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Full Length Research Paper

In vitro plant regeneration from Narbon Vetch (*Vicia narbonensis* L.) using cotyledonary node explants

Hayrettin Kendir, Nurdan Sahin-Demirbag, Khalid Mahmood Khawar*, and Muhammad Aasim

Department of Field Crops, Faculty of Agriculture, Ankara University Ankara, 06110, Diskapi, Turkey.

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Narbon vetch (*Vicia narbonensis* L.) is an agriculturally important forage plant that widely grows in an area extending from Central Europe to various parts of Asia. The study reports axillary shoot regeneration from cotyledonary 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). No shoot regeneration was recorded 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. All of which were morphologically normal and fertile and easily established under greenhouse conditions.

Key words: Narbon vetch, *Vicia narbonensis* L, axillary shoot regeneration, rooting.

INTRODUCTION

Narbon vetch (*Vicia narbonensis* L.), one of the agriculturally important species among vetches, has a natural distribution ranging from central Europe to Asia. It can be found in all regions of Turkey except Northern Anatolia (Davis and Plittman, 1970). It is winter-growing, drought and cold tolerant plant that does not lose its leaves following frost (El Moneim, 1989). It also shows adaptation in areas receiving 250 – 300 mm annual rainfall with low winter temperatures (El Moneim 1992).

Its use has been promoted as a replacement for fallow in the traditional barley-fallow rotation in the Eastern Mediterrenean (Oram and Belaid 1990). Narbon vetch has also been recommended as a forage crop in fallow years in dry farming areas of Turkey (Bakir, 1981).

The potential advantage of narbon bean as a dual purpose grain legume has stimulated the development of active breeding programmes at the International Centre for Agricultural Research in Dry Areas (ICARDA) Syria and at the Victorian Institute of Dry land Agriculture, Victoria, Australia (Bennet and Maxted 1997). During recent years, a number of breeding and agronomic studies have been conducted to introduce the plant into Turkish farming system. The plant has not been exploited extensively. It is self pollinated plant and may not express a variety of phenotypes differing in morphological and phytochemical characteristics. Tissue culture methods of narbon vetch could be effective in shorting evaluating or breeding period of new lines or cultivars. The main objective of the study was to develop a methodology for rapid multiplication through tissue culture for use in breeding programs.

*Corresponding author. E-mail: kmkhawar@gmail.

Abbreviations: BAP, 6 Benzylaminopurine; NAA, α Naphthalene acetic acid; IBA, Indole 3 butyric acid; TDZ, Thidiazuron.

MATERIALS AND METHODS

The seeds of *V. narbonensis*. was obtained from Osman Tosun Gene Bank, Department of Field Crops, Faculty of Agriculture, University of Ankara, Ankara, Turkey. The seeds were pre treated with 99% ethanol for 1 min by immersion followed by surface sterilisation with 50% commercial bleach (Ace- Turkey containing 5 - 6% sodium hypochlorite solution) in laminar flow hood by continuous stirring for 20 min. Thereafter, they were rinsed 3 x 5 min with sterile distilled water and germinated on 0.65% agar solidified MS

medium (Murashige and Skoog, 1962), containing 3% sucrose. Cotyledonary node explants were obtained from of 4 - 5 and 14 - 15 days old *in vitro* grown raised seedlings. They were cultured on MS medium containing 2 - 6 mg/l kinetin-0.1 mg/l indole-3-butyric acid (IBA) in 100 x 10 mm Petri dishes™.

All cultures were incubated at 24 ± 2°C in 16 h day length photoperiod. The pH of all cultures was adjusted to 5.6 - 5.8 before adding 0.65% agar (Duchefa) and autoclaving at 121°C, 118 kPa pressure for 20 min.

To induce roots, the shoots regenerated on all cultures were pulse treated with 50 mg/l IBA for 7 min. Thereafter, the treated shoots were transferred on MS medium containing ½ MS medium in magenta vessels GA7™ and incubated for 8 weeks.

