Investigations of *Trichoderma* spp. and *Beauveria bassiana* as biological control agent for *Xylotrechus arvicola*, a major insect pest in Spanish vineyards

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Abstract

*Xylotrechus arvicola* (Olivier) (Coleoptera: Cerambycidae) is an important pest in vineyards (*Vitis vinifera*) in the main wine-producing regions of Spain. Effective control of this pest is difficult due to the biology of this pest. Biological control agents (BCAs) have proven to be an effective tool in controlling and preventing the spread of a variety of plant pests and diseases. Consequently, the aim of the present study was to assess the capacity of different *Trichoderma* spp., isolated from various vineyards and one commercial isolate of *Beauveria bassiana* Vuillemin (Hypocreales: Cordycipitaceae), as BCAs of *X. arvicola*. Isolates of *Trichoderma* spp. and one isolate of *B. bassiana* were evaluated against *X. arvicola* eggs, larvae and adults. *Trichoderma harzianum* and *Trichoderma gamsii* demonstrated a good ovicidal control, 100.0% with *T. harzianum* and over 92.0% with *T. gamsii*. These *Trichoderma* strains achieved an over 65.0% larval mortality and 87.5% adult mortality. *B. bassiana* was the most effective treatment against *X. arvicola* larvae. These results confirm that *Trichoderma* spp. can be used to inhibit egg development. In addition, *Trichoderma* spp. and *B. bassiana* can help to prevent larvae boring into vines and to kill adults. Therefore, *Trichoderma* spp., especially *T. harzianum* and *T. gamsii*, and *B. bassiana* can be considered as highly effective BCAs of *X. arvicola* in vineyards.

Key words: biocontrol, xylophagous, *Vitis vinifera*, antagonist fungi, entomopathogen fungi

*Xylotrechus arvicola* (Olivier) (Coleoptera: Cerambycidae) is a cerambycid insect pest of *Vitis vinifera* (Ocete et al. 2002, 2010), which can spread in vineyards with different training systems (Rodríguez-González et al. 2016a). The larvae bore into the wood, producing galleries in the trunk and branches which favor the spread of wood diseases caused by fungi, e.g., *Diplodia seriata* (De Not) (Botryosphaeriales: Botryosphaeriaceae), *Formitiporia mediterranea* (Fisch) (Hymenochaetales: Hymenochaetaceae), *Eutypa lata* (Tul and Tul) (Xylariales: Diatrypeaceae), *Phomopsis viticola* (Sacc) (Diaporthales: Valsaceae), *Phaeococcomyces aleophilum* (Gams, Crous, Wingf., Mugnai) (Diaporthales: Togniniaceae), and *Phaeomoniella chlamydospora* (Crous and Gams) (Diaporthales: Togniniaceae) (Garcia-Benavides et al. 2013).

Females deposit eggs under the rhytidome or in cracks of wood vines (Peláez et al. 2002). Most of the hatching occurs 8 d after oviposition (Rodríguez-González et al. 2016b). From the first egg laying, the fecundity and viability of its eggs occurs over a longer period of time than their respective laboratory females’ fecundity (Rodríguez-González et al. 2016c). *X. arvicola* eggs are white or cream with a length of 1.8 mm and a width of 0.7 mm (Moreno 2005). The larvae are white and have an average size of 22 mm in the last stage (Moreno et al. 2003). Once the larva hatches from the egg, it bores galleries into the wood (Rodríguez-González et al. 2017a). For this reason, larvae are only reachable by insecticides the first 24 h after hatching and in most cases only adults and eggs stages are affected by plant-protection products (Rodríguez-González et al. 2016b).

The control of *X. arvicola* adults is complex, as they have a staggered emergence pattern (Garcia-Ruiz 2009). The emergence period ranges from June 15 to July 15 in vineyards of La Rioja (Spain) and can be extended until August 15 (Soria et al. 2013). Moreno (2005) described this period from March to late July in
vineyards of Valladolid (Castilla y León, Spain) and Biurrun et al. (2007) from May 14 to August 26 in plantations of Prunus spinosa L. in Navarra (Spain).

