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Impact of plant growth-promoting rhizobacteria on root colonization potential and life cycle of *Rhizophagus irregularis* following co-entrapment into alginate beads

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Running head: CO-ENTRAPMENT OF PGPR AND AMF

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Abstract

Aims: This study aimed at evaluating the impact of seven plant growth-promoting rhizobacteria (PGPR) on root colonization and life cycle of *Rhizophagus irregularis* MUCL 41833 when co-entrapped in alginate beads.

Methods and results: Two *in vitro* experiments were conducted. The first consisted in the immobilization of *R. irregularis* and seven PGPR isolates into alginate beads to assess the effect of the bacteria on the pre-symbiotic growth of the fungus. In the second experiment, the best-performing PGPR from experiment 1 was tested on its ability to promote the symbiotic development of the AMF in potato plantlets from three cultivars. Results showed that only one isolate identified as *Pseudomonas plecoglossicida* (R-67094) promoted germ tube elongation and hyphal branching of germinated spores during pre-symbiotic phase of the fungus. This PGPR further promoted the symbiotic development of the AMF in potato plants.

Conclusions: The co-entrapment of *P. plecoglossicida* R-67094 and *R. irregularis* MUCL 41833 in alginate beads improved root colonization by the AMF and its further life cycle under the experimental conditions.

Significance and impact of the study: Co-entrapement of suitable AMF-PGPR combinations within alginate beads may represent an innovative technology that can be fine-tuned for the development of efficient consortia-based bioformulations.

Keywords: AMF, co-entrapment, alginate beads, formulation, interaction, microbial inoculant, PGPR, potato, *Pseudomonas*, *Rhizophagus irregularis*.

Introduction

Fertilizers and plant protection products are two major inputs for increasing agricultural production, but their excessive use can have negative environmental and financial consequences (Wilson and Tisdell 2001). Most crops including potato (Maynard and Hochmuth 2007) are relatively sensitive in

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terms of yield under limited nutrient supply. One alternative to decrease the use of chemical inputs in agriculture is to harness soil microorganisms. Soils are natural reservoirs of beneficial microorganisms, which have a direct influence on soil fertility (Kennedy and Smith 1995). Certain microorganisms can improve plant growth via direct or indirect mechanisms (Velivelli *et al.* 2014a, 2014b; Kumar 2016). Among them, arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) have been extensively studied in the last decades. Promising results have been obtained with the co-inoculation of both organisms either under greenhouse (Budi *et al.* 1999; Gamalero *et al.* 2004) or field conditions (Adesemoye *et al*. 2008) suggesting the potential added-value of consortia versus single formulations (Berg 2009, Bhardwaj *et al.* 2014).

In the last decades, the market for microbial inoculants has noticeably increased (e.g. Berg 2009, Calvo *et al.* 2014). AMF and PGPR have been formulated using different techniques and applied in the field by spraying, coating seeds and/or entrapped within beads. In particular, entrapment technology has attracted much attention for its advantages over free cell formulations (Cassidy *et al.* 1996). Alginate beads can protect microbes from biotic and abiotic stresses and improve persistence and physiological activity as well as cell densities (Schoebitz *et al.* 2013). Entrapment also allows an easy delivery of the microbial inoculants at the site where they are needed and can be used in seed drills commonly used by farmers (De Jaeger *et al.* 2011). Entrapment in alginate has been reported in numerous studies with single isolated microorganisms (e.g. AMF – Declerck *et al.* 1996 or PGPR – Bashan *et al.* 2002), whilst much fewer studies reported on consortia. For instance, De Jaeger *et al.* (2011) and more recently Buysens *et al.* (2016) co-entrapped the AMF *R. irregularis* MUCL 41833 with the fungus *Trichoderma harzianum* MUCL 29707 into alginate beads for field trials, which resulted into increased potato yield (Buysens *et al.* 2016). Co-immobilization constitutes an unexploited biotechnology for microbial formulation (Vassilev *et al.* 2105)

In vitro cultivation represents a powerful tool to assess microbe-microbe interactions in a non-destructive way (De Jaeger *et al.* 2010). Although, even if it is naïve to extrapolate the obtained results to field conditions, data obtained *in vitro* could help us to unveil through direct observations the effects of PGPR on AMF within formulated alginate beads. In a previous experiment, Declerck *et*

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al. (1996) demonstrated that entrapment in alginate beads did not decrease the colonization capacity (evaluated as percentage of Potentially Infective Beads - % PIB) of *in vitro* produced spores of *Glomus versiforme*. One germinated spore per bead was sufficient to consider the bead as potentially infective. More recently, De Jaeger *et al.* (2011) succeeded in the co-entrapment of *Rhizophagus irregularis* MUCL 41833 with *Trichoderma harzianum*. They demonstrated that the %PIB of the AMF was not affected by the presence of *T. harzianum*. However, to our knowledge, the effects of PGPR on AMF co-entrapped in alginate beads have never been explored so far. Early reports suggested that some rhizobacterial species could stimulate arbuscular mycorrhizal symbiosis (e.g. Toro *et al.* 1997; Vosátka and Gryndler 2000; Gamalero *et al.* 2004, Pivato *et al.* 2009). Production of cost-effective microbial bioformulations will require the development of innovative technologies to improve their efficiency and applicability (Bashan 2014; Vassilev et al. 2015).

The aim of the present study was to evaluate the effect of seven PGPR isolates from potato plantations in Ecuador on the life cycle of *Rhizophagus irregularis* MUCL 41833 and on potato root colonization following co-entrapment into alginate beads. We selected potato as model plant because (1) it is the third most important food crop in the world after rice and wheat in terms of total production and human consumption (FAO 2015), (2) it is grown in various areas of EU and is central to the Andes, (3) it is usually cultivated under high-input systems to get acceptable yields (4) the PGPR tested were originally isolated from potato plantations in Ecuador. We hypothesized that certain PGPRs can promote the development of the AMF after co-encapsulation in alginate beads.

