PhD thesis

Szabó Károly Attila

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Relations between invasive weeds and aphids, they role as plant virus reservoirs

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Szabó Károly-Attila

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Name of Doctoral School: Plant Science

Scientific classification: Crop Science and Horticultural Science

School Director: Prof. Dr. Helyes Lajos

Supervisor: Prof. Dr. Balog Adalbert

Sapientia Hungarian University of Transylvania

Department of Horticulture

…….

Doctoral School Director

…… …….

Doctoral program leader Supervisor

1. INTRODUCTION

1.1. The role of invasive alien weeds

Invasive weeds represent a serious threat to world crop production as global trade expands and climatic conditions shift. In this light, it has recently been suggested that the losses to crop yield caused by invasive weeds and their infesting aphids (Hemiptera: Aphididae), may increase substantially within the EU over the next few years. Such weeds are particularly important because they not only serve as hosts for several local sap-feeding, virus-vectoring insects, especially aphids, but also because they may act as significant reservoirs of pathogenic plant viruses (Callaway & Maron, 2006).

Previous studies from Central Europe have reported 435 alien weed species from 82 plant families in the past 25 years. Most of these species, for example annual fleabane, *Erigeron annuus* (L.), Canadian horseweed, *Conyza canadensis* (L.), and goldenrod, *Solidago canadensis* L., occur in all types of habitat and are considered to be the most important and economically-relevant invasive weeds within the agro-ecosystem (Hejda, Pyšek, & Jarošík, 2009).

The aim of the present study was to: a) assess the population density of the most important invasive weed species when agricultural crops are managed with high-input fertilizers and chemical pesticides (high-input fields, HIF) and without chemical management (low-input fields, LIF); b) identify and compare population densities of the most important aphid species on dominant invasive weeds; and c), detect the most suitable weed as hosts for aphids under different cropping systems by conducting the peroxidase (POD) enzyme activation test during aphid feeding. POD-inducible weed plants would be lower quality hosts, and less likely to confer associational susceptibility to nearby crops because they would not support large aphid populations. Thus POD enzyme activation is a useful indicator of associational susceptibility or resistance to aphid colonization

2. MATHERIAL AND METHODS

2.1. Study area, focal weed and aphid species

Experiments were conducted during the crop growing (vegetative) seasons of 2015 and the 2016 in Central and Eastern Transylvania, Romania in order to assess the population density of the most important invasive weed species and infesting virus-vectoring aphids, both from low- and high-input agricultural crops.

Low-input, traditionally managed fields (LIF). This area belongs to a traditionally managed field (low-input) of the Saxon cultural region encompassing an area of 7,440 km² at altitudes between 230 and 800 mm above sea level (a.s.l.) and characterized by a landscape mosaic of different land-cover types (28% forest, 24% pasture, and 37% arable land, mostly maize, potato and alfalfa) (Zimmermann. et al, 2015). The farming practices in the studied area were predominantly small scale, with no chemical inputs and for subsistence purposes. One consequence of this land use is the exceptional biodiversity and natural value of the farming landscape (Akeroyd & Page, 2011).

High-input, conventionally managed fields (HIF). This study region contrasts the previously described region by large monocultures and farming landscapes with low levels of natural vegetation and heterogeneity (Eastern Transylvania). The area of about 5,500 km² was intensively treated with synthetic fertilizers and pesticides, major crops were maize, potato and alfalfa.

First, we selected two study sites in each region, these being 10 km in a fist and 15 km distant in a second region from each other. In each site we established two transects (at least at 1 km apart) of 10 m long × 1 m wide at an approximately equal distance of at least three major crops (maize, potato and alfalfa). In this way, each transect was surrounded by at least 8–10 ha of high-input, and at least 0.5–3 ha area of low-input, agricultural crops. Each transect was carefully measured and located using GPS. Second, inside each transect we placed ten 1 m2 quadrats. Each of these quadrats was further subdivided in 10 × 10 cm plots (100 for each quadrat) and all plants (native and invasive) were counted and their coverage estimated within them (Andújar, Ribeiro, Carmona, Fernández-Quintanilla, & Dorado, 2010). Third, ten invasive weed plant individuals from each quadrat were randomly collected in plastic bags. The number of invasive plants collected for each species from each quadrat mirrored the coverage of the species within the quadrat.

