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Pathogenicity of Entomopathogenic Fungi to the Green Peach Aphid *Myzus persicae* Sulzer (Aphididae) and the European Tarnished Bug *Lygus rugulipennis* Poppius (Miridae)

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ABSTRACT

To reduce economic losses, producers have no choice than controlling pests by chemical insecticides with their negative impact on the environment as well as on beneficial organisms. In this context, efficient and selective biological control agents such as entomopathogenic fungi should be integrated in pest management strategies. Screening experiments showed the pathogenicity of 16 fungal strains belong to the species *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, *B. brongniartii* (Sacc.) Petch, *Metarhizium anisopliae* (Metchnikoff) Sorokin, *Lecanicillium lecanii* (Zare and Gams) and *Paecilomyces fumosoroseus* Vassiljevsky against the two plant sap sucking pests, the green peach aphid, *Myzus persicae* Sulzerand the European tarnished bug, *Lygus rugulipennis* Poppius. Bioassays established on treated Chinese cabbage leaves and bean pods for *M. persicae* and *L. rugulipennis*, respectively, allowed to evaluate the most pathogenic strains for controlling each pest. The two strains ART41 and ART2580 of *B. bassiana* showed highest pathogenicity on *M. persicae* with a mortality rate of 92 and 98%, respectively, seven days post treatment. Mortality rates of *L. rugulipennis* reached 92 and 98% seven days after treatment for the *B. bassiana* strains ART2580 and ART360BB, respectively. These results should be confirmed in open field experiments by exposing both insects to the suggested *B. bassiana* strains.

Key words: Entomopathogenic fungi*; Beauveria bassiana*; *Metarhizium anisopliae;*; *Lecanicillium lecanii; Paecilomyces farinosus*; *Myzus persicae; Lygus rugulipennis*; Biological control.

INTRODUCTION

Chemical control with broad spectrum insecticides has resulted negative impacts on the environment as well as on beneficial organisms in the agroecosystem. Adverse effects of such pesticides on human health and environment are well documented (Yeo *et al.*, 2003), leading to a reduction of registered active ingredients for pest control. Furthermore, consumers exert an increasing pressure for reaching minimum pesticide residues in food (Vu *et al*., 2007). The use of Biological Control Agents (BCAs) should therefore be more widely integrated in strategies to control the damage caused by pests. Entomopathogenic fungi are particularly interesting BCAs since they are usually specific and consequently rarely harmful to beneficial organisms. Fungal pathogens have considerable advantage to avoid the problem of chemical residues and could be the replacements of choice for growers in order to reduce economic losses caused by pests.

Plant sap sucking insects such as aphids and true bugs are significant pests on arable and perennial crops. They are found throughout the world where they can build up high population densities. Moreover, they are frequently polyphagous and some of them are also powerful vectors of viral and bacterial plant diseases (Sylvester, 1980). Two major representatives of them are the green peach aphid, *Myzus persicae* Sulzer (Hemiptera: Aphididae) and the European tarnished bug, *Lygus rugulipennis* Poppius (Hemiptera: Miridae).

Myzus persicae (Down *et al*., 2009), feeds on a wide range of crops including peach trees as well as species of *Brassicae* and *Solanaceae*. It is generally the cause of decreased growth and necrosis of wounded tissues. It reproduces asexually in large numbers, up to 15 generations per year and may transmit at least 24 harmful plant viruses (*e.g.*,the potato Y virus or potato leaf roll virus and various mosaic viruses) as well as more than 100 plant diseases in more than 50 plant families including major crops such as potatoes, beans, sugar beet and brassics (Van Den Heuvel, 1991 and Yeo, 2000). Asexual reproduction in *M. persicae* potentially allowed it to quick develop resistances against the chemical insecticides (Bass *et al*., 2014). All those issues force to the development of alternative strategies against.

