Project:
“Novel fungal proteins as biopesticides for control of challenging invasive alien agricultural pests - FunContrAPest“

NKFIH, Hungary (project id 134356 SNN20) with lead Agency ARRS, Slovenia (project id J4-2543)

Scientific final report
(Period closing report 2021-11-01 - 2023 – 10 – 31, Project years 3)

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For
NFKIH

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Are project participants the same as those in the funding contract?

Yes

Changes and justification

NA

Is the research performed conforming to the research plan?

Yes

Changes and justification

NA

Are project participants the same as those in the funding contract?

Yes

Summary of results in Hungarian

Ezen pályázat a Jozef Stefan Intézet, a Ljubljanai Egyetem, a Szlovén Mezőgazdasági Intézet és a magyarországi MATE közös erőfeszítést tett, hogy biztonságos és hatékony biopeszticideket fejlesszen ki főbb invazív rovarkártevők ellen a mezőgazdaságban. 2020 és 2023 között magasabb rendű gombákból származó egyedi fehérjekomplexeket tanulmányoztunk, amelyek potenciálisan védekező funkciót tölthetnek be a rovarok ellen. Főbb vizsgált kártevők: az amerikai kukoricabogár (*Diabrotica v.virgifera*), a pettyesszárnyú muslica (*Drosophila suzukii*), az ázsiai márványospoloska (*Halyomorpha halys*) és a burgonyabogár (Leptinotarsa decemlineata). 26 bazídiumos és aszkomikóta gomba fehérjét jellemeztünk és teszteltünk. A kísérletek kimutatták, hogy a MOA lektin aktív a L. decemlineata lárváinak középső bélrendszerének peritrofikus mátrixán. Ezzel szemben a kukoricabogár mucin és adaptív emésztőenzim expressziója gátolta ezen hatást. Az AAG és SSA lektinek az fiatal *D. suzukii* egyedekre hatnak, de úgy tűnik, hogy nem befolyásolják a bélmátrixot. Egyik fehérje sem volt hatással a *H. halysra*. Egyik

biopeszticid jelölt sem hatott az Apis mellifera beporzóra, sem a *Heterorhabditis bacteriophora* és *Steinernema longicaudum* entomopatogén fonálférgekre. Összegezve, a projekt megalapozta a magasabb rendű gombákból származó új típusú biopeszticidek kifejlesztésének módszertani és tudományos alapjait. Pontosabban, a MOA fehérje, mint sikerrel kecsegtető biopeszticid további vizsgálódásának alapjait.

Summary of results in English

In this project, the Institute Jozef Stefan, University of Ljubljana, Agricultural Institute of Slovenia, and MATE University from Hungary joined efforts towards the development of safe, and effective biopesticides against major invasive insect pests in agriculture. From 2020 to 23, we studied unique protein complexes originating from higher fungi, some potentially of defence function against insects. We targeted the invasive corn rootworm (*Diabrotica v.virgifera*), spotted wing drosophila (*Drosophila suzukii*), marmorated stink bug *(Halyomorpha halys*) and potato beetle (*Leptinotarsa decemlineata*). In total, 26 proteins from *Basidiomycota* and *Ascomycota* fungi were characterized, and tested. Bioassays revealed that the lectin MOA is active on the peritrophic matrix of midgut guts of *L. decemlineata* larvae. In contrast, mucin or adaptive digestive enzyme expression hindered such effects in *D.v.virgifera*. The lectins AAG and SSA acted on immature *D. suzukii,* but seem not to affect the gut matrix. None of the proteins had any effect on *H. halys.* Positively, the candidate biopesticidial agents did neither affect the pollinators *Apis mellifera* nor the entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema longicaudum*. In conclusion, the project laid the methodological and scientific base for the development of novel types of biopesticides originating from higher fungi. Specifically, the protein MOA may be further studied as a biopesticidial or biotechnical agent.

(Project closing report (final report))
Description of the results

The here-reported project was a co-operation between research institutions from Slovenia and the MATE University, Godollo campus, Hungary, which run from 2020 to 2023. Slovenian and Hungarian lead scientists with strong complementary expertise as well as their talented young researchers joined efforts towards the development of new, safe, and effective bio-pesticides against major invasive alien insect pests.