Because plants contained aphid colonies, and the exact number of individual aphids was important, all bags were labelled and kept at low temperature (near 0–4 °C in a cool box), then returned to the laboratory, whereupon all samples were stored at −20 °C, and aphids counted and species identified (Blackman & Eastop, 2007; Dr. Szalay-Marzsó, 1969).

2.2. POD enzyme extraction and activity assays in weeds

Leaf samples used for enzyme analyses were collected each year from each abundant weed species per experimental field, sub-area and transect, starting from the first until the last assessment. Separate young leaves, all containing aphids, were collected from the weeds (n = 10 samples / 1 m2 sub-transect = 100 / transect). Samples were also held at −20 °C until enzyme extraction and activity assays.

2.3. Colonization experiment of aphids from weeds to crop plants

The experiment was performed during the vegetative period of 2017 by setting-up 40 blocks of the two most abundant weed species, *C. canadensis* and E. *annuus* and the most frequent crop plants cultivated (maize, potato and alfalfa). Specimens of *C. canadensis* and *E. annuus* of the same age (maturity) were collected in April from the field and potted in 8 litre pots.

Aphid numbers were assessed on both weed and crop plants of the same blocks starting from mid-May as follows: two randomly selected blocks (one with *C. canadensis* and one with *E. annuus* plants) were sampled by enclosing the infested plant in a transparent polythene bag and then cutting this free with scissors or a knife. On return to the laboratory, the entire content was stored at −20 °C and the next day all samples were carefully assessed for aphids and their respective numbers counted under a stereo microscope.

2.4. Colonization detection by aphids DNA analysis

Microsatellite-based analysis of aphids captured from weeds and cultivated plants was performed with two primers selected based on an article by (Wilson et al., 2004).

2.5. Virus detection from invasive weeds and crop plants

Leaf samples on which aphids had been found and counted were used for viral analyses from both weeds and crop plants (alfalfa, maize and potato) and both management systems, with small RNA analyses performed in 2017.

2.6. Total nucleic acid extraction

RNA was extracted using a phenol-chloroform method. Briefly, frozen plant material was homogenized in an ice-cold mortar, suspended in 650 µl of extraction buffer and mixed with an equal volume of phenol, and centrifuged for 5 mins. The aqueous phase was treated with equal volumes of phenol, chloroform, and isoamyl-alcohol (25:24:1), and after subsequent treatment with chloroform: isoamyl-alcohol (24:1), was precipitated with ethanol and then re-suspended in sterile water.

2.7. Small RNA library preparation

For small RNA HTS, small RNA was isolated from polyacrylamide gels involving RNA pools which were prepared by mixing equal amounts of RNA originating from different individuals, collection times (in the case of weeds) and from different species (in the case of crops). This pooling strategy allowed detection of any virus present in any of the sampled individual plants at any time of the survey.

2.8. Detection of viruses in invasive alien weeds and in crops

As the crops investigated belonged to different families, and as such hosted very different viruses, we investigated their viral patterning as a collective pool. In contrast, the invasive weeds were all members of the *Asteraceae*; hence virus diagnostics here were performed separately for each species concerned.

2.9. Data analyses

For weed data, the mean coverage in each 1 m2 quadrats was determined by averaging the plant values from 10 × 10 cm plot. No significant difference in weed coverage was detected between years. Therefore, data from the two years, collected on the same dates, were combined for the analyses.

