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Research Note

In Vitro Micropropagation of Sainfoin (*Onobrychis viciifolia* Scop.)

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Abstract: A method for *in vitro* micropropagation of sainfoin was developed. Following optimization of the growth regulator combinations in the media, large numbers of shoots were propagated from a single embryonic axis within 8 weeks on Murashige and Skoog (MS) media supplemented with combination of different 6-benzylaminopurine (BAP), indole-butyric acid (IBA) and α-naphthaleneacetic acid (NAA) concentrations. The highest number of shoot multiplication was obtained on the media containing 2 mg/l BAP and either 0.05 or 0.1 mg/l IBA, and on the media supplemented with 2 mg/l BAP and either 0.05, 0.1 or 0.5 mg/l NAA, or 8 mg/l BAP and 0.05 mg/l NAA. The highest shoot length was observed on a medium containing 2 mg/l BAP only. Excised shoots rooted at a frequency of 60 % within 4 weeks in half strength MS medium containing 1 mg/l IBA and grew into normal fertile plants.

Key Words: Sainfoin, *Onobrychis viciifolia*, micropropagation, shoot multiplication, shoot tip culture

In Vitro Koşullarda Korunga (*Onobrychis viciifolia* Scop.)'nın Hızlı Çoğaltımı

Özet: *In vitro* koşullarda korunga bitkisinin hızlı çoğaltımı için bir yöntem geliştirilmiştir. Büyüme ortamındaki bitki büyümesini düzenleyicilerin oranı optimize edildikten sonra, farklı konsantrasyonlardaki 6-benzylaniopurine (BAP), indole-butyric acid (IBA) ve a-naphthaleneacetic acid (NAA) ilave edilen Murashige ve Skoog (MS) ortamında, tek bir embriyodan, 8 hafta içerisinde çok yüksek oranda sürgün çoğaltımı elde edilmiştir. En yüksek sürgün çoğaltımı, 2 mg/l BAP ile IBA'nın 0.05 ve 0.1 mg/l'lik ortamlarından ve 2 mg/l BAP ile NAA'nın 0.05, 0.1 ve 0.5 mg/l'lik ortamlarından veya 8 mg/l BAP ile 0.05 mg/l NAA ortamından elde edilmiştir. En yüksek sürgün boyu yalnızca 2 mg/l BAP'nın bulunduğu ortamda gözlenmiştir. Elde edilen sürgünler 1 mg/l IBA içeren MS ortamında 4 hafta içerisinde %60 oranında köklendirilerek, normal bitkiler elde edilmiştir.

Anahtar Sözcükler: Korunga, *Onobrychis viciifolia,* mikroüretim, sürgün çoğaltımı, sürgünucu kültürü

Introduction

The forage legume sainfoin is an important crop well adapted to the dry and semi-dry regions of Turkey, where the cultivation of alfalfa is restricted by the environmental conditions. Sainfoin grows well in calcareous and chalky soils as well as in the soils with high water table (1, 2). As a member of *Leguminosae*, sainfoin improves the nitrogen content of the soil by fixing the atmospheric nitrogen. Its well-developed thick root system penetrates into the deeper layers of the soil, therefore, improves the organic matter of the soil and prevents soil erosion. Sainfoin is also a very palatable forage plant and since it does not induce bloat, strip grazing of green forage is possible. Furthermore, the lasting green colour of the foliage is aesthetically pleasing.

Many improved plant varieties has been regenerated using tissue and cell cultures techniques (3, 4). Although sainfoin is very important as a forage and soil improvement crop, it has received little attention for in vitro studies. Recently, a high frequency of adventitious shoot regeneration from a range of explants including mature (5) and immature embryos (6), leaflets, petioles and stems has been reported in our laboratories for sainfoin (7). Although adventitious shoot regeneration is a basic prerequisite for the application of genetic engineering to crop improvement, the production of plants from apical meristems and axillary buds has proven to be the most generally applicable and reliable method of *in vitro* micropropagation (4). This paper describes a rapid and efficient *in vitro* micropropagation system from seed explants of sainfoin.

Materials and Methods

Seed Sterilization and Germination

Healthy seeds of *Onobrychis viciifolia* Scop. of an ecotype (Elçi) largely cultivated in Turkey were surface-sterilized in 100 % commercial bleach (containing 6 %

sodium hypochlorite) plus few drops of Tween 20 for 30 min with a continuous stirring, then rinsed three times with sterilized distilled water. The seeds were germinated aseptically on half-strength Murashige and Skoog (MS ; 8) medium containing 3% sucrose and 0.7% agar in Magenta GA-7 culture vessels.

