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THE STUDY OF PHYTOHORMONES AND EXPLANTS ON CALLUS INDUCTION AND REGENERATION OF SAINFOIN (*ONOBRYCHIS SATIVA)*

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In this experiment, callus production and regeneration of Sainfoin (*Onobrychis sativa* var. Chadegan) were studied. The goal of this research was to establish a new method for micro proliferation of Sainfoin. Optimization of the growth regulator combinations in the Murashige and Skoog (MS) medium culture supplemented with combination of different 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxy acetic acid (2,4-D) concentrations was made. A large number of shoots was propagated from four explants. The biggest callus (5.47cm) was obtained on the MS medium containing 2,4-D (6 mgl^{-1}) + BAP (1 mgl^{-1}) from apical meristem explant. The large number of shoots was produced in the culture medium supplemented with BAP (4mg^{-1}) with average 1.6 plants per petri dish. The excised shoots were rooted in half strength MS medium containing indole-3-butyric acid (IBA) (1 mg^{-1}) and transferred to perlite which grew into normal fertile plants. This study showed that Sainfoin meristem explant, BAP and 2,4-D together were important for callogenesis and for regeneration only BAP was effective.

Keywords: Callus, regeneration, Sainfoin, MS medium

INTRODUCTION

Sainfoins (*Onobrychis sativa*) are Eurasian perennial herbs that have pale pink flowers and curved pods (Fig.1A). 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. It grows well in calcareous and chalky soils as well as in the soils with high water (Sancak, 1998). Sainfoin has needed no nitrogen fertilizer because there are many nitrogen stabilizing bacteria in its roots. It fattens livestock quicker than any other known forage. It has anthelmintic (anti-worm) properties and causes no bloat. Sainfoin is more tolerant to cold and drought; and more resistant to pests and diseases than alfalfa. Also, this plant is important in the honey industry. Breeding and biotechnology could be helpful in developing resistant varieties against different biotic and abiotic stresses with more flower size and yield. Regeneration via organogenesis or embryogenesis is the basis of tissue culture methods, and without regeneration it is impossible to produce transgenic plants. Efficient multiplication of this plant can offset lack of forage for growing livestock populations. Some of the varieties of Sainfoin can grow in regions having unfavorable conditions for alfalfa's growth. The most ideal regeneration systems for genetic transformation are direct and repetitive production of somatic embryos or de novo shoot organogenesis originating from single cells in the epidermis (Rugkhla and Jones, 1998). In a test, BAP at 22.2 µM supported the best combination of shoot quality and number of shoots produced (Neves *et al.*, 2001). BAP and 2, 4- D together increased embryo induction while these couldn't influence them without each other. Auxin and cytokinin together increased callus, while BAP alone increased regeneration in some meristemic parts (Bagheri and Safari, 2004). H6 medium comprising MS salts with 2,4-D (2mg^{-1}) and kinetin (0.5mg^{-1}) showed the greatest embryogenic efficiency (Lanas *et al.*, 2006). In somatic embryogenesis, somatic cells grow up as plants and this process is like zygotic embryogenesis (Gomes *et al*., 2006). Whereas genetic clones created through shoot organogenesis require further manipulation for root initiation, in somatic embryogenesis, complete plants with a bipolar axis, vascular system and functional meristems are produced in a single step (Bassuner *et al*., 2007). In a tissue culture test, induction of nodular meristemoids from 86% of the leaf cultures was achieved on MS medium with 4.0 µM BA and 1.0 µM NAA. High levels (78-100%) of induction were also achieved on WPM with different densities of BA (1.0-4.0 mgl^{-1}) and IBA (1.0-4.0 mgl^{-1}). The highest conversion of meristemoids into shoots was only 22% for 4.0 µM BA and 1.0 mgl^{-1} NAA on MS initiation medium (Moyo *et al.*, 2009). Recently, several reports have described shoot organogenesis was achieved through somatic embryo-like structures from different explants, mainly leaves and hypocotyls. In this research, a new method was developed for indirect organogenesis of Sainfoin, two phytohormones and 4 explants were applied, callus production and regeneration were examined.

