Vitis **55**, 83–93 (2016) DOI: 10.5073/vitis.2016.55.83-93

Egg development and toxicity of insecticides to eggs, neonate larvae and adults of *Xylotrechus arvicola*, a pest in Iberian grapevines

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Summary

***Xylotrechus arvicola* (Coleoptera: Cerambycidae) is a xylophagous species that is becoming an important pest in vineyards in the Iberian Peninsula. The most sensitive stages are adult and egg, but their neonate larvae can also be attacked during the first 24 h after hatching and before entering the wood. Adults were evaluated for seven days against the insecticides spinosad, *Beauveria bassiana*, imidacloprid and chlorpyrifos and neonate larvae (< 24 h) and eggs of different ages against the described insecticides, as well as flufenoxuron and pyriproxyfen. All insecticides were applied in a Potter tower at a maximum and minimum commercial dose, and showed significant differences both among insecticides as between the applied doses. Most of the hatching occurs eight days after oviposition by *X. arvicola* females. Chlorpyrifos had a quick and total control of eggs of different ages, neonate larvae and adults in both dosis applied, but its effectiveness could cause serious effects on other non-target species. Pyriproxifen and flufenoxuron had the best ovicidal control when the age of eggs increases and, *B. bassiana* also had a good ovicidal control, due its capacity to invade the eggs actively through their shell and proliferate inside them. Biological insecticides such as *B. bassiana* and spinosad, with a total control on adults and good rates of mortality of neonate larvae and eggs can be a great instrument to biological control of this pest.**

K e y w o r d s : *Xylotrechus arvicola*; eggs; neonate larvae; adults; Potter tower; toxic effect.

Introduction

Xylotrechus arvicola (Coleoptera: Cerambycidae) is a xylophagous species that is becoming an important pest in vineyard, with a great capacity to establish itself in new vineyards (Rodríguez-González 2014), causing the destruction of vines in the main wine-producing regions with Protected Denomination of Origin (PDO) of Iberian Peninsula wines, such as the vineyards of La Rioja Alta and Alavesa (Ocete and Del Tío 1996, Ocete and López 1999), Navarra (Ocete *et al.* 2002), Castilla-La Mancha (Rodríguez and Ocaña 1997), and Castilla y León (Ocete and López 1999, Peláez *et al.* 2001, Moreno *et al.* 2004). García Ruiz *et al.* (2012) described that the action of the larvae, associated with the spread of wood fungi, causes a direct damage (the larvae dig galleries that diminish the plant's capacity to transport sap by reducing the vascular area, resulting in smaller berries, which degrades wine quality by incrementing the proportion of berry skin in overall wine composition) and indirect damage (for fungal attack).

Their most sensitive stages are adult and egg, developed outside the vine plants which have been attacked. But their neonate larvae can also be attacked during the first 24 h after egg hatching has occurred and before they enter the wood. After mating, females of *X. arvicola* lay the eggs, concentrated in cracks or under the rhytidome in the vine wood. The fecundity and viability of eggs are extended over a long period (Rodríguez-González *et al.* 2016a). The location of the eggs enables the emerging larvae to get into the wood without any difficulty, making galleries inside the plant. The eggs are usually protected by the rhytidome or crack. The larvae, once inserted in the wood, are inaccessible to chemical compounds. Another problem is the treatment on *X. arvicola* adults, because it has a pattern of emergency which is very staggered in time (García-Ruiz 2009). Soria *et al.* (2013) described the emergency period between late June and mid-July in vineyards of La Rioja and can be extended until mid-August, Moreno (2005) described this period from March until the end of July in vineyards of Valladolid (Castilla y Leon) and Biurrun *et al.* (2007) in Navarre described the emergency period between 14 May and 26 August in plantations of *Prunus spinosa* L.

An integrated approach against this pest via adaptation of cultural techniques would to be remove the rhytidome (Peláez *et al.* 2006) and pruning the affected branches below the area of galleries (Ocete *et al.* 2004), but these cultural measures are not suitable for an indirect control of *X. arvicola* because it is expensive, and also it is destructive and not sustainable for the cultivation (Peláez *et al.* 2006). Alternative management techniques can be used, such as the change in the training system, using training systems with several branches in which, after the pruning of affected

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branches, it is easier and quicker to train new branches (bush wine training system). However, in vines conducted by trellis (as for example bilateral cordon training system with only two productive branches), if a branch is destroyed by a high level of *X. arvicola* attack, a longer period is needed to train a new branch, which decreases the vine production during this period. Rodríguez-González *et al.* (2016b) concluded that the renovation of attacked branches in vines is easier, with the bush vines training system, than in vines with bilateral cordon training systems.

Biological control techniques can be interesting, although the knowledge of *X. arvicola* natural enemies is still very low. *Xylotrechus* gender is parasitized by species of the Ichneumonidae family (Georgiev and Kolarov 1999, Reagel *et al.* 2012). Specifically, Vives-Noguera *et al.* (2000) described that their larvae can be parasitized by the ichneumonids *Xorides filiformis* Gravenhorst 1829 (Hymenoptera: Ichneumonidae), *Xorides rufipes* Gravenhorst 1829 (Hymenoptera: Ichneumonidae) and the braconid *Doryctes leucogaster* 1834 Nees (Hymenoptera: Braconidae) in forest ecosystems, although its low density suggests that they do not play an important role in their biological control (Georgiev and Kolarov 1999; García-Ruiz 2007). In *X. arvicola* eggs the entomopathogenic fungus *Beauveria bassiana* has been detected (García and Sanchez 2002).

To control *X. arvicola*, it is a priority to choose compounds with a different mode of action, with greater selectivity and less resistance, so as to minimize side effects on predators described by Peláez *et al.* (2012) or parasitoids described by Georgiev and Kolarov (1999), Reagel *et al.* (2012) and Vives-Noguera (2000) improving the environmental cost/benefit ratio of insecticide treatment. Specific compounds to control pests with a low eventual side effect in natural enemies would be entomopathogenic fungi as *Beauveria bassiana* and *Metarhizium anisopliae*, insurances against no object organisms and beneficial insects (Brinkman and Fuller 1999, Cottrell and Shapiro-Ilan 2003, Dunkel and Jaronski 2003). Biological insecticides, such as spinosad, provide a margin of safety for beneficial species or predators, as has been observed after treatment against the braconid parasitoid *Microplitis mediator* (Haliday), natural enemy of the cabbage moth *Mamestra brassicae* (Moens *et al.* 2012). Systemic insecticides such as imidacloprid have accumulated a low mortality after treatment against *Hyposoter didymator* (Thunberg) (Hymenoptera: Ichneumonidae), considered one of the most important native biocontrol agents of noctuids in Spain (Medina *et al.* 2007) as well as Pyriproxifen, with a low toxicity on mammals and high specificity. They are used instead of using non-selective compounds with natural enemies such as Sodium arsenite, which was used in the control of pests and diseases in vineyard, and it was eliminated because of its harmful effects on human health (García-Ruiz 2009) or Piricarb (Moens *et al.* 2012). Until today, no essay has been done in which different insecticides have been evaluated on the *X. arvicola* stages. Different phytosanitary strategies against this pest have been raised through the use of insecticides with a different mode of action, which have provided good results in other beetle insect pests.

