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Control of *Philaenus spumarius* (Linnaeus, 1758) using beneficial microorganisms and their metabolites in preventing the transmission of *Xylella fastidiosa* Wells *et al*. (1987)

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Riassunto

P. spumarius (Hemiptera, Aphrophoridae), è una specie altamente polifaga, diffusa nella Regione Olartica. Le ninfe e gli adulti sono "xylemomizi" e si alimentano su quasi tutte le parti delle piante. Questa specie è stata indicata come principale vettore del batterio *Xylella fastidiosa* sottospecie *pauca*, coinvolta nella "Sindrome da declino rapido delle olive" nella penisola salentina a causa della capacità dell'insetto di acquisire e inoculare il batterio da / verso diverse piante ospiti (Cornara *et al.,* 2017). Pertanto, il controllo di *P. spumarius* è un elemento chiave per limitare la trasmissione di *X. fastidiosa*.

Un approccio innovativo è focalizzato sulla ricerca di composti naturali, per lo sviluppo di strategie di biocontrollo, per la sicurezza umana, per contrastare l'uso di insetticidi di sintesi, l'inquinamento ambientale e gli effetti dannosi sugli organismi non bersaglio.

I funghi entomopatogeni sono un'importante fonte di molecole naturali, inclusi i composti organici volatili, che svolgono un ruolo importante come molecole segnale (semiochimici) nell'interazione tra microrganismi e insetti.

Allo scopo di identificare composti repellenti e patogeni nei confronti di *P. spumarius*, per ridurre l'acquisizione di *X. fastidiosa*, abbiamo effettuato biosaggi comportamentali e di patogenicità.

I biosaggi comportamentali sono stati condotti trattando germogli di *V. faba* con colture fungine in polvere, del genere *Trichoderma*. I test di patogenicità sono stati condotti, inoculando i funghi nella schiuma delle ninfe e, immergendo le ninfe e gli adulti sia in colture fungine in polvere sia nelle sospensioni conidiche fungine di specie appartenenti al genere *Trichoderma* Per., *Metarhizium* Sorokin, *Beauveria* Vuill. e *Lecanicillium* W. Gams & Zare.

I risultati del nostro studio mostrano che *T. citrinoviride* ITEM-4484 ha suscitato una risposta comportamentale negli adulti; entrambi i sessi sono in grado di discriminare tra piante e germogli di *V. faba* trattati e non trattati. In particolare, sia il numero medio di maschi che di femmine era significativamente maggiore sui germogli di controllo rispetto a quelli trattati. Le colture fungine in polvere inoculate nella schiuma prodotta dalle ninfe non hanno mostrato un'attività patogena.

I saggi di patogenicità, eseguiti immergendo ninfe e adulti, mostrano che la coltura in polvere e le sospensioni conidiche di *B. bassiana* SGB11B e *M. anisopliae* SGB1K hanno influenzato la loro sopravvivenza, mostrando un effetto dose e tempo-dipendente e causando mortalità già 24 ore dopo l'inizio del biosaggio. Inoltre, tra le specie di *Trichoderma* saggiate, *Trichoderma sp*. SAM 9, sia in sospensione fungina che in forma di surnatante, ha avuto un effetto letale

significativo sugli adulti già dopo le 24 ore. Osservazioni eseguite al microscopio evidenziano che tale effetto non sempre è legato alla presenza di micelio sulla cuticola, suggerendo che l'attività fungina antagonistica su ninfe e adulti potrebbe essere in parte dovuta alla presenza di metaboliti tossici secreti nel mezzo di coltura e presenti nella coltura in polvere e nelle sospensioni conidiche.

I risultati ottenuti meritano sicuramente di essere approfonditi con ulteriori biosaggi, in ambiente ristretto e in campo, in quanto l'uso di composti naturali con attività attrattiva/repellente o fagodeterrente e tossica è considerato uno dei mezzi più promettenti da introdurre nelle strategie di biocontrollo per limitare la trasmissione di *X. fastidiosa*.

Abstract

The meadow spittlebug, *P. spumarius* (Hemiptera, Aphrophoridae), is a highly polyphagous species widespread in the Holarctic Region. Nymphs and adults are "xylem-feeders" on nearly all parts of the plants. This species has been indicated as the main vector of the bacterium *Xylella fastidiosa* subspecies pauca involved in the "Olive Quick Decline Syndrome" in the Salento Peninsula (Southern Italy) due to the insect's capability of acquiring and inoculating the bacterium from/to different host plants (Cornara *et al*., 2017). Thus, the control of *P. spumarius* is a key element to limit the transmission of *X. fastidiosa*. An innovative approach is focused on the search of natural compounds of microbial origin, in the development of new biocontrol strategies, to contrast the use of synthetic insecticides for the human safety, and to limit the environmental pollution and harmful effects on non-target organisms. Entomopathogenic fungi are an important source of natural molecules including volatile organic compounds that play an important role as signaling (semiochemical) molecules in the relationship between microorganisms and insects. With the aim of identifying repellent and pathogenic compounds towards *P. spumarius*, to reduce the acquisition of *X. fastidiosa*, we carried out behavioral and pathogenicity bioassays. The former was conducted by treating *V. faba* plants and shoots with powder cultures of fungi belonging to the genus *Trichoderma*. The latter by inoculating fungi in the nymph spittle and by dipping nymphs and adults in powdered cultures and related conidial suspensions of different fungal species belonging to the genus *Trichoderma* Per., *Metarhizium* Sorokin, *Beauveria* Vuill. and *Lecanicillium* W. Gams & Zare already used in agriculture.

The results of our study, showed that *T. citrinoviride* ITEM-4484 elicited adult behavioral response, and that both sexes are able to discriminate between treated and untreated *V. faba* plants and shoots. In particular, both the mean number of males and females on the control shoots was significantly greater than that on the treated ones. Powder fungal cultures inoculated into nymph spittle did not shown a pathogenic activity. Pathogenicity bioassays, performed by dipping nymphs and adults, showed that powdered culture and conidial suspensions of both *B. bassiana* SGB11B and *M. anisopliae* SGB1K affected the survival of them, causing mortality already 24 hours after the start of the bioassay, and this effect is dose- and time-dependent.

Additionally, among the *Trichoderma* species tested, *Trichoderma* sp. SAM 9, both in fungal suspension and supernatant form, had a significant lethal effect on the adults already after 24 hours. The pathogenic activity of related supernatant and the observations that the lethal effect is not always linked to the presence of mycelium on the cuticle suggest that the antagonistic

activity on nymphs and adults of the tested fungi could be partly due to the presence of toxic metabolites secreted in the culture medium and present in the powdered culture and conidial suspensions.

The results obtained certainly deserve to be deepened with further suitable bioassays both in a confined and field environment, as the use of natural compounds with attractive/repellent or phagodeterrent and toxic activity is to be considered one of the most promising means to be introduce in environmentally friendly strategies to limit the transmission of *X. fastidiosa*.

Alla mia famiglia,

per avermi permesso di seguire

il vento del mio destino.

A.B;

tra le poche persone a saper donare

più di quanto riceve.

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1 INTRODUCTION

Human activities, especially the speed and volume of transportation, have accelerated the global expansion of invasive species because of a breakdown of natural barriers to dispersal, so much so that the distribution of invasive species appears to be restricted primarily by climatic factors (Capinha *et al.*, 2013). One activity highly impacted by invasive species is agriculture, where crop diversity has become gradually more homogeneous at the global scale (Khoury *et al.*, 2014), leading to a suite of shared pests and diseases. Invasions of exotic (non-native, non-indigenous) insects, and the diseases represent a major threat to the economy and environment of many countries.

Therefore, it is not surprising that some of the major current and future challenges to agriculture gravitate around the potential risks associated with the introduction of invasive species into new regions where they are absent. A recent and well-known example in southern Italy is the Gram-negative bacterium *Xylella fastidiosa* which was quickly spread by animal carriers once it arrived in the Salento peninsula.

1.1 *Xylella fastidiosa* Wells *et al.*, 1987

Xylella fastidiosa Wells *et al.*, (1987) is a Gram-negative bacterium whose cells are rod-shaped, 1.0-4.0 x 0.25-0.50 µm (Fig. 1), with a characteristic rippled cell wall, and without flagella (Wells *et al.*, 1987).

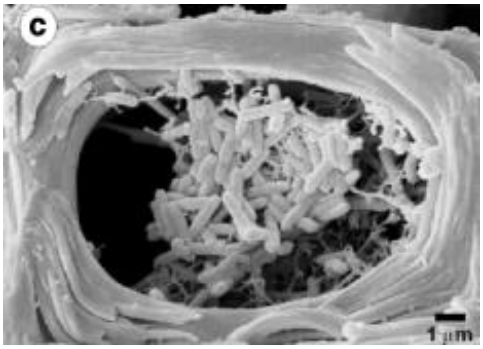


Fig. 1 - Scanning electron micrography showing morphological characteristics of *Xylella fastidiosa* and details of its pattern of xylem vessel colonization. C = *citrus* (Alves *et al.*, 2004).

The bacterium belongs to the Xanthomonadaceae family and is the only species of the genus. It is characterized by a high genetic variability, but the classification of the strains (subspecies) is mainly based on the specificity to host plants. The current classification of the *X. fastidiosa* species is based on two taxonomically valid subspecies, which present differences at the genetic level and in terms of host specificity (Schaad *et al.,* 2004; Schuenzel *et al.,* 2005; Bull *et al.,* 2012):

- *X. fastidiosa* subsp. *fastidiosa*: mainly associated with "Pierce's disease" on vines, but also able to infect the almond trees;
- *X. fastidiosa* subsp. *multiplex*: affects the almond trees and other fruit trees, olive trees and forest tree species.

Furthermore, three additional subspecies have been proposed:

- *X. fastidiosa* subsp. *pauca*: whose strains essentially attack citrus fruits and coffee plants;
- *X. fastidiosa* subsp. *sandy*: it mainly infects the oleander;
- *X. fastidiosa* subsp. *morus*: the most recently described, which infects mulberry trees (Nunney *et al.,* 2014b).

In addition to these, there are genetic variants that cannot be ascribed with certainty to any subspecies; these genotypes are presumably generated by homologous recombination between strains of different subspecies and testify to the great genetic plasticity of this bacterium.

Several molecular methodologies have been applied in the last two decades to characterize the isolates of *X. fastidiosa*. More recently, an MLST (multilocus sequence typing) scheme has been implemented to study the diversity of *X. fastidiosa* and to correctly evaluate the phylogenetic position of isolates (Yuan *et al.,* 2010).

X. fastidiosa, being an obligate parasite, lives and reproduces in the xylem vessels of the plants that parasitizes. It is a very slow-growing bacterium, difficult to isolate from xylem vessels and to be cultivated in vitro due to its non-uniform distribution within the plant, and the natural tendency of its cells to form biofilms and aggregates (Campanharo *et al.,* 2003). This bacterium infects numerous host plant species, on which it can be latent or induce mild to severe symptoms. The main diseases caused include Pierce's disease of grapevine (*Vitis vinifera* L.) (Davis *et al.,* 1978; Su *et al.,* 2013), citrus variegated chlorosis (Chang *et al.,* 1993), coffee leaf scorch (Li *et al.,* 2001), pecan leaf scorch (Sanderlin and Heyderich-Alger, 2000), phony peach (Wells *et al.,* 1983), plum leaf scald (Raju *et al.,* 1982), and almond leaf scorch (Mircetich *et al.,* 1976). To date, Pierce's disease of grapevine is present in the United States, from California to Florida, and in Central America. In some areas the disease is endemic and this rules out the cultivation of the grapevine; while, in other areas the disease

appears only periodically (Su *et al.*, 2013). *X. fastidiosa* has also been shown to be the causal agent of leaf scorch diseases in landscape plants such as oleander (Purcell *et al.*, 1999), mulberry (Hernandez-Martinez *et al.*, 2006) and oak (Barnard *et al.*, 1998).

1.2 Olive quick decline syndrome (OQDS)

In order to determine the etiology of this disease, systematic investigations started in October 2013, considering all the possible causes that could give rise to wilting and die-back symptoms, in relation to the extension of the phenomenon and to the different agronomic conditions present in the olive farms. Initially, the desiccations were associated with an increase in the infestation of leopard moth larvae (*Zeuzera pyrina*), always present in Salento and observed in all weakened trees, both as a recent and older attacks. More in-depth investigations have subsequently highlighted the presence of additional agents capable of causing wilt disease, in particular different fungal species of the genera *Phaeoacremomium*, *Phaemoniella*, *Pleumostomophora* and *Neofusicoccum*, whose penetration is favored by the trophic galleries of the larvae of *Z. pyrina*. For this reason, the described symptom picture has been called OQDS (Guario *et al.*, 2013), since at that stage of knowledge, it was hypotized that the different etiological agents identified contributed jointly in determining such a widespread and rapid symptomatic expression on centuries-old olive trees. The bacterial strain responsible for OQDS, identified on olive trees in Apulia, belongs to the subspecies *pauca*, and is genetically distinguished from the already known strains of the same subspecies that attack coffee (*Coffea* spp.) and citrus fruits (*Citrus* spp.) (Cariddi *et al.*, 2014). This is a newly identified strain, whose similarity was recently intercepted in Costa Rica on Oleander, Mango and Macadamia Nut. The phylogenetic analysis of the isolated strain in the province of Lecce has characterized the OQDS strain as belonging to *X. fastidiosa*, subspecies *pauca*. This strain, the only one so far reported in Salento, generally affects with comparatively greater severity the secular olive trees and has been found in constant association with OQDS symptomatology (Marcelletti *et al.*, 2016). The discovery of taxonomically close DNA suggests that Costa Rica is a possible center of origin for this strain, especially considering that in Europe high quantities of ornamental plants are imported annually; in natural conditions of infection, both the already known strains of the subspecies *pauca* and the strain OQDS have not been reported on vine plants; furthermore, infections on citrus plants have never been detected, even in plants intercropped with severely affected olive trees (Luvisi *et al.*, 2017). Although a large number of agricultural and ornamental species grown in the

southern Salento do not seem to be infected with *X. fastidiosa*, many plant species of native flora, particularly perennial shrubs, appear to be infected.

1.3 Vector insects and transmission of *X. fastidiosa*

During year 2015, approximately 2.300 km^2 of olive groves were estimated to be infected and, in recent years, new infection sites have developed, indicating that the disease keeps expanding over long distances, most probably due to the passive transport of infected vectors. At the moment, *Philaenus spumarius* (L.) is the main vector of *X. fastidiosa* in the infected areas of the Salento (Martelli, 2015).

The meadow spittlebug, *P. spumarius* (Hemiptera: Aphrophoridae), is a widespread species with a Holarctic distribution. Nymphs and adults are xylem-sap feeders on hundreds of host plants. In Europe, *P. spumarius* has never been considered a pest but, its status has changed dramatically since the discovery of *X. fastidiosa* in Apulia, where the meadow spittlebug has been recognized as the main vector of the bacterium. The vector infact can feed on infected plants, acquiring the bacterium, and then move on healthy plants, infecting them (Chatterjee *et al.,* 2008, Saponari *et al.,* 2014). Saponari *et al.,* (2014a) reported the first finding of field-collected *P. spumarius* positive for *X. fastidiosa* by PCR. The authors tested two Auchenorryncha species, collected from November 2013 to January 2014, *Euscelis lineolatus* (Brulle`) (Hemiptera: Cicadellidae) and *P. spumarius*, for *X. fastidiosa* by real-time PCR; only the latter was positive for the bacterium. The bacterium was isolated in culture and identified as a genotype of *X fastidiosa* subsp. *pauca*, molecularly identical to an isolate from Costa Rica. *P. spumarius* (meadow spittlebug), a froghopper quite common in the Salento area where it thrives on olive, was identified as the main vector (Martelli *et al.,* 2015).

However, it is thought that other vector insects, such as *Neophilaenus campestris* (Fallen) and *Euscelis lineolatus,* can spread the pathogen (Elbeaino *et al.,* 2014). The possible spread of the pathogen and the vector in the Mediterranean basin was studied through scientific previsional models. According to the model, spreading in Portugal, Spain, the rest of Italy, southern France, Corsica, Albania, Montenegro, Greece, and Turkey, as well as all the countries of North Africa and the Middle East has high chances to occur. The distribution of *X. fastidiosa* seems to be linked also to climatic constraints, which essentially affect its vectors, such as the colder winter temperatures that are expected to limit the spread of the pathogen beyond the northernmost countries of France (Bosso *et al.,* 2016). In anycase, the spread of *X. fastidiosa* and the presence of outbreaks in France, Corsica, Spain and Mallorca as foreseen by the predictive models prompted the Europe to adopt severe measures to slow the spread of the pathogen (EFSA PLH Panel, 2019).

P. spumarius is capable of inoculating the pathogen immediately after infectious nutrition, without the need for an incubation period; furthermore, infectivity can be maintained throughout life, but not through moulting. Transmission occurs because the bacterium is localized only in the first part of the digestive system, the bacterial cells, after being acquired, adhere to the walls of the foregut, in particular colonizing the precibarium, where they multiply and, without systemically infecting the insect, can be retained for the entire life of it (Almeida *et al.*, 2005). The relations between the bacterium and the vector are complex and involve the ability of the bacterium to probe the environment in which it lives and make a drastic change in gene expression. In particular, in the vegetable plant the bacterium actively expresses genes that facilitate the mobility and colonization of new xylem elements in order to invade the plant systemically. In the vector, instead, it actively expresses the genes responsible for the production of rubbery and adhesive substances, which lead to an adhesion of the cells to the walls of the anterior intestine and to the formation of biofilms (Chatterjee *et al.*, 2008). As for artificial inoculation, it has been proved that the vector is able to acquire and subsequently transmit the pathogen only if it feeds on a plant mechanically inoculated starting from *X. fastidiosa* cells in axenic (pure) culture using the technique of “needle inoculation”. On the contrary, if the vector feeds directly on cells of the bacterium from an axenic culture, added to the artificial nutrition substrate, it is unable to transmit them. This phenomenon bears witness to the "dioecious" cycle of the pathogen, which must necessarily pass from a plant infectious phase to an infecting vector phase to complete its cycle (Killiny and Almeida, 2009). The epidemiology of *X. fastidiosa* is ruled by ecological and behavioral factors that influence the probability of the vector's meeting with the bacterium for acquisition and subsequent inoculation to healthy plants. It is important to take into consideration the preference of host plants, of tissues (apical or basal shoots) (Daugherty *et al.*, 2011) and the number of nutrition bites (Daugherty *et al.*, 2009b). Among the characteristics of the host plant, the quantity of cells with which the bacterium infects the plant and the abundance of "saturated" xylem elements which contain *X. fastidiosa* cells in the "aggregation" phase, play an important role in the transmission “adhesion" (Hill and Purcell, 1997; Almeida *et al.*, 2005). The epidemiology of diseases caused by *X. fastidiosa* is therefore very variable and is the result of complex biotic and abiotic interactions involving the carrier species (in relation to the biological cycle and to voltinism, nutritional behavior, active displacement capacity), the genotype of the bacterium (in relation to the specific "host-range" of the different genotypes) the cultivated and spontaneous host plants (in relation to their susceptibility to multiplication of the bacterium and to their attractiveness for the vector). All these

interactions make it difficult to predict the epidemiology of plant diseases associated with the pathogenic bacterium. Further, despite the fact that only the insects feeding on xylem juices can transmit *X. fastidiosa*, a specificity of transmission is not proven and therefore all xylem-sap feeder insects are to be considered potential vectors.

