Review

Thirty Years of Research and Professional Work in the Field of Biological Control (Predators, Parasitoids, Entomopathogenic and Parasitic Nematodes) in Slovenia: A Review

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Abstract: This paper provides the first detailed presentation of research and professional activities in the field of biological control in Slovenia during the period of 1990–2020. It presents information on the important pioneering role of Prof. Dr. Lea Milevoj in biological control research in Slovenia, especially in regard to the inventorying and laboratory rearing of indigenous beneficial organisms, evaluation of the influence of food type on the feeding behaviour of beneficial organisms, participation in the first introduction of a natural enemy within the context of classical biological control in Slovenia, preparation of rules on the biological control of plant pests and publication of the first Slovenian monograph on biological control. The paper also describes the activities of Slovenian researchers in regard to entomopathogenic nematodes, especially related to the assessment of their presence and efficiency in suppressing harmful insects and the identification of indigenous parasitic nematodes associated with economically harmful slugs. The paper also notes some applicative and basic research projects pertaining to parasitoids, especially in terms of their function as natural enemies of aphids, and in regard to predators of harmful insects and mites, especially predatory mites. The main goal of these activities is to implement the use of as many natural enemies as possible in food and ornamental plant production systems to replace the use of plant protection products.

Keywords: biological control; Slovenia; chronology; legislation; application; research

1. Introduction

Biological control, i.e., a method of plant protection that involves the use of living beneficial organisms (predators, parasitoids, entomopathogenic nematodes, entomopathogenic fungi and bacteria, protozoa and viruses) to suppress or control populations of organisms harmful to plants to reduce the damage they would cause, is relatively well-known globally, yet its use is not widespread, as the chemical suppression of harmful organisms still prevails in most countries [1]. This environmentally friendly manner of suppressing harmful organisms requires more knowledge from the user and more systematic application than other methods, and its application is also more expensive than those of other plant protection products [2].

Biological control has an interesting and fairly long history; the ant *Oecophylla smaragdina* Fabricius, for example, was used in China in the third century to suppress a harmful organism on citrus fruits—the stink bug *Tessaratoma papillosa* (Drury) [3]. Despite the historical occurrence of various biological control activities, particularly in the USA in the 19th century, numerous professional sources mark the year 1888 as representing the beginning of biological control because two natural enemies of the cottony cushion scale (*Icerya purchasi* Maskell) were introduced from Australia to California in that

year, namely, the cottony cushion scale parasite, *Cryptochaetum iceryae* (Williston), and the ladybird beetle *Rodolia cardinalis* (Mulsant). In particular, the latter became a synonym for the efficiency of biological control [4].

Harmful organisms in agriculture are today suppressed by biological agents everywhere around the world, but the intensity and manner of their use and pertaining legislation, which both enables the use of natural enemies and restricts it, differ considerably among regions [5,6]. Our paper offers a chronological survey of activities in the field of the biological control of harmful organisms in Slovenia, a Middle European country with only two million inhabitants that has nevertheless maintained exceptionally rich research activities in this field over the last 30 years.

2. Beginnings and the Role of Prof. Dr. Lea Milevoj

Although the first documentation of work in the field of biological control in the area of present-day Slovenia dates back to 1870, when Viljem Schleicher presented in his book *Živali kmetijstvu in gozdarstvu koristne, s posebnim ozirom na zatiranje škodljivega mrčesa* (*Animals Useful in Agriculture and Forestry, with a Special Focus on the Suppression of Harmful Insects*) [7] the natural enemies of plant pests, which he categorised into four groups (mammals, birds, amphibians and insects), systematic research work in this field began only in independent Slovenia.

The pioneer of biological control in Slovenia is Prof. Dr. Lea Milevoj, today a retired professor in the field of phytomedicine, who began intensive work in this field at the beginning of the 1990s at the Biotechnical Faculty of the University of Ljubljana. Her prolific professional work can be divided into several segments, the most important of which is the inventorying and laboratory rearing of indigenous beneficial organisms. This work involved studying the influence of food type on the feeding behaviour of beneficial organisms, taking an active part in the first introduction of a natural enemy for the purpose of classical biological control in Slovenia, the preparation of the rules on biological plant protection (Official Gazette of the Republic of Slovenia no. 45/06), and the publication of the first Slovenian monograph on biological control.

