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BRIEF COMMUNICATION

Micropropagation and conservation of endangered species *Plantago algarbiensis* and *P. almogravensis*

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Abstract

Plantago algarbiensis and *P. almogravensis* are endemic Al tolerant species from the Western-centre of the Algarve region (South of Portugal) and Portuguese Southwest coast, respectively, which are in risk of global extinction. The aim of this work was to establish an efficient protocol to *in vitro* propagate these species using shoots obtained from *in vitro* germinated seeds. The best results in terms of multiplication response were afforded in Murashige and Skoog's (MS) medium supplemented with 6-benzyladenine (8.5 and 9.2 shoots per explant in *P. algarbiensis* and *P. almogravensis*, respectively). Shoots of both species showed a great rooting capacity (100 and 80 % for *P. algarbiensis* and *P. almogravensis*, respectively) that was not significantly influenced by the concentration of MS macronutrients or auxins. Plants were acclimatized to *ex vitro* conditions, exhibited normal development (survival rate of 95 and 80 % in *P. algarbiensis* and *P. almogravensis*, respectively), and were successfully reintroduced in their natural habitat.

Additional key words: acclimatization, Al hyperaccumulators, auxins, cytokinins, endemic species, plantlet production, rooting.

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Plantago algarbiensis Samp. and *P. almogravensis* Franco are endemic species from the Western-centre of the Algarve region (South of Portugal) and Portuguese Southwest coast, respectively. Both species are in risk of global extinction and were included in the Red List of Threatened Species (Walter and Gillet 1998). These species are legally protected under the European Habitats Directive 92/43/CEE and by the Portuguese law. The distribution of these species is restricted to a very small area and the number of individuals in each scattered population is very low.

Al-tolerant wild plants could be interesting sources of genes conferring tolerance (Ezaki *et al*. 2008). Recently, *P. almogravensis* was described as an Al hyperaccumulator (Branquinho *et al*. 2007). Results conducted with *P. algarbiensis* in *in vitro* cultures also indicate that this species accumulate Al (Martins 2008). Tissue culture techniques have previously been found useful in the selection of metal tolerant plants (Rout *et al*. 1999, Taddei *et al*. 2007, Gatti *et al*. 2008, Xu *et al*. 2008) and for their propagation in large-scale (Bidwell *et al*. 2001, Bhatia *et al*. 2002, Gatti *et al*. 2008, Xu *et al*. 2008). Moreover, micropropagation is a suitable tool for propagation of threatened species providing complementary conservation options (Filho *et al*. 2005, Kaliamoorthy *et al*. 2008, Dutra *et al*. 2008).

Micropropagation of other *Plantago* species, especially those with medicinal properties, was described in the literature (Mederos *et al*. 1997, Makowczyńska and Andrzejewska-Golec 2003, 2006, Budzianowska *et al*. 2004, Khawar *et al*. 2005, Makowczyńska *et al*. 2008), however, to the best of our knowledge, the *in vitro* propagation of *P. almogravensis* and *P. algarbiensis* has not been reported. Thus, the aim of this study was to establish an effective micropropagation protocol for these two species in order to produce large number of plants to reintroduce them in their natural habitat. Moreover, *in vitro* produced plants could be used in future to investigate in detail the Al accumulation capacity of these species.

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Abbreviations: BA - 6-benzyladenine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; KIN - kinetin; MS - Murashige and Skoog medium; ZEA - zeatin.

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Seeds of *P. algarbiensis* and *P. almogravensis* were collected from wild populations growing in Algoz and Odemira (Portugal), respectively. Collection was made from several plants to ensure the capture of a broad range of genetic variation. Seeds were surface sterilized with 15 % (v/v) commercial bleach (5 % sodium hypochlorite) and a few drops of *Tween-20* for 15 min and then washed three times in sterile water. Thereafter, seeds were germinated in test tubes containing 10 cm^3 of ¼ Murashige and Skoog (1962; MS) medium supplemented with 2 % (m/v) sucrose and solidified with 1 % (m/v) agar. pH was adjusted to 5.8 before autoclaving at 121 ºC and 1.1 kg cm^{-2} for 20 min. Cultures were incubated under a 16-h photoperiod at a photon flux density of 55 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps and a temperature of 25 ± 2 ºC.

After 2 months of germination, roots were discarded, and shoots were used in several multiplication assays. The effect of three cytokinins (6-benzyladenine - BA, kinetin - KIN and zeatin - ZEA) at 0.2 or 0.5 mg dm^{-3} was evaluated. MS medium without growth regulators was used as control. Shoots were cultured in 250 cm^3 Erlenmeyer flasks with 50 cm^3 of culture medium and capped with aluminum foil. After 6 weeks, shoot multiplication was assessed. In each assay 4 repetitions with 10 shoots each were tested.

