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Induction of different types of callus and somatic embryogenesis in various explants of Sainfoin (*Onobrychis sativa*)

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Abstract

To explore the potential for *in vitro* rapid regeneration of Sainfoin (*Onobrychis sativa*), different concentrations of 6-Benzylaminopurine (BAP), 1-naphthaleneacetic acid (NAA) and BAP combined with Indole-3-butyric acid (IBA) were evaluated for their effects on the induction of somatic embryos from leaf, stem and root explants. Different explants were cultured on MS medium supplemented with various concentrations (0, 0.5, 1, 1.5, 2, 2.5 and 3 mg/l) of each kind of hormone. Callus induction percentage, fresh weight, color and texture of the callus were assessed after 11 and 22 days of culture. The optimum medium for the proliferation of embryogenic calli from leaf and root explants was MS supplemented with 2.5 mg/L BAP and 0.5 mg/L NAA. Concentrations of 2.5 mg/L BAP and 1.5 mg/L IBA also had a remarkable effect on root and stem explants. The best concentration to produce callus from stem explants was 0.5 mg/L BAP and 1 mg/L IBA. Results of mean comparison showed that BAP and NAA were more effective on different explants than BAP and IBA. Results of the double staining method proved that somatic embryogenesis occurred in the most concentrations of BAP and NAA. Under microscopic observations, the different developmental stages of the embryos (globular, heart, torpedo and cotyledonary) were revealed together in callus cells, indicating that the most tested hormone combinations were effective for somatic embryogenesis formation in this species. Root explants formed torpedo and cotyledonary stages faster than leaf and stem explants in the most combinations. Most calli from root explants were cream colored and friable, while calli were compact and light green from leaf and stem explants. Some combinations gave direct regeneration and (3 mg/L BAP and 2 mg/L IBA) in stem explants and (0.5 mg/L BAP and 2.5 mg/L IBA) in leaf explants had the highest number of shoots with average of 21 and 27 shoots per callus. The developed protocol established the production of different callus types from leaf, stem and root explants and plant regeneration through somatic embryogenesis.

Keywords: *Onobrychis sativa*, callus induction, somatic embryogenesis, 6-Benzylaminopurine, 1-naphthaleneacetic acid, Indole-3-butyric acid.
Abbreviation: BAP, 6-Benzylaminopurine; NAA, 1-naphthaleneacetic acid; IBA, Indole-3-butyric acid; MS medium, Murashige and Skoog medium.

Introduction

Sainfoin (*Onobrychis sativa* Scop.) is one of the most important forage legumes which is appreciated by farmers due to high palatability and high nutritional value properties (Delgado *et al.*, 2008). It is a very palatable forage plant and, as animal feed hay, it has one major advantage over alfalfa since it does not induce bloat. It is capable of improving soil organic matter and nitrogen content by fixing atmospheric nitrogen. Roots of sainfoin penetrate the deeper layers of the soil and a supply great amount of organic matter when ploughed under. Sainfoin is also very beneficial in rotational agriculture, valuable in erosion control and grows well with forage grasses in mixture (Ozgen et al., 1998). To prevent extinction and derive maximum benefits from the indigenous plants of a nation, it is necessary to preserve the germplasm. Mass propagation by means of cell and tissue culture technique is a powerful tool for plant germplasm conservation and rapid clonal multiplication as well as for reforestation and forage improvement (Farnum et al., 1983; Biondi and Thorpe, 1982; Reddy et al., 2001). Breeding programmes are constrained by limited knowledge of genetic variation, a long period of reproductive maturity and long intervals in seed years, the impossibility of storing seeds for extended periods, and difficulties in vegetative propagation (Chalupa, 1995). Somatic embryogenesis is an efficient tool for rapid propagation, genetic transformation, somatic hybridization, and somaclonal variation (Gray and Purohit, 1991; Kuksova et al., 1997; Martinelli and Gribaudo, 2001). It also provides cytological and genetic stability of regenerated plants (Bennici et al., 2004). Although somatic embryogenesis may occur spontaneously in some plant species, it is usually induced in tissue culture medium potentially from almost any part of the plant body (Kantharajah and Golegaonkar, 2003; Siong et al., 2011; Ahmed et al., 2011). Although sainfoin is very important as a forage and soil improvement crop, it has received little attention for *in vitro* studies (Sankac, 1999). A high frequency of adventitious shoot regeneration from a range of explants including mature (Ozcan et al., 1996a) and immature embryos (Ozcan et al,. 1996b), leaflets, petioles and stems have been reported for sainfoin (Ozcan et al., 1998). Somatic embryogenesis is frequently regarded as the best system for propagation of superior genotypes (Kim, 2000), mostly because both root and shoot meristems are present simultaneously. Somatic embryos could be used for

