

# A new device for auto-disseminating entomopathogenic fungi against *Popillia japonica*: a study case

Claudia BENVENUTI<sup>1</sup>, Gian Paolo BARZANTI<sup>1</sup>, Leonardo MARIANELLI<sup>1</sup>, Giuseppino SABBATINI PEVERIERI<sup>1</sup>, Francesco PAOLI<sup>1</sup>, Giovanni BOSIO<sup>2</sup>, Davide VENANZIO<sup>2</sup>, Emanuela GIACOMETTO<sup>2</sup>, Pio Federico ROVERSI<sup>1</sup>

<sup>1</sup>CREA Research Centre for Plant Protection and Certification, Firenze, Italy

<sup>2</sup>Settore Fitosanitario e servizi tecnico-scientifici - Regione Piemonte, Torino, Italy

## Abstract

The Italian distribution range between Piedmont and Lombardy interested by the outbreak of the exotic Japanese beetle (JB) *Popillia japonica* Newman (Coleoptera Scarabaeidae) is getting wider. In the past, in the USA, where *P. japonica* is an exotic pest too, entomopathogenic fungi have been extensively used against larvae and adults of the Japanese beetle. We tested under laboratory and field conditions the effectiveness of a home-made 'attract-infect-release' device against *P. japonica* adults. The device was activated alternatively with the two commercial products (GranMet<sup>®</sup> and Met52<sup>®</sup>), available in Italy, containing *Metarhizium brunneum* Petch. During a series of fifteen-minute observations we recorded a mean permanence time of adult beetles inside the device of about 3 minutes with no difference between treatments. The number of *P. japonica* adult-borne conidia was assessed in the laboratory with no significant differences between treatments. For what concerns GranMet<sup>®</sup>, experiments on horizontal transmission showed 100% mortality by 19 days after treatment, while mortality associated with Met52<sup>®</sup> was 30-65%. In conclusion, our research showed that a 3-minute contact between a *P. japonica* adult and GranMet<sup>®</sup> was enough to permit the transmission of the infection to other individuals and eventually kill the insect. This result appears to be promising and, if confirmed in large field experiments, could add a new device for the biological control strategies against the Japanese beetle.

**Key words:** 'attract-infect-release', Japanese beetle, *Metarhizium brunneum*, EPF, horizontal transmission, biological control.

## Introduction

In recent years, microbiological control of pest populations has greatly developed due to the increasingly high restrictions applied to the use of chemical insecticides and the need of more environmentally friendly control strategies and improvement in food safety (e.g. EU Directive 2009/128/EC, 2009). The great number of researches devoted to Biological Control Agents (BCAs) has led to the identification of more than 750 different species of entomopathogenic fungi (EPF) from all around the world (Scheepmaker and Butt, 2010).

The device 'attract-infect-release' represent an effective way to spread a fungal inoculum while preserving its viability. Such devices, in fact, usually protect the inoculum against the effects of UV radiation and against leaching by rainfall. Moreover, specific lures attract target adult pests inside the trap assuring contact with the infecting substrate, and finally let the contaminated insects leave the device spreading out the inoculum within the pest population (Klein and Lacey, 1999; Maniania, 2002; Vega *et al.*, 2007; Francardi *et al.*, 2013; Lacey *et al.*, 2015). So, the advantages of this technique are: the selectivity of infection, the possibility of infecting a great number of individuals and the possibility of spreading the inoculum within the pest population through the contact between contaminated and healthy individuals (Maniania, 2002; Vega *et al.*, 2007; Quesada-Moraga *et al.*, 2008; Lacey *et al.*, 2015). The transmission of infecting conidia from one individual to another is also very important since it causes the spreading of infections and the induction of epizootics (Lacey *et al.*, 1994b; Heskett *et al.*, 2010). One more benefit that should not be underestimated is the possibility to bring the inoculum to the oviposition sites and the following

larval generation by means of vertical transmission, thus targeting niches sometimes hardly reached by biocides distributed with standard methods (Klein and Lacey, 1999; Vega *et al.*, 2007).

Auto-dissemination of EPF within a pest population is considered a good technique to control the Japanese beetle (JB), *Popillia japonica* Newman (Lacey *et al.*, 1994b; Klein and Lacey, 1999; Vega *et al.*, 2007).

JB is present in Ticino Natural Park (Northern Italy) since it was first discovered in 2014, raising considerable concern because of its potential impact on agricultural production in the surrounding area (Ahmad *et al.*, 1983; USDA/APHIS, 2015). Several studies focusing on the entomopathogenic nematodes (EPNs) to control JB larvae in the soil have been carried out to contrast the spreading of this dangerous quarantine pest in Italy (Mazza *et al.*, 2017; Paoli *et al.*, 2017a; 2017b; Marianelli *et al.*, 2018). On the other hand, at the moment of the study (June 2017), together with mechanical destruction of vegetation and use of chemicals in risk sites, only mass-trapping was applied against adult beetles in Italy, and other attract and kill devices were under experimentation (Regione Piemonte, 2017; Marianelli *et al.*, 2019).

