

MICROPROPAGATION OF *PLANTAGO MARITIMA* L. – A VANISHING SPECIES IN POLAND

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ABSTRACT

A vanishing species in Poland – *Plantago maritima* L. was regenerated in vitro from tips of shoots (obtained in vitro) and from different explants of 4-week-old seedlings: seedling tips, hypocotyls, cotyledons, roots. Murashige and Skoog basal medium, supplemented with 0.6 μ M indole-3-acetic acid in combination with cytokinins 6-benzyladenine, zeatin or kinetin, was used. The plants obtained in the result of micropropagation were normal in appearance. It was proved that *Plantago maritima* species was amenable to propagation from different kinds of explants. The method may be of significance for protection of sea plantain.

KEY WORDS: micropropagation, organogenesis, Plantaginaceae, root regeneration, sea plantain.

INTRODUCTION

A salt marsh plant, herbaceous perennial, sea plantain – *Plantago maritima* L. (Plantaginaceae) is a vanishing species in Poland (category “vulnerable” in Polish Red Book), under compact active protection (Piotrowska 2001; www). Many localities of sea plantain disappeared long time ago as a result of drainage, destruction of salt marshes or cessation of grazing. *P. maritima* nowadays grows in Poland only at the mouth of the Odra River and Bays of Puck and Gdansk (Piotrowska 2001).

P. maritima is sometimes used as soothing expectorant and stomachicum (Liebezeit et al. 1999). The phytochemical studies have shown that this plant contains several interesting metabolites: iridoid glucosides (aucubin, mellitoside, rehmannioside D), phenylethanoid glycosides (echinacoside), flavonoids, tannins, polysaccharides, sorbitol (More et al. 1972; Ahmad 1979; Liebezeit et al. 1999; Rønsted et al. 2000; Grubešić et al. 2005).

To the best of our knowledge, there was not any original paper on culture in vitro of *P. maritima*. There is only

a summary from congress on in vitro biology described propagation of this species as a model to study the role of polyol accumulation in salt tolerance (Chang and Locy 1996). Moreover, Liebezeit et al. (1999), describing *P. maritima*, suggested only that this species “can be of potential interest to biotechnologists”. Our preliminary experiments with *P. maritima* in vitro were presented in 3rd Country Congress of Biotechnology in Poznań (Makowczyńska and Andrzejewska-Golec 2007).

MATERIAL AND METHODS

Sources of explants

– 4-week-old *Plantago maritima* seedlings from seeds provided by the Jardine Botanique de la Ville et de l’Université, Besançon (France), because of legal protection of *P. maritima* in Poland (see: Introduction). Seeds were surface-sterilized by immersion in 2.0% sodium hypochlorite for 10 min. and then germinated (MS supplemented with 0.9 μ M KIN and 2.9 μ M GA₃) as it was previously described for the seeds of *P. asiatica* L. (Makowczyńska and Andrzejewska-Golec 2003);
– shoots derived from the in vitro culture.

Kind of explants

– from seedlings: tips (approximately 0.5 cm long), hypocotyls (approximately 0.5-1.0 cm long), roots (approximately 1 cm long), cotyledons (cultured whole);
– from shoots: tips (approximately 0.5 cm long);

Abbreviations

BA – 6-benzyladenine; 2,4-D – 2,4-dichlorophenoxyacetic acid; GA₃ – gibberellic acid; IAA – indole-3-acetic acid; IBA – indole-3-butyric acid; KIN – kinetin; MR – multiplication rate, evaluated as the mean number of shoots (or also and buds) on one explant at the end of the multiplication cycle (6 weeks); MS – Murashige and Skoog (1962) medium; NAA – naphthaleneacetic acid; TDZ – thidiazuron; ZEA – zeatin

Conditions of in vitro culture

Conditions were the same as it had been successfully used previously by the authors for micropropagation of other *Plantago* genus taxa: *P. asiatica* L. and *P. camtschatica* Link: MS medium solidified with 0.7% agar; pH adjusted to 5.6-5.9; $26 \pm 2^\circ\text{C}$; light $40 \mu\text{M}/\text{m}^2\text{s}$; humidity 80-90% (Makowczyńska and Andrzejewska-Golec 2003; Andrzejewska-Golec and Makowczyńska 2008).

