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Submerging Faba Beans in Water Improve *in vitro* Rooting of Microprpagated Shoots

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Abstract

A reliable shoot regeneration system for faba bean (Vicia faba L.) has not been reported to date to build a strong base for genetic transformation in broad bean. The present work reports a rapid, reproducible and proficient regeneration system for faba bean utilizing plumule and embryonic axis explants on MS medium containing 0.25 mg/l BAP +0.50, 0.25, 0.10, 0.05, 0.00 mg / l NAA following submerging of the seeds in distilled water for 24 hours. The explants showed a fast regeneration in 15 days time. These shoots were rooted on MS medium containing 1 mg/l IAA (15 d) and acclimatised on soil peat moss (10 days). The plantlet regeneration reported in this study accounts for effective micropropagation of faba bean. This protocol establishes a strong foundation and fundamental for future genetic transformation studies in faba beans under in vitro conditions.

Keywords*: in vitro*, mass propagation, *Vicia faba*, rooting, submerging

INTRODUCTION

Besides animal based proteins, a large number of proteins could also be obtained from edible grain legumes [1]. Broad beans; Beans, lentils, cowpea, chickpea and pea makes most important source of plant based proteins for both human consumption and animal feed [2, 3,4]. Faba bean (Vicia faba L. of Leguminosae are the third largest family of dicotyledonous plants protein ratios ranging from 25-35% [5]. V. fabagrows 0.5–1.8 m, have 10–25 cm long pinnate leaves, of a distinct glaucous grey-green color. The roots of these plants are well known for their role in biological fixation of atmospheric nitrogen [6; 7] and are often grown as a cover crop to prevent soil erosion.

Faba bean is highly susceptible to number of abiotic and biotic stresses that results in unstable yields. The faba bean plants have problem of self incompatibility that has led to the formation of limited genetic pool and slow progress in breeding of this plant [8,9; 10]. It is very difficult to to micropropagate or regenerate faba beans as the plants are highly recalcitrant [11; 12], eminating from the problems related to high release of phenolic compounds [13, 14, 15, 16, 17].

Previous studies confirm poor plantlets regeneration through direct or indirect somatic embryogenesis of faba bean [13, 18; 19; 20; 21; 22]. However, most of the reported protocols are not repeatable or has success in specific research laboratory conditions. This study was undertaken to develop a high speed, repeatable regeneration protocol in faba bean to establish efficient transformation system for crop breeding and study of related functional genomics aspects, thereafter.

MATERIALS and METHODS

Plant Material:The seeds of cv. Filiz 99 and Eresen 87 were used in the study that were obtained from the Aegean Agricultural Research Institute, Izmir.

Tissue Culture Methods

Growth regulators and storage conditions: All chemicals and growth regulators used in the study were obtained from SigmaAldrich Chemical Co. (St Lo MO, USA) and Duchefa Biochemie (Netherlands).

Ten (10) mg of NAA, IAA and BAP stock solutions were prepared by solving these with 1 N NaOH or Ethanol and then completing to 10 ml of water to give 1 mg / ml stock solution.

Basal media and culture conditions: MS mineral salts and vitamins [23] were used in the experiments.

Purified double distilled water was used in preparation of the medium after adjusting the pH of the basal medium to 5.6-5.8 using 1N NaOH or 1N HCl solidified with 8 g/l agar and supplemented with 30 g/l sucrose.

The basal medium for each treatments was sterilized using autoclave. All cultures were exposed to white fluorescent light (Preheat Daylight-42 µmol photons m-2s-1) under a 16 hour light photoperiod and 24 ± 1ºC temperature.

Surface Sterilization: The seeds of both cultivars were surface sterilized using 100% commercial bleach (Ace, Turkey) for 20 minutes with 5% NaOCl followed by 5 × 3 min rinsing with distilled water.

Germination of legume seeds in vitro: Post sterilization the seeds of both cultivars were subjected to submersion in double distilled water for 24 hours in a shaking incubator at room temperature at 190 rpm and 24 ° C. Thereafter, plumula and embryonic axes from the embryos were detached from by gently separating the cotyledons gently using scalpel blades under aseptic conditions. Subsequently, the explants were cultured on agar solidified MS medium containing 0.25 mg/l BAP +0.50, 0.25, 0.10, 0.05, 0.00 mg / l NAA(5 combinations)under 16 hours light photoperiod

Rooting: Regenerated shoots were cut and rooted on MS medium containing 1 mg / l IAA in magenta GA7 vessels. The agar was removed from the in vitro rooted plantlets under running tap water and the plantlets were submerged in water for 15-20 minutes [24] before transferring them to pots containing peat moss. Four to six weeks later rooting shoots were acclimatised in plastic pots by keeping them at room temperature (20-25 ± 2 ºC) in greenhouse for one week period.

