

Full Length Research Paper

## An efficient *in vitro* regeneration protocol for faba bean (*Vicia faba* L.)

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A reliable regeneration system for faba bean (*Vicia faba* L.) has been difficult to establish, delaying its genetic improvement. In the present work a rapid, reproducible and efficient regeneration method was developed for faba bean using single cotyledon explants with half embryonal axis. MS medium supplemented with 6  $\mu$ M TDZ (thidiazuron), 10  $\mu$ M 2-iP and 4  $\mu$ M kinetin induced 30 to 50 adventitious buds/shoots after two weeks of culture, which were elongated on MS medium supplemented with 6  $\mu$ M 2-iP and 2  $\mu$ M kinetin. With healthy and strong roots established, shoots were transferred to MS medium supplemented with 5  $\mu$ M IBA within 10 to 14 days. Shoots of 5 cm long were most suitable for rooting. Potting-mixture with good aeration and less capacity to retain water was most suitable for successful establishment of faba bean plantlets. Garden soil mixed with sand (gravel) and bio-manure (1:1:1) was most suitable for transplantation. TDZ promoted adventitious bud formation while 5  $\mu$ M IBA was most suitable for rooting, higher concentrations were toxic to plantlets. Aeration of the potting mixture was important for rapid micropropagation and successful establishment. The efficient regeneration protocol reported here allows for successful micropropagation of faba bean, which is essential for future genetic improvement of plants via transformation protocols.

**Key words:** *Vicia faba*, organogenesis, transplantation, TDZ, IBA and kinetin.

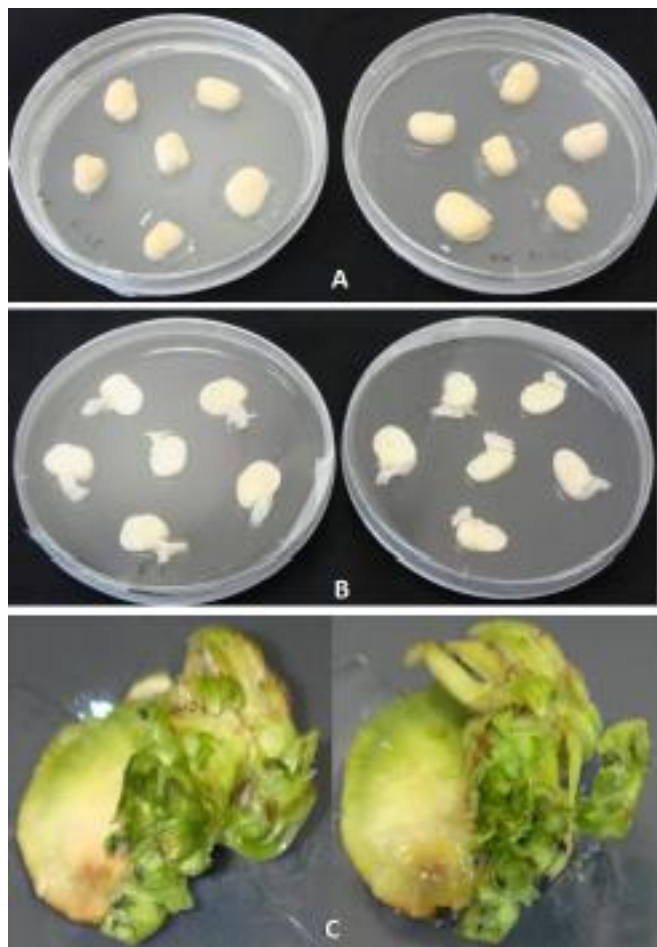
### INTRODUCTION

Legumes (*Leguminosae*) are the third largest family of dicotyledons. Faba bean (*Vicia faba* L.) belongs to the Fabaceae family and has many common names. It is one of the most important grain legumes in the world since it is an excellent source of protein, used for both human consumption and animal feed. Faba bean also plays an important role in biological fixation of aerial nitrogen (Duke 1981; Jelenić et al., 2000). This specie alone occupied nearly 3.2 x 10<sup>6</sup> ha worldwide in 1991 (FAO statistics, 1992) with a world production close to 4.5 million tons in 2004 (Gutierrez, 2006).

