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Full Length Research Paper

In vitro plant regeneration from Turkish Narbon Vetch (*Vicia narbonensis* L. var. *narbonensis* L.)

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Narbon vetch (*Vicia narbonensis* L.) is an important forage species among vetches of central Europe, Mediterranean, Near East, Ethiopia, central Asia and India. The study reports *in vitro* micropropagation of narbon vetch using cotyledon node, shoot tip and zygotic embryo explants on MS medium containing 0.25, 0.50, 0.75 and 1.00 mg/l BAP. The highest number of 4.42 shoots was recorded on cotyledon node explants, which was followed by significantly reduced number of 3.40 and 3.54 shoots per explant on shoot tip and zygotic embryo with two cotyledons, respectively. Zygotic embryo with two cotyledons explant was recalcitrant and slow in regeneration compared to other explants but any concentration of BAP was sufficient for multiple shoot regeneration. Shoot length decreased statistically with increase in each concentration of BAP in the regeneration medium. 65.0% shoots rooted on MS medium containing 0.5 mg/l IBA and newly regenerated plants were acclimatized in the greenhouse.

Key words: *In vitro*, Narbon vetch, regeneration, *Vicia narbonensis* L, shoot tip, cotyledon node, zygotic embryo with two cotyledons.

INTRODUCTION

Narbon vetch (*Vicia narbonensis* L.) is one of the important self pollinated forage species and has a natural distribution ranging from central Europe, Mediterranean, Near East, Ethiopia, central Asia and India. It can be found in all regions of Turkey, except Northern Anatolia (Davis and Plitman, 1970). It has two varieties namely narbonensis and serratifolia. It has a high level of resistance to aphids and as such shows good agronomic potential (Hernándo-Bermejo and León, 1994).

Narbon vetch has greater temperature and lesser humidity requirements, which make it possible to grow advantageously in warm dry areas. It tolerates cold, and is not damaged by frost. It can be used as forage crop, green manure and has great importance in crop rotation (Altinok, 2002; Altinok and Hakyemez, 2002). It has a potential to replace fallow land of traditional barley-fallow rotation in the Eastern Mediterrenean (Oram and Belaid, 1990) and is recommended as a forage crop in fallow years in dry farming areas of Turkey (Bakir, 1981). In recent years, a number of breeding and agronomic studies have been conducted to introduce the plant into Turkish farming system (Uzunmehmetoğlu and Kendir, 2006). However, the plant has not been exploited extensively (Kendir et al., 2008).

Legumes are always supposed to be less recalcitrant to tissue culture (Hammatt et al., 1986) and narbonensis is less recalcitrant among other grain legumes (Tegeder et al., 1996). Successful tissue culture of mesophyll protoplasts with low frequency of differentiated roots (Donn, 1978) shoot buds, somatic embryogenesis from epicotyls of young seedlings (Roupakias, 1985; Pickardt and Schieder, 1987), embryogenesis from shoot tips (Pickardt et al., 1989), induction of somatic embryogenesis from mature leaves (Albrecht and Kohlenbach, 1989), epicotyls, shoot tip (Tegeder et al., 1996), and shoot regeneration from cotyledon node (Kendir et al., 2008) in *V. narbonensis* has already been reported.

The objective of the study was to compare axillary shoot regeneration from cotyledon node, shoot tip and zygotic embryo with two cotyledons (seed with testa) to

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Abbreviations: BAP, 6-Benzylaminopurine; **IBA,** Indole 3 butyric acid.

develop rapid and reliable protocol for use in genetic transformation of *V. narbonensis*.

MATERIALS AND METHODS

The seeds of *V. narbonensis* var. *narbonensis* were obtained from Osman Tosun Gene Bank, Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara, Turkey. The seeds were surface sterilised with 70% commercial bleach (Ace- Turkey containing 5 - 6% sodium hypochloride) in laminar flow hood by continuous stirring for 10 min followed by 3 x 5 min rinsing with sterile bidistilled water.

Cotyledon node and shoot tip explants were obtained from 10 - 12 days old *in vitro* grown seedlings on MS medium (Murashige and Skoog, 1962) containing 0.5 mg/l BAP, supplemented with 3% sucrose and solidified with 0.65% agar (Duchefa- Germany). Thereafter, they and zygotic embryos with two cotyledons were cultured on MS medium containing 0.25, 0.50, 0.75 and 1.00 mg/l BAP in magenta vessels GA7™. All explants were also cultured on MS medium without plant growth regulators (control) to compare shoot regeneration. All cultures were incubated at 24 ± 2°C in 16 h light photoperiod. The pH of all cultures was adjusted to 5.6 - 5.8 before adding agar and autoclaving at 121°C, 118 kPa pressures for 20 min.

After 5 week on culture media, regenerated shoots were transferred to MS rooting medium in magenta vessels GA7™ containing 0.5 mg/l IBA. Rooted shoots (plantlets) were transferred to pots containing peat moss and acclimatized in the greenhouse. Acclimatization was done by covering pots with polyethylene bags to maintain initial humidity for 1 week and irrigated after every 2 days.