The cultures were continued in a growth cabinet for 6 weeks. For each treatment about 9-15 explants in 2 replicas were used.

Growth regulators used in experiments

– auxin: $0.6 \mu\text{M}$ IAA
 – cytokinins: 0.2, 0.4, 2.2, 4.4, 6.7, 8.9 μM BA – for shoots formed from tips of seedlings;
 2.2 and 4.4 μM BA – for shoots formed from tips of shoots cultured in vitro;
 0.9, 8.9, 22.2 μM BA, 0.9, 9.3, 23.2 μM KIN or 0.9, 9.1, 22.8 μM ZEA – for adventitious shoots regeneration from hypocotyls, cotyledons and roots

Rooting of shoots

MS alone or MS in combination with auxins: IAA, IBA or NAA (concentration – Table 3) were used.

Acclimatisation

Acclimatisation was conducted in flower pots for two months.

RESULTS

Shoot formation from seedling tips (Table 1; Fig. 1E)

The greatest multiplication rate MR of buds and shoots obtained by us for 6-week culture of seedlings tips of *P. maritima* was 8.8 ± 1.3 . However near 60% of buds and shoots were abnormal (less or more hydrous or deformed). The great MR of all shoots (normal and abnormal) was of 4.5 ± 0.9 , but in case of normal shoots – 3.8 ± 1 for concentration $6.7 \mu\text{M}$ of BA.

After the 6-week culture of seedling tips we observed roots only in low concentrations of BA: $0.4 \mu\text{M}$ – 15% and $0.2 \mu\text{M}$ – 50% rooted explants.

Shoots after 6 week culture obtained under 1.5 cm height.

Shoot formation on the tips of shoots derived from in vitro culture (Table 1; Fig. 1A)

We obtained 8.0 ± 1.5 MR of buds and shoots for tips of shoots derived from in vitro culture, but parts of buds and shoots were abnormal (less or more hydrous and deformed). MR of normal shoots was – 3.8 ± 0.9 . Shoots obtained on this way were higher than in case of seedling tips culture.

Shoot regeneration from hypocotyls, cotyledons, roots (Table 2; Fig. 1B-D)

We have obtained better results of MR for the hypocotyl, cotyledon and root explants than for tips. The highest MR – 28.3 ± 4.1 was obtained in case of root explants cultured on MS supplemented with $0.6 \mu\text{M}$ IAA and $22.8 \mu\text{M}$ ZEA. Adventitious shoots on these 3 kind of explants were regenerated directly and indirectly. Callus was formed especially on root explants: on 30-90% root explants, according to a kind and concentration of cytokinin (90% for $0.9 \mu\text{M}$ BA).

The rooting of shoots (without roots after 6-week culture) (Table 3)

The 4-week rooting was the most effective (nearly 90%) in the case of application of MS supplemented with auxin NAA in the concentration of $0.5 \mu\text{M}$, and the lowest (nearly 55%) – in the case of auxin IBA in the concentration of $2.5 \mu\text{M}$.

Acclimatisation

For two months of the acclimatisation in flower pots, 75% of plants regenerated from seedling tips and 40% of plants regenerated from other seedling explants survived. Plants blossomed in 2-month of the pot culture (Fig. 1E and F). Following adaptation the plants were transferred into the ground in the Garden of Medicinal Plants, Department of Pharmacognosy, Medical University of Łódź.

The plants obtained as a result of micropropagation were phenotypically normal with respect to leaf shape, spike constitution and growth features during growth stages (Fig. 1E).

TABLE 1. Effect of various concentration cytokinin BA and $0.6 \mu\text{M}$ IAA added to MS on shoot micropropagation of *Plantago maritima* from seedling tips and from shoot tips.

