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Full Length Research Paper

In vitro micro-propagation of indigenous chick pea (*Cicer arietinum* L.) cultivars, KK-1 and Hassan-2K

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Multiple shoot induction and plantlets regeneration ability from nodal explants of two indigenous cultivars of *Cicer arietinum* was investigated on MS+B5 medium supplemented with different concentration of Benzyl amino purine (BAP). Data were taken on shoot and root initiation, numbers, length (cm) and percent shooting for both varieties. Shoot initiation was best achieved on lower concentration (3 and 5 µM) while high concentration of BAP delayed shoot initiation in explants of both cultivars. Maximum shoot numbers per plant were recorded on higher BAP concentration (7 µM) while further increase in concentration was sub-optimal. BAP 3 and 5 µM produced maximum shoot (88 and 89%) in both cultivars. The shoot regenerated was further multiplied by sub-culturing on fresh medium. For root induction the elongated plantlets were transferred to MS media supplemented with different concentrations of IBA and NAA. Early rooting, maximum root numbers and root length were observed on IBA 2.5 µM and IBA 2.5 µM +NAA 0.25 Mg/L respectively. In all, IBA 2.5 µM gave excellent response for root induction.

Key words: Micro propagation, chick pea, shooting culture, multiple shooting.

INTRODUCTION

The leguminosae family is second in size only to the Gramineae (Aykroyod and Doughty, 1964). In addition, legumes are the main, and at times, the only source of protein and essential amino acid for the inhabitants of developing countries where protein deficiency is common (Mayer, 1976).

In legumes, chick pea is one of the most important grain legumes used for human food and animal feed in developing countries and is a rich source of dietary protein (Singh, 1990). Chickpeas are a good source of zinc, folate, phosphorus, iron and certain water soluble vitamins. They are also very high in dietary fiber and thus are a healthy food source of carbohydrates for persons with insulin sensitivity or diabetes (Hulse, 1991). In Asia, India is the largest producer of chick pea. Pakistan ranks second in terms of acreage under its cultivation (Hassan and Khan, 1991).

The heavy demand created by the pressure of increasing population in the developing world requires a tremendous scientific effort to meet the requirements of life. Since the conventional techniques employed in crop improvement may not keep pace with the demands of the increasing population (3 person/s) and decreasing land resources, the importance of *in vitro* technologies in crop improvement has great relevance.

More recently, plant biotechnology and molecular biology has emerged as spectacular discipline of life science. It has offered unprecedented opportunities and promises for the development of human resource and economic benefits (Sukapinda, 1993; Philip and Gamborg, 2005). Although many of the economically important plants have been improved regarding yield and productivity through genetic transformation and other cellular techniques, however certain plant species including legumes have generally proved notoriously recalcitrant due to the lack of reliable *in vitro* regeneration system (Flick et al., 1983; Barna and Wakhlu, 1993). Micropropagation offers the potential to produce thousands or even millions of plants per annum. Application of tissue culture techniques for genetic upgradation of economically important plants has been reported

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(Scowcraft and Ryan, 1985).

Among legumes, meristematic tissue has been used to regenerate plants. A number of investigators (Gosal and Bajaj, 1979; Kartha et al., 1973) have studied the regeneration potential of shoot apical meristems of chick pea on solid agar MS nutrient medium supplemented with various concentrations of 6-Benzylaminopurine (BAP) and 1-Naphthylacetic acid α (NAA) alone or in combination (Gosal and Bajaj, 1979). Looking at the potential and promises of the tissue culture technology, efforts have been directed for implementing this technology to improve productivity by developing the disease resistance and high yield varieties in chick pea. There are very few reports on efficient regeneration system which is a prerequisite of cellular and genetic manipulation. Development of highly reproducible and efficient regeneration protocol in chick pea is still awaited. Thus the present study was conducted to establish a reproducible protocol for *in vitro* micro propagation of indigenous cultivars of chick pea.

The present study was performed to investigate the direct effect of various concentrations of BAP, IBA and NAA alone and in combinations on the growth and development of explants of two indigenous chick pea cultivars KK-1 and Hassan-2K. The growth parameters such as days to shoot initiations, shoots number per plant, shoot length (cm), percent shooting , days to root initiations, roots number per plant and root length (cm) of transplanted plants were studied.