To control this pest through cultural measures, the rhizodrome can be removed from the vines, but this technique is expensive and unsustainable for the cultivation (Peláez et al. 2006). At this moment, there is a lack of information on field control of X. arvicular (Peláez et al. 2002). Nevertheless, some recent studies have evaluated several insecticides with different modes of action (Rodríguez-González et al. 2016b) or simulating semi-field conditions (spraying insecticides on pieces of vine wood where eggs and larvae of X. arvicular were placed) (Rodríguez-González et al. 2017a) against X. arvicular insects. Choosing compounds with different modes of action, with greater selectivity and lesser chance of developing resistance, is a priority to control insect pests. The use of fungi to control invertebrate pests minimizes side effects on the remaining organisms, the environment, and human health. Due to the aforementioned advantages, an increasing number of bio-control commercial products based on fungi are available or under development (Rodríguez-González et al. 2017b).

Information about natural predators of X. arvicular is still scarce (García-Ruiz et al. 2007), but the use of other types of microbial control agents (MCAs), such as fungi or bacteria, is a possibility that has been raised for its control. In previous studies, Rodriguez-González et al. (2016d, 2017b) have extracted, isolated and identified numerous Trichoderma spp. from vineyard substrates and vine wood (V. vinifera) affected by X. arvicular. Two of these isolated species (Trichoderma harzianum and Trichoderma citrinovirens) have shown a high reduction of egg development in X. arvicular. Trichoderma spp. has been described as a biological agent for the control of Lucanus cervus (Coleoptera: Lucanidae) larvae, reaching 83.1% of larval mortality (Ahlmadi et al. 2012), with the commercial product Trichodex (Makhteshim Ltd., Makhteshim-Agan of North America, Inc., NY) made from T. harzianum. Moreover, Hussein et al. (2013) reported that Trichoderma album caused 20% mortality of Rhyzopertha dominica (Coleoptera: Bostrichidae) adults at low spore concentration and a 100% mortality at high concentration 7 d after treatment.

The objective of this study is to evaluate various Trichoderma spp. isolated in different vineyards and one commercial isolate (GHA strain) of Beauveria bassiana Vuillemin (Hypocreales: Cordycipitaceae) against the most sensitive stages of X. arvicular, simulating cultivation conditions, in order to promote a newly developed microbial control option for X. arvicular.

Material and Methods

Insect Pest Collection and Rearing

X. arvicular adults were captured in vineyards belonging to a Protected Designation of Origin (PDO). PDO is a certification to distinguish quality schemes for agricultural products and foodstuff of a specific region (EC Reg. n. 1493/1999. 8 August 2009, OJC 187). The PDO of these vineyards is named ‘Tierra de León’ and the vineyards (42°08’14.9”N, 5°25’41.6”W) were located in Gordoncillo (León, Spain).

Interception traps (Crosstrap, Econex, Murcia, Spain), baited with ethanol as recommended by Rodriguez-González et al. (2017c), were used to capture X. arvicular adults. Captured adults were paired (one female and one male) and introduced into glass jars (80 mm in diameter and 100 mm high). The bottoms of the jars were covered with filter paper and substrates for oviposition (corrugated cardboard nets 120 x 40 mm rolled on themselves) and drinking bowls (cotton soaked in a solution of organic honey to 10% distilled water) were then placed on top. The eggs were placed into Petri dishes (90 mm diameter) and date of collection was noted. The Petri dishes were covered with aluminum foil to ensure dark conditions. X. arvicular adults and eggs were kept in a chamber under controlled temperature (24 ± 1°C) and humidity (60 ± 5%), and subjected to a photoperiod of 16:8 (L:D) h (luminous intensity of 1,000 µmol m⁻² s⁻¹).