2. Materials and Methods

Biological material

Plant growth-promoting rhizobacteria (PGPR)

Four PGPR isolates namely *Serratia sp.* (R-67091), *Pseudomonas plecoglossicida* (R-67094), *Pseudomonas granadensis* (R-67095) and *Pseudomonas extremaustralis* (R-49457) were isolated in the frame of the VALORAM (Valorizing Andean microbial diversity through sustainable

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intensification of potato-based farming systems) project; they are preliminary identified on the basis of 16S rRNA sequence analysis and by an in house MLSA data base. Three isolates: *Bacillus amyloliquefaciens* (LMG 24415) and *Bacillus subtilis* (LMG 24418 and LMG 24423) were obtained from the BCCM/LMG bacterial collection (http://bccm.belspo.be/about-us/bccm-lmg, Ghent, Belgium). The cultures were supplied in Petri plates (90 mm diam.) containing 40 g L^{-1} Tryptic Soy Agar (TSA) medium. All the isolates were obtained from the roots and rhizosphere of potato plantations in Ecuador and selected for their capacity to produce indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC deaminase) and to solubilize phosphorus and for their antagonistic activity against *Rhizoctonia solani* and *Phytophthora infestans* on plate essays. Table S1.

Arbuscular mycorrhizal fungi

A root organ culture (ROC) of *Rhizophagus irregularis* (Błaszk, Wubet, Renker and Buscot) C. Walker and A. Schüßler as ['irregulare'] MUCL 41833 was supplied by the Glomeromycota *in vitro* collection (GINCO – www.mycorrhiza.be/ginco-bel. The strain was grown in association with Ri T-DNA transformed carrot (*Daucus carota* L.) roots clone DC2 on Petri plates (90 mm diam.) containing the Modified Strullu Romand (MSR) medium (Declerck *et al.* 1998) solidified with 3 g L^{-1} Phytagel (Sigma-Aldrich, St. Louis, USA), following the method detailed in Cranenbrouck *et al.* (2005). The Petri plates were incubated in the dark in an inverted position for several months until thousands of spores were obtained.

Potato cultivars

In vitro produced potato plantlets (*Solanum tuberosum* L., cv. Bintje, c.v. Unica and cv. I-Fripapa) were provided by the "Station de Haute Belgique" in Libramont (Belgium) for the first cultivar and by the School of Biological Earth and Environmental Sciences of University College Cork (Ireland) for the two others. Nodal cuttings were sub-cultured on 4.412 g L^{-1} Murashige and Skoog (MS) medium (Duchefa, Biochemie. Haarlem, Netherlands) supplemented with 20 g L^{-1} sucrose, 3 g L^{-1} Phytagel and adjusted to pH 5.9 before sterilization (121°C for 15 min). Plantlets were kept in a growth chamber (21/18 °C day/night, relative humidity (RH) of 70%, photoperiod of 16 h d^{-1} and

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photosynthetic photon flux of 225 μmol m^2 s^{-1}) until use.

Co-entrapment of PGPR and AMF in alginate beads

The seven PGPRs were cultured at 28 °C in 250 ml flasks containing 50 ml Tryptic Soy Broth (TSB) at 30 g L^{-1}, and maintained under agitation at 150 rev. min^{-1} during 48 hours. The bacterial concentration of each PGPR was estimated following the method detailed in Reynolds (2011). Briefly, a standard curve was drawn for each isolate by plotting the number of CFU ml^{-1} obtained by the standard plate count method against the absorbance at 600 nm of several dilutions with a known amount of CFU ml^{-1}. Concentration of each culture was then adjusted to $3x10^8$ CFU ml^{-1} in a final volume of 30 ml of sterile TSB and centrifuged (14,000 *x g*, 15 min at 4°C) to recover the bacterial cells. The bacterial pellets were re-suspended into a Petri plate (90 mm diam.) containing 30 ml of sterilized (15 min, 121°C) sodium alginate (Sigma-Aldrich, St. Louis, USA) at 20 g L^{-1} (Declerck *et al.* 1996).

In parallel, a plug of gel from the ROC of *R. irregularis* MUCL 41833 containing thousands of spores was dissolved in 0.01 mol^{-1} citrate buffer (Doner and Bécard 1991). An approximate of 2000 healthy-looking spores was transferred to a 90 mm diam. Petri plate containing sterilized (121°C for 15 min) deionized water. Clusters of spores were separated with needles under a stereomicroscope (Olympus SZ61) at 45 X magnification to obtain individual spores. Hundreds of spores were finally transferred to the Petri plates containing the PGPR isolates. All combinations of the seven PGPRs and *R. irregularis* MUCL 41833 and a control (without PGPR) were entrapped in alginate beads via polymerization with calcium chloride. In experiment 1 below, each ~10 μL volume bead contained one single spore of *R. irregularis* MUCL 41833 and ~ $3x10^5$ CFU of the PGPR, while in experiment 2 below, each ~50 μL volume bead contained 25 ± 5 spores and ~ $3x10^5$ CFU of the PGPR. The number of AMF spores per bead in experiment 2 was quantified under a stereomicroscope (Olympus SZ61) at 45X magnification in ten ~50 μL droplets of the sodium alginate solution and the results averaged. The concentration of spores was adjusted to 25 ± 5 spores/bead by adding more spores or alginate to the solution.

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For the entrapment in alginate beads sodium alginate drops containing the AMF and the PGPR at the concentration detailed above were taken with a micropipette and dropped into a sterilized (121°C for 15 min) solution of 0.1 mol^{-1} $CaCl_2$ maintained under constant agitation during 30 minutes for polymerization. The alginate beads were subsequently sieved on a 1 mm sterile sieve and rinsed with sterilized (121 °C for 15 min) deionized water.

Experiment 1: Evaluation of the percentage of potentially infective beads following co-entrapment of R. irregularis MUCL 41833 with the seven PGPRs.

Each of the seven PGPRs were tested as follows: five beads each containing 1 single spore of AMF and $3x10^5$ CFU of the PGPR were plated into single Petri plates (90 mm diam.) containing 30 ml of warm (approx. 50°C) liquid MSR medium prior to solidification. The beads were thus embedded into the gel. Twenty Petri plates (each containing 5 beads were prepared and incubated in the dark at 27°C for 30 days. Each Petri plate was considered as a replicate.

The percentage of potentially infective beads (%PIB) originally developed by Declerck *et al.* (1996) to evaluate the percentage of beads containing at least one germinated AMF spore was adapted in the current study following De Jaeger *et al.* (2011) by evaluating the percentage of beads in which the hyphae from the germinated spore crossed the calcium alginate coating and developed into the medium. Data were plotted against time.

Hyphae length was further determined at day 30 on 20 potential infective beads (i.e. showing hyphal re-growth outside the alginate bead) randomly selected per treatment. Measurement was done from the edge of the bead to the hyphal tip under a stereomicroscope (Olympus SZ61 at 42X magnification) with an eyepiece reticle calibrated with a stage micrometer. The second and third order hyphae were counted and their length similarly measured as above. We selected this method instead of the measurement of the total length (i.e. from the spore subtending hyphae to the hyphal tip) because hyphal development (i.e. length and branching) outside the bead is a determinant factor for root colonization.