Kind of explants	BA (μM)	MR of buds and shoots $\pm\text{SE}$	Abnormal buds and shoots (%)	MR of all shoots $\pm\text{SE}$	Mean shoot length $\pm\text{SE}$ (cm)	MR of normal shoots $\pm\text{SE}$
Seedling tips *	0.2	6.1 ± 1.6	40.3	3.4 ± 0.7	1.8 ± 0.3	3.2 ± 0.7
	0.4	2.8 ± 1.3	58.8	0	0	0
	2.2	8.8 ± 1.3	58.5	2.9 ± 0.7	1.6 ± 0.2	2.3 ± 0.6
	4.4	6.0 ± 1.0	64.3	3.3 ± 1.1	2.0 ± 0.2	2.6 ± 0.8
	6.7	7.5 ± 1.5	58.3	4.5 ± 0.9	1.6 ± 0.1	3.8 ± 1.0
	8.9	3.8 ± 0.9	50.0	4.3 ± 0.3	1.7 ± 0.3	2.7 ± 0.3
Shoot tips *	2.2	8.0 ± 1.5	37.0	4.2 ± 0.9	2.7 ± 0.2	3.8 ± 0.9
	4.4	5.9 ± 1.1	64.5	3.0 ± 0.8	2.7 ± 0.5	2.0 ± 0.4