The **idea of the project** was to lay the scientific base for the development of novel types of bio-pesticides originating from edible mushrooms which contain a large variety of unique protein complexes, many yet unknown, and some supposedly having defence functions against insects. They may offer novel approaches to pest management, even under harsh climatic conditions due to their stability. We, therefore, searched for and studied such proteins, described them, and tested them against major invasive insect pests and non-targets.

Main targets were the invasive western corn rootworm (*Diabrotica v. virgifera*, Coleoptera), which is a beetle whose larvae damage maize roots, the spotted wing drosophila (*Drosophila suzukii*, Diptera) which is a fly that destroys fruits and berries, the brown marmorated stink bug (*Halyomoprha halys*, Hemiptera) which attacks fruits and nuts but is also a problem in urban areas, and the Colorado potato beetle (*Leptinotarsa decemlineata*, Coleoptera: Chrysomelidae), an “old” but often still unsolved problem in potato production. The studied non-targets were honeybees and biocontrol agents such as entomopathogenic nematodes.

The research teams were from Dr. Jerica Sabotič from Jožef Stefan Institute (IJS) of Slovenia (project coordinator, higher fungi protein expert), Dr. Jaka Razinger from the Plant Protection Department of the Agricultural Institute of Slovenia (KIS-PPD) (*Drosophila*, *Halyomorpha*, *Leptinotarsa*), Dr. Janez Prešern from the Animal Production Department (KIS-APD) (bee expert), Dr. Nada Žnidaršič from the Biotechnical Faculty, University of Ljubljana (UL-BF) (insect microscopy expert), as well as from Dr. Stefan Toepfer (*Diabrotica* expert, Principal Investigator, Hungary) with young scientist Dr. Szabolcs Toth (both Plant Protection Institute NVI, MATE, Hungary, formerly SZIE-NVI) as well as Dr. Marta Ladanyi from the Mathematics and Basic Science Institute (MATI) of MATE. The project had involved a number of MSc students (e.g. Tanja Zupan, Sergej Praček, IJS) and young scientists (Primož Žigonas, KIS-PPD, Dr. Szabolcs Toth, MATE-NVI, Urban Bogataj, Polona Mrak (UL-BF), Lenka Jerele KIS-APD), and several senior researchers of the respective teams.

The project was mainly **funded** by ARRS Slovenia and co-funded by NKFIH. The project has started on 1 November 2020. The project kick-off meeting was held on 10 November 2020. Technical review-planning meetings were held on 24 February and 17 November 2021, as well as 23 February, 19 April, and 28 November 2022. The final project meeting was held on 10 October 2023. Through those meetings and frequent exchanges between project partners, we have strengthened cross-border research, institutional cooperation and scientific excellence of Slovenia and Hungary. This final project-end report covers the 3 project years.

The project participants remained the same as mentioned in the funding contracts. The research has been performed according to the research plan. No major deviations occurred, but one extra target pests had been added compared to originally planned. The Colorado potato beetle (*Leptinotarsa decemlineata*, Coleoptera) was additionally studied due to its surprising susceptibility to the fungal protein MOA-lectin, serving as a positive control in comparative studies with the other target organisms of this project.

Workpackage 1 (WP1) dealt with preparation of biological test materials and digital tools, the first lead by IJS, the latter led by the MATE team. During the project, IJS has discovered and/or described 26 proteins (20 originally proposed) (WP1, Deliverable D1.1, Milestone 1). These are from Basidiomycota such as from *Macrolepiota procera*, *Clitocybe nebularis, Agaricus bisporus* (Agaricaceae), *Marasmius oreades* (Marasmiaceae),*Coprinopsis cinerea* (Psathyrellaceae), *Xerocomellus chrysenteron* and *Boletus edulis* (Boletaceae), *Agrocybe aegerita* and *A. cylindracea* (Strophariaceae), *Rhizoctonia solani* (Ceratobasidiaceae), *Sclerotium rolfsii* (Atheliaceae), *Laetiporus sulphureus* (Fomitopsidaceae); or from Ascomycota such as from *Aleuria aurantia* (Pyronemataceae), *Sordaria macrospora* (Sordariaceae), *Sclerotinia sclerotiorum* (Sclerotiniaceae) (Table 1). Of those, 6 appeared to be cysteine or serine protease inhibitors and 20 are galactose, mucin or fucose binding lectins. Next to the 26 proteins, also 11 mutants of 6 of the lectins were produced. In detail, the proteins were referred to as AAL, CML1, CCL2, CGL2 and 1 mutant, CGL3, CCP1, MOA and 4 mutants, TAP1, PIC, Clt, Mcp1, Mcp3, Mcp4, MpL and 1 mutant, CNL and 2 mutants, AAG and 1 mutant, BELbeta, SSA and 2 mutants, BEL, XCL, ABL, CC1G, LSL, PSL, SRL, ACG, RSA. For example, Mcp macrocypin 1, 3, 4 are protease inhibitors from the fungus *Macrolepiota procera*, Clt clitocypin from *Clitocybe nebularis,* and CCP cocaprin 1 from *Coprinopsis cinerea*. IJS were able to clone, express and purify those proteins.

