S ARE ALSO CONSERVED IN CPGV-R5. ONLY C
PRESENTS DIFFERENCES, AND AMONG THESE ONLY TWO ORFS (ORF25 AND ORF26) HARB
CHANGES. THE MOST SIGNIFICANT DIFFERENCES ARE PRESENTED IN TABLE 1. THE DETAIL
IE-I SEQUENCES FOR EACH VIRUS IS PRESENTED IN FIGURE 1. PREVIOUS WORK (EBE
SUGGESTED AN ASSOCIATION BETWEEN PE38 (ORF24) VARIATION AND VIRUS ABILITY T
RESISTANCE. FIGURE 1 DETAILS THE DIFFERENCES FOUND AT THE PE38 (CPGV-M1 ORF 24
THE VARIOUS ISOLATES ANALYZED IN THIS PAPER. THE MAIN DELETION PREVIOUSLY DES
I01, IS ALSO CONSERVED IN CPGV-R1.8 AND ITS DERIVATIVE, CPGV-R5. TWO SPECIFIC AMI
CHANGES DIFFERENTIATE THESE LAST ISOLATES FROM ALL THE OTHERS, K355Q AND Q362

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Functional characterization of serine/threonine protein kinase gene (AMV197) of *AMSACTA MOOREI* entomopoxvirus

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Abstract: WE REPORT HERE THE FUNCTIONAL CHARACTERIZATION OF A SERIN/THREONINE (SE KINASE GENE (ORF AMV197) OF *Amsacta moorei* ENTOMOPOXVIRUS (AMEV). A RECOMBINANT VIRUS LACKING AMV197 (AMV-PK/*gfp*) INITIATED VIRAL DNA REPLICATION 6 HOUR EARLIER THA PARENTAL AMEV. HOWEVER, THE RECOMBINANT VIRUS YIELDED FIVE-FOLD LOWER PROGE EXPRESSED AMV197 GENE OF AMEV BY BAC-TO-BAC EXPRESSION SYSTEM YIELDED A 72 K HOMODIMERIC PROTEIN. PROTEIN KINASE SUBSTRATE PROFILING BY PEPTIDE MICROARRAY 80 OF 1248 SUBSTRATES BELONG TO 28 PROTEIN KINASE FAMILY WERE PHOSPHORYLATED BY PROTEIN. WHILE AMV197 WAS KNOWN TO PHOSPHORYLATE BOTH SERINE AND THREONINE EXPRESSED PROTEIN KINASE ALSO PHOSPHORYLATED PROBES WITH TYROSINE RESIDUES INDICATE THAT AMV197 IS AN ACTIVE PROTEIN KINASE AND PHOSPHORYLATES SEVE SUBSTRATES. HOWEVER, FURTHER EXPERIMENTS ARE NEEDED TO IDENTIFY THE EXACT ROL IN AMEV REPLICATION.

Key words: *Amsacta moorei* ENTOMOPOXVIRUS (AMEV), PROTEIN KINASE, PEPTIDE MICROARRA

Transcriptional analysis of CpGV isolates in *CYDIA MOLESTA*

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Abstract: THE ORIENTAL FRUIT MOTH *Cydia molesta* IS AN INSECT OF THE TORTRICIDAE FAMILY. ITS ORIGIN IS IN CHINA BUT TODAY IT IS A MAJOR PEST IN NEARLY ALL STONE FRUIT GROWING AR EUROPE, SOUTH AND NORTH AMERICA, THE MIDDLE EAST, NEW ZEALAND AND AUSTRALIA). PRINCIPAL HOST PLANT OF THE FRUIT MOTH IS PEACH BUT THE LARVAE FEED ALSO ON APPLE, QUIN CHERRY, NECTARINE AND PEAR. *Cydia molesta* IS ALSO A CLOSELY RELATED SPECIES TO THE CODLING MOTH *Cydia pomonella,* A MAJOR PEST IN APPLE PRODUCTION. THE CODLING MOTH IS CONTROLLED BY T *Cydia pomonella* GRANULOVIRUS (CPGV), WHICH IS OF GREAT IMPORTANCE FOR CODLING MOTH CO IN BOTH ORGANIC AND INTEGRATED POME FRUIT PRODUCTION. THOUGH *C. molesta* AND *C. pomonella* ARE CLOSELY RELATED, THE INFECTION SUCCESS OF *C. molesta* BY CONVENTIONAL CPGV, SUCH AS CPGV-M, IS NOT HIGH. RECENTLY A CPGV ISOLATE, TERMED V22 (MADEXTWIN, ANDERMA BIOCONTROL), HAD BEEN SELECTED AND SHOWED IMPROVED EFFICACY FOR *C. molesta*. FOR A BETTER UNDERSTANDING OF THE INFECTION PROCESS OF CONVENTIONAL CPGV-M AND THE IMPROVE V22 IN *C. molesta*, A COMPARATIVE TRANSCRIPTION ANALYSIS OF THESE TWO VIRUSES IN *C. mole* PERFORMED. THE TRANSCRIPTION OF SELECTED GENES (*granulin, lef-8, mcp*) OF CPGV-M WAS ANALYZED BY REVERSE TRANSCRIPTION QUANTITATIVE PCR (RT-QPCR). IT WAS FOUN TRANSCRIPTION LEVELS ARE LOW COMPARED TO THOSE OF *C. pomonella*. IN FURTHER EXPERIMENTS WILL COMPARE THE TRANSCRIPTIONAL LEVEL OF THESE GENES OF CPGV-V22 *C. pomonella* AND *C. molesta.*

Key words: CPGV, *Cydia pomonella*, *Cydia molesta*, TRANSCRIPTOME

Insect pathogens and entomoparasitic nematodes
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An examination of stress-related activation of SeMNPV in covertly infected *SPODOPTERA EXIGUA*

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Abstract: THE AIM OF THIS STUDY WAS TO EVALUATE THE EFFECT OF DIFFERENT STRESS FACT INFECTED *Spodoptera exigua* LARVAE IN TERMS OF NUCLEOPOLYHEDROVIRUS (NPV) ACTIVATION. FOR SURVIVORS THAT HAD INGESTED OCCLUSION BODIES OF *S. exigu*a NUCLEOPOLYHEDROVIRUS (SEMNPV) WERE MATED AND THE SUBSEQUENT GENERATION (F1) INFECTION BY VIRUS ACTIVATION IN THE SECOND INSTAR L LABORATORY AND FIELD CONDITIONS. IN THE LABORATORY, A NUMBER OF TREATMENTS WE CHEMICAL STRESSORS, INOCULATION WITH HETEROLOGOUS *Bacillus thuringiensis* SPORES AND CRYSTALS. BOTH, PARENTAL AND F1 ADULTS WERE CONFIRMED TO HARBOR THE INFECTION BY QPCR. VIRUS A WAS OBSERVED IN INSECTS TREATED WITH 0.1% COPPER SULPHATE, 1% IRON SULPHATE, AND 1 SELENITE, RESULING IN 12%, 15%, AND 41% MORTALITY DUE TO SEMNPV, RESPECTIVELY, WHERE WITH SYMPTOMS OF VIRAL INFECTION WERE REGISTERED IN VIRUS-FREE CONTROLS. NO EFFEC MORTALITY WAS DETECTED AFTER INOCULATION WITH HETEROLOGOUS VIRUS. FIELD TRIALS ARTIFICIAL INFESTATION OF PEPPER CROPS IN EXPERIMENTAL GREENHOUSES USING *S. exigua* LARVAE TO EVALUATE COPPER SULFATE AND SODIUM SELENITE AS ACTIVATION FACTORS. VER MORTALITY (< 5%) WAS OBSERVED IN THOSE LARVAE TREATED IN FIELD CONDITIONS.

Key words: NPVS REACTIVATION, STRESS FACTORS, *Spodoptera exigua* MULTIPLE NUCLEOPOLYHEDRO

Introduction

RECENTLY STUDIES ON BACULOVIRUS TRANSMISSION REPORTED A HIGH PREVALENCE OF INFECTIONS IN LEPIDOPTERAN POPULATIONS SUCH AS *Spodoptera exigua* (CABODEVILA *et al.*, 2011A). SPONTANEOUS NUCLEOPOLYHEDROVIRUS (NPV) OUTBREAKS MIGHT EXPLAIN THE INITIA EPIZOOTICS IN HOST POPULATIONS. HOWEVER, VERY LITTLE IS KNOWN ABOUT THE MEC TRIGGERS COVERT INFECTIONS TO BECOME PATENT FATAL INFECTIONS. VIRUS ACTIVAT TO STRESS CONDITIONS FOR LARVAE THAT EXPERIENCE HIGH DENSITIES DURING REARING EXTREME TEMPERATURES OR CERTAIN CHEMICAL TREATMENTS (CABODEVILA *et al.*, 2011B; 1998; KUKAN, 1999). INVESTIGATING THE FACTORS INVOLVED IN VIRUS REACTIVATION MAY CONTRIBUTE TO THE DEVELOPMENT O BIOLOGICAL CONTROL USING NPV-BASED BIOPESTICIDES. THE AIM OF THIS STUDY WAS T EFFECT OF DIFFERENT TYPES OF TREATMENTS AS ACTIVATION FACTORS IN COVERTLY *S. exigua* LARVAE, IN BOTH LABORATORY AND FIELD CONDITIONS.

Material and methods

INSECT AND VIRUS

A VIRUS-FREE COLONY OF *Spodoptera exigua* MAINTAINED IN THE INSECTARY OF UNIVERSIDAD PÚBLIC
NAVARRA WAS USED FOR THIS EXPERIMENT. THE VT-SEAL1 STRAIN OF SEMNPV WAS
INOCULATE *S. exigua* LARVAE (CABODEVILLA *et al*., 2011A).

COVERT INFECTION INDUCTION AND QPCR VIRUS DETECTION

COVERT INFECTIONS WERE ESTABLISHED IN *S. exigua* VIRUS-FREE CULTURES ACCORDING TO THE MET
DESCRIBED BY CABODEVILLA *et al*. (2011B). BRIEFLY, FOURTH INSTAR (L_4) VIRUS-FREE LARVAE WERE
SUBLETHALLY INFECTED WITH OCCLUSION BODIES (OBS) OF THE VERTICALLY TRANSMI
SEAL1. A GROUP OF LARVAE WERE TREATED SIMILARLY EXCEPT THAT THE INOCULUM DID
THIS LINEAGE WAS USED AS CONTROL. ADULT SURVIVORS TO THE VIRUS CHALLENGE W
SUBSEQUENT GENERATIONS (F_1) TESTED FOR VIRUS ACTIVATION IN THE SECOND INSTAR (L_2) BY
COVERTLY INFECTED LARVAE WITH CHEMICALS OR ENTOMOPATHOGENS. GROUPS OF 24 LA
BY DROPLET-FEEDING WITH ONE OF THE FOLLOWING GROUPS OF TREATMENT: I) CHE
COPPER SULFATE (1%-0. 1%), 1% IRON SULFATE, HYDROXYLAMINE (1-0.1%), 2% TINOPAL
SODIUM SELENATE, OR 1 PPM PARAQUAT DICHLORIDE; II) CHALLENGED WITH: *Chrysodeixis chalcites*
NPV (NON-PERMISSIVE), *Mamestra brassicae* NPV (PERMISSIVE), SEMNPV-USED, *Bacillus*
thuringiensis SPORES, *Bt* CRYSTAL, MIXED *Bt* SPORES & CRYSTALS (1:1); AND III) REARING TEMPERATU
OF 18 °C AND 28 ºC. NPV MORTALITY WAS REGISTERED BY CHECKING CADAVERS FOR TH
OBS USING A PHASE-CONTRAST MICROSCOPE. TO CONFIRM TRANSGENERATIONAL T
INFECTION A GROUP OF L_1 LARVAE WERE REARED TO ADULTS AND TESTED FOR AMPLIFICATIO
SPECIFIC GENE *DNA polymerase* BY QPCR USING SYBR BASED METHOD (CABODEVILLA *et al*.,
2011B).

FIELD TRIALS

TREATMENTS THAT HAD PROVED TO BE EFFECTIVE ACTIVATION FACTORS IN THE LABO
WERE TESTED IN FIELD CONDITIONS. THREE EXPERIMENTAL GREENHOUSES OF 100 M^2 AREA USED OF
INSTALLATIONS OF IFAPA (INSTITUTO DE INVESTIGACIÓN Y FORMACIÓN AGRARIA Y PESQU
SPAIN) WERE PLANTED WITH PEPPER CROPS USING A PLANTATION FRAME 0.5 × 1 M. EACH
WAS SPLIT INTO FOUR PLOTS IN WHICH ONE OF THE FOLLOWING FOUR TREATMENTS WA
COPPER SULPHATE, II) 1 PPM SODIUM SELENATE, III) BT-BASED INSECTICIDE (FLORBAC, BA
WATER CONTROL. THE OFFSPRING OF SUBLETHAL INFECTED ADULTS (100% POSITIVE FOR
FOR ARTIFICIAL INFESTATIONS. EGG MASSES WERE PLACED ON THE THREE CENTRAL PLA
RATE OF 200 EGGS PER PLANT. ONCE MOST OF THE LARVAE REACHED SECOND INSTARS,
APPLIED TO PLANTS USING A HAND-HELD SPRAYER. AFTER 48 H POST TREATMENT A TOTA
PLOT WERE COLLECTED FROM THE THREE CENTRAL PLANTS AND CONFINED INDIVIDUA
CUPS PROVIDED WITH DIET AND REARED IN THE LABORATORY UNTIL DEATH OR PUPATION

Results and discussion

REACTIVATION OF SEMNPV BY STRESSOR FACTORS IN LABORATORY CONDITIONS

ALL OF THE F_1 TESTED ADULTS (N = 27) WERE CONFIRMED TO HARBOR THE VIRUS BY QPCR, SU
HIGH PREVALENCE OF PERSISTENT INFECTION IN LARVAE SUBJECTED TO ACTIVATOR
MORTALITY WAS OBSERVED IN 0.1% COPPER SULFATE, 1% IRON SULFATE, AND 1-PPM SO
TREATMENTS THAT RESULTED IN 12%, 15%, AND 41% VIRUS MORTALITY, RESPECTIVEL
LARVAE WITH SYMPTOMS OF VIRAL INFECTION WERE REGISTERED IN VIRUS-FREE CONT

REMAINING CHEMICAL TREATMENTS CASED VIRUS ACTIVATION. REPORTED SIMILAR RESULTS ON THE ACTIVATION OF OCCULT VIRUS BY FEEDING *Lymantria dispar* LARVAE ON DIET CONTAINING 0.6% COPPER SULFATE. COPPER IRON AND SELENIUM ARE ESSENTIAL MICROELEMENTS RE FUNCTIONS AND THE IMMUNE SYSTEM (CHATURVEDI *et al*., 2004). THEY HAVE BEEN DESCRIBED AS INVOLVED IN INMUNOMODULATION THAT INFLUENCE THE COURSE OF THE OUTCOME OF INFECTIONS. THEREFORE THE CHEMICALS USED HERE THAT RESULTED IN ACTIVATION O BE ACTING AS PHYSIOLOGICAL STRESSORS.

NO EFFECT OF ENTOMOPATHOGEN INOCULATION WAS OBSERVED IN SUBLETALLY SINCE ONLY THOSE VIRUSES WHICH A PERMISSIVE SPECIES (MBMNPV AND SEMNPV) RESULTED IN 64% AND 17% OF NPV MORTALITY, RESPECTIVELY. HOWEVER, NUMEROUS REPORTED THE TRIGGERING EFFECT OF HETEROLOGOUS VIRUSES IN THE ACTIVATIO INFECTIONS, INCLUDING *S. exigua* (MURILLO *et al*., 2011).

REACTIVATION OF SEMNPV BY CHEMICAL STRESSORS IN FIELD CONDITIONS

FOURTH INSTAR LARVAE COLLECTED FROM PLANTS TREATED WITH COPPER SULPHATE SHOWED VERY LOW LEVELS OF NPV-INDUCED MORTALITY; 1.4-2.4%, RESPECTIVELY. PESTICIDE WAS INCLUDED AS A CONTROL SINCE THIS PESTICIDES USED IS HIGHLY EXTEN CONTROL CROP SYSTEMS IN ALMERIA. THIS PATHOGEN RESULTED IN 2.8% NPV M GREENHOUSE TREATED LARVAE. NO VIRUS MORTALITY WAS OBSERVED IN LARVAE FROM

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Insect pathogens and entomoparasitic nematodes
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Functional analysis of *CHILO* iridescent virus zinc-binding matrix metalloproteinase gene

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Abstract: BASEMENT MEMBRANES THAT SURROUND THE TISSUES OF LEPIDOPTEROUS LAR PHYSICAL BARRIER TO THE MOVEMENT OF VIRUSES. THEREFORE, ONE OF THE POTENTIAL USING THE ENZYMES THAT DISRUPT THE BASEMENT MEMBRANE PROTEINS IN BIOLOGICAL AGRICULTURAL PEST INSECTS. MATRIX METALLOPROTEINASES ARE ZINC-DEPENDENT ENDO HAVE THE COMBINED CAPACITY TO DEGRADE ALL THE COMPONENTS OF THE *Chilo* EXTRACELLULAR IRIDESCENT VIRUS (CIV) GENOME ENCODES A 264 AMINO ACID PROTEIN (ORF 165R) CON ZINC-DEPENDENT MATRIX METALLOPROTEINASE (MMP) DOMAIN WITH OVER 40% AMINO AC IDENTITY TO A LARGE GROUP OF ORGANISMS INCLUDING PRIMARILY INSECTS OF THE *Drosophila* SPECIES. CIV-MMP HOMOLOG WAS CLONED AND A RECOMBINANT ACMNPV BACMID THAT EXPRESS MMP UNDER THE *Autographa californica* MULTIPLE NUCLEOPOLYHEDROVIRUS POLYHEDRIN PROMOTE WAS CONSTRUCTED. RECOMBINANT BACMID WAS PRODUCED AND TRANSFERRED TO SF-9 C LEVEL EXPRESSION OF RECOMBINANT PROTEIN. EXPRESSED PROTEIN WAS PURIFIED FROM S 96 HOUR POST INFECTION. WESTERN BLOT ANALYSIS OF THE PROTEIN RESULTED IN A 34 KDA P CIV-MMP PROTEIN DIGESTED DYE-IMPREGNATED COLLAGEN (AZOCOLL). THE ENZYMATIC ACTI INHIBITED BY METALLOPROTEINASE INHIBITOR EDTA. THESE RESULTS SUGGEST THAT THE CIV-M HOMOLOG ENCODES A FUNCTIONAL METALLOPROTEINASE WHICH CAN BE UTILIZED IN BIOLOGIC LEPIDOPTERON PESTS.

Key words: *Chilo* IRIDESCENT VIRUS, CIV, METALLOPROTEINASE, BACMID, GENE EXPRESSION

Soil pests

Latest field results on the biological control of *DIABROTICA VIRGIFERA VIRGIFERA* with nematodes

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Abstract: THE ENTOMOPATHOGENIC NEMATODE *Heterorhabditis bacteriophora* HAS BEEN TESTED SUCCESSFULLY AGAINST LARVAE OF THE WESTERN CORN ROOTWORM (*Diabrotica virgifera virgifera*) FOR THE LAST 5 YEARS IN HUNGARY, AUSTRIA AND ITALY. WHEN APPLIED AT DOSES OF 1.5 X 10^9 NEMATODES HA^{-1} THE RESULTS HAVE BEEN COMPARABLE TO THOSE OBTAINED WITH CHEMICAL SEED NEONICOTINOIDS OR APPLICATION OF GRANULAR INSECTICIDES CONTAINING THE PYRETHROI AT HIGHER DOSE OF 2 X 10^9 NEMATODES HA^{-1} THE RESULTS WERE MORE STABLE AT CONTROL BETWE AND 90%. ALTHOUGH THE DIFFERENCES ARE REMOTE, IN COMPARISON TO CHEMICAL INSEC NEMATODES USUALLY PROVIDED HIGHER REDUCTION OF ADULTS WHEREAS LESS ROOT RECORDED FOR CHEMICAL INSECTICIDES. THE EFFECT OF NEMATODES IS EQUALLY HIGH WH DURING SAWING OF THE MAIZE OR AT OCCURRENCE OF THE LARVAE APPROXIMATELY 6 W DIFFERENT APPLICATION TECHNIQUES HAVE BEEN TRIED. SEED DRESSING AND GRANULAR A CAUSED PROBLEMS UNDER COMMERCIAL CONDITIONS. LIQUID APPLICATIONS INTO THE DRIL LITRE WATER HAVE PROVIDED OPTIMAL CONDITIONS FOR NEMATODE ESTABLISHMENT AND P THE OCCURRENCE OF THE LARVAE. ARTICLE 55 OF THE NEW EU REGULATION (EC) NO 1107/20 PLACEMENT OF PLANT PROTECTION PRODUCTS ON THE MARKET EXPLICITLY IMPLIES THE PR USE OF NON-CHEMICAL AND NATURAL ALTERNATIVES. DIRECTIVE 2009/128/EC (SUD) AIMS T THE SUSTAINABLE USE OF PESTICIDES. ARTICLE 14 LINES OUT THAT "THE MEMBER STATE NECESSARY MEASURES TO PROMOTE LOW PESTICIDE-INPUT PEST MANAGEMENT, GIVING POSSIBLE PRIORITY TO NON-CHEMICAL METHODS, SO THAT PROFESSIONAL USERS OF PESTICI PRACTICES AND PRODUCTS WITH THE LOWEST RISK TO HUMAN HEALTH AND THE ENVIRONM CONTROL INDUSTRY IS PREPARING TO SUPPLY THE MARKETS WITH THE NECESSARY AM ENTOMOPATHOGENIC NEMATODE *H. bacteriophora*. IN 2011, THE FIRST PRODUCT (DIABACT®) BASED ON THIS NEMATODE WAS INTRODUCED. ALTHOUGH EU MEMBER STATES SHOULD GIVE PRIORI CHEMICAL MANAGEMENT OF *Diabrotica* IN ACCORDANCE WITH THE SUD, MEMBER STATES PROVID EMERGENCY AUTHORISATIONS (ARTICLE 53, REGULATION (EC) NO 1107/2009) FOR CHE INSECTICIDES TO CONTROL THE PEST. ARTICLE 53 ALLOWS USE ONLY "WHERE SUCH A ME NECESSARY BECAUSE OF A DANGER WHICH CANNOT BE CONTAINED BY ANY OTHER REASONA CONCLUSION, WE SUGGEST IMPLEMENTING AND EXECUTING OF EU REGULATIONS/DIRE ACCORDANCE WITH CONSUMER DEMANDS. CONSISTENT ENFORCEMENT OF EUROPEAN LEGIS LEAD TO PREFERENCE FOR NON-CHEMICAL CONTROL, PREVENT THE USE OF PROBLEMATIC PROMOTE LOW PESTICIDE-INPUT MANAGEMENT THUS CONTRIBUTING TO IMPLEMENTATIO MAIZE PRODUCTION.

Key words: WESTERN CORN ROOTWORM, *Heterorhabditis bacteriophora*, APPLICATION TECHNIQUE, REGULATION

Insect pathogens and entomoparasitic nematodes
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Development of new formulations for soil pest control

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Abstract: IN FORMULATION SCIENCE, THERE ARE FEW SYSTEMATIC INVESTIGATIONS ON ENCAPS
BIOLOGICALS WITH REGARD TO MATERIALS, METHODS AND TECHNOLOGY FOR MASS PRODUCTI
WORK WAS TO DEVELOP NOVEL MECHANICALLY STABLE CAPSULE SYSTEMS WITH INCREASED PE
THIS END, WE TESTED DIFFERENT METHODS WITH SEVERAL BIOPOLYMERS, COMBINATIONS OF
LIGNIN AS CAPSULE ADDITIVE. CAPSULE SYSTEMS WERE PREPARED BY IONIC GELATION, THERMAL
COACERVATION AND ADDITIONAL BEAD COATING. IN SELECTED CAPSULES, LIGNIN WAS INCO
BASED ON SINGLE BIOPOLYMERS WERE ABLE TO FORM STABLE SPHERICAL CAPSULES, E.G. ALGINA
AND GELATIN. CAPSULES BASED ON COMBINATIONS OF POLYMERS ALSO SHOWED STABLE CAPSU
ALGINATE/GELATIN, ALGINATE/LIGNIN AND SEC/PDADMAC. ADDITIONALLY, LIGNIN WAS USED S
ADDITIVE IN SEC HOLLOW BEADS. FIRST EXPERIMENTS INDICATE SIGNIFICANT DIFFERENCES
DEGRADABILITY AND THUS PERSISTENCE IN SOIL WITH DIFFERENT CAPSULE SYSTEMS. THESE NOV
WITH INCREASED PERSISTENCE ARE SUITABLE FOR DELIVERY OF BCAS INTO THE SOIL.

Key words: FORMULATION, ENCAPSULATION, IMMOBILIZATION, ENTOMOPATHOGENIC FUNGI, E
BEADS, BIOLOGICAL CONTROL, AGGREGATION, *Metarhizium anisopliae*, BIO-INSECTICIDE

Introduction

THE EU-FUNDED PROJECT INBIOSOIL WILL EXPLORE IN DETAIL THE RECENTLY DISCOVERE
EFFECTS BETWEEN ENTOMOPATHOGENIC FUNGI (EPFS), ENTOMOPATHOGENIC NEMATODE
SEMIOCHEMICALS BY DEVELOPING INNOVATIVE CO-FORMULATIONS, MAKING USE OF ST
FROM NATURE. THE FORMULATIONS TO BE DEVELOPED FIRST AIM AT OVERCOMING THE
IN THE APPLICATION OF BIOCONTROL AGENTS (BCAS) LIKE EPF, E.G. HANDLING, LOW SHE
ESTABLISHMENT IN SOIL. FORMULATION METHODS SUCH AS ENCAPSULATION OFFER A
PROBLEMS.

FOR THE ENCAPSULATION OF BCAS ONLY CONVENTIONAL ALGINATE BEADS WE
ACCORDING TO STANDARD OR UNECONOMIC METHODS. TO DATE NOBODY INVESTIGATED
ALL 20 DIFFERENT AVAILABLE ALGINATES ON EFFICACY. BESIDES, CAPSULES BASED ON O
HOLLOW BEADS AND COATED CAPSULES (VEMMER & PATEL, UNPUBLISHED) HAVE NO
SUCCESSFULLY SO FAR. ALSO, THE NOVEL FORMULATION TREND OF MIXING POLYMER
PHYSICOCHEMICAL AND BIOCHEMICAL PROPERTIES INTO ONE FORMULATION WI
CHARACTERISTICS SUCH AS IMPROVED RE-SWELLING AT HIGH MECHANICAL STRENGTH W

EPF SUCH AS *Metarhizium anisopliae* OR *Beauveria bassiana* HAVE BEEN FORMULATED BY
ENCAPSULATION IN CONVENTIONAL ALGINATE BEADS SUPPLEMENTED WITH NUTRIENTS
PEREIRA & ROBERTS, 1991; MOORE & CAUDWELL, 1997; GERDING ET AL., 2007; JACZ
et al., 2010). BUT
ESTABLISHMENT IN SOIL IS STILL SLOW AND BIOMASS CONTENT TO HIGH, MAKING T
UNECONOMIC. THAT IS WHY WE AIM AT DEVELOPING NOVEL CAPSULES CONTAINING EPF
OF THE EU-FUNDED PROJECT INBIOSOIL. THE AIM OF THIS WORK WAS TO DEVELO
MECHANICALLY STABLE CAPSULE SYSTEMS WITH INCREASED PERSISTENCE IN SOIL. TO TH
SEVERAL BIOPOLYMERS, COMBINATIONS OF BIOPOLYMERS AND LIGNIN AS CAPSULE ADDI

Material and methods

IONIC GELATION

BEADS WERE FORMED BY DRIPPING POLYMER SOLUTIONS WITH A CONCENTRATION OF 2% LINKING SOLUTION CONTAINING 1.25% Ca^{2+} IONS. DIFFERING POLYMER CONCENTRATIONS WERE USED GELATIN BLOOM 280 IN COMBINATION WITH ALGINATE AND WITH XANTHAN. HERE, CON 2%, 5% AND 0.5% WERE USED, RESPECTIVELY.

THERMAL GELATION

BEADS WERE FORMED BY DRIPPING A WARM BIOPOLYMER SOLUTION INTO ICE-COLD $CaCl_2$ SOLUTION. CONCENTRATION OF GELATIN BLOOM 280 WAS 20% AND OF GELATIN BLOOM 280 IN COMBIN ALGINATE WERE THE SAME AS USED ABOVE. GUAR GUM AND GELLAN GUM WERE USED A OF 1%.

COMPLEX COACERVATION

FOR THE PRODUCTION OF HOLLOW BEADS A SOLUTION OF A POLYELECTROLYTE WAS DRIP OF ANOTHER POLYELECTROLYTE WITH COUNTER CHARGES.

BEAD COATING

COATED CA-ALGINATE BEADS WITH A SECOND LAYER OF ALGINATE WERE PRODUCED CA-ALGINATE BEADS INTO A 0.8% ALGINATE SOLUTION.

Results and discussion

POLYMER SCREENING

CAPSULE FORMATION BY IONIC GELATION WAS EVALUATED FOR 14 SCREENED BIOPO POLYMER COMBINATIONS. THERMAL GELATION WAS EVALUATED FOR 3 BIOPOLYMERS A COMBINATION. POLYMERS SHOWING INSTABLE OR NO CAPSULE FORMATION WITH TH FURTHER EVALUATED USING COMPLEX COACERVATION.

EIGHT OF THE SCREENED BIOPOLYMERS WERE ABLE TO FORM STABLE SPHERICAL CAP IONIC OR THERMAL GELATION (FIGURE 1 A). ALL THREE BIOPOLYMER COMBINATIONS WI FORM STABLE SPHERICAL CAPSULES, DEMONSTRATING THAT THESE GELATION METHODS PRODUCE CAPSULES BASED ON TWO DIFFERENT POLYMERS (FIGURE 1 B).

DIFFERENCES IN GELATION PROPERTIES AND FUNCTIONAL GROUPS OF THE BIO RESPONSIBLE FOR THE FORMATION OF DIFFERENT ASSEMBLY LEVELS. THE FUNCTIONAL G BIOPOLYMERS ARE TYPICAL FOR EACH BIOPOLYMER. IN SHORT, ALGINATE HAS CARBOXYL NEGATIVE CHARGE, SO IT IS ABLE TO FORM CAPSULES BY USING IONIC GELATION. CHITO HAND HAS AMINE GROUPS WHICH ARE POSITIVELY CHARGED, SO THIS POLYMER IS NOT CAPSULES WITH ANY COUNTER ION, BUT WITH THE COMPLEX COACERVATION METHOD. C VIA COMPLEX COACERVATION CLEARLY SHOWED TWO BIOPOLYMER COMBINATIONS WH FORM STABLE AND SPHERICAL HOLLOW BEADS BASED ON TWO POLYMERS WITH DIFFERE CHARACTERISTICS (FIGURE 2).

ALL BIOPOLYMERS HAVE CHARACTERISTICS BEYOND THEIR CAPSULE FORMING P EXAMPLE, PECTIN DERIVATES MAY SERVE AS CAPSULE MATRIX BUT AT THE SAME TIME AS AND MAY ADDITIONALLY IMPROVE RESWELLING OF DRIED CAPSULES (DATA WILL BE SHOW

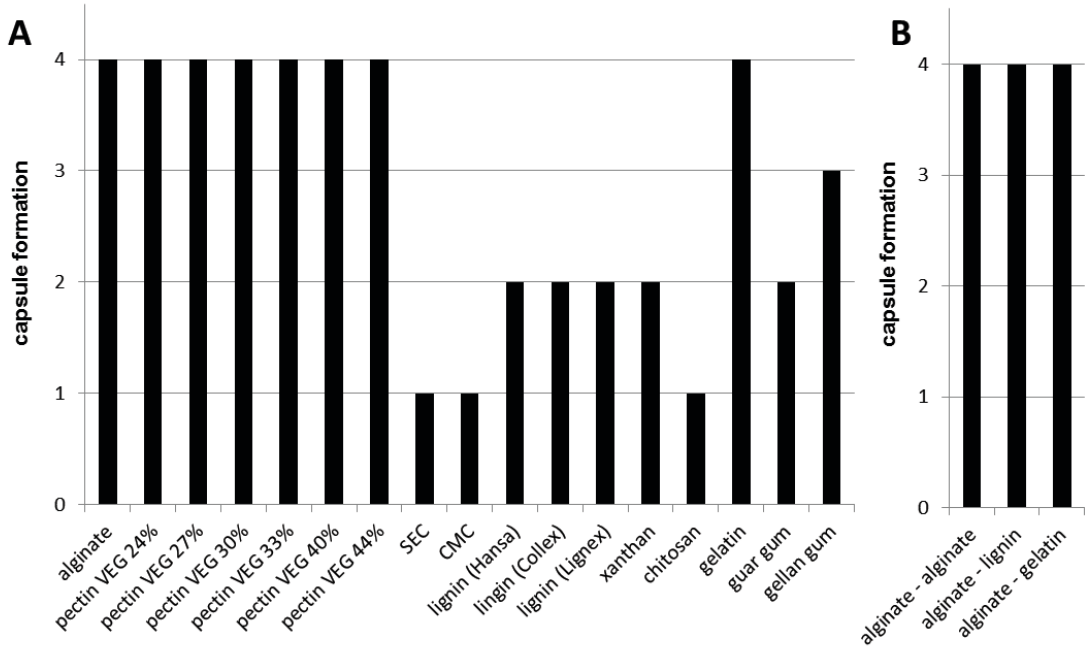

Figure 1. Evaluation of capsule formation using ionic and thermal gelation. (A) single biopolymers, (B) polymer combinations. Legend for capsule formation: 1 = no capsule formation, 2 = instable capsule formation, 3 = no spherical capsule, 4 = spherical capsule formation. VEG = degree of esterification.

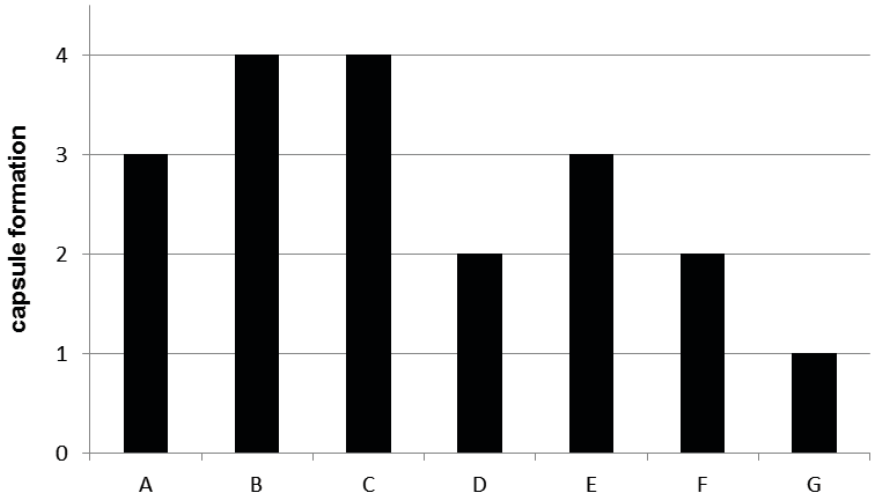

Figure 2: Evaluation of capsule formation using complex coacervation. A: 2% SEC/2% PDADMAC, B: 3% SEC/5% PDADMAC, C: 2% SEC/2% PDADMAC + lignin, D: 2% chitosan/2% SEC, E: 1% chitosan/5% lignin, F: 2% PDADMAC/2% SEC, G: 2% CMC/2% PDADMAC. Samples were prepared by dripping a solution of the first polymer into a solution of the second one. Legend for capsule formation: 1 = no capsule formation, 2 = instable capsule formation, 3 = no spherical capsule, 4 = spherical capsule formation.

CAPSULE SYSTEMS WITH PR LIKE GOOD MECHANICAL STA BILITY AND PERTI BE ACHIEVED BY COMBINING DIFFERENT BIOPOLYMERS LIKE ALGINATE A FURTHERMO E, CHITOSAN WITH ANTIMICROBIAL AND LIGNIN WITH POOR BIODEGR FOR AN EFFECTIVE CAPSULE SYSTEM TO INCREASE THE PERSISTENCE OF EN FIRST EXPERIMENTS IN SOIL SHOWED SIGNIFICANT DIFFERENCES IN BIODEGRADABILITY AND PERSISTENCE IN SOIL WITH DIFFERENT CAPSULE SYSTEMS

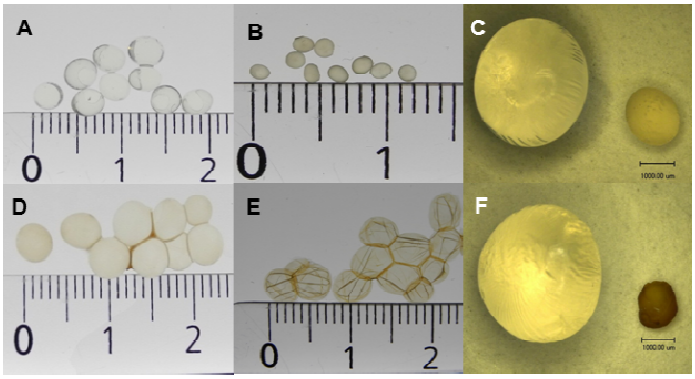

FIGURE 3: INFLUENCE OF DRYING ON HYDROGEL CAPSULE. A: ALGINATE-GELATIN CAPSULE (HOLLOW BEAD), B: DRIED CA-ALGINATE-GELATIN CAPSULE, C: MOIST ALGINATE-GELATIN CAPSULE, D: MOIST SEC-LIGNIN CAPSULE (HOLLOW BEAD), E: DRIED SEC-LIGNIN CAPSULE, F: MOIST DRIED CA-ALGINATE-GELATIN CAPSULE. D & E: PICTURES TAKEN WITH SCOPE, MAGNIFICATION 30X.

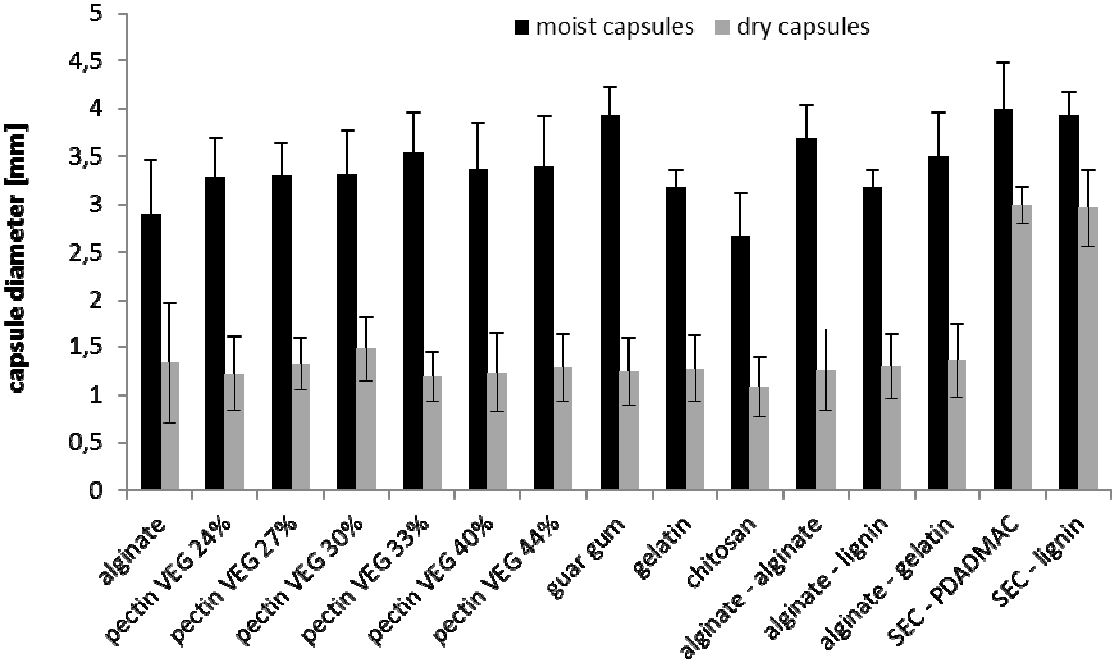

FIGURE 4: INFLUENCE OF DRYING ON CAPSULE SIZE. CAPSULE H AT ROOM TEMPE

DRYING OF HYDROGEL CAPSULES RESULTS IN A REDUCTION OF SIZE CORRELATED TO
DEPENDING ON THE USED BIOPOLYMERS THE SHRINKING OF THE CAPSULES DIFFERS IN F
(FIGURE 3). FOR EACH OF THE DIFFERENT CAPSULE SYSTEMS SCREENED IN FIGURE 1 TH
MOIST AND DRY CAPSULES (FULL BEADS) WAS MEASURED. 13 OF 15 CAPSULES LOST ABO
VOLUME AFTER DRYING, WITH THE EXCEPTION OF THE HOLLOW BEADS CONTAINING SEC.
THIN FILMS RETAINING 75% OF THEIR DIAMETER (FIGURE 4). HERE, FILLERS WILL BE INVES

TO CONCLUDE, THESE NOVEL CAPSULE SYSTEMS WITH INCREASED PERSISTENCE A
DELIVERY OF FUNGAL BCAS INTO THE SOIL. IN FURTHER EXPERIMENTS PROMISING F
ADDITIVES WILL BE ANALYSED BY MEASURING CHARACTERISTIC PROPERTIES LIKE PAR
SIZE DISTRIBUTION, DIFFUSION PROPERTIES, MECHANICAL STABILITY, RESWELLING,
BIOLOGICAL DEGRADABILITY AND TOXICITY. ADDITIONALLY, WE WILL DEVELOP ENCAPSU
REDUCED BIOMASS CONTENT, HIGH SURVIVAL, LONG SHELF-LIFE, SLOW OR CONTROLLED
A “DEPOT” RESULTING IN INCREASED ESTABLISHMENT IN SOIL, LONGER PERSISTENC
NUMBER OF APPLICATIONS. THE FORMULATED BIOMASS WILL BE CHARACTERIZED BY V
DATA WILL BE SHOWN. FURTHERMORE, WITHIN THE INBIOSOIL PROJECT THE FORMULATE
TESTED FOR EFFICACY AGAINST TARGET INSECTS SUCH AS WIREWORMS, WESTERN CORN
AND BLACK WINE WEEVIL LARVAE. ADDITIONALLY THE INFLUENCE OF THE FORMULATED
BENEFICIAL INSECTS WILL BE EVALUATED.

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Click beetles disperse widely across farmland: what else do we need to know?

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Abstract: FOR MANY YEARS WIREWORM RESEARCH FOCUSSED ON THE DISTRIBUTION OF LARVAE IN FIELD CROPS. THE DEVELOPMENT OF SEX PHEROMONE LURES HAS FACILITATED THE STUD DISTRIBUTIONS OVER GREATER SPATIAL SCALES BUT THE ASSUMPTION THAT THESE WOULD MONITORING TECHNIQUE HAS NOT BEEN FULFILLED AND IT IS NOW CLEAR THAT WE CANNOT BE C THE TRAP COUNTS ACTUALLY MEAN. THIS SECONDARY FOCUS ON ADULT MALES NEGLECTS THE C BEHAVIOURS IN AGRICULTURAL LANDSCAPES. UNDERSTANDING THESE IS ESSENTIAL TO THE I MANAGEMENT STRATEGIES IN AN ERA OF DECLINING INSECTICIDE AVAILABILITY. IN THIS PAPE WHAT IS KNOWN ABOUT THE MOVEMENT OF CLICK BEETLES ACROSS FARMLAND AND IDENTIFY NEED TO BE FILLED IF WE ARE TO DEVELOP AREA-WIDE MANAGEMENT STRATEGIES.

Key words: ELATERIDAE, *Agriotes*, CLICK BEETLES, WIREWORMS, DISPERSAL, SPATIAL DISTRIBUTION, A MANAGEMENT

Introduction

THE HISTORY OF WIREWORM PEST RESEARCH SHOWS THAT THE FOCUS HAS BEEN ON CONT IN A CROP. HOWEVER, RESEARCH INTO WIREWORM CONTROL HAS BEEN RESTRICTED BY T OF LARVAL IDENTIFICATION AND THEIR CRYPTIC SOIL HABITAT. RECENTLY, PROGRESS H ROUTINE IDENTIFICATION OF LARVAE THROUGH THE USE OF MOLECULAR METHODS (*et al.*, 2008; STAUDACHER *et al.*, 2011). THIS HAS ENCOURAGED CONSIDERATION TO BE GIVEN TO THE EC INDIVIDUAL SPECIES WITHIN REGIONAL PEST COMPLEXES. THE DEVELOPMENT OF SPECI PHEROMONES FOR A RANGE OF CLICK BEETLES (*et al.*, 2003; AND SUBSEQUENT PAPERS) OPENED UP THE POSSIBILITY THAT A SIMPLER METHOD THAN MONITORING WIREWORMS WAS AVA OVERCOME THE PROBLEMS OF DIRECTLY ESTIMATING WIREWORM NUMBERS IN THE SO APPROACH WAS CHALLENGED BY (2008) ON THREE GROUNDS. FIRSTLY, FOR ANY ADU COHORT TO REFLECT THAT OF THE WIREWORM POPULATION EACH ANNUAL COHORT SHO EQUAL IN SIZE. THE SECOND ASSUMPTION IS THAT THE DISTRIBUTION OF ADULT SEX P CATCHES SHOULD BE RELATED TO THAT OF THE WIREWORMS THEMSELVES. FINALLY, UN RESPONSES IN RELATION TO THE TRAPPING SYSTEM ARE SIMILAR FOR THE DIFFERENT SP SPECIFIC RELATIONSHIPS WOULD NEED TO BE DEVELOPED TO GUARANTEE ROBUST PRE THESE THREE ASSUMPTIONS SURVIVED THE COMPARISON WITH (2012) AND BLACKSHAW SUBSEQUENT STUDY REVEALED SUBSTANTIAL INTERSPECIFIC DIFFERENCES IN SEX PHE RATES OF *Agriotes lineatus*, *A. obscurus* AND *A. sputator* (HICKS & BLACKSHAW, 2009) WHICH IMPLIED POTENTIAL DIFFERENCES IN WALKING BEHAVIOURS FOR THESE THREE SPECIES.

SINCE WIREWORM MOVEMENT THROUGH SOIL IS LIMITED (SCHALLER *et al.*, 2010) MIGRATION OF NEW AREAS, AND ADDITION OF A NEW GENERATION TO AN EXISTING POPULATION, DISPERSAL BY THE ADULT CLICK BEETLES. THUS DIFFERENCES IN WALKING BEHAVIOU IMPORTANT TO THE SPREAD ACROSS AND DISTRIBUTION IN AGRICULTURAL LANDSCAP

SUMMARISE THE EVIDENCE FOR DISPERSAL AND THEN ADDRESS THE ISSUE OF WHAT WE
ORDER TO CONTEMPLATE AN AREA-WIDE MANAGEMENT STRATEGY FOR WIREWORMS.

Click beetle dispersal

THERE HAS ONLY BEEN ONE PUBLISHED STUDY TO DATE THAT DIRECTLY INVESTIGATED H
BEETLES CAN BE FOUND FROM THE SITE OF LARVAL FEEDING. SCHALLER *et al.* (2009) CAPTURED AN ADULT
MALE *A. obscurus* IN A SEX PHEROMONE TRAP AT LEAST 80 M FROM WHERE IT HAD TO HAV
LARVA. THIS DISTANCE WAS SIMILAR TO THE MAXIMUM ESTIMATED FOR *A. obscurus* (151 M) AND
A. lineatus (82 M) OVER 45 DAYS IN A MARK-RELEASE-RECAPTURE (MRR) STUDY (HIC
BLACKSHAW, 2008). THUS, IT APPEARED THAT ADULT MALE DISPERSAL WAS SOMEWHAT L

THIS VIEW SUPPORTED THE CONCLUSION REACHED BY BLACKSHAW & VERNON (200
WAS, GENERALLY, SPATIAL STABILITY IN *A. lineatus* AND *A. obscurus* POPULATIONS AT THE LANDSCAPE
SCALE OVER THREE YEARS. AT FIELD SCALES, HOWEVER, DIFFERENCES IN THE SPATIAL DI
TWO SPECIES BECAME APPARENT WITH DYNAMIC CHANGE OVER THE ADULT ACTIVITY P
& VERNON, 2008). AT THE TIME THIS WAS ATTRIBUTED TO ADVECTION OF THE SEX PHERO
PROGRESSIVE OVERLAPPING OF INDIVIDUAL TRAP ATTRACTION ZONES, WITH THE GREAT
A. lineatus (AS REPORTED BY HICKS & BLACKSHAW, 2008) CONTRIBUTING TO AN EARLIER BI
POPULATION SPATIAL STRUCTURE.

RESULTS SHOWING THAT THE ATTRACTION ZONE OF *Agriotes* SEX PHEROMONES MIGHT BE LIMITED TO
A FEW METRES (SUFYAN *et al.*, 2011) SUGGEST THAT IT WAS NOT THE PHEROMONES *per se* THAT WERE
CAUSING THE BREAKDOWN IN SPATIAL STRUCTURE REPORTED BY BLACKSHAW & VERNO
THERE WAS AN ADDITIONAL INTRINSIC FACTOR ARISING OUT OF ADULT DISPERSAL BEHA
EXPLANATION IS THAT ADULT MALE CLICK BEETLES ARE MOVING MUCH FURTHER THAN
AND THAT SEX PHEROMONE TRAPS CAPTURE BEETLES ORIGINATING FROM A RANGE OF D
HAPPEN TO BE IN THE IMMEDIATE VICINITY. THIS WOULD ALSO EXPLAIN THE LACK OF DI
IN MRR STUDIES REPORTED BY HICKS & BLACKSHAW (2008) FOR *A. lineatus*, FOR *A. obscurus* AND
A. sputator AND BY KISHI *et al.* (2003) FOR *Melanotus okinawensis*; THE DIRECTION FROM WHICH
INDIVIDUALS ARE CAUGHT MAY NOT BE THE SAME AS THE DIRECTION FROM THEIR RELEA

IF THIS IS THE CASE, THEN IT CAN BE EXPECTED THAT ADULT CLICK BEETLES WILL
DISTRIBUTED ACROSS AGRICULTURAL LANDSCAPES AND NOT RESTRICTED TO AREAS WH
RECOVERED. BLACKSHAW & HICKS (2012) REPORTED A STUDY INTO THE DISTRIBUTION
A. lineatus, *A. obscurus* AND *A. sputator* IN AN AGRICULTURAL LANDSCAPE USING TRANSECTS OF SEX P
TRAPS AT 100M SPACING. THE SAMPLED AREA COVERED A RANGE OF CROPS AND LAND CO
THAT ALL THREE SPECIES WERE PRESENT IN EACH FIELD BUT THAT THERE WERE INTERSE
THEIR DISTRIBUTIONS. IN A SEPARATE, BUT RELATED STUDY (BATCHELOR *et al.*, 2012) EACH FIELD WAS
SAMPLED TO ASSESS WIREWORM NUMBERS. IN THE FIELDS COVERED BY THE TRANSECTS,
RESTRICTED TO PERMANENT AND TEMPORARY (LEY) GRASS AND NONE WERE FOUN
CULTIVATED SOILS. FURTHERMORE, NO *A. lineatus* LARVAE WERE RECOVERED DESPITE THERE BEING
OF THIS SPECIES THAN EITHER OF THE OTHERS. THIS PROVIDES STRONG EVIDENCE
CONTENTION THAT ADULT CLICK BEETLES ARE HIGHLY MOBILE EVEN WHEN DISPERSING

A SURVEY OF ADULT MALE (USING SEX PHEROMONE TRAPS) AND WIREWORM (USING S
DISTRIBUTIONS IN 97 ORGANIC FIELDS ACROSS SIX FARMS IN THE UK ALSO SHOWED THA
STRONG SPATIAL ASSOCIATIONS BETWEEN ADULTS AND LARVAE OF THE SAME SPECIE
2012).

A SECOND CONCLUSION TO BE DRAWN FROM BOTH STUDIES IS THAT NOT ALL THE BEETLES
CAPTURED IN A FIELD ORIGINATED THERE. THIS IMPLIES THAT THERE ARE REFUGIA IN
THAT THE SPATIAL DYNAMICS OF THESE PESTS MIGHT BE OF THE SOURCE: SINK MODEL W

THE RECIPIENT OF INFLOWING BEETLES/EGGS. THIS HYPOTHESIS IS ENTIRELY CONSISTENT
OF BLACKSHAW & VERNON (2006) AND THE OBSERVATION OF PROBABLE EDGE EFF
PHEROMONE TRAP COUNTS ATTRIBUTABLE TO THE MOVEMENT OF MALE CLICK BEETLES
THE FIELD MARGIN (BLACKSHAW & VERNON, 2008).

THE POTENTIAL FOR UNCROPPED AREAS TO ACT AS RESERVOIRS FOR CROP INVASION
MRR IN TWO EXPERIMENTAL SITES AT AGASSIZ, BRITISH COLUMBIA. EACH WAS 72 M X 72 M
IN WHEAT AND THE OTHER KEPT AS BARE FALLOW. IN EACH FIELD, A NUMBER OF PI
DEPLOYED AND MARKED *A. obscurus* MALE BEETLES RELEASED FROM SEVERAL LOCATIONS ALON
THE FOUR SIDES OF EACH FIELD. TRAPS WERE CHECKED AND EMPTIED AFTER DIFFERE
INDIVIDUALS WERE CAUGHT 1 M FROM THE FIELD EDGE WITHIN 1 H OF RELEASE AND CAP
FROM THE RELEASE POINT WERE OBSERVED AFTER 19 H. UNMARKED (NATURALLY OCC
BOTH SEXES WERE ALSO RECOVERED FROM TRAPS ACROSS BOTH FIELDS. GENERALLY
MALES CAPTURED THAN FEMALES AND THEY WERE CAUGHT EARLIER IN THE SEASON.

CONCURRENT RELEASES OF MARKED *A. lineatus* AND *A. obscurus* BEETLES WERE MADE FROM
THE CENTRE OF THE FIELD AND A SIGNIFICANT DIFFERENCE IN THEIR RESPECTIVE TRA
THIS REINFORCES THE VIEW THAT THERE ARE BEHAVIOURAL DIFFERENCES THAT INFLU
ACROSS FARMLAND. MORE IMPORTANTLY, WE ALSO FOUND SIGNIFICANT DIFFERENCES
FEMALE *A. obscurus* RELEASES FROM THE FIELD CENTRE, SUGGESTING THAT WE CANNOT NEC
KNOWLEDGE OF FEMALE DISPERSAL AND SPATIAL DISTRIBUTIONS FROM THAT OF MALES.

Conclusions

MALE CLICK BEETLES ARE WIDESPREAD IN AGRICULTURAL LAND AND SUBSTANTIALLY
PREVIOUSLY THOUGHT. THERE IS EVIDENCE FOR INTERSPECIFIC DIFFERENCES IN BE
INFLUENCE THE RATE AT WHICH THEY DISPERSE. UNCROPPED AREAS WILL ACT AS REFUG
AND ADULTS MOVE OUT OF THESE INTO ADJACENT FARMLAND, WHETHER IT HAS A POT
ALTHOUGH NOT YET CONCLUSIVE, EMERGING EVIDENCE INDICATES THAT THERE
DIFFERENCES IN DISPERSAL BEHAVIOUR WHICH MAY AFFECT FIELD COLONISATION RATES

TO DATE, FIELD STUDIES HAVE CONCENTRATED ON ADULT MALES LARGELY BECAUSE
OF SEX PHEROMONES THAT ENABLE THEM TO BE EASILY TRAPPED AT A LOCATION AND FO
BE COLLECTED FOR MRR STUDIES. GIVEN THAT WIREWORMS DO NOT MOVE FAR THE
(SCHALLHART *et al.*, 2011), THE CRITICAL BEHAVIOUR FOR WHERE THEY ARE TO BE FOUND WIL
THE OVIPOSITING FEMALE. WE HAVE SHOWN THAT WE CANNOT NECESSARILY EXTRAPOL
FEMALE DISPERSAL BEHAVIOURS BUT WE ALSO KNOW VERY LITTLE ABOUT WHEN MA
TIMING OF OVIPOSITION, THE OVIPOSITION PERIOD AND ADULT LONGEVITY OR EVEN
EMERGENCE. FURTHERMORE, EVEN IF WE KNEW ALL THIS, WE STILL LACK THE ABILITY
FROM THE SOIL IN ORDER TO TEST HYPOTHESES.

