Metarhizium brunneum-7 with Phytophagous Mites

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We investigated *Metarhizium brunneum*-7 interactions in three systems of phytophagous mites and their respective plant hosts: Volkamer lemon (*Citrus volkameriana*) and the citrus rust mite *Phyllocoptruta oleivora*, common bean (*Phaseolus vulgaris*) and the two-spotted spider mite *Tetranychus urticae*, and spring onion (*Allium cepa*) and the bulb mite *Rhizoglyphus robini*. All three mite species were susceptible to directly applied *M. brunneum*-7 conidia. Results obtained using the standard method for studying endophytic colonization vs. live confocal imaging of plant tissues using the GFP-transformed fungus differed markedly, demonstrating that microscopy validation was more definite than the standard process of recovery from plant tissue. Endophytic colonization was observed in conidium-infiltrated citrus leaves and in roots of onion plants treated with soil-drenched conidia, but not in common bean treated by either spray or drench of conidia. Endophytic colonization of citrus leaves did not affect the citrus mite population. Drench application in common bean reduced two-spotted mite population. Similarly, drench application in onion reduced bulb mite population. This study demonstrates *M. brunneum*-7 interactions with citrus, onion and their mite pests, and the importance of live-imaging techniques in studying endophytic interaction.

Microbial control entomopathogenic fungus Hypocreales live confocal imaging
Rhizoglyphus robini Phyllocoptruta oleivora Tetranychus urticae Spray application
Drench application

1. Introduction

For decades the worldwide strategy for pestiferous mite (Acari) control has relied mainly on synthetic acaricides (organophosphates, carbamates, pyrethroids, and macrocyclic lactone [1]), and these precluded the use of alternative strategies such as biocontrol agents like predatory mites and insects [2][3][4]. As many conventional mite products are being withdrawn from markets, banned, or are no longer effective due to resistance of the mites, these and other biocontrol agents are now becoming more relevant [1][5][6].

Entomopathogenic fungi belonging to the order Hypocreales play central roles in the control of pestiferous mite populations [7]. Entomopathogenic fungi are typically applied repeatedly, ensuring a high number of infective units aimed at suppressing the pest population. However, the fungal conidia are highly sensitive to environmental conditions, such as UV radiation, and have to be properly dispersed and fixed in the plant canopy to demonstrate significant pest-control efficiency [8][9][10][11]. Persistence of entomopathogenic fungi in the environment depends on habitat architecture, host behavior, and environmental factors. In the soil, *Metarhizium* spp. propagules can persist for several years [12][13]. In the crop canopy, persistence was only 16–28 days for *Beauveria bassiana* conidia [12][14][15] and 3 days for *M. anisopliae* sprayed on corn [16]. In recent years, a number of studies have demonstrated the establishment of *Metarhizium* spp. in various associations with plants, resulting in a secondary effect on successive generations of pest populations [17][18][19]. Two of the major associations studied were rhizosphere competence and endophytic interactions of entomopathogenic fungi with various plants [20][21]. Both of these associations play an ecological role in diverse plant–pest and plant–pathogen systems, protecting plants against insects, mites, and pathogens, and have mostly a positive effect on plant growth [18][19][22].

Fungal endophytes are defined as plant-inhabiting fungi that, at some time during their lives, colonize internal tissues without causing apparent harm to their hosts [23]. Endophytes have a variety of positive effects on different aspects of their hosts’ ecological fitness, such as growth enhancement and increased tolerance to biotic and abiotic stresses [24]. Some endophytes are known to induce resistance of their host by priming of the host defense system, while others are secreting secondary metabolites that influence pathogens in a direct or indirect way as antibiosis or induce defense accordingly. In other cases, the endophytes are producing plant hormones or fixing nitrogen that promotes plant growth. Endophytes are known to improve plant resistance to drought, salinity, and other abiotic stresses. However, the mode of action is not known in all cases, but in some it is assumed that there is involvement of secondary metabolites secretion and plant defense system priming [24]. Endophytic entomopathogenic fungi, such as *Metarhizium* spp. and *Beauveria* spp., are known to inhibit the growth and reproduction of a wide range of herbivores from different feeding guilds. These include members of the Coleoptera, Diptera, Lepidoptera, and Orthoptera [18][25][26][27].