The MATE team has, in collaboration with all partners, also developed an open-source bioassay data visualisation tool via the Shiny R surface https://tszfreeac.shinyapps.io/funcontrapestshinyapp_v2023/, WP1, D 1.2). It visualises all experiments conducted with the fungal proteins in the project, as well as results. It currently contains 562 observations on *D. v. virgifera* larvae, 324 on *D. v. virgifera* adults, 82 on *D. v. virgifera* eggs, 157 on *D. suzukii* eggs 120 on *D. suzukii* adults, 156 on *L. decemlineata* larvae, and 142 on *H. halys* nymphs, but can easily be expanded with more data or other targets.

Table 1 Characteristics of higher fungi proteins discovered and/or described, and then tested on target invasive insect pests by Slovenian and Hungarian scientists during the FunContrAPest project between 2020 and 2023.

Treatment (Code)	Origin	Size	Fold	Function
Proteins				
Macrocypin 1 (Mcp1)	*Macrolepiota procera* (Basidiomycota: Agaricaceae)	19.1 kDa 169 aa	β-trefoil (3H6Q)	Cysteine proteases: C1/C13
Macrocypin 3 (Mcp3)		19.0 kDa 167 aa	β-trefoil (3H6Q)	Cysteine proteases: C1/C13
Macrocypin 4 (Mcp4)		18.7 kDa 167 aa	β-trefoil (3H6Q)	Cysteine and serine proteases: C1/S1
Clitocypin (Clt)	*Clitocybe nebularis* (Basidiomycota: Agaricaceae)	16.7 kDa 150 aa	β-trefoil (3H6R)	Cysteine proteases: C1/C13
Cocaprin 1 (Ccp1)	*Coprinopsis cinerea* (Basidiomycota: Psathyrellaceae)	16.1 kDa 138 aa	β-trefoil (7ZNX)	Cysteine and aspartic proteases: C1/A1
Cospin (PIC)		16.7 kDa 150 aa	β-trefoil (3N0K)	Serine proteases: S1 (trypsin)

