

AGROBACTERIUM-MEDIATED TRANSFORMATION OF COTYLEDONARY NODE OF *VICIA FABA* L.

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ABSTRACT

Broomrape represents one of the most intractable parasitic weed problems for faba bean in Mediterranean region. It causes enormous yield losses, and few control measures are effective. Recently, a novel strategy based on parasite-induced expression of a selective sarcotoxin IA polypeptide in transgenic tobacco was used to control broomrape. Generation of transgenic plants with broomrape resistance requires an efficient genetic transformation system. The present study explored the possibility of faba bean plant regeneration via direct organogenesis from cotyledonary node explants to improve transgenic efficiency. We studied the effect of some factors (genotype, *Agrobacterium* strains, Acetosyringone and thiols products). Cotyledonary nodes of two cultivars 'Lobab' and 'Aquadulce' were inoculated and co-cultivated with two *A. tumefaciens* strains, EHA101 and AGL1, both carrying the pBIBhyg vector containing sarcotoxin IA and hptII genes. The results revealed a significant effect of genotype, genotype x acetosyringone interaction and addition of thiol compounds in co-cultivation medium on frequency of hygromycin resistant shoots of faba bean. Thus, the highest frequency of hygromycin resistant shoots was 38.3% with 'Aquadulce' inoculated by AGL1 and co-cultivated in medium with cysteine and without acetosyringone. The integration of *sarcotoxin IA* gene construct in transgenic plants was confirmed by PCR analysis.

Key words: *Vicia faba*, cotyledonary node, genetic transformation, *Sarcotoxin IA* gene, *Orobanche*.

INTRODUCTION

Faba bean (*Vicia faba* L.) is an important food legume, widely grown and consumed, especially in China, North African countries, parts of Europe and North and South Americas. For both humans and livestock, it provides high quality, lysine-rich proteins, carbohydrates and fibres. It is also rich in carotenoids, vitamins and essential minerals including iron, magnesium, potassium, zinc, copper and selenium. Faba beans have also been shown to have lipid-lowering effects and may also be a good source of antioxidants and chemo preventive factors (Ray and Georges, 2010). However, the total area in the world dedicated to faba bean cultivation is declining (Jensen et al., 2010) from 5 million ha in 1965 to 2.5 million in 2008. The total production was 4.3 Mt from

2.5 Mha, which is relatively small compared with soybean and pea world production (262 Mt and 10 Mt, respectively) (FAOSTAT, 2010).

The main reason for lower area and production is the unreliable yields, mainly due to susceptibility of the crop to pests, diseases and parasitic weeds such as broomrape. In fact, broomrape causes severe yield losses in faba bean production in the West Asia, North Africa and Mediterranean regions (Stoddard et al., 2010).

Broomrapes (*Orobanche* spp.) are especially destructive and affect large areas of the globe, including many developing nations, where they have potential to greatly decrease yield and quality of host crops (Westwood et al., 2012). Despite efforts aimed at their control, broomrape persists and is expanding his negative impact on crop productivity

(Parker, 2009). The best long-term strategy for limiting damage by *Orobanche* is the development of resistant cultivars that could deal with this serious constraint (Cubero, 1991). However, generation of resistant crops through conventional means has been challenging and slow (Gnanasambandam et al., 2012). Genetic engineering may offer new insights and strategies to facilitate progress toward this goal. For improving resistance to *Orobanche*, this new approach was investigated in tobacco by using *sarcotoxin IA* gene from the flesh fly *Sarcophaga pregrina* as a pest-specific toxin (Hamamouch et al., 2005).

Transformation of faba bean has been difficult through the *Agrobacterium*-mediated approach (Böttinger et al., 2001). The present study explored the possibility of faba bean regeneration via direct organogenesis from cotyledonary node explants (Khalafalla and Hattori, 1999; Abdelwahd et al., 2008). To improve faba bean genetic transformation efficiency we studied the effect of some key factors (genotype, *A. tumefaciens* strains and use of additives in co-cultivation medium). Then the improved protocol will be used to integrate a *sarcotoxin IA* polypeptide gene, under HMG2 promoter, in faba bean in order to enhance host resistance to *Orobanche*.

MATERIAL AND METHODS

Plant material

Faba bean seeds (*Vicia faba* L.) cvs. 'Lobab' and 'Aquadulce', obtained from National Agricultural Research Institute (INRA), Morocco, were used for this experiment.

