

Somatic embryogenesis, callus production, and plantlet growth in sainfoin (*Onobrychis viciifolia* Scop.)

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Abstract Plant parts (internode segments, young leaves, apical-axillary meristems, petioles and immature inflorescences) isolated from the fresh, full-leaved and juicy sainfoin (*Onobrychis viciifolia* Scop.) were used as explants in this study. The explants were cultured on B5 medium with 5.4 μM or 10.7 μM NAA combined with 0, 0.9, 1.4, or 2.3 μM of kinetin and 0, 0.9, 1.3, or 2.2 μM of BAP to determine the effects of explant types and medium compositions on the callus induction and plant regeneration. Somatic embryogenesis and organogenesis, as well as direct shoot regeneration from the explants, were observed depending on the explant types and medium composition. The effect of the phytohormone type and concentration on the callus induction rate was under explant dependency. The highest frequency of shoot regeneration was achieved from apical-axillary meristems on B5 medium with 10.7 μM NAA and 2.3 μM kinetin.

Keywords sainfoin; phytohormones; explant; somatic embryogenesis

INTRODUCTION

The *Onobrychis* genus, sometimes also known as “holy clover” (Lu et al. 2000), encompasses about a hundred species. It is native to Anatolia and West Asia. Sainfoin, *Onobrychis sativa* L. or *Onobrychis viciifolia* Scop., is the most widespread species of this aggregate. It is a tetraploid, perennial, cross-pollinated forage legume, which combines persistency, drought resistance, and high palatability with valuable protein and non-bloating properties (Ahuja et al. 1983). Bloat is a serious problem for ruminants, particularly for beef cattle and dairy cows grazing on the pasture with bloat-causing forage species such as lucerne and white clover. Bloat-safe legume species such as sainfoin contain foliar condensed tannins, which play a substantial role in preventing bloat (Li et al. 1993). Tannins also have an important role in the assimilation process of forage proteins by animals (Pupilli et al. 1989). Sainfoin also improves soil fertility because of its nitrogen fixing ability, and has an earlier growth habit than lucerne in spring providing good forage yield for both cattle and honey bees.

Breeding sainfoin cultivars for high yields of good quality forage with valuable proteins and providing resistance against some insect pests such as *Bembesia scopiger* and *Sphenoptera carceli* is of great importance for animal nutrition (Elci et al. 1996). Breeding for desired traits is a long process because of the complex cytology and out-breeding nature of sainfoin. Many studies have been conducted to shorten the breeding process by using tissue culture techniques. Finding and selecting promising new variants from the present gene pool and propagating them faster by *in vitro* techniques is now possible. Several studies revealed high frequency shoot regeneration for sainfoin. However, the initial materials for most of these investigations are based on germinated seeds. Therefore, it is hardly possible to maintain genetic stability of a variety in this cross-pollinated species. Propagation of selected individuals using vegetative explants is required to generate a stable population.

The objective of this study was to identify an efficient propagation system from different vegetative explants of sainfoin (*Onobrychis viciifolia* Scop.) on varying medium compositions.

MATERIAL AND METHODS

A sainfoin ecotype (*Onobrychis viciifolia* Scop.), with a high level of *in vitro* regeneration capacity as determined by a previous study (Çelıktaş 2001), was used as a donor plant for the present study. Donor plants were planted into pots (250 mm in diameter 180 mm in height) filled with a soil + manure + sand (3:1:1) mixture and placed in a greenhouse. The pots were fertilised weekly with a 0.1% solution of a fertiliser mixture containing 8% N + 8% P₂O₅, and were watered regularly. The shoots were harvested at the two-node stage and cold-treated at +4°C overnight. The surface was initially sterilised for 1 min in 70% ethanol and then in a sodium hypochlorite solution (1.5% available chlorine) with two drops of Tween-80 per 100 ml solution for 10 min, followed by rinsing four times in sterile distilled water. Internodes, young leaves, apical-axillary meristems, petioles of the young leaves and immature inflorescences from the shoots were removed as explants and cultured on the basal medium of B5 (Gamborg et al. 1968) supplemented with 3% sucrose and different combinations of 5.4 µM or 10.7 µM naphthalene acetic acid (NAA) and 0, 0.9, 1.4, 2.3 µM of kinetin or 0, 0.9, 1.3, 2.2 µM of 6-benzylaminopurine (BAP). The medium was adjusted to pH 5.8 with 1 N KOH or 1 N HCl and solidified with 0.8% agar. The inorganic compounds of the medium were autoclaved at 121°C, 1.4 kg cm⁻² for 15 min, while the organics were filter-sterilised. Four explants (c. 2–5 mm long depending on the explant types) were cultured in each petri dish (60 × 15 mm) with 7 ml of medium.