The vector species of *X. fastidiosa* are well known in America, a continent that represents the area of original distribution of the bacterium and where the main vectors are the "sharpshooter" (Cicadellidae Cicadellinae), while the other xylem-sap feeder have a secondary role. The vector species of *X. fastidiosa* have been listed almost entirely by Redak *et al.,* (2004) which includes 37 Cicadellidae (leafhoppers) and 5 Cercopidae (spittlebugs) among the known vectors (Paiao *et al.,* 2002; Krell *et al.,* 2007). In Italy and in Europe the Cicadellidae are extremely poorly represented, while the species of the other groups of xylem-sap feeder vectors, spittlebugs and cicadas are much more numerous (De Jong, 2013). Among the spittlebugs are 26 species of the Aphrophoridae family including *P. spumarius* (De Jong, 2013). From field investigations carried out recently in the olive orchards of Salento, four xylem-sap feeder candidate species have been identified, *P. spumarius*, *Neophilaenus campestris*, *Cercopis sanguinolenta* (Scopoli) and *Cicada orni* L. (Elbeaino *et al.,* 2014; Cornara and Porcelli 2014) although to date only for *P. spumarius* the ability to transmit *X. fastidiosa* has been definitively proven (Saponari *et al.,* 2014; Cornara *et al.,* 2017; 2019; EFSA PLH Panel, 2019).

The characteristic alterations and symptoms of OQDS (Fig. 2) consist in a scattered drying of twigs and small branches present mainly on the upper part of the foliage. These symptoms subsequently extend to the rest of the crown and to the other branches giving it a burnt appearance. The desiccation of the tissue starts from the tip of the leaves and progresses towards the petiole, extending soon to the whole leaf blade. The most severely affected olive trees are the centuries-old trees of the indigenous cultivars “Cellina di Nardò” and “Ogliarola Salentina”; farmers try to recover the trees through a drastic rejuvenation and massive pruning to stimulate new growth. The colonization of tracheae (xylem vessels) by *X. fastidiosa* results from the migration of bacterial cells from one vessel to another. The bacterium multiplies, its colonies obstruct the vessels and hinder the absorption of water, giving rise to the symptoms shown by most of the infected trees. An extensive program of mechanical inoculations and vector-mediated transmission experiments has been launched in the last 2 years, and continuously implemented, in an attempt to reveal the role of *X. fastidiosa* in OQDS and to quantify threatened crops due to severe expansion epidemic that threatens southern Italy. Plants of different species, including a large number of olive cultivars, have also been tested

by field experiments in the infected area outlined, exposing uninfected plants to the natural inoculum pressure (Saponari *et al*., 2017).

Comparison of bacterial infection rates derived from mechanical inoculation and vector transmission in field experiments showed a perfect consistency with regard to susceptibility of different host species, as follows:

- high rates of systemic infection for olive trees, with some differences in relation to different cultivars, oleanders and *Poligala myrtifolia*;
- there was a low colonization of host plants such as cherry and almond trees;
- no bacterial infestation and colonization were detected on vines, citrus fruits, apricot, peach and plum.

The pathogenicity of the strain Co.Di.R.O. isolated in Salento has been demonstrated by the severity of the symptoms found in host plants, particularly on olive, which has been identified as the plant species most sensitive to the disease (Saponari *et al.*, 2017).

Fig. 2 - Desiccation of the crown, browning of the branches and stem (Source SFR Puglia Region)

1.4 INSECTS

1.4.1 *Philaenus spumarius* (L.)

The meadow spittlebug *P. spumarius* belongs to the order Hemiptera, superfamily Cercopoidea, family Aphrophoridae. It is a very common and widespread species in Italy, it has a livery with very variable colors and sizes ranging from 5 to 7 mm, the females are slightly larger than the males. The head is equal in width to the pronotum which is rounded. The ocelli are as far from each other as they are from the eyes and the antennae are placed on the gena between the eyes. The species belonging to Afrophoridae are sap-feeders with a characterisitc piercing and sucking mouthparts, consisting of the lower lip or rostrum, which contains, at rest, 4 mouth stylets, two mandibular and two maxillary ones. The union of the maxillary stylets gives rise to the formation of two channels, one of which is dorsal for food ingestion, and the other ventral for the injection of saliva. The posterior tibiae have two hard

spines and a smaller crown of thorns around the apex. The species is not prominently pubescent.

The name “spittlebug” derives from the characteristic foamy liquid that is produced in the nymphs by mixing air and a secretion produced by glands located between the 7th and 8th abdominal sternitis; air bubbles are formed by caudal appendages and a ventral tube formed by abdominal wipers (from 4th to 9th) folded downwards (Yurtsever, 2000).

Since the nymphs live in masses of spittle, they are commonly called spittlebugs, but are also known as froghoppers from their adult leaping ability (Chinery *et al.*, 1993). The nymphs derive their nourishment from xylem elements by piercing them with their stylets and sucking the sap. A spittlebug nymph usually rests head downward on the plant, and as the spittle forms, it flows down over and covers the nymph, providing the nymph with a moist habitat. Adults are free living individuals and do not produce spittle. The meadow spittlebug was commonly called *Philaenus leucophtalmus* (L.) in the early literature, as for example in Severin (1950) and Weaver and King (1954). The taxonomical confusion was solved when, in 1961, the International Commission of Zoological Nomenclature decided for the only valid specific name of *P. spumarius* (Yurtsever, 2000). Now, it is well known that this ubiquitous, common and locally very abundant insect is the main vector of the bacterium *X. fastidiosa* in the Apulia region of Italy and it has the potential to spread it in all the other European regions where the pathogen is present. Nevertheless, since the meadow spittlebug has never been considered an agricultural pest in Europe before the introduction of *X. fastidiosa*, its biology, ecology and ethology have never been investigated continuously and in a comprehensive way.

1.4.2 Geographical range of *P. spumarius*

P. spumarius is highly polyphagous and occurs in most of the terrestrial habitats (Stewart and Lees, 1996). The exploitation of a wide range of hosts belonging to monocotyledonous and dicotyledonous may have been the leading factor promoting the geographical expansion of the species. Thus, it is widely distributed, covering most of the Palearctic regions and extending to Nearctic, as well as most of the temperate regions of earth and oceanic islands (Drosopoulos and Asche, 1991; Stewart and Lees, 1996; Drosopoulos and Remane, 2000). In Europe its distribution ranges from northern Lapland to the Mediterranean basin, including Turkey. It has been reported for North Africa, several parts of the former Soviet Union, Afghanistan, Japan, USA, Canada, Azores, Hawaii and New Zealand (Yurtsever, 2000). The meadow spittlebug was probably introduced in new continents, as North America, as

overwintering eggs in straw stubble (Whittaker, 1973). Its distribution in Europe and worldwide has been summarized by EFSA (2015). In Greece, Drosopoulos and Asche (1991) reported *P. spumarius* at an altitude ranging from the sea level to more than 2000 meters above sea level. Climate change may significantly have affected the distribution of *P. spumarius*: Karban and Strauss (2004) suggested that the species northward shift in California since 1988 is related to variations in humidity and temperature.

In Europe, *P. spumarius* is not considered a major pest and rarely causes severe damage (Whittaker, 1973, Buczacki *et al.,* 1991). However, it has been regarded as an economic pest of crops and other cultivated plants in America. It mainly causes two types injury to plants.

The first one is as a vector of some plant diseases. It has been reported that *P. spumarius* transmits the virus of Pierce's disease of grapevines from diseased to healthy vines and some other plants, which may serve as reservoirs of the virus (Delong *et al.,* 1950). It has also been reported that the species may be a vector of peach yellows and little peach disease and may be a carrier of the plum mite (Mundinger, 1946).

The second type of injury is its directly harmful effect on plants. The nymphs cause the main injury. Indeed, *P. spumarius* nymphs may take up to 280 times their own fresh weight of the plant sap in 24 hours (Horsfield, 1978). Heavy infestations of the nymphs and adults on plants cause serious damage, leading to reduction and losses in crop yield. Spittlebug injuries weaken the infested plants and cause significant deformation which results in delayed plant maturity and reduced forage yield. Damaged fields give relatively poor second-crop yield and the effects of the injuries may even persist to affect yield in the following year.

Either as nymph or adult, *P. spumarius* is a xylem feeder. The spittlebug ingests considerable amount of sap from the main transpiration stem without causing vessels cavitation, overcoming dramatically high tension reaching -10 bars (Wiegert, 1964; Horsfield, 1978; Crews *et al.,* 1998; Malone *et al.,* 1999; Watson *et al.,* 2001; Ponder *et al.,* 2002). Nymphs and adults feed preferentially on actively growing parts (Mundinger, 1946; Wiegert, 1964). Nitrogen-fixing legumes and other plants with high amino acids concentration in the xylem sap [*Medicago sativa* L. (1753), *Trifolium* sp. L., *Vicia* spp. L., and *Xanthium strumarium* L. (1753)] are the preferred hosts (Horsfield, 1977; Thompson, 1994). Overall, *P. spumarius* seems to prefer plants that transport fixed nitrogen as amino acids and amides than those that transport fixed nitrogen as ureides (Thompson, 1994). Nymphal excretion rate has been proven to be positively correlated with amino acids concentration in the xylem sap (Horsfield, 1977). Collectively, nymphs and adults thrive on various plants in habitats moist enough to provide them with sufficient humidity to keep them alive, such as meadows, abandoned

fields, waste grounds, roadsides, streamsides, hayfields, marshlands, parks, gardens and cultivated fields (Yurtsever, 2000).

Gulijeva (1961) reported cereals, Asteraceae, legumes and Lamiaceae as the most favorable hosts. Ossiannilsson (1981) states that *P. spumarius* is the most polyphagous insect currently known, with a host lists that exceed 1000 plants. Dicotyledonous plants tend to be used more often than monocotyledonous (Wiegert, 1964; Halkka *et al.*, 1967, 1977).

1.4.3 Biology of *P. spumarius*

P. spumarius is an univoltine and hemimetabolous insect in most of its range (Halkka, 1962), although Drosopoulos and Asche (1991) believed that the species may be partly bivoltine in certain parts of Greece. After the overwintering, egg hatching begins in April and, after five nymphal instars, adults appear in June. Adults start to mate soon after the emergence, and copulating pairs can be seen throughout the summer. Adults exist at least until October. Males appear before females and, with the summer season advancing, the number of males decreases compared to females (Edwards, 1935; Halkka, 1964; Drosopoulos and Asche, 1991) since males generally do not survive as long as females (Stewart and Lees, 1996). Oviposition commences in early September, females are induced to lay eggs by the short daylight and the low temperature, and then eggs undergo overwintering diapause. Before the hatching in spring, this diapause is broken by exposure to a chill period with temperature lower than 5°C for about 100 days (West and Lees, 1988). Two are the key factors regulating *P. spumarius* development: humidity and temperature. There are certain temperature thresholds, which play an important role on the egg hatching and nymphal developmental stages (Chmiel and Wilson, 1979; Zajac and Wilson, 1984), that temperature influences could modify the egg hatching and developmental rates of these periods. The low temperature causes severely delayed development of the nymphs (Ahmed and Davidson, 1950). Development to adulthood requires a sum of 700-800 day-degrees above 5°C (Halkka, and Halkka, 1990). Hence, in cooler climatic conditions development to the adult stage takes longer. The phenology of the life cycle of *P. spumarius* may be different in certain parts of its global distribution, because the species encounters an extensive range of climatic conditions due to its wide distribution. However, variations of the life cycle are not fundamentally different (Weaver and King, 1954; Lavigne, 1959; Whittaker, 1973; Halkka and Halkka, 1990; Drosopoulos and Asche, 1991; Booth, 1993; Lees and Novel, 1993; Loukas and Drosopoulos,

1992). In North Europe, gravid females begin to lay eggs in late summer. While in the climatic conditions of southern Italy the eggs are laid at the beginning of winter and occur during the February hatching. Figure 3 shows the life cycle of *P. spumarius* in the typical climatic conditions of southern Italy. The number of eggs laid varies but an individual female may produce up to 350-400 eggs (Yurtsever, 1997). The eggs are laid in groups of one to 30 elements glued together, by means of the secretion of the colleter glands, by a hardened foamy cement. In laboratory conditions, each nymphal stage takes about 10 days, and adults usually appear approximately 50 days after the hatching. Females may mate a few days after emergence. The life cycle is completed by the oviposition stage. The first plants on which nymphs are observed are those exhibiting dense lateral growth, thus limiting air movements and having a higher RH (relative humidity). Furthermore, nymphs tend to congregate on closely apposed surfaces where the humidity can be maintained at high levels, as noticed both in field and laboratory conditions using *Sonchus* sp. L. as a rearing plant (Morente *et al.*, 2018). As reported by Weaver and King (1954), early in the morning nymphs can be found at the tip of the plant, but as the temperature rises, the masses dry and they leave them to move down on the plant.

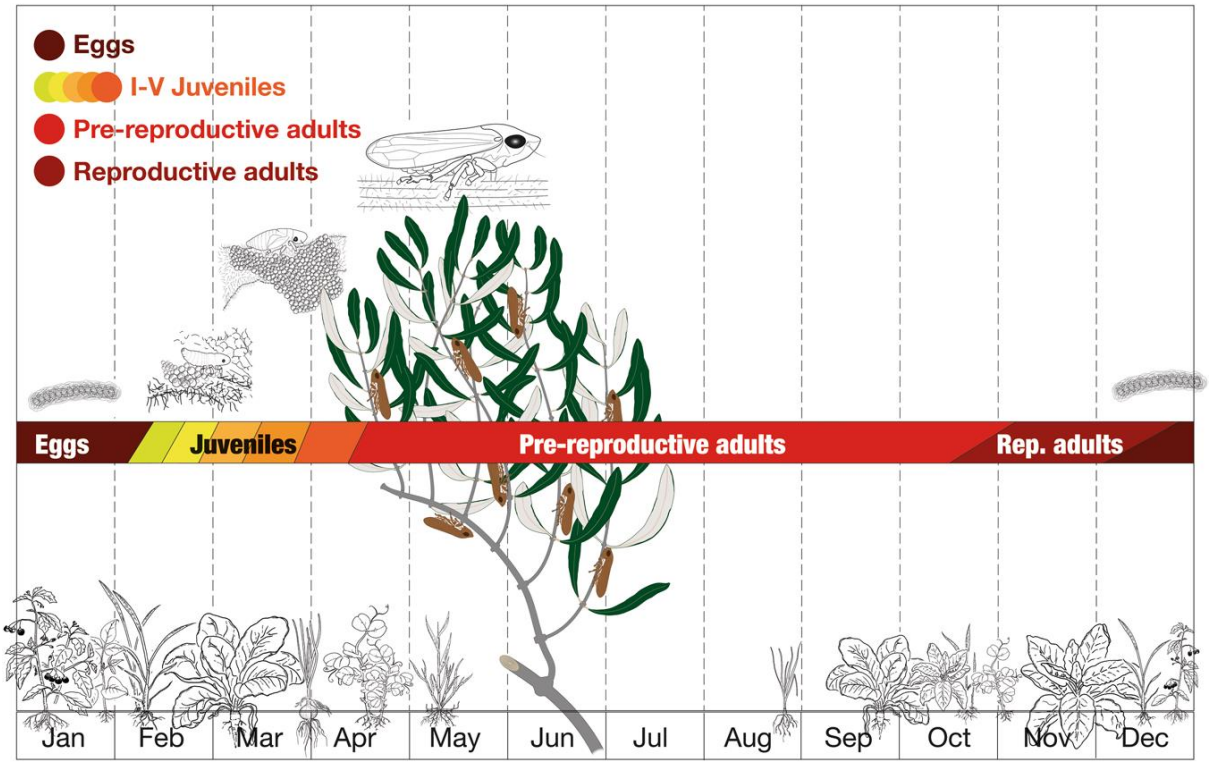


Fig. 3 - Vector life-cycle in relation to host plants phenology in olive orchard (Fierro, 2019).

Eggs are about 1 mm long, and 0.35 mm wide, elongated, ovoid and tapering in shape (Fig. 4). Egg is yellowish white and has a dark orange pigmented spot in the shell at one end. If the egg is fertilised this orange spot gets bigger and a black coloured, lid-like formation develops on it in about 90 days (Yurtsever, 1997). This lid-like black formation clearly indicates that the eggs are ready for hatching. The young nymph leaves the egg through the black lid. The black lid doesn't develop if the egg is not fertilised or if it is unhealthy; the orange spot still remains but the egg turns brown and eventually shrivels. In optimum conditions, hatching takes place after approximately 20 days and the first instar appears. The first mature eggs are visible in the ovaries of female individuals since the end of August and increase until November (Weaver, 1951).

The nymphs (1st and 2nd instar), not very mobile, have a completely different appearance from adults. They are yellowish-orange in their early instar and gradually darker until they become green (nymphs); wing pads begin to develop on third instar nymphs. The nymphs are abundantly covered with spittle, which takes on the function of protection. Adults usually stay in the spittle mass until the cuticle is hard and fully pigmented. However, they may occasionally leave their spittle mass earlier. Adults (Fig. 5A, 5B) are fully mature approximately ten days after leaving the spittle and females may mate several times thereafter.

