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Effect of Rhizosphere Bacteria and Endomycorrhizal Fungi on the Growth of Christmas Rose (*Helleborus niger* L.)

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Summary

Micropropagated and vegetative (by rhizome cuttings) propagated plants of Christmas rose (*Helleborus niger* L.) were tested for their responses to the inoculation with microorganisms in order to optimize root growth and development. In our experiment, plants were inoculated with arbuscular endomycorrhizal fungi or/and *Agrobacterium radiobacter*. The investigation indicated that inoculation with *A. radiobacter* had significantly positive effect on growth and development of plants multiplicated by *in vitro* techniques as compared to the dual inoculation. This study indicates that, biotization can be beneficial to plant growth in *in vitro* plant production systems but inocula have to be carefully selected for this purpose.

Key words. *Agrobacterium radiobacter* – biotization – growth – arbuscular mycorrhizal fungi – multi-microbal biotization – plantlets – rooting

Introduction

Christmas roses (*Helleborus niger* L.) are becoming very popular as late winter flowering perennials (Susek et al. 2005). Micropropagation is becoming the most widely used technique in a large scale production of Christmas roses because classical vegetative production based on rhizome cuttings is time-consuming and can not always guarantee success (Seyring 2002; Dhooghe and van Labeke 2007). Inadequate root development and activity are the most common causes for failure of young vegetatively propagated plants.

Plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal (AM) fungi are able to colonize plant roots and stimulate plant growth when applied to seed, tubers or roots (Vessey 2003; Smith and Read 2008). Recent studies indicate that improvement of plant growth and health can be achieved by either inoculating selected strains of beneficial microorganisms (separately or in combination) or by applying cultural practices that favour development of indigenous beneficial rhizosphere microbial populations in soils and suppress pathogenic ones (Binet et al. 2007; Avis et al. 2008).

We decided to investigate the possibility of introducing beneficial microorganisms (biotization) during the acclimatization period of *H. niger* plants propagated through rhizome cuttings and material obtained by *in vitro* multiplication. Microbial inoculation is intended to be an efficient and a relatively simple procedure, which should be incorporated into nursery practice. The main objective of this investigation was to identify beneficial rhizosphere microorganisms which are efficient in promoting root development and growth of Christmas roses.

Materials and Methods

Plant material

In the first experiment, Christmas roses (originating from Slovenian local populations) were propagated from mother plants which had been growing in the greenhouse for two years. New plants were obtained by vegetative propagation based on rhizome cuttings. These cuttings were transplanted into 1 L plastic pots filled with sterile substrate (peat: perlite: compost mix, (60: 20: 20, v:v:v)) and inoculated or not with beneficial rhizosphere microorganisms. Young developing plants were transferred directly in greenhouse conditions (18–25 °C day-night, 70 % relative humidity and natural photoperiod). During spring and summer, plants were watered daily with distilled water mixed with fertilizers (a commercial solution Plantprod® 20:20:20 N:P:K), whereas during autumn and winter, watering took place once or twice a week, depending on needs.

In the second experiment, the *in vitro* propagated plantlets (originating from French National Institute for Agricultural Research – Antibes) were transplanted from the agar medium into 200 ml plastic pots filled with sterile substrate (peat: perlite: compost mix, (60:20:20, v:v:v)), inoculated or not with beneficial microorganisms. The transplanted plantlets were placed into misting

tunnel (having 100 % relative humidity) for four weeks before being transferred to the greenhouse under the same conditions as in the first experiment.

Microorganisms

The experiments were conducted with commercial products of AM fungi and bacteria (M+ for endomycorrhizal fungi, A+ for *Agrobacterium radiobacter* and M+A for the mixture). A homologated commercial product of endomycorrhizal fungi (Endorize, Agrauxine) was incorporated in to the disinfected substrate at the rate of 5 % (v:v). The commercial *Agrobacterium radiobacter* strain K1026 (Nogall, Bio Care Technology) was applied to the substrate at the dilution of 10^6 cfu g^{-1}. This bacterial inoculum is recognized in Australia, USA and Europe to prevent crowngall.