Extraction and Isolation of Fungal Isolates

Seven fungal isolates were used for testing. One of these fungal isolates (T. harzianum) was taken from the mycological collection of the ‘Diagnostic Laboratory of Plant Pests and Diseases’ located at the Higher and Technical School of Agricultural Engineering of the Universidad de León.

The other six fungal isolates were extracted from various substrates in different vineyards (V. vinifera) planted with Tempranillo and Prieto Picudo cultivars, located in Gordoncillo (León, Castilla y León, Spain), in the PDO ‘Tierra de León’. The methodology used for the extraction of the six fungal isolates from the soil is described below. Five soil samples were taken from different points of the vineyard. For each sample, the surface of the soil was removed and the sample was taken from a depth of 10 cm. All collected soil samples were mixed and dried in a metal tray (200 mm long x 200 mm wide and 50 mm high) at a temperature 30 ± 1°C for 4 d. The dry sample was passed through a 2-mm light sieve. Subsequently, the soil sample was ground on a mortar until a thin powder was obtained. A 50 ml suspension was made for each sample, consisting of 45 ml of 1% hydroxyethyl cellulose and 5 g of soil. The obtained dilutions were left under mechanical agitation (IKA RT 15, IKA Werke GmbH & Co. KG, Germany) for 1 h at a temperature of 26 ± 1°C. From each dilution (9 ml of 1% hydroxyethyl cellulose and 1 ml 10⁻³) were taken a hundred microliters in three sets (10⁻², 10⁻³ and 10⁻⁴) with two replicates each one. After that, they were seeded in Petri dishes (90 mm diameter) with Rose Bengal-Chloramphenicol Agar medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), with three replicates per dilution, and incubated for 3 wk at 24°C. The plates were checked after 11 and 18 d of incubation, and samples that visually resembled Trichoderma growth were re-cultivated on Rose Bengal-Chloramphenicol Agar medium. Once the plates sporulated, preparations were made for identification under a microscope.

If they were found to have Trichoderma isolates, they were grown in Potato-Dextrose-Agar (PDA) medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and stored as a 50% glycerol spor suspension.

Identification of Fungal Isolates

Nucleic Acid Extraction and Manipulation

Genomic DNA was isolated from 100 mg of mycelia from each fungal isolate of Trichoderma. The manufacturer’s protocol for fungi of the Nucleospin Plant II kit (Machery-Nagel, Düren, Germany) was used. Extracts were diluted in 50 μl of sterile water. A NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) was used to estimate DNA concentration.

PCR Amplification and Sequencing

Amplifications were performed using 50 ng of template DNA in a final volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 M for each dNTP, 400 nM for each primer and 1.5 U of DreamTaq DNA polymerase (Thermo Scientific, Wilmington, DE). The primer pair ITS5-ITS4 was used

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to amplify nuclear rDNA-ITS regions. PCR products were first purified by the Nucleo Spin Extract II kit (Machery-Nagel, Düren, Germany) and were then sequenced using primer ITS4 and the kit BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the automatic capillary sequencer ABI 3130xl (Applied Biosystems) according to the manufacturer’s instructions. Sequences were introduced in databases such as the NCBI Genbank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) to identify the fungi.

Collection and Growth of Fungal Isolates

Once all fungal isolates were isolated and identified, they were grown on PDA. Fungal cultures were incubated at controlled conditions (temperature, 24 ± 1°C; humidity, 60 ± 5%; and a photoperiod of 16:8 (L:D) h (luminous intensity of 1,000 μmol m⁻²s⁻¹) for 7 d. Twenty milliliters of distilled and autoclaved water were poured over the Petri dishes to remove the spores. Then, medium’s surface was scrubbed with a brush and a suspension of 1 × 10⁶ spores per milliliter was obtained using a microscope (magnification of 200×) and a Neubauer chamber. The spore suspension was poured in Eppendorf tubes (1.5 ml) (Eppendorf AG, Hamburg, Germany) and stored at 20 ± 1°C for some hours until its use.

