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Original article

Effect of endophytic entomopathogenic fungi on soybean *Glycine max* (L.) Merr. growth and yield

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ABSTRACT

The soybean is a crop of economic importance and has a great number of potential pests which cause significant economic losses. The entomopathogenic fungi *Beauveria bassiana*, *Metarhizium anisopliae* and *Metarhizium robertsii* are important biological control agents, which can live as endophytes within plants and causes no apparent damage to the host. The aims of this study were to assess whether the entomopathogenic fungi *B. bassiana, M. manisopliae* and *M. robertsii* are able to colonize soybean plants as endophytes by using different inoculation techniques; and assess if these fungi produce any effect on the growth and yield of soybean plants under field conditions. We demonstrate the effectiveness of three inoculation methods (foliar spray, seed immersion and root immersion) to establish fungal entomopathogens as endophytes. Percentage of recovery for the different fungal strains was higher after 7 days of inoculation, through the organ that was in direct contact with the fungus during the inoculation. *B. bassiana* LPSc 1098 inoculated by leaf aspersion was the most successful strain. It was also demonstrated for the first time that inoculation with *B. bassiana* promoted the growth and increased the yield of soybean plants under filed conditions, with no adverse effects observed in the inoculated plants.

1. Introduction

The soybean *Glycine* max (L.) Merril is native to China and belongs to the family Fabaceae. Due to its oils and proteins, which are widely used in the production of food for animals and humans, soybean is currently cultivated worldwide. Soybean is affected from plant emergence to grain maturity by a great diversity of pest arthropods, which usually limit the growth and yield of this crop. The main management strategy of these pests is focused on the use of pesticides however, they also have direct effects on humans and the environment. Entomopathogenic fungi including *Beauveria bassiana* (Bals-Criv.) Vuill. (Hypocreales: Cordicypitaceae), *Metarhizium anisopliae* (Metschn.) Sorokin and *Metarhizium robertsii* Bisch., Rehner & Humber (Hypocreales: Clavicipitaceae) are used as biocontrol agents worldwide (Vega et al., 2012). Most research on these fungi has focused on the development of inundative methods, however, their endophytic behavior indicates that the ecology of these microorganisms is far beyond the fungus-insect interaction. Endophytic fungi can live inside plants and, in general, do not cause visible damage to the host (Gurulingappa et al., 2010). Several species of entomopathogenic fungi occur naturally in different plant species (Vega, 2008). Others have been artificially introduced into plants by different inoculation techniques, such as foliar spray, stem injection, root and seed immersion, and soil drenching (Akello and Sikora, 2012; Gurulingappa et al., 2010; Jaber and Enkerli, 2016; Castillo Lopez and Sword, 2015; Parsa et al., 2013; Quesada Moraga et al., 2014a; Russo et al., 2015), being able to colonize the plants either locally (Wearn et al., 2012; Yan et al., 2015) or systemically (Gurulingappa et al., 2010; Quesada Moraga et al., 2006; Russo et al., 2015). Recently, many studies have demonstrated that some species are able to play a wider role in nature than previously thought, for example, as promoters of plant growth through the increasing of root length, dry and wet

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weight, foliar area, seed germination, plant height, yield and even the nutritional status (Akello et al., 2008a,b; Castillo Lopez and Sword, 2015; Greenfield et al., 2016; Griffin et al., 2005; Kabaluk and Ericsson, 2007; Liao et al., 2014; Ownley et al., 2004, 2008; Qayyum et al., 2015; Sánchez Rodríguez et al., 2015; Sasan and Bidochka, 2012; Vega, 2008; Vega et al., 2009).

The aims of this study were to (1) assess whether the entomopathogenic fungi *B. bassiana, M. anisopliae* and *M. robertsii* are able to colonize soybean plants as endophytes either locally or systematically by using different inoculation techniques; and (2) use the most efficient inoculation technique and the most frequent fungal isolate recovered from inoculated plants under laboratory conditions, to assess if these fungi produce any effect on the growth and yield of the soybean plants under field conditions.

2. Material and methods

2.1. Experiment-I

2.1.1. Fungal strains and inoculum preparation

Sixteen strains were used in the experiments including fourteen of *B. bassiana*, one of *M. anisopliae* and one of *M. robertsii*. All strains of entomopathogenic fungi were obtained from the fungal collection of "Instituto Spegazzini" (LPSc), La Plata, Argentina and preserved by freezedrying (lyophilization) technique. To confirm the identity of all strains, previously characterized on the basis of morphological characters according to taxonomic keys of Humber (2012), molecular techniques were used. DNA was extracted from fungal cultures developed on potato dextrose liquid medium after 7 days incubation at 25 °C in darkness using the DNA easy Plant Mini kit (Qiagen, Hilden, Germany). Amplification of the internal transcribed spacers (ITS) was carried out using the universal primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCC TCCGCTTATTGATATGC-3′) (White et al., 1990). Polymerase-chain reactions (PCRs) were carried, amplicon sizes were checked by electrophoresis and purified PCR products were sent to Sequencing Service – CERELA (Tucumán, Argentina) for sequencing in both directions. The sequences obtained were edited using the program BioEdit version 7.0.9.0 (Hall, 1999) and submitted to the National Center for Biotechnology Information (NCBI) GenBank database for gene annotation. Sequences are available under the accession numbers: *Beauveria bassiana* MG712618 (LPSc 1060), MG712619 (LPSc 1061), MG712620 (LPSc 1062), MG712624 (LPSc 1063), MG712621 (LPSc 1066), MG712622 (LPSc 1080), MG712623 (LPSc 1081), MG712625 (LPSc 1083), MG712626 (LPSc 1086) and MG712627 (LPSc 1156). While *M. anisopliae* LPSc 907, *M. robertsii* LPSc 963, *B. bassiana* LPSc 902, 1067, 1082 and 1098 were previously determined with GeneBank accession numbers KT163258, KJ772494, KT952326, KF500409, KJ7722495 and KT163259 respectively.