Lygus rugulipennis, the European tarnished plant bug, is a very polyphagous insect able to feed on more than 400 plant species. It is highly mobile and usually presents at high population densities. It is an

indigenous species to Europe, which is becoming a pest of growing concern, in reason of economic increasing damage to a wide variety of agricultural crops from Southern Europe (Accinelli *et al.*, 2005) to Northern Europe (Petrova *et al*., 2010) that includes for instance eggplant, strawberries, lettuce, peach trees as well as Scots pines (Holopainen and Rikala, 1990). Feeding injuries caused deformations and necrotic stripes on the leaves due to salival enzymes that cause aesthetic damage, flower and fruit abortion.

Control relies mainly on broad spectrum insecticides such as pyrethroids with the known inconvenience of negative impacts on the environment and conservative biological control available through natural enemies (predatory and parasitoid species) (Accinelli *et al.*, 2005). Methods, such as the inundative and inoculative release of natural enemies as well planting of trap crops and microbial control with the fungus *Beauveria bassiana* did not yield effective results in open field conditions, according to several authors (Kovach,1996; Steinkraus and Tugwell, 1997; Noma and Strickler, 1999). Biocontrol options available for similar phytophagous Mirids in the US, with a focus on a close American species *L. linolearis* were reviewed by Ruberson and Williams (2000). However, the application of fungi might be a more reliable alternative control strategy where it proved to be efficient against *M. persicae* (Hesketh *et al*., 2008; Rashki *et al*., 2009 and Shan and Feng, 2010).

Although, many Zygomycetes species are known in aphids (Barta and Cagan, 2006), most successful entomopathogenic fungi belong to the genera *Beauveria* and *Metarhizium* of the Hypocreales order (Ascomycetes). Many strains have been isolated within the species of these two genera and new ones are regularly obtained from mummified insects (Kuske *et al*., 2011). About 70% of all current fungal formulations registered or developed are based on *Beauveria* or *Metarhizium* spp. The virulence of 23 strains of *M. anisopliae* and *M. acridium* were compared against the green peach aphid and the recorded mortalities ranged from 10 to 95% (Shan and Feng (2010). Testing of 15 *B. bassiana* isolates on *Aphis fabae*, documented that the mortality rates were higher than 80% (Hesketh *et al*., 2008). In the same work, aphid species could also be ranked in function of their sensitivity to the different fungi, from the most sensitive *Sitobion avenae*, followed by *M. persicae, Acyrthosiphon pisum, Aphis fabae* and *Rhopalosiphum padi*. Alike, Yeo *et al*. (2003) evaluated the pathogenicity of certain strains as *B. bassiana, L. lecanii, M. anisopliae* and *P. fumusoresus* on *A. Fabae* and *M. persicae*. *A. fabae* was more sensitive to fungal infections than *M. persicae* and *L. lecanii* was the most efficient to fungus. Reports on the use of entomopathogenic fungi against *L. rugulipennis* in the literature are very scarce; in spite it has been causing damages on several crops in Europe in the past years (Xu *et al*., 2014). Meanwhile, the experimental use of entomopathogenic fungi against *L. lineolaris* which has a very similar behaviour species to *L. rugulipennis,* is well documented (Fleury *et al*. 2006). Liu *et al*. (2002) compared the efficacy of several isolates belonging to the genera *Beauveria*, *Paecilomyces*, *Metarhizium*, *Mariannaea* and *Hirsutella* and found two strains of *B. bassiana* and other one of *M. anisopliae* that exhibited a significant higher mortality on *L. lineolaris* nymphs. Sabbahi *et al*. (2008) found that a *B. bassiana* isolate caused mortality up to 78% on *L. lineolaris* adults. Al-Mazra'awi *et al*. (2006) used an isolate of *B. bassiana* to control *L. lineolaris* in greenhouse sweet pepper. Propagules were disseminated by bumble bees and were found to cause mortality up to 45% in the pest population.

The objective of this study was to test the pathogenicity of several strains of the fungi *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, *B. brongniartii* (Sacc.) Petch, *Metarhizium anisopliae* (Metchnikoff) Sorokin, *Lecanicillium lecanii* (Zare and Gams) and *Paecilomyces fumosoroseus* Vassiljevsky on the two model of pests, *M. persicae* and *L. rugulipennis* in order to identify potential BCAs, which could also be used against other sucking insect pests and provide a sustainable strategy to control their damages on horticultural crops.

