Arbuscular mycorrhizal fungi and micropropagation of *Ranunculus asiaticus* L.: a useful alliance?

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Abstract

The present work is aimed at providing new information about the role of arbuscular mycorrhizal fungi (AMF) in improving the acclimatization and establishment of micropropagated plants of *Ranunculus asiaticus* L. Micropropagated plantlets of *R. asiaticus* were multiplied and rooted in vitro. Ex vitro plantlets were inoculated with three different commercial inocula, based on arbuscular mycorrhizal fungi: (1) *Funneliformis mosseae* (GM); (2) *F. mosseae*, *Rhizophagus intraradices* and *Glomus* sp. (MIX); (3) *F. mosseae*, *R. intraradices*, *Viscospora viscosa*, plant growth promoting rhizobacteria (MF) and saprotrophic fungi. A two-step inoculation method has been carried out: mycorrhizal inoculum was added to pots filled with peat substrate at acclimatization phase and a further inoculation occurred when plants were transplanted to field conditions. Plants growth was followed all over the culture cycle till harvesting the tuberous roots. Our findings showed that ex vitro plantlets of *R. asiaticus* already form arbuscular mycorrhizae during the acclimatization phase. MF inoculum had the lowest root colonization levels, while the MIX and GM-inocula provided satisfactory results with high levels of root colonization. Once in the field, MIX-treated plants showed an earlier flowering, an improved flower production and somewhat better rhizome yield at the end of the culture cycle if compared to the control (untreated plants). GM-treated plants performed in a way not significantly different from the control. MF-treated plants evidenced the worst performance with a low flowering efficiency. From our results it can be argued that ex vitro *R. asiaticus* plantlets can be colonized by AM fungi; however, different AMF species in the inoculum can affect the performance of plants when transferred to the field.

Keywords: acclimatization, arbuscular mycorrhiza, ex vitro performance, in vitro, micropropagated plants

INTRODUCTION

Ranunculus asiaticus L. is an ornamental geophyte prized for its showy flowers. Plant tissue culture is an attractive alternative to the traditional propagation methods (Beruto, 2002). Under Mediterranean conditions, the cut flower production is carried out through the tuberous roots harvested at the end of the cultivation cycle of ex vitro plantlets. The production can be greatly influenced by tuberous roots size and quality and this, in turn, is influenced by the quality standard of the ex vitro plantlets (Beruto et al., 2009; Cerveny et al., 2011).

In this frame, the application of arbuscular mycorrhizal fungi (AMF) could play an important role for enhancing the quality and the sustainability of the production. In fact, these fungi are beneficial symbionts that act as plants' biofertilizers'. They are able to grow inside plant roots improving plant nutrition, growth and stress resistance; in exchange they benefit from plant's photosynthates (Smith and Read, 2008). Thanks to these characteristics, AMF application is becoming an increasingly adopted practice in agriculture. In particular, AMF inoculation is viewed with growing interest during ex vitro growth phase that represents a highly stressful stage for micropropagated plantlets (Pospíšilová et al., 1999).

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In this situation, AMF have already proved to be able to reduce plant stress and to enhance plant surviving and production (Declerck et al., 2002; Fortunato et al., 2005). However, to our knowledge, little information is available about the ability of AMF to colonize *R. asiaticus* roots and the effect on plant establishment and growth.

The present work is aimed at providing more information about the ability of arbuscular mycorrhizal (AM) fungi to enter in symbiosis with micropropagated plants of *Ranunculus asiaticus* L. and to affect plant acclimatization and in vivo establishment.

MATERIALS AND METHODS

Plant material and experimental design

Micropropagated plantlets of *Ranunculus asiaticus* L. 'Juny' (breeder: Biancheri Creation, Camporosso, IM, Italy) were multiplied and in vitro rooted, according to the protocol suggested by Beruto and Debergh (2004).

Plantlets were acclimatized in a climatic chamber (22°C, relative humidity 90%, light intensity 80 µmol m^{-2} s^{-1} and 16-h photoperiod), in 30 mL pots filled with peat substrate. After 6 weeks, plants were transferred to an unheated greenhouse, in raised benches with 70% pomix and 30% peat substrate previously steam sterilized. During cultivation the usual practices for nutrition and irrigation were followed (Tesi, 1985); no pesticides were applied. Planting density was 18 plants m^{-2}, arranged according to a 'quinconce' scheme (Crescini, 1959). At the end of the cultural cycle (about 30 weeks), the irrigation stopped and tuberous roots were harvested after two additional weeks.