Ten replications (dishes) for each explant-medium composition combination were applied. The cultures were incubated at 26 ± 1°C under cool white fluorescent light irradiance of 2000 lux with 16-h photoperiod. The number of explants producing callus and the number of shoots per explant were recorded after 6 weeks of culture and the shoot regeneration rate was computed. Callus and the regenerated shoots were then transferred onto MS (Murashige & Skoog 1962) inorganic + B5 vitamins + 1.1 µM NAA + 8.8 µM BAP. The regenerated shoots were then excised and rooted in half strength MS medium with 2% sucrose + 9.8 µM IBA and solidified with 0.3% gelrite. The rooted plantlets (80–100 mm long) were scored and the number of plantlets per explant was determined (referred to as “plant regeneration rate”) before they were transferred to compost.

The data were analysed by ANOVA using Duncan and LSD multiple-comparison and *t*-tests. The software MSTATC was used. Data given in percentages were subjected to arcsine $\sqrt{x}$ transformation before statistical analysis.

RESULTS AND DISCUSSION

Callus induction was best achieved from the immature inflorescences ($P \leq 0.05$) and apical-axillary meristems (Table 1). This result could be explained by very intensive mitotic cell division in both plant parts as expected. Effects of NAA concentrations on the callus induction from explants were significantly different. The callus induction from the immature inflorescences was best achieved at 10.7 µM NAA but the effect of 5.4 µM NAA was similar, while 5.4 µM NAA was better for apical-axillary meristems. The region synthesised and the amount, the target tissue, and active growth period used for phytohormones are different. The apical-axillary meristems

Table 1 Average rate (%) of callus induction from explants of sainfoin cultured on B5-medium with two concentrations of naphthalene acetic acid (NAA). Means of combinations of explant and NAA concentration with different letters are significantly different at the $P \leq 0.05$ level, as analysed by Duncans' Multiple Range test.

Explants	NAA concentration		Average
	5.3 µM	10.7 µM	
Internode	3.1cd	2.1d	2.6b
Leaf	14.6bc	3.1cd	8.9b
Apical-axillary meristem	33.3a	15.6bcd	24.5a
Petiole	6.3cd	3.1cd	4.7b
Immature inflorescences	27.1ab	36.5a	31.8a
Average	16.9	12.1	14.5

are rich in terms of auxin, cytokinin, and gibberellic acid content, while the mature leaves, the stem segments, and the root contain lower amounts (Noggle & Fritz 1976). Consequently, differential responses of explants to the NAA concentrations may be attributed to endogenous hormone levels of explants. Wernicke et al. (1986) reported that increased auxin concentrations prevented meristematic cell divisions, resulting in decreased reactions. Similar findings were reported by several authors (Hiroshi & Zhuomeng 1993; Ozcan et al. 1996). Kinetin was found to be a better cytokinin type than BAP ($P \leq 0.05$) (Table 2). The effect of cytokinin concentrations varied depending on the explant used. The immature inflorescences produced higher rates of callus induction at 2.3 μM kinetin while the 0.9 μM was better for the apical-axillary meristems.

