Selection of Infective Arbuscular Mycorrhizal Fungal Isolates for Field Inoculation

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Abstract

Arbuscular mycorrhizal (AM) fungi play a key role in host plant growth and health, nutrient and water uptake, plant community diversity and dynamics. AM fungi differ in their symbiotic performance, which is the result of the interaction of two fungal characters, infectivity and efficiency. Infectivity is the ability of a fungal isolate to establish rapidly an extensive mycorrhizal symbiosis and is correlated with pre-symbiotic steps of fungal life cycle, such as spore germination and hyphal growth. Here, different AM fungal isolates were tested with the aim of selecting infective endophytes for field inoculation. Greenhouse and microcosm experiments were performed in order to assess the ability of 12 AM fungal isolates to produce spores, colonize host roots and to perform initial steps of symbiosis establishment, such as spore germination and hyphal growth. AM fungal spore production and root colonization were significantly different among AM fungal isolates. Spore and sporocarp densities ranged from 0.8 to 7.4 and from 0.6 to 2.0 per gram of soil, respectively, whereas root colonization ranged from 2.9 to 72.2%. Percentage of spore or sporocarp germination ranged from 5.8 to 53.3% and hyphal length from 4.7 to 79.8 mm. The ordination analysis (Redundancy Analysis, RDA) showed that AM fungal isolates (used as explanatory variables) explained about 60% of the whole variance and their effect on fungal infectivity variables was significant ($P = 0.002$). The biplot clearly showed that variables which might be used to detect infective AM fungal isolates were hyphal length and root colonization. Such analysis may allow the detection of the best parameters to select efficient AM fungal isolates to be used in agriculture.

Key-words: arbuscular mycorrhizas, hyphal length, root colonization, spore germination, AM fungal infectivity, sporulation.

Introduction

Arbuscular mycorrhizas are mutualistic associations between the roots of about 80% of plant species and soil fungi of the phylum Glomeromycota (about 150 species) (Schüßler and Schwarzott, 2001; Smith and Read, 2008). Fungal hyphae spread into the soil from mycorrhizal roots and improve the uptake effectiveness of poorly mobile nutrients such as P, Zn and Cu and of mineral forms of N and K. The enhanced nutrient uptake by mycorrhizal plants compared to non-mycorrhizal plants is due to fungal hyphae that grow beyond the area of root nutrient depletion and within soil micropores, increasing the absorbing surface and the access to inorganic and organic forms of P and N. Moreover, arbuscular mycorrhizal (AM) fungi provide protection to host plant against root and shoot pathogens (Newsham et al., 1995). AM fungal symbionts are obligate biotrophs and, after establishing functional symbioses with host plants, produce spores in the soil which are able to germinate and grow, but are unable to produce extensive mycelia and to complete their life cycle in the absence of their hosts (Giovannetti and Avio, 2002). Although AM symbionts cannot be grown in axenic culture and

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knowledge of their biology is limited, some of the factors affecting their development have been studied (Giovannetti and Avio, 2002). Because of the complexity of the interactions between fungal isolates and host/soil/environmental factors, no generalization is possible. Nevertheless, the criteria to be followed for the selection of suitable endophytes is the result of the interaction of two fundamental characters, infectivity and efficiency.

Infectivity has been defined as the ability of a fungal isolate to establish rapidly an extensive mycorrhizal colonization in the roots of a host plant and is correlated with complex soil-fungus-host factors affecting fungal life cycle stages, *i.e.* spore germination, non symbiotic germling growth, host recognition, differentiation of infection structures and root colonization (Giovannetti and Gianinazzi-Pearson, 1994). The efficiency of different fungal endophytes cannot be measured simply in terms of host growth responses, since many factors, such as the uptake of P and other poorly mobile nutrients, linked to extraradical fungal networks development in the soil and to the rate of P adsorption, translocation and transfer from hyphae to host cells, contribute to determine their symbiotic performance (Giovannetti and Avio, 2002).

Field utilization of AM fungi depends primarily on the availability of highly infective isolates. Although different kinds of fungal propagules may affect infectivity, most studies have investigated the ecology and physiology of spores, as they represent the most frequently used propagules in laboratory and field experiments.

Therefore the parameters to be taken into account when selecting for infectivity mainly concern spore-related factors such as spore germination, pre-symbiotic growth of spore germlings and intraradical growth.