proliferative propagation or for gene transfer procedures in sainfoin (*Onobrychis sativa*) if adequate methods for initiation and development could be devised. Celiktas et al., (2006) reported somatic embryogenesis in sainfoin (*Onobrychis viciifolia* Scop.) from leaf and immature inflorescence explants. Several investigators have worked extensively on plant regeneration through somatic embryogenesis in different Sainfoin cultivars (Arcioni and Mariotti, 1983; Gu, 1987; Pupilli et al., 1989). The limiting step to the successful use of modern techniques in genetic improvement of the major crops has not been the transgene insertion itself, but rather the regeneration of viable plants from the transgenic explants material (Murphy, 2003). Thus, prior to successful agri-biotechnological research on crops, reliable callus induction and efficient *in vitro* regeneration system is urgently required (Abdellatef et al., 2008). Phenotypic variation among plants regenerated from *in vitro* growth condition is referred to as somaclonal variation (Garcia and Martinez, 1995). The extent of phenotypic variation is usually determined as the percentage of plants showing aberrations from the parental plant for one or more defined characteristics. Successful use of *in vitro* techniques for producing somaclonal variants depends on the establishment of an efficient method for regenerating a large number of plants indirectly from an intervening callus stage (Skirvin et al., 1994). Raghawa Ram and Nabors (1984) reported the necessity of a cytokinin and an auxin for the production of callus. The present study describes the procedures for the establishment of different types of callus and induction of somatic embryogenesis from various explants of sainfoin by using different concentrations of BAP, NAA and IBA.

Results

Aseptic seed germination and callus observation

The best sterilization procedures were achieved when 50% chlorox (outside the laminar-1min) and 70% of alcohol (inside the laminar-1min) were utilized, respectively. After 1-2 weeks, almost 100% of seeds were germinated. Explant sources, such as leaf, stem and root segments were derived from aseptic seedlings. Generally callus was formed after11 days from this species. There were various types of callus observed such as compact, bubbly and friable. The different colors seen were green, cream, white, brown and light green. Direct plant regeneration was achieved from stem and leaf explants cultured on MS supplemented with IBA and BAP after 22 days of culture. The best hormone concentrations were chosen based on the highest percentage and fresh weight of callus. Results of callus induction from leaf explants are shown in Tables 1 and 2. In these Tables, hormone concentrations which resulted in more than 0.1 g callus weight and 50% callus in the second record were chosen from 98 concentrations for comparison. Stem explants which produced more than 0.1 g callus after 22 days were chosen for assessment in Tables 3 and 4. Table 5 and 6 are drawn based on some concentrations which had root explants more than 0.25 g in the second subculture. Leaf and stem explants in some concentrations of BAP and IBA produced shoots directly. These combinations and number of shoots are displayed in Table 7. While there was a little growth extension on the explants, however, no callus production was observed during the culture period in the control (that is, basal medium without growth regulator).

Somatic embryogenesis expression

Embryogenic tissue was distinguished easily by double staining method. All embryogenic cells of different explants had large nuclei and dense cytoplasms. These nuclei stained intense bright red with acetocarmine. Strands in the cytoplasm also showed an affinity for acetocarmine and stained bright red. Figure1 shows formation of somatic embryos from leaf, root and stem explants, respectively.

Callus induction from leaf explants

Most of the leaf explants only grown bigger in size after 11 days. Callus initiation occurred from the cut ends of the explants during 3 weeks of culture in most of the leaf explants. The induction percentage, texture and color of callus and fresh weight was different on different media (Tables 1 and 2). The concentration of 2.5 mg/L BAP and 0.5 mg/L NAA was the first to induce callus production after 11 days with 82%. In combination of BAP and IBA, the MS media with 3 mg/l BAP alone also had the highest callus production after 11 days (79%). In combination of BAP and NAA, a remarkable increase in average fresh weight of calli was obtained (2.5 mg/L BAP and 0.5 mg/L NAA) with 0.461 g and cells were in torpedo stage (Fig. 2D). The concentrations of 3 mg/L BAP and 0.5 mg/L NAA and 2 mg/L BAP and 1.5 mg/L NAA produced more callus (high weight) and were in cotyledonary and globular stages, respectively. In this concentration (BAP, NAA), different types of color and texture were observed and most callus were compact and light green (Fig. 2A). Different stages of embryogenesis development were observed but the most of the calli were in the globular stage (Table 1). In combination of BAP and IBA, the highest fresh weight observed in 1 mg/L BAP and 2 mg/L IBA, with 0.422g in pre-embryo stage. The primary callus was greenish after 11 days but grew rapidly into light green and compact callus after 22 days. Most of the calli were in pre-embryo stage and the induction percentage of calli ranged from 47% to 98% among the different concentrations (Table 2). Mean comparison between BAP, NAA and BAP, IBA showed, there was no significant difference between two combinations after 11 days but fresh weight and callus percentage were higher in BAP, NAA after 22 days with 0.249g and 89.9%, respectively.