Experiment 2: Impact of the best PGPR from Experiment 1 on R. irregularis MUCL 41833

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extraradical mycelium development, spore production and root colonization of potato plantlets

The best performing PGPR promoting %PIB and hyphal elongation and branching of *R. irregularis* MUCL 41833 in Experiment 1 was selected to evaluate its effects on the extraradical mycelium (ERM) length, spore production and percentage of root length colonization (%RLC) following association with three potato cultivars.

Three beads, each containing 25 ± 5 spores of the AMF and $3x10^5$ CFU of the PGPR were plated in Petri plates (90 mm diam.) in 30 ml of warm (50 °C) MSR medium lacking sucrose and vitamins. Before solidification of the MSR medium, the alginate beads were sorted evenly in the Petri plates. Petri plates were incubated during seven days in the dark at 27 °C. Afterwards, Half-closed Arbuscular Mycorrhizal (H-AMP) systems were set up with the three potato cultivars as described in Voets *et al.* (2005). Briefly, a hole (± 2 mm diam.) was made in the side and the lid of the Petri plates. A ten-day-old rooted nodal explant of potato was inserted in the hole with the roots plated on the medium and the shoot extending outside the Petri plate via the hole. The roots developed in the vicinity of the alginate beads. Petri plates were sealed with parafilm and the holes plastered with silicon grease. H-AMP systems were refilled weekly with sterilized MSR medium lacking sucrose and vitamins and kept during nine weeks in a growth chamber under the same conditions as above. Six treatments were thus considered with ten replicates (i.e. H-AMP systems): beads containing *R. irregularis* MUCL 41833 alone or in combination with *Pseudomonas plecoglossicida* R-67094 inoculated to three potato cultivars (i.e. Bintje, Fripapa, and Unica): $Bin^{+R67094}$, $Bin^{-R67094}$, $Fri^{+R67094}$, $Fri^{-R67094}$, $Uni^{+R67094}$, $Uni^{-R67094}$.

ERM development and dynamics of spore production

The ERM development was estimated at week 4 and 9 as described in Declerck *et al.* (2003). Briefly, a 10 mm grid of lines was marked on the bottom of the Petri plates. The total number of hyphae/gridline intersects was evaluated under a stereomicroscope (Olympus SZ61 at 45 X magnification) and used to estimate the total length of ERM with the formula: $R = \pi NA/2H$, where N is the number of intersections, A the area in which the hyphae lies, H the total length of the straight

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lines, and *R* the total length of hyphae in the Petri plate.

The number of newly produced spores per H-AMP system was further evaluated at week 5, 6, 7, 8, 9 according to Declerck *et al.* (2001). The number of spores was counted in three 1 cm^2 cells of the grid above. The cells were distributed along a transect from upper to mid and lower part of the Petri plates. The same cells in the grid were always followed for each Petri plate. The number of spores was subsequently extrapolated to the entire Petri plate.

Potato root colonization

The potato plantlets were harvested after nine weeks. The roots were stained according to Urgiles *et al.* (2009) with slight modifications. Briefly, the roots were cut into 1 cm length segments, cleared with 10% KOH at 60°C for 30 minutes, rinsed three times with tap water, acidified in 10% HCl for 1 minute and stained with 0.05% methyl blue in 90% lactic acid for 30 minutes at 60°C. Root pieces were then distained in 50% lactic acid overnight to get rid of the excess of methyl blue. Thirty randomly selected root pieces (1 cm length) from each replicate were finally mounted on microscope slides (ten per slide) and analysed for AMF colonization under a bright-field light microscope (Leitz Wetzlar, Germany) at 100X and 400X magnifications. The frequency of colonization (%F) was determined by the formula: %F = (*N* - *no*/ N) x100, where N is the number of observed fragments and n0 is the number of fragments without traces of colonization. The root length colonization (%RLC) also known as intensity of colonization was estimated using colonization classes where: 0 = 0%, 1 ≤ 1%, 2 = 2-10%, 3 = 11-50%, 4 = 51-90, 5 > 90% assigned to each root fragment depending on the percentage of the root length colonized by AMF (Trouvelot *et al.* 1986). The final RLC% was calculated as an average of 30 root pieces using the formula: $\%RLC = (95n^5+70n^4+30n^3+5n^2+n)/N$; where n^5, n^4, n^3, n^2, n is respectively the number of fragments scaled as 5, 4, 3, 2, 1 and N is the total number of fragments observed.

Statistical analyses

All variables were tested for homogeneity of variances and normality using the tests after Levene and Kolmogorov-Smirnov, respectively. The results of Experiment 1 were submitted to one-way

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ANOVA. The data of percentages were first arcsin square root transformed to fulfil the requirements for a parametric analysis. The results of experiment 2 were analysed via a two-way ANOVA to test the effects of cultivars and the presence of the bacteria. Simple pair comparisons were carried out with T test (P<0.05). The data were analysed using the software SPSS Statistics 20.

Results

Experiment 1: Evaluation of the percentage of potentially infective beads following co-entrapment of R. irregularis MUCL 41833 with the seven PGPR

The seven PGPR developed within the beads and in the surrounding MSR medium within 48h of incubation. The first germinated spores of *R. irregularis* were observed within the beads after 3 days of incubation in the control (i.e. in absence of bacteria) treatment as well as and in presence of most bacteria. However, in the treatments containing the bacterial isolate *Pseudomonas plecoglossicida* R-67094 and *Pseudomonas granadensis* R-67095 it was not possible to visualize the spores and thus to ascertain spore germination, due to a dense bacterial growth inside the beads. The first hyphal regrowth outside the alginate coating were observed after 4 - 5 days of incubation. The %PIB was evaluated every 5 days starting day 5 until day 30 and plotted against time. The %PIB varied with the bacterial isolate and time of observation (Table 1). In the presence of the isolate *Serratia* sp. R-67091, *P. granadensis* R-67095, *P. extremaustralis* R-49547 and *Bacillus subtilis* LMG 24418, the %PIB was null at any time of evaluation. These isolates were subsequently excluded from the statistical analysis and only the isolates *P. plecoglossicida* R-67094, *B. amyloliquefaciens* LMG 24415 and *B. subtilis* LMG 24423 and the control were considered (Table 1). No significant differences in %PIB were noticed between the treatments at day 5, 10 and 15 (Table 1). Similarly, at day 20, 25 and 30, no significant differences were noticed in %PIB between the *P. plecoglossicida* R-67094 treatment and the control treatment. Conversely, the %PIB of both these treatments was significantly higher as compared to the treatments with *B. amyloliquefaciens* LMG 24415 and *B. subtilis* LMG 24423 at days 25 and 30 and at day 20 for *P. plecoglossicida* R-67094. No significant difference in %PIB was observed between the treatments *B. amyloliquefaciens* LMG 24415 and *B. subtilis* LMG 24423 at any

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time of observation. Both treatments significantly inhibited the %PIB as compared to the control at day 25 and 30 (Table 1).