THIS KNOWLEDGE OF FEMALE BEHAVIOUR IS ESSENTIAL IF WE ARE TO DEVELOP NEW
STRATEGIES THAT ACT TO LIMIT PEST NUMBERS IN FIELD CROPS THROUGH AREA-WIDE
POTENTIAL APPROACH THAT COULD BE CONSIDERED WOULD BE TO DISRUPT MATI
WIDESPREAD USE OF SEX PHEROMONES – BUT ONLY IF WE CAN GET THE TIMING RIGHT. DE
FEMALE SPECIFIC LURE WOULD ALSO ALLOW US TO CONTEMPLATE A PUSH-PULL STRATEG
FROM FEMALES AT THE CRITICAL MATING PERIOD.

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Distribution and abundance of *AGRIOTES USTULATUS* L. adults on pheromone traps in four regions in Croatia

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Abstract: DURING SEVERAL YEARS OF INVESTIGATIONS, THE DISTRIBUTION AND THE ABUNDAN*Agriotes ustulatus* IN FOUR DIFFERENT REGIONS OF CROATIA WERE RESEARCHED WITH THE AIM TO CORREL WITH THE PREVAILED CLIMATIC CONDITIONS. ADULTS OF *Agriotes ustulatus* WAS CAPTURED BY PHEROMONE TRAPS (CSALOMON) ON 17 FIELDS DISTRIBUTED AT SEVEN LOCALITIES IN FOUR DIFFERENT RE ACCORDING TO THE CLIMATIC DATA. THE HIGHEST DOMINANCE OF *A. ustulatus* WERE RECORDED IN THE WARMEST COUNTY, COUNTY OF VUKOVAR-SRIJEM AND SPECIES WAS CLASSIFIED AS EUDOMIN*Agriotes ustulatus* WAS SUBDOMINANT AT LOCALITY ČAKALTY WHERE THE AVERAGE TEMPERATURE WAS THE LO COMPARING TO THE OTHER LOCALITIES.

Key words: ABUNDANCE, *Agriotes ustulatus* L., CROATIA, DISTRIBUTION, PHEROMONE TRAPS

Introduction

GENUS *Agriotes* BELONGS TO THE FAMILY ELATERIDAE (COLEOPTERA), WHICH IS CHARAC LARGE NUMBER OF GENERA. IN CROATIA, THE MOST IMPORTANT SPECIES ARE: *Agriotes lineatus* L., *Agriotes sputator* L., *Agriotes obscurus* L., *Agriotes brevis* CAND. AND *Agriotes ustulatus* SCHALL. (MACELJSKI, 2002). *A. ustulatus* IS THE LARGEST ONE AND OVERWINTER ONLY AS LARV LARVAE DEVELOP OVER TWO YEARS, PUPATE DURING MAY, AND ADULT FORMS OCCUR BE SEPTEMBER (BAŽOK, 2007; FURLAN, 1996; HONEKI & FURLAN, 1995). IT REQUIRES TWO OR CALENDAR YEARS FOR FULL DEVELOPMENT IN CROATIA (MACELJSKI, 2002). IN CROATIA, T FLIGHT IS IN LATE JUNE AND EARLY JULY (BAŽOK, 2010; ŠTRBAC, 1983). *A. ustulatus* IS THE MEDITERRANEAN SPECIES, ENCOUNTERED IN CENTRAL, SOUTHERN AND EASTERN E 1997). IN CROATIA, THIS SPECIES DOMINATES ON THE FIELDS OF SLAVONIA AND BARANJA THE EASTERN PART (MACELJSKI, 2002; ŠTRBAC, 1983). BAŽOK (2007) STATES, THAT THIS REPRESENTED IN NORTHWEST CROATIA AT MEDIUM TO HIGH POPULATION DENSITY. TH THESE INVESTIGATIONS WAS TO DETERMINE THE DISTRIBUTION AND ABUNDANCE OF *A. ustulatus* IN DIFFERENT REGIONS OF CROATIA AND CORRELATE THE ABUNDANCE WITH THE PREVAILED IN EACH REGION.

Material and methods

FIELD DATA

FROM 2001 TO 2005, PHEROMONE TRAPS TARGETING THE FIVE MOST IMPORTANT *Agriotes* SPECIES (*A. lineatus, A. sputator, A. obscurus, A. brevis* AND *A. ustulatus*) WERE SET IN TWO FIELDS IN THE REGION OF ZAGREB (LOCALITIES ČAKOVEC AND ORAHOVICA). FROM 2007 TO 2010, PHEROMONE TRAPS TARGETING THE SAME SPECIES WERE SET IN FIELDS IN REGION OF KOPRIVNICA-KRIŽEVCI (FERDINANDOVAC) AND REGION OF VIROVITICA-PODRAVINA (THREE FIELDS IN TEREZIN

FIELDS IN BANKOVCI). DURING THE YEARS 2007 AND 2008, ALL FIVE SPECIES WERE MO
REGION OF VUKOVAR- SRIJEM (TWO FIELDS IN BOšNJACI AND THREE FIELDS IN TOVARNIK)
FIELDS WERE INVOLVED IN THE INVESTIGATION AND ACCORDING TO THE CLIMATIC AND
WERE GROUPED INTO FOUR MAIN REGIONS (COUNTIES) AND SEVEN DIFFERENT MICRO-REGI

PHEROMONE TRAPS

TO COLLECT *A. brevis*, *A. lineatus*, *A. sputator* AND *A. obscurus* CSALOMONYATLORF FUNNEL TRAPS WERE USED AND FOR *A. ustulatus* CSALOMON VARB3 TRAPS WERE USED. THE MONITORING PERIOD OF *A. brevis*, *A. sputator*, *A. lineatus* AND *A. obscurus* WAS FROM THE 18TH TO THE 32ND WEEK OF THE YEAR, AND THAT OF *A. ustulatus* FROM THE 23RD TO THE 32ND WEEK OF THE YEAR. TRAPS WERE INSPECTED ONCE A WEEK. DURING EACH WEEKLY OBSERVATION PERIOD ALL BE WERE COLLECTED FROM THE TRAPS AND COUNTED. PHEROMONE VIALS WERE REPLACED E

DATA ANALYSIS

ADULT POPULATION DENSITIES AT TRAPPED LOCALITIES WERE CLASSIFIED ACCORDIN CATEGORIES SET BY *FURLAN et al.* (2001) AS FOLLOWS: HIGH = MORE THAN 500 ADULTS PER TRAP SEASON; MEDIUM = BETWEEN 50 AND 500 ADULTS PER TRAP PER SEASON; LOW = LESS THA PER TRAP PER SEASON; NO = NO SPECIMENS. THESE LIMIT VALUES ARE NOT CONSIDERED THRESHOLDS. BASED ON THE TOTAL INDIVIDUAL NUMBER OF FIVE SPECIES AND THE INDIV EACH PARTICULAR SPECIES THE DOMINANCE WAS CALCULATED FOR EACH FIELD AND YE WAS CALCULATED WITH BALOGH'S FORMULA (CIT. BALARIN, 1974). THE RESULTS (EUDOMIN SUBDOMINANT, RECEDENT, SUBRECEDENT) WERE CLASSIFIED ACCORDING TO TISCHLER A BALARIN, 1974). CLIMATIC CONDITIONS ABOUT AVERAGE AIR TEMPERATURE AND RAINFA WERE TAKEN FROM THE NEAREST METEOROLOGICAL STATIONS. DATA ON CLICK BEETLE INDICES AND VALUES OF COLLECTED METEOROLOGICAL ELEMENTS WERE ANALYZED BY GDM SOFTWARE) WITH MEAN SEPARATION USING DUNCAN MULTIPLE RANGE TEST (DMR). I THE DATA WERE TRANSFORMED BY LOG (X+1) TRANSFORMATION BEFORE THE ANALYSIS.

Results and discussion

THE SIGNIFICANT DIFFERENCES IN CLIMATIC CONDITIONS, AMONG ALL FOUR COUNTIES, ARE

TABLE 1. CHARACTERISTICS OF THE CLIMATIC CONDITIONS AT DIFFERENT LOCALITIE DURING THE PERIOD OF INVESTIGATION.

COUNTY	MICRO-REGION (LOCALITY)	PERIOD OF INVESTIGATION	AVERAGE AIR TEMPERATURE (°C) ± SD	TOTAL AMOUNT OF RAINFALL ± SD
ZAGREB	OBOROVO	2001-2005	11.38 ± 0.61 B	1018.58 ± 211.64 A
	ČAZMA		11.24 ± 0.63 B	885.02 ± 111.08 AB
KOPRIVNICA-KRIŽEVCI	FERDINANDOVAC	2007-2010	11.33 ± 0.36 B	860.5 ± 224.07 ABC
VIROVITICA-PODRAVINA	TEREZINOPOLJE		11.5 ± 0.52 B	903.68 ± 281.32 AB
	BANKOVCI		11.48 ± 0.43 B	909.95 ± 281.32 AB
VUKOVAR- SRIJEM	BOšNJACI	2007-2008	13.05 ± 0.07 A	742.15 ± 155.21 BC
	TOVARNIK		13.05 ± 0.07 A	645.85 ± 184.06 C
LSD P =0.05			0.557	217.586

COUNTY OF VUKOVAR-SRIJEM WAS THE COUNTY WITH THE HIGHEST AVERAGE
(13.1 °C) AND THE LOWEST AMOUNT OF RAINFALL, ESPECIALLY THE LOCALITY TOVARN
AMOUNT OF RAINFALL WAS ESTABLISHED IN THE COUNTY OF ZAGREB, LOCALITY OBO
AMOUNT OF RAINFALL IN THE COUNTY OF KOPRIVNICA-KRIžEVCI AND THE COUNTY O
PODRAVINA WAS BETWEEN ONES MENTIONED ABOVE. THE AVERAGE TEMPERATURES IN
ZAGREB, THE COUNTY OF KOPRIVNICA-KRIžEVCI AND THE COUNTY OF VIROVITICA-POD
LOWER COMPARING TO THE COUNTY OF VUKOVAR-SRIJEM.

THERE WAS S SIGNIFICANT DIFFERENCE IN THE AVERAGE CAPTURE OF *A. ustulatus* AMONG COUNTIES AND LOCALITIES (TABLE 2). IN THE COUNTY OF ZAGREB, LOCALITY OBOROVO, THE *A. ustulatus* INDIVIDUALS CAPTURED PER PHEROMONE TRAP WAS HIGH (OVER 500 BEETLES PER SEASON) WITH THE EXCEPTION IN 2005, WHEN THAT NUMBER WAS MEDIUM. IN THE SAME COUNTY AT THE LOCALITY ČAZMA, THE NUMBER OF *A. ustulatus* INDIVIDUALS WAS LOW, EXCEPT IN 2003, WHEN IT WAS MEDIUM. MEAN AVERAGE CAPTURE OF *A. ustulatus* IN COUNTY OF ZAGREB WAS 1295.21 BEETLES PER FIELD AT LOCALITY OBOROVO AND 30.66 BEETLES PER FIELD AT LOCALITY ČAZMA. IN OTHER TWO COUNTIES (COUNTY OF KOPRIVNICA-KRIžEVCI, COUNTY OF VIROVITICA-PODRAVINA) THE POPULATION WAS CLASSIFIED AS MEDIUM, WITH MEAN AVERAGE CAPTURE BETWEEN131.68 AND 243.78 INDIVIDUALS PER LOCALITY. IN THE COUNTY OF VUKOVAR-SRIJEM AT BOTH LOCALITIES DURING THE YEAR 2001 HIGH POPULATION WAS ESTABLISHED, WHILE IN 2008 THE POPULATION WAS MEDIUM. THE MEAN AVERAGE CAPTURE PER FIELD WAS HIGHER AT TOVARNIK LOCALITY (519.08 INDIVIDUALS) THEN AT BOšNJACI LOCALITY (176.07 INDIVIDUALS).

TABLE 2. CLASSIFICATION OF *A. ustulatus* POPULATION DENSITY ACCORDING TO (2011) *et al.* AN BASED ON THE AVERAGE CAPTURE OF ADULTS ON PHEROMONE TRAP/FIELD.

COUNTY	MICRO-REGION (LOCALITY)	MEAN AVERAGE CAPTURE/ FIELD	CLASSIFICATION OF ADULT POPULATION LEVEL								
			2001	2002	2003	2004	2005	2007	2008	2009	2010
ZAGREB	OBOROVO	1295.21 A*	H**	H	H	H	M				
	ČAZMA	30.66C	L	L	M	L	L				
KOPRIVNICA-KRIžEVCI	FERDINANDOVAC	131.68 B							M	M	M M
VIROVITICA-PODRAVINA	TEREZINOPOLJE	243.78B							M	M	M M
	BANKOVCI	142.11 B							M	M	M M
VUKOVAR-SRIJEM	BOšNJACI	176.07 B							H	M	
	TOVARNIK	519.08 AB							H	M	
LSD P = 0.05%		0.542 T***									

* MEANS FOLLOWED BY SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT ACCORDING TO DUN
(DMR) TEST (P = 0.05); **H – HIGH = MORE THAN 500 ADULTS/ TRAP/SEASON; M – MEDIUM = BETWE
AND 500 ADULTS/TRAP; L – LOW = LESS THAN 50 ADULTS /TRAP /SEASON;* ** = LSD IS REPORTED IN
DATA UNITS (DATA WERE TRANSFORMED USING $\log(x+1)$ TRANSFORMATION; X- NO DATA AVAILABLE

THE HIGHEST DOMINANCE INDICES OF *A. ustulatus* WERE RECORDED IN THE WARMEST COUNTY COUNTY OF VUKOVAR-SRIJEM (FIGURE 1) WHAT CORRESPONDS WITH STATEMENT OF MAC AND ŠTRBAC (1983). THESE RESULTS PARTIALLY CORRESPOND WITH THE RESULTS OF TI (FURLAN, 1996; FURLAN, 1998; TACKENBERG *et al.*, 2011) THAT THE SPECIES PREFERS HIGHER TEMPERATURES. DESPITE LOWER AVERAGE TEMPERATURES, IN THE COUNTY OF VIROV SPECIES WAS CLASSIFIED AS EUDOMINANT. THIS COUNTY IS CHARACTERIZED WITH SANDY (1998) STATES THAT *A. ustulatus* PREFERS SANDY SOIL WITH LITTLE CLAY. IN SPITE OF VERY HIGH AT LOCALITY OBOROVO THE DOMINANCE INDEX WAS 47%. THE CAPTURE OF OTHER SI LOCALITY WAS VERY HIGH AS WELL. ALTHOUGH THE TEMPERATURES WERE LOWER THAN CROATIA, OBOROVO WAS CHARACTERIZED WITH HIGH AMOUNT OF RAINFALL, WHAT IS A FOR THIS SPECIES (FURLAN 1996; FURLAN, 1998). *A. ustulatus* WAS SUBDOMINANT AT LOCALITY ČAČMA WHERE THE AVERAGE TEMPERATURE WAS THE LOWEST COMPARING TO THE OTHER LOCALIT

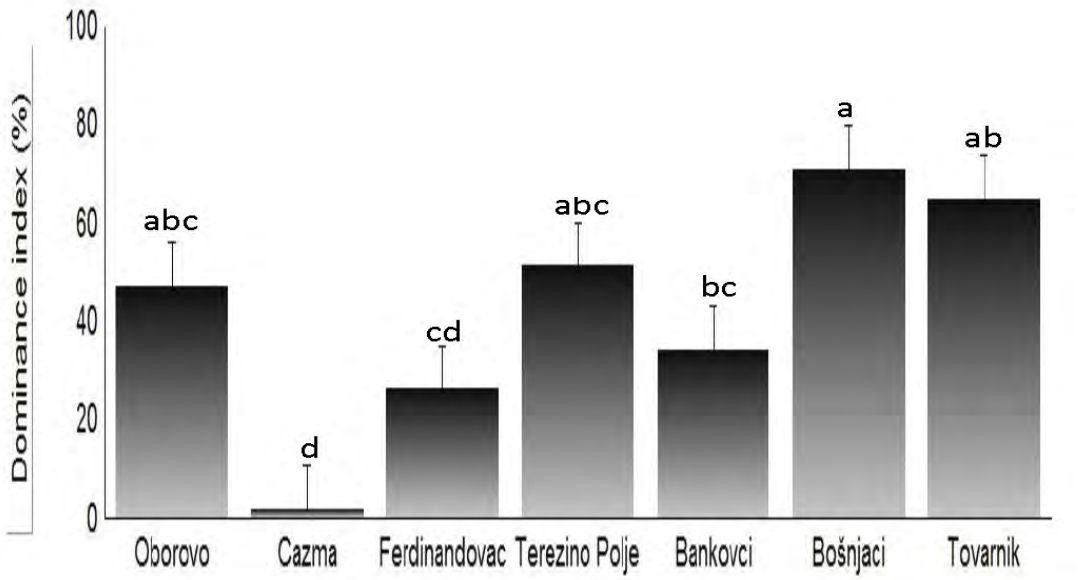

FIGURE 1. THE DOMINANCE INDICES OF *A. ustulatus* AT DIFFERENT LOCALITIES (MICRO-REGIONS) CROATIA, LSD (P = 0.05) = 29.096.

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Efforts to develop female-targeted attractants for click beetles – a summary

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Abstract: AN OVERVIEW IS GIVEN ON RECENT RESEARCH EFFORTS TO DEVELOP ATTRACTANT COM
OF ATTRACTING FEMALE CLICK BEETLES.

Key words: CLICK BEETLES, COLEOPTERA, ELATERIDAE, *Agriotes*, FEMALE ATTRACTANT, FLORAL LURE, PHEROMO

Introduction

MONITORING POPULATIONS OF ADULTS OF PEST CLICK BEETLES IS LARGELY BASED ON T
PRODUCED SEX PHEROMONES (E.G. FURLAN *et al.*, 2007). THIS HAS THE DISADVANTAGE THAT "TRA
CATCHES OF MALES MUST USUALLY BE INTERPRETED IN TERMS OF THE BEHAVIOUR OF
ADDING TO THE COMPLEXITY OF THAT INTERPRETATION" (WALL, 1985). CAPTURE OF FEMA
PROVIDE A BETTER OPPORTUNITY FOR: 1) MORE PRECISE MONITORING, LEADING TO
DECISION MAKING ON TIMING OF CONTROL STRATEGIES AGAINST A GIVEN PEST SPECIES;
MASS TRAPPING BY CATCHING GRAVID FEMALES FOR DIRECT POPULATION REDUCTION; 3
APPLICATION OF THE LURE-AND-KILL METHOD; 4) AN OPPORTUNITY TO ASSESS EGG CONT
FERTILITY OF CAPTURED FEMALES.

Material and methods

IN THE COURSE OF THE EXPERIMENTS, INTERNATIONALLY ESTABLISHED AND WIDESPRE
USED. PLEASE REFER TO DESCRIPTIONS OF MATERIAL AND METHODS IN THE REFERENCES

Results and discussion

FLOWER-VISITING SPECIES

INSECTS LOCATING A FLOWER ARE AIDED BY AN ARRAY OF VISUAL AND CHEMICAL STIM
FLOWER-VISITING CLICK BEETLES CHEMICAL COMMUNICATION BETWEEN FLOWERS A
COULD BE EXPLOITED TO DEFINING FEMALE-TARGETED ATTRACTANTS. INDEED, IN *Agriotes ustulatus*,
WHICH CAN FREQUENTLY BE SEEN FEEDING ON FLOWERS (*E.G.* ANETHUM), A
MALES), AND THIS ATTRACTION CAN BE SIGNIFICANTLY INCREASED BY THE ADDITION O

COMPOUNDS (TÓTH *et al.*, 2011). FURTHER RESULTS INDICATE THAT THE NUMBER OF FEMAL
CAPTURED INCREASED DRAMATICALLY WHEN THIS FLORAL ATTRACTANT WAS APPLI
PHEROMONE IN THE SAME TRAP, COMPARED TO THE CATCH IN TRAPS WITH THE FLORAL
et al., 2009).

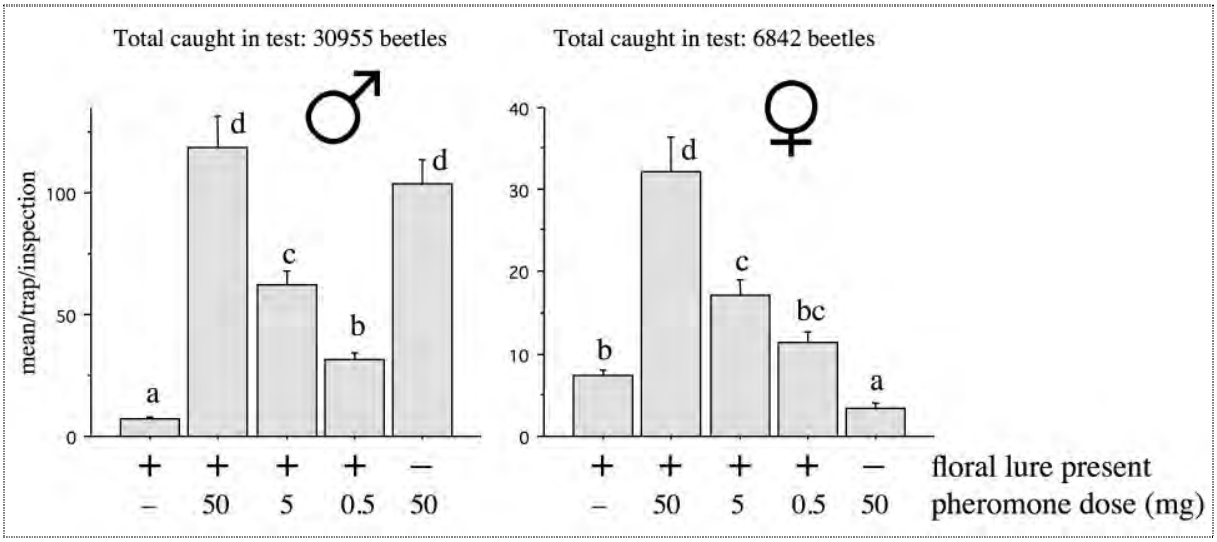

FIGURE 1. CATCHES (MEAN + SE) OF *Agriotes ustulatus* BEETLES IN TRAPS BAITED WITH THE
PHEROMONE ONLY, THE FLORAL LURE ONLY, AND DUAL BAITED TRAPS WITH THE FLORA
DOSES OF THE PHEROMONE IN A TRAPPING TEST IN KNEZHA, BULGARIA. (DATA FRO
T. B. TOSHOVA, M. SUBCHEV, D. I. VELCHEV, UNPUBLISHED). COLUMNS WITH THE SAME LE
ONE DIAGRAM ARE NOT SIGNIFICANTLY DIFFERENT BY ANOVA, STUDENT-NEWMAN-KEUL

IT WAS INTERESTING THAT FEMALE NUMBERS CAUGHT INCREASED WITH INCREAS
PHEROMONE IN DUAL-BAITED TRAPS (FIGURE 1), GIVING MORE EVIDENCE THAT TH
UNEQUIVOCALLY HAS A FAVOURABLE INFLUENCE ON FEMALE CATCHES OF *A. ustulatus*. FEMALES TARGETED
TRAPS FOR CATCHING *A. ustulatus* EQUIPPED WITH A DUAL PHEROMONE PLUS FLORAL LURE ARE
AVAILABLE ON THE MARKET FOR USE BY BOTH GROWERS AND EXPERTS.

NON-FLOWER-VISITING SPECIES

SIMILAR PERSPECTIVES ARE OPEN IN THE STUDY OF CHEMICAL COMMUNICATION BETW
MATERIAL AND SPECIES WHICH FEED ON GREEN LEAVES OF PLANTS. IN THE DEVELOPM
TARGETED LURE FOR *Limonius* (WHICH CANNOT BE OBSERVED FEEDING ON FLOWERS, INSTEAD, FE
GREEN LEAVES OF WEEDS), THE INITIAL IDEA WAS GIVEN BY A COMMONLY USED METHO
TRAPS" FOR COLLECTING BOTH SEXES OF ADULTS OF CLICK BEETLE SPECIES (FURLAN, 200
CM PLASTIC SHEETS PUT ON BARE SOIL IN AREAS KNOWN TO BE INFESTED WITH WIRE
SHEETS BEING COVERED WITH FRESH FOLIAGE OF DIFFERENT GRAMINEAE AND/OR LEG
ADULT BEETLES CONGREGATE BELOW THE FOLIAGE AND CAN EASILY BE COLLECTED
SAME METHOD WAS REPORTED TO BE EFFICIENT FOR *Agriotes* SPP. (TÓTH *et al.*, 2005; FURLAN
italicum (GRAMINEAE) AND *Medicago sativa* (LEGUMINOSAE) FOLIAGE, MOST ABUNDANT CONSTITU
OF THE TYPICAL HABITAT OF *A. brevis* IN ITALY (L. FURLAN, PERS. COMM.). ASSUMING THAT PLA
DERIVED VOLATILES ARE RESPONSIBLE FOR THE AGGREGATION OF BEETLES UNDER T
PARTIALLY, PRELIMINARY FIELD TESTS WITH *A. brevis* (FURLAN *et al.*, 2004; YELTSOV, 2005) THIS TRAP TYPE IS IN WIDE
USE IN PHEROMONE TRAPPINGS OF CLICK BEETLES IN EUROPE) BAITED WITH A COUPL

M. sativa OR *L. italicum* WERE SET UP. TRAPS CONTAINING SHOOTS OF EITHER PLANT SPECI
SIGNIFICANTLY MORE *A. brevis* THAN EMPTY CONTROL TRAPS (VUTS *et al.*, 2011).

CONSEQUENTLY, VOLATILES WERE COLLECTED FROM *L. sativa* AND *L. italicum* SHOOTS, AND STRUCTURE ELUCIDATION OF COMPOUNDS ELICITING RESPONSES FROM THE ANTENNA *A. brevis* IN GC-FID/EAD STUDIES WAS ATTEMPTED. SO FAR WE IDENTIFIED 5 COMPOUNDS IN VOLATIL FROM *L. italicum*, AND 9 COMPOUNDS FROM *M. sativa*. THE COMPOUNDS (*Z*)-3-HEXENYL ACETATE (WHICH WAS THE DOMINANT CONSTITUENT) AND METHYL BENZOATE WERE PRESENT IN BOTH PLANT SPECIES.

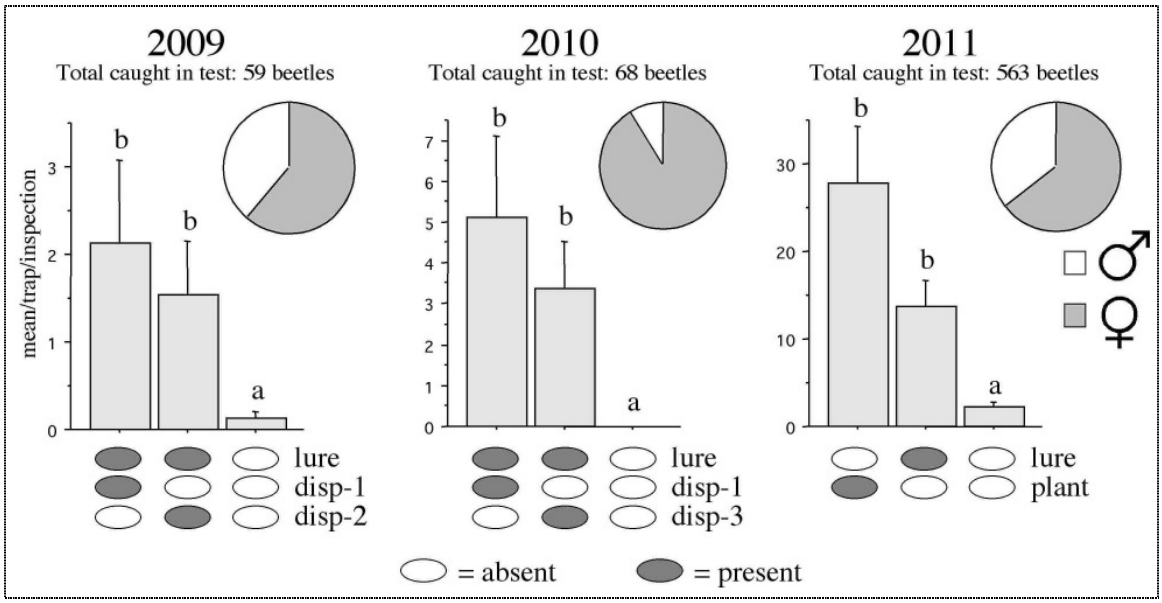

FIGURE 2. CATCHES (MEAN + SE) OF *Agriotes brevis* IN TRAPS BAITED WITH BLENDS OF COMPOUNDS IDENTIFIED FROM VOLATILES OF *Lolium italicum* AND *Medicago sativa* PLANTS FORMULATED IN DIFFERENT DISPENSERS IN TRAPPING TESTS IN VENETO, ITALY (DATA FROM M. TÓTH, L. F UNPUBLISHED). DISP-1 = POLYETHYLENE CAPSULE; DISP-2 = POLYETHYLENE BAG WITH DE 3 = POLYETHYLENE CAPSULE WITH PIECE OF DENTAL ROLL. COLUMNS WITH THE SAME L DIAGRAM ARE NOT SIGNIFICANTLY DIFFERENT BY ANOVA, STUDENT-NEWMAN-KEULS, P =

IN PRELIMINARY FIELD TESTS IN 2009, A BLEND OF THE 5 *Lolium* COMPOUNDS FORMULATED IN TWO DIFFERENT TYPES OF DISPENSERS CAUGHT SIGNIFICANTLY MORE *A. brevis* THAN UNBAITED TRAPS, AND A CONSIDERABLE PERCENTAGE OF THE CAPTURE WERE FEMALES (FIGURE 2, GREY PIE CHA CONFIRMED IN 2010 BY USING A REDUCED BLEND OF ONLY 4 COMPONENTS OCCURRING F *italicum* OR *M. sativa* VOLATILES (OR BOTH), WHEN AGAIN HIGHER CATCHES WERE RECORD TRAPS WITH BOTH DISPENSER TYPES TESTED. IN 2011, TRAPS BAITED WITH THE SYNTHET LURE CAUGHT AGAIN HIGHER NUMBERS THAN UNBAITED TRAPS, HOWEVER, ONLY CA. H IN TRAPS BAITED WITH NATURAL *L. italicum* SHOOTS.

IN CONCLUSION, ALTHOUGH THE FIRST RESULTS ARE HIGHLY PROMISING, FURTHER IMPROVEMENT IS NEEDED TO DEVELOP A LURE BASED ON SYNTHETIC GREEN-LEAF SEM PRACTICAL USE IN CLICK BEETLE CONTROL.

Acknowledgements

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DO02-244/2008 OF THE BULGARIAN NATIONAL SCIENTIFIC FUND TO MS.

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New perspectives for wireworm control based on an improved understanding of their feeding ecology

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Abstract: WIREWORMS, THE SOIL-DWELLING LARVAE OF CLICK BEETLES (COLEOPTERA: ELATERIDAE), ARE FOUND THROUGHOUT THE WORLD AND THEY DAMAGE A WIDE SPECTRUM OF ARABLE AND VEGETABLE CRO UNFORTUNATELY, THESE INSECTS ARE HARD TO CONTROL USING INSECTICIDAL-, BIOLOGICAL-, AND CULTIVATION-BASED CONTROL MEASURES, AS THEIR BEHAVIOUR AND OCCURRENCE IN THE SOIL COLUMN I HARD TO PREDICT. DEVELOPING ALTERNATIVE MANAGEMENT TACTICS IS THUS ROOTED IN A SOUN UNDERSTANDING OF THE BIOLOGY AND ECOLOGY OF THESE INSECTS. IN THIS CONTEXT, THE KNOWLEDGE THE FEEDING ECOLOGY OF WIREWORMS IS A KEY ASPECT. FORTUNATELY, WITHIN THE LAST FEW YEA CONSIDERABLE PROGRESS HAS BEEN MADE IN THIS RESEARCH AREA AS NOVEL TECHNOLOGY ALLOWED EXAMINING THE FEEDING BEHAVIOUR OF WIREWORMS IN BOTH MESOCOSM AND FIELDING EXPERIMENTS. THIS WORK EXAMINED, WHICH PLANT SPECIES ARE PREFERENTIALLY ATTACKED, WHEN FEEDING OCCUI AND HOW ENVIRONMENTAL PARAMETERS AFFECT WIREWORM FEEDING BEHAVIOUR. IN THIS TALK WE WI SYNTHESIZE THE CURRENT UNDERSTANDING OF THE FEEDING ECOLOGY OF WIREWORMS AND IDENTIFY, HOW THIS KNOWLEDGE CAN BE EMPLOYED TO IMPROVE THE CONTROL OF THESE PESTS.

Key words: ELATERIDAE, PEST CONTROL, *Agriotes* SPP., DIET

Posters

Exploratory use of geometric morphometrics in the identification of wireworm species

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Abstract: WIREWORMS ARE CLICK BEETLE LARVAE FROM THE GENUS *Agriotes* (COLEOPTERA: ELA
THAT CAUSE CONSIDERABLE DAMAGE TO FIELD CROPS. *Agriotes* SPECIES RECORDED IN
CENTRAL EUROPE ARE FOUND IN ARABLE LAND IN CONTINENTAL CROATIA AND CAN CAUSE S
ECONOMIC YIELD LOSSES. THE IDENTIFICATION OF THESE LARVAE TO THE SPECIES LEVEL IS DIFI
CLASSICAL TAXONOMIC MEASUREMENTS. OUR STUDY EXPLORES THE USE OF SPECIES-
MORPHOLOGICAL CHARACTERS (I.E. SPECIFIC SPIRACLES PLACED ON THE NINTH ABDOMINAL
CERTAIN STRUCTURES OF THE MANDIBLE) THAT WILL ENABLE THE USE OF GEOMETRIC MO
METHODS FOR DIAGNOSTIC PURPOSES. GEOMETRIC MORPHOMETRICS (GM) IS THE QUA
MEASUREMENT, ANALYSIS AND INTERPRETATION OF SHAPE VARIATION IN ORGANISMS. THE AP
GM IN TAXONOMY AND SYSTEMATIC IS NOVEL AND HAS THE POTENTIAL TO PROVIDE INFORM
SHAPE VARIATION THROUGH THE RELATIVE POSITION OF ANATOMICAL LANDMARKS. GM
PREVIOUSLY USED IN WCR POPULATION ANALYSES, *Diabrotica virgifera virgifera* AND *D. virgifera dorsalis* SPECIES COMPLEX,
TORTRICIDAE AND GEOMETRIDAE SPECIES ANALYSES AND OTHER IMPORTANT AGRICULTURA
THE AIM OF THIS STUDY WAS TO EXPLORE THE USE OF LANDMARK-BASED MORPHOMETRIC AN
SIMPLE METHOD TO DISCRIMINATE AMONG SPECIES IN MIXED WIREWORMS POPULA
APPROXIMATELY 10 LANDMARKS WERE USED IN SPECIES DISCRIMINATION OF FIVE *Agriotes* SPECIES,
INCLUDING *A. sputator, A. lineatus, A. brevis, A. obscurus, A. ustulatus,* RANDOMLY COLLECTED ON
ARABLE LAND ACROSS CROATIA. EACH LANDMARK WAS DIGITISED AND IMPORTED IN MORPHOJ SOFTW
FOR FURTHER STATISTICAL ANALYSES. STATISTICAL PROCEDURES USED IN THIS STUDY WERE
PROCRUSTES ANALYSES, DISCRIMINANT FUNCTION ANALYSES, PRINCIPAL COMPONENT ANALYSE
CANONICAL VARIATES ANALYSES. MORPHOMETRIC RESULTS WILL BE VERIFIED BY POLYMERA
REACTION (PCR) ANALYSES OF *Agriotes* SPECIES USING DIAGNOSTICS PRIMERS PUBLISHED BY
STAUDACHER *et al.* [2011] IN BULL. ENTOMOL. RES. 101: 201-210 (2011). WE DEMONSTRATE THAT GM
TECHNIQUES HOLD PROMISE AS A DIAGNOSTIC TOOL FOR DISCRIMINATING BETWEEN MORPH
CRYPTIC TAXA OF THE *Agriotes* SPECIES COMPLEX.

Key words: WIREWORMS, GEOMETRIC MORPHOMETRICS, SPECIES IDENTIFICATION

Insect pathogens and entomoparasitic nematodes
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Development of novel biocontrol encapsulation techniques for garlic extracts: first results

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Abstract: THE AIM OF THE WORK PRESENTED HERE IS TO DEVELOP NOVEL ECO-EFFICIENT EN TECHNIQUES FOR BIOACTIVE INGREDIENTS USED IN BIOLOGICAL PEST CONTROL FOR THE EU PROJ PROCESS FOR PRODUCTION OF SMALL-SCALE ALGINATE BEADS CONTAINING GARLIC EXTRACT B SELF-CONSTRUCTED TECHNICAL ENCAPSULATION EQUIPMENT WAS DEVELOPED. THE ENCAPSU ACTIVE INGREDIENTS AGAINST OXYGEN AND OTHER OUTSIDE INFLUENCES, THUS ENHANCING SH SLOW RELEASE EFFECT. THE CAPSULE SIZE PRODUCED WITH THIS TECHNOLOGY CAN BE VARIED DESIRED PRODUCT – BETWEEN 4 AND 600 µM. PARTICLES ARE STABLE AND SPHERICAL.

Key words: ALGINATE BEADS, GARLIC EXTRACT, AIR ATOMIZATION, ENCAPSULATION, FORMU CONFUSE AND KILL, BIOLOGICAL CONTROL, SEMIOCHEMICALS, BIOINSECTIDE, WIREWORMS, WEST

Introduction

RECENT STUDIES HAVE SHOWN THAT, BENEATH OTHER BENEFICIAL EFFECTS, GARLIC (*Allium sativum*) HAS BIOINSECTICIDAL ACTIVITY AND THUS CAN BE USED FOR INSECT PEST CONTROL (FENG-L *et al.*, 2009). SOME OF THE ACTIVE COMPONENTS CONTAINED IN GARLIC (E.G. ALLICIN) ARE SENSITIVE EXTERNAL FACTORS (E.G. OXYGEN, LIGHT), HIGHLY VOLATILE AND INSOLUBLE IN WATER OR CONTROLLED RELEASE SYSTEMS THAT CAN STABILIZE SENSITIVE COMPONENTS OF NOVEL CAPSULE FORMULATIONS, ARE NEEDED.

TO THIS END INVESTIGATIONS OF RELEASE KINETICS (E.G. SLOW OR CONTROLLED REL FROM A DEPOT) ARE CRUCIAL. THE RELEASE OF ACTIVE INGREDIENTS CAN BE EITHER CO BY ENVIRONMENTAL CONDITIONS (E.G. TEMPERATURE, HUMIDITY ETC.) AND ENCAPSU PROPERTIES. HEREBY EFFICACY CAN BE ENHANCED AND APPLICATION COSTS CAN BE R DECREASED NUMBER OF APPLICATIONS. PRELIMINARY STUDIES ON LAB SCALE HAVE S JUICE CAN BE ENCAPSULATED IN BEADS TO SLOW DOWN THE RELEASE OF ACTIVE INGR *Phytophthora* (SLUSARENKO *et al.*, 2008).

THE AIM OF THIS WORK WAS TO DEVELOP A NOVEL PROCESS FOR THE PRODUCTION OF BASED ON BIODEGRADABLE MATERIALS.

Material and methods

ENCAPSULATION OF GARLIC EXTRACT

A SOLUTION OF 1% SODIUM ALGINATE AND GARLIC EXTRACT PROVIDED BY NEEM BIOTEC THROUGH A NOZZLE WITH SELF-CONSTRUCTED TECHNICAL ENCAPSULATION EQUIPMEN INTO A CALCIUM CHLORIDE SOLUTION, WHERE THEY HARDENED. PARTICLE SIZES FROM OBTAINED, THUS OFFERING A WIDE RANGE OF APPLICATION OPTIONS. WHEN DRIED, PAR FURTHER REDUCED, OFFERING THE OPTION OF REACHING NANO SCALED PRODUCTS.

Results and discussion

AS ENCAPSULATION MATERIAL FOR THE GARLIC EXTRACT, CONTAINING THE OXYGEN-S
ALLICIN, THE HYDROPHILIC BIOPOLYMER HYDROGEL CALCIUM ALGINATE WAS
ENCAPSULATION MATRIX DUE TO ITS LOW OXYGEN PERMEABILITY, LOW TOXICITY AND
(E.G. HEUSKIN *et al.* 2012).

AIR-ATOMIZATION METHOD FITS THE PROPERTIES OF THE SENSIBLE GARLIC EXTRACT
ARE AVOIDED AND REACTION CONDITIONS ARE GENTLE. IN ORDER TO OPTIMIZE BOTH P
PARTICLE SIZE DISTRIBUTION, SEVERAL HOLLOW CONE NOZZLES WERE TESTED. THE C
FINEST CAPSULES WITH THE BEST PARTICLE SIZE DISTRIBUTION WAS USED.

FURTHER OPTIMIZATION OF PARTICLE SIZE DISTRIBUTION WAS ACHIEVED BY INSTA
HORIZONTALLY RATHER THAN VERTICALLY. THIS WAY, DROPLETS OF LARGER SIZE, HA
THAN SMALLER DROPLETS, COULD BE SORTED OUT, AS SHOWN IN FIGURE 1.

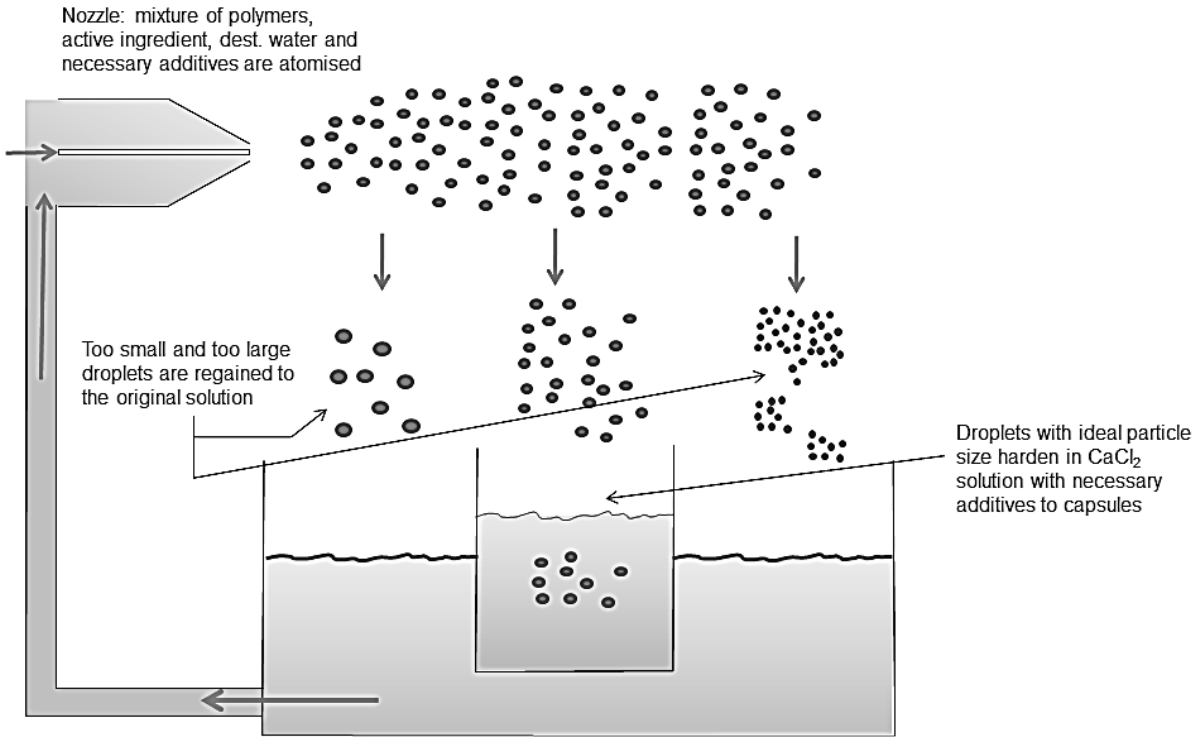

FIGURE 1. OPTIMIZED AIR-ATOMIZATION METHOD, OFFERING REDUCED PARTICLE SIZE
FRACTIONING.

THE FOLLOWING PICTURES (FIGURE 2) SHOW THE EFFECT OF THESE FIRST OPTIMISA
PARTICLE SIZE DISTRIBUTION FOR CALCIUM ALGINATE BEADS.

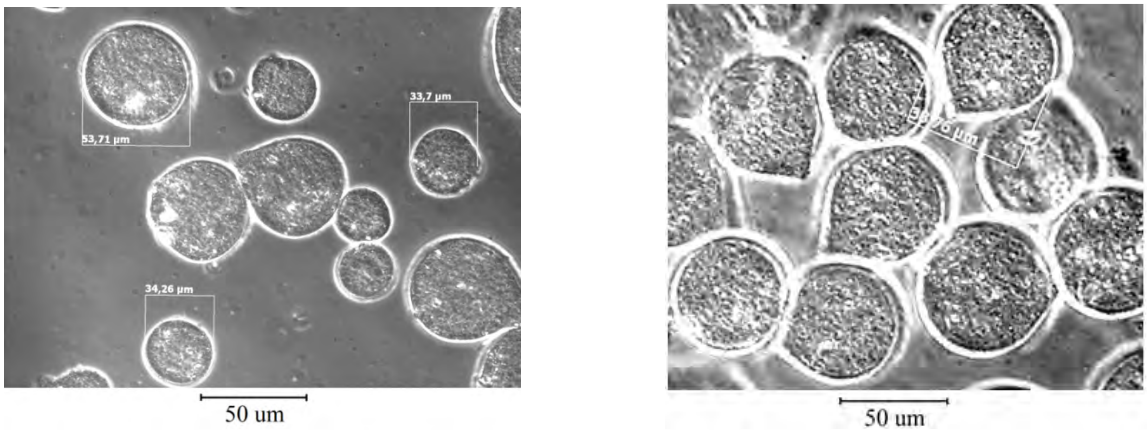

FIGURE 2. INHOMOGENEOUS (LEFT) AND OPTIMIZED (RIGHT) PARTICLE SIZE DISTRIBUTION.

ALSO, THE DISTANCE BETWEEN NOZZLE AND CALCIUM CHLORIDE SOLUTION WAS INC
TO OFFER A PROPER TIME OF SPHERICAL DROPLET FORMATION. BY THIS, AN OPTIMAL PA
OBTAINED. FIGURE 3 SHOWS THE EFFECT OF A LOWER DISTANCE BETWEEN NOZZLE AND C
$CaCl_2$ SOLUTION, AS
WELL AS A STABLE PARTICLE OF SPHERICAL FORM AS A RESULT.

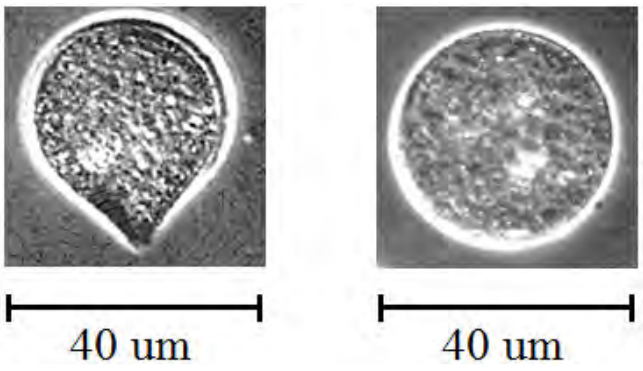

FIGURE 3. NON-IDEAL (LEFT) AND OPTIMIZED SPHERICAL (RIGHT) PARTICLE FORM.

FOR THE FORMATION OF SMALL-SCALED PARTICLES (ABOUT 4 µM), A TENSIDE WAS
$CaCl_2$ SOLUTION, SINCE OTHERWISE PARTICLES WERE UNABLE TO PENETRATE THE SOLUT
LOW WEIGHT AND SIZE.

APART FROM THE PHYSICAL PROPERTIES, SENSORY EVALUATION SHOWED THAT TYPIC
WAS DECREASED BY ENCAPSULATION COMPARED TO PURE GARLIC EXTRACT INDICATI
EFFECT OF ALLICIN, ONE OF THE LEAD COMPONENTS.

FUTURE EXPERIMENTS WILL DEAL WITH MEASUREMENTS OF EXACT PARTICLE SIZ
DISTRIBUTION AND PHYSICAL STABILITY OF CAPSULES. FURTHERMORE, DRYING OF CAPS
SCALE WILL BE INVESTIGATED.

WITH REGARD TO APPLICATION, NOVEL CO-FORMULATIONS WITH SEMIOCHEMIC
PESTICIDAL AGENTS WILL BE DEVELOPED, IMPLEMENTING A “CONFUSE AND KILL” OR “A
STRATEGY. EFFICACY OF THE CAPSULES OBTAINED WITH THE DEVELOPED METHODS WIL
WORK PARTNERS OF THE EU PROJECT INBIOSOIL AGAINST WIREWORMS ON POTATO, W
ROOTWORM ON MAIZE, BLACK VINE WEEVIL ON STRAWBERRIES AND SCIARIDS IN GROWIN

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The project ATTRACT: Protection of crops from soil-borne insect pests with a novel attract and kill strategy

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Abstract: THE PROJECT ATTRACT TARGETS THE DEVELOPMENT OF A NOVEL ATTRACT-AND-KILL STRA PROTECTION OF CROPS FROM SOIL-BORNE INSECT PESTS. THE AIM IS THE DESIGN OF A PLANT PROTECT WITH AN INNOVATIVE FORMULATION BASED ON CO_2 EMITTING SOURCES AS AN ATTRACTIVE COMPOUND AND ENVIRONMENTALLY FRIENDLY INSECTICIDAL COMPOUNDS.

Key words: ATTRACT-AND-KILL, PEST CONTROL, WIREWORM, WESTERN CORN ROOTWORM, BLACK VINE W

Introduction

LARVAE OF HERBIVOROUS INSECTS (E.G. WIREWORMS, WESTERN CORN ROOTWORM, BLACK VI CAUSE SEVERE LOSSES IN MANY CROPS (POTATO, MAIZE, STRAWBERRY). A CONTROL OF THESE SOIL INSECTICIDES IS SEVERELY RESTRICTED OR HAS RECENTLY BEEN ABANDONED. THE PROJECT AIMS AT DEVELOPING INNOVATIVE ATTRACT AND KILL FORMULATIONS WHICH CAN BE PRODUCE SCALE AND CAN THEN BE USED AS NOVEL CONTROL STRATEGIES AGAINST SOIL-BORNE INSE CONVENTIONAL AS WELL AS ORGANIC FARMING SYSTEMS. BY ATTRACTING LARVAE TO CONTAINING A KILL COMPOUND (FIGURE 1) INSECTICIDE APPLICATIONS OR OTHER CONTROL ST BE REPLACED, THE AMOUNT OF INSECTICIDES CAN BE MINIMIZED AND THE ENVIRONMENT AND H FARMERS AND CONSUMERS CAN BE PROTECTED.

Outlook

IN THE PROJECT ATTRACT NOVEL FORMULATIONS (CAPSULES, GRANULES, MATRICES) BASED ON CO_2 SOURCES WILL BE DEVELOPED AND TESTED UNDER PRACTICAL CONDITIONS IN ORDER TO LURE L FROM PLANT ROOTS. IN THESE ATTRACT FORMULATIONS PLANT-BASED ENVIRONMENTA INSECTICIDAL COMPOUNDS, SUCH AS NEEM AND QUASSIN WILL BE INCORPORATED IN MULTIPH MULTILAYER SYSTEMS WITH ADDITIVES . THESE FORMULATIONS WILL BE OPTIMIZED IN EFFICAC LAB, GREENHOUSE AND FIELD EXPERIMENTS. FIRST DATA ON ENCAPSULATION AND EFFICACY WILL

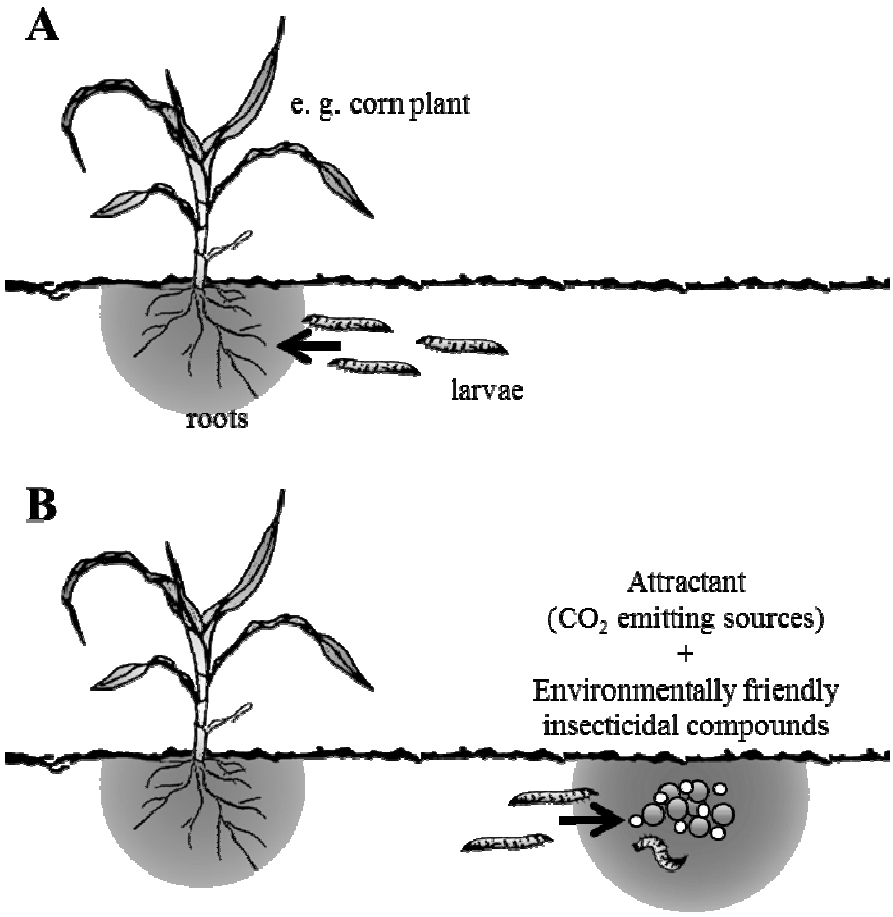

FIGURE 1. LARVAE USE CO_2 TO LOCATE THE ROOTS OF LIVING CORN PLANTS (A). LARVAE ARE KILLED BY CO_2 EMITTING SOURCES AND ARE KILLED BY AN ENVIRONMENTALLY FRIENDLY INSECTICIDE (B).