3. Inventorying and Laboratory Study of Beneficial Organisms

Milevoj was aware that in order to undertake more intensive work on natural enemies in Slovenia, she needed to first acquire information on the local occurrence of these species. Such information was very scarce until the 1990s. At the beginning of this decade, she thus found a large number of beneficent species on various plant species, including the predatory midge *Aphidoletes aphidimyza* (Rondani) [8] and the parasitoids *Aphidius matricariae* Haliday, *Diaeretiella rapae* (M' Intosh) [9] and *Aphelinus asychis* Walker [10]. She was interested in identifying the natural enemies of aphids, and to this end, she meticulously studied the insect fauna associated with colonies of cotton aphids (*Aphis gossypii* Glover) [11] and with aphids in apple orchards [12] and cereal crops [13]. Her laboratory experiments involved rearing natural enemies of aphids [14] and the common green lacewing (*Chrysoperla carnea* [Stephens]) [15], while her study into the influence of food type (aphids) on the feeding behaviour of adult seven-spot ladybirds (*Coccinella septempunctata* L.) [16] and the influence of insecticides on the parasitoid *Diaeretiella rapae* [17] were topical at that time.

4. The Rules on the Biological Control of Plant Pests

The activities of Milevoj in the preparation of the first Slovenian rules on biological control—The rules on biological plant protection (Official Gazette of the Republic of Slovenia, št. 45/06) [18]—deserve special attention. These rules include lists of indigenous and alien species recommended for biological control; conditions for the introduction, rearing and application of indigenous and alien species for biological control; conditions relating to professional and technical qualifications or conditions associated with the premises, equipment and personnel used for the introduction, rearing or application of indigenous and alien species for biological control; and the contents of requests for authorisation for the introduction and use of alien species or for the rearing of beneficial organisms. Particularly

important from a research perspective are two lists that are directly linked to the so-called European and Mediterranean Plant Protection Organization (EPPO) positive list (the list of biological control agents widely used in the EPPO region), which include the species of natural enemies that are commercially available in the EPPO region (Appendix I) or were successfully introduced into this region as a result of classical biological control (Appendix II) [19]. The peculiarity of the Slovenian lists is that they contain only species from the EPPO positive list, which contains more than 100 species. The list of indigenous species suitable for biological control thus contains species that are common in the natural environment in Slovenia due to their autochthony or because they were intentionally or unintentionally introduced into Europe from other continents in the past. These organisms have proved to be efficient natural enemies of harmful organisms in Europe while not having unwanted effects on indigenous natural enemies.

The list of indigenous species of organisms suitable for biological control, i.e., organisms that can be used in plant production in Slovenia, includes those species whose presence in Slovenia has been confirmed in writing in a scientific article by experts or by an accredited diagnostic laboratory. The list currently includes 29 species of biological control agents—19 predators, 6 parasitoids and 4 entomopathogenic nematodes. Following the publication of a scientific article or the issuance of a certificate from a diagnostic laboratory indicating the detection of a new beneficial species for Slovenia, a research organisation (this has so far always been the Biotechnical Faculty of the University of Ljubljana) prepares an application to change the status of an alien organism that is then submitted to the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection, which issues a decision regarding changing the lists of indigenous and alien species for biological control after the evaluation of the documentation. By being included on the list of indigenous species suitable for biological control, a beneficial organism can be used for the biological control of plant pests. The number of species of biological control agents that can be used in Slovenia in everyday agricultural practices, therefore, directly depends on the activities of domestic (and foreign) experts when establishing the presence of beneficent species. Preparations created on the basis of entomopathogenic fungi, bacteria and viruses are regulated in the same way as plant protection products, which are regulated by the Plant Protection Products Act (Official Gazette of the Republic of Slovenia, no. 83/12) [20].