As numerous species of phytophagous mites infest plants of economic importance worldwide [4], sustainable solutions for mite control are highly needed. Still, only a few studies have been conducted on the effects of the method of entomopathogenic fungi conidia application and endophytic establishment on phytophagous mite populations [22][28]. We hypothesized that: (i) foliar spray and root drench application of *M. brunneum*-7 (Mb7) will result in localized and systemic colonization of plant tissues, respectively; (ii) endophytic or rhizospheric colonization of plants with Mb7 will affect mite populations; (iii) mites that are susceptible to Mb7 through direct contact with its conidia will also be affected by feeding on Mb7-colonized plant tissues. We assessed the hypotheses by studying the interactions of *Metarhizium brunneum* isolate Mb7 in three plant systems that include different phytophagous mites: (i) Volkamer lemon (*Citrus volkameriana*) and the citrus rust mite *Phyllocoptruta oleivora* (Ashmead); (ii) common bean (*Phaseolus vulgaris*) and the two-spotted spider mite *Tetranychus urticae* Koch; and (iii) spring onion (*Allium cepa*) and the bulb mite *Rhizoglyphus robini* Claparede. In each fungus–mite system, the effect of Mb7 application (spray, infiltration, or drench) on the studied mites was investigated. The study objectives were to: (i) localize and describe Mb7 colonization of the studied plants following application of conidia, and (ii) determine the efficacy of Mb7 for mite suppression when applied directly vs. when present as an endophyte or in the rhizosphere of the host plant.

2. Development and Finding

Here, we assessed the interactions of Mb7 with three phytophagous mites in three different plant systems including a monocot and two dicots. The plant colonization by Mb7 was evaluated by CLSM [29] and plant tissue recovery [30]. We observed differences in results obtained using the classical method of evaluating endophytic colonization by re-isolation i.e., confirmation by tissue recovery and the actual endophytic colonization rates confirmed by live, direct imaging of plant tissue by CLSM. The latter proved to be a reliable tool for monitoring the early stages of Mb7 interactions with plant tissue (as an endophyte or as a rhizosphere resident) and for characterizing the local and systemic colonization of plant tissues. Visualization of fungal entomopathogens expressing GFP was claimed as a valuable methodology with great impact on endophytism study, which still relies on cultural methods (based on the review by Vega [19]).

By CLSM we observed endophytic colonization of citrus leaves and onion roots with Mb7, reported here for the first time (based on reviews by Jaber and Ownley, and Bamisile [18][25]). The unsuccessful colonization of bean leaves with Mb7 and the fact that conidia were not observed germinating on the bean leaves could be a result of adverse conditions that do not support development and plant colonization for Mb7 on bean leaves [19]. Another explanation could be incompatibility of the *M. brunneum* strain used in this study with bean leaves, the presence of non-stimulatory compounds on the surface of those leaves [10][31] or presence of trichomes forming a physical barrier between the spores and the plant surface [32]. In a recent study, 10 different strains of *Metarhizium* did not establish endophytic association with bean plant within a 20-day period although rhizosphere colonization was detected for all the 10 strains [33]. The association described in our study, performed on a single strain, cannot indicate whether it is dictated by the plant host or by the fungal strain. Yet it is reasonable that some fungi exhibiting endophytic lifestyle in one plant may not establish in others. This selective inoculation known as specificity in pathogen host relations may be true for beneficial interactions as well.

The Mb7 strain in our study showed high degree of pathogenicity toward the three mite hosts in direct application. Entomopathogenic fungi play a central role in the natural control of mite populations [7][34][35] and in previous studies Mb7 was already confirmed as a pathogen of ticks (Acari: Ixodidae) [29]. This study describes, for the first time, the pathogenicity and efficacy of *M. brunneum*-7 against the citrus rust mite under laboratory conditions and the bulb mite under laboratory and greenhouse conditions. The only entomopathogenic fungi known to naturally attack the citrus rust mite are *Hirsutella thompsonii* [35] and *Meira* spp. [36]. Commercial products based on *H. thompsonii* have been used to reduce mite infestations. *Meira* spp., mainly *Meira geulakonigii*, have been identified as plant endophytes that reduce mite populations not by direct colonization, but through the production of toxic metabolites such as argovin [34][37]. In regard to the bulb mite, previous studies have evaluated its susceptibility to entomopathogenic fungi, including *Hirsutella* spp., *Isaria fumosorosea, Metarhizium* spp., and entomopathogenic nematodes [38][39][40]; but only one strain of a *Metarhizium* sp. isolated in Israel was found virulent [40].