For root induction, individual shoots with identical size, harvested at the end of the multiplication stage in MS medium with 0.2 mg dm^{-3} BA, were used. During this phase the effect of MS and ½ MS medium supplemented with 0.2 or 0.5 mg dm^{-3} indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) was evaluated. Shoots were grown in 500 cm^3 Erlenmeyer flasks, containing 80 cm^3 of medium, capped with aluminium foil and rooting was evaluated after 6 weeks. To test each medium 3 repetitions with 10 shoots each were used.

At the end of rooting period, plantlets of both species were removed from the culture flasks and the roots were cleared of agar to prevent pathogenic contamination. Plantlets were transplanted to 350 cm^3 plastic pots containing a mixture of peat and *Vermiculite* (3:1, v/v). The acclimatization took place in a plant growth chamber at 25 ºC and a 16-h photoperiod (100 µmol m^{-2} s^{-1}, *Grow-Lux F18W/GRO* lamps). The relative humidity, initially at 98 %, was decreased to 70 % during the acclimatization period. After 4 months of acclimatization micropropagated plants were planted in natural conditions.

The data were subjected to analysis of variance (*ANOVA*) with the general linear model procedure (*SPSS* statistical package for *Windows*, release *15.0*; *SPSS Inc*., Chicago, IL, USA) to assess treatment differences and interactions. Significant differences between means were determined using Duncan's new multiple range test (P = 0.05). Before analysis of percentage data, arcsin square root transformation was used.

After 2 months of germination 80 and 58 % seedlings were obtained for *P. algarbiensis* and *P. almogravensis*, respectively. Embryos grew into normal seedlings without morphological abnormalities and the entire shoots were used in the multiplication assays. Cultures of both species had a great multiplication capacity in all tested media even without cytokinins (Table 1, Fig. 1*A*,*B*).

Table 1. Effect of cytokinin type and concentration on multiplication rate, number of shoots and leaves and longest leaf length of *P. algarbiensis* and *P. almogravensis* shoots. Control - medium without growth regulators. Means ± SE of 4 replications with 10 shoots. *, **, ***: significant at $P < 0.05$, 0.01 and 0.001, respectively. Values followed by the same letter are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Plant species	Cytokinin	[mg dm^{-3}]	Multiplication [%]	Shoot number	Leaf number	Length [cm]
P. algarbiensis	control	-	55.00 ± 2.89 b	4.45 ± 0.69 b	27.91 ± 2.70 abc	5.33 ± 0.42 bc
	KIN	0.2	55.00 ± 6.45 b	4.00 ± 0.53 b	27.33 ± 3.46 abc	5.40 ± 0.53 abc
		0.5	57.50 ± 4.79 b	3.74 ± 0.44 b	26.73 ± 3.52 abc	4.62 ± 0.35 c
	ZEA	0.2	65.00 ± 2.89 b	4.04 ± 0.55 b	30.25 ± 3.75 ab	6.10 ± 0.43 ab
		0.5	67.50 ± 2.50 b	4.67 ± 0.60 b	33.57 ± 4.46 a	6.57 ± 0.38 a
	BA	0.2	80.00 ± 4.08 a	8.50 ± 1.09 a	19.00 ± 1.24 c	4.89 ± 0.38 bc
		0.5	82.50 ± 4.79 a	7.21 ± 0.96 a	22.79 ± 1.48 bc	5.25 ± 0.34 bc
P. almogravensis	control	-	65.00 ± 6.45 b	6.38 ± 1.28 a	36.58 ± 2.96 bc	4.13 ± 0.30 a
	KIN	0.2	77.50 ± 4.79 ab	5.74 ± 0.63 a	31.10 ± 1.86 c	3.81 ± 0.18 ab
		0.5	72.50 ± 8.54 ab	5.17 ± 0.66 a	35.86 ± 3.49 bc	3.07 ± 0.23 c
	ZEA	0.2	87.50 ± 4.79 ab	7.31 ± 1.10 a	45.86 ± 2.12 a	3.87 ± 0.21 ab
		0.5	67.50 ± 7.50 b	7.00 ± 0.76 a	35.85 ± 2.83 bc	3.36 ± 0.22 bc
	BA	0.2	90.00 ± 7.07 a	9.22 ± 1.15 a	44.22 ± 2.54 ab	3.44 ± 0.19 abc
		0.5	90.00 ± 5.77 a	7.11 ± 0.88 a	38.08 ± 3.39 abc	3.18 ± 0.28 bc
Plant species (A)			***	**	***	***
Cytokinin type (B)			***	***	**	***
Cytokinin conc. (C)			ns	ns	ns	ns
A × B × C			ns	ns	ns	ns

Table 2. Effect of MS macronutrients concentration (MS total and ½ MS) and auxins (IAA and IBA) on *in vitro* rooting, root number and longest root length of *P. algarbiensis* and *P. almogravensis* micropropagated shoots. Controls - media without growth regulators. Means ± SE of 3 replications with 10 shoots. Means followed by the same letter are not significantly different at $P < 0.05$ according to DMRT.

