High frequency multiple shoot regeneration from cotyledon with half embryonic axes explants of chickpea (Cicer arietinum L.)

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ABSTRACT **– Chickpea is an important legume crop used almost all over the world. Development of new efficient and reliable tissue culture protocols is imperative for improvement of breeding and genetic transformation studies. It is highly recalcitrant in nature and there is an urgent need to develop a regeneration protocol that can ensure easy multiple and healthy shoots that could be used for transformation studies. We have developed an efficient multiple shoot induction protocol in chickpea variety C 235 using cotyledom with half embryonic axes explants. Different shoot induction medium containing thidiazuron (TDZ), benzyl aminopurine (BAP), indol butyric acid (IBA) and naphthalene acetic acid (NAA) were evaluated for their effect on multiple shoot induction from the explants. The shoots were elongated on GA_3 containing medium and grafted on the rootstocks. The grafted plants were successfully acclimatized.**

Keywords **– Chickpea, *Cicer arietinum*, benzylaminopurine, naphthalene acetic acid, multiple shoot regeneration.**

I. INTRODUCTION

Chickpea (*Cicer arietinum L.*), one of the earliest cultivated edible legumes are mainly grown in the Mediterranean regions, South Asia, Western Asia and Australia. Though significant progress has been made in chickpea production in last decades, it is not yet able to supply the demand made by the highly growing population of our country. Hence, to further increase the production of chickpea, it is required to minimize effects of several constraints which decrease the production of chickpea. Conventional breeding has contributed significantly in dramatic improvements in chickpea production, but it is limited mainly by the availability of desired traits in chickpea germplasm. Modern biotechnology techniques provide new opportunities to enhance the germplasm of several crop plants (Sharma and Ortiz, 2000). However, an efficient and reliable multiple shoot regeneration protocol is a prerequisite for the application of genetic transformation studies. Shoot regeneration in chickpea have been reported by different scientists through direct and indirect organogenesis and somatic embryogenesis using diverse explants (Sagare et al., 1993; Suhasini et al., 1994; Kar et al., 1996; Polisetty et al., 1997; Jayanand et al., 2003; Chakraborti et al., 2006). However, regeneration *via* somatic embryogenesis is not the commonly used route as no strong, repeatable protocol is available for regeneration in several important legumes including chickpea. The effective regeneration in chickpea has been possible mainly through the use of explants based on cotyledonary nodes or shoot apices obtained from seedling explants. The present study was performed with an objective to develop a simple and high frequency multiple shoot regeneration protocol amenable for *Agrobacterium* mediated genetic transformation, so that the method could be applied for the routine transformation protocols.

II. MATERIALS AND METHODS

A. Plant Material and Explant Preparation

Seeds of chickpea (*Cicer arietinum* L.) cultivar C 235 were used for the study. Prior to sterilization, mature seeds were screened manually, damaged seeds were removed and only the light

coloured uniform seeds were used for the experiments. Seeds were washed in running tap water and kept in water with 2-3 drops of tween 20 for 15 minutes. Then, the seeds were sterilized with a quick rinse with 75% alcohol followed by treatment with 1% sodium hypochlorite for 10 min. Finally, seeds were washed thoroughly with sterile distilled water several times and soaked overnight. The germinated seeds were bifurcated from the centre with a fine scalpel and seed coats were removed. Thus, two cotyledon with half embryonic axes explants were prepared from each seedling.

B. Culture Media and Growth Conditions

MS medium were used in all culture media in the study. All regeneration media were supplemented with 3% (w/v) of sucrose; pH was maintained at 5.8 and solidified with 0.8% agar. All the autoclavable growth regulators were added to the medium before autoclaving and non-autoclavable plant growth regulators were sterilized by filtering through a 0.2 µm membrane and added to the media after cooling of the autoclaved media. Cultures were kept in a culture room maintained at 22±1^{o} C with 16 h light/ 8h dark photoperiod.