Since the Italian infestation is primarily located in a Natural Park, we have looked for a control strategy against JB adults alternative to chemicals, and took into consideration the already tested and more eco-friendly entomopathogenic fungi (Lacey *et al.*, 1994b; 1995; 2015). Horizontal transmission of *Metarhizium anisopliae* (Metchnikoff) Sorokin was already evaluated in laboratory trials, with dry conidia outperforming water suspensions (Quesada-Moraga *et al.*, 2008) and several studies assessed the susceptibility of JB adults to this fungus (Lacey *et al.*, 1994b; Giroux *et al.*, 2015; Behle and Goett, 2016). The genus *Metarhizium* Sorokin

(Hypocreales Clavicipitaceae) has recently been subjected to a thorough taxonomic revision and today the former *M. anisopliae sensu lato* species is known to be a complex that comprises at least four species: *Metarhizium pingshaense* Chen et Guo, *M. anisopliae sensu stricto*, *Metarhizium robertsii* Bischoff, Rehner et Humber, and *Metarhizium brunneum* Petch (Bischoff *et al.*, 2009). Only two *Metarhizium*-based commercial products, GranMet® and Met52®, are available in Italy, both in dry formulation and registered for soil applications only. Moreover, only GranMet® is registered against JB. These two products are both based on the same single strain of *M. brunneum* alternatively indicated as Bipesco5 or F52 (Mayerhofer *et al.*, 2015).

Based on all these considerations, our aim was to test the possibility of infecting JB adults by attracting beetles inside a simple device baited with the standard double lure attractant and activated with a commercially available *Metarhizium*.

First step of our work was to set up and test in the field a home-made ‘attract-infect-release’ device alternatively activated by GranMet® and Met52®. To verify the possibility of initiating epizootics within the pest population, the number and germinability of JB adult-borne conidia were then assessed in the laboratory, as well as the effectiveness of horizontal transmission.

## Materials and methods

### ‘Attract-infect-release’ device and field bioassays

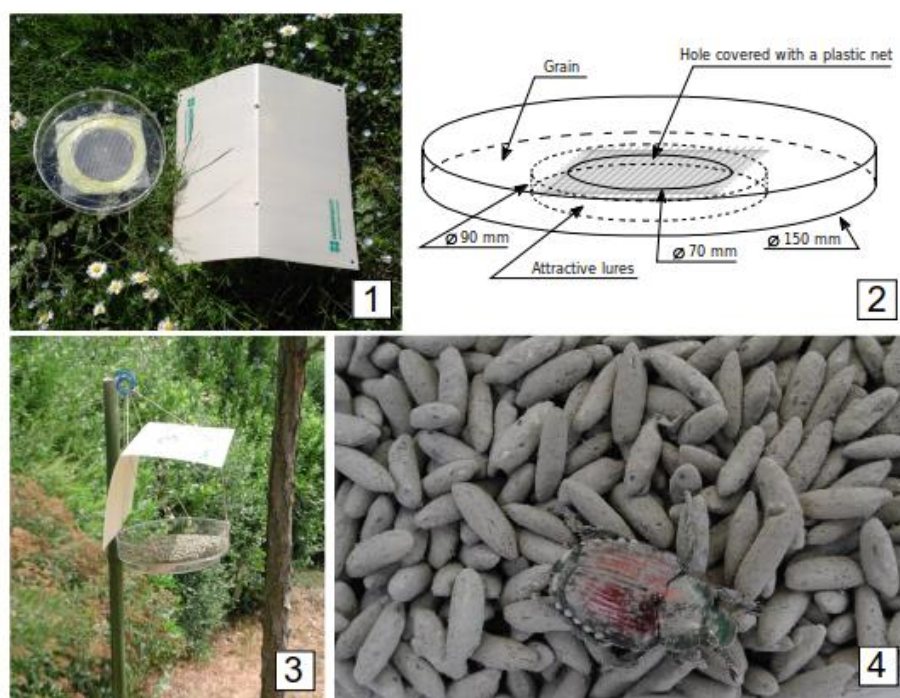
The work was carried out from 27<sup>th</sup> to 29<sup>th</sup> of June 2017, at Villa Picchetta, Cameri, which is located in a JB-infested area of Novara province (Italy, Piedmont region, 45°30'N 08°41'E). Climatic data information

were obtained from the records of Cameri weather station.

The ‘attract-infect-release’ device (figure 1-1) consisted of the bottom half of a large plastic Petri dish (150 mm diameter) with a 70 mm diameter hole in the centre. This hole was covered by a fine plastic net (mesh 1 × 1 mm) and was coupled on the bottom with the base of a 90 mm plastic Petri dish (figure 1-2) in order to accommodate a commercial lure dispenser (Trécé Inc., Adair, OK, USA) that combines JB sex pheromone [(R,Z)-5-(1-decenyl) dihydro-2(3H)-furanone] and floral attractant (phenethyl propionate:eugenol:geraniol, 3:7:3). This lure is specific to both JB males and females (Chen *et al.*, 2013). The 150 mm dish was filled with a thin layer (80 grams) of either a bioinsecticide (GranMet®, Agrifutur srl, Alfianello (BS), Italy, or Met52®, Novozymes BioAg Ltd, Saskatoon, Canada) or commercial brown rice as untreated control. A pale-yellow plastic panel was set above the device working as a ‘roof’ (figure 1-3).