Here, different AM fungal isolates were tested, with the aim of detecting the best parameters allowing the selection of infective endophytes for field inoculation.

Materials and methods

Fungal material
Twelve AM fungal isolates were used as fungal material. Details of the isolates are given in Table 1.

Greenhouse experiment

This experiment allowed the assessment of the ability to produce spores and colonize host roots of the AM fungal isolates tested.

Experimental setup. Seeds of *Medicago sativa* cv. Messe and *Trifolium alexandrinum* cv. Tigri were sown in 600 ml plastic pots containing a mixture (270:270 ml) of soil and Terragreen (calcinated clay, OILDRI, Chicago, IL, USA). The mixture was steam-sterilized to kill naturally occurring AM fungi. Pots were inoculated with 60 ml of crude inoculum (mycorrhizal roots and soil containing spores and extraradical mycelium) of one of the 12 fungal isolates. In this way, possible differences in AM fungal colonization ability of the 12 isolates were balanced by using such high amounts of inoculum (10% by volume). All the pots received 100 ml

Table 1. Geographic origin and inoculum source of AM fungal isolates used in the present work.

Fungal Species	Isolate code[a,b]	Geographic origin	Donor	Original inoculum supplier
Acaulospora spinosa	MN405D	Minnesota, USA	H. Gamper	INVAM, Morgantown, W. Va.
Glomus caledonium	IMA2 = BEG20	Hartfordshire, UK	M. Giovannetti	Rothamsted, Exp. St., UK
Glomus clarum	FL979A	Florida, USA	H. Gamper	INVAM, Morgantown, W. Va.
Glomus coronatum	IMA3 = BEG28	Tuscany, Italy	M. Giovannetti	IMA, Pisa, Italy
Glomus etunicatum	UK208	UK	H. Gamper	INVAM, Morgantown, W. Va.
Glomus geosporum	CI102	China	H. Gamper	INVAM, Morgantown, W. Va.
Glomus mosseae	BEG25	West Sussex, UK	M. Giovannetti	IIB, Canterbury, UK
Glomus mosseae	FL156	Florida, USA	M. Giovannetti	INVAM, Morgantown, W. Va.
Glomus mosseae	IMA1	Kent, UK	M. Giovannetti	Rothamsted, Exp. St., UK
Glomus mosseae	NB114	Namibia	M. Giovannetti	INVAM, Morgantown, W. Va.
Glomus spp.	AD 1	Abu Dhabi	M. Giovannetti	IIB, Canterbury, UK
Scutellospora calospora	NC128	N. Caroline, USA	H. Gamper	INVAM, Morgantown, W. Va.

[a]BEG, Bank of European Glomales; [b]IMA, International Microbial Archives; [c]Abbreviations: INVAM, International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi; IIB, International Institute of Biotechnology.

of a filtrate, obtained by sieving a mixture of the 12 inocula and of agricultural soil from *M. sativa* and *T. alexandrinum* fields, through a sieve with pore diameter of 50 μm, to ensure a common microflora to all treatments. After emergence, seedlings of *M. sativa* and *T. alexandrinum* were thinned to 10. Plants were grown for three months in greenhouse, supplied with tap water as needed and fertilized with half-strength Hoagland's solution once a month. The experiment was a completely randomized design with 12 inoculum treatments (each fungal isolate) and 5 replicates.

Assessment of spore production and host root colonization. At harvest, three months after emergence, sporocarps and spores were extracted from a subsample of 50 g of soil by each pot culture (*n* = 5) by wet-sieving and decanting, down to a mesh size of 100 μm, flushed into Petri dishes, manually collected with forceps and counted under a Wild dissecting microscope (Leica, Milano, Italy). Mycorrhizal colonization were then assessed on root samples from each pot culture after clearing and staining (Phillips and Hayman, 1970), utilizing lactic acid instead of lactophenol, by using the gridline intersect method (Giovannetti and Mosse, 1980).

Microcosm experiment

This experiment allowed the assessment of the ability of the AM fungal isolates tested to perform initial steps of symbiosis establishment, such as spore germination and hyphal growth.

Experimental set-up. The experimental model is the 'sandwich system' devised for investigating the early stages of AM fungal life cycle (Giovannetti et al., 1993a). Sporocarps or single spores (at least 20 propagules for each membrane) were germinated in the dark at 24 °C between two 47-mm-diameter cellulose nitrate Millipore™ membranes (pore diameter 0.45 μm) placed on acid-washed, sterile quartz grit (2-5 mm) in 14-cm-diameter Petri dishes. The experiment was a completely randomized design with 12 inoculum treatments (each fungal isolate) and at least 5 replicates.

