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ISSN 1028-8880

Pakistan Journal of Biological Sciences

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Pakistan Journal of Biological Sciences 11 (7): 1009-1014, 2008
ISSN 1028-8880

Optimizing Regeneration Condition in Chickpea (*Cicer arietinum* L.)

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Abstract: In this study, multiple shoot induction and whole plant regeneration from decapitated embryo axes of three chick peal genotypes including MCC252, MCC283 and MCC505 were evaluated on modified Murashige and Skoog's medium (MMS) which, its vitamins were replaced by vitamins of B5 medium, supplemented with varied concentration of thidiazuron (0.1, 0.2 mg L^{-1}) or 6-benzylaminopurin (1, 2 mg L^{-1}) or zeatin (1, 2 mg L^{-1}) treatments. BAP was found to be the most effective cytokinin in normal multiple shoot induction. Shoots were elongated on growth regulator-free medium and then rooted on two media containing 1/4 MMS salts and B5 vitamins + 3% sucrose + 0.8% agar with indol-3-butyric acid (0.4 or 1 mg L^{-1}). The highest rooting frequency resulted in a medium including 0.4 mg L^{-1} IBA. It was found that different shoot induction media also positively affected rooting, where a medium with 2 mg L^{-1} BAP in MCC252/MCC505 and a medium with 2 mg L^{-1} zeatin in MCC283 were the best media in shoot induction that produced high frequency, thick spread roots. Plantlets were preliminary acclimatized in liquid medium (1/4 MMS salts and B5 vitamins + 3% sucrose + 0.4 mg L^{-1} IBA) for 7 to 14 days, then transferred to pots filled by cocopit: perlite (1:1) and kept in a growth chamber until their shoots and roots were well developed. This resulted in more than 70% survival rate.

Key words: Multiple shoot, acclimatization, 6-benzylaminopurin, thidiazuron, indol-3-butyric acid

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important grain legume in West Asia, Northeast Africa, South Europe, South-Central America and Australia (FAOSTAT Report, 2007). This plant is a good source of carbohydrate (48.2-67.6%), protein (12.4-31.5%), starch (41-50%), fat (6%) and nutritionally important minerals (Ignacimuthu and Prakash, 2006) that are employed in human nutrition and feed for livestock, also as a factor of biological nitrogen fixation in soil. However, the production of this crop has remained low because of its susceptibility to biotic stresses like Ascochyta blight, Fusarium and insect pests (Heliothis), also abiotic stresses such as saltiness, drought and cold (Kiran *et al*., 2005). Conventional breeding methods for stress resistance are often cost and time consuming, limited to lack of proper gene in gene pool and none crossing inter-species. Therefore, using tissue culture and genetic engineering are unavoidable for increasing yield, resistance to stresses and high quality products (Bajaj, 1990). Transgenic chickpea has already been produced by *Agrobacterium tumefactions* mediated with *nptII*, *GUS*, *bar*, α-amylase, *Cry1Ac* and *Aspartat kinase* genes (Krishnamurthy *et al*., 2000; Polowick *et al*., 2004; Sanyal *et al*., 2005), but the transformation frequency was low due to chickpea recalcitrant to regeneration (Senthil *et al*., 2004). Totally chickpea regeneration has been investigated was four methods namely organogenesis without callus phase (Srivastava *et al*., 2001; Batra *et al*., 2002; Singh *et al*., 2002), somatic embryogenesis without callus phase (Murthy *et al*., 1996; Kiran *et al*., 2005), organogenesis from callus (Surya *et al*., 1992; Barna and Wakhlu, 1994; Aminuddin and Singh, 1995; Huda *et al*., 1999) and somatic embryogenesis from callus (Barna and Wakhlu, 1993; Eapen and George, 1994; Sagare *et al*., 1993; Kumar *et al*., 1994, 1995).

The Organogenesis without callus phase using multiple shoot induction has been the most successful.

The reported regenerated protocols are not commonly acceptably repeatable because of the dependency of complete regeneration to variety of factors such as genotype, explants, growth regulator and physical factors like temperature, humidity, photoperiod and irradiance. In addition, root induction on the formed shoots and acclimatization of plantlets in open environment were not highly successful; therefore, the current research has been undertake to optimize and

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increase regeneration under *in vitro* conditions using a multiple shoot method. Rooting and acclimatization of plantlets in an open environment were also investigated.

