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Full Length Research Paper

Use of an adsorbent and antioxidants to reduce the effects of leached phenolics in *in vitro* plantlet regeneration of faba bean

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Development of a reliable *in vitro* regeneration protocol is necessary to facilitate genetic transformation of faba bean. However, leaching of phenolics from the explants of most genotypes of faba bean to the culture medium causes browning, and eventually kills the explants, hindering *in vitro* regeneration. This study is aimed to minimize the effect of phenolics and to identify the most suitable types of explants for *in vitro* regeneration. We pre-treated faba bean seeds in polyvinylpyrrolidone (PVP), then cultured different types of explants on tissue culture media supplemented with an adsorbent (activated charcoal) and antioxidants (ascorbic acid, cysteine and silver nitrate). Our results showed that treating the over night soaked seed (after removing the seed coat) with PVP solution (1000 mg/l) for 1 h, followed by culturing in Murashige and Skoog medium (MS medium) with 3% (w/v) sucrose, 0.8% (w/v) agar, 2 mg/l 6-benzylaminopurine and 2 mg/l thidiazuron, supplemented with ascorbic acid (1 mg/l) or activated charcoal (10 g/l), greatly reduced lethal browning in explants and improved shoot regeneration. The shoots rooted on half-strength MS medium supplemented with 0.5 mg/l α-naphthaleneacetic acid. The cotyledonary node is the most suitable type of explant for regeneration. Regenerated plantlets were successfully established in pots and set seeds in the green house.

Key words: Adsorbent, antioxidants, faba bean, *in vitro* regeneration, phenolics.

INTRODUCTION

Faba bean (*Vicia faba* L.) is an important cool season legume in West Asia, North Africa, Southern Europe, China, and Ethiopia. In developing countries people eat the faba bean as a vegetable, green or dried, fresh or canned. It is often eaten for breakfast in the Middle East, the Mediterranean region, China and Ethiopia (Bond et al., 1985). In industrialized countries, faba beans are mainly fed to animals.

Conventional breeding approaches (Bond, 1987) can be complemented by *in vitro* techniques for genetic improvement of crops (Smith, 1992). Genetic improvement of crops *in vitro* depends mostly on the ability of plant tissue to regenerate into whole plants (Smith, 1992). A reliable *in vitro* regeneration system for faba bean is, therefore, a pre-requisite for applying biotechnological tools for genetic improvement, such as *Agrobacterium*-mediated genetic transformation.

Extensive efforts to develop efficient regeneration methods for many legumes have resulted in a large number of *in vitro* protocols (Moss, 1992). However, faba bean is one of the least-studied legumes and only a few reports describe *in vitro* culture (Busse, 1986; Khalafalla and Hattori, 1999; Tegeder et al., 1995; Selva et al., 1989). Faba bean is considered one of the most recalcitrant legumes with respect to *in vitro* regeneration and genetic transformation. Therefore, *in vitro* regeneration has mainly been by organogenesis or somatic embryogenesis (based on the *de novo* regeneration from calli). But *de novo* regeneration of faba bean from calli in *Agrobacterium*-mediated transformation is time-comsum-

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ing and relatively inefficient (Böttinger et al., 2001).

The regeneration protocols reported in the literature are seldom repeatable because they did not address the problem of the leaching of phenolics into the medium by most genotypes of faba bean (Busse, 1986; Khalafalla and Hattori, 1999; Tegeder et al., 1995; Selva et al., 1989). The phenolics cause very high explant mortality and have a negative effect on regeneration (Bieri et al., 1984; Selva et al., 1989). Here, we report a rapid and reproducible *in vitro* regeneration method by organogenesis for faba bean. In this system the culture media are supplemented with an adsorbent and antioxidants to reduce the effect of leached phenolics on explant regeneration.

MATERIALS AND METHODS

Plant material and preparation of explants

We washed seeds of the faba bean variety 'Défès' (one of the phenolics-leaching varieties) in running tap water. Next, we sterilized the washed seeds by immersing them, first in 70% (v/v) ethanol for 1 min, and then in 30% (v/v) CLOREX (12% sodium chlorite; Age Company, Casablanca, Morocco) with a few drops of Tween 20, for 10 min. After rinsing the seeds with sterile distilled water for 1 min, we soaked them in sterile distilled water overnight. We removed the seed coats, and treated the seeds with polyvinylpyrrolidone (PVP) solution (1000 mg/l) for 1 h. We then left the seed to germinate on half-strength MS medium (Murashige and Skoog, 1962) under low light conditions (40 µmol $m^{-2}s^{-1}$) at 22°C for six days.