Experiment was carried out in 3 replications containing 6 explants and repeated twice (4 x 6 x 2 = 48 explants). Data was analyzed with SPSS 16.0 program using one way ANOVA and the post hoc tests were performed using Duncan's Multiple Range test. Data given in percentages were subjected to arcsine transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS

Early shoot regeneration was observed on cotyledon node explants followed by regeneration on shoot tip explants. Zygotic embryos with two cotyledon explants started late regeneration compared to the other two explants. The results showed no interaction among explants and concentrations of BAP. Different concentrations BAP also did not affect frequency of shoot regeneration from three explants. However, shoot induction behavior of three explants varied; which ranged 69.17 to 95.83% (Table 1). Maximum frequency of shoot regeneration (95.83%) was recorded on cotyledon node explant. Zygotic embryo with two cotyledons had lower shoot regeneration potential with significantly lower shoot regeneration frequency of 69.17%.

Similarly, no effect of the concentrations of plant growth regulators was recorded on the explants for the number of shoots per explants. However, number of shoots per explants was significantly different among explants. Maximum number of 4.42 shoots per explants was recorded on cotyledon node explants (Figure 1a, Table 2), with marked reduction in the number of shoots per explant on the other explant. The shoot regeneration from shoot tip and zygotic embryo with two cotyledons was statistically similar with 3.4 and 3.54 shoots per explants respectively (Figure 1b, c).

The shoot length from different explants did not show an interaction of plant growth regulators with the explants. However, each concentration of plant growth regulators and the explants behaved variably. The results showed that shoot length decreased with each increase in the concentration of plant growth regulators. Maximum shoot length was recorded on MS medium containing 0.25 mg/l BAP, which reduced to 3.167 cm on 1 mg/l BAP (Table 3). Comparing explants, maximum shoot length was recorded on cotyledon node explants followed by the shoot length on shoot tip and zygotic embryo with two cotyledons.

Rooting and acclimatization

Regenerated shoots started rooting in the rooting medium containing 0.5 mg/l IBA within 10 days (Figure 1d) and 65.0% of transferred shoots developed roots in three weeks time (Figure 1e). They were transferred to pots and successfully acclimatized in the greenhouse.

DISCUSSION

Efficient *in vitro* regeneration of narbon vetch under *in vitro* conditions is an important step toward improvement of this neglected but important forage legume. The protocol provides an alternative and rapid mean for the improvement of narbon vetch through tissue culture.

Shoot regeneration behavior of different explants used in the experiment showed no shoot regeneration from cotyledon node or shoot tip explants on plant growth regulator free MS medium. The three explants used in the study showed they had different shoot regeneration potential, which resulted in different number of shoots per explants and variable shoot length from the shoots regenerated on each explant. No callus induction was observed on any explants on any concentration of BAP with visible variable shoot induction behavior from each explant.

Least shoot regeneration and shoot length were recorded on zygotic embryo explants. That might be due to the harder seed coat which inhibited direct contact and intake of plant growth regulators which ultimately resulted in lower shoot regeneration and shoot length. Once the seed coat ruptured and cotyledon node of zygotic embryo got in touch with the medium, multiple shoot regeneration started from the cotyledon node that increased in shoot length. However, it could not compete with other explants where no such problem existed. Diallo et al. (2008) reported maximum plant regeneration from *in vitro* grown explants with two entire cotyledons in cowpea.

Previously, Kendir et al. (2007) has reported axillary

Table 1. Effects of various concentrations of BAP on frequency of shoot regeneration from cotyledon node, shoot tip and mature embryo with two cotyledons of Narbon Vetch.

Explant	BAP					
	0 (mg/l)	0.25 (mg/l)	0.50 (mg/l)	0.75 (mg/l)	1.0 (mg/l)	Mean
Cotyledon node	0.00	100.00	100.00	91.67	91.67	95.83 a
Shoot tip	0.00	100.00	88.89	91.67	94.44	93.75 a
Zygotic embryo with two cotyledons	0.00	70.00	73.33	66.67	66.67	69.17 b
Mean	0.00	90.00	87.41	83.33	84.26	86.25

Mean values within a column followed by different letters are significantly different at the 0.05 probability level using Duncan's multiple range test.

Table 2. Effects of various concentrations of BAP on number of shoots per explant from cotyledon node, shoot tip and zygotic embryo with two cotyledons of Narbon Vetch.

Explant	BAP					
	0 (mg/l)	0.25 (mg/l)	0.50 (mg/l)	0.75 (mg/l)	1.0 (mg/l)	Mean
Cotyledon node	0.00	4.67	4.412	4.08	4.53	4.42 a
Shoot tip	0.00	2.67	2.70	4.43	3.79	3.40 b
Zygotic embryo with two cotyledons	0.00	3.43	3.93	3.67	3.13	3.54 b
Mean	0.00	3.59	3.68	4.06	3.82	3.79

Mean values within a column followed by different letters are significantly different at the 0.05 probability level using Duncan's multiple range test.