5. Two Examples of Classical Biological Control

The first example of the introduction of an alien beneficial organism for inoculative release to suppress a recently released harmful alien organism in Slovenia is that of the predatory parasitoid wasp *Neodryinus typhlocybae* (Ashmead) for the biological suppression of the citrus flatid planthopper (*Metcalfa pruinosa* [Say]). The introduction of this natural enemy was carried out in Nova Gorica in 1998 as an integral part of the master's thesis research performed by Ivan Žežlina [21] under the supervision of Milevoj. The numbers of the citrus flatid planthopper, which was an important polyphagous pest in the 1990s, especially in the Slovene Littoral, declined significantly in the years following this introduction. This decline was mainly attributed to *N. typhlocybae* but likely also due to some extent to indigenous natural enemies, which recognised the citrus flatid planthopper as a suitable host approximately ten years following its introduction in Slovenia. The introduction of the predatory parasitoid wasp from Italy was relatively simple, as the rules on the biological control of plant pests had not been implemented.

Another example of classical biological control is the introduction of the parasitoid wasp *Torymus sinensis* Kamijo for the suppression of the chestnut gall wasp (*Dryocosmus kuriphilus* Yasumatsu). This introduction was carried out in 2015, although the process leading up to it took considerable time because the Department of Agronomy of the Biotechnical Faculty, which played an important role in the procedure as the authorised agent, was required to prepare an extensive assessment of the risk to nature prior to this introduction. The conditions for carrying out the assessment of risk and the manner in which the assessment was performed are defined in the rules on the carrying-out of the

assessment of risk to nature and the acquisition of authorisation (Official Gazette of the Republic of Slovenia, no. 43/02) [22]; an integral component is also the list of authorised agents for carrying out the assessment of risk to nature prior to the introduction and reintroduction of alien plants and animals into nature or the rearing of alien wildlife animals. The assessors of risk had the most misgivings regarding the potential nontarget effects of the parasitoid wasp on indigenous parasitoids of the gall wasp, yet these were proved unwarranted in subsequent research [23]. Positive results from introducing *T. sinensis* occurred a year after its introduction, i.e., a considerable improvement in the health of sweet chestnut trees (*Castanea sativa* Mill.).

6. The First Slovenian Monograph on Biological Control and Other Professional and Pedagogic Activities

Milevoj is also the author of the first Slovenian monograph on biological control, publishing the book *Biological Control of Pests in Greenhouses* in 2011 [24]. This book presents the main approaches used in biological control, the legal bases for this approach, reasons for using biological control in greenhouses, important natural enemies and harmful organisms that can be suppressed with this technique, and the compatibility of natural enemies and plant protection products. The book contains numerous practical instructions and other useful information for the application of biological control agents, which Milevoj acquired as a many-year Slovenian member of the EPPO Panel on Biological Control Agents.

During the period of 1999–2001, Milevoj published a series of papers about the biological control of natural enemies classified into groups of cultivated plants and locations of their production in the then-prominent Slovenian scientific professional journal *Sodobno kmetijstvo* (*Modern Agriculture*). She presented the possibilities of this manner of suppressing harmful organisms on fruiting vegetables [25], brassicas [26], salad plants [27] and other groups of cultivated plants, as well as in orchards [28] and greenhouses [29]. Her 1998 paper discussing prospects for biological control in Slovenia [30] has currently drawn great attention. She foresaw seven years before the implementation of the rules on biological control of plant pests that, of the three essential approaches to biological control, those feasible for the circumstances in Slovenia are the protection and facilitation of antagonists under natural conditions and (partly) the introduction of antagonists for inoculative release, known as classical biological control. At that time, she believed that the third approach—the rearing and release of beneficial species—could not yet be implemented, primarily because laboratories able to sequence massive numbers of beneficial organisms had not yet been established.

We wish to impart the knowledge acquired over years of intensive research in the field of biological control to agricultural advisors and students of agriculture, who need or will need, during the course of their work, information regarding the possible application of natural enemies for the suppression of harmful organisms. To this end, the Biotechnical Faculty organised four workshops (in 2006, 2009, 2010 and 2015) on biological control, which provided information on natural enemies and possibilities regarding their use for suppressing harmful organisms in Slovenia [31–34].

The subject of biological control, which has been taught at the Biotechnical Faculty as an independent elective course since 2008, is also a popular choice among students for research projects; thus, 19 bachelor's theses in university and higher professional programmes, 15 bachelor's theses in first-cycle Bologna study programmes, 7 master's theses in pre-Bologna study programmes, 12 master's theses in second-cycle Bologna study programmes and 2 doctoral dissertations on this topic have been defended at the Department of Agronomy [35,36].

