IN VITRO PROPAGATION OF COMMON BEAN (*Phaseolus vulgaris* L.)

Ninh Thị Thảo[1*], Nguyễn Thị Phương Thảo[1], Fathi Hassan[2], Hans Jörg Jacobsen[2]

[1]Faculty of Biotechnology, Hanoi University of Agriculture;
[2]Faculty of Natural Sciences, University of Hannover

Email: ntthao@hua.edu.vn*

Received date: 06.07.2013 Accepted date: 25.08.2013

ABSTRACT

The study was conducted to establish the protocol for *in vitro* regeneration of common bean (*Phaseolus vulgaris* L.). Cotyledonary nodes excised from 7-day *in vitro* seedlings were used as primary explants. The optimum treatment for shoot induction was MS salts and B5 vitamins (MSB5) medium supplemented with 11.1μM BAP and 0.5μM α-NAA, where 86.7% of cotyledonary nodes induced shoots with an average of 3.1 shoots/explant after 4 weeks of culture. Elongated, well developed individual shoots with 3-6 leaves were transferred to rooting medium containing 4.9μM of IBA. The average rooting frequency was 53.3% with a mean of 1.7 roots/shoot after 4 weeks of culture. 80% rooted plantlets survived during acclimatization period in greenhouse.

Keywords: BAP, *in vitro* regeneration, *Phaseolus vulgaris* L., α-NAA

Quy trình nhân giống *in vitro* cây đậu cô ve (*Phaseolus vulgaris* L.)

TÓM TẮT

Nghiên cứu được tiến hành nhằm xây dựng quy trình nhân giống *in vitro* cây đậu cô ve (*Phaseolus vulgaris* L.). Đốt lá mầm từ cây con *in vitro* 7 ngày tuổi được sử dụng làm vật liệu nuôi cấy khởi đầu. Môi trường thích hợp cho tái sinh chồi từ đốt lá mầm là môi trường khoáng MS và vitamin B5 (MSB5) bổ sung 11,1μM BAP and 0,5μM α-NAA cho tỷ lệ mẫu tái sinh chồi đạt 86,7% với hệ số nhân chồi đạt 3,0 chồi/mẫu cấy sau 4 tuần nuôi cấy. Chồi sinh trưởng phát triển tốt, có từ 3-6 lá được chuyển sang môi trường ra rễ MSB5 chứa 4.9μM IBA, tỷ lệ chồi ra rễ đạt 53,3%, số rễ trung bình đạt 3,5 rễ/chồi sau 4 tuần. Tỷ lệ cây sống khi chuyển ra thích nghi ngoài vườn ươm đạt 80%.

Từ khóa: BAP, *Phaseolus vulgaris* L., tái sinh *in vitro,* α-NAA.

1 INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) provides as much as 15% of the total daily calories and greater than 30% of the daily protein intake per day in numerous countries (FAO: http://faostat.fao.org). Despite the nutritional importance, the bean productivity in some areas has been declining and does not correspond with its potential productivity. The yield in average, in West Asia and North America, Latin America and African countries are 600, 1100-1500 and 500-600 kg/ha, respectively, while the potential yield is over 4000 kg/ha (Aragao and Rech, 2001).

Beside the conventional breeding techniques, genetic transformation has been made to obtain improved common bean. However, transformation in common bean is still problematic because the common bean, like other legumes, is generally recalcitrant to *Agrobacterium*-mediated transformation due to poor plant regeneration in tissue culture (Svetleva et al., 2003; Zambre et al., 2005; Colpaert et al., 2008; Arellano et al., 2009). The recalcitrance towards *in vitro* regeneration in common bean is explained by the inability to heal faster from the wounding and the production of excessively secondary callus tissue at the excision site (Kwapata et al., 2010).

Moreover, rooting of *in vitro* regenerated shoots is also very challenging since common bean shoots produce high amount of callus tissue that blocked root formation and phenolic compounds that causes death of tissues due to tissue oxidation (Arnaldos et al., 2001).

Most of the protocols for *in vitro* regeneration of common bean are based on direct organogenesis or shoot development from different types of explants, e.g. shoot apical meristems, cotyledonary and primary leaf node explants, petiole etc. (reviewed by Nagl et al., 1997 and Veltcheva et al., 2005). However, these procedures yield very low regeneration efficiency (Ahmed et al., 2002). Another approach has been done through indirect *in vitro* regeneration. To date, there are only four reports on indirect regeneration of *P. vulgaris*. However, the frequency of shoot regeneration from callus is extremely low (Arellano et al., 2009; Mahamune et al., 2011) or highly depends on cultivars (Zambre et al., 1998; Mohamed et al., 1993).