Figure 4 – Eggs of *P. spumarius*

Fig. 5A – Adult of *P. spumarius* just emerged

Fig. 5B – Adult of *P. spumarius*

1.4.4 Composition and function of the spittle produced by the nymphs of *P. spumarius*

The spittle (Fig. 6) produced by the nymphs of *P. spumarius* has been confirmed as a mixture of liquid excretions from their intestines, mixing proteins and saccharides secreted by their Malpighian tubules (Ziegler and Ziegler, 1957). It is generated by a back-and-forth contraction of the abdomen (Marshall, 1965; 1966; 1973; Mello *et al*., 1987; Guilbeau, 1908; Chen *et al*., 2018). In detail, it a mixture that flows into the caudal appendages and mixes with the mucus secreted by the Batelli glands at the 7th‑8th abdominal segments, and is eventually excreted from the anus. The spittle surrounding Cercopoidea nymphs during development has the role of protecting them against harmful effects (Fig. 6, 7).

Fig. 6 - Spittle in the field and in the laboratory of nymphs of *P. spumarius*

Fig. 7 - Spittle surrounding *P. spumarius* nymph

Nymphal spittlebugs, synthesize mucopolysaccharides and proteins in specialized Malpighian tubules and mix these products with air bubbles trapped in the fluid eliminated from the alimentary canal, forming a saliva-like froth where the nymphs immerse themselves (Lomer *et al.*, 1993; Rakitov, 2002). *P. spumarius* and other Cicadomorpha and Cicadellidae have four Malpighian tubules arising from the junction of the midgut and hindgut, and each tubule consists of five morphologically distinct regions (Rakitov, 2002; Zhong *et al.*, 2015). An anterior-most segment, taking part in the composition of the filter chamber and, being enveloped by a cellular sheath; an intermediate duct, connecting anteriorly with the anterior-most segment; a proximal segment, connecting anteriorly with the intermediate duct; a terminal segment with ends attached to half of the inflated rectum; and a distal segment, situated between the proximal region and the terminal segment. However, no studies concerned with comparison of Malpighian tubules between nymphs and adults within species of any Cicadomorpha insects have been published, so far. The nymphal behaviors of Cicadomorpha are closely related to the Malpighian tubules that perform a modified glandular function during nymphal stages (Rakitov, 2002). Guilbeau (1914) stated that the anal excretion of nymphal spittlebugs by it self cannot be capable of holding air bubbles. Previous investigations suggested that the Malpighian tubule proteinaceous and carbohydrate secretions with adhesive properties account for stabilizing the froth bubbles (Pesson, 1956; Marshall, 1966a, b). Qinglong (2015) indicates that Malpighian tubules of nymphal spittlebugs can secrete proteinaceous products by stabilizing foam bubbles. Until the study by Gabe (1962), the occurrence of mucocomplexes other than chitin in insects was thought to be

uncommon (Day, 1949). They are, however, usually produced in quantity only by the salivary glands and reproductive organs in some insects. Very small quantities of histochemically characterized mucocomplexes of various categories are produced by the Malpighian tubules of a number of insect species of several orders (Gabe, 1962). In most cases, the functional significance of the mucocomplex is unknown. In cercopid nymphs the production of a mucoprotein has been attributed to the proximal segments of the Malpighian tubules. The evidence for this is slender and is based upon the results of three histochemical tests carried out by Pesson (1956). Secretory activity in the Malpighian tubules of cercopid nymphs has been shown to be associated with spittle-production (Marshall, 1964b) and the presence of the same polysaccharide in Malpighian tubules and spittle has been demonstrated by histochemical and chromatographical methods (Marshall, 1966b). Thus, a possible role for large quantities of mucocomplex produced in the nymphs cercopid Malpighian tubules could be as a surfactant in the spittle masses within which the nymphs live. Proximal segments of the Malpighian tubules of cercopid nymphs contain large quantities of a highly negatively charged mucopolysaccharide. The compound appears to contain sulphate groups and possibly carboxyl groups. These results are in accord with the observations of a previous paper (Marshall, 1966b) in which glucuronic acid, glucosamine, glucose, and rhamnose were tentatively identified in proximal segment hydrolysates by means of paper chromatography. It seems probable that the proximal segment mucopolysaccharide is a polymer containing glucuronic acid, acetylglucosamine, glucose, and rhamnose units, some or all of which are sulphated. The cercopid mucocomplex is strikingly different from these in that it is periodic acid/Schiff negative and highly negatively charged. A highly charged mucopolysaccharide such as this could be expected to have the requisite ionic and viscosity properties to function as a very efficient surfactant and could thus be responsible for the maintenance of bubble stability in spittle. The Malpighian tubules of insects are primarily concerned with the excretion of the waste products of metabolism and the regulation of ion and water balance. However, in a variety of insects they appear to carry out accessory functions which are quite unrelated to excretion or osmoregulation (Wigglesworth, 1965). This is particularly true of cercopoid nymphs. Protein synthesis and secretion is certainly a function of the nymphs Malpighian tubules and ceases in both machaerotid and cercopid adults. It therefore seems likely that it is related to the peculiar modes of nymphs life. However, it must be borne in mind that the feeding site of the adults appears to be different to that of the nymphs (Hagley, 1965) and it is possible that the protein functions as an excretory product in the nymphs stage but not in the adult. Smith and Littau (1960) have suggested that the secretion of protein in the

form of brochosomes from the Malpighian tubules of cicadellids is a possible excretory mechanism. In the Cercopoidea, the Malpighian tubules display a different regional specialization and are active as glands only at the nymphal stage. During this period, the inflated proximal segment of the tubule part not hidden within the filter chamber produces large quantities of an acid mucopolysaccharide1 (Pesson 1956; Marshall 1964b, 1965, 1966a), while the more distal, nodulose segment produces a proteinaceous secretion (Marshall, 1973). In adult spittlebugs, the tubules function exclusively as excretory organs (Marshall and Cheung, 1973). The composition of the spittle is complex (Kato, 1958; Mello *et al.*, 1987), and the exact functions of its different components are poorly understood. In regions characterized by a hot and humid climate, no mould has ever been observed on the foam on the field, suggesting the presence of some antifungal elements (Chang *et al.*, 2019).

1.5 CONTROL METHODS

1.5.1 Sustainable agriculture

Until about fifteen years ago, the control of the harmful insects in agriculture relied almost exclusively on the use of synthetic products. The use of increasing quantities of crop protection products has increased the risk of contamination of agricultural products, with detrimental effects on human health and the environment.

Furthermore, the active ingredients normally used determined the selection of resistant populations of insects, fungi, bacteria and viruses with the consequent inevitable necessity to increase the doses to obtain a containment effect. This has led to pollution and high levels of resistance in several key parasites (van Lenteren, 2000). In this situation, there was a strong need to develop eco-sustainable alternative agricultural techniques thanks to which, in many countries, it has been possible to significantly improve the quality of agricultural products and to drastically decrease environmental pollution.

1.5.2 Description of phytosanitary measures against *P. spumarius*

The control of vectors is very often the activity of choice to obtain immediate and effective results in limiting the spread of phytopathogenic organisms as well as mitigating the diseases. An regulation was issued to deal with the phytosanitary risk associated with the spread of *X. fastidiosa* (CDPC 225/2015) which lists the intervention plans to be implemented. For example, the multiplication material must be carefully selected from sources known to be free

from disease, for new plantations certification of plant material is required through the application of effective indexing procedures.
(http://cartografia.sit.puglia.it/doc/Piano_operativo_*Xylella*_approvato%2018_03_2015.pdf.)
Moreover, the regulation requires to carry out agronomic interventions against the juvenile stages of the vector and to control spontaneous herbaceous weeds and to forbid to move outside the delimited areas, or within them, any plant material specified in art. (Movements of specified plants within the Union - Ordinance of the CDPC 225/2015). A serious obstacle to the slowdown of the OQDS spread has been the lack of registered and approved pesticides for the control of *P. spumarius* on olive trees. Since 2014, when *P. spumarius* was identified as the vector of the bacterium, despite being faced with an unparalleled phytosanitary emergency in Apulia, no formulation was available for the control of this insect, except for brief periods in when 120-day temporary authorizations were granted, for one formulation based on sweet orange essential oil and one based on acetamiprid (Dongiovanni *et al.,* 2018). Recently, a formulation based on acetamiprid (Epik SL), different formulations based on deltamethrin (Decis) and the essential oil of sweet orange (containing the active ingredient limonene) obtained the definitive registration for the control of spittlebug on olive trees. Therefore, the effectiveness evaluation of other active substances for the spittlebug control is strongly necessary. In the case of *P. spumarius* we refer to an integrated control strategy based on two main components: the control of nymphs and the control of adults. Excellent control of the nymphs of *P. spumarius*, which live almost on spontaneous herbs or weeds in the olive groves, can be obtained by resorting to mechanical interventions, such as shredding, milling and plowing, with the great advantage of affecting the population of the potential carrier when it not yet infective and with a very limited impact on the agro-ecosystem. For the control of adult spittlebugs, it is necessary to follow the provisions and further instructions, prepared on the basis of scientific acquisitions and biology of verified vector insects. In addition, monitoring of vector insects must be carried out and, in the presence of consolidated populations, the application of insecticide programs must be considered. In the period January - April mechanical operations must be carried out for the elimination of spontaneous herbaceous plants in order to reduce the population of the nymphs of vector insects, by means of tillage, preferably with milling, or shredding of herbs, or weed burning, or insecticide treatments in areas with difficult access. In the period May - August at least two insecticidal applications must be carried out on the crops to control the adult stages of the vector. In the period September - December, the insecticidal interventions required by the "eco-sustainable

standards for phytosanitary defense" issued by the Apulia Region must be carried out, in order to control the adult stages of the vector.

1.5.3 Integrated management and sustainable control prospects

Integrated pest management strongly relies on effective sampling and surveillance methods. Unfortunately, to date, an effective method for *P. spumarius* sampling is still missing. Sweep net is the most common method used for adult collection; however, as remarked by Purcell *et al.*, (1994), sweep net is a poorly effective tool for sampling insects from a tree canopy, in contrast with its high efficacy on the ground cover. Although sweep net is the tool largely used to collect *P. spumarius*, other methods, namely minicage (biocenometers), pitfall traps, sticky traps, aerial suction traps, beat tray, and tanglefoot bands have been tested (Cornara *et al.*, 2019). However, all these methods were proven to be less effective than sweep net (Weaver and King, 1954; Lavigne 1959; Wilson and Shade, 1967; Novotny 1992; Pavan, 2000; Bleicher *et al.*, 2010). Recent studies on the fine structure of antennal sensilla of the spittlebug allowed to identif chemoreceptors (Ranieri *et al.*, 2016; Germinara *et al.*, 2017). Although the presence of olfactory receptors among the antenna is limited, it is possible that *P. spumarius* responds to olfactory attractants or repellents, e.g. volatile organic compounds (VOCs), thus providing new tools for monitoring and control. A recent research (Ganassi *et al.*, 2020) evaluating *P. spumarius* adult behavioral responses towards essential oils and related plants, pointed out a complex and varied situation, in which male and females respond differently to the same volatile blend coming from either essential oils or entire plant. This is probably a result of the different role played by the same stimuli in the ecology of males and females. Nevertheless, these results are a first step to the development of innovative strategies, alternative to the use of synthetic pesticides, for the sustainable control of *P. spumarius*. The recent changes determined by the EU legislation on agrochemicals have changed the set of active substances that can be used in the control of pathogens and pest. The chemical struggle, however, poses serious problems of eco-sustainability and environmental and food safety; moreover, it does not respond to the needs of organic production, which represents a significant share of the olive production in the area affected by the OQDS.

1.5.4 Alternative control methods

Alternative methods for the control of crops include the creation and use of new biotechnologies, that is methodologies that use living organisms or substances and / or metabolites from these organisms, their genes and / or gene products (Lorito and Cervone, 2004). This definition greatly expands the classical concept of biological control against pests harmful to agricultural production and includes: i) the use of semiochemicals to attract or remove the insect; ii) the creation of products deriving from living organisms, such as insecticidal metabolites; iii) the application of antibiotic secondary metabolites of fungal origin; iv) the use of living organisms used as they are.

1.5.5 Entomopathogenic fungi

The entomopathogenic fungi are natural pathogens in insect populations and comprise a wide range of morphologically, phylogenetically, and ecologically diverse fungal species (Araújo and Hughes, 2016). Moreover, some of them are endophytes, also antagonists of some fungal pathogens, colonizers of the rhizosphere and promoters of plant growth (Lacey *et al.*, 2015). Their main characteristic, unlike other insect pathogens such as bacteria or viruses, is the ability to infect the hosts by penetrating through the cuticle without need to be ingested. Therefore, they have a great potential to control sucking insects (e. g; aphids, leafhoppers, stink bugs, thrips) or disease vectors (e.g; mosquitoes, kissing bugs, flies) (Mannino *et al*; 2019). Entomopathogenic fungi produce a diverse array of bioactive compounds known as secondary metabolic compounds, and some of these have shown to possess insecticidal activity and might be used as biopesticides. The characterization of bioactive metabolites is a very important step to obtain potentially useful molecules. Numerous fungal microorganisms are pathogens not only of many species of insects but also of nematodes, and are able to control the natural populations of these by limiting their spread. Entomopathogenic fungi have been mass-produced as biopesticides since the 1970s and they are among the most used entomopathogenic microorganisms for biological control (Lacey *et al.*, 2015). In particular, species belonging to the genus *Metarhizium* Sorokin., *Beauveria* Vuill., *Lecanicillium* W. Gams & Zare. and *Trichoderma* Pers. already represent important entomopathogenic and nematopathogenic species most used for biocontrol in agriculture (Ganassi *et al*, 2010; Brunner-Mendoza *et al*, 2019; Keswani *et al*, 2019).

1.5.5.1 *Trichoderma* Pers.

The fungi of the genus *Trichoderma* Pers. are the most studied and used biocontrol agents worldwide, they produce beneficial effects in plant which are able to increase plant growth and health, as well as agricultural production thus providing great livelihood to the world population (Lorito *et al.,* 2010). The species of the genus *Trichoderma* help to maintain soil fertility and are able to offer general crop protection, together with other microorganisms, through various mechanisms of action. Many studies have shown the ability of some strains to produce lytic enzymes and antibiotics and to trigger a defense response on the plant, through which the "immune system" of plant organisms is pre-alerted and thus more rapidly end efficiently activated in response to the attacks by some pathogens and to abiotic stresses (Lorito *et al.,* 1993; Sanz *et al.,* 2004). This makes the members of *Trichoderma* genus the most widely used fungal active principles in bio-formulations intended for biological control of plant diseases, worldwide (Woo *et al.,* 2014). In recent years, the potential of *Trichoderma* species for biocontrol of pests has grown in scientific interest and in the number of reports. *Trichoderma* species are ubiquous and characterized by a great metabolic versatility, which makes these fungi able to establish complex trophic and ecological relationships with other organisms that share their same ecological niche, including insects and nematodes. A major role of secondary metabolites produced by *Trichoderma* in these interactions is more and more apparent. Some metabolites have toxic effects against insects (Singh and Prakash, 2015), and possibly act synergistically with extracellular enzymes, such as chitinases, proteases and lipases in the course of entomoparasitic and entomopathogenic activities. Other molecules have the role of signaling compounds and may act as semiochemicals, thus influencing insect behavior such as oviposition (Geetha *et al*., 2003), choice of host plant (Evidente *et al*., 2008, 2009; Ganassi *et al*., 2016) or aggregation (Xiong *et al*., 2019). In this regard, both attractive and repellent effects on different insects have been reported for *Trichoderma* strains and *Trichoderma* metabolites, which might eventually lead to the development of biorational products (Altomare, 2020).

1.5.5.2 *Metarhizium* Sorokin

A large number of species have been identified in the genus *Metarhizium* Sorokin, they consist mainly of entomopathogenic fungi and are ascribable to ascomycetes. Many species are adapted to a remarkable diversity of hosts. Aphids have been a target focus, since they are susceptible to natural fungal epizootics (Milner, 1997). For example, *M. acridum* and *M. album* are specialized in parasitizing orthoptera and hemiptera compared to other more generalist species, such as *M. robertsii* and *M. anisopliae* (Wang *et al.*, 2012). Soil ecology studies of *M. anisopliae* show that this species has a remarkable ecological and metabolic versatility; in fact, it is able to colonize the rhizosphere, adhere to the surface of the roots and parasitize insects. *M. anisopliae* is able to live as an insect parasite, spread in the hemolymph and internal organs, and occupy the ecological niche of soil saprophytes.

1.5.5.3 *Beauveria* Vuill.

Among the insect pathogenic fungi, those belonging to the *Beauveria* Vuill. genus have assumed a fundamental role in the management and protection of crops. Among them in particular, *Beauveria bassiana* is used in many bio-formulations, as an aqueous suspension of conidia, to protect crops and forest species from harmful arthropods. *B. bassiana* is a cosmopolitan species adapted to life in the soil, and a entomopathogen of many species of arthropods. About 40% of the myco-insecticides on the market are based on *Beauveria* spp. (Faria and Wraight, 2007). The fungus infects the host through the contact of conidia on the arthropod body, subsequently it secretes enzymes, lytic substances and applies mechanical pressure to penetrate the exoskeleton and spread in it. Once the exoskeleton has penetrated, the fungus is able to differentiate into individual cells (blastospores) that have the ability to spread very quickly in the hemolymph and resist the immune system of the insect (Humber, 2008; Xiao *et al.*, 2012). During the colonization of the host, a wide range of toxic metabolites, specialized in the suppression of the insect immune system, and in the destruction of internal tissues are produced by the fungus, ultimately leading to the death of the host (Xiao *et al.*, 2012). Once the death of the insect has occurred, mycelium erupts from the cuticle of the mummified insects, producing conidia.