To obtain the conidial suspensions, different isolates of each species were cultivated onto potato dextrose agar (PDA) (Britania®) and incubated at 25 °C in darkness. After 15 days, conidia were harvested by scrapping them off the Petri dishes and transferred to 10 ml of 0.01% (v/v) Tween 80 (polyoxyethylene sorbitan monolaurate) (Merck®). The suspension was filtered and homogenized by shaking for 10 min. Conidial concentration was determined by using a Neubauer chamber and adjusted to 1×10^8 conidia/ml (Gurulingappa et al., 2010). The conidial viability of each isolate was evaluated according to Greenfield et al. (2016), and in all cases, the mean conidial viability was >95%.

2.1.2. Soybean plants substrates

Seeds of the variety DM3810 (Don Mario, Argentina) were used for all experiments. The seeds were surface-sterilized (Posada et al., 2007) and were transferred to plastic pots (330 cm^3) containing a mixture of equal parts of earth-perlite-vermiculite (1: 1: 1), used as planting substrate. The substrate was tindalized in an autoclave for 45 min at 121 °C. Sterilization was performed three times with 24-h intervals between each process (Quesada Moraga et al., 2014b). Plants were produced and maintained under greenhouse conditions (25 ± 2 °C, 12:12 LD photoperiod) until used.

2.1.3. Inoculation methods and determination of endophytic colonization

Three methods of inoculation were tested: leaf aspersion, root immersion and seed immersion, according to Russo et al. (2015).

Plant colonization by different fungal isolates was evaluated after 7, 14, 21, and 28 days of inoculation. Each plant was separated into roots, shoot and leaves, and surface-sterilized by successive immersions in 70% ethanol for 2 min, sodium hypochlorite (55 g Cl/L of commercial bleach) for 2 min and finally rinsed twice with sterile distilled water. To determine the efficiency of the surface sterilization method, surface-sterilized stem pieces were plated onto solid PDA (Reddy et al., 2009). The absence of fungal or bacterial growth was considered indicative of a successful sterilization technique.

Each plant organ was cut with a sterile scalpel into pieces of 1 cm^2. Six pieces of each plant organ were placed onto 20 ml of PDA medium with 2 ml of antibiotics (5g streptomycin and 0.25 g chloramphenicol/200 ml) (Vega et al., 2008). All dishes were maintained at 25 °C in a growth chamber and examined after 10 days of incubation. A total of 1920 plants and 34,560 plant pieces were examined (120 plants and 2160 plant pieces for each fungal strain inoculated). Data were expressed as the frequency of colonization (FC) = (number of plant pieces colonized/total number of plant pieces examined) × 100 (Petrini and Fisher, 1986).

2.2. Experiment-II

2.2.1. Effect of fungal inoculation on plant growth and yield

To determine if the entomopathogenic fungus has any effect as endophyte on the soybean growth and yield, we selected leaf aspersion as inoculation technique because it proved to be the most efficient, and *B. bassiana* strain LPSc 1098 because it was the most frequently recovered from the inoculated plants under laboratory conditions. Plants were obtained according to the methodology described in Experiment I. After two weeks, all plants were inoculated and maintained in a greenhouse for 7 days before transferring them (according to the sowing time of the crop) to a 10 × 10 m field plot, in a randomized way and forming six rows of 10 plants each. Plants transferred to the field were previously checked for endophytic colonization according to Russo et al. (2015). The fungal effect on plant growth and yield was determined after five months, when plants completed their annual cycle. The following parameters were analyzed: total plant height, number of branches per plant, number of pods per branch and per plant, pod weight per branch and per plant, number of seeds per pod, per branch and per plant, seed weight per branch and per plant, yield (Diestéfano and Gadbán, 2010), and germinative capacity of seeds, according to the International Association of Seed Analysis (ISTA, 2007), following the protocol (modified) of Luna and Iannone (2013).

This experiment was performed in Alberti city, Buenos Aires province, Argentina (35° 1′ 53″ S-60° 16′ 49″ W). The mean annual rainfall was 1000 mm and the mean temperature was 16 °C (www.trigoklein.com.ar/estacion-meteorológica). Soybean is one of the main crops in this area, arriving at physiological maturity in optimal conditions. In this experiment, we used 30 inoculated plants and 30 controls. Controls were inoculated with a 0.01% (v/v) Tween 80 (Merck®) solution without the addition of fungal inoculum.

2.3. Statistical analyses

Inoculation techniques (Experiment-I), plant organs, time and frequency of colonization of the different strains were compared with a three-way Analysis of Variance (ANOVA) and the mean differences compared by Tukey's test ($p < 0.05$) using InfoStat (2004).

To stabilize the variance, percent values were transformed to arcsine. Differences between the germinative capacity, the yield, and each growth parameter analyzed of plants inoculated with *B. bassiana* and non-inoculated (controls) (Experiment-II) were compared with a t-test.

3. Results

3.1. Experiment-I. Recovery of entomopathogenic fungi as endophytes

No growth of entomopathogenic fungi was observed in the non-inoculated controls (data not shown). All techniques of inoculation introduced successfully the strains of *B. bassiana* into soybean plants while the seed immersion technique was unsuccessful for introducing *M. anisopliae* and *M. robertsii* (Table 1). ANOVA results of the comparison between inoculation techniques, organs and time for each fungal strain are shown in Table 2.