The rooted plantlets were transferred to potting mixtures containing equal ratios of peat moss vermiculite and perlite. Plants in plastic pots were maintained in the greenhouse at 24 ± 2°C under natural light and irrigated every 2 days for 2 weeks.

Each treatment had 4 replicates (Petri dishes) containing 5 explants and all experiments were repeated twice (4 x 5 x 2 = 40 explants). Data was analyzed with SPSS 14.0 using one way ANOVA and the post hoc tests were performed using Duncans Multiple Range test. Data given in percentages were subjected to arcsine transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS

Shoot induction

Shoot induction behavior of 4 - 5 and 14 - 15 days old explants varied sharply. Both explants showed swellings after 6 - 7 days of culture on MS medium containing 2 - 6 mg/l kinetin and 0.1 mg/l IBA. However, the swellings on 4 - 5 days old explants were numerous compared to 14 - 15 days old explants. After further 3 - 4 days of culture, explants 4 - 5 days old cotyledon node explants showed development of shoot meristems and then small shoots (Figure 1a,b). The shoot meristems developed in to variable number of shoots after eight weeks of culture. However, the swellings on 14 - 15 days old explants began to show chlorosis leading to browning and death of explants. Whereas, no such behavior was recorded in 4 - 5 days old cotyledon node explants.

No shoot regeneration was recorded on MS medium (control). Analysis of variance results showed significant variation in the frequency (%) of shoot regeneration, mean number of shoots per explant and mean shoot length on all combinations of plant growth regulators (Table 1). Frequency of shoot regeneration showed a variation of 46.67 to 93.33%. The highest frequency of 93.33% shoot regeneration was recorded on MS medium containing 4 mg/l kinetin-0.1 mg/l IBA. All other combinations were inhibitory and had negative effect on the frequency of shoot regeneration and number of shoots per explant. The highest number of 3.85 shoots per explant was also recorded on the same concentration of kinetin-IBA (Figure 1c). It was closely followed by 3.56 shoots per explant on MS medium containing 3 mg/l kinetin -0.1 mg/l IBA. The longest shoots were also recorded on these two culture media with shoot length of 2.11 and 2.06 cm respectively.

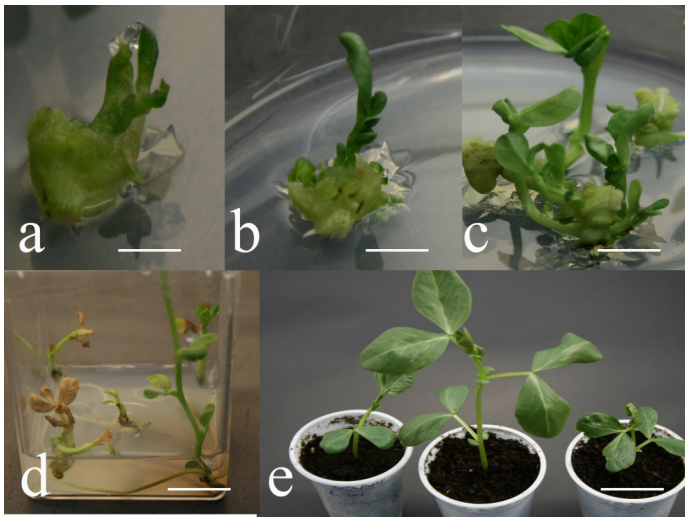

Figure 1. Axillary shoot regeneration from cotyledon node explant of *V. naronensis*. (a, b) development of shoot meristems and small shoots on cotyledon node explant. (c) The highest number of 3.85 shoots per explant on MS medium containing 4 mg/l kinetin- 0.1 mg/l IBA. (d) Rooting of pulse treated shoots with 50mg/l IBA for 7 min. (e) Establishment of plants in the greenhouse. Bar Figure 1a, b, c, d = 0.5 cm, Figure 1e = 4 cm.