Unfortunately, faba bean is susceptible to environmental conditions, biotic and abiotic stresses, and

has unstable yields. Difficulties in pollination control and a limited genetic pool has led to slow progress in crop varietal improvement (Bond, 1987; Bond et al., 1985; Selva et al., 1989). Legumes such as faba bean appear to be recalcitrant in *in vitro* regeneration (Khalafalla and Hattori, 2000; Anwar, 2007; Anwar et al., 2008, 2010), due to difficulties regenerating from callus and the release of phenolic compounds. As a result, studies on *in vitro* culture of faba bean are difficult (Böttinger et al., 2001). Regeneration of faba bean via indirect somatic embryogenesis was reported by Griga et al. (1987) using cotyledons on media supplemented with 2,4-D. Somatic embryos at the globular to early torpedo stage were released, but their development terminated at the late torpedo stage. In tissue culture, regenerated plants from cultured cells are known to exhibit genetic and epigenetic changes collectively called somaclonal variations

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**Figure 1.** Regeneration of faba bean. (A) Seeds de-coated after 14 h imbibition on shoot induction medium (SIM) (MS medium supplemented with 6  $\mu\text{M}$  TDZ, 10  $\mu\text{M}$  2-iP and 4  $\mu\text{M}$  kin), (B) Faba bean (4 days after incubation of de-coated seeds on SIM) used to obtain explants for regeneration and transformation; (C) Two-week-old culture of embryonal axis with single cotyledon, showing emergence of adventitious buds (12 and 15 days after incubating four-day-old cultures from SIM on SIM).

(Razdan, 1994). Direct shoot organogenesis and establishment of plantlets from different explants of faba bean has been reported (Taha and Francis, 1990; Tegeder et al., 1995; Böttinger et al., 2001). Plantlets have been developed through direct somatic embryogenesis and from callus using different explants of faba bean (Griga et al., 1987; Albrecht and Kohlenbach, 1989; Pickardt et al., 1989; Al-Aref and Elithy, 2003; Veltcheva et al., 2005; Hamdi and Hattori, 2006; Bahgat et al., 2008).

Despite several reports of successful regeneration, legumes are considered highly recalcitrant (Shri and Davis, 1992; Vani and Reddy, 1996; Khalafalla and Hattori, 2000; Rizvi and Singh 2000; Polowick et al., 2004). Surprisingly, the majority of reported faba bean regeneration protocols are either not repeatable or

successful only in certain research laboratories, suggesting that faba bean regeneration is highly recalcitrant. This study was undertaken to fine-tune the protocol for high speed regeneration in faba bean and establish a reproducible, efficient and time saving *in vitro* system in faba bean. The broad aim of this activity is to establish efficient transformation system for crop manipulation and related functional genomics aspects.

## MATERIALS AND METHODS

### Explant preparation and media for induction of adventitious buds/shoots

Seeds of faba bean (*Vicia faba* L.) cv. Gazira 2, Giza 402 and Kamlin (gifted by ICARDA, Egypt and Australia) were treated with 0.2% chlorax for 5 min, rinsed thoroughly with double distilled water followed by treatment with 70% ethanol for 3 min and 0.1% mercuric chloride solution for 5 min. The seeds were then washed three times with sterile double-distilled water under aseptic conditions prior to soaking overnight. Sixteen-hour imbibed seeds were de-coated and germinated on shoot induction medium (SIM) (Figure 1A), consisting of MS medium (Murashige and Skoog, 1962) supplemented with 6  $\mu\text{M}$  TDZ, 10  $\mu\text{M}$  2-iP and 4  $\mu\text{M}$  kinetin, for 4 d (Figure 1B). Explants consisting of a single cotyledon with half an embryonal axis with plumular and radicular ends were excised, the resulting 4 day-old seedlings were inoculated on SIM for 8 to 15 days (Figure 1C). Explants were subsequently transferred to MS basal medium (that is, devoid of growth regulators) for 12 to 15 days (Figure 2).

### Elongation of shoots

The adventitious buds/multiple shoots induced from explants were excised from the bunch without callus or globular structures and cultured on shoot elongation medium (SEM) consisting of MS medium supplemented with 6  $\mu\text{M}$  2-iP and 2  $\mu\text{M}$  kinetin, for 10 days. They were then routinely sub-cultured after 10 to 15 d on SEM.

### Rooting

After several preliminary experiments, elongated shoots varying in length from 5 cm were used because they were most suitable for rooting. Five cm long shoots were transferred to semi-solid MS medium supplemented with 5  $\mu\text{M}$  IBA. All cultures were maintained at  $25\pm 2^\circ\text{C}$  with a light/dark cycle of 16/8 h. The white fluorescent light had an intensity of  $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

### Transplantation

Plantlets were carefully removed from tubes, roots were thoroughly washed with tap water and transferred to small pots filled with soil, sand and manure in equal proportions. Each pot with plantlet was covered with a transparent polythene bag for 7 days (Figure 6A). After 3 days, the corners of the bags were cut. Four days later the bags were removed. After seven more days, the plants were carefully transferred to large pots containing soil, sand and manure in equal proportions. Plants were acclimatized, and showed normal growth and development (Figure 6B).