MATERIALS AND METHODS

Explant source

Seedlings of two chick pea cultivars, Hassan 2K and KK-1, sown in soil in separate trays under green house conditions, were used to raise plantlets through *in vitro* micro-propagation. The basal MS (Murrashige and Skoog, 1962) media along with Gamborg B_5 vitamins were used.

Explant selection

Young chick pea nodal buds excised with great care from two different cultivars of chick pea (Hassan 2K) and (KK-1) were used as explants.

Explants sterilization and culturing

The cut explants of chick pea were washed with clean water for 30 min then put in a sterilized flask or universal bottle. The mercuric chloride ($HgCl_2$) at a concentration of 0.04 - 0.06% was used for surface sterilization of these explants. After 5 - 6 min shaking with $HgCl_2$ explants were rinsed 5 - 7 times with sterilized distilled water in a laminar flow cabinet for the removal of any traces of $HgCl_2$. Completely sterilized buds were used for culturing. Under sterilized condition of laminar flow cabinet these buds were cultured in sterilized media test tubes near the burner. A spirit lamp was used for the sterilization of forceps. The cultured test tubes of explants were kept in a growth chamber at 27°C and 65 - 70% humidity with a photoperiod of 16/8 h.

Culture medium

BAP 112.5 mg/100 ml water (5 mM BAP), IBA (Indole-3-butyric acid) 40 mg/100 ml water, NAA 50 mg /100 ml water were used as a stock solution.

BAP 1 ml l^{-1} (5 µM BAP), 0.6 ml l^{-1} BAP (3 µM BAP), 1.4 ml l^{-1} BAP (7 µM BAP) and 2 ml l^{-1} BAP (10 µM BAP) was added from their stock solution in MS media for induction of shoots. After the successful initiation of shoots using shoot induction growth regulators in MS media, plantlets were transferred to rooting media. IBA 1 ml l^{-1} (2 µM IBA), 1.25 ml l^{-1} IBA (2.5 µM IBA) and 1.50 ml l^{-1} IBA (3 µM IBA) or NAA 0.5 ml l^{-1} (0.25 mg/l) from their stock solution in MS media were used for root development.

Transplantation into soil

Upon complete shoot and root development in explants, the plantlets were transferred to soil and kept under controlled environment for several weeks with periodic watering of the culture. Finally these were transferred to a green house for further development.

RESULTS AND DISCUSSION

Effect of BAP on multiple shoot formation

The results of days to shoot initiation of chick pea explants cultured on MS+B5 media with growth regulators BAP (6-Benzyl-Amino-Purine) at different concentrations revealed that explants of KK-1 variety treated with control media and media containing 10 µM BAP took longer to initiate shooting (12.8 and 7.5 days) and explants cultured with 3 and 5 µM BAP, respectively required much shorter time to initiate shoot formation (3.1 and 3.6 days). Similarly, the explants of Hassan-2K variety on control and 10 µM BAP required 14.5 and 8.5 days, respectively, to initiate shoots and only 3.9 days when media contained 3 µM BAP (Table 1). The results indicate that the BAP level in the media is an important factor influencing the shoot induction in the explants of KK-1 and Hassan-2K varieties; these results are similar to those reported by Shagufta et al. (2007).

Maximum number of shoots per plant (6 and 5.8) were observed in the explants of KK-1 variety treated with 7 µM BAP and 10 µM BAP, whereas, minimum shoot number per plant (2.3 and 2.5) were observed in explants treated with 3 µM BAP and control, respectively. While in Hassan-2K variety maximum shoot number (4.1) were observed in explants cultured with 5 µM BAP and minimum number of shoots (1.8) with 3 µM BAP. Elke et al. (1994) has demonstrated that shoots number per plant increases with increasing BAP (6-Benzyl-Amino-Purine), Franklin et al. (1998) obtained maximum of 49 shoots on 3.0 mg/l BAP with seedling explants which is a combined cotyledonary node and shoot tip and only five shoots with cotyledonary node. Similar results were obtained by Polisetty et al. (1997) in chick pea. The results of shoot

Table 1. Effect of different BAP concentrations on multiple shoot formation from nodal explants of KK-1 and Hassan -2K.