Agaricus bisporus lectin (ABL)	*Agaricus bisporus* (*Agaricaceae,* Agaricales, Agaricomycetes, Basidiomycota)	143 aa, homo-tetramer	beta-sandwich / cytolysin-like (1Y2T)	Two distinct glycan binding sites, one for Gal-beta-1,3-N-GalNAc and another for Gal-beta-1,3-N-GlcNAc
Agrocybe aegerita galectin (AAG)	*Cyclocybe aegerita (formerly Agrocybe aegerita)* (*Strophariacea,* Agaricales, Agaricomycetes, Basidiomycota)	162 aa, homo- dimer	beta-sandwich / ConA-like (2ZGL)	specific binding of sulfated and sialyl Thomsen-Friedenreich disaccharide (Galbeta1-3GalNAcalpha-O-) / function in mycelial differentiation, possible fruiting initiatior
Aleuria aurantia lectin (AAL)	*Aleuria aurantia* (*Pyronemataceae,* Pezizales, Pezizomycetes, Ascomycota)	313 aa, homo- dimer	beta-propeller (1OFZ)	binding fucose with strongest preference for the alpha-1,6-fucosylated glycans
Boletus edulis lectin (BEL)	*Boletus edulis (Boletaceae, Boletales, Agaricomycetes, Basidiomycota)*	143 aa, homo-tetramer	beta-sandwich / cytolysin-like (3QDS)	Two glycan binding sites, one for Gal-beta-1,3-N-GalNAc or GalNAc and another for GlcNAc
Clitocybe nebularis lectin (CNL)	*Clitocybe nebularis* (*Clitocybaceae,* Agaricales, Agaricomycetes, Basidiomycota)	149 aa, homo- dimer	beta-trefoil (3NBC)	binding terminal, non-reducing GalNAc-containing carbohydrates including N,N'-diacetyllactosediamine/LDN (GalNAcbeta1-4GlcNAc, LacdiNAc). Specific also for carbohydrates containing GlcNAc or Galbeta1-4GlcNAc at the reducing end
Coprinopsis cinerea galectin 2 (CGL2)	*Coprinopsis cinerea (Psathyrellaceae,* Agaricales, Agaricomycetes, Basidiomycota*)*	150 aa, homo-tetramer	beta-sandwich / ConA-like (1UL9)	binding beta-galactosides, specifically Gal-beta-1,4-Fuc-alpha-1,6-GlcNAc modification of N-glycan cores; also binding Gal-beta1,4-Glc; Gal-beta1,4-GlcNAc; Gal-beta1,4-GlcNAc; Gal-beta1,3-GalNAc
Coprinopsis cinerea galectin 3 (CGL3)	*Coprinopsis cinerea (Psathyrellaceae,* Agaricales, Agaricomycetes, Basidiomycota*)*	164 aa, homo-tetramer	beta-sandwich / ConA-like (2R0F)	binding chitooligosaccharides (oligomers of beta1-4-linked N-acetyl-glucosamines) and GalNAc beta 1-4GlcNAc (LacdiNAc)
Coprinopsis cinerea lectin (CC1G)	*Coprinopsis cinerea* (*Psathyrellaceae,* Agaricales, Agaricomycetes, Basidiomycota)	787 aa	putative beta-trefoil	unknown
Coprinopsis cinerea lectin 2 (CCL2)	*Coprinopsis cinerea* (*Psathyrellaceae,* Agaricales, Agaricomycetes, Basidiomycota)	142 aa, homo- dimer	beta-trefoil (2LIE)	specific binding of alpha1,3 fucosylated N-glycan cores
Coprinopsis cinerea mucin binding lectin 1 (CML1)	*Coprinopsis cinerea (Psathyrellaceae,* Agaricales, Agaricomycetes, Basidiomycota*)*	127 aa, homo-hexamer - trimer of - dimers	beta-sandwich / agaromycete-like (6ZRW)	biding to oligosaccharides containing fucose in alpha1–2, alpha1–3, and alpha1–4, but not alpha1–6 linkage
Laetiporus sulphureus lectin (LSL)	*Laetiporus sulphureus* (*Laetiporaceae*, Polyporales, Agaricomycetes, Basidiomycota)	315 aa, homo-hexamer	beta-trefoil chimeric with a pore-forming domain (1W3A)	binding Gal-beta-1-4Glc, Gal-beta-1-4GlcNAc
Macrolepiota procera lectin (MpL)	*Macrolepiota procera* (*Agaricaceae,* Agaricales, Agaricomycetes, Basidiomycota)	141 aa, homo- dimer	beta-trefoil (4ION)	binding terminal, non-reducing N-acetyllactosamine (Galβ1–4GlcNAc, LacNAc)
Marasmius oreades agglutinin (MOA)	*Marasmius oreades* (*Marasmiaceae,* Agaricales, Agaricomycetes, Basidiomycota)	293 aa, homo- dimer	beta-trefoil chimeric with a proteolytic domain (2IHO)	binding Gal-alpha1,3-Gal-beta, Gal-alpha1,3GalNAc-beta;