Culture media, *in vitro* culture and acclimatization

We used MSBT media (Khalafalla and Hattori, 1999) composed of MS medium (Murashige and Skoog, 1962), 3% (w/v) sucrose, 0.8% (w/v) agar, 2 mg/l 6-benzylaminopurine (BAP) and 2 mg/l thidiazuron (TDZ), and supplemented either with 10 g/l activated charcoal, 200 mg/l cysteine as suggested for chickpea by Sanyal et al. (2005), 0.02 g/l silver nitrate also suggested for chickpea by Sanyal et al. (2005) or 1 mg/l ascorbic acid as suggested for orchids by Arditti and Ernst (1993), according to the treatments. Initial study with supplementation of MSBT with activated charcoal 5 g/l, 10 g /l and 20 g/l, revealed that regeneration is better at 10 g/l supplementation. In order to increase firmness of the media, agar concentration was increased to 1% for the shoot sub-culturing at later stage of regeneration. We adjusted the pH of the media to 5.6 - 5.7 with 1 N NaOH or HCl then autoclaved the cultures at 121˚C and 1.06 kg/cm^2 for 20 min. The filter sterilized BAP, TDZ, ascorbic acid and cysteine were added after autoclaving the media.

When the seeds germinated (after six days), we excised the explants, the cotyledonary nodes, cotyledonary buds and epicotyls, and cultivated them on MSBT supplemented with an antioxidant or adsorbent. Cultures were sub-cultured onto fresh media prepared the same way after 15 days. After 45 days we counted the number of shoots per explant and measured the length of the shoots.

We harvested the shoots (3 - 4 cm) and transferred them onto half-strength MS medium supplemented with 0.5 mg/l α-naphthaleneacetic acid (NAA) (rooting medium). After 15 days we recorded the percentage rooting.

Plantlets with well-developed shoots and roots were removed from the culture medium, the roots were washed gently in warm tap water (35 ˚C) to remove any adhering solid media, and the plantlets were transferred to plastic pots (1.5 l capacity) filled with peat moss. Potted plantlets were covered with a transparent polythene bag (to ensure high humidity) and kept in the green house (16:8 h of light and dark photoperiod, 25°C). We gradually acclimatized the plantlets to normal field conditions. After two days, we cut the corner of the polythene bags. Then, we cut the top of the bags at two-day intervals, so that the hole gradually became larger. Finally, after 10 days we removed the polythene bags completely to acclimatize plants to normal field conditions. After seven days, we transferred the acclimatized plants to bigger pots (2/3 normal soil and 1/3 peat moss) and put them in a green house under normal day-length conditions.

Statistical analysis

We replicated each treatment three to five times and cultured one explant per plate (the treatments with unequal replications). Average number of shoots/explant and shoot length average (cm) were recorded. The results were analyzed using Proc GLM (SAS version 9.1). Analysis of variance (ANOVA) was used to test statistical significance.

RESULTS

The results show that treating the overnight soaked seed with PVP solution (1000 mg/l) for 1 h followed by culture in MSBT to which ascorbic acid or activated carbon or silver nitrate had been added completely eliminated browning in explants (Table 1). Seeds not treated with PVP did show browning.

However, explants from seed treated with PVP, followed by culture on MSBT medium with or without cysteine showed a high percentage of browning. The results of the adsorbent and antioxidant treatments on the number of shoots regenerated and shoot length per explant are presented in Table 2. Figure 1 shows some stages of *in vitro* regeneration.

We studied regeneration of cotyledonary nodes, cotyledonary buds and epicotyls. We observed that the type of explant had a significant effect on the mean number of shoots (ANOVA; $F=26.39$, d.f. = 2, 48; $P < 0.0001$) and the length of shoots regenerated (ANOVA; $F= 5.30$, d.f =2, 48; $P < 0.0083$). In general, cotyledonary node explants are the best for regeneration in faba bean (100% regeneration with a mean of 5.9 plants/explant), followed by cotyledonary buds (100% regeneration with a mean of 1.85 plants/explant) and epicotyls (9.6% regeneration with a mean of 1.25 plants/explant).

Ascorbic acid, activated charcoal, cysteine and silver nitrate had significant effects on the number of shoots (ANOVA; $F=5.2$, d.f = 4, 48; $P < 0.0015$) and the length of shoots regenerated per explant (ANOVA; $F= 11.43$, d.f = 4, 48; $P < 0.0001$). In general, explants cultured in MSBT media supplemented with ascorbic acid had the highest number of shoots after 45 days (average 5.1 plants per explant; Table 2). The control regenerated an average 4.8 plants per explant (only MSBT), activated charcoal an average 3.1 plants per explant, and cysteine and silver nitrate the fewest. However, the length of the shoots after 45 days differed between the treatments and the control.