Nine of such devices were placed on the ground in a grid (40 × 40 m, 20 meters from each other) according to a Latin square experimental design with two treatments and a control in each row and column. Devices were set at a height of 1.5 meters off the ground hanging from bamboo poles.

As the devices were set in place, the first ten individuals visiting those activated with GranMet® and the first ten visiting those activated with Met52® (20 insects in total, no insects were collected from the control devices) were captured after being in touch with the inoculating substrate. Insects were singly picked up when leaving the device, stored inside a sterile 15 ml screw-capped plastic tube, and brought to the lab to count the number of conidia attached to the insect body and assess the proportion of conidia successfully germinating.



**Figure 1.** The ‘attract-infect-release’ device (1-2); the same positioned in the field (3). A JB adult inside the device walking on the infecting substrate and dusted with *Metarhizium* conidia (4).

To assess the day-light flight activity of JB, devices were also checked at three different times (morning, 11:00; midday, 13:00; afternoon, 15:00) and the number of adults present in each device at that moment (snapshot) was recorded too (Lacey *et al.*, 1994a). The number of insects visiting and leaving the device (visit & fly) during a fifteen-minute time lapse and the duration of permanence inside the device were also recorded. The sex of the beetles visiting the devices was not determined to avoid any interference with the test (sexing needs specimen handling). However, sex ratio can be considered approximately 50% since it is well known that the selected lures attract both males and females in the same way (Klein *et al.*, 1981; Switzer *et al.*, 2009; Chen *et al.*, 2013). To bypass the possible effects of the position within the grid, the experiment was repeated during three following days, modifying the spatial disposition of devices. In this way, each treatment was repeated in each point of the grid.

### Laboratory bioassays

Adults collected from GranMet<sup>®</sup>- and Met52<sup>®</sup>-activated devices were washed one by one in 1 ml of a 0.02% Tween<sup>®</sup> 80 water solution shaken with a vortex mixer (¾ speed) for five minutes. The concentration of conidia in the washing solution was then determined by a Thoma-Zeiss haemocytometer (Inglis *et al.*, 2012). The germination rate at 24 °C of these conidia was determined after 24 hours according to Liu *et al.* (2003). Spores with a germ tube longer than their width were considered as germinated (Hywel-Jones and Gillespie, 1990).

### Horizontal transmission trials

Virgin adults from pupae collected in the natural environment of locations where commercial EPNs or EPF were never used, were reared in 50 ml screw-capped plastic tubes containing 15 ml of wet soil and used in the transmission trials. Emerging adults were transferred separately into plastic cups covered with a perforated screw cap and filled with fresh hazel (*Corylus avellana* L.) leaves as food supply. All the experiments were performed at room temperature (about 24 °C).

Horizontal transmission during mating between infected and uninfected adults was studied according to the following scheme: infected male x uninfected female (MixF), uninfected male x infected female (MxFi), infected male x infected female (MixFi), and uninfected male x uninfected female (MxF) as a control (Quesada-Moraga *et al.*, 2008). Each thesis consisted of ten couples of insects. Batches of ten virgin male and ten virgin female JB adults were separately exposed to the bioinsecticide inside plastic boxes with perforated lid containing 80 grams of either GranMet<sup>®</sup> or Met52<sup>®</sup>. Control adults were exposed to uninfected commercial brown rice. After three minutes (average time of activity inside a device in the field) couples of insects were formed according to the previous scheme. Each couple was isolated for one hour at room temperature (about 24 °C) inside a plastic cup covered with a perforated screw cap and filled with fresh hazel leaves as a food supply. Couples were then separated, and each adult

was isolated into a plastic cup as described above and fed with fresh hazel leaves (three times per week) until death. Mortality was daily assessed till the end of the experiment (21 days). Dead adults were first processed by external sterilization in a 1% sodium hypochlorite water solution and three rinses in sterile distilled water; then they were separately placed in Petri dishes lined with sterile moistened filter paper. Successful fungal infection was confirmed by external sporulation of the fungus from insect cadavers (Llácer *et al.*, 2013).

### Data analysis

The percentage of adult survival was corrected using Abbott's formula (Abbott, 1925) to obtain the mortality rate due to the pathogen infection. Survival data throughout the trial were processed with Kaplan-Meier survival analysis; to compare curves, the Kaplan-Meier Log Rank pairwise over strata procedure was used. The linear interpolation showed LT<sub>50</sub> and LT<sub>90</sub> (Marcus and Eaves, 2000). Upon checking the assumption of normality (test of Kolmogorov-Smirnov), data related to adults visiting the 'attract-infect-release' device were analysed using one-way analysis of variance (ANOVA). Data concerning the number and germinability of conidia transported by insects conformed to assumption of normality and were processed by means of Student's *t* test (significance at *P* = 0.05). SPSS 15.0 software was used for statistical analysis.