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IPM (Fungi/Bacteria)

IPM microbial control based strategies

Combined use of entomopathogenic fungi and their extracts to improve the control of the cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae)

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Abstract: THIS WORK HAS EVALUATED THE ACTIVITY OF ENTOMOPATHOGENIC FUNGI AND THEIR EXTRAC AGAINST *Spodoptera littoralis* LARVAE, A VERY HARMFUL POLYPHAGOUS PEST. TWENTY-SIX *Metarhizium* SPP. AND *Beauveria* SPP. ISOLATES AND THEIR CRUDE EXTRACTS WERE EVALUATED AGAINS SECOND INSTAR *S. littoralis* LARVAE. AMONG *Beauveria* ISOLATES, THE HIGHER MORTALITY RATES AND LOWER SURVIVAL TIMES WERE IN THE RANGE OF 75-80% AND 8.7-9.6 DAYS RESPECTIVELY. AMONG *Metarhizium* ISOLATES, THE HIGHER MORTALITY RATES AND LOWER SURVIVAL TIMES WERE IN THE RANGE 55-60% AND 9.0 DAYS RESPECTIVELY. THE CRUDE EXTRACTS FROM THE MOST VIRULENT ISOLATES, 6 FRO *Beauveria* AND 2 FROM *Metarhizium*, WERE OBTAINED IN ADAMEK'S LIQUID MEDIUM AND BIOASSAYED AGAINST SECOND INSTARS IN ALFALFA LEAF DISC EXPERIMENTS. THE EXTRACT *Metarhizium* ISOLATES EAMB 09/01-SU AND EAMA 01/58-SU CAUSED THE HIGHEST MORTALITY RATES, 80.0 AND 66.6%, AND LOWEST AST VALUES, 5.1 AND 4.4 DAYS, RESPECTIVELY. COMBINED TREATMENTS OF FUNGAL SUSPENSIONS OF ISOLATES EAMB 09/01-SU AND EAMA 01/58-SU AND THEIR EXTRACTS CAUSED HIGHER MORTALITY RATES THAN THE SINGLE ONES, IN A DOSE-DEPENDENT MANNER, WITH MORTA RATES REACHING 100% FOR EAMB 09/01-SU ISOLATE AND ITS EXTRACT AT AND 76.0% MORTALITY FOR EAMA 01/58-SU, AND ITS EXTRACT AT . THESE RESULTS HIGHLIGHT THE POTENTIAL OF *S. littoralis* INTEGRATED CONTROL STRATEGY BASED ON THE COMBINED USE OF ENTOMOPATHOGENIC FUNGI AND THEIR EXTRACTS.

Key words: BIOLOGICAL CONTROL, SYNERGISM, METABOLITES, *Metarhizium*, *Beauveria*

Insecticidal activity of a semi-purified extract from *Metarhizium brunneum* (Ascomycota: Clavicipitaceae) against the red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae)

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Abstract: THE RED PALM WEEVIL, *Rhynchophorus ferrugineus* (OLIVIER) (COLEOPTERA: CURCULIONIDAE), IS CURRENTLY CONSIDERED THE MOST DAMAGING PESTS OF PALMS WORLDWI SPREAD EXTENSIVELY FROM ITS ORIGIN MAINLY BY TRADING OF INFESTED PALM TREES AND OF EUROPE, MANY PREVENTATIVE AND CURATIVE PROCEDURES, MOSTLY CHEMICAL, HAVE BEEN IMP WITH VARIABLE DEGREES OF SUCCESS TO ERADICATE *R. ferrugineus*. AND CONTAIN HOWEVER, THESE TECHNIQUES HAVE BEEN HAMPERED BY ENVIRONMENTAL CONCERNS RELATED TO THE USE O AND LEGISLATION RESTRICTING THEIR USE. FOR THIS REASON, THERE IS AN INCREASING INTERES NATURAL ENEMIES OF *R. ferrugineus* WITH EMPHASIS IN ENTOMOPATHOGENIC FUNGI (EPF), WHICH HAVE PROVIDED ENCOURAGING RESULTS AS MICROBIAL CONTROL AGENTS OF THIS PEST. NEVERT HAVE ALSO SHOWN TO BE A POORLY STUDIED SOURCE OF INSECTICIDAL COMPOUNDS OF NA THE AIM OF THIS STUDY WAS TO DETERMINE THE INSECTICIDAL ACTIVITY OF THE CRUDE *Metarhizium brunneum* EAMB 09/01-SU STRAIN AGAINST *R. ferrugineus* AND TO PURIFY THE ACTIVE FRACTIONS. THE CRUDE EXTRACT CONTAINING LOW-MOLECULAR-WEIGHT SECONDARY META SEPARATED INTO DIFFERENT FRACTIONS BY ADJUSTING THE ACETONITRILE/WATER RATIO IN ELUTION BUFFER OF THE SEMI-PREPARATIVE HPLC. SUBSEQUENTLY, THIS EXTRACT *per os* WAS EVALUA AGAINST *R. ferrugineus* ADULTS AND LARVAE. THE F5B AND F6 FRACTIONS SHOWED HIGH ORAL TOX AND THIS IS THE FIRST EVIDENCE OF INSECTICIDAL ACTIVITY OF FUNGAL COMPOUN *R. ferrugineus*.

Key words: ENTOMOPATHOGENIC FUNGI, CURCULIONIDAE, METABOLITE, BIOLOGICAL CONTROL

Insect pathogens and entomoparasitic nematodes
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Subterranean control of an arboreal pest: EPNs and EPFs for FCM

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Abstract: CONTROL MEASURES AGAINST THE FALSE CODLING MOTH (FCM), *Thaumatotibia leucotreta*, HAVE TRADITIONALLY IGNORED THE SOIL-BORNE PUPAL STAGE. RECENT TRIALS WITH ENTOMOPAT (EPNS) AND ENTOMOPATHOGENIC FUNGI (EPFS) HAVE TARGETED THIS LIFE-STAGE. APP *Heterorhabditis bacteriophora* TO A CITRUS ORCHARD FLOOR REDUCED *C. leucotreta* INFESTATION OF FRUIT BY UP TO 81%. CONSERVATION OF *S. zealandica* THROUGH NON-USAGE OF A NEMATICIDE ALSO RESULTE DRAMATICALLY LOWER FRUIT INFESTATION. DOSE-RESPONSE AND EXPOSURE TIME-RESPONSE B THE THREE MOST PROMISING FUNGAL ISOLATES AGAINST *T. leucotreta* PUPAE IN ORCHARD TRIALS SHOWED PERSISTENCE OF THESE FUNGI IN ORCHARD SOIL FOR AT LEAST SIX MONTHS.

Key words: *Thaumatotibia leucotreta*, ENTOMOPATHOGENIC NEMATODES, *Heterorhabditis*, ENTOMOPATHOGENIC FUNGI, *Beauveria bassiana*, *Metarhizium anisopliae*

Introduction

FALSE CODLING MOTH (FCM), *Thaumatotibia* (= *Cryptophlebia*) *leucotreta* (MEYRICK) (LEPIDOPTERA: TORTRICIDAE), IS AN IMPORTANT PEST OF CITRUS AND OTHER CROPS I (NEWTON, 1998). IT HAS TRADITIONALLY BEEN CONTROLLED BY TARGETING THE ABOVE-G THESE INCLUDES THE EGG, WHICH IS LAID ON THE FRUIT (E.G. USING EGG PARASITOIDS AN REGULATORS); THE LARVAL STAGE, BETWEEN HATCHING AND PENETRATION INTO TH GRANULOVIRUS AND CHEMICALS); AND THE NOCTURNALLY ACTIVE MOTH (E.G. USING M ATTRACT AND KILL, AND THE STERILE INSECT TECHNIQUE) (MOORE & HATTINGH, 2012). RE HAS FOCUSSED ON TARGETING THE PREVIOUSLY IGNORED SOIL-BORNE PUPAL STAGE. TH WITH EPNS AND EPFS. RESEARCH WITH EPNS IS IN THE FINAL STAGE OF FIELD WORK, WH WITH EPFS IS STILL RELATIVELY NEW. HOWEVER, BOTH GROUPS OF PATHOGENS ARE S POTENTIAL AS ADDITIONAL WEAPONS AGAINST *T. leucotreta*.

Material and methods

Entomopathogenic nematodes: introduction

THIS TRIAL WAS CONDUCTED ON KLAWERVLEI FARM IN THE WESTERN CAPE PROVINCE (32º21’26”S 18º55’82”E). FOUR PALMER NAVEL ORANGE ORCHARDS OF APPROXIMATELY 1 H WITH MICRO SPRINKLER IRRIGATION, WERE USED. COMMERCIALLY FORMULATED, S *Heterorhabditis bacteriophora* INFECTIVE JUVENILES (IJS) (E-NEMA, GERMANY) WERE APPLIED

27 SEPTEMBER 2011 (SPRING) IN THREE OF THE ORCHARDS AND ONE WAS LEFT AS AN UNT
EPNS WERE APPLIED TO THE SOIL USING A SPRAY MACHINE IN TWO OF THE ORCHARDS (14
HA: 10 AND 20 IJS CM^{-2} RESPECTIVELY) AND THROUGH THE IRRIGATION SYSTEM (10 000 IJS CM^{-3}
WATER) IN THE OTHER – ALL FOLLOWED BY 6 H IRRIGATION.

BEFORE APPLICATION OF EPNS AND AT 1, 4 AND 8 WEEKS AFTER APPLICATION, MON
CONDUCTED TO DETERMINE PRESENCE OF EPNS IN THE SOIL. SIX SMALL CAGES, EA
T. leucotreta (SENTINEL) LARVAE, WERE PLANTED PER TREATMENT. CAGES WERE REMOV
WEEK AND *T. leucotreta* LARVAE WERE COUNTED, RECORDED AS ALIVE OR DEAD AND INFE
EPNS) OR NOT.

T. leucotreta PHEROMONE TRAPS WERE HUNG AND MONITORED WEEKLY FROM 6 OCTO
UNTIL THE TRIAL WAS TERMINATED ON 15 MARCH 2012. WEEKLY FROM 22 DECEMBER
MARCH 2012, ALL FRUITS WHICH HAD DROPPED FROM 10 DATA TREES IN THE MIDDLE OF
BLOCK WERE RETRIEVED AND ASSESSED (DISSECTED AND INSPECTED) FOR *T. leucotreta* INF

Entomopathogenic nematodes: conservation

ON CROCODILE VALLEY ESTATE IN MPUMALANGA PROVINCE (25º28'39"S 31º03'59"E), THE
OCCURENCE OF *H. zealandica* IN THE SOIL WAS DETERMINED TO BE HIGH. RUGBY (CADUSAFOS) (
ME (EW)) (FMC CHEMICALS, USA), A LOCALLY AVAILABLE NEMATICIDE, WAS APPLIED TO
A 1 HA BLOCK OF WASHINGTON NAVEL ORANGE TREES; AN ADJACENT BLOCK OF THE SA
UNTREATED AND USED AS A CONTROL. RUGBY WAS APPLIED TO THE SOIL UNDERNEATH
20 ML (A.I.) M^{-2} AND FOLLOWED BY IRRIGATION. NATURAL OCCURRENCE OF EPNS AND THE
CADUSAFOS APPLICATION ON THESE EPNS WAS DETERMINED AS DESCRIBED FOR THE PREV
WAS DONE BEFORE APPLICATION AND 2, 4 AND 8 WEEKS POST-APPLICATION. MONITO
INFESTATION BY *T. leucotreta* LARVAE WAS ALSO INITIATED IMMEDIATELY AFTER APPLICATION (
2012), AS DESCRIBED ABOVE, AND CONTINUED UNTIL 25 APRIL 2012.

Entomopathogenic fungi: bioassays

GOBLE *et al.* (2010) IDENTIFIED 62 POTENTIALLY USEFUL EPF ISOLATES FROM IN AND ARO
ORCHARDS IN THE EASTERN CAPE PROVINCE OF SOUTH AFRICA. TWELVE ISOLATES WE
GOBLE *et al.* (2011) AS HAVING POTENTIAL FOR CONTROL OF *T. leucotreta* AND FRUIT FLIES.

IN THIS STUDY, EIGHT OF THESE ISOLATES (AND TWO COMMERCIAL ISOLATES)
INVESTIGATED IN THE FORM OF CONCENTRATION DOSE-RESPONSE BIOASSAYS, USING THI
(1×10^4, 1×10^5 AND 1×10^6 CONIDIA ML^{-1}). 1 ML FUNGAL SUSPENSION (5 ML) WAS MIXED WITH
AUTOCLAVED SAND (50 G) IN PETRI DISHES. TWENTY FIFTH-INSTAR LARVAE, READY TO PU
NEXT 24 HOURS, WERE PLACED ON THE SAND AND INCUBATED AT 26 °C (12:12 H, L:D). AFT
THE PUPAE WERE REMOVED AND PLACED ON STERILE SAND AND INCUBATED AS BEFOR
FIRST EMERGENCE, THE NUMBER OF DEAD PUPAE AS WELL AS EMERGED AND DEAD ADU
DEAD ADULTS AND PUPAE WERE SURFACE STERILISED IN 70% ETHANOL AND PLACED O
THAT MYCOSIS COULD BE OBSERVED. THE PROCEDURE WAS REPLICATED FOUR TIMES
PROBAN (VAN ARK, 1995) WAS USED TO DETERMINE THE LC_{30} AND LC_{50} VALUES FOR EACH FUNGAL
ISOLATE INVESTIGATED.

FOR EXPOSURE TIME-RESPONSE BIOASSAYS, TWO CONCENTRATIONS WERE INVESTIGA
(1×10^7 CONIDIA ML^{-1}) AND THE LC_{50} FOR EACH CONCENTRATION, FIFTH-INSTAR LARVAE WERE EXPOSED TO
INOCULATED SOIL FOR DIFFERENT TIME PERIODS (1, 3, 5 AND 7 DAYS). AN UNTREATED CON
FOR EACH TIME PERIOD. LOGIT ANALYSIS WAS USED TO DETERMINE THE LT_{30} AND LT_{50} VALUES FOR EACH
FUNGAL ISOLATE INVESTIGATED. THE COMMERCIAL ISOLATES WERE NOT INCLUDED.

Entomopathogenic fungi: field persistence

RICE OVERGROWN WITH EACH OF THREE FUNGAL ISOLATES (0.5 G PER ISOLATE) WAS SEPA
ISOLATE WITH 100 G AUTOCLAVED ORCHARD SOIL. THIS WAS THEN PLACED INTO NET B
FEW CENTIMETRES BELOW THE SOIL UNDER CITRUS TREES AT MOSSLANDS FARM IN T
PROVINCE (33°24′26″S 26°26′E). EACH MONTH FOR SIX MONTHS, FOUR BAGS OF EACH ISOLA
CONTROL WERE COLLECTED. LABORATORY ASSAYS WERE CONDUCTED USING 50 G SOIL F
FROM THE CONTROL, FOLLOWING THE PROCEDURE DESCRIBED ABOVE. IN ADDITION, CFU
UNIT) COUNTS WERE PERFORMED FOR EACH NET BAG INCLUDING THE CONTROL. NON-N
ANALYSED USING THE KRUSKAL-WALLIS NON-PARAMETRIC TEST AFTER TRANSFORMATIO
TO A MULTIPLE MEAN RANK TEST. NORMALLY DISTRIBUTED DATA WERE ANALYSED BY A
BY TUKEY'S POST-HOC TEST. LINEAR REGRESSION ANALYSIS WAS USED TO TEST FOR COR
THE MONTHLY CFU COUNT AND MYCOSIS PERCENTAGE.

Results and discussion

Entomopathogenic nematodes: introduction

THE LOWEST *T. leucotreta* TRAP CATCHES AND FRUIT INFESTATION WERE RECORDED IN THE TW
WHERE EPN PERSISTENCE WAS GOOD, I.E. THE 10 IJS CM^{-2} SPRAY AND THE 20 IJS CM^{-2} IRRIGATION.
RELATIVE TO THE UNTREATED CONTROL, FCM INFESTATION OVER THIS PERIOD WAS RE
AND 54.55% BY THE 10 IJS CM^{-2} TREATMENT AND THE IRRIGATION TREATMENT, RESPECTIVELY.
THE 20 IJS CM^{-2} SPRAY TREATMENT (WHICH CAN BE CONSIDERED AS A SECOND UNTREA
BECAUSE NO SURVIVAL OF EPNS WAS RECORDED BEYOND ONE WEEK) INFESTATION WA
80.95% AND 76.19%, RESPECTIVELY. EPN SURVIVAL IN THIS ORCHARD WAS POOR, AS THE
SYSTEM IN THE ORCHARD WAS DEFICIENT, RESULTING IN INADEQUATE SOIL-MOISTURE
APPLICATION. TRAP CATCHES AND FRUIT INFESTATION WERE SIGNIFICANTLY HIGHER IN T
(THE HIGH-DOSE SPRAY TREATMENT) THAN THE OTHER TWO EPN TREATMENTS (P<0.05). TR
FRUIT INFESTATION BETWEEN THE MICRO SPRINKLER-APPLIED TREATMENT AND THE UN
NOT DIFFER SIGNIFICANTLY (P > 0.05).

Entomopathogenic nematodes: conservation

BEFORE APPLICATION OF THE NEMATICIDE, EPN LEVELS IN THE TWO ORCHARDS WERE SIM
FOUR WEEKS AFTER APPLICATION, MEAN (± SE) PERCENTAGE INFESTATION OF SENTINE
LARVAE WITH *H. zealandica* IN THE UNTREATED CONTROL WAS 19.5 ± 8.9% AND 24.7 ± 12.3%
RESPECTIVELY, WHILE IN THE NEMATICIDE-TREATED BLOCK IT WAS 1.9 ± 1.9% AND 3
RESPECTIVELY. RUGBY SIGNIFICANTLY REDUCED THE LEVEL OF EPNS IN THE SOIL, BUT T
RECOVERED BY EIGHT WEEKS AFTER TREATMENT.

CONSEQUENTLY, MEAN (± SE) NUMBER OF FRUIT INFESTED WITH *T. leucotreta* LARVAE PER TREE PER
WEEK FOR THE 13 WEEKS FROM IMMEDIATELY POST-APPLICATION OF THE NEMATICI
(3 FEBRUARY TO 25 APRIL 2012), WAS 0.09 ± 0.03 IN THE CONTROL BLOCK AND 0.22 ± 0.06
TREATED BLOCK. ALTHOUGH INFESTATION WAS MORE THAN 2.4 TIMES HIGHER IN THE N
THAN UNTREATED BLOCK, THE DIFFERENCE WAS NOT STATISTICALLY SIGNIFICANT (P > 0.0

Entomopathogenic fungi: bioassays

THE THREE ISOLATES WHICH GENERALLY CAUSED THE LOWEST *T. leucotreta* PUPAL
MORTALITY AND LOWEST LC_{50} AND LC_{90} VALUES ARE LISTED IN TABLE 1. THE COMMERCIAL PRODUC
NOT FARE WELL IN COMPARISON (TABLE 1), HOWEVER, NEITHER OF THEM ARE RE
RECOMMENDED FOR USE AGAINST *T. leucotreta*.

TABLE 1. LETHAL CONCENTRATIONS (LC_{50} AND LC_{90}) FOR THE THREE MOST PROMISING FUNGAL ISOLA
AND TWO COMMERCIAL ISOLATES.

Species	Isolate	Lethal Concentration [CONIDIA ML^{-1}]	
		LC_{50}	**LC_{90}**
M. anisopliae	G 11 3 L6	6.26×10^{5}	1.91×10^{7}
M. anisopliae	FCM AR 23 B3	1.92×10^{6}	1.67×10^{8}
B. bassiana	G AR 17 B3	1.98×10^{5}	1.02×10^{7}
B. bassiana	ECO-BB®	2.16×10^{6}	1.92×10^{10}
M. anisopliae	ICIPE 69	2.60×10^{7}	2.08×10^{10}

LOGIT ANALYSIS INDICATED THAT IT WOULD REQUIRE A MINIMUM OF 5 AND A MAXIMU
TO OBTAIN AN LT_{50} AND LT_{90} RESPECTIVELY AT THE LOWEST CONCENTRATION WHILST AT THE HIGHE
CONCENTRATION, 1×10^{7} CONIDIA ML^{-1}, A MINIMUM EXPOSURE TIME OF 4 AND A MAXIMUM OF 9 DAY
WAS REQUIRED TO OBTAIN AN LT_{50} AND LT_{90}, RESPECTIVELY.

Entomopathogenic fungi: field persistence

FOR ALL ISOLATES, INCLUDING THE COMMERCIAL ISOLATES TESTED, A LARGE INITIAL DEC
IN THE NUMBER OF CFU PER GRAM OF SOIL OVER THE FIRST MONTH. CFU NUMBERS
THEREAFTER. AFTER SIX MONTHS IN THE FIELD, ALL FUNGAL ISOLATES WERE STILL PR
RELATIVELY LOW NUMBERS, WITHIN THE SOIL (G 11 3 L6 – 6.14×10^{4} CFU G^{-1}; FCM AR 23 B3 –
1.46×10^{3} CFU G^{-1}; G AR 17 B3 – 2.71×10^{4} CFU G^{-1}; ECO-BB® - 2.93×10^{1} CFU G^{-1} AND
ICIPE 69 – 9.42×10^{2} CFU G^{-1}). THE GREATEST DECREASE IN CFU OBTAINED FOR ECO-BB®
WITH THE LEAST FOR G 11 3 L6.

AVERAGE PERCENTAGE MYCOSIS VARIED GREATLY FOR ALL ISOLATES OVER THE SIX
SOME CASES, EVEN THOUGH A DECREASE IN THE NUMBERS OF CFU WAS RECORDED, THE AVERAGE
PERCENTAGE MYCOSIS STILL INCREASED. FOR EXAMPLE, FOR ISOLATE G 11 3 L6, EVEN THO
COUNT RECORDED AT WEEK 4 WAS LOWER THAN THAT RECORDED AT WEEK 0, THE PERCENTAGE MYCOSIS STILL
INCREASED SIGNIFICANTLY FROM 59.9% TO 92.3%.

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Do plant-associated insect toxin producing pseudomonads have the potential for the biocontrol of insect pests?

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Abstract: THE EXCELLENT ROOT COLONIZERS *Pseudomonas protegens* AND *Pseudomonas chlororaphis* WERE SO FAR MAINLY KNOWN FOR THEIR ABILITY TO PRODUCE ANTIFUNGAL COMPOUNDS A TO SUPPRESS SOIL-BORNE PLANT DISEASES. IN CONTRAST TO OTHER BIOCONTROL PSEUDOMONADS THESE SPECIES ARE ALSO ABLE TO PRODUCE A POTENT INSECT TOXIN (FIT) AND TO DISPLAY ORAL AND INJE ACTIVITY AGAINST INSECTS. *Pseudomonas* BASED PRODUCTS AGAINST BACTERIAL AND FUNGAL PLANT DISEASES ARE ALREADY ON THE MARKET. IF *Pseudomonas* BIOCONTROL AGENTS COULD BE USED AGAINST BOTH, PLANT DISEASES AND PLANT PESTS THIS WOULD OF COURSE BE HIGHLY INTERESTING DEVELOPMENT OF A NEW KIND OF BIOCONTROL PRODUCT. WE ARE THEREFORE CURRENTLY EVALUATI POTENTIAL OF FIT TOXIN PRODUCING PSEUDOMONADS FOR THE BIOLOGICAL CONTROL OF INSECT PESTS FIT PRODUCING MODEL STRAINS *P. protegens* CHA0 AND *P. chlororaphis* PCL1391, WHICH ARE HIGHLY EFFECTIVE AGAINST FUNGAL DISEASES, DISPLAY ALSO HIGH ORAL ACTIVITY AGAINST DIFFERENT LEPIDOPTERAN INSECT PESTS. CHA0 IS ALSO ORALLY ACTIVE AGAINST THE APHID *Acyrtho pisum*, BUT IS NEITHER TOXIC TO THE LARGE EARTH BUMBLEBEE *Bombus terrestris* AN IMPORTANT POLLINATOR NOR TO LARVAE OF THE MOSQUITO *Aedes aegypti*. CHA0 CAN ESTABLISH HIGH POPULATIONS IN LARVAE OF THE ROOT WEEVIL *Otiorhynchus sulcatus* AND OF THE EUROPEAN COCKCHAFER (*Melolontha melolontha)* AND CHANGE THE COMPOSITION OF THE LARVAE'S OWN BACTERIAL FLO WHEN FED TO LARVAE OF *O. sulcatus*, CHA0 SURVIVES THE PUPAL STAGE AND CAN BE RECOVERED FROM HATCHED ADULTS. ADDITIONAL EXPERIMENTS SHOWED THAT CHA0 IS COMPATIBLE WITH THE BI FUNGUS *Metarhizium anisopliae*. TAKEN TOGETHER OUR RESULTS SUGGEST THAT FIT PRODUCING PSEUDOMONADS CAN ESTABLISH AND SURVIVE VERY WELL IN INSECTS, BUT THAT HIGH VIRULENCE MIG SPECIFIC FOR CERTAIN INSECT GENERA.

Key words: *Pseudomonas protegens, Pseudomonas chlororaphis*, FIT INSECT TOXIN

Insect pathogens and entomoparasitic nematodes
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Untangling insect pathogenicity in plant-associated pseudomonads by a combination of comparative genomics and bioassays

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Abstract: *Pseudomonas* BACTERIA DEMONSTRATE IN A STUNNING WAY THE METABOLIC AND ECOLOGICA DIVERSITY OF BACTERIAL LIFESTYLES. THEIR ECOLOGY MAY DIFFER DRAMATICALLY, EVEN BETWEEN CLO RELATED STRAINS AND INCLUDE PATHOGENIC AND BENEFICIAL BACTERIA. THE *Pseudomonas fluorescens* GROUP HARBORS MANY ROOT-ASSOCIATED BIOCONTROL AGENTS THAT SUPPRESS SOIL-BORNE FUNGAL OF MANY DIFFERENT CROPS. REMARKABLY, STRAINS OF THE SPECIES *Pseudomonas protegens* AND *Pseudomonas chlororaphis* ALSO DISPLAY POTENT ORAL INSECTICIDAL ACTIVITY TOWARDS LEPIDOPTERA INSECT LARVAE. INSECTICIDAL ACTIVITY IS TRIGGERED IN PART BY THE FIT INSECT TOXIN AND UNKN GACA REGULATED TRAITS. THE MAIN AIM OF THE PRESENTED STUDY IS TO DISCOVER THE TRAITS ENA PLANT-ASSOCIATED PSEUDOMONADS NOT ONLY TO ANTAGONIZE FUNGAL PATHOGENS BUT ALSO TO COLONIZE AND KILL INSECTS. TO THIS END, 15 STRAINS OF FLUORESCENT PSEUDOMONADS WERE CHARACTERIZED FOR THEIR BIOCONTROL ACTIVITY AGAINST ROOT PATHOGENS, AS WELL AS T INSECTICIDAL ACTIVITY AND THEIR ABILITY TO MULTIPLY IN INSECT LARVAE. WHILE MOST STRAINS WERE TO COLONIZE INSECT LARVAE UPON INGESTION, THE LETHAL ORAL ACTIVITY WAS RESTRICTED TO ST *P. protegens* AND *P. chlororaphis*. THE EXPERIMENTAL APPROACH WAS COMPLEMENTED BY A COMPARATIVE GENOMIC SURVEY OF THE 15 FLUORESCENT PSEUDOMONADS, TO YIELD AN IMPROVE UNDERSTANDING OF INSECT PATHOGENICITY AND THE CONTRIBUTING VIRULENCE FACTORS. WE FOUND T REMARKABLE 37% OF PREDICTED PROTEIN CODING GENES SHARED BY *P. protegens* AND *P. chlororaphis*, BUT ABSENT IN OTHER STRAINS OF THE *P. fluorescens* GROUP, COULD BE POTENTIALLY ASSOCIATED WITH INSECT VIRULENCE. OUR COMBINED APPROACH OF GENOMIC SURVEY AND BIOASSAY REVEALS INTRIGUING ASPECTS ON INSECT ASSOCIATION AND INSECT PATHOGENESIS OF PLANT-ASSOC PSEUDOMONADS AND IDENTIFIES SEVERAL *Pseudomonas* STRAINS WITH POTENT DUAL ACTIVITY AGAINST ROOT PATHOGENS AND INSECT PESTS.

Key words: *Pseudomonas* SPECIES, PLANT ASSOCIATION, GENOMICS, INSECT VIRULENCE

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Colorado potato beetle (*Leptinotarsa decemlineata* Say) – control strategies in organic farming using biological insecticides (azadirachtin, *Bacillus thuringiensis* var. *tenebrionis*, pyrethrum and spinosad)

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Abstract: FIELD EXPERIMENTS USING DIFFERENT CONTROL AGENTS FOR COLARADO POTATO BEET
THAT ALL TESTED APPLICATION STRATEGIES WERE EFFECTIVE. TIME-SHIFTED APPLICATION OF NE
AND *Bacillus thuringiensis* VAR. *tenebrionis* (*B.t.t.*) (NOVODOR FC) AS WELL AS DOUBLE TREATMENT WITH *B.t.*
ACHIEVED UNDER OPTIMAL WEATHER CONDITIONS GAVE AN EFFECTIVENESS LEVEL OF OVER
YIELDS. SURPRISINGLY, A SINGLE APPLICATION OF SPINOSAD (SPINTOR) ALSO PROVED TO BE
(> 80%) IN THE THREE YEARS STUDIED IN SPITE OF THE DIFFICULT STUDY CONDITIONS IN 2009. DUE
AND A HIGH EFFICACY OF SPINOSAD IT IS LIKELY FARMERS WOULD PREFER THIS PLANT PRO
CONSIDERING RESISTANCE OF THE COLORADO POTATO BEETLE IT IS RECOMMENDED TO CHAN
EVERY YEAR.

Key words: COLORADO POTATO BEETLE, ORGANIC FARMING SPINOSAD, *Bacillus thuringiensis*

Introduction

INGERMANY, THE INCREASING DEMAND FOR ORGANICALLY GROWN POTATOES COULD NO
THE ACREAGE HAS BEEN INCREASED TO MORE THAN 8200 HECTARES (ZMP, 2008). TWO
REASONS FOR THIS DEFICIT ARE YIELD LOSSES CAUSED BY EARLY SUMMER DROUGHT AND
BY THE COLORADO POTATO BEETLE. RECENT INCREASES IN POTATO BEETLE OCCURRENCE
GERMANY CAN BE ATTRIBUTED TO INCREASED AREA SIZE, REGIONAL CONCENTRATION
CROP ROTATION, AND INCREASING RESISTANCE OF THE POTATO BEETLE TO PYRETHROID
MANY AREAS, PREVENTIVE MEASURES DO NOT SUFFICE TO PREVENT POTATO BEETLE DA
CAN AND SHOULD BE USED TO PREVENT ECONOMIC LOSSES IN THESE CASES – EVEN IN
IN ORDER TO DEVELOP A STRATEGY FOR SUSTAINABLE CONTROL OF THE COLORADO POT
FARMING, THE JULIUS KÜHN INSTITUTE LAUNCHED A SERIES OF FIELD TESTS AT AN EU-
FARMING SITE (CONTROL NO. D-ST-043-4829) LOCATED IN DAHNSDORF, GERMANY. THES
FOCUSED ON THE EFFICACY, COMBINABILITY, OPTIMAL TIMING OF APPLICATION OF BIOLO
APPROVED FOR USE IN ORGANIC FARMING. THIS ARTICLE IS A CONTINUATION OF RESUL
PUBLISHED BY KÜHNE *et al.* (2012).

Material and methods

THESTUDIES WERE CONDUCTED IN ACCORDANCE WITH THE SPECIFICATIONS IN EPPO GUI
(3). THE TESTS WERE CONDUCTED USING A BLOCK DESIGN WITH FOUR REPETITIONS OF

VARIANT AND ONE UNTREATED CONTROL. THE PLOT SIZE FOR EACH TREATMENT VARIANT WAS 24 M². THE EXPERIMENT WAS CONDUCTED IN 3 YEARS. THE NUMBER OF POTATO BEETLES AND THE PERCENTAGE OF FEEDING DAMAGE WERE ASSESSED WEEKLY ON THE SAME 10 RANDOMLY SELECTED AND MARKED POTATO PLANTS PER TREATMENT; THIS MADE IT POSSIBLE TO ESTIMATE THE VARIANCE ATTRIBUTABLE TO LOCAL COLONISATION OCCURRENCE AND TO PERFORM AN ANALYSIS OF INFESTATION DYNAMICS PER PLANT.

IN PRACTICE, TREATMENT IS USUALLY NECESSARY IN POTATO FIELDS WHEN THE AVERAGE BEETLES DETECTED IS ONE EGG MASS OR 10 LARVAE (L1, L2) PER PLANT ON 5 PLANTS FROM 5 LINES DISTRIBUTED ACROSS A GIVEN FIELD. IN ADDITION TO SCOUTING, SIMULATIONS (SIMULATION: LEPTINOTARSA), A COMPUTER-AIDED DECISION SUPPORT TOOL DEVELOPED BY THE OFFICE OF THE GERMAN STATES (ZEPP), WERE PERFORMED IN ALL THE YEARS STUDIED. SIMULATION WAS USED TO MODEL THE POPULATION DYNAMICS (MAXIMUM OCCURRENCE OF DEVELOPMENTAL STAGES) OF THE COLORADO POTATO BEETLE IN ORDER TO DETERMINE THE OPTIMAL TIMING OF TREATMENT. THE TEMPERATURE-SUM METHOD TO CALCULATE THE POPULATION DYNAMICS OF THE POTATO BEETLE FROM THE FIRST SIGN OF EGG-LAYING TO THE EMERGENCE OF FIRST INSTAR LARVAE. THE DATE OF EGG-LAYING IN THE FIELD WAS USED AS THE INPUT VALUE FOR MODEL CALCULATION OF THE TIME OF MAXIMUM OCCURRENCE OF FIRST INSTAR LARVAE. LOCAL WEATHER DATA ARE ALSO NEEDED FOR THE FORECAST. CALCULATIONS WERE BASED ON WEATHER DATA OBTAINED FROM THE WEATHER STATION LOCATED DIRECTLY IN THE FIELD AT DAHNSDORF.

TABLE 1. BIOLOGICAL INSECTICIDES USED TO CONTROL THE COLORADO POTATO BEETLE

	NeemAzal-T/S	**Novodor FC**	**SpinTor**
ACTIVE SUBSTANCE CONCENTRATION	10 G L^{-1} AZADIRACHTIN (NEEM)	20 G L^{-1} *Bacillus thuringiensis* VAR. TENEBRIONIS (*B.t.*)	480 G L^{-1} SPINOSAD
FOR CONTROL OF	LARVAE	LARVAE	BEETLES AND LARVAE
HAZARD SYMBOL	NONE	XI – IRRITATING	N – DANGEROUS FOR THE ENVIRONMENT
WATER POLLUTION CONTROL	NO MINIMUM DISTANCE TO SURFACE WATERS REQUIRED	NO MINIMUM DISTANCE TO SURFACE WATERS REQUIRED	MAINTAIN MINIMUM DISTANCE TO SURFACE WATERS; VERY TOXIC TO FISH, FISH BAIT AND ALGAE
BEE PROTECTION	NON-TOXIC TO BEES	NON-TOXIC TO BEES	TOXIC TO BEES
BENEFICIAL INSECTS (BIS)	NOT HARMFUL TO BIS EXCEPT HOVERFLIES (*Episyrphus balteatus*)	SLIGHTLY HARMFUL TO SEVEN-SPOT LADYBIRDS (*Coccinella septempunctata*)	SLIGHTLY HARMFUL TO SEVEN-SPOT LADYBIRDS (*Coccinella septempunctata*); HARMFUL TO EGG PARASITOIDS (*T. dendrolini*)
APPLICATION VOLUME (L/HA)	2.5	3.0 (L1 TO L2) 5.0 (L3 TO L4)	0.05
WATER VOLUME (L/HA)	400	500	400
PRICE PER UNIT	55 € L^{-1}	21 € L^{-1}	375 € L^{-1}
TREATMENT COSTS	16 € HA^{-1}	16 € HA^{-1}	16 € HA^{-1}

THE DIFFERENT BIOLOGICAL INSECTICIDE TREATMENT VARIANTS (TABLE 1) WERE AI
TO THE MANUFACTURER'S INSTRUCTIONS AT THE OPTIMAL APPLICATION TIMES AND UNI
CONDITIONS (NO DIRECT SUNLIGHT, WIND SPEED < 1 M/S, AND TEMPERATURE < 20 °C) EAC
2009, DURING WHICH THE TREATMENTS HAD TO BE POSTPONED BECAUSE THE POTATO B
NOT REACH THE THRESHOLD LEVEL DURING THE OPTIMAL TREATMENT TIME FRAME PRED

THE FIRST EGG MASSES WERE FOUND ON 26 MAY 2009, BUT TREATMENT WAS NOT NEC
5 WEEKS LATER (5 JULY 2009). THE REASON FOR THIS DELAY WAS A COLD SNAP AT THE BEG
LAYING IN LATE MAY 2009, WHICH PROLONGED THE OVIPOSITION PERIOD BY SEVERAL WE
RESULTED IN EXTENSION OF THE HATCHING PERIOD, AND THE BEETLE COUNTS DID NOT E
THRESHOLD UNTIL NEARLY 3 WEEKS LATER THAN IN THE PREVIOUS YEARS OF THE STUDY
2009 TREATMENTS WERE NOT ADMINISTERED UNTIL THE 7TH AND 10TH OF JULY, WHICH
THE PREDICTED OPTIMAL TREATMENT TIME FRAME SET BY SIMLEP3. FURTHERMORE, A
OCCURRING SHORTLY AFTER THE SECOND TREATMENT DATE SEVERELY IMPAIRED TH
TREATMENT PRODUCTS. BECAUSE THE 2009 PLANT SURVEYS WERE ALSO PERFORMED LA
YEARS.

LATE BLIGHT (*Phytophthora infestans*) WAS TREATED AS NEEDED USING A COPPER-BASED PRO
(CUPROZIN FLÜSSIG, 750 G L^{-1} COPPER PER TREATMENT) THROUGHOUT THE ENTIRE TEST SITE I
THE AMOUNT OF PURE COPPER USED WAS 2.25 KG HA^{-1} IN 2008 AND 2009, 1.5 KG HA^{-1} IN 2010.
SUFFICIENT CONTROL OF LATE BLIGHT WAS ACHIEVED IN ALL YEARS EXCEPT 2009.

Results and discussion

THE RESULTS OF OUR STUDIES SHOWED THAT ALL APPLICATION STRATEGIES WERE EFFE
COLORADO POTATO BEETLE (TABLE 2).

TABLE 2. CONTROL OF THE COLORADO POTATO BEETLE BY BIOLOGICAL INSECTICIDES A
DAHNSDORF, GERMANY (BRANDENBURG), DEGREE OF EFFECTIVENESS (%) BASED ON THE
AREA LOSS ATTRIBUTABLE TO FEEDING BY THE POTATO BEETLE 24-25 DAYS AFTER THE F
INCREASE IN YIELD (DT HA^{-1}) RELATIVE TO THE YIELDS IN THE UNTREATED CONTROLS (UC). * ST
SIGNIFICANT DIFFERENCE TO THE UNTREATED CONTROLS (TUKEY; P < 0.05). ACTIVE ING
Bacillus thuringiensis VAR. *tenebrionis* (*B.t.t.*) 20 G L^{-1} (NOVODOR FC), NEEM 10 G L^{-1}
(NEEMAZAL-T/S), AND SPINOSAD 480 G L^{-1} (SPINTOR).

Year	1st treatment (T1)	Dose (l ha^{-1})	2nd treatment (T2)	Dose (l ha^{-1})	Time of 2nd treatment	Effectiveness (%)	Increase in yield (dt ha^{-1})
2008	*B.t.t.*	3	*B.t.t.*	5	T1 + 4 DAYS	78 *	54 *
2009	*B.t.t.*	3	*B.t.t.*	5	T1 + 3 DAYS	37 *	40 *
2010	*B.t.t.*	3	*B.t.t.*	5	T1 + 4 DAYS	88*	52
2008	NEEM	2.5	*B.t.t.*	3	T1 + 4 DAYS	82 *	70 *
2009	NEEM	2.5	*B.t.t.*	5	T1 + 3 DAYS	43 *	53 *
2010	NEEM	2.5	*B.t.t.*	5	T1 + 4 DAYS	82 *	21
2008	SPINOSAD	0.05	NONE	NONE	NONE	82 *	103 *
2009	SPINOSAD	0.05	NONE	NONE	NONE	83 *	37 *
2010	SPINOSAD	0.05	NONE	NONE	NONE	87 *	17

TIME-SHIFTED APPLICATION OF NEEM (NEEMAZAL TS) AND *B.t.t.* (DIPEL ES OR FC) LEADS TO THE RELATIVELY RAPID CESSATION OF FEEDING FOLLOWING INGESTION. NEEM SHOULD BE ADMINISTERED BEFORE *B.t.t.* WHENEVER THE TIME-SHIFTED COMBINATION STRATEGY IS USED. THIS APPROACH ACHIEVED AN EFFECTIVENESS LEVEL OF OVER 80% AND INCREASED YIELDS BY 1 T HA^{-1} IN THREE OUT OF FOUR YEARS STUDIED (2006 TO 2008). IN 2009, THE TWO-TIME TREATMENT WITH *B.t.t.* ALSO ACHIEVED COMPARABLY GOOD RESULTS. HOWEVER, A SECOND APPLICATION OF ACTIVE INGREDIENT WITHIN A GIVEN YEAR IS NOT RECOMMENDED IN PRACTICE DUE TO RESISTANCE DEVELOPMENT (KÜHNE *et al*., 2012).

SURPRISINGLY, A SINGLE APPLICATION OF SPINOSAD (SPINTOR) ALSO PROVED TO BE VERY EFFECTIVE (> 80%) IN THE THREE YEARS STUDIED IN SPITE OF THE DIFFICULT STUDY CONDITIONS IN 2009. THE DEGREE OF EFFECTIVENESS WAS VERY HIGH (2009) IN THE SPINOSAD GROUP (83%) COMPARED WITH 43% (NEEM + *B.t.t.*) AND 37% (*B.t.t.* + *B.t.t.*) IN THE OTHER TWO GROUPS (STATISTICALLY SIGNIFICANT). THIS DID NOT RESULT IN HIGHER YIELDS THAN IN THE OTHER TWO GROUPS. THE REASON FOR THIS WAS THE SPREAD OF LATE BLIGHT, WHICH COULD NOT BE ADEQUATELY MANAGED IN 2009, RESULTING IN 2.0 T HA^{-1}. HOWEVER, THE INCREASE IN YIELD OF THE DIFFERENT TREATMENT VARIANTS COMPARED WITH THE CONTROLS WAS STATISTICALLY SIGNIFICANT (RANGE 2.0 TO 3.1 T HA^{-1}). THE YIELD DIFFERENCES BETWEEN THE UNTREATED CONTROL AND THE TREATMENT VARIANTS ARE NOT STATISTICALLY SIGNIFICANT. THE REASON LIES IN THE SEVERE DROUGHT, THE HIGH BLOCK VARIANCES MEANT.

THE COST OF TREATMENT WAS LOWER WHEN USING SPINOSAD WITH 136 € HA^{-1} COMPARED TO NEEM AND *B.t.t.* BY 277 € HA^{-1} AND SHIFTED *B.t.t.* APPLICATION BY 203 € HA^{-1}.

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Nematodes

Insect pathogens and entomoparasitic nematodes
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Update on life cycle of entomopathogenic nematodes

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Abstract: ENTOMOPATHOGENIC NEMATODES (EPN) ARE WITHIN THE GENERA *Heterorhabditis* AND *Steinernema*, WHICH ARE CLOSELY ASSOCIATED WITH ENTEROBACTERIA OF THE GENERA *Photorhabdus* AND *Xenorhabdus*, RESPECTIVELY. THE LIFE CYCLE OF THE NEMATODES WILL BE REVIEWED. WITH HELP OF A VIDEO THE DEVELOPMENT OF *Heterorhabditis bacteriophora* IN LARVAE OF THE CORN ROOTWORM WILL BE FOLLOWED (E-NEMA, 2013). THE ADULT NEMATODES (HERMAPHRODITIC) EGGS INTO THE INSECT CADAVER FROM WHICH OFFSPRING DEVELOP INTO A SECOND AD AMPHIMICTICS (FEMALE AND MALE) AND DAUER JUVENILES (DJS). ON DAY 6 AFTER INFECTI HATCH INSIDE THE UTERUS AND DEVELOP TO DAUER JUVENILES (DJ), FEEDING ON THE CONTENT (ENDOTOKIA MATRICIDA). THE RESULTING DJS HAVE TO LOAD THEMSELVES WIT SYMBIONT. IN THE PAST, WE SUGGESTED THAT THE PRE-DAUER STAGES LOADED THE MOMENT THE INTESTINE OF THE ADULT NEMATODE DISSOLVED (JOHNIGK & EHLERS, HERMAPHRODITE IS STILL ALIVE INGESTING HAEMOLYMPHE WITH A MIX OF MICRO-ORG THEORY WAS DISPROVEN *et al.* (2008) INVESTIGATED TRANSMISSION OF CELLS OF THE SYMBI IN DETAIL. ALREADY DURING THE J4 STAGE THE SYMBIONT CELLS ARE FORMING A BIOFILM OF THE RECTAL INTESTINAL CELLS AND ARE THEN TRANSFERRED INTO THE RECTAL GLAN GLAND CELL VACUOLES THE SYMBIONT CELLS SEEM TO DIVIDE AND FILL THE VACUOLES. TH HAVE HATCHED INSIDE THE UTERUS, DESTROY THE UTERUS TISSUE AND THEN GET ACC BODY CAVITY. ALMOST AT THE SAME TIME THE RECTAL GLAND CELLS LYSE AND RELEASE TH THE SYMBIONT CELLS COLONIZE THE BODY CAVITY OF THE MOTHER. THE J1 ARE THUS EX SYMBIONT CELLS AND ARE ABLE TO LOAD THEMSELVES MUCH BEFORE THE INTESTINE OF TH LYSES DURING ENDOTOKIA MATRICIDA.

EPN ARE ABLE TO GROW AND REPRODUCE ON NON-SPECIFIC SYMBIOTIC BACTERIA, BU TRANSMIT THEM IN THE DAUER JUVENILE. OTHER THAN HETERORHABDITIDS DJS, WHICH H IN THE ANTERIOR PART OF THEIR INTESTINE, DJS OF THE GENUS *Steinernema* FORM A RECEPTACLE (FORMERLY VESICLE) TO HARBOUR CELLS OF THEIR SPECIFIC SYMBIONT. HEIDI GOODRICH WORKERS HAVE INVESTIGATED THE RELATION BETWEEN *Xenorhabdus* BACTERIA AND (BHASIN *et al.*, 2012). FEW BACTERIAL CELLS ENTER INTO THE RECEPTACLE DURING DJ FORM THEN OUTGROW TO AN INITIATING POPULATION TO 30 TO 200 BACTERIAL CELLS, ENTIR RECEPTACLE. CELLS FIRST ADHERE TO A NEMATODE-DERIVED ANUCLEATE CLUSTER OF CALLED THE INTRAVESICULAR STRUCTURE (IVS). NEMATODE INTESTINE LOCALIZATION (NIL) C, HAVE BEEN IDENTIFIED IN *X. nematophila* THAT CONTRIBUTE TO SPECIFICITY AND ARE NOT DETE OTHER *Xenorhabdus* SPP. NILB IS A SURFACE EXPOSED OUTER MEMBRANE PROTEIN WHOSE EX IS REPRESSED BY NILR AND GROWTH IN NUTRIENT-RICH MEDIUM. MUTANT NILB STRA RETAINED IN THE RECEPTACLE. STUDIES WITH GFP BACTERIA REVEALED THAT CELLS FI ANTERIOR INTESTINAL CELLS DURING DEVELOPMENT OF THE FIRST JUVENILE STAGES AT INTESTINAL VALVE (PIV) ANTERIOR TO THE INTESTINAL EPITHELIUM. THE INTESTINE THEN *X. nematophila* CELLS REMAIN AT THE PIV. THE ANTERIOR INTESTINE CONSTRICTION THEN R AND COLONIZING BACTERIA OCCUPY THE RECEPTACLE. *Photorhabdus* SPP. SPECIFIC BINDING TO NEMATODE EPITHELIA HAS ALSO REPORTED FROM *H. bacteriophora*, *H. megidis*, *S. carpocapsae* AND *S. feltiae*, HOWEVER, BINDING TO

THE POSTERIOR INTESTINE CELLS HAS NOT BEEN REPORTED IN STEINERNEMATIDS AND
JUVENILES DURING ENDOTOKIA MATRICIDA IS NOT YET UNDERSTOOD IN STEINERNEMATID
NEW FINDINGS HAVE BEEN REPORTED BY THE GROUP OF NELSON SIMOES ON EPN
MECHANISMS (JING *et al.*, 2010; YOU-JIN *et al.*, 2009; 2010). EPN VIRULENCE IS DEPENDING ON THE
ABILITY OF THE NEMATODES TO INVADE HOSTS AND TO OVERCOME INSECT DEFENCES.
SUPPORTED BY THE RELEASE OF ENZYMES. TRANSCRIPTS ANALYSIS OF *S. carpocapsae* IS IDENTIFY
GENES ENCODING FOR PROTEASES (JING *et al.*, 2010; YOU-JIN *et al.*, 2009; 2010), WHICH HAVE BEEN
DETECTED TO PLAY A KEY ROLE IN NEMATODE PENETRATION INTO INSECTS. AMONG THE G
BE INVOLVED IN INSECT IMMUNE SUPPRESSION, EFFECTORS WERE IDENTIFIED, WHICH ARE
HUMORAL AND CELLULAR DEFENCES OF THE HOST INSECT ENABLING THE DJS AND THEIR RE
TO ESTABLISH IN THE HAEMOCEL (BALASUBRAMANIAN *et al.*, 2009; 2010).

Key words: LIFE CYCLE, SYMBIOSIS, VIRULENCE MECHANISMS, *Heterorhabditis bacteriophora*, *Photorhabdus luminescens*, *Steinernema carpocapsae*, *S. feltiae*, *Xenorhabdus nematophila*, *X. bovienii*

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Insect pathogens and entomoparasitic nematodes
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Aiming to eradicate small hive beetle *Aethina tumida* using entomopathogenic nematodes

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Abstract: THE SMALL HIVE BEETLE (*Aethina tumida*) IS AN ENDEMIC PARASITIC PEST AND SCAVENGER O COLONIES OF SOCIAL BEES INDIGENOUS TO SUB-SAHARAN AFRICA. IN THIS REGION THE BEETLES DAMAGE ON STRONG COLONIES SINCE THE BEES HAVE DEVELOPED STRATEGIES TO COMBAT *A. tumida* HAS SINCE 'ESCAPED' FROM ITS NATIVE HOME AND HAS RECENTLY INVADED AREAS S AMERICA AND AUSTRALIA WHERE ITS ECONOMIC IMPACT ON THE APICULTURE INDUSTRY HAS COMMERCIALLY AVAILABLE ENTOMOPATHOGENIC NEMATODES WERE SCREENED FOR THEIR PO BEETLE LARVAE. THE NEMATODES *Steinernema kraussei* AND *S. carpocapsae* PROVIDED EXCELLENT CONTROL WITH 100% MORTALITY OF LARVAE BEING OBTAINED. DELAYED APPLICATIONS OF THE NEMATODES ENTERING SAND TO PUPATE ALSO PROVIDED EXCELLENT CONTROL FOR UP TO 3 WEEKS. THE IN SUPPORTS THE DEVELOPMENT OF CONTINGENCY PLANS SHOULD *A. tumida* EVER OCCUR IN THE UK OR EUROPE.

Key words: *Aethina tumida*; *Apis mellifera*; BIOLOGICAL CONTROL; ENTOMOPATHOGENIC NEMATODES

Introduction

IN ITS NATIVE RANGE THE SMALL HIVE BEETLE, *Aethina tumida* MURRAY, COLEOPTERA: NITIDULIDAE) (SHB) IS AN OCCASIONAL PARASITE AND SCAVENGER OF HONEY BEE COLONIES INDIGE SAHARAN AFRICA (NEUMANN & ELZEN, 2004). HOWEVER, AS AN INVASIVE SPECIES IT HA MUCH ECONOMIC DAMAGE, AND SINCE 1996 HAS BECOME ESTABLISHED IN NORTH A AUSTRALIA. THE SHB HAS YET TO BE REPORTED IN EUROPE, SOUTH AMERICA OR ASIA (CU *et al.*, 2013).

THE SHB LIFECYCLE CONSISTS OF A PUPATION STAGE THAT OCCURS OUTSIDE THE H SURROUNDING SOIL. BOTH LARVAE AND PUPAE CAN BE FOUND IN THE SOIL. THEREFO OPPORTUNITY FOR CONTROL MEASURES TO BE APPLIED AT THIS STAGE THAT WILL NOT HA THE BEES IN THE HIVE. BEEKEEPERS HAVE TRADITIONALLY USED PESTICIDES CONTAININ CONTROL LARVAE AND PUPAE IN THE SOIL (HOOD, 2004). HOWEVER, CONTINUED USE OF RISE TO RESISTANCE AND UNDESIRABLE SIDE EFFECTS ON BOTH HONEY BEES AND HUMAN BROWN, 2009). THEREFORE, THERE IS MUCH DEMAND TO IMPROVE THE RANGE OF PRODU FOR THE CONTROL OF THE LARVAE AND PUPAE STAGES. SUCH ALTERNATIVE CONTR ENTOMOPATHOGENIC NEMADODES (EPN), WHICH HAVE SUCCESSFULLY BEEN USED A INVERTEBRATE PESTS. IN REGARDS TO *A. tumida*, THE INFECTIVITY OF THREE SPECIES OF NEMATOD TOWARDS WANDERING LARVAE (THE LARVAL STAGE THAT IS ACTIVELY SEEKING A PUPATI PREVIOUSLY TO BE MODERATE (CABANILLAS & ELZEN, 2006).

Material and methods

Insect rearing

Aethina tumida WERE CULTURED AND MAINTAINED AS DESCRIBED BY CUTHBERTSON *et al.* (2008) UNDER STRICT QUARANTINE CONDITIONS. FINAL INSTAR (WANDERING) LARVAE WERE USED FO TRIALS. THE CONTROL AGENTS USED ARE ALL COMMERCIALLY AVAILABLE PRODUCTS IN EUROPE AND COMPRISED 3 SPECIES: *Steinernema feltiae* (NEMASYS), *S. kraussei* (NEMASYSL), *S. carpocapsae* (CAPSANEM). THE IMPACT OF DIRECT AND IN-DIRECT EXPOSURE ALONG WIT APPLICATION OF THE AGENTS SHOWING MOST POTENTIAL WERE INVESTIGATED IN SEPA (CUTHBERTSON, 2012).

Direct exposure of larvae to control agents

FOR DIRECT EXPOSURE TRIALS, INDIVIDUAL WANDERING LARVAE WERE DIPPED IN REC RATES OF THE NEMATODE PRODUCTS (10,000 INFECTIVE JUVENILES ml^{-1}) FOR 5 SECONDS.

Indirect exposure of larvae to control agents

FOR INDIRECT EXPOSURE, 7 CM DIAMETER BY 15 CM TALL PLASTIC CONTAINERS WERE F (8% MOISTURE CONTENT). 50 ML OF CONTROL PRODUCT (NEMATODE) WAS ADDED OVER TH SAND AT THE SAME DOSE RATES AS IN THE DIRECT TRIALS. ONCE THE SOLUTION HAD SO SAND, TEN WANDERING LARVAE WERE ADDED TO THE SURFACE. THE CONTAINERS W TREATMENTS WERE MAINTAINED FOR 6 WEEKS IN ORDER TO ALLOW ADULT BEETLES TO WAS CALCULATED AS THE NUMBER OF BEETLES THAT FAILED TO EMERGE.

Delayed application of nematodes against beetle larvae

DELAYED APPLICATION TRIALS USING THE TWO NEMATODE SPECIES THAT GAVE THE BES WERE CONDUCTED. TEN WANDERING SHB LARVAE WERE ADDED TO A CONTAINER. FOLL FIRST BATCH OF NEMATODE SOLUTION WAS ADDED. THEN AT WEEKLY INTERVALS, NEM WERE ADDED TO LARVAE INFESTED CONTAINERS. CONTROL CONTAINERS RECEIVED 50 ML

Results and discussion

DIRECT EXPOSURE DEMONSTRATED A SIGNIFICANT TREATMENT EFFECT ON THE WANDE COMPARED TO THE CONTROL ($P < 0.001$) (FIGURE 1). THE NEMATODES SHOWED PROMISE; *S. carpocapsae* ACHIEVED SIGNIFICANTLY HIGHER MORTALITY THAN *S. kraussei* AND *S. feltiae* ($P < 0.05$), WHICH IN TURN ACHIEVED SIGNIFICANTLY HIGHER MORTALITY THAN THE C DISSECTING THE LARVAE, NEMATODES FREELY EMERGED FROM THE BODY CAVITY CONFIR TO INFECT THE LARVAE. IT HAS BEEN STATED THAT SUSCEPTIBILITY OF INSECTS TO CONT DECLINES WITH INCREASING INSECT SIZE. THIS HAS BEEN DEMONSTRATED WITH MERMI AGAINST MOSQUITO LARVAE. HOWEVER, AS NEMATODES ENTER THROUGH THE NATURA LARVAE, GAUGLER & MOLLOY (1981) SHOWED THAT SUSCEPTIBILITY WAS A FUNCTION OF L LARGER LARVAE BEING MORE SUSCEPTIBLE TO NEMATODE INFECTION, SIMPLY DUE TO TH EASIER FOR NEMATODES TO ENTER THE NATURAL OPENINGS.