Statistical Evaluation of Data

All experiments were established and tested according to the factorial design with 3 replications in Magenta GA7 vessels or Petri dishes containing 5 explants in each treatments. All exprerimental data was analysed using univariate analysis of SPSS 20 for Windows statistical program for social sciences. Application of Duncan Multiple Range Test was performed for post hoc comparisons. All percentage values were arcsine transformed before "statistical analysis" Following Snedecor and Cochran [25].

RESULTS

***In vitro* experiments**

Initiation of shoot initials was noted on the explants after 2-3 days followed by rapid growth of shoots in two weeks time. Thereafter, the data were subjected to analysis of variance that showed a significant ($p>0.01$) interaction among plant growth regulators, cultivars and explants for all parameters.

The Duncan test results for determining the significance level of these interactions are given in Table 1. The rate of shoot induction (%) on the embryo axis of the Cv. Eresen 87 ranged 80 to 100%. The maximum percentage of shoot induction was noted on MS medium containing 0.25 mg/l BAP + 0.5 mg/l NAA, 0.25 mg/l BAP + 0,25 mg/l NAA, 0.25 mg/l BAP + 0.1 mg/l NAA and 0.25 mg/l BAP + 0.05 mg/l NAA. Maximum shoot induction was noted on MS medium containing 0.25 mg/l BAP + 0.25 mg/l NAA on plumule explant.

Embryonic axes of Cv. Filiz 99 showed 25-100% shoot induction. Such that maximum shoot induction was noted on MS medium containing 0.25 mg/l BAP. Shoot regeneration on plumule explants also ranged %25 - 100 % with maximum shoot regenration on 0.25 mg/l BAP + 0,25 mg/l NAA and 0.25 mg/l BAP + 0.1 mg/l NAA.

Table 1. Effects of various concentrations of BAP+NAA on shoot induction percentage from plumule and embryonic axis explants of faba bean

Treatments		Shoot induction percentage (%)			
		Eresen 87		Filiz 99	
BAP (mg/l)	NAA (mg/l)	Embryonic axis	Plumule	Embryonic axis	Plumule
0.25	**0.50**	100.00a	66.67c	86.67b	80.00b
0.25	**0.25**	100.00a	86.67a	25.00d	100.00a
0.25	**0.10**	100.00a	86.67a	73.33c	100.00a
0.25	**0.05**	100.00a	53.33cd	80.00bc	50.00c
0.25	**0.00**	80.00b	73.33b	100.00a	25.00d

*Each value is the mean of 3 replications with 10 explants each

**Values within a column followed by different letters are significantly different at the 0.05 level

The number of shoots per explant on the embryo axis explants of cv. Eresen 87 ranged 1.0 to 2.40 (Fig. 1 a, Table 2). The highest number of shoots was obtained on MS medium containing 0.25 mg / l BAP. The minimum number of shoots was noted on MS medium containing 0.25 mg / l BAP and 0.25 mg / l NAA. The number of shoots per explant ranged 1.0 to 1.77 on plumule explants and the highest number of shoots was observed on MS medium containing 0.25 mg / l BAP.The minimum number of shoots was obtained on MS medium containing 0.25 mg/l BAP -0.05 mg/l NAA.

Table 2. Effects of various concentrations of BAP+NAA on Number of shoots per explant from plumule and embryonic axis explants of faba bean

Treatments		Number of shoots per explant			
		Eresen 87		Filiz 99	
BAP (mg/l)	NAA (mg/l)	Embryonic axis	Plumule	Embryonic axis	Plumule
0.25	**0.50**	1.60b	1.10d	1.40b	1.40c
0.25	**0.25**	1.00d	1.50b	1.00c	1.40c
0.25	**0.10**	1.57bc	1.40c	1.00c	1.00d
0.25	**0.05**	1.70b	1.00e	1.62a	1.70b
0.25	**0.00**	2.40a	1.77a	1.47b	2.00a

*Each value is the mean of 3 replications with 10 explants each

**Values within a column followed by different letters are significantly different at the 0.05 level

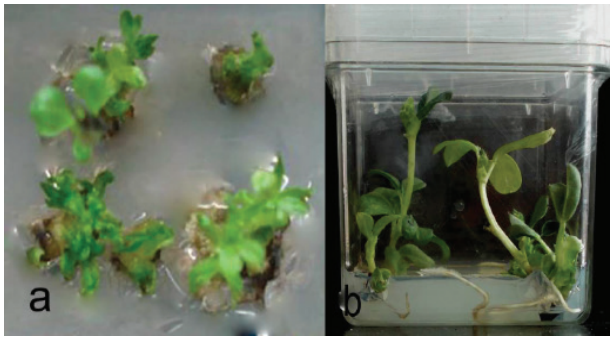

Figure 1. A. Axillary shoots regenerated from plumule explants on MS medium containing 0.25 mg / l BAP and 0.1 mg / l NAAB. Rooting of the killed shoots in medium containing 1 mg / l IAA