In this study, an efficient and reproducible regeneration protocol was developed via organogenesis from cotyledonary nodes arising from 7-day seedlings, which might be suitable for genetic transformation of common bean via *Agrobacterium tumefaciens.*

2 MATERIALS AND METHODS

2.1. Seed sterilization and explant preparation

After washing with running tap water, common bean seeds of "GS012" cultivar were rinsed twice with sterile deionized water, surface sterilized by immersion in 70% ethanol for 1 min, vigorously shaken and then immersed for 20 min in 6% NaOCl with some drops of Tween 20. Unwrinkled seeds were rinsed four times with sterile deionized water and soaked overnight in sterile water. Subsequently, seeds were germinated on MSB5 medium containing MS (Murashige and Skoog, 1992) macro and micro salts and B5 (Gamborg et al., 1968) vitamin mixture, then cotyledonary node (CN) was excised from the 7-day seedling (Figure 1).

2.2. Plant culture medium and culture conditions

MSB5 was used as plant culture medium, fortified with different combinations of growth regulators depending on the cultural purposes and stages. Medium was solidified by 6.0 g/l plant agar and adjusted to pH 5.8 prior to autoclaving for 15 min at 121^0C. Growth regulators were filter-sterilized and added to the medium after autoclaving and cooling down to 50–60^0C. Cultures were kept at 22 ± 2^0C under cool white fluorescent lights with a 16 h photoperiod.

2.3. Shoot induction

Cotyledonary node explants were cultured on MSB5 medium supplemented with different concentrations of cytokinins (BAP, TDZ) and the combination of optimum concentration of BAP or TDZ with various concentrations of α-NAA. Six explants per vessel in five replications were designed. The percentage of explants that regenerated adventitious shoot buds, and the number of shoots per explant were recorded after four weeks of culture.

2.4. Rooting

Elongated, well developed individual shoots with 3-6 leaves were transferred to rooting medium containing different concentrations (0, 2.5, 4.9 and 7.4µM) of IBA. Six explants per vessel in five replications were designed in this experiment. The percentage of rooted shoots, the number of roots per shoot and the root length were recorded after four weeks.

2.5. Acclimatization of rooted plantlets

80 rooted plantlets were removed from vessels, the agar medium was removed from the roots by direct rinsing under running tap water, and then washed rooted plantlets were transferred to small pots containing Profit substrate. The potted plants were covered with plastic foil to avoid high transpiration maintained at 26±1^0C. From 7 to 10 days, depending on plants status, the plastic foil was a little bit opened so that the plants were able to adapt to the outside environment (step by

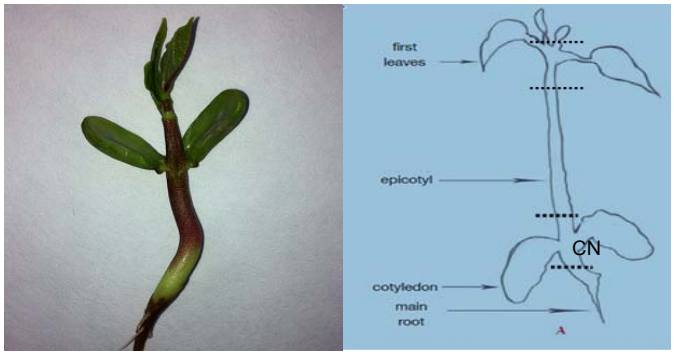


Figure 1. Scheme representation of cotyledonary node (CN) explants from 7-day seedling

step), and it was totally opened when they were two weeks old. The acclimatized plants were kept in the greenhouse until harvesting.

2.6. Data analysis

One way analysis of variance (ANOVA) was conducted to test for significance at 0.05 level of probability using R program version 2.13.0. If the ANOVA test results led to a conclusion that the group means differ, then further comparison of means which was took place, using the LSD test, $P \leq 0.05$.

3 RESULTS

3.1. Effect of BAP and TDZ on shoot organogenesis from cotyledonary nodes

The regeneration efficiency was 70-83.3% after 4 weeks of culture depending on the types and concentrations of BAP and TDZ compared to 60% in control treatment (Figure 2).