TABLE 1. CONCENTRATION OF DIFFERENT PLANT GROWTH REGULATORSS IN DIFFERENT SHOOT INDUCTION MEDIUM

SIM	PGRs (mg/L)			
	TDZ	BAP	IBA	NAA
SIM 1	0.5	-	0.05	-
SIM 2	1.0	-	0.05	-
SIM 3	1.5		0.05	-
SIM 4	-	1	-	0.05
SIM 5		2	-	0.05
SIM 6	-	3	-	0.05
SIM 7	-	2	0.1	-
SIM 8	-	2	-	0.1

BAP, IBA and NAA were added in different concentrations and combinations (Table 1) in the basal medium to prepare eight different shoot induction medium (SIM). Three replications were made for each experiment and fifty explants were cultured in each replication. The explants were sub-cultured to the same medium after every one week. Data for percent regeneration response and mean number of shoots per explant were recorded after four weeks of culture of the explants on SIM.

(C) Histological Study

Explants were fixed and dehydrated and embedded in paraffin wax and blocks were prepared. Longitudinal sections of 12 µm thickness were cut from these blocks using a rotatory microtome. The sections were fixed on glass slides, stained with safranin and fast green and mounted with DPX mountant.

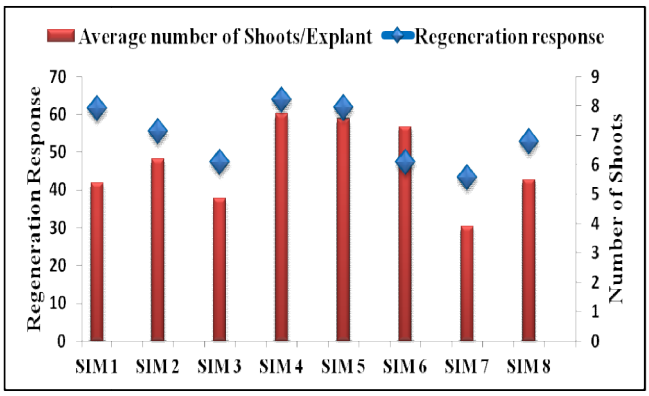
III. RESULT AND DISCUSSION

A. Effect of Different PGRs on Shoot Induction

Direct shoot regeneration was observed from cotyledon with half embryonic axes explants without any callus formation on MS medium containing various concentrations of TDZ, BAP, IBA and NAA. When shoot regeneration was recorded on explants cultivated on different SIM media, it was observed that mean number of shoots per explants was significantly different on different media. From

the different concentrations and combinations tried, 1 mg/L BAP and 0.05 mg/L NAA (SIM 4) was proved as the most effective in producing highest number of multiple shoots from the explants. This combination of growth regulators produced 7.72 shoots per explant. Maximum regeneration response was also observed in the same medium. Hence, SIM 4, which was found best for multiple shoot induction from the explants, were used for all the regeneration experiments.