In the biological control of other Coleoptera described as pests, entomopathogenic fungi, such as *Beauveria bassiana*, have been able to infect and kill all stages of the coffee borers *Xylotrechus quadripes* Chevrolat (Coleoptera: Cerambycidae), *Acalolepta cervinus* Hope (Coleoptera: Cerambycidae) (Jia-Ning and Rong-Ping 2002) or the red oak borer *Enaphalodes rufulus* (Coleoptera: Cerambycidae) eggs (Meyers *et al.* 2013). *B. bassiana* also has controlled the larvae and adults of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) (Mitsuaki *et al.* 2002), *Saperda populnea* (L.) (Coleoptera: Cerambycidae) (Eken *et al.* 2006). Insecticides from natural derivatives such as spinosad have been used effectively in adult control of *Rhyzopertha dominica* L. (Coleoptera: Bostrychidae) (Athanassiou *et al.* 2011), *Hypocryphalus mangiferae* Stebbing (Coleoptera: Scolytidae) (Saeed *et al.* 2011), *Sitophilus granarius* (Coleoptera: Curculionidae), *Cryptolestes ferrugineus* Stephens (Coleoptera: Laemophloeidae) (Athanassiou *et al.* 2011) and the fruit fly *Ceratitis capitata* (Diptera: Tephritidae) eggs (Adán *et al.* 1996). Inhibitors of embryogenesis such as pyriproxyfen have demonstrated lethal effects on larvae and sublethal front adult effects of *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) (Planes *et al.* 2013) and in *Callosobruchus maculatus* Fabricius 1775 (Coleoptera: Bruchidae) eggs (Abo-Elghar *et al.* 2003).

In the chemical control of other beetles described as pests, insecticides such as imidacloprid have been used, which has proven useful in the embryonic control of insect pests of the order Homoptera, Coleoptera, Lepidoptera, Diptera, Hemiptera and Hymenoptera (Bostanian *et al.* 2010). Imidacloprid also has shown toxicity against *Plectrodera scalator* (F.) (Coleoptera: Cerambycidae) and *Anoplophora glabripennis* Motschulsky (Coleoptera: Cerambycidae) larvae (Poland *et al.* 2006), and against *Acalolepta vastator* (Coleoptera: Cerambycidae) (Goodwin 2005), *Hypocryphalus mangiferae* Stebbing (Coleoptera: Scolytidae) (Saeed *et al.* 2011) and *Anoplophora glabripennis* Motschulsky (Coleoptera: Cerambycidae) adults (Poland *et al.* 2006). Chlorpyrifos has been classified moderately toxic against all stages of *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) (Planes *et al.* 2013) and this insecticide has reduced populations of adults of various bark beetles such as *Acalolepta vastator* (Coleoptera: Cerambycidae) (Goodwin 2005) and *Aubeonymus mariaefranciscae* Roudier (Coleoptera: Curculionidae) (Marco and Castañera 1996). Flufenoxuron, which acts on the immature insect stages ("Insect growth regulators", IGRs) has shown good control on *Gonipterus scutellatus* Gyllenhal (Coleoptera: Curculionidae) (Perez-Otero *et al.* 2003, Santolamazza-Carbone and Fernández de Ana-Magan 2004).

The control carried out by insecticides on different insect stages (direct contact) can give an idea of the insecticide that will perform a better control in a field application. Wang *et al.* 2016 evaluated the residual efficacy of four liquid insecticides against *Cimex lectularius* (Hemiptera: Cimidae) on different surfaces and demonstrated that the best residual efficacy of insecticides was obtained in wood. Vine wood can act as a support for insecticides with good residual effect (as for example, *B. bassiana*), providing an

indirect contact with the insect and being able to control the pest. Our group demonstrated (Rodríguez *et al.* 2014) that when different insecticides were applied on eggs and larvae of *X. arvicola* located on branches and trunks of *Vitis vinifera,* the biological insecticides showed a good residual effect. The resistance of insect pest to insecticides is a problem that is ever-increasing and that is related to the frequency of applications and with increasing dosages, resulting in an increase of chemical residues on the hosts of the insects. To prevent the development of this resistance, integrated pest management (IPM) strategies are being used. IPM appears to be a sustainable alternative to the traditional management of pests and is based on the use of safer and more selective insecticides, biological control, and other cultivation techniques and is defined as an economically viable strategy that combines several methods of control to reduce pest populations to tolerable levels, minimizing the effects on people's health and the environment (Ripa *et al.* 2008). Therefore, the aim of the study was to evaluate for the first time in laboratory conditions the susceptibility to insecticides with different modes of action against the sensitive stages of *X. arvicola*, eggs, neonate larvae (< 24 h) and adults, determining which is the insecticide with better results on each stage in order to promote an integrated management of the pest.

Material and Methods

Insects and experimental conditions: *X. arvicola* eggs used in tests were obtained by pairing adults captured in vineyards using interception traps (CROSS-TRAP®, Econex, Murcia, Spain). Adults were matched and put into glass jars (80 mm in diameter and 100 mm high) covering the bottom partly with filter paper in which substrates for oviposition and bowls for drinking (cotton soaked in a solution of organic honey to 10 % in distilled water) were placed. Oviposition substrates (corrugated cardboard nets 120 x 40 mm) were reviewed daily. The eggs were extracted and placed into 55 mm diameter Petri dishes. These plates were covered with aluminium foil ensuring complete darkness. The neonate larvae were obtained after 7-8 d and were extracted with the help of a brush. Collection dates were noted. *X. arvicola* stages (adults, eggs and neonate larvae), before and after the application of the treatments, were kept in a chamber with controlled temperature (24 ± 1 °C), humidity (60 ± 5 %), and subjected to a photoperiod of 16 h of light (luminous intensity of 1000 lux) and 8 h of darkness.

Experiment 1 - *X. arvicola* eggs day of hatching: In order to know accurately the day when most egg hatching occurs after the oviposition, in 2012 *X. arvicola* females were captured from three DO wine-producing regions [(Ribera del Duero (field 1, n = 57 females), Toro (field 2, n = 8 females) and Tierra de León (field 3, n = 22 females)]. Adult insects were paired (one female and one male) and put into glass jars as described previously. Oviposition substrates were reviewed daily and the eggs were extracted into Petri dishes. The egg-laying collection and egg hatching dates were noted. The dead eggs observed in Petri dishes (broken, shrinking, shrivelled or dried) were discarded for insecticide testing.