Shoot Multiplication

After germination, two-third of each cotyledons and the radicle were discarded and intact embryonic axes were placed onto the shoot multiplication medium in 125 ml glass jars containing 40 ml medium. The shoot multiplication medium consisted of MS medium, 3% sucrose and 0.7% agar, 2-8 mg/l 6-benzylaminopurine (BAP) and 0.05-0.5 mg/l indole-butyric acid (IBA) or 0.05-0.5 mg/l α-naphthaleneacetic acid. The medium pH was adjusted to 5.6 with 1N NaOH or 1N HCl before autoclaving at 121°C, 1.4 kg/cm2 for 20 min. The cultures were kept at 26 ± 1 °C under cool white fluorescent light with a 16 h photoperiod. The number of shoots per explant and shoot length were scored after seven weeks of culture initiation. Each treatment had 4 replicates consisting of five 125 ml glass jars containing one explants. Data were statistically analysed by Duncan's multiple range test. Well-developed shoots were excised and rooted in agar-solidified half-strength MS medium supplemented with 1 mg/l IBA in Magenta GA-7 culture vessels. Rooted shoots were then transferred to pots containing compost.

Results and Discussion

Due to the outbreeding nature of sainfoin, plants propagated by sexual methods are highly heterogeneous and therefore, asexual (vegetative) propagation has great importance for preserving uniformity and unique characteristics of this crop (3). Probably, the most widespread technique for vegetative propagation is reproduction by actively growing pieces of shoots, called cuttings. Our previous *in vivo* studies showed that rooting of cuttings and establishment of rooted plantlets occurred at a very low frequency and appeared to be main problem in vegetative propagation of sainfoin (unpublished results). However, many plant species have been multiplied or propagated exclusively by *in vitro* techniques. In this study, the effect of varying concentrations of cytokinins and auxins in the growth media on *in vitro* micropropagation from seed explants of sainfoin was investigated.

In order to establish an efficient in vitro micropropagation system for sainfoin, intact embryonic axis explants were cultured on MS basal media supplemented with combination of different BAP, IBA and NAA concentrations. Prolific shoot initials from pre-existing apical and axillary meristems were detected within 2-3 weeks on all explants cultured. These shoot initials later developed into large number of shoots. *In vitro* micropropagation of sainfoin has not been reported before. However, high level of shoot multiplication was achieved from seed explants of other legumes such as *Phaseolus coccineus* L. (9), alfalfa (10) and chickpea (11). The results of the previous studies and the present work clearly reveal the micropropagation potential of seedling explants. In this work only 1-2 shoots were developed in some explants. However, early removal of these shoots enhanced shoot proliferation, as has been reported for pea (12). It was also shown in the current study that when apical and axillary meristems from micropropagated shoots were isolated and subcultured, similar shoot multiplication rates were observed.

The optimal combinations of auxins and cytokinins in the medium are perhaps the most critical factor enhancing shoot multiplication on a particular explant. In the present study, the number of shoots per explant and shoot length also varied dramatically with the varying levels of BAP, IBA and NAA levels in the basal media (Table 1 and 2; $p<0.05$). In the presence of IBA, the highest shoot multiplication was obtained on media containing 2 mg/l BAP and either 0.05 or 0.1 mg/l IBA (Table 1;

Table 1. *In vitro* micropropagation of sainfoin from embryonic axis after 8 weeks in culture on MS media containing various concentrations of BAP and IBA

Growth Regulators (mg/l) BAP	IBA	Mean no. of shoots/Explant		Shoot length (mm)	
2	0	6.0	d[1]	40.9	a
2	0.05	22.0	ab	28.4	bc
2	0.1	26.2	a	24.5	bcd
2	0.5	12.7	cd	30.7	b
4	0.05	13.2	cd	25.7	bc
4	0.1	15.33	bc	26.3	bc
4	0.5	8.5	cd	25.7	bc
8	0.05	9.2	cd	16.9	cd
8	0.1	12.2	cd	18.1	cd
8	0.5	10.8	cd	14.0	d

[1]Values within a column followed by different letters are significantly different at 0.05 probability level using Duncan's multiple range test.

Table 2. *In vitro* micropropagation of sainfoin from embryonic axis after 8 weeks in culture on MS media containing various concentrations of BAP and NAA

Growth Regulators (mg/l) BAP	NAA	Mean no. of shoots/Explant		Shoot length (mm)	
2	0.05	20.3	ab[1]	21.6	ab
2	0.1	24.0	a	29.9	a
2	0.5	18.6	ab	24.3	ab
4	0.05	13.0	bc	18.5	bc
4	0.1	12.8	bc	17.4	bcd
4	0.5	7.0	c	16.9	bcd
8	0.05	17.8	ab	11.4	cd
8	0.1	10.8	bc	10.1	cd
8	0.5	13.1	bc	9.1	d

[1]Values within a column followed by different letters are significantly different at 0.05 probability level using Duncan's multiple range test.