The positive effect of TDZ or BAP on the regeneration of shoots has been reported in common bean. According to Dang and Wei (2009), the optimal BAP concentration for *in vitro* bud induction of *P.vulgaris* Hacaidou No.1 was 22.2μM, where 71.9% of cotyledonary nodes induced buds after 4 weeks of culture, while lower (4.4 - 13.3μM) and higher (26.6μM) concentrations of BAP were less effective. Mohamed et al. (1992) reported that the highest frequency (74%) of embryo axis explants of most common bean tested lines developed multiple shoot buds on medium with 5 or 10μM BAP.

It was observed from our results that, at equal concentration, TDZ was slightly more efficient than BAP on shoot formation from cotyldeonary nodes of common bean (4.5μM TDZ compared to 4.4μM BAP) (Figure 2). Mohamed-Yasseen and Splittstoesser (1990) reported that 0.1μM TDZ and 5μM BAP had similar effects on shoot induction from soybean cotyledonary nodes. To explain this phenomenon, Dang and Wei (2009) supposed that TDZ may exert its influence by modifying the metabolism of endogenous cytokinins. Trigiano and Gray (2010) also proposed that the cytokinin exhibiting activity of TDZ at low concentration as 10pM relative to the effective range of amino purine cytokinins (1-10μM) such as BAP and kinetin.

Throughout the experiment, we observed that the formation of the shoots was always associated with callus from the cut surface of the explant (Figure 4). Callus induction was pronounced on all media, except the control treatment without hormone. As had been observed by other researchers, a combination of callus and phenolic compounds is naturally produced as a defense mechanism following wounding to aid in healing of plant tissues and to prevent entry of micro-organisms. However, the presence of callus could be the greatest limiting factor in shoot formation and rooting of *in vitro* shoots of *P. vulgaris* (Arnadols et al., 2001). This problem was also observed in many previous studies when cotyledonary nodes or leaf petioles of common bean were regenerated

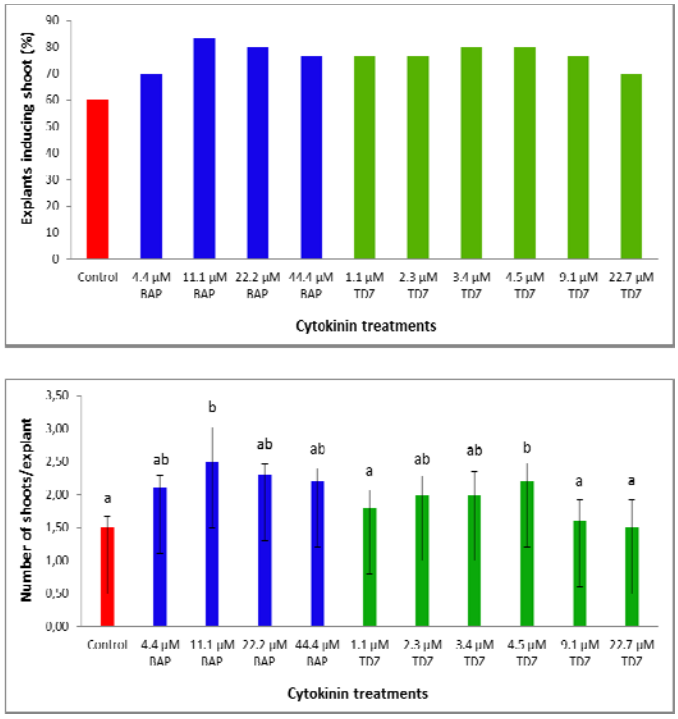


Figure 2. Effect of BAP and TDZ on shoot organogenesis from cotyledonary nodes. Letter differences within a hormone indicate statistically significant differences, LSD test, P ≤ 0.05

on medium containing hormones (Mohamed et al., 1992a; Veltcheva et al., 2005).

3.2. Effect of α-NAA in combination with 11.1µM BAP or 4.5µM TDZ on shoot organogenesis from cotyledonary nodes

There were no differences between media supplemented with BAP/α-NAA and BAP alone or TDZ/α-NAA and TDZ alone, as regards to percentage of explants producing shoots (Figure 3A), but slightly different regarding the number of shoots induced from one explant (Figure 3B).

