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Rapid in vitro plant regeneration from cotyledonary node explants in Cicer arietinum L

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***Abstract*— In the present study, we have developed a rapid and efficient multiple shoot induction protocol for chickpea, an important crop legume. Explants prepared from mature seeds germinated on 6-benzylaminopurine (BAP) supplemented medium, were cultured on MS medium fortified with different combinations of BAP and indole butyric acid (IBA) for multiple shoot induction. Preculture of seeds in BAP significantly enhanced the frequency of multiple shoot induction from the explants. Shoots were elongated in gibberellic acid (GA_3) containing medium and were grafted on root stocks prepared from the same cultivar of chickpea.**

***Keywords*— Benzylaminopurine; chickpea; *Cicer arietinum*; cotyledonary node explants**

I. INTRODUCTION

Chickpea is cultivated in more than 50 countries and ~75% of the chickpea production is in South Asia, and India is the largest chickpea producing country. The major constraints associated with chickpea production are susceptibility to insect pests, fungal diseases, and low tolerance to drought and temperature extremities. Production of pulses has lagged behind the highly increasing population growth rate, which has resulted into a reduction in per capita availability of pulses. In 2005, the per capita availability of pulses was 32 g/day while it was 69 g/day in 1961 [1-5]. Production of resistant chickpea varieties, against these stresses is limited through conventional breeding due to unavailability of sufficient level of resistance in the gene pool. An alternative strategy is to transfer of desired genes through genetic engineering from sources, which are not possible to introduce through conventional breeding. However, success of the gene transfer technologies largely depends on availability of a rapid, efficient and reliable *in vitro* plant regeneration protocol. In chickpea, several regeneration protocols have been reported either through direct shoot organogenesis [6-11] or indirectly through callus formation [12-15]. In legumes, cotyledonary nodes from mature seeds have been found very responsive for induction of multiple shoots [16-20].

Here we have described an efficient approach for high frequency multiple shoot induction from mature seed-derived explants, which may be further utilized for *Agrobacterium*- mediated genetic transformation of this important food legume crop.

II. MATERIALS AND METHODS

A. Plant Material

Seeds of chickpea (*Cicer arietinum* L.) cultivars DCP 92-3 were used for the study. Seeds were first washed in running tap water and kept in water having 2-3 drops of tween 20 for 15 minutes. Seeds were then sterilized with a quick rinse with 75% alcohol followed by treatment with 1% sodium hypochlorite for 10 min. Seeds were then washed thoroughly with sterile distilled water several times and soaked overnight. Prior to germination, seed coats were removed with the help of scalpel, for proper germination of the seeds.

B. Culture Media and Growth Conditions

MS salts [11] and B5 vitamins [8] were used in all culture media in the study. All working media were supplemented with 3% (w/v) of sucrose; pH was maintained at 5.8 and after adjusting the volume, solidified with 0.8% agar and autoclaved. Non-autoclavable plant growth regulators were sterilized by filtering through a 0.2 µm membrane and added to the media when the temperature had cooled to 50-60^{o}C. All cultures were kept in a culture room maintained at 23±1^{o} C with 16 h light/ 8h dark photoperiod. Light intensity was kept 120µmol$^{m-2\ s-1}$ from cool white fluorescent tubes.

C. Germination of Seeds and Explant preparation

After removing the seed coat, seeds were kept for germination on MS medium supplemented with 2 mg/L BAP for 10 days. Cotyledonary node explants were excised from 10 day old aseptically germinated seedlings and used as the experimental material. For explants preparation, all the apical and axillary buds and root tips were removed, the seedling is bifurcated and two explants were prepared. The explants were cultured on different combinations of BAP and IBA to establish the best medium for multiple shoot induction. All experiments were repeated thrice. Thirty seeds were cultured, about 20-25 seeds showed uniform germination and only they were used for further observation and data collection. The explants were scored after four weeks of culture for determining the percentages of seedlings showing shoot regeneration and the number of regenerated shoots from each cotyledonary node explant.

D. Shoot Induction from the Explants

Cotyledonary node explants were incubated in Petri dishes containing different shoot induction medium (SIM), to test their efficiency in producing multiple shoots from the explants, prepared from the seeds precultured with 2 mg/L BAP for 10 days. SIM contained MS medium supplemented with different combinations and concentrations of BAP and IBA (Table 1). Explants were transferred to fresh medium after every seven days. Best shoot induction medium was then used in all further experiments.

