

SHORT COMMUNICATION

Endophytic colonisation of tobacco, corn, wheat and soybeans by the fungal entomopathogen *Beauveria bassiana* (Ascomycota, Hypocreales)

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(Received 24 June 2014; returned 5 August 2014; accepted 28 October 2014)

We demonstrate the effectiveness of three inoculation methods (foliar spray, seed immersion and root immersion) in establishing fungal the entomopathogen *Beauveria bassiana* as an endophyte in tobacco, corn, wheat and soybean. Colonisation of leaves by *B. bassiana* was assessed 7, 14, 21 and 28 days post-inoculation. There were significant differences ($p < 0.001$) in endophytic colonisation among the different inoculation techniques.

Keywords: *Beauveria bassiana*; endophyte; *Zea mays*; *Glycine max*; *Triticum aestivum*; *Nicotiana tabacum*

Beauveria bassiana (Balsamo) Vuillemin (Ascomycota: Hypocreales) is a cosmopolitan fungal entomopathogen that can live as an endophyte in plants, without causing any symptoms (Van Bael et al., 2005). According to Rodriguez, White, Arnold, and Redman (2009), it is classified as Clavicipitaceous, class 1, Type III in its role as an endophyte. Landa et al. (2013) observed hyphae of *B. bassiana* in leaf and stem intercellular spaces, and in determined zones of the parenchyma tissue of the main leaf vein, but not in the vascular tissues. However, Quesada-Moraga, Landa, Muñoz-Ledesma, Jiménez-Díaz, and Santiago-álvarez (2006), previously, reported fungal growth into the xylem vessels observed by scanning electron microscopy. In some cases, colonisation of plant tissues by *B. bassiana* has been demonstrated to provide protection against insects and to inhibit insect development and establishment (Cherry, Banito, Djegui, & Lomer, 2004; Ownley, Pereira, Klingeman, Quigley, & Leckie, 2004; Vega et al., 2008). The aim of the present work was to evaluate the most appropriate method to inoculate *B. bassiana* as an endophytic fungus and to assess its stability over time in tobacco (*Nicotiana tabacum*), corn (*Zea mays*), wheat (*Triticum aestivum*) and soybeans (*Glycine max*).

B. bassiana (LPSC 1067) from the culture collection of the Spegazzini Institute, La Plata, Argentina was chosen based on its laboratory efficacy against Argentinian pest insects (Pelizza, Eliades, Saparrat, et al., 2012; Pelizza, Eliades, Scorsetti,

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Cabello, & Lange, 2012). It was identified using both molecular and morphological data. For molecular identification, PCR was used to amplify the Internal Transcribed Spacer (ITS) rDNA region (White, Bruns, Lee, & Taylor, 1990). The PCR and fragment purification and sequencing were performed according to Canel, Wagner, Stenglein, and Ludemann (2013). Similarities of the fragment with previously published sequence data were examined with BLASTn (Altschul, Gish, Miller, Myers, & Lipman, 1990) in GenBank (NCBI, <http://www.ncbi.nlm.nih.gov/BLAST/>). The sequence generated in this study was submitted to GenBank (accession number KF500409).

Conidia were obtained from cultures maintained on potato dextrose agar (PDA; Britania S.A., Buenos Aires, Argentina) for 10 days at 25°C in darkness. Conidia were harvested and placed in test tubes containing 0.01% (v/v) aqueous solution of Tween 80 (Merck®). Suspensions were adjusted to 1×10^8 conidia mL⁻¹ according to Gurulingappa, Sword, Murdoch, and Mc Gee (2010) using a Neubauer hemocytometer.

Seeds of the four plant species were surface sterilised according to Brownbridge, Reay, Nelson, and Glare (2012). For seed inoculation, seeds of each crop were immersed in 10 mL of a *B. bassiana* conidial suspension for 24 h. They were then dried on sterile tissue paper in a sterile laminar flow cabinet for 30 min, sown in 330 cm³ plastic pots at a depth of 4 cm, filled with a sterile mixture of perlite, vermiculite, soil, equally ground (1:1:1) and maintained in greenhouse at 25°C and a photoperiod of 12-12h L-D. Control seeds were immersed in a conidia-free solution of 0.01% Tween 80.

For leaf spray and root immersion, seeds were planted in pots (330 cm³) filled with a mixture of substrate performed as mentioned above. A glass hand sprayer (30 mL capacity) was used to spray each seedling with 3 mL conidial suspension (Posada, Aime, Peterson, Rehner, & Vega, 2007). Control plants were sprayed with a conidia-free solution of Tween 80 0.01%.