MATERIALS AND METHODS

Fungi

Sixteen strains of entomopathogenic fungi belonging to the following species: *Beauveria bassiana, Metarhizium anisopliae, Lecanicillium lecanii* and *Paecilomyces farinosus* were provided by Agroscope. They had been isolated from dead insects at several locations across Switzerland (Table 1). Genetic identity of these strains was checked through PCR amplification of the ITS rDNA region with ITS1 and ITS 4 primers (White *et al*., 1990) and sequencing of the PCR product following routine laboratory protocols (Dagno *et al*., 2011). Resulting sequences were registered in GenBank (Benson *et al*. 2013). Fungi were grown in the dark at room temperature (20 ± 3°C) on a solid medium of potato glucose agar (PGA; potato 4 g/L; glucose 20 g/L; agar 15 g/L; pH 5.4) for sporulation and on Sabouraud medium for conservation (Fleury *et al*., 2013). Spores were suspended by scraping Petri dish cultures in ultrapure sterile water with 0.02% Tween 20. All spore suspensions were homogenised at a final

concentration of 10^7 spores per ml, used for all conducted experiments.

Insects

Initial populations of *M. persicae* and *L. rugulipennis* were provided by Agroscope and originated from long-time laboratory cultures. The two insect species were reared in climatic chambers at 23±2°C, with a relative air humidity of 70% and under 16 h light / 8 h dark photoperiod with a light intensity of approximately 20 000 lux provided by cool white fluorescent (Sylvania LUXLINE PLUS F58W/840 – T8, 1500 mm) and purple photosynthetic lamps (Sylvania GROLUX F58W/GRO, 1500 mm). Population of *M. persicae* was reared on Chinese cabbage leaves (*Brassica rapa* subsp. *pekinensis* cv "Tip Top") whereas *L. rugulipennis* was kept on commercial green bean pods (*Phaseolus vulgaris*). Both cabbage and bean plants were grown under similar climatic conditions.

ITest procedure

The study was conducted in two phases. In a first screening phase, mortality of all 16 strains of entomopathogenic fungi was assessed. Strains that caused on average mortality higher than 70% in all assays were retained for further examination. In the second phase, these strains were tested in a confirmation experiment in order to approve their high pathogenicity.

For *M. persicae*, a Chinese cabbage leaf disc (Ø 23 mm) was dipped in a spore suspension of 10^7 conidia ml^{-1} for 5 seconds and in order to dry it was then laid on a blotting paper humidified with 400 µL ultrapure sterile water in a small plastic box (32x17x19mm). After 30 min, a single individual at the 2nd or 3rd nymphal instars was deposited on the surface of the leaf disc with the help of a fine brush. Thereafter, the plastic boxes were placed into the climatic chambers at previously described conditions. In order to assess the pathogenicity of different spore solutions, a negative and positive control was set-up in parallel. The negative control consisted of leaf discs that were dipped in ultrapure sterile water whereas the leaf discs of the positive control dipped in an pirimicarb insecticide solution of 0.04%. Each treatment consisted of each 50 individuals in a plastic box . Moreover, each treatment was repeated at 3 different dates.

For *L. rugulipennis*, common bean pods were inoculated by immersion for one hour in a 50 mL suspension of 10^7 conidia ml^{-1} for each isolate and thereafter allowed to dry out at room temperature for 30 min. Subsequently, each bean pod was transferred into a Petri dish (Ø 90 mm) and five individuals of the 2nd and 3rd nymphal instars were added. Finally, Petri dishes were dislocated into the climatic chambers and stored at the previously defined conditions. Again, the negative control consisted of bean pods that were immersed in ultrapure sterile water whereas bean pods of the positive control were immersed in lambda-cyhalothrin insecticide solution of 0.04%. Each treatment consisted of 4 Petri dishes counting altogether 20 individuals and each treatment was repeated at 3 different dates.

For both insect species, mortality of individuals was recorded at 3, 5 and 7 days after initial application of conidial treatments.