1.5.5.4 *Lecanicillium* W. Gams & Zare

Lecanicillium lecanii (Zimmermann) Gams & Zare (formerly *Verticillium lecanii* (Zimm.) Viegas) is an important entomopathogenic fungus of scale insects. Fungi belonging to the *Lecanicillium* genus have long been utilised, especially in greenhouses, for the biological control of some aphid species (Rabasse and van Steenis 1999), and some strains are available for the production of commercial products (Jackson *et al.,* 1985). Fungi belonging to this genus are known to be pathogens of aphids (Feng *et al.,* 1990; Askary *et al.,* 1998, 1999; Steenberg and Humber 1999; Fournier and Brodeur 2000; Alavo *et al.,* 2001; Safavi *et al.,* 2002; Shah and Pell, 2003; Sugimoto *et al.,* 2003; Loureiro and Moino 2006; Asman 2007; Kim *et al.,*, 2007; Vu *et al.,* 2007; Diaz *et al.,* 2009) and consequently they have been considered as candidates for biological control. Ganassi *et al* (2010) showed that the *L. lecanii* strain ITEM 3757 influenced the survival and reproduction of aphids of the species *S. graminum* and that this effect is dose-dependent. In addition to entomopathogenic effects on aphids, *L. lecanii* has also been used to control thrips and whiteflies (Schreiter *et al.,* 1994; Van Driesche and Bellows, 1996; Askary *et al.,* 1999; Gindin *et al.,* 2000; Cuthbertson and Walters, 2005; Kim, 2007; Ganassi *et al.,* 2010).

2 AIM OF THE RESEARCH

Despite the preventive measures put in place, the relentless spread of the bacterium, after a few years, reached the territories of the provinces of Bari and Taranto and still represents one of the main threats to the production of olives and olive oil in the Apulia region. To this end, the present study aims to identify methods for the biocontrol of *P. spumarius*, based on the use of entomopathogenic fungi with possible repellent activity and low environmental impact, which can be effectively used on organic crops as well as on conventional crops. The work aims to control the populations of nymphs and adults of *P. spumarius*. In particular, the research aimed at identifying repellents that may reduce the acquisition of *X. fastidiosa* inoculum by the adults and thus the consequent transmission of the pathogen to healthy plants. The microbial products and application methodologies developed by the project could represent important elements in the context of a multiple strategy for the prevention of infection of olive trees and for the containment of OQDS.

3 MATERIALS AND METHODS

3.1 Maintenance of *Philaenus spumarius* in laboratory conditions

P. spumarius nymphs and later, adult morphs were collected from field of *Medicago sativa*, *Hedysarum coronarium*, *Foeniculum vulgare*, *Sonchus oleraceus* and *Cichorium intybus* located near Ripabottoni, Tufara, Gambatesa and Campobasso. Due to limited information on the insect's optimal reproductive conditions in laboratory (Morente *et al*., 2018), we collected *P. spumarius* from the field for the whole period during which the bioassays were carried out (March – July – November). The nymphs were transferred, with a paint brush, to 2-days old *Vicia faba* plants. The nymphs quickly surrounded themselves with a spittle-like foam (Fig. 8-9) until they reached the adult stage. The adult morphs, unlike the nymphs, could be reared on older plants (Figure 10-11). The spittlebugs were maintained into 47.5 x 47.5 x 93 cm, 150 x 150 mesh nylon netting cages with two access openings closed by gauze (Bug-Dorm-2400 71 Insect Rearing Tent, MegaView Science Co. Ltd., Taiwan) at 23±2°C, 70±5% relative humidity (R.H.), and 14:10 L:D photoperiod to obtain a mass-rearing. In these conditions, adults emerged after about 25 days.

Prior to be used in bioassays, adults were identified utilizing the hind leg morphology, since in *P. spumarius* the number of apical spines is less ten (7-8), both in tibia and in tarsus (Elbeaino *et al.,* 2014). The sex of the insects was determined by observing their genitalia with a stereomicroscope.

Fig. 8 and Fig. 9
P. spumarius maintained in laboratory conditions

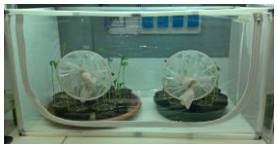


Fig. 10 and Fig. 11
P. spumarius maintained in laboratory conditions

3.2 Fungal isolates tested

The powdered fungal cultures of *Trichoderma citrinoviride* Bisset ITEM 4484, *Trichoderma atrobrunneum* F.B. Rocha, P. Chaverri & W. Jaklitsch ITEM 908 (formerly *T. harzianum* Rifai ITEM 908, Fanelli *et al.*, 2018), *T. atrobrunneum* ITEM 908-5, *Trichoderma minutisporum* Bisset SAM 56, and *Trichoderma* sp. SAM 9 and the methanolic extract of *Trichoderma* sp. SAM 9 were supplied by the Institute of Food Production Sciences (CNR, Bari).

Each isolate was cultured in solid state fermentation on 200 g of autoclaved rice kernels previously adjusted to 45% moisture in a 500-ml erlenmeyer flask and inoculated with 10 ml of an aqueous suspension containing approximately 10^7 conidia per millilitre. The cultures were incubated at 25°C under an L12:D12 cycle for 4 weeks. The harvested culture material was dried in a forced draft oven at 35°C for 48 h, finely ground in a laboratory mill Mulino Cyclone (International PBI, Milano, Italy) to particles ≤ 0.2 mm and stored at 4°C until use. The colony-forming units (CFU) per gram of each powdered fungal culture were determined by the dilution-plate method as follows: *Trichoderma* sp. SAM 9, 6.0 x 10^5 CFU/g; *T. minutisporum* SAM 56, 3.3 x 10^5 CFU/g; *T. citrinoviride* ITEM 4484, 9.7 x 10^4 CFU/g; *T. atrobrunneum* ITEM 908, 3.0 x 10^3 CFU/g; *T. atrobrunneum* ITEM 908-5, 3.4 x 10^6 CFU/g.

The culture extract of *Trichoderma* sp. SAM 9 was obtained by processing and extracting the fungal culture on sterile rice kernels. A 20 g sample of dried culture was extracted in a blender with 100 ml of methanol-1% aqueous NaCl (55+45) for 3 min., filtered through filter paper (Whatman No. 1), and 50 ml of the filtrate were transferred into a separatory funnel and defatted twice with 50 ml of n-hexane. The upper n-hexane layer was discarded and the methanol layer was extracted with three 30 ml portions of methylene chloride. The methylene chloride portions were collected, combined, evaporated to dryness; the residue was dissolved in 1 ml of methanol and stored at +4 °C until use.

Beauveria bassiana (Balsamo-Crivelli) Vuillemin SGB7004, *Metharizium anisopliae* (Metchnikoff) Sorokin SGB1K and *Lecanicillium lecanii* (Zimm.) Zare and Gams SGB4711 in form of powdered fungal cultures and conidial suspensions were supplied by MS Biotech S.p.A. (Larino CB). *B. bassiana* SGB7004 at 1.9 x 10^9 CFU/g; *M. anisopliae* SGB1K at 3.2 x 10^7 CFU/g and *L. lecanii* SGB4711 at 4.5 x 10^9 CFU/g., coming from MS Biotech S.p.A. Mycotech collection, were cultivated by transferring a piece of sporified culture in Petri dishes containing suitable agarized culture substrate, and incubated at 26° C for 7 days. From the obtained cultures, a single monoclonal fungal colony was obtain for each strain. The

single colony was transferred in a Petri dish containing the same culture substrate, and incubated at 26° C until complete sporification. The fungal conidia were removed adding distilled water directly to the plates and the conidial suspension obtained was stored at 4° C.

3.3 Behavioral bioassays

Behavioral bioassays were carried out on *P. spumarius* adults using different powdered fungal cultures. In a first type of bioassay, 30 mg of powdered culture of each *Trichoderma* isolate were suspended in 10 ml of distilled water and stirred for 10 minutes to obtain the following suspensions: *T. citrinoviride* ITEM 4484, 2.91 x 10^2 CFU/ml; *T. atrobrunneum* ITEM 908, 9 x 10^2 CFU/ml; *T. atrobrunneum* ITEM 908-5, 1.02 x 10^4 CFU/ml; *T. minutisporum* SAM 56, 9.9 x 10^2 CFU/ml; and *Trichoderma* sp. SAM 9, 1.8 x 10^3 CFU/ml. The concentration of powdered *Trichoderma* cultures used in this test was the highest applicable without obstructing the dispenser nozzle. A suspension of non-inoculated ground rice kernels at the same concentration as the *Trichoderma* cultures was used as control. Each suspension was then transferred into a dispenser and sprayed on *V. faba* plants.

The bioassays were carried out utilizing 7 days old *Vicia faba* plants, with 12 leaves for plant, cultivated in the laboratory conditions in plastic pots (6 cm diameter; 12 cm height). Before the plants were treated, each pot was covered with parafilm to avoid that volatile compounds from the topsoil interfered with insect choices. Each plant was sprayed with 10 ml fungal suspension or with the control suspension, until complete wetting of the plant and then air-dried for 15 min before use. Four plants, two sprayed with fungal suspension and two with control suspension, were placed into plexiglass cages (size: 42 cm length, 60 cm width, 37 cm depth), the two plants treated with fungal suspension on one side and the two mock plants on the other side, 18 cm apart. Each cage was provided with two access openings (10 cm diameter) in the front door, closed by sleeve canvas to allow insect manipulation. In each of the other cage sides one opening (16 cm diameter), closed by gauze, was present to allow air exchanges. The bioassays were conducted at 23 ± 2 °C, 70 ± 5% relative humidity (R.H.), and 14:10 L:D photoperiod. Ten insects were placed in the center of the bottom of the cage, and their position was recorded: 12, 24 and 48 h from the start of the bioassay. Testing of each treatment (powdered fungal cultures and control) was replicated three times for a total of 30 males and 30 females. Significant differences between the observed and expected frequency of the insects choosing the fungal suspension and the corresponding control was determined using the Chi-square test with the Yate's continuity correction for small sample sizes (Sokal

and Rohlf 1981). The data obtained from behavioural bioassay were also utilized to calculate the Feeding Preference Index calculated as FPI = (C-T/C+T) × 100, where: C= the number of *P. spumarius* counted on control; T = the number of *P. spumarius* counted on treated. FPI may vary from -100 (complete preference for treated) to + 100 (complete preference for control), with 0 meaning no effect (Powell *et al.*, 1997).

Based on the results obtained in the above behavioral bioassay, a second bioassay was carried out with a different methodology. In this bioassay the rice culture of *T. citrinoviride* ITEM 4484 was tested at the concentrations of 5.82×10^3, 2.91×10^3, 1.45×10^3, 7.2×10^2 and 3.6×10^2 CFU/ml (that is 60, 30, 15, 7.5 and 3.75 mg/ml, respectively) in 12 cm-diameter glass Petri dishes. *Vicia faba* shoots, 5-7 cm long, grown in the laboratory in plastic pots (diameter 6 cm, height 12 cm) were utilized. Suspensions of fungal culture and non-inoculated rice (control) were prepared in 45 ml of distilled water to completely submerge the shoots, and then gently shaken for 10 seconds. Then, the shoots were placed on wet filter paper in Petri dishes. Each dish contained two shoots, one treated with the test suspension and one dipped in the control suspension, arranged in parallel at a distance of 4 cm (Fig. 12). Bioassays with fungal suspensions were preceded by two sets of experiments. The first set evaluated the behavioural response of insects to rice (60 mg/ml) *vs*. water. In the second one, the response of insects to rice/rice (60 mg/ml) was assessed. To avoid interactions among insects during the choice, only one adult (male or female) was placed between the two shoots and its position was recorded after 1, 3, 6, 12 and 24 hours, starting from the initial access which started less than 1 hour after the shoot excision. For each bioassay, 5 insects for treatment or control were tested and the test were repeated six times for a total of 30 males and 30 females. The raw data obtained from the behavioral bioassay feeding preference tests with Petri dishes were analyzed by the Generalized Linear Model (GLM) for repeated measures (over time) procedure and compared by using a test of within-subject effects. The Bonferroni test was used to assess whether the mean number of *P. spumarius* on treated shoots and the number of *P. spumarius* on the control shoots, over time, were significantly different. All the statistical analyses were performed by Statistical Package for Social Sciences (SPSS), version 25.0 for Windows software (SPSS Inc., Chicago, IL).

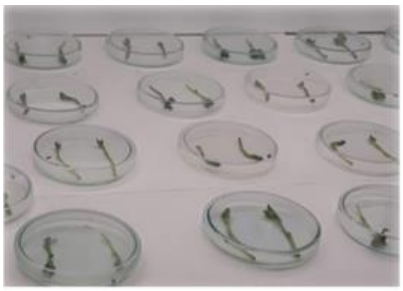


Fig. 12 - *V. faba* shoots treated with *T. citrinoviride* ITEM 4484 and control suspension, placed on wet filter paper in Petri dishes.

Fig. 13 – *P. spumarius* adult in Petri dish with two *V. faba* shoots, one treated with *T. citrinoviride* ITEM 4484 suspension and the other with rice one.

3.3.1 Results of behavioral bioassays on plants in observation cages

Results of behavioral bioassays on *P. spumarius* adults carried out with *V. faba* plants in observation cages are summarized in table 1. Only the fungal culture of *T. citrinoviride* ITEM-4484 showed a significant phagodeterrent effect, over the duration of the bioassay (χ^2=5.760, df=1, P<0.05; χ^2=7.041, df=1, P<0.01; χ^2=9.480, df=1, P<0.01), limitedly to males; on the contrary, the fungus showed a slight attractive effect on females. *T. atrobrunneum* ITEM 908 showed attractive effect on both males and females.

Table 1. Behavioural response of *P. spumarius* adult females and males to fungal cultures of different *Trichoderma* spp. isolates, sprayed on *V. faba* plants.

Females	**12 h**					**24 h**					**48 h**				
Fungal culture concentration CFU/ml	**N1 (%)[1]**	**♀(%) attracted by fungi**	**FPI %**	**$\chi 2^a$ (df)**	***P***	**N (%)[1]**	**♀(%) attracted by fungi**	**FPI %**	**$\chi 2^a$ (df)**	***P***	**N (%)[1]**	**♀(%) attracted by fungi**	**FPI %**	**$\chi 2^a$ (df)**	***P***
***T. atrobrunneum* ITEM 908** *vs.* rice	53,33	75.00	- 50.0	3.062 (1)	*P*>0.05	60.00	55.55	-11.11	0.005 (1)	*P*>0.05	73.33	63.63	- 27.27	1.113 (1)	*P*>0.05
***T. atrobrunneum* ITEM 908-5** vs rice	90.00	44.44	11.11	0.148 (1)	*P*>0.05	90.00	44.44	11.11	0.050 (1)	*P*>0.05	93.33	42.85	14.28	0.000 (1)	*P*>0.05
***T. minutisporum* SAM 56** vs rice	56.66	52.94	-5.88	0,00 (1)	*P*>0.05	66.66	50.00	0.00	0.050 (1)	*P*>0.05	70	47.61	4.76	0.000 (1)	*P*>0.05
***Trichoderma* sp. SAM 9** vs rice	76.66	69.56	-39.13	2.782 (1)	*P*>0.05	86.66	69.23	-38.46	3.115 (1)	*P*>0.05	86.66	46.15	7.69	0.038 (1)	*P*>0.05
***T. citrinoviride* ITEM 4484** *vs* rice	76.66	65.21	-30.43	1.565 (1)	*P*>0.05	83.33	68.00	-36.00	2.560 (1)	*P*>0.05	73.33	59,09	-18.18	0.409 (1)	*P*>0.05

Males	**12 h**					**24 h**					**48 h**				
Fungal culture concentration CFU/ml	**N2 (%)[1]**	**♂(%) attracted by fungi**	**FPI %**	**$\chi 2^{a}$ (df)**	***P* value**	**N (%)[1]**	**♂(%) attracted by fungi**	**FPI %**	**$\chi 2^{a}$ (df)**	***P* value**	**N (%)[1]**	**♂(%) attracted by fungi**	**FPI %**	**$\chi 2^{a}$ (df)**	***P* value**
***T. atrobrunneum* ITEM 908** *vs* rice	83.33	64.00	-28.0	1.440 (1)	*P*>0.05	73.33	77.27	-54.54	5.500 (1)	***P*<0.05**	73.33	59.09	-18.18	0.409 (1)	*P*>0.05
***T. atrobrunneum* ITEM 908-5** vs rice	83.33	48.00	4.00	0.000 (1)	*P*>0.05	93.33	39.28	21.42	0.892 (1)	*P*>0.05	93.33	42.85	14.28	0.321 (1)	*P*>0.05
***T. minutisporum* SAM-56** vs rice	53.33	50.00	0.00	0.062 (1)	*P*>0.05	63.33	57.89	-15.78	0.201 (1)	*P*>0.05	63.33	63.15	-26.31	0.842 (1)	*P*>0.05
***Trichoderma* sp. SAM 9** *vs* rice	60.00	61,11	-22.2	0.500 (1)	*P*>0.05	66,66	70.00	-40.00	0.941 (1)	*P*>0.05	76.66	69.56	-39.13	2.782 (1)	*P*>0.05
***T. citrinoviride* ITEM-4484** *vs* rice	83.33	24.00	52.00	5.760 (1)	***P*<0.05**	80	20.83	58.33	7.041 (1)	***P*<0.01**	90.00	18.51	62.96	9.480 (1)	***P*<0.01**

- N1 indicates the percentage of *P. spumarius* females that made their choice within 15 min;
- N2 indicates the percentage of *P. spumarius* males that made their choice within 15 min.
- Females (%) attracted by the tested stimulus indicates the percentage of *P. spumarius* females that chose the plants with the stimulus, among those that carried out a choice at 12, 24, 48h.
- Males (%) attracted by the tested stimulus indicates the percentage of *P. spumarius* males who chose the plants with the stimulus, among those that carried out a choice 12, 24, 48h.

FPI = Feeding Preference Index

Chi-square test with the Yate's continuity correction for small sample sizes was used to determine significant difference between the observed and expected frequency of the insects choosing the stimulus (fungal culture) and the control (rice).

3.3.2 Results of the activity of *T. citrinoviride* ITEM 4484 on *P. spumarius* adult behaviour carried out on *Vicia faba* shoots

Behavioral bioassay data were analyzed with the repeated GLM measurement procedure. This analysis assessed whether the interaction between the test conditions (treated or untreated) and the changes over time in the number of *P. spumarius* individuals that visited the treated shoots or the control ones were statistically significant. The time-treatment interaction indicated that the number of *P. spumarius* for the treated shoots and the number of *P. spumarius* for the control shoots changed over time. In our bioassays, a significant time-treatment interaction effect indicated that the number of *P. spumarius* counted on treated shoots increased with time, over the duration of the experiment. The Bonferroni test was used to assess whether the average number of *P. spumarius* on the treated shoot was significantly lower than that on the corresponding control shoot, over time, indicating a phagodeterrent effect of the suspension. The results of the phagodeterrent effect of *T. citrinoviride* ITEM 4484 are shown in Table 2.