**Figure 2.** Regeneration of faba bean-two-week-old culture of embryonal axis with single cotyledon, showing emergence of shoots from adventitious buds (10 days after incubating 20 day-old cultures from SIM on MS).

## RESULTS AND DISCUSSION

In our study, TDZ (6  $\mu\text{M}$ ) in combination with 2-iP (10  $\mu\text{M}$ ) and kinetin (4  $\mu\text{M}$ ) was optimal for expansion of the meristematic zone followed by adventitious bud/shoot induction. MS medium supplemented with 6  $\mu\text{M}$  TDZ, 10  $\mu\text{M}$  2-iP and 4  $\mu\text{M}$  kinetin induced 30 to 50 adventitious buds/shoots after two weeks culture. Subsequently, induction adventitious bud/shoot were transferred into MS medium supplemented with 6  $\mu\text{M}$  2-iP and 3  $\mu\text{M}$  kinetin for 10 days for further multiplication and elongation. In general, legumes such as chickpea and faba bean appear to be recalcitrant in *in vitro* regeneration (Khalafalla and Hattori, 2000; Anwar et al., 2008, 2009, 2010). Adventitious buds/shoots induced from axillary meristem/nodal buds have been reported in many plants, with most responding better to thidiazuron (TDZ) than other cytokinins (Faisal et al., 2005; Faisal and Anis, 2006; Khan et al., 2006; Hussain and Anis, 2006, 2009; Siddique and Anis 2008; Jahan and Anis 2009). In the faba bean cultivars studied here, the response of single cotyledon explants with embryonal axes to adventitious bud/shoot induction and development differed with different concentrations and combinations of plant growth regulators. The time of exposure, and the type and concentration of cytokinin, affects adventitious bud/shoot induction and subsequent development/growth rate of shoots produced (Michael and Hornbuckle, 1999; Michael and Christianson, 2000; Catharina et al., 2003; Hamdi and Hattori, 2006, 2007; Abdelwahd et al., 2008; Anwar et al., 2008, 2010). TDZ is a substituted phenyl urea [1-phenyl-3-(1,2,3-thidiazol-5-yl)urea] with potential for shoot organogenesis in many plant systems including legumes (Murthy et al., 1998; Shan et al., 2000; Onamu et al., 2003; Ganeshan et al., 2003; Mroginski et al.,

2004; Sharma et al., 2005; Chen and Chang, 2006; Radhika et al., 2006; Hamdi and Hattori, 2006, 2007; Abdelwahd et al., 2008; Anwar et al., 2008, 2010). TDZ is the most promising cytokinin for shoot induction in legumes (Malik and Saxena, 1992; Barna and Wakhlu, 1993; Huetteman and Preece, 1993; Tegeeder et al., 1995; Murthy et al., 1996; Khalafalla and Hattori, 2000; Rizvi and Singh 2000; Jayanand et al., 2003; Senthil et al., 2004; Tewari-Singh et al., 2004; Kiran et al., 2005; Anwar et al., 2008, 2010). TDZ was most effective inducing healthier shoots when used at lower concentration ( $\sim 10$   $\mu\text{M}$ ) compared to higher concentrations (Malik and Saxena 1992; Murthy et al., 1995, 1996, 1998; Jiang et al., 2005; Anwar et al., 2008, 2010). Although the exact mechanism of action of TDZ is not yet clear, it is believed to be involved in regulating endogenous levels of various growth regulators (Malik and Saxena, 1992). Using TDZ with a purine ring containing cytokinins such as kinetin, BAP and 2-iP promoted healthy shoot growth (Eisinger, 1983; Mroginski and Kartha, 1984; Radhika et al., 2006; Anwar et al., 2008, 2010). In order to avoid any negative impact of TDZ on shoot formation, explants were transferred immediately after shoot induction to a medium devoid of TDZ (Figures 1 and 2). Cytokinins such as 2-iP and kinetin are well-known to promote rapid shoot multiplication (Jayanand et al., 2003; Kiran et al., 2005; Anwar et al., 2008, 2010).