Assessment of spore and sporocarp germination and hyphal growth. Spore germination was assessed after 14 days by staining with 0.05% trypan blue in lactic acid, and mycelial length was measured under a Wild dissecting microscope by using the grid-line intersect method (Giovannetti and Mosse, 1980).

Statistics and data analyses

Data were compared using a one-way (fungal treatment as factor) analysis of variance (ANOVA). Data were ln- and arcsine-transformed when needed to fulfil the assumptions of the ANOVA, which was carried out according to a completely randomized design. Tukey B procedure was used for comparing means. All statistics were performed with the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Means and standard errors (S.E.) given in tables are for untransformed data. Ordination analysis (Redundancy Analysis, RDA) was carried out in Canoco for Windows v. 4.5 (ter Braak and Smilauer, 2003) in order to investigate the influence of the AM fungal isolates (used as explanatory variables) on fungal physiological parameters (used as response variables).

Results and discussion

Greenhouse experiment

Assessment of spore production and host root colonization. Three month after inoculation, spore density ranged between 0.78 ± 0.25 and 7.43 ± 1.20 spores/g of soil (*S. calospora* NC128 and *G. Geosporum* CI102, respectively) and the total mean was 3.51 ± 0.47 (Tab. 2). Numbers of spores per gram of soil of *A. spinosa* MN405B, *G. caledonium* IMA2 = BEG20, *Glomus* spp. AD 1 and *S. calospora* NC128 significantly differed from values assessed for *G. geosporum* CI102 (*P* < 0.01) (Tab. 2). Sporocarp density ranged between 0.63 ± 0.23 and 2.01 ± 0.66

Table 2. Number of spores of eight arbuscular mycorrhizal fungal isolates produced after three months in symbiosis with *Medicago sativa* and *Trifolium alexandrinum*.

Isolate	Spore density (n/g of soil)
Acaulospora spinosa MN405B	2.32 ± 0.98[a] a
Glomus caledonium IMA2=BEG20	2.67 ± 0.65 a
Glomus clarum FL979A	n.a.[b]
Glomus coronatum IMA3=BEG28	3.88 ± 0.61 ab
Glomus etunicatum UK208	4.60 ± 1.66 ab
Glomus geosporum CI102	7.43 ± 1.20 b
Glomus spp. AD 1	2.92 ± 0.83 a
Scutellospora calospora NC128	0.78 ± 0.25 a
Total	3.51 ± 0.47

[a]Values are means ± SE of five replicates for each treatment; values in the column not followed by the same letters are significantly different (*P* < 0.01); [b]not assessed.

spores/g of soil (*G. mosseae* FL156 and *G. mosseae* NB114, respectively) and total mean was 1.13 ± 0.20 (Tab. 3). Interestingly, significant intraspecific differences were observed between *G. mosseae* FL156 and *G. mosseae* NB114 (*P* < 0.05) (Tab. 3).

Our data on spore production were comparable to those obtained by Giovannetti et al. (1988) by using *Glomus monosporum* with *Allium cepa* and *Trifolium pratense* as host plants (0.6-3.7 spores/g of soil) and by Moreira et al. (2007) by using diverse AM fungi in symbiosis with different host plants (0.2-1.5 spores/g of soil). Differences in AM fungal sporulation have been reported by several authors when different plants were used as hosts, both in laboratory (Daft and Hogarth, 1983; Hetrick and Bloom, 1986; Koomen et al., 1987; Hung and Sylvia, 1988; Bever et al., 1996; Carrenho et al., 2002) and in field conditions (Black and Tinker, 1979; Schenck and Kinloch, 1980; McGraw and Hendrix, 1984; Moreira et al., 2007). Moreover, sporulation was significantly affected by harvest time (Bever et al., 1996), season (Fowler and Antonovics, 1981; Gemma et al., 1989; Bentivenga and Hetrick, 1991; Hetrick et al., 1994), edaphic factors (Gilden and Tinker, 1981; Stahl and Smith, 1984; Louis and Lim, 1988; Boerner, 1990; Stahl et al., 1990; Stahl and Christensen, 1991), and AM fungal geographic origin (Fox and Morrow, 1981; Burdon, 1987).