The length of germ tube, 2nd and 3rd order hyphae as well as total hyphal length of the AMF significantly differed between the treatments (Table 2). Whatever the parameter, length was significantly increased in the treatment with *P. plecoglossicida* R-67094 as compared to the three other treatments. Similarly, the length of germ tube of the control treatment was significantly increased as compared to the treatments with *B. amyloliquefaciens* LMG 24415 and *B. subtilis* LMG 24423, while no differences between these three treatments was noticed for length of 2nd and 3rd order hyphae and total length of hyphae (Table 2).

The number of 2nd, 3rd and total number of hyphal branches was significantly increased for the treatment with *P. plecoglossicida* R-67094 as compared to the control, *B. amyloliquefaciens* LMG 24415 and *B. subtilis* LMG 24423 treatments. No significant differences were noticed between these three later treatments for any of the parameters (Table 2).

Experiment 2: *Impact of the best PGPR from Experiment 1 on R. irregularis MUCL 41833 ERM development, spore production and root colonization of potato plantlets*

The first hyphae of *R. irregularis* MUCL 41833 growing outside the beads were observed after five days of incubation in presence as well as in absence of *P. plecoglossicida* R-67094 (Figure S1). The beads were incubated during seven days before association to potato plantlets. At that time, 100% of the Petri plates contained at least one potentially infective bead. After two weeks of association, the first contacts between hyphae and roots were observed with the three potato cultivars regardless of the presence of *P. plecoglossicida* R-67094.

At week 4, a significant effect of "*P. plecoglossicida* R-67094" was noticed on the ERM length of the AMF, while no effect of the "potato cultivar" or the interaction (R-67094 *x* potato cultivar) was observed (Table 3). The ERM length in the treatments Bin$^{+R67094}$ and Fripapa$^{+R67094}$ were significantly shorter as compared with their respective controls, while no significant difference was noticed

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between Uni$^{+R67094}$ and Uni$^{-R67094}$ (Figure 1). At week 9 a significant effect of "potato cultivar" as well as "*P. plecoglossicida* R-67094" was noticed on ERM length, while no effect for their interaction (Table 3). Contrarily to week 4, the ERM length of the AMF in the treatment Bin$^{+R67094}$ was significantly larger (P=0.003) as compared to its respective control Bin$^{-R67094}$. The relative increase in ERM due to *P. plecoglossicida* R-67094 was 54,6%. No significant differences were observed between the treatments Fri$^{+R67094}$ and Fri$^{-R67094}$ (P=0.168) or Uni$^{+R67094}$ and Uni$^{-R67094}$ (P=0.354) (Figure 1). The ERM length at week 9 also differed significantly among cultivars. Pairwise comparisons showed significant differences between cultivars Bintje and Unica (P=0.013) and Fripapa and Unica (P=0.001) but not Bintje and Fripapa (P=0.346) regardless of the presence of *P. plecoglossicida* (data not shown).

The first spores of *R. irregularis* MUCL 41833 were observed four weeks following association with the potato plants and increased gradually from week 5 onwards, whatever the cultivar and presence/absence of *P. plecoglossicida* R-67094 (Figure 2).

The effect of the factor "potato cultivar" on the number of spores was significant at weeks 6 and 8 (Table 4), while the effect of the factor "*P. plecoglossicida* R-67094" was significant at weeks 7 and 9. No effects of the interaction potato cultivar *x P. plecoglossicida* R-67094 was observed at any time of spore enumeration (Table 4). Regardless of the potato cultivar, the number of spores of the AMF in presence of *P. plecoglossicida* R-67094 was superior to the control, starting at week 6 (Figure 2). Pairwise comparisons within the cultivar Bintje showed a significantly higher number of spores of the AMF in the treatment with *P. plecoglossicida* R-67094 at weeks 7 (P=0.025), 8 (P=0.016) and 9 (P=0.005), with relative increments of 47, 53 and 66%, respectively as compared to its respective control. Within cultivar Fripapa, a significant increase at week 9 (P=0.022) in presence of *P. plecoglossicida* R-67094 with a relative increment of 44% was noticed, while no significant increase was noticed with cultivar Unica at any time (Figure 2). Regardless of the presence of *P. plecoglossicida* R-67094, the production of spores also differed significantly among cultivars. Pairwise comparisons at week 9 showed that *R. irregularis* produced significantly (P=0.010) more spores (44% higher) when associated to cultivar Fripapa, compared to the cultivar Bintje. Similarly,

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the cultivar Unica produced significantly (P=0.047) more spores compared with the cultivar Bintje (50% higher), but not differences between cultivars Unica and Fripapa were detected (P=0.663), data not shown.

At the end of the experiment (week 9) microscopic observations of the roots revealed the presence of intraradical hyphae, arbuscules and vesicles/spores in the three cultivars either in the presence as well as absence of *P. plecoglossicida* R-67094. A significant effect of the factors "potato cultivar" and "*P. plecoglossicida* R-67094" was observed on the %F and %RLC, while no effect of their interaction (Table 4). Pairwise comparisons within cultivars showed that in presence of *P. plecoglossicida* R-67094, the %F was higher independently of the potato cultivar but differences were only significant for cultivars Fripapa (P=0.025) and Unica (P=0.001) (Figure 3). Similarly, the %RLC was significantly affected by the factors "potato cultivar" and "*P. plecoglossicida* R-67094", while not for their interaction (Table 4). Pairwise comparisons within the same cultivars showed that the presence of *P. plecoglossicida* R-67094 significantly increased the %RLC in the cultivars Bintje (P=0.029), Fripapa (P<0.0001) and Unica (P=0.001) compared to the respective controls (Figure 3).