SE – standard error of the mean

For each treatment about 20-30 explants were used

* see: Material and methods

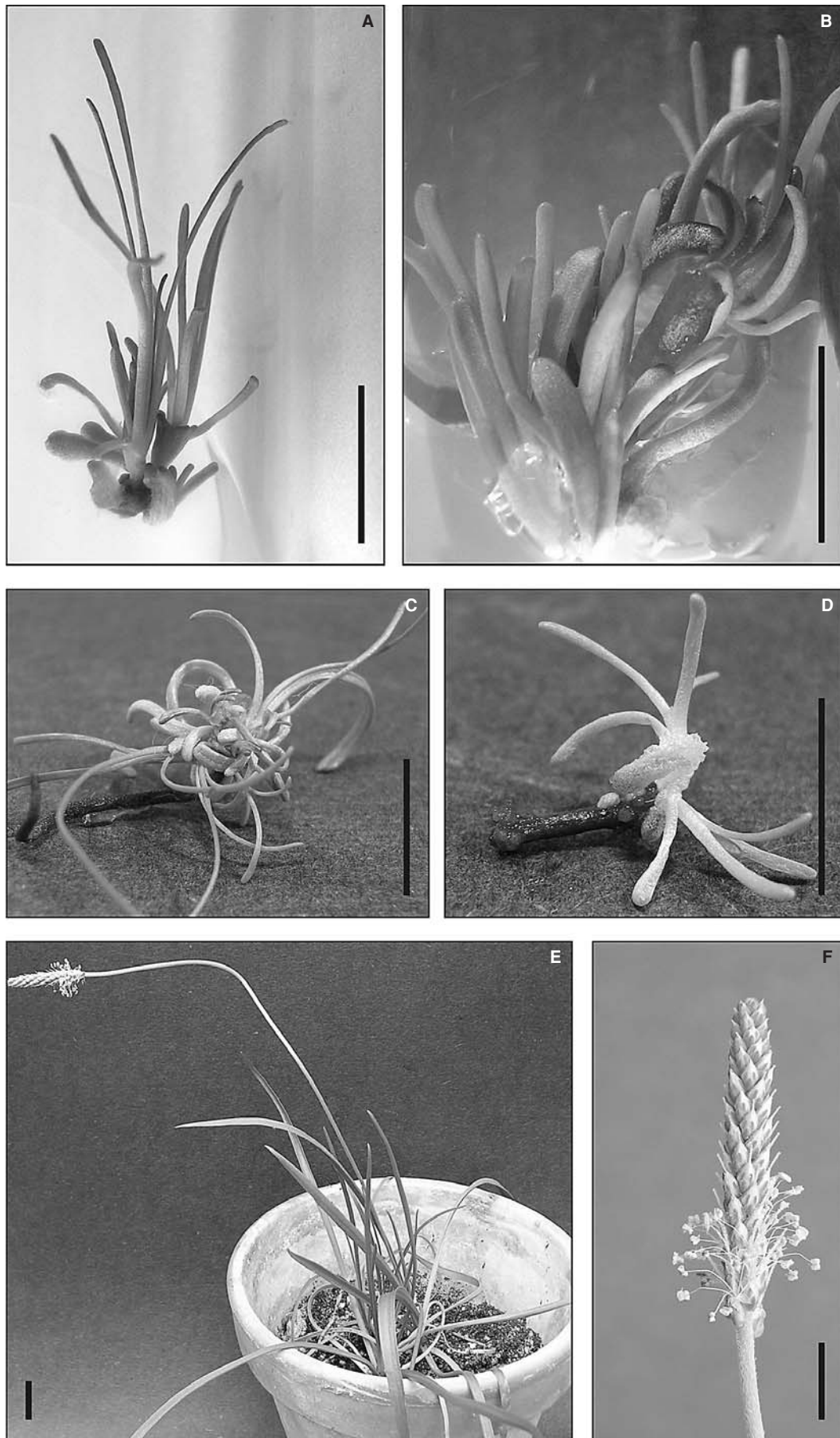


Fig. 1. In vitro propagation of *Plantago maritima* L. A – micropropagation via shoot-tip method. MS + 2.2 μ M BA + 0.6 μ M IAA; B – organogenesis on the cotyledon. MS + 8.9 μ M BA + 0.6 μ M IAA; C – organogenesis on the cotyledon. MS + 2.2 μ M BA + 0.6 μ M IAA; D – organogenesis on the hypocotyl. MS + 2.2 μ M BA + 0.6 μ M IAA; E – 2-month blooming plant in vitro regenerated from seedling tips; F – spike of these plant (E). Bar 1 cm.

TABLE 2. Effect of cytokinin and kind of explants on shoot micropropagation from various explants of *Plantago maritima*. MS supplement with 0.6 µM IAA and various kind of cytokinins.

Kind of cytokinins	µM	Root			Hypocotyl			Cotyledon		
		Explants with buds and shoots (%)	MR of buds and shoots ±SE	Buds and shoots regenerated indirectly (%)	Explants with buds and shoots (%)	MR of buds and shoots ±SE	Buds and shoots regenerated indirectly (%)	Explants with buds and shoots (%)	MR of buds and shoots ±SE	Buds and shoots regenerated indirectly (%)
		BA	100	18.2±2.9	93.4	95.0	21.9±3.1	22.4	85.0	24.6±3.7
	100	9.2±1.8	62.0	95.0	10.1±1.7	38.2	65.0	19.6±4.0	0	
	75.0	7.3±1.3	100	75.0	6.1±1.0	2.2	70.0	15.3±2.8	5.6	
KIN	0.9	100	16.0±1.9	47.3	100	14.9±1.9	85.7	14.8±2.5	6.8	
	9.3	100	13.6±2.2	41.1	100	10.6±1.5	85.7	17.8±3.1	0	
	23.2	95.5	13.6±2.1	22.5	78.9	12.0±0.7	73.9	18.9±5.8	2.2	
ZEA	0.9	95.5	13.9±2.4	64.0	100	13.5±1.7	100	15.8±2.6	9.2	
	9.1	100	22.5±3.1	62.4	95.0	13.3±0.9	95.0	14.0±2.7	0	
	22.8	100	28.3±4.1	50.4	100	14.4±1.3	95.0	19.6±2.4	12.6	

SE – standard error of the mean

For each of treatment about 16-30 explants were used

TABLE 3. Percentage of the adventitious shoot rooting. MS medium

Kind of auxin (µM)	Week of culture			
	1	2	3	4
Control without auxin	16.2	41.9	45.2	48.4
IAA	0.6 2.9	33.3 27.6	75.0 55.2	75.0 62.1
IBA	0.5 2.5	46.9 6.5	65.6 35.5	75.0 41.9
NAA	0.5 2.7	27.6 0	82.8 61.3	86.2 67.7

DISCUSSION

There is a little information in the literature on in vitro culture of *P. maritima*. It is only a short summary from Congress on In Vitro Biology in San Francisco in 1996 year (Chang and Locy 1996). Authors regenerated *P. maritima* exclusively on leaf explants. Instead we multiplied this plant from different explants: seedling tips, shoot tips, roots, cotyledons and hypocotyls. Chang and Locy (1996) used MS basal salt, Gamborg B5 vitamins, 2% sorbitol, 0.6 µM NAA and 8.9 µM BA for direct regeneration of adventitious shoots on leaf explants. The optimum medium for callus growth contained the same basal salt and vitamins with 2% sucrose, 4.5 µM 2,4-D, 2.3 µM KIN and 1g/l activated carbon. Callus transferred to the same medium containing 5 g/l activated carbon readily regenerated shoots. They obtained plants normal in appearance. These plants flowered and produced normal fertile seeds.