Rhizoctonia solani agglutinin (RSA)	*Rhizoctonia solani* (*Ceratobasidiaceae,* Cantharellales, Agaricomycetes, Basidiomycota)	137 aa, homo- dimer	beta-trefoil (4G9M)	binding terminal Gal/GalNAc
Sclerotinia sclerotiorum agglutinin (SSA)	*Sclerotinia sclerotiorum* (*Sclerotiniaceae,* Helotiales, Leotiomycetes, Ascomycota)	153 aa, homo- dimer	beta-trefoil (2X2S)	binding non-reducing terminal GalNAc or Gal with preference for the alpha over beta anomer
Sclerotium rolfsii lectin (SRL)	*Agroathelia rolfsii* - *(*formerly *Sclerotium rolfsii)* (*Amylocorticiaceae*, Amylocorticiales, Agaricomycetes, Basidiomycota)	143 aa, homo- dimer	beta-sandwich / cytolysin-like (2OFC, 4YLD)	probably acts as signaling molecule in the germination process of sclerotial bodies; binding Galβ1-3GalNAc-O-linked TF antigen and second binding site for GlcNAc
Sordaria macrospora transcript associated with perithecial development (TAP1)	*Sordaria macrospora* (*Sordariaceae,* Sordariales, Sordariomycetes, Ascomycota)	143 aa	beta-sandwich	binding Gal-beta-1,3-GalNAc, no second binding site for GlcNAc
Xerocomus chrysenteron lectin (XCL)	*Xerocomellus chrysenteron* (formerly *Xerocomus chrysentheron*) (*Boletaceae,* Boletales, Agaricomycetes, Basidiomycota)	143 aa, homo-tetramer	beta-sandwich / cytolysin-like (1XI0)	binding GalNAc, TF antigen (beta-D-Gal(1-3)-D-GalNAc) and second binding site for GlcNAc

WP2 dealt with testing toxicity and other effects of novel proteins against *D. v. virgifera,* led by MATE – NVI. A continuous rearing of a non-diapause colony of *D. v. virgifera* had been established and maintained throughout the project, with more than 30 rearing cycles during the three project years (D 2.1.). In addition, 20000 adult insects have been field-collected every year (so-called wild strain), and 100 to 250000 eggs produced for rearing of different stages for experimentation.

The MATE team conducted 11 sets of artificial diet-based bioassays including several dose-response assays under semi-sterile laboratory conditions on neonate larvae of *D.v.virgifera*, 7 sets of experiments on adult beetles, and 5 sets of experiments on eggs (D 2.2.,2.3). For some of the 26 proteins, dose-reponse assays were conducted.

Artificial diet assays with neonates were conducted using 96-well tissue culture plates with 8 wells with one larva per treatment per each of 6 to 7 plates per assay. Mortality, stunting or deformation, and length of larvae have been assessed 3 and 5 days after 20ul top treatments of proteins to the diet.

Artificial diet assays with adults were conducted using 6-well tissue culture plates with usually 2 wells with 3 beetles per each of 4 plates per treatment per assay. Mortality, feeding and sublethal effects have been assessed 1,3,5,7 days after 40ul top treatments of proteins to diet cores.

Egg assays were conducted by dipping ready-to-hatch eggs into 200ul treatments, and subsequently assessing hatching rates and neonate mortality 1,3,5, and 7 days post treatment.

The results showed that the 26 proteins tested seem not to have major effects on *D. v. virgifera* life stages (Toepfer, Toth, Ladanyi, Sabotic, et al. 2024, *Insects*, submitted). We confirmed the inhibition by mycocypins of the cysteine catalytic type proteolytic activities in gut extracts of larvae and adults. The inhibition of pGlu-Phe-Leu-hydrolysing activity was stronger than that of Z-Phe-Arg-hydrolysing activity. Mycocypins and cospin resisted long-term proteolytic digestion, whereas cocaprin 1 was digested. Bioassays with overlaid artificial diet revealed no effects of proteins on neonatal mortality or stunting, and no effects on adult mortality. Immersion of eggs in protein solutions had little effect on egg hatching or mortality of hatching neonates. Microscopic analysis of the peritrophic matrix and apical surface of the midguts revealed the similarity between larvae of *D.v.virgifera* and the chrysomelid *L. decemlineata,* which are sensitive to these inhibitors. The resistance of *D.v.virgifera* to fungal protease inhibitors is likely due to effective adaptation of digestive enzyme expression to dietary protease inhibitors. For more detailed explanations of tolerance of *D.v.virgifera* to those proteins, see results in WP6.