Callus induction from root explants

Although induction of callus and regeneration are not difficult from root explants in most species, however, sainfoin was very strenuous to produce callus. During the first 16 days, the root explants formed callus in most concentrations of BAP, NAA and BAP, IBA. In combination of BAP and NAA, two concentrations had the highest fresh weight. Whole explants in the media with 2.5 mg/L BAP and 0.5 mg/L NAA formed callus and average fresh weight was 0.875g in cotyledonary stage (Fig. 2E). Concentrations of 1.5 mg/L BAP and 3 mg/L NAA was also effective with average of 0.814 g in torpedo stage (Table 3).In combination of BAP and IBA, the response of explants were better in concentration of 3 mg/L BAP and 0.5 mg/L IBA and this combination produced 0.751g which was the highest fresh weight in the cotyledonary stage (Table 4). Browning of the explants was observed in some concentrations but most calli were light green and cream although white and green callus also appeared. All textures were friable and different stages of embryogenesis were displayed during the 3 weeks culture period.

Table 1. Effect of BAP and NAA combinations on induction of various colors, textures, weight and callus percentage from leaf explants.

		Callus%					
BAP	NAA	11Days	22Days	Weight(g)	Color	Texture	Embryo Stage
2.5	0	12 f	96 a	0.136 gh	Wight	FR	PE
3	0	34 def	100 a	0.112 h	Light G	BU	TR
2	0.5	75 ab	100 a	0.183 e-h	Light G	FR	TR
2.5	0.5	82 a	91 abc	0.461 a	Light G	CO	TR
3	0.5	45 cde	80 cd	0.383 ab	Green	CO	CT
0.5	1	50 bcd	96 a	0.237 d-g	Green	CO	PE
1	1	19 ef	55 e	0.348 bc	Green	CO	GL
0	1.5	51 bcd	98 a	0.255 c-f	Light G	CO	PE
0.5	1.5	54 bcd	78 cd	0.201 e-h	Light G	CO	PE
2	1.5	69 abc	99 a	0.381 ab	Light G	BU	GL
2.5	1.5	32 edf	87 abc	0.208 d-h	Green	CO	GL
3	1.5	67 abc	68 d	0.276 cde	Green	CO	GL
2	2	74 ab	97 a	0.238 d-g	Light G	CO	PE
0	2.5	21 e	97 a	0.171 e-h	Light G	FR	PE
2.5	2.5	36 ed	94 ab	0.162 fgh	Green	CO	GL
3	2.5	9 f	81 bcd	0.312 bcd	Light G	CO	GL
3	2.5	9 f	81 bcd	0.312 bcd	Light G	CO	GL
1	3	14 f	78 cd	0.167 fgh	Wight	CO	GL
2	3	13 f	94 ab	0.196 e-h	Light G	CO	GL
Mean		40.3 A	87.9 A	0.249 A			

FR: Friable, CO: Compact, BU: Bubbly, PE: Pre Embryo, GL: Globular, CT: Cotyledon, TR: Torpedo, G: Green. The means of the populations with same small letters were not significantly different as per Duncan's multi-range test at P<0.05

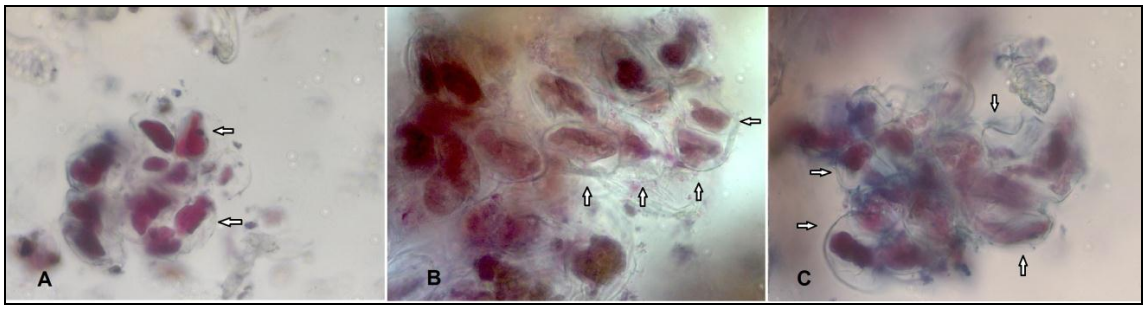

Fig 1. Embryogenic callus from double staining method observed with camera lens, magnification of 40x. The white arrows show somatic embryogenesis cell with red nucleus. A. Leaf explant cells; B. Root explant cells; C. Stem explant cells.

In comparing two different combinations, the average of callus percentage after 11 and 22 days were not significant but combination of BAP, NAA had the higher fresh weight and callus size with average of 0.472g. Faster callus growth and more callus production was observed in BAP, IBA since most explants had passed the globular stage after 22 days (Fig 2B). Some concentrations with only one kind of hormone caused abnormal root production. For example, some abnormal roots were observed in concentration with only 2.5 mg/L BAP, 0.5 mg/L IBA or 2 mg/L NAA (Fig 2G).