## Results

### 'Attract-infect-release' device

A sunny weather with mean temperature of 22°C (min 16 °C - max 28 °C), 72% RH and up to 12 Km/h wind was registered during the experiments.

As regards the number of beetles in the 'snapshots', no statistical difference emerged between control, GranMet<sup>®</sup> and Met52<sup>®</sup> (*F* = 0.00, *df* = 2, *P* = 0.99) (table 1).

No statistical difference emerged between the different times of the day (morning, midday and afternoon) as well (*F* = 2.06, *df* = 2, *P* = 0.13).

### Laboratory bioassays

There were no significant differences in the average number of conidia present on insects infected in the 'attract-infect-release' devices (t-test: *t* = -0.84, *df* = 21, *P* = 0.41) (table 1). A highly significant statistical difference was observed for the germination rate of conidia: 52% vs. 5% for GranMet<sup>®</sup> and Met52<sup>®</sup>, respectively (independent samples t-test: *t* = 6.50, *df* = 8, *P* = 0.00).

### Horizontal transmission trials

A mortality of 10% was recorded in the control (MxF), while 100% of mortality was achieved within 19 days for all trials associated with GranMet<sup>®</sup> (MixFi, 9 days; MxFi, 16 days; MixF, 19 days). At the end of the experiment (21 days after treatment), a 100% mortality was never achieved for trials associated with Met52<sup>®</sup>. In this case, a final cumulative mortality of 65%, 45% and 30% for MixFi, MxFi and MixF,

**Table 1.** ‘Attract-infect-release’ devices: number of insects in the ‘snapshot’; number of ‘visit & fly’ events and mean permanence time in minutes; number of conidia ( $\times 10^6$ ) collected by insects that visited the devices.

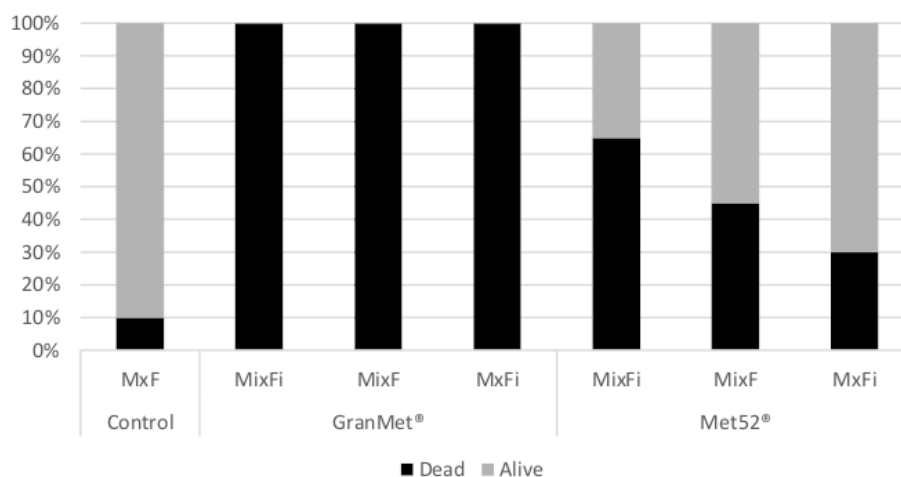
‘Snapshot’ - Number of insects		Mean $\pm$ SE
	Control	7.29 $\pm$ 1.52
	GranMet <sup>®</sup>	7.18 $\pm$ 1.79
	Met52 <sup>®</sup>	7.22 $\pm$ 1.50
	Morning	5.14 $\pm$ 1.21
	Midday	9.62 $\pm$ 1.86
	Afternoon	6.92 $\pm$ 1.57
‘Visit & fly’		Mean $\pm$ SE
	N <sup>o</sup> of events/device	23.66 $\pm$ 0.65
	Permanence time (N = 21) (minutes)	3 $\pm$ 0.79
‘Attract-infect-release’ N <sup>o</sup> of conidia $\times 10^6$		Mean $\pm$ SE
	GranMet <sup>®</sup> (N = 10)	1.74 $\pm$ 0.57
	Met52 <sup>®</sup> (N = 10)	2.45 $\pm$ 0.59

respectively (figure 2), was recorded. All dead individuals showed external sporulation of *Metarhizium* once incubated in wet-chamber and no significant difference between numbers of dead males and females occurred ( $P = 0.75$ ; data not shown). However, survival analysis gave highly significant differences for GranMet<sup>®</sup> (Log Rank test:  $\chi^2 = 55.21$ ,  $df = 3$ ,  $P = 0.00$ ) and Met52<sup>®</sup> experiments (Log Rank test:  $\chi^2 = 13.22$ ,  $df = 3$ ,  $P = 0.00$ ) (table 2). In particular, MxF treatment significantly differed from MixFi. MixF and MxFi showed an outcome with intermediate values between MxF and MixFi, in both GranMet<sup>®</sup> and Met52<sup>®</sup> (table 3); however, no statistical difference emerged between them.