The number of shoots per explant varied from 1.0 to 1.62 in shoots obtained from embryonic axis explants of cv. Filiz 99. The number of shoots per explant varied from 1.0 to 1.70 shoots obtained from plumule explants of cv. Filiz 99. The highest number of shoots was observed on MS medium containing 0.25 mg / l Bap- 0.05 mg / l NAA and the lowest number of shoots was observed on MS medium containing 0.25 mg/l BAP + 0.25 mg/l NAA and 0.25 mg/l BAP with 0.1 mg/l NAA. The number of shoots per explant ranged from 1.00 to 2.00 on plumule explants.

Embryonic axis explants of cv. Eresen 87, elongated shoots in range of 1.57 to 5.93 cm (Table 3). The longest shoots were noted on MS medium containing with 0.25 mg/l BAP + 0.1 mg/l NAA. The shortest shoots were observed on MS medium containing 0.25 mg/l NAA using 0.25 mg/l BAP. Whereas, the shoots regenerated on plumule explants had shoot length rangeof 1.80 to 5.67 cm.

Table 3. Effects of various concentrations of BAP+NAA on shoot length from plumule and embryonic axis explants of faba bean

Treatments		Shoot length (cm)			
		Eresen 87		Filiz 99	
BAP (mg/l)	NAA (mg/l)	Embryonic axis	Plumule	Embryonic axis	Plumule
0.25	**0.50**	2.50c	5.30b	3.13a	5.50a
0.25	**0.25**	1.57d	5.67a	1.00d	2.20d
0.25	**0.10**	5.93a	1.80e	1.90c	2.70c
0.25	**0.05**	2.10c	4.50c	1.67cd	1.50e
0.25	**0.00**	3.37b	2.70d	2.80b	4.00b

*Each value is the mean of 3 replications with 10 explants each
**Values within a column followed by different letters are significantly different at the 0.05 level

The longest shoots on embryonic axis and plumule explants of cv. Filiz 99 ranged 1.00 to 3.13 cm and 1.50 to 5.50 cm on cv. Eresen 87 respectively.

The longest shoots on cv. Eresen87 embryonic axis and plumule explant was noted on 0.25 mg / l BAP + 0.10 mg / l NAA and 0.25 mg / l BAP + 0.25 mg / l NAA respectively.

In the shoots obtained from the embryonic axis and plumule explants of cv. Filiz 99 the longest shoots were noted on MS medium containing 0.25 mg / l BAP + 0.50 mg / l NAA.

15 d later, the shoots from both explants were successfully rooted on MS medium containing 1 mg / l IAA.

Thereafter 10 d the newly rgenerating plantlets were successfully acclimatised in peat moss in greenhouse.

DISCUSSION

In general, faba bean appear to be recalcitrant in in vitro regeneration [11,12]. In the faba bean cultivars studied here, the response of plumule and embryonic axes explants were used to developin vitro regeneration using 0.25 mg/l BAP+7 different concentrations of NAA. The time of exposure, and the type and concentration of cytokinin, affects adventitious bud/shoot induction and subsequent development/ growth rate of shoots produced [12, 21, 26; 27; 28; 29]. The plant growth regulators induced rapid shoot regeneration in 15 days followed by rooting(15 days) along and acclimatisation in 10 days.

Both BAP and NAA are important plant growth regulators used by number of researchers for shoot regeneration in number of plant species [31, 32, 33]. The results of this study confirm that these plant growth regulartors are also effective for regeneration on cv. Filiz99 and Eresen 87 of Broad bean with rapid growth of shoots. A major hurdle that limits in vitro regeneration of legumes including Broad bean is hinderance of in vitro regenerated shoots to root that compel number of researchers working on legumes to switch to grafting as an alternative approach [34,35, 36,37,38,39]. The results of this study approves that grafting could be avoided if the regenerating shoots are rooted after two weeks of regeneration. The roots developed on the cut ends of shoots were vigorous longer and had no problem on acclimatisation of plants. These plantlets had no difficulty during acclimatisation in pots and greenhouse.

For transplantation of legume plantlets, various potting mixtures have been used: Peat moss [30]; is most often encouraged for the purpose. All plants acclimatised in 10 days time. The results are in agreement with Mokhtarzadeh et al. [40], who also preferred highly recalcitrant lavender species using peat moss and achieved 100%success in transplantation.

This will definitely help in developing batter faba genotypes suitable for local and regional ecosystem and enhancing faba role in conservation agriculture in arid and semiarid regions.

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