Proc. of Int. Conf. on Emerging Trends in Engineering & Technology, IETET

Direct in Vitro Shoot Regeneration of Cicer Arietinum L. from Decapitated Embryo Axes Explants

Jaya Srivastava[1]and Pallavi Sinha[2]
[1]Mahtma Gandhi Chitrakoot Gramodaya Viswavidyalaya, Chitrakoot-Satna (M. P.)
[2]Geeta Institute of Management and Technology
srivastavapp75@gmail.com
*Corresponding author E mail: srivastavajaya2003@gmail.com

***Abstract*—This study reports a protocol for plant regeneration from cultured explants of chickpea *Cicer arietinum* L. via direct organogenesis using decapitated embryonic axes explants. Morphologically normal healthy plants were regenerated directly without an intervening callus phase from the explants. Explants prepared from overnight soaked seeds were cultured on Murashige and Skoog (MS) medium fortified with different concentrations of benzylaminopurine (BAP), indolbutyric acid (IBA) and naphthalene acetic acid (NAA). The type and concentration of plant growth regulators influenced the frequency of multiple shoot regeneration. 2 mg/L BAP and 0.05mg/L IBA was the most effective combination for high frequency multiple shoot induction. Individual shoots produced were aseptically excised and sub cultured in the media fortified with 1 mg/L GA_3 for shoot elongation. The elongated shoots were grafted on the rootstocks prepared from the seeds of the same cultivar.**

Index Terms*— Decapitated embryonic axes, multiple shoots, Benzylaminopurine, *Cicer arietinum.

I. INTRODUCTION

Grain legumes are the major source of proteins for more than two billion people worldwide. The demand for grain legumes has been increasing due to the increase in population in developing countries for whom grain legumes are the major source of protein requirement and also due to the growing trend towards more vegetarian diets. Among the grain legumes, chickpea occupies the first position with regard to the total cultivation area in India. Grain legumes are also very useful due to their capacity for nitrogen enrichment of soil through symbiosis with rhizobium. Yield has been improved in grain legumes in last decades qualitatively and quantitatively through conventional breeding. However, lack of resistant genes in the gene pool limits the success of conventional breeding approaches and several abiotic and biotic stresses still remains a major cause of significant loss in the yield of chickpea. The advances made in genetic engineering techniques have clearly demonstrated the possibility of transfer of foreign genes to the desired crop while preserving the existing characteristics of the genotypes. An efficient and reliable *in vitro* regeneration method is the major need for the successful employment of the transgenic approach. Plant regeneration requires large number of totipotent cells, capable of transformation through DNA addition, efficient regeneration of healthy plants, have genotype independency, and can be manipulated easily under in vitro conditions. Most

Grenze ID: 02.IETET.2016.5.39

transformation systems use a regeneration system which starts with physical isolation of explants followed by exposing them to suitable plant growth regulators to activate a new developmental pathway, either somatic embryogenesis or organogenesis. In most crops, this strategy was found successful, usually accomplished by optimizing the type of explants, growth regulators used and the nutritional and physical conditions for its *in vitro* culture. In grain legumes, protocols for regeneration have been demonstrated through direct organogenesis from pre-existing meristems, leaflet callus and immature explants (Kartha *et al*., 1981; Islam *et al*., 1994; Barna and Wakhlu, 1994). In chickpea, somatic embryogenesis and regeneration of shoots *via* direct organogenesis has also been reported from leaflets and embryo axis (Dineshkumar *et al*., 1995; Pathak and Hamzah, 2008; Mehrotra *et al*., 2011). The objective of the present study was to develop an efficient protocol for multiple shoot regeneration using embryonic axes explants, which may be employed for routine chickpea transformation experiments.