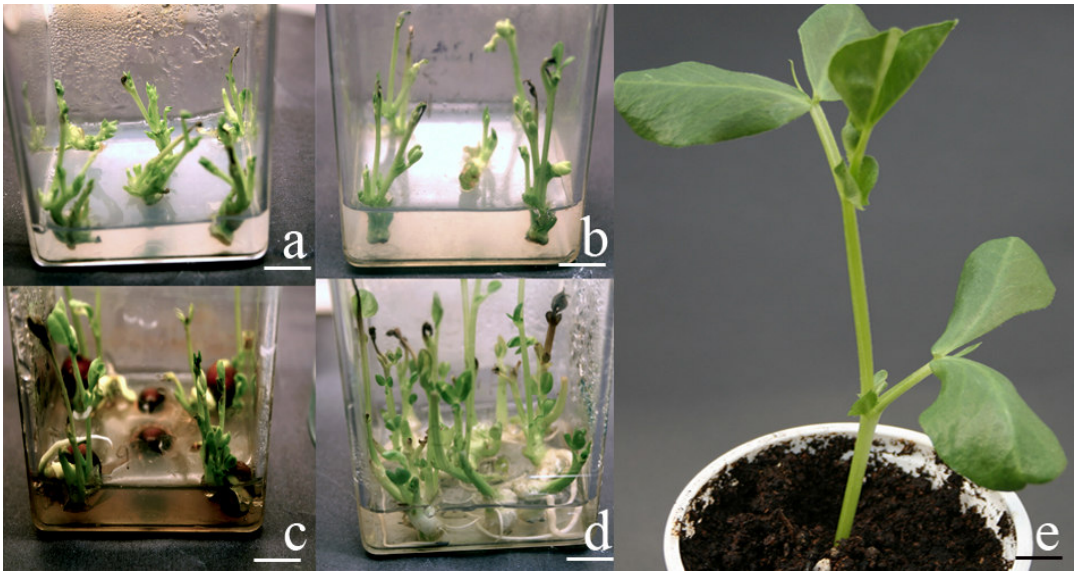

Figure 1. Shoot regeneration from (a) cotyledon node (b) shoot tip (c) zygotic embryo with two cotyledons (d) rooting on MS medium containing 0.5 mg/l IBA (e) acclimatized plant. Bar, Figure 1 a, b, c, d = 1 cm, Figure 1e = 1.5 cm.

shoot regeneration from cotyledon node explants obtained from *in vitro* raised seeds of 4 - 5 and 14 – 15 days old seedlings on MS medium containing 2 - 6 mg/l kinetin-0.1 mg/l indole-3-butyric acid (IBA). There was no shoot regeneration on 14 - 15 days old cotyledon node explants. Whereas, 4 - 5 days old cotyledon node explants showed high regeneration potential with the highest number of 3.85 shoots per explant, with mean shoot length of 2.11 cm and shoot regeneration frequency of 93.33%. The shoots obtained from all regeneration media could be easily rooted after pulse treatment with 50 mg/l IBA for 7 min.

Table 3. Effects of various concentrations of BAP on shoot length from cotyledon node and shoot tip and mature embryo with two cotyledons of Narbon vetch.

Explant	BAP					
	0 (mg/l)	0.25 (mg/l)	0.50 (mg/l)	0.75 (mg/l)	1.0 (mg/l)	Mean
Cotyledon node	0.00	5.17	4.73	3.80	3.73	4.36 a
Shoot tip	0.00	4.57	3.47	3.30	3.23	3.64ab
Zygotic embryo with two cotyledons	0.00	4.10	3.40	3.43	2.53	3.37 b
Mean	0.00	4.61 a	3.87 ab	3.51 b	3.17 b	3.79

Mean values within a column followed by different letters are significantly different at the 0.05 probability level using Duncan's multiple range test.

Khalafalla and Hattori (1999) obtained high number of shoots in *Vicia faba* using different concentrations of BAP-TDZ. Sancak et al. (2000) used immature cotyledons and embryo axes of *V. pannonica* using BAP-NAA. Similarly, Taha and Farncis (2004) and Erdogan et al. (2005) obtained high shoot regeneration from *V. ervilia* using immature embryo and cotyledon explants on MS medium using various concentrations of TDZ. Fakhrai et al. (1989) obtained successful shoot culture on stem, leaves, roots and cotyledons of *V. faba*.

Concentration of plant growth regulators significantly affected the shoot length of all explants. Shoot length decreased with each increase in BAP concentration which showed the inhibitory effect of BAP on shoot length. Statistically similar shoot length from zygotic embryos and shoot tip explants showed the same but poor efficiency of these explants compared to cotyledon node explant. Cotyledon node showed more positive response to all BAP concentrations for mean number of shoots per explant compared to other explants.

On the basis of results, it is concluded that cotyledon node explants showed better response than shoot tip and zygotic embryos with two cotyledons on all concentrations of BAP used in the experiment. Also, shoot lengths were negatively affected by each increase in the BAP concentrations. The shoots rooted easily on MS media containing 0.5 mg/l IBA in agreement with Kendir et al. (2007).

The simple, efficient and rapid protocol of shoot regeneration opens way for genetic transformation work for future use in Narbon and other vetches.

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