Experiment 1: Evaluation of Fungal Isolates Against X. arvicola Eggs

The selected fungal isolates were applied to eggs of different ages (eggs from 1 to 3 d old since the egg laying of the X. arvicola female). Five sets of 10 eggs were used for each fungal isolate treatment. Each set (placed in a Petri dish) had previously been covered with sterile filter paper (90 mm in diameter) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and moistened with 1 ml of autoclaved distilled water.

A Potter Tower (Burkard Scientific Limited, Uxbridge, United Kingdom) (Potter 1952) of manual loading was used for the application of the fungal isolates. One milliliter of fungal isolate suspension was applied over Petri dishes at a pressure of 40 kPa in each spray (a deposit of 0.004 ± 0.0004 ml/cm²).

One milliliter of spore suspension (1 × 10⁶ spores per milliliter) of each Trichoderma spp. strain was applied directly to eggs. After every application the Petri dishes were sealed with two layers of Parafilm and incubated in a chamber under controlled conditions (temperature, 24 ± 1°C; humidity, 60 ± 5% and no light). After the application, daily monitoring of eggs was carried out for 15 d of every treatment. This monitoring consisted of visualizing the eggs using a stereoscopic zoom microscope (Nikon SMZ 645 10X/22, Tokyo, Japan) every day. Those eggs which had an external mycelium and/or green spores were counted as infected eggs (that is to say, eggs inhibited, degraded or colonized by fungal isolate that suppressed the metamorphosis of the larvae in their inner and their subsequent egg hatching). The assay was repeated a second time the following year.

Experiment 2: Evaluation of Fungal Isolates Against X. arvicola Adults and Larvae

A factorial design with two factors was used in this experiment, including two parts of the vine (branch or trunk) and fungal isolates (seven treatments divided into six fungal isolates and distilled water as control) with four repetitions. The vines’ samples (Tempranillo variety in Double Cordon Royat training system) were gathered from vineyards located in the PDO ‘Tierra de León’. The samples were separated into trunks and branches. These samples were classified in similar sizes and sections, for trunks (6 cm long and 4 cm diameter, that suppose a surface of 75.36 cm²) and, for branches (6 cm long and 3 cm diameter, that suppose a surface of 56.52 cm²). To avoid a rapid drying of the samples, these were immersed in liquid paraffin to a depth of 10 mm by each end.

The selected treatments were the five isolates with the best inhibitory capacity shown in experiment 1 to which a commercial strain (GHA strain) of B. bassiana (Bassi WP, Massó SA, Barcelona, Spain) was added such a new treatment more because it has shown to control this insect pest on branches and trunks in previous studies (Rodríguez-González et al. 2017a). The six fungal isolates (1 × 10⁷ spores per milliliter) and distilled water (control) were sprayed over vine samples. To spray the treatments, a manual diffuser (0.10 liter) (Plastic Forte SL, Alicante, Spain) was used at drip point. Then, the vine samples were deposited into glass jars with 80 mm in diameter and 100 mm in height. Filter paper was used to cover the bottom of the jars. Finally, six X. arvicola adults and four X. arvicola larvae were placed per replicate (24 adults and 16 larvae by treatment, four replicates by treatment), with a brush, on vines samples. After treatment application, the glass jars with the vine samples and the insects were kept under controlled conditions (temperature, 24 ± 1°C; humidity, 60 ± 5%, and photoperiod of 16:8 (L:D) h (luminous intensity of 1,000 μmol m⁻²s⁻¹). The glass jars were checked daily until the last adult died, screening adult mortality (male and/or female). Four and a half months later, midway of the larval cycle described for this species in the field (Moreno 2005), vines samples were opened with an axe, and larvae found within the wood were counted and extracted. The assay was repeated a second time the following year.

Experiment 3: Isolation and Growing of Fungal Isolates From X. arvicola Adults in Petri Dishes

Every time an adult died, it was extracted from the glass jar, and transferred to press-seal low-polylethylene bags (76 mm × 57 mm × 50 μm thick; Transpack, Southampton, United Kingdom) in which the sex of the insect, day of death and treatment in which the insect was introduced were noted. The bags with the dead insects were stored at −20 ± 1°C until placed in Petri dishes.