Results revealed that 6  $\mu\text{M}$  TDZ in combination with 10  $\mu\text{M}$  2-iP and 4  $\mu\text{M}$  kinetin was optimal for expansion of the meristematic zone (Figure 1B) followed by adventitious bud/shoot induction (Figure 1C). MS medium supplemented with 6  $\mu\text{M}$  TDZ, 10  $\mu\text{M}$  2-iP and 4  $\mu\text{M}$  kinetin induced 30 to 50 adventitious buds/shoots after two weeks culture compared with only 5.9 shoots or



**Figure 3.** Regeneration of faba bean-shoots on shoot elongation medium (MS medium with 5  $\mu\text{M}$  2-iP and 2  $\mu\text{M}$  kinetin), 15 to 20 days after shoot transfer from SIM.

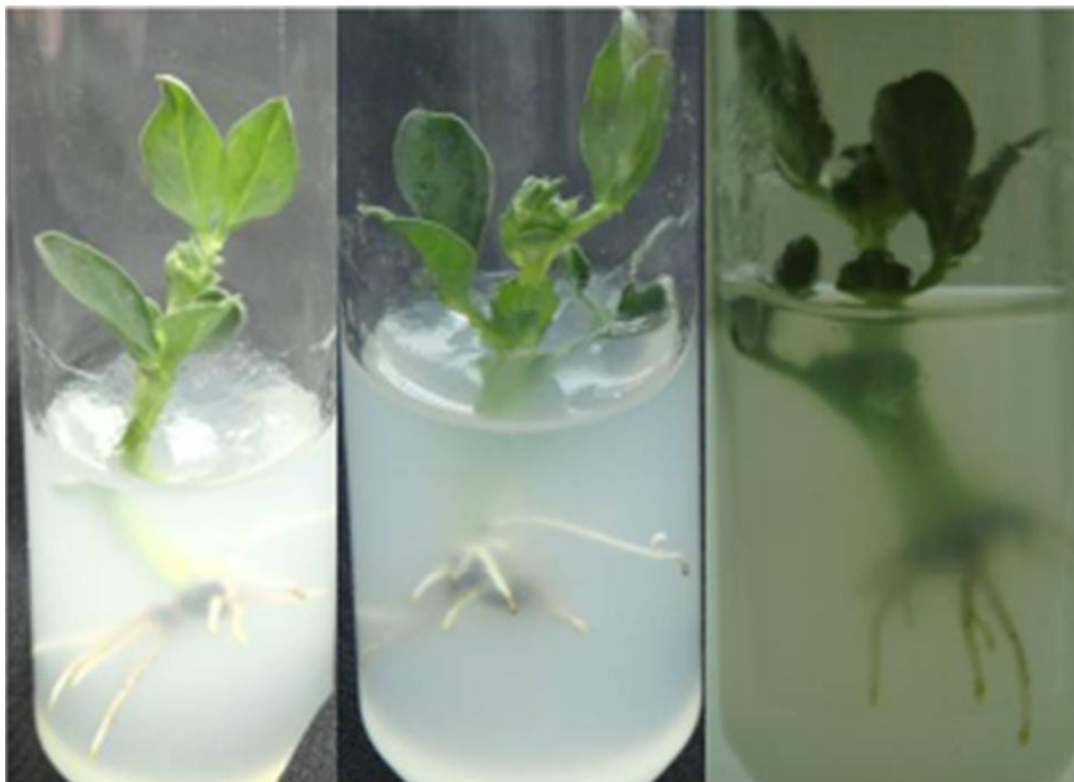


**Figure 4.** 24 and 28 day old shoots showing *in vitro* flowering.

plants per explant observed by Abdelwahd et al. (2008). The induced multiple shoots were excised from the bunch, without callus or globular structures, and cultured

on shoot elongation medium (SEM) consisting of MS medium supplemented with 6  $\mu\text{M}$  2-iP and 3  $\mu\text{M}$  kinetin for 10 days (Figure 3), before being routinely sub-cultured after 10 to 15 days on SEM. A major hurdle that limits *in vitro* regeneration of legumes is the induction and development of a strong root system. This limitation compelled several researchers to investigate grafting as an alternative (Krishnamurthy et al., 2000; Bottinger et al., 2001; Sarmah et al., 2004; Senthil et al., 2004; Sanyal et al., 2005; Chakraborti et al., 2006). However, grafting is time consuming, requires special skills and success rates vary. Roots induced from cut ends of shoots of faba bean were shorter in length in semi-solid medium, similar results have been observed by others (Cournac et al., 1991; Jackson et al., 1991; Ebrahim and Ibrahim, 2000; Hazarika, 2006). During the present investigation, higher concentrations and/or longer exposure of explants in TDZ-containing media inhibited rooting, similar results have been observed by others (Khalafalla and Hattori, 2000; Anwar et al., 2008, 2010). Rooting was also observed in half MS supplemented with 0.5  $\text{mg L}^{-1}$  NAA (Abdelwahd et al., 2008). In our experiments, 5 cm long shoots were most suitable for rooting when transferred to MS medium supplemented with 5  $\mu\text{M}$  IBA (Figure 5). Some shoots flowered when retained in tubes for longer (Figure 4).