Table 1. Effect of supplementation of ascorbic acid, cysteine, silver nitrate, and activated charcoal on browning of explants during *in vitro* regeneration of faba bean using cotyledonary node, epicotyl and cotyledonary buds as explants. The seeds were pre-treated with PVP and subsequently the explants were isolated and cultured.

Products supplemented to MSBT[1]	Number of explants tested				Over all percentage of explant browning[2]
	Cotyledonary bud	Cotyledonary node	Epicotyl	Total	
MSBT (control)	13	23	16	52	13.46
Ascorbic acid	13	12	23	48	0
Cysteine	9	14	5	28	17.8
Silver nitrate	6	24	12	42	0
Activated charcoal	8	20	12	40	0

[1]MSBT = MS medium supplemented with 2 mg/l of BAP, 2 mg/l of thidiazuron (TDZ) and 3% (w/v) sucrose.
[2]Recorded after 7 days of culturing.

Table 2. Effect of supplementation of ascorbic acid, cysteine, silver nitrate, and activated charcoal on regeneration of faba bean using cotyledonary node, epicotyl and cotyledonary buds as explants.

Products supplemented to MSBT [1]	Average number of shoots/explant			Shoot length average (cm)		
	Cotyledonary node	Epicotyl	Cotyledonary bud	Cotyledonary node	Epicotyl	Cotyledonary bud
MSBT [1] (control)	8.55	0.688	0.71	0.58	0.30	0.79
Ascorbic acid	9.65	2.394	2.83	0.45	0.77	0.78
Cysteine	4.33	0.000	2.45	0.64	0.00	0.27
Silver nitrate	1.99	0.000	0.67	0.44	0.00	1.16
Activated harcoal	4.20	1.41	2.87	1.97	0.60	1.42

[1]MSBT = MS medium supplemented with 2 mg/l of BAP, 2 mg/l of thidiazuron (TDZ) and 3% (w/v) sucrose.

In general, shoots were shorter and weaker in the control, and in the treatments with cysteine and silver nitrate. Type of explant and phenol adsorbent/antioxidant treatment had significant effect on the number of shoots (ANOVA; $F=2.31$, d.f =8, 48; $P<0.0347$) and length of shoots (ANOVA; $F= 3.04$, d.f =8, 48; $P<0.0076$). Cotyledonary node explants cultured in media to which ascorbic acid was added gave the most shoots (Figures 1A and 1B), followed by the control (only MSBT). The longest shoots were observed in the treatment with activated charcoal, and the next longest in the ascorbic acid treatment. In general, shoots regenerated in media supplemented with activated charcoal were more vigorous and looked normal and green (Figures 1C and 1D), followed by the treatment with ascorbic acid. In the other treatments and the control, shoots were weak and stunted, demonstrating that not all the antioxidants and adsorbents are useful in reducing the effect of phenolics and improving regeneration in faba bean.

After transferring the regenerated healthy and vigorous shoots to rooting media (MS/2 supplemented with 0.5 mg/l of NAA), good rooting was observed within 2 weeks of transfer (Figure 1E). The overall average rooting was 60% of the shoots transferred from activated charcoal and ascorbic acid treatments; however, the rooting was highest (80%) in shoots regenerated on MSBT medium supplemented with activated charcoal.

Finally, the regenerated plants were transferred to pots. The plants were acclimatized by gradually enlarging holes in the plastic bags that covered the pots, in order to reduce relative humidity. We observed 100% success at this stage. The plants were well established in pots (Figure 1F) flowered (Figure 1G) and set seeds in the green house.

DISCUSSION

Several authors have reported methods for *in vitro* regeneration of faba bean (Busse, 1986; Selva et al., 1989; Khalafalla and Hattori, 1999; Tegeder et al., 1995). However, these regeneration methods were developed for specific varieties. They do not solve the problem of phenolic compounds killing explants. Therefore, our study examined the effects of a polyphenol adsorbent and antioxidants on regeneration in different types of explant (epicotyl, cotyledonary nodes and cotyledonary buds) and identified the adsorbent, antioxidant and type of explant that would be most effective for *in vitro* regeneration of this crop.