WP3 dealt with testing toxicity and other effects of novel proteins against *D. suzukii,* led by KIS-PPD. A continuous rearing of *D. suzukii* had been established and maintained throughout the project, with around 1000 individuals reared per year (D 3.1.). Novel screening methods were developed through integrating test agents such as proteins into *D. suzukii* artificial diet for larvae, pupae and adults. All 26 novel proteins have been tested in several replicates against all life stages of this pests, and two of them (AAG and SSA) showed some activity (D. 3.2 to 4.3, Milestone 3, yet unpublished). Further experiments evaluated dose-responses and suggested a development-hindering mode of action on the pest larvae and potentially their moulting (D. 3.3). Microscopy of mid-guts conducted by UL-BF did reveal that the guts of *D. suzukii* are different from *D. melanogaster*, but no direct effects of the two proteins on the gut membrane structures were observed.

WP4 dealt with testing toxicity and other effects of novel proteins against *H. halys,* led by KIS-PPD. A continuous rearing of *H. halys* had been established and maintained throughout the project, with around 1000 individuals reared per year (D 4.1). 26 novel proteins have been tested in several replicates against nymphs and adults of this pests using treated leaf disc assays, but no activity of any was found. Subsequently, doses were increased for the proteins that showed effects on other of the test insects, but still no effects were found on *H. halys*. Nevertheless, there is still potential in the future to control *H. halys* by higher-fungi proteins, once new ones would be discovered.

In addition, KIS-PPD tested the novel proteins against lab-reared and field-collected *L. decemlineata* larvae using leaf disc assays. Assays confirmed the activity of the fungal lectin MOA (IJS and KIS-PPD, D 3.2 to 4.3, Milestone 3). MOA is likely disturbing glycolipids in the midgut. Microscopy of guts conducted by UL-BF, revealed that the matrix of membranes of the mid guts of *L. decemlineata* was disturbed (UL-BF, D 6.1, 6.2, Milestones 4, 6).

WP5 dealt with assessing potential undesirable side effects of novel fungal proteins, potentially used in biopesticides, on non-target organisms. The tested non-targets in this project were pollinators, i.e. *Apis mellifera* (by KIS-APD) and biocontrol agents, i.e. entomopathogenic nematodes (by MATE team).
Non-target effects such as mortality or behavioural changes have not been detected on bees in colonies, that had been reared and tested by KIS-APD. Those tested had been conducted with 7 major tested proteins, that were of interest according to the results from other WPs, namely MOA, CGL2, AAL, CCL2, CCP1, SSA, AAG (KIS-APD, D 5.1, Milestone 5). The lack of effects of the candidate fungal proteins on bees is promising with regard to further potential developments for biopesticidial pest management solutions.

Methods for testing higher-fungi proteins on entomopathogenic nematodes, another group of beneficials, had been established (D 5.2). Subsequently, all 26 proteins have been tested for their potential effects on survival and/or pathogenicity of two commercial nematode species of large morphological difference, this is on *Heterorhabditis bacteriophora* and *Steinernema longicaudum* (both Rhabditida). Three sets of experimentations have been conducted with the 2 nematodes, using immersion treatments in 96-well plates under semi-sterile conditions. In addition, infection of *Galleria mellonella* larvae with surviving nematodes was tested in 6-well plates. Results revealed that none of the fungal proteins seemed to have major effect on those nematodes, neither on survival, nor on their pathogenicity (not yet published), despite of some reports of effects on *C. elegans* and plant parasitic nematodes. This is not surprising as the infective juvenile stages of the entomopathogenic nematodes, that live freely in the soil (as opposed to the other stages living inside of insects), are not feeding stages. Thus, they cannot ingest any test agent, which may limit some of their potential undesirable effects. The resistance of *D.v.virgifera* to fungal protease inhibitors is likely due to effective adaptation of digestive enzyme expression to dietary protease inhibitors, as well as due to the layer of mucin in the gut, something unusual for insects. In general, our studies have given another set of evidence that entomopathogenic nematodes are highly resistant or tolerant to other plant protection agents like proteins and can therefore be combined for an integrated pest management approach.