The weed frequency data were tested using Poisson-distributed errors and residuals for normality of errors (Kolmogorov-Smirnov test) and for equality of variance (Levene's test). Because residuals did not meet the assumption of normality, we used the non-parametric Kruskal-Wallis- and Mann- Whitney U test to compare variables..

All aphid species were correlated with particular weed species. In the case of one individual weed plant hosting two aphid species, the percentage of the species were considered. This was the case in only 7% of all the samples examined. It was then determined how cropping system

differentially affected associational susceptibility to the two aphidspecies, *B. helichrysi* and *A. solani*. General linear modelling was used with mean aphid abundance on *S. annua*, *E. canadensis* and *S. canadensis* as response variable. Initial analyses indicated no difference between study years and aphid abundance averaged across study years were analysed. The model included cropping system type (HIF vs LIF), aphid species (*B. helichrysi* and *A. solani*) and their interaction as explanatory variables. Because aphid abundance is a discrete variable, Poisson-distributed errors were assessed. Aphid abundances on *S. annua* was normally distributed, so factorial ANOVA was used, followed by Tukey testing. Effects of weed plants on *B. helichrysi* colonization toward each crop plant (maize, potato and alfalfa) were tested using repeated measures MANOVA. Interactions were then compared using χ^2 tests on the differences between the covariance matrices and by the root mean square error of approximation.

Thereafter, POD values were compared between cropping system type (HIF vs LIF) for *E. annuus*. POD enzyme data were analyses by pair t-test.

2.10. Data analyses and bioinformatics

These pools were used for small RNA library preparation (6 libraries in total) using Truseq Small RNA Library Preparation Kit (Illumina, USA) and our modified protocol (Czotter et al. 2018). Samples were sequenced using HiScan2000 by UD Genomed (Debrecen, Hungary) 50 bp, single end. Fastq files of the sequenced libraries were deposited to the GEO and can be accessed through series accession number GSE132755 .

Principal Components Analysis (PCA) was used to identify the proportion of variation in each PCA axis (Aphids density and treatments) explained by each virus distribution. We then used the average count of each virus reads numbers detected and log10 transformed from each weed and crop plant sample grouping as response variables, and used aphid abundance as component 1 (PCA axis1) and treatment (LIF vs. HIF) as component 2 (PCA axis 2) scores for each virus reads as independent variables. RNA and DNA viruses were analysed separately, and the only one insect virus detected (Helicoverpa zea nudivirus 2, HzNV-2) was not considered in our analyses. PCA covariance analyses were run using Community Analysis Package 4 (Pisces Conservation Ltd).

Virus diagnosis was determined by small RNA HTS. For bioinformatics analysis of the HTS results, we used CLC Genomic Workbench. Briefly: For trimming, quality control and QC reports, embedded protocols in CLC Genomic Workbench were employed. For virus diagnostics, we followed two strategies and used CLC Genomic Workbench: we built longer contigs from the non-redundant

reads using assembler of CLC (*de novo* assembly) and compared the resultant contigs using BLAST to the NCBI Reference Genomes of plant viruses downloaded from GenBank. In parallel, we directly mapped contigs to Reference Genomes of those viruses, which were represented at least with one contig in any of the libraries with map to the reference tool of CLC Genomic workbench. Virus presence was recorded if at least two parameters (virus specific contig was present and/or normalized redundant virus specific read count was > 200, and/or coverage of the virus genome was > 60%) were reached.

3. RESULTS

3.1. Dominant invasive weed species and their variations between management systems

Three invasive weed species were dominant during the two years field assessment. *Erigeron annuus* was the most frequent weed, and dominated both LIF (97.5%) and HIF regimes (84.5%). Two other invasive weed species were present at lower densities. *Solidago canadensis* was only present in LIF, with an average coverage of 2.5%. No other invasive weeds were detected under this management system during the assessment *Conyza canadensis* was only present under HIF with an average coverage of 15%. Dominance of *E. annuus* was significant under both LIF and HIF.