Callus induction from stem explants

Average of callus production was less than 10% in some concentrations of BAP and NAA after 11 days but mean of this parameter reached 81.4% after 22 days. Concentration of 2.5 mg/L BAP and 1.5 mg/L NAA had the highest fresh weight with 0.503 g in the torpedo stage and 0.5 mg/L BAP and 1 mg/L NAA was the second highest with 0.451 g in the shoot stage (Fig. 2F). There were no significant differences among other combinations (Table 5). Most calli were green and compact and five different stages of embryogenesis were observed at the same time in this combination (Fig. 2C,F). BAP was more effective than IBA for callus production in combinations of BAP and IBA because the concentrations of 2 mg/L BAP and 0 mg/L NAA and 1mg/L BAP and 0.5 mg/L NAA had the highest fresh weight (Table 6). There were no significant differences between BAP, NAA and BAP, IBA for fresh weight, callus production after 11 and 22 days but combinations of BAP and NAA were better. Most calli were green and compact in both combinations but some concentrations showed light green and friable in BAP and IBA.

Direct regeneration from leaf and stem explants

The different concentrations of BAP and IBA had significant influence on direct shoot regeneration from stem and leaf explants. Among the various concentrations of BAP and IBA, the combination of 2 mg/L BAP and 3 mg/L IBA had the highest percentage (92%) of direct shoot regeneration from leaf explants. This concentration also had maximum number of shoots with average of 21. Concentrations of 2.5 mg/L BAP and 0.5 mg/L IBA and 1.5 mg/L BAP and 1.5 mg/L IBA were also remarkable in percentage of direct shoot production with 68% and 67%, respectively (Table 7).

In the stem explants, the best concentration was 0.5 mg/L BAP and 2.5 mg/L IBA for average of shoot and direct shoot

Table 2. Effect of BAP and IBA combinations on induction of various colors, textures, weight and callus percentage from leaf explants.

		Callus%					
BAP	IBA	11Days	22Days	Weight(g)	Color	Texture	Embryo Stage
0.5	0	8 f	91 ab	0.109 ef	Light G	CO	PE
2.5	0	9 ef	92 ab	0.136 de	Wight	FR	PE
3	0	36 b-e	98 a	0.110 ef	Light G	BU	TR
1	0.5	54 abc	87 abc	0.136 de	Light G	CO	PE
2	1	54 abc	92 ab	0.232 bcd	Light G	FR	TR
0.5	1.5	41 bcd	86 abc	0.128 e	Green	CO	PE
1	1.5	19 def	78 bcd	0.267bc	Light G	CO	PE
2.5	1.5	3 f	59 ef	0.144 de	Green	CO	PE
1	2	17 def	57 ef	0.422 a	Light G	CO	PE
1.5	2	52 abc	76 bcd	0.149 de	Light G	CO	PE
2	2	61 ab	83 abc	0.171 de	Light G	CO	PE
1	2.5	77 a	83 abc	0.208 cde	Light G	CO	TR
3	2.5	8 ef	47 f	0.168 de	Light G	CO	PE
0	3	79 a	89 ab	0.122 e	Green	CO	PE
0.5	3	52 abc	75 bcd	0.117 ef	Green	CO	PE
1	3	31 def	56 ef	0.121 e	Light G	CO	PE
1.5	3	12 ef	64 de	0.022 f	Light G	BU	PE
2	3	74 a	91 ab	0.308 b	Light G	CO	PE
3	3	15 def	71 cde	0.111 ef	Light G	CO	PE
Mean		36.9 A	77.6 B	0.167 B			

FR: Friable, CO: Compact, BU: Bubbly, PE: Pre Embryo, TR: Torpedo, G: Green. The means of the populations with same small letters were not significantly different as per Duncan's multi-range test at P<0.05.