E. Elongation of Shoots and Grafting

Explants with multiple shoot buds were kept for elongation and were transferred to fresh medium every two weeks. For proper elongation of the multiple shoots, different media containing different concentrations of BAP and GA3 were tested. After elongation, shoots were grafted to the rootstocks in greenhouse. For preparation of rootstocks, seeds of same cultivar were germinated in autoclaved soil mix (peat, perlite and vermiculite in equal proportions) on small pots in the greenhouse. Rootstocks were prepared by removing the shoots from 5-7 days-old seedlings and by making a 3-4 mm perpendicular cut at the tip. The scion (regenerated shoot) was

prepared very carefully and inserted into a cut made in rootstock. Grafted shoot was covered with a polybag to maintain high humidity. The polybags were punctured after 7-8 days to acclimatize plants to low moisture conditions. After complete adaptation, polybags were removed.

TABLE I. COMBINATION AND CONCENTRATIONS OF PLANT GROWTH REGULATORS IN DIFFERENT SIM AND SEM MEDIA

Media	Plant Growth Regulators		
	BAP	***IBA***	***GA3***
SIM 1	1	0.05	-
SIM 2	2	0.05	-
SIM 3	3	0.05	-
SIM 4	1	0.1	-
SIM 5	2	0.1	-
SIM 6	3	0.1	-
SEM 1	-	-	0.5
SEM 2	-	-	1.0
SEM 3	0.5	-	0.5
SEM 4	0.5	-	1.0
SEM 5	1.0	-	0.5
SEM 6	1.0	-	1.0

III. RESULTS AND DISCUSSION

A. Effect of Seed Pretreatment

BAP promoted growth of the axillary meristems and hence its supplementation in pretreatment medium significantly increased the frequency of shoot induction from axillary meristem explants. In plant species which show strong apical dominance, removal or injury of terminal bud is necessary to stimulate the axillary buds to grow out into a shoot [3]. Positive effect of cytokinins, when used for pretreatment of seeds, has already been reported in several legumes [12, 24]. When cytokinins are exogenously provided in the medium, they enhance proliferation of meristematic cells and thus increase the formation of bud primordia increasing number of shoots originating [5]. BAP has also been found a very effective cytokinin for shoot induction in several plant species including chickpea [10, 15].

B. Effect of Plant growth Regulators on Multiple Shoot Induction

Six different combinations of BAP and IBA were tested for multiple shoot induction from cotyledonary node explants, prepared from seeds precultured in MS medium supplemented with 2 mg/L BAP for ten days. From the different BAP + IBA concentrations used, 2 mg/L BAP and 0.05 mg/L IBA (SIM 2) was proved as the most effective in producing highest number of multiple shoots. This combination produced 10.2 shoot per explant. Maximum regeneration response was observed in SIM 1 (1 mg/L BAP + 0.05 mg/L IBA), however, it was not significantly superior than regeneration response of SIM 2. Hence, SIM 2 was found as the best medium for multiple shoot induction from cotyledonary node explants. Lower regeneration response and number of multiple shoot was observed in SIM 3 which contain 3 mg/L BAP without any change in IBA concentration. Thus, any further increase in the concentration of BAP did not improve the frequency of regeneration and number of multiple shoots produced. Further, when the explants were cultured on medium containing high concentration of BAP (3 mg/L) some negative effects of this cytokinin was observed like browning of shoot tips and decaying of the shoots. Reduction in number of multiple shoots produced was observed in the SIMs, which contain higher IBA concentration.

Several scientists have used BAP alone for the induction of multiple shoots [4] , while some have reported that BAP with a low concentration of IBA or NAA have a synergistic effect on shoot regeneration [6, 21]. Combination of more than one cytokinin as well as cytokinin and auxin combination has been successfully employed in different legumes including chickpea [4, 6]. Sharma and Amla [17] observed that number of shoots produced per explant could be significantly increased by supplementing the media with TDZ and IBA in comparison to using TDZ alone. Differences in the requirements of growth regulators might be due to differences in endogenous levels of various growth hormones or relative sensitivity of them for different hormones [7]. Successful application of tissue culture technique often requires efficient production of multiple shoots. Due to continuous availability of cytokinin, explants develop axillary buds which could grow directly into shoots. During explant preparation, excision of apical and axillary buds and radicle tip promotes formation of high number of multiple shoots [15]. The cotyledons were not removed from the explants because they have are very supportive for multiple shoot production from mature seed derived explants [19].