MATERIALS AND METHODS

The present study was conducted in laboratory of Science and Research Branch, Islamic Azad University, Tehran, Iran during spring and summer 2006 to study and developed a new method for indirect organogenesis of Sainfoin (*Onobrychis sativa* var. Chadegan).

***Seed sterilization and germination*:** Seeds of Sainfoin were surface sterilized in 100% commercial bleach (containing 6% sodium hypochlorite) plus few drops of Tween 20 for 30 minutes with a continuous stirring, then rinsed three times with sterilized distilled water (Sancak, 1998) and inoculated in petri dishes containing agar solution (containing 7 gl^{-1} agar and 30 gl^{-1} sucrose). Explants were obtained from Sainfoin seedlings grown in plant growth chambers (Controlled Environments Ltd., Manitoba, Canada) at 25±2°C under 16 hours photoperiod at a photosynthetic photon flux density of 100 $\mu mol.m^{-2}.s^{-1}$ provided by cool white fluorescent light (Moyo *et al.,* 2009). After 7 to 10 days, seedlings grew 6 to 10 cm tall. Seedlings were cut in to 5×5 mm^2 segments and the explants were comprised of cotyledon leaf, apical meristem, stem and roots.

***Callus induction*:** MS medium (Murashige and Skoog, 1962) was supplemented with vitamins, sucrose (30 gl^{-1}), myo-inositol (0.1 gl^{-1}), polyvinylpyrrolidone (3 g^{-1}) and plant growth regulators was adjusted to pH 5.6 before the addition of the gelling agent (8 gl^{-1} Agar Bacteriological, Agar No.1, Oxoid Ltd., Bastingstoke, England). The medium was then autoclaved at 121°C and 15 psi for 20 min. MS medium was poured in polystyrene petri dishes (100 × 15 mm2) for callus induction and shoot regeneration. Grown callus was transferred to petri dishes after 37-40 days. Callus was divided into two pieces of size $5\times5\times5mm^3$. The callus parts were transferred to new MS medium supplemented with the hormones for regeneration. Five callus of each were incubated in the photoperiod chamber for a month at 25±2°C, and then number of plantlets was noted. The callus production experiment was run as Factorial Completely Randomized Design with three factors and repetitions. The factors were explants (4 kinds), BAP and 2,4-D. The densities of BAP and 2, 4-D were (0, 0.5, 1, 1.5 mg^{-1}) and (0, 2,4, 6 mgl^{-1}), respectively. The plant growth regulators were obtained from Sigma–Aldrich, St. Louis, USA.

***Regeneration*:** The regeneration was the next stage of the experiment. Callus was divided into two pieces of equal size ($5\times5\times5mm^3$). The callus parts were transferred to new MS medium supplemented with the hormones for regeneration. Five callus of each were incubated in the photoperiod chamber for a month at 25±2°C, and then a number of plantlets were noted (Fig. 1C).

The BAP densities were 0, 1.5 and 4 mgl^{-1} and 2,4-D concentrations were 0, 0.1 and 3 mgl^{-1}. Then, regenerated plantlets rooted with hormonal treatment (IBA) in half MS medium for 4 weeks (Fig.1D).