The GLM analysis of the data obtained in bioassays with *T. citrinoviride* ITEM 4484, at all concentrations tested, for both males and females, revealed time × treatment interaction effects. On the contrary, in bioassays with H_2O-Rice and Rice-Rice no time × treatment interaction effect was found. Over time, the average number of insects on treated shoots, at all the concentrations tested, was significantly smaller than the number of insects on the control shoots, but no significant differences was found for H_2O-Rice and rice-rice (Bonferroni test).

Table 2. Effect of *T. citrinoviride* ITEM 4484 on the feeding preference of *P. spumarius* adults carried out on *Vicia faba* shoots

Tested stimuli	**GLM (time × treatment)**[a]		**Bonferroni test**[b]		
			Mean treatment	**Mean control**	
Males					
H_2O-Rice (60mg/ml)	$F_{4,40} = 0.422$	P>0.05	1.500± 0.242	2.067 ± 0.242	ns
Rice-Rice (60mg/ml)	$F_{4,40} = 0.493$	P>0.05	1.567 ± 0.251	1.600 ± 0.251	ns
***Trichoderma citrinoviride* ITEM 4484**					
$5{,}82 \times 10^3$	$F_{4,40} = 5.912$	P<0.05	0.233 ± 0.207	2.867 ± 0.207	**
$2{,}91 \times 10^3$	$F_{4,40} = 5.294$	P<0.05	0.267 ± 0.228	1.967 ± 0.228	**
$1{,}45 \times 10^3$	$F_{4,40} = 1.288$	P<0.05	0.567 ± 0.210	2.467 ± 0.210	**
$7{,}2 \times 10^2$	$F_{4,40} = 1.343$	P<0.05	0.733 ± 0.394	2.333 ± 0.394	*
$3{,}6 \times 10^2$	$F_{4,40} = 3.441$	P<0.05	0.767 ± 0.310	3.067 ± 0.310	**
Females					
H_2O-Rice (60mg/ml)	$F_{4,40} = 0.422$	P>0.05	1.467 ± 0.285	1.967 ± 0.285	ns
Rice-Rice (60mg/m)	$F_{4,40} = 0.378$	P>0.05	1.800 ± 0.178	1.500 ± 0.178	ns
***Trichoderma citrinoviride* ITEM 4484**					
$5{,}82 \times 10^3$	$F_{4,40} = 11.855$	P<0.0001	0.300 ± 0.245	2.433 ± 0.245	**
$2{,}91 \times 10^3$	$F_{4,40} = 7.041$	P<0.0001	0.300 ± 0.375	2.600 ± 0.375	**
$1{,}45 \times 10^3$	$F_{4,40} = 2.857$	P<0.05	0.667± 0.378	2.533 ± 0.378	**
$7{,}2 \times 10^2$	$F_{4,40} = 5.060$	P<0.05	0.467 ± 0.288	2.567 ± 0.288	**
$3{,}6 \times 10^2$	$F_{4,40} = 1.025$	P<0.05	1.167 ± 0.261	2.100 ± 0.268	*

[a]Values of $P < 0.05$ for GLM indicate that the interaction between either test conditions (treated or control) and the change over time was statistically significant. Values of $P > 0.05$ for GLM indicate that the interaction between either test conditions (treated or control) and the change over time was not statistically significant.

[b]The average number of insects on treated shoot and the number of insects on the control shoot over the duration of the assay were analyzed and adjusted with Bonferroni test for the number of comparisons. In each treatment, significant difference between the means are indicated with *($P < 0.05$) or with ** ($P < 0.01$), ns indicates a not significant difference.

3.3.3 Discussion behavioral bioassays

Until recent times, *P. spumarius* was not considered a serious pest and rarely caused severe damages (Whittaker 1973, Buczacki *et al* 1991). However, at present, it is regarded as the main vector of *X. fastidiosa*, (Martelli *et al.*, 2015; Cornara *et al.*, 2019). The identification of behaviorally-active natural compounds, interfering in *P. spumarius* host plant selection processes, could be of great practical interest in developing sustainable control strategies towards the spittlebug to prevent sap sucking from olive plants and the spread of the bacterial pathogen from infected to healthy plants. Moreover, attractant compounds could be used to develop suitable monitoring systems for this pest.

Our results of behavioral bioassays, with detached shoots in glass Petri dishes, indicate that *P. spumarius* adult males and females can discriminate between shoots treated with *T. citrinoviride* ITEM-4484 and untreated ones and that the powdered fungal culture clearly influenced the feeding preference of males and females. Indeed, at all the concentrations tested, both the mean number of males and females on the control leaves was significantly greater than that on the treated leaves.

The different effect of *T. citrinoviride* ITEM-4484 on *P. spumarius* in behavioral bioassays carried out with plants in cages, which was slightly attractive to females, could be due to a non-uniform distribution of the powdered fungal culture on plants.

Fungal species of the genus *Trichoderma* are well known to produce volatile and non-volatile organic compounds, derived from both primary and secondary metabolism, involved in numerous biological activities (Ganassi *et al.*, 2007; Korpi *et al.*, 2009; Morath *et al.*, 2012; Jelen *et al.*, 2014; Hung *et al.*, 2015; Ganassi *et al.*, 2016; Lee *et al.*, 2016; Zelinger *et al.*, 2016; Li *et al.*, 2019), some of which are of great agricultural importance (Mathivanan *et al.*, 2008; Mukherjee *et al.*, 2012). Volatile organic compounds, produced by *Trichoderma* spp., belong to different groups of chemical compounds as hydrocarbons, aromatics, amines, thiols, and terpenes (Korpi *et al.*, 2009; Lemfack *et al.*, 2014) and *P. spumarius* male and female peripheral olfactory systems proved to be able to perceive a broad range of VOCs, in spite of the low number of antennal sensory structures (Germinara *et al.*, 2017).

However, even non-volatile compounds could be perceived by *P. spumarius.* In this regard, Ganassi *et al.*, (2007; 2016) and Evidente *et al.*, (2009) demonstrated that metabolites produced by *Trichoderma* species, including *T. citrinoviride* ITEM-4484, function as signal molecules that can modify aphid feeding preferences resulting in a strong phagodeterrent activity, and that taste cells, located on aphid tarsomeres, were involved in the perception of these ones.

Trichoderma spp. live in soil and grow saprophytically on wood, bark and many other organic substrates. As a consequence, these fungi exibit a high adaptability to various ecological conditions and a great metabolic versatility (Atanasova *et al.*, 2013b; Holzlechner *et al.*, 2016; Kubicek *et al.*, 2011). The isolates that were used in our tests, having been originally isolated from samples of soils or plant debris, might be introduced in agrarian ecosystems with good chances of successful establishment, and without any environmental risk that might be possibly associated to the introduction of alien fungal species or isolates. Moreover, the tested fungal isolates were applied in the form of ground dried cultures grown in solid state fermentation on rice kernels. Solid state fermentation on cereal grains is one of the most used methods for the production of fungal biomass of biopesticides and is also fully compatible with organic farming and other forms of environmentally friendly agriculture.

4 MATERIALS AND METHODS

4.1 Fungal pathogenicity bioassays by inoculation into nymph spittle

The bioassays were conducted utilizing 25 mg of powdered culture of different fungal isolates. Cultures were suspended in 1 ml of distilled water and then shaken for 10 minutes. The tested fungi were *T. minutisporum* SAM 56 (8.25 x 10^3 CFU/ml), *T. citrinoviride* ITEM 4484 (2,425 x 10^3 CFU/ml), *T. harzianum* ITEM 908 (7.5 CFU/ml), *T. harzianum* ITEM 908-5 (8.5 x 10^4 CFU/ml), *Trichoderma* sp. SAM 9 (1,5 x 10^4 CFU/ml), *B. bassiana* SGB7004 (4.75 x 10^7 CFU/ml), *M. anisopliae* SGB1K (8.00 x 10^5 CFU/ml) and *L. lecanii* SGB4711 (1.35 x 10^8 CFU/ml). Finely ground rice (25 mg) was used as control for the fungi of the genus *Trichoderma*, *B. bassiana* SGB7004 and *L. lecanii* SGB4711, and bran (25 mg) for *M. anisopliae* SGB1K. The bioassays were performed using 5-10 cm high *V. faba* plants grown in plastic pots (diameter 6 cm, height 12 cm) on a laboratory bench. Ten plants were used for each treatment and for controls. The plants were placed into 47.5 x 47.5 x 47.5 cm, 150 x 150 mesh nylon netting cages with two access openings closed by gauze (Bug-Dorm-2400 71 Insect Rearing Tent, MegaView Science Co. Ltd., Taiwan) (Fig. 14). Five nymphs from the third to fourth stage were transferred with a paintbrush on each plant, and they surrounded themselves with the spittle (Fig. 14). Twenty microliters of powdered fungal culture or of control suspensions were inoculated with a Gilson pipette into the spittle surrounding the nymphs. The spittle and the insects were observed daily under a stereomicroscope for five days. The bioassays

were conducted at room temperature (23° C), 70% relative humidity and 14:10 (L: D) photoperiod.

Fig. 14 - Third to fourth stage nymphs transferred on *V. faba* plant. Twenty microliters of powdered fungal culture or of control suspensions were inoculated into the spittle surrounding the nymphs.

4.1.1 Results of fungal pathogenicity bioassays by inoculation of nymph spittle

Daily observations of the spittle inoculated with the fungal suspensions under a stereomicroscope allowed to record the lack of mycelial hyphae on the surface of the spittle. All the nymphs inside the spittle reached the adult stage and both, nymphs and adults, did not show signs of fungal development on the cuticle.

5 MATERIALS AND METHODS

5.1 Preliminary evaluation of *P. spumarius* spittle's antimicrobial activity

In collaboration with the plant Pathology Laboratory of the Department of Agriculture, Environment and Food of Molise University, preliminary tests were conducted on fresh spittle picked up in order to evaluate its possible antimicrobial activity.

The protective role of *P. spumarius* (meadow spittlebug) spittle has been discussed in a number of papers for many decades. Both the nymphs and adults of most spittlebugs are xylem feeders, but only the nymphs produce spittle. The content of spittle is derived from the xylem but it is further elaborated in two ways: the fluid portions comprises an anal secretion into which the insect introduces numerous air bubbles by means of caudal appendage (German, 1921) the retention of the air bubbles and viscosity of the spittle mass is maintained by a mucilaginous substance which is produced from the abdominal Batelli glands (Kershaw, 1914).

5.1.1 *Philaenus spumarius* spittle

Fresh *P. spumarius* spittle was collected, using a sterile syringe, from *P. spumarius* nymphs fed on *V. faba* and was stored in 1 ml sterile glass tube.

The activity was tested against two phytopathogenic bacteria: *Pseudomonas syringae* pv. *tomato* strain DC3000 and *Xanthomonas campestris* pv. *pelargonii* (from the collection of the Plant Pathology laboratory of Department of Argricoltural, Enviromental and Food Science of Molise University) and against the fungi: *L. lecanii* SGB4711, *B. bassiana* SGB11B, *M. anisopliae* SGB1K, *T. atrobrunneum* 908, *T. atrobrunneum* 908-5, *T. citrinoviride* ITEM 4484, *T. minutisporum* SAM 56, and *Trichoderma* sp. SAM 9. Furthermore, the spittle was tested towards the phytopathogenic fungi *Fusarium oxysporum* f. sp. *lycopersici* and *Alternaria alternata* (from the collection of the Plant Pathology laboratory of Department of Agricultural, Environmental and Food Science of Molise University).

5.1.2 Spittle antibacterial assay

For each bacterium, 100 µl of cell suspension (1 x 10^8 CFU/ml) were inoculated in Petri dish containing 15 ml of Nutrient Agar (NA) and spread with 2-3 mm glass beads. Nine filter paper disks (Ø 5mm) were placed on the agar surface of the Petri dish, as showed in Fig. 15. Twenty microliter of spittle (PSS) were inoculated on each filter paper disk forming a first line; on the second line, disks were loaded with 20 µl of sterile H_2O as negative control; and on a third line disks were loaded with 20 µl of an antibiotics mix solution (Ampicillin 100.000 ppm and

Streptomycin 250.000 ppm) (Amp + Stept) as positive control. The plates were incubated at 28 °C in the dark for 48 h. The antibacterial activity was calculated by measuring the diameter of the inhibition area around the filter paper disk.

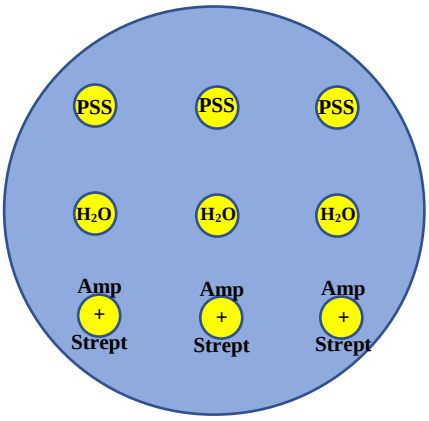


Fig. 15 - Spittle antibacterial assay. Filter paper disk disposition on the agar surface in Petri dish. PSS = *P. spumarius* spittle; H_2O = Negative control; Amp+Strept = Positive control.

5.1.3 Spittle antifungal assay

For each fungus, 100 µl of conidial suspension (1 x 10^4 CFU/ml) were inoculated in Petri dish containing 15 ml of Potato Dextrose Agar (PDA) and spread with 2-3 mm glass balls. Nine filter paper disks (Ø 5mm) were placed on the agar surface of the Petri dish as showed in Fig. 16. Twenty µl of spittle (PSS) were inoculated on each filter paper dish forming a first line; on a second line, the disks were loaded with 20 µl of sterile H_2O as negative control; and on a third line the disks were inoculated with 20 µl of a 10% Zn formulate solution as positive control. The plates were incubated at 24 C° in the dark for 5 day. The antifungal activity was calculated by measuring the diameter of the inhibition area around the filter paper disk.

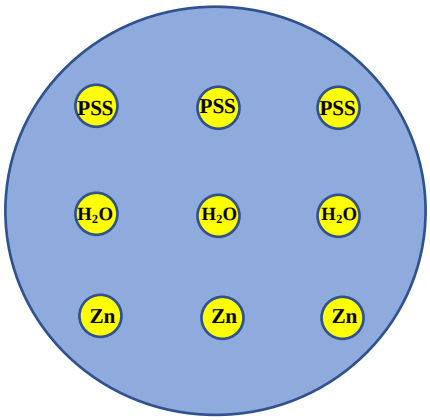


Fig. 16 - Spittle antifungal assay. Filter paper disk disposition on agar surface in Petri dish. PSS = *P. spumarius* spittle; H_2O = Negative control; Zn = Positive control.

5.1.4 Results of spittle antibacterial assay

Antibacterial activity was not detected by this assay. However, further investigations are needed to evaluate a possible activity in liquid cultures of bacteria or using ultra-concentrated insect spittle.

5.1.5 Results of spittle antifungal assay

Antifungal activity was not detected after 5 days of incubation, however, in the samples 2, 3 and 6 an alteration of fungal growth, probably due to a reduction of sporulation, was apparent (Fig. 17).

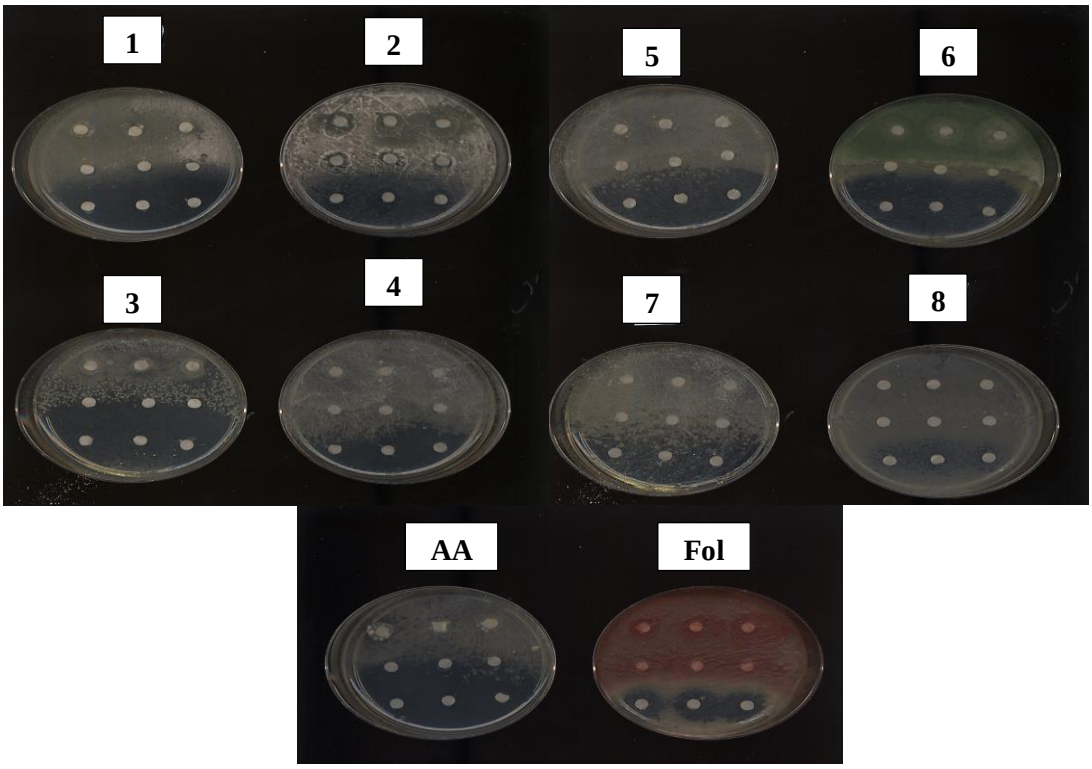


Fig. 17 – Antifungal activity of *P. spumarius* spittle after 5 days of incubation with the following strains: 1) *Lecanicillium lecanii* SGB4711; 2) *Beauveria bassiana* SGB11B; 3) *Metharizium anisopliae* SGB1K; 4) *Trichoderma atrobrunneum* 908; 5) *Trichoderma atrobrunneum* 908-5; 6) *Trichoderma citrinoviride* ITEM 4484; 7) *Trichoderma minutisporum* 56; 8) *Trichoderma* sp. *SAM 9*; AA) *Alternaria alternata*; Fol) *Fusarium oxysporum* f.sp. *lycopersici*.