Bacterial strains and gene constructs

The pBIBhyg vector containing the *sarcotoxin IA* genes construct was kindly provided by Dr James H. Westwood, Virginia Tech, USA. The plasmid was transformed into two *Agrobacterium tumefaciens* strains EHA101 and AGL1 (kindly provided by Dr Kan Wang, Iowa State University, USA) by electroporation. This plasmid also contains selection gene (*hptII*) which confers resistance to hygromycin. *A. tumefaciens* strains containing this construct were used for genetic transformation of faba bean genotypes.

Preparation of *Agrobacterium* suspension

For preparation of the bacterial suspension culture, 1 ml of *A. tumefaciens* strains EHA101 and AGL1 were allowed out from a -80°C glycerol stock culture and precultivated on Yep solid medium containing antibiotics (100 mg/l kanamycine + 30 mg/l rifampicine) at 28°C for 1 to 2 days. Single colonies of the bacteria (EHA101 and AGL1) were grown overnight on a shaker at 28°C and 250 rpm in YEP liquid medium containing appropriate antibiotics. The suspension was used for inoculation once the *A. tumefaciens* cell culture reached density of 0.8 at $\text{OD}_{600\text{nm}}$. *Agrobacterium* suspension was diluted with MS liquid medium amended with 2 mg/l Tidiazuron (TDZ) and 2 mg/l Benzylaminopurine (BAP) (MSTB) and with or without 100 μM of acetosyringone (AS).

Explants preparation and co-cultivation

Healthy and uniform seeds of 'Lobab' and 'Aquadulce' cultivars were surface-sterilized by 70% ethanol for 1 min, followed by soaking in 30% sodium hypochlorite (bleach) with a few drops of Tween 20 for 20 min and subsequently rinsed 3 times with sterile distilled water. Then, seeds were soaked in sterile distilled water overnight. The peeled seeds were soaked in 1 g/l PVP solution for 1 hour to reduce production of phenols and cultured on half MS/2 medium supplemented with MS vitamins (Murashige and Skoog, 1962). Cotyledonary node explants were excised from 6 days old in vitro seedlings, by removing both cotyledons and excising both epicotyls and hypocotyls approximately 1 and 2 mm respectively from above and below the nodal region with a sharp razor blade wetted by the *Agrobacterium* strain. Then the explants were incubated in the *Agrobacterium* suspension for 30 min.

Experimental design and statistical analysis

A completely randomised design was used with 2 genotypes (Lobab and Aquadulce), 2 *Agrobacterium* strains (EHA101 and AGL1), 3 media for co-cultivation (MSTB medium as control, MSTB supplemented with 50 mg/l ascorbic acid, and

MSTB supplemented with 100 mg/l cysteine) and 2 levels of acetosyringone (with and without). Each test was performed twice. For each treatment we used at least fifteen explants per replication. Cotyledonary nodes were incubated separately with the two *Agrobacterium* strains in the bacterial suspension, with or without AS (100 µM) for 30 min. In order to enhance the efficiency of transformation via *Agrobacterium*, the wetted explants were co-cultivated on solidified MSTB medium (control) or MSTB supplemented with 50 mg/l ascorbic acid or 100 mg/l cysteine for 3-4 days in dark at 25°C. After co-cultivation, the explants were subsequently washed thoroughly with sterile distilled water and cefotaxim 400 mg/l and placed on solidified MS basal salt medium supplemented with MS Vitamins, 2 mg/l of (TDZ) and (BAP) and 400 mg/l cefotaxim for 1 week. The explants were subsequently transferred to regeneration and selective medium containing MS basal salts and vitamins, 2 mg/l TDZ, 2 mg/l BAP, 200 mg/l cefotaxim and 3 mg/l hygromycin for selection. Every 2-3 weeks the cultures were transferred to fresh medium, for 2-4 months. After that, healthy resistant shoots were transferred to elongation medium (MS) and then to rooting medium (MS with 0.5 mg/l Naphthalenacetic acid (NAA)). The genetic transformation of plants was confirmed by molecular analysis.

The percentage of regenerating explants, the number of newly formed shoots by explant, and the percentages of selected shoots in selective medium were counted. Data were analysed using analysis of variance (ANOVA) for a completely randomised design. Duncan's new multiple range test was used to separate the means of significant effect (DMRT).