## Discussion and conclusion

In protected areas, such as the Ticino Natural Park, Integrated Pest Management is probably the best tool to preserve crops minimizing both environmental pollution and the impact on non-target organisms. Even if it cannot be considered totally safe (Toledo-Hernandez *et al.*, 2016), *M. anisopliae s. l.* seems to pose a very low threat to pollinators and beneficial insects as reported by several authors (Alves *et al.*, 1996; Hamiduzzaman *et al.*, 2012; Smagghe *et al.*, 2013).

Several authors verified the efficacy of EPF, and that of *M. anisopliae s. l.* in particular, against JB adults (Lacey *et al.*, 1994b; Giroux *et al.*, 2015).



**Figure 2.** Cross contamination trials: percentages of mortality.

**Table 2.** Horizontal transmission trials: results of the Kaplan-Meier survival analysis (average survival time  $\pm$  standard error) and mortality percentages corrected by Abbott.

Treatment	Ast $\pm$ SE	GranMet <sup>®</sup>			Met52 <sup>®</sup>			Abbott
		LT50* days	LT90* days	Abbott	Ast $\pm$ SE	LT50* days	LT90* days	
Control MxF	19.90 $\pm$ 0.76	n.d.	n.d.	n.d.	19.90 $\pm$ 0.76	n.d.	n.d.	n.d.
MixFi	7.50 $\pm$ 0.19	6.6	8.6	100%	15.52 $\pm$ 1.39	13.0	n.d.	61%
MixF	9.15 $\pm$ 1.29	8.4	16.0	100%	18.70 $\pm$ 1.18	n.d.	n.d.	22%
MxFi	9.15 $\pm$ 1.22	8.5	15.5	100%	18.55 $\pm$ 1.26	n.d.	n.d.	39%

\* Lethal time (LT<sub>50</sub> and LT<sub>90</sub>) obtained by linear interpolation according to Marcus and Eaves, 2000.

**Table 3.** Horizontal transmission trials: Mantel-Cox Log Rank test results.

	Control MxF		MixFi		MixF	
	$\chi^2$	P value	$\chi^2$	P value	$\chi^2$	P value
<b>GranMet®</b>						
Control MxF (a)	-	-	-	-	-	-
MixFi (b)	38.210	0.000	-	-	-	-
MixF (c)	35.429	0.000	4.084	0.043	-	-
MxFi (c)	33.752	0.000	4.468	0.035	0.067	0.796
<b>Met52®</b>						
Control MxF (a)	-	-	-	-	-	-
MixFi (b)	10.730	0.001	-	-	-	-
MixF (ac)	1.465	0.266	4.097	0.026	-	-
MxFi (bc)	5.448	0.020	1.736	0.188	1.265	0.261

Within columns, these followed by the same letter are not significantly different from each other.

Our study was set up to test the effectiveness of the *Metarhizium* commercial products available in Italy against JB. In this perspective, and since our previous preliminary lab experiments showed a low efficacy against JB larvae in soil, the development of an alternative technique compared to soil application is extremely important. This is the first time in Italy that a trial with EPF has been performed targeting adults. Taking a cue from literature reports, our study has deeply analysed the actual possibility of distributing inocula through direct contact between contaminated and healthy adults (Klein and Lacey, 1999; Vega *et al.*, 2007; Kaya and Vega, 2012; Lacey *et al.*, 2015) validating this assumption as a tool for the adult control.

The lack of significant differences in the number of visits between the control devices and the treated ones, highlighted the absence of repellence by *Metarhizium*-based commercial products on JB. This aspect is crucial for the success of the proposed control method and can be considered as a prerequisite for the development of a new commercial device to control JB adults. Furthermore, non-target insects were never found inside the devices during our observations, thus being a proof of the selectivity of the attractant lures.

We analysed the ability to transport and spread conidia in both male and female JB adults, observing no difference between sexes. Under our experimental conditions a significant difference in the germination rate of transported conidia was observed; such a difference could explain the higher mortality caused by GranMet® compared to Met52®.

Our tests confirmed that the inoculum can be transmitted from one infected adult to a healthy one during copulation, thus supporting the idea of an auto-dissemination of the fungus within the pest population that can induce epizootics. Fungi transmission can be increased by the behaviour of both male and female adults that usually display multiple matings and feed on host plants in an aggregated way (Fleming, 1972; Tigreros *et al.*, 2010). Moreover, long lasting matings (till 2 hrs) and the occurrence of homosexual pairings (Barrows and Gordh, 1978) may enhance the spreading of the EPF infection by multiplying contacts between adults.

In this study we verified that, upon a three-minute contact with the inoculum, adults carry enough conidia

to transmit the infection to other individuals and eventually be killed by the fungus. This was particularly evident in GranMet® applications that yielded 100% mortality by 19 days.

The hand-crafted 'attract-infect-release' device tested in this experiment is simple in structure and easy to set up. Additional studies are necessary to assess duration and functionality of the device over time and possibly improve the prototype. Further studies are also being carried out to isolate and test indigenous fungal strains in order to avoid the spreading of a single alien strain by preserving the local biodiversity.