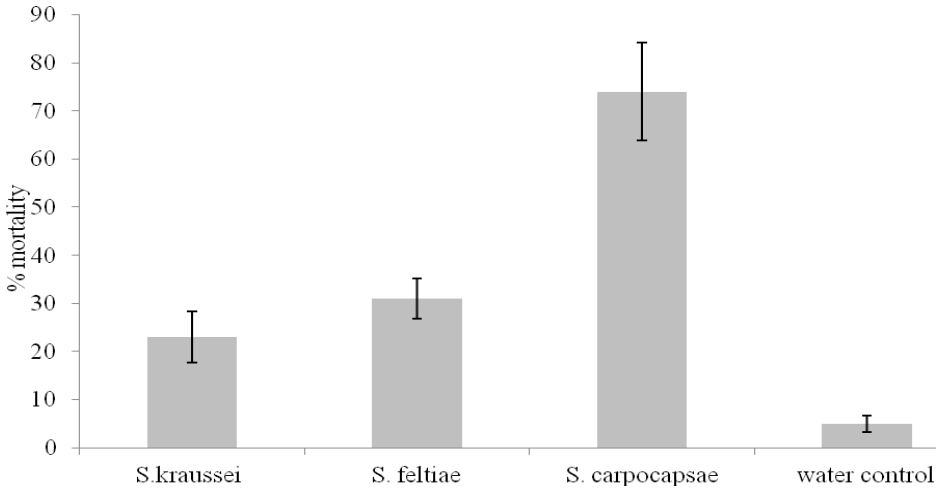

FIGURE 1. IMPACT OF DIRECT EXPOSURE OF CONTROL AGENTS ON *Aethina tumida* WANDERING LARVAE AFTER 2 WEEKS. ERROR BARS REPRESENT THE 95% CONFIDENCE INTERVALS (CUTHBERTSON *et al.*, 2012).

TREATING THE SAND BEFORE ADDING THE LARVAE EXPOSES THE SHB TO THE BIOC DURING PUPATION, AND MORE CLOSELY SIMULATES HOW BEEKEEPERS MIGHT APPLY SUCH FIELD. INDIRECT EXPOSURE DEMONSTRATED A SIGNIFICANT TREATMENT EFFECT ON SH COMPARED TO THE CONTROL ($P < 0.001$). TREATING THE SAND PRODUCED EXCELLENT RESULT *S. kraussei* AND *S. carpocapsae* WHERE TOTAL MORTALITY OF *A. tumida* WAS ACHIEVED. NO ADULTS EMERGED FROM EITHER OF THESE TWO TREATMENTS. *S. feltiae* ALSO SIGNIFICANTLY HIGHER MORTALITY THAN THE CONTROL ($P < 0.05$, FIGURE 2).

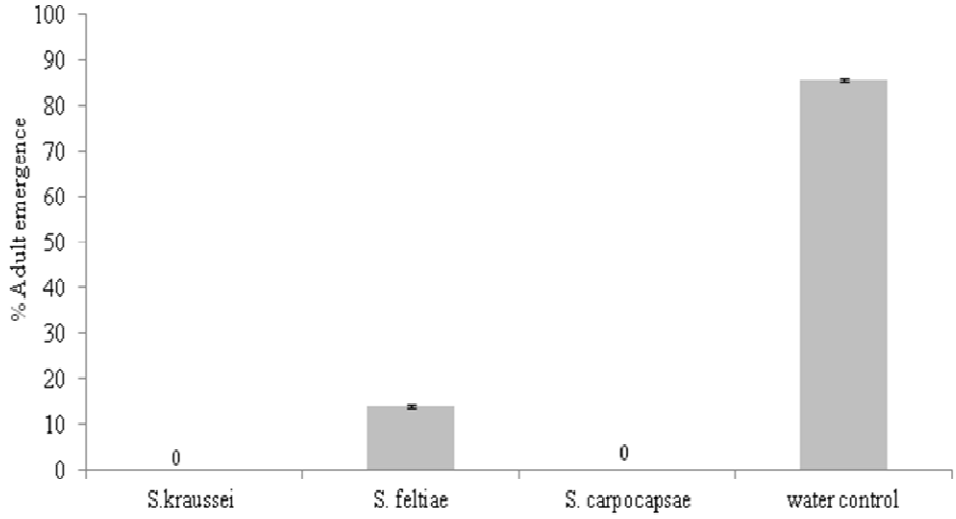

FIGURE 2. IMPACT OF IN-DIRECT EXPOSURE OF CONTROL AGENTS ON *Aethina tumida* WANDERING LARVAE. ERROR BARS REPRESENT THE 95% CONFIDENCE INTERVALS (CUTHBERTSON *et al.*, 2012).

FOLLOWING DELAYED APPLICATION OF THE NEMATODES, SIGNIFICANT REDUCTION
EMERGENCE WAS OBTAINED (PAIRED *t*-TEST; FIGURE 3) FOR UP TO 3 WEEKS FOLLOWING LARVAE ENTERI
SAND TO PUPATE (CUTHBERTSON *et al*., 2012).

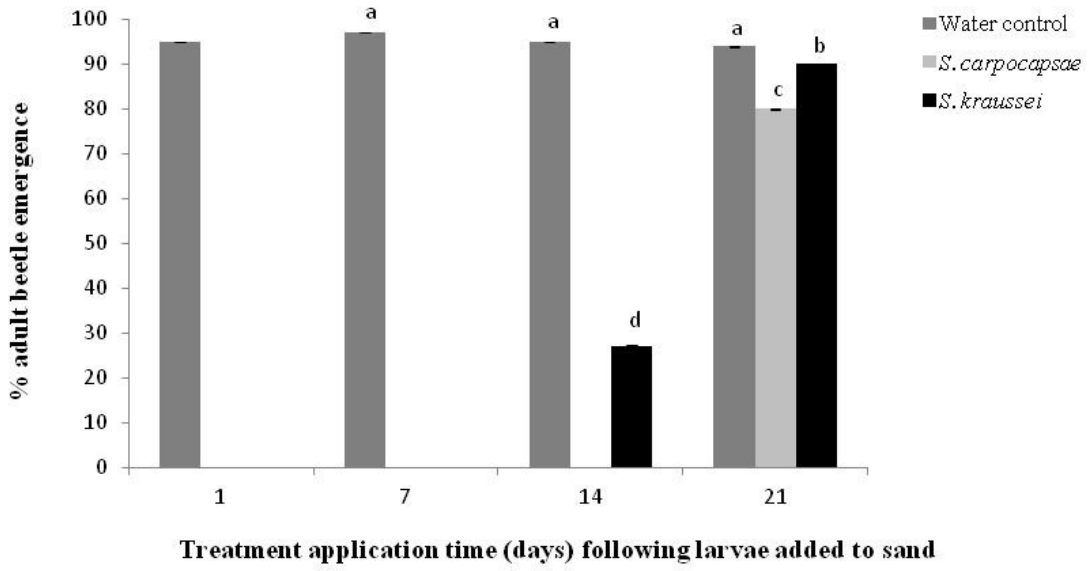

FIGURE 3. IMPACT OF DELAYED APPLICATIONS OF ENTOMPATHOGENIC NEMATODES ON *Aethina tumida* WANDERING LARVAE FOLLOWING THEIR SUBMERGENCE IN SAND POTS. BEETLE EMERGEN
WEEKS FOLLOWING LARVAE BEING ADDED TO SAND. ERROR BARS REPRESENT THE
INTERVALS (CUTHBERTSON *et al.*, 2012).

THE DATA FROM OUR SCREENING TRIALS ARE CONSISTENT WITH THOSE OF CABANILL
AND ELLIS (2010), WHO DEMONSTRATED THAT *Aethina tumida* LARVAE AND PUPAE ARE SUSCEPTIBLE TO
ENTOMOPATHOGENIC NEMATODES. IN OUR STUDY NEMATODE EFFICACY VARIED WITH NE
OUR TRIALS DEMONSTRATE THAT COMMERCIALLY AVAILABLE ENTOMOPATHOGENIC NE
CAN INFEST AND KILL *Aethina tumida* WANDERING LARVAE. FURTHERMORE, THESE PRODUCTS ARE
ACROSS EUROPE, AND SO HAVE THE POTENTIAL TO BE USED AS CONTROL AGENTS SHOU
BEETLE EXPAND ITS RANGE TO THIS CONTINENT (CUTHBERTSON *et al*., 2013).

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Insect pathogens and entomoparasitic nematodes
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The development of mollusc-parasitic nematode *Phasmarhabditis hermaphrodita* (Nematoda: Rhabditidae) in different substrates

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Abstract: THE EFFECT OF DIFFERENT GROWING SUBSTRATES ON THE DEVELOPMENT OF THE FAC
PARASITE *Phasmarhabditis hermaphrodita* HAS BEEN STUDIED IN A SERIES OF LABORATORY EXPERIMENTS.
LABORATORY AND NEMASLUG STRAINS OF *P. hermaphrodita* WERE REARED IN AGAR PLATES ON HOMOGENIZED PI
KIDNEY, THE HOMOGENIZED BODIES OF *Deroceras reticulatum*, *Arion lusitanicus*, AND *Galleria mellonella*,
THE FAECES OF *D. reticulatum* AND *A. lusitanicus*, OR LEAF COMPOST. DEVELOPMENT TIME, YIELD, LIPID
RESERVES, AND THE BODY LENGTH OF FEMALES AND DAUER LARVAE WERE ASSESSED. *P. hermaphrodita* STRAINS
WERE ABLE TO GROW AND REPRODUCE ON ALL TESTED SUBSTRATES. HOWEVER, YIELDS WERE MA
ANIMAL SUBSTRATES. LIPID CONTENT AND BODY SIZE VARIED ACROSS THE SUBSTRATES, HOWEVER
PRODUCED NORMAL SIZED INDIVIDUALS WITH NORMAL LIPID CONTENT. IT THUS SEEMS THAT TH
SUBSTRATE IS EXPRESSED MAINLY IN YIELD. HIGH AND LESS VARIABLE YIELDS AND FASTER DEVEL
AND NEMASLUG STRAINS, IN COMPARISON WITH THE LABORATORY STRAIN, WERE PROBABLY
BACTERIAL ASSOCIATES. THE DRAMATIC DIFFERENCES IN YIELDS ON ANIMAL SUBSTRATES, IN COM
PLANT TISSUE, ILLUSTRATE THE EVOLUTIONARY ADVANTAGE OF THE ASSOCIATION OF NEMATODES W

Key words: SLUG PARASITIC NEMATODES, LIPID RESERVES, PROGENY PRODUCTION, DEVELOPMENT TIM

Introduction

Phasmarhabditis hermaphrodita IS A BACTERIOPHAGOUS NEMATODE THAT DOES NOT LIVE IN A
ASSOCIATION WITH ONLY ONE SPECIES OF BACTERIA AS ENTOMOPATHOGENIC NEMATODES (
IS ASSOCIATED WITH, AND ABLE TO FEED ON, MANY BACTERIAL SPECIES (Vellai *et al.*, 2003; Page & Wilson
2010) THAT ARE COMMON IN ITS HABITAT. BACTERIAL SPECIES SIGNIFICANTLY
P. hermaphrodita PROGENY PRODUCTION AND DAUER JUVENILES (DJS) QUALITY (Wilson *et al.*, 1995A)
AND ARE RESPONSIBLE FOR THE PATHOGENICITY OF NEMATODE-BACTERIA COMPLEXES TOW
(WILSON *et al.*, 1995B).

APART FROM THE PARASITIC CYCLE *P. hermaphrodita* ALSO HAS A NECROMENIC LIFE CYCLE
(MENGERT, 1953), AND HAS BEEN SHOWN TO REPRODUCE ON EARTHWORMS (Tan & Grewal, 2001), LEAF
LITTER (MACMILLAN *et al.*, 2009) AND SLUGS OR SLUG FAECES HOMOGENATES (TAN & GREW

AS FOUND PREVIOUSLY GROWING SUBSTRATE INFLUENCED THE REPRODUCTIVE CAPACI
PRODUCTION OF *P. hermaphrodita* (TAN & GREWAL, 2001; Ruiu *et al.*, 2009). THE INFLUENCE OF
GROWING SUBSTRATE HAS BEEN THOROUGHLY STUDIED IN EPNS AND OTHER NEMATODES
COMPOSITION (E.G. LIPIDS, PROTEINS ETC.) AND ORIGIN (PLANT VS. ANIMAL) IS AN IMPORTA
NEMATODE GROWTH AND REPRODUCTION AND MAY DETERMINE THE FINAL YIELD (FRIEDM
SIZE (HOOPER *et al.*, 1999; YANG *et al.*, 1997) AND LIPID CONTENT OF INFECTIVE JUVENILES (ABU
HATAB & GAUGLER, 1999) OR DEVELOPMENT (EHLERS & SHAPIRO-ILAN, 2005).

IN THE PRESENT STUDY WE ASSESSED THE INFLUENCE OF DIFFERENT GROWING S
BACTERIA ON THE *P. hermaphrodita* STRAINS AND THE DIFFERENCES BETWEEN THEM. WE FOCUS
THE DEVELOPMENT TIME, YIELD, SIZE, AND LIPID RESERVES OF FEMALES AND DAUER LARVA

Material and methods

THREE STRAINS OF *P. hermaphrodita* WERE USED IN THIS STUDY, THE NEMASTAR® STRAIN, THE LABORATORY STRAIN DERIVED FROM THE COMMERCIAL STRAIN, AND THE WILD STRAIN ISOLATED FROM AN *Arion* SP. CADAVER. SLUGS OF THE SPECIES *Arion lusitanicus* AND *Deroceras reticulatum* WERE COLLECTED USING BUDDE DEVICE (CZECH REPUBLIC). *Galleria mellonella* LARVAE WERE OBTAINED FROM LABORATORY CULTURE.

THE INFLUENCE OF DIFFERENT SUBSTRATES ON THE *P. hermaphrodita* STRAINS WAS OBSERVED ON 2% PURE AGAR PLATES IN PETRI DISHES, DIAMETER 55 MM. SUBSTRATES USED WERE HOMOGENIZED PIG KIDNEY, STERILIZED SLUG FAECES, THE HOMOGENIZED BODIES OF *A. lusitanicus*, *D. reticulatum*, AND *G. mellonella*, AND LEAF COMPOST. THE HOMOGENIZED SLUGS AND *G. mellonella* WERE PREPARED FROM INDIVIDUALS THAT WERE AUTOCLAVED AND HOMOGENIZED WATER.

A 0.02 G PIECE OF EACH SUBSTRATE WAS PLACED ON THE PLATE AND TWENTY *P. hermaphrodita* IN 20 µL WATER WERE PIPETTED ONTO THE SUBSTRATE. THE EXPERIMENT WAS PERFORMED AT 15 °C, AND IN TWO SERIES, EACH CONSISTING OF 10 REPLICATES. THE FIRST SERIES WAS CHECKED DAILY, AND THE DEVELOPMENT OF THE POPULATION OBSERVED UNDER A STEREOMICROSCOPE. THE FIRST APPEARANCE OF MATURE FEMALES WAS RECORDED. TWENTY MATURE FEMALES WERE COLLECTED FOR LIPID STAINING (WEAVER *et al.*, 1997) AND MEASUREMENT OF LENGTH. THE OTHER SERIES WAS OBSERVED EVERY DAY, AND THE DEVELOPMENT WAS RECORDED, UNTIL THE FOOD WAS DEPLETED AND THE NEW DJS EMERGED. THE DJS WERE COLLECTED AND COUNTED, AND 20 FROM EACH DISH WERE USED FOR THE MEASUREMENTS OF THE LIPID CONTENT AND BODY LENGTH. THE WHOLE EXPERIMENT WAS REPEATED TWICE IN TIME.

THE ASSOCIATED BACTERIA OF ALL TESTED NEMATODE STRAINS WERE ISOLATED BOTH FROM STERILIZED DJS (WILSON *et al.*, 1995A) AND FROM HOMOGENIZED STERILE PIG KIDNEY 3 DAYS AFTER INOCULATION WITH SURFACE STERILISED DJS. BACTERIA WERE IDENTIFIED IN THE SPECIALIZED LAB AT THE CCM (CZECH COLLECTION OF MICROORGANISMS).

Results and discussion

ALL THE TESTED SUBSTRATES SUPPORTED THE GROWTH AND REPRODUCTION OF THE THREE TESTED STRAINS OF *P. hermaphrodita*. THESE RESULTS ARE IN ACCORDANCE WITH THE *P. hermaphrodita* CONCEPT THAT IS ABLE TO LIVE AND REPRODUCE ON VARIOUS ORGANIC MATERIALS (MENGERT, 1953; TAN & GRAE *et al.*, 2006).

YIELD IN OUR STUDY WAS CLEARLY AFFECTED BY SUBSTRATE. SIMILARLY TO THE RESULTS WITH EPNS (YANG *et al.*, 1997) *P. hermaphrodita* IN OUR STUDY PRODUCED UP TO 20-30 FOLD MORE PROGENY ON SUBSTRATES BASED ON ANIMAL TISSUE THAN ON FAECES AND COMPOST. THESE CLEARLY ILLUSTRATE THE BENEFIT OF THE ASSOCIATION OF THE NEMATODE WITH INVERTEBRATE ON EPNS MEDIUM COMPOSITION HAS BEEN SHOWN TO AFFECT THE AMOUNT AND QUALITY OF BACTERIA AND IN TURN NEMATODE YIELD (ABU HATAB & GAUGLER, 2001). THE SAME EXPECTATION BE APPLIED TO OUR OBSERVATIONS.

IN EPNS THE LIPID CONTENT HAS BEEN SHOWN TO VARY WITH THE QUALITY OF THE SUBSTRATE (ABU HATAB & GAUGLER, 1999). IN OUR STUDY WE FOUND NEGLIGIBLE DIFFERENCES IN THE LIPID CONTENT OF THE FEMALES AND, TO A LESSER EXTENT, THE DJS OF ALL TESTED STRAINS ACROSS THE SUBSTRATES. THIS OBSERVATION SUGGESTS THAT UNLIKE EPNS (YANG *et al.*, 1997) *P. hermaphrodita* CAN PRODUCE FULL QUALITY DJS ON A VARIETY OF SUBSTRATES.

THERE WAS AN APPARENT VARIATION IN FEMALE AND LARVAL LENGTH ON DIFFERENT SUBSTRATES. THIS FINDING FITS WELL WITH THE STUDIES PERFORMED ON EPNS (YANG *et al.*, 1997). SIMILARLY,

P. hermaphrodita FEMALES REARED 0N DEAD SLUGS WERE USUALLY BIGGER THAN FEMAL
BACTERIAL CULTURE (H0PPER, 1999). YANG *et al.* (1997) HAVE SH0WN THAT THE IJS 0F EPNS
GR0W LARGER 0N ANIMAL SUBSTRATES. SIMILARLY, WE HAVE SH0WN THAT, IN GENERAL
PIG KIDNEY AND H0M0GENIZED *Galleria* WERE LARGER, WHILE IN SLUG FAECES AND C0MP0ST SH
FEMALES AND DJS WERE PR0DUCED, ESPECIALLY IN THE NEMASLUG STRAIN.

THERE WERE APPARENT DIFFERENCES IN THE RESP0NSE 0F THE TESTED STRAIN
SUBSTRATES. WE ASSUME THAT AT LEAST S0ME 0F THESE DIFFERENCES C0ULD BE DUE
THE C0MP0SITI0N 0F BACTERIAL ASS0CIATES. AS EXPECTED THE NEMASLUG STRAIN C0NTA
osloensis 0NLY. THE LAB0RAT0RY STRAIN L0ST ITS 0RIGINAL BACTERIAL ASS0CIATE D
CULTURING. ITS PRESENT ASSOCIATES *Acinetobacter* SP., *Alcaligenes faecalis*, *Bacillus cereus* AND
Stenotrophomonas SP. PR0BABLY 0RIGINATE FR0M N0N-STERILE SLUGS USED F0R NE
PR0PAGATI0N IN THE LAB0RAT0RY. THE WILD STRAIN HARB0URED A QUITE SIMILAR BAC
TH0UGH HAVING *Pseudomonas putida* IN ADDITI0N.

WE SUPP0SE, THE L0WER YIELD AND SL0WER DEVEL0PMENT 0F THE LAB0RAT0R
C0MPARIS0N WITH THE NEMASLUG ARE PR0BABLY DUE T0 THE ASS0CIATED BACTER
DIFFERENCES BETWEEN THE LAB0RAT0RY AND WILD STRAIN ARE QUESTI0NABLE AS THE
VERY SIMILAR BACTERIAL ASSEMBLAGES WITH THE 0NLY DIFFERENCE BEING IN THE PRESEN
THE WILD STRAIN. THESE BACTERIA HAVE BEEN SH0WN T0 SUPP0RT WELL THE
P. hermaphrodita (WILS0N *et al.*, 1995B). H0WEVER, FURTHER RESEARCH IS NECESSARY T0 SH0V
R0LE 0F BACTERIAL ASS0CIATE IN THESE EXPERIMENTS.

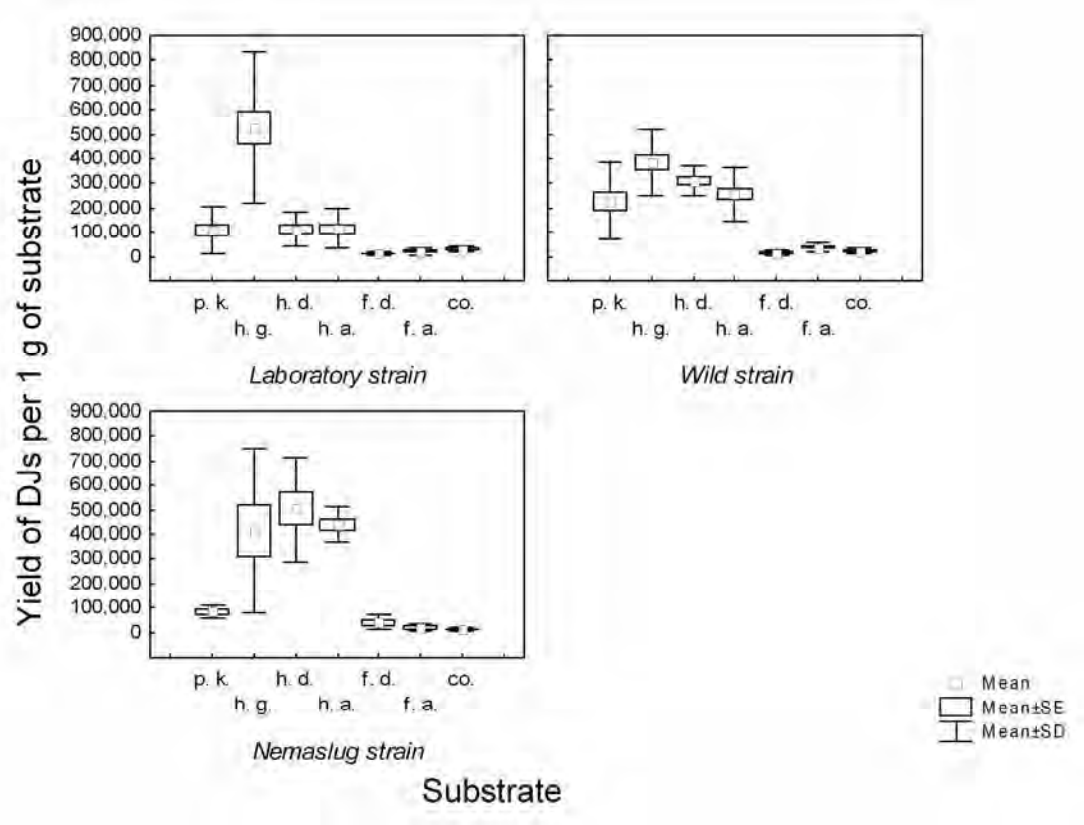

FIGURE 1. DAUER JUVENILES YIELD 0F THE WILD, LAB0RAT0RY AND NEMASLUG
Phasmarhabditis hermaphrodita 0N DIFFERENT SUBSTRATES.
LEGEND: P.K. PIG KIDNEY, H.G. H0M0GENIZED *Galleria*, H.D. H0M0GENIZED *D. reticulatum*, H.A.
H0M0GENIZED *A. lusitanicus*, F.D. FAECES 0F *D. reticulatum*, F.A. FAECES 0F *A. lusitanicus*, C0. LEAF
C0MP0ST.

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New nematodes associated to *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae): preliminary description

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Abstract: SEVERAL NEMATODES, JUVENILES AND ADULTS, WERE FOUND DISSECTING *Rhynchophorus ferrugineus* PUPAE AND ADULTS FROM INFESTED *Phoenix canariensis* EXEMPLAR IN BARI (ITALY). INSECT WAS INTACT EXTERNALLY BUT INNER TISSUES WERE COMPLETEL NEMATODES WERE COLLECTED USING THE WATER TRAP METHOD AND TOTAL DNA WAS EXT EACH INDIVIDUAL. THE 18S RDNA, THE ITS CONTAINING REGION AND THE MITOCHONDRIAL CYT OXIDASE I (COI) WERE AMPLIFIED AND SEQUENCED. ITS-RFLP ANALYSIS WERE ALSO OBTA BLAST SEARCH REVEALED THAT NUCLEOTIDES SEQUENCES ARE SIMILAR (93%) TO *Koerneria* SP. RS1982 (NEMATODA: DIPLOGASTRIDAE). NEMATODES BELONGING TO DIPLOGASTRIDAE ARE C ASSOCIATED WITH INSECTS, WITH DIFFERENT TYPES OF ASSOCIATION DEPENDING ON DIPLOG *Koerneria* SPP. ARE FREQUENTLY ASSOCIATED WITH STAG AND DUNG BEETLES. CHARACTERIZAT ARE NOW STILL IN PROGRESS FOR THE SPECIES IDENTIFICATION. OUR FUTURE PURPOSE IS TO CL OF ASSOCIATION BETWEEN THIS SPECIE AND THE RED WEEVIL AND THE EVENTUAL ROLE AS NAT AGENT.

Key words: *Rhynchophorus ferrugineus*, NEMATODE, *Koerneria sp.*, NUCLEOTIDE SEQUENCE, IDENTIFICATION

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The role of bacterial symbionts in the competition of entomopathogenic nematode species

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Abstract: COMPETITION BETWEEN ENTOMOPATHOGENIC NEMATODE (EPN) SPECIES IS STILL A LARG
TOPIC. PREVIOUS RESEARCH HAS SHOWN THAT IN THE COMPETITION WITHIN ONE INSECT H
Steinernema affine STRONGLY DOMINATES *S. kraussei* AND SUGGESTED A POSSIBLE ROLE OF SYMBIOTI
BACTERIA IN THE COMPETITION. IN PRESENT STUDY, *S. affine* AND *S. kraussei* AND THEIR SYMBIONTS WERE REARED
IN DIFFERENT COMBINATIONS ON WOUTS AGAR PLATES, AND NEMATODE DEVELOPMENT WAS O
PROGENY FROM THESE COMBINATIONS WAS HARVESTED AND BODY SIZE AND LIPID CONTENT OF
(IJS) WERE ASSESSED. *S. affine* WAS ABLE TO DEVELOP, MATURE AND PRODUCE VIABLE PROGENY ON TH
OF *S. kraussei*. INTERESTINGLY, THERE WAS NO DIFFERENCE IN THE DURATION OF THE CYCLE O
POTENTIAL, IJ SIZE AND LIPID CONTENT OF *S. affine* REARED ON THEIR OWN SYMBIONT AND SYMBIONT
S. kraussei. ON THE OTHER HAND, *S. kraussei* DEVELOPED AND REPRODUCED WELL ONLY ON ITS OWN SYM
THESE EXPERIMENTS EXPLAINED THE PREVIOUSLY OBSERVED DOMINANCE OF *S. affine* OVER *S. kraussei*.
RESEARCH WITH MORE EPN SPECIES IS PLANNED TO FURTHER CLARIFY THE TOPIC.

Key words: *Steinernema*, *Xenorhabdus*, COMPETITION

Introduction

INTERSPECIFIC COMPETITION OF ENTOMOPATHOGENIC NEMATODES (EPNS) IS STILL AN UNDERSTUDIED AREA
DESPITE ITS IMPLICATIONS FOR THE USE OF EPNS IN BIOLOGICAL CONTROL. LABORATO
REVEALED, THAT EPNS DO NOT AVOID CO-INFECTING HOSTS TOGETHER WITH H
(KOPPENHÖFER *et al.*, 1995; KOPPENHÖFER & KAYA, 1997; JAŠKOVÁ & MRÁČEK, 2009) AND NATURAL
MULTIPLE INFECTION HAS BEEN ALSO OBSERVED (BOVIEN, 1937). THUS THERE IS A POTEN
INTER-SPECIFIC INTERACTIONS BETWEEN SYMPATRIC ENTOMOPATHOGENIC NEMATOD
BELIEVED THAT THE OUTCOME OF THE COMPETITION INTENSITY AND ITS IMPACT ON
DEPENDS ON THE INOCULUM SIZE OF NEMATODES. *S. affine* HAS BEEN SHOWN TO SUPPRESS
S. kraussei IN CO-INFECTED *Galleria mellonella* REGARDLESS THE INOCULUM SIZE OR THE RATIO BETWE
NEMATODES (JAŠKOVÁ & MRÁČEK, 2009) OR HOST SPECIES (JAŠKOVÁ & MRÁČEK, 2010), SUGGESTING A
POSSIBLE ROLE OF BACTERIAL SYMBIONT IN THIS INTERACTION.

IN PRESENT STUDY, THE ROLE OF SYMBIOTIC BACTERIA IN THE COMPETITION BETWE
S. affine AND *S. kraussei* WAS INVESTIGATED ON WOUTS AGAR PLATES AND INFECTIONS OF *G. mellonella*.

Material and methods

Nematode and bacteria preparation and rearing

S. affine AND *S. kraussei* ORIGINATING FROM ONE LOCALITY WERE SELECTED FOR THE EX
AXENIC 1ST INSTAR LARVAE OF BOTH STRAINS WERE PREPARED ACCORDING TO (KAYA & STO

SYMBIOTIC BACTERIA OF BOTH STRAINS WERE ISOLATED FROM HAEMOLYMPH OF THE *G. mellonella* THAT WAS STREAKED ON NBTA AGAR PLATES. SINGLE COLONIES WERE THEN T THE LIQUID YS MEDIUM AND INCUBATED 2 D ON ORBITAL SHAKER AT 25 °C PRIOR TO EXPE

THEN THE LARVAE OF BOTH STRAINS WERE SEPARATELY AND IN MIXTURE REARED O SYMBIOTIC BACTERIA ON WOUTS AGAR PLATES. THE PLATES WERE CHECKED DAILY AND OF THE NEMATODES WAS RECORDED.

The assessment of the progeny quality

IJS WERE HARVESTED FROM THE PLATES AND THEIR BODY LENGTH AND MAXIMAL B MEASURED UNDER LIGHT MICROSCOPE. THE LIPID CONTENT WAS ASSESSED ACCORDIN *et al.* (1997).

Infections

IJS HARVESTED FROM HETEROXENIC COMBINATIONS WERE SURFACE STERILISED ACCO EHLERS (1998) AND THE RETENTION OF THE BACTERIA WAS TESTED BY THE INFECTIONS *G. mellonella* AND BY PLACING THE IJS TO THE STERILE YS MEDIUM FOR 48 H.

MIXED INFECTIONS OF *S. affine* IJS FROM MONOXENIC AND HETEROXENIC COMBINATIONS MONOXENIC *S. kraussei* WERE PERFORMED, AND THE OUTCOME OF THE INFECTION WAS ASSESS

Results and discussion

Wouts agar plates

THERESULTS FROM THE WOUTS AGAR TESTS ARE SUMMARISED IN THE TABLE 1. AS E NEMATODES GROWN AND REPRODUCED ON THEIR ORIGINAL BACTERIAL SYMBIONTS. HOWEVER, OBSERVED, WHEN THE NEMATODES WERE REARED ON THE EACH OTHER'S SY *S. affine* WAS ABLE TO GROW AND REPRODUCE ON THE SYMBIONT OF *S. kraussei*, THE SAME WAS NOT TRUE FOR THE LATTER. WHEN REARED ON *S. affine* SYMBIONT PART OF *S. kraussei* LARVAE DEVELOPED TO PIGMY ADULTS THAT DIED AFTER SEVERAL DAYS WITHOUT FURTHER REP

TABLE 1. DEVELOPMENT OF BOTH NEMATODES ON SYMBIONTS IN DIFFERENT COMBINATIONS.

NEMATODE	BACTERIA	ADULTS	REPRODUCTION	DURATION (DAYS)
S. affine	XB A	YES	YES	12
S. affine	XB K	YES	YES	12
S. affine	BOTH	YES	YES	12
S. kraussei	XB K	YES	YES	11
S. kraussei	XB A	YES*	NO	-
S. kraussei	BOTH	YES	NO	-
BOTH	BOTH	YES	YES	12[A]

*ONLY DWARF ADULTS; [A] ONLY *S. affine*

WHEN BOTH BACTERIA WERE AVAILABLE, *S. kraussei* MAINLY DEVELOPED TO ADULTS, BUT SUBSEQUENT REPRODUCTION WAS OCCASIONAL WITH NO IJ PRODUCTION. IT THUS S SYMBIONTS OF *S. affine* IS TOXIC FOR *S. kraussei*. THIS EXPLAINS THE UNIVERSAL DOMINANCE OF T

LATTER SPECIES IN MIXED INFECTIONS. ON THE CONTRARY, *S. affine* IS INHIBITED BY THE SYMBIONT OF *S. kraussei* (SEE FURTHER). THE MIXED TREATMENT WITH BOTH BACTERIA AND NEMATO THE INFECTION OF THE HOST, WHERE ALSO TWO NEMATODES AND TWO BACTERIA ARE P *S. affine* REPRODUCED.

Progeny assessment

NO DIFFERENCES WERE OBSERVED IN BODY SIZE AND LIPID CONTENT OF *S. affine* JUVENILES GROWN SYMBIOTICALLY AND APOSYMBIOTICALLY (FIGURE 1) AND IN THE DURATION OF THE CY FROM WOUTS AGAR PLATES WERE IN GENERAL SMALLER IN COMPARISON TO LA *G. mellonella* (FIGURE 1).

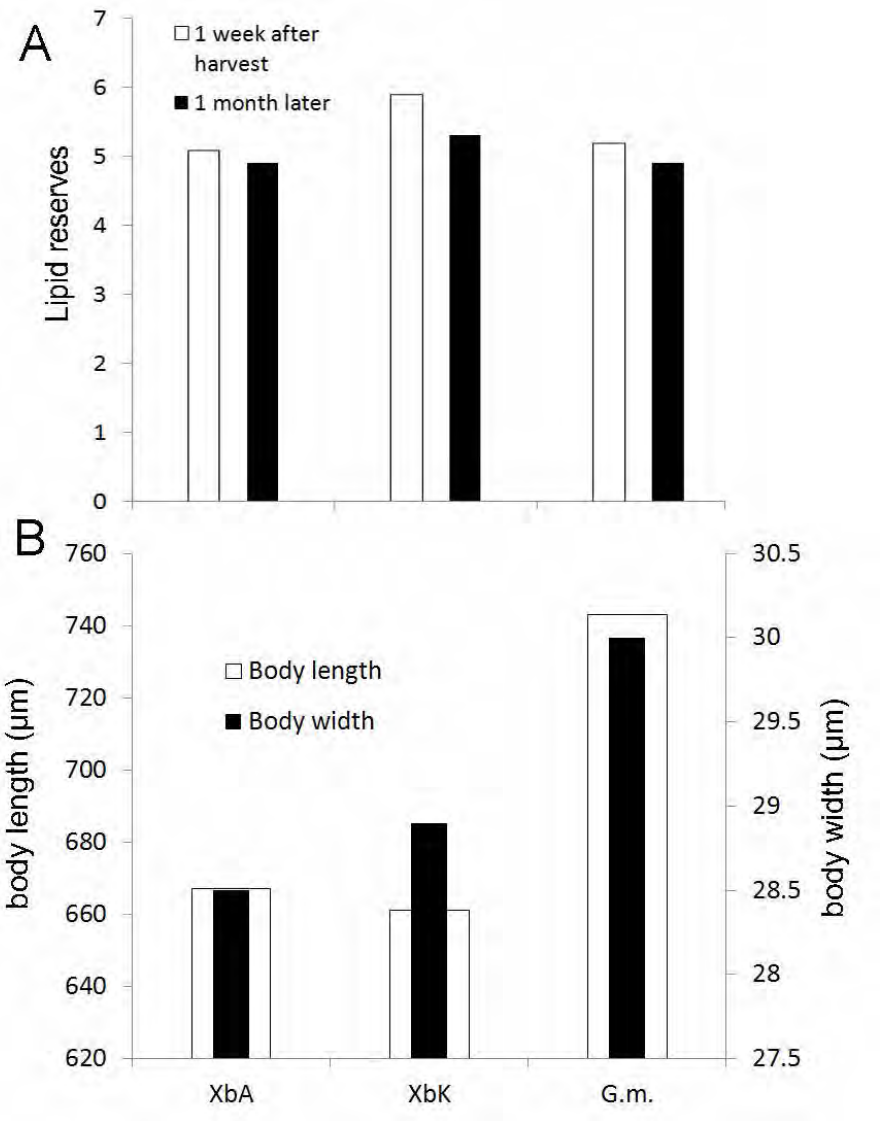

FIGURE 1. LIPID RESERVES (A) AND BODY LENGTH AND WIDTH (B) OF *S. affine* JUVENILES GROWN ON THEIR ORIGINAL SYMBIONT (XBA), SYMBIONT OF *S. kraussei* (XBK) AND REARED IN *G. mellonella* (G.M.).

Infections of G. mellonella

AS WAS EXPECTED, INCUBATION IN YS MEDIUM AND EXPERIMENTAL INFECTION OF *G. mellonella* SHOWED NO RETENTION OF *S. kraussei* SYMBIONT IN *S. affine* IJS. INTERESTINGLY, IN THE MIXED INFECTIONS *S. affine* IJS FROM HETEROXENIC COMBINATION WITH *S. kraussei* WERE THE SYMBIONT OF *S. affine* WAS MISSING, *S. affine* STILL DOMINATED (DATA NOT SHOWN). THIS FA SUGGESTS ALSO A ROLE OF THE NEMATODE IN THE COMPETITION. HOWEVER, FURTHER R EPN SPECIES IS NEEDED TO CLARIFY THIS TOPIC.

Acknowledgements

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Research and development for a nematode-based biological control solution for western corn rootworm in maize

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Abstract: 10 YEARS OF JOINT EFFORTS IN RESEARCH AND DEVELOPMENT HAVE LED TO A NE
BIOLOGICAL CONTROL SOLUTION FOR ONE OF THE MOST DESTRUCTIVE MAIZE PESTS, THE WEST
Diabrotica virgifera virgifera LECONTE (COLEOPTERA: CHRYSOMELIDAE).

Key words: ENTOMOPATHOGENIC, INSECT PARASITIC NEMATODES, INUNDATIVE BIOLOGICAL CONT

Introduction

THE WESTERN CORN ROOTWORM (WCR), *Diabrotica virgifera virgifera* LECONTE (COLEOPTERA: CHRYSOMELIDAE), IS ONE OF THE MOST DESTRUCTIVE PESTS OF MAIZE IN NORTH AM UNIVOLTINE SPECIES WITH EGGS THAT OVERWINTER IN THE SOIL. AFTER MAIZE HAS GER HATCH, AND ITS THREE LARVAL LIFE STAGES FEED ON MAIZE ROOTS, OFTEN CAUSING YIELD LOSSES. ADULTS CAN OCCASIONALLY REDUCE YIELDS THROUGH INTENSIVE SILK LAST 25 YEARS, IT HAS MOVED INTO EUROPE CAUSING MAJOR PROBLEMS IN MAIZE.

BETWEEN 2004 AND 2008, CABI, THE UNIVERSITY OF NEUCHÂTEL, THE FARMER ASS LANDI REBA IN BASEL, THE PLANT PROTECTION DIRECTORATE IN HODMEZOVASARHEI RECKENHOLZ-TÄNIKON, THE UNIVERSITY OF KIEL, AND THE NEMATODE PRODUCERS SCHWENTINENTHAL AND ANDERMATT BIOCONTROL AT GROSSDIETSWIL, LAID THE SC NEMATODE-BASED BIOLOGICAL CONTROL PRODUCTS AGAINST WCR (CABI, 2008). BETWE 2008, A NUMBER OF INSTITUTIONS REVIEWED BIOLOGICAL CONTROL OPTIONS AGAINST PROPOSED THEM TO THE EUROPEAN COMMISSION (DIABR-ACT, 2007; CABI, 2008). BETWEE AND 2012, THE LANDWIRTSCHAFTLICHES TECHNOLOGIEZENTRUM STUTTGART, CULT-TEC THE AUSTRIAN AGENCY FOR HEALTH AND FOOD SAFETY IN VIENNA, THE CEREAL RESEA SZEGED, SAGEA CENTRO DI SAGGIO S.R.L., CABI, AND OTHERS IMPROVED APPLICATION TE AIMING FOR THE FARMER-FRIENDLIEST AND LEAST-COSTLY METHOD (CABI, 2012).

Achievements of research so far

Review of biological control options against WCR

SEVERAL NATURAL ENEMY SPECIES OR GROUPS APPEARED PROMISING CANDIDATES FOR WITH DIFFERENT ECOLOGICAL RATIONALES. RESEARCH PROPOSED TO PURSUE: (1) DEVE BIOLOGICAL CONTROL PRODUCTS, PARTICULARLY MASS-PRODUCED ENTOMOPATHOGE FUNGI; (2) UNDERSTANDING SPECIFIC NATURAL ENEMIES OF DIABROTICINA THROUGHO INCLUDING POTENTIAL CLASSICAL BIOLOGICAL CONTROL AGENTS; AND (3) ENHANCIN THROUGH CULTURAL PRACTICES. DETAILS IN TOEPFER *et al.* (2009); PILZ *et al.* (2008); DIABR-ACT (2007); TOEPFER *et al.* (2009); PILZ *et al.* (2008).

Nematode screening in laboratory

SCREENINGS EXPERIMENTS IN PETRI DISHES ON FILTER PAPER OR IN SAND, AS WELL A
CONTAINERS WITH SAND OR SOIL AND MAIZE REVEALED THAT *H. bacteriophora*, *H. megidis*, *Steinernema feltiae*, *S. arenarium*, AND *S. kraussei* ARE HIGHLY VIRULENT AGAINST WCR LARVAE. *S. abassi* WAS FOUND INTERMEDIATE. *S. carpocapsae* AND *S. glaseri* APPEARED LESS VIRULENT. DETAILS IN TOEPFER *et al.* (2009), KURTZ *et al.* (2009), HILTPOLD *et al.* (2010).

Nematode screening in the field

PLANT SCALE FIELD EXPERIMENTS WITH ARTIFICIAL WCR INFESTATION AND INTO-SOIL FLUIDS OF DIFFERENT NEMATODE SPECIES DURING SOWING OR LATER IN JUNE, *H. bacteriophora* AND *H. megidis* ARE HIGHLY EFFECTIVE AGAINST WCR LARVAE (I.E. UP TO 8 AND IN PREVENTING DAMAGE TO MAIZE ROOTS (I.E. UP TO 80%), AND THIS LARGELY TO T AS PESTICIDES. *S. feltiae* APPEARED SLIGHTLY LESS EFFECTIVE. DETAILS IN TOEPFER *et al.* (2008), TOEPFER *et al.* (2009); HILTPOLD *et al.* (2010).

Scientific pre-requisites

Instar susceptibility of target

BIOASSAYS WITH DIFFERENT LIFE STAGES OF WCR AND DIFFERENT NEMATODES REVEAL INSTARS AND EVEN PUPAE ARE EFFECTIVELY KILLED BY *H. bacteriophora*, *H. megidis* AND *St. feltiae*. ADULTS APPEARED LESS SUSCEPTIBLE. DETAILS IN KURTZ *et al* (2009).

Orientation of nematodes to target

NEMATODES WERE FOUND TO ORIENT TOWARDS WCR-DAMAGED MAIZE ROOTS USING T ORGANIC VOLATILE COMPOUND (E)-β-CARYOPHYLLENE AS AN ORIENTATION CUE FOR LARVAE. CARYOPHYLLENE MIGHT BE PARTICULARLY IMPORTANT FOR *H. megidis* AND LESS FOR *H. bacteriophora*. OTHER AUTHORS MENTION THAT CARYOPHYLLENE IS OF LITTLE TO NO IM NEMATODES. DETAILS IN RASMANN *et al.* (2005), HILTPOLD *et al.* (2008); ANBESSE *et al.* (2013).

Maize hybrid importance

THERE ARE HARDLY ANY HINTS THAT THE CHOICE OF MAIZE HYBRIDS IS IMPORTANT FO CONTROL WITH NEMATODES. SOME HYBRIDS HAVE LOST THE CAPABILITY TO EMIT THE NE (E)-β-CARYOPHYLLENE; HOWEVER, MOST EUROPEAN MAIZE HYBRIDS EMIT CARYOPHYLLENE UPON LARVAL FEEDING. DETAILS IN RASMANN *et al.* (2005); HILTPOLD *et al.* (2008; 2010).

Establishment and persistence of nematodes

FIELD EXPERIMENTS REVEALED THAT APPLIED NEMATODES ESTABLISH AT RELATIVELY L OF MAIZE FIELDS; BUT, THAT THEY SURVIVE MORE THAN TWO MONTHS, WHICH IS LO EFFECTIVELY KILL ALL THREE LARVAL INSTARS. NEMATODES WERE FOUND TO PROPAGAT THE FIELD, A BIG ADVANTAGE OVER PESTICIDES. DETAILS IN KURTZ *et al.* (2009); PILZ *et al.* (2011A).

Soil importance

FIELD TRIALS SHOWED THAT *H. bacteriophora* CAN EFFECTIVELY KILL WCR LARVAE IN A WIDE RANG SOILS IN MAIZE FIELDS. AS WCR LARVAE ARE USUALLY MOST DAMAGING IN DENSE SOIL EFFICACIES OF NEMATODES WERE FOUND HIGHER IN DENSE SOILS THAN IN LIGHT, E.G DETAILS IN GRABENWEGER *et al.* (2010); TOEPFER *et al.* (2010D); PILZ *et al.* (2011A).

Non-target effects

ENTOMOPATHOGENIC NEMATODES ARE RESTRICTED TO ARTHROPODS, THUS THERE IS NO HUMANS. NEMATODES ARE KNOWN TO BE SLIGHTLY HOST SPECIFIC ON INSECT GROUPS.

EXPERIMENTS REVEALED ONLY MINOR EFFECTS OF TREATMENTS ON NON-TARGET POPULA
BE A RESULT OF THE GENERALLY POOR ARTHROPOD DIVERSITY IN SOILS OF INTENSIVE F
MAIZE, AS WELL AS OF THE APPLICATION OF NEMATODES INTO RELATIVELY NARROW SOI
THE TARGET. DETAILS IN BABENDREIER *et al.* (2004); KUHLMANN AND TOEPFER (2009); TOEPFER AND KUHLMANN (2010); TOEPFER (2011); TOEPFER *et al.* (2011); TOEPFER AND KUHLMANN (2012); TOEPFER AND KUHLMANN (2013); TOEPFER AND KUHLMANN (2002).

Application of nematodes

Where?

NEMATODES WERE SUCCESSFULLY APPLIED THROUGH FLUID SOLID STREAM SPRAYS, M
SEED COATING INTO SOIL AT SOWING, OR THOUGH FLUID SOLID STREAM SPRAYS OR GRA
TO YOUNG MAIZE PLANTS, OR THROUGH FLUID NARROW FLAT SPRAYS APPLIED WITH L
ROWS OF SMALL PLANTS. DETAILS AND MORE OPTIONS IN TOEPFER *et al.* (2010A); TOEPFER (2011); TOEPFER AND KUHLMANN (2013).

When?

NEMATODES WERE SUCCESSFULLY APPLIED INTO SOIL AT SOWING (MID APRIL TO EARLY
EUROPE), THIS IS, A FEW WEEKS BEFORE WCR EGG HATCHING; AS WELL AS INTO OR ON
ROWS OF YOUNG MAIZE PLANTS (MID TO LATE MAY IN CENTRAL EUROPE). FIELD APPLIC
ADULTS, I.E. IN JULY OR AUGUST, HAVE NEVER BEEN ATTEMPTED. DETAILS IN TOEPFER *et al.* (2010A); TOEPFER (2011).

Formulation

NEMATODES CAN BE APPLIED AGAINST WCR LARVAE PREFERABLY JUST DILUTED IN
GRANULES, SEED COATINGS, CAPSULES AND OTHER OPTIONS NEED FURTHER RESEARCH. D
al. (2010ABC); HILTPOLD *et al.* (2012).

Need of water

FIELD EXPERIMENTS REVEALED THAT THE NEED OF WATER DURING APPLICATION IS VAR
ON THE SOIL TYPE, WHETHER CONDITIONS, AND APPLICATION TECHNIQUES. CURRENTLY A
TO 400 L WATER HA^{-1} ARE ADVISED FOR FLUID STREAM SPRAYS OF NEMATODES INTO SOILS, AND
OF 800 TO 1000 L HA^{-1} FOR NARROW STREAM SPRAYS ONTO THE SOIL OR PLANTS. DETAILS THR
CENTRO DI SAGGIO S.R.L. (2010, PERS. COMM.); TOEPFER *et al.* (2010A).

Farmer friendly application techniques

FLUID AND MICRO-GRANULAR APPLICATIONS AS WELL AS SEED COATING WITH NEM
TECHNICALLY POSSIBLE WITH AVAILABLE FARMER MACHINERIES; AND ALL ACHIEVED
LARVAE AS WELL AS ROOT DAMAGE PREVENTION. CURRENTLY MOST PROMISING AND MO
STREAM SPRAY APPLICATION INTO THE SOIL AT SOWING, USING SOWING MACHINES WITH
APPLY NEMATODES BEHIND THE SOWING WHEEL AND PRIOR THE SOIL-CLOSING WHE
TOEPFER *et al.* (2010C); SAGEA CENTRO DI SAGGIO S.R.L. (2010, PERS. COMM.); CULT-TEC (2012).

Field scale efficacy and dosage-efficacy response

FIELD SCALE TRIALS USING FARMER MACHINERY REVEALED THAT *H. bacteriophora* IS ABLE TO REACH
CONTROL EFFICACIES OF WCR LARVAE TO THE SAME EXTENT AS SOIL INSECTICIDES AND
COATINGS. ON MULTIPLE YEAR, SITE AND MACHINERY AVERAGE, CONTROL EFFICACIES A
BETWEEN 30 AND 80%. NEMATODES CAN ALSO SIGNIFICANTLY PREVENT ROOT DAMAGES,
TO SOIL INSECTICIDES AND INSECTICIDE SEED COATINGS. A DOSE-EFFICACY RESPONSE CU
ESTABLISHED, BUT PRELIMINARY RESULTS SUGGEST THAT THE OPTIMAL DOSE OF NEM
SOMEWHAT BETWEEN 2 AND 3 BILLION HA^{-1} IN MAIZE FIELD. DETAILS IN TOEPFER *et al.* (2009; 2011B); TOEPFER *et al.* (2010B); SAGEA CENTRO DI SAGGIO S.R.L. (2010, PERS. COMM.).

Products

H. bacteriophora AND *H. megidis* PRODUCTS ARE AVAILABLE FROM SEVERAL BIOCONTROL CO
AND CAN BE APPLIED, WITHOUT RESTRICTIONS, IN COUNTRIES WHERE ENTOMOPATHOGE
NOT NEED REGISTRATIONS AND WHERE THE PRODUCTS CONSIDER SPECIES THAT ARE NA
IN GERMANY. ONE OF THE PRODUCTS (DIABEM™) IS, FOR EXAMPLE, REGISTERED IN AUSTRIA.

Legislation

WITH THE BANNING OF SEVERAL INSECTICIDES FOR SEED COATINGS DUE TO THEIR BEE T
RECENT DISCUSSIONS ON A NUMBER OF SOIL PESTICIDES IN MAIZE, FARMERS NEED ALTE
MOREOVER, THE EUROPEAN DIRECTIVE ON SUSTAINABLE USE OF PESTICIDES REQUESTS F
COUNTRIES TO PREFER ALTERNATIVE PEST CONTROL OPTIONS. ENTOMOPATHOGENI
EXCEPTIONALLY SAFE BIOCONTROL AGENTS; THUS THEY ARE EXEMPTED FROM REGIST
EUROPEAN COUNTRIES, IN OTHERS THEY NEED REGISTRATION. DETAILS IN EHLERS (20
COMMISSION, (2008; 2009); DELOS *et al.* (2011); GILL *et al.* (2012); CRESSEY (2013).

Conclusions

JOINT RESEARCH AND DEVELOPMENT EFFORTS HAVE LED TO A NEMATODE-BASED BI
SOLUTION FOR WESTERN CORN ROOTWORM IN MAIZE, WHICH IS NOW READY FOR USE AND

Acknowledgements

THE SUCCESSFUL DEVELOPMENT OF A BIOLOGICAL CONTROL SOLUTION FOR WESTERN CO
10-YEAR COLLABORATIVE EFFORT OF MANY PARTNERS (PLEASE REFER TO: CABI (2008; 201
RELIED ON PUBLIC FUNDING (SWISS COMMISSION FOR TECHNOLOGY AND INNOVATION OF
FOR PROFESSIONAL EDUCATION AND TECHNOLOGY; A SPECIFIC SUPPORT ACTION 'POL
RESEARCH' THROUGH THE 5TH FRAMEWORK PROGRAMME; THE MINISTRY FOR RURAL AREA
CONSUMER PROTECTION OF THE STATE OF BADEN-WÜRTTEMBERG, GERMANY; AND THE FE
OF AGRICULTURE OF GERMANY), AND TO SOME EXTENT ON FUNDING FROM LANDI
SWITZERLAND, E-NEMA GMBH GERMANY, IN-KIND CONTRIBUTIONS OF FARMERS, AND MA

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Posters

Development of a method to establish entomopathogenic nematodes (EPN) in arable soils by using farm-suitable field equipment

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Abstract: ONE MAJOR PROBLEM OF THE UTILIZATION OF ENTOMOPATHOGENIC NEMATODES AGAINST PESTS IN ARABLE CROPS IS THAT TOPICAL SPRAYING WITH USUAL SPRAY EQUIPMENT IS EFFECTIVE BECAUSE OF THE RISK OF DRYING UP OF THE EPN BEFORE THEY CAN ACT AGAINST PESTS. FURTHERMORE, THE STAGES ACTIVE ON THE CROP PLANTS ARE IN MANY CASES LESS AGAINST EPN, HAVE A SHORT ACTIVITY PERIOD AND ARE OFTEN PROTECTED BY LEAVES SO THAT T HIT BY SPRAYING. MANY PESTS HOWEVER STAY MORE OR LESS INACTIVE FOR A LONGER T PUPATION, OVERWINTERING, ESTIVATION) IN ARABLE SOILS. IN THIS PERIOD OF INACTIVITY CONSIDERABLY SUSCEPTIBLE REGARDING ATTACKS BY EPN AND/OR ENTOMOPATHOGENIC FUNGI GOAL IS TO FIND A TECHNIQUE, SUITABLE FOR NORMAL FIELD APPLICATION WHICH HELPS TO EST PARTICULAR EPN-POPULATIONS FOR LONGER TERM IN ARABLE SOILS SO THAT THEY ARE PRESEN PEST ORGANISMS ENTER THE SOIL FOR PUPATION, OVERWINTERING OR AESTIVATION. TO ACH TESTED THE APPLICABILITY OF THE SO CALLED CULTAN-TECHNIQUE. THIS TECHNIQUE WAS DEVEL INJECT A CONCENTRATED AMMONIUM SOLUTION BY HIGH PRESSURE INTO THE SOIL WITH THE SUBTERRANEAN BALL-LIKE DEPOSIT IS FORMED FROM WHICH NITROGEN IS SLOWLY RELEASED ADJACENT SOIL.