The combination of 11.1µM BAP and 0.5µM α-NAA produced the highest number of shoots with 3.1 shoots/explant. However, further increase in α-NAA concentrations led to decrease in the number of shoots. The same pattern was detected in case of TDZ/α-NAA combinations. The explants cultured on medium supplemented with 4.5µM TDZ and 0.005/0.01µM α-NAA induced the highest number of shoots per explant (2.3 shoots/explant). Increasing the α-NAA concentration over 0.01µM α-NAA, and combining with 4.5µM TDZ decreased the number of shoots induced from one explant (Figure 3B).

It was observed that shoots induced from media containing BAP or BAP/α-NAA combinations had better quality with more leaves than those from media containing TDZ or TDZ/α-NAA combinations. Moreover, the formation of shoot was associated with callus formation at the cut ends of explants on all tested media, even on medium without hormone (Figure 4).

The combination of plant growth regulators was usually more effective than single one for the development of shoot (Mallikarjuna and Rajendrudu, 2007). Lamseejan et al. (1992)

observed that the highest number of complete plantlets was obtained from cotyledonary nodes of *P. vulgaris*, when the explants were cultured on the medium containing 0.02µM BAP combined with 0.03µM α-NAA compared to media supplemented with BAP alone. It also was indicated that 2µM TDZ combined with 0.6µM α-NAA benefited the process of shoot initiation from petiole explants of common bean (Veltcheva et al., 2005). Ahmed et al. (2002) showed the efficiency of regeneration from intact seedlings and cotyledonary node explants reached 100%, when they were cultured on MS medium added with 4.4µM BAP and 0.5µM α-NAA. Kwapata et al. (2010) suggested that the most efficient growth regulator combination for shoot proliferation of common bean was a combination of 11.1µM BAP and 0.5µM α-NAA, which produced a mean number of 12 multiple shoots per explant in all tested cultivars. However, our results showed that the trend in the response of common bean cotyledonary nodes on shoot formation was not consistent with the increase of α-NAA concentration in either BAP/α-NAA or TDZ/α-NAA combinations (Figure 3).