### Acknowledgements

Claudia Benvenuti and Gian Paolo Barzanti contributed equally to the present work.

This work was supported by Piedmont Region Grant/Award number D. n. 1161 - 29/11/2016.

### References

- ABBOTT W. S., 1925.- A method of computing the effectiveness of an insecticide.- *Journal of Economic Entomology*, 18 (2): 265-267.
- AHMAD S., STREU H. T., VASVARY L. M., 1983.- The Japanese beetle: a major pest of turfgrass.- *American Lawn Applicator*, 3 (2): 4-10.
- ALVES S. B., MARCHINI L. C., PEREIRA R. M., BAUMGRATZ L. L., 1996.- Effects of some insect pathogens on the Africanized honey bee, *Apis mellifera* L. (Hym., Apidae).- *Journal of Applied Entomology*, 120: 559-564.
- BARROWS E. M., GORDH G., 1978.- Sexual behaviour in the Japanese beetle, *Popillia japonica*, and comparative notes on sexual behaviour of other scarabs (Coleoptera: Scarabaeidae).- *Behavioral Biology*, 23: 341-354.
- BEHLE R. W., GOETT E. J., 2016.- Dosage response mortality of Japanese beetle, masked chafer, and June beetle (Coleoptera: Scarabaeidae) adults when exposed to experimental and commercially available granules containing *Metarhizium brunneum*.- *Journal of Economic Entomology*, 109 (3): 1109-1115.
- BISCHOFF J. F., REHNER S. A., HUMBER R. A., 2009.- A multi-locus phylogeny of the *Metarhizium anisopliae* lineage.- *Mycologia*, 101 (4): 512-530.