MATERIALS AND METHODS

Plant materials: Seeds of three genotypes of chickpea including MCC252, MCC253 and MCC505 were obtained from Ferdowsi University of Mashhad seed bank. Seeds were rinsed for 30 sec in 70% ethanol and then washed with sterilized distilled water, treated with 2% hypochlorite sodium for 15 min, rinsed 3 times for 10 min with sterilized distilled water in order to ease the dissecting embryo axes (EAs) and removal of shoot and root apices of EAs. Sterilized seeds were cultured on modified Murashige and Skoog's medium (MMS), whose vitamins were replaced by vitamins of B5 medium (Gamborg *et al.*, 1968) for 72 h.

Induction multiple shoots: Decapitated EAs were cultured in different MMS media which were supplemented by TDZ (0.1, 0.2 mg L^{-1}), BAP (1, 2 mg L^{-1}), Zeatin (1, 2 mg L^{-1}) + 3% sucrose + 0.8% Agar, pH = 5.8 separately as Growth Regulators (GR) treatments named MT_1, MT_2, MB_1, MB_2, MZ_1 and MZ_2 hereafter. These cultures were incubated under 16 h photoperiod and 30 µmol m^{-1} sec^{-1} irradiance.

The shoots were formed from cut shoot apex and cotyledon node of explants after 7 days and were then sub-cultured on a free-GR medium in 10 cm Petri dishes. In order to reduce brownish phenomena due to phenolic compounds, sub-culture was performed once every 10 days instead of 14 days.

In the third subculture, for elongation and increase in numbers, the formed shoots were dissected out and transferred with original explants in a new medium. The number of regenerated shoots was investigated after 10, 14 and 23 days (Fig. 1).

Rooting: Regenerated shoots were transferred in 1/4 MMS + 3% sucrose + 0.8% Agar which were supplemented with IBA (0.4, 1 mg L^{-1} named MI_1 and MI_2 treatments), pH = 5.8 in 10 cm Petri dishes 10 days after the third subculture. These cultures were incubated under 16 h photoperiod and 30 µmol m^{-1} sec^{-1} irradiance. Plantlets were investigated 10 and 14 days after culture (Fig. 1).

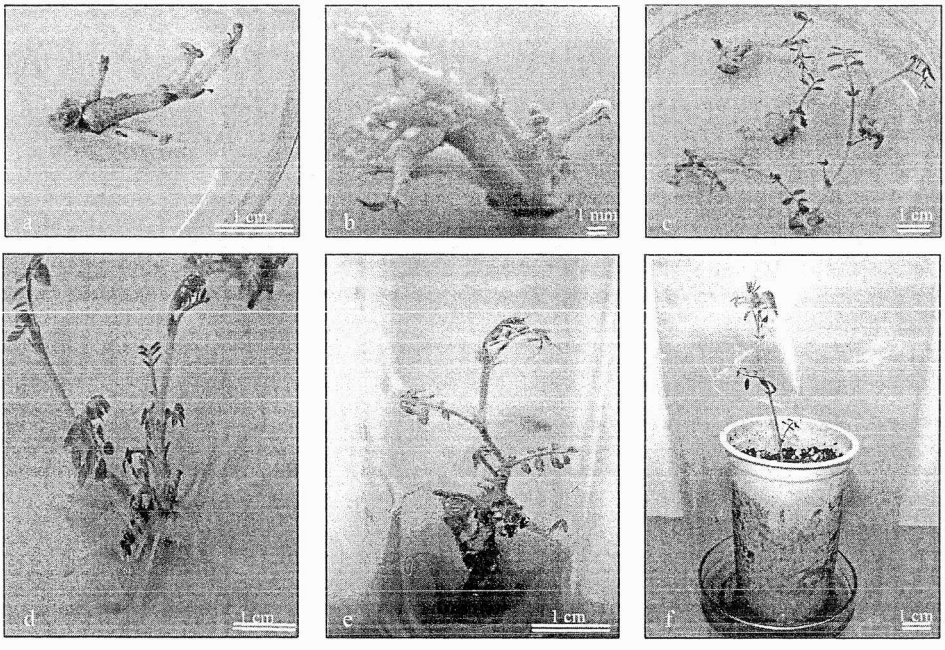

Fig. 1: Multiple shoot induction, rooting and acclimatization of chickpea. (a) shoot induction cotyledon node and cut shoot apiece on MMS + 2 mg L^{-1} BAP, (b) increasing of shoot numbers after sub-cultures on GR-free MMS, (c) elongation of dissected out shoots from original explants on GR-free MMS, (d) produced spread and thick roots on 1/4 MMS + 0.4 mg L^{-1} IBA, (e) plantlets in liquid medium containing 1/4 MMS + 0.4 mg L^{-1} IBA on Paper Bridge and (f) plantlets to small pots containing perlite: cocopit (1:1)