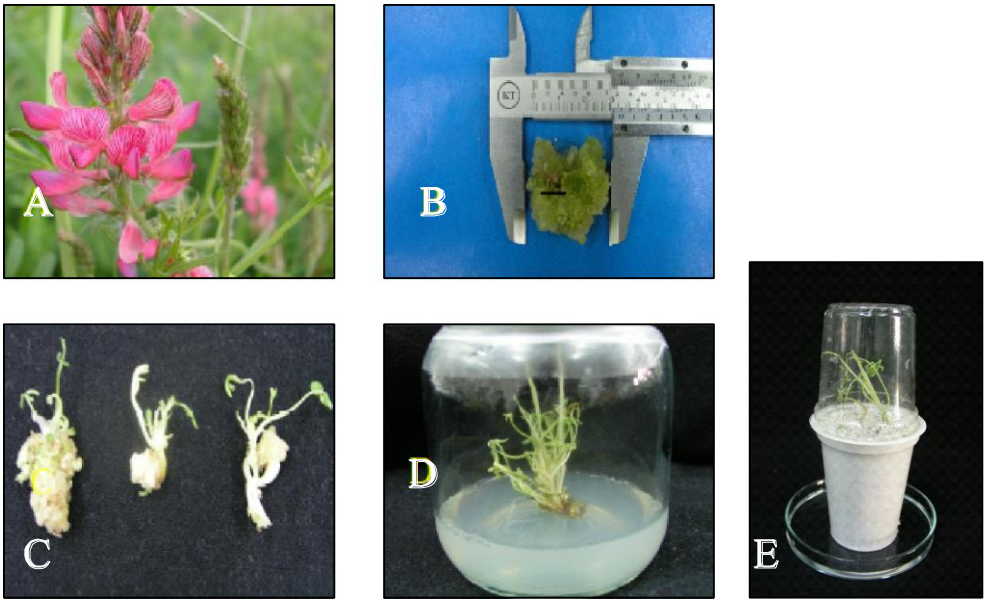

Figure 1. A. Sainfoin's (*Onobrychis sativa* var. Chadegan) flower; B. A well developed callus of Sainfoin in MS medium containing two hormones, Bar = 1cm; C. Regeneration of Sainfoin's calluses; D. Root production of plantlet in half-MS medium supplemented with IBA (9 $mg1^{-1}$); E. Acclimatization of a Sainfoin plantlet in pots covered with a plastic bag.

Table 1. Callus production of Sainfoin (*Onobrychis sativa* var. Chadegan). Explants after 30 days in MS supplemented with hormonal treatments.

Number	BAP (mg.l^{-1})	2, 4- D (mg.l^{-1})	Explant	Mean number of Callus size cm)
1	0	0	Cotyledon leaf	0.75 H-J*
2		2	″	4.36 A-E
3		4	″	1.79 D-J
4		6	″	4.90 A-D
5	0.5	0	″	0.00 J
6		2	″	0.00 J
7		4	″	2.60 A-J
8		6	″	4.40 A-F
9	1	0	″	0.00 J
10		2	″	4.92 A-D
11		4	″	5.41 A
12		6	″	5.13 A-C
13	1.5	0	″	0.94 G-J
14		2	″	4.97 A-D
15		4	″	4.43 A-F
16		6	″	3.72 A-H
17	0	0	Apical meristem	0.00 J
18		2	″	4.75 A-D
19		4	″	4.83A-D
20		6	″	5.11 A-C
21	0.5	0	″	0.00 J
22		2	″	2.78 A-J
23		4	″	2.99 A-J
24		6	″	0.38 IJ
25	1	0	″	4.68 A-E
26		2	″	4.91 A-D
27		4	″	3.65 A-H
28		6	″	5.48 A
29	1.5	0	″	0.00 J
30		2	″	4.99 A-D
31		4	″	5.28 AB
32		6	″	5.07 A-C
33	0	0	Stem	1.05 G-J
34		2	″	4.16 A-G
35		4	″	0.00 J
36		6	″	1.49 E-J
37	0.5	0	″	2.86 A-J
38		2	″	1.29 F-J
39		4	″	1.27 F-J
40		6	″	2.11 B-J
41	1	0	″	3.31 A-I
42		2	″	4.79 A-D
43		4	″	4.94 A-D
44		6	″	3.34 A-I
45	1.5	0	″	1.49 E-J
46		2	″	3.00 A-J
47		4	″	4.71 A-E
48		6	″	4.69 A-E
49	0	0	Root	2.11 B-J
50		2	″	3.78 A-H
51		4	″	1.49 E-J
52		6	″	0.00 J
53	0.5	0	″	1.43 F-J
54		2	″	1.99 C-J
55		4	″	2.11 B-J
56		6	″	2.11 B-J
57	1	0	″	1.49 E-J
58		2	″	4.46 A-F
59		4	″	2.67 A-J
60		6	″	4.11 A-G
61	1.5	0	″	4.07 A-G
62		2	″	4.40 A-F
63		4	″	3.02 A-J
64		6	″	5.21 A-C