3.1.1. Leaf aspersion

The highest number of isolates was recovered from leaves after 7 days inoculation. In general, there was a significant decrease in the percentage of colonized pieces over time. *B. bassiana* strain LPSc 1098 was the most successful strain, because it showed 100%, 90% and 45% of recovery from leaves, stems, and roots, respectively, after 7 days of inoculation; values that were not obtained for any of the other strains tested (Table 1).

The two isolates belonging to *Metarhizium* spp. were re-isolated from leaves, stems and roots, but with colonization rates of 60%, 40% and 16% respectively (Table 1).

3.1.2. Root immersion

B. bassiana strains LPSc 1080, LPSc 1156, LPSc 1061, LPSc 1082 and LPSc 902 and *Metarhizium* strains LPSc 963 and LPSc 907 showed the highest percentages of isolation (55–71%) from roots after 7 days of inoculation. *B. bassiana* strain LPSc 1062 exhibited the highest frequency of leaf colonization. No isolates of entomopathogenic fungi were registered after 28 days of inoculation (Table 1).

3.1.3. Seed immersion

B. bassiana strains LPSc 1081, LPSc 1082, LPSc 1061 and LPSc 1086 were re-isolated from roots and stems after 7 days of inoculation, with a mean percentage of 40% and 16.6%, respectively. In the case of *B. bassiana* strains LPSc 1063, LPSc 1066 and LPSc 1083, they were able to colonize the roots and stems but not the leaves after 7, 14 and 21 days of inoculation, exhibiting higher percentages of colonization than the previously mentioned strains.

The other fungal strains were introduced through the seeds and were able to colonize roots, stems, and leaves, even though, after 28 days of inoculation, the only strain re-isolated from roots, stems and leaves was *B. bassiana* LPSc 1067 (Table 1).

3.2. Experiment-II: effect of fungal inoculation on plant growth and yield

The entomopathogenic fungus *B. bassiana* promoted the growth of soybean plants, since all growth parameters assessed after inoculation were significantly higher than in the controls: plant height ($T = 4.49$; $df = 58$; $p < 0.0001$), number of branches per plant ($T = 4.38$; $df = 58$; $p < 0.0001$), weight of the pods per branch ($T = 3.71$; $df = 58$; $p = 0.0005$), weight of the pods per plant ($T = 4.85$; $df = 58$; $p < 0.0001$), number of pods per branch ($T = 2.87$; $df = 58$; $p = 0.0057$), number of pods per plant ($T = 4.32$; $df = 58$; $p < 0.0001$), number of seeds per pod ($T = 3.48$; $df = 58$; $p = 0.0009$), number of seeds per branch ($T = 4.54$; $df = 58$; $p < 0.0001$), number of seeds per plant ($T = 4.88$; $df = 58$; $p < 0.0001$), seed weight per branch ($T = 1.57$; $df = 58$; $p = 0.05$), seed weight per plant ($T = 4.17$; $df = 58$; $p < 0.0001$) and yield ($T = 2.67$ $df = 4$; $p = 0.0456$) (Fig. 1). The mean germinative capacity in inoculated plants was significantly higher ($T = 8.55$, $df = 4$, $p < 0.0010$) than in the controls.

4. Discussion

This study demonstrated that the three inoculation techniques used, i.e. leaf aspersion, root and seed immersion, successfully introduced different strains of *B. bassiana* into soybean plants. On the contrary, strains of *M. anisopliae* and *M. robertsii* were able to establish endophytically exclusively by leaf aspersion and root immersion. The sterilization efficiency was tested by incubating a piece of vegetal tissue onto a solid medium (McKinnion et al., 2017; Reddy et al., 2009). In contrast, most studies to date have tested the efficiency of sterilization by pipetting aliquots of the final water sterilization onto a solid medium (Greenfield et al., 2016; Parsa et al., 2013; Posada and Vega, 2005; Tefera and Vidal, 2009; Vidal and Jaber, 2015), however, this approach may not be an adequate control due to dilution effects and potential failure to remove epiphytes. Plant cuticles are multi-dimensional and hydrophobic so, these surfaces can potentially protect epiphytic microorganisms during submersion and a single viable colony forming unit could yield a false 'endophyte' positive. In particular, viable conidia are typically found adhering to the plant surface rather than floating freely in the rinse solution, even when using surfactants (Schulz and Boyle, 2005).

As in the case of poppies (Quesada Moraga et al., 2006), beans (Parsa et al., 2013) and sorghum (Tefera and Vidal, 2009), leaf aspersion was the most efficient technique for the inoculation of different fungal strains into soybean plants. The greatest recovery of *B. bassiana* and *Metarhizium* spp. was obtained from leaves after 7 days of inoculation. On the other hand, when the inoculation was performed in the roots or seeds, the entomopathogenic fungi were mostly re-isolated from roots and in less proportion from leaves. This might be the result of a higher colonization frequency in plant organs next to the inoculum than in ones distant to the application place (Greenfield et al., 2016). It is important to mention that the percentage of isolation decreased over time. This result is in agreement with Parsa et al. (2013) in beans, Greenfield et al. (2016) in manioc and Brownbridge et al. (2012) in pine trees. On the contrary, Batta (2013) showed that in rape plants the entomopathogenic fungi *Metarhizium* sp. and *Beauveria* sp. were mainly isolated after 4 weeks of inoculation. However, in contrast to our results in which leaf inoculation was a successful technique, some studies showed the greatest recovery of *B. bassiana* in coffee by direct injection (Posada et al., 2007) and in tomato through the inoculation of roots (Qayyum et al., 2015), indicating that leaves are not appropriate routes of entry for fungal colonization. The low recovery of *B. bassiana* from leaves could be due to specific cuticular components on the leaf and the lack of stomata on the adaxial side. It is possible that the main components on the leaf cuticle, waxes and cutin, might have a detrimental effect on conidium germination (Posada et al., 2007).