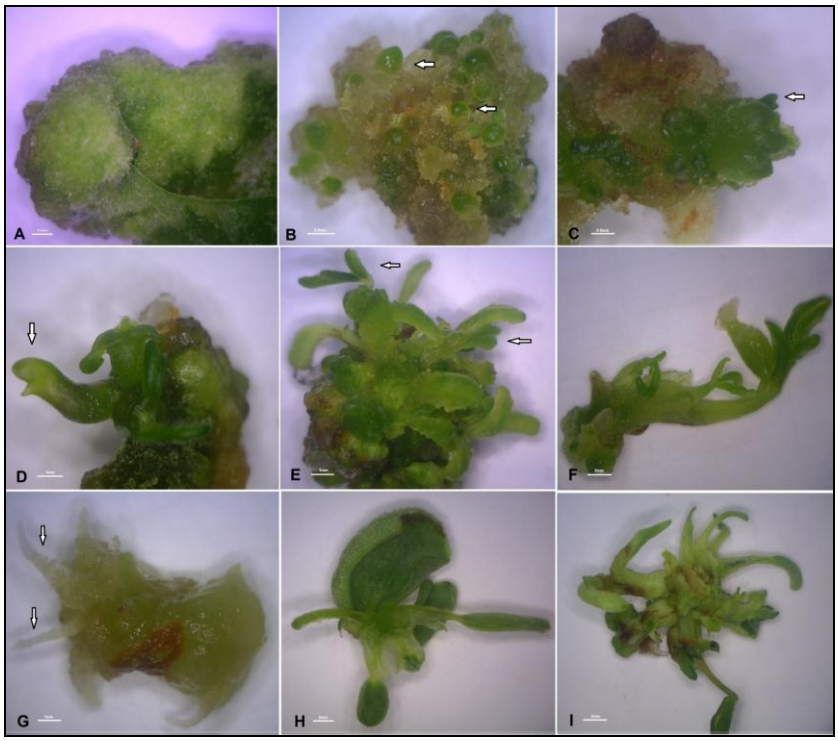

Fig 2. Stages of somatic embryogenesis in *Onobrychis sativa* (A-F). A. Pre-embryo stage and compact callus in leaf explants; B. Globular stage and friable callus in root explants; C. Heart-shaped stage in stem explants; D. Torpedo stage in leaf explant; E. cotyledonary embryo stage in root explants; F. Shoot production from callus in stem explants; G. Abnormal roots in root explants; H. Shoot formation from leaf explants; I. Shoot formation from stem explants. Arrows show different stages of the embryogenesis.

percentage with 27 and 77%. Some concentrations produced callus and direct shoots at the same time. For both goals (callus and shoot production), concentration of 1.5 mg/L BAP and 1 mg/L IBA was more effective. All these calli were compact and at pre-embryo stage with green and light green colors.

Discussion

In vitro micropropagation of sainfoin has not been reported a lot before. Although some researches have assessed a few combinations of BAP, NAA and 2,4-D (Karamian and Ranjbar, 2008; Saglam, 2010; Avci et al., 2010). However, high level of shoot multiplication was achieved from seed explants of other legumes such as alfalfa (Ozgen et al., 1997) and chickpea (Polisetty et al., 1997). The present study has established a protocol for different types of callus induction and formation of somatic embryos until conversion into normal plantlets of sainfoin. Many factors including the choice of growth regulators and explants were responsible for successful somatic embryogenesis (Luo et al., 1999). A wide investigation with 98 different concentrations of BAP, IBA and NAA was carried out. Results of the different concentrations showed that combinations of BAP and NAA was better than BAP and IBA for callus production and BAP and IBA was necessary to regenerate this species directly from leaf and stem explants. In the current investigation, MS medium supplemented with 2.5 mg/L BAP and 0.5 mg/L NAA was chosen as the best medium for leaf explants due to high fresh weight, callus percentage and its effective formation of embryogenesis stages in combination of BAP and NAA. Genetic improvement through transgenic technology is impended due to non-availability of efficient regeneration system in many grain legumes (Chandra and Pental, 2003). Embryogenic system offers an ideal tool for in vitro production and selection of transgenic plants (Christou, 1997). Such methods were available only for few grain legumes such as *Cajanus cajan* (Anbazhagan and Ganapathi, 1999) Chickpea (Kiran et al., 2005) and Horsegram (Varisai et al., 2004). Different plant species or cultivars will react differently to different hormone regime. In previous studies, the most researchers used 2,4-D for callus induction. Siong et al., (2011) obtained embryogenic callus and somatic embryogenesis from *Brassica oleraceae* hypocotyls and leaf explants whereas, Ahmed et al., (2011) used picloram, 2,4-D, NAA with ascorbic acid to induce somatic embryogenesis from *Phyla nodiflora* (L.) Greene. In combination of BAP and IBA, although concentration of 1 mg/L BAP and 2 mg/L IBA gave the highest fresh weight however, 2 mg/L BAP and 1 mg/L IBA is recommended because of its significant results in leaf explants. Concentration of 2.5 mg/L BAP and 0.5 mg/L NAA was also the best concentration in root explants for callus formation. Combination of 2 mg/L BAP and 1.5 mg/L IBA can be recommended not only for root explants but also for stem explants. Except 0.5 mg/L BAP and 1 mg/L NAA, other combinations of BAP and NAA had remarkable results in callus formation. Root explants developed to torpedo stage faster than leaf and stem explants, and also formation of callus was faster in root explants. Most of the calli were friable and light green in root explants, while in stem and leaf were compact and green. To regenerate directly from leaf explants, two concentrations of the BAP and IBA were required (2.5 mg/L BAP and 0.5 mg/L IBA and 3 mg/L BAP and 2 mg/L IBA). MS supplemented with 0.5 mg/L BAP and 1 mg/L IBA and also 0.5 mg/L BAP and 2.5 mg/L IBA had the best results in direct regeneration percentage and number of shoots formation from stem explants.