Scanning electronic microscopy

Dead insects were fixed according Andrès-Barrao *et al*. (2012) using glutaraldehyde and osmic acid before gold embedding. Observations were made with a scanning electronic microscope JEOL JSM7001F (JEOL, Tokyo, Japan).

Table (1): List of the sixteen entomopathogenic fungal strains from Agroscope evaluated against *Myzus persicae* and Lygus *rugulipennis* under laboratory conditions

Agroscope Reference Strain Number	Species	Host species	Origin
ART9	*Beauveria bassiana*	*Agelastica alni* (Chrysomelidae)	unknown
ART10	*Beauveria bassiana*	*Agelastica alni* (Chrysomelidae)	Canton Zürich, Switzerland
ART15	*Paecilomyces farinosus*	*Lepidoptera* sp.	Canton Thurgau, Switzerland
ART18	*Beauveria bassiana*	*Corythuca ciliate* (Tingidae)	Canton Ticino, Switzerland
ART19	*Beauveria bassiana*	*Corythuca ciliate* (Tingidae)	Canton Ticino, Switzerland
ART38	*Beauveria brongniartii*	*Oulema* sp. (Chrysomelidae)	Reckenholz, Switzerland
ART41	*Beauveria bassiana*	*Meligethes* sp. *(Nitidulidae)*	Stammheim, Switzerland
ART47	*Metarhizium anisopliae*	*Cetonia* sp. *(Scarabaeidae)*	Altnau, Switzerland
ART57	*Metarhizium anisopliae*	*Cetonia* sp. (Scarabaeidae)	Buchs, Switzerland
ART234	*Beauveria bassiana*	*Ips typographus* (Curculionidae)	Tösstock, Switzerland
ART252	*Metarhizium anisopliae*	*Amphimallon majalis* ((Scarabaeidae)	Davos, Switzerland
ART332	*Metarhizium anisopliae*	*Carabidae* sp.	Flumserberge, Switzerland
ART360	*Lecanicillium lecanii*	*Aphis rumicis* (Aphididae)	Watt, Switzerland
ART360BB	*Beauveria bassiana*	unknown	unknown
ART372	*Beauveria bassiana*	*Carabidae* sp.	Hindelbank, Switzerland
ART2580	*Beauveria bassiana*	*Meligethes* sp. (Nitidulidae)	Augwil, Switzerland

Statistical analyses

Data were analyzed by the software R (statistical computing). The test khi^2 ($\chi 2$) was used to observe significant differences. Abbott formula was used to calculate the effective corrected mortality compared to the negative control (Abbott, 1925):

$$M_{corr} = \frac{M_t - M_c}{100 - M_c} \times 100$$

Where: Mcorr = corrected mortality (%), Mt = Mortality in the treated group and Mc = mortality in the control.

RESULTS AND DISCUSSION

Confrontation of *Myzus persicae* to entomopathogenic fungi

Lethality screening of the 16 strains showed that seven days after their application, the two strains ART41 and ART2580 of *B. bassiana* provoked an average mortality higher than 70%. Screening of 13 strains is shown on figure (1). Moreover, on at least two dates the mortality of these two strains was higher than 85% (Figure 1). They were consequently retained for further testing, aiming to confirm the observed pathogenicity.

Likewise, ART41 and ART2580 caused an average mortality of 92 and 98%, respectively, after seven days in the conformation experiment (Figure 2). Statistical analysis showed that these results were significantly different as compared to the negative control ($P \leq 0.01$). Moreover, these two strains were quickly mortal. For instance already three days after their application, mortality reached 64% for ART41 and 82% for ART2580 and then jumped at 5 days to 94 and 100%, respectively (Figure 3). After 7 days, insect bodies were completely covered by mycelia and fruchtifications budding as shown in (Figure 4).

Confrontation of *Lygus rugulipennis* to entomopathogenic fungi

Screening experiment allowed retaining the *B. bassiana* strains of ART19, ART234, ART360BB and ART2580 as well as the *M. anisopliae* strain ART252 from the 16 tested isolates. Confirmation experiment showed that, in particular the two strains ART360BB and ART2580 were highly pathogenic to *L. rugulipennis,* with mortality rates reached 98.3 and 91.7%, respectively (Figure 5). These mortality rates differed significantly from the negative control ($P<0.01$*). Seven days after treatment, fungal growth was also observed on dead bodies of *L. rugulipennis*.

