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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 *Plantago afra* on MS medium containing various concentrations of BAP + NAA, BAP + IBA, Kinetin + IBA and TDZ+IBA. Multiple shoot regeneration was observed from both explants with the highest shoot regeneration per explant (23.17) on MS medium containing 0.91 µM TDZ – 0.98 µM IBA from hypocotyls. Shoots regenerated on MS medium containing 0.91 µM TDZ – 0.98 µM IBA were best rooted on MS medium containing 3.22 µM NAA. Rooted plantlets were transferred to soil, acclimatized and transferred to the greenhouse for flowering and seed set.

Key Words: *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, family Plantaginaceae is a stem less annual herb with leaves arranged alternately in a basal rosette from the centre of which grows an erect non showy spike with wind pollinated flowers. Black psyllium is native to the western Mediterranean region, North Africa, and West Asia and is now cultivated in southern France and Spain (Budavari, 1996; Leung & Foster, 1996). According to the monograph published by World Health Organization (WHO, 1999) on psyllium seed covering; *P. afra*, *P. indica*, *P. ovata*, and *P. asiatica*, the seeds constituents include lipids with unsaturated fatty acids (5-10%), 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 great medical use in both conventional and traditional systems of medicine throughout Asia, Europe, and North America (Bradley, 1992). Limited literature on Plantago tissue culture is available (Mederos 1994; Mederos *et al*., 1997-98, Khawar *et al*., 2005). The goal of study reported here was to establish an efficient plant regeneration and micropropagation system from hypocotyl and cotyledon explants of *P. afra*. Its improvement through tissue culture techniques can lead to develop commercially improved varieties. So the present study optimized conditions for tissue culture of black psyllium for subsequent use in varietal development and genetic transformation programs.

MATERIALS AND METHODS

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. Selected vigorous and healthy seeds were used for germinating on MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.7% agar (Sigma agar type A) contained in 100x10 mm petridishes sealed with Stretch film® and germinated for one week.

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

The pH of each medium was adjusted to 5.6-5.8 with 1N NaOH or 1N HCl before addition of agar and autoclaving (1.4 $kgcm^{-2}$ pressure of at 121°C for 20 minutes). All experiments were carried out under sterile conditions. Cultures were incubated at temperature of 24 ± 2 °C provided by Sylvania ® Grolux fluorescent tubes giving a light intensity of 42 $\mu mol.m^{-2} s^{-1}$ 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 by Duncan's multiple range test.

RESULTS AND DISCUSSION

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

Shoot regeneration. Concentration 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 on all media with subsequent shoot development. Shoot primordia were observed on all media from both explants with considerable growth after 15 days (Fig. 1b). Mean number of shoots per explant ranged from 0.33 to 13.17 on cotyledons and 1.17 to 23.17 on hypocotyl explants after 30-45 days (Fig. 1c,d) (Table I). It was found that 2.22 µM BAP with 1.34 µM NAA promoted and 1.11 µM BAP with 1.34 µM NAA suppressed shoot regeneration on cotyledon and hypocotyls explants but variably. 1.11 µM BAP with 0.98 µM IBA or 1.16 µM Kinetin with 0.98 µM IBA were suppressive compared to 1.11 µM BAP with 0.09 µM IBA or 1.16 µM Kinetin with 0.09 µM IBA which promoted shoot regeneration. In case of TDZ, 0.91 µM TDZ with 0.98 µM IBA was promoted and 0.91 µM TDZ with 0.09 µM IBA inhibited shoot regeneration.

No regeneration was observed from cotyledons and 2.92 shoots per explant were noted on hypocotyls under no treatment on MS medium. However, shoot regeneration was better on hypocotyls over cotyledons on all media showing that hypocotyls had better regeneration potential. *In vitro* plant regeneration of P. ovata was attempted through somatic embryogenesis 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 use of additives such as coconut water and casein hydrolysate promoted large-scale *In vitro* somatic embryogenesis of *P. ovata*. Makowczynska and Andrzejewska (2000) observed early stages of direct somatic embryogenesis in *P. asiatica* by light microscopy and found two phenomenons of somatic organogenesis and somatic embryogenesis in calli simultaneously. Similarly Khawar *et al*. (2005), recorded shoot regeneration from hypocotyls and cotyledon explants of *P. lanceolata* with various concentrations of BAP+IBA.