Three different AMF formulations have been applied: (1) a single isolate inoculum containing *Funneliformis mosseae* (code: GM); (2) a mix inoculum containing three isolates *F. mosseae*, *Rhizophagus intraradices* and *Glomus* sp. (code: MIX) and (3) a composite inoculum containing AMF fungi propagules of *F. mosseae*, *R. intraradices*, *Viscospora viscosa* species and plant growth promoting rhizobacteria (code: MF). The first and second formulations were provided by MycAgro Lab. (Bretenière, France) and the last one by C.C.S. Aosta Srl (Aosta, Italy). No treated plants served as a control. Details about inoculation are given below.

For each treatment, 30 ex vitro plantlets were considered. A dozen of ex vitro plantlets $treatment^{-1}$ were taken to evaluate the mycotrophic status of roots, the biomass production at the end of the acclimatization phase and the AMF colonization level during the cultivation cycle under greenhouse conditions. The remaining ex vitro plantlets $treatment^{-1}$ were used to assess the agronomical performance at the end of acclimatization phase, during the cultural cycle and at harvesting time of tuberous roots. Three replications were taken into account.

Plant biomass evaluation

At the end of the acclimatization phase, plantlets were evaluated for their fresh and dry weight and water content. Plantlets were cleaned up from soil residues and weighted to determine the fresh weight (FW). For dry weight (DW) evaluation, plantlets have been dried for 24-36 h at 80°C. Water content (WC%) was inferred from ((FW-DW)/FW) × 100.

Arbuscular mycorrhizal inoculation

The AMF treatments were applied according to a two-step inoculation method. The first inoculation took place at the beginning of the acclimatization phase. The ex vitro rooted plantlets were planted in an acclimatization substrate made of AMF inoculum: peat substrate (20% v/v). The second AMF inoculation was performed at transplanting to the greenhouse: about 10 g of inoculum was added into the soil of the planting hole of each plant.

Morphological observation of roots

The observations were carried out at the end of the acclimatization stage, one month after transplanting under greenhouse condition (vegetative phase of plant development)

and three months later on at flowering time. The roots free from soil were washed and stained with a solution of 0.1% cotton blue in lactic acid overnight and then de-stained washing the samples 6-8 times with lactic acid. The roots were cut into small fragments (about 1 cm each) and mounted onto microscope slides with some drops of lactic acid. About 90 fragments were observed for each sample. AMF colonisation intensity in the root cortex and arbuscule presence was determined according to Trouvelot et al. (1986). Mycorrhization frequency (the percentage of root fragments showing fungal colonization- F%), AMF colonization intensity (the percentage of the root system area showing fungal colonization – M%) and the presence of arbuscules (the percentage of the root system area showing presence of arbuscules – A%) percentages were calculated for each plant.

Statistical analysis

All data were analysed by means of analysis of variance (ANOVA) and post-hoc compared using the Tukey's test with PAST statistical program (version 3.01; Hammer et al., 2001).

RESULTS AND DISCUSSION

Mycorrhization efficiency

Morphological analysis on roots at the end of the acclimatization phase highlighted no presence of AM fungal structures in control plants (ex vitro plantlets untreated); at this stage, a great difference in the level of root colonization was detected among the three different AMF inoculum treatments. The highest colonization levels were observed for the treatments based on the GM single species inoculum (F%=55%) followed by the MIX inoculum composed by three different AMF species (F%=28.33%). The lowest values were registered in the plants inoculated with the MF inoculum (F%=5.33%) (Table 1). The quantity of fungal structure in the roots of plants treated with GM and MIX inocula showed a constant increment during the experiment, reaching about 90% frequency and 66% intensity at the flowering stage (22 weeks after first AMF inoculation and four weeks of culture under greenhouse conditions). This observation suggests a good symbiotic success of both GM and MIX inoculum that deeply colonized root apparatus of the plants where they have been inoculated. On the contrary, the application of MF inoculum results in poor root colonization levels confirming the trend presented at the end of the acclimatization (Table 1).