The two strains of *B. bassiana, e.g.*, ART41 and ART2580, were found highly pathogenic to *M. persicae* under the given laboratory setting. Moreover, the strains ART2580 together with the strain ART360BB were also highly lethal to *L. rugulipennis*. These strains have the potential to be integrated in biological control strategies and should therefore be examined in more details, since the application of fungal entomopathogens has been frequently used and represents a sustainable strategy to control insect pests (Roy *et al*., 2010). Entomopathogenic fungi also harbour the large advantage that they can be applied in an inoculative manner, since they are frequently soil inhabitants and may survive in winter conditions (Nielsen *et al*., 2003). Moreover, Ye *et al*. (2005) revealed that entomopathogenic fungi such as *B. bassiana* might increase the efficacy of insecticides such as imidacloprid. This fact provides new perspectives in the way of applying BCAs and novel strategies to successfuly combat the emerging problem of resistance towards pesticides.

The experimental setting, namely bioassays with treated cabbage leaf-discs or bean pods, proved to be adequate for determining pathogenicity caused by the entomopathogenic fungi. The bioassays were reproducible and obtained results were consistent over several successive dates. The bioassays therefore allowed for the evaluation of the efficacy of the 16 tested strains on two major sucking pest insects of agricultural and horticultural crops, *M. persicae* and *L. rugulipennis*. An interesting fact is that with the exception of one strain, all were collected on mummified coleopterans, and nonetheless had a significant effect on homopterous insects. Experiments were conducted at the temperature of 23°C, which is adequate to insect development and to fungal growth. This is also a temperature, which is close to natural conditions in late spring and summer in Palaearctic regions and was previously described adequate to screen fungal entomopathogens against *M. persicae* (Yeo *et al*., 2003). In order to cope with outdoors conditions (*e.g.*, UVA, UVB, and relative humidity) and to be successful, the pathogenicity of strains must be high and their impact should be immediate.This requirements prompted us to select strains through our screening experiments, which caused mortality rates higher than 70%.

Amnuaykanjanasin *et al*. (2013) studied the histopathogenesis of *M. persicae* individuals infected by *B. bassiana* and showed that fungal penetration preceded through the integument of the less-resistant leg intersegmental membrane and invasion of natural openings. Formation of hyphae and extensive hyphal colonization of the insect body occurred in 3 days after inoculation. This is congruent with obtained results where mortality appeared 3 days after inoculation. Although the strain ART360 was collected from the aphid *Aphis runicis* (Table 1), it had a surprisingly low pathogenicity on *M. persicae* in the bioassays (Fig. 1). Other studies on *L. lecanii*