Table I. Mean shoot regeneration from cotyledon and hypocotyl explants of *P. afra* after 8 weeks of culture per replication on 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] ± 0.34 ab[2]	11.00 ± 0.83 bc
1.11	1.34	0.42 ± 0.27 d	9.75 ± 0.30 bc
BAP (µM)	IBA (µM)		
1.11	0.98	0.33 ± 0.01 d	7.33 ± 0.61 c
1.11	0.09	2.00 ± 0.13 c	2.00 ± 0.05 c
Kinetin (µM)	IBA (µM)		
1.16	0.98	1.82 ± 0.19 c	1.17 ± 0.03 d
1.16	0.09	2.58 ± 0.33 c	2.67 ± 0.69 cd
TDZ (µM)	IBA (µM)		
0.91	0.98	13.17 ± 0.92 a	23.17 ± 0.84 a
0.91	0.09	3.67 ± 0.74 bc	21.92 ± 0.79 a
Control (MS medium)		0.00 ± 0.00 d	2.92 ± 0.09 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.

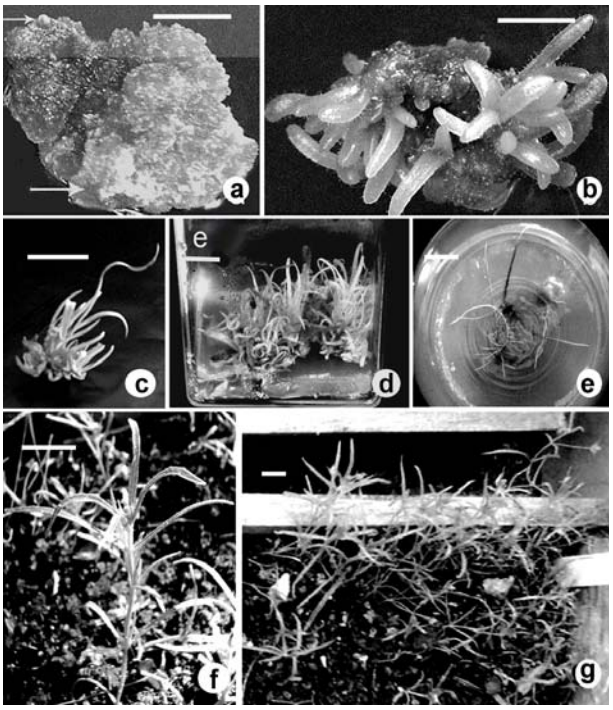
Table II. *In vitro* rooting of *P. afra* using varied concentrations of NAA.

NAA (µM)	Frequency of root regenerating explants (%)	Mean Number of regenerated roots/explant	Mean shoot length (cm)
1.07	58.33[1] ± 0.66 bc[2]	1.25 ± 0.07 d	2.33± 0.26 b
2.15	33.33 ± 0.37 c	1.00 ± 0.11 d	3.00 ± 0.04 a
3.22	83.33 ± 0.67 ab	5.75 ± 0.14 a	3.33 ± 0.39 a
4.30	91.67 ± 0.45 a	2,75 ± 0.37 b	2.33 ± 0.12 ab
5.37	58.33 ± 0.67 bc	2.25 ± 0.64 c	1.33 ± 0.14 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.

Rooting. Healthy growing green shoots regenerated on hypocotyl (from 0.91 µM TDZ + 0.98 µM IBA) rooted under all 5 combinations of NAA in MS media (Table II). No carry over effect of TDZ was observed and the shoots were rooted easily in contradiction to Preece *et al.,* (1987) in Hibiscus rosa-sinensis L., Yusnita *et al.,* (1990) in *Cercis canadensis* L., and Gray and Benton, (1991) in muscadine grape. It seemed TDZ had no effect on rooting in line with

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 on MS medium containing 3.22 µM NAA (f,g) adaptation under greenhouse conditions. Bar = 1 cm.



Fasolo *et al.* (1989) and Preece *et al.* (1991), as the highest rooting per shoot (5.75) was observed on MS medium containing 3.22 µM NAA (Fig. 1e) 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 on MS medium containing 4.30 µM NAA. Rooted plantlets were very difficult to handle because of very fragile roots and difficulty in removing agar from the roots of rooted shoots (plantlets). It is supposed that this might be due to genotype or due to interaction of TDZ with NAA. A great care was taken during this process to avoid any damage to roots. In spite of this only 35 % plantlets could be successfully transferred to soil mix in wooden crates. All of the transferred plants were not difficult to acclimatize and were transferred to the greenhouse (Fig. 1f, g) for flowering and seed set.

As such this paper reports a successful and reliable protocol for optimization of conditions for *in vitro* propagation of *P. afra* using cotyledons and hypocotyl explants and achieved the aim of study in terms of *In vitro* regeneration. We find, that the described protocol could be utilized on commercial scale and offers a considerable economic potential.

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