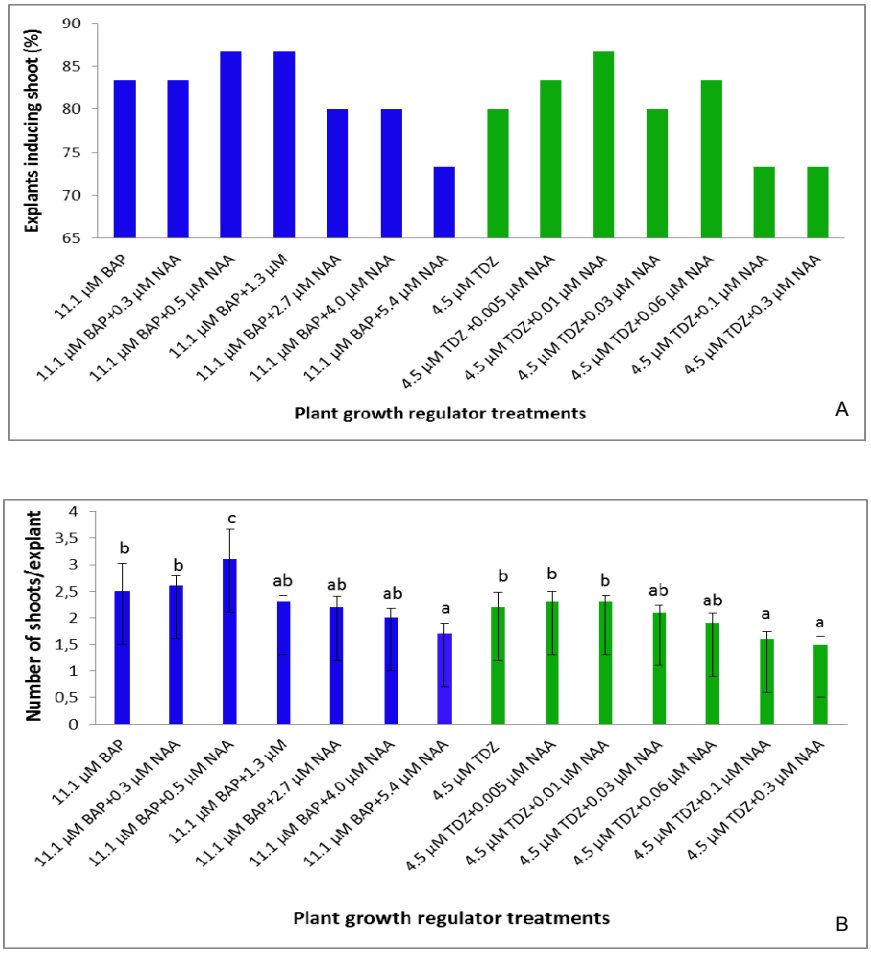


Figure 3. Effect of α-NAA in combination with 11.1µM BAP and 4.5µM TDZ on shoot organogenesis from cotyledonary nodes. Letter differences within a hormone combination indicate statistically significant differences, LSD test, $P \leq 0.05$.

3.3. Effect of IBA on *in vitro* rooting of regenerated shoots

Single shoots was cultured on MSB5 medium containing different concentrations of IBA showed differences in rooting response (Table 1). An average of 32.1 - 53.3% shoots were rooted with 0.6 - 1.7 roots/shoot. The root induction was observed within 10-15 days after culture but complete root development took 4 weeks before transferring the plantlets to acclimatize in greenhouse.

The best rooting response was achieved on medium supplemented with 4.9µM IBA which gave 1.7 roots/shoot. The lower concentration (2.5µM) led to poor or no root development, but the leaves were observed larger (Figure 5). High concentration of IBA (7.4µM) gave roots with a frequency of 1.23 roots/shoot and longer roots but the shoot development did not perform well. Overall, IBA had the effect on root establishment since no root was induced on IBA-free medium, and the best concentration of IBA was 4.9µM, which resulted in 53.3% of shoots with roots induction, 1.7 roots/shoot with the mean length of root was 9.1cm.

IBA is an important factor in root initiation, and its varying concentrations significantly affect the root regeneration in legumes. For rooting of *in vitro* shoots, 2.5µM IBA was used in chick pea (Khan et al., 2010) or 1µM IBA in soybean (Nedev et al., 2007).

The greatest limiting factor in rooting of *in vitro* regenerated shoots of *P. vulgaris* is its propensity to produce high amount of callus that blocked root formation as well as phenolic compounds that caused death of tissues due to tissue oxidation (Zambre et al., 1998; Arnaldos et al., 2001). In order to overcome the problems from phenolic compounds, Kwapata et al. (2010) added 15 mg/l active charcoal and 30 mg/l silver nitrate into shoot induction medium and the base of each shoot was dipped in IBA