The low values of *S. calospora* spore production detected in this work were similar to those reported by Bever et al. (1996), ranging from 0.15 to 0.35 spores/g of soil. Other authors showed low (0.02-0.16 spores/g of soil) or no sporulation of different *Scutellospora* species (Bever et al., 1996; Moreira et al., 2007), suggesting possible sporulation difficulties of such genus in pot conditions.

The isolate of *A. spinosa* utilised in our work produced a higher number of spores (2.3 spores/g of soil) compared to data reported for different isolates of *Acaulospora* spp., showing spore density values ranging from 0.002 to 0.17 spores/g of soil, in symbiosis with *Panicum* and *Plantago* in greenhouse conditions (Bever et al., 1996) and from 0.02 to 0.6 spores/g of soil, in symbiosis with *Araucaria angustifolia* in field conditions (Moreira et al., 2007).

G. etunicatum was shown to produce a high

Table 3. Number of sporocarp of four arbuscular mycorrhizal fungal isolates, belonging to *Glomus mosseae* species, produced after three months in symbiosis with *Medicago sativa* and *Trifolium alexandrinum*.

Isolate	Sporocarp density (n/g of soil)
Glomus mosseae BEG25	0.90 ± 0.09[a] ab
Glomus mosseae FL156	0.63 ± 0.23 a
Glomus mosseae IMA1	0.97 ± 0.05 ab
Glomus mosseae NB114	2.01 ± 0.66 b
Total	1.13 ± 0.40

[a]Values are means ± SE of five replicates for each treatment; values in the column not followed by the same letters are significantly different (*P* < 0.05).

number of spores/g of soil, ranging from 18.6 to 152.8 , in symbiosis with *Panicum miliaceum*, whereas lower values (from 0.22 to 1.54 spores/g of soil), comparable to spore densities reported by this work, were observed in symbiosis with *Sorghum vulgare* (Silva et al., 2005).

The high values of spore density detected here for *G. geosporum* are not comparable with those previously reported by Snyder et al. (2001).

G. mosseae, in associations with different cultivars of *M. sativa* and with *Paspalum notatum*, showed values of 1.32-3.65 spores/g of soil (Douds et al., 1998), which were comparable with *G. mosseae* spore densities assessed here. Interestingly, in this work a variability within the worldwide distributed species *G. mosseae* was detected, suggesting that sporulation is not a species-level feature in AM fungi.

Percentages of root colonization ranged between 2.86 ± 1.39 and 72.17 ± 6.03 (*S. calospora* NC128 and *G. clarum* FL979A, respectively) and the total mean was 39.33 ± 2.74 (Tab. 4). Results allowed the discrimination of AM fungal isolates tested into three groups, with low, medium and high infectivity: *S. calospora* NC128 (low infectivity); *G. etunicatum* UK208, *G. mosseae* IMA1, *A. spinosa* MN405B, *G. caledonium* IMA2 = BEG20, *G. coronatum* IMA3 = BEG28, *G. mosseae* FL156, *G. mosseae* NB114, *G. geosporum* CI102 and *Glomus* spp. AD 1 (medium infectivity); *G. clarum* FL979A and *G. mosseae* BEG25 (high infectivity) (Tab. 4). Interestingly, significant differences were also observed among *G. mosseae* isolates (Tab. 4).

Here, no correlation was found between spore density and root colonization (data not shown).

Table 4. Percentage of root colonization of *Medicago sativa* and *Trifolium alexandrinum* grown for three months in symbiosis with twelve arbuscular mycorrhizal fungal isolates.

Isolate	Root colonization (%)
Acaulospora spinosa MN405B	36.98 ± 6.38[a] bc
Glomus caledonium IMA2=BEG20	27.35 ± 4.22 bc
Glomus clarum FL979A	72.17 ± 6.03 d
Glomus coronatum IMA3=BEG28	41.24 ± 2.65 bc
Glomus etunicatum UK208	22.26 ± 2.46 ab
Glomus geosporum CI102	50.64 ± 3.78 cd
Glomus mosseae BEG25	68.54 ± 8.05 d
Glomus mosseae FL156	34.56 ± 3.48 bc
Glomus mosseae IMA1	24.17 ± 5.13 ab
Glomus mosseae NB114	42.00 ± 4.34 bc
Glomus spp. AD 1	49.17 ± 5.84 cd
Scutellospora calospora NC128	2.86 ± 1.39 a
Total	39.33 ± 2.74

[a]Values are means ± SE of five replicates for each treatment; values in the column not followed by the same letters are significantly different ($P < 0.001$).