Following 3 weeks of culture, highly embryogenic and compact callus and somatic embryos were obtained from the apical-axillary meristems (Fig. 1A) while soft and moist callus emerged from the cut

edge of the internode segments. This reaction was particularly pronounced when internodal segments were placed onto the medium vertically instead of horizontally. In contrast, horizontal placing was a better method for the petioles. The young leaves were more reactive when their tips were cut before culturing. At the fourth week of culture, somatic embryos started to emerge from the epidermal layer of internodes and easily differentiated to shoots within 6 weeks (Fig. 1B). Similarly, Gu (1987) reported the differentiation of somatic embryos into shoots was highest from young leaf and internode explants. In addition, NAA induced this differentiation (Orczyk 1992). Sainfoin has an imparipinnate leaf structure in which the pulvini of leaflets are meristematic and very easy to isolate, and thus could be considered as a promising explant (Çelıktaş 2001).

The highest mean number of shoots per explant was obtained from the apical-axillary meristems (Table 3). However, the regeneration varied depending on the cytokinin type and concentration.

Table 2 Average rate (%) of callus induction from explants of sainfoin cultured on B5-medium with four concentrations of two cytokinins. Means of combinations of explant-cytokinin type-cytokinin concentration with different letters are significantly different at the $P \leq 0.05$ level, as analysed by Duncans' Multiple Range test. BAP, 6-benzylaminopurine.

Explants	Kinetin				BAP			
	0 μM	0.9 μM	1.4 μM	2.3 μM	0 μM	0.9 μM	1.3 μM	2.2 μM
Internode	0.0e	4.2e	0.0e	8.3e	0.0e	8.3e	0.0e	0.0e
Leaf	0.0e	8.3e	20.8b-e	8.3e	8.3e	8.3e	16.7b-e	0.0e
Apical axillary meristem	20.8b-e	54.2a	20.8b-e	45.8a-c	25.0b-e	16.7c-e	12.5de	0.0e
Petiole	0.0e	4.2e	20.8b-e	0.0e	0.0e	0.0e	12.5de	0.0e
Immature inflorescences	8.3e	45.8a-c	50.0ab	62.5a	45.8a-c	12.5de	12.5de	16.7c-e
Average		19.2a*				9.8b		

*Means of cytokinin types are significantly different as analysed by t -test.

Table 3 Average rate (number of shoots per explant) of shoot regeneration from explants of sainfoin cultured on B5-medium with four concentrations of two cytokinins. Means of combinations of explant-cytokinin type-cytokinin concentration with different letters are significantly different at the $P \leq 0.05$ level, as analysed by Duncans' Multiple Range test. BAP, 6-benzylaminopurine.

Explants	Kinetin				BAP				Average
	0 μM	0.9 μM	1.4 μM	2.3 μM	0 μM	0.9 μM	1.3 μM	2.2 μM	
Internode	0.4c-e	0.2de	0.4c-e	0.1de	0.2de	0.3c-e	0.4c-e	0.0e	0.2c
Leaf	0.1de	0.8b-e	0.8b-e	0.2de	0.1de	0.5c-e	0.3c-e	0.3de	0.4c
Apical-axillary meristem	1.5b-e	1.8bc	3.5a	3.6a	1.0b-e	2.0b	0.4c-e	0.7b-e	1.8a
Petiole	1.3b-e	0.9b-e	0.9b-e	0.3c-e	0.4c-e	1.6b-d	0.3c-e	0.9b-e	0.8b
Immature inflorescences	0.1de	1.4b-e	0.7b-e	0.3c-e	0.8bc	0.0e	0.4c-e	0.0e	0.5bc