3.2. Aphids and their abundances on invasive weeds

The two important aphid species were detected in high densities on all three dominant invasive weeds. The most frequent species was *B. helichrysi*; its abundance was high and dominated the most frequent weed, *E. annuus* under both HIF and LIF regimes (LIF-*B. helichrysi* and LIF-*A. solani* LIF-*B. helichrysi* and HIF-*A. solani*). The next most abundant aphid was *A. solani*, also present on *E. annuus* plants under both management systems; its density was significantly higher under LIF compared with HIF. Higher density of *B. helichrysi* was detected on *S. canadensis* under LIF but its density varied greatly between assessment data. Furthermore, the dominance of the *B. helichrysi* on *C. canadensis* was detected under HIF conditions. A very low number of other important aphid species were detected, i.e. about 12 individuals of *T. trifolii*. were collected on *S. canadensis*.

The high invasive weed density harbours a concomitantly higher aphid population density comprising local species. More precisely, a 13% higher coverage difference of *E. annuus* in LIF further resulted in a significantly higher (about 30% higher) *B. helichrysi* aphid abundance under this

management system. The same trend can also be detected for *A. solani*, where the 13% higher coverage of E. *annuus* resulted in an increase of about 85% for this aphid species under LIF compared to HIF.

3.3. POD enzyme activity on invasive weeds under aphids' feedings

No observable differences in POD enzyme activity were detected for *S. annua* at 5 μmol min−1 · mg protein−1 unit between HIF and LIF regimes. When the POD activity was compared for the 10 μmol min–1 · mg protein–1 unit aliquot sample, there was a significantly higher enzyme activity, suggesting a significantly higher stress by aphids feeding on *E. annuus* in LIF system. Higher POD enzyme activity at both 5 and 10 μmol unit was detected on *C. canadensis* than on *S. canadensis*, again indicating higher stress as a result of aphid feeding; however, because of low samples numbers no statistics were performed here.

3.4. Colonization of aphids from weeds to crop plants

The number of *A. solani* were low and colonies persisted in five blocks on *C. canadensis* only, which shows a very similar trend with field observations of only 7% of *A. solani* detected together with *B. helichrysi*. The *B. helichrysi* colonies persisted in all blocks on both weed plants. Therefore, comparisons were, made separately for those blocks where both aphid species were present, and separately for those where only the *B. helichrysi* persisted. Colonization of *B. helichrysi* from *C. canadensis* was significant on all crop plants, with a higher number of aphids detected on maize. Low or no colonization of *A. solani* was detected from this weed to crop plants, hence no statistics were here possible. By comparing the colonization of *B. helichrysi* from both weed species, again a significant effect toward all crop plants was detected. The number of aphids on maize was significantly higher when maize was in close vicinity with *E. annuus*. No differences in aphid abundance were detected for potato and alfalfa when these plants were in close vicinity with *E. annuus* or *C. canadensis*. The colonization experiment also revealed that *E. annuus* and *C. canadensis* can be considered suitable host plants for both aphid species examined, especially for *B. helichrysi*. Significant colonization from both weeds toward the most important crop plants of this last aphid species were detected.

3.5. Plant viruses in invasive weeds and crop plants

Sequencing of the small RNA libraries resulted 9.4-21 million raw reads. After trimming and quality control we obtained 9.2-20.4 million reads, which represented 2.019.811-7.192.941 individual sequences. Using overlapping stretches of these reads, we were able to construct *de novo* 3553-19038 longer contigs. Virus derived contigs were annotated following BLAST searches.(1.-2. Tabel)