OUR IDEA WAS, THAT THIS TECHNIQUE COULD BE USED TO APPLY IN PARTICULAR EPN INTO THE SOIL LAYERS SO THAT THEY ARE BETTER PROTECTED AGAINST DRYING UP AND ARE ABLE TO RE ALTERNATIVE FOOD ITEMS IN PERIOD THE RELEVANT LIFE STAGES OF PESTS ARE NOT PRESENT. BY SOIL, IT CAN BE ASSUMED THAT THEY PRESUMABLE HAVE A BETTER CHANCE TO SURVIVE AND IN COMPARISON TO APPLICATION BY NORMAL SPRAY EQUIPMENT. FOR THE INJECTION OF EPN SOIL WE MODIFIED THE CULTAN-TECHNIQUE BY USING A WATER SOLUTION OF WITH THE CONCENTRATION OF EPN WHICH IS RECOMMENDED FOR NORMAL SPRAY APPLICATION FOR PRO ARE ALREADY ON THE MARKET.

IN THE FIRST PRELIMINARY TESTS WHICH ARE DEMONSTRATED HERE THE TECHNIQUE WA ORGANIC WINTER OILSEED RAPE, BECAUSE IT IS THE ARABLE CROP WITH THE MOST PEST ORGAN LEAST ONE LIFE STAGE STAYING FOR A LONGER PERIOD IN THE SOIL. THE INJECTION-TECHNIQU AS AUTUMN AND SPRING APPLICATION EITHER ALONE OR AS COMBINATION. IT WAS COMPAR RANDOMIZED BLOCK DESIGNED FIELD EXPERIMENT WITH A TOPICAL SPRAY APPLICATION OF (*Steinernema feltiae*), EPF (*Beauveria bassiana*) AND SPINOSAD, AND AN UNTREATED CONTROL. FIRST RESULTS OF THESE PRELIMINARY TESTS AND EXPERIENCES WITH THIS TECHNIQUE ARE REPORTE

Key words: *Steinernema feltiae*, *Beauveria bassiana*, CULTAN-TECHNIQUE, APPLICATION, OILSEED RAPE

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Biosafety analysis of the *Bacillus pumilus* 15.1 strain through a *Caenorhabditis elegans* pathogenicity assay

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Abstract: USING A *Caenorhabditis elegans* PATHOGENICITY ASSAY WE EVALUATED THE BIOSAFETY
B. pumilus 15.1 STRAIN, A RECENTLY ISOLATED BACTERIA ACTIVE AGAINST LARVAE OF THE MEDI
Ceratitis capitata. IN THE STUDY WE EVALUATED THE TOXICITY OF THIS STRAIN TOWARD THE NEM
WITH OTHER *B. pumilus* STRAINS AND COMPARED ITS TOXICITY WITH A NON-PATHOGENIC STRAIN (*Escherichia coli* OP50) AND A PATHOGENIC ONE (*Burkholderia cepacia*). AFTER THIS STUDY, WE CONCLUDED THAT *B. pumilus* 15.1 IS A SAFE STRAIN AND COULD NOT REPRESENT A PROBLEM TO BE USED AS A BIOLOGICAL CON

Key words: *Caenorhabditis elegans*, PATHOGENICITY ASSAY, *Ceratitis capitata*, *Bacillus pumilus*

Introduction

Bacillus pumilus 15.1 STRAIN HAS BEEN RECENTLY DESCRIBED AS ACTIVE AGAINST LAR
MEDITERRANEAN FRUIT FLY, *Ceratitis capitata*, ONE OF THE WORST PEST FOR FRUITS AND VEGETA
WORLD-WIDE. THE TOXICITY MECHANISM OF THIS STRAIN IS COMPLETELY UNKNOWN,
ELUCIDATING IT ARE ON PROGRESS. *B. pumilus* 15.1 COULD BE USEFUL IN THE FUTURE FOR DEVELOPI
BIOINSECTICIDE AGAINST THIS PEST. FOR THAT REASON, AND SINCE *B. pumilus* SPECIES IS NOT
CONSIDERED AS A HEALTH RISK, WE DECIDED TO EVALUATE THE BIOSAFETY OF THIS
C. elegans PATHOGENICITY ASSAY.

C. elegans (MAUPAS, 1900) IS A SOIL NEMATODE THAT FEEDS ON MICROORGANISM AND
WELL STUDIED ORGANISM MODEL FOR THE STUDY OF MICROBIAL PATHOGENICIT
MECHANISMS (ABALLAY & AUSUBEL, 2002; *et al.*, 2010), GIVEN IT IS A EUKARYOTIC
ORGANISM WITH A VERY SIMPLE BIOLOGICAL CYCLE AND EASY TO MAINTAIN UN
CONDITIONS.

HERE WE DESCRIBE THE PATHOGENICITY ASSAYS PERFORMED ON *C. elegans* FED ON THE STRAIN
B. pumilus 15.1 AND OTHER BACTERIAL STRAINS WITH KNOWN PATHOGENICITY IN ORDER
BIOSAFETY OF THE STRAIN.

Material and methods

Bacterial strains and culture conditions

Bacillus pumilus 15.1, *B. pumilus* M1, *Bacillus thuringiensis* VAR. *kurstaki*, *Escherichia coli* OP50, AND *Burkholderia cepacia* WERE CULTURED ON LB PLATES AT 30 °C. WHEN CULTURE

LIQUID MEDIUM, 3 ML OF LB WERE PLACED IN A 15 ML TEST TUBE AND INCUBATED OVE
ORBITAL SHAKER (240 RPM) AT 30 °C.

Pathogenicity assay

C. elegans WAS GROWN ON THE *E. coli* OP50 STRAIN CULTURED ON PDA (POTATO DEXTROSE AGAR
SIGMA, 15 G L^{-1} AGAR, 20 G L^{-1} DEXTROSE AND 4 G L^{-1} POTATO EXTRACT) PLATES (NAM *et al.*, 2007).
WHEN NEMATODES WERE NEEDED FOR THE ASSAY, PDA PLATES WERE DISRUPTED AND W
OF RUNNING WATER AND FILTERED THROUGH A 90 MESH SIEVE. THE NEMATODE SUSPENSION WAS ALLOWED T
SEDIMENT FOR 3 H IN A DECANTATION FLASK. THEN, THE SUPERNATANT WAS FILTERED T
SIEVE TO OBTAIN THE NEMATODES. NEMATODES WERE TRANSFERRED INTO A 50 ML CO
WITH 17 ML OF WATER SUPPLEMENTED WITH 1 G L^{-1} STREPTOMYCIN AND AMPICILLIN AND INCUBATED FOR 2 H IN ORDER
ELIMINATE THE *E. coli* OP50 STRAIN. ONCE CONCENTRATED, 1 ML OF NEMATODE SUSPENSIO
TRANSFERRED TO A FUJIWARA SLIDE AND OBSERVED UNDER A NIKON SMZ800 MICROSCOP
NEMATODES WERE SELECTED. FIVE PREADULTS WERE PLACED ON PDA PLATES WITH A L
STRAIN. TO GET THIS BACTERIAL LAWNS, 24 H BEFORE THE PATHOGENICITY ASSAY, 50 µL OF AN OVERNIGHT
CULTURE OF THE BACTERIAL UNDER STUDY WAS EVENLY DISTRIBUTED ON THE SURFAC
PLATE AND INCUBATED OVERNIGHT AT 30 °C. ONCE THE 5 PREADULTS WERE PLACED O
LAWN, PLATES WERE INCUBATED AT 20 °C. IN EACH ASSAY, EVERY STRAIN WAS TESTE
PLATES AND THE ASSAY WAS REPEATED TWICE.

Nematode counting

EVERY DAY, EACH PLATE WAS OBSERVED UNDER THE MICROSCOPE AND THE NUMBER OF
PER PREADULTS AND ADULTS WAS COUNTED AND REGISTERED. AT THE END OF THE EX
POPULATION OF NEMATODES IN EACH ASSAY WAS DETERMINED. FOR THAT, AGAR FR
DISRUPTED, WASHED AND FILTERED THROUGH A 90 MESH SIEVE AS PREVIOUSLY DESCRIBED. NEMATODE
WERE RECOVERED IN A FINAL VOLUME OF 20 ML OF WATER. ONE MILLILITRE OF THE SUS
TO DETERMINE THE NUMBER OF INDIVIDUALS UNDER THE MICROSCOPE AND TOTA
NEMATODES IN EACH PLATE WAS EXTRAPOLATED.

Results and discussion

IN ORDER TO DETERMINE THE BIOSAFETY OF THE *B. pumilus* 15.1 STRAIN, WE PERFORMED A *C. elegans*
PATHOGENICITY ASSAY PREVIOUSLY DESCRIBED BY Ben Arfa *et al.* (2013) TOGETHER WITH
B. pumilus 15.1 STRAIN, THE *E. coli* OP50 WAS USED AS NEGATIVE CONTROL OF PATHOGEN
AND *Burkholderia cepacia* AS POSITIVE CONTROL (PALLERONI & HOLMES, 1981; YABUUCHI *et al.*,
1992). IN ADDITION, SEVERAL OTHER STRAINS WERE INCLUDED IN THE ASSAY, AS *Bacillus thuringiensis*
kurstaki, AND TWO OTHER *B. pumilus* STRAINS. IN THE STUDY WE FOLLOWED THE POPULATION D
OF *C. elegans* BY COUNTING THE NUMBER OF EGGS, JUVENILES/PREADULTS AND ADULTS
(TABLES 1, 2 AND 3).

IN ADDITION, WE RECORDED THE TOTAL NUMBER OF INDIVIDUALS 7 DAYS AFTER THE
ASSAY (FIGURE 1).

TABLE 1. NUMBER OF EGGS (MEANS AND STANDARD DEVIATION, SD) REGISTERED IN PDA
TIME.

	24 h		48 h		72 h		96 h	
Strain	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
***B. pumilus* 15.1**	1.25	2.58	10.62	6.30	64.125	26.88	293.75	155.56
***B. pumilus* 15.1C**	0	0	23.1	15.41	63	37.06	206	138.86
***B. pumilus* M1**	1.25	1.71	14.12	9.97	84.375	47.46	219	109.14
B. t.* var. *kurstaki	0	0	3.75	0	2.875	4.67	2.875	4.67
***E. coli* OP50**	0.125	0.33	2	0	161.5	115.82	239	143.3
B. cepacia	0	0	0	0	0	0	0	0

TABLE 2. NUMBER OF JUVENILE PER PREADULTS (MEANS AND SD) REGISTERED IN PDA P
TIME.

	24 h		48 h		72 h		96 h	
Strain	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
***B. pumilus* 15.1**	3.25	2.98	10.25	8.65	58.12	20.24	352.25	60.43
***B. pumilus* 15.1C**	3.87	4.98	16.12	5.62	71.25	26.77	255.12	131.62
***B. pumilus* M1**	8.50	8.83	12.62	5.65	73.87	24.33	427.5	245.80
B. t.* var. *kurstaki	1.00	1.50	1.12	1.61	2.75	3.63	2.75	3.86
***E. coli* OP50**	2.25	2.77	25.12	16.18	129.25	84.70	379.25	41.03
B. cepacia	0.50	0.70	1.12	1.53	0	0	0	0

TABLE 3. NUMBER OF ADULTS (MEANS AND SD) REGISTERED IN PDA PLATES ALONG TIME.

	24 h		48 h		72 h		96 h	
Strain	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
***B. pumilus* 15.1**	5.60	0.80	5.00	1.35	14.75	4.99	103.12	32.59
***B. pumilus* 15.1C**	5.00	0	14.87	14.97	25.12	24.07	88.00	41.98
***B. pumilus* M1**	5.60	1.20	5.37	1.67	17.75	6.19	86.62	36.13
B. t.* var. *kurstaki	5.00	0.63	1.12	0	1.50	2.34	0.62	1.31
***E. coli* OP50**	5.00	0	10.5	15.67	27.75	9.76	83.75	62.77
B. cepacia	5.00	0.63	0.87	0.74	0	0	0	0

RESULTS SHOWED THAT UNDER THE CONDITIONS USED, *E. coli* OP50 SUPPORT A NEMATODE POPULATION OF 1208 (± 299) INDIVIDUALS PER PLATE AFTER 7 DAYS. IN THE SAME WAY, *B. pumilus* 15.1, *B. pumilus* 15.1C, AND *B. pumilus* M1, SUPPORTED A HIGH NEMATODE POPULATION OF 725 (± 19 734 (± 312), AND 825 (± 246) NEMATODES PER PLATE, RESPECTIVELY. *B. thuringiensis*, A STRAIN CONSIDERED AS SAFE AND EXTENSIVELY USED AS BIOLOGICAL CONTROL, SUPPORT POPULATION OF 62 (± 23) INDIVIDUALS PER PLATE, WHILE *B. cepacia*, CONSIDERED AS A PATHOGEN DID

NOT SUPPORT NEMATODE PROLIFERATION. AFTER THIS STUDY WE CONCLUDED THAT *B. pumilus* 15.1 IS A NON-PATHOGENIC STRAIN AND COULD POTENTIALLY BE USED AS A BIOLOGICAL SAFE WAY.

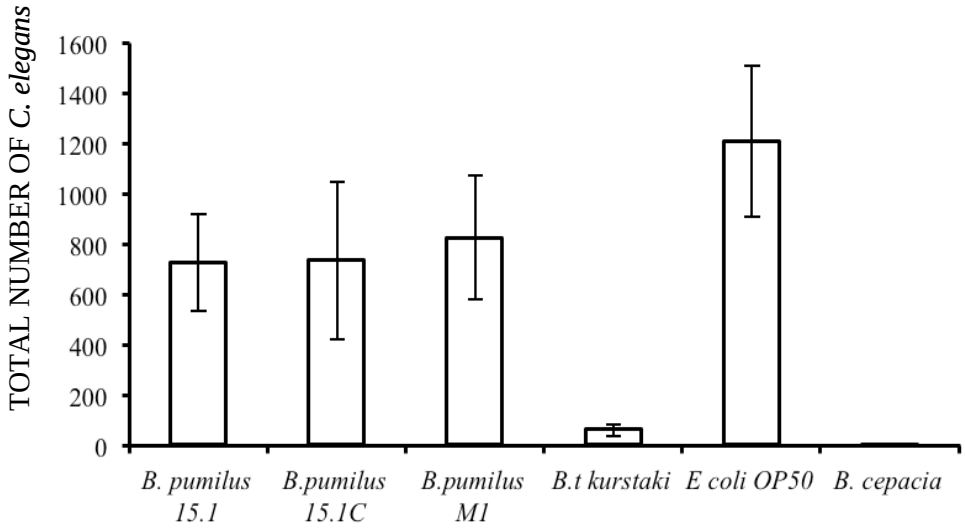

FIGURE 1. *Caenorhabditis elegans* PATHOGENICITY ASSAY. TOTAL NUMBERS OF INDIVIDUA *C. elegans* 7 DAYS AFTER THE BEGINNING OF THE ASSAY.

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The indigenous entomopathogenic nematode searching results at different agrocenosis of Georgia

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Abstract: SOME KIND OF SOIL IS A HABITAT FOR ENTOMOPATHOGENIC NEMATODES (EPNS), WHICH MA BE CONSIDERED AS POTENTIAL BIOLOGICAL CONTROL AGENTS TO VARIOUS PEST INSECTS IN D AGROECOSYSTEMS OF GEORGIA. THE INVESTIGATIONS OF SOIL SAMPLES FOR SEARCHING AND ISOLAT LOCAL EPN STRAINS HAVE BEEN CONDUCTED. DETERMINATION OF INVASIVE ABILITY OF ISOL NEMATODES HAS BEEN CARRIED OUT ACCORDING TO GENERALLY ACCEPTED METHODS IN NEMATOLOGY. THE INFECTIVITY OF ISOLATES ON LABORATORY INSECT CULTURES, THE GREATER WAX *Galleria mellonella,* AND THE MEAL WORM, *Tenebrio molitor,* HAS BEEN APPROVED. AS A RESULT OF MULTIPLE RESEARCHES, THE NEW MODEL OF NEMATODE DIRECT MIGRATION HAS BEEN ELABORATED, GIVES POSSIBILITY TO OBTAIN MORE INFECTIVE JUVENILES (IJS) FROM SOIL DURING A SHORT PERIOD. T EXPERIMENTS WERE CONTINUED ON ESTABLISHMENT OF NEW ISOLATES INVASIVE ABILITY. 100 STRAIN I, 100-150 IJS OF STRAIN II, AND 100-120 IJS OF STRAIN III WERE USED FOR CONTAMINATION 10 *G. mellonella* LARVAE OF AVERAGE SIZE. THE LAST INSTARS OF 10 *T. molitor* LARVAE OF WERE INFECTED BY 150 IJS OF ALL EXPERIMENTAL STRAINS. A TYPICAL PATTERN OF NEMATODE PATHOLO BEEN OBTAINED AND IJS WERE APPLIED TO THE TEST INSECTS IN NEXT TRIALS OF BIOASSA COMPARATIVE VIRULENCE HAS BEEN DETERMINED BETWEEN STRAIN I, STRAIN II AND STRAIN PRELIMINARY RESULTS SHOW PERCEPTIVITY OF NEW APPROACH ISOLATION NEMATODES FOR SEARCHIN EPN LOCAL STRAINS, WHICH ARE PRODUCED *in vivo* FOR IDENTIFICATION.

Key words: ENTOMOPATHOGENIC NEMATODES, VIRULENCE, *Galleria mellonella, Tenebrio molitor*

Insect pathogens and entomoparasitic nematodes
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Field evaluation of entomopathogenic nematodes for controlling fall webworm *Hyphantria cunea* (Lepidoptera: Arctiidae) in West Georgia

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Abstract: THE PRESENT WORK DEALS WITH RESULTS OF APPLICATION OF ENTOMOPATHOGENIC N
GENUS *Steinernema* (*S. carpocapsae, S. thesami* AND *Steinernema* SP.) AGAINST THE HARMFUL PEST OF THE
FOREST AND AGRICULTURAL CROPS, *Hyphantria cunea* (LEPIDOPTERA: ARCTIIDAE) DISTRIBUTED IN GEORGIA. FI
EXPERIMENTS WERE CARRIED OUT IN AUGUST OF 2012 ON PRIVATE PLOTS OF GURIA REGION OF T
IN HAZELNUT PLANTATIONS DISEASED WITH PEST'S LARVAE. A HIGH PERCENTAGE OF MORTALITY
TO 98.3% WAS OBSERVED IN ALL EXPERIMENTS AS A RESULT OF ENTOMOPATHOGENIC NEMAT
AMONG THE SPECIES USED, THE EFFICIENCY OF *Steinernema* SP. SPECIES WAS SPECIALLY NOTICED. HIGH
EFFICIENCY OF THE TREATMENT WAS ALSO PROMOTED BY OPTIMUM CLIMATIC CONDITIONS (TEM
AND HYGROMETRY = 99%).

Key words: ENTOMOPATHOGENIC NEMATODES, BIOFORMULATION, *Hyphantria cunea*

Introduction

THE AMERICAN WHITE WEBWORM OR FALL WEBWORM (FWW) *Hyphantria cunea* DRURY (LEPIDOPTERA: ARCTIIDAE) IS A VERY HARMFUL QUARANTINE PEST. IT IS DISTRIBUTED I
THE PEST IS A POLYPHAG INSECT. IT HAS BEEN ESTABLISHED THAT THE SPECIES DAMAGE
PLANT SPECIES IN GEORGIA (EDILASHVILI, 2002). AS FWW IS ALSO AN URBAN INSECT, TH
THIS PEST NEEDS SPECIAL BIOFORMULATIONS (ENTOMOPATHOGENIC FUNGI, VIRUSES, BA
AND OTHER ORGANISMS) WHICH ARE SAFE FOR HUMAN AND ENVIRONMENT. NUMEROUS E
BEEN CARRIED OUT USING THE MENTIONED BIOFORMULATIONS, WHICH SHOW THAT
FLUCTUATES WITHIN THE RANGE 55-98% (BURJANADZE *et al.*, 2012; GORGADZE *et al.*, 2011;
GORGADZE, 2000; EDILASHVILI , 2002; LORTKIPANIDZE *et al.*, 2010).

THE OBJECTIVE OF THE PRESENT INVESTIGATION WAS TO STUDY THE EFF
ENTOMOPATHOGENIC NEMATODES BELONGING TO THE GENUS *Steinernema* (*S. carpocapsae, S. thesami* AND *Steinernema* SP.) AGAINST FALL WEBWORM AT OPTIMUM CONDITIONS IN THE

S. carpocapsae INTRODUCED TO GEORGIA IS ASSOCIATED WITH A SPECIFIC SYMBIOTI
Xenorhabdus nematophila, WHEREAS LOCAL FORMS, *S. thesami* AND *Steinernema* SP.,
WHICH BELONG TO THE *S. affine/intermedium* GROUP ARE ASSOCIATED WITH THE SYMBIOTIC BAC
Xenorhabdus bovienii. SPECIES OF BACTERIA ASSOCIATED WITH LOCAL NEMATODES H
IDENTIFIED AT THE LABORATORY OF DIVERSITY, GENOME, AND MICROORGANISMS-INSEC
(DGIMI, INRA) OF THE NATIONAL INSTITUTE OF AGRONOMIC RESEARCH OF MONTPELLIER UNIVERSITY
(FRANCE).

Material and methods

INFECTIVE JUVENILES OF (S.) *Sf* (S.) *carpocapsae, S. thesami* AND *Steinernema* SP. WERE REARED ON LARVAE OF *Galleria melonella* AND *Bombyx mori* (VEREMCHUK, 1986; DUTKY, 1964). FIELD EXPERIMENTS WERE CARRIED OUT IN THE SECOND DECADE OF 2012 IN GURIA REGION (W HAZELNUT PLANTATIONS DISEASED WITH FWW LARVAE. THE PEST PRODUCES TWO GENER SEASON IN THE MENTIONED REGION – IN MAY AND AUGUST. THE WARMEST MONTH AUGU FOR EXPERIMENTS. CONCENTRATED NEMATODES WERE TRANSPORTED IN ICEBOX IN C MORTALITY OF NEMATODES DUE TO TRANSFER TO LONG DISTANCE (300 KM). BEFOR EXPERIMENTS AND TREATMENT OF BEFOREHAND CHOSEN EXPERIMENTAL AND CONTROL OF PESTS PER M^2 AREA OF BRANCHES WAS EVALUATED. THE NUMBER OF PESTS PER M^2 VARIED FROM 65 TO 289. EXPERIMENTS WERE CARRIED OUT AS FOLLOWS: ONE CONTROL PLANT WITHOU APPLICATION AND 3 EXPERIMENTAL PLANTS (ONE WITH *S. carpocapsae*, THE SECOND ONE WITH *S. thesami* AND THE THIRD ONE WITH *Steinernema* SP.). ALL SUSPENSIONS USED IN TRIALS CONTAINED EQ CONCENTRATION OF NEMATODES (2500 ± 120 NEMATODES ML^{-1}). THE TREATMENT OF EXPERIMENTAL PLANTS BY NEMATODE SUSPENSION WAS PERFORMED USING THE HAND APPARATUS OF T IN EVENING HOURS, IN CLOUDY WEATHER AT 28 °C TEMPERATURE AND 99% RELATIVE HUMIDITY MONITORING OF TREATED PLANTS AND ACCOUNTING OF DEAD PESTS WAS MADE ON 3RD, 5TH AND 7TH DAYS AFTER SPRAYING ACCORDING TO THE METHOD BY ABBOTT (ABBOTT, 1925).

Results and discussion

CHECKING OF SPRAYED PLANTS 14 HOURS AFTER TREATMENT SHOWED THAT THE NEM WAS NOT DRIED OUT ON LEAF SURFACES, ESPECIALLY ON THE LOWER SIDES OF LEAVES WERE ASSEMBLED IN COLONIES. WHILE EXAMINING SUCH LEAVES ONLY LIVING AND A INVASIVE JUVENILES WERE REVEALED. NONE OF INDIVIDUALS OF LARVAE WAS DEAD. PASSIVE CONDITION, THOUGH REACTED ON IRRITANT.

TWENTY HOURS AFTER TREATMENT WITH ENTOMOPATHOGENIC NEMATODES, DAM CAUSED BY PESTS WAS REDUCED, WHILE MORTALITY RATE OF PESTS WAS SIGNIFICANTL THE 3RD DAY POST-TREATMENT.

ONLY THREE DAYS AFTER TREATMENT, MORE THAN 90% OF THE PEST LARVAE WERE DE NEMATODE SPECIES USED. WHERE SUSPENSION OF *S. carpocapsae* WAS USED FOR SPRAYING, 94.3% MORTALITY OF PEST'S LARVAE HAS BEEN STATED ON THE EXPERIMENTAL PLANT; ON THE 5TH DAY MORTALITY REACHED 98.1%; AND ON THE 7TH DAY THE MORTALITY RATE OF PESTS WAS ALMOST NOT CHAN AVERAGE MORTALITY RATE IN THIS VARIANT OF EXPERIMENT WAS 96.8% (FIGURE 1).

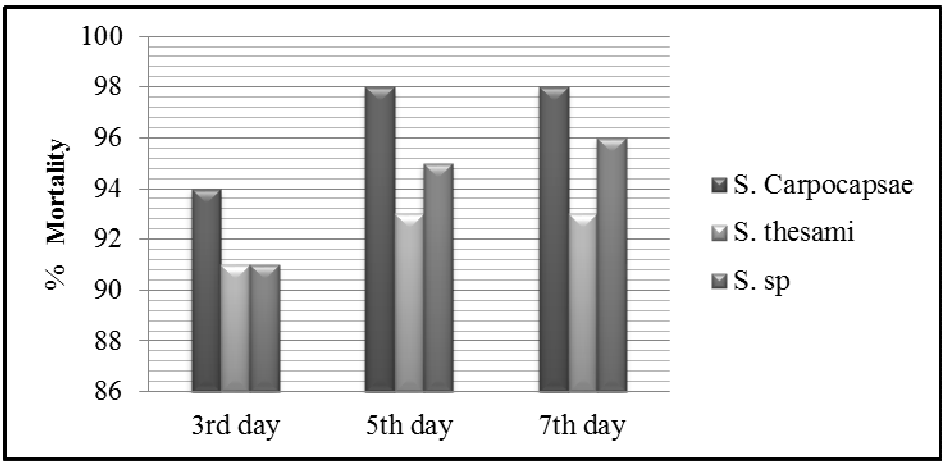

FIGURE 1. MORTALITY OF *Hyphantria cunea* 3RD - 4TH INSTAR LARVAE AFTER APPLICATION O ENTOMOPATHOGENIC NEMATODE SUSPENSION OF THE GENUS *Steinernema* (250 - 120 NEMATODES/ML WATER) UNDER FIELD CONDITIONS (TEMPERATURE: 28 °C, HUMIDITY: 99%).

SIMILAR RESULTS OF MORTALITY WERE OBTAINED FOR *S. thesami* IN BIOCONTROL. THE HIGHEST MORTALITY RATE OF 93% WAS REACHED 5 DAYS AFTER TREATMENT.

WHEN *Steinernema* SP. WAS TESTED AGAINST FWW, THE MORTALITY RATE WAS 95% ON 7TH DAY AFTER APPLICATION, SIMILAR TO THE OTHER TESTED NEMATODES *S. carpocapsae* AND *S. thesami*. IN THE UNTREATED CONTROL EXPERIMENTS, NO MORTALITY OF THE PEST WAS OBSERVED UNDER THE BINOCULAR MICROSCOPE, 22-36 INDIVIDUALS OF DEVELOPED 4TH - 5TH INSTAR *Steinernema* NEMATODES WERE OBSERVED IN EACH DEAD LARVA OF FWW.

IN ALL EXPERIMENTS WHERE *S. carpocapsae, S. thesami* AND *Steinernema* SP. WERE USED FOR THE BIOLOGICAL CONTROL OF FWW, HIGH MORTALITY LEVELS WERE OBSERVED. IT IS W SPECIAL EFFICIENCY OF A NEW *Steinernema* SPECIES SP. AGAINST THE PEST. HIGH EFFICIENCY OF TH USED FORMULATIONS SEEMS TO BE FAVOURED BY OPTIMAL CLIMATIC CONDITIONS HUMIDITY, ETC.) DURING THE EXPERIMENT. THESE PARAMETERS ARE OF GREAT IMPO ACTIVITY AND EFFICIENCY OF ENTOMOPATHOGENIC NEMATODES.

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Insect pathogens and entomoparasitic nematodes
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Feeding activity and survival of slug *Arion lusitanicus* (Gastropoda: Arionidae) exposed to the rhabditid nematode, *Phasmarhabditis hermaphrodita* (Nematoda: Rhabditidae)

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Abstract: SLUGS ARE IMPORTANT PESTS OF CULTIVATED PLANTS IN CROATIA. IN MANY SITES SLUG *Arion lusitanicus* (MABILLE, 1868) (GASTROPODA: ARIONIDAE) HAS BECOME THE MOST FREQUENT SPECIES VERY HARD TO CONTROL BY CHEMICAL MOLLUSCICIDES. SINCE 1996 A BIOLOGICAL MOLLUSC NEMATODE *Phasmarhabditis hermaphrodita* (SCHNEIDER, 1859) (NEMATODA: RHABDITIDAE) HAS BEEN FORMULATED AS AN EFFECTIVE PRODUCT FOR SLUG CONTROL. IN ORDER TO ESTABLISH FEEDING OF ADULT *A. lusitanicus* SPECIMENS EXPOSED TO PARASITIC NEMATODE *P. hermaphrodita* AND TO COMPARE ITS EFFICIENCY TO EFFICIENCY OF CHEMICAL MOLLUSCICIDES, A LABORATORY EXPERIMENT WAS P SPECIMENS OF *A. lusitanicus* (FEEDED ON LETTUCE LEAVES IN FLOWER POTS) WERE EXPOSE *P. hermaphrodita*, METALDEHYDE AND METHIOCARB TREATMENTS. FOOD CONSUMPTION OF SLUG SP MEASURED DAILY. SURVIVAL OF SLUGS WAS OBSERVED TO THEIR DEATH, BUT IN THE FIRST 30 TH DAY OF INVESTIGATION, CHEMICAL MOLLUSCICIDE TREATMENTS WERE FOUND TO DIFFER SIGNIFICANTLY PRODUCT AND CONTROL. AT THE TREATMENTS TREATED BY NEMATODES, DAILY LEAF AREA CO REDUCED AND WAS SIGNIFICANTLY DIFFERENT FROM THE CONTROL TREATMENT. FOOD CONSUMP BOTH CHEMICAL AND BIOLOGICAL TREATMENTS BUT THE MOST OF *A. lusitanicus* SPECIMENTS OF AND CONTINUED TO FEED. TO THE END OF THE SECOND WEEK OF INVESTIGATION, FOOD CONSUMP ALL TREATMENTS AND WAS MAINLY UNIFORM WITH NO SIGNIFICANT DIFFERENCES BETWEEN TRE EXPERIMENT, THE SLUGS WERE DYING WITHIN THE PERIOD OF 3 TO 30 D AT THE TREATMENTS TRE *P. hermaphrodita* OR IN THE PERIOD OF 9 TO 24 D AT THE TREATMENTS TREATED BY METALD METHIOCARB. BECAUSE THE TOLERANCE LEVEL TO SLUG DAMAGES IN LETTUCE MARKET IS EFF RESULTS INDICATE A FAILURE OF BIOLOGICAL PRODUCT BASED ON *P. hermaphrodita* IN CONTROL OF OF *A. lusitanicus* AS WELL AS A FAILURE OF CHEMICAL MOLLUSCICIDES. THESE DATA POINT AT A G INTEGRATED CONTROL OF SLUG DAMAGE WHICH MUST INCLUDE CULTURAL AND DIFFERENT MEASURES, NOT ONLY CHEMICAL CONTROL.

Key words: *Arion lusitanicus*, MOLLUSCICIDES, PARASITIC NEMATODES, *Phasmarhabditis hermaphrodita*, SLUGS, SLUG CONTROL

Introduction

Arion lusitanicus (MABILLE, 1868) IS THE SLUG SPECIES WHICH HAS SPREAD WITH AN ALARMI MANY SITES IN CROATIA, WHERE IT IS CONSIDERED AS A SERIOUS PEST. IT DAMAGES MA AND ORNAMENTALS BUT ALSO OILSEED RAPE, MAIZE AND SUNFLOWERS. IN CEN *A. lusitanicus* IS THE MAJOR PEST SLUG SPECIES AND THE MOST SALES OF MOLLUSCICIDES I AND GARDEN MARKET CAN BE ATTRIBUTED TO THIS SPECIES (WEIDEMA, 2006). CURRENT C FOR *A. lusitanicus* IN CROATIA RELY ON CHEMICAL MOLLUSCICIDES, WHICH ARE OFTEN INEF CAN HARM NON-TARGET ORGANISMS. *Phasmarhabditis hermaphrodita* (SCHNEIDER, 1859) IS A NEMATODE THAT PARASITES MANY SLUGS AND SNAILS. IN 1994 IT WAS FORMULATED I MOLLUSCICIDE (*et al.*, 1996) WHICH IS USED AS INUNDATIVE BIOCONTROL AGENT. THE NEM

IS APPLIED ONTO SOIL, WHERE IT SEEKS OUT SLUGS AND INFECTS THEM. INFECTION RA
FEEDING INHIBITION AND LATER KILLS THE SLUGS. THERE ARE SOME INDICATIONS THAT I
SPECIES SUSPECTIBILITY. THE *P. hermaphrodita* DECREASES WITH BODY SIZE AND THAT
P. hermaphrodita CANNOT KILL OR INHIBIT FEEDING OF *A. lusitanicus* INDIVIDUALS OF >1 G WEIGHT
(GRIMM, 2002; SPEISER *et al.*, 2001). IN ORDER TO ESTABLISH THE EFFICACY OF *P. hermaphrodita* IN
CONTROL OF ADULT SPECIMENS OF *A. lusitanicus*, THE LABORATORY EXPERIMENT WAS PERFORMED
ZAGREB IN AUTUMN 2009.

Material and methods

LABORATORY EXPERIMENT WAS PERFORMED IN PERIOD OCTOBER 26 TO NOVEMBER 9, 2009
MAKSIMIR, CROATIA. THERE WERE SIX TREATMENTS IN THE EXPERIMENT: (1) UNTREATE
METALDEHYDE PELLETS (5% ACTIVE INGREDIENT, RECOMMENDED RATE), (3) METHIOCA
ACTIVE INGREDIENT, RECOMMENDED RATE), (4) *P. hermaphrodita* (30 NEMATODES $^{-2}$ CM
RECOMMENDED RATE), (5) *P. hermaphrodita* (15 NEMATODES $^{-2}$ CM) AND (6) *P. hermaphrodita* (15
NEMATODES $^{-2}$ CM + *P. hermaphrodita* (15 NEMATODES $^{-2}$ CM APPLIED ONE WEEK FOLLOWING THE
FIRST APPLICATION). THERE WERE FOUR REPLICATES OF EACH TREATMENT. EVERY
REPRESENTED BY FLOWER POT (21 CM IN DIAMETER) REPLETED BY POTTING SOIL. ONE ADU
A. lusitanicus (WEIGHT 2 G) WAS PLACED ALONG WITH ONE LETTUCE LEAF IN EVERY FLOWER
LEAVES WERE CHANGED EVERY DAY. COMMERCIAL FORMULATION OF *P. hermaphrodita*
(PHASMARHABDITIS – SYSTEM; SUPPLIER: BIOBEST N.V., BELGIUM) WAS USED IN THE EXP
THE NEMATODES WERE STIRRED IN WATER AND WERE APPLIED TO THE FLOWER POTS US
"ROSE". PELLETS OF MOLLUSCICIDES WERE BROADCASTED BY HAND ON THE SOIL SURFAC
SLUG FEEDING WAS ASSESSED BY MEASURING OF THE LETTUCE LEAF AREA EATEN BY
MILLIMETER PAPER) AFTER 14 D. FOR THE PURPOSE OF INTERPRETING THE RESULTS, AIR TEMPERA
MEASURED ON MEASUREMENT STATION IN ZAGREB - MAKSIMIR. DATA WERE SUBJECTED TO
ANOVA AND DUNCAN'S NEW MRT (P = 0.05). SIMULTANEOUSLY, MONITORING OF APPEAR
BEHAVIOR CHANGES AND SURVIVAL OF SLUGS WAS CONDUCTED. AFTER MEASUREMENT
AFTER 14 D THE SURVIVAL OF SLUGS IN EACH REPLICATE WAS OBSERVED TO THEIR DEATH

Results and discussion

OVERVIEW DATA ABOUT FOOD CONSUMPTION OF *A. lusitanicus* SPECIES IN A LABORATORY
EXPERIMENT MEASURED FOR 14 D ARE PRESENTED IN FIGURE 1. FOOD CONSUMPTION CAUS
WAS EVIDENTED ON ALL TREATMENTS. THE DATA OBTAINED ON BIOLOGICAL PRODUC
DIFFERENT THEN THE DATA REPORTED BY GLEN AND GREWAL (2000) AND *et al.* (2001, 2003) INDICATED
THAT SLUGS INFECTED BY NEMATODES CEASE TO FEED IMMEDIATELY, HOWEVER, FOOD CONSUMPTION
OF *A. lusitanicus* ON TREATMENTS TREATED BY CHEMICAL MOLLUSCICIDES WAS FOUND
SIGNIFICANTLY FROM BIOLOGICAL PRODUCT TREATMENTS AND CONTROL (FIGURE 1). IT W
BECAUSE NEMATODES NEED A FEW DAYS TO BEGIN PARASITIZATION AND DISABLING T
NEMATODE TREATMENTS, DAILY LEAF AREA CONSUMPTION, IN THE FIRST WEEK OF EXPE
REDUCED AND WAS SIGNIFICANTLY DIFFERENT FROM THE UNTREATED CONTROL. FROM THE 12
OF FOOD CONSUMPTION ASSESSMENT STATISTICALLY SIGNIFICANT DIFFERENCES WERE DE
8, 10 AND 11, WHEN SIGNIFICANTLY LESS FOOD CONSUMPTION WAS MEASURED ON ALL
BIOLOGICAL TREATMENTS COMPARED TO THE UNTREATED CONTROL. TOWARDS THE EN
FROM DAY 12 TO 14, POOR FEEDING OF SLUGS ON LETTUCE LEAVES WAS EVIDENT IN ALL T
CONSUMPTION WAS OFTEN UNIFORM AND THERE WERE NO SIGNIFICANT DIFFERENCES BE

EXPOSURE OF SLUGS TO LOW TEMPERATURES MAY LEAD TO REDUCED FEEDING, THEREFOR
WERE COMPLETED AFTER 14 D.

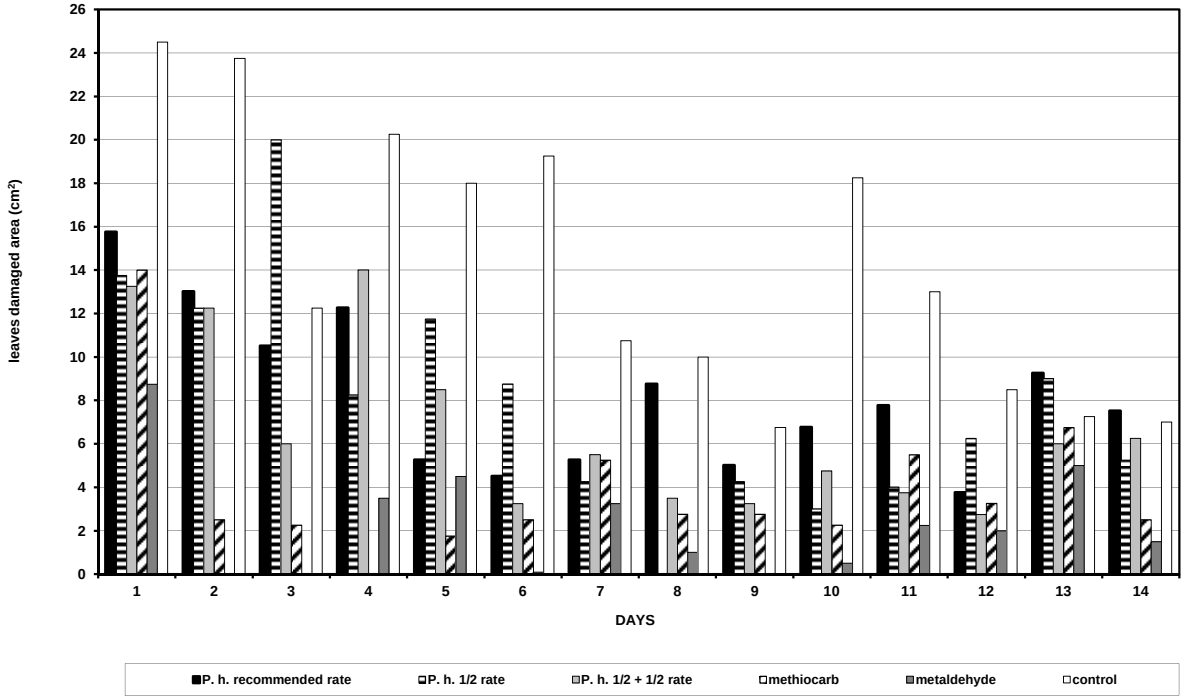

FIGURE 1. MEAN FOOD CONSUMPTION OF *Arion lusitanicus* IN FEEDING EXPERIMENT.

MONTORING OF THE SURVIVAL OF SLUGS (FEEDED BY LETTUCE) WAS CONTINUED FROM
30. DURING THE EXPERIMENT, THE SLUGS DIED BETWEEN DAY 3 TO DAY 30 WHEN TRE
P. hermaphrodita AND BETWEEN DAY 9 AND DAY 24 WHEN TREATED WITH METALDEH
METHIOCARB. GLEN (2000) AND GREWAL *et al.* (2001, 2003) REPORTED THAT SLUGS INFECTED BY
NEMATODES DIE WITHIN 4 TO 20 D, DEPENDING ON TEMPERATURE AND ABUNDANCE OF
POPULATION, WHAT IS COMPARABLE WITH RESULTS IN THIS INVESTIGATION. IN A 14-DA
GRIMM (2002) REPORTED SIGNIFICANT MORTALITY (ABOUT 30-50%) OF *A. lusitanicus* SPECIMENS OF
0.24 G OR SMALLER, WHILE THE MORTALITY OF BIGGER SPECIMENS (0.45 G) WAS LESS THA
DAILY TEMPERATURES MEASURED IN MAKSIMIR WERE 11.7 °C IN OCTOBER 2009 AND 8
NOVEMBER. THE MEASURED TEMPERATURES WERE LOW, WHICH COULD BE THE REASO
COMPARED TO LITERATURE DATA, A BIT LONGER TIME WAS NEEDED FOR SLUGS TO DIE A
OF NEMATODES. THE OPTIMUM TEMPERATURE FOR THE DEVELOPMENT OF *P. hermaphrodita* NEMATODE
IS ABOUT 17 °C (GLEN *et al.*, 1996) SO IT IS OBVIOUS THAT THE TEMPERATURE CONDITIONS DUR
EXPERIMENT WERE NOT OPTIMAL. HOWEVER, NEMATODES DEVELOP IN THE TEMPERA
5-20 °C, INDICATING THAT NEMATODES IN SLUGS WERE DEVELOPING SLOWER DURING TH
THIS COULD BE THE REASON, WHY THE ACHIEVED EFFICIENCY IN REDUCING OF FOOD CON
LOWER. DURING THE EXPERIMENT, CHANGES ON THE BODY OF *A. lusitanicus* SPECIMENS IN FORM
OF A SWOLLEN MANTLE AND DAMAGES ON THE EPIDERMIS HAVE BEEN NOTICED. THIS W
BEFORE (TAN & GREWAL, 2001; GRIMM, 2002). GRIMM (2002) AND SPOSEL *et al.* (2010) REPORTED
THAT *P. hermaphrodita* CAN NOT KILL OR INHIBIT FEEDING OF *A. lusitanicus* OF MORE THAN 1 G WEIGHT.
THEIR FINDINGS ARE NOT IN ACCORDANCE WITH THE RESULTS OBTAINED IN THIS INVESTI
TO THE MEASURED FOOD CONSUMPTION, IT IS EVIDENT THAT NONE OF THE TREATMENTS

OF ADULT FORMS OF *A. lusitanicus* EFFECTIVELY. THIS HAS BEEN OBSERVED BY MANY PRODUCER
LETTUCE AND OTHER VEGETABLES. IN ACCORDANCE WITH OUR RESULTS, THE USE OF CHE
MOLLUSCICIDES NEED TO BE COMPLEMENTED BY CULTURAL METHODS, SUCH AS PHYS
ALTERNATIVE METHODS OF SLUG CONTROL.

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New insights to insect response to the infection by nematobacterial complex

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Abstract: ENTOMOPATHOGENIC NEMATODES (EPNS) OF THE GENERA *Heterorhabditis* AND *Photorhabdus* ARE OBLIGATE AND LETHAL INSECT PARASITES. IN RECENT YEARS THEY HAVE BEEN USED INCREASINGLY AS BIOCONTROL AGENTS. THESE EPNS ARE SYMBIOTICALLY ASSOCIATED WITH BACTERIA OF THE GENUS *Photorhabdus*. THE BACTERIAL SYMBIONTS ARE ESSENTIAL TO KILL THE HOST (WITHIN 24-48 H) AND DIGEST ITS TISSUES TO PROVIDE NUTRIENTS FOR THEMSELVES AS WELL FOR EXPANDING *Drosophila* LARVAE ARE SUITABLE INSECT HOSTS AND PART OF THE TRIPARTITE MODEL SYSTEM (*Drosophila*, NEMATODES, BACTERIA), DNA BEFORE TO SHOW THE IMPORTANCE OF HAEMOLYMPH CLOTTING AND EICOSANOIDS DURING

WE USED THE WELL-ESTABLISHED TRIPARTITE MODEL (*Drosophila*, NEMATODES, BACTERIA), DNA CHIPS AND BIOINFORMATIC TOOLS TO COMPARE GENE EXPRESSION IN NON-INFECTED AND LARVAE. WE FOCUSED ON THE EARLY TIME POINT OF NEMATODE INFECTION AND THEREFORE *Drosophila* LARVAE USING *H. bacteriophora* HARBOURING GFP-LABELLED *P. luminescens* BACTERIA. INFECTED (GFP POSITIVE) LARVAE WERE COLLECTED 6 HOURS AFTER INFECTION.

WE DETECTED APPROXIMATELY 650 GENES WHOSE EXPRESSION WAS SIGNIFICANTLY INDUCED BY NEMATOBACTERIAL INFECTION CAUSED BY *H. bacteriophora* AND *P. luminescens*. MOST OF THEM ARE UPREGULATED UPON INFECTION INCLUDING MAINLY THE GENES INVOLVED IN ANTIMICROBIAL DEVELOPMENT. BASED ON GENE ONTOLOGY ANNOTATION WE IDENTIFIED SEVERAL PATHWAYS COULD BE INVOLVED IN SEALING AND REPAIRING THE WOUND CAUSED BY INVADING NEMATODE.

WE COMPARED THESE RESULTS WITH THE AVAILABLE DATA FOR OTHER INFECTION TYPES BACTERIA AND PARASITIC WASPS. SMALL GROUP OF GENES WERE COMMON FOR ALL TYPES OF INFECTION AND APPROXIMATELY 25 GENES WERE OVERLAPPING IN EACH PAIRWISE COMPARISON FOCUSED ON THE GENES EXPRESSED IN THE HAEMOCYTES AND FAT BODY, RESPECTIVELY SUBJECTED SELECTED CANDIDATE GENES TO FUNCTIONAL TESTS. WE TESTED THE EFFECT OF KNOCKDOWN OF SELECTED GENES FOR THE SUSCEPTIBILITY OF FLIES TO THE NEMATOBACTERIA THE OVERLAP BETWEEN THE PROTECTIVE GENES AND GENES INDUCED BY THE NEMATOBACTERIA WAS NOT COMPLETE. THEREFORE, WE ASSUME THAT ONLY A FRACTION OF THE GENES IMPORTANT PROTECTION OF INFECTED LARVAE FROM DEATH ARE INDUCED BY THE NEMATOBACTERIAL

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Key words: *Drosophila*, IMMUNITY, *Heterorhabditis*, *Photorhabdus*

Insect pathogens and entomoparasitic nematodes
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Compatibility of five different entomopathogenic nematode (Nematoda: Rhabditida) species with registered insecticides and fungicides under laboratory conditions

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Abstract: TO INCREASE OUR KNOWLEDGE ON THE SUSCEPTIBILITY OF ENTOMOPATHOGENIC NE
SPECIES TO AGROCHEMICALS, THE COMPATIBILITY OF THE INFECTIVE JUVENILES (IJS) OF THE *Steinernema feltiae*
S. carpocapsae, *S. kraussei*, *Heterorhabditis bacteriophora* AND *H. downesi* WITH 6 CHEMICAL, ONE
PLANT-BASED AND ONE BIO-INSECTICIDE, AND 13 SYNTHETIC ORGANIC AND TWO INORGANIC
INVESTIGATED UNDER LABORATORY CONDITIONS. THE EFFECT OF DIRECT IJ EXPOSURE TO INSEC
HOURS WAS TESTED IN PETRI DISHES AT 15, 20 AND 25 °C. IN OUR EXPERIMENT WE DETERMIN
COMPATIBILITY OF *S. feltiae* WITH ACTIVE INGRIDIENTS AZOXYSTROBIN, AZADIRACHTIN, *Bacillus thuringiens*
kurstaki AND IMIDACLOPRID. THE PRESENT STUDY SHOWED THAT *S. carpocapsae* AND *S. kraussei* ARE SENSITIVE
TO ALL TESTED INSECTICIDES, *H. bacteriophora* IS SENSITIVE ONLY TO ABAMECTIN AND LUFENURO
NEMATODE *H. downesi* SIGNIFICANTLY SUFFERED THE HIGHEST MORTALITY WHEN INFECTIVE JUVENIL
WITH ACTIVE INGREDIENTS (A. I.) TEBUCONAZOLE, SPIROXAMINE, AND TRIADIMENOL. BASED ON
CONCLUDE THAT COMPATIBILITY IS NOT ONLY A SPECIES-SPECIFIC BUT ALSO A STRAIN-SPECIFIC C

Key words: ENTOMOPATHOGENIC NEMATODES, COMPATIBILITY, INSECTICIDES, FUNGICIDES

Introduction

ENTOMOPATHOGENIC NEMATODES (EPNS) (STEINERNEMATIDAE AND HETERORHABDITIDA
AS BIOLOGICAL CONTROL AGENTS TO SUPPRESS A VARIETY OF ECONOMICALLY IMPORT
(LAZNIK & TRDAN, 2011). EPN ARE OFTEN APPLIED TO SITES AND ECOSYSTEMS THAT ROUT
OTHER INPUTS THAT MAY INTERACT WITH NEMATODES, INCLUDING CHEMICAL PESTICID
SOIL AMENDMENTS (DE NARDO & GREWAL, 2003). IT IS OFTEN DESIRABLE TO KNOW IF A
BE TANK-MIXED OR APPLIED SIMULTANEOUSLY WITH ANOTHER PESTICIDE TO SAVE TIME
FOR COMPATIBILITY WITH INTEGRATED PEST MANAGEMENT (IPM) AND INTEGRATED
SYSTEMS (GREWAL, 2002).

EPN INFECTIVE JUVENILES (IJS) CAN TOLERATE SHORT-TERM EXPOSURE (2-24 H) TO MA
AND BIOLOGICAL INSECTICIDES, FUNGICIDES, HERBICIDES, FERTILIZERS, AND GROWTH F
THUS BE TANK-MIXED AND APPLIED TOGETHER (DE NARDO & GREWAL, 2003; LAZNIK *et al.*, 2012).
HOWEVER, GENERALIZATIONS CANNOT BE MADE BECAUSE THE NEMATODES` SUSCEPTIE
SEVERAL FACTORS, SUCH AS SPECIES, STRAIN, AGROCHEMICAL FORMULATIONS AND
(GREWAL, 2002; LAZNIK, 2012).

TO INCREASE THE KNOWLEDGE OF THE EPN SPECIES AND STRAIN SUSCEPTIBILITY TO
(INSECTICIDES AND FUNGICIDES) AND TO EXPLORE THE EFFECT OF THEIR MECHANISMS O
THESE ORGANISMS, THE AIM OF THIS STUDY WAS TO SELECT SOME COMMERCIAL IN
FUNGICIDES CURRENTLY USED IN SLOVENIA FOR INTEGRATED CROP PROTECTION AND
EFFECTS ON THE SURVIVAL OF IJS FROM NATIVE SLOVENIAN EPN STRAINS OF *Steinernema feltiae*

(FILIPJEV), *S. carpocapsae* WEISER, AND *Heterorhabditis bacteriophora* POINAR AND COMMERCIAL STRAINS [BECKER UNDERWOOD, *S. feltiae*, *S. carpocapsae* AND *S. kraussei* (STEINER); KOPPERT B.V: *S. Feltiae*] AT DIFFERENT TEMPERATURES UNDER LABORATORY CONDITIONS, THEREBY D SUITABILITY IN IPM PROGRAMS.

Material and methods

Pesticides

IN THE PRESENT STUDY, 8 COMMERCIAL INSECTICIDES AND 15 COMMERCIAL FUNGICID AGAINST DIFFERENT INSECT PESTS AND PATHOGENS IN SLOVENIA WERE EVALUATED (TAB

TABLE 1. TRADE NAMES, ACTIVE INGREDIENTS, AND CONCENTRATIONS (CONCENTR.) OF TESTED IN THIS STUDY.

Trade Name	Active ingredient	Test Concentr.	Trade Name	Active ingredient	Test Concentr.
ALIETTE FLASH	PHOSETHYL-AL	3.75 G L^{-1}	SCALITHANE	DINOCAP	0.4 ML L^{-1}
BELLIS	BOSCALID & PYRACLOSTROBIN	0.8 G L^{-1}	TATTOO	MANCOZEB & PROPAMOCARB	3 ML L^{-1}
CLARINET	FLUQUINCONAZOLE & PYRIMETHANIL	1.5 ML L^{-1}	TELDOR SC 500	FENHEXAMID	2 ML L^{-1}
CUPRABLAU-Z	COPPER HYDROXIDE & ZINC	4 G L^{-1}	VERTIMEC	ABAMECTIN	1.25 ML L^{-1}
DITHANE M-45	MANCOZEB	2.5 G L^{-1}	MATCH 050 EC	LUFENURON	2 ML L^{-1}
FALCON EC-460	TEBUCONAZOLE & SPIROXAMINE & TRIADIMENOL	0.4 ML L^{-1}	DELFIN WG	*B. thuringiensis* VAR. *kurstaki*	0.75 G L^{-1}
FOLPAN 80 WDG	FOLPET	150 ML L^{-1}	CHESS 50 WG	PYMETROZINE	0.6 G L^{-1}
PEPELIN	SULPHUR	6 G L^{-1}	NEEMAZAL-T/S	AZADIRACHTIN	3 ML L^{-1}
POLYRAM DF	METIRAM	1.2 G L^{-1}	CONFIDOR 200 SL	IMIDACLOPRID	0.75 ML L^{-1}
PREVICUR 607 SL	PROPAMOCARB	2.5 ML L^{-1}	KARATE ZEON 5 CS	LAMBDA-CIHALOTRIN	0.15 ML L^{-1}
RIDOMIL GOLD PLUS 42.5 WP	COPPER HYDROXIDE & METALAXYL-M	4 G L^{-1}	PIRIMOR 50 WG	PIRIMICARB	0.6 G L^{-1}
QUADRIS	AZOXYSTROBIN	1 ML L^{-1}	CONTROL	DISTILLED WATER	

Nematodes

EPNS WERE REARED AND PREPARED AS DESCRIBED EARLIER (PEČNIK *et al.*, 2012). SIX EPN STRAINS WERE INCLUDED IN THE INSECTICIDAL EXPERIMENT. THE COMMERCIAL PREPARATIONS *Steinernema feltiae*), NEMASYS C (*S. carpocapsae*) AND NEMASYS L (*S. kraussei*) WERE OBTAINED FROM BECKER UNDERWOOD (LITTLEHAMPTON, UNITED KINGDOM). ALL C

(*S. feltiae* B30, *S. carpocapsae* C101 AND *Heterorhabditis bacteriophora* D54 WERE ISOLATED FROM THE SOIL IN SLOVENIA (LAZNIK & TRDAN, 2011). FOUR EPN STRAINS WERE INC FUNGICIDAL EXPERIMENT. THE COMMERCIAL PREPARATION OF *S. feltiae* (S. FELTIAE) WAS OBTAINED FROM KOPPERT B.V. (BERKEL EN RODENRIJS, THE NETHERLANDS). ALL OF THE OTHER ISOLATED FROM THE SOIL: *S. feltiae* C76 AND *S. carpocapsae* C67 WERE ISOLATED IN SLOVENIA (LAZNIK & TRDAN, 2011), WHILE *Heterorhabditis downesi* 3173 WAS ISOLATED IN HUNGARY (TÓTH, 2006).