Experiment 2 - Toxicity of insecticides to different *X. arvicola* stages: Eggs of four different ages (1-2, 3-4, 5-6 and 7-8 day-old), neonate larvae and adults were selected to be tested against insecticides. For each age (of egg) and insecticide treatment, 5 replicates of 10 or 20 eggs were used. For neonate larvae and insecticide treatment, 5 replicates of 10 larvae were used. And finally, for adults and insecticide treatment, 4 replicates of 4 adults were used in each one, and the adults were placed one hour after applying insecticides on Petri dishes. Each replication was placed in a Petri dish. In the covers, 4 holes of 5 mm diameter (20 mm^2) were made to avoid the lethal chamber effect. Daily monitoring was carried out from the application of every treatment, counting the inhibition in the eggs (the eggs were shrank or decreased, suppressing the emergence of larvae and whose metamorphosis was altered) and the mortality in larvae and adults. For the insecticides application, a Potter Tower (Burkard Scientific Limited, Po Box 55 Uxbridge, Middx UB8 2RT, U.K.; Potter 1952) of manual loading coupled to an air compressor was used. The insecticide dissolution volume used in each spray was 1 mL, being applied on Petri dishes to 40 kPa pressure.

Insecticides: Commercial formulations of the following insecticides were tested for activity against *X. arvicola* eggs of different age, neonate larvae and adults: spinosad (Spintor® 480 CC, Dow Science Ibérica S.A.; 48 g of a.i. per liter) at 25 $mL \cdot hL^{-1}$ and 20 $mL \cdot hL^{-1}$, *Beauveria bassiana* (Bassi® WP, Massó S.A.; 22 g of 'active ingredient' (a.i. per 100 g) at 125 $g \cdot hL^{-1}$ and 62.5 $g \cdot hL^{-1}$, imidacloprid (Confidor® 20 LS, Bayer Crop Science S.L.; 20 g of a.i. per liter) at 100 $mL \cdot hL^{-1}$ and 50 $mL \cdot hL^{-1}$, chlorpyrifos (Cúspide® 48, Massó S.A.; 48 g of a.i. per liter) at 200 $mL \cdot hL^{-1}$ and 150 $mL \cdot hL^{-1}$, pyriproxyfen (ATOMINAL® 10 EC, Massó S.A.; 10 g of a.i. per liter) at 75 $mL \cdot hL^{-1}$ and 50 $mL \cdot hL^{-1}$ and flufenoxuron (KIMLUX®, Sapec Agro S.A.U. 10 g of a.i. per liter) at 100 $mL \cdot hL^{-1}$ and 50 $mL \cdot hL^{-1}$. All the insecticides were applied at maximum and minimum commercial dose (Tab. 1) and diluted or suspended in distilled water, which was used as the control treatment. All used insecticides are active by contact and ingestion, except chlorpyrifos that in addition is also active *via* inhalation (gas phase).

Data analysis: Data were subjected to variance analysis. Mean comparisons were performed using the Tuckey test to examine differences ($P < 0.05$) among insecticides and doses. All analyses were performed using in SAS version 9.1.2 software (SAS Institute Inc., 2004, Cary, NC, USA).

Results

Experiment 1 - *X. arvicola* eggs day of hatching: The daily percentage of *X. arvicola* hatched eggs after the oviposition (Fig. 1). The highest egg hatching occurs on the eighth day (32.5 %) after the oviposition for all tested fields. Regarding these fields, on the eighth day, the eggs laid by females captured on field 2 had the higher number of eggs hatched (40.1 %), being significantly different ($F_{2,84} = 3.465$, $P = 0.036$) than the eggs laid by females

Table 1

Insecticides, actives substances, doses of six insecticides in vineyard

Insecticide	Active substance	Dose/s authorized in Spain	
		Maximun	Minimum
Spintor® 480 CC [a]	Spinosad (480 g L^{-1})	0.025 L·hL^{-1}	0.020 L·hL^{-1}
Bassi® WP [a]	Beauveria bassiana (220 g L^{-1})	125 g·hL^{-1}	62.5 g·hL^{-1}
Confidor® 20 LS [a]	Imidacloprid (200 g L^{-1})	0.100 L·hL^{-1}	0.050 L·L^{-1}
Cúspide® 48 [a]	Chlorpyrifos (480 g L^{-1})	0.200 L·hL^{-1}	0.150 L·hL^{-1}
KIMLUX® [b]	Flufenoxuron (100 g L^{-1})	0.100 L·hL^{-1}	0.050 L·hL^{-1}
ATOMINAL® 10 EC [a]	Pyriproxifen (100 g L^{-1})	0.075 L·hL^{-1}	0.050 L·hL^{-1}

[a] Authorised doses in Spain (MAGRAMA 2012).
[b] Authorised doses in Spain (MAGRAMA 2015).

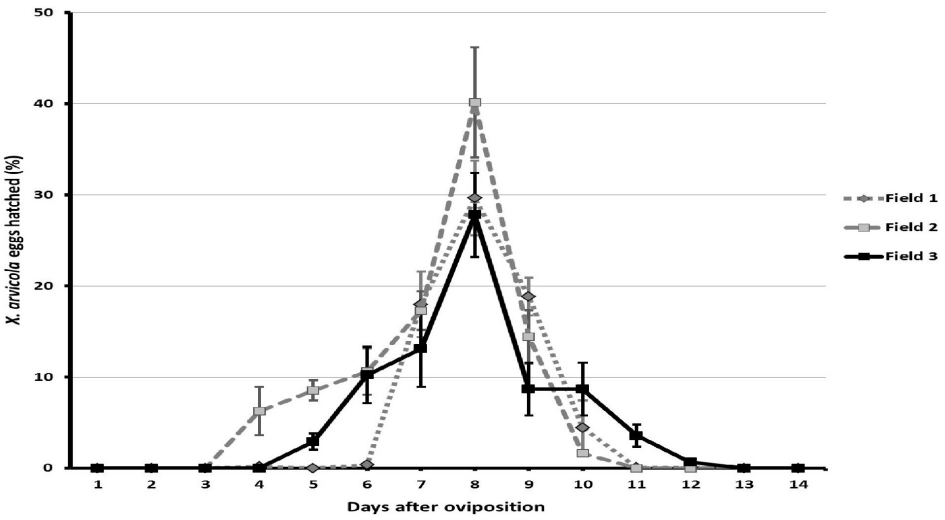

Fig. 1: Percentages of hatched eggs each day after oviposition for females of *X. arvicola* from different fields. Upper and lower error bars are represented (ANOVA, Tukey; $P \geq 0.05$).

from field 1 (29.6 %) and field 3 (27.8 %). The accumulated emergence rates on the eighth day were 48.1 % (field 1), 82.8 % (field 2) and 54.0 % (field 3). On day fourteen, the accumulated emergence rates were 71.5 % (field 1), 98.8 % (field 2) and 75.6 % (field 3).