1. Tabel- RNA viruses

	Vírus name	**Genus**	**Genom**	**Host plant**	**Spreading**
PVY	Potato virus Y	Potyvirus	RNA	*Solanaceae* familie more than 60 species	Aphids*: Myzus persicae, Myzus ornatus, Macrosiphum euphorbiae, Aulacorthum circumflexum, Aphis nasturtii, Aphis gossypii, Brachycaudus helichrysi*, és szaporító anyaggal
ClYVV	Clover yellow vein virus	Potyvirus	RNA	6 families(*Fabaceae, Cucurbitaceae, Solanaceae, Poaceae*) 25 species	*Myzus persicae, Acyrthosiphon pisum, Aulacorthum solani* és *Macrosiphum euphorbiae*
ZTMV	Zucchini tiger mosaic virus	Potyvirus	RNA	*Cucurbitaceae*	L. Xiao and et.al.. S. E. Webb.et.al. 2016 in China and Florida, Da Wang et. al. 2019 Hawaii descriebed, vector unknown
PVS	Potato virus S	Carlavirus	RNA	*Solanaceae* family	*Myzus persicae* (Wetter & Völk, 1960); *Aphis frangulae, A. nasturtii, Macrosiphum euphorbiae*
PVM	Potato virus M	Carlavirus	RNA	*Solanaceae* és *Chenopodiaceae*	*Myzus persicae*
	Vírus name	**Genus**	**Genom**	**Host plant**	**Spreading**
PVX	Potato virus X	Potexvirus	RNA	*Solanaceae*	Szöcske: *Melanoplus differentialis,*

					Tettigonia viridissima Gomba: *Synchytrium endobioticum,* mechanikai átvitel
OVX	Opuntia virus X	Potexvirus	RNA	*Opuntia Cactaceae, Chenopodium quinoa*	Koenig R. et. al 2004, firs recorded, unknown vectors
BLRV	Bean leafroll virus	Luteovirus	RNA	65 species, *Vicia, Pisum, Medicago, Trifolium, Lathyrus és Trigonella*	*Acyrthosiphon pisum*
SCBMV	Squash chlorotic leaf spot virus	Picornavirales	RNA	*Cucurbitaceae*	Mech spread, white flies

2. Tabel- DNA viruses

	Vírus name	**Genus**	**Genom**	**Host plant**	**Spreading**
TVCV	Tobacco vein clearing virus	Solendovirus	DNA	*Nicotiana* species	By seed
SPSMV-1	Sweetpotato symptomless mastrevirus 1	Mastrevirus	DNA	Sweetpotato	Cicada
DMV	Dahlia mosaic virus	Caulimovirus	DNA	*Dahlia* fajok, *Asteraceae, Solanaceae, Chenopodiaceae, Amaranthaceae*	13 aphis species *Aphis fabae, Myzus persicae* és a *Macrosiphum euphorbiae*
SPuV	Soybean mild mottle pararetrovirus	Caulimovirus	DNA	Soybean	Junping Han et. al. 2012
PBCoV	Pineapple bacilliform comosus virus	Caulimovirus	DNA	Pineapple	Scales
SCBGDV	Sugarcane bacilliform Guadeloupe D virus	Caulimovirus	DNA	Sugarcane	By seed
HzNV-2	Helicoverpa zea nudivirus 2	Baculovirus	DNA	*Helicoverpa zea*	During replication

4. CONCLUSSION

Here we showed that associational susceptibility can be detected between the most frequent weed and crop plants under the different crop management regimes.

The two important aphid species were detected in high densities on all three dominant invasive weeds. The most frequent species was *B. helichrysi*; its abundance was high and dominated the most frequent weed, *S. annua* under both HIF and LIF regimes. The next most abundant aphid was *A. solani*, also present on *E. annuus* plants under both management systems; its density was significantly higher under LIF compared with HIF. High invasive weed density harbours a concomitantly higher aphid population density comprising local species. A very low number of other important aphid species were detected, of *Macrosiphum* spp. were collected (Francis et al., 2010; Srinivasan & Alvarez, 2011).