Compatibility test

COMPATIBILITY TEST WAS MADE ACCORDING TO LAZNIK *et al.* (2012).

Statistical analyses

STATISTICAL ANALYSES WAS MADE ACCORDING TO LAZNIK *et al* (2012).

Results and discussion

IN OUR EXPERIMENT WE DETERMINED THE BEST COMPATIBILITY OF *S. feltiae* WITH ACTIVE INGRIDIENTS AZOXYSTROBIN, AZADIRACHTIN, *B. thuringiensis* VAR. *kurstaki* AND IMIDACLOPRID (TABLE 2 AND TABLE 3). THE PRESENT STUDY SHOWED THAT *S. carpocapsae* AND *S. kraussei* ARE SENSITIVE TO ALL TESTED INSECTICIDES, WHILE *H. bacteriophora* IS SENSITIVE ONLY TO ABAMECTIN AND LUFENURON (TAB NEMATODE *H. downesi* SIGNIFICANTLY SUFFERED THE HIGHEST MORTALITY WHEN INFECTIV WERE MIXED WITH A. I. TEBUCONAZOLE, SPIROXAMINE, AND TRIADIMENOL (TABLE 2).

MOST PREVIOUS STUDIES OF THE COMPATIBILITY OF NEMATODES WITH CHEMICA CONDUCTED AS LABORATORY BIOASSAYS WITH DIRECT EXPOSURE OF NEMATODES TO PES DESEO, 1990; GORDON *et al.*, 1996; LAZNIK *et al.*, 2012). THE LARGE VARIABILITY BETWEEN PESTICIDES FROM THE SAME CHEMICAL GROUP IN THEIR COMPATIBILITY WITH EPN EXTRAPOLATION OF DATA BETWEEN PRODUCTS UNRELIABLE (ROVESTI & DESEO, 199 CANDIDATE PRODUCT FOR AN IPM SYSTEM SHOULD BE TESTED INDIVIDUALLY. SIMILAR COMPATIBILITY DATA BETWEEN DIFFERENT NEMATODE SPECIES OR EVEN STRAINS IS (LAZNIK *et al.*, 2012).

THE RESULTS OF THE PRESENT STUDY AND THOSE OF PREVIOUS INVESTIGATIONS GREWAL, 2003; LAZNIK *et al.*, 2012) IN WHICH THE COMPATIBILITY OF PLANT PROTECTION PR WITH EPN WAS EVALUATED REVEALED THAT COMPATIBILITY IS SPECIES-SPECIFIC. THE REVEALED THAT AZADIRACHTIN AND PIRIMICARB DID NOT AFFECT *S. feltiae* AND VIABILITY *H. bacteriophora* NEMATODES. HOWEVER, PREVIOUSLY MENTIONED ACTIVE INGREDIENTS DI VIABILITY OF *S. carpocapsae* AND *S. kraussei*. LAZNIK *et al.* (2012) RECENTLY REPORTED THAT COMPATIBILITY OF EPN WITH PESTICIDES (FUNGICIDES) IS NOT ONLY A SPECIES-SPECIFI STRAIN-SPECIFIC CHARACTERISTIC. SIMILAR CONCLUSIONS WERE ALSO OBTAINED IN T NAMELY, THE ACTIVE INGREDIENTS AZADIRACHTIN, *B. thuringiensis* VAR. *kurstaki* AND IMIDACLOPRID DID NOT AFFECT THE VIABILITY OF THE DOMESTIC *S. feltiae* B30 STRAIN. IN CONTRAST, THE BEFORE-MENTIONED INSECTICIDES SIGNIFICANTLY REDUCED THE NUMBER OF LIVING IJS OF A COM OF THE SAME TESTED EPN SPECIES.

Table 2. Percent change in the survival of different EPN strains after incubation with 15 different fungicides at 15 °C, 20 °C and 25 °C for 24 h.

Trade name	Change in nematode survival after exposure to chemicals for various durations at different temperatures (%)											
	S. feltiae strain C76			*S. feltiae* strain Entonem			*S. carpocapsae* strain C67			*H. downesi* strain 3173		
	15 °C	20 °C	25 °C	15 °C	20 °C	25 °C	15 °C	20 °C	25 °C	15 °C	20 °C	25 °C
Aliette flash	- 59.9b*	- 20.8ab	- 37.8bc	- 46.3cd*	- 35.5cd	- 11.5a	- 63.0def*	- 22.3bcd	+ 7.9ab	+ 22.9a	- 38.2de	- 59.3bcde
Bellis	- 58.6b	+ 1.4a	+ 1.5ab	- 33.7bc	- 39.4cde	+ 1.2a	- 38.5bcd	- 22.9bcd	- 48.7cdef	+ 17.2a	- 24.5cde	- 49.2bcde
Clarinet	- 68.2b	+ 2.8a	- 38.4bc	- 28.1abc	- 18.8abc	- 27.8a	- 27.8ab	+ 63.3a	- 58.9ef	+ 9.0a	- 34.0de	- 51.4bcde
Cuprablau-Z	- 65.1b	- 8.4a	- 2.1ab	- 38.8bcd	- 53.3def	- 12.7a	- 37.6bcd	- 21.0bcd	- 14.7abc	+ 9.0a	+ 3.8abcd	- 69.6cdef
Dithane M-45	- 98.2cd	- 98.6c	- 92.8de	- 100.0e	- 98.6h	- 99.6b	- 89.6fg	- 65.4de	- 41.9cdef	- 64.7bc	- 52.8e	- 72.7def
Falcon EC-460	- 100.0d	- 100.0c	- 100.0e	- 100.0e	- 100.0h	- 100.0b	- 100.0g	- 100.0e	- 100.0g	- 100.0c*	- 100.0f	- 100.0f
Folpan 80 WDG	- 61.4b	- 32.0ab	- 41.0bc	- 43.2cd	- 45.6de	- 9.1a	- 35.2bcd	+ 2.5abcd	- 24.5bcde	- 7.4ab	+ 14.1abc	- 60.5bcde
Pepelin	- 60.8b	- 8.4a	- 24.4b	- 25.4abc	- 64.5efg	- 4.8a	- 46.2bcde	- 33.4bcd	- 17.0abcd	+ 0.8ab	- 16.1bcde	- 68.4cde
Polyram DF	- 65.7b	- 17.4ab	- 28.8b	- 39.7bcd	- 58.5defg	- 13.5a	- 53.2cde	+ 17.6ab	+ 13.9a	+ 4.1a	- 13.2bcde	- 64.3bcde
Previcur 607 SL	- 72.8bcd	- 7.6a	- 7.3ab	- 41.5bcd	- 18.5abc	+ 0.0a	- 20.8ab	+ 32.0ab	- 47.6cdef	+ 22.9a	- 20.3bcde	- 44.8bcde
Ridomil Gold Plus 42.5 WP	- 77.2bcd	- 68.0bc	- 87.6cde	- 66.9d	- 79.1fgh	- 98.0b	- 74.3efg	- 50.3cde	- 50.2cdef	- 22.9a	- 33.5de	- 67.0cde
Quadris	- 13.9a	+ 5.6a	+ 26.9a	- 22.3abc	- 32.7bcd	- 14.7a	- 23.2ab	- 24.9bcd	- 50.2cdef	+ 25.4a	- 17.9bcde	- 42.7bcd
Sabithane	- 70.7bc	- 37.5ab	- 47.2bcd	- 34.3bc	- 82.6gh	- 9.1a	- 49.6bcde	- 66.0de	- 75.1fg	+ 22.2a	+ 45.3a	- 75.2ef
Tattoo	- 52.2b	+ 13.9a	- 25.9b	- 14.5ab	- 6.3ab	+ 0.4a	+ 2.13a	- 0.7abcd	- 43.1cdef	+ 26.2a	- 4.7bcd	- 41.4bc
Teldor SC 500	- 59.3b	- 2.8a	- 13.5ab	- 27.5abc	-38.0cde	- 23.8a	- 41.9bcd	+ 8.4abc	- 52.8def	- 16.4ab	+ 25.0ab	- 34.2b
Control (water)	100.0a	100.0a	100.0ab	100.0a	100.0a	100.0a	100.0a	100.0abcd	100.0 ab	100.0ab	100.0abcd	100.0a

*Values were significantly different ($P \leq 0.05$) in Tukey's multiple range tests. Small letters indicate that statistically significant differences were observed between the control treatment and fungicide treatments with the same EPN strain at the same temperature.

TABLE 3. CORRECTED MORTALITY RATES (%) OF INSECTICIDAL-TREATED EPN STRAI
TEMPERATURES 6 HRS AFTER EXPOSURE. MEANS WITHIN A ROW FOLLOWED BY DIFFE
SIGNIFICANTLY DIFFERENT (P < 0.05, TUKEY'S TEST). SFB30 – *Steinernema feltiae* STRAIN B30; SFBU – *Steinernema feltiae* STRAIN BECKER UNDERWOOD; SCC101 – *Steinernema carpocapsae* STRAIN C101; SCBU – *Steinernema carpocapsae* STRAIN BECKER UNDERWOOD; SKBU – *Steinernema kraussei* STRAIN BECKER UNDERWOOD; HBD54 – *Heterorhabditis bacteriophora* STRAIN D54.

		Corrected mortality rates (%) of insecticidal-treated EPN strains at different temperatures 6 hrs after exposure							
		Treatments							
EPN strain	**Temp. (°C)**	**Aba-mectin**	**Azadir-achtin**	**B. t. var. kurstaki**	**Imida-cloprid**	**Lambda-cihalotrin**	**Lufen-uron**	**Pirimi-carb**	**Pymetro-zine**
SFB30	15	54.2 B	0.0 A	7.4 A	0.0 A	0.0 A	10.5 A	0.0 A	9.2 A
	20	20.8 B	0.0 A	0.0 A	0.0 A	0.0 A	3.0 A	0.0 A	0.0 A
	25	0.0 A	0.0 A	0.0 A	0.0 A	0.0 A	31.4 B	0.0 A	0.0 A
SFBU	15	86.3 G	25.0 E	25.8 E	28.3 E	10.2 C	14.5 D	4.4 B	39.1 F
	20	86.5 C	1.1 A	4.6 B	7.6 B	0.0 A	0.2 A	0.0 A	0.0 A
	25	90.3 D	8.3 B	30.1 C	6.4 B	0.2 A	0.0 A	1.4 A	0.0 A
SCC101	15	8.0 B	32.9 C	25.1 C	0.0 A	0.0 A	0.0 A	1.2 A	0.0 A
	20	0.2 A	13.1 B	12.5 B	0.5 A	15.1 B	23.6 B	0.0 A	0.0 A
	25	19.5 C	58.4 F	6.5 B	42.2 E	28.3 D	54.4 F	26.5 CD	39.7 E
SCBU	15	1.3 A	19.2 BC	18.3 B	14.5 B	16.5 B	22.2 BC	30.8 C	16.5 B
	20	0.0 A	9.9 B	25.2 C	5.5 AB	18.3 B	8.4 B	0.0 A	10.2 B
	25	0.0 A	0.0 A	0.0 A	0.0 A	0.0 A	7.4 B	0.0 A	0.0 A
SKBU	15	33.6 D	13.5 B	22.2 C	2.7 A	28.5 C	49.4 F	27.4 C	43.4 E
	20	25.9 D	3.6 A	0.0 A	0.0 A	14.9 B	19.9 C	13.3 B	0.0 A
	25	21.4 D	0.0 A	8.4 B	18.3 CD	19.5 CD	0.0 A	8.2 B	14.6 C
HBD54	15	30.3 C	0.0 A	26.9 C	14.5 B	0.5 A	0.0 A	0.0 A	0.0 A
	20	16.7 A	0.0 A	0.0 A	0.0 A	0.0 A	0.0 A	0.0 A	0.0 A
	25	3.4 A	19.0 B	2.4 A	0.0 A	16.1 B	0.0 A	0.0 A	13.7 B

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Susceptibility of *Phytodecta fornicata* (Coleoptera: Chrysomelidae) to *Heterorhabditis bacteriophora*

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Abstract: THE INFECTIVITY OF *Heterorhabditis bacteriophora* FOR ADULT STAGE OF *Phytodecta fornicata* WAS EVALUATED IN THE LABORATORY. TWO DIFFERENT NEMATODE CONCENTRATIONS (1000 A JUVENILES (IJS) PER ADULT) WERE TESTED AT TEMPERATURES OF 22 °C AND 30 °C. MORTALITY CHECKED AT 3 D POST INFECTION (P.I.); EMERGENCE OF IJS FROM CADAVERS WAS NOTED AT 8 AN THE FIRST TIME, THE EXPERIMENT DEMONSTRATED THAT *H. bacteriophora* APPLIED IN RATE OF 1000 IJS PER BEETLE IS CAUSING 100% MORTALITY OF *P. fornicata*, AND THAT IJS DEVELOPED INSIDE ADULT INSECT CADAVER EFFECTS OF TEMPERATURE AND CONCENTRATION OF NEMATODE PREPARATION WERE OF LESS IM OF IJS FROM CADAVERS WAS OBTAINED BUT WITH LOW POPULATION LEVEL. THE TIME OF EMER TEMPERATURE DEPENDENT. IN ORDER TO DEVELOP COST EFFECTIVE AND SUSTAINABLE CONTI MORTALITY STUDIES ON INDIVIDUALS, WITH OTHER SPECIES AND STRAINS, AND ASSAYS WIT ENVIRONMENTS ARE NEEDED.

Key words: *Phytodecta fornicata*, *Heterorhabditis bacteriophora*, MORTALITY, EMERGENCE

Introduction

IN SOUTHERN EUROPE, DAMAGES ON LUCERNE (*Medicago sativa*) CAUSED BY THE LUCERNE LEAF BEETLE *Phytodecta (Gonioctena) fornicata* (BRÜGGEMANN) (COLEOPTERA: CHRYSOMELIDAE) HAVE BE REPORTED SINCE EARLY 1910S AND 1920S (JABLONOWSKI, 1921; KNECHTEL, 1922). LATELY, LI PUBLISHED REGARDING THIS PEST HAS BEEN MAINLY REPORTED FROM BULGARIA, IT CROATIA. *P. fornicata* IS A MONOPHAGOUS PEST, FEEDING ON LEAF, LEAF BUDS AND STEMS O THIS PEST CAUSES DEFOLIATING OF THE PLANTS RESULTING IN MAJOR CROP LOSSES. K (2005) REPORTED YIELD LOSS OF 30-50% IN THE FIRST MOWING OF LUCERNE. SOME AGR MEASURES, SUCH AS EARLIER MOWING CAN BE APPLIED IN ORDER TO REDUCE CROP POPULATION. RECENTLY, NEW BIOTECHNOLOGICAL APPROACHES HAVE BEEN PROPOSED PEST (NINKOVIĆ *et al.*, 2007). INSECTICIDAL APPLICATION IS OFTEN ADVISED SINCE SEVERAL COMPOUNDS WERE REPORTED AS EFFECTIVE. HOWEVER, ENVIRONMENTALLY FRIENDL NEEDED. ENTOMOPATHOGENIC NEMATODES (EPNS) HAVE PROVEN THEIR EFFICIENCY I COLEOPTERAN PESTS IN DIFFERENT CROPS AND IN LUCERNE (KOPPENHÖFER *et al.*, 1995; 2000). EPN ARE RESISTANT TO WIDELY USED PESTICIDES (KOPPENHÖFER, 2000) AND THIS CHARACTERISTIC IS AN ADVANTAGE AS IT PROVIDES A GREAT POTENTIAL FOR BIOPESTICIDE DEVELOPMENT AND PROTECTION MEASURE. SO FAR, NO REPORTS ARE PUBLISHED REGARDING *P. fornicata* SUSCEPTIBILITY ENTOMOPATHOGENIC NEMATODES.

THE AIM OF THIS PAPER IS TO DETERMINE INFECTIVITY OF ENTOMOPATHOGENI *Heterorhabditis bacteriophora* AGAINST LUCERNE LEAF BEETLE.

Material and methods

BEETLES OF *Phytodecta fornicata* WERE COLLECTED IN LUCERNE FIELDS AT THE AGRICULTURA OSIJEK, CROATIA IN APRIL 2011. EXPERIMENT WAS CONDUCTED IN LABORATORY BETWEEN MAY 9, 2011. TEN BEETLES WERE PLACED ON WET FILTER PAPER WITH LUCERNE LEAFS I SUSPENSIONS OF 10000 AND 20000 INFECTIVE JUVENILES (IJS) OF *H. bacteriophora* (1000 AND 2000 IJS PER BEETLE), RESPECTIVELY, WERE PIPETTED IN EACH PETRI DISH. EXPERIMENT WAS D TEMPERATURE REGIMES: IN A CLIMATE CHAMBER AT 30 °C AND AT ROOM TEMPERATUR TREATMENT WAS REPLICATED FOUR TIMES AND INCLUDED UNTREATED CONTROL DISHES WAS OBTAINED FROM KOPPERT B. V. (BERKEL EN RODENRIJS, THE NETHERLANDS). T MORTALITY, INSECTS WERE CHECKED ON DAY 3 POST EPN APPLICATION (P.I.). TEN CAD PLACED ON FOUR WHITE TRAPS (WHITE, 1927) DEPENDING ON THE NEMATODE TREATME WERE KEPT IN CLIMATE CHAMBER AT 30 °C AND OTHER TWO TRAPS AT ROOM TEMPERATU WHITE TRAPS, THE EMERGING IJS WERE HARVESTED AND COUNTED ON AT 8 AND 11 D P. VARIANCE (PROC GLM) AND MEANS SEPARATION WITH TUKEY'S TEST (SAS 9.2; SAS INS CAREY, NC, USA) WERE APPLIED FOR DATA OF MORTALITY OF INSECTS. DATA WERE ARCSI PRIOR TO ANALYSIS.

Results and discussion

MORTALITY RATES OF LUCERNE LEAF BEETLE ARE PRESENTED IN TABLE 1. IN BOTH NEMATODE T ALL BEETLES WERE FOUND TO BE DEAD AND MORTALITY WAS 100% ALREADY AT LOWER I CONTROL DISHES MORTALITY OF LEAF BEETLES WAS 20 AND 22.5%, RESPECTIVELY, DEP TEMPERATURE REGIME. STATISTICALLY SIGNIFICANT DIFFERENCE WAS OBSERVED BET NEMATODE TREATMENTS. INSECTS HAD SIMILAR MORTALITY RATES UNDER BOTH TEMPE DID NOT STATISTICALLY DIFFER. THE LABORATORY MORTALITY OF *P. fornicata* SHOWED THAT *H. bacteriophora* IS A CANDIDATE FOR USE IN LUCERNE INTEGRATED PEST MANAGEMENT. ARE NO REPORTS ON *P. fornicata* THESE ARE THE FIRST FINDINGS. MORTALITY STUDIES USING V STEINERNEMATID AND HETERORHABDITID SPECIES HAVE BEEN DONE WITH SUCC CHRYSOMELIDS (ELLERS-KIRK *et al.*, 2000; TRDAN *et al.*, 2008; LAZNIK *et al.*, 2010) AND OTHER *Phytodecta* SPECIES (TOMALAK, 2009).

TABLE 1. MORTALITY OF *Phytodecta fornicata* CAUSED BY *Heterorhabditis bacteriophora*.

Treatment (IJ beetle^{-1})	Mortality (%)	
	30 °C	22 °C
CONTROL	22.5A	20A
1000	100B	100B
2000	100B	100B

VALUES IN COLUMNS MARKED WITH DIFFERENT LETTER ARE STATISTICALLY DIFFERENT ($P < 0.05$).

COUNTS OF HARVESTED IJS FROM WHITE TRAPS REVEALED THE EFFECT OF TEMPERAT EMERGENCE OF NEMATODES FROM CADAVERS (FIGURE 1). EIGHT DAYS POST TREATM NUMBER OF IJS (42 IJS IN TOTAL) WAS OBTAINED FROM THE TRAPS KEPT AT 30 °C, WHILE O

RECOVERED FROM TRAPS KEPT AT RATURE. THREE DAYS LATER, NO IJS WERE
TRAPS IN CLIMATE CHAMBER WHILE 50 IJS IN TOTAL WERE RECOVERED FRO
TEMPERATURE. THE NEMATODE BEHAVIOURAL ECOLOGY, CHANGING CONDITIONS
ENVIRONMENT AS WELL AS FOOD AVAILABILITY ARE FACTORS THAT DETERMINE IJS
EMERGENCE (LACEY & GEORGIS, 2012).

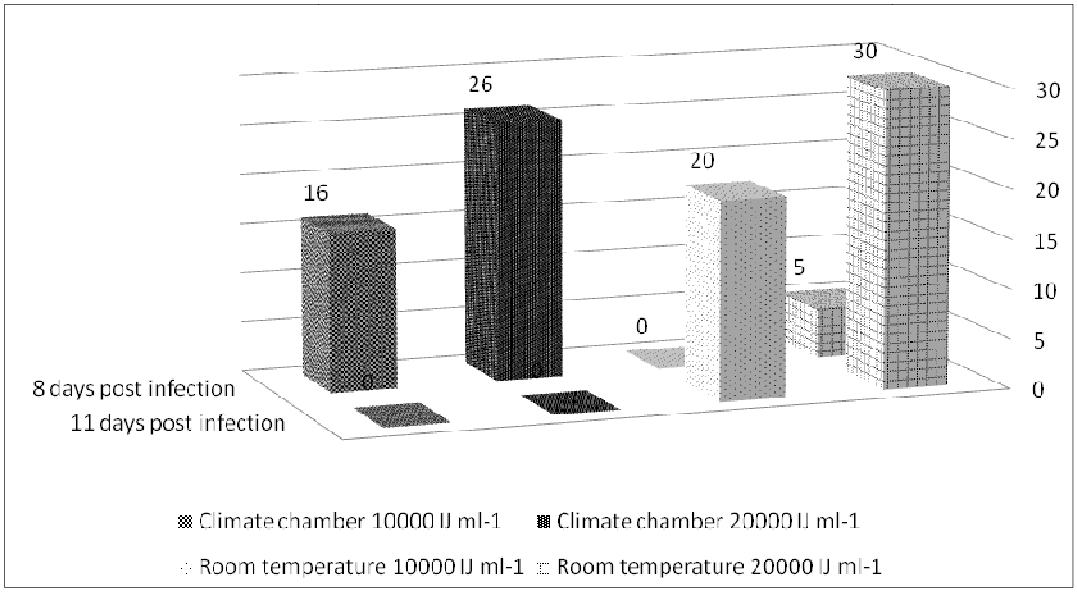

FIGURE 1. HARVESTED IJS FROM LUCERNE LEAF.

THE DEVELOPMENT AND EMERGENCE OF IJS FROM WERE EXPECTED IN HIGHER NU
RESULTS INDICATE HARVEST OF IJS SHOULD HAVE BEEN DONE AFTER LONG
CADAVERS ON WHITE TRAPS SINCE THE POPULATION OF EPN IN THE INSECT
TIME, AND/OR IN DIFFERENT ENVIRONMENTS. EMERGENCE OF IJS IS USUALLY
WITHIN 8 DAYS P. I. (O'LEARY *et al.*, 1998). THESE RESULTS MAY INDICATE THAT A SMALL PORTION
OF NEMATODES WAS INFECTIOUS TO PENETRATE AND RESCUE, I.E. THERE MIGHT
BEEN A HIGHER PERCENTAGE OF NON-INFECTIOUS EPN IN THE PREPARATION. THIS MIGHT BE THE
WHY WE COLLECTED LOW LEVEL OF THE CADAVERS.

FOR THE FIRST TIME, THE EXPERIMENT SHOWED THAT *H. bacteriophora* APPLIED IN RATE OF
1000 IJS PER BEETLE IS CAUSING 100% MORTALITY OF *P. fornicata* AND THAT IJS DEVELOP INSIDE
CADAVERS OF *P. fornicata*. THE EFFECT OF CONCENTRATION OF NEMATODE PREPARATION IS
IMPORTANCE, AND FUTURE STUDIES SHOULD INVOLVE LOWER CONCENTRATIONS
IN ORDER TO DEVELOP COST-EFFECTIVE AND SUSTAINABLE CONTROL. FURTHER MORTALITY STUDIES
ON INDIVIDUALS, WITH OTHER EPN SPECIES AND STRAINS, AND ASSAYS WITH DIFFERENT
ENVIRONMENTS ARE NEEDED.

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Insect pathogens and entomoparasitic nematodes
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The susceptibility of mulberry moth to infection by entomopathogenic nematodes, *Heterorhabditis bacteriophora* and *Steinernema carpocapsae*

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Abstract: THEMULBERRY MOTH, *Glyphodes pyloalis,* IS CONSIDERED AS AN URBAN PEST AND THERE APPLICATION OF ENVIRONMENTALLY SAFE MEANS FOR MULBERRY TREES PROTECTION IS RECOM ENTOMOPATHOGENIC NEMATODE (EPN) SPECIES *Heterorhabditis bacteriophora* AND *Steinernema carpocapsae* ARE IMPORTANT AS A BIOLOGICAL CONTROL AGENTS (GL THE SUSCEPTIBILITY OF *G. pyloalis* TO INFECTION BY *H. bacteriophora and S. carpocapsae* INFECTIVE JUVENILES (IJ) WAS TESTED UNDER LABORA CONDITIONS. INDIVIDUALS OF IV INSTAR LARVAE WERE COLLECTED FROM MULBERRY TREES (VILLAGE DIGOMI). NEMATODE SUSPENSIONS AT A CONCENTRATION OF 1500 IJS/ML WERE USED F MULBERRY LEAVES. AFTER 72 H, THE MORTALITY OF *G. pyloalis* CAUSED BY *H. bacteriophora* WAS 54%, WHEREAS *S. carpocapsae* CAUSED 76% MORTALITY. THE RESULTS SUGGEST THAT NEMATODE SUSP *H. bacteriophora* AND *S. carpocapsae* CAN BE USED TO CONTROL *G. pyloalis in* URBAN PLOTS.

Key words: MULBERRY MOTH, *Glyphodes pyloalis, Heterorhabditis bacteriophora, Steinenema carpocapsae*

Introduction

THE MULBERRY MOTH *Glyphodes pyloalis* (WALKER) (LEPIDOPTERA: PYRALIDAE) HAVE BEEN FOUN THE LEAVES OF MULBERRY TREES IN KAKHETI (EAST GEORGIA) (*G. pyloalis* CATALOGUE, 2008). THE PEST INSECT IS DISTRIBUTED IN USA (FLORIDA, MISSISSIPPI, AND VIRGINIA STATES), MEXICO, IN IRAN, IN REPUBLIC OF CENTRAL ASIA AND AZERBAIJAN. IT IS SPECIALIZED AS A MONOP THE LEAVES OF MULBERRY TREES (FIG. 1). *G. pyloalis* IS CONSIDERED AS AN URBAN PEST AND THEREFORE THE APPLICATION OF ENVIRONMENTALLY SAFE MEANS FOR MULBERRY RECOMMENDED. AMONG ENTOMOPATHOGENIC NEMATODES (EPNS), *Heterorhabditis* SPECIES *bacteriophora* AND *Steinernema carpocapsae* ARE IMPORTANT BIOLOGICAL CONTROL AGENTS (GL *et al*., 2000). THE STRAINS OF *H. bacteriophora* AND *S. carpocapsae* WERE INTRODUCED IN GEORGIA FROM GERMANY, E-NEMA COMPANY AND THEN EPN HAS BEEN MASS PRODUCED SUC BOTANY-ZOOLOGY LABORATORY IN TBILISI. ENTOMOPARASITIC NEMATODES OF THE FAMIL HETERORHABDITIDAE AND STEINERNEMATIDAE HAVE BEEN KNOWN FOR DECADES AS EFFECTIVE AGENTS OF INSECT PESTS. THESE NEMATODES CAN ACTIVELY LOCATE, INFECT AND KIL INSECT SPECIES. ONLY THE THIRD STAGE JUVENILE (INFECTIVE OR DAUER STAGE) CAN SU INSECT HOST AND MOVE FROM ONE INSECT TO ANOTHER. INSECT MORTALITY, DUE TO NI IS CAUSED BY A SYMBIOTIC BACTERIUM (*Photorhabdus luminescens and Xenorhabdus nematophilus).* THE INFECTIVE JUVENILES (IJS) CARRY THE SYMBIOTIC BACTERIA IN THE INTESTINES AND RELEASE THEM INTO THE INSECT HAEMOCOEL. THE BACTERIA CELLS PROLIFERATE AND EVENTUALLY INSECT HOST (USUALLY WITHIN 24 H) (KAYA *et al*., 1997).

FIGURE 1. *G. pyloalis* LARVAE ON MULBERRY LEAVES.

Material and methods

100 INDIVIDUALS OF IV INSTARS LARVAE WERE COLLECTED FROM MULBERRY TREES IN DIGOMI) AND TRANSFERRED TO THE BOTANY AND ZOOLOGY LABORATORY OF THE DEPA SCIENCES AND HEALTH CARE AT CONDITIONS OF 24-25 °C AND 70-74% RELATIVE H NEMATODES SUSPENSIONS OF 1500 IJ/ML WAS USED FOR THE TREATMENT OF MULBERRY LE (FIGURES 2 & 3). THE CULTIVATION OF *H. bacteriophora* AND *S. carpocapsae* WAS PERFORMED UNDER CONTROLLED CONDITIONS AT 24-25 °C AND 70-74% RELATIVE HUMIDITY ON CATERPILLARS LAST INSTAR WAX MOTH, *Galleria mellonella*, USING STANDARD TECHNIQUES (KAYA *et al.*, 1997). INSECT MORTALITY WAS DETERMINED AFTER 48 AND 72 H. THE MORTALITY WAS CORRECTED FOR CONTROL MORTALITY USING THE FORMULA OF ABBOTT (1925). DEAD LARVAE OF *G. pyloalis* WERE TRANSFERRED FROM PETRI DISHES INTO THE WHITE TRAPS, WHERE THE REPRODUCTION OF *H. bacteriophora* AND *S. carpocapsae* STARTED (WHITE, 1927). CONTROLS WERE TREATED WITH S WATER. THE PRELIMINARY EXPERIMENTS ON THE SUSCEPTIBILITY OF *G. pyloalis* TO *H. bacteriophora* AND *S. carpocapsae* HAVE BEEN CARRIED ACCORDING TO (SOLTER *et al.*, 2010) (TABLES 2-6).

Results

DATA OF EPNS *H. bacteriophora* AND *S. carpocapsae* CONCERNING MULBERRY CATERPILLAR – *Glyphodes pyloalis* ARE PRESENTED. THE INVASIVE LARVAE WERE DETECTED AFTER 48 H. MORTALITY OF *Glyphodes pyloalis* LARVAE AFTER 72 H WAS 54% WHEN TREATED WITH *H. bacteriophora* AND 76% WHEN TREATED WITH *S. carpocapsae* (FIGURE 7).

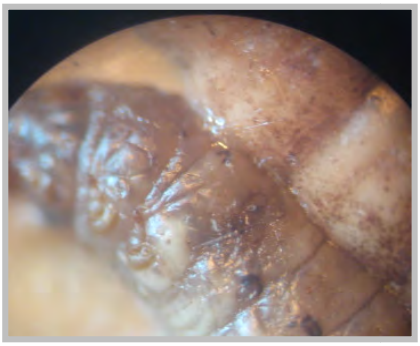

FIGURE 2. *G. pyloalis* LARVAE INFECTED WITH *H. bacteriophora*.

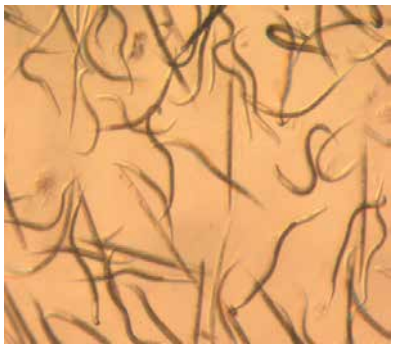

FIGURE 3. *H. bacteriophora* ISOLATED FROM *G. pyloalis* LARVAE.

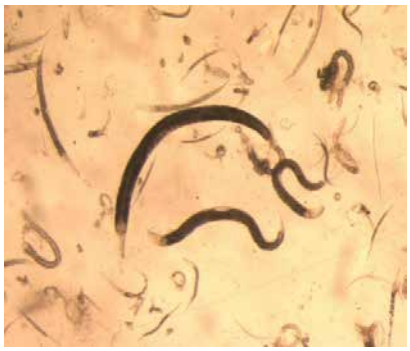

FIGURE 4. *H. bacteriophora* ISOLATED FROM *G. pyloalis* LARVAE-ET.

FIGURE 5. *G. pyloalis* LARVINFECTED WITH *S. carpocapsae*.

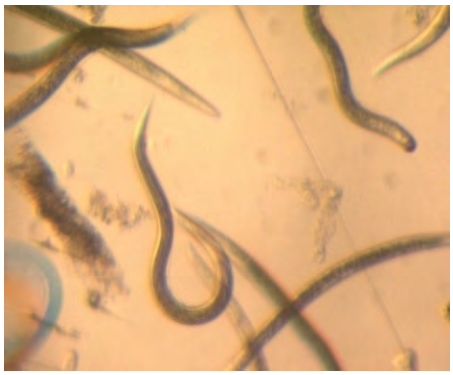

FIGURE 6. *S. carpocapsae* ISOLATED FROM *G. pyloalis* LARV.

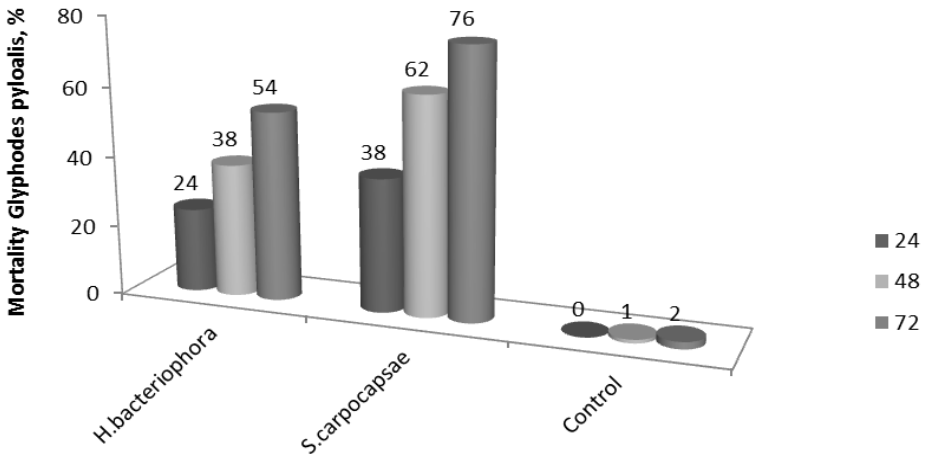

FIGURE 7. THE MORTALITY (%) OF *G. pyloalis* LARVAE INFECTED WITH *H. bacteriophora* AND *S. carpocapsae* AFTER 24, 48 AND.

Conclusions

THESE PRELIMINARY INVESTIGATIONS PROPOSE THE POSSIBILITY OF INVASION BY *G. pyloalis* IN AREAS OF *H. bacteriophora* AND *S. carpocapsae*. THIS GIVES THE POSSIBILITY TO USE THE NEMATOD *H. bacteriophora* AND *S. carpocapsae* TO CONTROL *G. pyloalis* IN URBAN PLOTS IN THE FUTURE.

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Insect pathogens and entomoparasitic nematodes
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Attract and kill against western corn rootworm larvae with entomopathogenic nematodes

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Abstract: THE WESTERN CORN ROOTWORM (WCR) *Diabrotica virgifera virgifera* LECONTE (COLEOPTERA: CHRYSOMELIDAE) IS A SERIOUS MAIZE PEST IN THE US CORN BELT AND IN CENTRAL EUROPE. BIOLOGICAL CONTROL WITH ENTOMOPATHOGENIC NEMATODES (EPNS) A DWELLING LARVAL STAGES HAS PROVEN TO BE EFFECTIVE UNDER FIELD CONDITIONS. THE IM OF THIS APPROACH IS, HOWEVER, CURRENTLY HAMPERED BY HIGHER COSTS COMPARED TO CONTROL OPTIONS.

ATTRACT AND KILL MECHANISMS MAY INCREASE THE CHANCE FOR A CONTACT BETWEEN A KILL SUBSTANCE AND HAVE BEEN SHOWN TO IMPROVE EFFICACY OF THE KILLING AG MECHANISM MIGHT ALSO HELP IN REDUCING NEMATODE APPLICATION DENSITIES, WHILST H HIGH CONTROL LEVELS, AND WOULD REDUCE COSTS FOR BIOLOGICAL CONTROL OF WCR LAR

IN THIS STUDY WE USED A COMBINATION OF CO_2 AS ATTRACTANT SEMIOCHEMICAL KNOWN TO BE USED BY WCR LARVAE IN HOST PLANT LOCATION, AND THE ENTOMOPATHOGENIC N *Heterorhabditis bacteriophora* AS THE KILLING AGENT (PROVIDED BY E-NEMA, SCHWENTINENTAL). THE RELEASE OF THE ATTRACTANT CO_2 WAS ENCAPSULATED (DRINKING CAPSULES) TO ENSURE A LONG AND SLOW RELEASE OF THE SEMIOCHEMICAL. THE NEMATODES WERE MA WITH THE CAPSULES.

A NON-DESTRUCTIVE OBSERVATION DEVICE WAS USED TO EXAMINE THE SPATIAL INFECT 2ND INSTAR WCR LARVAE BY EPN AND TO ASSESS THE EPN INFECTION RATE OVER A PERIOD THIS DEVICE CONSISTS OF A THIN SOIL LAYER (45 CM X 30 CM X 6 MM) EMBEDDED BETWEEN TW SHEETS, WHICH WERE DIVIDED INTO 60 GRIDS WITH 10 VERTICAL AND 6 HORIZONTAL LAYERS 4.5 CM X 5 CM) TO LOCALIZE AND QUANTIFY LARVAL INFECTIONS. A MAIZE PLANT WAS G WEEKS IN THE DEVICE AND WCR LARVAE WERE THEN PLACED 7 CM DEEP INTO THE SOIL 15 C FROM THE MAIZE STEM. THE ATTRACT AND KILL COMPONENTS (CAPSULES AND EPNS) WERE CM AWAY FROM THE MAIZE STEM. TO ASSESS THE EFFICACY OF THIS ATTRACT AND KILL CONVENTIONAL TREATMENT WAS ALSO SET UP WITH THE CURRENT APPLICATION SCEN APPLYING THEM DIRECTLY AT THE MAIZE STEM.

THE RESULTS SHOWED THAT EPNS INFECT WCR LARVAE MORE THAN 5 CM FROM THE AI POINT OF THE EPNS IN AN ATTRACT AND KILL AND A CONVENTIONAL TREATMENT. THIS INDI LARVAE EITHER AVOID OR EMIGRATE OUT OF EPN INFECTED SOIL PARTS, THUS RECOGNIZIN UPON CONTACT OR THROUGH VOLATILES RELEASED BY EPNS. IN BOTH TREATMENTS THE FIR EPNS WAS MEASURED 2 DAYS AFTER RELEASE OF WCR LARVAE IN THE DEVICE AND WAS SI HIGHER AFTER 7 DAYS THROUGH A COMBINATION OF EPN WITH CO_2 AS COMPARED TO A CONVENTIONAL APPLICATION OF EPNS. CONSEQUENTLY A COMBINATION OF EPNS AND SEMI USED IN HOST FINDING COULD HELP TO REDUCE APPLICATION RATES AND COSTS OF BIOLO WCR LARVAE WITH EPNS, MAKING THIS STRATEGY MORE COMPETITIVE WITH REGARD TO CONTROL OPTIONS.

Key words: *Heterorhabditis bacteriophora, Diabrotica virgifera virgifera,* ENCAPSULATION, APPLICATION TECHNIQUE

Bacteria

Poster

Insect pathogens and entomoparasitic nematodes
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Cloning strategy for recovering phage-displayed Cry1Aa13 mutants from phages with affinity towards proteins present in the gut of *Ceratitis capitata*

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Abstract: USING THE PHAGE DISPLAY TECHNIQUE, A POOL OF PHAGES FROM A LIBRARY OF BA EXPRESSING CRY1AA13 TOXINS WITH MODIFIED LOOPS 2 AT THE DOMAIN II WAS SELECTED THAT TOWARD PROTEINS PRESENT IN THE GUTS OF THE MEDITERRANEAN FRUIT FLY, *Ceratitis capitata*. THE SEQUENCES OF THE HYPERVARIABLE REGIONS OF THE *in vivo* SELECTED PHAGES WERE ANALYSED AND AN ALMOST IDE SEQUENCE WAS OBTAINED IN ALL OF THE SELECTED PHAGES. THOSE PHAGES BEARING TOXINS WILD TYPE TOXIN AT THE LOOP 2 WERE SELECTED IN ORDER TO RECOVER THE CRY1AA13 MUTAI DESCRIBE THE CLONING STRATEGY DESIGNED AND USED TO CLONE THE TOXINS FROM THE PHAG BE EXPRESSED.

Keys words: *Ceratitis capitata*, PHAGE DISPLAY, CRY TOXINS, *in vitro* EVOLUTION

Introduction

CRY TOXINS FROM *Bacillus thuringiensis*, HAVE BEEN WIDELY STUDIED AND USED FOR THEIR ABIL CONTROL PEST AND VECTOR INSECTS. CRY TOXINS ARE VERY SPECIFIC TO THE TARGET I GOOD ALTERNATIVE TO CHEMICAL CONTROL OF PESTS AND VECTORS (FREDERICI, 2005). NUMBER OF NATURAL CRY TOXINS ARE REPORTED (CRICKMORE, 2013), THERE IS NOT AN ALL INSECTS OF INTEREST. IN ADDITION, THE RESISTANCE PHENOMENON HAS BEEN OI INSECTS TOWARD CRY TOXINS (JURAT-FUENTES *et al.*, 2002), REQUIRING NEW TOXINS WITH NOVEL SPECIFICITIE

ONE OF THE MOST POWERFUL TECHNIQUES FOR *in vitro* EVOLUTION OF PROTEINS IS THE PHAGE DISPLAY OF MUTANT LIBRARIES. IN THIS SYSTEM, THE VARIANTS ARE EXPRESSED ON THE ALLOWING THEM TO INTERACT WITH OTHER PROTEINS AND BE RETAINED ON IMM MOLECULES. THOSE VIRUS PARTICLES THAT DO NOT BIND ARE REMOVED BY WASHING, A CAN BE RECOVERED AND AMPLIFIED IN *E. coli* (NELSON, 2004).

USING THIS MOLECULAR TOOL, WE SELECTED A POOL OF PHAGES FROM A LIBRARY (COURTESY OF PROF. D. J. ELLAR, UNIVERSITY OF CAMBRIDGE), DISPLAYING ON THEIR SU IN LOOP 2 OF DOMAIN II OF A CRY1AA13 TOXIN (PIGOTT *et al.*, 2007), WITH AFFINITY TO PROTEINS PRESENT IN THE GUT OF ADULTS OF *C. capitata* (DOMINGUEZ *et al.*, 2011). HERE, WE REPORT THE CLONING STRATEGY USED TO OBTAIN THE MUTANT TOXINS PRESENT IN THE SELECTED EXPRESS THE NOVEL TOXINS.

Material and methods

Obtaining DNA phages

PHAGES WERE AMPLIFIED BY PROPAGATION IN LIQUID CULTURE (DOMINGUEZ *et al.*, 2010). THIS PROCESS WAS REPEATED UNTIL WE OBTAINED A PHAGE SUSPENSION WITH 10^{11} PLAQUE FORMING UNITS (PFU). BACTERIOPHAGE DNA WAS EXTRACTED WITH DNEASY BLOOD AND TISSUE KIT (QIAGEN).

Cry gene PCR amplification

THE GENE CODING FOR THE COMPLETE CRY TOXIN WAS OBTAINED BY PCR USING P (5'-AATTTAGATCTAGACGAAAGGGCATCGC-3') AND TD2 (5'-AATTCCCGGGCTATT CTAAATCATATTC-3') (FIGURE 2). THE AMPLIFIED FRAGMENT ALSO CONTAINED THE P*lac* PROMOTER (INDUCIBLE BY IPTG). AMPLIFICATION CONDITIONS WERE: 10 MIN AT 95 °C, 30 CYCLES OF 1 MIN, 53 °C FOR 1 MIN, AND 72 °C FOR 1 MIN, AND EXTENSION AT 72 °C FOR 10 MIN.

Cloning and selection of positive clones

THE TD1-TD2 FRAGMENTS WERE INTRODUCED INTO THE PGEM-T PLASMID ACCORDI MANUFACTURER'S INSTRUCTIONS. TRANSFORMANTS WERE PLATED ON LB + 0.5 MM IPTG XGAL + 100 MG ML^{-1} AMPICILLIN. WHITE COLONIES WERE PICKED ONTO A FRESH LB PLATE WI IPTG AND AMP TO DOUBLE CHECK THAT THEY WERE WHITE. WHITE COLONIES WERE COLONY PCR USING PRIMERS A2F (5'-CCCGTACTTGTCTCATTAACTGG-3') AND A2R (5'-GGAAGGCAAGTTGGTCGTTAGG-3') AND USING THE FOLLOWING CONDITIONS: 10 MIN 95 °C, 30 CYCLES 95 °C FOR 30 S, 52 °C FOR 30 S AND 72 °C FOR 2 MIN, FINAL EXTENSION AT 72 °C FOR 10 MIN.

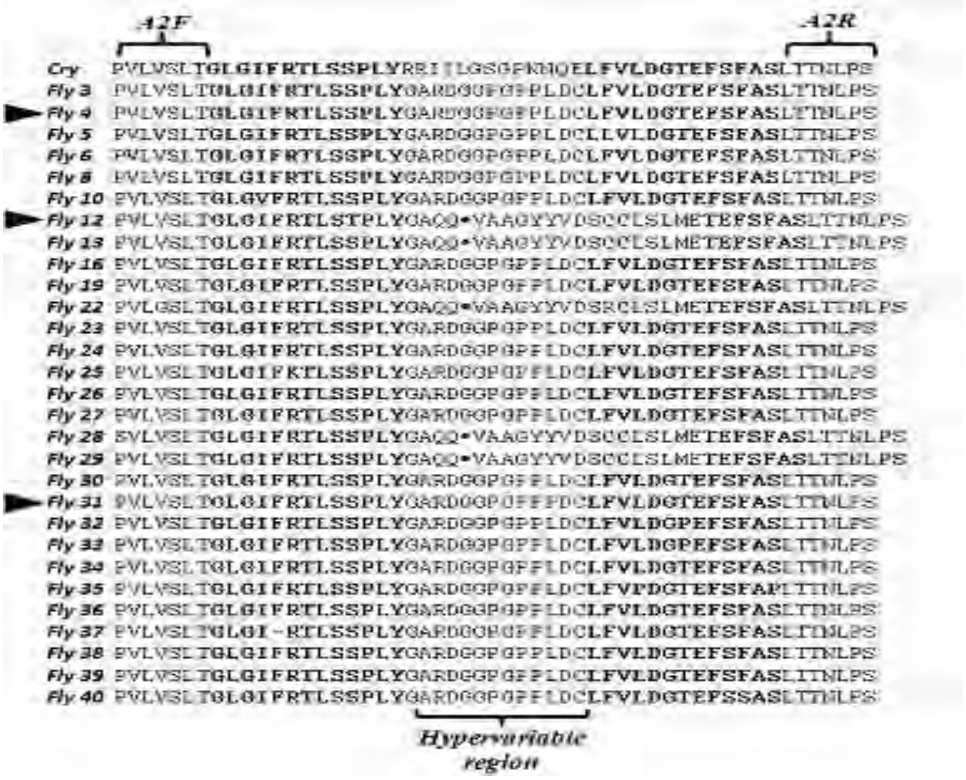

FIGURE 1. HYPERVARIABLE REGIONS AT LOOP 2 OF THE CRY1AA13 WITH SEQUENCES FROM THE PHAGES WITH AFFINITY TO PROTEINS PRESENT IN THE GUT OF *C. capitata*.

Confirmative PCR

TO CONFIRM THE PRESENCE OF THE COMPLETE TOXIN ON THE POSITIVE CLONES, PLASMID
USING A MINI PREP KIT (QIAGEN) AND USED AS TEMPLATE IN A PCR WITH TD1-A2R (10
95 °C, 30 CYCLES OF 95 °C FOR 1 MIN, 57 °C FOR 1 MIN AND 72 °C FOR 2 MIN 30 S, AND FINAL
EXTENSION AT 72 °C FOR 10 MIN) AND A2F-TD2 (10 MIN AT 95 °C, 30 CYCLES OF 95 °C FOR
56 °C FOR 50 S AND 72 °C FOR 1 MIN, AND A FINAL EXTENSION AT 72 °C FOR 10 MIN).

Results and discussion

AFTER CLONING AND SEQUENCING THE HYPERVARIABLE REGION OF THE SELECTED PHA
LIBRARY (PIGOTT, 2006), WE OBSERVED THAT MOST OF THE TOXINS PRESENT IN THE S
SHOWED THE SAME AMINO ACID SEQUENCE (FIGURE 1). THREE PHAGES WERE C
REPRESENTATIVES OF THE CRY1AA13 MUTANT TOXINS SELECTED: FLY 4, FLY 12 AND FLY 3

ALL THE SEQUENCES OBTAINED FROM THE SELECTED PHAGES WERE COMPLETELY DI
CRY1AA13 WILD TYPE. FROM THE 29 SEQUENCES ANALYSED, 5 SHOWED A STOP CODON I
HYPERVARIABLE REGION. 21 SEQUENCES SHOWED THE SAME HYPERVARIABL
(GARDGGPGPPLDC), WITH THE EXCEPTION OF ONE (FLY 31), OUT OF THE 20 (FLY 25, FLY
30, FLY 32, FLY 33, FLY 35, FLY 37, FLY 40) SHOWED A MUTATION OUTSIDE THE HYPERVARIA
REGION. AFTER THE ANALYSIS, PHAGE FLY 4 (GARDGGPGPPLDC), FLY 31 (GARDGGPGPPPDC)
AND FLY 12 WERE SELECTED FOR CLONING.

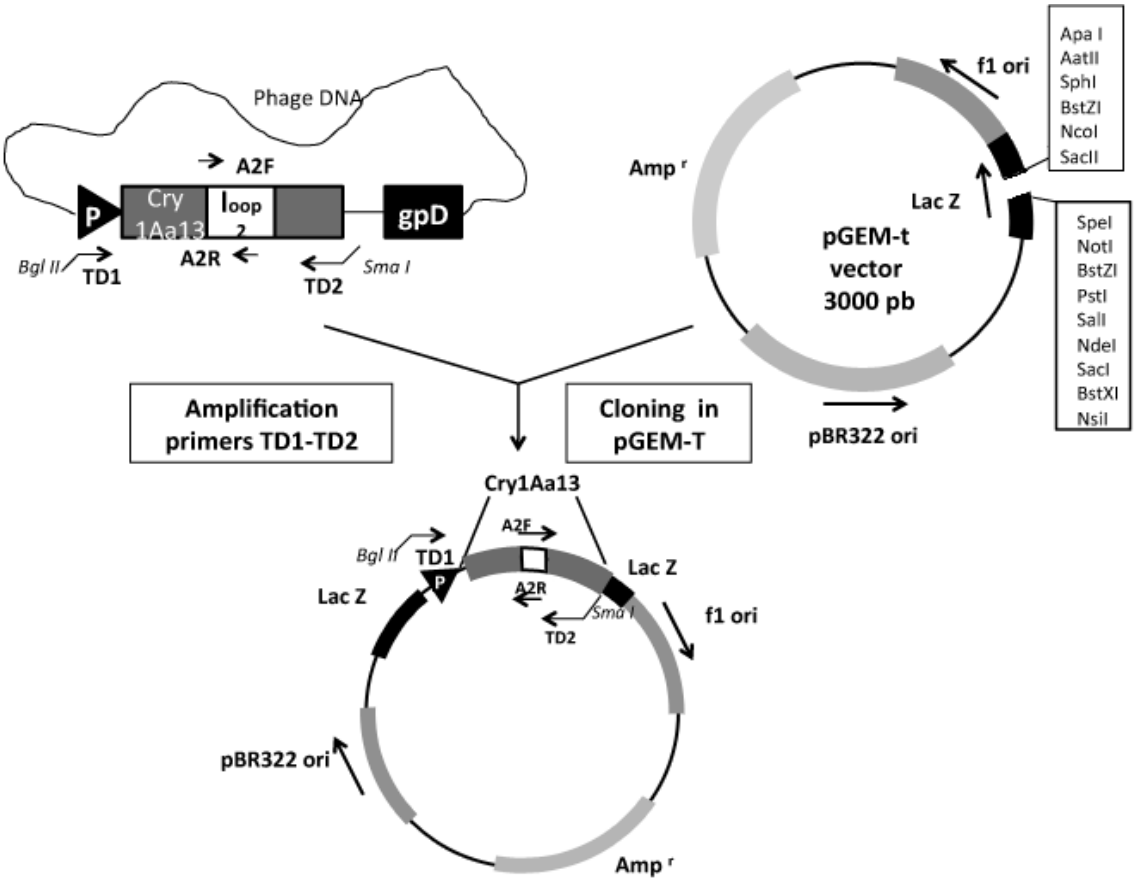

FIGURE 2. CLONING STRATEGY OF THE GENE INTO PGEM-T FROM SELECTED PHAGES.

Obtaining mutant toxins from the selected phage

THE *cry* MUTANT GENES FROM FLY 4, FLY 12 AND FLY 31 PHAGES WERE OBTAINED BY PC
TD1-TD2 PRIMERS AND TOTAL PHAGE DNA AS A TEMPLATE FOLLOWING THE STRATEGY SH
2. THE PRIMERS WERE DESIGNED TO AMPLIFY THE *cry1Ac13* GENE INCLUDING THE INDUCIBLE
P_{lac} PROMOTER. THE AMPLIFIED FRAGMENTS WERE ANALYSED IN A 1% AGAROSE GEL AN
SHOWED THE EXPECTED SIZE OF 2000 BP (FIGURE 3).