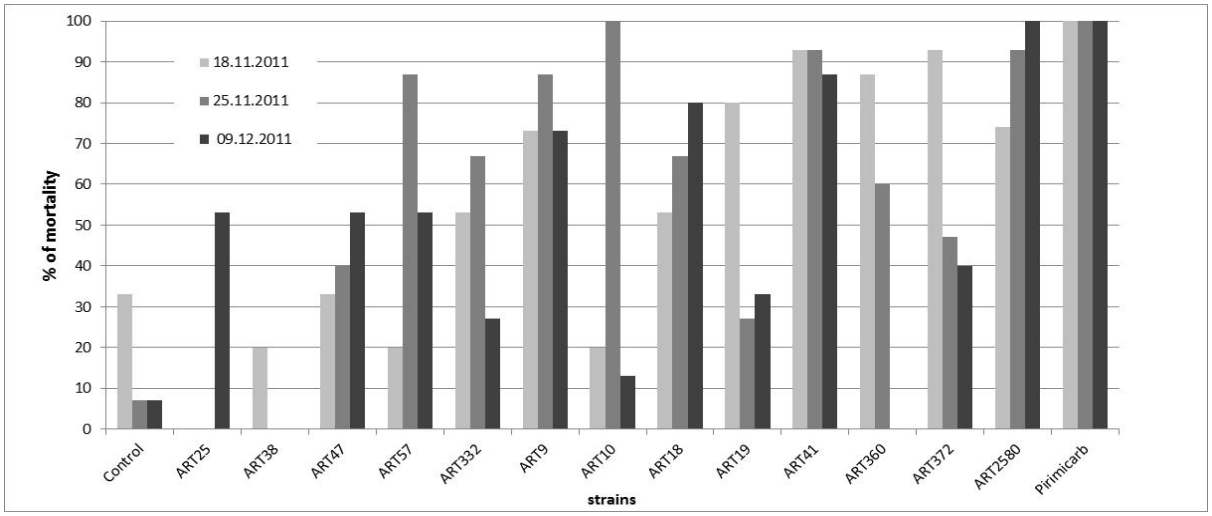

Fig. (1): Screening of 13 strains from Agroscope: mortality (%) on *M. persicae* 7 days after treatment in 3 successive repetitions.

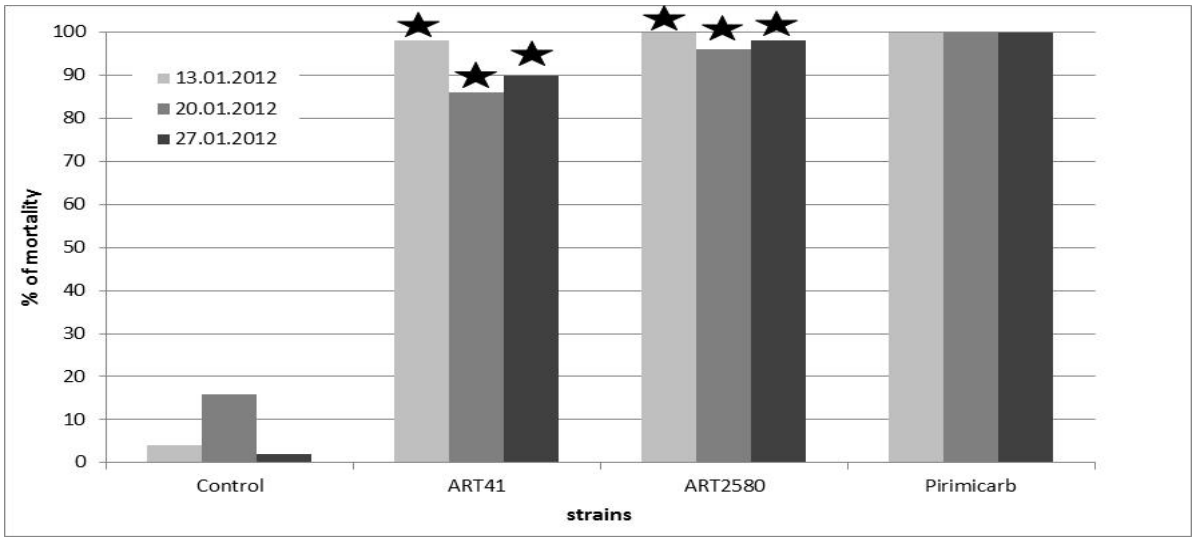

Fig. (2): Mortality (%) on *M. persicae* 7 days after treatment (khi2; * $P \leq 0.01$) in 3 successive repetitions, with *B. bassiana* strains No 41 and No 2580.