TABLE II. EFFECT OF DIFFERENT SIM ON REGENERATION RESPONSE AND MULTIPLE SHOOT INDUCTION FROM THE EXPLANTS

SIM	Regeneration response (%)	Average number of shoots per explant*
1	68	6.1
2	64	10.2
3	48	5.8
4	65	4.2
5	52	4.5
6	42	3.8

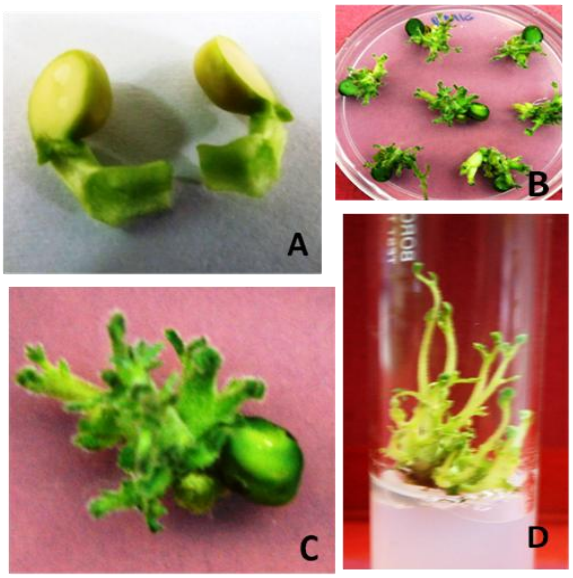
[a.] Data recorded after four weeks culture of the explants on SIM

C. *Shoot Elongation And Grafting*

Use of BAP and IBA led to the formation of healthy shoots in the present study, which synchronously increased in number. However, this combination failed to initiate the elongation of shoots. Multiple shoot buds produced were treated with low dose of GA_3 (1 mg/L) for further development and elongation. Gibberellins are the most commonly used growth regulator for elongation of shoots. In the present study also, GA_3 was found effective for chickpea shoot elongation.

For elongation of shoots, SEM 4 (0.5 mg/L BAP + 1 mg/L GA_3) was found as the best medium. Gibberellins promote elongation of shoots but shoots elongated in high dose of GA_3 are often weak. These shoots show low survival rate after grafting. Shoots elongated in SEM 4 were healthy and show high survival rate after grafting. Optimally elongated shoots were grafted on rootstocks after 20-25 days incubation in elongation medium. Root stocks prepared from 5-6 days germinated seedlings gives proper support to the graft. In chickpea and other legumes, rooting is very difficult with different growth regulators and takes long incubation. Significant inhibition of root formation has been observed in shoots regenerated on BAP containing medium for more than four weeks [14]. Grafting when performed with proper care give sufficiently high number of regenerated shoots. Successful grafting has been reported for various legumes including chickpea [1, 11, 23].

Fig. 1. Regeneration of Shoots from Cotyledonary node explants



A. Cotyledonary node explants B. Induction of shoots from explants, C. Multiple shoots produced from the explants, D. Longitudinal section of explants showing induction of shoots E. Elongation of shoots in GA_3 containing medium

TABLE III. EFFECT OF DIFFERENT SEM ON ELONGATION OF SHOOTS FROM COTYLEDONARY NODE EDXPLANTS

SEM	Mean shoot length (cm)*
1	1.8
2	2.4
3	1.9
4	3.4
5	2.1
6	2.7

[b.] Data recorded after four weeks culture of the explants on SEM

Thus, we have presented a simple, rapid and efficient regeneration system for chickpea. We recommend the use of cotyledonary node explants, prepared from seeds precultured on 2 mg/L BAP for 10 days and culture of these explants on 2 mg/L BAP and 0.05 mg/L IBA. This regeneration system could be efficiently used for *Agrobacterium* mediated genetic transformation of this important legume crop of the semi-arid tropics.

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