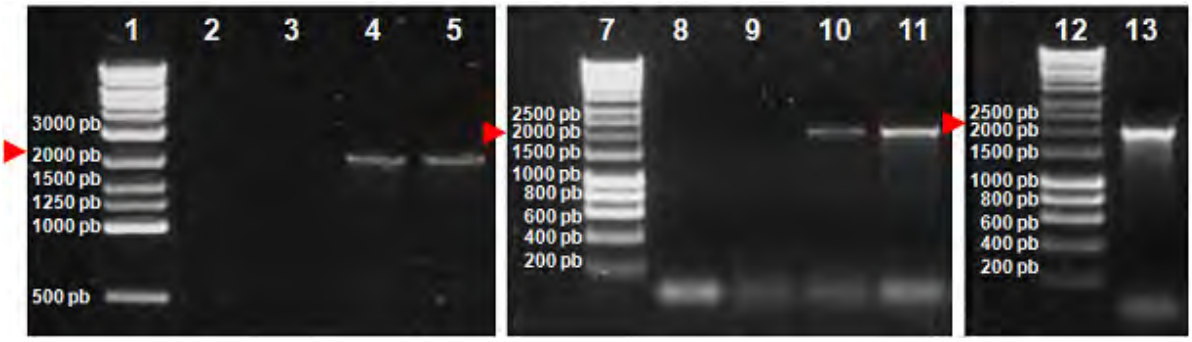

FIGURE 3. AGAROSE GEL (1%) OF THE TD1-TD2 PCR FRAGMENTS AMPLIFIED FROM DIFFERE
LANES 1, 7, 12: MOLECULAR WEIGHT MARKER (PB = BASE PAIRS), LANES 3 AND 8: EMI
(NEGATIVE CONTROL), LANES 4 AND 9: CP2 PHAGE (POSITIVE CONTROL) CONTAINING W
TOXIN, LANES 5: FLY 12 PHAGE, LANE 11: PHAGE FLY 4, LANE 13: PHAGE FLY 31.

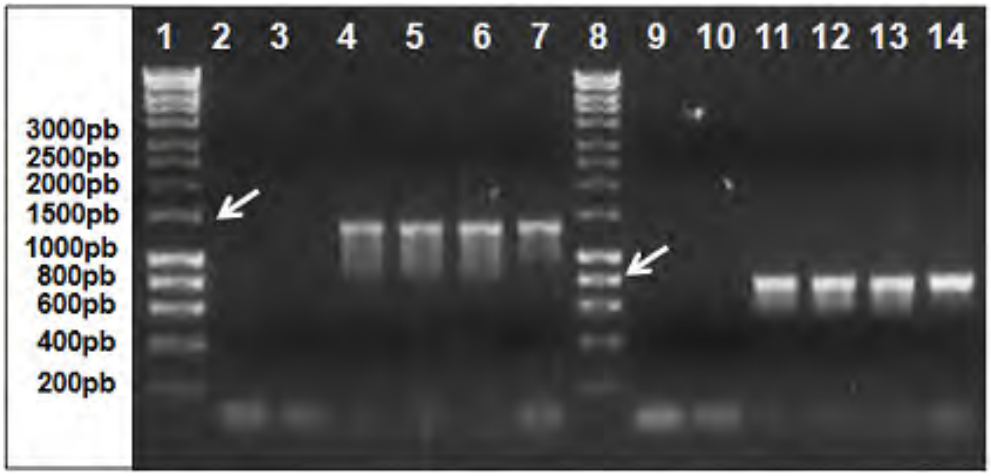

FIGURE 4. AGAROSE GEL (1%) OF THE TD1-A2R (LANES 2-7) AND A2F-TD2 (LANES 9-14) PC
LANES 2 AND 9: MASTER MIX; LANES 3 AND 10: PGEM-T RECIRCULARICED (NEGATIVE CON
AND 11: CP2 PHAGE (POSITIVE CONTROL); LANES 5 AND 12: PFLY 4 CLONE; LANES 6 AND 1
CLONE; LANES 7 AND 14: PFLY31 CLONE; LANES 1 AND 8: MOLECULAR WEIGHT MARKERS (PB

Cloning and screening of the Cry1Aa13 mutant toxins

THE AMPLIFIED TD1-TD2 FRAGMENTS WERE INTRODUCED INTO PLASMID PGEM-T FOLL
STRATEGY DETAILED IN FIGURE 2. WHITE COLONIES WERE SCREENED BY COLONY PCR WI
AND A2R TO CONFIRM THAT THEY WERE POSITIVE (DATA NOT SHOWN). THE POSITIVE
SELECTED FOR FURTHER ANALYSIS.

Clone analysis

TO CONFIRM THAT THE POSITIVE CLONES CONTAINED THE COMPLETE CRY GENE, TW
PERFORMED, ONE WITH THE PAIR OF PRIMERS TD1-A2R, THAT AMPLIFIED A FRAGMENT
CONTAINING THE P_{LAC} PROMOTER, THE N-TERMINAL END OF THE MUTANT TOXIN AND THE LO
DOMAIN II OF CRY GENE, AND ANOTHER ONE WITH THE PAIR OF PRIMERS A2F-TD2, THAT
800 BP FRAGMENT CONTAINING THE BEGINNING OF THE LOOP 2 UNTIL THE C-TERMINAL E
AMPLICONS OBTAINED FROM THE CLONES SHOWED THE SAME SIZE AS IN THE POSI
INDICATING THAT THE PLASMIDS PFLY 4, PFLY 12 AND PFLY31 CONTAINED THE COMPL
(FIGURE 4).

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Insect pathogens and entomoparasitic nematodes
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Efficacy evaluation of different *Bacillus thuringiensis* sv *kurstaki* strain EG2348 formulations against *Malacosoma neustrium* (Lepidoptera: Lasiocampidae)

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Abstract: CORK OAK FOREST PROTECTION AND MANAGEMENT REQUIRE CONTINUOUS MONITORI
MOTH SPECIES. AMONG THESE, THE EUROPEAN TENT CATERPILLAR, *Malacosoma neustrium* L., CAN CAUSE
WIDESPREAD AND EXTENSIVE DEFOLIATION OF HOST PLANTS, AND THE IMPLEMENTATION
MANAGEMENT PROGRAMS BECOMES NECESSARY. SUSTAINABLE CONTROL METHODS MAY INC
ENTOMOPATHOGENIC MICRORGANISMS, SUCH AS *Bacillus thuringiensis* SEROVAR *kurstaki* (*Btk*). HOWEVER, THE
FORMULATION OF THE MICROBIAL CONTROL AGENTS CAN BE A KEY FACTOR FOR THE SUCC
PROGRAMS. THE RESULTS OF AN EFFICACY TRIAL WITH DIFFERENT FORMULATIONS OF *Btk* STRAIN EG 2348 AGAINST
LARVAE OF *M. neustrium* CONDUCTED IN A CORK OAK FOREST IN SARDINIA (ITALY) ARE REPORTED. IN
COMMERCIAL AND AN EXPERIMENTAL FORMULATION OF *Btk* STRAIN EG 2348 WERE TESTED (HENCEFORTH RAPAX®
AND RAPAX EXPERIMENTAL) IN COMPARISON WITH TWO *Btk*-BASED REFERENCE PRODUCTS (FORAY® 48B
DELFIN®). BOTH FORMULATIONS OF *Btk* STRAIN EG 2348 PROVED TO BE EFFECTIVE IN CONTROLLING T

Key words: *Malacosoma neustrium*, *Bacillus thuringiensis*, MICROBIAL CONTROL, FOREST

Introduction

Malacosoma neustrium L. (LEPIDOPTERA: LASIOCAMPIDAE) IS A UNIVOLTINE SPECIES OVERWI
IN EGG MASSES. LARVAE HATCH FROM EGGS IN SPRING. EARLY-INSTAR LARVAE FEED G
GATHER ON PLANT FOLIAGE TO CONSTRUCT WHITE WEBBINGS (TENTS) AT MAJOR BRANC
INSTAR LARVAE ARE SOLITARY AND FEED ALL OVER THE CROWN (MERLINO *et al.*, 2004). DURING
OUTBREAKS, LARVAE CAN CAUSE WIDESPREAD AND EXTENSIVE DEFOLIATION OF HOS
IMPLEMENTATION OF AN APPROPRIATE MANAGEMENT PROGRAM BECOMES NECESSAR
LENTINI, 2007).

SUSTAINABLE MANAGEMENT STRATEGIES OF THIS PEST MAY INCLUDE THE USE OF EN
MICRORGANISMS, SUCH AS *Bacillus thuringiensis* SEROVAR *kurstaki (Btk)*-BASED PRODUCTS (MARTIN
& BONNEAU, 2006). THE ACTIVE INGREDIENT IS REPRESENTED BY A MIXTURE OF BACTERI
PARASPORAL CRYSTALS CONTAINING INSECTICIDAL CRY TOXINS ACTING BY INGESTION AI
STRAINS FOR THEIR POTENTIAL AGAINST DIFFERENT TARGET INSECTS (CRICKMORE, 20
STRAIN CHARACTERISTICS, THE FORMULATION OF THE MICROBIAL CONTROL AGENT CAN
SUCCESS OF APPLICATION PROGRAMS, ESPECIALLY IN FORESTS BECAUSE OF THE NE
THOROUGH COVERAGE ON BIG-SIZED TREES AND OVER WIDE AREAS (SATINDER *et al.*, 2011).

THE RESULTS OF AN EFFICACY TRIAL WITH TWO DIFFERENT FORMULATIONS OF *Btk* STRAIN EG 2348
AGAINST LARVAE OF *M. neustrium*, CONDUCTED IN A CORK OAK FOREST IN SARDINIA, ARE REPOR
TRIAL WAS CONDUCTED IN COMPLIANCE WITH GOOD EXPERIMENTAL PRACTICE (GE
ESTABLISHED BY THE EUROPEAN AND MEDITERRANEAN PLANT PROTECTION ORGANIZA
1/210(1), REF. EFFICACY EVALUATION OF INSECTICIDES – DEFOLIATORS OF FOREST TREES).

Material and methods

Tested treatments

IN THE TRIAL, TWO FORMULATIONS OF *Btk* STRAIN EG 2348 WERE TESTED: A COMMERCIALLY AVAIL
SUSPENSION CONCENTRATE® (RAPAX) AND AN EXPERIMENTAL AQUEOUS FLOWABLE FORMUL
(RAPAX EXPERIMENTAL), BOTH FROM CBC (EUROPE) SRL, ITALY. BOTH FORMULATIONS W
COMPARISON TO TWO COMMERCIAL *Btk*-BASED REFERENCE PRODUCTS, RESPECTIVELY FORAY 4
(VALENT BIOSCIENCE CORPORATION) AND DELFIN® (CBC (EUROPE) SRL), AND AN UNTREATED CONTROL. THE T
Btk STRAIN EG 2348-BASED FORMULATIONS WERE TESTED AT TWO DIFFERENT APPL
(RESPECTIVELY 1.0 AND 1.5 L/HA), WHILE THE *Btk*-BASED REFERENCE PRODUCTS WERE APPLIED AT
RECOMMENDED LABEL RATES (TABLE 1). FOR ALL TESTED PRODUCTS, A PRELIMINARY LA
AGAINST EARLY-INSTAR *M. neustrium* LARVAE WAS CONDUCTED TO VERIFY THEIR EFFICACY.

TABLE 1. TESTED TREATMENTS AND APPLIED RATES.

Treatment	Active ingredient (strain)	Concentration[1] a. i. (%)	Formulation[2]	Applied rate
UNTREATED CONTROL	-	-	-	-
RAPAX®	*Btk* EG2348	7.5	SC	1.5 L/HA
RAPAX®	*Btk* EG2348	7.5	SC	1.0 L/HA
RAPAX EXP.	*Btk* EG2348	7.5	AF	1.5 L/HA
RAPAX EXP.	*Btk* EG2348	7.5	AF	1.0 L/HA
DELFIN®	*Btk* SA11	6.4	WG	750 G/HA
FORAY 48B®	*Btk* HD1	2.1	AF	3.0 L/HA

[1] A. I. = ACTIVE INGREDIENT; [2] SC, SUSPENSION CONCENTRATE; AF, AQUEOUS FLOWABLE FORMULATION
WATER DISPERSIBLE GRANULE

Experimental design and assessments

THE TRIAL WAS CONDUCTED IN 2012 IN A CORK OAK FOREST NEARBY PLOAGHE-CHIARAM
SARDINIA, ITALY). THE ACTUAL PRESENCE OF *M. neustrium* IN THE STUDY FOREST WAS VERIFIED THE
PREVIOUS WINTER VIA MONITORING AND COUNTS OF EGG MASSES.

THE EXPERIMENTAL DESIGN CONSISTED IN A COMPLETELY RANDOMIZED BLOCK D
REPLICATES PER TREATMENT (PLOT SIZE: 1 TREE). ALL CORK OAK TREES USED IN THE TRI
SIZE (APPROXIMATELY 5 M IN HEIGHT AND WITH 7 M FOLIAGE PROJECTION DIAMETER), A
COMPARABLE INITIAL *M. neustrium* INFESTATION LEVEL (MEAN NUMBER OF TENTS/TREE: 3).

ALL TREATMENTS WERE APPLIED ON 11 MAY, WHEN THE MAJORITY OF LARVAE WE
DEVELOPMENTAL STAGE (ALMOST EXCLUSIVELY 2ND, 3RD INSTAR LARVAE) USING A MOTORIZED KNAPSA
SPRAYER FOR EXPERIMENTAL TRIALS (M3 SERIES, CIFARELLI SPA, ITALY).

TO VERIFY WHETHER PEST DISTRIBUTION WAS HOMOGENEOUS AMONG TREATMEN
TREATMENT APPLICATION, IN EACH PLOT, THE NUMBER OF *M. neustrium* LARVAE PRESENT IN THE TENTS AND
IN GROUPS ON BRANCHES WAS COUNTED. AFTER TREATMENT APPLICATION, INSTEAD

ALREADY SWITCHED TO A SOLITARY BEHAVIOUR ON FOLIAGE. THEREFORE, TO ESTIMATE DIFFERENT TREATMENTS, THE NUMBER OF LARVAE PRESENT ON EIGHT 30 CM LONG RANDOMLY SELECTED ON EACH TREE, WAS COUNTED ONE AND TWO WEEKS AFTER TREA FURTHERMORE, TWO WEEKS AFTER TREATMENT APPLICATION, *M. neustrium* DEFOLIATION CAUSED LARVAE WAS ESTIMATED BY ASSIGNING PERCENT DEFOLIATION VALUES ACCORDING TO 2% WITH TREE DEFOLIATION < 5%, 7.5% WITH DEFOLIATION RANGING FROM 5-10%, 15 DEFOLIATION RANGING FROM 11-20%, 25.5% WITH DEFOLIATION RANGING FROM 21-30%, 3 DEFOLIATION RANGING FROM 31-45%, 53% WITH DEFOLIATION RANGING FROM 46-60%, 7 DEFOLIATION RANGING FROM 61-80%, AND 90.5% WITH DEFOLIATION > 80%.

THE NUMBER OF LARVAE/TREE (PRELIMINARY ASSESSMENT), THE NUMBER OF LARVAE THE PERCENTAGE OF DEFOLIATION WERE COMPARED ACROSS TREATMENTS USING FOLLOWED BY STUDENT-NEWMAN-KEULS' TEST FOR POST-HOC COMPARISONS OF MEANS.

Results and discussion

ALL TESTED PRODUCTS PROVED TO BE EFFECTIVE IN THE LABORATORY AGAINST *M. neustrium* LARVAE (DATA NOT REPORTED). THIS EFFICACY WAS CONFIRMED UNDER OPEN FIELD CONDITIONS: ALL SIGNIFICANTLY REDUCED THE NUMBER OF *M. neustrium* LARVAE IN COMPARISON TO THE UNTREAT CONTROL (TABLE 2).

TABLE 2. NUMBER OF *M. neustrium* LARVAE/TREE, NUMBER OF LARVAE/8 BRANCHES AND P DEFOLIATION (M ± SE) IN THE TESTED TREATMENTS AT THE DIFFERENT ASSESSMENTS. (COLUMN FOLLOWED BY DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT (SNK-TEST: P < 0.05)

Treatment	N. larvae/tree	N. larvae on 8 branches/tree		Defoliation (%)
	11 May	18 May	25 May	25 May
UNTREATED CONTROL	150.8 ± 19.6 A	17.8 ± 0.8 A	22.3 ± 1.4 A	71.8 ± 10.8 A
RAPAX® (1.5 L/HA)	179.0 ± 36.0 A	2.0 ± 0.6 BC	2.0 ± 0.4 C	5.4 ± 3.4 B
RAPAX® (1.0 L/HA)	169.5 ± 31.3 A	5.3 ± 1.3 B	2.5 ± 0.7 C	12.0 ± 4.5 B
RAPAX EXP. (1.5 L/HA)	104.5 ± 24.3 A	1.3 ± 0.6 C	1.8 ± 0.5 C	2.0 ± 0.0 B
RAPAX EXP. (1.0 L/HA)	247.5 ± 55.3 A	5.5 ± 1.7 B	5.3 ± 0.9 B	11.5 ± 2.3 B
DELFIN®	181.3 ± 24.4 A	3.0 ± 0.4 BC	2.5 ± 0.3 C	4.8 ± 1.6 B
FORAY 48B®	150.8 ± 20.2 A	2.0 ± 0.4 BC	1.8 ± 0.3 C	2.0 ± 0.0 B

BOTH ONE AND TWO WEEKS AFTER TREATMENT APPLICATION, THE RAPAX EXPERIMENTAL SHOWED SIGNIFICANTLY HIGHER INFESTATION LEVELS THAN THE HIGHE WHILE NO SIGNIFICANT DOSE-RESPONSE EFFECT EMERGED FOR RAPAX. EXCEPT FOR RAPA AT THE LOWER RATE AT THE FINAL ASSESSMENT, THE EFFICACY IN REDUCING THE NUMB LARVAE OF BOTH FORMULATIONS OF *M. neustrium* WAS ALWAYS COMPARABLE TO THAT OF TH BASED REFERENCE PRODUCTS. HOWEVER, THIS LOWER EFFICACY AND THE SIGNIFICAN EFFECT OBSERVED FOR THE AQUEOUS FLOWABLE FORMULATION SHOULD BE CONSID BECAUSE OF THE SLIGHT THOUGH NOT SIGNIFICANTLY HIGHER MEAN INITIAL INFESTA TREATED WITH THE LOWER RATE OF RAPAX EXPERIMENTAL (TABLE 1).

PERCENT DEFOLIATION LEVELS TWO WEEKS AFTER TREATMENT APPLICATION WERE S IN UNTREATED CONTROL PLOTS THAN IN TREATED PLOTS, WITH DIFFERENCES AMONG TR SIGNIFICANT (TABLE 2). MEAN PERCENT DEFOLIATION EXCEEDED 70% IN THE UNTREATEI IT WAS EQUAL TO OR BELOW 5% FOR RAPAX® AND RAPAX EXPERIMENTAL AT 1.5 L HA[1] FOR FORAY 48B® AND DELFIN®. SLIGHTLY THOUGH NOT SIGNIFICANTLY HIGHER DEFOLIATION VALUES W FOR RAPAX AND RAPAX EXPERIMENTAL AT 1.0 L HA[1] (MEAN = 12%).

Bt-BASED FORMULATIONS HAVE BEEN SUCCESSFULLY USED TO CONTROL TENT CATE THE PAST (VAN DER LAAN & WASSINK, 1962). OVER TIME DIFFERENT FORMULATIONS WI EFFICACY HAVE BEEN DEVELOPED BY THE INDUSTRY (LORD, 2005). LADURNER *et al.* (2011) INVESTIGATED THE EFFICACY OF DIFFERENT FORMULATIONS OF *Bt* STRAIN EG 2348 AGAINST THE TOMATO LEAF MINER, *Tuta absoluta*, ON TOMATO, AND IN THEIR STUDIES THE SUSPENSION CONCENTI BE MORE EFFECTIVE THAN THE WETTABLE POWDER. UNDER OUR TRIAL CONDITIONS CONCENTRATE AND THE AQUEOUS FLOWABLE FORMULATION, APPLIED AS A BROADCAS THE GROUND, SEEMED TO SHOW COMPARABLE AND HIGH EFFICACY AGAINST *M. neustrium*. HOWEVER, FURTHER RESEARCH IS NEEDED TO EVALUATE THE SAME FORMULATIONS ALSO WITH EQUIPMENT, MOST COMMONLY USED ON LARGE FORESTS AND WHERE ALSO OTHER DEFOLIATORS CAN BE FOUND (SMITLEY & DAVIS, 1993; LUCIANO & LENTINI, 2007).

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Development of a new bio-insecticide for controlling lepidopteran pests

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Abstract: TURKEY HAS BEEN USED TO BE A SELF-SUFFICIENT COUNTRY IN TERMS OF AGRICU PRODUCTS, BUT TODAY IT IS IMPORTING AGRICULTURAL PRODUCTS FROM MANY OTHER COUNTRIES MOST IMPORTANT REASONS FOR THIS IS TO NOT BE RELIANT ON THE EFFECTIVE CONTROL ECONOMICALLY IMPORTANT PLANTS. THE INSECTS BELONGING TO THE ORDER LEPIDOPTERA ARE MOST HARMFUL INSECT GROUPS IN OUR COUNTRY. MEMBERS OF THIS GROUP CAUSE SERIOUS DA AGRICULTURAL AND FORESTED AREAS AS WELL AS IN WAREHOUSES. SO FAR, EFFORTS TO CONTROL PESTS HAVE MAINLY INVOLVED THE USE OF CHEMICAL INSECTICIDES, PARTICULARLY INSEC INHIBITORS. HOWEVER, THESE AGENTS CAN HAVE UNDESIRABLE SIDE-EFFECTS ON HUMANS, PL OTHER ANIMAL SPECIES, PARTICULARLY PREDATORS AND PARASITOIDS OF LEPIDOPTERAN PESTS. T IS NECESSARY TO FIND ALTERNATIVE AND ENVIRONMENTALLY FRIENDLY CONTROL METHODS. IN WE PROPOSE TO DEVELOP A BIOLOGICAL PREPARATION (BIO-INSECTICIDE) AGAINST LEPIDOPTERA USING AN INSECTICIDAL ISOLATE OF *Bacillus thuringiensis* SUBSP. *kurstaki*. OUR RESULTS SHOWED THAT THE ISOLATE HAS MAXIMUM GROWTH AT 30 °C, AT PH 7 IN TRYPTIC SOY BROTH CONTAINING 1 ITS SPORULATION WAS SUPPORTED IN SYNTHETIC MEDIUM AND THE BACTERIAL CELL SUSPE PRODUCED IN PILOT FERMENTER. A POWDER BIO-PESTICIDE WAS PRODUCED USING THIS CELL SUSPE AND NECESSARY FORMULATION MATERIALS IN THE SPRAY DRYER. THE PHYSICAL AND BIOLOGICAL LIKE WETTABILITY, SUSPENSIBILITY, PARTICLE SIZE, MOISTURE CONTENT, AND VIABLE SPORE FORMULATED POWDER WERE DETERMINED AND NOTED AS 30 S, 80%, 25 µM, 8% AND 1.3×10^{11} CFU g^{-1} DW, RESPECTIVELY. INSECTICIDAL ACTIVITY OF THE PRODUCT AGAINST *Thaumetopoea pityocampa*, *Plodia interpunctella* AND *Lobesia botrana* LARVAE IN LABORATORY CONDITIONS WERE INVESTIGATED. MORTALITY RESULTS WERE IDENTIFIED AS 48% AGAINST *T. pityocampa*, 90% AGAINST *L. botrana* AND 90% AGAINST *P. interpunctella*. TOXICITY/PATHOGENICITY ASSAYS OF THE DRIED POWDER ON EUKARYOTIC HOSTS WERE PERFORMED ON RATS. SUBSEQUENTLY, BLOOD, FECES AND LUNG SAMPLE WERE INVESTIGATED FOR THE PRESENCE OF *B. thuringiensis* SPORES.

Key words: *Bacillus thuringiensis*, LEPIDOPTERA, BIO-PESTICIDE

Insect pathogens and entomoparasitic nematodes
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Bioluminescence determination of antibacterial activity of *Bombyx mori* and *Galleria mellonella* haemolymph

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Abstract: WE DESCRIBE AN ANTIBACTERIAL ASSAY BASED ON BIOLUMINESCENCE OF TWO GE BACTERIA *Photorhabdus luminescens* AND TRANSFORMED *Escherichia coli*, WHICH CAN BE USED FOR A REAL-TIME MEASUREMENT OF ANTIBACTERIAL ACTIVITY. WE OBSERVED A SIGNIFICANT DOSE-DEPEN BIOLUMINESCENCE USING BOTH BACTERIAL SPECIES DURING ONE HOUR AFTER EXPOSURE TO *Bombyx mori* OR *Galleria mellonella* HAEMOLYMPH. THE HUMORAL ORIGIN OF THE ANTIBACTERIAL ACTIVITY OBSER HAEMOLYMPH WAS CONFIRMED IN HAEMOLYMPH PLASMA WITHOUT HAEMOCYTES. ANTIBAC OPERATING AGAINST GRAM NEGATIVE BACTERIA WAS MEASURED IN UNAFFECTED INSECT LAR SEPTIC INJURY; INCREASED ANTIBACTERIAL ACTIVITY IN HAEMOLYMPH WAS DETECTED IN THE CONFIRMS INDUCIBILITY OF ANTIMICROBIAL AGENTS. THIS METHOD CAN BE WIDELY USED FOR ANTIBACTERIAL ACTIVITY IN INSECTS AND SUPPOSEDLY IN OTHER INVERTEBRATES.

Key words: ANTIBACTERIAL ACTIVITY, *Galleria mellonella*, *Bombyx mori*, BIOLUMINESCENT BACTERIA

Introduction

BIOLUMINESCENCE IS THE PRODUCTION AND EMISSION OF LIGHT BY LIVING ORGANISMS; OCCURRING FORM OF CHEMOLUMINESCENCE WHERE ENERGY IS RELEASED BY ENZYMA REACTION IN THE FORM OF LIGHT (IN BACTERIA MAXIMUM 490 NM).

THE GENUS *Photorhabdus* INCLUDES TERRESTRIAL GRAM NEGATIVE BACTERIA, WHICH A FOUND IN ASSOCIATION WITH ENTOMOPATHOGENIC NEMATODES *Heterorhabditis* SPP. UPON ENTERING AN INSECT HOST NEMATODES RELEASE BACTERIAL CELLS FROM THEIR INTESTINAL T ESTABLISH A LETHAL SEPTICAEMIA IN THE HOST (FFRENCH-CONSTANT *et al.*, 2003).

SIMILARLY TRANSFORMED *Escherichia coli* K12 ARE CAPABLE OF LIGHT PRODUCTION. IT CONTA PLASMID WITH THE COMPLETE LUXABCDEAMP OPERON ORIGINATING FROM *Photorhabdus* LEUMI BELONG TO THE EXPRESSION OF BACTERIAL LUCIFERASE, WHICH USES ALONG-CHAIN ALDEHYDE AS GENERATION OF LIGHT (ATOSUO *et al.*, 2012).

INSECT IMMUNITY INVOLVES BOTH HUMORAL AND CELLULAR ACTIVITIES IN THE INSECT RELY ON HAEMOCYTES WHICH PERFORM PHAGOCYTOSIS, ENCAPSULATION A HUMORAL FACTORS INCLUDE ESPECIALLY HIGHLY POTENT ANTIMICROBIAL PEPTIDES ENZYME PHENOLOXIDASE. SEVERAL OF THE CELLULAR REACTIONS (CLOTTING, NODU ENCAPSULATION) ACTIVATE PHENOLOXIDASE AND THEREFORE A VISIBLE MELANISATION.

THE AIM OF THIS STUDY WAS TO ANALYSE ANTIBACTERIAL ACTIVITY OF INSECT HAE DIRECT REAL TIME MEASUREMENT OF CHANGES IN BIOLUMINESCENCE PRODUCED BY *E. coli* K12.

Material and methods

Bacterial suspensions

Photorhabdus luminescens, SUBSP. *kayaii* WAS INOCULATED IN LB MEDIUM AFTER ISOLATION F
FRESH SURFACE STERILIZED CADAVERS OF *Galleria mellonella* INFECTED BY THE ENTOMOPATHOGENIC
NEMATODE *Heterorhabditis bacteriophora*. TRANSFORMED *E. coli* K12 RESISTANT TO AMPICILLIN
WITH LUXABCDEAMP GENES WAS USED AS *E. coli* CULTURE. MEDIA CONTAINED 100 $\mu g \cdot ml^{-1}$ AMPICILLIN.
BACTERIAL STOCKS WERE PREPARED AFTER CULTIVATION IN LIQUID BROTH MEDIUM BY A
DENSITY 1.0 (AT 400 NM) FOR *P. luminescens* AND 0.25 (AT 620 NM) FOR *E. coli* USING SPEKOL 11
(CARL ZEISS).

Luminometry

BIOLUMINESCENCE OF BACTERIAL SUSPENSIONS AFTER EXPOSITION TO INSECT HAE
MEASURED DURING ONE HOUR USING LUMINOMETER LM01-T (IMMUNOTECH) AT 25 °C (*P. luminescens*) OR 37 °C (*E. coli*). THE LIGHT EMISSION DURING REACTION IS POSITIVELY CORRELA
BACTERIAL VIABILITY (ATOSUO *et al.*, 2012). RESULTS ARE EXPRESSED IN RELATIVE LIGHT U

Bacterial viability measurements

COLONY FORMING UNITS (CFU) WERE COUNTED IN *E. coli* SUSPENSION AND THEN 30 MIN AFTER
ADDITION OF HAEMOLYMPH. BOTH BACTERIA SUSPENSION AND BACTERIA TREATED W
WERE DILUTED LOGARITHMICALLY AND PLATED ON DISHES WITH NUTRIENT AGAR. NUME
DETERMINED AFTER OVERNIGHT INCUBATION AT 37 °C.

Haemolymph collection

Bombyx mori LARVAE (5TH INSTAR, 5 DAYS OLD) WERE REARED ON MULBERRY *Galleria mellonella*
LARVAE (7TH INSTAR, 3-4 DAYS OLD) WERE OBTAINED FROM LABORATORY CULTURES MAIN
ARTIFICIAL DIET (HAYDAK, 1936) AT 29 ± 1 °C IN CONSTANT DARKNESS. HAEMOLYMPH WAS
BY PROLEG AMPUTATION AND POOLING INTO COOLED EPPENDORF TUBE WITH SEVE
PHENYLTHIOUREA (PTU) AS ANTICOAGULANT.

Antimicrobial peptides induction

B. mori LARVAE WERE PRICKED LATERALLY THROUGH THE PROLEG WITH NEEDLE DIPPED
SALINE OR BACTERIAL SUSPENSION OF *P. luminescens*). UNTREATED LARVAE WERE USED AS A
CONTROL. AFTER FIVE HOURS OF INCUBATION HAEMOLYMPH WAS COLLECTED FOR
ANTIBACTERIAL ACTIVITY AS DESCRIBED ABOVE.

Results and discussion

INSECT HAEMOLYMPH SHOWS HIGH ANTIBACTERIAL ACTIVITY. WE OBSERVED A SIGNIFIC
THE BIOLUMINESCENCE SIGNAL IN SAMPLES CONTAINING *B. mori* HAEMOLYMPH. CONCENTRATIONS OF
HAEMOLYMPH RANGING FROM 10% TO 40% WERE TESTED AGAINST *E. coli* (FIGURE 1A) AND
P. luminescens (FIGURE 1B). FOR SUBSEQUENT EXPERIMENTS 20% WAS SELECTED AS AN
DILUTION OF HAEMOLYMPH, IT SUPPRESSES APPROXIMATELY 50% OF BACTERIA IN 30 MIN.

TO VERIFY THAT ANTIBACTERIAL ACTIVITY IS BASED ON HUMORAL FACTORS,
PREPARATIONS OF 20% HAEMOLYMPH SAMPLES WERE TESTED. FRESHLY COLLECTED HA
COMPARED TO HAEMOCYTE-FREE HAEMOLYMPH AND HAEMOLYMPH STORED AT –20 °C FO
TESTED SAMPLES SHOWED COMPARABLE ANTIBACTERIAL ACTIVITY (BIOLUMINESCEN
WITHIN 3% IN EXPERIMENTS USING *E. coli* AND 10% WITH *P. luminescens* IN ALL THREE HAEMOLYMPH

SAMPLES) BOTH AGAINST *E. coli* (FIGURE 2A) AND *P. luminescens* (FIGURE 2B) LEADING TO THE ASSUMPTION THAT THE MEASURED ANTIBACTERIAL ACTIVITY WAS CAUSED BY HUMOR AGAINST GRAM NEGATIVE BACTERIA.

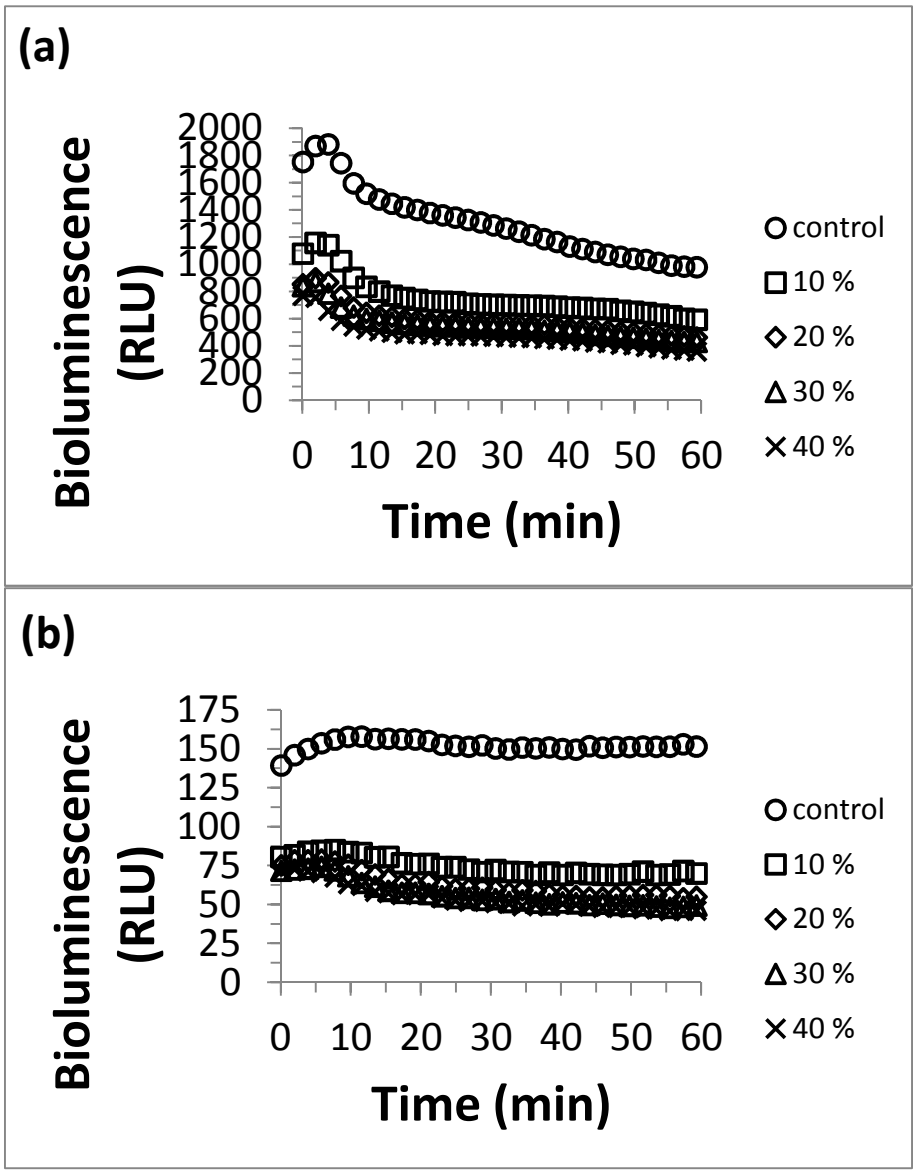

FIGURE 1DEPENDENCE OF ANTIBACTERIAL ACTIVITY AGAINST *E. coli* (A) AND BIOLUMINESCENT *P. luminescens* (B) ON *B. mori* HAEMOLYMPH CONCENTRATION EXPRESSED AS A DECLIN BIOLUMINESCENCE IN RELATIVE LIGHT UNITS (RLU).

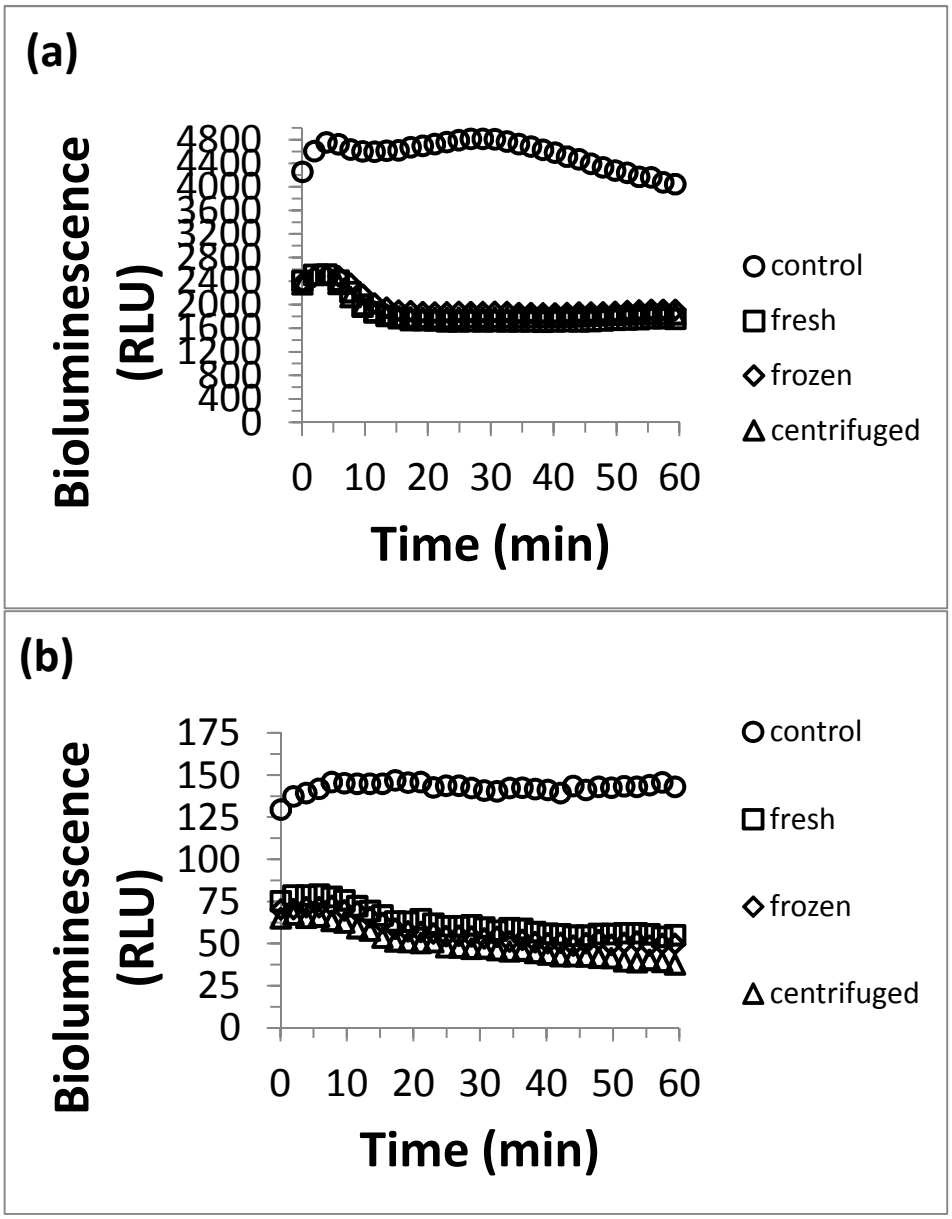

FIGURE 2. ANTIBACTERIAL ACTIVITY AGAINST *E. coli* (A) OR *P. luminescens* (B) IN 20% FRESH, CENTRIFUGED AND FROZEN HAEMOLYMPH OF *G. mellonella* EXPRESSED AS A DECLINE OF BIOLUMINESCENCE IN RELATIVE LIGHT UNITS (RLU).

HAEMOLYMPH OF *G. mellonella* WAS ALSO USED. SIMILARLY TO HAEMOLYMPH FROM *B. mori*, HAEMOLYMPH FROM *G. mellonella* SHOWED STRONG ANTIBACTERIAL ACTIVITY AGAINST BOTH *E. coli* AND *P. luminescens*, APPROXIMATELY 50% DECREASE IN BACTERIAL BIOLUMINESCENCE SIGNAL IN 30 MIN WAS

TO HIGHLIGHT THE PRACTICAL USE OF THE ASSAY, UNTREATED LARVAE WERE COMPARED WITH LARVAE PRICKED WITH A BACTERIAL SUSPENSION TO INDUCE ANTIBACTERIAL ACTIVITY. *B. mori* LARVAE PRICKED BY *E. coli* OR *P. luminescens* SHOWED HIGHER ANTIBACTERIAL ACTIVITY FIVE HOURS AFTER PRICKING. INCREASE OF ANTIBACTERIAL ACTIVITY WAS REFLECTED IN DECREASED *E. coli* BIOLUMINESCENCE THAT WAS SIGNIFICANTLY DIFFERENT COMPARED TO UNTREATED CONTROL OR LARVAE PRICKED WITH STERILE NEEDLE (FIGURE 3; 45% BIOLUMINESCENCE DECLINE IN 30 MIN). INSECTS TREATED WITH *E. coli* WERE

ANTIBACTERIAL RESPONSE (76% BIOLUMINESCENCE DECLINE IN 30 MIN) THAN LARVAE TR
INSECT PATHOGEN *P. luminescens* (63% BIOLUMINESCENCE DECLINE IN 30 MIN).

CFU COUNTS SHOWED APPROXIMATELY 30% DECREASE IN VIABILITY OF BACTERIA 3
TREATMENT WITH HAEMOLYMPH, WHEREAS DECREASE IN LUMINESCENCE SIGNAL W
SUGGESTING THAT ANTIBACTERIAL FACTORS IN HAEMOLYMPH HAVE PARTLY ONLY BACTE

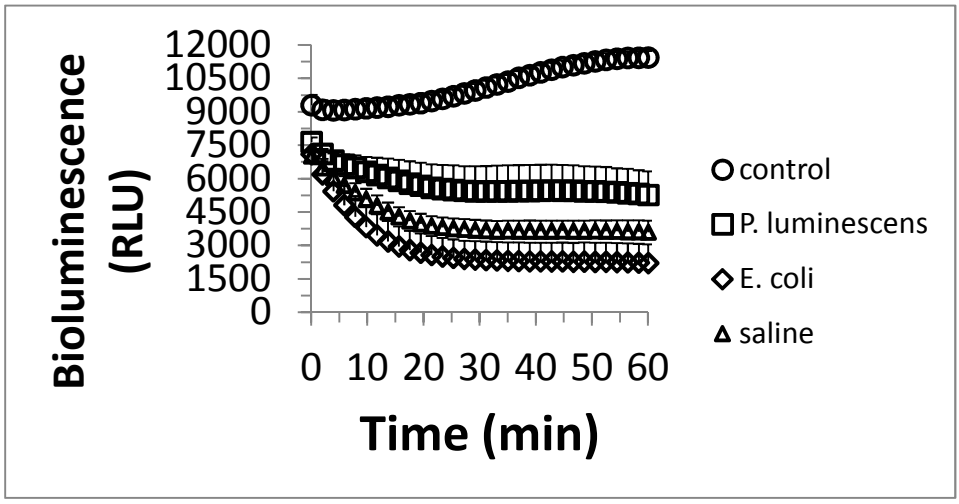

FIGURE 3. INFLUENCE OF SEPTIC INJURY ON ANTIBACTERIAL ACTIVITY AGAINST
HAEMOLYMPH *B. mori* LARVAE WERE PRICKED BY STERILE NEEDLE (SALINE), *E. coli* SUSPENSION OF
P. luminescens. DECLINE OF BIOLUMINESCENCE IS EXPRESSED IN RELATIVE LIGHT UNITS (RLU

INSECTS DO NOT HAVE A COMPLEMENT AS VERTEBRATES THUS MOSTLY AMPS ARE
RESPONSIBLE FOR BACTERICIDAL EFFECT. MOST OF THE AMPS DETECTABLE IN THE HA
MICROBIAL INFECTION ARE PRODUCED WITHIN A FEW HOURS BY THE FAT BODY, HAEMO
SPECIFIC TISSUES (LEMAITRE & HOFFMANN, 2007). APART FROM INDUCED AMPS SYNTHES
ALSO CONSTITUTIVE LEVEL OF AMPS PRESENT IN HAEMOLYMPH. BIOLUMINESCENCE CA
NEW, FAST AND REAL-TIME METHOD FOR ASSESSMENT OF HAEMOLYMPH ANTIBACTERIAL

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Intramolecular cleavage at the loop between α3-helix and α4-helix is critical for cytotoxic activity of Cry8Da

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Abstract: CRY8DA FROM *Bacillus thuringiensis galleriae* SDS-502 HAS THE TOXICITY AGAINST BOTH LARVA AND ADULT *P. japonica*. CRY8DA IS PROCESSED INTO THREE FRAGMENTS (64 KDA, 54 KDA AND 8 KDA) JUICE OF *P. japonica*. FRAGMENTS OF 54 KDA AND 8 KDA ARE DERIVED FROM THE CLEAVAGE OF FRAGMENT AT THE LOOP BETWEEN α3-HELIX AND α4-HELIX IN DOMAIN I. BINDING ASSAYS SHOWED THAT TH 54 KDA FRAGMENT BOUND TO BOTH LARVAE AND ADULT *P. japonica* BRUSH-BORDER MEMBRANE VESICLES WHILE THE 64 KDA AND 8 KDA FRAGMENTS DID NOT. WE CONSTRUCTED A PROTEASE-RESISTANT MUTAN WHICH R^{163} ON THE LOOP WAS CHANGED TO R^{163}. TO DIRECTLY INVESTIGATE WHETHER INTRAMOLECULAR CL CRITICAL FOR INSECTICIDAL ACTIVITY OF CRY8DA, WE PERFORMED CYTOTOXIC ASSAYS AGAINS CELLS (MECS) PREPARED FROM *P. japonica* ADULT USING PURIFIED UNCLEAVED (64 KDA) AND INTRAMOLECU CLEAVED (MIXTURE OF 54 KDA AND 8 KDA) CRY8DA TOXIN. CYTOTOXIC ASSAY SHOWED MECS W BY ONLY INTRAMOLECULAR CLEAVED CRY8DA TOXIN. INTRAMOLECULAR CLEAVED CRY8DA OLIGOMERIC STRUCTURE AFTER INCUBATION WITH MGCS. THESE RESULTS STRONGLY SUPPOR CLEAVAGE AT THE LOOP BETWEEN α3-HELIX AND α4-HELIX IS CRITICAL FOR TOXICITY OF CRY8DA

Key words: CRY8DA, INTRAMOLECULAR CLEAVAGE, *P. japonica*, MECS, 54 KDA

Introduction

Bacillus thuringiensis (BT) IS A ROD SHAPED, GRAM-POSITIVE, SPORE FORMING BACTERIU PRODUCES PARASPORAL CRYSTAL (CRY) PROTEINS DURING SPORULATION. SINCE THE CR SHOW INSECTICIDAL ACTIVITY TO SPECIFIC SPECIES WITHIN THE ORDERS LEPIDOPTE COLEOPTERA, ESPECIALLY LARVAE OF THESE INSECTS, BT IS WIDELY USED IN PEST CONTR OF COLEOPTERAN PESTS SUCH AS LARVAE OF FAMILY SCARABAEIDAE, WHICH DAMAGE GRASS AND OTHER HORTICULTURAL AND AGRICULTURAL PLANTS, IS DIFFICULT, BECAUS WHERE SPRAYABLE BT FORMULATION IS HARD TO REACH THE TARGET INSECTS. THERE FIND A BT CRY PROTEIN THAT EFFECTIVELY CONTROLS BOTH LARVAE AND ADULTS O REPORTED THAT CRY8DA AND CRY8DB HAVE TOXICITY AGAINST NOT ONLY LARVAE E JAPANESE BEETLE (*Popillia japonica* NEWMAN).

CRY8DA IS PROCESSED TO 64 KDA, 54 KDA AND 8 KDA FRAGMENTS BY *P. japonica* ADULT JUICE. THE FRAGMENTS OF 54 KDA AND 8 KDA ARE DERIVED FROM INTRAMOLECULAR C 64 KDA FRAGMENT AT THE LOOP BETWEEN α3-HELIX AND α4-HELIX OF DOMAIN I. BINDING ASSAYS SHOWED THAT THE 54 KDA FRAGMENT BOUND TO BOTH *P. japonica* LARVAE AND ADULT BRUSH-BORDER MEMBRANE VESICLES (BBMV) WHILE 64 KDA AND 8 KDA FRAGMENTS DID NOT (YAMAGUCHI *et al.*, 2010). THIS INTRAMOLECULAR CLEAVAGE INDUCES SPECIFIC BINDING OF CRY3A TOXIN A TARGET INSECTS (VACHON *et al.*, 2008). THUS, INTRAMOLECULAR CLEAVAGE AT THE LOOP IS CO FEATURE AMONG ONLY BEETLE ACTIVE CRY PROTEINS AND IS SEEMS TO BE IMPORTA TOXICITY.

IN THIS PAPER, WE CONSTRUCTED A PROTEASE-RESISTANT MUTANT 8DA-R163A, WH
FROM R^{163} TO A^{163}. WE ALSO PERFORMED CYTOTOXIC ASSAY AGAINST MECS FROM ADULT *P. japonica*
USING INTRAMOLECULAR CLEAVED OR NONE INTRAMOLECULAR CLEAVED CRY8DA TOXI

Material and methods

Preparation of midgut epitherial cell (MECs)

MECS WERE PREPARED FROM DISSECTED MIDGUTS OF ADULT *P. japonica*. MIDGUTS FROM EXCISED TO
REMOVE THE PERITROPHIC MEMBRANE AND FOOD CONTENTS WERE WASHED WITH 10 M
PBS, PH 7.4. MIDGUTS WERE PLACED IN A PETRI DISH CONTAINING 10 MM GLUCOSE IN PBS
WITH 1000 U ML^{-1} COLLAGENASE (WAKO PURE CHEMICAL INDUSTRIES). AFTER GENTLY SHAI
AT 25 °C, DISSOCIATED MECS WERE CENTRIFUGED FOR 3 MIN AT 500 ×G AND THE RESULTIN
RESUSPENDED IN PBS. THE CELLS WERE WASHED BY CENTRIFUGATION SEVERAL TI
SUPERNATANT WAS CLEAR.

Cytotoxic assay

CYTOTOXICITY OF INTRAMOLECULAR CLEAVED CRY8DA TOXIN (54+8 KDA) AND UNCL
TOXIN (64 KDA) WAS ASSESSED BY MEASURING ATP AMOUNTS OF LIVE MECS. MECS AND
TOXINS WERE INCUBATED IN A WELL OF 96 WELL-PLATE (IWAKI) FOR 120 MIN AT 25 °C
INCUBATION, ATP AMOUNT OF LIVE MECS WERE MEASURED WITH CELLTITER-GLO™
CELL VIABILITY ASSAY (PROMEGA) AND GLOMAX™ 96 MICROPLATE LUMINOMETER (PROMEGA)
ACCORDING TO MANUFACTURER'S PROTOCOL. ALSO CRY8DA TOXIN TREATED MECS WERE
THE MICROSCOPY.

Detection of oligomer

TO CONFIRM CRY8DA TOXIN FORM OLIGOMER WHEN TOXIN KILLS MECS, WE TRIED TO DI
OFCRY8DA TOXIN. MECS WERE INCUBATED WITH INTRAMOLECULAR CLEAVED CRY8DA TO
AT 25 °C IN PBS. AFTER THE INCUBATION, MECS WERE HARVESTED BY CENTRIFUGATION
5 MIN, 4 °C) AND WASHED TWO TIMES WITH PBS. MECS WERE SUSPENDED WITH 0.5% TRITO
IN PBS AND SOLUBILIZED FOR 10 MIN AT 25 °C. AFTER THE INCUBATION, SOLUBLE AN
MATERIALS WERE FRACTIONATED BY CENTRIFUGATION (20,000 XG, 15 MIN, 4 °C). INSOL
WAS SUSPENDED WITH 6 M UREA, 2 M THIOUREA, 2% CHAPS IN 40 MM TRIS-HCL, PH 6.8. SOL
AND INSOLUBLE FRACTIONS WERE SUBJECTED TO 8% SDS-PAGE AND PROTEINS WERE T
PVDF MEMBRANE. CRY8DA PROTEINS ON MEMBRANES WERE DETECTED BY MONOCLONAL
ANTIBODY OF CRY8DA TOXIN AND SUPERSIGNAL WEST PICO CHEMILUMINESCENT SUBSTRA

Results and discussion

Cytotoxicity of Cry8Da toxins against MECs

TO DIRECTLY INVESTIGATE INTRAMOLECULAR CLEAVAGE AT THE MODIFIED BETWEEN R^{163}-A^{163} IS
CRITICAL FOR INSECTICIDAL ACTIVITY OF CRY8DA, WE PERFORMED CYTOTOXIC ASSAY
PREPARED FROM ADULT *P. japonica* USING PURIFIED UNCLEAVED (64 KDA FRAGMENT) A
INTRAMOLECULAR CLEAVED (54 KDA AND 8 KDA FRAGMENTS) CRY8DA TOXIN. CRY TOX
OF MIDGUT EPITHELIAL CELLS BY MAKING PORES ON PLASMA MEMBRANE OF MIDGUT EP
TARGET INSECTS, WHICH LEAD TO INTOXICATION OF TARGET INSECTS. THEREFORE CYT
MECS CAN DEMONSTRATE INTRAMOLECULAR CLEAVAGE OF CRY8DA TOXIN IS CRITICA
ACTIVITY OF CRY8DA. CELL VIABILITY OF MECS INCUBATED WITH INTRAMOLECULAR C

TOXIN (54+8 KDA) REDUCED TO 20% (FIGURE 1). ALSO MICROSCOPIC OBSERVATION SHOWE
AND CELL BURST, WHICH IS TYPICAL SYMPTOM OF PORE FORMING TOXIN ATTACK, WHEN
WITH INTRAMOLECULAR CLEAVED CRY8DA TOXIN. OTHERWISE UNCLEAVED CRY8DA TO
SIGNIFICANT REDUCTION OF CELL VIABILITY OF MECS AND CYTOPATHIC EFFECTS SUCH
THESE RESULTS CLEARLY SHOWED INTRAMOLECULAR CLEAVAGE AT THE LOOP BETWE
HELIX IS CRITICAL FOR INSECTICIDAL ACTIVITY OF CRY8DA.

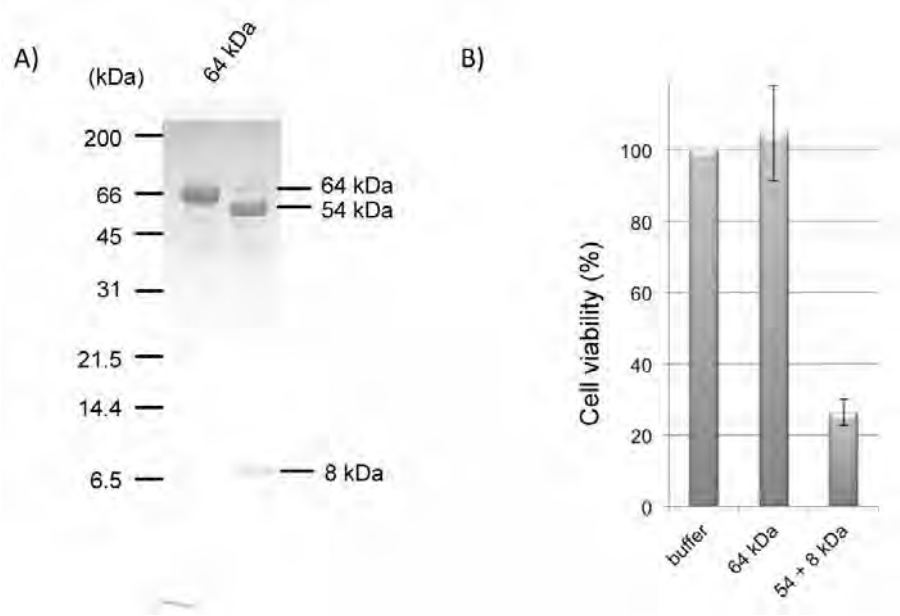

FIGURE 1. PURIFICATION OF CRY8DA TOXINS AND CYTOTOXIC ASSAY AGAINST MECS P
ADULT *P. japonica*. A) GUT JUICE TREATED CRY8DA WERE PURIFIED BY ANION EXC
CHROMATOGRAPHY AND GEL FILTRATION CHROMATOGRAPHY. PURIFIED TOXINS WERE S
SDS-PAGE. B) MECS PREPARED FROM ADULT *P. japonica* WERE TREATED WITH UNCLEAVED (64 KDA
FRAGMENT) OR CLEAVED (MIXTURE OF 54 KDA AND 8 KDA) CRY8DA TOXIN AND CELL V
RECORDED. BLACK BARS INDICATE STANDARD DEVIATION.