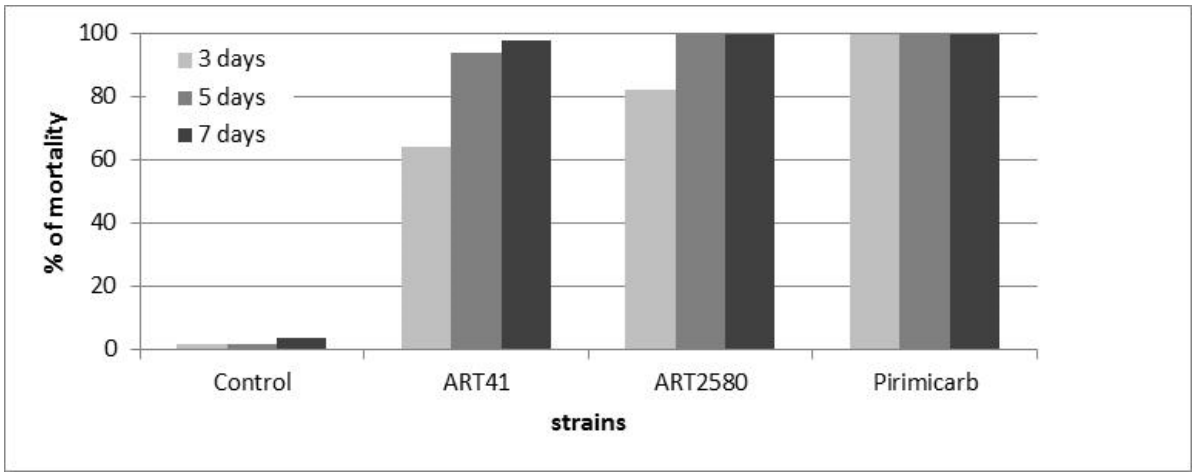

Fig. (3): Pathogenicity of the most two efficient entomopathogenic strains against *M. persicae*.

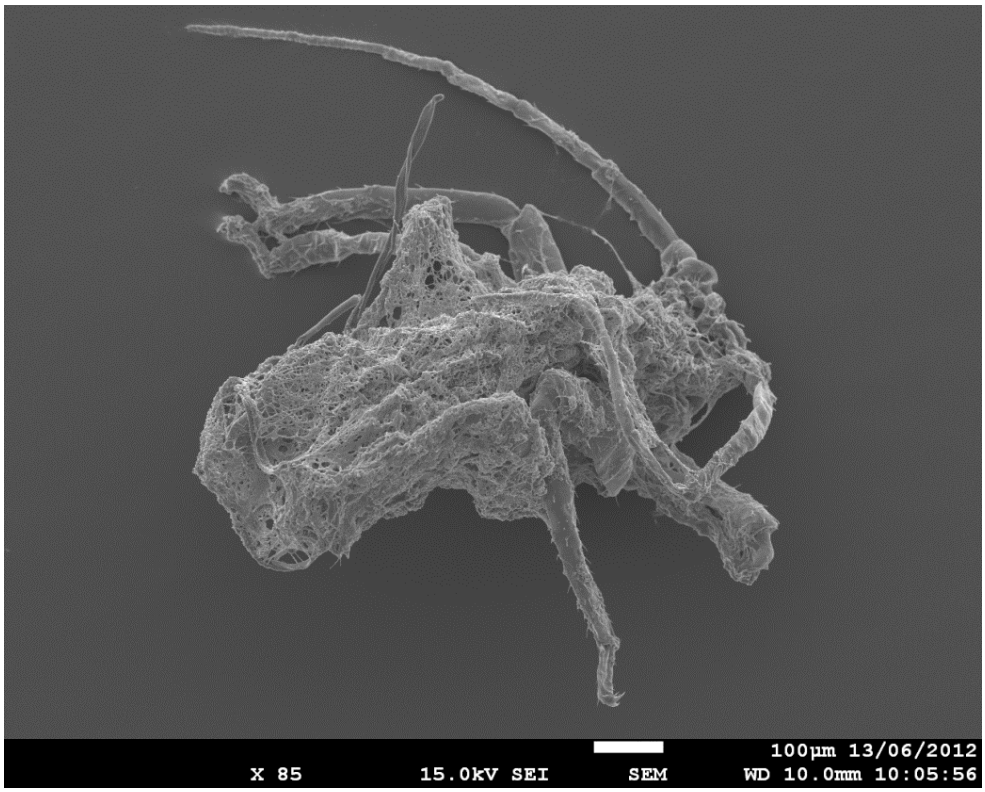

Fig. (4): Scanning electron microscopy of *M. persicae* invaded by the strain Bb 2580, dead body sampled 7 days after treatment.