Discussion

The growing need for environmental-friendly and cost-effective agricultural practices is supporting the use of a broad range of beneficial microorganisms comprising bacteria and fungi. Among these are the AMF. However, even though the inoculation of plants with these fungi is a well-known practice, their formulation with a reliable and consistent effect under field conditions is still a problem for their wider exploitation (Rodriguez and Sanders 2015). Within soil, numerous plant growth-promoting microrganisms work synergistically to improve plant growth (Barea *et al.* 2002, 2005; Saxena *et al.* 2006; Adesemoye *et al.* 2008). Considering that associations between bacteria and AMF have been reported to promote mycorrhizal symbiosis (Gamalero *et al.* 2004; Artursson *et al.* 2006; Pivato *et al.* 2009; Ramasamy *et al.* 2011), formulations that include adequate combinations of bacteria and AMF may open novel opportunities for microbial formulation (Adesemoye *et al.* 2008, Adesemoye and Kloepper 2009; Malusá *et al.* 2012).

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In the present study, all the seven bacteria entrapped in beads, were able to develop inside as well as to proliferate outside the beads. A similar observation was made for the AMF in the absence of bacteria. The fungus was able to germinate and grow outside the bead as earlier reported by Declerck *et al.* (1996) with *Glomus versiforme* and by De Jaeger *et al*. (2011) with *R. irregularis* MUCL 41833.

In presence of bacteria the AMF behaved differently. Indeed, six out of the seven PGPR tested in our study strongly decreased or completely inhibited the %PIB and thus impacted the AMF. This was not totally surprising, since members of several species of *Bacillus* (Swain *et al.* 2009; Govindasamy *et al.* 2010) and *Pseudomonas* (de Bruijn *et al.* 2007; Weller 2007; Raaijmakers *et al.* 2010; Beneduzi *et al.* 2012) have been reported to produce compounds able to suppress the growth of a wide range of bacteria, fungi oomycetes, protozoa, nematodes and plants. These compounds include broad-spectrum antibiotics, lactic acid, lytic agents and numerous types of exotoxins as well as bacteriocins (Riley and Wertz 2002). All the PGPRs used in the present study showed antagonistic effects against *R. solani* and *P. infestans* (Table S1). Therefore, it cannot be excluded that these isolates also produced compounds with inhibitory effects on spore germination and hyphal elongation of *R. irregularis* MUCL 41833 and hence decreased substantially the %PIB. Interestingly, a number of studies also reported that some PGPR can stimulate mycorrhizal symbiosis (Budi *et al.* 1999, Fernandez *et al.* 2011). This was obviously the case for *P. plecoglossicida* R-67094, which was the only isolate that favoured the development of *R. irregularis.* Curiously this isolate also showed antagonistic effects against *R. solani* (38.6%) and *P. infestans* (25.3%) in dual culture plates (Table S1). It is possible that *P. plecoglossicida* R-67094 produces antifungal compounds but at doses not affecting AMF as well as stimulatory compounds active on the fungal symbiont. Even if the %PIB was not significantly increased in presence of this bacterium as compared to the control, a significant stimulatory effect was noticed on the pre-symbiotic hyphal growth and branching of the AMF suggesting the release of some stimulatory compounds such as exudates or volatiles by the bacteria. Fernández *et al.* (2011) also reported a promoting effect of two IAA producing *Paenibacillus* strains on the pre-symbiotic development of germinated spores of *Glomus intraradices* but a little impact on germination.

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However, earlier studies showed the stimulatory effect of the volatiles geosmin, 2-methyl isoborneol and CO_2 produced by *Streptomyces*, *Streptosporangium*, and *Nocardia* (Actinobacteria) on the germination of spore of *Gigaspora margarita* (Carpenter-Boggs *et al.* 1995). More recently, *Streptomyces* sp., *S. griseus* and *S. albogriseus* were reported to increase germination of *G. margarita* spores probably via the release of gaseous compounds although the presence of diffusible exudates could not be excluded (Aliasgharzad *et al.* 2012). Although a number of studies have reported on the effects of different bacteria on germination and root colonization of AMF, the specific compounds and the mechanisms involved remain almost unknown. The stimulatory effects of *P. plecoglossicida* R-67094 on hyphal elongation and branching is likely to increase the chances of contact between hyphae and roots of a suitable host.

Interestingly, *P. plecoglossicida* R-67094 also increased the production of a profuse extraradical mycelium bearing numerous spores and improved root colonization of the AMF when associated to potato plants. Similar results were obtained with IAA-producing *Paenibacillus* strains (*P. rhizosphaerae* and *P. favisporus*); but only *P. rhizosphaerae* strain improved the symbiotic development of the AMF *G. intraradices*. However, the relation between IAA production and AMF development could not be proven with certainty (Fernández *et al.* 2011). Our results are in agreement with the findings of Barea *et al.* (1998), who tested the effect of three fungal antagonistic *Pseudomonas* strains (F113, F113G22 and F113 (pCU203)) on the arbuscular mycorrhiza formation. They found a positive effect of the first two strains on spore germination, mycelial growth and root colonization of *Funneliformis mosseae* in tomato plants. Similarly, Pivato *et al.* (2009) found a strong stimulatory effect of *Pseudomonas fluorescens* C7R12. This bacterial strain promoted the pre-symbiotic and symbiotic development of *F. mosseae* but not of *G. rosea* in *M. truncatula and L. esculentum* plants indicating a specific effect.

The above-mentioned results are in favor of the hypothesis that *P. plecoglossicida* R-67094 functions as mycorrhiza helper bacteria (MHB). The term MHB has been defined as the group of bacteria able to "*promote the establishment of symbiosis by stimulating mycelial extension; increasing root–fungus contacts and colonization; and reducing the impact of adverse environmental conditions on the*

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mycelium of the mycorrhizal fungi" (Frey-Klett *et al.* 2007). MHB have evolved selective mechanisms of interaction with the surrounding microbial communities such as favoring mycorrhizal association but competing with root pathogens that might threaten their habitat (Frey-Klett *et al.* 2007). Mechanisms for AMF spore germination seem to include the production of volatiles and CO_2 (Carpenter-Boggs *et al.* 1995). Another commonly observed characteristic of MHB is the stimulation of lateral root formation (Frey-Klett *et al.* 2007). This effect was also reported with IAA or other signal-producing PGPR impacting root architecture (Vacheron *et al.* 2014). The changes on root morphology were not evaluated in the present study. However, it is likely that IAA producing *P. plecoglossicida* R-67094 stimulated lateral root formation and subsequent increase the number of entry points at which plant and fungus interacted.

