PROLIFIC ADVENTITIOUS SHOOT REGENERATION FROM BLACK PSYLLIUM (*PLANTAGO AFRA* L.)

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Abstract. Adventitious shoots were regenerated from *in vitro* cultured cotyledon and hypocotyl explants excised from one-week-old seedlings of *P. afra* on MS medium, containing various concentrations of BAP + NAA, BAP + IBA, Kinetin+IBA and TDZ+IBA. Multiple shoot regeneration was observed in both explants, with highest shoot regeneration per hypocotyl explant (23.17) in MS medium containing 0.91 µ M TDZ – 0.98 µM IBA. Shoots regenerated in MS medium containing 0.91 µM TDZ – 0.98 µM IBA were best rooted in MS medium containing 3.22 µM NAA. Rooted plantlets were transferred into soil and after acclimatization were transferred to the greenhouse for flowering and seed set.

***Keywords*:** *Plantago afra, in vitro* shoot regeneration, rooting.

***Abbreviations*:** BAP: 6 benzylaminopurine, IBA: Indole 3 butyric acid, MS: Murashige and Skoog basic salts and vitamins, TDZ: Thidiazuron.

INTRODUCTION

Plantago afra, or black psyllium, belonging to the Plantaginaceae family, is an annual stemless herb with leaves, arranged alternately in a basal rosette, from the centre of which an erect not visible spike arises. It has wind-pollinated flowers. Black psyllium is native to the western Mediterranean region, North Africa, and West Asia and is now being cultivated in Southern France and Spain (Budavari, 1996; Leung and Foster, 1996; Wichtl and Bisset, 1994).

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The World Health Organization (WHO) has published a monograph on psyllium seed, covering *P. afra, P. indica, P. ovata, and P. asiatica* (WHO, 1999). Constituents of the seeds include 5–10% lipids with unsaturated fatty acids, sterols, and mucilaginous polysaccharide (10–15%), consisting of xylose, galacturonic acid, arabinose, and rhamnose residues (Bruneton, 1995; ESCOP, 1997).

Psyllium has a long history of medical use in both conventional and folk-traditional systems of medicine throughout Asia, Europe, and North America (Bradley, 1992). The seed mucilage is believed to act as a soothing lubricant and to absorb toxins from the digestive tract. In folk therapies throughout the world, it is used to treat mainly chronic constipation, diarrhea and alleviate problems of bladder, kidney, urethritis and hemorrhoids (Newall et al., 1996). It is also believed to lower blood cholesterol, when included in a diet, and the seeds - to regulate intestinal peristalsis (Wichtl and Bisset, 1994). Psyllium seed also lowers blood cholesterol levels and LDL cholesterol in cases of hyper-cholesterol, without significant changes in triglycerides and HDL cholesterol. It is also believed that the seed reduces peak levels of blood glucose by delaying intestinal absorption of sugar (ESCOP, 1997).

Very limited literature on *Plantago* tissue culture is available (Mederos 1994; Mederos et al., 1997-98; Khawar et al., 2005). The aim of the present study was to optimize conditions for morphogenesis from hypocotyl and cotyledon explants of black psyllium for subsequent usage in various development programs.

MATERIALS AND METHODS

Seed was given from Prof. Dr. Neşet Arslan, Department of Field Crops, University of Ankara, Turkey. Seeds were surface sterilized with 20% commercial bleach (Axion, Turkey) for 4 minutes, without using magnetic stirrer. Thereafter, they were rinsed 3 times with sterile distilled water. Only vigorous and healthy seeds were used for germinating in MS medium (Murashige and Skoog, 1962), supplemented with 3% sucrose, 0.7% agar (Sigma agar type A), contained in 100 X 10 mm Petri dishes sealed with Stretch film ® and germinated for one week.

The regeneration medium consisted of 2.22 or 1.11 м M 6-benzylaminopurine (BAP) and 1.34 µ M Naphthalene acetic acid (NAA), 1.11 µ M BAP with 0.98 or 0.09 м M IBA, 1.16 µ M Kinetin with 0.98 or 0.09 µ M IBA and 0.91 µ M TDZ with 0.98 or 0.9 µ M IBA in MS media (Table 1). Scoring was done after 8 weeks of culture being contained in Magenta (GA7®) vessels. Regenerated shoots (10-20 mm in length) from media containing 0.91 µ M TDZ with 0.98 µ M IBA were rooted in MS medium, containing 1.07, 2.15, 3.22, 4.30 or 5.37 µ M NAA (Table 2).