II. Materials And Metods

A. Plant material and culture conditions:

Mature seeds of Indian chickpea cultivar DCP 92-3 were first washed in tap water and kept in water with 3-4 drops of Tween 20 for 15 min. Seeds were then treated with a quick rinse of 75% alcohol followed by treatment with 1% sodium hypochlorite for 5 min. Seeds were then washed properly with sterile distilled water several times and soaked in autoclaved water for overnight. Next days the seeds were used for explants preparation. The embryos were separated from the cotyledons and the shoot and root apices were cut with a sharp scalpel. The MS salts and vitamins (Murashige and Skoog, 1962) were used in all the culture media in present study. The pH of the culture media was adjusted to 5.8 and then 0.8% (w/v) agar was added to it before autoclaving. The nonautoclavable plant growth regulators used in the culture media were sterilized by filtering through a 0.2 µm membrane and mixed to the media after autoclaving when the temperature was cooled to 50-60ºC. All cultures were maintained in a growth chamber at 23 ± 1ºC with 16/8 h (light/dark) photoperiod. The decapitated embryonic axes were incubated in Petri dishes containing different shoot induction medium to test their efficiency in producing multiple shoots from the explants. The media contained MS basal medium supplemented with different combinations of BAP, NAA, and IBA as shown in Table 1. The explants were transferred to fresh medium every week. The best medium was used in all further experiments to regenerate the plants.

III. Elongation And Grafting Of Shoots

The explants with multiple shoots were kept for elongation on MS medium devoid of any PGR supplementation and transferred to fresh medium every 2 weeks. However, some of the shoots that did not elongate on this medium were transferred to a medium supplemented with 1 mg/L GA_3. Shoots, which were properly elongated, were removed at regular intervals to support the growth of the developing buds. To prepare the rootstocks, seeds of the same cultivar were germinated and after 5 to 7 days the shoot tip of the seedling was removed and a 2 - 3 mm perpendicular cut were made at the tip of the shoot base. The scion was prepared carefully and inserted into the cut made in the rootstock. The pots were covered with a polybag which was punctured after 7-8 days to slowly acclimatize the plants to low moisture greenhouse conditions. The plants were watered with a quarter-strength Hoagland's nutrient solution until they were well established. When survived, the plants were transferred to earthen pots (18 cm) filled with soil mix (peat, perlite, and vermiculite in equal proportions 1:1:1) and grown to maturity.

IV. Results And Discussion

Eight different combinations of BAP, NAA, and IBA were evaluated for multiple shoot induction from the decapitated embryonic axes explants prepared from overnight soaked seeds. Excision of the apical and radicle tip promoted the formation of a high number of multiple shoots from the axillary meristems containing cells, highly competent for regeneration when provided with proper growth regulators (Polisetty *et al*., 1996). The maximum shoot regeneration was observed on the SIM 2 medium. In the present study among the different combinations of growth regulators tested combination of BAP and IBA were found better than the BAP and NAA combination for multiple shoot induction from embryonic axes. Further, among the different BAP + IBA concentrations evaluated a media containing 2 mg/L BAP and 0.05 mg/L IBA (SIM2) was found as the most effective for producing maximum number of healthy multiple shoots and this shoot

induction medium produced an average 9.36 shoots per embryonic axes explant. However, if the concentration of BAP was increased further, the production of multiple shoots was adversely affected qualitatively and quantitatively. High concentration of BAP showed many negative effects like browning and shoot tip decay. It has been observed by other workers also that increasing concentrations of cytokinines may increase the number of shoots produced but significantly reduces the shoot length and subsequent rooting (Gulati and Jaiwal, 1990; Prakash *et al*., 1994; Polisetty *et al*., 1996; Subhadra *et al*., 1998). The *in vitro* production of multiple shoots is much useful for application of efficient transformation protocols. Growth regulators present in the regeneration media plays an important role hence our interest is to find out the plant growth regulators which could promote healthy multiple shoot formation from the explants. The phytohormone added to the shoot induction medium not only affects the number of shoots regenerated, it also significantly affects the quality and length of the shoots regenerated. The results showed that all the factors analysed, concentration and type of phytohormones significantly affected the morphological responses (Table 1)

TABLE I. EFFECT OF DIFFERENT SHOOT INDUCTION MEDIUM ON PERCENT REGENERATION AND MULTIPLE SHOOT INDUCTION IN CHICKPEA CULTIVAR DCP92-3