Ten days after the inoculation, a reduction of the sporulation around the filter paper disks treated with PSS, compared to the control, was apparent for all the tested fungi (Fig. 18)

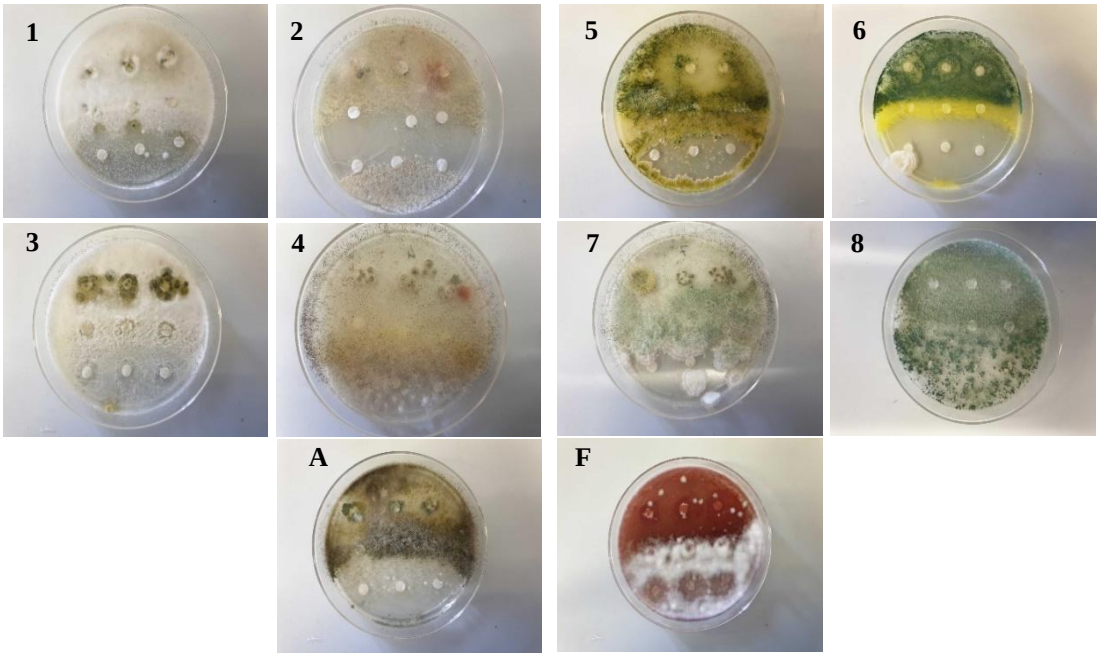


Fig. 18 - Antifungal activity of *P. spumarius* spittle after 10 days of incubation with the following strains 1) *Lecanicillium lecanii* SGB4711; 2) *Beauveria bassiana* SGB11B**;**3) *Metharizium anisopliae* SGB1K; 4)*Trichoderma atrobrunneum* 908*;* 5) *Trichoderma atrobrunneum* 908-5*;* 6) *Trichoderma citrinoviride* ITEM 4484; 7) *Trichoderma minutisporum* SAM 56; 8) *Trichoderma* sp. SAM 9; AA) *Alternaria alternata;* Fol) *Fusarium oxysporum* f.sp. *lycopersici*.

6 MATERIALS AND METHODS

6.1 Pathogenicity bioassays with powdered fungal cultures on *P. spumarius* nymphs and adults

In these bioassays the pathogenicity of powdered fungal cultures toward *P. spumarius* nymphs and adults was evaluated.

In the bioassays with nymphs, at third stage, the fungal cultures were tested at 60 mg/ml corresponding to the following fungal propagule concentrations: *B. bassiana* SGB11B, 1.14 x 10^8 CFU/ml; *M. anisopliae* SGB1K, 1.92 x 10^6 CFU/ml; and *L. lecanii* SGB4711, 3.24 x 10^8 CFU/ml. In addition, the conidial suspensions of *B. bassiana* SGB11B at the concentration 9.1 x 10^8 CFU/ml, and of *M. anisopliae* SGB1K 1.5 x 10^7 CFU/ml were also tested.

In the bioassays with adults, the powdered fungal cultures were tested at 2.91 x 10^3, 5.82 x 10^3, 1.16 x 10^4 CFU/ml for *T. citrinoviride* ITEM 4484; 9.0 x 10, 1.8 x 10^2, 3.6 x 10^2 CFU/ml for *T. atrobrunneum* ITEM 908; 1.02 x 10^5, 2.04 x 10^5, 4.08 x 10^5 CFU/ml for *T. atrobrunneum* ITEM 908-5; 5.7 x 10^7, 1.14 x 10^8, 2.28 x 10^8 CFU/ml for *B. bassiana* SGB11B; 9.6 x 10^5, 1.92 x 10^6, 3.8 x 10^6 CFU/ml for *M. anisopliae* SGB1K and 1.62 x 10^8, 3.24 x 10^8, 6.48 x 10^8 CFU/ml for *L. lecanii* SGB4711 corresponding respectively to: 30, 60, and 120 mg/ml. In addition, the conidial suspensions of *B. bassiana* SGB11B at 5.4 x 10^8 CFU/ml, of *M. anisopliae* SGB1K 1.9 x 10^6 CFU/ml, and of *L. lecanii* SGB4711 2.2 x 10^8 CFU/ml were also tested. *Trichoderma* sp. SAM 9 was tested at 4.5 x 10^3, 9 x 10^3, 1.8 x 10^4, 3.6 x 10^4, 7.2 x 10^4 CFU/ml (7.5, 15, 30, 60 e 120 mg/ml) and *T. minutisporum* SAM 56 at 9.9 x 10^3, 1.98 x 10^4, 3.96 x 10^4, 4.95 x 10^4, 5.94 x 10^4 CFU/ml (30, 60, 120, 150 and 180 mg / ml). Powdered rice was used as control, except in bioassay with *M. anisopliae* where powdered bran was used. The powdered cultures and controls were suspended in 1 ml of distilled water and 0.01% Tween 40 as wetting agent. The suspensions were shaken for 1 minute.

Trichoderma sp. SAM 9 was also tested using its water extract, obtained suspending the fungal culture, at 4.5 x 10^3, 9 x 10^3, 1.8 x 10^4, 3.6 x 10^4, 7.2 x 10^4 CFU/ml (7.5, 15, 30, 60 and 120 mg/ml), in 1 ml sterile distilled water and 0.01% Tween 40, shaken for 10 min and then centrifuged at 3000 rpm for 2 minutes. Also, a 1,77% (v/v) solution of the methanolic extract of *Trichoderma* sp. SAM 9, obtained by diluting 0.177 ml of methanolic extract in 10 ml of 0.01% Tween-40, was tested 1.77 % methanol in 0.01% Tween-40 was used as control.

The bioassays were conducted using 10 x 10 cm plastic Petri dishes, whose lids had a round opening closed with a very thin nylon net for ventilation. The dishes were provided with a plastic septum with three 5 mm-diameter holes, which divided the dish into two compartments. In the

compartment between the septum and the edge of the box, wet wadding and three *V. faba* seeds with shoots were placed; the shoots were inserted in the holes of the septum. The wet wadding was placed to ensure the availability of water to prevent shoots dehydration.

A single adult insect, or a single nymph, was dipped for 10 sec. into the fungal culture suspensions, conidial suspensions or into the supernatant suspension and then laid onto filter paper to drain the excess liquid. Then, the insect was transferred into the Petri dishes (Fig. 19A-19B).

For adult insects, five adults were transferred in each dish. Six replicated plates (30 adults total) were prepared for each fungal treatment and for each control.

For the nymphs, six nymphs were transferred in each of five replicated dishes (30 nymphs total). Insects were checked under a stereomicroscope 24, 48, 72, 96, and 120 h after the start of the bioassay. The tests were conducted at room temperature (approx. 25° C), 90% relative humidity, and 14:10 (L: D) photoperiod.

Fig. 19A - Nymphs transferred to a Petri dishes

Fig. 19B - Adult transferred to a Petri dishes

The raw data on *P. spumarius* adult survival, obtained after 24, 48, 72, 96 and 120 hours after the start of the bioassay, and on nymph survival, after 24, 48, and 72 hours were analyzed using the General Linear Model (GLM) for repeated measures (over time) procedure and compared by using a test of within-subjects effects. The Bonferroni test was used to assess whether the mean number of insects that survived to exposure to the same concentration of powdered fungal cultures, conidial suspension or fungal supernatant suspension and the number of insects that survived in controls at different times of exposure, were significantly different.

The percentages mortality of *P. spumarius* adults were reported as a mean of values, starting from six replicates, corrected with Abbotts' formula (Abbott, 1925). To calculate LC50 and LC90 with a 95% confidence interval, data obtained in the bioassays with adults were corrected using the Abbotts' formula, transformed into arcsine / proportion values and then subjected to probit regression analysis.

The percentages mortality of *P. spumarius* nymphs were reported as a mean of values, starting from five replicates, corrected with Abbots' formula (Abbott, 1925).

6.2 Results of pathogenicity tests with powdered fungal cultures on *P. spumarius* nymphs and adults

The results of the pathogenicity bioassays carried out with powdered fungal cultures of *B. bassiana* SGB11B, *M. anisopliae* SGB1K and *L. lecanii* SGB4711 against *P. spumarius* nymphs, expressed as percent mortality corrected using Abbott's formula are shown in Table 3. In *B. bassiana* SGB11B and *L. lecanii* SGB4711 bioassays the control mortalities were around 30% and only in *M. anisopliae* SGB1K bioassays control mortality was 0%.

Both *B. bassiana* SGB11B and *M. anisopliae* SGB1K powdered culture suspensions and conidial suspensions were active on the nymphs, already 24 hours after the start of the bioassay. The higher mortality values, corresponding to 81.333% ± 0.816 and 88.667% ± 7.870 for *B. bassiana* SGB11B powdered culture and conidial suspensions respectively, and to 58.667± 3.742 for *M. anisopliae* SGB1K conidial suspension, were reached after 48h. With regard to *M. anisopliae* SGB1K powdered culture, the higher nymph mortality value was reached after 72h. The *L. lecanii* SGB4711 powdered culture suspension was active only after 48 hours from the start of the bioassay, resulting in 39.333% ± 3.055 nymph mortality. With regard to *B. bassiana* SGB11B and *L. lecanii* SGB4711, the mortality values obtained after 72h (75.000 ± 7.906; 22.000 ± 6.442) were lower than these after 48h because of mortality recorded in controls.

Table 3. Pathogenic activity of *B. bassiana* SGB11B, *M. anisopliae* SGB1K and *L. lecanii* SGB4711 against *P. spumarius* nymphs

***Beauveria bassiana* SGB11B powdered culture suspension**			
Concentration CFU/ml	Mortality%[a] mean ± SE 24 h	Mortality%[a] mean ± SE 48 h	Mortality%[a] mean ± SE 72 h
1.14×10^8	23.667 ± 5.812	81.333 ± 0.816	75.000 ± 7.906
***Beauveria bassiana* SGB11B conidial suspension**			
9.1×10^8	56.667 ± 4.082	88.667 ± 7.870	88.667 ± 7.870
***Metharizium anisopliae* SGB1K powdered culture suspension**			
1.92×10^6	60.000 ± 4.082	86.667 ± 3.333	90.000 ± 4.082
***Metharizium anisopliae* SGB1K conidial suspension**			
1.5×10^7	23.333 ± 4.082	58.667± 3.742	58.667± 3.742
***Lecanicillium lecanii* SGB4711 powdered culture suspension**			
3.24×10^8	0.000 ± 0.000	39.333 ± 3.055	22.000 ± 6.442

[a]The nymphs mortality percentages are the means of five replicates, each one carried out with 6 individuals, corrected using Abbotts's formula.

The raw data obtained by pathogenicity tests with *B. bassiana* SGB11B, *M. anisopliae* SGB1K and *L. lecanii* SGB4711 suspensions on nymphs, were analyzed using the GLM repeated measures procedure and Bonferroni test. GLM assessed whether the interaction between both test conditions (treatment and control) and the changes over the time of the number of nymphs survived to exposure to fungal suspensions and the number of survived control nymphs was statistically significant (Table 4). The Bonferroni test was used to assess whether the mean number of nymphs that survived to exposure to fungal suspensions was significantly smaller than the mean number of survived nymphs in control, over time, indicating a lethal effect of the fungal suspensions. The Bonferroni test revealed that the mean number of adults exposed to *B. bassiana* SGB11B, *M. anisopliae* SGB4711, and to *L. lecanii* SGB1K at the concentrations tested, was significantly smaller than the number of respective controls, over time (Table 4).

Table 4. Pathogenic effect of *B. bassiana* SGB11B, *M. anisopliae* SGB1K and *L. lecanii* SGB4711 against *P. spumarius* nymphs

Concentration	**GLM (time × treatment)[a]**		**Bonferroni test[b]**		
			Mean treatment	**Mean control**	
Beauveria bassiana SGB11B 1.14x 10^8 CFU/ml	$F_{2,16} = 19.846$	*P* <0.01	1.800 ± 0.120	5.00 ± 0.120	**
Metarhizium anisopliae SGB1K 1.92 x 10^6 CFU/ml	$F_{2,16} = 41.714$	*P* <0.01	1.267 ± 0.137	6.000 ± 0.137	**
Lecanicillium lecanii SGB4711 3.24 x 10^8 CFU/ml	$F_{2,16} = 20.222$	*P* <0.01	4.133± 0.200	5.200 ± 0.200	**

GLM values describe the effect of time on survival of the nymphs
ns not significant; *P<0.05; **P<0.01

[a]Values of P>0.05 for GLM indicate that the interaction between the two conditions (treated and control) and the change over time was not statistically significant. Values of P<0.05 for GLM indicate that the interaction between the two conditions (treated and control) and the change over time was statistically significant

[b]Differences between the means of the number of survived nymphs at different powdered fungal cultures in each of the experimental treatments and those of the number of related controls over time were analyzed and adjusted with Bonferroni test for the multiple of comparisons.

The results of pathogenicity bioassays carried out on *P. spumarius* adults, utilizing powdered *B. bassiana* SGB11B, *M. anisopliae* SGB1K and *L. lecanii* SGB4711 cultures at different concentrations, and respective conidial suspensions, expressed as percentage mortality corrected using Abbott's formula, are shown in Table 5. In most bioassays the control mortalities was well below 10% and only in some bioassays it reached a maximum of 10%.

B. bassiana SGB11B powdered culture suspension, at the highest concentrations tested (1.14 x 10^8 and 2.28 x 10^8 CFU/ml), caused respectively 50.000% ± 4.472 and 70.000% ± 4.472 adult mortality 96 hours after the start of the bioassay, while the conidial suspension caused 30.000% ± 4.472 adult mortality at the concentration 5.4 x 10^8 CFU/ml. One hundred and twenty hours after the start of the bioassay, mortality reached 93.333% ± 6.667, 96.667% ± 3.333 and 86.667% ± 6.667 respectively. *M. anisopliae* SGB1K powdered culture suspensions, at the lowest concentration (9.6 x 10^5 CFU/ml), and at the highest one (3.84 x 10^6 CFU/ml), caused 10.000 ± 4.472 and 47.500 ± 5.439 adult mortality percentage respectively, after 96h; the percent mortality values increased to 33.333% ± 4.216 and 81.667% ± 3.801 after 120h. At the intermediate concentration (1.92 x 10^6 CFU/ml) for *M. anisopliae* SGB1K powdered culture and at 1.9 x 10^6 CFU/ml for conidial suspension, mortality percentage values were 6.667 ± 4.216 and 40.000 ± 5.164 after 96h; 66.667 ± 6.667 and 45.000 ± 3.416 after 120h.

L. lecanii SGB4711 powdered culture suspensions, at 5.4 x 10^8 CFU/ml caused 10.000 ± 4.472, 13.333 ± 4.216 and 26.667 ± 4.216 mortality percentage values after 72, 96, and 120h from the start of the bioassay, while conidial suspension, at 2.2 x 10^8 CFU/ml caused 10.000 ± 4.472, 23.333 ± 6.146, 33.333 ± 6.667, 56.667 ± 3.333, 58.333 ± 1.667 mortality percentage values after 24, 48, 72, 96, and 120h. The percentage of dead adults after treatment with *B. bassiana* SGB11B (2.28 x 10^8 CFU/ml), *M. anisopliae* SGB1K (3.84 x 10^6) and *L. lecanii* SGB4711 (5.4 x 10^8) on which the fungal infection occurred after 120 hours after the start of treatment, was 38.46%, 37.03% and 61.54% respectively.

Table 5. The pathogenic activity of *B. bassiana* SGB11B, *M. anisopliae* SGB1K, *L. lecanii* SGB4711 powdered fungal culture suspensions and related conidial suspensions, against *P. spumarius* adults.