Detection of oligomer

CRY TOXINS HAVE BEEN CHARACTERIZED AS PORE FORMING TOXINS. COMMON MODE OF
FORMING TOXINS IS BINDING TO THE RECEPTOR MOLECULE(S) ON TARGET CELL, OLIGO
INSERTION OF PART OF THE TOXIN INTO THE PLASMA MEMBRANE. CRY1AB TOXIN BI
RECEPTOR, CADHERIN LIKE PROTEIN, AND THEN CRY1AB TOXIN IS REMOVED BY A MEMBRA
BOUND PROTEINASE. LOSS OF α-HELIX INDUCED OLIGOMERIZATION OF CRY1AB TOXIN. OLIGOM
CRY1AB TOXIN DETACH CADHERIN LIKE PROTEIN AND BIND TO A SECOND RECEPTOR, GP
OR ALP FOLLOWED BY INSERTION TO PLASMA MEMBRANE TO FORM PORE (BRAVO *et al.*, 2007).
OLIGOMERIC STRUCTURE OF CRY8DA TOXIN WAS DETECTED FROM TRITON X-100 INSOLU
MECS AFTER INCUBATION LIKE IN THE CASE OF CRY1AB TOXIN (FIGURE 2). PREVIOUS STU
THAT FRAGMENTS OF 54 KDA AND 8 KDA STILL FORM A TOXIN COMPLEX AFTER ACTIVATI
THE 54 KDA FRAGMENT OF CRY8DA TOXIN BINDS TO *P. japonica* BMV. THE 8 KDA FRAGMENT
RESPONDING TO α-HELIX-3 OF DOMAIN I DID NOT. THIS SUGGESTS THAT THE 8 KDA
FRAGMENT FROM THE 54 KDA FRAGMENT IS A TRIGGER OF OLIGOMERIZATION LIKE CRY1
MECS STARTS BLEBBING AFTER TOXIN EXPOSURE WITHIN 30 MIN. THIS MEANS THAT TH
CRY8DA TOXIN MAKES TOXIC PORES IN THE PLASMA MEMBRANE OF MECS. THUS, THE MO

OF CRY8DA HAS SIMILARITY WITH OTHER CRY TOXINS, SUCH AS CRY1AB. WE SHOWE
DIFFERENCE BETWEEN LARVAE AND ADULT (YAMAGUCHI *et al.*, 2010). CRY1AB TOXIN
REQUIRES COMPLICATED RECEPTOR INTERACTION AS DESCRIBED ABOVE. RECEPTOR IDEN
TO UNDERSTAND OVERALL MODE OF ACTION OF CRY8DA AGAINST *P. japonica*.

IN THIS STUDY WE SHOWED THAT THE INTRAMOLECUAR CLEAVAGE AT THE LOOP BET
α4-HELIX OF DOMAIN I IS CRITICAL FOR INSECTICIDAL ACTIVITY. ALSO, CRY8DA MAKES
OLIGOMERS ON MECS PREPARED FROM ADULT *P. japonica*.

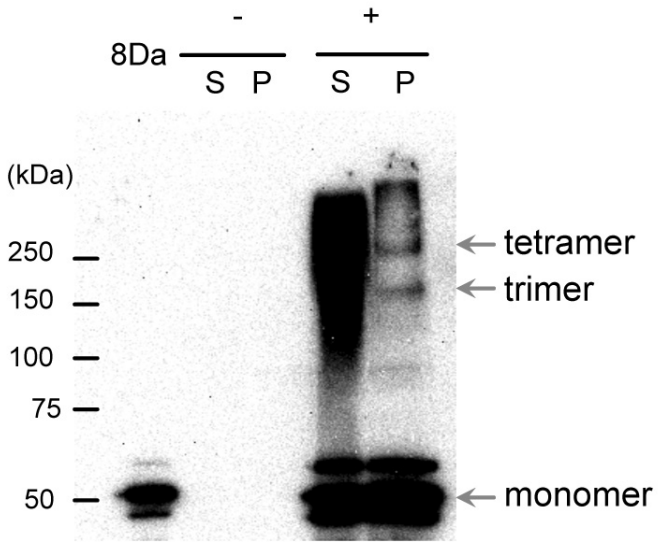

FIGURE 2. DETECTION OF CRY8DA OLIGOMERIC STRUCTURE. CRY8DA TREATED (+) OR N
MGCS WERE COLLECTED AND SOLUBILISED WITH TRITON X-100. SOLUBLE FRACTIONS (S)
FRACTIONS (P) WERE SUBJECTED TO SDS-PAGE, WESTERN BLOTTING FOLLOWED BY DET
CRY8DA SERUM.

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Electron microscope and genetic analysis of an intracellular bacterium associated with the common rough woodlouse, *Porcellio scaber* (Isopoda, Porcellionidae)

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Abstract: THE COMMON ROUGH WOODLOUSE, *Porcellio scaber*, IS A COMMON AND WIDESPREAD ISOPOD SPECIES OF WESTERN AND NORTHERN EUROPE. A PREVIOUSLY UNKNOWN INTRACELLULAR BA IDENTIFIED IN A DISEASED *Porcellio* LARVA. MICROSCOPIC STUDIES REVEALED THE SUBCELLULAR S CHARACTERISTIC OF INFECTION BY *Rickettsiella*-LIKE BACTERIA. MOLECULAR PHYLOGENETIC ANALYSIS BASE 16S RIBOSOMAL RNA ENCODING GENE DEMONSTRATED THAT THE WOODLOUSE PATHOGEN BELON TAXONOMIC GENUS *Rickettsiella* (GAMMAPROTEOBACTERIA; LEGIONELLALES). MOREOVER, GENETIC ANALYSIS MAKES IT LIKELY THAT THIS NEW PATHOTYPE SHOULD BE CONSIDERED A MEMBER OF TH *armadillidii* COMPLEX", I.E. A GROUP OF *Rickettsiella* BACTERIA FOUND MAINLY IN TERRESTRIAL ISOPO *R. armadillidii* IS CURRENTLY PLACED IN SYNONYMY WITH THE NOMENCLATURAL TYPE SPECIES *popilliae*. THE PRESENT STUDY DOES NOT LEND SUPPORT TO THIS SYNONYMIZATION.

Key words: *Porcellio scaber*, `*Rickettsiella armadillidii*´, *Rickettsiella popilliae*, INTRACELLULAR PATHOGENS, ENTOMOPATHOGENIC BACTERIA, TRANSMISSION ELECTRON MICROSCOPY (TEM), 16S

Introduction

THE GAMMA-PROTEOBACTERIAL GENUS *Rickettsiella* (PHILIP) COMPRISES INTRACELLULAR PATHOGENS WIDE RANGE OF ARTHROPODS THAT TYPICALLY MULTIPLY IN VACUOLAR STRUCTURES AND ARE FREQUENTLY ASSOCIATED WITH PROTEIN CRYSTALS. CURRENTLY, FOUR RECO THE NOMENCLATURAL TYPE SPECIES *Rickettsiella popilliae* (DUTKY & GOODEN) AS WELL AS *Rickettsiella grylli* (VAGO & MARTOJA), *Rickettsiella stethorae* (HALL & BADGLEY), AND *Rickettsiella chironomi* (WEISER) – AND NUMEROUS PATHOTYPES ARE DISTINGUISHED WIT GENUS (FOURNIER & RAOULT, 2005).

Rickettsiella-LIKE BACTERIA FROM NUMEROUS INSECTS AND ARACHNIDS HAVE BEE MORPHO- AND HISTOPATHOLOGICALLY, AND INTRACELLULAR BACTERIA FROM, E.G., CR AS WELL AS COLEOPTERAN AND DIPTERAN INSECTS HAVE GENETICALLY BEEN DEMONS THE GENUS *Rickettsiella* (FOURNIER & RAOULT, 2005 AND REFERENCES THEREIN). MORE *Rickettsiella*-LIKE BACTERIA HAVE BEEN REPORTED TO OCCUR IN CRUSTACEANS AS, E.G. (VAGO *et al.*, 1970; CORDAUX *et al.*, 2007) AND FRESHWATER AMPHIPODS (FIELDER, 1974; LARSSON, 1982). INFECTION OF HEMOCYTES AND MIDGUT GLANDS (HEPATOPANCREAS) AP RULE IN CRUSTACEANS, AS IS THE ABSENCE OF WELL-DEFINED PROTEIN CRYSTALS. MOLECULAR TAXONOMIC ANALYSES HAVE MOTIVATED ASSIGNMENT OF SEVERAL ISOPO GENUS *Rickettsiella* (GAMMAPROTEOBACTERIA) (CORDAUX *et al.*, 2007), WHEREAS *Rickettsiella*-LIKE BACTERIA FROM FURTHER CRUSTACEANS, INCLUDING WOODLICE, HAVE GENETICALLY GENUS *Coxiella* (COOPER *et al.*, 2007) AND THE ORDERS RICKETTSIALES OR COXIELLALES (KOSTANJSEK *et al.*, 2004).

Porcellio scaber (LATREILLE, 1804) (ISOPODA, PORCELLIONIDAE) IS THE MOST COMMON
OF WOODLOUSE, KNOWN FROM CENTRAL AND WESTERN EUROPE, THE UNITED KINGDOM
COLONIZED NORTH AMERICA, SOUTH AFRICA AND AUSTRALIA. THE COMMON ROUGH WO
IN A WIDE RANGE OF HABITATS AND IS CHIEFLY FOUND UNDER STONES, AND ON ROTTING
ONLY LITTLE IS KNOWN ABOUT NATURAL DISEASES OF WOODLICE. THE PRESENT WO
INFECTION WITH AN INTRACELLULAR BACTERIUM OF THE GENUS *Rickettsiella* IN *P. scaber*.

Material and methods

SPECIMENS OF THE COMMON ROUGH WOODLOUSE, *Porcellio scaber*, WERE COLLECTED IN MARCH 2012
FROM A GARDEN AT MAINTAL, FRANKFURT/MAIN REGION, GERMANY, WHERE ALIVE AND
WERE FOUND UNDER STONES AND WOODEN BOARDS DISTRIBUTED OVER AN AREA OF
METERS. IN A DEAD HYPERTROPHIED LARVA OF THIS ISOPOD SPECIES, INFECTION WITH
Rickettsiella-LIKE
BACTERIA WAS DETECTED BY LIGHT AND ELECTRON MICROSCOPY. FOR MEASUREMENTS OF THIS PATHOGEN,
NEGATIVELY STAINED PREPARATIONS, USING 2.0% SODIUM PHOSPHOTUNGSTATE IN *aqua bidest.* WERE
EXAMINED BY ELECTRON MICROSCOPY. THE AVERAGE SIZES OF *Rickettsiella*-LIKE NEGATIVELY STAINED
BACTERIA WERE DETERMINED USING “IMAGESP SOFTWARE” (TROENDLE, MOORENWIES, G

THE ALMOST COMPLETE 16S RRNA ENCODING GENE WAS AMPLIFIED FROM INFECTED
TISSUE AND SEQUENCED. ALIGNMENT WITH ORTHOLOGOUS SEQUENCES, PAIRWISE P-D
CONSTRUCTION AS WELL AS PHYLOGENETIC RECONSTRUCTION USING NEIGHBOR JOINING
EVOLUTION (ME) ALGORITHMS WERE PERFORMED BY MEANS OF THE MEGA 4 SOFTWARE
CORRESPONDING MAXIMUM LIKELIHOOD (ML) PHYLOGENY WAS RECONSTRUCTED USIN
PROGRAM. IRRESPECTIVE OF THE METHOD OF PHYLOGENETIC RECONSTRUCTION EMPI
DISTRIBUTION BASED MODEL OF RATE HETEROGENEITY ALLOWING FOR EIGHT RATE CAT
AND TREE TOPOLOGY CONFIDENCE LIMITS WERE EXPLORED IN NON-PARAMETRIC BOOTS
1,000 PSEUDO-REPLICATES. A CONSENSUS TREE WAS GENERATED FROM THE DIFFERENT PH
THE PHYLIP 3.6 SOFTWARE TOOL.

Results and discussion

IN SQUASH PREPARATIONS OF INFECTED *P. scaber*, TISSUE ONLY BACTERIA DANCING IN RAPID
BROWNIAN MOVEMENT WERE OBSERVED WITH PHASE CONTRAST MICROSCOPY AS
Rickettsiella DISEASE. NEGATIVELY STAINED BACTERIA IN ELECTRON MICROSCOPY WERE RO
AN AVERAGE SIZE OF APP. 590 NM IN LENGTH AND 270 NM IN WIDTH (FIGURE 1), I.E. A CELL
SIMILAR TO THAT MEASURED FOR *Rickettsiella* BACTERIA FROM INSECTS. IN CONTRAST TO OTHER K
Rickettsiella-INFECTIONS, NO WELL-DEFINED ASSOCIATED CRYSTALS COULD BE DETECTED I
THIS ISOPOD. THE TYPICAL APPEARANCE OF *Rickettsiella*-LIKE BACTERIA APPEARS IN
CONSISTENT WITH THE MAIN CYTOLOGICAL FEATURES OF THE LIFE CYCLE AS DESCRIBED
(1967) FOR INSECT PATHOGENIC *Rickettsiella* BACTERIA. THE ABSENCE OF STRUCTURALLY WELL-DEFI
MEMBRANE-BOUNDED CRYSTALS IN *P. scaber* THAT ARE A CHARACTERISTIC FEATURE OF INFECTION I
Rickettsiella (KLEESPIES *et al.*, 2011), HAVE ALSO BEEN OBSERVED IN PREVIOUS STUDIES OF IN
BY *Rickettsiella*-LIKE BACTERIA FROM ISOPODS, E.G., THE ARMADILLIDIID, *Armadillidium vulgare*
(LATREILLE, 1804) (ISOPODA, ARMADILLIDIIDAE) (VAGO *et al.*, 1970; FEDERICI *et al.*, 1974).

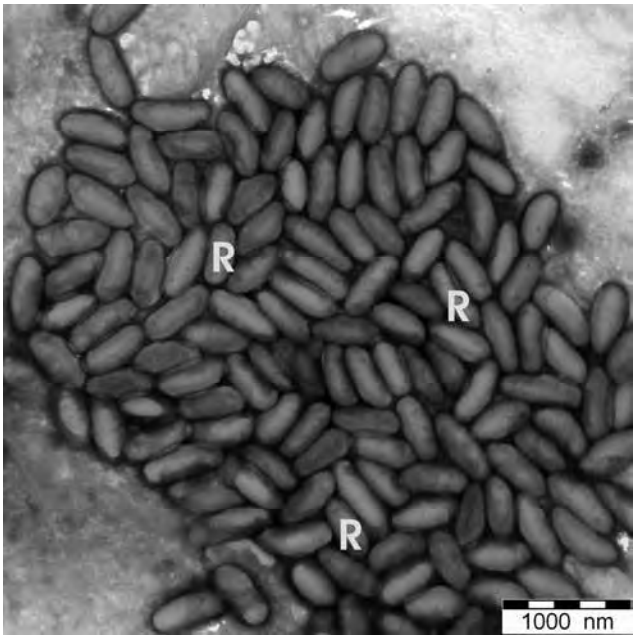

FIGURE 1. ELECTRON MICROGRAPH OF *Rickettsiella* BACTERIA (R) ISOLATED FROM *Porcellio scaber*, NEGATIVELY STAINED WITH SODIUM PHOSPHOTUNGSTATE.

THREE INDEPENDENT AMPLIFICATION EXPERIMENTS FROM *P. scaber* GENERATED THE IDENTICAL 16S RRNA GENE SEQUENCE (GENBANK ACCESSION NUMBER JX406180). WHI CONSENSUS SEQUENCE WAS USED AS BLASTN QUERY, 16S RRNA GENE SEQUENCES FROM A *Rickettsiella* BACTERIA WERE IDENTIFIED AS BEST HITS. CONSISTENTLY, THE MAXIMUM PHYLOGENY (FIGURE 2A) RECONSTRUCTED FROM THE ALIGNMENT OF 16S RRNA GENES BACTERIA FIRMLY PLACES THE NEW BACTERIUM AMONG THE PREVIOUSLY DESC PROTEOBACTERIA *Rickettsiella* SPECIES AND PATHOTYPES. IMPORTANTLY, THE CLADE COMPRI CONSIDERED *Rickettsiella* BACTERIA RECEIVES MAXIMAL (100%) BOOTSTRAP SUPPORT IN THE M WELL AS IN THE CORRESPONDING ME AND NJ PHYLOGENIES (DATA NOT SHOWN). A CO VIEW IS GIVEN BY THE EXTENDED MAJORITY RULE CONSENSUS TREE COMBINING THE RI ALTERNATIVE APPROACHES (FIGURE 2B). THUS, THE NEW BACTERIUM CAN CONSISTENTL THE TAXONOMIC GENUS *Rickettsiella*.

CONCERNING THE RELATIVE TAXONOMIC POSITION OF THIS NEW SPECIMEN WITH *Rickettsiella*, IT IS OBVIOUS FROM THE PHYLOGENIES PRESENTED IN FIGURE 2 THAT THE S STUDY CLUSTERS TIGHTLY WITH SEVERAL PATHOTYPES FROM TERRESTRIAL ISOPOD REPRESENTATION OF THE *R. armadillidii* COMPLEX" RECEIVES MAXIMUM BOOTSTRAP SUPPORT AN LOCATED IN A SISTER POSITION WITH RESPECT TO *R. popilliae*. IN ORDER TO EXPLORE THE INFRAGENERIC TAXONOMIC POSITION OF THE *P. scaber* PATHOGEN IN MORE QUANTITATIVE TERMS PAIRWISE 16S SEQUENCE IDENTITIES WERE ESTABLISHED BY CONSTRUCTION OF A P-DISTANCE OF SEQUENCES FROM WITHIN THE *R. armadillidii* COMPLEX" WERE FOUND >99% IDENTICAL, WHEREA THE RESPECTIVE VALUES WITH RESPECT TO *Rickettsiella* BACTERIA, INCLUDING THE *R. popilliae* PATHOTYPES, RANGED BETWEEN 97% AND 98% (FIGURE 2). IN TERMS OF A SEQUENCE THRESHOLD OF 98.5% AS APPLIED FOR SPECIES DELINEATION WITHIN THE ORDER CHLA VALUES WOULD HAVE TO BE TRANSLATED INTO A CO-SPECIATION OF THE ISOPOD PATHOG

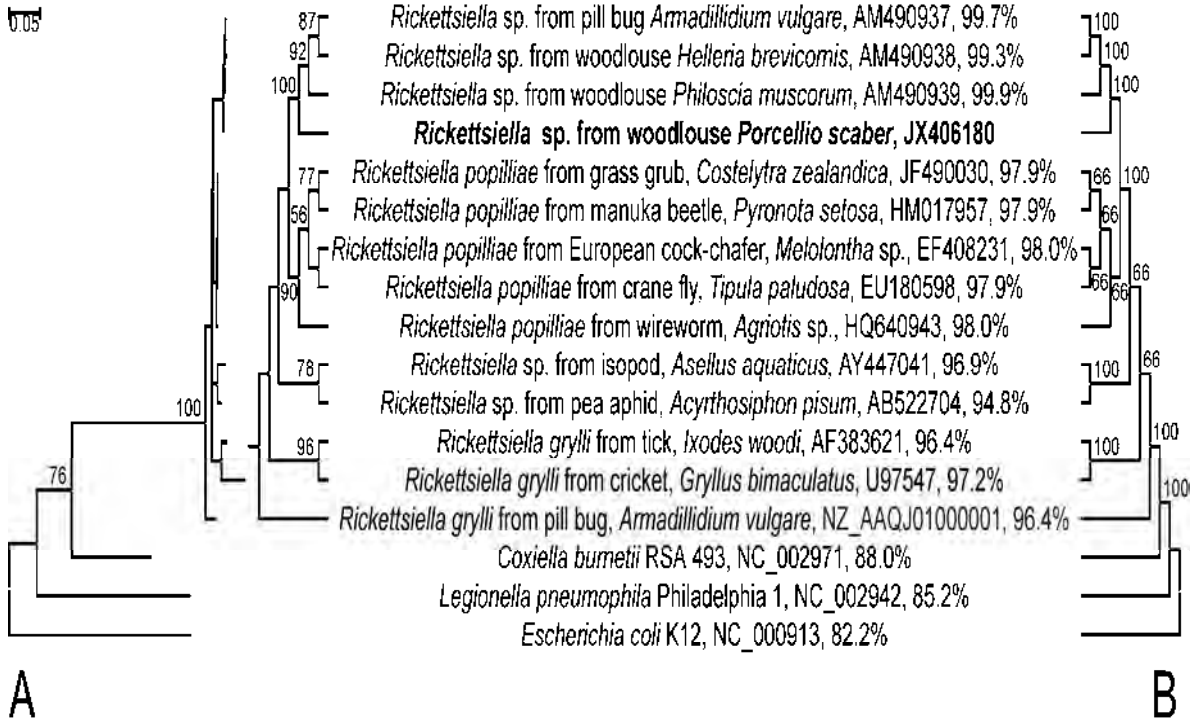

FIGURE 1 **(A)** MAXIMUM LIKELIHOOD 16S RRNA GENE-BASED PHYLOGENY OF THE BACTER
Legionellales; FOR ENHANCED RESOLUTION, THE *Rickettsiella* CLADE HAS BEEN EXPANDED INTO A
CLADOGRAM. BRANCHES ARE LABELLED BY GENUS, SPECIES, ORIGINAL HOST, GENE
NUMBERS, AND PAIRWISE SEQUENCE IDENTITY VALUES WITH RESPECT TO THE *P. scaber* TYPE
NUMBERS ON BRANCHES DESIGNATE BOOTSTRAP SUPPORT PERCENTAGES > 50%. TREES HA
USING *E. coli* AS TECHNICAL OUTGROUP. THE SIZE BAR INDICATES A 5% RELATIVE SEQUENCE
(B) EXTENDED MAJORITY RULE CONSENSUS TREE COMBINING THE RESPECTIVE ML, ME,
NUMBERS ON BRANCHES DENOTE SUB-STRUCTURE FREQUENCIES ACROSS TREES.

IN CONCLUSION, THE RESULTS OF THE PRESENT STUDY FIRSTLY DEMONSTRATE T
PATHOGEN ASSOCIATED WITH *P. scaber* BELONGS TO THE TAXONOMIC GENUS *Rickettsiella*
(GAMMAPROTEOBACTERIA, LEGIONELLALES) AND APPEARS BOTH MORPHOLOGICALLY
CLOSELY RELATED TO FURTHER WOODLOUSE-ASSOCIATED BACTERIA FORMING TH
COMPLEX". IN CONTRAST, THE CURRENTLY ACCEPTED INCLUSION OF THIS PATHOTYPE
RECOGNIZED SPECIES *R. popilliae* IS NOT SUPPORTED BY OUR DATA. HOWEVER, A RESPECTIVE TAX
ASSIGNMENT SHOULD NOT BE BASED ON 16S RRNA GENE DATA ALONE.

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Miscellaneous

Poster

Impact of various oilseed rape productions on biological potential of endogaeic active ground beetles (Coleoptera: Carabidae)

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Abstract: GROUND BEETLES ARE ONE THE MOST IMPORTANT FAMILY OF ARTHROPOD PREDATO CROPS. COMPARED TO CENTRAL EUROPEAN COUNTRIES THAT HAVE MOST FREQUENTLY LIS SPECIES IN OILSEED RAPE FIELDS, IN CROATIA NOTHING IS KNOWN ABOUT GROUND BEETLES ACTIVITY. A TRIAL WAS SET UP ON THREE DIFFERENT OILSEED RAPE PRODUCTIONS: C (PLOUGHING, FULL SEED DRESSING, INTENSIVE APPLICATION OF PESTICIDES AND FERTILIZERS), (MULCHING, NO SEED DRESSING, REDUCED INPUT OF PESTICIDES AND FERTILIZERS, 3 M WIDTH STRIP ALONG EACH SIDE OF THE FIELD) AND ORGANIC (PLOUGHING, NO SEED DRESSING, PEAS A ENTIRE FIELD ROUNDED WITH 3 M WIDTH TRAP CROP STRIP, NO FERTILIZERS AND PESTICIDES A AIM OF THIS RESEARCH WAS TO INVESTIGATE ENDOGAEIC ACTIVITY AND DENSITY OF GROUN THE IMPACT OF DIFFERENT OILSEED RAPE PRODUCTION SYSTEMS ON THEIR APPERANCE. END WERE USED FOR MONITORING ENDOGAEIC ACTIVITY AND SAMPLING PREDATORY ARTHROPOD WERE PUT ON EACH PRODUCTION SYSTEM AND ON INTEGRATED AND ORGANIC TRAP CROP S FOUR ADDITIONAL TRAPS. DURING 2011, MONITORING WAS CONDUCTED FROM FEBRUARY 10 T SAMPLES WERE TAKEN EVERY TWO WEEKS. RESULTS SHOWED THAT THE LEVEL OF ENDOGAEIC A GROUND BEETLES WAS HIGHEST IN ORGANIC PRODUCTION WITH 26.5 INDIVIDUALS PER TRAP IN OF THE FIELD AND 27.25 INDIVIDUALS PER TRAP IN THE TRAP CROP STRIP. IN THE CENTR CONVENTIONAL FIELD NUMBER OF GROUND BEETLES INDIVIDUALS PER TRAP WAS 21.63. IN INT SYSTEM LEVEL OF ENDOGAEIC ACTIVITY WAS THE LOWEST WITH 7.5 INDIVIDUALS PER TRAP IN THE THE FIELD AND 17.25 INDIVIDUALS PER TRAP IN THE TRAP CROP STRIP. THIS PRESENTATION RESULTS ABOUT ENDOGAEIC ACTIVE GROUND BEETLES IN DIFFERENT MANAGED OILSEED R CROATIA.

Key words: ENDOGAEIC TRAPS, GROUND BEETLES, OILSEED RAPE, CROATIA

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Ground beetles (Coleoptera: Carabidae) in sugar beet fields as the base for conservation biological control

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Abstract: THE FAUNA AND ABUNDANCE OF GROUND BEETLES (COLEOPTERA, CARABIDAE) IN ARA
AN INDICATOR OF INFLUENCE OF DIFFERENT AGRICULTURAL MEASURES ON BIODIVERSITY. THE A
TO DETERMINE GROUND BEETLE FAUNA ABUNDANCE AND FREQUENCY IN TWO FIELDS WITH DIF
INSECTICIDE APPLICATION PRACTICE, AND TO DETERMINE DIFFERENCES IN TOTAL NUMBE
INDIVIDUALS, COLLECTED WITH TWO CAPTURING METHODS. THE STUDY WAS CONDUCTED IN 2
PART OF CROATIA (COUNTY OF VUKOVAR-SRIJEM). BEETLES WERE COLLECTED IN A PERIOD OF T
(APRIL- SEPTEMBER) BY SETTING FOUR MODIFIED PITFALL TRAPS AIMED TO COLLECT ABOVE GR
PROBES (WB PROBE® TRAP, TRECE INC.) AIMED TO COLLECT ENDOGEIC FAUNA IN EACH FIELD. NIN
SPECIES AND EIGHT GENERA WERE IDENTIFIED IN THE STUDY. *Pseudophonus rufipes* (DE
GEER 1774) AND *Bembidion* SP. (LATREILLE 1802). BOTH ARE CLASSIFIED AS EUDOMINANT. THE MOST F
SPECIES WAS *P. rufipes* CLASSIFIED AS CONSTANT (71.42%) AND THE MOST FREQUENT GENUS WAS *Bembidion* SP.
(38.04%) CLASSIFIED AS ACCESSORY. THERE WAS NO SIGNIFICANT DIFFERENCE BETWEEN FIEL
NUMBER OF ESTABLISHED SPECIES AND/OR GENUS NO MATTER IF THEY WERE CAPTURED BY PI
SIGNIFICANTLY MORE INDIVIDUALS WERE CAPTURED IN PITFALL TRAPS ON THE FIELD NO. 1 (33.3
NO. 2 (8.8), RESPECTIVELY. OPPOSITE, SIGNIFICANTLY FEWER INDIVIDUALS WERE CAPTURED WI
FIELD NO. 1 (0.5) THAN ON THE FIELD NO. 2 (6.6), RESPECTIVELY.

Key words: ABUNDANCE, AGRICULTURAL PRACTICE, CROATIA, FREQUENCY, GROUND BEETLES, SUG

Introduction

AMONG MANY OTHER ARTHROPOD SPECIES, GROUND BEETLES ARE USUALLY CONSIDE
INDICATIVE ORGANISMS FOR ASSESSMENT ECOLOGICAL EFFECTS OF DIFFERENT AGRI
THEY ARE KNOWN AS IMPORTANT PREDATORY ORGANISMS OF THE SOIL LIVING P
SUNDERLAND, 1996; SUNDERLAND, 2002). SUGAR BEET IS HIGHLY SENSITIVE CROP TO WE
DISEASES. COMPARING TO OTHER ARABLE CROPS, THE AMOUNT OF PESTICIDES USED FO
CONTROL IS HIGHER. PEST CONTROL PRACTICE AND PESTICIDE APPLICATION PRACTICE
CONDITION AND FARMERS EXPERIENCE. WE HYPOTHESIZED THAT THE NUMBER OF GROU
AND INDIVIDUALS DEPEND ON THE PESTICIDE APPLICATION PRACTICE ON EACH PARTICU
OF OUR STUDY WAS 1) TO DETERMINE AND TO ANALYZE GROUND BEETLES ABUNDANCE
TWO FIELDS WITH DIFFERENT HERBICIDE AND INSECTICIDE APPLICATION PRACTICE, AN
DIFFERENCES IN TOTAL NUMBER OF SPECIES AND INDIVIDUALS, COLLECTED WITH TWO CA

Material and methods

THE STUDY WAS CONDUCTED DURING THE VEGETATION SEASON 2012 ON TWO SUGAR BEET
LOCATED IN THE EASTERN PART OF CROATIA, VILLAGE OF TOVARNIK (COUNTY OF VUKOV

TABLE 1. BASIC INFORMATION ON EXPERIMENTAL FIELDS.

FIELD NO	FIELD SIZE	PRE CROP	HERBICIDES			INSECTICIDES		
			ACTIVE INGREDIENT	DOSE G/HA OR ML/HA (TOTAL)	NO. OF APPLI-CATIONS	ACTIVE INGREDIENT	DOSE G/HA OR ML/HA (TOTAL)	NO. OF APPLI-CATIONS
1	130	WHEAT	DESMEDIFAM	184	3	CHLORPYRIPHOS	900	APPLIED
			FENMEDIFAM	184		CYPERMETHRIN	45	ON THE
			KLOPIRALID	135		CHLORPYRIPHOS	850	EDGES
			TRIFLUSULFURON	10		CYPERMETHRIN	42.5	1
2	4.05	WHEAT	DESMEDIFAM	56	2	LAMBDA-CYCHALOTHRIN	5	2
			FENMEDIFAM	72		CHLORPYRIPHOS	850	
			ETOFUMESAT	88		CYPERMETHRIN	42.5	
			KLOPIRALID	60				

ABOVE GROUND ARTHROPODS WERE SAMPLED BY USING PITFALL TRAPS. EACH TRA
PLASTIC CUP 11 CM IN DIAMETER AND 9 CM DEEP. A CLAY RING WAS PLACED AROUND
FACILITATE SOIL DWELLING ENTOMOFAUNA INTO TRAPS. WATER SOLUTION (5%) OF SOD
CASTED TO THE PITFALL TRAP FOR CONSERVATION OF CAPTURES AND TO PREVENT ESC
THE ENDOGEIC FAUNA WAS COLLECTED WITH PERFORATED PROBE (Ø = 35 MM, H = 440 M
PERFORATIONS: 4 MM X 2 MM) (WB PROBE®, TRECE INC.). FOUR PITFALL TRAPS AND FO
PROBES PER PLOT WERE PLACED DIAGONALLY ACROSS EACH FIELD, STARTING AT LEAST 2
BOUNDARY TO MINIMIZE EDGE EFFECT. THE DISTANCE BETWEEN PITFALL TRAP AND EN
2 M AND THE DISTANCES AMONG PITFALL TRAPS WERE AT LEAST 30 M. SAMPLES WERE TA
BETWEEN APRIL 19 AND SEPTEMBER 11. ALL GROUND BEETLES WERE IDENTIFIED TO TH
WHEN POSSIBLE TO THE SPECIES LEVEL, USING THE FOLLOWING IDENTIFICATION KEYS
BECHYNE (1974); HARDE & SEVERA (1984); CASALE & KRYZHANOVSKIJ (2003). BASED ON TH
NUMBER OF COLLECTED INDIVIDUALS AND THE INDIVIDUAL NUMBER OF THE EACH PARTI
DOMINANCE WAS CALCULATED. THE RESULTS (EUDOMINANT, DOMINANT, SUBDOMI
SUBRECEDENT) WERE CLASSIFIED ACCORDING TO TISCHLER & HEYDEMAN (CIT. BALA
FREQUENCY WAS CALCULATED WITH THE BALOGH'S FORMULA (CIT. BALARIN, 1974). TO
SPECIES AND TOTAL NUMBER OF INDIVIDUALS CAPTURED IN PITFALL TRAPS AND PROBES
ANOVA ANALYSIS (ONE-WAY, $P = 0.05$) TO DETERMINE DIFFERENCES BETWEEN FIELDS. FOR M
SEPARATION DUNCAN'S MULTIPLE RANGE TEST WAS USED.

Results and discussion

SEVENTEEN TAXA WERE IDENTIFIED THROUGHOUT THE STUDY, WITH NINE IDENTIFIED TO
EIGHT TO GENUS LEVEL (TABLE 2). *Pseudophonus rufipes* WAS EUDOMINANT AND THE MOST ABUNDAN
SPECIES IN THE STUDY. GENUS *Bembidion* SP. WAS ALSO EUDOMINANT AND THE SECOND MO

SPECIES. FREQUENCY *P. rufipes* WAS CONSIDERED AS CONSTANT, *Bembidion* SP. WAS CLASSIFIED AS ACCESSORY. ALL OTHER TAXA WERE CLASSIFIED AS ACCIDENTAL.

ESTABLISHED SPECIES OF GROUND BEETLES CORRESPOND WITH THOSE IDENTIFIED IN IN CROATIA (BALARIN, 1974; ŠERUGA, 1973; DURBEŠIĆ, 1987; DURBEŠIĆ *et al*., 2006; BAŽOK *et al*., 2007; KOS *et al*., 2010).

TABLE 2. TOTAL NUMBER OF CAPTURES, ABUNDANCE AND FREQUENCY OF GROUND BEETL FIELDS, TOVARNIK, 2012.

NO	NAME OF SPECIES/ GENUS	C*	A**	F***
1.	*Nebria* SP. (LATREILLE, 1802)	3	2.27 C	9.52 D
2.	*Acupalpus (Acupalpus) parvulus* (STURM, 1825)	3	2.27 C	4.76 D
3.	*Agonum (Agonum) muelleri* (HERBST, 1784)	6	4.55 C	9.52 D
4.	*Agonum (Europhilus) fuliginosum* (PANZER, 1809)	7	5.30 B	23.80 D
5.	*Amara* SP. (BONELLI, 1810)	10	7.58 B	14.28 D
6.	*Bembidion* SP. (LATREILLE, 1802)	26	19.70 A	38.09 C
7.	*Calosoma (Campalita) auropunctatum auropunctatum* (HERBST, 1784)	3	2.27 C	9.52 D
8.	*Carabus (Oreocarabus) glabratus glabratus* (PAYKULL, 1790	3	2.27 C	14.28 D
9.	*Chlaenius* SP. (BONELLI, 1810)	6	4.55 C	19.04 D
10.	*Cylindera (Cylindera) germanica* (LINNE, 1758)	2	1.52 C	9.52 D
11.	*Dyschirius* SP. (BONELLI, 1810)	4	3.03 C	4.76 D
12.	*Platynus* SP. (BONELLI, 1810)	1	0.76 D	4.76 D
13.	*Poecilus (Poecilus) cupreus* (LINNE, 1758)	6	4.55 C	1904 D
14.	*Pseudoophonus (Pseudoophonus) rufipes* (DE GEER, 1774)	40	30.30 A	71.42 B
15.	*Pterostichus (Morphnosoma) melanarius* (ILLIGER, 1798)	4	3.03 C	19.04 D
16.	*Tachyta* SP. (KIRBY, 1837)	7	5.30 B	19.04 D
17.	*Tachys* SP. (DEJEAN, 1821)	1	0.76 D	4.76 D
	TOTAL	132	100.00	-

C* (TOTAL CAPTURE ON BOTH FIELDS);
A**(ABUNDANCE (PERCENT AND RANK: A-EUDOMINANT (10% <); B-DOMINANT (5%-10%); C-SUBD (1%-4.99%); D-RECEDENT (0.5%-0.99%); E-SUBRECEDENT (0.01%-0.49%));
F*** (FREQUENCY (PERCENT AND RANK (A-EUCONSTANT (75%-100%); B-CONSTANT (50%-75%); C-A (25%-50%); D-ACCIDENTAL (0.1%-25%).

INVESTIGATION DID NOT PROVIDE SIGNIFICANT DIFFERENCES BETWEEN FIELDS IN SP (TABLE 3). AVERAGE NUMBER OF INDIVIDUALS CAPTURED IN PITFALL TRAP WAS SIGNIFI THE FIELD NO. 1 THAN IN THE FIELD NO. 2. ALTHOUGH SEED TREATMENT AND INSECTIC AGAINST SUGAR BEET WEEVIL WERE SIMILAR ON BOTH FIELDS, IN THE FIELD NO. 2 INS APPLIED TWICE ON THE WHOLE SURFACE WHILE IN THE FIELD NO. 1, FIRST APPLICATION WAS CONDUCTED ONLY ON THE EDGES. THE MORE INTENSIVE TILLAGE WAS CARRIED OUT AND HERBICIDES WERE APPLIED IN THREE *vs.* TWO APPLICATIONS IN THE FIELD NO. 2 THAN

TILLAGE AND WEED CONTROL WERE DISCUSSED BY THIELE (1977) AS THE MAIN FA
NUMEROUSNESS AND RICHNESS OF THE BENEFICIAL FAUNA.

TABLE 3. RESULTS OF ANOVA FOR NUMBER OF SPECIES AND INDIVIDUALS PER TRAP FOR PIT
PROBE, TOVARNIK, 2012.

	NUMBER OF TAXA PER TRAP		NUMBER OF INDIVIDUALS PER TRAP	
	PITFALL TRAP	PROBE	PITFALL TRAP	PROBE
FIELD NO. 1	5.5	0.8	33.3 A*	0.5 B
FIELD NO. 2	5.0	1.5	8.8 B	6.8 A
LSD	N.S.	N.S.	10.97	5.73

* MEANS FOLLOWED BY THE SAME LETTER ARE NOT SIGNIFICANTLY DIFFERENT ACCORDING T
RANGE TEST (P = 0.05).

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Impact of *Entomophaga maimaiga* on gypsy moth populations in Bulgaria

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Abstract: THE ENTOMOPATHOGENIC FUNGUS *Entomophaga maimaiga* HUMBER, SHIMAZU AND SOPER (ENTOMOPHTORALES) (ENTOMOPHTORACEAE) WAS INTRODUCED INTO THREE POPULATIONS (*Lymantria dispar* L., LEPIDOPTERA: EREBIDAE) IN BULGARIA IN 1999. AFTER THE FIRST STRONG EPI 2005, THE SPECIES WAS INTRODUCED IN SIX OUTBREAK POPULATIONS OF GYPSY MOTH IN DIFFEREN COUNTRY FROM 2008 TO 2011. DUE TO THE RESULTING FUNGAL EPIZOOTICS, THE CALAMITIES BULGARIA WERE TOTALLY SUPPRESSED. THE PATHOGEN INCREASED ITS IMPACT BY A NATURAL F IT IS NOW PRESENT IN NEARLY ALL REGIONS OF THE COUNTRY IN WHICH *L. dispar* OCCURS.

Key words: GYPSY MOTH, *Entomophaga maimaiga*, BULGARIA, BIOLOGICAL CONTROL

Introduction

THE GYPSY MOTH (*Lymantria dispar* L., LEPIDOPTERA: EREBIDAE) PERIODICALLY CAUSES SEV DAMAGE IN DECIDUOUS FORESTS IN SEVERAL CENTRAL AND EASTERN EUROPEAN COUNT THE USA WHERE IT WAS INTRODUCED IN THE END OF THE 19TH CENTURY. IN BULGARIA, OAK STANDS OF DIFFERENT AGE WERE INFESTED OVER LONG PERIODS OF TIME (PILARSKA *et al.*, 2007). REPEATED DEFOLIATIONS AND DECREASE GROWTH CAUSE A PHYSIOLOGICAL WEAKENING OF THE HO INCREASING THEIR SUSCEPTIBILITY TO INFESTATIONS OF WOOD BORERS AND PLANT PA REDUCE THE PEST DENSITY AND CONTROL GYPSY MOTH POPULATIONS, BROAD SPEC INSECTICIDES AND THE BACTERIAL PATHOGEN *Bacillus thuringiensis* VAR. *kurstaki* (*Btk*) WERE USED. DUE TO A LACK OF HOST SPECIFICITY, THESE METHODS AFFECT AQUATIC ORGANISMS SPECIES WITHIN THE ORDER LEPIDOPTERA, AND THUS REDUCE BIODIVERSITY IN FOR (MILLER, 1990).

THE ENTOMOPATHOGENIC FUNGUS *Entomophaga maimaiga* HUMBER, SHIMAZU & SOPER (ENTOMOPHTHORALES: ENTOMOPHTHORACEAE) WAS DESCRIBED AS A HOST SPECIFI *L. dispar* FROM JAPAN (SOPER *et al.*, 1988). IT WAS INTRODUCED INTO THE USA IN THE BEGINNING THE 20TH CENTURY. SINCE THEN, IT SUCCESSFULLY REDUCED GYPSY MOTH DENSITY IN SEVE 1999, *E. maimaiga* WAS SUCCESSFULLY INTRODUCED INTO BULGARIA FROM THE USA (PILA *et al.*, 2000). THEREAFTER IT CAUSED EPIZOOTICS AND MORTALITY IN FOUR OUTBREAK POPUL MOTH, LOCATED 30-70 KM FROM THE INTRODUCTION SITES (PILARSKA *et al.*, 2006).

IN THIS PAPER WE PRESENT RESULTS OF RECENT INTRODUCTIONS OF *E. maimaiga* IN GYPSY MOTH POPULATIONS IN BULGARIA AND ON THE IMPACT OF THE FUNGUS ON THE PEST.

Material and methods

FROM 2008 TO 2011, SIX INTRODUCTIONS OF *E. maimaiga* WERE PERFORMED IN OUTBREAK POPULATIO
OF *L. dispar* IN OAK FORESTS IN DIFFERENT PARTS OF THE COUNTRY (TABLE 1). TWO OF THE
WERE CONDUCTED DURING THE SPRING, FOUR IN THE FALL. BEFORE USE, THE INOCULUM
SOIL FOR NOT LESS THAN 9 MONTHS UNDER NATURAL CONDITIONS.

TABLE 1: MAIN CHARACTERISTICS OF STUDIED AREAS, *L. dispar* DENSITY AND ORIGIN OF *E. maimaiga*

LOCALITY	STATE FOREST (HUNTING) ENTERPRISE	GEOGRAPHICAL COORDINATES	ALTITUDE, M A.S.L.	TREE SPECIES[A]	DENSITY OF *L. dispar*[B]	DATE OF INTRODUCTION	ORIGIN OF *E. maimaiga*
SADIEVO	NOVA ZAGORA	42º31.783'N; 026º08.901'E	151	Q.R.	83	28.03.2008	BULGARIA
ASSENOVO	G. ORYAHOVITSA	43º17.695'N; 026º04.051'E	401	Q.C.	78	18.11.2009	USA
SLAVYANOVO	POPOVO	43º17.090'N; 026º08.834'E	345	Q.C.	89	18.11.2009	USA
RUETS	TARGOVISHTE	43º12.119'N; 026º37.950'E	312	Q.C.; C.B.	76	18.11.2010	BULGARIA
DALGACH	TARGOVISHTE	43º12.966'N; 026º42.478'E	193	Q.RU.; T.P.	86	18.11.2010	BULGARIA
SOLNIK	S. ORYAHOVO	42º54.268'N; 027º44.296'E	202	Q.F.; Q.C.	183	05.04.2011	USA

[A] – C.B. – *Carpinus betulus* L.; Q.C. – *Quercus cerris* L.; Q.F. – *Quercus frainetto* TEN.; Q.R. – *Quercus robur* L.; Q.RU. – *Quercus rubra* L.; T.P. – *Tilia platyphyllos* SCOP.
[B] – EGG MASSES PER 100 TREES

FOR THE RELEASE OF INOCULUM, WITHIN EACH EXPERIMENTAL PLOT OF 100 X 100 M, FI
SITES WERE ESTABLISHED – ONE CENTRAL AND FOUR CIRCLES 50 M FROM THE CENTER
NORTH, SOUTH, EAST AND WEST. EACH CIRCLE CONTAINED AT LEAST FIVE TREES. THE FU
WERE CONDUCTED BY MIXING CRUSHED INFECTED LARVAE CONTAINING *E. maimaiga* RESTING SPORE
WITH SOIL, AND DISPERSING THE MIXTURE AROUND THE BASE OF 5 TO 10 TREES. THE BASE
WATERED WITH 4-5 LITERS OF WATER TO ACHIEVE ADEQUATE HUMIDITY.

TO MONITOR LARVAL DENSITY, IN EACH STUDY SITE BURLAP BANDS WERE PLACED
(INCLUDING THE TREATED TREES) AT A HEIGHT OF 1.3 M FROM THE GROUND. *L. dispar* LARVAE C
COLLECTED FROM THE BURLAP BANDS 2-3 TIMES PER MONTH FROM EARLY MAY TO
TRANSPORTED TO THE LABORATORY, WHERE THEY WERE REARED ON FRESH OAK FOLIA
THE FOLIAGE WAS CHANGED DAILY, DEAD GYPSY MOTH LARVAE WERE PLACED IN PET
MOISTURIZED FILTER PAPER AT 20 °C FOR 5-7 DAYS AND THEN REFRIGERATED AT 5 °C UN
EVALUATION. EACH CADAVER WAS DISSECTED INDIVIDUALLY AND OBSERVED UNDER LI
125X MAGNIFICATION FOR THE PRESENCE OF CONIDIA OR AZYGOSPORES OF *E. maimaiga*.

TO ESTIMATE THE INFLUENCE OF THE FUNGAL INFECTION ON THE DENSITY OF TH
POPULATION, THE NUMBER OF EGG MASSES ON 100 TREES IN EACH STUDY SITE WAS COUN

Results and discussion

Impact of E. maimaiga on L. dispar in Bulgaria

THE INTRODUCTION IN SADIEVO LOCALITY WAS CONDUCTED WITH DEAD GYPSY MOTH L
DURING THE EPIZOOTIC IN 2005 IN THE VILLAGE OF KREMEN. AFTER THE INTRODUCTION, I
E. maimaiga INFECTED AND KILLED 87.5% OF THE FIFTH AND SIXTH INSTAR *L. dispar* LARVAE. THE
REDUCTION OF EGG MASSES WAS 96.4% AND NO EGG MASSES WERE RECORDED TWO YEARS

INTRODUCTIONS OF *E. maimaiga* IN ASSENOVO AND SLAVYANOVO AREA WERE CONDUCTED W
INOCULUM FROM THE USA. AS A RESULT OF THE INTRODUCTION EPIZOOTICS OCCU
EXPERIMENTAL SITES IN 2010. MORTALITY OF YOUNG LARVAE REACHED 44-55%, WHEREAS
THE LATE INSTAR LARVAE WAS 95-98%; NO DEFOLIATIONS WERE OBSERVED IN THE STAN
OF THE EGG MASSES WAS 55.1-81.8%, AND 100% A YEAR LATER. INTERESTINGLY, IN 2010, GY
EPIZOOTICS CAUSED BY *E. maimaiga* WERE RECORDED NOT ONLY IN ASSENOVO AND SLAVYANOV
ALSO IN MANY OTHER AREAS IN THE ADJACENT FORESTS OF SFE GORNA ORYAHOVITSA A
ENTERPRISE (SHE) POPOVO.

INTRODUCTIONS OF *E. maimaiga* IN RUETS AND DALGACH IN THE REGION OF SFE TARGOVI
WERE CONDUCTED WITH A MIXED INOCULUM FROM THE EPIZOOTIC NEAR SLAVYANOVO (
SUMMER 2010) AND SOFIA (COLLECTED IN 2005 NEAR KREMEN, STORED IN SOIL SUBSTRAT
YEARS). IN MAY AND JUNE 2011 FREQUENT AND HEAVY RAINFALL OCCURRED IN TH
TARGOVISHTE. WE SUPPOSE THAT THIS FAVOURED THE *E. maimaiga* AND RESULTED IN
AN EPIZOOTIC THAT KILLED ALMOST ALL MIDDLE AND LATE INSTAR LARVAE OF THE PE
EXPERIMENTAL SITES WAS NOT OBSERVED AND NO EGG MASSES OF *L. dispar* WERE RECORDED.
FURTHERMORE, CONIDIAL INFECTIONS WERE REGISTERED IN LARVAE IN MANY AREAS NEA
BELIEVE THAT THIS CAUSED THE RAPID SUPPRESSION OF THE OUTBREAK IN THE
NORTHEASTERN BULGARIA, WHERE THE STRONGEST GYPSY MOTH INFESTATIONS IN BU
REPORTED IN THE PAST.

FOR THE INTRODUCTION IN SOLNIK, INOCULUM FROM THE USA WAS RELEASED IN APR
LATE SPRING OF 2011, 80.4% MORTALITY OF THE LATE INSTAR GYPSY MOTH LARVAE WAS R
RELEASE SITE. THE REDUCTION OF GYPSY MOTH EGG MASSES WAS 77.6% IN 2011 AND 86.3%

IN 2011, ON THE BLACK SEA COAST IN THE REGION OF SHE NESSEBAR (30-50 KM FROM
STRONG DEFOLIATION OF OAK FORESTS BY *L. dispar* WAS REGISTERED. IN 2012, HOWEVER, GYPSY MOTH IN
THIS REGION AS WELL AS ALL OVER CENTRAL BLACK SEA COAST WAS SUPPRESSED BY
SEEN IN OTHER AREAS, ADJACENT TO RELEASE PLOTS (E. G. POPOVO, SEE ABOVE), WE B
DEMONSTRATES THE SELF-DISSEMINATING CAPACITY OF THE FUNGUS *E. maimaiga* WELL KNOWN
CAN SPREAD MORE THAN 100 KM IN ONE SEASON, (HILTON *et al* 1994).

Infestations of Bulgarian forests by L. dispar

FIGURE 1 PRESENTS DATA OF THE FOREST AREA INFESTED BY DIFFERENT *L. dispar* GRADATIONS
PERIOD OF 60 YEARS. BEFORE THE FIRST INTRODUCTION OF *E. maimaiga* IN BULGARIA IN 1999, 492 TO
1,028 THOUSAND HA OF FORESTS WERE AFFECTED BY THE PEST EACH DECADE, AND AN
REACHED FROM 150,000 TO 370,000 HA. AFTER THE INTRODUCTION OF *E. maimaiga*, NO LARGE-SCALE
PEST CALAMITIES WERE OBSERVED AND THE GYPSY MOTH´S ANNUAL INFESTATION ARE
25,000 HA, ONLY 2-5% OF THE INFESTATION LEVELS OBSERVED BEFORE THE INTRODUCTION

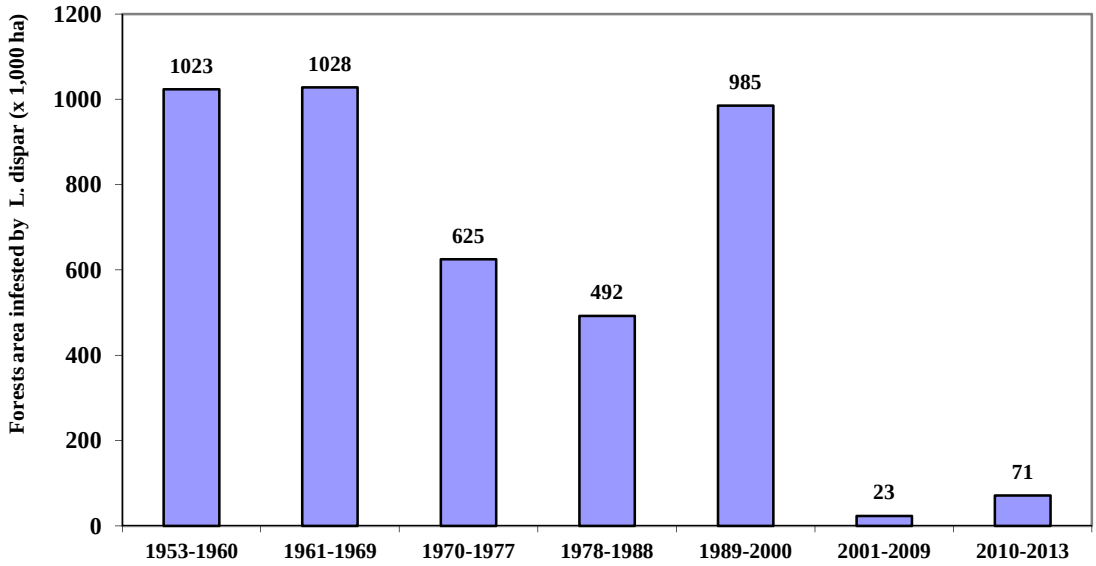

FIGURE 1: FOREST AREA INFESTED BY *L. dispar* IN BULGARIA DURING THE PERIOD 1953-2013

FROM 2010 TO 2013, GYPSY MOTH IS UNDERGOING ANOTHER OUTBREAK, BUT ONLY 71 FOREST WERE SEVERELY AFFECTED. BY EXTRAPOLATION WE ESTIMATE THAT BY THE EN GRADATION (EXPECTED FOR 2017/18), THE TOTAL FOREST AREA AFFECTED BY GYPSY MOTH 150,000 HA, CORRESPONDING TO ONLY 15-30% OF THE INFESTATION LEVELS OBSERV GRADATIONS BEFORE THE ESTABLISHMENT OF *E. maimaiga*.

THE DECREASE OF *L. dispar* DAMAGES TO THE FOREST AFTER A ONE-TIME INTRODUC *E. maimaiga* SHOWS THAT THE PATHOGEN EFFECTIVELY REDUCES AND REGULATES THE PES THE INTRODUCTION OF *E. maimaiga* IN BULGARIA, CHEMICAL CONTROL OF GYPSY MOTH WAS USED SMALL AREAS ONLY, DISMISSING THE PREVIOUS PRACTICE OF LARGE-SCALE USE OF CHEMICAL INSECTICIDES. *E. maimaiga* IS EXPANDING ITS RANGE IN THE BALKAN COUNTRIES (GEO *et al.*, 2012) AND IN THE NEAR FUTURE IT IS EXPECTED TO SPREAD INTO OTHER AREAS EUROPE. THE HIGH VIRULENCE AND SPECIES SPECIFICITY OF *E. maimaiga* AND ITS ABILITY TO REDUCE *L. dispar* DENSITY CHARACTERIZE THE FUNGUS AS AN EFFECTIVE, ECONOMICAL AND ENVIR BIOLOGICAL CONTROL OPTION FOR *L. dispar*.

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