The pH of each medium was adjusted to 5.6-5.8 with 1N NaOH or 1N HCl, before the addition of agar, and autoclaving under the pressure of 1.4 kg/cm^2 at 121^{o}C for 20 minutes. All experiments were carried out under sterile conditions.

Table 1: Mean values of shoot regeneration from cotyledon and hypocotyl explants of *P. afra* after 8 weeks of culture, per replication in MS medium containing various concentrations of BAP and IBA.

Plant growth regulators (mg/l)		**Mean number of shoots per replication**	
BAP (µM)	NAA (µM)	Cotyledon	Hypocotyl
2.22	1.34	9.32[1] ab[2]	11.00 bc
1.11	1.34	0.42 d	9.75 bc
BAP (µM)	IBA (µM)		
1.11	0.98	0.33 d	7.33 c
1.11	0.09	2.00 c	2.00 c
Kinetin (µM)	IBA (µM)		
1.16	0.98	1.82 c	1.17 d
1.16	0.09	2.58 c	2.67 cd
TDZ (µM)	IBA (µM)		
0.91	0.98	13.17 a	23.17 a
0.91	0.09	3.67 bc	21.92 a
Control (MS medium)		0.00 d	2.92 c

[1] Each value is the mean of 4 replications with 5 explants.
[2] Values within a column followed by different letters are significantly different at 0.01 level of significance using Duncans Multiple Range Test.

Table 2. *In vitro* rooting of *P. afra* using various concentrations of NAA.

NAA (µM)	Frequency of root regenerating explants (%)	Mean number of regenerated roots/explant	Mean shoot length (cm)
1.07	58.33[1] bc[2]	1.25 d	2.33 b
2.15	33.33 c	1.00 d	3.00 a
3.22	83.33 ab	5.75 a	3.33 a
4.30	91.67 a	2,75 b	2.33 ab
5.37	58.33 bc	2.25 c	1.33 b

[1] Each value is the mean of 4 replications with 4 explants.
[2] Values within a column followed by different letters are significantly different at 0.01 level of significance using Duncans Multiple Range Test.

All cultures were incubated at 24 $\pm$ 2 ^{o}C, provided by Sylvania ^{R} Grolux fluorescent tubes, giving light intensity of 42 mol.m^{-2} s^{-1} and photoperiod of 16 h in the growth chamber.

Statistical analysis

Each treatment, containing 5 explants in regeneration and 4 explants in rooting experiments, was replicated 4 times and repeated twice. Significance was determined by one-way ANOVA, using SPSS for Windows (v. 11. SPSS Inc USA). Data given

in percentages were subjected to arcsine (√X) transformation (SPSS v. 11) before statistical analysis and differences between the means were compared using Duncan's multiple range test.

RESULTS AND DISCUSSIONS

Callus formation and precocious root development

Roots precocious development during callus formation is undesirable in tissue culture, which not only reduces the quality of callus, but also hinders shoot regeneration. Precocious roots or root initials were observed during early growth stages in both explants in MS media, containing 1.11 or 2.22 µ M BAP with 1.34 µ M NAA before the development of shoot primordia. Under aseptic conditions, they were removed to achieve callus (Fig 1a) of good quality and better shoot regeneration in both explants. No precocious rooting was observed in regeneration medium, containing BAP-IBA, Kinetin-IBA or TDZ-IBA. It was concluded that the presence of BAP with NAA promoted precocious root development in black psyllium, which could be suppressed by replacing NAA with IBA. Results are in agreement with Khawar et al. (2005), who recorded similar observation in *P. lanceolata.*

Shoot regeneration

Concentrations of different plant growth regulators in the media significantly ($p<0.01$) influenced shoot regeneration from cotyledons and hypocotyl explants in concentration and explant dependent manner. Callus formation was observed in all media with subsequent shoot development. Shoot primordial were observed in all media from both explants with considerable growth after 15 days (Fig 1 b). Mean number of shoots per explant ranged from 0.33 to 13.17 in cotyledons, and from 1.17 to 23.17 in hypocotyl explants after 30 - 45 days (Fig. 1 c, d; Table 1). It was found that 2.22 µ M BAP with 1.34 µ M NAA promoted, and 1.11 µ M BAP with 1.34 µ M NAA suppressed shoot regeneration of cotyledon and hypocotyls explants. 1.11 µ M BAP with 0.98 µ M IBA or 1.16 µ M Kinetin with 0.98 µ M IBA were suppressive compared to 1.11 µ M BAP with 0.09 µ M IBA or 1.16 µ M Kinetin with 0.09 µ M IBA, which promoted shoot regeneration. In case of TDZ, 0.91 µ M TDZ with 0.98 µ M IBA regeneration was promoted, and 0.91 µ M TDZ with 0.09 µ M IBA inhibited shoot regeneration.