Metarhizium brunneum soil drenches improved wheat yield and reduced the damage caused by the elaterid, *Limonius californicus* [41]. Furthermore, foliar applications of *M. brunneum* have been shown to lead to transient endophytic colonization and high mortality rates of the moth *Spodoptera littoralis* and the whitefly *Bemisia tabaci* in alfalfa, tomato and melon [42][43]. Based on our results, in which direct application of Mb7, but not endophytic leaf colonization, reduced citrus rust mite populations, we conclude that leaf-endophytic Mb7 most likely does not secrete any acaricidal metabolites, reducing mite numbers only by direct contact of the mites with the conidia and ultimately infection. The results of our study are not in accordance with those of Resquín-Romero et al. [44] and Garrido-Jurado et al. [43], who observed additional mortality of *Spodoptera littoralis* larvae that fed on endophytically colonized plants, as well as insect cadavers and leaf discs in which destruxin A was found. We suggest that the differences between the results of those studies and our findings stem from differences in the experimental system. Further study of the secondary metabolites of Hypocrealean entomopathogenic fungi as endophytes in general, and of *M. brunneum* in particular, may contribute to elucidating the involvement of secondary metabolites in these specific scenarios.

Management of soil pests is not only challenged by a lack of efficient pesticides, but also by a lack of appropriate sampling methods for assessing field populations and predicting outbreaks, a lack of integrated management alternatives, and limited knowledge of pest biology and ecology. The bulb mite is a common soil dweller [45] that attacks onion (*A. cepa*), garlic (*Allium sativum*), lily (*Lilium longiforum*), and ruscus (*Danae racemosa*) in Israel [46][47], and is considered an important pest of these crops worldwide. This mite is not an easy target for chemical pesticides and natural enemies, as it lives in the soil on rotting plant tissue, between bulb scales and inside sprouts. Hence, a management strategy capable of reaching its hidden locations is needed. The results of the current study demonstrate the susceptibility of the bulb mite to Mb7 and the efficacy of drench applications of Mb7 in reducing the bulb mite populations infesting potted onions. We speculate that the mechanisms leading to reduced bulb mite numbers in this scenario include: (i) enrichment of the rhizosphere with Mb7 conidia, which facilitates direct contact of mites with conidia and ultimately infection; (ii) endophytic establishment of Mb7 in the roots, which could engender conidiogenesis, thereby increasing the amount of conidial inoculum; and (iii) establishment of Mb7 in the complex bulb tissues.

In studies conducted on onion bulbs, mites were observed to be attracted to *Fusarium*-infested bulbs and to become established on them, due to the emission of alcohols that attracted them [45][47]. Other studies have shown that this mite can transfer various microorganisms through its alimentary tract [45][48]. Several studies reported antagonistic effect of endophytic entomopathogenic fungi on plants pathogens (review in Vega [19]) and more specifically *M. anisopliae* effectively reduced *Fusarium oxysporum* infection in onion bulbs [49]. The fact that Mb7 was observed developing and endophytically colonizing bulb roots gives rise to questions such as: (i) what are the interactions among the members of the onion rhizosphere microflora?; (ii) are these mites protected from pathogens due to their establishment in niches that are rich in microflora, and as such have developed resistance to pathogens?; and (iii) does the establishment of *M. brunneum* in the rhizosphere impair the establishment of phytopathogenic fungi?

Although we studied a single strain, our observations indicate that Mb7 was readily colonizing endophytically the onion root, a monocot plant, but following drench application to bean root, a dicot plant, Mb7 was detected as a rhizosphere resident with no endophytic colonization of the roots. Similar observations were reported for *Metarhizium* species with a clear preference for monocots than to dicots plants [33]. Our results showed lower abundance of *T. urticae* females on bean plants with Mb7 as a rhizosphere resident, whereas males, immatures, and egg numbers were not affected. As females are the main dispersing life stage of spider mites [50], it is likely that female *T. urticae* were repelled by chemical foliar cues, possibly induced by the Mb7 resident in the rhizosphere. Future studies should address questions regarding plant responses, such as changes in chemical cues; induced resistance and formation of active metabolites, which can effect pests; and the mechanism that enables endophytic establishment of *M. brunneum* in microflora-enriched niches, such as bulbs infested with phytopathogenic microorganisms.

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