Independently of the presence of *P. plecoglossicida* R-67094, spore production, ERM development and root colonization varied markedly among the potato cultivars suggesting a clear role of the plant genotype on AMF symbiotic development. Voets *et al.* (2005) reported similar observations with five potato cultivars associated with *R. irregularis* MUCL 43194 under *in vitro* culture conditions. They noticed that spore production varied markedly among the cultivars. Similarly, inoculation of five durum wheat cultivars with *R. irregularis* DAOM 197198 in a greenhouse experiment under low and medium soil fertility resulted in different levels of root colonization, as well as different plant growth benefits among cultivars. The authors concluded that differences in mycorrhiza formation and function among cultivars depend on their degree of compatibility with the AMF (Singh *et al*. 2012). Although we did not evaluate plant growth in our experiment, numerous studies have reported that plant species (e.g. Klironomos 2003; Tawaraya 2003) as well as cultivars within a same species (e.g. Declerck *et al.* 1995; Vosátka and Gryndler 2000; Linderman and Davies 2003; Estaún *et al.* 2010) can respond differently to inoculation with AMF. Mycorrhizal dependency has been closely related with root characteristics such as fibrousness (in different primitive and modern wheat cultivars (Hetrick *et al*. 1992)) and the volume of root system, length and density of root hairs (in banana cultivars (Declerck *et al*. 1995). In order to evaluate the impact of differential of AMF development observed among potato cultivars in our *in vitro* experiment (e.g. AMF compatibility and mycorrhizal

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dependency) additional tests need to be conducted under greenhouse and field conditions.

Interestingly, in the presence of *P. plecoglossicida* R-67094 there was also an effect of the bacteria on the AMF symbiotic development suggesting either a direct effect of the bacteria on the fungus or an indirect effect on the AMF via the plant. In either of both scenarios it is most probable that *P. plecoglossicida* R-67094 behave as a MHB by promoting mycorrhizal development in the pre-symbiotic as well as the symbiotic phase.

The results of the present study demonstrated the importance of a preliminary selection of AMF-PGPR combinations prior to formulation. The co-entrapment in alginate beads of *P. plecoglossicida* R-67094 and *R. irregularis* MUCL 41833 had a positive effect on the mycorrhizal symbiosis in potato plants. *P. plecoglossicida* has been reported as a dominant bacterial taxon in the rhizosphere of banana (Naik *et al.* 2008) and sugar cane (Rameshkumar *et al.* 2012). Furthermore the fact that this PGPR was also isolated from potato plantations in Ecuador in combination together with its capacity to solubilize phosphorus, produce IAA and act as antagonist against soil-borne pathogens (Naik *et al.* 2008; Rameshkumar *et al.* 2012) makes it a good candidate for microbial formulations (Jha *et al.* 2009) for potato cropping. Our study represents a promising step towards the formulation of context-adjusted microbial inoculants aimed to improve the establishment of arbuscular mycorrhizal inoculum. Further steps will be required to fine-tune alginate co-encapsulation technology, especially regarding shelf-life of inocula. Additionally greenhouse and field essays will bring new light on the effect of encapsulated microorganisms on potato growth.

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Conflict of Interest:

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No conflict of interest declared

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Tables and Figures:

Table 1. Percentage of potentially infective beads (%PIB) estimated as the % of beads with germinated spores of *R. irregularis* MUCL 41833 showing hyphal regrowth crossing the calcium coating of the bead in presence or absence (i.e. the control) of different PGPRs of plant growth promoting rhizobacteria.

	Percentage of Potentially Infective Beads (%PIB)					
	5	**10**	**15**	**20**	**25**	**30**
P. plecoglossicida R-67094	1.8 ± 1.3^{a}	3.6 ± 1.7^{a}	10 ± 2.5^{a}	33.1 ± 4.4^{a}	58.3 ± 6.5^{a}	68.6 ± 6.8^{a}
B. amyloliquefaciens LMG 24415	0^{a}	0.9 ± 0.9^{a}	5.2 ± 3^{a}	11.2 ± 3.9^{bc}	11.2 ± 3.9^{b}	12.1 ± 4.3^{b}
B. subtilis LMG 24423	0.9 ± 0.9^{a}	2.7 ± 1.5^{a}	6.4 ± 2^{a}	6.4 ± 2.0^{c}	6.4 ± 2^{b}	6.4 ± 2.0^{b}
Control	0^{a}	0.9 ± 0.9^{a}	10.2 ± 3^{a}	23 ± 3.7^{ab}	47.4 ± 5.1^{a}	56.5 ± 6.2^{a}

Values represent Means ± SE of 20 replicates. Values in the same column followed by identical letter did not differ significantly ($P<0.05$, Tukey's test).

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Table 2. Hyphal length and number of hyphal branches of spores of *R. irregularis* MUCL 41833 entrapped in alginate beads in presence or absence (i.e. the control) of different PGPRs of plant growth promoting rhizobacteria.

	Hyphal length (mm)				**Number of hyphal branches**		
	Germ tube	2nd order branches	3rd order branches	Total length	2nd order	3rd order	Total branches
P. plecoglossicida R-67094	26.1 ± 1.2[a]	6.1 ± 0.9[a]	6.2 ± 1.5[a]	38.5 ± 2.3[a]	15 ± 1.5[a]	26.5 ± 4.5[a]	41.4 ± 5.5[a]
B. amyloliquefaciens LMG 24415	1.7 ± 0.5[c]	0.3 ± 0.1[b]	0.2 ± 0.1[b]	2.2 ± 0.5[b]	2 ± 0.3[b]	2.1 ± 0.5[b]	4.1 ± 0.6[b]
B. subtilis LMG 24423	0.4 ± 0.2[c]	0[b]	0[b]	0.4 ± 0.2[b]	0[b]	0[b]	0[b]
Control	6.9 ± 0.9[b]	0.5 ± 0.2[b]	0.02 ± 0.0[b]	7.4 ± 0.9[b]	1.5 ± 0.5[b]	0.2 ± 0.2[b]	1.7 ± 0.6[b]

Values represent Means ± SE of 10-20 replicates (The isolates LMG 24415 and LMG 24423 decreased %PIB and hence a low number of spores were available for evaluations). Values in the same column followed by identical letter did not differ significantly (P<0.05, Tukey's test).

Table 3. Two-way ANOVA for main effects of "potato cultivar", "*P. plecoglossicida* R-67094" and their interaction on the length of the extraradical mycelium (ERM) of *R. irregularis* MUCL 41833, 4 and 9 weeks following co-entrapment in alginate beads with/without *P. plecoglossicida* R-67094 and association to three potato cultivars (Bintje, Fripapa and Unica).