Once all insects died, dead adults within the same treatment were grouped and put on Petri dishes with Rose Bengal-Chloramphenicol agar medium, where number of cadavers showing Trichoderma growth was recorded. Insect cultures were incubated at controlled conditions (temperature, 24 ± 1°C; humidity, 60 ± 5%, and photoperiod of 16:8 (L:D) h (luminous intensity of 1,000 μmol m⁻²s⁻¹) for 7 d. The identification of the fungal isolates obtained in each insect was again performed using the methodology described in this article in section ‘Identification of Fungal Isolates’.

Statistical Analysis

Seven fungal isolate treatments with five replicates were used to complete experiment 1, following a General Linear Model (GLM) procedure which was processed with an ANOVA. Daily differences (P < 0.05) among fungal isolate treatments were analyzed by mean comparisons with the least significant difference (LSD) test.

Seven fungal isolate treatments with five replicates were used to complete experiment 2, following a GLM procedure which was processed with an ANOVA. Fungal isolate differences (P < 0.05) on same insect stages (dead adults showing signs of infection or extracted larvae) were analyzed by mean comparisons with the least significant difference (LSD) test.
All analyses were performed using the SAS version 9.1.2 software (SAS Institute, Cary, NC). The Abbott’s formula (Abbott 1925) was used to correct the inhibited eggs data in experiment 1.

Results
Identification of Fungal Isolates of Trichoderma Extracted From Vineyards’ Substrates
Following, the Trichoderma species isolated in the different substrates are described (in brackets, the number of fungal isolate assigned). From the substrates of the Prieto Picudo variety, the fungal isolates were Trichoderma gamsii (T066) and T. gamsii (T067). From the substrates of the Tempranillo variety, the fungal isolates were T. gamsii (T068), T. koniangbra (T069), T. gamsii (T070), and T. gamsii (T071).

Evaluation of Trichoderma spp. Against X. arvicola Eggs (Experiment 1)
Significant differences were observed between different fungal isolates and X. arvicola eggs (Fig. 1). Best performing fungal isolates were T. harzianum (T019) (Fig. 2a) and T. gamsii (T071) from the day 13 to the end of the experiment, at day 15. T019 and T071 infection percentages were not significantly different to T. gamsii (T067) from 5 d to 15 d. T. gamsii (T070) was not significantly different from T. gamsii (T066). T. gamsii (T068) and T. koniangbra (T069) were the treatments with the lowest infection percentage of the experiment on the 15th day, being significantly lower to T. harzianum (T019), T. gamsii (T071), and T. gamsii (T067) on days 14 (N = 10; F = 13.19; df = 7,32; P ≤ 0.001) and 15 (N = 10; F = 12.69; df = 7,32; P ≤ 0.001). Control treatment egg infection was significantly lower to the rest of the treatments from the 5 d (N = 10; F = 4.15; df = 7,32; P = 0.002).

Evaluation of Trichoderma spp. and B. bassiana Against X. arvicola Adults and Larvae (Experiment 2)
Significant differences were found among the number of X. arvicola dead adults in contact with fungal isolates of Trichoderma spp. and B. bassiana (by toxic effect) and the number of X. arvicola larvae extracted from wood (indirect effect).

B. bassiana was the fungal isolate which obtained the highest larvae mortality (only a 25.0% of inoculated larvae were extracted from the wood), not significantly different to T. gamsii (T066, T070 and T071) and T. harzianum (T019) treatments. T. gamsii (T067) had the lowest control over larvae inoculated in the wood (68.7% of inoculated larvae), resulting not to be significantly greater than the control treatment. Finally, the control treatment was significantly (N = 4; F = 5.50; df = 6,21; P = 0.001) different to all other treatments with 93.7% of inoculated larvae (Fig. 2b) being extracted, making it the treatment with the highest larval survival.