Evaluation of growth variables

In vitro and *in vivo* propagated plants were harvested after 4 and 12 months of culture, respectively. Following the harvest survival rate and fresh biomass of whole plants and roots were recorded. After drying at 105 °C for 24 h, dry biomass of whole plants and roots was measured.

In order to evaluate endomycorrhizal infection, fine roots (1–3 g) were cleared in KOH 10 % for 1 h and stained with trypan blue (PHILIPPS and HAYMAN 1970). Mycorrhizal infection was assessed using the TROUVELOT et al. (1986) method. This method enables the evaluation of frequency of infection (F%):

$$F\% = 100(N-no)/N;$$

where 'N' represents the number of root fragments observed, and 'no' the number of root fragments without mycorrhizal colonization.

Experimental design and statistical analysis

The experiments with in vivo propagated plants were based on 40 plants for each treatment and biotization of *in vitro* propagated plants were based on 20 plants per treatment. Plants were arranged in a randomised block design, in four replications. Results were analysed by ANOVA (Analysis of variance) and compared using the Newman-Keuls test ($P < 0.05$).

Results

Endomycorrhizal infection

All inoculated *in vitro* and *in vivo* plants had a low level of infection (F % less than 5 %) whether they were inoculated or not with *A. radiobacter*.

Biotization of in vivo *plants*

Analyses of plant survival rates, which were evaluated after 12 months of growth, indicated that the highest survival rate was obtained for the endomycorrhizal inoculation (21 survival plants) but the differences among treatments were not significant (Table 1). There were also no significant effects on plant dry weight. However, *A. radiobacter* inoculation significantly increased the root/whole plant rate in comparison to that of control plants and to the binary mixtures containing endomycorrhizal fungi and *A. radiobacter* (Table 1).

Biotization of in vitro *plants*

Differences in the survival rate (between inoculated and non-inoculated individuals) of plants originating from the *in vitro* technique were observed after 4 months of growth in a sterile peat-perlite-compost substrate. *A. radiobacter* had the highest stimulatory effect on survival rate (the survival rate was 1.38 higher as compared to the control plants) (Table 2). The lowest percentage of survival of these plants was observed with the mixture of both microorganisms. The highest growth was obtained with *A. radiobacter* inoculation. *A. radiobacter* significantly ($P < 0.05$) stimulated plant and root growth compared to the inoculation with the mixture of microorganisms (Table 2). However, after the four-months period, there were no significant effects from the bacterial and endomycorrhizal inoculations on plant growth in comparison to the control treatment (Table 2).

Root/whole plant ratios were modified by inoculation with microorganisms. The root/whole plant ratio of *in vitro* plants inoculated with *A. radiobacter* and with both

Table 1. Survival rate and whole plant/root ratio of the Christmas rose plants developed by rhizome cuttings (*in vivo*) inoculated with *Agrobacterium radiobacter* (strain K1026) and a commercial product of endomycorrhizal fungi.

Treatment	Survival rate N = 40	Survival rate (%)	Dry weight of whole plant (g)	Dry weight of roots (g)	Root/whole plant ratio
Control	18 a[+]	45.0	8.93 a	7.47 a	0.83 bc
M+	21 a	52.5	9.30 a	8.28 a	0.88 ab
A+	20 a	50.0	12.76 a	11.54 a	0.91 a
MA+	17 a	42.5	10.95 a	9.09 a	0.81 c

[+] Values followed by the same letter are not significantly different ($P < 0.05$)
N: plant number; M+ inoculated with endomycorrhizal fungi; A+ inoculated with *Agrobacterium radiobacter*; MA+ inoculated with mixture of endomycorrhizal fungi and *Agrobacterium radiobacter*

Table 2. The effects of inoculation with *Agrobacterium radiobacter* (strain K1026) and a commercial product of endomycorrhizal fungi on Christmas rose plants developed by *in vitro* technique.