	ERM length (mm)	
Treatments	**Week 4**	**Week 9**
Potato Cultivar	0.800	**0.002****
R-67094	**<0.001*****	**0.017***
Potato Cultivar *x* R-67094	0.441	0.589

Significant effects are marked with * for P<0.05, ** for P<0.01, and *** for P<0.001

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Table 4. Two-way ANOVA for main effects of "potato cultivar", "*P. plecoglossicida* R67094" and their interaction on the number of spores (from week 5 to 9) and root colonization of *R. irregularis* MUCL 41833.

	Spore production (weeks)					% Colonization	
	5	6	7	8	9	*%F*	*%RLC*
Potato cultivar	0.052	**<0.001*** **	0.060	**0.026***	0.068	**<0.001*** **	**<0.001*** **
R-67094	0.251	0.273	**0.027***	0.122	**0.005****	**0.001****	**<0.001*** **
Potato cultivar *x* R-67094	0.914	0.863	0.668	0.945	0.994	0.054	0.131

Significant effects are marked with * for P<0.05, ** for P<0.01, and *** for P<0.001

Figure 1. Length of extraradical mycelium (ERM) of *Rhizophagus irregularis* MUCL 41833 grown in presence/absence of *P. plecoglossicida* R-67094, 4 (a) and 9 (b) weeks following association with potato cultivars Bintje, Fripapa and Unica. The bars represent means ± SE of treatments without (white) and with (grey) *P.plecoglossicida* (R-67094), n=10. T tests were carried out within each cultivar to compare the mean values at each time point with the respective control treatment. * P<0.05, **P<0.01, P<0.001.

Figure 2. Spore production of *Rhizophagus irregularis* MUCL 41833 in presence/absence of *P. plecoglossicida* R-67094 and associated to potato cultivars Bintje (a), Fripapa (b) and Unica (c) from week 5 to 9. The bars represent means ± SE of treatments without (white) and with (grey) *Pseudomonas plecoglossicida* (R-67094), n=10. T tests were carried out within each cultivar to compare the mean values at each time point with the respective control treatment. * P<0.05, **P<0.01, P<0.001.

Figure 3. Frequency (a) and root length colonization (b) of *R. irregularis* MUCL 41833 grown in presence/absence of *P. plecoglossicida* R-67094, 9 weeks following association with potato cultivars Bintje, Fripapa and Unica. The bars represent means ± SE of treatments without (white) and with (grey) *Pseudomonas plecoglossicida* (R-67094), n=10. T tests were carried out within each cultivar to compare the mean values in the presence of R-67094 with the respective control treatment. * P<0.05, **P<0.01, P<0.001.

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Legends for the Supporting Information

Table S1. List of PGPR isolates used for entrapment with *R. irregularis* MUCL 41833.

[a] EC1–4 = Ecuador, field 1–4

[b,c] *In vitro* tests to determine biocontrol against *R. solani* and *P. infestans*, IAA and ACC production, and P solubilization were carried out in University College Cork (UCC).

Percentage biocontrol was calculated with the formula: (C1-C2/C1)×100, where C1=total fungal growth of the control and C2=measured fungal growth in the presence of rhizobacteria. Values are means of five replicates.

IAA: indole-3-acetic acid production (mg mL^{-1}). Values are means of two replicates.

PO_4^{3}−sol. Phosphate solubilization. Isolates were rated on a scale of 0 to 5 depending on the halo diameter as follows: 0=no activity, 1=1-5 mm, 2=6-10 mm, 3=11-15 mm, 4=16-20 mm, 5=>20 mm. Values are means of four replicates.

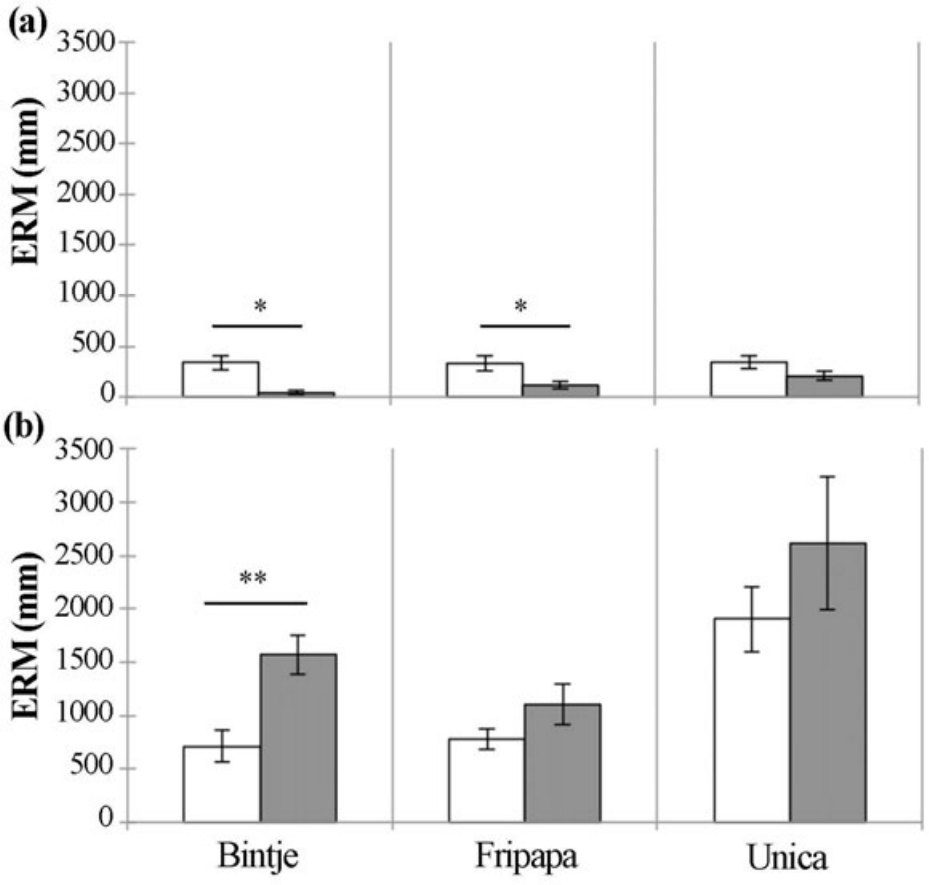
ACC: 1-aminocyclopropane-1-carboxylate deaminase activity (nmol (α-ketobutyrate) mg^{-1} h^{-1}). Values are means of three replicates.

nd, no data

Figure S1. Alginate beads containing the AMF *Rhizophagus irregularis* MUCL 41833 in combination with *P. plecoglossicida* isolate R-67094 (a) or alone (b). The profuse growth of the PGPR within the alginate beads (a) made it difficult to visualize the spores inside the beads compared to the control (b). Bar = 1000μm.