No regeneration was observed in cotyledons, and 2.92 shoots per explant were noted in hypocotyls under no treatment in MS medium. However, shoot regeneration was better on hypocotyls over cotyledons in all media showing that hypocotyls had better regeneration potential.

Through a somatic embryogenesis, *In vitro* plant regeneration of *P. ovata* was

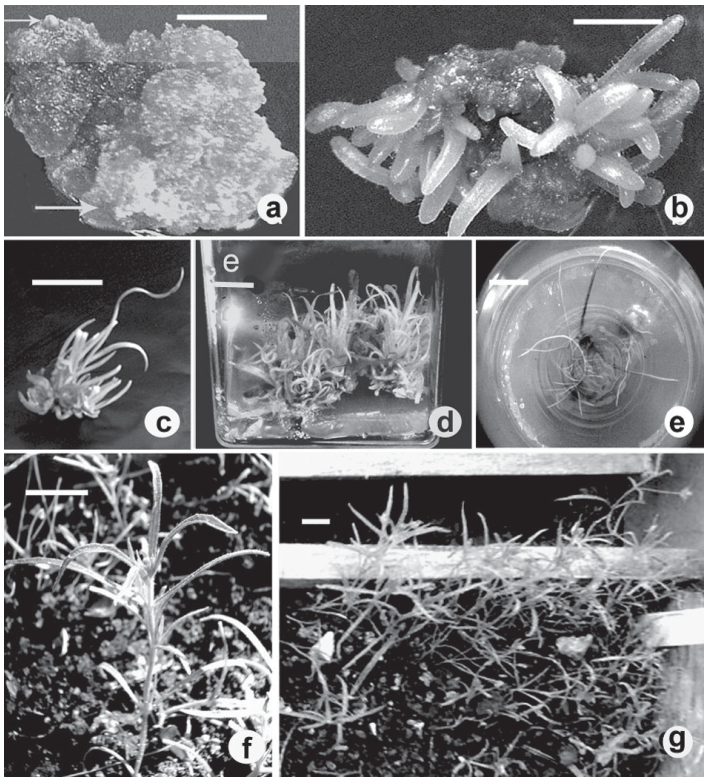

Fig.1. Shoot proliferation of *P. afra* (a) development of callus from hypocotyls (b), shoot primordia from callus after 15 days of culture, (c) adventitious shoot development after 30 and (d) 45 days of culture (e), rooting in MS medium containing 3.22 µM NAA (f, g) adaptation under greenhouse conditions. Bar = 1 cm.

achieved by Das and Sen-Raychaudhuri (2001). Casein hydrolysate and coconut water were used in different concentrations in MS medium along with 1-naphthaleneacetic acid and N^6 benzyladenine to increase the amount of callus and number of somatic embryos. Results indicated that optimum concentrations of casein hydrolysate and coconut water are useful for promoting the growth of embryogenic cultures. The appliance of additives, such as coconut water and casein hydrolysate, promoted large-scale *in vitro* somatic embryogenesis of *P. ovata*. Makowczynska and Andrzejewska (2000) observed the early stages of direct somatic embryogenesis in *P. asiatica* using light microscopy and found two simultaneous phenomena in calli - somatic organogenesis and somatic embryogenesis. Similarly, Khawar et al. (2005) recorded shoot regeneration from hypocotyls and cotyledon explants of *P. lanceolata* under various concentrations of BAP+IBA.

Rooting

Healthy growing green shoots regenerated from hypocotyl (from 0.91 µM TDZ + 0.98 µM IBA) were rooted under all 5 combinations of NAA in MS media (Table 2). No carry over effect of TDZ was observed and it was not difficult to root the shoots. However, the highest rooting per shoot (5.75) was observed in MS medium containing 3.22 µM NAA (Fig. 1 e) with mean shoot length of 3.33 cm and frequency of 83.33%. This was followed by 2.75 roots with mean root length of 2.33 cm in MS medium containing 4.30 µM NAA. Rooted plantlets were very difficult to handle because of their fragile roots. It was also difficult to remove agar from the roots of rooted shoots (plantlets). A great care was taken during this process to avoid any damage of the roots. Nevertheless, only 35 % plantlets could be successfully transferred to soil mix in wooden crates. All of the plants transferred were easily acclimatized and were transferred to the greenhouse (Fig. 1 f, g) for flowering and seed set.

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