7. Activities in the Past 15 Years

7.1. Entomopathogenic Nematodes

Research on entomopathogenic nematodes at the Biotechnical Faculty intensified in 2004, when we became responsible for the national research project "Usage of entomopathogenic nematodes in plant

protection-method optimisation”, in which we began carrying out the first research into the efficiency of these biological control agents under laboratory conditions. Entomopathogenic nematodes were still considered alien organisms at that time in Slovenia, and their outdoor use was not permitted. In the first laboratory study, we thus confirmed the effects of entomopathogenic nematodes on adult specimens of the granary weevil (*Sitophilus granarius* [L.]) and the sawtoothed grain beetle (*Oryzaephilus surinamensis* [L.]) [37,38] and then against the rice weevil (*Sitophilus oryzae* [L.]) [39], cabbage flea beetles [40], the cereal leaf beetle (*Oulema melanopus* [L.]) [41], thrips *Hercinothrips femoralis* [Reuter] [42], larval and adult specimens of the Colorado potato beetle (*Leptinotarsa decemlineata* [Say]) [43], and larvae of the common cockchafer (*Melolontha melolontha* [L.]) [44], while in greenhouses, we identified satisfactory effects of these biological control agents on the western flower thrips (*Frankliniella occidentalis* [Pergande]) [45] and the greenhouse whitefly (*Trialeurodes vaporariorum* [Westwood]) [46]. Under laboratory conditions, we also confirmed nontarget effects of entomopathogenic nematodes on natural enemies, namely, with the example of the two-spot ladybird (*Adalia bipunctata* [L.]) and common green lacewing [47].

By sampling soil in different regions of Slovenia, we confirmed the presence and autochthony of five species of entomopathogenic nematodes, four of which (included on the EPPO positive list) were added to the list of indigenous species of organisms for biological control on the basis of published scientific articles indicating their detection. Among the species that can now be used in Slovenia to control harmful insects are *Steinernema feltiae* (Filipjev) [48], *Steinernema carpocapsae* (Weiser) [49], *Steinernema kraussei* (Steiner) [50] and *Heterorhabditis bacteriophora* (Poinar) [51]. The development from modest beginnings in the field of entomopathogenic nematodes to their implementation in food production systems was described in a monograph [52].

The establishment of the autochthony of the mentioned entomopathogenic nematode species enabled their testing for the control of harmful insects in the field; they were initially fairly successful against the Colorado potato beetle in a potato field [53] and the cereal leaf beetle in a wheat crop [54], while their use did not prove particularly efficient for controlling white grubs (Scarabaeidae) in grassland soils [55,56]. We also carried out a separate set of research projects addressing the simultaneous application of entomopathogenic nematodes and plant protection products, which was intended to save time and money in application, increase the efficiency of both plant protection products and entomopathogenic nematodes, and reduce the risk of developing resistance to plant protection products. In connection to this work, we tested the ability of entomopathogenic nematodes to survive when mixed with fungicides [57], insecticides [58], acaricides [59] and herbicides [60]; the results of all our research projects and numerous related studies were published in a monograph chapter [61].

After 2013, we also focused our research on the chemotactic responses of entomopathogenic nematodes to volatile substances released by plants damaged by herbivores. We thus assessed the reactions of nematodes to substances released from damaged roots of maize [62], potato tubers [63], roots of *Brassica nigra* [64], and roots and leaves of hemp [65]. Some interesting multitrophic interactions among plants, harmful soil insects and entomopathogenic nematodes have been recently presented in review papers [66,67].