GRAPH 1. EFFECT OF DIFFERENT SIM ON REGENERATION AND MULTIPLE SHOOT INDUCTION FROM CHA EXPLANTS



Among the cytokines, TDZ was found less favourable for the CHA explants as compared to BAP. Further, combinations of BAP and IBA was found less favourable for multiple shoot regeneration and resulted in less number of shoots per explants compared to the explants cultured on medium containing BAP and NAA. Increasing the concentration of NAA (SIM 8) did not show any further improvement in regeneration response and multiple shoot regeneration (Graph 1). When concentration of NAA was kept constant and concentration of BAP was increased upto 2 mg/L (SIM 5), it decreased the number of multiple shoots and regeneration response. Further increase in BAP concentration to 3 mg/L (SIM 6) significantly decreased the regeneration response and number of multiple shoots produced. However, BAP concentration upto 2-3 mg/L have been reported to be effective for multiple shoot induction from explants prepared from nodes (Polisetty et al., 1997; Srivastava et al., 2012). In the present protocol, increase in the concentration of BAP more than 1 mg/L did not improved the frequency of regeneration and production of multiple shoots. When the explants were cultured high concentration of BAP, several negative effects of this cytokinin was observed like browning and decaying of the shoots. Several workers have reported that use of BAP alone is effective for the induction of multiple shoots (Brandt and Hess, 1994), while some observed that BAP with a low concentration of auxin (IBA or NAA) show a synergistic effect on shoot regeneration (Chakraborti et al., 2006; Subhadra et al., 1998). Cytokinin and auxin combination as well as combination of more than one cytokinin has been successfully used in different legumes (Chakraborti et al., 2006; Sharma and Amla, 1998). This difference in requirement of growth regulators was may be due to differences in endogenous levels of different growth regulators in the explants or relative sensitivity of the explants for different hormones (Chandra et al., 1993). The cotyledons have been reported to be very supportive for multiple shoot production from mature seed derived explants hence they were not removed from the explants (Singh et al., 2002).

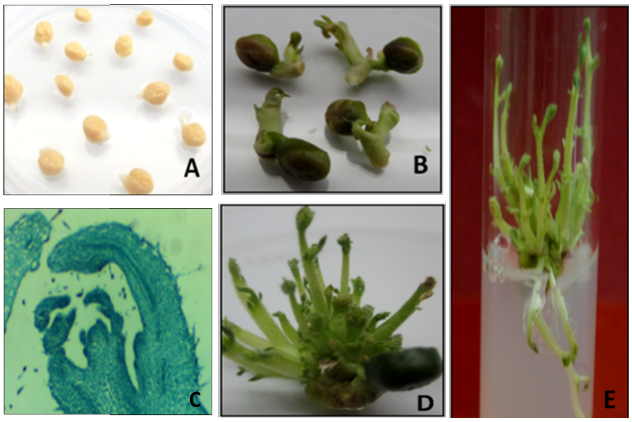


Fig1. A. Cotyledon with half embryonic axes explants **B.** Induction of shoots from explants, **C.** Longitudinal section of explants showing development of shoot apical meristem and leaf primordia D. Multiple shoots produced from the explants, **D**. Elongation of shoots in GA_3 containing medium

B. Shoot Elongation and Grafting

With the use of BAP and NAA, we obtained healthy multiple shoots, which synchronously increased in number. However, this combination did not promote the elongation of shoots. Hence to obtain properly elongated shoots multiple shoots produced were treated with 1 mg/L GA_3. Gibberellins, the most commonly used growth regulator for shoot elongation, was found effective for chickpea shoot elongation in the present study also. However, shoots elongated in high dose of GA_3 are often weak and show low survival rate after grafting. Shoots were grafted on rootstocks after 3-4 subcultures in elongation medium. Root stocks were prepared from 5-6 days germinated seedlings. In legumes, including chickpea, rooting has been found very difficult with different growth regulators by different workers. Inhibition of root formation has been observed in shoots regenerated on BAP containing medium (Polanco and Ruiz, 1997). Grafting when performed properly give sufficiently high number of regenerated plants. Successful grafting has been reported in chickpea by different scientists (Yadav and Singh, 2012; Krishnamurthy et al., 2000).

IV. CONCLUSION

Thus, in the present paper we have presented a simple and efficient regeneration system for chickpea cultivar C 235. We conclude that the use of cotyledon with half embryonic axes prepared from overnight soaked seeds and culture of these explants on 1 mg/L BAP and 0.05 mg/L NAA for four weeks give high number of multiple shoots. The shoots elongate properly in 1 mg/L GA3. Shoots were produced through direct organogenesis and on callus phase was observed in histological study. The development of new repeatable and reliable tissue culture protocols is very important for the improvement of breeding and genetic transformation studies. This regeneration system could also be used for *Agro bacterium* mediated genetic transformation of this important legume crop.

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