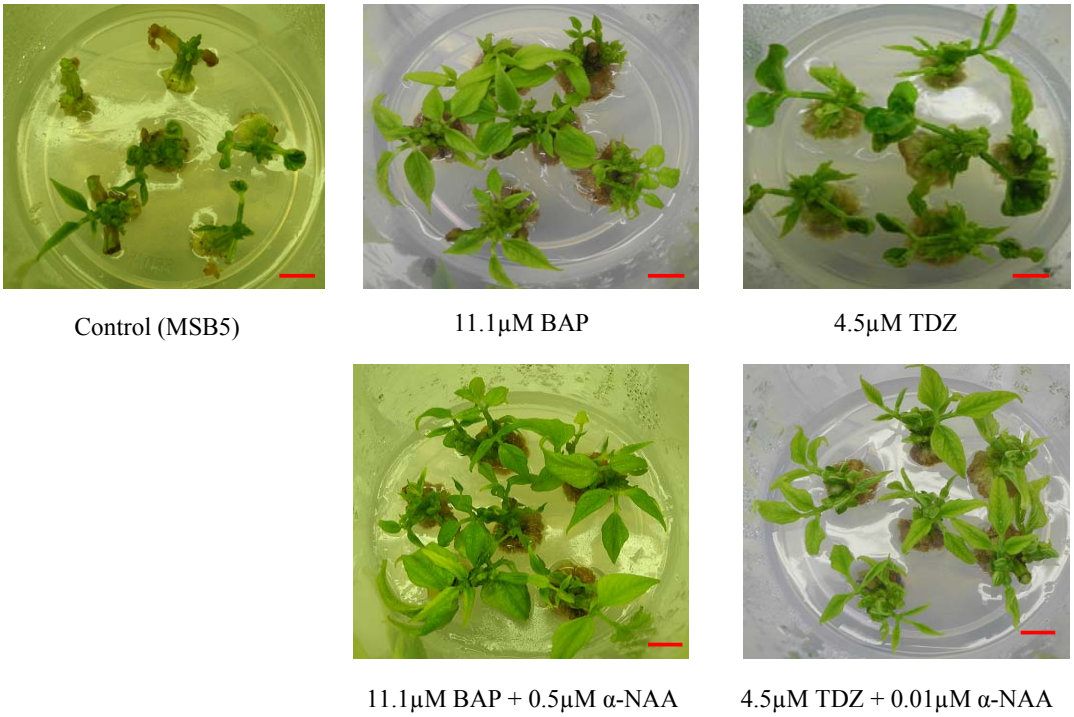


Figure 4. Shoot regeneration from cotyledonary nodes on MSB5 medium supplemented with BAP, TDZ, BAP/α-NAA and TDZ/α-NAA compared to the control medium after 4 weeks of culture. The red bar represents 1cm

Table 1. Effect of IBA on *in vitro* rooting of regenerated shoots

IBA concentration	% rooting	Mean of No. roots/shoot	Mean length of roots (cm)
0	0	0	0
2.5µM	32.1	0.6 ± 0.03a	6.9 ± 0.08a
4.9µM	53.3	1.7 ± 0.03b	9.1 ± 0.17b
7.4µM	46.7	1.2 ± 0.08c	10.2 ± 0.24c

Ghi chú: Mean ± SD followed by the same letters within the column indicates no significant differences between the treatments, LSD Test, P ≤ 0.05.

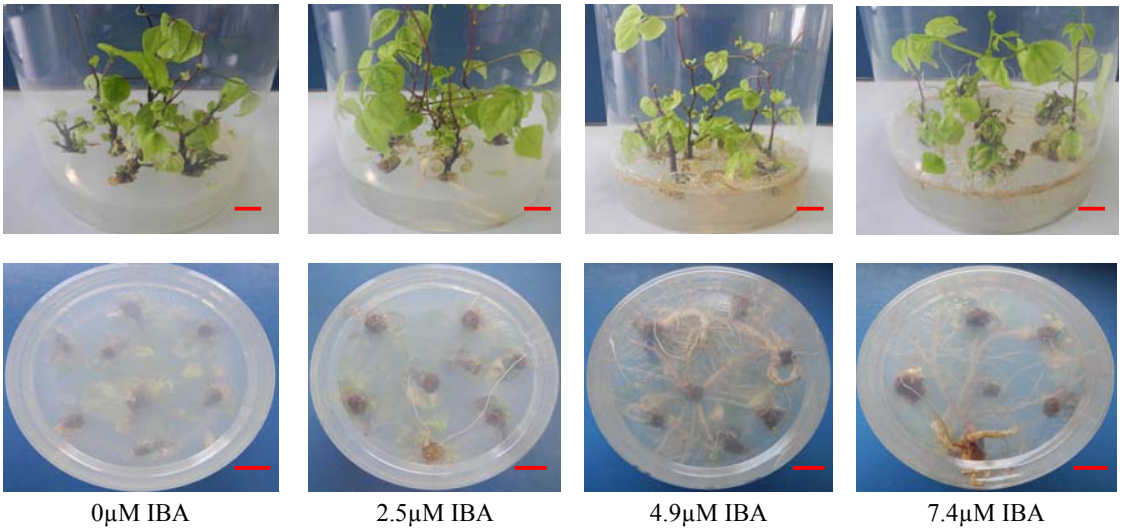


Figure 5. Effect of IBA on *in vitro* rooting of regenerated shoots after 4 weeks of culture. The red bar represents 1 cm

solution for 30 seconds before transferring them onto rooting medium. To establish a better rooting system for *P. vulgaris*, Zambre et al. (1998) grafted *in vitro P. vulgaris* shoots on the *P. acutifolius* rootstocks.

3.4. Acclimatization of rooted plantlets

Plantlets were successfully hardened with 80% survival after acclimatization in greenhouse. The survived plants were fertile and set seeds (Figure 6).