Variety	BAP concentration (µM)	Days to shoot initiation (Mean±SE)	Shoot number (Mean±SE)	Shoot length (cm) (Mean±SE)	Percent shooting
KK-1	Control	12.1 ± 0.757a	2.5 ± 0.341c	3.56 ± 0.396c	57
	BAP 3	3.1 ± 0.314d	2.3 ± 0.300c	5.92 ± 0.183a	88
	BAP 5	3.6 ± 0.340d	4.7 ± 0.213b	5.14 ± 0.344b	80
	BAP 7	4.8 ± 0.326c	06 ± 0.258a	4.73 ± 0.207b	76
	BAP 10	7.5 ± 0.453b	5.8 ± 0.249a	3.66 ± 0.483c	65
Hassan -2K	Control	14.5 ± 0.860a	2.3 ± 0.335b	4.57 ± 0.412d	42
	BAP 3	3.9 ± 0.233d	1.8 ± 0.200c	6.59 ± 0.203a	65
	BAP 5	4.5 ± 0.372d	4.1 ± 0.179a	6.62 ± 0.178a	89
	BAP 7	5.6 ± 0.371c	4.1 ± 0.233a	5.73 ± 0.197b	83
	BAP 10	8.5 ± 0.543b	2.6 ± 0.427b	5.16 ± 0.188c	71

Data was collected after 25 days of culture. Each value is a mean of ten replicate with standard error (Mean±SE); a,b,c: Mean with same superscript are not significantly different from each other at 5% level by Duncan' new multiple range test.

Table 2. Effect of different concentrations and combinations of IBA and NAA on regenerated roots of two chick pea cultivars.

Variety	Auxins concentration	Days to root initiation (Mean±SE)	Root numbers (Mean±SE)	Root length (cm) (Mean±SE)
KK-1	IBA 2.0 µM	14.1 ± 0.277b	1.8 ± 0.233d	3.60 ± 0.529c
	IBA 2.5 µM	11.6 ± 0.163d	3.6 ± 0.305c	6.10 ± 0.224a
	IBA 3.0 µM	15.2 ± 0.200a	4.3 ± 0.221b	4.87 ± 0.362b
	NAA 0.25 Mg/L	14.5 ± 0.166b	3.2 ± 0.416c	2.58 ± 0.303d
	IBA 2.5 µM+ NAA 0.25 Mg/L	13.0 ± 0.333c	5.10 ± 0.326a	5.96 ± 0.185a
Hassan -2K	IBA 2.0 µM	14.8 ± 0.326b	1.9 ± 0.241d	2.64 ± 0.434d
	IBA 2.5 µM	13.1 ± 0.277c	4.8 ± 0.260b	4.47 ± 0.510b
	IBA 3.0 µM	14.5 ± 0.372b	3.5 ± 0.166c	4.87 ± 0.616b
	NAA 0.25 Mg/L	15.5 ± 0.341a	5.2 ± 0.371b	3.53 ± 0.302c
	IBA 2.5 µM+ NAA 0.25 Mg/L	13.3 ± 0.495c	6.3 ± 0.221a	6.36 ± 0.176a

Data was collected after 25 days of culture. Each value is a mean of ten replicate with standard error (Mean±SE); a,b,c: Mean with same superscript are not significantly different from each other at 5% level by Duncan' new multiple range test.

length showed that while maximum shoot length of 5.92 and 6.62 cm were obtained in KK-1 and Hassan-2K cultivars respectively at 3 - 5 µM BAP, further increases in the BAP concentration were inhibitory to shoot length in both cultivars. Shagufta et al. (2007) also reported that after an initial stimulation with BAP, the shoot length decreases with increasing BAP concentration. Similar results were obtained in chick pea by Altaf and Ahmed (1986) and Rao and Chopra (1989). The data presented in (Table 1) showed that in KK-1, BAP at 3 µM and 5 µM levels produced shoots in most explants (88 and 80%) and at 10 µM BAP produced shoots in considerably fewer explants (65%). In Hassan 2K, a somewhat higher range of 5 - 7 µM BAP produced shoots in most explants (89 and 83%), respectively. Singh et al. (2002) has also reported a similar response to BAP in multiple shooting and percent shooting.