Materials and methods

Plant materials

The present work was carried out at Institute of Biological Sciences, University of Malaya, Kuala Lumpur. Seeds of *Onobrychis sativa* existing from natural resources of Iran were selected. Different concentrations of hormones were assessed in two separate experiments using factorial design plot with 30 replications which is standard in tissue culture research to decrease the error and enhance the accuracy.

Surface sterilization

The seeds were rinsed in distilled water for 20 minutes with addition of 1-2 drops of Tween-20. The seeds were sterilized by rinsing in sodium hypochlorite (chlorox) solution of 70%, 50%, 30% and 10% for 5 min each. The seeds were then soaked three times in sterile distilled water for 5 min. They were surface sterilized with 70% alcohol in the laminar flow. Finally the seeds were rinsed again with sterile distilled water three times.

Aseptic seed germination

Stock solutions of MS (Murashige and Skoog, 1962) media used in this study were prepared by dissolving the constituents amount of salt, iron and vitamin solution in 1L of distilled water and kept in dark-colored bottles in a refrigerator. The recommended amount of each stock solution was added to distilled water up to 75% of final volume required for the medium preparation. To prepare the Murashige and Skoog medium (MS), 3% (w/v) sucrose and 0.75% (w/v) agar were added to stock solution. The pH of the medium was adjusted to 5.6-5.8 using 1 N NaOH or 1N HCl. Autoclaving was carried out at 120 ℃ and 20 psi for 20 min. The laminar air flow cabinet was sterilized by exposing to ultraviolet radiation for at least 1 h before seeds inoculation. The seeds were cultured in control medium (MS basal) and transferred to a growth room and maintained at 20 ± 2 ℃ and 16 h photoperiod and 8 h dark period. Light was supplemented using white fluorescent tube at a photosynthetic photon flux density of 40-45 μmol m^{-2} sec^{-1}.

Explant preparation and culture

After 3-4 weeks of incubation, various explants of sainfoin such as root, leaf and stem were cut into small pieces (2-3 mm) from aseptic seedlings using fine sterile forceps with a sharp sterile blade. To determine the best auxin and cytokinin combination, the explants were inoculated in MS medium fortified with different concentrations of BAP, NAA and BAP, IBA in two separate experiments. Different concentrations (0, 0.5, 1.0, 1.5, 2, 2.5 and 3 mgl^{-1}) of each plant growth regulator were combined together and a total of 98 different combinations were examined. All the explants were maintained for 3 weeks in growth room at 22 ℃, 70% humidity and 16 h light photoperiod provided by cold fluorescent lamps.

Identification of embryogenic callus

After 3 weeks of culture, double staining technique was used to distinguish embryogenic cells from non-embryogenic cells. Two steps procedure (Preparation of 2% Acetocarmine and 0.5% Evan's Blue) were done for this method 2 g of carmine was weighed out and added to acid solution of 45%.

Table 3. Effect of BAP and NAA combinations on induction of various colors, textures, weight and callus percentage from root explants.

		Callus%					
BAP	NAA	11Days	22Days	Weight(g)	Color	Texture	Embryo Stage
2.5	0	26 c	91 b	0.433 cde	Wight	FR	CT
1.5	0.5	100 a	100 a	0.541 bc	Light B	FR	GL
2	0.5	100 a	100 a	0.269 e	Light B	FR	GL
2.5	0.5	100 a	100 a	0.875 a	Light G	FR	CT
3	0.5	100 a	100 a	0.489 cd	Light G	FR	GL
0.5	1	100 a	100 a	0.446 cde	Light G	FR	GL
2.5	1	89 a	100 a	0.369 cde	Light G	FR	GL
3	1	100 a	100 a	0.424 cde	Light G	FR	GL
1.5	1.5	83 ab	100 a	0.346 de	Light G	FR	GL
2.5	1.5	100 a	100 a	0.468 cd	Cream	FR	GL
3	1.5	100 a	100 a	0.705 ab	Light G	FR	CT
0.5	2	81 ab	100 a	0.345 de	Light G	FR	CT
2.5	2	100 a	100 a	0.430 cde	Cream	FR	GL
1.5	2.5	100 a	100 a	0.331 de	Light G	FR	GL
2.5	2.5	78 ab	100 a	0.342 de	Light G	FR	PE
3	2.5	28 c	99 a	0.342 de	Light G	FR	PE
0.5	3	83 ab	100 a	0.535 bc	Light G	FR	PE
1.5	3	63 b	100 a	0.814 a	Light G	FR	TR
3	3	100 a	100 a	0.479 cd	Light G	FR	PE
Mean		85.8 A	99.4 A	0.472 A			

FR: Friable, PE: Pre Embryo, GL: Globular, CT: Cotyledon, TR: Torpedo, G: Green, B: Brown,The means of the populations with same small letters were not significantly different as per Duncan's multi-range test at P<0.05.

Table 4. Effect of BAP and IBA combinations on induction of various colors, textures, weight and callus percentage from root explants.