Experiment 2: Toxicity of insecticides to different *X. arvicola* stages:

- Eggs: Chlorpyrifos had a total ovicidal control on all *X. arvicola* egg ages evaluated in both doses applied, differing significantly from the rest of insecticides on eggs 1-2 day-old (maximum dose) and eggs 7-8 day-old (minimum dose) (Tab. 2, lowercase letters). Chlorpyrifos did not show significant differences among eggs of different ages (Fig. 2). *B. bassiana* (97.0 % of eggs inhibited) show the best ovicidal control on older eggs (7-8 day-old) at maximum dose (significantly different from younger eggs, Fig. 2), not being significantly different from chlorpyrifos (100 %) and pyriproxyfen (95 %), but significantly higher than imidacloprid (86 %), flufenoxuron (83 %), spinosad (77 %) and the control treatment (13 %) (Tab. 2, lowercase letters). Pyriproxifen had the best ovicidal control at maximum dose on eggs 7-8 day-old, inhibiting a 95 % of eggs, not being significantly different from chlorpyrifos (100 %), but significantly higher than imidacloprid (86 %), flufenoxuron (83 %), spinosad (77 %) and the control treatment (13 %) (Tab. 2, lowercase letters). Imidacloprid (92.0 % of eggs inhibited) show the best ovicidal control at maximum dose on eggs 5-6 day-old, only significantly different from flufenoxuron (78 %) and the control treatment (16 %) (Tab. 2, lowercase letters). imidacloprid showed the worst ovicidal control over the youngest eggs (1-2 day-old), being significantly different from the rest of egg ages (Fig. 2). Flufenoxuron had the highest ovicidal control on eggs 3-4 and 7-8 day-old at maximum dose, inhibiting 82 % and 83 % of eggs respectively. These values were significantly different from the two best insecticides in the same eggs ages evaluated (chlorpyrifos and pyriproxyfen) (Tab. 2, lowercase letters). Spinosad was able to inhibit 82 % of 5-6 day-old eggs, being the best ovicidal control at maximum dose, significantly different from the best insecticide in the same egg age evaluated [chlorpyrifos (100 %)]. Spinosad, at maximum commercial dose, was

Table 2

Inhibition (%) ± Standard Error of *X. arvicola* eggs of different ages exposed during 7 days to different toxicity insecticides at maximum and minimum commercial dose and different exposure periods

Insecticides	Maximum Dose				Minimum Dose
Egg Stage	1-2 days [b]	3-4 days [c]	5-6 days [c]	7-8 days [d]	7-8 days [d]
Chlorpyrifos	100.00 ± 0.00a [a]	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00aA[e]	100.00 ± 0.00aA[e]
Pyriproxyfen	87.00 ± 4.35b	92.00 ± 2.00ab	88.00 ± 3.74bc	95.00 ± 1.58aA	63.00 ± 4.35cB
B. bassiana	86.00 ± 5.56b	80.00 ± 3.16c	86.00 ± 2.44bc	97.00 ± 2.00aA	87.00 ± 5.83bA
Flufenoxuron	81.00 ± 2.44bc	82.00 ± 3.74c	78.00 ± 4.89c	83.00 ± 4.63bcA	75.00 ± 5.91bcA
Imidacloprid	77.00 ± 4.84c	86.00 ± 2.44bc	92.00 ± 3.74ab	86.00 ± 1.87bA	79.00 ± 2.91bA
Spinosad	48.00 ± 4.06d	78.00 ± 5.83c	82.00 ± 2.00bc	77.00 ± 4.63cA	75.00 ± 3.87bcA
Control	12.00 ± 2.54e	8.00 ± 3.74d	16.00 ± 2.44d	13.00 ± 2.54dA	11.00 ± 4.00dA
F	38.78	46.43	28.49	92.37	29.90
df	(6.28)	(6.28)	(6.28)	(6.28)	(6.28)
P	< 0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a] Data are expressed as percentage ± standard error. When followed by the same lowercase letter, there are no significant differences among insecticides for the same egg age (ANOVA, Tukey; $P \geq 0.05$).

[b] Initial number of *X. arvicola* eggs were 20 per replicate and 5 replicates were considered per treatment on eggs 1-2 days old.

[c] Initial number of *X. arvicola* eggs were 10 per replicate and 5 replicates were considered per treatment on eggs 3-4 and 5-6 days old.

[d] Initial number of *X. arvicola* eggs were 20 per replicate and 5 replicates were considered per treatment on eggs 7-8 days old.

[e] Data are expressed as percentage ± standard error. When followed by the same capital letter, there are no significant differences between insecticides at different dose in the seventh day (ANOVA, Tukey; $P \geq 0.05$).

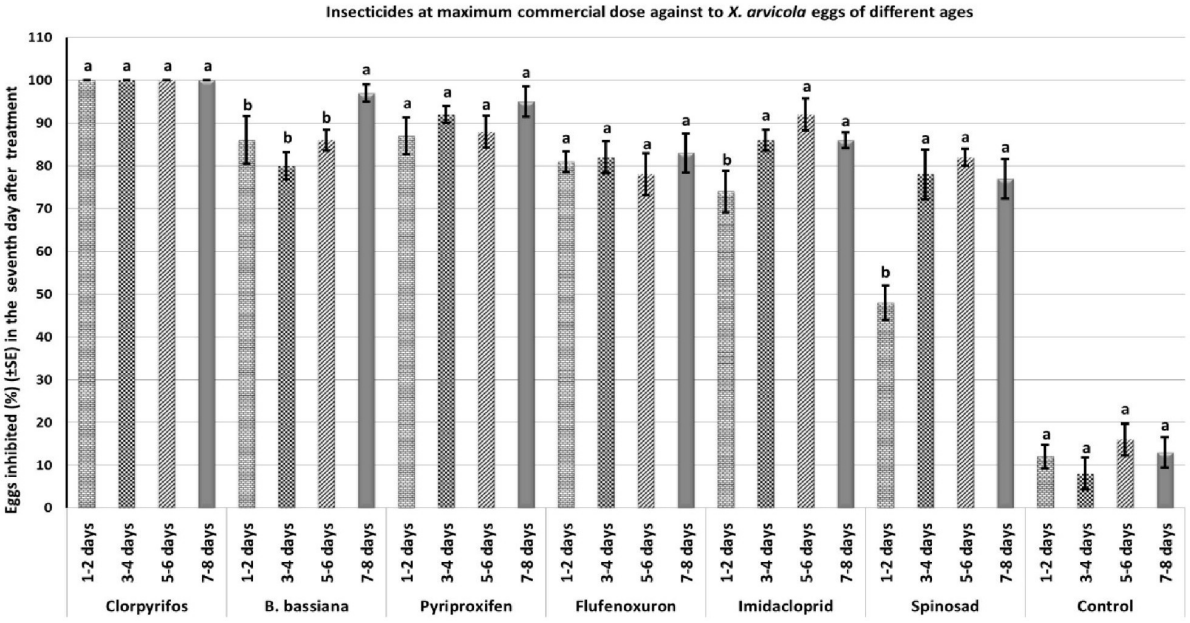

Fig. 2: *X. arvicola* eggs of different ages inhibited by different insecticides at maximum commercial dose in the seventh day after treatment. Upper and lower error bars are represented. Bars with the same lowercase letter, are not significantly different among eggs of different ages in the same insecticide (ANOVA, Tukey; $P \geq 0.05$).

the insecticide with the worst ovicidal control on eggs 1-2 (48 %), 3-4 (78 %) and 7-8 day-old (77 %). Also it had the worst ovicidal control at minimum dose over 7-8 day-old eggs (75 %) (Fig. 2 and Tab. 2 lowercase letters). The control treatment had a low ovicidal control, differing significantly from the rest of insecticides on all egg ages evaluated in both doses applied (Tab. 2, lowercase letters). When evaluated the applied doses for each insecticide on eggs 7-8 day-old on the seventh day after the treatment, only Pyriproxifen showed significant

differences between maximum (95 %) and minimum (63 %) commercial dose (Tab. 2, capital letters).