Isolation and Growing of Fungal Isolates From X. arvicola Adults in Petri Dishes (Experiment 3)
Percentage of fungal growth on X. arvicola dead adults was significantly different depending on the insect’s treatment provenance.

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**Fig. 1.** Fungal virulence of *Trichoderma* spp. on *Xylotrechus arvicola* eggs during 15 d on Petri dishes and statistical data. Different lower case letters indicate significant differences among fungal isolates for the same day.
Significant differences were found among the \( X. \) \textit{arvicola} dead adults with a growth of \( T. \) \textit{harzianum} and \( B. \) \textit{bassiana} on their body after their cadavers were placed over Petri dishes. An 87.5% of dead adults showed growth of \( T. \) \textit{harzianum} (T019), significantly different (\( N = 4; F = 15.39; \text{df} = 6,21; P \leq 0.001 \)) from the other fungal isolates, except with \( T. \) \textit{gamsii} (T071) and \( B. \) \textit{bassiana} (87.5% and 83.5% of dead adults showed growth of \( T. \) \textit{gamsii} and \( B. \) \textit{bassiana}, respectively). Among fungal isolates, \( T. \) \textit{gamsii} (T071) was the only one to show external growth on dead adults on the surface of vine wood (Fig. 3a), whereas the \( B. \) \textit{bassiana} commercial strain also showed growth of the fungus over dead adults on Petri dish (Fig. 3b).

The fungal isolates \( T. \) \textit{gamsii} (T066 and T070) and the control, with 29.1%, 20.7%, and 0.0% adults, respectively, were the treatments with the lowest growth of \( T. \) \textit{harzianum} spp. over dead adults, significantly lower from the other fungal isolates.

### Discussion

\( T. \) \textit{harzianum} (T019) can be considered to have a total ovicidal control on \( X. \) \textit{arvicola} eggs when compared to previous studies (Alahmadi et al. 2012; Rodríguez-González et al. 2016d, 2017b). Furthermore, \( T. \) \textit{harzianum} demonstrated to be an effective fungal isolate to control \( X. \) \textit{arvicola} adults and larvae under semi-field conditions (sprayed on vine wood samples). Cracks on vine rhytidome promoted the adherence of fungal spores which then actively invaded and penetrated the cuticle of \( X. \) \textit{arvicola} adults and developed inside and outside of their bodies. A similar mechanism of action occurred in the case of the larvae (spores invade and penetrate through their hemocele and proliferate inside the larva). To present, only one study had analyzed the potential of \( T. \) \textit{harzianum} as a bio-control agent against an insect pest. This study, described by Alahmadi et al. (2012), showed that Trichodex (Makhteshim Ltd., Makhteshim-Agan of North America, Inc., NY), a commercial product based on \( T. \) \textit{harzianum}, can effectively control beetles. Trichodex showed a high performance on the control of \( L. \) \textit{cervus} (Linnaeus) (Coleoptera: Lucanidae) larvae, managing to achieve a larval mortality of 83.1% on day 19 after product application.

\( T. \) \textit{gamsii} (T071) showed a high fungal virulence effect on \( X. \) \textit{arvicola} eggs and, was the best fungal isolate to control \( X. \) \textit{arvicola} adults and larvae on the present study. The mortality percentages exhibited by \( T. \) \textit{gamsii} (T071) in our experiments improved those described by Razinger et al. (2014), who used three isolates of \( T. \) \textit{gamsii} over \( D. \) \textit{radicum} L. (Diptera: Insecta) larvae obtaining a mortality of 11.5, 32.1, and 20.1%, 14 d after application. \( T. \) \textit{gamsii} (T066 and T067) showed inferior larvae mortality percentages compared to \( T. \) \textit{gamsii} (T071), yet comparable to results described by Razinger et al. (2014). These data confirm that \( T. \) \textit{gamsii} isolated from vineyards of \textit{Tempranillo} variety attacked by \( X. \) \textit{arvicola} can be used to infect eggs and, suppress larvae emergence, showing its high potential as a pest BCA. Different \( T. \) \textit{gamsii} isolates showed differences on their control capacity on \( X. \) \textit{arvicola} individuals. This might be due to the fungus isolate prospecting point on the vineyard. A variety of edaphic conditions appeared on the prospecting vineyard, owing to its large extension, and thus, conditioning the characteristics expressed by each fungus strain. In consequence, each \( T. \) \textit{gamsii} isolate might have produced different
secondary metabolites which can influence their control capacity on *X. arivcola*.