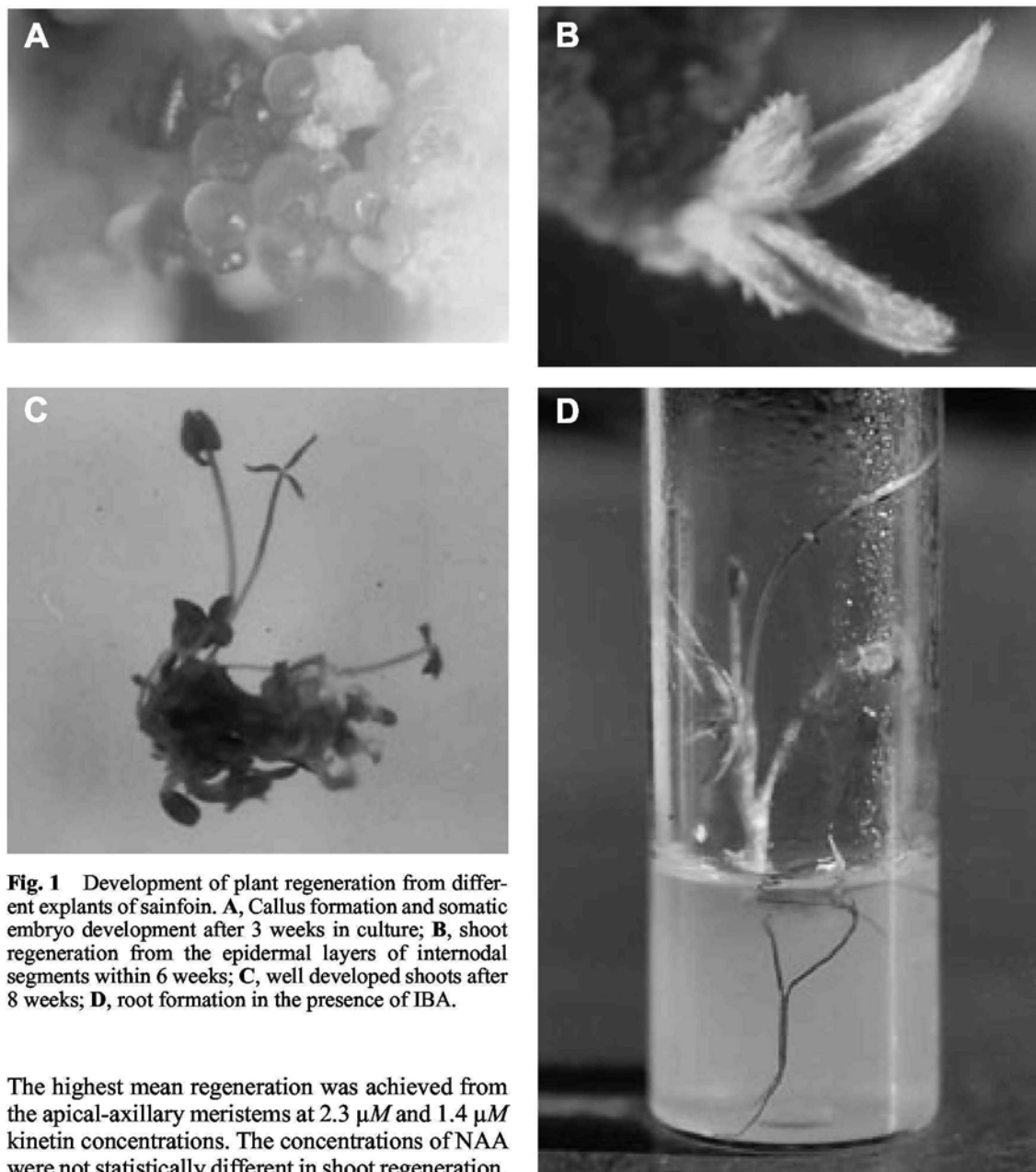


Fig. 1 Development of plant regeneration from different explants of sainfoin. **A**, Callus formation and somatic embryo development after 3 weeks in culture; **B**, shoot regeneration from the epidermal layers of internodal segments within 6 weeks; **C**, well developed shoots after 8 weeks; **D**, root formation in the presence of IBA.