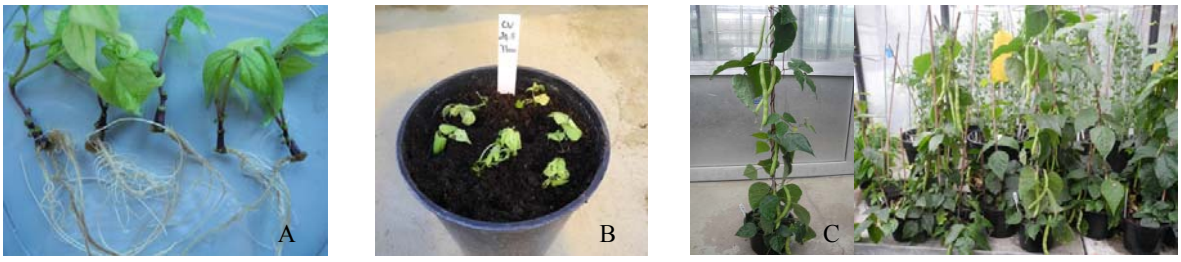


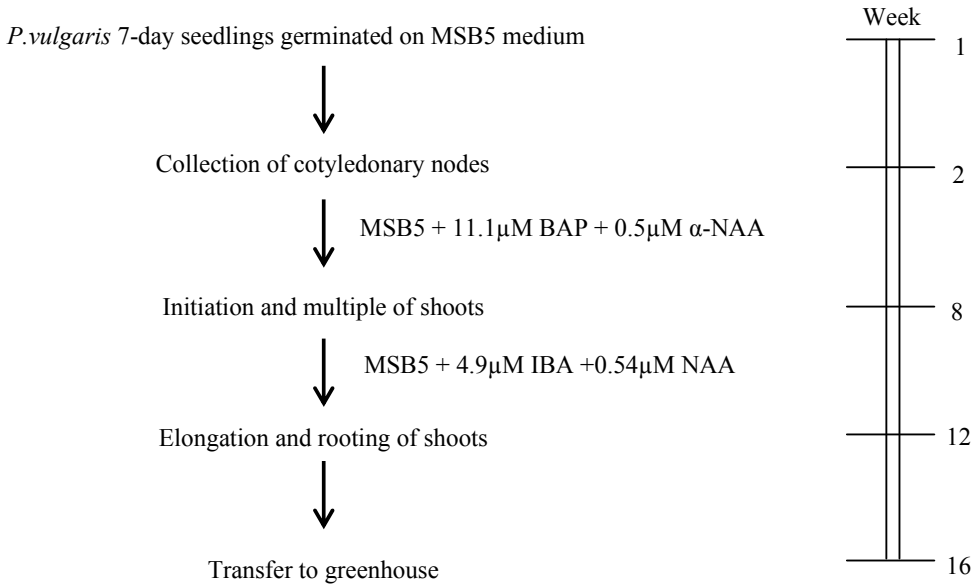
Figure 6. Acclimatization of *P. vulgaris in vitro* plantlets:
A) Plantlets derived from in vitro culture; B) Plants transferred to the Profi-substrate C) Mature plants setting pods in greenhouse

4. CONCLUSIONS

A protocol for *in vitro* regeneration from cotyledonary nodes of 7 day-seedlings of common bean was developed.

Shoot induction medium was MSB5 + 11.1µM BAP + 0.5µM α-NAA, on which 86.7% of explants induced shoots with an average of 3.1 shoots/explant. 53.3% of shoots rooted with a mean of 1.7 roots/shoot on root induction medium MSB5 + 4.9µM IBA. 80% rooted plantlets survived during acclimatization in greenhouse.

The entire procedure starting from shoot induction to establishing a plant under greenhouse conditions took approximately 3-4 months:



REFERENCES

Ahmed, E., Ahmed, E. E., Bisztray, G. Y. D. and Velich I. (2002). Plant regeneration from seedling explants of common bean (*P. vulgaris* L.). Acta Biologica Szegediensis 46: 27-28.

Aragao, F. J. L. and Rech, E. L. (2001). Transgenic Common Bean (*P. vulgaris*). Biotechnology in Agriculture and Forestry 47: 269-283.

Arellano, J., Fuentes, S. I., Castillo-Espana, P. and Hernandez, G. (2009). Regeneration of different cultivars of common bean (*P. vulgaris* L.) via indirect organogenesis. Plant Cell, Tissue and Organ Culture 96:11–18.

