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Ex vitro Survival and Early Growth of *Alpinia purpurata* Plantlets Inoculated with *Azotobacter* and *Azospirillum*

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Abstract: The survival rate, shoot and root dry mass, shoot number, plant growth, stem height and diameter, number of leaves and root length were measured in micropropagated plantlets of *Alpinia purpurata* (Red ginger) inoculated with *Azospirillum* sp. 11B and *Azotobacter* sp. Pachaz 008 at 10^7, 10^8 and 10^9 cells cm^{-3} using a complete randomized experimental design. Inoculation of *A. purpurata* plantlets with the *Azospirillum* sp. 11B or *Azotobacter* sp. PACHAZ 008 strains induced larger stem diameter, root dry mass, number of shoots and increased their survival rate from 77 to 100% compared to plantlets without inoculation, while other plant characteristics were not affected.

Key words: *Alpinia purpurata*, *ex vitro*, micropropagation, *Azospirillum*, *Azotobacter*

INTRODUCTION

Red ginger (*Alpinia purpurata* Vieill) is a rhizomatous perennial plant from tropical America belonging to the Zingiberaceae family. *A. purpurata* is an exotic plant that grows in several parts of the world with an inflorescence very much sought after for its ornamental value (Rolf and Faria, 1995; Berry and Kress, 1991). Nevertheless, the market value of the plant is limited for its low natural propagation rate and due to some pests; in the field ants, banana aphids and mealy bugs (Hara *et al*., 1996) often damage the red ginger inflorescences. *In vitro* micropropagation might be an alternative technique to produce diseases-free plantlets for direct commercialization or germplasm storage (Cassalls and O´Herlihy, 2003; Dekkers *et al*., 1991). However, the low survival rate of the plantlets in the acclimatizing phase is still a major impediment limiting the use of *in vitro* culture propagation. *In vitro* culture of *A. purpurata* often results in the formation of plantlets with abnormal morphology, anatomy and physiology. After *ex vitro* transfer, these plantlets are easily impaired by sudden changes in environmental conditions and therefore need a period of acclimatization to correct abnormalities (Pospíšilová *et al*., 1999).

Baptisia tinctoria plantlets were inoculated with an arbuscular mycorrhizal fungi strains to increase their survival rate in the acclimatizing phase (Grotkass *et al*., 2000). Similar results were reported for the micropropagated grape (Hare Krishna *et al*., 2005). Srinath *et al*. (2003) found that micropropagated *Ficus benjamina* plantlets co-inoculated with *Glomus mosseae*, *Trichoderma harzianum* and *Bacillus coagulans*, were significantly higher with larger shoot, root and total plant biomass, larger shoot and root phosphorus content and more root colonization with *Glomus* compared to un-inoculated plants.

Larger accumulation of nutrients (Ca, Mg, Fe, B, Mn, Zn and Cu) in *Vicia faba* L. (faba bean) plants was a direct consequence of the growth-promoting effect of *Azotobacter* and *Azospirillum* strains in combined inoculation with *Rhizobium leguminosarum* (Rodelas *et al*., 1999). Additionally, shoot and root dry matter content significantly increased compared to control plants inoculated only with *R. leguminosarum*. These results indicate that inoculation of micropropagated plantlets with soil microorganisms can promote plant growth and their survival in the field. In this study, the effect of inoculation of *A. purpurata* with *Azotobacter* sp. Pachaz 008 and *Azospirillum* sp. 11B, two free-living

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nitrogen-fixing bacteria, on survival rate and plant growth during acclimatizing of plantlets obtained from micropropagation was investigated.

MATERIALS AND METHODS

Explants source and micropropagation: Inflorescences from *A. purpurata* Vieill plants were disinfected in 70% ethanol for 15 min, immersed in 3% aqueous calcium hypochlorite solution for 15 min and washed three times with sterile distilled water. The interbracteal buds were removed and placed in 150 ml bottles containing 30 mL Murashige and Skoog (1962) (MS) medium supplemented with sucrose (40 g dm^{-3}) and solidified with 2.6 g dm^{-3} Phytagel. Bottles with one explant each were incubated under cool white fluorescent light (45.8 μmol m^{-2} sec^{-1}) and 12 h photoperiod at 26-28°C for one month.