***Beauveria Bassiana* SGB11B powdered culture suspension**					
Concentration CFU/ml	Mortality%[a] mean ± SE 24 h	Mortality%[a] mean ± SE 48 h	Mortality%[a] mean ± SE 72 h	Mortality%[a] mean ± SE 96 h	Mortality%[a] mean ± SE 120 h
5.7×10^7	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
1.14×10^8	0.000 ± 0.000	13.333 ± 4.216	16.667 ± 6.146	50.000 ± 4.472	93.333 ± 6.667
2.28×10^8	6.667 ± 4.216	13.333 ± 4.216	16.667 ± 6.146	70.000 ± 4.472	96.667 ± 3.333
***Beauveria Bassiana* SGB11B conidial suspension**					
5.4×10^8	0.000 ± 0.000	0.000 ± 0.000	16.667 ± 3.333	30.000 ± 4.472	86.667 ± 6.667
***Metharizium anisopliae* SGB1K powdered culture suspension**					
9.6×10^5	0.000 ± 0.000	3.333 ± 3.333	6.667 ± 4.216	10.000 ± 4.472	33.333 ± 4.216
1.92×10^6	0.000 ± 0.000	0.000 ± 0.000	6.667 ± 4.216	6.667 ± 4.216	66.667 ± 6.667
3.84×10^6	0.000 ± 0.000	6.667 ± 4.216	6.667 ± 4.216	47.500 ± 5.439	81.667 ± 3.801
***Metharizium anisopliae* SGB1K conidial suspension**					
1.9×10^6	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	40.000 ± 5.164	45.000 ± 3.416
***Lecanicillium lecanii* SGB4711 powdered culture suspension**					
1.35×10^8	0.000 ± 0.000	3.333 ± 3.333	3.333 ± 3.333	3.333 ± 3.333	3.333 ± 3.333
2.7×10^8	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
5.4×10^8	0.000 ± 0.000	6.667 ± 4.21	10.000 ± 4.472	13.333 ± 4.216	26.667 ± 4.216
***Lecanicillium lecanii* SGB4711 conidial suspension**					
2.2×10^8	10.000 ± 4.472	23.333 ± 6.146	33.333 ± 6.667	56.667 ± 3.333	58.333 ± 1.667

[a]The adult mortality percentages are reported as mean values of four replicates, each carried out with 5 adults, corrected using Abbotts's formula.

The raw data obtained by pathogenicity tests with *B. bassiana* SGB11B, *M. anisopliae* SGB1K and *L. lecanii* SGB4711 suspensions, and their conidial ones were analyzed using the GLM repeated measures procedure and Bonferroni test.

GLM assessed whether the interaction between both test conditions (treatment and control) and the changes over the time of the number of adults survived to exposure to fungal and conidial suspensions and the number of survived control adults was generally statistically significant (Table 6). The Bonferroni test was used to assess whether the average number of adults that survived to exposure to fungal and conidial suspensions was significantly smaller than the average number of survived adults in control, over time, indicating a lethal effect of the fungal and conidial suspensions. The Bonferroni test revealed that the average number of survived adults exposed to *B. bassiana* SGB11B at 1.14×10^8 and 2.28×10^8 CFU/ml, its conidial suspension at 5.4×10^8 CFU/ml, to *M. anisopliae* SGB4711at all tested concentrations, its conidial suspension, and to *L. lecanii* SGB1K tested at 5.4×10^8 CFU/ml was significantly smaller than the number of survived individuals in the respective controls, over time (Table 6).

Table 6. Pathogenic effect of *B. bassiana* SGB11B, *M. anisopliae* SGB1K and *L. lecanii* SGB4711 against *P. spumarius* adults

Concentration (CFU/ml)	**GLM (time × treatment)[a]**		**Bonferroni test[b]**		
			Mean treatment	**Mean control**	
Beauveria bassiana SGB11B					
5.7×10^7			5 ± 0.000	5 ± 0.000	ns
1.14×10^8	$F_{4,40} = 61.947$	$P < 0.01$	3.200 ± 0.115	5.000 ± 0.115	**
2.28×10^8	$F_{4,40} = 151.964$	$P < 0.01$	3.133 ± 0.087	5.000 ± 0.087	**
Conidial suspension					
5.4×10^8	$F_{4,40} = 151.964$	$P < 0.01$	3.667 ± 0.064	4.967 ± 0.064	**
Metarhizium anisopliae SGB1K					
9.6×10^5	$F_{4,40} = 19.146$	$P < 0.01$	4.467± 0.087	5.000± 0.087	
1.92×10^6	$F_{4,40} = 76.250$	$P < 0.01$	4.200 ± 0.097	5.000± 0.097	**
3.84×10^6	$F_{4,40} = 57.922$	$P < 0.01$	3.500 ± 0.095	4.800 ± 0.095	**.
Conidial suspension					**
1.9×10^6	$F_{4,40} = 53.409$	$P < 0.01$	4.867 ± 0.094	5.000± 0.094	
Lecanicillium lecanii SGB4711					**
1.35×10^8	$F_{4,40} = 1.000$	$P > 0.05$	0.667 ±0.288	4.767 ± 0.288	ns
2.7×10^8			5 ± 0.000	5 ± 0.000	ns
5.4×10^8	$F_{4,40} = 8.000$	$P < 0.01$	4.433 ±0.085	5.000± 0.085	**

GLM values describe the effect of time on survival of the adults

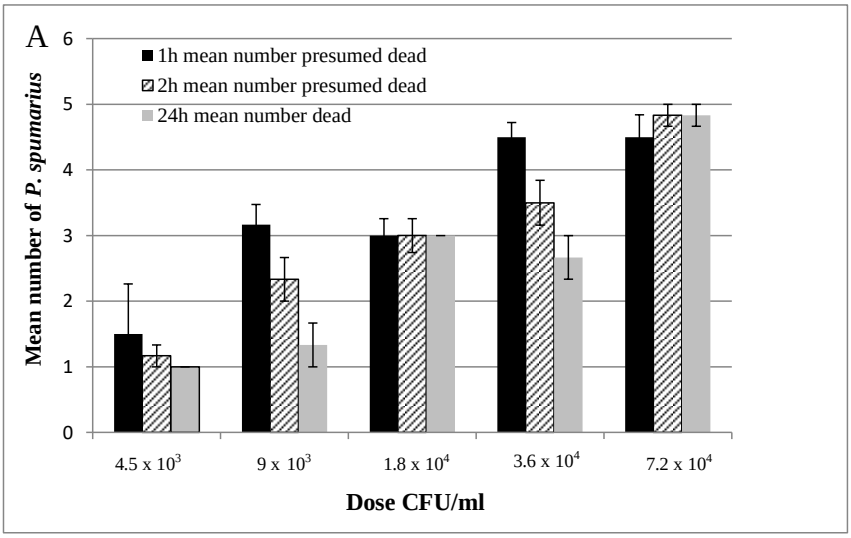
ns = not significant; *P<0.05; **P<0.01

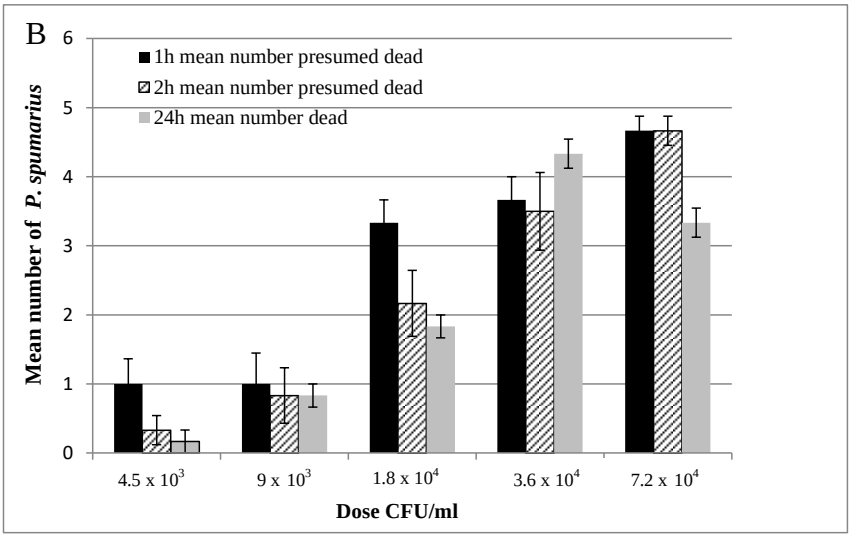
[a]Values of P>0.05 for GLM indicate that the interaction between the two conditions (treated and control) and the change over time was not statistically significant. Values of P<0.05 for GLM indicate that the interaction between the two conditions (treated and control) and the change over time was statistically significant

[b]Differences between the means of the number of survived adults at different powdered fungal cultures and related supernatant suspensions in each of the experimental treatments and those of the number of related controls over time were analyzed and adjusted with Bonferroni test for multiple comparisons.

Immediately after the treatment of *P. spumarius* adults with the powdered culture of *Trichoderma* sp. SAM 9, with its supernatant suspensions or methanolic extract, presumed dead insects were observed (Figure 20). This state of apparent death was precided by tremors. The average number of presumed dead insects recorded after the first hour, both for the treatment with powdered culture suspensions (1.50 ± 0.764, 3.17 ± 0.307, 3.00 ± 0.258, 4.50 ± 0.224, 4.50 ± 0.342) and with related supernatant ones (1.00 ± 0.365, 1.00 ± 0.447, 3.33 ± 0.333, 3.67 ± 0.333, 4.67 ± 0.211), in general increased with increasing concentration (Fig. 20A, 20B). Furthermore, in general the average number of presumed dead individuals recorded after the first

hour decreased with time, excepted after immersion in the culture suspension at 7,2 x 10^4 where, it was similar to the average number of dead insects at 24 hours (4.50 ± 0.342; 4.83 ± 0.17) and after immersion in supernatant (7,2 x 10^4), where it was lower. Although the methanolic extract was not active (average number of dead after 24 hours 1.167 ± 0.307) in the first two hours, an average number of 2.5 ± 0.224 insects affected by tremor and apparently dead was recorded subsequently, and then decreased at 24 hours (1.167 ± 0.307) (Fig. 20C). As for *T. minutisporum* SAM 56 although the powdered culture tested at different concentrations was not active, presumed dead insects were observed in the first hour (Fig. 20D).





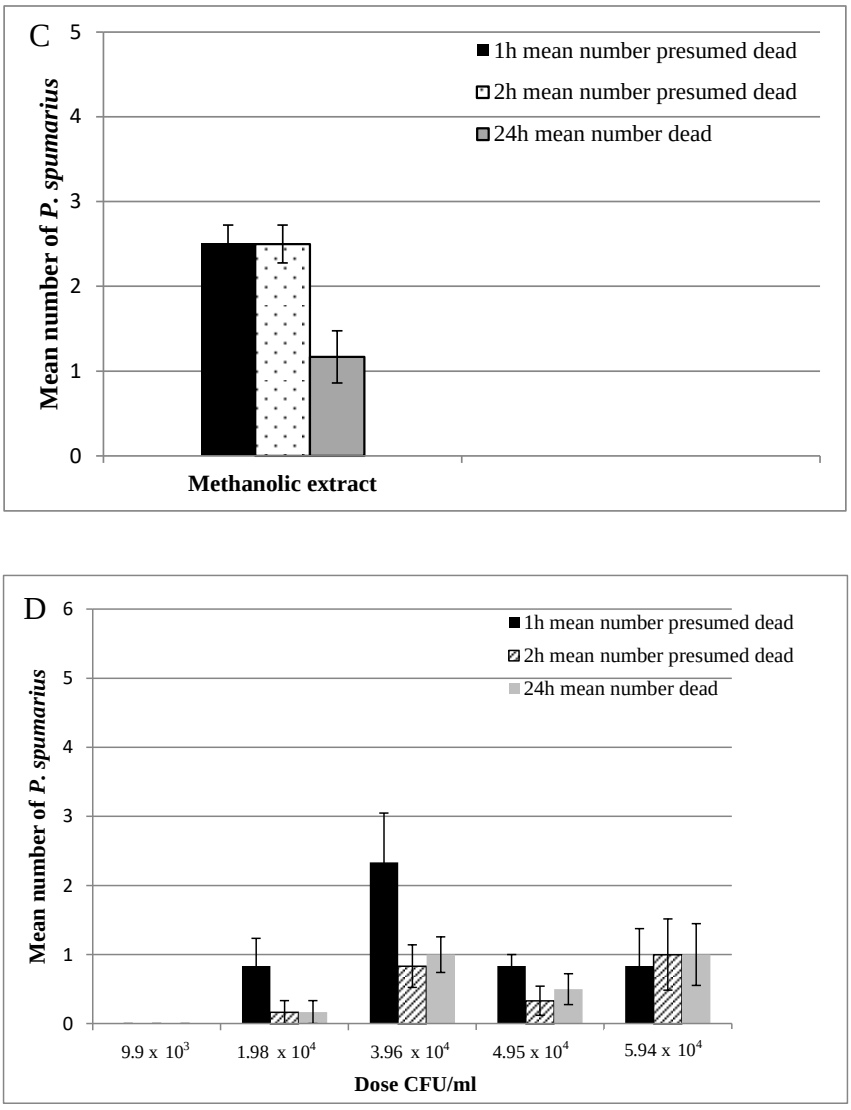


Fig. 20 - Number of *P. spumarius* individuals dead or presumed dead, after treatment with different concentrations of *Trichoderma* sp. SAM 9 powdered culture (A) or its supernatant suspensions (B), with methanolic extract at different exposure times (C), or with different concentrations of *T. minutisporum* SAM 56 powdered culture (D).

The results of the pathogenicity bioassays carried out with powdered culture of *Trichoderma* sp. SAM 9 and related supernatant suspension, at different concentrations, are reported in table 7.

The raw data obtained by pathogenicity tests were analyzed using the GLM repeated measures procedure and Bonferroni test. GLM assessed whether the interaction between both test conditions (treatment and control) and the changes over the time of the number of adult survived to exposure to suspensions of *Trichoderma* sp. SAM 9 and the number of survived control adults was generally not statistically significant (Table 7). The Bonferroni test was used to assess whether the mean number of adults that survived to exposure to fungal suspensions was significantly smaller than the mean number of survived adults in control, over time, indicating a lethal effect of the suspensions. This test revealed that the mean number of adults exposed to fungal suspensions at the concentration: 1.8×10^4, 3.6×10^4 and 7.2×10^4 CFU/ml was significantly smaller than the number of correspondent controls, respectively, over time (Table 8). The GLM analysis of the data obtained with the supernatant solutions obtained by *Trichoderma* sp. SAM 9 suspensions at the concentrations 4.5×10^3, 9×10^3, 1.8×10^4, 3.6×10^4 and 7.2×10^4 CFU/ml do not reveal time × treatment interaction effects. The Bonferroni test revealed that the average number of adults survived to supernatant solutions obtained by *Trichoderma* sp. SAM 9 suspensions tested at the concentration 1.8×10^4, 3.6×10^4 and 7.2×10^4 CFU/ml was significantly smaller than the number of corresponding control over time (Table 8). The time taken for *Trichoderma* sp. SAM 9 powdered culture and supernatant suspensions to kill 50% and 90% of *P. spumarius* adults, is shown in Table 2.

The results obtained by pathogenicity bioassays with fungi of the Genus *Trichoderma*, expressed as percentage mortality corrected using Abbott's formula, are summarized in table 9.

For *Trichoderma* sp. SAM 9, the mortality values recorded with the powdered culture and surnatant suspension increased with increasing concentration and with exposure time (Table 9). For *T. minutisporum* SAM 56, *T. atrobrunneum* ITEM 908, *T. atrobrunneum* ITEM 908-5 and *T. citrinoviride* ITEM 4484 the highest mortality values were recorded at the highest concentration and after 120 hours. The percentages of dead adults after treatment with *T. atrobrunneum* ITEM 908 (3.6×10^2 CFU/ml) and *T. citrinoviride* ITEM 4484 (1.16×10^4 CFU/ml) that resulted in fungal infection after 120 hours from the treatment, was 40.00% and 45.45% respectively.

Table 7. Pathogenic effect of *Trichoderma* sp. SAM 9 against *P. spumarius* adults

Concentration CFU/ml)	**GLM (time × treatment)[a]**		**Bonferroni test[b]**		
			Mean treatment	**Mean control**	
***Trichoderma* sp. SAM 9**					
4.5×10^3	$F_{4,40} = 0.300$	$P > 0.05$	3.867 ± 0.369	4.767 ± 0.369	n.s.
9×10^3	$F_{4,40} = 0.857$	$P > 0.05$	3.567 ± 0.410	4.767 ± 0.410	n.s.
1.8×10^4	$F_{4,40} = 0.385$	$P > 0.05$	1.867 ± 0.225	4.767 ± 0.225	**
3.6×10^4	$F_{4,40} = 2.843$	$P > 0.05$	1.500 ± 0.299	4.767 ± 0.299	**
7.2×10^4	$F_{4,40} = 1.000$	$P > 0.05$	0.167 ± 0.203	4.767 ± 0.203	**
Supernatant obtained *Trichoderma* sp. SAM 9					
4.5×10^3	$F_{4,40} = 1.129$	$P > 0.05$	4.567 ± 0.216	4.767 ± 0.216	n.s.
9×10^3	$F_{4,40} = 1.129$	$P > 0.05$	3.900 ± 0.397	4.767 ± 0.397	n.s.
1.8×10^4	$F_{4,40} = 0.385$	$P > 0.05$	3.033 ± 0.320	4.767 ± 0.320	**
3.6×10^4	$F_{4,40} = 1.000$	$P > 0.05$	0.667 ±0.288	4.767 ± 0.288	**
7.2×10^4	$F_{4,40} = 0.897$	$P > 0.05$	1.467 ± 0.352	4.767 ± 0.352	**

GLM values describe the effect of time on survival of the adults is not significant; *P<0.05; **P<0.01

[a]Values of P>0.05 for GLM indicate that the interaction between the two conditions (treated and control) and the change over time was not statistically significant. Values of P<0.05 for GLM indicate that the interaction between the two conditions (treated and control) and the change over time was statistically significant

[b]Differences between the means of the number of survived adults at different *Trichoderma* sp.SAM 9 powdered culture and related supernatant solutions in each of the experimental treatments and those of the number of related controls over time were analyzed and adjusted with Bonferroni test for the multiple of comparisons.

Table 8. Pathogenic activity of *Trichoderma* sp.SAM 9 and supernatant suspension obtained by *Trichoderma* sp.SAM 9 at different concentration against *P. spumarius* adults.