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

**Means, in each column and for each treatment, followed by similar letter(s) are not significantly different at 5% of probability, level –using Duncan's Multiple Range Test.*

The plantlets were transferred to pots (covered with a plastic bag to prevent wilting for a few days) containing Peat moss, acclimatized in a greenhouse and then transplanted to soil (Sancak, 1998). The data on callus production and regeneration was recorded to analysis of variance (ANOVA). The least significant difference test at 5% probability level was used for the mean separation.

RESULTS AND DISCUSION

The data emphasized the fact that Sainfoin has a good capacity of *in vitro* callogenesis and regeneration (Fig. 1B). Many factors including the choice of growth regulators and choice of explants are responsible for successful callus production and regeneration. In the study of callus production about the leaf cotyledon explant, the greatest callus (5.41cm) was produced in existence of two hormones with high ratio 2,4-D to BAP, and the smallest callus produced in lack of 2,4-D (Table 1). These results confirm the previous findings of Bagheri and Safari (2004).

About the apical meristem, the greatest callus (5.48cm) was produced in presence of two hormones with high ratio of 2,4-D to BAP, and no callus was produced in the lack of two phytohormones or 2,4-D (Table 1). The results of this study were in agreement with the findings of Lanas *et al.* (2006). Next, about the stem explant, the greatest callus (4.94cm) grew in with high ratio of 2,4-D to BAP, and the smallest callus (1.05cm) produced in the lack of two growth plant regulators or BAP. These findings are in line with the finding of Moyo *et al.* (2009). Finally, in the root explant, maximum size of callus (5.21cm) was shown in existence of two hormones 2,4-D and BAP, and no callus was produced in the lack of BAP or 2,4-D. These results confirm the previous finding of Bassuner *et al*. (2007) and Gomes *et al.* (2006). The consideration on regeneration defined that BAP, 2, 4-D and BAP × 2, 4-D had sense in 1% probable levels. BAP had the most effect on regeneration (Fig. 2, No. 7). Addition of BAP and 2,4-D increased regeneration (Figure 2). The BAP had positive and 2,4-D negative effect on regeneration, and interaction effect between two hormones was negative (Fig. 2). These results are in line with some scientist's point of view as Bagheri and Safari (2004) and Lanas *et al.*, 2006).

The well developed shoots were transferred and cultured on half-strength MS medium supplemented with IBA (1 mgl^{-1}). More than 60% of shoots rooted on this medium within four weeks.

***Conclusions*:** Many factors including the choice of growth regulators and choice of explants were responsible for successful callus production and regeneration. The increase of 2,4-D (4 or 6 mgl^{-1}) ratio to BAP in each explant raised callus production of Sainfoin. BAP and 2,4-D had positive effect on the callus production. The Shoot meristemic explants produced the greatest callus.

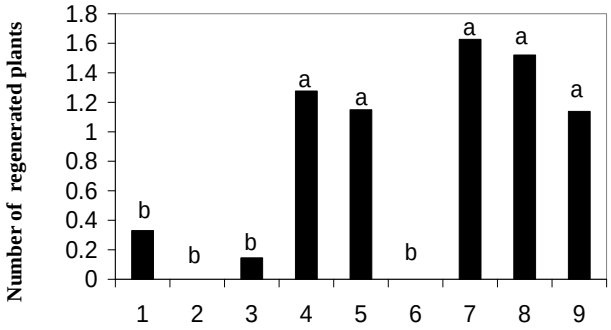

Figure 2. Effect of BAP × 2, 4-D interaction on regeneration of Sainfoin levels BAP × 2,4-D (mg^{-1}) hormonal interaction
1: (0, 0), 2: (0, 0.1), 3: (0, 3), 4: (1.5, 0), 5: (1.5, 0.1), 6: (1.5, 3), 7: (4, 0) , 8: (4, 0.1) , 9: (4, 3).

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