WP6 dealt with elucidating the mode of action on selected pest-protein pairs (UL-BF, IJS). Morphological changes by the fungal lectin MOA were observed in the matrix of the mid-gut of *L. decemlineata* larvae using electron microscopy (D. 6.1, 6.2). Microscopic analysis of the peritrophic matrix and apical surface of the midguts revealed the similarity between larvae of *D.v.virgifera* and *L. decemlineata*. However, changes in mid gut morphology were not observed for *D.v.virgifera* larvae or adults although it is from the chrysomelid

family as well. In situ assays revealed that MOA may target glycolipids in guts of *L. decemlineata* and also *D.v.virgifera*, but this special mucin as an unusual compound of insect guts, may hinder such effects in *D.v.virgifera* (D. 6.3, and 6.4, not yet published). No changes of the midgut membrane of *D. suzukii* by AAG or SSA proteins were observed, thus they may be of another, not yet known, mode of action in the midgut.

WP7 aimed at laying the base for potential future biopesticide product development. Therefore, the MATE teams reviewed microbials including biostimulants for potential effects on *D.v.virgifera* (published Tarigan, Toth, Toepfer, et al. 2022 Bc Sci Tech 31, and Tarigan, Toth, Turoczi, Toepfer, et al. 2023 IOBC Bull. 162). We also analysed biotic and abiotic factors that affect the efficacy of plant protection measures against *D.v.virgifera,* including temporal considerations (published Toth, Toepfer, et al. 2022 Agronomy 12). We have established a model on the effects of novel and established pest management options in agricultural settings typical to central Europe (D 7.1, Milestone 7, not yet published). Spatio-temporal population dynamics lattice model included agricultural input factors, biological input factors and the efficacy of plant protection products such as biopesticides, with an output of risks of *D.v.virgifera* populations passing a threshold. Results revealed that plant protection products may not need a high control efficacy at certain maize production schemes, something that is positive with regard of developing future pest management solutions. For example, at 50% crop rotation of maize, only an about 60% effective agent would be needed to keep risk low for the farmers, and at 40% rotation, 75% effective agents would be needed. In general, density of maize fields plays a much less role than the rotation of maize or the efficacy of a plant protection product.

Future biopesticide development strategies may be based on MOA when it comes to *L. decemlineata* management, either through a biopesticidial or biotechnological approach (D7.2). A biopesticidial approach against the larvae of *D.suzukii* remains challenging, even if AAG and SSA proteins may be considered. This is because those larvae are concealed inside the fruits, and any treatment with a pest management agent, regardless of chemical or biological, is a challenge. As for *D.v.virgifera* and *H. halys*, this project did not find any higher-fungi protein with major effects on those pests and pest stages that may warrant the immidate development of pest management solutions. However, it is proposed to continue to explore the variety of unique protein complexes.

WP8 dealt with project management and evaluation as well as dissemination. Project reports had been regularly prepared as per donor demands (D 8.2). Project meetings have been regularly organized as mentioned above (D 8.1). A project workspace had been established on the next cloud server of IJS accessible to all project partners. In addition, the open-source bioassay data visualisation tool serves project dissemination, awareness raising and visibility (D 8.3) via the Shiny R surface https://tszfreeac.shinyapps.io/funcontrapestshinyapp_v2023/). The project progress and results have been disseminated at several conferences (D8.3). Examples are presentations at 67th, 68th and 69th Hungarian Plant Protection Days, at the 15th Slovenian conference on Plant Protection, the European Biotechnology Congress, and through a symposium organised by the project on “Alternative strategies of plant protection against invasive insect pests” in Ljubljana, Slovenia on 28 September 2022 (https://www.ijs.si/project/FunContrAPest_MiniSymposium_AbstractBook_2022.pdf),

The outputs of the Hungarian MATE team are: a presentation in 2021, 5 presentations and 3 articles in periodicals in 2022, a presentation and a scientific paper in 2023, a manuscript submitted to a scientific journal in 2023, and 2 further manuscripts in preparation by the MATE team.

In conclusion, the project progressed as it had been proposed. We succeeded in laying the methodological base for future developments of new integrated pest managements solutions for a set of invasive target pests. We also succeed in setting the base for potential biopesticide developments with the application of higher fungi proteins against *L. decemlineata*. In the mid-term, this may lead to providing farmers with more options ultimately leading to safer food and better protection of nature.