Plant species	Medium	Auxin	[mg dm^{-3}]	Rooting [%]	Root number	Length [cm]
P. algarbiensis	MS	control	-	96.67 ± 3.33 a	4.17 ± 0.36 c	23.85 ± 1.00 a
		IAA	0.2	93.33 ± 3.33 a	4.68 ± 0.53 c	13.11 ± 0.90 b
			0.5	96.67 ± 3.33 a	7.45 ± 0.74 b	12.29 ± 1.01 b
		IBA	0.2	96.67 ± 3.33 a	10.55 ± 1.25 a	13.61 ± 0.80 b
			0.5	96.67 ± 3.33 a	8.66 ± 0.73 ab	12.97 ± 0.77 b
	½ MS	control	-	93.33 ± 6.67 a	6.46 ± 1.15 b	11.81 ± 0.82 a
		IAA	0.2	93.33 ± 3.33 a	8.39 ± 0.80 b	9.75 ± 0.86 a
			0.5	96.67 ± 3.33 a	7.93 ± 0.94 b	9.57 ± 0.63 a
		IBA	0.2	96.67 ± 3.33 a	8.45 ± 0.79 b	11.52 ± 0.75 a
			0.5	100.00 ± 0.00 a	14.77 ± 1.89 a	9.41 ± 0.62 a
P. almogravensis	MS	control	-	46.67 ± 14.53 a	8.29 ± 1.86 a	5.95 ± 1.16 a
		IAA	0.2	43.33 ± 14.53 a	8.00 ± 1.74 a	7.77 ± 2.00 a
			0.5	66.67 ± 8.82 a	11.25 ± 1.86 a	6.26 ± 1.06 a
		IBA	0.2	63.33 ± 12.02 a	13.58 ± 1.92 a	6.07 ± 0.78 a
			0.5	63.33 ± 17.64 a	11.79 ± 2.07 a	4.77 ± 0.92 a
	½ MS	control	-	50.00 ± 10.00 a	8.27 ± 1.88 a	10.86 ± 1.59 a
		IAA	0.2	73.33 ± 8.82 a	12.91 ± 1.37 a	10.51 ± 1.08 a
			0.5	80.00 ± 5.77 a	14.79 ± 1.43 a	9.20 ± 1.07 a
		IBA	0.2	70.00 ± 0.00 a	16.57 ± 2.34 a	9.24 ± 1.00 a
			0.5	80.00 ± 11.55 a	12.58 ± 1.90 a	5.31 ± 0.88 b

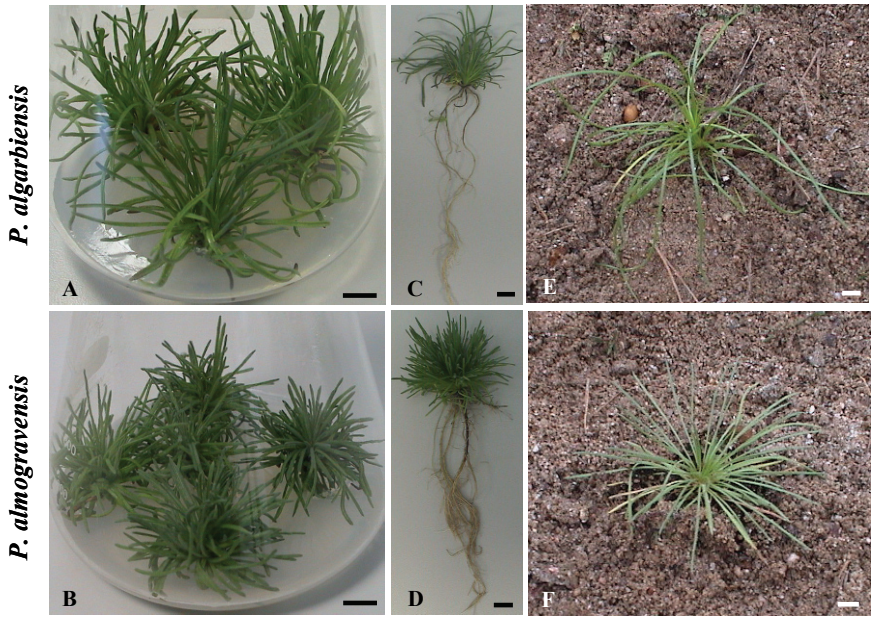

Fig. 1. Micropropagation of *P. algarbiensis* and *P. almogravensis*. *A*, *B* - shoots at the end of the multiplication phase (*bar* = 1 cm); *C*, *D* - rooted shoots after 6 weeks in ½ MS medium (*bar* = 1 cm); *E*, *F* - plants reintroduced in the nature (*bar* = 2 cm).