- Neonate larvae (maximum commercial dose): Chlorpyrifos had a total control of this stage with a mortality of 100 % from the 2nd to the 7th day and was the insecticide that had the best results, not differing significantly from flufenoxuron and imidacloprid. Flufenoxuron (98 %) and imidacloprid (96 %) had practically a total control of this stage, not differing significantly from chlorpyrifos. Pyriproxifen had a good larvae control capacity with 96 % mortality, being significantly different from chlorpyrifos. *B. bassiana* achieved a larvae mortality of 86 % on the 7th day and was the insecticide with the 2nd worst result for larvae mortality, not significantly different from spinosad (the worst insecticide in this stage) nor with pyriproxyfen, insecticide with larvae activity. Spinosad showed larvae mortality of 84 % on the 7th day and was the insecticide with the lowest larvae mortality, significantly different from the rest of insecticides, except *B. bassiana*. The control treatment on neonate larvae produced a low larval mortality of 18 %, differing significantly from the rest of insecticides from the first day after the application of treatments (Fig. 3).
- Neonate larvae (minimum commercial dose): Chlorpyrifos and flufenoxuron had a total control of this stage, both insecticides had a mortality of 100 %, differing significantly from pyriproxyfen, imidacloprid and *B. bassiana*. Pyriproxifen (92 %) and imidaclorpid (90 %) had a good larvae control capacity, being significantly different from chlorpyrifos and flufenoxuron. *B. bassiana* achieved a larvae mortality of 88 % on the 7th day and was the biological insecticide with the second worst result for larvae control, significantly different from spinosad but not differing significantly from pyriproxyfen and imidacloprid. Spinosad had a low larvae mortality of 62 % on the 7th day and was the biological insecticide with the worst larvae mortality, significantly different from the rest of insecticides. The control treatment produced a low larval mortality of 18 %, differing significantly from the rest of insecticides from the first day after the application of treatments (Fig. 4). Between insecticides and commercial doses applied against *X. arvicola* neonate larvae on

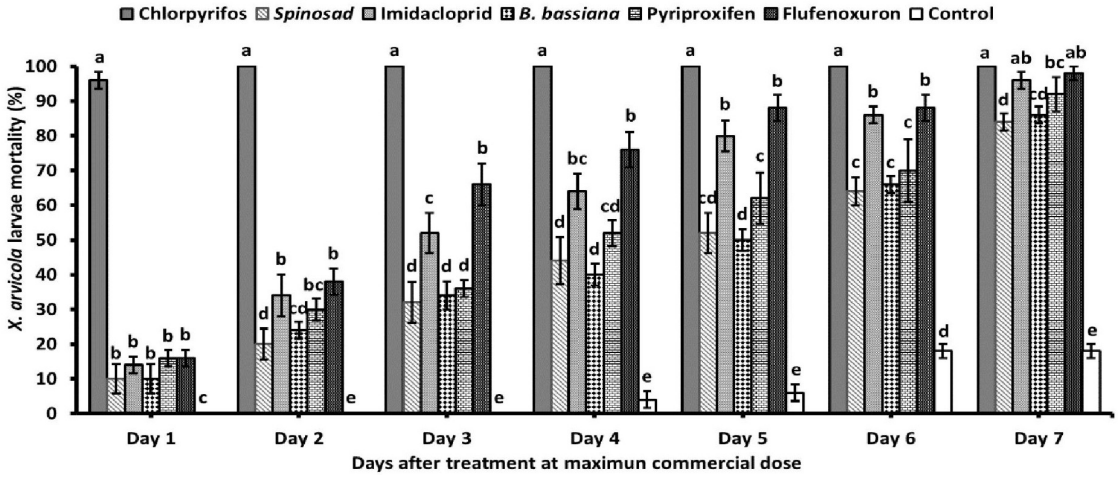

Fig. 3: Mean (± SE) *X. arvicola* larvae mortality when treated with Chlorpyrifos (200 mL·hL^{-1}), spinosad (25 mL·hL^{-1}), imidacloprid (100 mL·hL^{-1}), *B. bassiana* (125 g·hL^{-1}), pyriproxifen (75 mL·hL^{-1}), Flufenoxuron (100 mL·hL^{-1}) and control treatment. Values accompanied by the same letter are not significantly different; lowercase letters among insecticides for the same day, Tuckey test, $P \leq 0.05$.

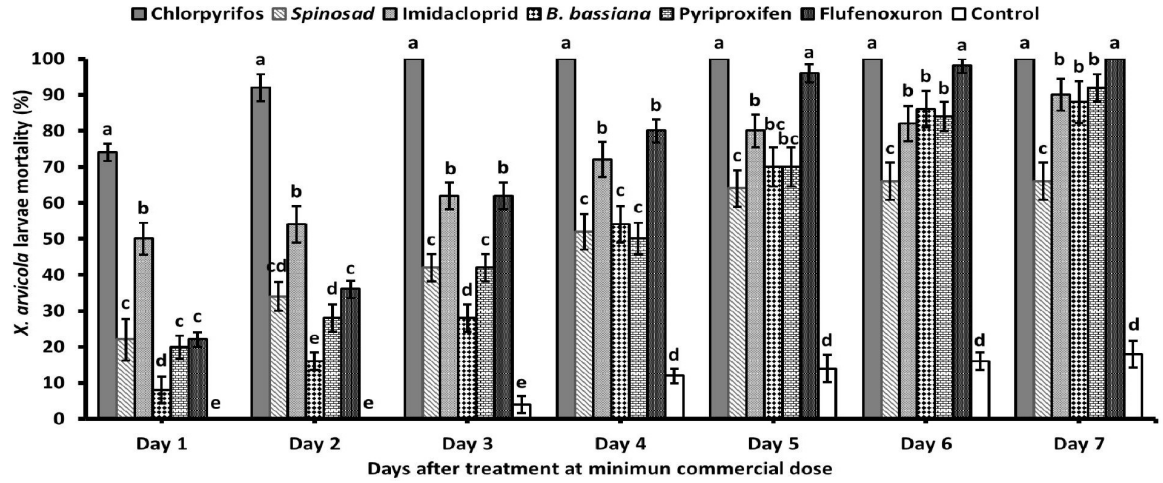

Fig. 4: Mean (± SE) *X. arvicola* larvae mortality when treated with Chlorpyrifos (150 mL·hL^{-1}), spinosad (20 mL·hL^{-1}), imidacloprid (50 mL·hL^{-1}), *B. bassiana* (62.5 g·hL^{-1}), pyriproxifen (50 mL·hL^{-1}), Flufenoxuron (50 mL·hL^{-1}) and control treatment. Values accompanied by the same letter are not significantly different; lowercase letters among insecticides for the same day, Tuckey test, $P \leq 0.05$.

the seventh day after treatment, there were no significant differences between insecticides, except spinosad which had significant differences between maximun (84 %) and minimun (66 %) commercial dose.