More precisely, a 13% higher coverage difference of *E. Annuus i*n LIF further resulted in a significantly higher (about 30% higher) *B. helichrysi* aphid abundance under this management system. The same trend can also be detected for *A. solani*, where the 13% higher coverage of *E. annuus* resulted in an increase of about 85% for this aphid species under LIF compared to HIF. Altogether these results also show that the response to cropping system varied according to the aphid species concerned, possibly due to the difference in host plant preference of the two aphid species (i.e. *A. solani* was less polyphagous than *B. helichrysi*), a scenario also supported in the case of lower *A. solani* density on weeds in the field and low colony persistence during the colonization experiment.

The colonization experiment also revealed that *E. annuus* and *C. canadensis* can be considered suitable host plants for both aphid species examined, especially for *B. helichrysi*. Significant colonization from both weeds toward the most important crop plants of this last aphid species were detected (Popkin et al., 2017).

Following this revised analytical approach, the number of viruses detected dropped to 16, differentiated as nine RNA and seven DNA viruses (1-2 Tabels). The distribution of both RNA and DNA viruses varied greatly between crops and invasive weeds, both under LIF and HIF regimes. In addition, aphid abundance and the management systems used directly influence plant virus distribution between weeds and crop plants. While RNA virus distribution (mostly transmitted through stylet-borne mechanisms) was more influenced by aphid density, DNA virus distribution (transmitted less by aphids, probably more by mechanical means) between weeds and crops was by contrast predominantly influenced by the management system involved.

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6. NEW SCIENTIFIC RESULTS

According to the results, the following new scientific results can be defined:

1. I confirmed that invasive weeds (*Erigeron annuus, Conyza canadensis*) harbors a rich aphid fauna from local species.
2. Using genomial DNA analyses I proved that high colonization exists between invasive weeds and crop plants.
3. I showed that maize can be a suitable host for the aphid *B. helichrysi.*
4. New plant viruses, undetected until now, were presented in invasive weeds.
5. Crop management or its absence influenced invasive weeds (*Erigeron annuus, Conyza canadensis*) density, and their inhabiting aphids which influences the virus infection of the neighboring crops.
6. I presented for the first time the existence of an artificial insect pathogen virus in invasive weeds (*Erigeron annuus, Conyza canadensis*).
7. It was presented that plant virus detection differs between treatments, while RNA based viruses are more frequent in HIF, DNA based viruses occurs more widely in LIF.

Publication list

ISI papers

1. **Szabó KA**, Várallyay E, Demian E, Kiss J, Bálint J, Loxdale HD and Balog A 2019). Local aphid species infestation on invasive weeds affects virus infection of nearest crops under different management systems. Frontiers in Plant Science 11(684); https://doi.org/10.3389/fpls.2020.00684. (**IF. 4,106**). Scimago rank **D1**.
2. **Szabó KA,** Kiss J, Bálint J, Kőszeghi Sz, Loxdale HD, Balog A. 2019). Low and high input agricultural fields have different effects on pest aphid abundance via different invasive alien weed species. NeoBiota 43, 27-45. doi: 10.3897/neobiota.43.31553 (**IF. 2,488**). Scimago rank **D1**.
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Papers not related with PhD works

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1. Turoczi, B., Bakonyi, J., **Szabó, KA**., Bálint, J., Máthé, I., Lányi, Sz., **Balog, A***. 2020). In vitro and in vivo effect of poplar bud extracts on Phytophthora infestans: a new effective biological method in potato late blight control. Plants 9(2), 217; https://doi.org/10.3390/plants9020217. (**IF. 2,632)**. Scimago rank **D1**.
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4. Bálint J., **Szabó KA**, Tófalvi B., Puia C., Balog A. 2016) Comparing diseases resistance of local and international plum cultivars (Prunus domestica) from Eastern Transylvania, Romania. Journal of Plant Diseases and Protection 123: 317. doi:10.1007/s41348-016-0048-6 (**IF. 0,485**). Scimago rank **Q3.**
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