Beauveria bassiana, M. anisopliae and *M. robertsii* were successfully established as endophytes in soybean plants. As observed by Brownbridge et al. (2012) and Parsa et al. (2013), inoculation

Table 1
Mean (±SEM) percentage colonization of entomopathogenic fungi in leaves, stems and roots by different inoculation techniques (seed inoculation, leaf spray and root immersion), at 7, 14, 21 and 28 days. Different letters within the same strain indicate significative differences (Tukey test, $p < 0..05$).

Strain	Days	Leaf aspersion			Seed immersion			Root immersion		
		Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
	7	45 ± 5.6 defhi	90 ± 5.8 ij	100 ± 0 J	51.3 ± 8.8 efghi	36.7 ± 7.2 bcdefg	18.7 ± 11.3 abcd	40 ± 10.6 cdefgh	43.3 ± 11.2 cdefgh	40 ± 10.6 cdfgh
B. bassiana LPSc 1098	14	43.3 ± 5.1 defghi	66.7 ± 7 ghi	100 ± 0 J	16.7 ± 7.5 abcde	20 ± 8.2 abcde	23.3 ± 10.9 abcdef	40 ± 10.6 cdefgh	46.7 ± 6 defgh	5 ± 2 ab
	21	35 ± 4.6 cdefghi	73.3 ± 3.7 hij	83.3 ± 7.9 ij	13.3 ± 7.4 abcd	11.7 ± 7.9 abc	15 ± 7.6 abcd	0 ± 0 a	0 ± 0 a	0 ± 0 a
	28	0 ± 0 a	31. 7 ± 7.2 abcdefg	70 ± 5 ghi	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	7	31.6 ± 8 abcd	66.7 ± 4 bcd	80 ± 6 d	38.3 ± 4 abcd	40 ± 6 abcd	43.3 ± 6 abcd	33.3 ± 4 abcd	40 ± 2 abcd	33.3 ± 2 abcd
B. bassiana LPSc 1067	14	25 ± 7 abcd	25 ± 3 abcd	76.7 ± 4 cd	38.3 ± 4 abcd	33.3 ± 5 abcd	23.3 ± 5.5 abcd	25 ± 8.3 abcd	18.3 ± 9.4 abc	35 ± 5 abcd
	21	16.6 ± 7 abc	25 ± 2 abcd	50 ± 2 abcd	20 ± 0 abc	23.3 ± 4 abcd	23.3 ± 1.2 abcd	0 ± 0 a	23.3 ± 4.5 abcd	15 ± 7 abc
	28	16.6 ± 2 abc	25 ± 4 abcd	31.7 ± 6 abcd	8.3 ± 0 ab	15 ± 4 abc	10 ± 6.7 ab	0 ± 0 a	0 ± 0 a	10 ± 1 ab
	7	36.6 ± 4 cde	83.3 ± 6 fg	88.3 ± 5 g	33.3 ± 0.5 cde	3.3 ± 1 ab	0 ± 0 a	30 ± 1 bcde	30 ± 7 bcde	26.7 ± 4 abcde
B. bassiana LPSC 1086	14	18. 3 ± 7 abcd	46.6 ± 5 de	58.3 ± 5.6 efg	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	36.7 ± 2 cde	13.3 ± 4.6 abc
	21	0 ± 0 a	0 ± 0 a	16.6 ± 7.4 abcd	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	28	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	7	66.6 ± 4.4 fghi	86.7 ± 6 J	81.7 ± 6 ij	61.3 ± 7.3 efghij	31.7 ± 7 abcdef	13.7 ± 6 abcd	71.7 ± 7 ghij	36.7 ± 7 abcdef	40 ± 6 abcdef
B. bassiana LPSc 1080	14	40 ± 8 abcdef	66.7 ± 2.6 ghij	78.3 ± 6 hij	45.3 ± 3.3 abcdef	36 ± 6 abcdefg	23.7 ± 4.8 abcdef	16.7 ± 5 abcde	36.7 ± 3 abcdef	35 ± 6 abcdef
	21	6.7 ± 2 abc	53.3 ± 1.3 defgh	48.3 ± 2.9 cdefgh	0 ± 0 a	0 ± 0 a	0 ± 0 a	6.7 ± 1 ab	16.7 ± 3 cdefgh	16.7 ± 2 abcde
	28	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	7	20 ± 7.7 abcdef	60 ± 3 fgh	71.7 ± 6 h	71.7 ± 5 gh	58.3 ± 7.1 fgh	0 ± 0 a	40 ± 8 bcdefg	46.7 ± 8 cdefgh	40 ± 9.6 bcdefgh
B. bassiana LPSc 1063	14	11.7 ± 3.6 abcd	36.7 ± 4 abcdefg	53.3 ± 6 efgh	13.3 ± 5.9 abcd	25 ± 6.5 abcef	0 ± 0 a	15 ± 6.3 abcde	35 ± 7 abcdefg	13.3 ± 4.3 abcd
	21	8.3 ± 2.2 abc	30 ± 5 abc	15 ± 2 abcd	10 ± 2 ab	10 ± 3 abc	0 ± 0 a	3.3 ± 1 ab	8.3 ± 3.7 ab	0 ± 0 a
	28	3.3 ± 1.1 ab	5 ± 2.5 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	7	43.3 ± 8.6c	46.7 ± 6.9cd	83.3 ± 4.9f	45 ± 7 cd	31.7 ± 3.8c	0 ± 0 a	45.7 ± 8.2cd	45.3 ± 8.7cd	33.3 ± 1c