Effect of auxins on root regeneration

The root initiation varied significantly with various concentrations of rooting hormones. The mean value of KK-1 variety presented in (Table 2) showed that maximum days to root initiation (15.2 and 14.5 days) was observed in explants treated with 3 µM IBA and 0.25 mg/l NAA, whereas, minimum days to rooting (11.6 and 13 days) was recorded in explants with 2.5 µM IBA and 0.25 mg/l NAA in combination with 2.5 µM IBA. In Hassan 2K, minimum days to rooting (13.1 and 13.3 days) was also observed in explants treated with 2.5 µM IBA and 2.5 µM IBA + 0.25 mg/l NAA (Table 2) and maximum days to rooting (15.5) was observed in explants cultured in 0.25 mg/l NAA. It is inferred from the given results that IBA is an important factor in root initiation, and its varying concentrations and combinations significantly effect the

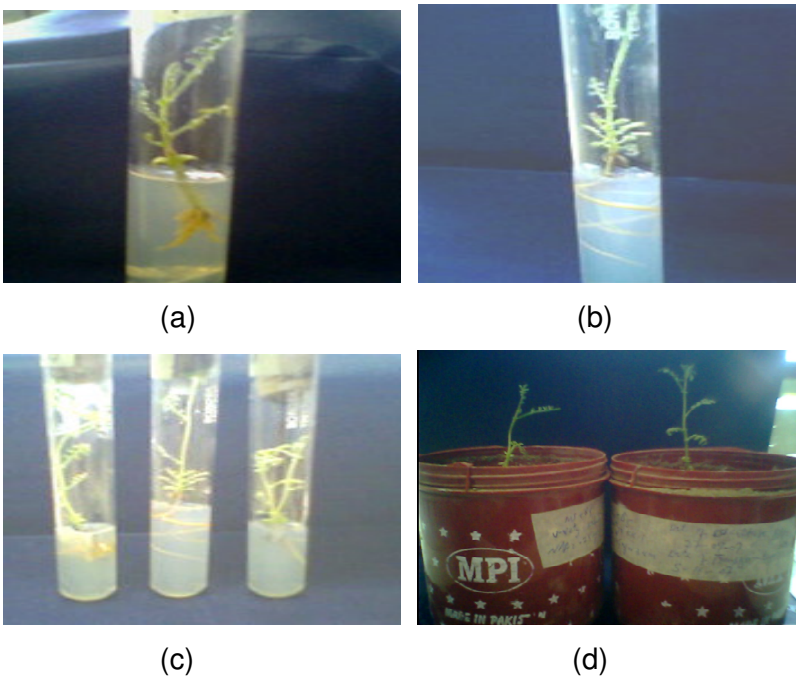

Figure 1. a) Multiple shooting and root initiation in KK-1 cultured on M.S medium. b) Shoot formation and regenerated roots in Hassan-2K cultured on M.S + IBA 2.5µM. c) Shoot proliferation and elongation of roots of KK-1 after 20 days of culture on rooting medium containing IBA. d) *In vitro* regenerated plantlets of both KK-1 and Hassan -2K transferred to soil under controlled environment.

root regeneration in chick pea cultivars. Islam et al. (2005), obtained similar results. For rooting of *in vitro* raised shoots, 0.1% IBA was used in soybean (Buising et al., 1994) and IAA in common bean (Kartha et al., 1981). It was reported that half strength MS medium induced maximum rooting in cowpea (Kulothungan, 1997). The data presented on the number of roots per plant show a maximum number of roots (5.10) and average root numbers (4.3 and 3.6) at IBA 2.5 µM IBA + 0.25 mg/l NAA, 3.0 µM IBA and 2.5 µM IBA, respectively, while a minimum number of roots (1.8) at 2.0 µM IBA in explants of KK-1; and in Hassan-2K also the similar results were obtained for maximum and minimum root numbers. Islam et al., 2005 and Sujatha et al., 2007 have observed similar hormonal interaction between IBA and NAA effect in rooting response of chick pea varieties. Data pertaining to root length (Table 2) show that maximum root length (6.10 cm) and minimum root length (2.58 cm) were recorded in explants of KK-1 variety treated with 2.5 µM IBA and 0.25 mg/l NAA, respectively. Similarly, maximum root length (6.36 cm) and minimum root length (2.64 cm) were recorded in explants of Hassan-2K variety cultured in 2.5 µM IBA + 0.25 mg/l NAA and 2.0 µM IBA, respectively. It is inferred from the present results that different concentrations and combinations of IBA and NAA significantly affect root length in chick pea cultivars and root length response depend both on rooting hormones and genotypes of chick pea. Similar results were obtained by Elke et al. (1994) and Islam et al. (2005).

Conclusion

An efficient protocol is developed for raising plantlets and clonal propagation of both indigenous cultivars of chick pea KK-1 and Hassan-2K as they appear to be replaced by the imported cultivars. The protocol described in the present study is reproducible and can be used in future for further developments of the crop.

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