Treatment	Survival rate N = 20	Survival rate (%)	Dry weight of whole plant (mg)	Dry weight of roots (mg)	Root/whole plant ratio
Control	13 bc	65	53.2 ab	38.4 ab	0.73 a
M+	14 b	70	54.4 ab	37.4 ab	0.69 ab
A+	18 a	90	87.2 a	57.1 a	0.65 b
MA+	11 c	55	40.3 b	25.7 b	0.64 b

[+] Values followed by the same letter are not significantly different ($P < 0.05$)
N: plant number; M+ inoculated with endomycorrhizal fungi; A+ inoculated with *Agrobacterium radiobacter*; MA+ inoculated with mixture of endomycorrhizal fungi and *Agrobacterium radiobacter*

micro-organisms was significantly lower than the one of non inoculated plants (Table 2).

Discussion

The results reported in this paper show that inoculation with a single microorganism could have positive effects on the growth of *Helleborus* plants, especially on root growth as compared to the dual inoculation. The effects on plant growth generally depend on the type of microorganism and the microbial mixture introduced into the substrate (Dunabeitia et al. 2004). In the case of *Helleborus niger*, it seemed that microorganisms used separately were most efficient.

A. radiobacter was found to be stimulating the growth and the survival rate of plants, and could be considered as an effective plant growth promoting agent for Christmas rose plants multiplied by micropropagation technique (*in vitro*). The dry weight of a whole *in vitro* plant was significantly higher when compared to the inoculation with the microorganism mixture. In the same experiment, the survival rate of plants originated from the *in vitro* technique and growing in the substrate inoculated with *A. radiobacter* was 90 % (the survival rate of control plants was 65 %). The survival rate of plants multiplied by rhizome cuttings was lower (17 to 21 survival plants on 40 plants). Although the survival rates of different treatments were not significantly different, the highest values were obtained when plants were inoculated with one microorganism (A+ and M+ treatments). It is important to note that *A. radiobacter* and endomycorrhizal fungi mixtures may have negative effects on the rate of plant survival and plant growth. Similar effects were observed by Cordier et al. (2000) on micropropagated strawberries. Among the main reasons which could explain these results could be an unbalanced ratio between the release of organic substances by plants (mainly as root exudates which act as either signals or growth substrates) and root-associated biota (combination of two microorganisms).

The level of root colonization by AM fungi was low. This was not due to the quality of the inoculum as it was checked on other plants such as leek, raspberry and artichoke and gave satisfactory results (Gianinazzi et al. 2003). However, the 5 % dilution of the inoculum in the growth substrate might have been too high. In addition, the nutrient solution (NPK 20-20-20) used in this experiment has been shown to inhibit mycorrhization of raspberry plants, probably because of its high P content whilst a NPK 15-15-30 solution was compatible with root colonization by AM fungi (Gollotte et al. 2009). However, Christmas roses may also have a low mycorrhizal dependency, this would have to be checked under growth conditions adapted for AM fungi. Therefore, the application of multimicrobial substancies to plants requires knowledge and understanding of the (in)compatibility systems between different beneficial micro-organisms in their interactions within the mycorrhizosphere, rhizosphere, rhizoplane and the endorhizosphere.

The root/whole plant ratio could be modified by microorganisms for the plants obtained by micropragation. The ratio of inoculated *in vitro* plant was reduced. This has been already reported for other *in vitro* inoculated plant species (Guillemin et al. 1992). Inoculated plants showed a greater increase in shoot production than root production, so that micropropagated plant with beneficial micro-organisms possess a more efficient underground absorbing system (Binet et al. 2007, Kapoor et al. 2008, Padilla et al. 2006). This modification of root/whole plant ratio was not observed for Christmas rose plants multiplied by rhizome cuttings (*in vivo* plants). Moreover the biotization of *in vivo* plants with *A. radiobacter* (A+) increased the root/whole plant ratio. This change of biomass repartition is not favourable for the aerial shoot production of Christmas rose.

This study confirmes that plant biotization could be a promising technique to improve the quality of *in vitro* plants and to ensure an appropriate development within the context of sustainable horticulture (Guillemin et al. 1997, Cordier et al. 2000). Different responses to microbial treatments suggest that micro-organisms have to be screened before being used (Guillemin et al. 1992). Moreover, the application of multi-microbial biotization also requires the understanding of the (in)compatibility among different beneficial microorganisms and their interactions.

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