B. bassiana LPSc 1066	14	25 ± 4.4 bc	46.7 ± 6.9cd	71.7 ± 3.5 def	31.7 ± 4.6c	25 ± 3.7 bc	0 ± 0 a	43.3 ± 5 cd	30 ± 6.1c	0 ± 0 a
	21	0 ± 0 a	28.3 ± 4.3c	48.3 ± 3.8 cde	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	28	0 ± 0 a	0 ± 0 a	10 ± 5 ab	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	7	10 ± 3.5 ab	83.3 ± 7.8 fg	86.7 ± 4.8 fg	5 ± 3.5 a	13.3 ± 3.3 ab	26.7 ± 3 abcd	68.3 ± 2.9 defg	40.7 ± 5.1 bcd	40.3 ± 1 bcd
B. bassiana LPSc 1156	14	13.3 ± 5.9 ab	60 ± 2.2 cdefg	81.7 ± 4 fg	23.3 ± 6 abc	16.7 ± 7 ab	28.3 ± 6.1 abcd	26.7 ± 2 abc	33.3 ± 2.8 abcd	33.3 ± 3.33 abcd
	21	0 ± 0 a	35 ± 5.5 abcd	48.3 ± 6.5 bcde	0 ± 0 a	0 ± 0 a	0 ± 0 a	18.3 ± 8.4 ab	21.7 ± 7.4 abc	20 ± 6.9 abc
	28	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	7	40 ± 7.5 cdefg	63.3 ± 8.5 gh	73.3 ± 9 h	56.7 ± 8.3 efg	31.7 ± 6.3 cdefg	0 ± 0 a	41.7 ± 8 cdefg	28.3 ± 6 bcde	16.7 ± 5.5 abc
B. bassiana LPSc 1060	14	13.3 ± 2.2 abcd	40 ± 8.3 cdefg	46.7 ± 7.7 efgh	43.3 ± 7.5 defg	23.3 ± 3.6 bcdef	0 ± 0 a	35 ± 8 cdefg	16.7 ± 4.6 abcde	0 ± 0 a
	21	0 ± 0 a	21.7 ± 5.5 bcde	18.3 ± 5.2 abcde	18.3 ± 2 abcd	0 ± 0 a	0 ± 0 a	6.7 ± 2 ab	0 ± 0 a	0 ± 0 a
	28	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	7	61.7 ± 5 fg	66.7 ± 4 gh	95 ± 3.5h	50 ± 0 defg	25 ± 0 abcdef	0 ± 0 a	66.3 ± 9.3 gh	55.3 ± 7.7 defg	55 ± 5 defg
B. bassiana LPSc 1061	14	48.3 ± 9 defg	46.7 ± 8 cdefg	50 ± 6.9 defg	0 ± 0 a	0 ± 0 a	0 ± 0 a	35 ± 8.4 bcdefg	26.7 ± 5.6 abcdef	23.3 ± 5.6 abcde
	21	18.3 ± 7.2 abcd	11.7 ± 6 abc	13.3 ± 3 abc	0 ± 0 a	0 ± 0 a	0 ± 0 a	6.7 ± 2.7 ab	3.3 ± 1.2 ab	3.3 ± 1 ab
	28	0 ± 0 a	1.7 ± 0 a	3.3 ± 1 ab	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	7	20 ± 5.4 abcdef	20 ± 5.4 abcdef	61.7 ± 5 g	18.3 ± 6 abcde	21.7 ± 3.4 abcdef	21.7 ± 2 abcdef	40 ± 3 bcdefg	43.3 ± 6 bcdefg	60 ± 4 fg
B. bassiana LPSc 1062	14	0 ± 0 a	20 ± 5.4 abcdef	51.7 ± 2 efg	16.7 ± 3 abcd	11.7 ± 2 abc	11.7 ± 2 abcd	31.7 ± 4 bcdefg	46.7 ± 5.9 defg	15 ± 2 abcd
	21	0 ± 0 a	8.3 ± 2 a	25 ± 3.7 bcdefg	13.3 ± 3 abcd	11.7 ± 2 abc	10 ± 1 ab	0 ± 0 a	0 ± 0 a	0 ± 0 a
	28	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	7	30 ± 2 bcde	45 ± 4 defg	78.3 ± 6.1h	30 ± 0 cdef	16.6 ± 0 bcde	0 ± 0 a	68.3 ± 5.2 gh	51.7 ± 5.2 efg	48.3 ± 5 efg
B. bassiana LPSc 1082	14	13.3 ± 3 abc	35 ± 7 cdef	53.3 ± 5.9 fgh	0 ± 0 a	0 ± 0 a	0 ± 0 a	33.3 ± 3 cdef	21.7 ± 2.2 bcde	0 ± 0 a
	21	0 ± 0 a	18.3 ± 3.8 abcd	18.3 ± 3.2 abc	0 ± 0 a	0 ± 0 a	0 ± 0 a	5 ± 1 ab	0 ± 0 a	0 ± 0 a
	28	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	7	46.7 ± 4.1 de	73.3 ± 2.7 ef	73.3 ± 2.7 ef	76.7 ± 7.1f	48.3 ± 3 de	0 ± 0 a	20 ± 0 bc	6.6 ± 0 ab	0 ± 0 a
B. bassiana LPSc 1083	14	36.7 ± 3.3cd	56.7 ± 5.6 def	71.7 ± 2.5 ef	40 ± 4 cd	18.3 ± 7.6 bc	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	21	20 ± 2.1 bc	46.7 ± 4.1 de	50 ± 3.5 de	6.7 ± 1.7 ab	6.7 ± 2 ab	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	28	6.7 ± 2 ab	6.7 ± 1.7 ab	6.7 ± 1.7 ab	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a