The highest mean regeneration was achieved from the apical-axillary meristems at $2.3 \mu\text{M}$ and $1.4 \mu\text{M}$ kinetin concentrations. The concentrations of NAA were not statistically different in shoot regeneration. Nevertheless, kinetin was found to be superior to BAP, but this superiority varied depending on the NAA concentrations (Table 4). Pupilli et al. (1989) reported that the shoot regeneration of sainfoin could be increased by combining 2,4-dichlorophenoxyacetic acid (2,4-D) or NAA with kinetin. This finding is in agreement with our results. On the other hand, our previous studies on the same species showed that the NAA + BAP combination was the best for a good regeneration system (Çelıktaş 2001). This contrast

could be because of the medium used. However, the most effective plant growth regulator combination and dosages can result in great variation between individuals of same species and even between the explants. Plants have various types and amounts of metabolites and endogenous hormones at different developmental stages on different organs, leading to different *in vitro* requirements.

When the regenerated shoots were transferred to the micro-propagation medium, a large number of shoots were propagated within 8 weeks (Fig. 1C). Well developed shoots were excised and cultured on half-strength MS medium supplemented with 9.8 μ M indole butyric acid (IBA). In the first week on this medium, pre-callus initiation was observed at the root region. Ozcan et al. (1996) reported similar observations for the same species.

Apical-axillary meristems produced more plantlets, while the others showed no statistical difference (Table 5). Kinetin was a better cytokinin type for all explants; however, the effect of BAP combined with 5.4 μ M NAA was similar (Table 6).

The most shoots rooted within 4 weeks on half-strength MS medium supplemented with 9.8 μ M IBA (Fig. 1D). The solidifying agent also affected the rooting (Grum et al. 1998). The solidifier gelrite produced a high rooting percentage. However, the quality of roots was poor because of the initial callus formation. Avenido & Hattori (1999) reported that indole acetic acid (IAA) added to rooting medium also caused callus formation prior to rooting. The rooted shoots were then transferred to pots containing compost and grown in polyethylene bags for 4–5 days for acclimatisation, and later placed in a growth chamber. Consequently, the highest frequency of shoot regeneration was achieved from apical-axillary

Table 4 Average rate (number of shoots per explant) of shoot regeneration from explants of sainfoin cultured on B5-medium with two concentrations of naphthalene acetic acid (NAA) and two cytokinins. Means of combinations of NAA concentration-cytokinin type with different letters are significantly different at the $P \leq 0.05$ level as analysed by LSD test. BAP, 6-benzylaminopurine.

	Kinetin	BAP	Average
5.4 μ M NAA	0.8a	0.9 a	0.8
10.7 μ M NAA	1.1a	0.2 b	0.6
Average	0.9a*	0.5 b	0.7

*Means of cytokinin types are significantly different as analysed by *t*-test.

Table 5 Average rate (number of plantlets per explant) of plant regeneration from explants of sainfoin cultured on B5-medium with two cytokinins. Means of combinations of explant-cytokinin type with different letters are significantly different at the $P \leq 0.05$ level as analysed by Duncans' Multiple Range test. BAP, 6-benzylaminopurine.

Explants	Kinetin	BAP	Average
Internode	0.1bc	0.2bc	0.1c
Leaf	0.2bc	0.0c	0.1c
Apical axillary meristem	1.2a	0.5b	0.8a
Petiole	0.4bc	0.4bc	0.4b
Immature inflorescences	0.3bc	0.2bc	0.3bc
Average	0.5a*	0.3b	0.4

*Means of cytokinin types are significantly different as analysed by *t*-test.

Table 6 Average rate (number of plantlets per explant) of plant regeneration from explants of sainfoin cultured on B5-medium with two concentrations of naphthalene acetic acid (NAA) and two cytokinins. Means of combinations of NAA concentration-cytokinin type with different letters are significantly different at the $P \leq 0.05$ level as analysed by LSD test. BAP, 6-benzylaminopurine.