- Adults (maximum commercial dose): Chlorpyrifos and the biological insecticides spinosad and *B. bassiana* had a total control of this stage (mortality of 100 %), but showed no significant differences with imidacloprid which achieved a mortality of 93.7 % in the 7th day of evaluation. Chlorpyrifos and spinosad showed total control on the 3rd day of evaluation. The low mortality rates obtained in the control treatment (12.5 %) differed significantly from the rest of insecticides evaluated also from the 1st day after the application of treatments (Fig. 5).
- Adults (minimum commercial dose): Chlorpyrifos, spinosad and *B. bassiana* had a total control of this stage (100 % mortality), but showed no significant differences with imidacloprid which achieved a mortality of 93.7 % on the 7th day of evaluation. Chlorpyrifos and spinosad showed a total control on the 2nd day of evaluation. The low mortality rates obtained in the control treatment (12.5 %) difered significantly from the rest of insecticides evaluated also from the 4th day after the application of treatments (Fig. 6). Between insecticides and commercial doses applied against *X. arvicola* adults, there were no significant differences in the mortality accumulated in the 7th day after treatment.

Discussion

For *X. arvicola* the evolution of hatching eggs is shown after oviposition. Data obtained showed that it is from the sixth day after the oviposition when the majority of eggs begin to hatch, being between the 7th and the 8th day when the greater number of egg hatching is registered. Therefore, it is necessary to know the effects of available insecticides on eggs at these ages to prevent their eclosion and the sub-

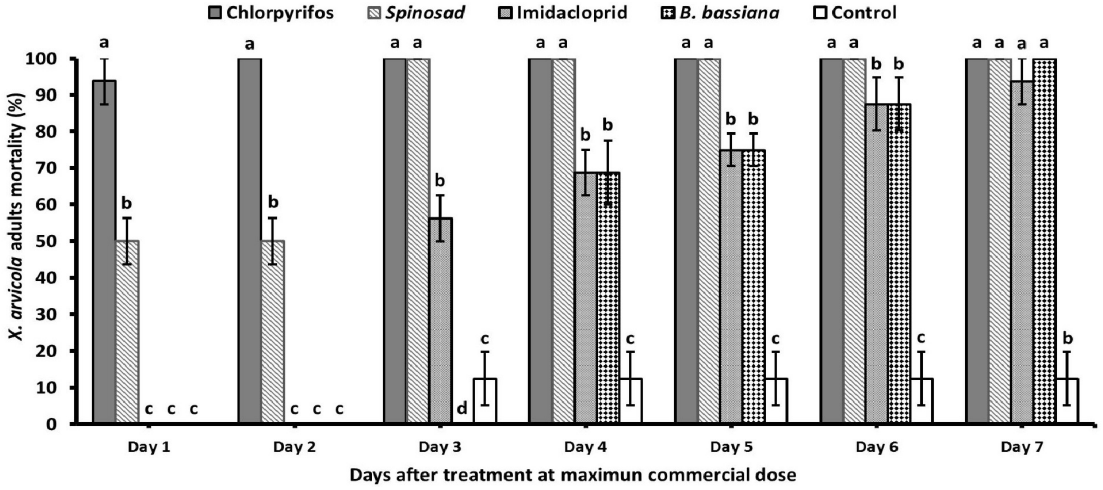

Fig. 5: Mean (± SE) *X. arvicola* adults mortality when treated with Chlorpyrifos (200 mL·hL^{-1}), spinosad (25 mL·hL^{-1}), imidacloprid (100 mL·hL^{-1}), *B. bassiana* (125 g·hL^{-1}) and control treatment. Values accompanied by the same letter are not significantly different; lowercase letters among insecticides for the same day, Tuckey test, $P \leq 0.05$.

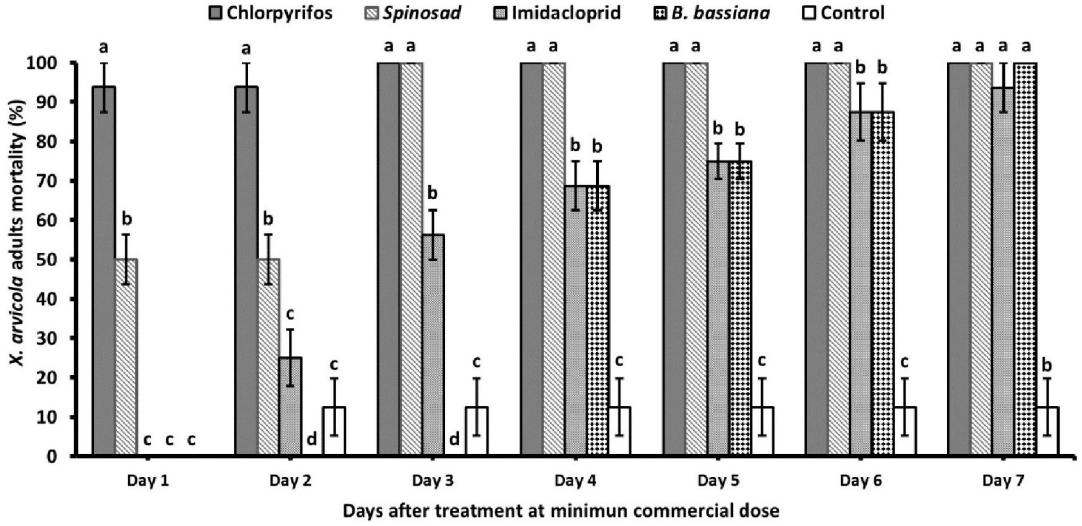

Fig. 6: Mean (± SE) *X. arvicola* adults mortality when treated with Chlorpyrifos (150 mL·hL^{-1}), spinosad (20 mL·hL^{-1}), imidacloprid (50 mL·hL^{-1}), *B. bassiana* (62.5 g·hL^{-1}) and control treatment. Values accompanied by the same letter are not significantly different; lowercase letters among insecticides for the same day, Tuckey test, $P \leq 0.05$.

sequent penetration of larvae into the wood, where their control will be very difficult.