In our work multiplication of *P. maritima* through seedling tips is first described, but seedling tips were used with success for multiplication of another taxa of the genus *Plantago*: *P. asiatica* L., *P. camtschatica* Link, *P. ovata* Forsk. (Barna and Wakhlu 1988; Pramanik et al. 1995; Makowczyńska and Andrzejewska-Golec 2003; Andrzejewska-Golec and Makowczyńska 2008). In case of multiplication of these three plantain, as in our present work, also MS medium with cytokinin BA and auxin IAA (and also KIN or NAA) were used. The best effect (MR = 18) obtained Pramanik et al. (1995) for *P. ovata* in case MS with 1.1 µM IAA and 22.2 µM IBA. In our previous study concerning *P. camtschatica* a better effect was obtained for the culture of tips of shoots derived from in vitro than for culture of seedling tips (Andrzejewska-Golec and Makowczyńska 2008). Now we obtained similar effects for seedling tips and shoot tips of *P. maritima*. We have obtained better results of MR for hypocotyl, cotyledon and root explants than for tips. The best effect (MR = 28.3±4.4) we obtained in case of root explants cultured on MS supplemented with 0.6 µM IAA and 22.8 µM ZEA. Khawar et al. (2005) obtained MR near 25 for cotyledon and near 19 for hypocotyl in case of *Plantago lanceolata* (MS supplemented with 1.34 µM NAA and 3.33 µM BA). Instead Budzianowska et al. (2004) on average 4.38 new rosetts from leaf segments and 4.0 from root explants obtained for *P. lanceolata* (MS medium supplemented 11.42 µM IAA and 9.29 µM KIN). Sarihan et al. (2005) used the hypocotyl explant of *P. afra* L. and obtained MR of 23.17

(MS supplemented with 0.91 μM thiazuron TDZ and 0.98 μM IBA).

Adventitious shoots on the explants of *P. maritima* were regenerated directly and indirectly. Also *P. asiatica* was regenerated directly and indirectly from hypocotyls, cotyledons and roots (Makowczyńska 2006; Makowczyńska et al. 2008).

A very high multiplication result (68.5 shoots per explant) in the case of first nodes from the top healthy shoots (derived from hypocotyl explants) used as explants cultured on MS with 0.45 μM TDZ and 0.09 μM IBA from other species of the genus *Plantago* – *P. lanceolata* L. was obtained by Khawar et al. (2005). Shoots were formed here only indirectly.

On the part of the shoots of *P. maritima*, obtained as a result of organogenesis during 6-week culture, adventitious roots developed spontaneously. This phenomena was often observed during micropropagation of the genus *Plantago* taxa (Makowczyńska 2006; Andrzejewska-Golec and Makowczyńska 2008). Rooting of the shoots during shoot micropropagation allows to omit the stage of rooting after the shoot micropropagation.

A great part of the shoots obtained for micropropagation of *P. maritima* were abnormal. Observed by us hydrous shoots it is abnormality often observed in in vitro culture (Wawrosch et al. 1999; Piątczak and Wysokińska 2003). This anomalous behaviour can be attributed to the excess of the growth regulators in the explants or supplied by the medium (Wawrosch et al. 1999). These anomalous shoots were rejected and only well-developed shoots were used for rooting and acclimatisation and plant obtained from there shoots were phenotypically normal.

Survival young plants of *P. maritima* was comparatively high and dependent on explant sources. Survival of the *P. camtschatica* was lower, but also dependent on sources of explants: 14% for plants regenerated from seedling tips and 27% for plants regenerated from other seedling explants (Andrzejewska-Golec and Makowczyńska 2008).

We have successfully demonstrated an efficient and reliable procedure for regeneration of *P. maritima* via different explants. This species was amenable to propagation from different kind of explants. Micropropagation of this plant may be an alternative method of its culture for different purposes. This method may have significance for protection of sea plantain. Micropropagation as alternative cultivation system for other vanishing plants among others in case of *Atropa belladonna* L., *Centaurium erythraea* Rafn, *Drosera* sp., *Gentiana* sp. (Zenkteler 1971; Momčilović et al. 1997; Królicka et al. 2001; Piątczak and Wysokińska 2003) was described.

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