*T. komlangbra* (T069) showed the lowest ovicidal efficiency on *X. arivcola* eggs. Until now, no records exist of an entomopathogenic activity of this fungus, whereas activity over plant diseases has been studied almost two decades ago (Samuels et al. 1998).

In experiment 2, larval mortality obtained by *B. bassiana* can be considered high compared to Mitsuki et al. (2002) for the larval control of *Anoplophora glabripennis* (Coleoptera: Cerambycidae), obtaining a mortality of 50.0%. However, Meyers et al. (2013) described a higher performance of *B. bassiana* (strain GHA) on *Enaphalodes rufulus* (Coleoptera: Cerambycidae) larvae, achieving a total control 4 d after the treatment. Insect larvae are generally more susceptible than adults to entomopathogenic fungi due to their thinner cuticle (Rodríguez-González et al. 2016b). Albeit our results on *X. arivcola* adult mortality using *B. bassiana* are analogous to Meyers et al. (2013) in the control of *E. rufulus* adults, Jia-Ning and Rong-Ping (2002) report a higher mortality (90.0%) on *Xylotrechus quadrripes* (Coleoptera: Cerambycidae). Rodríguez-González et al. (2016b) have described that entomopathogenic fungi have the capacity to invade living insects through their cuticle and proliferate inside them, making these entomopathogenic fungi a unique and highly effective tool for insect pest control. In our study, *X. arivcola* cadavers did not show growth on *B. bassiana* until they were placed on Petri dishes with Rose Bengal-Chloramphenicol Agar medium, which is in agreement with previous data on *E. rufulus* adults as listed by Meyers et al. (2013). Frequently, this type of fungi proliferates in the insect’s hemocoel without showing external growth of the fungus mycelium (Shimazu 1994). It is not until the death of the insect that the infection symptoms become conspicuous. The symptoms may vary depending on the temperature, relative air humidity, etc.; Dunkel and Jaronski (2003) explain that *B. bassiana* is a safe BCA for nontarget organisms and beneficial insects (predators, parasitoids and honeybees) in the field. These characteristics make *B. bassiana* an attractive organism to develop commercial products to control insect pests (Liu and Bauer 2006).

In conclusion, the isolated *T. harzianum* (T019) and *G. illiricum* (T071) may be suitable to control *X. arivcola* because they have demonstrated a good capacity to colonize eggs, 100.0% with *T. harzianum* (T019) and, over 92.0% with *G. illiricum* (T071). When these *Trichoderma* spp. were sprayed on vine wood surface, more than 65.0% of inoculated larvae died and could not bore into the vine wood. In addition, 87.5% (in both fungal isolates) of dead adults showed external growth of these *Trichoderma* spp. The control effect demonstrated by *T. gamsii* (T068) and *T. koningbra* (T069) (experiment 1) and the toxic/indirect effect demonstrated by *T. gamsii* (T066, T067 and T070) (in both experiments) were lower than the other *Trichoderma* spp. All these *Trichoderma* spp. isolated from vineyards can be used to inhibit egg development. Also, *Trichoderma* spp. and *B. bassiana* can help to prevent larval boring into vine wood and to kill adults. These *Trichoderma* spp., and especially *T. harzianum* (T019) and *T. gamsii* (T071), and *B. bassiana* can be considered as highly effective BCAs of *X. arivcola* in vineyards.

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References Cited