Although differences in AM fungal root colonization, when different plants were used as hosts in different edaphic conditions, have been reported by several authors (Giovannetti et al., 1988; van der Heejden and Sanders, 2002; Smith and Read, 2008), root colonization represents a fundamental parameter to be studied in order to detect the most infective endophytes for field inoculation.

In this work the high values of root colonization showed by *G. mosseae* BEG25 were comparable with the values reported by Hart and Reader (2001). By contrast, other isolates of *G. mosseae*, IMA1, FL156 and NB114, showed low root colonization. Intraspecific variability, reported by several authors for parameters related to infectivity and efficiency (Smith et al., 2000; Munkvold et al., 2004; Avio et al., 2006), suggested that root colonization might be related more to isolates than to species, as showed for *G. intraradices* species by Hart and Reader (2001). Results obtained in this work for *A. spinosa* are similar to those reported by Hart and Reader (2001) for the same species, whereas values found for *G. geosporum*, showing a medium-high infectivity, are not comparable.

Microcosm experiment

Assessment of spore and sporocarp germination and hyphal growth. Spores of *A. spinosa, G. caledonium, G. coronatum* and *S. calospora* did not germinate in our experimental conditions. Germination percentages ranged between 5.83 ± 3.00 and 53.33 ± 4.94 (*G. etunicatum* UK208 and *G. clarum* FL979A, respectively) and the total mean, excluding the AM isolates showing no germination, was 22.71 ± 2.72 (Tab. 5). *Glomus* spp. AD 1, *G. geosporum* CI102, *G. etunicatum* UK208, *G. mosseae* FL156, *G. mosseae* IMA1 and *G. mosseae* NB114 were characterised by low germination percentages, whereas *G. clarum* FL979A, *G. mosseae* BEG25 showed high germination ability (Tab. 5).

Table 5. Percentage of spore or sporocarps germination and hyphal length per spore or sporocarp of twelve arbuscular mycorrhizal fungal isolates.

Isolate	Percentage of germination (%)	Hyphal length per spore or sporocarp (mm)
Acaulospora spinosa MN405B	0	-
Glomus caledonium IMA2=BEG20	0	-
Glomus clarum FL979A	53.33 ± 4.94[a] d	31.16 ± 4.00[b] ab
Glomus coronatum IMA3=BEG28	0	-
Glomus etunicatum UK208	5.83 ± 3.00 a	36.22 ± 35.17 a
Glomus geosporum CI102	12.50 ± 3.35 ab	12.09 ± 3.14 ab
Glomus mosseae BEG25	41.67 ± 2.79 cd	79.81 ± 9.73 b
Glomus mosseae FL156	10.00 ± 1.83 ab	4.67 ± 1.41 a
Glomus mosseae IMA1	29.17 ± 7.90 bc	12.86 ± 6.13 a
Glomus mosseae NB114	20.00 ± 3.87 abc	43.83 ± 14.39 ab
Glomus spp. AD 1	9.17 ± 3.00 ab	75.65 ± 21.11 ab
Scutellospora calospora NC128	0	-
Total	22.71 ± 2.72	37.01 ± 6.50

[a,b]Values are means ± SE of six replicates for each treatment; values in each column not followed by the same letters are significantly different ($P \leq 0.001$).