(*continued on next page*)

Table 1 (*continued*)

Strain	Days	Leaf aspersion			Seed immersion			Root immersion		
		Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
B. bassiana LPSc 1081	7	0 ± 0 a	51.7 ± 7 fg	76.7 ± 5.6h	30 ± 0 cdef	20 ± 0 bc	0 ± 0 a	53.3 ± 4.1 fg	53.3 ± 4.1 fg	30 ± 3.1 cde
	14	0 ± 0 a	41.7 ± 6.2 efg	65 ± 6.3 gh	0 ± 0 a	0 ± 0 a	0 ± 0 a	53.3 ± 4.1 fg	43.3 ± 2.7 efg	20 ± 5.4 bc
	21	0 ± 0 a	28.3 ± 3.5 cdef	36.7 ± 2.1 def	0 ± 0 a	0 ± 0 a	0 ± 0 a	16.7 ± 0.1cd	5 ± 1 a	6.7 ± 2.2 ab
	28	0 ± 0 a	0 ± 0 a	3.3 ± 2.2 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
B. bassiana LPSc 902	7	31.7 ± 6 abcd	65 ± 5 bcd	75 ± 5.6 d	38.3 ± 7 abcd	46.7 ± 6 abcd	43.3 ± 5.6 abcd	55 ± 3 bcd	40.3 ± 5 abcd	40 ± 2 abcd
	14	25 ± 4 abcd	25 ± 2 abcd	66.7 ± 5.6cd	38.3 ± 2.2 abcd	33.3 ± 2 abc	23.3 ± 3.3 abc	40 ± 1 abcd	36.7 ± 3.9 abcd	25 ± 3.4 abc
	21	16.7 ± 3 abc	25 ± 2 abcd	25 ± 5.4 abcd	0 ± 0 a	11.7 ± 3.8 ab	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	28	16.7 ± 3 abc	16.7 ± 1 abc	23.3 ± 3 abcd	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
M. anisopliae LPSc 907	7	16.7 ± 0.1b	41.7 ± 6.2c	56.7 ± 7.2 d	0 ± 0 a	0 ± 0 a	0 ± 0 a	66.6 ± 0.4 d	33.3 ± 0.2c	16.7 ± 0.1b
	14	0 ± 0 a	26.7 ± 3.6 bc	41.7 ± 5.1c	0 ± 0 a	0 ± 0 a	0 ± 0 a	33.3 ± 0.2c	16.7 ± 0.1b	0 ± 0 a
	21	0 ± 0 a	0 ± 0 a	18.3 ± 3.8b	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	28	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
M. robertsii LPSc 963	7	26.7 ± 3.6 bc	38.3 ± 6.4cd	48.3 ± 4.3 de	0 ± 0 a	0 ± 0 a	0 ± 0 a	66.6 ± 0.4 e	16.7 ± 0.1b	0 ± 0 a
	14	16.6 ± 1 a	20 ± 3.3b	26.7 ± 5 bc	0 ± 0 a	0 ± 0 a	0 ± 0 a	66.6 ± 0.4 e	16.7 ± 0.1b	0 ± 0 a
	21	0 ± 0 a	0 ± 0 a	23.3 ± 3.6 bc	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	28	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a

of both seeds and roots by entomopathogenic fungi did not reduce seed germination or affect plant growth and we did not observe any damage to roots (data not shown). In agreement with these results, Posada and Vega (2005) and Tefera and Vidal (2009) demonstrated that colonization of the different plant organs differed between *Beauveria* and *Metarhizium*. The reason for the greatest colonization found in leaves and roots is yet unclear but might show differences in physiological conditions or in the microbial content between plant organs. Although we used sterile substrates and seeds, we were unable to guarantee that plants were free of native endophytes (Posada and Vega, 2005; Quesada Moraga et al., 2009; Vega, 2008).

In this study, we demonstrated by using the seed and root immersion techniques that as endophytes, the entomopathogenic fungi are able to move throughout the soybean plant tissues, entering through the roots, stems, and leaves. Likewise, the leaf aspersion technique showed the ability of entomopathogenic fungi to enter the plant through these organs, move throughout the different tissues and consequently been isolated from stems, roots, and leaves. The colonization of different plant organs indicated that these fungi are able to move throughout the plant systemically (Akutse et al., 2013; Ownley et al., 2008; Quesada Moraga et al., 2009).

The low inoculum recovery after 28 days of inoculation might be due to the competition with other fungi and bacteria in the system or the host response to fungal colonization. Consequently, there was no balance in the coexistence of both organisms (endophytic relationship), which lead to the growth inhibition of the entomopathogenic fungus (Posada et al., 2007). It is also possible that the efficiency of sterilization methods used minimized the recoverage of fungal propagules (Brownbridge et al. 2012; Quesada Moraga et al., 2009). Considering that we used small sections of the plant organs, the chosen procedure should be optimized for the host plant with respect to the type of tissue and its sensitivity, as stated by Brownbridge et al. (2012). On the other hand, the entomopathogenic fungus *B. bassiana* positively affected all the growth parameters evaluated. Posada and Vega (2006) obtained similar results in coffee seedlings, without registering harmful impacts on plant health, whereas Griffin et al. (2005), Ownley et al. (2004), and Ownley et al. (2008) observed that the application of *B. bassiana* as an endophyte in tomato and cotton plants produced a significant increase in the height of these crops. Castillo Lopez and Sword (2015) found an increase in certain growth parameters of cotton plants, such as dry weight and size of the reproductive structures, in response to the inoculation of *B. bassiana* and *Purpureocillium lilacinum* (Thom) Samson (Hypocreales: Ophiocordycipitaceae). Likewise, Greenfield et al. (2016) observed an increase in the growth of cassava plants after inoculation with *B. bassiana* and *M. anisopliae*. Qayyum et al. (2015) inoculated two different strains of *B. bassiana* in tomato plants and observed that one of them promoted plant growth while the other caused a delay in the growth and development of the plants and a reduction in the size of the fruits.