Arnaldos, T. L., Munoz, R., Ferrer, M. A. and Calderon, A. A. (2001). Changes in phenol content during strawberry (*Fragaria x ananasa*, cv. Chandler) callus culture. Plant Physiology 113: 315-322.

Colpaert, N., Tilleman, S., van Montagu, M., Gheysen, G. and Terryn, N. (2008). Composite *P. vulgaris* plants with transgenic roots as research tool. African Journal of Biotechnology 7: 404-408.

Dang, W. and Wei, Z. M. (2009). High frequency plant regeneration from the cotyledonary node of common bean. Biologia Plantarum 53: 312-316.

Gamborg, O. L., Miller, R. A. and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research 50: 151-158.

Khan, S., Ahmad, M., Ghaffar, M., Fida, M. and Swati, Z. A. (2010). *In vitro* micro-propagation of indigenous chick pea (*Cicer arietinum* L.) cultivars, KK-1 and Hassan-2K. African Journal of Agricultural Research 5: 2934-2938.

Kwapata, K., Sabzikar, R., Sticklen, M. B. and Kelly, J. D. (2010). *In vitro* regeneration and morphogenesis studies in common bean. Plant Cell, Tissue and Organ Culture 100: 97–105.

Lamseejan, S., Tonguthaisri, T., Wongpiyasati, A., Unsrisong, S., Jonglaekha, N., and Kumpai, S. (1992). Induction of multiple shoots from cotyledonary node of red kidney bean (*P. vulgaris* L.). Natural Sciences 26: 227-233.

Mahamune, S. E., Bansode, R. P., Sangle, S. M., Waghmare, V. A., Pandhure, N. B. and Kothekar, V. S. (2011). Callus induction from various explants of French bean (*P. vulgaris* L.). Journal of Experimental Sciences 2: 15-16.

Mallikarjuna, K. and Rajendrudu, G. (2007). High frequency *in vitro* propagation of Holarrhena antidysenterica from nodal buds of mature tree. Biologia Plantarum 51: 525-529.

Mohamed, M. F., Coyne, D. P., Read, P. E. (1993). Shoot organogenesis in callus induced from pedicel explants of common bean (*P. vulgaris* L.). Journal of American Society for Horticulture Science 118: 158–162.

Mohamed, M. F., Read, P. E. and Coyne D. P. (1992). Plant regeneration from *in vitro* culture of embryonic axis explants in common and tepary beans. American Society of Horticulture Science 117: 332-336.

Mohamed-Yasseen, Y. and Splittstoesser, W. E. (1990). Regeneration of soybean [*Glycine max* (L) Merr.] from the seedling apex, stem node, cotyledonary node, and cotyledons. Plant Growth Regulator Society of America 18: 203 – 210.

Murashige T., Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco cultures. Plant Physiology 15:473–497.

Nagl W., Ignacimuthu, S. and Becker, J. (1997). Genetic engineering and regeneration of *Phaseolus* and *Vigna*. State of the art and new attempts. Plant Physiology 150: 625–644.

Nedev, T., Todorova, R., Kosturkova, G., Akitha Devi, M. K., Sakthivelu, G., Giridhar, P., Rajasekaran, T., Ravishankar, G. A. (2007). Variation in *in vitro* morphogenic response to growth regulators in soybean genotypes from India and Bulgaria. Bioautomation 8: 193 – 200.

Svetleva, D. L., Veltcheva, M. R. and Bhowmik, G. (2003). Biotechnology as a useful tool in common bean (*P. vulgaris* L.) improvement. Euphytica 131: 189-200.

Trigiano, R. N. and Gray, D. N. (2010). Plant Tissue Culture, Development, and Biotechnology.

Veltcheva, M. R. and Svetleva, D. L. (2005). *In vitro* regeneration of *P. vulgaris* L. via organogenesis from petiole explants. Journal of Central European Agriculture 6: 53-58.

Zambre, M. A., Clerq, J. and Vranova, E. (1998). Plant regeneration from embryo derived callus in *P. vulgaris* L. (common bean) and *Phaseolus acutifolius* A. Grey (tepary bean). Plant Cell Reports 17: 626–630.

Zambre, M., Goossens, A., Cardona, C., Van Montagu, M., Terryn, N. and Angenon, G. (2005). An efficient and reproducible *Agrobacterium*-mediated transformation systems for cultivated *Phaseolus acutifolius* (tepary bean). Theoretical and Applied Genetics 110: 914-924.