Figure 1. Redundancy analysis biplot showing five AM fungal isolates (*Glomus geosporum* CI102; *Glomus mosseae* IMA1, NB114, BEGG25; *Glomus* spp. AD 1), used as explanatory variables (up-triangles), in order to investigate their influence on four fungal physiological parameters (hyphal length, arbuscular mycorrhizal root colonization, spore density and spore germination) used as response variables (rows).
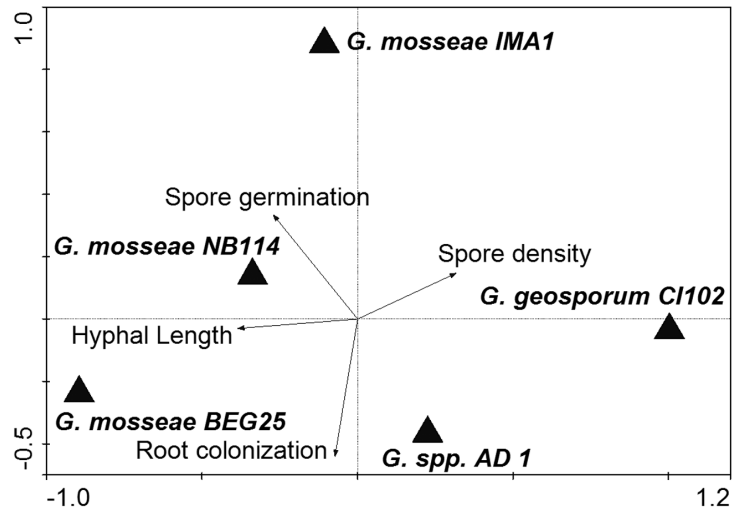
Germination is the first step of AM fungal life cycle and an important character to take into account when selecting endophytes for pratical applications, since it shows a high degree of variation among isolates (Smith and Read, 2008). The environmental parameters influencing spore germination are represented by pH, temperature, moisture, mineral and organic nutrients, presence of host/nonhost plants and microorganisms (Giovannetti, 2000). Interestingly, significant differences were observed within *G. mosseae* species (Tab. 5). Our data confirm previous results on the large variability of spore and sporocarp germination within *G. mosseae*, both *in vivo* and *in vitro* (Giovannetti et al., 2003). The absence of germination of *A. spinosa*, *G. caledonium*, *G. coronatum* and *S. calospora* spores confirms previous data on the erratic germination ability of such species in an *in vitro* system (Declerck et al., 2005).

Hyphal length per spore or sporocarp ranged between 4.67 ± 1.41 and 79.81 ± 9.73 mm (*G. mosseae* FL156 and *G. mosseae* BEG25, respectively) and the total mean was 37.01 ± 6.50 mm (Tab. 5). Significant differences were observed between *G. etunicatum* UK208, *G. mosseae* FL156, *G. mosseae* IMA1 and *G. mosseae* BEG25 (Table 5).

Germlings of AM fungi are able to elongate and originate a coenocytic mycelial network generally extending no more than 50-200 mm (Giovannetti and Avio, 2002). Our data are comparable with such hyphal lengths, which have been reported to be affected by several vari-ables, such as AM fungal isolates (Hepper and Smith, 1976; Hepper, 1979; Graham, 1982; Siqueira et al., 1982; Gianinazzi-Pearson et al., 1989; Tommerup, 1983; Giovannetti et al. 2003; Bago et al., 1998; Logi et al., 1998), host plants (Giovannetti et al. 1993b) and edaphic conditions (Giovannetti and Avio, 2002; Smith and Read, 2008). Interestingly, we found variability within *G. mosseae* species, confirming previous data reported by Avio et al. (2009).

Main patterns in AM fungal infectivity traits

The RDA, performed in order to investigate the influence of AM fungal isolates on infectivity parameters, such as spore production, host root colonization, spore germination and hyphal growth, showed that the identity of the different AM fungi (used as explanatory variables) explained about 60% (I and II axes) of the whole variance and that their effects on fungal infectivity traits was statistically significant ($P = 0.002$) (Fig. 1). In detail, Monte Carlo permutation test showed that the infectivity parameters of *G. geosporum* CI102 and *Glomus* spp. AD 1, differed significantly and were also different from those of the other fungal isolates (*G. mosseae* BEG25, IMA1, NB114) ($P < 0.05$). The biplot clearly suggested that variables useful for the detection of the most infective AM fungal isolates are represented by hyphal length, the most discriminating variable on the first axis, and root colonization, the most discriminating variable on the second axis (Fig. 1).

Several authors have previously assessed sin-

gle or multiple variables in order to evaluate AM fungal infectivity (Giovannetti and Avio, 2002). Here, for the first time, the power of four of the main variables commonly utilized to detect infectivity, was evaluated by using a multivariate approach. Our results showed that root colonization and hyphal length were able to discriminate AM fungi better than spore production and germination, which are affected by environmental factors.

Conclusion

The utilization of AM fungi in the field primarily relies on the availability of highly infective isolates. Spores and sporocarps are mostly utilised as inoculum sources in laboratory and field experiments, though they show high variability in germination, pre-symbiotic growth and intraradical growth, which greatly affect the infectivity and efficiency of AM propagules. In this work we detected the best parameters for the selection of infective endophytes for field inoculation, which are represented by hyphal length and root colonization.

Acknowledgment

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