Shoot Induction Medium	BAP	IBA	NAA	Percent Regeneration response	Average number of shoots/ explants
SIM 1	1	0.05	-	66.67	7.41
SIM 2	2	0.05	-	66.03	9.36
SIM 3	3	0.05	-	50	6.58
SIM 4	1	-	0.05	40.6	4.86
SIM 5	2	-	0.05	45.97	4.39
SIM 6	3	-	0.05	41.97	4.15
SIM 7	2	0.1	-	46.67	4.64
SIM 8	2	-	0.1	53.34	3.45

Among the different cytokinins evaluated for multiple shoot regeneration, BAP has been reported very effective in several legumes including chickpea (Kar *et al*., 1997; Polowick *et al*., 2004). While some workers have used only BAP for multiple shoot induction (Brandt and Hess, 1994) some have reported that if a low concentration of auxin is used with BAP show a synergistic effect on shoot regeneration and more number of shoots were produced (Subhadra *et al*., 1998; Chakraborti *et al*., 2006). Combination of cytokinins was also reported better than using a single cytokinin for multiple shoot induction (Jayanand *et al*., 2003).
Among different shoot elongation medium, the medium containing 0.5 mg/L BAP + 1mg/L GA_3 was found as the best medium. Shoots elongated in this medium were healthy and show high survival rate after grafting. A low dose of BAP further supports the development of new shoot buds continuously arising from the explants. Gibberellins have been proved efficient for elongation of the shoots, developed after prolong culture on cytokinin containing medium in chickpea as well other crops.

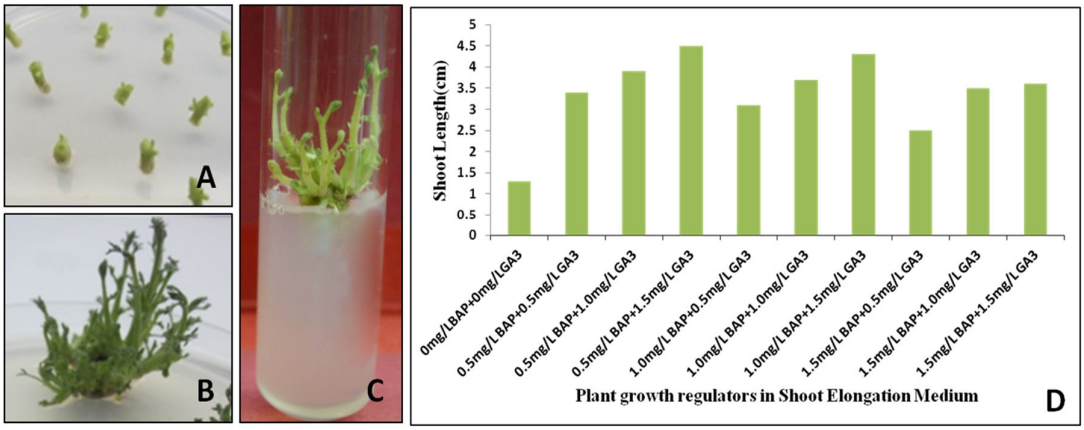

Figure1. Shoot regeneration from decapitated embryonic axes explants (A) Inoculated explants (B) Multiple shoot regeneration from the explants (C) Elongation of shoots. (D) Effect of different plant growth regulators on shoot elongation