Genomic DNA isolation and PCR analysis

Genomic DNA was extracted from young leaves of both non transformed and transformed plants by using modified CTAB (cetyltrimethyl ammonium bromide) method (Rogers and Bendich, 1988). Polymerase

chain reaction (PCR) was performed to detect the presence of the *HptII* in putative T₀ transformants. *HptII* coding regions was amplified by using primer combinations: HPT-5':(ATGAAAAAGCCTGAACTCACC) and HPT-3': (CTATTCCTTTGCCCTCGGAC).

The PCR reaction mixtures (10 µl) contained about 60 ng of genomic DNA, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 U Taq DNA polymerase, and 10 µM of each gene specific primers. DNA from non-transformed (control) plants was included in the experiments as negative control to discriminate from possible contamination. The amplification was carried out with initial denaturation at 95 °C for 5 min, followed by 35 cycles of amplification, each consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min, and final extension for 6 min at 72 °C for detection of *hptII* gene amplification. Amplified DNA fragments were analysed by electrophoresis on 1% agarose gel, visualized by ethidium bromide staining and photographed under ultraviolet light in a gel documentation system.

RESULTS AND DISCUSSION

The intent of this study was to develop an efficient protocol in order to improve *Agrobacterium* mediated transformation of cotyledonary nodes of *V. faba* by manipulating some factors: faba bean genotypes, *Agrobacterium* strains, the composition of co-cultivation medium and the presence of acetosyringone, in order to integrate sarcotoxin IA gene. A chimeric hygromycin phosphotransferase gene (*hptII*) was used as the selectable marker and hygromycin as selection agent.

The results revealed significant effect of genotype, of genotype × acetosyringone interaction and of co-cultivation medium additioned with thiol compounds, on transformation efficiency (as measured by hygromycin resistant shoot/ inoculated explants) of faba bean (Table 1).

Table 1. Effect of different factors (genotype, strain, +/- acetosyringone and thiol products in co-cultivation medium) on hygromycin resistant shoots efficiency of faba bean

Source	df	Mean square	Pr > F
Genotype	1	17697.88474	<.0001
Genotype × strain	1	198.17354	0.0587
Acetosyringone	1	1004.79301	0.2346
Genotype × Acetosyringone	1	090.70993	0.0375
Strain × Acetosyringone	1	418.13081	0.4430
Co-culture medium	2	5172.81912	0.0008
Genotype × medium	2	12.32177	0.9828
Genotype × strain × acetosyringone × medium	2	82.35360	0.8903

+/-: Absence or presence of acetosyringone

Effect of genotype on the number of shoots regenerated from the transformed explants and on transformation efficiency

The analysis of variance clearly showed a significant ($P < 0.002$) difference between cultivars with respect to average number of regenerated shoots per explants. The proliferation of shoots from the cotyledonary nodes was higher in Lobab variety compared to Aquadulce. On the other hand, Aquadulce was the most responsive genotype with respect to transformation efficiency and was significantly different ($P < 0.0001$) from Lobab (Table 1). Indeed, the percentage of regenerated shoots having resistance to selective agent was 33.4% in Aquadulce variety, while it was 18.7% in Lobab, which expressed a good proliferation. This shows clearly that Lobab variety is less susceptible to *Agrobacterium*. These results are in agreement with those reported by Svabova et al. (2005), who showed a different sensitivity to *A. tumefaciens*, between cultivars of pea. Meurer et al. (1998) also reported that the infection and regeneration of cotyledonary node tissue of soybean was dependent on varieties used. This phenomenon, i.e. genotype-dependent transformation, was also frequently reported in other crops (Sonntag et al., 2001; Heeres et al., 2002). This different degree of susceptibility of cultivars to specific *Agrobacterium* strains is regulated by mechanisms of attachment and DNA-transfer, and also by the ability of plants to produce different inducer molecules. The differences in the *vir* gene expression in different hosts affect their sensitivity to *Agrobacterium*

infection. Low level of *vir* gene expression can make a plant recalcitrant, by virtue of the inability of the bacterium to synthesize and transfer sufficient T-DNA strand essential for a successful infection (Karami et al., 2009).

Effect of *Agrobacterium* strain on faba bean transformation

Several researchers reported that *Agrobacterium* strains play a critical role in legume transformation (Dang and Wei, 2007; Akcay et al., 2009). Moreover, Böttinger et al. (2001) showed that the strain EHA101 was more effective than strain EHA105 for transformation of faba bean. In this study, the results did not show any significant effect of *Agrobacterium* strains on transformation of faba bean, when we used two disarmed *Agrobacterium* strains (EHA101 and AGL1) (Table 1).