- CHEN R.-Z., KLEIN M. G., SHENG C.-F., LI Y., LI Q.-Y., 2013.- Male and female *Popillia quadriguttata* (Fabricius) and *Protaetia brevitarsis* (Lewis) (Coleoptera: Scarabaeidae) response to Japanese beetle floral and pheromone lures.- *Journal of Asia-Pacific Entomology*, 16: 479-484.
- CITO A., MAZZA G., STRANGI A., BENVENUTI C., BARZANTI G. P., DREASSI E., TURCHETTI T., FRANCARDI V., ROVERSI P. F., 2014.- Characterization and comparison of *Metarhizium* strains isolated from *Rhynchophorus ferrugineus*.- *FEMS Microbiology Letters*, 355: 108-115.
- EU DIRECTIVE 2009/128/EC, 2009.- Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides.- [online] URL: <https://eur-lex.europa.eu/eli/dir/2009/128/oj> (accessed, August 7, 2018).
- FLEMING W. E., 1972.- Biology of the Japanese beetle.- *United States Department of Agriculture. Technical Bulletin*, 1449: 1-129.
- FRANCARDI V., BENVENUTI C., BARZANTI G. P., ROVERSI P. F., 2013.- Autocontamination trap with entomopathogenic fungi: a possible strategy in the control of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera Curculionidae).- *Redia*, 96: 57-67.
- GIROUX F., LAVALLÉE R., BAUCE É., GUERTIN C., 2015.- Susceptibility of the Japanese beetle, *Popillia japonica* (Newman) (Coleoptera: Scarabaeidae), to entomopathogenic Hypocreales fungi.- *Phytoprotection*, 95: 1-6.
- HAMIDUZZAMAN M. MD., SINIA A., GUZMAN-NOVOA E., GOODWIN P. H., 2012.- Entomopathogenic fungi as potential biocontrol agents of the ecto-parasitic mite, *Varroa destructor*, and their effect on the immune response of honey bees (*Apis mellifera* L.).- *Journal of Invertebrate Pathology*, 111: 237-243.
- HESKET H., ROY H. E., EILENBERG J., PELL J. K., HAILS R. S., 2010.- Challenges in modelling complexity of fungal entomopathogens in semi-natural populations of insects.- *BioControl*, 55 (1): 55-73.
- HYWEL-JONES N. L., GILLESPIE A. T., 1990.- Effect of temperature on spore germination in *Metarhizium anisopliae* and *Beauveria bassiana*.- *Mycological Research*, 94 (3): 389-392.
- INGLIS D. G., ENKERLI J., GOETTEL M. S., 2012.- Laboratory techniques used for entomopathogenic fungi: Hypocreales, pp. 189-253. In: *Manual of techniques in invertebrate pathology (2<sup>nd</sup> ed.)* (LACEY L. A., Ed).- Academic Press, Amsterdam, The Netherlands.
- KAYA H. K., VEGA F. E., 2012.- Scope and basic principles of insect pathology, pp. 1-12. In: *Insect pathology (2<sup>nd</sup> ed.)* (VEGA F. E., KAYA H. K., Eds).- Academic Press, Amsterdam, The Netherlands.
- KLEIN M. G., LACEY L. A., 1999.- An attractant trap for auto-dissemination of entomopathogenic fungi into populations of the Japanese beetle *Popillia japonica* (Coleoptera: Scarabaeidae).- *Biocontrol Science and Technology*, 9: 151-158.
- KLEIN M. G., TUMLINSON J. H., LADD JR. T. U., DOOLITTLE R. E., 1981.- Japanese beetle (Coleoptera: Scarabaeidae): response to synthetic sex attractant plus phenethyl propionate: eugenol.- *Journal of Chemical Ecology*, 7 (1): 1-7.
- KOPPENHÖFER A. M., WU S., 2017.- Microbial control of insect pests of turfgrass, pp. 331-341. In: *Microbial control of insect and mite pests* (LACEY L. A., Ed).- Academic Press, Amsterdam, The Netherlands.
- LACEY L. A., AMARAL J. J., COUPLAND J., KLEIN M. G., 1994a.- The influence of climatic factors on the flight activity of the Japanese beetle (Coleoptera: Scarabaeidae): implications for use of a microbial control agent.- *Biological Control*, 4: 298-303.
- LACEY L. A., MARTINS A., RIBEIRO C., 1994b.- The pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* for adults of the Japanese beetle, *Popillia japonica* (Coleoptera: Scarabaeidae).- *European Journal of Entomology*, 91: 313-319.
- LACEY L. A., AMARAL J. J., COUPLAND J., KLEIN M. G., SIMOES A. M., 1995.- Flight activity of *Popillia japonica* (Coleoptera: Scarabaeidae) after treatment with *Metarhizium anisopliae*.- *Biological Control*, 5: 167-172.
- LACEY L. A., GRZYWACZ D., SHAPIRO-ILAN D. I., FRUTOS R., BROWNBIDGE M., GOETTEL M. S., 2015.- Insect pathogens as biological control agents: back to the future.- *Journal of Invertebrate Pathology*, 132: 1-41.
- LIU H., SKINNER M., BROWNBIDGE M., PARKER B. L., 2003.- Characterization of *Beauveria bassiana* and *Metarhizium anisopliae* isolates for management of tarnished plant bug, *Lygus lineolaris* (Hemiptera: Miridae).- *Journal of Invertebrate Pathology*, 82: 139-147.
- LLÁCER E., SANTIAGO-ÁLVAREZ C., JACAS J. A., 2013.- Could sterile males be used to vector a microbiological control agent? The case of *Rhynchophorus ferrugineus* and *Beauveria bassiana*.- *Bulletin of Entomological Research*, 103: 241-250.
- MANIANIA N. K., 2002.- A low-cost contamination device for infecting adult tsetse flies, *Glossina* spp., with the entomopathogenic fungus *Metarhizium anisopliae* in the field.- *Biocontrol Science and Technology*, 12: 59-66.
- MARCUS R., EAVES D. M., 2000.- Statistical and computational analysis of bioassay data, pp. 249-293. In: *Bioassay of entomopathogenic microbes and nematodes* (NAVON A., ASCHER K. R. S., Eds).- CABI Publishing, Wallingford, UK.
- MARIANELLI L., PAOLI F., TORRINI G., MAZZA G., BENVENUTI C., BINAZZI F., SABBATINI PEVERIERI G., BOSIO G., VENANZIO D., GIACOMETTO E., PRIORI S., KOPPENHÖFER A. M., ROVERSI P. F., 2018.- Entomopathogenic nematodes as potential biological control agents of *Popillia japonica* (Coleoptera, Scarabaeidae) in Piedmont Region (Italy).- *Journal of Applied Entomology*, 142: 311-318.
- MARIANELLI L., PAOLI F., SABBATINI PEVERIERI G., BENVENUTI C., BARZANTI G. P., BOSIO G., VENANZIO D., GIACOMETTO E., ROVERSI P. F., 2019.- Long-lasting insecticide-treated nets: a new integrated pest management approach for *Popillia japonica* (Coleoptera: Scarabaeidae).- *Integrated Environmental Assessment and Management*, 15: 259-265.
- MAYERHOFER J., LUTZ A., WIDMER F., REHNER S. A., LEUCHTMANN A., ENKERLI J., 2015.- Multiplexed microsatellite markers for seven *Metarhizium* species.- *Journal of Invertebrate Pathology*, 132: 132-134.
- MAZZA G., PAOLI F., STRANGI A., TORRINI G., MARIANELLI L., SABBATINI PEVERIERI G., BINAZZI F., BOSIO G., SACCHI S., BENVENUTI C., VENANZIO D., GIACOMETTO E., ROVERSI P. F., POINAR G. O. Jr., 2017.- *Hexameris popilliae* n. sp. (Nematoda: Mermithidae) parasitizing the Japanese beetle *Popillia japonica* Newman (Coleoptera: Scarabaeidae) in Italy.- *Systematic Parasitology*, 94: 915-926.
- MORRIS E. E., GREWAL P. S., 2011.- Susceptibility of the adult Japanese beetle, *Popillia japonica* to entomopathogenic nematodes.- *Journal of Nematology*, 43 (3-4): 196-200.
- PAOLI F., MARIANELLI L., BINAZZI F., MAZZA G., BENVENUTI C., SABBATINI PEVERIERI G., BOSIO G., VENANZIO D., GIACOMETTO E., KLEIN M., ROVERSI P. F., 2017a.- Effectiveness of different doses of *Heterorhabditis bacteriophora* against *Popillia japonica* 3<sup>rd</sup> instars: laboratory evaluation and field application.- *Redia*, 100: 135-138.
- PAOLI F., MARIANELLI L., TORRINI G., MAZZA G., BENVENUTI C., BOSIO G., VENANZIO D., TARASCO E., KLEIN M., ROVERSI P. F., 2017b.- Differential susceptibility of *Popillia japonica* 3<sup>rd</sup> instars to *Heterorhabditis bacteriophora* (Italian strain) at three different seasons.- *Biocontrol Science and Technology*, 27: 439-444.