For root immersion, we used seedlings three weeks after emergence. Each seedling was removed from the pot and rinsed three times with sterile distilled water. The ends of the roots were cut for better absorption and placed individually in test tubes with 2 mL of a conidial suspension. The roots of control plants were submerged in 0.01% Tween 80 only. Treated plants and controls were left for 24 h at 25°C, and a photoperiod of 12 h and then replanted in the same pots.

Seedlings in all treatments were watered as needed. For each inoculation technique, 10 replicates were used, and the experiment was replicated on three different dates. Ten non-inoculated seedlings were used as control.

The final rinse water was plated on PDA media and incubated for 10 days at 25°C to determine whether the sterilisation process was successful in eliminating epiphytic micro-organisms. If we observed fungal growth, we did not consider the corresponding samples for analyses. The leaves were dried on sterile paper towels in a laminar flow cabinet and the edges were cut to remove dead tissue ensuing from the sterilisation process. Six pieces of leaves of each treated plant and six pieces of leaves of each control plant were used. The leaves pieces of approximately 1 cm² were placed on each Petri dish containing PDA with 0.1% stock antibiotics consisting of 0.02 g each of tetracycline, streptomycin and penicillin.

The presence or absence of *B. bassiana* growth was recorded after 10 days at 25°C. A total of 160 plants and 960 plant pieces were examined. The data are expressed

as colonisation frequencies, where colonisation frequency = (number of plant pieces colonised/total number of plant pieces) × 100 (Petrini & Fisher, 1986). The colonisation frequency data (expressed as percentages) were angular transformed to stabilise the variances. The transformed data were analysed using [analysis of variance (ANOVA)] performed with the software Version InfoStat, 2001.

B. bassiana was never recorded in the control plants. All the inoculation techniques were successful in establishing *B. bassiana* as an endophyte in the four plant species, but we observed differences in efficacy among the different inoculation techniques over time. The inoculation method significantly affected the colonisation of leaves in all species at 7, 14 and 21 days but not at 28 days (Table 1).

For tobacco seedlings, the technique that resulted in the highest colonisation was the leaf spray, with 100% colonisation of leaves seven days post-inoculation, decreasing to 2.2% at 28 days post-inoculation (Figure 1a). For seed inoculation and root immersion techniques, seven days post-inoculation the percentage of colonisation obtained was 15 and 5%, respectively. At 21 and 28 days post-inoculation, no sign of the fungus was observed.

For wheat seedlings, the highest colonisation was obtained with the leaf spray, with values ranging from 40% at 14 days to 1.7% at 28 days (Figure 1b). For root immersion and seed inoculation, highest colonisation (8.3% and 3.3%, respectively) was obtained at seven days.

For corn seedlings, the highest colonisation was obtained with the leaf spray technique at 7 and 14 days, with 7.8% and 7.2% colonisation, respectively. The highest colonisation at 21 and 28 days was obtained with seed inoculation and root immersion, with 2.8% and 2.2%, respectively (Figure 1c).

For soybean seedlings, the highest colonization was obtained from leaf sprays, with 24% colonization at 7 days. At 14 days post-inoculation, the highest colonization was obtained with root immersion, with 10.5% and for seed inoculation the highest colonization was obtained at 7 days with 14% (Figure 1d).

Statistical analysis showed that at 7 and 14 days post-inoculation, the plant species factor and the inoculation techniques factor, as well as the interaction between them (species × techniques), were highly significant ($p < 0.0001$; Table 1). However, at 21 days post-inoculation, the species factor was not significant ($p > 0.0001$), but the technique factors and the interaction were highly significant ($p < 0.0001$; Table 1). At 28 days post-inoculation, the technique factor, species factor and the interaction (species × techniques) were not significant ($p > 0.0001$; Table 1).

Table 1. Results of ANOVA for species factor, techniques factor and the interaction between both factors (species × techniques).

	7 days			14 days			21 days			28 days		
	df	F	p	df	F	p	df	F	p	df	F	p
Species	3	52,51	<0.0001	3	30,91	<0.0001	3	2,15	0.0937	3	5,52	0.0010
Techniques	2	107,46	<0.0001	2	97,29	<0.0001	2	20,82	<0.0001	2	1,53	0.2186
Species × techniques	6	39,82	<0.0001	6	38,11	<0.0001	6	6,31	<0.0001	6	0,38	0.8899

Significant at the $p < 0.001$ probability level.