Growth parameters and agronomical performance

Dry weight at the end of the acclimatization phase was not affected by the AMF treatments. Vice versa, a somewhat influence on FW and WC% was observed as a function of the AMF applied (Table 2). In particular, compared to the control, GM and MIX treatments showed a little increase in WC% while for MF-treated plants a reduction of FW and WC% at the end of the acclimatization period was scored. This could account for the different root colonization levels (Table 1) since from literature (Gholamhoseini et al., 2013) it is known that sunflower plants colonized by *F. mosseae* and *Glomus hoi* showed an increased water uptake capacity. An improved AMF mediated water uptake capacity could be pivotal for reducing ex vitro plant water stress during acclimatization, favouring a higher acclimatization success and an incremented survival rate. However our results, in contrast with experiments on other micropropagated plants (Borkowska, 2002; Santos et al., 2010), showed no evidence of a significant increment in plant survival rate. The absence of a significant increment in ranunculus survival rate at the end of acclimatization could be ascribed to an already high percentage of plant survival due to a combination of plant genotype characteristics and well established acclimatization protocols (Table 2).

Table 1. AMF colonization levels for *Ranunculus asiaticus* ('Juny') plants according to Trouvelot et al. (1986). Different AMF inoculations were used: a single isolate inoculum containing *Funneliformis mosseae* (code: GM); a mix inoculum containing three isolates *F. mosseae*, *Rhizophagus intraradices* and *Glomus* sp. (code: MIX) and a composite inoculum containing AMF fungi propagules of *F. mosseae*, *R. intraradices*, *Viscospora viscosa* species and plant growth promoting rhizobacteria (code: MF). Untreated plants served as control. Observation were carried out at the end of the acclimatization phase (6 weeks after in vivo transfer); one month after transplanting under greenhouse conditions (vegetative phase of plant development) and three months later at flowering time.

AMF treatment	(F%)	(M%)	(A%)
		End of acclimatization phase	
Control	0	0	0
GM	55±13.2 b	24.05±0.6 b	8.51±3.6 a
MIX	28.33±15.3 ab	5.75±2.8 a	1.08±1.7 a
MF	5.33±9.2 a	4.73±8.2 a	3±5.1 a
		Greenhouse cultivation at vegetative phase of plant development	
Control	0	0	0
GM	92±6.9 b	71.1±8.1 b	67.8±9.9 b
MIX	52±18.3 a	26.2±18.3 a	21±14.3 a
MF	24±14 a	2.2±3.7 a	0.7±1.3 a
		Greenhouse cultivation at flowering phase	
Control	0	0	0
GM	85.3±10 b	66.6±13.8 b	65.8±13.9 b
MIX	91.8±7.3 b	66.9±22.8 b	60.9±29.6 b
MF	16±20 a	15±9.1 a	12.77±11.1 a

Means followed by the same letter do not differ significantly at $p<0.05$, according to Tukey's test. F% – mycorrhization frequency: percentage of root fragments showing fungal colonization; M% – mycorrhization intensity: percentage of the root system area showing fungal colonization; A% – arbuscule abundance: percentage of the root system area showing presence of arbuscules.

Table 2. Growth parameters of micropropagated *R. asiaticus* ('Juny') plantlets at the end of acclimatization phase. Ex vitro plantlets were treated with different AMF inocula (see text and Table 1) at the beginning of the acclimatization phase. Untreated ex vitro plantlets served as control. The acclimatization phase lasted 6 weeks.

	FW plantlet^{-1} (g)	DW plantlet^{-1} (g)	WC%	Plant survival (%)
Control	1.03±0.16 b	0.12±0.02 a	88.5 b	94.5 a
GM	1.29±0.17 b	0.13±0.02 a	89.5 c	95.6 a
MIX	1.20±0.13 b	0.13±0.02 a	89.1 bc	98.9 a
MF	0.84±0.13 a	0.11±0.02 a	87.0 a	96.7 a

Means followed by the same letter do not differ significantly at $p<0.05$, according to Tukey's test.

Plantlets shifted to greenhouse conditions started to show flower induction after 10 weeks. At this time, the percentage of plants with flower buds was different for the different treatments: 57% of MIX-treated plants were induced and this percentage was significantly higher compared to the control plants (37%) and GM inoculated plants (37%). MF-treated plants showed only 3.7% of flower induced plants. Two weeks later, about the full flower induction was reached for all the treatments, being the MF-treated plants the worst performing (Table 3).

Table 3. Percentage of flower induction for plantlets shifted to greenhouse after acclimatization. Data were scored after 10 weeks of culture under greenhouse conditions and flower induction was followed for a further two weeks. Plantlets were treated with different AMF inocula (see text and Table 1) at the beginning of the acclimatization phase and at transplanting to the greenhouse.