Phenolics are secondary metabolites that modulate plant development (Arnaldos et al., 2001) and protect plants against biotic and abiotic stresses (Kefeli et al., 2003; Conceicao et al., 2006; Fan et al., 2006). However,

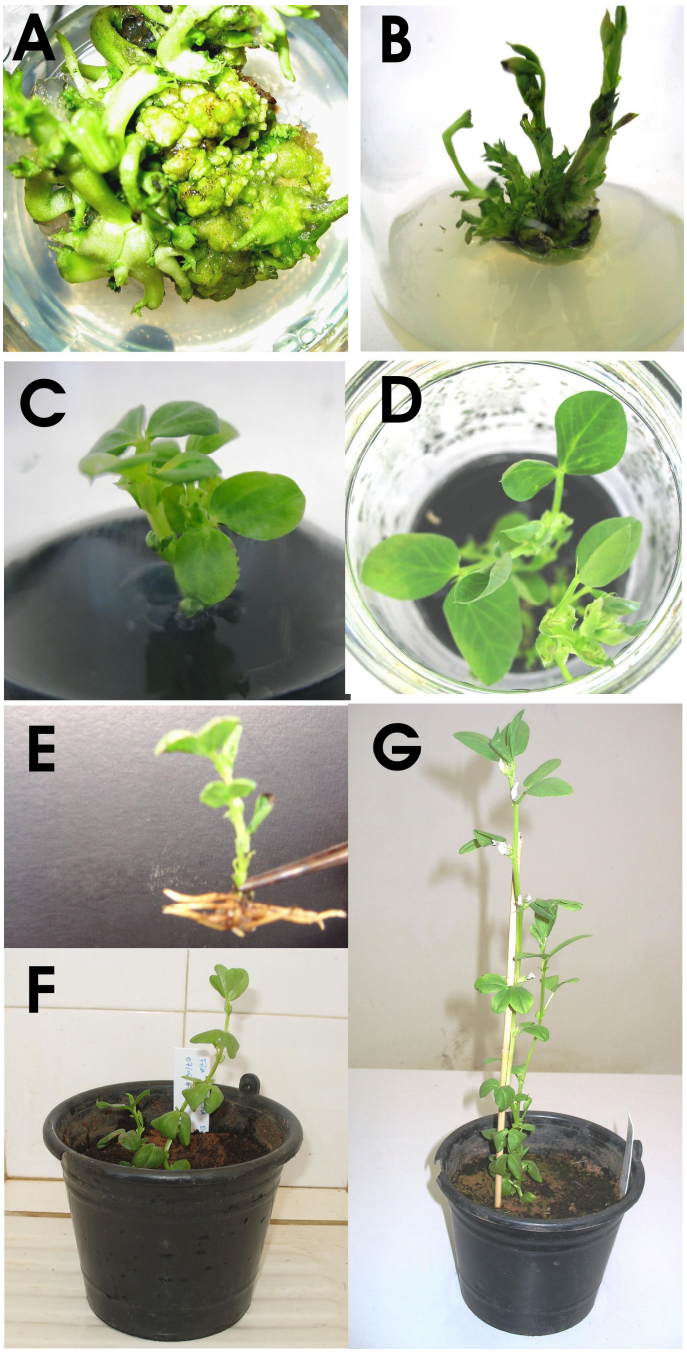

Figure 1. Plant regeneration via organogenesis in faba bean. **A** and **B**, shoot regeneration on medium supplemented with ascorbic acid; **C** and **D**, shoot regeneration on medium supplemented with activated charcoal; **E**, regenerated plantlet with roots ready for planting in soil and acclimatization; **F**, acclimatized regenerated plant; and **G**, regenerated plant in soil.

many authors have observed that phenolics also generate toxic compounds in plant tissue culture media. These toxic compounds negatively affect *in vitro* regeneration of some tree species (Laukkanen et al., 1999; Dibax et al., 2005; Toth et al., 1994) and crop species (Sharada et al., 2003; Prajapati et al., 2003), including faba bean (Bieri et al., 1984; Selva et al., 1989). When explants are cut they exude phenolic compounds that readily oxidize. Oxidized phenolic compounds inhibit enzyme activity and darken the culture medium. The explants brown or blacken, and die (Laukkanen et al., 1999; Compton and Preece, 1986; Lainé and David, 1994; Arnaldos et al., 2001).

Several authors have suggested solutions to minimize the lethal browning or blackening of explants caused by phenolic compounds in plant tissue culture. These include treating explants with ascorbic acid (Arditti and Ernst, 1993) and/or adding a polyphenol adsorbent, such as activated charcoal (Arditti and Ernst, 1993), or antioxidants, such as cysteine (Sanyal et al., 2005), ascorbic acid (Arditti and Ernst, 1993), PVP (Lainé and David, 1994) or silver nitrate (Sanyal et al., 2005), to culture media. Therefore, we tried these chemicals in this study to minimize the lethal browning of explant in faba bean *in vitro* regeneration.