7.2. Parasitic Nematodes

When inventorying the natural enemies of plant pests in Slovenia, we searched for parasitic nematodes in roundback slugs (Arionidae), which form the most important group of economically harmful slugs in this region. The most widespread species in Slovenia are *Arion vulgaris* Moquin-Tandon, *Arion hortensis* (Férussac) and *Arion rufus* (L.), which have been most intensively studied for the presence of parasitic nematodes [68]. We wanted to find *Phasmarhabditis hermaphrodita* (A. Schneider), which is the most efficient and available species on the market in Europe. Among more than a thousand slugs collected from all around the country, we did not find the mentioned species, but we found other species of parasitic nematodes. The most frequent was *Alloinema appendiculatum* Schneider [69], which is unfortunately not as efficient as *P. hermaphrodita* and consequently not commercially available. In 2018, we found a new species of parasitic nematode in roundback slugs in Slovenia, namely, *Phasmarhabditis*

papillosa (A. Schneider (unpubl.). In preliminary laboratory research, it proved more efficient than *A. appendiculatum*, and it has already been subjected to intensive research in regard to its applicability as a biological control agent for the suppression of economically harmful roundback slugs and keelback slugs (Limacidae). Among the latter, the most common species in Slovenia are *Deroceras reticulatum* (O. F. Müller) and *Limax maximus* L.

7.3. Predators

Studies on predators performed over the first decade of the 30-year period had a particular focus on ladybugs (Coccinellidae) and the common green lacewing, as already stated in the third chapter, while Milevoj confirmed the presence of some economically interesting predatory bugs (*Anthrenus nemoralis* [Fabricius], *A. nemorum* [L.], *Amblyseius cucumeris* [Oudemans] and *Typhlodromus pyri* Scheuten), the aphid midge (*Aphidoletes aphidimyza*), the marmalade hoverfly (*Episyrphus balteatus* [De Geer]) and the predatory bugs *Macrolophus pygmaeus* (Rambur), *Orius majusculus* (Reuter) and *Picromerus bidens* (L.) [18] in Slovenia before the implementation of the rules on biological plant protection.

Here, we should also mention the inventorying of thrips, which involved the detection of 9 species of the genus *Aeolothrips* and 17 species of the genus *Haplothrips* and *Scolothrips longicornis* Priesner [70] on the basis of studying published professional sources and the collection of new samples in Slovenia. These species are representatives of predatory genera that feed primarily on different developmental stages of thrips and mites. The majority of our research was dedicated to the species *Aeolothrips intermedius* Bagnall, which is a ubiquitous species in Slovenia and neighbouring countries [71]. This predator was detected on 30 plant species in 16 botanical families. However, it always appeared in mixed populations with phytophagous and facultative phytophagous species of insects and with 18 species of thrips. It was established that this predator appears in relatively low numbers on the vegetative parts of plants, although these parts have an abundance of its potential prey, which indicates the great importance of pollen as an alternative food source for this species. Another of our articles also describes the first detection of the predatory thrips species *A. gloriosus* Bagnall [72], which we found in olive blossoms and whose larvae, in particular, are known to feed on pollen and small arthropods. None of these species are available commercially in Europe; one of the reasons for this is the difficulties associated with the massive rearing of thrips from the genus *Aeolothrips*. In 2018, Kos et al. [73] were the first to report the detection of a natural enemy of the red spider mite (*Tetranychus urticae* Koch), namely, the predatory gall midge *Feltiella acarisuga* (Vallot). Here, we should also note the significance of volatile substances released by damaged plants in affecting the activities of the two-spot ladybird (*Adalia bipunctata* L.) and common green lacewing [74], and the influence of temperature on cannibalism rates among larvae of the latter species [75].

The inventorying of predatory mites (Phytoseiidae) has also received particular attention over the last decade as some of them are known to be very efficient natural enemies of smaller harmful insects and mites. As in the case of thrips, we found by studying the available professional sources and new samples collected in Slovenia, and with the very helpful cooperation of the French acarologists Kreiter and Douin in 2018–2019 that the fauna of predatory mites in Slovenia includes 36 species, 20 of which belong to the subfamily Amblyseinae, 4 to the subfamily Phytoseiinae, and 12 to the subfamily Typhlodrominae. Among the 22 species first recorded in Slovenia in 2018–2019, at least 8 are known biological control agents of plant pests [76–78]. In recent years, the predatory mites *Amblyseius andersoni* (Chant), *A. californicus* (McGregor), *A. barkeri* (Hughes) and *Euseius gallicus* Kreiter and Tixier were added to the list of indigenous species of organisms for biological control on the basis of published scientific articles.