Gibberellins promote elongation of shoots but shoots elongated on high dose of GA_3 often have large internodal regions and are very thin and weak. These shoots generally show low survival rate when grafted on rootstocks. Optimally elongated shoots have distinct nodes and internodes and have an average length of 4-5 cm. Unless the shoots grafted were very weak more than 70% grafting efficiency was obtained. However, even after proper fusion of the graft to the rootstock, 30% of the shoots died during hardening process. As rooting in chickpea generally takes prolong incubation and do not show very good frequency of rooting. Significant inhibition of root formation from shoots regenerated on BAP containing medium for more than 4 weeks have been reported (Polanco and Ruiz, 1997). Grafting when performed with proper care give high number of regenerated shoots in lesser time duration and phytohormone treatments are also not needed (Krishnamurthy *et al*., 2000; Bean *et al*., 1997). In conclusion, we have presented here a simple and efficient regeneration system for chickpea. We recommend the use of decapitated embryonic axes explants prepared from overnight soaked seeds and culture of these explants on 2 mg/L BAP and 0.05 mg/L IBA. The multiple shoots produced were elongated in BAP and GA_3 containing medium and successfully grafted on the rootstocks. Through histological study of the regenerating explants we have shown that this regeneration system could be efficiently employed for *Agrobacterium*-mediated genetic transformation of this important legume crop.

ACKNOWLEDGMENT

The research fellowship awarded to Srivastava J. by the Council of Scientific and Industrial Research, New Delhi, is gratefully acknowledged. All the lab work was conducted at Biotechnology Unit, Indian Institute of Pulses Research, Kanpur.

REFERENCES

[1] Barna, K.S. and Wakhlu, A.K.: Plant Cell Rep., 13: 510-513 (1994).
[2] Bean, S.J., Gooding, P.S., Mullincaux, P.M. and Davies, D.R.: Plant Cell Rep., 16: 513-519 (1997).
[3] Brandt, E.B. and Hess, D.: In Vitro Cell. Dev. Biol. Plant., 30: 75-80 (1994).
[4] Carman, J.G.: In vitro Cell. Dev. Biol., 26:746-753 (1990).
[5] Chakraborti, D., Sarkar, A. and Das, S.: Plant Cell Tiss. Org. Cult., 86:117-123 (2006).
[6] Dineshkumar, V., Kirti, P.B., Sachan, J.K.S. and Chopra, V.L.: Plant Sci., 109:207-13 (1995).
[7] Gulati, A. and Jaiwal, P.K.: Plant Cell Tiss. Org. Cult., 23: 1-7 (1990).
[8] Islam, R., Malik, T., Husnain, T. and Riazuddin, S.: Plant Cell Rep., 13:561-563 (1994).
[9] Jayanand, B., Sudarsanam, G. and Sharma, K.K.: In Vitro Cell. Dev. Biol., 39: 171–179 (2003).
[10] Kar, S., Basu, D., Das, S., Ramakrishnan, N.A., Mukherjee, P., Nayak, P. and Sen, S.K.: Transgenic Res., 6:177-175 (1997).
[11] Kartha, K.K., Pahl, K. and Leung, N.L.: Can. J Bot., 59:1671-1679 (1981).
[12] Krishnamurthy, K.V., Suhasini, K., Sagare, A.P., Meixner, M., de Kathen, A., Pickardt, T. and Schieder, O.: Plant Cell Rep., 19:235-240 (2000).
[13] Mehrotra, M., Singh, A.K., Sanyal, I., Altosaar, I. and Amla, D.V.: Euphytica., 182:87-102 (2011).
[14] Murashige, T. and Skoog, F.: Physiologia Plantarum., 15:473-497 (1962).
[15] Pathak, M.R. and Hamzah, R.Y.: Plant Cell Tiss. Org. Cult., 93:65-67 (2008).
[16] Polanco, M.C. and Ruiz, M.L.: Plant Cell Rep., 17: 22-26 (1997).
[17] Polisetty, R., Patil, P., Deveshwar, J.J., Khetarpal, S. and Chandra, R.: Indian J Exp. Biol., 34:806-809 (1996).
[18] Polowick, P.L., Baliski, D.S. and Mahon, J.D.: Plant Cell Rep., 23:485-491 (2004).
[19] Prakash, S.N., Pental, D. and Bhalla-Sarin, N.: Plant Cell Rep., 13: 623-627 (1994).
[20] Subhadra, Vashishat, R.K., Chowdhury, J.B., Singh, M. and Sareen, P.K.: Indian J Exp. Biol., 36:1276-1279 (1998).