Spinosad showed a low ovicidal capacity against all eggs. Its highest inhibition (82 %) occurred when spinosad was applied on eggs 5-6 day-old, very similar to that described by Adán *et al.* (1996) on *Ceratitis capitata* (Diptera: Tephritidae) eggs (79 % of eggs inhibited). The low percentage of inhibition obtained by spinosad (48 %) on eggs 1-2 day-old, matches as described by Bostanian *et al.* (2010), where the application of spinosad inhibited 38.9 % on *Neoseiulus fallacis* (Acari: Phytoseiidae) young eggs. Spinosad showed better ability to control *X. arvicola* adults (100 % mortality) than larvae applied on both doses (84 % and 66 % mortality). Adán *et al.* (1996) described a larval control of *Ceratitis capitata* (Diptera: Tephritidae) from 84 % when spinosad had been pulverized directly on them, a value similar to that obtained in the trial when spinosad was applied at maximum dose. Long exhibitions (21 d) to spinosad against *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) larvae only achieved a mortality of 89.4 % (Podizi-Metaxa and Athanassiou 2013). Spinosad also had high susceptibility against *Ceratitis capitata* (Diptera: Tephritidae) (Adán *et al.* 1996), *Rhyzopertha dominica* L. (Coleoptera: Bostrychidae) and *Sitophilus oryzae* (Coleoptera: Curculionidae) adults (Athanassiou *et al.* 2011). While Podizi-Metaxa and Athanassiou (2013) evaluated the capacity of spinosad on adults of *Prostephanus truncatus* Horn (Coleoptera: Bostrychidae) only achieved a mortality of 91.4 % respectively, 7 d after treatment. Saeed *et al.* (2011) showed the potential of spinosad against *Hypocryphalus mangiferae* Stebbing (Coleoptera: Scolytidae), one of the most destructive beetle pests of mango trees, but only obtaining a mortality rate of 90 %. Results showed that spinosad decreased the survival on *X. arvicola* eggs when their age advanced, not achieving the desired values and always bettered by the other insecticides evaluated. Still being under their inhibition values, spinosad can be used to reduce the density of eggs that complete their development and subsequently the emergence of neonate larvae, increasing the ability to control the pest. Spinosad is a moderately toxic insecticide against *Coleoptera larvae*, and this toxicity is lower than the conventional insecticides (Galvan *et al.* 2005). Spinosad is a relatively new insecticide of biological origin as compared to traditional insecticides, controlling some pests that have presented resistance to organophosphates and pyrethroids in field conditions and has shown low toxicity for natural enemies of insect pests, so it could be used in strategies for the integrated control of *X. arvicola*, where potential predators described by Peláez *et al.* (2012) or parasitoids described by Georgiev and Kolarov (1999), Reagel *et al.* (2012) and Vives-Noguera (2000) can play a significant role. The selectivity of this insecticide is attributed to changes in nutrition, behaviour of predators (Tillman and Mulrooney 2000, Mori and Gotoh 2001) and parasites (Williams *et al.* 2003), its mode of action and its bioactivation, which could provide a margin of safety for these beneficial species or predators, improving the environmental cost/benefit ratio.The inhibitory activity of *B. bassiana* was very good, its mean inhibition rate being above 80 %, and reaching to 97 % for eggs 7-8 day-old. These ovicidal controls exceed those described by Ren *et al.* (2009) to treat *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) eggs and nymphs, which only achieved an inhibition of 72.9 %, but lower than those obtained by Meyers *et al.* (2013) when treating red oak borer *Enaphalodes rufulus* (Coleoptera: Cerambycidae) eggs obtaining a total control. The larval control obtained by *B. bassiana* against *X. arvicola* neonate larvae at both doses (86 and 88 %), can be considered high. The infected and killed larvae turned pink, rigid to the touch, as well as *Enaphalodes rufulus* (Coleoptera: Cerambycidae) neonate larvae described by Meyers *et al.* (2013). Mitsuaki *et al.* (2002) described *B. bassiana* (strain F-263) for the larval control of *Anoplophora glabripennis* (Coleoptera: Cerambycidae) with a mortality of 50 %, Meyers *et al.* (2013) on *Enaphalodes rufulus* (Coleoptera: Cerambycidae) larvae with *B. bassiana* (strain GHA) which obtained a total control in 4 d after treatment. *X. arvicola* neonate larvae did not die at the moment of application, but several days later when the hyphae of the fungus penetrated the interior of the hemocele of the larvae. The results obtained in both doses with *B. bassiana* against *X. arvicola* neonate larvae are lower than those obtained by Jia-Ning and Rong-Ping (2002) on *Acalolepta cervinus* (Coleoptera: Cerambycidae) and *Xylotrechus quadripes* (Coleoptera: Cerambycidae) neonates larvae, with mortalities of 93 % and 98.7 % respectively during 15 d of exposure. *B. bassiana* showed a more virulent and faster pathogenic effect than *Saperda populnea* (Coleoptera: Cerambycidae) larvae, causing mortalities of 100 % in the 4th day (Eken *et al.* 2006). The larvae of insects, generally, are more susceptible than adults to these types of fungi, since they are thinner than the adult cuticle. *B. bassiana* showed complete control over *X. arvicola* adults, but did not show external growth of the fungus, as the adults of *Enaphalodes rufulus* (Coleoptera: Cerambycidae) described by Meyers *et al.* (2013). It is not uncommon for entomopathogenic fungi to proliferate in the hemocele of insects and not show external growth of the fungus; not so with the contaminants involving the insecticides, such as enteric bacterias, which can propagate through the hemolymph of the insect causing the death of this (Shimazu 1994). Total control obtained on *X. arvicola* adults in both doses exceeds the mortalities obtained on *Xylotrechus quadripes* (Coleoptera: Cerambycidae) with a mortality of 90 % (Jia-Ning and Rong-Ping 2002) and *Enaphalodes rufulus* (Coleoptera: Cerambycidae) which achieved mortalities of 80 % (Meyers *et al.* 2013). The capacity of the entomopathogenic fungi to invade actively live insects through their cuticle and proliferate inside them, make them unique and highly effective tools for the management of insect pests. Other trials with *B. bassiana* on Coleoptera describes a reduction in the diet of *Ootheca mutabilis* Shalberg (Coleoptera: Chrysomelidae) and *Cylas puncticollis* Boheman (Coleoptera: Curculionidae) adults (Ekesi 2001) and is attributed to the production of toxic substances and/or mechanical breaking of the internal structure of insects by the growth of the hyphae of the fungus. *B. bassiana* is considered an insurance against no object organisms and beneficial insects such as predators, parasitoids and honeybees in the field (Brinkman and Fuller 1999, Cottrell and Shapiro-Ilan 2003, Dunkel and Jaronski