- QUESADA-MORAGA E., MARTIN-CARBALLO I., GARRIDO-JURADO I., SANTIAGO-ÁLVAREZ C., 2008.- Horizontal transmission of *Metarhizium anisopliae* among laboratory populations of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae).- *Biological Control*, 47: 115-124.
- REGIONE PIEMONTE, 2017.- *Popillia japonica* - Piemonte. Aggiornamento dicembre 2017.- [online] URL: [https://www.regione.piemonte.it/web/sites/default/files/media/documenti/2018-12/risultati\\_piano\\_azione\\_2017.pdf](https://www.regione.piemonte.it/web/sites/default/files/media/documenti/2018-12/risultati_piano_azione_2017.pdf);
- SCHEEPMAKER J. W. A., BUTT T. M., 2010.- Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil in relation to risk assessment and in accordance with EU regulations.- *Biocontrol Science and Technology*, 20 (5): 503-552.
- SHAPIRO-ILAN D. I., BRUCH D. J., LACEY L. A., 2012.- Principles of epizootiology and microbial control, pp. 29-72. In: *Insect pathology (2<sup>nd</sup> ed.)* (VEGA F. E., KAYA H. K., Eds).- Academic Press, Amsterdam, The Netherlands.
- SMAGGHE G., DE MEYER L., MEEUS I., MOMMAERTS V., 2013.- Safety and acquisition potential of *Metarhizium anisopliae* in entomovectoring with bumble bees, *Bombus terrestris*.- *Journal of Economic Entomology*, 106 (1): 277-282.
- SWITZER P. V., ENSTROM P. C., SCHOENICK C. A., 2009.- Behavioral explanations underlying the lack of trap effectiveness for small-scale management of Japanese beetles (Coleoptera: Scarabaeidae).- *Journal of Economic Entomology*, 102 (3): 934-940.
- TIGREROS N., JADHAV R., KOWLES K. A., NATHAN B. P., SWITZER P. V., 2010.- Physiological status of male and female *Popillia japonica* (Coleoptera: Scarabaeidae) affects mating and grouping behaviour.- *Environmental Entomology*, 39 (3): 892-897.
- TOLEDO-HERNANDEZ R. A., RUIZ-TOLEDO J., TOLEDO J., SANCHEZ D., 2016.- Effect of three entomopathogenic fungi on three species of stingless bees (Hymenoptera: Apidae) under laboratory conditions.- *Journal of Economic Entomology*, 109 (3): 1015-1019.
- USDA/APHIS, 2015.- *Managing the Japanese beetle: a homeowner's handbook*.- United States Department of Agriculture/Animal and Plant Health Inspection Service, [online] URL: [https://www.aphis.usda.gov/plant\\_health/plant\\_pest\\_info/jb/downloads/JBhandbook.pdf](https://www.aphis.usda.gov/plant_health/plant_pest_info/jb/downloads/JBhandbook.pdf) (accessed, August 8, 2018).
- VEGA F. E., DOWD P. F., LACEY L. A., PELL J. K., JACKSON D. M., KLEIN M. G., 2007.- Dissemination of beneficial microbial agents by insects, pp. 127-146. In: *Field manual of techniques in invertebrate pathology: application and evaluation of pathogens for control of insects and other invertebrate pests (2<sup>nd</sup> ed.)* (LACEY L. A., KAYA H. K., Eds).- Springer, Dordrecht, The Netherlands.

**Authors' addresses:** Gian Paolo BARZANTI (corresponding author, [gianpaolo.barzanti@crea.gov.it](mailto:gianpaolo.barzanti@crea.gov.it)), Claudia BENVENUTI, Leonardo MARIANELLI, Giuseppino SABBATINI PEVERIERI, Francesco PAOLI, Pio Federico ROVERSI, CREA Research Centre for Plant Protection and Certification, via Lanciola 12/A, loc. Cascine del Riccio, 50125 Firenze, Italy; Giovanni BOSIO, Davide VENANZIO, Emanuela GIACOMETTO, Settore Fitosanitario e servizi tecnico-scientifici, Regione Piemonte, c/o Environment Park, Pal. A2, via Livorno 60, 10144 Torino, Italy.

Received January 4, 2019. Accepted August 28, 2019.