However, it seems that various strains of *Agrobacterium* don't have the same potential depending on host genotype. Our results showed that the use of strain EHA101 to inoculate Aquadulce variety gave higher frequency of hygromycin resistant shoots than using AGL1. However, AGL1 strain was more effective than EHA101 for transformation of Lobab variety. With strain EHA101, the rate of selected shoots of Aquadulce on hygromycin medium was improved by 15.8% compared to AGL1 (Figure 1).

Karami et al. (2009) reported that *A. tumefaciens* has different ability to infect different species of plants and in some cases, even has different ability to infect different genotypes of the same species.

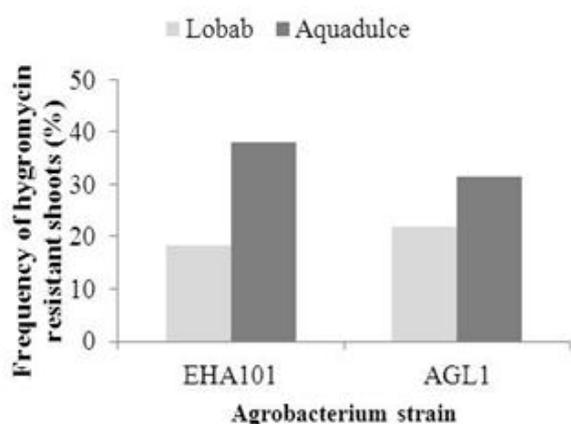


Figure 1. Genotype – Agrobacterium strain interaction effect on frequency of hygromycin resistant shoots on selective medium

Effect of acetosyringone on transformation efficiency

As one of the phenolic compounds secreted by wounded tissues, acetosyringone (AS) is a potent inducer of *Agrobacterium vir* genes (Stachel et al., 1986), thus enhancing *Agrobacterium*-mediated transformation (Kumar et al., 2010). However, in our experiments, no significant effect on faba bean transformation efficiency was found ($P < 0.2346$) when 100 μ M acetosyringone was added in co-culture medium with *Agrobacterium* strains. This outcome is consistent with the results of Nadolska-Orczyk and Orczyk (2000), where AS

treatment did not improve transformation efficiency of pea with EHA105 strain.

Svabova and Griga (2008) also recorded distinct results for pre-cultivation treatment of *Agrobacterium* with AS, which was ineffective in peas. In contrast to other crops, including some legumes, it seems that faba bean is less sensitive to AS, because this species can release a significant amount of *vir*-specific endogenous phenolic inducers. Thus, there is no need to add acetosyringone to improve genetic transformation efficiency of this species. Or perhaps, there is a relatively low AS concentration (less than 100 μ M AS used) stimulating *vir* gene of *Agrobacterium* in faba bean, as reported in pea (Svabova and Griga, 2008).

Effect of antioxidants on transformation

The results revealed a significant effect ($P < 0.0007$) of antioxidants on faba bean genetic transformation. Cysteine and ascorbic acid significantly increased transformation efficiency of faba bean. Cysteine gave the highest percentage of hygromycin resistant shoots (33.6%) followed by ascorbic acid treatment. Hence, it can be concluded that the application of L-cysteine or ascorbic acid are effective for faba bean transformation. This beneficial effect of cysteine on transformation efficiency was more noted on Lobab variety (Figure 2).

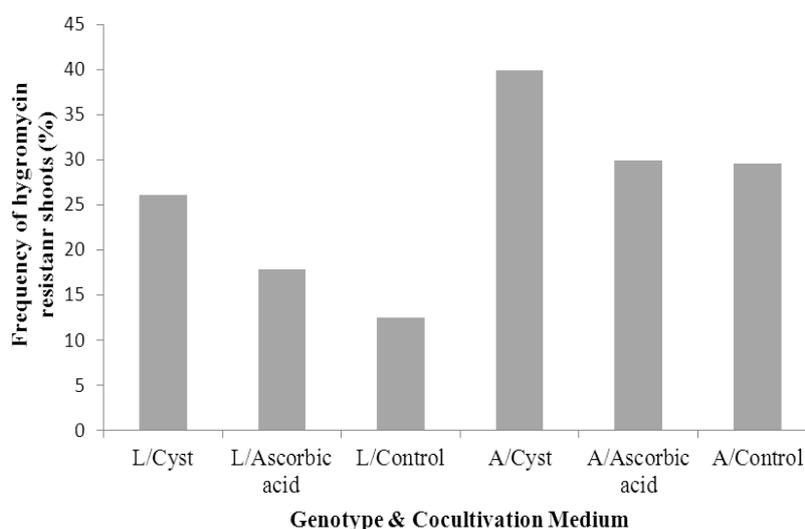


Figure 2. Genotype- cocultivation medium interaction effect on frequency of hygromycin resistant shoots on selective medium
L: Lobab; A: Aquadulce; Cyst: Cysteine