Acclimatization: Plantlets were transferred and placed in a liquid medium containing 1/4 MMS + 3% sucrose + 0.4 mg L^{-1} IBA on Paper Bridge for 7 to 14 days. Then cotton plugs of culture tubes were loosened and removed 2 to 3 days later and plantlets were transferred to small pots, which contained perlite: cocopit (1:1) and put in to a growth chamber. In order to protect humidity and better establishment, pots were covered by polythene bags and irrigated by distilled water.

A solution includes 1/4 MS salts was prepared to nurture plants. A hydroponics culture system was used as a second method to acclimatization. In this system, plantlets with distributed shoots and roots were directly put in a Hoagland nutritious solution and covered by transparent glass or polythene bag (Fig. 1).

Statistical analysis: Seven replicates for each treatment were considered. Replicated data from all experiments were statistically analyzed using a completely randomized design. Mean values were evaluated at a $p<0.01$ level of significance using Duncan's multiple-range test. Arcsine transformation was applied to convert rooting data to normal distribution.

RESULTS AND DISCUSSION

Multiple shoot induction: In all three genotypes on six GR treatments, shoots were completely produced. Genotypes, plant growth regulators and days after culture affected the number of induced shoots in each explant (Fig. 2). The Different responses of the genotypes to shoot regeneration have also been reported in other studies (Sheila *et al.*, 1991; Polisetty *et al.*, 1997; Singh *et al.*, 1997, 2002).

The number of formed shoots in 10, 14 and 23 days after culture showed that shoot generation has an ascending trend during growth time. In this study, the maximum number of shoots were obtained from MCC505 in MT_2 (14.35) after 23 days. TDZ with varied concentration from 0.1 to 11 mg L^{-1} as an efficient cytokinin has been used in multiple shoot induction (Malik and Saxena, 1992; Murthy *et al.*, 1996; Sharma and Alma, 1998) have gained high frequency shoots in MS containing 0.2 mg L^{-1} TDZ with an average highest number in genotype ICC4951 (9.7 shoots per explants) and lowest in ICC11531 (3.8 shoots per explants). In our research the number of induced shoots in each explant on 0.2 mg L^{-1} TDZ in all genotypes was more than these reported above. Nevertheless, these shoots were small, compressed and abnormal which caused problems in root induction. For the MCC505 genotype in MB_1 and MB_2, shoots not only were induced

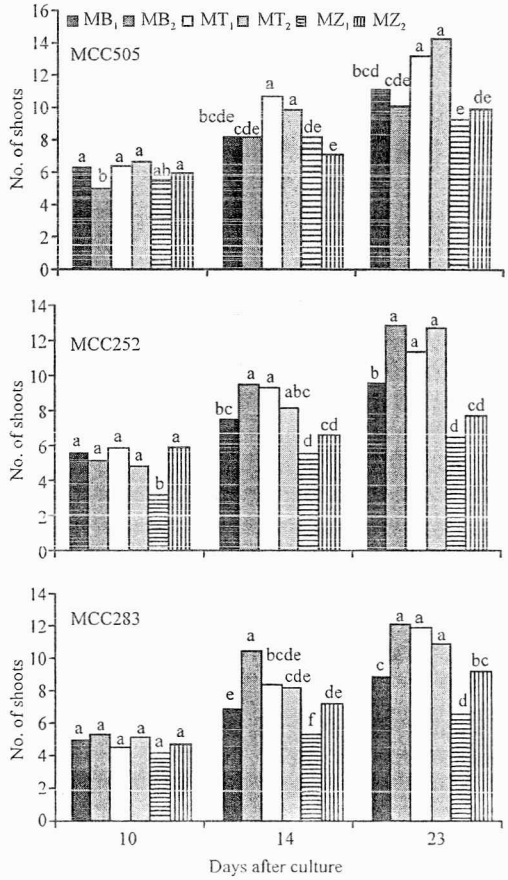

Fig. 2: Response of genotypes to shoot regeneration in different media over time. Bars indicated by the same letters are not significantly different at $p<0.01$ (Duncan's multiple-rang test)

in a sufficient number, but also their lengths were long enough. In MZ_1 and MZ_2, the number of induced shoots were lower than the former four media but with normal appearance (average by 3 cm).