Rooting

The highest numbers of shoots were recorded on MS medium containing 4 mg/l kinetin-0.1 mg/l IBA. Therefore, these were pulse treated with 50 mg/l IBA for 7 min. Root initiation occurred after 2 weeks of culture after pulse treatment with rooting frequency of 80%. (Figure 1 d).

Acclimatisation

These were transferred to 10 cm pots and acclimatised in the greenhouse (Figure 1d) for seed set. No problem was observed in acclimatization of *in vitro* regenerated plantlets in the greenhouse. Although more detailed agronomic studies are still needed to confirm the fidelity of the system, 90% of the regenerated plants survived. All of which were morphologically normal and fertile.

DISCUSSION

Knowledge of conditions favoring efficient *in vitro* regeneration of narbon vetch plantlets under *in vitro* conditions is an important step toward the improvement of this important plant species. The protocol provides an alternative mean for the improvement of narbon vetch through tissue culture. Only few successful experiments have been reported for *V. narbonensis.* Donn (1978) used 2,4-D to regenerate callus derived from mesophyll protoplasts that differentiated roots in low frequency. Pickardt and Schieder (1987) induced shoot buds and

Table 1. Effects of various concentrations of kinetin-IBA on axillary shoot regeneration from cotyledon node explant of Narbon vetch.

Treatments		Frequency (%) of shoot egeneration	Mean number of shoots per explant	Mean shoot length (cm)
Kinetin (mg/l)	IBA (mg/l)			
2	0.1	46.67 c	2.58 bc	1.92 ab
3	0.1	46.67 c	3.56 ab	2.06 a
4	0.1	93.33 a	3.85 a	2.11 a
5	0.1	78.33 ab	2.50 c	1.69 ab
6	0.1	66.67 bc	2.11 c	1.23 b
MS (control)		0.00	0.00	0.00

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

somatic embryogenesis from epicotyls of young seedlings. Pickardt et al. (1989) described embryogenesis from shoot tips using 2,4-D. Whereas, Albrecht and Kohlenbach (1989) described a method for the induction of somatic embryogenesis in callus cultures, using explants from mature leaves of *V. narbonensis* using low concentrations of picloram and benzylaminopurine.

No shoot regeneration was recorded on plant growth regulator free MS medium. This showed that the explants needed a specific stimulus to regenerate shoots. An increase in the frequency of shoot regeneration, mean number of shoots per explant and mean shoot length was recorded in ascending order, when the culture medium contained 2 - 4 mg/l kinetin and 0.1 mg/l IBA. Higher concentrations of 5 and 6 g/l kinetin-0.1 mg/l IBA were inhibitory and resulted in sharp decline in the frequency of shoot regeneration, mean number of shoots per explant and mean shoot length.

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), 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 *Vicia faba.*

Explants obtained from 4 - 5 day-old seedlings cultured on medium containing kinetin–IBA gave the highest number of shoots, compared to those obtained from 14 - 15 days old seedlings, which did not did not perform well indicating that age of explants is an important factor. The differential effect of various concentrations of kinetin on the induction of multiple shoots has already been reported for *Vicia faba* (Mohamed et al., 1992), and soybean (Kim et al., 1990). Similarly, *in vitro* culture response of cotyledon node was found to be influenced by age in agreement with Sears and Deckard, (1982), Mathias and Simpson (1986) and the concentration of plant growth regulators in the medium.

The pulse treated shoots rooted very easily and the plantlets were transferred to pots and acclimatized in the greenhouse, once desired number of roots were obtained. Previously, Erdogan et al. (2005) rooted *in vitro* regenerated shoots of *V. ervillia* on MS medium containing 2.0 mg IBA/l. Whereas, Sancak et al. (2000) rooted the regenerated shoots of *V. pannonica* in half-strength MS medium supplemented with 5 µM indole-3-butyric acid (IBA).

It is hoped that the protocol will be useful for clonal multiplication and utilization in breeding activities of individual genotypes belonging to *Vicia* species from diverse geographic origins for very successful tissue culture in the future. Moreover, it is expected that the protocol would provide the potential for genetic transformation of this plant for improved plant characteristics.

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