Shoots were transferred to similar bottles containing 30 cm^3 MS medium supplemented with sucrose 40 g dm^{-3}; myo-inositol, 0.1 g dm^{-3}; nicotinic acid, 1 mg dm^{-3}; pyridoxine, 1 mg dm^{-3}; thiamine, 1 mg dm^{-3}; glycine, 2 mg dm^{-3}; bencylaminopurine, 5 mg dm^{-3} and solidified with 2.6 g L^{-3} Phytagel (Tiwari *et al*., 2002). Bottles were incubated under cool white fluorescent light (45.8 μmol m^{-2} sec^{-1}) and 12 h photoperiod at 26-28°C for one month.

New shoots were transferred to newly prepared above mentioned medium after two and four weeks and then after two, three and four months. The plantlets were thus kept on the above mentioned MS medium for a total of four months. Plantlets were then rooted for one month in MS medium supplemented with 0.5 mg dm^{-3} A-Naphthalene Acetic Acid (NAA) and incubated under the same conditions.

***Ex vitro* inoculation:** Well-rooted plantlets were washed with water to remove the medium and then inoculated with different concentrations of *Azotobacter* sp. Pachaz 008 or *Azospirillum* sp. 11 B according to a completely aleatorized experimental design (Table 1). Ten plants for each treatment were used. A soil-agrolite mixture (3:1), previously sterilized in an autoclave at 1 kg cm^{-2} for 20 min (Declerck *et al*., 2002), was added to 500 cm^3 containers and the plantlets were planted in the mixture to acclimatize.

The plantlets were covered with a nylon mesh for two weeks to reduce water loss and irrigated with potable water each three days. One month after inoculation, plants were harvested, the soil washed from the roots and plant and stem height, stem diameter, number of leaves, root length, aerial and root mass dry, number of shoots and percentage of survival determined. Plant characteristics and survival rate were subjected to one-way analysis of variance using PROC GLM (SAS, 1989) to test for significant differences between treatments ($p<0.05$).

RESULTS

Azospirillum and *Azotobacter* induced a higher survival rate in micropropagated *A. purpurata* plantlets (Table 1). The survival of the plantlets was bigger when increasing the quantity of bacterial inoculums. The survival was increased from 13 to 23%, depending on the treatment, regarding the un-inoculated plantlets. The plantlets inoculated with 10^8 or 10^9 cells of *Azospirillum* had 100% of survival.

Likewise, the stem diameter of the plantlets inoculated with anyone of the two strains used in this study was bigger, between 25 to 52%, than that of the un-inoculated plantlets.

On the other hand, with the inoculation of 10^8 or 10^9 cells of *Azospirillum* was found that the stem diameter, dry mass of root and the number of buds were increased significantly; the other treatments did not show differences regarding the un-inoculated plantlets.

Finally, in the variables plant height, shaft longitude, root longitude, dry weight of the aerial part and number of leaves, did not meet any effect with the bacterial inoculation.

Table 1: Survival rate, plantlet height, stem diameter, root and stem length (cm), aerial and root dry mass (g) and number of leaves and shoots in *Alpinia purpurata* Vieill micropropagated plants inoculated with different numbers of *Azotobacter* and *Azospirillum* (n = 30)