Another major hurdle limiting legume such as chickpea and faba bean regeneration is the establishment of *in vitro* raised plantlets in pots or the field. This is another reason why grafting is preferred by many research groups. Shorter plantlets are better able to withstand transplantation shock and establish better in pots. It has been reported that root to shoot ratio plays an important role in successful establishment of *in vitro* raised legume plantlets (Subhan et al., 1998; Anwar 2007; Anwar et al.,



**Figure 5.** Regeneration of faba bean—12 days after transfer of elongated shoots on MS medium supplemented with 5  $\mu$ M IBA (note strong root system).



**Figure 6.** Plantlets transferred to pots for acclimatization; **A.** Plantlets transferred to pots with a mixture of soil, sand and bio-manure in equal proportions. Freshly transferred plantlets covered with polythene bags. **B.** Plants of faba bean established in pots (two week after transfer) with a mixture of soil, sand and bio-manure in equal proportion.

2008, 2009, 2010). Understandably, shorter shoot area/length reduced excessive water loss leading to rapid loss in turgidity of plantlets (Munns, 2002). During our study, plantlets with 3 to 6 cm long shoots survived better

than plantlets with longer shoots which often failed to establish even when shoots were thicker, as they tended to collapse and die within a few days after transplantation.

For transplantation of legume plantlets, various potting mixtures have been used: Peat moss (Abdelwahd et al., 2008); normal soil (Bahgat et al., 2008); autoclaved/sterile soil (Indurker et al., 2007); non-autoclaved/non-sterile soil, soilrite, manure, vermiculite, soilrite + garden soil (1:1), soilrite + garden soil + manure (1:1:1) and commercial soil mixture (Sunshine No. 4, Sun Gro Horticulture, Bellevue) (Polowick et al., 2004). In all cases, the rate of successful establishment was very low or not mentioned. We attribute failure in these particular cases to excess moisture from the high water holding capacity of the potting mixtures. During our investigations, we found that potting mixture containing garden soil mixed with sand (gravel) and bio-manure in the ratio of 1:1:1 (Figures 6A and B) was most suitable for cent percent transplantation success. Transplantation of *in vitro* raised plantlets and their successful establishment in pots is shown in Figures 6A and B (Figure 6A, initially covered with transparent polythene bag). Legumes are well known for their susceptibility to flooding and excess moisture. Under high moisture conditions, chickpea is prone to fungus and wilt diseases (Yadav et al., 2006). During our investigations, autoclaved soil mixture was not essential for establishing *in vitro* raised plantlets of faba bean. We realized that improving aeration/porosity of potting mixture by mixing sand was highly beneficial, as reported by Anwar et al., (2008, 2009, 2010). Good drainage is necessary because even short periods of flooding reduced growth and increased susceptibility to root and stem rots, and a heavy rainfall season reduced yields due to disease outbreaks and stem lodging from excessive vegetative growth (Yadav et al., 2006).

Another important factor that often limits regeneration and successful establishment of *in vitro* raised plantlets of faba bean is the season (that is, time of the year) when transplantation is attempted, during the present investigation, the best and cent percent established success followed by good seed set which occurred when plantlets were transplanted during October and November (that is, beginning of winter).

## Conclusion

In summary, a simple protocol was developed for successful regeneration and establishment of *in vitro* raised plantlets of faba bean. Successful establishment depends upon two basic factors, namely (1) shoot length of plantlets and root to shoot ratio; and (2) using potting mixture with good aeration and lesser capacity to retain water. During our investigations, all plantlets with shoot lengths ranging from 3 to 6 cm, potted in soil mixed with sand (gravel) and bio-manure in equal proportion, established perfectly. Using this protocol, future studies should be able to develop transgenic faba beans for biotic and abiotic stress tolerance. Successful transgenic

approach will also open a new avenue for faba functional genomics and crop manipulation. This will definitely help in developing better faba genotypes suitable for local and regional ecosystem and enhancing faba role in conservation agriculture in arid and semiarid regions.

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