The positive impact of cysteine observed on transformation efficiency of faba bean was also reported in many crops and legume (Mehrotra and Goyal, 2012; Ceasar and Ignacimuthu, 2011; Wang and Xu, 2008; Olhoft and Somers, 2001; Olhoft et al., 2003). L-cysteine is a compound that may increase the frequency of transformed cells either by acting as a nutritional supplement, or by acting through its thiol group as antioxidant or inhibitor of wound- and pathogen-defence responses. Similarly, it limits enzymatic browning and necrosis of wounded (in vitro cultured) and *Agrobacterium*-infected explants (Olhoft and Somers, 2001).

In our experiments cysteine concentration (100 mg l^{-1}) significantly increased the percentage of resistant shoots in selective medium, which may be theoretically a result of its antioxidant action. Faba bean is characterized by intense production of phenolic compounds, as compared to some other grain legume such as pea (Svabova et al., 2005) and thus there is need for treatment with antioxidant compounds to improve faba

bean transformation in some varieties, as we have already shown in a previous publication (Abdelwahd et al., 2008).

Molecular analysis of the transgenic plants

PCR analysis, done to confirm the integration of hygromycinphosphotransferase (hpt II) gene, on genomic DNA of 77 randomly selected hptII expressing plants and one non-transformed plant, was carried out using hptII gene-specific primers. The expected fragment was successfully amplified from all transgenic plants (Figure 3). However, this fragment was not amplified in the non-transformed plant (Figure 3, lane 1) and in not transgenic plants. The data indicated that transgenic plants expressing hpt II gene were successfully produced via *Agrobacterium* mediated transformation of faba bean with 4.56% for transformation efficiency, as measured by hygromycin expressing shoots number/total of inoculated explants.

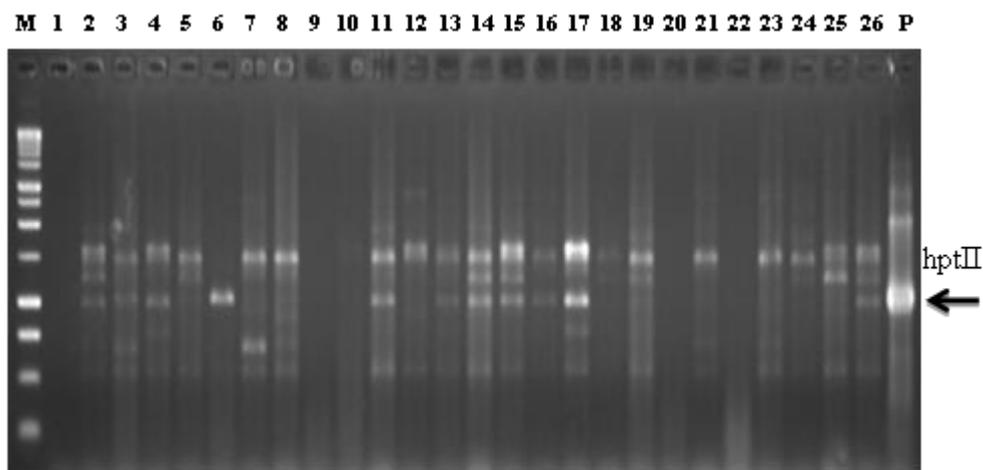


Figure 3. Confirmation of the presence of the hygromycin resistance gene (hptII) in transformed faba bean plants (2-26). Ethidium bromide-stained agarose gel showing products of hptII amplification from transgenic plants using hpt-specific primers. L1: non-transformed faba bean plant, p: Plasmid, M: Marker.

Integration of *sarcotoxin IA* gene

In order to control *Orobanche*, we introduced sarcotoxin IA gene in faba bean cotyledonary node using *Agrobacterium*. Among the hygromycin resistant transformants, 16 lines were used for PCR analysis. The result showed that PCR amplification yielded a DNA fragment of

sarcotoxin IA gene with the expected size (0.2 kb) in only 2 hygromycin expressing shoots (Figure 4). These results indicate that sarcotoxin IA gene was integrated in these plants with 0.5% of transformation efficiency (number of lines caring sarcotoxin transgene/total of inoculated explants * 100).