2003) which makes it more attractive to conventional insecticides (Liu and Bauer 2006). Imidacloprid had a low inhibition capacity on young eggs (74 % on eggs 1-2 day-old), but obtaining better results when the egg age advanced (86 % on eggs 3-4 and 7-8 day-old). All of them demonstrated a lower ovicidal control at this stage than described by Bostonian *et al.* (2010) on *Neoseiulus fallacis* (Acari: Phytoseiidae) eggs where a 100 % inhibition achieved. Imidacloprid applied to both doses was toxic against *X. arvicola* neonate larvae (values above 90 %), as is described against *Anoplophora glabripennis* Motschulsky (Coleoptera: Cerambycidae) and *Plectrodera scalator* (F.) (Coleoptera: Cerambycidae) larvae, achieving a mortality after 14 weeks of 60 % and 100 %, respectively (Poland *et al.* 2006). The Coleoptera Cerambycidae larvae require long periods against the insecticide so they die until inserted in the wood (Poland *et al.* 2006). Some *X. arvicola* neonate larvae continue to feed themselves and being exposed to the insecticide, not dying until after 7 d. This is typical of many wood drilling larvae, which are able to survive having had little or no food for long periods of time (Linsley 1943, Smith 1962, Haack and Slánský 1986). On adults, imidacloprid in both doses also showed toxic effect as in other cerambycidae adults, as for example *Anoplophora glabripennis* (Poland *et al.* 2006) or *Acalolepta vastator* (Coleoptera: Cerambycidae) (Goodwin 2005). Saeed *et al.* (2011) evaluated imidacloprid against *Hypocryphalus mangiferae* Stebbing (Coleoptera: Scolytidae) adults, reducing the population by 50 % more quickly, 32 h from the application of the treatment, against to 72 h that it took to achieve similar reduction in our essay with both doses.

Chlorpyrifos was the insecticide that had total control on all *X. arvicola* eggs evaluated. Much higher values described by Planes *et al.* (2013) on *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) eggs, 62.8 %. Chlorpyrifos also exercised a total control over *X. arvicola* neonate larvae in both doses, similar to *Cacopsylla melanoneura* Forster (Hemiptera: Psyllidae) larvae (Angeli *et al.* 2009). While Planes *et al.* (2013) obtained worse larval control on *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) larvae, achieving a reduction of 50 % on the population after 40 d. Against *X. arvicola* adults in the first 24 h (mortality of 93.7 % at both doses), exceeded the control described by Saeed *et al.* (2011) against *Hypocryphalus mangiferae* Stebbing (Coleoptera: Scolytidae) with a mortality of 50 %, and that described by Planes *et al.* (2013) on *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) with a mortality of 71.6 %. Chlorpyrifos also had total control over *Aubeonymus adults mariaefranciscae* Roudier (Coleoptera: Curculionidae) (Marco and Castañera 1996). The results showed the great capacity of chlorpyrifos for total control of *X. arvicola* neonate larvae and adults when applied at both commercial doses. Quickly achieved total mortalities can be attributed to the mode of action and its compounds, with nerve toxins (Tomlin 1994). The difference in toxicity among the traditional insecticides (chlorpyrifos) and the other insecticides evaluated in laboratory, suggest that systemic insecticides (imidacloprid) or biological insecticides (spinosad or *B. bassiana*), are less effective in *X. arvicola* control during the first days, but reached a final value similar to that described for organophosphates, being an alternative insecticide for safety and environmental issues. Side effects caused by chlorpyrifos against non-target insect populations, are important. Although chlorpyrifos shows high levels of efficiency in a large number of species, it is also known to cause mortalities in beneficial populations (Corso 1988). The substitution of chemicals such as pyrethroids and the organophosphors may allow the protection of natural enemies, such as the clerid described by Peláez *et al.* (2012) for *X. arvicola* neonate larvae and adults.

The ovicidal insecticide flufenoxuron obtained acceptable values, its inhibition varying from 78 % to 83 %, increasing with the egg age. On eggs that were about 1-2 day-old (81 %), the value obtained was higher than that obtained by Santolamazza-Carbone and Fernández de Ana-Magan (2004) against *Gonipterus scutellatus* Gyllenhal (Coleoptera: Curculionidae) 1-2 d-old eggs (74.5 % eggs inhibited), but in double evaluation period (15 d). The great susceptibility of *X. arvicola* neonate larvae front of flufenoxuron, agrees with what was described by Santolamazza-Carbone and Fernández de Ana-Magan (2004), who treated *Gonipterus scutellatus* Gyllenhal (Coleoptera: Curculionidae) neonates larvae, obtained a mortality of 100 %, from 24 h after treatment after 7 d of exposure. IGR's (Insect Growth Regulators) are more toxic to immature insect stages than adult stages, including many Coleoptera families (Staal 1975, Peleg 1983, Parrella and Murphy 1998). Choosing the ideal time period to apply flufenoxuron against different *X. arvicola* stages is particularly important bearing in mind that its main activity is ovicidal and larvicidal.

Pyriproxyfen showed better ovicidal capacity than its analogue insecticide flufenoxuron, obtaining an inhibition of 87 % on eggs 1-2 day-old, very similar to that exposed by Abo-Elghar *et al.* (2003) to treat *Callosobruchus maculatus* Fabricius 1775 (Coleoptera: Bruchidae) 0-24 h old eggs on bean seeds, with an inhibition of 91.9 %. pyriproxyfen could be used as a highly effective product on *X. arvicola* neonate larvae, obtaining a high degree of control on both doses applied, similar to that described by Planes *et al.* (2013) on *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae), in which a larval mortality of 89.9 %. Abo-Elghar *et al.* (2003) evaluated the residual activity of pyriproxyfen by processing bean seeds which fed adults of *Callosobruchus maculatus* Fabricius 1775 (Coleoptera: Bruchidae), which observed their residual activity on their different biological parameters. Its rate of oviposition was reduced and later a total suppression of the emergence of adults (100 % larval mortality). Pyriproxifen is an insecticide that has good activity on many families of insect at immature stages, allowing it to be used as an effective tool against the immature *X. arvicola* stages, due to the low toxicity to mammals and high specificity.

The low larvae mortality obtained with distilled water was not produced by the application of distilled water, but that it is produced by the starvation of larvae (larvae can not feed). Concerning adults, the mortality was also low, and these deaths were due to lack of food or senescence of adults introduced to the test.

In conclusion, most of the hatching occurs eight days after oviposition by *X. arvicola* females. Chlorpyrifos shows

a quick and total control of *X. arvicola* eggs of different age, neonate larvae and adults in both doses applied (however it would only be recomended at minimum commercial dose in vineyards with very high rates of infestation) but its effectiveness collides with the serious effects on other non-target species and its use may be prohibited in the near future in many countries. Biological insecticides such as *B. bassiana* and spinosad should be preferably used in the field; these biological insecticides show a total control on adults and good rates of mortality of neonate larvae and eggs. The mode of action of *B. bassiana* and spinosad could provide a margin of safety for beneficial species in field, limiting the environmental impact.

Acknowledgements

The study was carried out thanks to the Project (*Xylotrechus arvicola*, técnicas de seguimiento y control en el cultivo de la vid; VA/090137/S21) financed by the Rural Development Programme in Castilla y León - co-financed by the European Agricultural Fund for Rural Development (FEADER). I would also like to thank to the Crop Protection Unit of the School of Agricultural Engineering of the Polytechnic University of Madrid, especially to P. Del Estal and P. Medina, and the Department of Engineering and Agricultural Sciences of the Higher School and Technical Agricultural Engineering and the Institute of Environment, Natural Resources and Biodiversity of the University of León.

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Received July 1, 2015