Our results showed that the yield increased significantly in inoculated plants, which is in agreement with the results of field studies obtained in onion inoculated with entomopathogenic fungi (Kabaluk and Ericsson, 2007). It has also been demonstrated for root endophytic fungi as *Piriformospora indica* Sav.Verma, Aj. Varma, Rexer, G.Kost & P.Franken (Sebacinales: Sebacinaceae) to promote growth and yield in soybean plants (Bajaj et al., 2015, 2017a, b).

Unlike Quesada Moraga et al. (2014b), who found that *B. bassiana* can be transferred vertically in poppy plants, our results could not demonstrate this effect. Even though, we observed that the germinative capacity of seeds in plants inoculated with *B. bassiana* was considerably higher than in non-inoculated plants.

Table 2
Results of ANOVA for species factor, technique factor and their interaction. Significant at the $p < 0.001$ probability level.

	Technique			Organ			Time			Technique * organ			Technique * time			Organ * time			Technique * organ * time		
	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p
B.bassiana LPSc 1098	201.52	2	**0.0001**	28.13	2	**<0.0001**	81.4	3	**<0.0001**	26.5	4	**<0.0001**	5.43	6	**<0.0001**	2	6	0.0646	3.17	12	**0.0003**
B.bassiana LPSc 1067	9.26	2	**0.0001**	7.12	2	**0.0009**	16.18	3	**<0.0001**	3.23	4	**0.0128**	0.11	6	0.9949	0.77	6	0.5920	0.88	12	0.5656
B.bassiana LPSc 1086	61.67	2	**<0.0001**	8.1	2	**0.0004**	107.8	3	**<0.0001**	15.51	4	**<0.0001**	16.48	6	**<0.0001**	3.41	6	**0.0028**	5.02	12	**<0.0001**
B.bassiana LPSc 1080	35.41	2	**<0.0001**	20.84	2	**<0.0001**	93.9	3	**<0.0001**	2.8	4	**0.0260**	11.15	6	**<0.0001**	5.97	6	**<0.0001**	4	12	**<0.0001**
B.bassiana LPSc 1063	9.43	2	**0.0001**	8.47	2	**0.0003**	70.23	3	**<0.0001**	12.03	4	**<0.0001**	1.14	6	0.3408	0.78	6	0.5826	5.1	12	**<0.0001**
B.bassiana LPSc 1066	110.75	2	**<0.0001**	7.32	2	**0.0008**	339.78	3	**<0.0001**	81.88	4	**<0.0001**	21.99	6	**<0.0001**	17.27	6	**<0.0001**	8.77	12	**<0..0001**
B.bassiana LPSc 1156	46.82	2	**<0.0001**	30.65	2	**<0.0001**	106.47	3	**<0.0001**	15.43	4	**<0.0001**	12.66	6	**<0.0001**	5.68	6	**<0.0001**	2.36	12	**0.0065**
B.bassiana LPSc 1060	34.01	2	**<0.0001**	12.85	2	**<0.0001**	136.9	3	**<0.0001**	34.18	4	**<0.0001**	6.02	6	**<0.0001**	3.54	6	**0.0021**	5.87	12	**<0.0001**
B.bassiana LPSc 1061	117.8	2	**<0.0001**	0.48	2	0.6223	102.76	3	**<0.0001**	2.58	4	**0.0371**	26.68	6	**<0.0001**	0.5	6	0.8071	1.18	12	0.2984
B.bassiana LPSc 1062	3.06	2	**0.0482**	8.66	2	**0.0002**	69.51	3	**<0.0001**	11.52	4	**<0.0001**	4.47	6	**0.0002**	1.74	6	0.1101	3.52	12	**0.0001**
B.bassiana LPSc 1082	129.51	2	**<0.0001**	2.52	2	0.0819	135.86	3	**<0.0001**	26.16	4	**<0.0001**	37.91	6	**<0.0001**	2.06	6	0.0572	5.21	12	**<0.0001**
B.bassiana LPSc 1083	461.75	2	**<0.0001**	10.45	2	**<0.0001**	130.17	3	**<0.0001**	51.32	4	**<0.0001**	41.09	6	**<0.0001**	7.35	6	**<0.0001**	10.32	12	**<0.0001**
B.bassiana LPSc 902	7.66	2	**0.0006**	1.13	2	0.3258	43.32	3	**<0.0001**	4.09	4	**0.0030**	0.73	6	0.6241	0.51	6	0.8021	0.91	12	0.5332
M.anisopliae LPSc 907	377.16	2	**<0.0001**	4.19	2	**0.0160**	450.57	3	**<0.0001**	137.91	4	**<0.0001**	116.18	6	**<0.0001**	9.98	6	**<0.0001**	30.46	12	**<0.0001**
M.robertsii LPSc 963	308.93	2	**<0.0001**	11.42	2	**<0.0001**	302.69	3	**<0.0001**	171.78	4	**<0.0001**	93.5	6	**<0.0001**	28.22	6	**<0.0001**	40.38	12	**<0.0001**