MCC252 and MCC283 genotypes produced maximum number of shoots in MB_2 (12.75), (12.17) and minimum number in MZ_1 (6.42), (6.53), respectively (Fig. 2).

Finally, results of this research showed that in spite of the different reactions of genotypes in six media, BAP was the best GR. TDZ is an expensive GR that is not economic and produced abnormal shoots, so BAP could be used as efficient GR with economic cost in multiple shoot induction. Polisetty *et al.* (1997) also reported BAP efficiency in multiple shoot induction. They have introduced 7.5 µmol (1.68 mg L^{-1}) and 2.5 µmol (0.56 mg L^{-1}) concentration of BAP as a proper GR. In many reports, 2 mg L^{-1} BAP only or composed with an auxin, was the best GR in shoot induction (Sheila *et al.*,

1991; Polisetty *et al.*, 1997; Singh *et al.*, 1997; Sharma and Alma, 1998; Chaturvedi and Chand, 2001; Shalini *et al.*, 2001).

Rooting: The highest percent of rooting in all genotypes was obtained from 0.4 mg L^{-1} IBA: 65% in MCC252, 54% in MCC283 and 53% in MCC505 (Fig. 3). Singh *et al.* (2002) reported that 0.5 mg L^{-1} and higher IBA concentrations were not favorable due to callus induction in the base of shoots, which caused the production of low amount and weak roots. MCC505 had higher rooting (49%) in MI_2 and then MCC252 (43%) and MCC283 (29%). It should be mentioned that MCC505 is only genotype with a positive response in MI_2, which showed no significant difference from the MI_1 treatment results.

In this study, genotypes showed different responses to rooting which were related to the shoot induction media (Fig. 4). Polisetty *et al.* (1997) have reported that regenerated shoots in 12 μmol BAP (2.7 mg L^{-1}) produced more roots than other BAP concentration. Fernandez-Romero *et al.* (1998) also showed that the increase of BAP caused 35% reduction of rooting. Even the duration of culture in medium containing GR, could affect rooting. Singh *et al.* (2002) have shown that small and compressed shoots will be generated from explants when settled down in medium with 0.2 mg L^{-1} TDZ more than 7 days, which caused problems in shoot elongation and root induction.

In the MCC252 genotype, higher and lower frequency rooting was obtained from induced shoots on MZ_2 and MT_2 78 and 22%, respectively. Rooting percentage in induced shoots from other media were 68% (MB_2), 57% (MB_1), 62% (MZ_1) and 37% (MT_1). In the MCC283 genotype, induced shoots on MZ_2 had a maximum rooting amount (85%) and minimum amount on MB_1 (17%). Rooting percentage of induced shoots in other media were 50% (MZ_1), 37% (MB_2), 32% (MT_1) and 24% (MT_2). In the MCC505 genotype higher and lower frequency rooting was obtained from induced shoots on MB_2 (84%) and MT_2 (9%) and other media were 75% (MZ_1), 72% (MZ_2), 45% (MB_1) and 25% (MT_1) (Fig. 4).

In multiple shoot induction, MB_1 for MCC505 and MB_2 for MCC252/MCC283 were the best media, but these induced shoots did not produce high frequency rooting. In MCC252, rooting of induced shoots on MZ_2 was not significantly different from MB_2 (Fig. 4), therefore in MCC252, MB_2 had optimal results in shoot regeneration, which produced proper roots. Nevertheless, in MCC283, the rooting of induced shoots on MZ_2 and MB_2 showed significant difference, so in this genotype; MZ_2 was a suitable medium for shoot regeneration.