NAA concentrations	Kinetin	BAP	Average
5.4 μ M	0.4a	0.4a	0.4
10.7 μ M	0.5a	0.1b	0.3

meristems on B5 medium with 10.7 μ M NAA and 1.4 or 2.3 μ M kinetin.

In conclusion, the procedure described here is rapid, simple and valuable for both synthetic and artificial seed production. The results of the study showed that a large number of shoots from desirable sainfoin individual plants could be propagated for the synthetic seed production of cross-pollinated species such as sainfoin within a very short time via apical-axillary meristems by optimising the auxin and cytokinin types and concentrations in the medium.

REFERENCES

- Ahuja PS, Lu DY, Cocking EC, Davey MR 1983. An assessment of the cultural capabilities of *Trifolium repens* L. (white clover) and *Onobrychis viciifolia* Scop. (sainfoin) mesophyll protoplasts. *Plant Cell Reports* 2: 269–272.
- Avenido RA, Hattori K 1999. Differences in shoot regeneration response from cotyledonary node explants in Asiatic *Vigna* species support genomic grouping within subgenus *Ceratotropis* (Piper) Verdc. *Plant Cell, Tissue and Organ Culture* 58: 99–110.
- Çeliktas N 2001. Research on using *in vitro* techniques in the sainfoin (*Onobrychis sativa* L.) breeding. Unpublished PhD thesis, Institute of Natural and Applied Sciences of Çukurova University, Adana, Turkey. 219 p.
- Elci S, Ekiz H, Sancak C 1996. The problems of sainfoin (*Onobrychis* sp.) production in Turkey. 3rd National Congress of Pasture and Forage Plants. Erzurum, Turkey.
- Gamborg O, Miller R, Ojima K 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* 50: 151–158.
- Gu Z 1987. Callus culture of sainfoin (*Onobrychis viciifolia*) and plant regeneration through somatic embryogenesis. *Annals of Botany* 60(3): 309–313.
- Grum M, Camloh M, Rudolph K, Ravnkar M 1998. Elimination of seed-borne bacteria by thermotherapy and meristem culture. *Plant Cell, Tissue and Organ Culture* 52: 79–82.
- Hiroshi N, Zhuomeng Y 1993. Tissue culture of sainfoin. *Proceedings of the XVII International Grassland Congress*. Pp. 1044–1045.
- Li YG, Tanner GJ, Delves AC, Larkin PJ 1993. Asymetric somatic hybrid plants between *Medicago sativa* L. (alfalfa, lucerne) and *Onobrychis viciifolia* Scop. (sainfoin). *Theoretical Applied Genetics* 87: 455–463.
- Lu Y, Sun Y, Foo LY, McNabb WC, Molan AL 2000. Phenolic glycosides of forage legume *Onobrychis viciifolia*. *Phytochemistry* 55: 67–75.
- Murashige T, Skoog F 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473–497.
- Noggle GR, Fritz GJ 1976. *Introductory plant physiology*. Englewood, New Jersey, Prentice-Hall Inc. Pp. 502–544.
- Orczyk AN 1992. Somatic embryogenesis of agriculturally important lupin species (*Lupinus angustifolius*, L. *albus*, L. *mutabilis*). *Plant Cell, Tissue and Organ Culture* 28: 19–25.
- Ozcan S, Yıldız M, Sancak C, Ozgen M 1996. Adventitious shoot regeneration in sainfoin (*Onobrychis viciifolia* Scop.). *Turkish Journal of Botany* 20: 497–501.
- Pupilli FD, Amiani F, Pezzotti M, Arcioni S 1989. Plant regeneration from callus protoplasts of *Onobrychis viciifolia* Scop. (sainfoin). *Plant Science (Limerick)* 63(1): 87–94.
- Wernicke W, Grost J, Molkovits L 1986. The ambiguous role of 2,4-dichlorophenoxyacetic acid in wheat tissue culture. *Physiologia Plantarum* 68: 597–602.