		Callus%					
BAP	IBA	11Days	22Days	Weight(g)	Color	Texture	Embryo Stage
2.5	0	35 d	100 a	0.412 b-d	Wight	FR	CT
3	0.5	39 d	95 b	0.751 a	Green	FR	CT
0.5	1	64 c	100 a	0.283 f	Light G	FR	CT
2	1	100 a	100 a	0.332 ef	Cream	FR	TR
2.5	1	100 a	100 a	0.441 b-e	Light G	FR	CT
0	1.5	78 abc	86 c	0.311 ef	Light G	FR	PE
0.5	1.5	100 a	100 a	0.375 edf	Light G	FR	GL
1.5	1.5	100 a	100 a	0.473 bcd	Green	FR	CT
2	1.5	71 bc	100 a	0.552 b	Light G	FR	CT
2.5	1.5	100 a	100 a	0.331 ef	Brown	FR	GL
2	2	83 abc	100 a	0.505 bc	Cream	FR	GL
0	2.5	100 a	100 a	0.325 ef	Cream	FR	PE
2.5	2.5	100 a	100 a	0.336 ef	Cream	FR	GL
0	3	100 a	100 a	0.463 bcd	Wight	FR	GL
0.5	3	91 ab	100 a	0.413 c-f	Light G	FR	PE
1	3	84 abc	100 a	0.339 ef	Light G	FR	PE
1.5	3	100 a	100 a	0.312 ef	Cream	FR	PE
2	3	100 a	100 a	0.317 ef	Cream	FR	PE
2.5	3	83 abc	100 a	0.428 cde	Light G	FR	GL
Mean		85.6 A	99 A	0.405 B			

FR: Friable, PE: Pre Embryo, GL: Globular, CT: Cotyledon, TR: Torpedo, G: Green, The means of the populations with same small letters were not significantly different as per Duncan's multi-range test at P<0.05.

The solution was stirred until boiling for 5 min. To prepare Evan`s Blue solution, 0.5 g of Evan's Blue was measured and swirled to mix properly to 100 ml distilled water. Small pieces of the callus (3-5 mm) were placed on clean glass slides and few drops of 2% acetocarmine were added until all calli were submerged and divided with forceps into very small pieces. The slides were held over a low flame for a few seconds. After rising, three drops of 0.5% Evan`s Blue were added and samples were washed again 2-3 times. One or two drops of glycerol were added to stained cells to prevent from drying. Finally, the slides were examined under Axiocam microscope (Axioskop).

Parameters Measured – Fresh Weight and callus Percentage

After 11 days in maintenance medium, percentage of the callus was measured through average of 10 samples. After 22 days, most explants changed to callus. Fresh weight and callus percentage of leaf, root and stem explants were determined. For confirmation of embryogenic callus, the prepared slides were observed under microscope and assessed based on Sharma and Sharma (1980) method. Different callus textures (compact, bubbly and friable) were evaluated after 3 weeks.

Table 5. Effect of BAP and NAA combinations on induction of various colors, textures, weight and callus percentage from stem explants.

		Callus%					
BAP	NAA	11Days	22Days	Weight(g)	Color	Texture	Embryo Stage
1.5	0.5	39 b-e	67 cde	0.195 c	Light G	FR	GL
2.5	0.5	4 h	96 ab	0.186 c	Green	CO	CT
3	0.5	8 gh	89 abc	0.135 c	Green	CO	CT
0.5	1	18 e-h	92 ab	0.451 b	Light G	CO	SH
1	1	38 b-e	100 a	0.169 c	Green	CO	CT
1.5	1	0 h	32 f	0.129 c	Green	CO	TR
2	1	0 h	57 de	0.154 c	Green	CO	TR
2.5	1	29 c-h	84 abc	0.171 c	Green	CO	TR
3	1	9 gh	69 cde	0.154 c	Green	CO	CT
2	1.5	41 b-e	100 a	0.175 c	Light G	FR	HA
2.5	1.5	0 h	52 ef	0.503 a	Green	CO	TR
1.5	2	64 b	93 ab	0.174 c	Green	CO	GL
2	2	94 a	100 a	0.201 c	Light G	CO	PE
2.5	2	57 bc	93 ab	0.165 c	Green	CO	TR
0	2.5	37 b-g	100 a	0.119 c	Light G	FR	PE
1.5	2.5	53 bcd	56 de	0.248 c	Light G	FR	GL
0.5	3	2 h	75 bcd	0.126 c	Green	CO	GL
1	3	8 fgh	93 ab	0.165 c	Wight	FR	CT
2	3	24 d-h	100 a	0.159 c	Light G	FR	GL
Mean		27.6 A	81.4 A	0.201 A			

FR: Friable, CO: Compact, PE: Pre Embryo, GL: Globular, HA: Heart, CT: Cotyledon, TR: Torpedo, G: Green, The means of the populations with same small letters were not significantly different as per Duncan's multi-range test at P<0.05.