$p<0.05$; Figure 1a, 1b); whereas, shoot multiplication using NAA was best achieved on a range of media supplemented with 2 mg/l BAP and either 0.05, 0.1 or 0.5 mg/l NAA, or 8 mg/l BAP and 0.05 mg/l NAA (Table 2; $p<0.05$; Figure 1c). In general, high BAP levels reduced the number of shoots per explants. The previous studies in legumes have also confirmed the great importance of plant growth regulators on *in vitro* shoot multiplication (7, 10).

Similar to shoot numbers, shoot length was also influenced significantly by different BAP, IBA and NAA concentrations in the media (Table 1 and 2; $P<0.05$). The highest shoot length was observed on a medium containing 2 mg/l BAP only. In general, IBA containing media produced longer shoots than the media supplemented with NAA. Shoots stimulated by IBA showed sturdy growth with smaller leaves and better rooting capacity.

Well developed shoots were excised and cultured on half-strength MS medium supplemented with 1 mg/l IBA. More than 60% of shoots rooted on this medium within

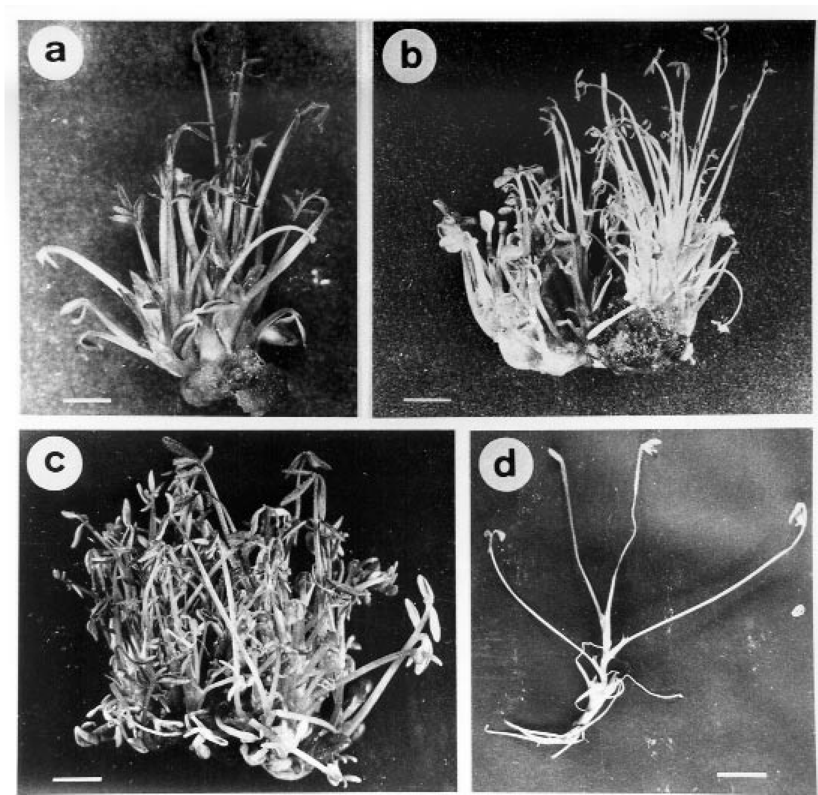

Figure 1. *In vitro* micropropagation of sainfoin from embryogenic axis. (a) Shoot development after 4 weeks in culture on a medium containing 2 mg/l BAP and 0.1 mg/l IBA. (b) Shoot development after 8 weeks in culture on a medium containing 2 mg/l BAP and 0.1 mg/l IBA. (c) Shoot development after 8 weeks in culture on a medium containing 2 mg/l BAP and 0.1 mg/l NAA. (d) Root formation on regenerated shoots after 4 weeks in culture. Bar = 0.5 cm in a; 1 cm in b and c; 1.5 cm in d.

four weeks (Figure 1d). Rooted plantlets were then transferred to pots (covered with a plastic bag for a few days to prevent wilting) containing compost and established later under greenhouse conditions.

In conclusion, the results of this study show that following the optimization of cytokinin and auxin combinations in the media, large numbers of shoots can be propagated from a single embryonic axis within 8 weeks. Furthermore, apical and axillary meristems from these shoots can be isolated and subcultured on micropropagation medium for further shoot multiplication. Thus, desirable genotypes can be micropropagated in large numbers within a short period of time.

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