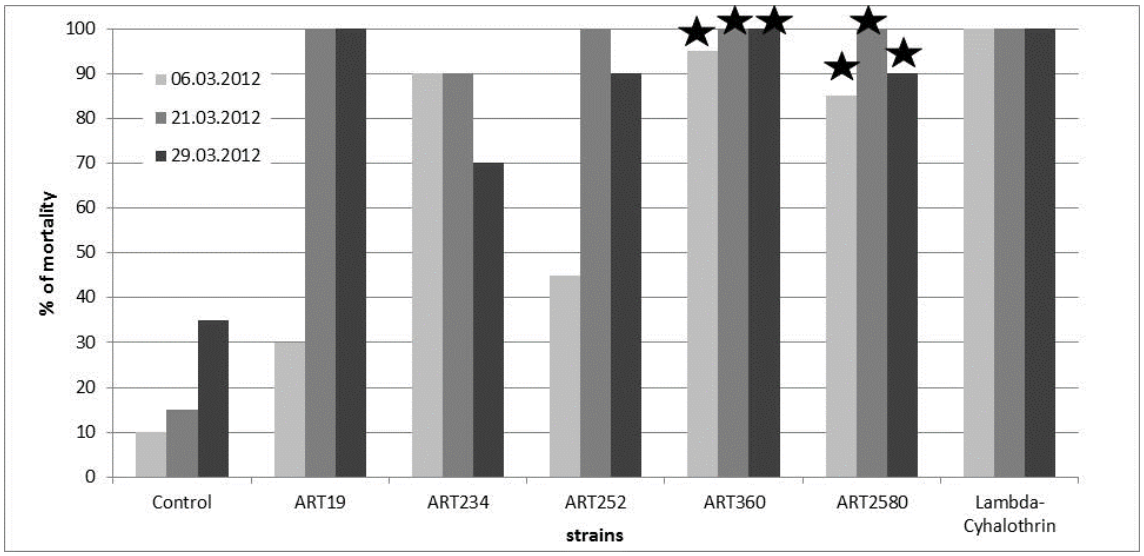

Fig. (5): Mortality (%) on *L. rugulipennis* 7 days after treatment (khi2; * $P \leq 0.01$) on 3 successive repetitions with strains No 19, 234, 252, 360BB and 2580.

showed that ART360 strain had higher or at least as high pathogenicity towards aphids than *B. bassiana* (Vu *et al*., 2007 and Hesketh *et al*., 2008). Overall, this stresses where there is a high variability among different strains within the same species and that findings can difficultly be compared if the strain is not specified. Nonetheless, it could be concluded that the two *B. bassiana* strains ART41 and ART2580 had a strong potential for the biological control of *M. persicae* and are therefore promising BCAs.

Obtained results on *L. rugulipennis* confirmed previous observations recorded on its similar behaviour with the species of *L. lineolaris*. The entomopathogenic fungus, *B. bassiana* at the concentration of 10^7 conidia per ml caused high mortality reached up to 98% for the most effective two strains, which was comparable to mortality observed on *L. lineolaris* (Steinkraus and Tugwell, 1997 and Sabbahi *et al*., 2008). Moreover, the two strains were rapidly effective and killed substantial percentages of nymphs already three days after application and in line with the findings of Steinkraus and Tugwell (1997). Overall, we are confident that with high pathogenic *B. bassiana* strains ART2580 and ART360BB to *L. rugulipennis* , they have the

potential to become future BCAs against this pest and maybe even against *L. lineolaris*.

Conclusion

This work fits in the present efforts to find biological alternatives to pesticides, in particular against some sucking pests in agriculture and horticulture (Yeo, 2000). Further laboratory works should include the combined application of these 3 strains on *M. persicae* and *L. rugulipennis*, since an increase in the diversity of the fungal treatment could results in a faster efficacy and a higher reliability of such applications to field conditions. It has also been scheduled that these promising results should be confirmed in open field experiments by exposing *M. persicae* and *L. rugulipennis* to the *B. bassiana* strains ART41, ART360BB and ART2580.

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