7.4. Parasitoids

After Milevoj, more intensive studies of the parasitoids of harmful insects continued only in the second half of this century's first decade, when Professor Željko Tomanović from Belgrade piqued

our interest in this issue [79–82] and later on in parasitoids of aphids. In the first field study of this kind, Kos et al. (2007) found 18 species of aphids on plants in a garden ecosystem, and parasitism was observed in as many as 14 of these species. Seventeen species of parasitoids of aphids from 8 genera were identified, namely, *Aphidius*, *Binodoxys*, *Diaeretiella*, *Ephedrus*, *Lipolexis*, *Lysiphlebus*, *Monoctonus* and *Praon*. The first detection of the parasitoid *Encarsia formosa* Gahan, an important natural enemy of the glasshouse whitefly (*Trialeurodes vaporariorum* [Westwood]) [83], was confirmed soon after this, followed by the detection of the parasitoid *Aphidius ervi* Haliday. The latter was found on different species of aphids collected on nine species of cultivated plants [84]. The following period involved more intensive work in the molecular identification of aphid parasitoids [85–87] and research on the interactions between plants, aphids, their parasitoids and hyperparasitoids [88]. As a result of these studies, *Diglyphus isaea* (Walker) and *Praon volucre* (Haliday) were added to the list of indigenous species of organisms for biological control over the last ten years. Many more parasitoids have been found in Slovenia by systematically inventorying beneficent species, but they cannot be included on the list, because they are not a part of the EPPO positive list (examples of this are the parasitoid *Anisopteromalus calandrae* [Howard] [89], which is a natural enemy of storage beetles, and the parasitoid *Cotesia glomerata* [L.] [90], a natural enemy of caterpillars of Pieridae) or because the executive branch of government did not issue a positive opinion regarding their inclusion on the list. This holds true for the egg parasitoid *Trichogramma brassicae* Bezdenko, for example, for which the executive branch issued the opinion that its presence in different regions of the country should be assessed despite the fact that it was detected at a location in Slovenia [91].

After the first appearance and soon very high abundance of the chestnut gall wasp, which threatened to completely end the cultivation of sweet chestnut in Slovenia, the sampling of indigenous parasitoids of this harmful organism and other gall wasps intensified with an aim to determine whether the introduction of the alien parasitoid *T. sinensis* did in fact endanger the survival of indigenous parasitoids. Soon after the emergence of the chestnut gall wasp in Slovenia, we predicted that the only appropriate and safe (for indigenous parasitoids) manner to reduce its harmfulness was to introduce *T. sinensis* [92], which was also confirmed by many years of research following the introduction of the natural enemy [23,93].

8. Conclusions

The short 30-year history of biological control in Slovenia is an excellent example of the very good cooperation between a profession and the legislative branch of the government, as it contains all elements of planned preparation for the implementation of biological control in food production in both the professional and legislative fields. Prof. Dr. Lea Milevoj, the pioneer of biological control in Slovenia, became aware of the importance of this environmentally acceptable manner of the suppression of harmful organisms in agriculture very early in its development, and she undertook intensive research and professional work in the 1990s to set the foundations for this field in Slovenia. Due to her exceptional general knowledge of plant protection and agriculture, she was an outstanding member of the expert group involved in the preparation of the rules on the biological control of plant pests, which also became a model for some other European countries. The most important component of these rules, which are considered to be very stringent by some people, is the list of indigenous species of organisms for biological control, which includes only those beneficial species that are indigenous or ubiquitous in Slovenia (the latter, for example, include *Aphelinus mali* [Haldeman] and *Encarsia formosa*) and that are also included on the EPPO positive list. This criterion is an additional safeguard that practically prevents the use of beneficent species that could in any way endanger the common domestic flora and fauna. The validity of this statement is, to a great extent, confirmed by the fact that after the implementation of the rules on the biological control of plant pests in Slovenia, classical biological control allowed for the use of only one beneficent species, yet this species proved highly efficient in suppressing a harmful organism that would otherwise likely destroy all sweet chestnut trees in Slovenia over the course of a few years. Our future work will involve the continued inventorying of beneficent

species to expand the list of indigenous species for use in biological control. Slovenian food producers will thus have at their disposal agents to reduce the numbers of and, consequently, the damage caused by plant pests.

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