Table 6. Effect of BAP and IBA combinations on induction of various colors, textures, weight and callus percentage from stem explants

		Callus%					Embryo
BAP	IBA	11Days	22Days	Weight(g)	Color	Texture	Stage
2	0	0 e	17 e	0.330 a	Light G	CO	PE
2.5	0	0 e	89 ab	0.110 c	Cream	FR	PE
3	0	0 e	71 a-d	0.103 c	Cream	FR	PE
1	0.5	17 cde	93 ab	0.302 a	Green	FR	CT
3	0.5	52 ab	69 a-d	0.137 bc	Green	CO	CT
0	1	8 de	13 e	0.102 c	Green	CO	PE
2	1	26 cd	83 ab	0.155 bc	Light G	CO	CT
0.5	1.5	49 ab	97 a	0.105 c	Green	CO	PE
1.5	1.5	24 cd	97 a	0.159 bc	Green	CO	GL
2	1.5	64 a	97 a	0.138 bc	Light G	FR	TR
1.5	2	49 ab	81 abc	0.102 c	Green	CO	GL
2	2	24 cd	78 abc	0.111 c	Green	CO	HA
0	2.5	18 cde	46 d	0.172 bc	Light G	FR	PE
1	2.5	13 cde	53 cd	0.158 bc	Green	CO	CT
2.5	1	0 e	96 ab	0.108 c	Light G	FR	GL
2.5	2.5	18 cde	76 abc	0.155 bc	Light G	CO	SH
1.5	3	26 cd	67 bcd	0.133 bc	Green	CO	PE
2	3	61 a	96 ab	0.125 bc	Light G	CO	PE
2.5	3	32 bc	79 abc	0.184 b	Light G	CO	GL
Mean		25.3 A	73.5 A	0.152 A			

FR: Friable, CO: Compact, PE: Pre Embryo, GL: Globular, HA: Heart, CT: Cotyledon, TR: Torpedo, G: Green, The means of the populations with same small letters were not significantly different as per Duncan's multi-range test at P<0.05.

Colors of callus were also noted. Five different stages of somatic embryogenesis were observed using Dinocapture camera and photos were taken. Stem and leaf explants produced shoots directly in some concentrations of BAP, NAA and IBA and average of shoot number were calculated from 10 explants.

Statistical analysis

The two ways ANOVA was performed for each experiment and means were compared using Duncan's multiple range tests ($p< 0.01$) through SAS 9.2 software. Standard Division (SD) of the callus percentage and weight were less than ±0.5 and ±0.07 in all Tables, respectively.

Conclusion

The results of this study show that large numbers of shoots can be propagated from the optimization of cytokinin and auxin combinations in the media within 3-4 weeks. Furthermore, apical and axillary meristems from these shoots can be isolated and subcultured on micropropagation medium

Table 7. Effect of BAP and IBA combinations on direct shoot formation from leaf and stem explants

BAP	IBA	Callus% 11Days	Callus% 22Days	Direct	Weight(g)	Color	Texture	Embryo Stage	Number Shoot
LEAF									
2.5	0.5	18 a	24 bc	68 ab	0.051 b	Light G	CO	PE	12
3	0.5	5 bc	17 bc	31 c	0.031 b	Light G	CO	PE	7
0.5	1	17 a	36 b	24 c	0.195 a	Green	CO	PE	3
1.5	1	12 ab	57 a	31 c	0.021 b	Light G	CO	PE	9
1.5	1.5	1 c	3 c	67 ab	0.008 b	Green	CO	PE	4
2	1.5	9 b	12 c	51 bc	0.005 b	Green	CO	PE	8
3	2	7 bc	9 c	92 a	0.005 b	Light G	CO	PE	21
0	2.5	5 bc	11 c	21 c	0.008 b	Light G	CO	PE	5
STEM									
0.5	0.5	21 a	43 a	19 b	0.053 bc	Light G	CO	PE	3
0.5	1	0 c	9 b	57 a	0.024 c	Green	CO	PE	20
1	1	6 bc	19 b	52 a	0.106 ab	Green	CO	PE	2
1.5	1	12 b	16 b	64 a	0.141 a	Green	CO	PE	15
2.5	1.5	11 b	18 b	26 b	0.060 bc	Green	CO	PE	4
0.5	2.5	0 c	14 b	77 a	0.039 c	Green	CO	PE	27
2	2.5	8 bc	48 a	23 b	0.140 a	Light G	CO	PE	18
3	3	0 c	9 b	62 a	0.015 c	Light G	CO	PE	9

The means of the populations with same small letters were not significantly different as per Duncan's multi-range test at P<0.05.

for further shoot multiplication. Thus, desirable genotypes can be micropropagated in short period of time. The success in raising plants through somatic embryogenesis has opened up the possibility for large-scale clonal propagation of sainfoin. The somatic embryogenic lines have been used for genetic transformation and somatic hybridization which are the most advanced techniques for genetic improvement of varieties.

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