	Inoculation rate	Survival rate	Plantlet highest	Stem diameter	Length (cm)		Dry mass (g)		No. of	
Bacterium	(cells cm^{-3})	(%)	(cm)	(cm)	Stem	Root	Aerial	Root	Shoots	Leaves
Azotobacter	10^{-7}	90	16.74	0.60	7.24	8.53	0.045	0.035	0.6	4.5
	10^{-8}	92	16.58	0.66	8.33	8.05	0.041	0.035	0.7	4.5
	10^{-9}	94	16.16	0.64	7.78	7.88	0.039	0.044	0.9	4.6
Azozpirillum	10^{-7}	96	16.64	0.65	7.31	6.90	0.038	0.043	2.5	5.3
	10^{-8}	100	16.52	0.70	7.33	7.49	0.032	0.067	1.9	4.5
	10^{-9}	100	16.25	0.73	7.43	8.45	0.032	0.071	2.0	4.6
None		77	15.32	0.48	7.04	6.84	0.031	0.043	0.5	4.3
Least significant difference ($p<0.05$)		6	1.47	0.10	1.53	2.00	0.016	0.019	0.6	1.2

DISCUSSION

The survival rate of 100% for inoculated plantlets in this experiment was higher than reported for other plants. A survival rate of only 70% was reported for *ex vitro* wild strawberry plants (Bhatt and Dhar, 2000). On average, 83% *in vitro* and *ex vitro* rooted plantlets of wild citrus trees (*Citrus halimii* Stone) survived after being transferred to a soil mixture consisting of soil, sand and organic material (1:1:1) (Normah *et al*., 1997). Ninety percent of *Acacia mearnsii* plantlets survived when acclimatized in transparent plastic containers under greenhouse conditions (Sascha *et al*., 1998).

Our method to increase survival of *A. purpurata* plantlets was less complicated than methods reported before, as it did not involve modifications of the *in vitro* conditions. Voráèková *et al*. (1998), for instance, used higher sucrose concentrations in the culture media of wheat (*Triticum aestivum*) and rape (*Brassica napus*) plantlets to increase survival rates. Additionally, non special culture conditions were required to increase survival rates, such as ventilation to promote *in vitro* hardening of micropropagated carnation shoots (Majada *et al*., 2000), or special light qualities as for strawberry (*Fragaria x ananassa* Duch.) (Duong *et al*., 2003).

For other hand, the increment in the dry weight of the root of the inoculated plants could explain its biggest rate of survival. This effect might be due to an increased uptake of minerals or N. Increased uptake of minerals in sorghum, wheat and soybean when inoculated with *Azospirillum* has been reported (Pacovsky *et al*., 1985; Bashan *et al*., 1990) and inoculation of wheat (*Triticum aestivium*) plants with *A. brasilense* increased plant growth by stimulating N uptake (Saubidet *et al*., 2002). At maturity, the inoculated wheat plants showed a larger biomass and grain yield, protein and N content than the un-inoculated, plants.

Rooting is an important factor to successfully acclimatize plantlets (Pruski *et al*., 2000). α-Naphthalene acetic acid (1.25-5.0 μM) has been added to *Phyllanthus urinaria* (Euphorbiaceae), a medicinal plant to promote rooting (Catapan *et al*., 2002). Regenerated plants were successfully acclimatized and 91% of the *P. urinaria* plantlets survived under *ex vitro* conditions (Catapan *et al*., 2002). The increased root mass is also beneficial for germplasm storage. For example, when *Miscanthus xogiformis* Honda Giganteus shoot cultures were stored *in vitro* on proliferation or rooting medium for up to 27 weeks, plants survived storage much 5 better on rooting medium than on proliferation medium (Hansen and Kristiansen, 1997).

Increases in root dry mass in teak plants (*Tectona grandis*) (Tiwari *et al*., 2002) has been related to the synthesis and excretion of plant-growth regulators and vitamins B in the rhizosphere by *Azospirillum* (Rodelas *et al*., 1993). The specific response of *A. purpurata* plantlets after inoculation with *Azospirillum*, but not with *Azotobacter*, could be indicative of specificity between the plant and the microorganisms (Mrkovaèki *et al*., 1997).

It was concluded that the survival rate of *A. purpurata* plantlets increased after inoculation with *Azospirillum* or with *Azotobacter*. This appeared to be related to the fact that the strains used, promoted root formation. As such, the inoculation of micropropagated plantlets with *Azospirillum* 11B or *Azotobacter* Pachaz 009 is a simple procedure to increase survival of *A. purpurata* plantlets for commercial purposes.

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