The success of *in vitro* regeneration of faba bean depends on the type of explant used. Previous tissue culture studies used individual seedlings (Busse, 1986), micro-cuttings (Selva et al., 1989), auxiliary buds (Selva et al., 1989), cotyledonary nodes (Khalafalla and Hattori, 1999) and protoplasts (Tegeder et al., 1995) as explants. These studies correlated regeneration efficiency to explant genotypes and the culture conditions, such as the effect of hormones, the composition of the medium, or other physical conditions. These methods did not compare the effect of different types of explants on regeneration. Therefore, we studied regeneration efficiency of cotyledonary nodes, cotyledonary buds and epicotyls. We observed that the type of explant had a significant effect on regeneration. In general, cotyledonnary node explants are the best for regeneration in faba bean (100% regeneration with a mean of 5.9 plants/explant). Although there are no studies on the effect of different types of explants of faba bean on the efficiency of regeneration, several authors have reported highly efficient organogenesis in explants from cotyledonary nodes in other legumes, such as soybean (Shan et al., 2005), pea (Tzitzikas et al., 2004) and chickpea (Sanyal et al., 2005).

Our results showed that treating the overnight soaked seed with PVP solution (1000 mg/l) for 1 h followed by culture in MSBT to which ascorbic acid (an antioxidant) or activated charcoal (an adsorbent) had been added completely eliminated browning in explants. Therefore, activated charcoal and ascorbic acid are useful and effective in managing the problem of phenolics and improving regeneration in faba bean. The other chemicals are not effective in managing the problem of phenolics, even though they are useful and effective in other plant species, indicating species specificity in the role of adsorbents and antioxidants in reducing browning and improving *in vitro* regeneration. For example, in Bermuda grass, pretreatment of explants with ascorbic

acid (2 g/l), followed by culturing in MS medium supplemented with polyvinylpolypyrrolidone (PVPP) (6 g/l), greatly reduced browning and improved regeneration (Qu and Chaudhury, 2001).

In trees and woody plants, studies have suggested that antioxidants, such as PVP, can be added to the media to reduce the oxidation, and thus browning, in cultured tissue (Gupta et al., 1980; Tyagi et al., 1981; Zhong et al., 1995). However, PVP did not have any effect on phenolics when added to media in the *in vitro* regeneration of *Curculigo orchioides* (Prajapati et al., 2003). As we observed in our study, Strosse et al. (2004) reported that adding ascorbic acid to the media inhibited the exudation of phenols in banana tissue culture. Further more, Rout et al. (2001) reported highest percentage of cultures with multiple shoots in *Lawsonia inermis* when the media supplemented with ascorbic acid, supported our observation in faba bean. Sanyal et al. (2005) reported that adding the antioxidants cysteine and silver nitrate improved the maximum recovery of chickpea plantlets *in vitro* after agro-inoculation. Similarly, Strosse et al. (2004) reported that addition of cysteine to the growth media reduced explant blackening in banana tissue culture. However, we did not observe similar improvements in our study.

Sharada et al. (2003) and Prajapati et al. (2003) found that adding activated charcoal to the culture medium prevented the effect of leached phenolics that hindered regeneration of *Celastrus paniculatus* and *C. orchioides* respectively, supporting our results. Furthermore, Dibax et al. (2005) found that, in addition to suppressing phenolics and thus browning, adding activated charcoal to the culture medium for regenerating *Eucalyptus* enhanced the elongation of shoots and made the leaves dark green and vigorous. This again supports our findings.

On the basis of our results, we conclude that treating the overnight soaked seed (after removing the seed coat) with PVP solution (1000 mg/l) for 1 h, followed by culturing in MSBT supplemented with ascorbic acid or activated carbon, greatly reduces the lethal browning or blackening of explants caused by the release of phenolic compounds; it also enhances shoot regeneration of faba bean. The cotyledonary node is the best type of explant for regeneration. To obtain vigorous plantlets, the MSBT medium should be supplemented with activated charcoal. If, on the other hand, the aim is to produce explants with more shoots, culture the explants in MSBT medium supplemented with ascorbic acid. To get more shoots that are also vigorous, sub-culture explants initially in MSBT supplemented with ascorbic acid and then in MSBT supplemented with activated charcoal.

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