	Weeks (#) of greenhouse cultivation		
	10	11	12
Control	37.0±5.2 b	96.3±2.6 b	98.2±2.6 b
GM	37.0±6.9 b	88.9±4.54 b	98.2±2.6 b
MIX	57.4±6.9 c	96.3±2.62 b	100.0±0.0 b
MF	3.7±5.24 a	51.8±10.5 a	85.2±2.6 a

Means followed by the same letter do not differ significantly at $p<0.05$, according to Tukey's test.

Blooming started after 13 weeks of greenhouse culture and lasted 6 weeks. During this period, plants treated with MIX inoculum (334 flowers treatment^{-1}; 6.19 mean number of flowers plant^{-1}; stem length 34,5±0.3 cm) showed a better performance compared to the control (276 flowers treatment^{-1}; 5.80 mean number of flowers plant^{-1}; stem length 32,7±0.3cm) and GM plants (314 flowers treatment^{-1}; 5.81 mean number of flowers plant^{-1}; stem length 32,7±0.3 cm). Plants treated with MF inoculum showed the lowest flower production (232 flowers × treatment; 4.30 mean number of flowers plant^{-1}; 29,2±0,3 cm). At the end of the cultural cycle, no significant variations for tuberous root weight were detected among the different treatments tested (Table 4).

Table 4. Growth and flowering parameters of ex vitro *R. asiaticus* '*Juny*' plantlets during greenhouse cultivation phase.

	Flowers treatment^{-1} (#)	Flowers plant^{-1} (average)	Stem length (average; cm)	Tuberous roots weight plant^{-1} (g)
Control	276	5.80±0.80 a	32.7±0.3 b	6.7±2.6 a
GM	314	5.81±1.31 ab	32.7±0.3 b	6.9±3.0 a
MIX	334	6.19±0.05 b	34.5±0.3 c	7.8±2.9 a
MF	232	4.30±0.65 a	29.2±0.3 a	6.7±2.3 a

Means followed by the same letter do not differ significantly at $p<0.05$, according to Tukey's test. FW and DW = Fresh and dry weight plantlet^{-1}; WC%= water content (%) plantlet^{-1} (see Materials and Methods).

Interestingly, the overall positive effect of MIX inocula on ranunculus flowering is in accordance to previous findings which pointed out that AM fungi can improve plant commercial traits without increasing the need of fertilization (Scagel 2004; Garmendia and Mangas 2012). However, our experiment showed that inoculum formulations can differently colonize plant roots and affect the following growth. Particularly, GM inoculum, that in this study resulted to be the fastest root colonizer, showed no significant variations in the following plant growth parameters compared to control plants. On the other side, MF inoculum, that resulted unable to deeply colonize ranunculus plant roots, showed a detrimental effect on plant growth. Therefore, the combination of AMF species in each inoculum seems to represent a fundamental factor determining the final effect on plants. In particular, the presence of the same species (*F. mosseae*) in all the tested inocula, highlights that probably during the symbiosis a synergic effect occurred, allowing only one inoculum combination (MIX) to better perform over the other formulations (GM and MF). So far, the different performances of AMF inocula are often referred to a functional complementarity of the present species (Jakobsen et al., 1992; Smith et al., 2000). Therefore, is reasonable to affirm that fungal assemblages present in the MIX inoculum was probably the better assorted inoculum used in our experiment, although we cannot excluded that other AMF combinations could perform even better.

CONCLUSION

The results of this study confirm that *Ranunculus asiaticus* micropropagated plants can form arbuscular mycorrhizal symbiosis. This symbiotic relationship can influence ranunculus growth and flower production. Our data showed that AMF root colonization and the effect generated on plant growth can greatly vary, depending on the different commercial inoculum tested. As supported by previous literature (Mirabelli et al., 2009; Yadav et al., 2013), our findings confirm that the use of AMF inocula can be a powerful tool to improve the acclimatization phase and the further in vivo growth of ranunculus micropropagated plants.

However, the AMF formulations can perform differently. Future researches will focus on screening and selecting more targeted and performing inoculum formulations. With this aim, our goal is to widen the AMF species by rescuing new isolates from natural environments. The availability of a larger pool of characterized AMF species and the possibility to rearrange them in ad hoc inocula, will represent a further step toward a sustainable and environmentally friendly floriculture.

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