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Agrobacterium-mediated transformation protocol, based on direct shoot regeneration from cotyledonary node, has been used for the first time in faba bean (Figure 5). The integration of *sarcotoxin IA* gene in faba bean was confirmed by PCR analysis. This transgene strategy was recently designed to enhance host resistance to *Orobanche* based on parasite-induced expression of a selective *sarcotoxin IA* polypeptide (Aly et al., 2006). Transgenic tobacco plants harbouring the *sarcotoxin IA* gene under the regulation of the HMG2 promoter showed enhanced level resistance to *Orobanche aegyptiaca* (Hamamouch et al., 2005). The expression of this gene will be studied by detection of the transcript of transgenic faba bean by RT-PCR and by physiological test to see the impact of this gene on *Orobanche*-faba bean interaction.

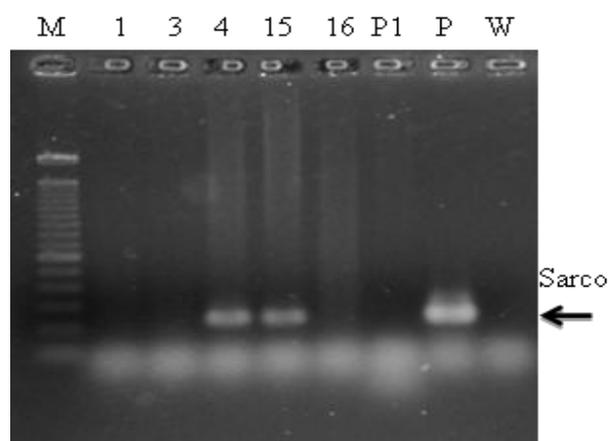


Figure 4. Integration of *sarcotoxin IA* gene in transgenic faba bean lines: PCR amplification of the transgene using primers specific to *sarcotoxin IA* showing a product around the expected size of 189 bp (see arrow) in L04 and L15 but not in the non-transformed (NT) line (L1). P: *pBIBhyg* plasmid. P1: *pLEA3* plasmid not containing *sarcotoxin IA* gene, w: sterile distilled water.

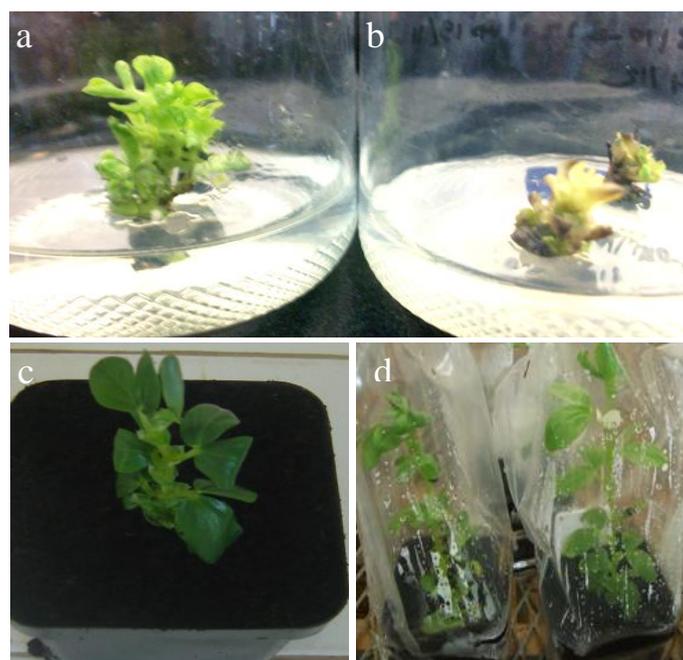


Figure 5. Transformation and production of faba bean transgenic plants using CN as starting materials
a: selection of regenerated adventitious shoots on hygromycin containing medium;
b: non-transformed shoots on selective medium; **c** and **d:** acclimatized transformed plant.

CONCLUSIONS

The genetic transformation protocol based on direct organogenesis of shoots from cotyledonary nodes provides an alternative approach for faba bean transformation and holds potential for integrating some genes of agronomical significant value, such as

sarcotoxin IA gene to control *Orobanche* in faba bean.

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