	Concentration (CFU/ml)	LC50[b] (95% CL)	LC90[c] (95% CL)	Slope ± SE	Intercept ± SE	R^2
***Trichoderma* sp. SAM9** Time						
24h	4.5×10^3 9×10^3 1.8×10^4 3.6×10^4 7.2×10^4	50.414 (35.879-67.451)	135.845 (107.446-195.817)	0.026±0.002	1.119±0.133	0.843
48h	4.5×10^3 9×10^3 1.8×10^4 3.6×10^4 7.2×10^4	50.414 (35.879-67.451)	135.845 (107.446-195.817)	0.026±0.002	1.119±0.133	0.843
72h	4.5×10^3 9×10^3 1.8×10^4 3.6×10^4 7.2×10^4	43.836 (28.694-59.689)	129.415 (102.086-187.552)	0.026±0.002	1.309±0.131	0.843
96h	4.5×10^3 9×10^3 1.8×10^4 3.6×10^4 7.2×10^4	42.903 (27.517-58.711)	129.092 (101.670-187.812)	0.026±0.002	1.344±0.146	0.810
120h	4.5×10^3 9×10^3 1.8×10^4 3.6×10^4 7.2×10^4	40.903 (24.324-57.200)	131.559 (102.585-196.120)	0.025±0.002	1.448±0.121	0.851
Supernatant obtained by *Trichoderma* sp. SAM 9 Time						
24h	4.5×10^3 9×10^3 1.8×10^4 3.6×10^4 7.2×10^4	201,903 (169,603 - 256,114)	363.957 (298.060-483.782)	0.043±0.004	0.603±0.410	0.819
48h	4.5×10^3 9×10^3 1.8×10^4 3.6×10^4 7.2×10^4	162.720 (135.215 - 207.265)	321.462 (261.766-431.270)	0.053±0.005	1.428±0.484	0.827
72h	4.5×10^3 9×10^3 1.8×10^4 3.6×10^4 7.2×10^4	98.917 (82.335-119.914)	209.861 (177.453-262.092)	0.079±0.007	2.223±0.656	0.850
96h	4.5×10^3 9×10^3 1.8×10^4 3.6×10^4 7.2×10^4	65.956 (53.477-79.272)	198.691 (171.547-238.967)	0.066±0.004	5.403±0.391	0.919

[b]Concentration of the suspension of *Trichoderma* sp. SAM 9 powdered culture and of the supernatant suspension of *Trichoderma* sp. SAM 9 powdered culture that kills 50% of the exposed adult.

[c]Concentration of the suspension of *Trichoderma* sp. SAM 9 powdered culture and of the supernatant suspension of *Trichoderma* sp. SAM 9 powdered culture that kills 90% of the exposed adult.

Table 9. Pathogenic activity of *Trichoderma* sp. SAM 9, *T. atrobrunneum* ITEM 908, *T. atrobrunneum* ITEM 908-5, *T. citrinoviridae* ITEM 4484, *T. minutisporum* SAM 56 on *P. spumarius* adults.

Concentration CFU/ml	Mortality%[a] mean ± SE 24 h	Mortality%[a] mean ± SE 48 h	Mortality%[a] mean ± SE 72 h	Mortality%[a] mean ± SE 96 h	Mortality%[a] mean ± SE 120 h
***Trichoderma* sp. SAM 9**					
4,5 x 10^3	16.667 ± 3.333	16.667 ± 3.333	20.000 ± 5.164	17.500 ± 3.594	21.667 ± 1.054
9 x 10^3	20.833 ± 0.833	20.833 ± 0.833	27.500 ± 4.031	29.167 ± 5.231	29.167 ± 5.231
1,8 x 10^4	58.333 ± 1.667	58.333 ± 1.667	58.333 ± 1.667	64.167 ± 4.549	64.167 ± 4.549
3,6 x 10^4	58.333 ± 5.426	58.333 ± 5.426	75.833 ± 3.270	75.000 ± 3.162	75.000 ± 3.162
7,2 x 10^4	96.667 ± 3.333	96.667 ± 3.333	96.667 ± 3.333	96.667 ± 3.333	96.667 ± 3.333
***Trichoderma* sp. SAM 9 surnatant**					
4,5 x 10^3	3.333 ± 3.333	10.000 ± 4.472	6.667 ± 4.216	6.667 ± 4.216	6.667 ± 4.216
9 x 10^3	16.667 ± 3.333	23.333 ± 3.333	20.000 ± 5.164	20.000 ± 5.164	20.000 ± 5.164
1,8 x 10^4	36.667 ± 3.333	36.667 ± 3.333	33.333 ± 6.667	40.833 ± 4.549	40.833 ± 4.549
3,6 x 10^4	86.667 ± 4.216	86.667 ± 4.216	85.833 ± 4.549	85.833 ± 4.549	85.833 ± 4.549
7,2 x 10^4	66.667 ± 4.216	100.000 ± 0.000	100.000 ± 0.000	100.000 ± 0.000	100.000 ± 0.000
***Trichoderma* sp. SAM 9 methanolic extract**					
1.77%	23.33 ± 6.146	26.667 ± 6.667			
***Trichoderma minutisporum* SAM 56**					
9.9 x 10^3	13.333 ± 4.216	16.667 ± 3.333	6.667 ± 4.216	6.667 ± 4.216	3.333 ± 3.333
1.98 x 10^4	10.333 ± 4.472	6.667 ± 4.216	6.667 ± 4.216	10.833 ± 4.902	13.333 ± 6.667
3.96 x 10^4	20.333 ± 5.164	20.000 ± 5.164	20.000 ± 5.164	20.000 ± 5.164	23.333 ± 8.028
4.95 x 10^4	0.000 ± 0.000	6.667 ± 4.216	13.333 ± 6.667	16.667 ± 6.146	20.000 ± 5.164
5.94 x 10^4	0.000 ± 0.000	3.333 ± 3.333	16.667 ± 6.146	23.333 ± 8.028	23.333 ± 8.028
***Trichoderma harzianum* ITEM 908**					
90	3.333 ± 3.333	3.333 ± 3.333	6.667 ± 4.216	6.667 ± 4.216	13.333 ± 4.216
1.8 x 10^2	3.333 ± 3.333	3.333 ± 3.333	6.667 ± 4.216	6.667 ± 4.216	13.333 ± 4.216
3.6 x 10^2	3.333 ± 3.333	6.667 ± 4.216	6.667 ± 4.216	10.000 ± 4.472	16.667 ± 6.146
***Trichoderma harzianum* ITEM 908-5**					
1.02 x 10^5	3.333 ± 3.333	6.667 ± 4.216	6.667 ± 4.216	6.667 ± 4.216	10.000 ± 4.472
2.04 x 10^5	0.000 ± 0.000	6.667 ± 4.216	6.667 ± 4.216	6.667 ± 4.216	10.000 ± 4.472
4.08 x 10^5	0.000 ± 0.000	6.667 ± 4.216	6.667 ± 4.216	16.667 ± 3.333	33.333 ± 6.667
***Trichoderma citrinoviride* ITEM 4484**					
2.91 x 10^3	10.000 ± 4.472	23.333 ± 3.333	26.667 ± 4.216	26.667 ± 4.216	33.333 ± 4.216
5.82 x 10^3	10.000 ± 4.472	23.333 ± 3.333	26.667 ± 4.216	30.000 ± 4.472	36.667 ± 6.146
1.16 x 10^4	16.667 ± 6.164	26.667 ± 4.216	30.000 ± 4.472	33.333 ± 4.216	36.667 ± 6.146

[a]The adult mortality percentages were reported as the average values of four replicates, each setup with 5 adults, corrected using Abbotts's formula.

6.3 Discussion pathogenicity tests with powdered fungal cultures on *P. spumarius* nymphs and adults

The powdered fungal cultures of *B. bassiana* SGB11B, *M. anisopliae* SGB1K, and *L. lecanii* SGB4711 inoculated into spittle did not shown lethal activity against *P. spumarius* nymphs. Furthermore, no fungal development was observed, either in the spittle or on the cuticle of both nymphs and adults obtained by nymphs. The lack of fungal activity could have different explanations. One could be the presence of compounds with antimicrobial activity inside the spittle, while another could be a physically unsuitable environment for fungal growth. This result is also supported by findings of Chang *et al*. (2019), who reported that mould growth was not observed on the froth of *Poophilus costalis* (Walker) (Hemiptera: Cercopoidea: Aphrophoridae) under natural conditions.

Based on the results obtained in the antimicrobial activity assays performed with spittle, neither antibacterial nor antifungal effects of *P. spumarius* spittle were found, however, the results of our *in vitro* experiments showed that the spittle reduced the sporulation of some fungi at 10 days post inoculation. This aspect needs to be further investigated to understand whether it is due to a biochemical inhibition of sporulation or to a physical mechanism that reduced spore formation. The mucilaginous composition of the spittle might have surface-active activity, which can play a physical role in the displacement of the conidia at the border of the spittle drop. Once the spittle becomes harder, the hyphae cannot penetrate this surface, and fungal growth is hindered. To investigate more in depth these aspects, the development of an ultra-concentration protocol, able to concentrate the spittle at least 5-fold, followed by an experiment in liquid culture, are envisaged.

The results of pathogenic bioassays showed that *B. bassiana* SGB11B, *M. anisopliae* SGB1K, although to a lesser extent, *L. lecanii* SGB4711 powdered culture, affected the survival of *P. spumarius* nymphs and adults when they were dipped into fungal suspensions: the effect was dose- and time-dependent. *M. anisopliae* SGB1K powdered culture suspension resulted in a high percentage of mortality of nymphs already 24 hours after the beginning of the bioassay, even at a lower concentration than *B. bassiana* SGB11B and *L. lecanii* SGB4711. Regarding the conidial suspensions, both were active against nymphs, although *B. bassiana* SGB11B caused a higher mortality, compared to *M. anisopliae* SGB1K. Regarding *P. spumarius* adults, *B. bassiana* SGB11B powdered culture suspension, at the highest concentrations, was active 96 hours after the start of the bioassay, while *B. bassiana* SGB11B, *M. anisopliae* SGB1K conidial suspensions, and *M. anisopliae* SGB1K powdered culture suspensions at the higher concentrations showed a slightly delayed effect, that started after 120 hours. *L. lecanii* SGB4711 conidial suspension was active, after 96 hours. *Trichoderma* sp. SAM 9 both in the form of

fungal suspension and of supernatant, had a lethal effect on adults already after 24 hours. In this regard, the quick lethal activity that was exhibited by the culture of *Trichoderma* sp. SAM 9 and also maintained by its supernatant, in the absence of fungal spores and mycelia, strongly supports the hypothesis that at least part of the lethal effect was due to fungal metabolites produced and released in the culture medium during the fungal growth. In this regard, the eventual isolation and the identification of the active compound(s) produced by *Trichoderma* sp. SAM 9 might provide new active principles for the development of biorational products for *P. spumarius* control. Numerous observations under the stereomicroscope, conducted on dead *P. spumarius* nymphs and adults, after the treatment with powdered fungal culture and conidial suspensions, highlighted that the lethal effect was not always related with the development of mycelium on the cuticle. This was particularly evident in the treatments of adult insects with *Trichoderma* sp. SAM 9, both in the form of powdered fungal culture and supernatant, which caused tremors in the early hours and death already after 24 hours.

Previous enzymatic tests (data not shown), conducted on *B. bassiana* SGB11B and *M. anisopliae* SGB1K revealed a marked chitinase activity of *B. bassiana*, but not in *M. anisopliae*. The absence of this activity in *M. anisopliae* SGB1K, however, did not affect the entomopathogenic activity, as its pathogenicity has been widely demonstrated in bioassays carried out not only on *P. spumarius* but also on the mite *Tetranycus urticae* (Koch) (Sonia Petrarca, unpublished 2016-2017). The antagonistic activity of the tested fungi on nymphs and adults could be due not only to the parasitic development of mycelium on the insect cuticle (Fig. 21), observed only on a few specimens, but also to toxic metabolites produced during the axenic growth of the fungal strains and inglobated into the culture medium, and which therefore may be present in the powdered culture and conidial suspensions.

Fig. 21 Fungal mycelium development on *P. spumarius* nymphs and adults. (A) *M. anisopliae* SGB1K, (B) *B. bassiana* SGB11B,(C) *T. harzianum* ITEM 908 and , (D) *L. lecanii* SGB4711 .

It is well known that fungi utilised in biocontrol (Butt *et al.*, 2016) including *Trichoderma* spp. and the entomopathogenic fungi *B. bassiana*, *M. anisopliae* and *L. lecanii*, secrete a wide range of metabolites, at least in vitro (Molnár *et al*., 2010; Gibson *et al*., 2014; Li *et al*., 2019). The most notable metabolites of *Beauveria* species include bassianin, bassiacridin, bassianolid, tenellin, and oosporein, whereas *Metarhizium* species produce cyclosporine, swainsonine, and 39 congeners of the cyclic peptide destruxin (Gibson *et al.*, 2014; Molnár *et al.*, 2010; Wang *et al.*, 2012; Ortiz-Urquizza & Keyhani and Donzelli & Krasnoff 2013). Some of these compounds have been detected in vivo and have been linked with virulence and specificity (Amiri-Besheli, Khambay, Cameron, Deadman, & Butt, 2000; Kershaw, Moorhouse, Bateman, Reynolds, & Charnley, 1999). *Trichoderma* species are ubiquitous and have evolved the capability to interact with different organisms that occupy their ecological niche, including insects. Cases of entomoparasitic behviour by *Trichoderma* spp. have been reported (Ganassi *et al.*, 2004; Shakeri and Foster, 2007) and, likewise in mycoparasitism, this activity is likely related to secretion of bioactive secondary metabolites, besides lytic enzymes. So far, only a small number of secondary metabolites that are involved in the interactions between *Trichoderma* spp. and insects have been described. Some of these have semiochemical properties and act as phagodeterrent or attractive compounds. These include the phagodeterrent substances citrantifidiene, citrantifidiol, bisorbicillinoids and long chain alcohols (Evidente *et al.*, 2008, 2009; Ganassi, 2016). On the contrary, non-identified secondary metabolites attractive to the mosquito *Culex quinquefasciatus*, vector of the disease "West Nile fever" are produced by *Trichoderma virens* (Geetha *et al*., 2003). Other metabolites have insecticidal effect. In the methanolic extract of a *Trichoderma asperellum* strain with high insecticidal efficacy on mosquito larvae of the genus *Anopheles*, the insecticidal substances 2,3-dihydro-thiopene, p-cymene, α -pinene, hexadecanoic acid, 8-methyl quinoline, linoleic acid and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-Pyran-4-one were identified (Podder and Ghosh, 2019). However, in consideration of the huge and largely unexplored metabolic versatility of members of this genus, more and more compounds with a potential use for control of insect pests are expected to be discovered in the next future.

7 CONCLUSIONS

The behavioural bioassays pointed out that *T. citrinoviride* ITEM-4484 elicited *P. spumarius* adult response. The bioassays carried out with *V. faba* shoots clearly indicated that both sexes are able to discriminate between treated and untreated plants, in particular, the mean number of both males and females on the control leaves was significantly greater than the mean number on the treated leaves. Consequently, it is possible to affirm that *T. citrinoviride* ITEM-4484 can influence the feeding preference of *P. spumarius* males and females. In this regard, some secondary metabolites were proved to have semiochemical properties acting as phagodeterrent or attractive compounds (Evidente *et al.*, 2008, 2009; Ganassi, 2016). The research conducted so far, certainly deserve to be deepened and developded with further suitable bioassays both in laboratory and field environment, as the use of compounds with attractive, repellent or phagodeterrent activity is to be considered one of the most promising means to be introduced in a multi-tool environmental-friendly strategy aimed to limit the transmission of *X. fastidiosa*.

B. bassiana SGB11B, *M. anisopliae* SGB1K, and *L. lecanii* SGB4711 fungal species, entomopathogenic activity of which is well known, did not shown lethal activity when inoculated into *P. spumarius* nymph spittle and no fungal development was observed either in the spittle or on the cuticle of nymphs and derived adults. The absence of mycelium could be due to the antimicrobial activity of compounds contained into the spittle or to the lack of nutrients required for the fungal development; lastly, the spittle could represent an unsuitable physical environment for fungal growth.

No antibacterial or antifungal properties of *P. spumarius* spittle were proved, though, *in vitro* experiments proved that the spittle reduced the sporulation of some fungi at 10 days post inoculation. This aspect needs be studied in depth, with appropriate methods, to understand whether it is due to a biochemical inhibition of sporulation or to a physical mechanism that reduced spore formation. Conversely, *B. bassiana* SGB11B and *M. anisopliae* SGB1K, tested as culture and conidial suspensions, affected the survival of nymphs and adults, and this effect was dose- and time-dependent. In particular, *M anisopliae* SGB1K powdered culture suspension caused a high percentage of mortality in nymphs already at 24 hours, even at a low concentration. *B. bassiana* SGB11B conidial suspensions instead, caused a higher mortality, compared to *M. anisopliae* SGB1K. *B. bassiana* SGB11B powdered culture suspension and *L. lecanii* SGB4711 conidial suspension, at the highest concentrations, were active towards *P. spumarius* adults, 96 hours after the beginning of the bioassay. While *B. bassiana* SGB11B, *M. anisopliae* SGB1K conidial suspensions, and *M. anisopliae* SGB1K powdered culture suspensions, at the higher concentrations, showed a slightly delayed activity, starting after 120

hours. *Trichoderma* sp. SAM 9, both in the form of fungal suspension and of supernatant, exhibited a lethal effect on adults already after 24 hours.

The lethal effect, especially the one caused by *Trichoderma* sp. SAM 9, was not always related with the development of mycelium on the cuticle and this could be partially due to fungal metabolites released in the culture medium, and present in the powdered culture and conidial suspensions. Previous enzymatic tests showed a marked chitinase activity of *B. bassiana* (Master Thesis, 2016-2017) but is well known that fungi utilised in the biocontrol produce a wide range of metabolites (Molnár *et al*., 2010; Gibson *et al*., 2014; Butt *et al.*, 2016; Li *et al*., 2019), and some of these compounds have been linked with virulence and specificity (Amiri-Besheli, Khambay, Cameron, Deadman, & Butt, 2000; Kershaw, Moorhouse, Bateman, Reynolds, & Charnley, 1999). Although less known in this respect, *Trichoderma* species also interact with insects, and entomoparasitic behviour by *Trichoderma* spp. has been reported (Ganassi *et al.*, 2004; Shakeri and Foster, 2007) and this activity is likely related to secretion of bioactive secondary metabolites.

The laboratory bioassays carried out so far have shown that the fungi tested have a good potential to be used in organic farming and other forms of environmental-friendly agriculture. Moreover, despite that *Trichoderma* spp. are well known as efficient mycoparasites and already largely in use for plant protection against fungal diseases and certain nematodes (Woo et al., 2014), their potential in the biocontrol of insects is still poorly understood. This research contributes to broaden the knowledge on *Trichoderma* spp. biological activities so as to lead to a wider use of these beneficial microbials and metabolites thereof also for the control of harmful insects.

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