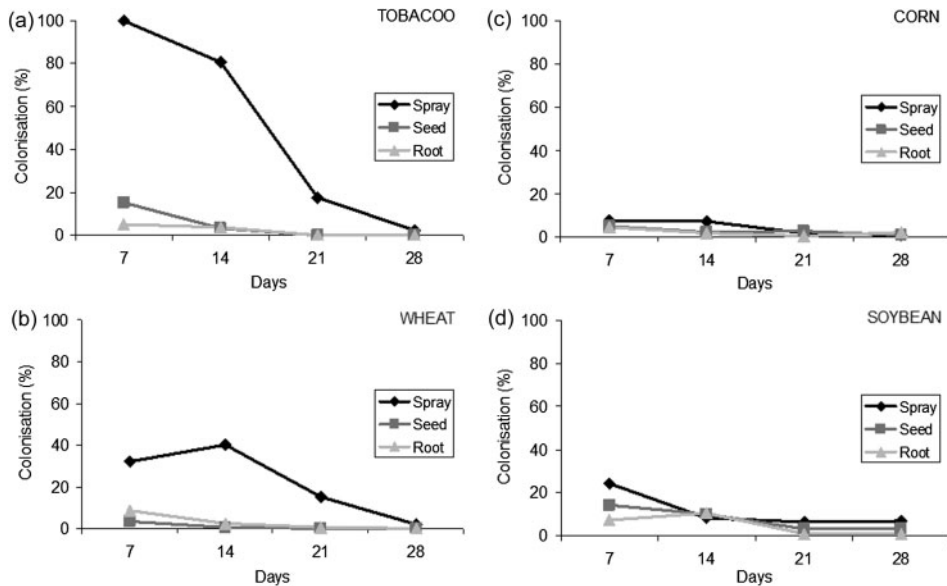


Figure 1. Recovery percentage of *B. bassiana* in leaves by different inoculation techniques (seed inoculation, leaf spray and root immersion), at 7, 14, 21 and 28 days. (a) tobacco, (b) wheat, (c) corn and (d) soybean.

We observed that *B. bassiana* strain LPSC 1067 can be successfully inoculated as an endophyte in tobacco, corn, wheat and soybean seedlings, by means of leaf spray, seed inoculation and root immersion. Previous studies have shown the ability of *B. bassiana* to become established as an endophyte in corn (Cherry et al., 2004), opium (*Papaver somniferum*; Quesada-Moraga et al., 2006), cocoa (*Theobroma cacao* L.; Posada & Vega, 2005), pine (Brownbridge et al., 2012), tomato (*Lycopersicon esculentum* Mill.; Ownley et al., 2004), banana (*Musa paradisiaca* L.; Akello et al., 2007), coffee (*Coffea arabica* L.; Posada & Vega, 2006; Posada et al., 2007) and sorghum (*Sorghum bicolor* Kuntze; Reddy, Ali Khan, Devi, Sharma, & Reineke, 2009; Tefera & Vidal, 2009) following artificial inoculation.

Endophytic colonisation by *B. bassiana*, however, depends on the inoculation method, fungal isolate and plant species. In coffee, the highest post-inoculation recovery of *B. bassiana* occurred by direct injection of plants (Posada et al., 2007); in banana tissue culture, by dipping plants in conidial suspension (Akello et al., 2007); in opium poppy (Quesada-Moraga et al., 2006) and corn (Cherry et al., 2004) by foliar application; and in tomato by seed coating (Ownley et al., 2008).

Our results agree with Lewis, Bruck, Gunnarson, and Bidne (2001) and Tefera and Vidal (2009), who did not observe negative effects on corn and sorghum plants when inoculated with *B. bassiana*. The ability to recover *B. bassiana* in the four treated plant species decreased with time. A possible explanation for the low recovery we recorded 28 days post-inoculation could be a result of competition from other fungi and bacteria in the system, leading to inhibition of *B. bassiana* growth. It is also possible that the surface sterilisation method used on small sections of plant material may have inhibited outgrowth (Brownbridge et al., 2012), or could be

caused by the host response to the fungus (Posada et al., 2007). This study provides the basis for further investigations, which should focus on the virulence of the endophytic *B. bassiana* against mainly insect pests of tobacco, corn, wheat and soybean plants.

Acknowledgements

We thank Dr Fernando Vega for critical reading of the manuscript.

Funding

This study was partially supported by Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET- PIP0009), Comisión de Investigaciones Científicas de la provincia de Buenos Aires (CICPBA), Universidad Nacional de La Plata (UNLP, 11/N 651) and Rizobacter Argentina S.A.

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