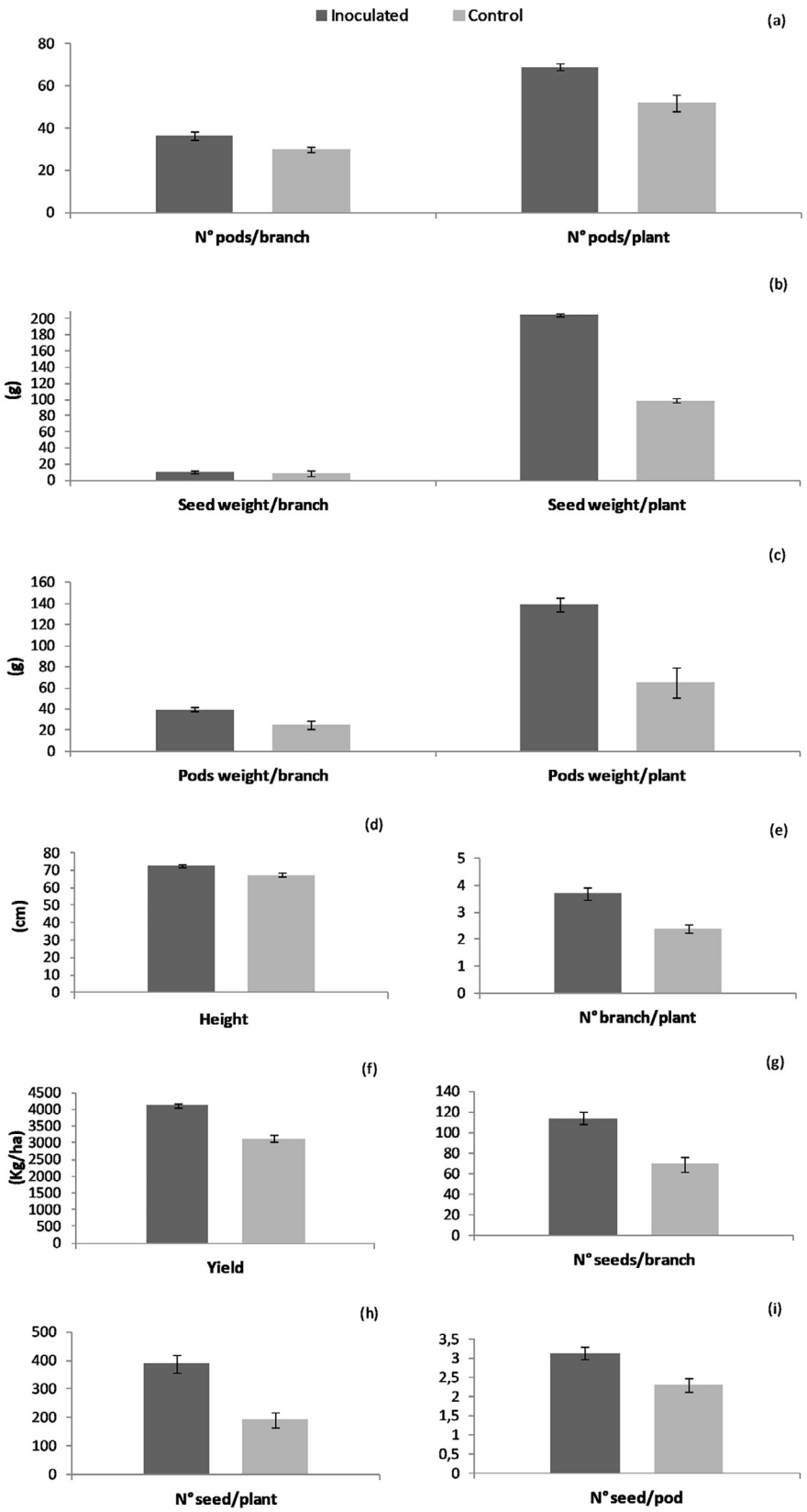

Fig. 1. Growth parametres in soybean plants: (a) N° pods/branch and plant, (b) seed weigth/branch and plant (g), (c) pods weigth /branch and plant (g), (d) heigth (cm), (e) N° branch/ plant, (f) yield (kg/ha), (g) N° seed/branch, (h) N° seed/plant and (i) N° seed/pod. Bars indicate ±SEM.

Regarding the mechanisms related to the promotion of plant growth, previous studies suggested that *B. bassiana* could reduce the damage caused by insect pests and/or act as an antagonist against certain pathogens (Ownley et al., 2008). Other studies conducted mainly with endophytic and non-entomopathogenic fungi, have suggested that the increase in plant growth can be either due to the production of growth hormones (auxins, gibberellins, and cytokinins) or an increase in the fixation of soil nutrients (Castillo Lopez and Sword, 2015).

Although the aim of the present study was not elucidating the mechanisms that promoted the growth of soybean plants in response to the colonization by the entomopathogenic fungus *B. bassiana* as an endophyte, it demonstrated a significant increase in the growth and yield of inoculated plants, without adverse effects observed in their development.

5. Conclusions

Our study demonstrated for the first time that the entomopathogenic fungi *B. bassiana*, *M. anisopliae* and *M. robertsii* could associate endophytically with soybean plants. The greatest recovery of the different fungal strains occurred after 7 days inoculation, through the organ that was in direct contact with the fungus during the inoculation. We found that *B. bassiana* LPSc 1098 inoculated by leaf aspersion is a promising isolate increasing fitness of soybean plants under field conditions.

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MLR and SP conceived and designed research. MC performed morphological determination; CM and AT conducted molecular analysis; MLR, ACS and NA conducted laboratory experiments; MLR and MFV conducted fiel work. MLR, SP and ACS analyzed data. MLV wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest

There is no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jksus.2018.04.008.

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