Regardless the cytokinin type and concentration, the multiplication percentage ($P < 0.001$) and the mean number of shoots ($P < 0.01$) were significantly higher in *P. almogravensis*.

In *P. algarbiensis* both multiplication percentage and mean number of shoots were significantly higher in media with BA, regardless the concentration (Table 1). As compared with the control, shoots of *P. almogravensis*

also showed a multiplication percentage significantly higher in media with BA, however, no significant differences were observed in the mean number of shoots (Table 1).

Regardless the multiplication medium, shoots of *P. algarbiensis* had longer leaves, while *P. almogravensis* had higher number of leaves. In *P. algarbiensis* no significant differences were observed between all the media tested and the control, regarding the mean number of leaves per shoot. Moreover, in this species only in medium containing 0.5 mg dm^{-3} ZEA the length of the longest leaf was significantly higher than the control. The mean number of leaves of *P. almogravensis* shoots cultured in medium with 0.2 mg dm^{-3} ZEA was significantly higher than the value obtained in the control, although no differences were observed between these results and those observed in media with BA. In relation to the leaf length, in this species differences were not observed between the control and the media containing 0.2 mg dm^{-3} KIN, ZEA and BA.

Spontaneous rooting was observed during the multiplication phase in both species. In *P. algarbiensis* rooting percentage was not influenced by the presence of cytokinins, while in *P. almogravensis* rooting was significantly higher in medium with 0.5 mg dm^{-3} KIN or 0.2 mg dm^{-3} BA. The good rooting results observed during multiplication are probably due to the high contents of endogenous plant growth regulators (Centeno *et al*. 1996). The produced plantlets could be directly acclimatized without passing through a rooting phase. Likewise, shoot production and rooting in one-phase was also observed for *Thapsia garganica* (Makunga *et al*. 2003), *Pinguicula lusitanica* (Gonçalves *et al*. 2008) and *Crithmum maritimum* (Grigoriadou and Maloupa 2008).

In order to improve the rooting frequencies fully developed shoots, with similar size, were selected and used in a range of rooting assays. As expected rooting occurred in the absence of auxins (control) and, surprisingly, auxins did not improve rooting significantly (Table 2). These results are not in agreement with those observed by Mederos *et al*. (1997) in *Plantago major* where no rooting was obtained in medium without auxins. However, similar results were reported by Grigoriadou and Maloupa (2008) in *Crithmum maritimum* where IBA at several concentrations did not improve rooting.

Contrarily to previous results in several species (Gonçalves and Romano 2005, Gonçalves *et al*. 2008, Zhang *et al*. 2008), in this study the concentration of MS macronutrients did not affect rooting frequency significantly (Table 2). As observed during shoot proliferation, *P. algarbiensis* showed higher rooting frequencies (93 - 100 %) when compared with *P. almogravenis* (47 - 80 %). The mean number of roots developed by *P. algarbiensis* shoots was significantly affected by MS strength and auxin type, with ½ MS and IBA being the most effective. The highest number of roots was afforded in ½ MS supplemented with 0.5 mg dm^{-3} IBA (Table 2). On the contrary, the longest roots were obtained in MS medium without auxins (Table 2). In *P. almogravensis* the mean number of roots was not affected by any of the factors tested whereas root length was significantly higher in ½ MS media, except when supplemented with 0.5 mg dm^{-3} IBA. In both species, the number and length of the roots were higher in shoots rooted during the rooting phase than in shoots spontaneously rooted during the multiplication phase (data not shown). Additionally, greater rooting frequencies were attained during the rooting assays. Therefore, we can conclude that an independent rooting phase must be included in the micropropagation protocols of both *Plantago* species.

In vitro produced plantlets (Fig. 1*C*,*D*) were transplanted to substrate in pots and higher survival rates were achieved after 6 weeks of transplantation (95 and 80 % for *P. algarbiensis* and *P. almogravensis*, respectively). The micropropagated plants produced in this work were successfully transferred into the field conditions exhibiting good performance (Fig. 1*E*,*F*). Plans are underway to reintroduce a high number of *in vitro* produced plants in selected locations in their natural habitat.

This work describes for the first time two effective protocols for the mass propagation of *P. algarbiensis* and *P. almogravensis* providing a valuable contribution to the preservation of these species.

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