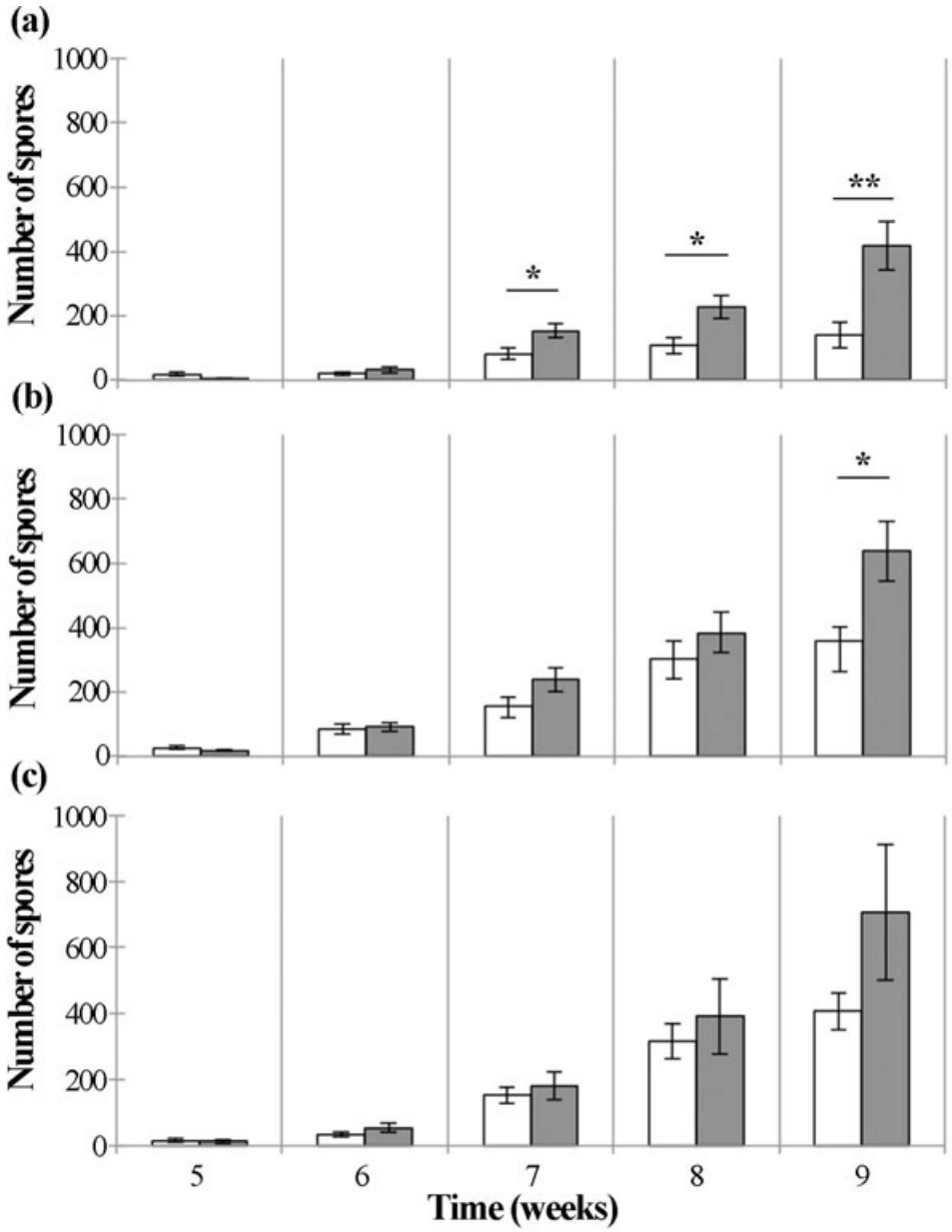
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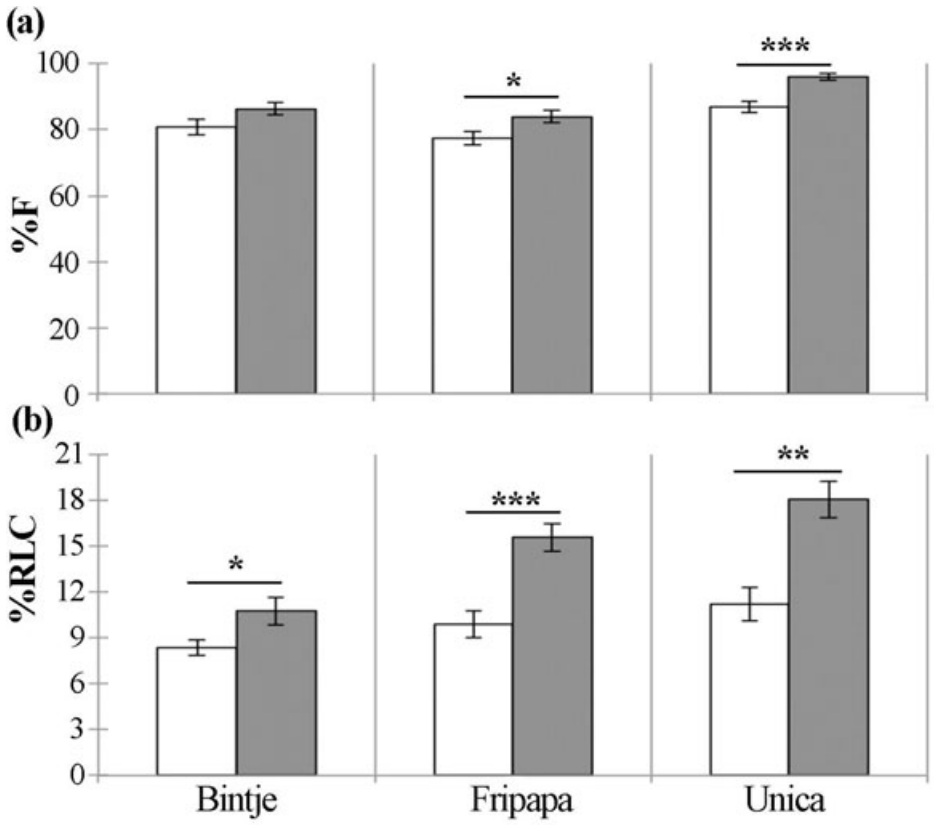


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