Exploitation (Project closing report (final report))

Can your results lead to economic exploitation after further research and development?

Yes

The project laid the methodological and scientific base for the development of novel types of bio-pesticides originating from higher fungi that contain a large variety of unique protein complexes, many yet unknown. Those may offer novel approaches to pest management, and due to their stability, even under harsh climatic conditions. Specifically, the higher-fungi lectins MOA, AAG, SSA may warrant further basic and applied research and development towards a biopesticidial or biotechnical solution for Colorado potato beetle *L. decemlineata* and, to some extent, the spotted wing drosophila *D. suzuki*. Although, no highly effective higher-fungi protein has yet been discovered for the control of western corn rootworm *D. v.virgifera* and brown marmorated stink bug *H. halys*, we suggest continuing to study unique protein and numerous complexes of higher fungi to develop new approaches to pest control.

Any steps taken for economic exploitation?

No

Description of the steps for exploitation in Hungarian

NA

Description of the steps for exploitation in English

NA

Publications and presentations (Project closing report (final report))

Toepfer, S., Szabolcs Toth, Tanja Zupan, Urban Bogataj, Nada Žnidaršič, Marta Ladanyi, Jerica Sabotič (2024) Diabrotica v. virgifera seems not affected by entomotoxic protease inhibitors from higher fungi. MDPI Insects (in press) (NKFIH acknowledged) IF 3

Toepfer S., Toth S, Zupan T, Bogataj U, Žnidaršič N, Ladanyi M, Peternel T, Sabotič J. (2023) Diabrotica v. virgifera resists the effects of entomotoxic fungal protease inhibitors in food. European biotechnology congress. 4 – 6 October 2023 . Ljubljana, Slovenia, (NKFIH acknowledged) (Poster)

Tarigan S.I., Toth, Sz., Szalai, M:, Turoczi, G., Toepfer, S. (2023) Microbial biostimulants registered for maize with potential side-effects on its insect pests. 28th International Working Group of Ostrinia and other maize pests (IOBC / IWGO) Conference, Nairobi, Kenya, 2 to 4 May 2023, (NKFIH acknowledged) (Oral presentation)

Toth Sz, Toepfer, Sz, Razinger, J, Primoz Z, Modic, Sp, Ladanyi M, Sabotic J. (2023) Higher fungi lectins proteins against the maize pest Diabrotica v. virgifera. (Magasabb rendu gombakbol szarmazo lektinek a kukorica rovarkartevo, Diabortica v. virgifera (Coleoptera: Chrysomelidae) ellen. 69th Hungarian Plant Protection Days. 69. Novenyvedelmi tudomanyos napok. Budapest, Hungary, 21 February 2023, p. 56 (NKFIH acknowledged) (Oral presentation) p. 26.

Tarigan, S. I., Toth, S., Szalai, M., Kiss, J., Turoczi, G., & Toepfer, S. (2022). Biological control properties of microbial plant biostimulants. A review. Biocontrol Science and Technology, 1-21., DOI: 10.1080/09583157.2022.2129589 (NKFIH acknowledged) IF 1.8

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Proceedings Symposium: Alternative strategies of plant protection against invasive insect pests, 28 September 2022. Publisher CIP – Kataložni zapis o publikaciji, Narodna in univerzitetna knjižnica, Ljubljana, Slovenia, COBISS.SI-ID 123364867, ISBN 978-961-264-231-0, p. 6-7. (proceedings) https://www.ijs.si/project/FunContrAPest_MiniSymposium_AbstractBook_2022.pdf (NKFIH acknowledged)

Toth, Sz, Toepfer, S., Szalai, M., Kiss J. (2022) Challenges in controlling the maize pest Diabrotica v. virgifera (Coleoptera: Chrysomelidae) under field conditions. Proceedings Symposium: Alternative strategies of plant protection against invasive insect pests, 28 September 2022. Publisher CIP – Kataložni zapis o publikaciji, Narodna in univerzitetna knjižnica, Ljubljana, Slovenia, COBISS.SI-ID 123364867, ISBN 978-961-264-231-0, p. 10-11. https://www.ijs.si/project/FunContrAPest_MiniSymposium_AbstractBook_2022.pdf (proceedings) (NKFIH acknowledged)

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