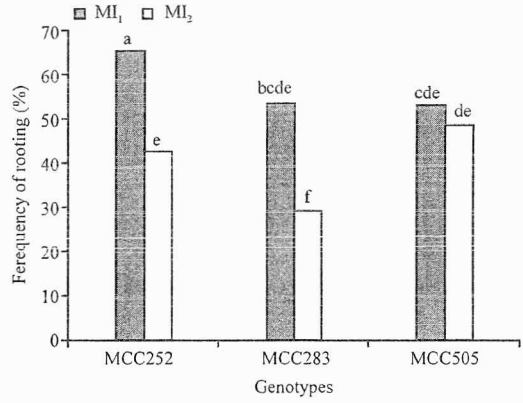

Fig. 3: Response of genotypes to the rooting in medium containing IBA. Bars indicated by the same letters are not significantly different at $p<0.01$ (Duncan's multiple-rang test)

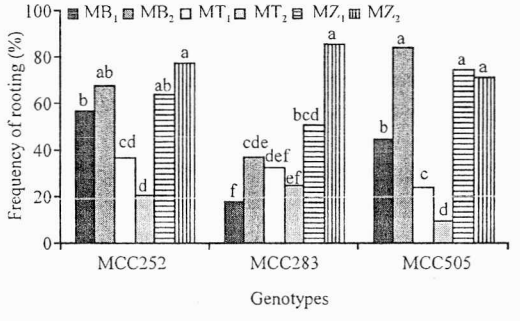

Fig. 4: The effect of shoot induction media on rooting. Bars indicated by the same letters are not significantly different at $p<0.01$ (Duncan's multiple-rang test)

In MCC505, shoot regeneration results were not significantly different on MB_1 and MB_2, so MB_2 was considered the best medium, which produced proper roots. Almost in all of the three genotypes, lower rooting has observed on MT_1 and MT_2 that confirmed difficult rooting due to small, compressed and abnormal induced shoots.

In this study, shoots produced thin and long roots on MMS GR-free medium in sequential subcultures. These roots were transferred to rooting medium that produced thick and spread roots. Singh *et al.* (2002) reported GR-free as a proper medium in rooting and the adding more than 1 mg L^{-1} IBA would suppress rooting. However, Islam and Riazuddin (1993) investigated that roots were produced with delay, lower rooting frequency (24%) and number of roots/shoots (3.5) in free-hormone medium. They also showed that formed roots were longer than other media, but were thin and weak.

We showed that the rooting frequency significantly increased during time (increase from 41% after 10 days to 55% after 14 days).

Effect of temperatures (18 and 24°C) on rooting has been investigated by Singh *et al.* (2002). Their results showed that rooting frequency per shoot on 18°C was significant higher than 24°C. We suggest that if environmental conditions of growth chamber are similar to natural growth of chickpea, regeneration could be better; however, more researches is necessary to obtain the optimal temperature. In this research, nitrogen and osmotic potential was decreased using 1/4 MMS. This resulted improvement root regeneration. The same results have been reported by Islam and Riazuddin (1993), Sharma and Alma (1998), Chaturvedi and Chand (2001) and Singh *et al.* (2002).

Acclimatization: Plantlets, which were transferred to Hoagland nutritional solution, remained green for 7 days, gradually died, whereas shoot and root systems were well developed when plantlets were transferred in liquid medium on Paper Bridge after 7 days to 14 days and finally transferred to pots. These plantlets were properly acclimatized and finally 70% survived after three weeks. Two earlier reports using a similar method showed noticeable increase of survival frequency (Polisetty *et al.*, 1996; Singh *et al.*, 2002). In several studies, plantlets were transferred to pots directly and obtained different results. Sheila *et al.* (1991) had reported that plantlets were acclimated and even matured, whereas Huda *et al.* (2003) reported 35% survival and other research showed that plantlets remained green for 10-12 days, but did not get established in the soil and eventually died (Chaturvedi and Chand, 2001).

CONCLUSION

In spite of different response of interested genotypes of chickpea on media include variety of GR treatments, BAP was the most proper hormone for multiple shoot induction.

Low concentration of IBA (0.4 mg L^{-1}) and decreasing salts using 1/4 MMS resulted proper and favorite rooting. In addition, different shoot induction media showed significant affection on this process; so that the medium contains 2 mg L^{-1} BAP was the best medium for shoot induction in MCC505 and MCC252 genotypes which these shoots produced the highest frequency of roots. Nevertheless, in MCC283 genotype, shoots that was induced on medium with 2 mg L^{-1} zeatin have the highest rooting.

Initial adopting of plantlets on liquid medium during 7 to 14 days and then transferring to pots was the best acclimatization method.

In order to develop regeneration protocols with reasonable irritation and success in gene transferring projects, investigating morphological and agricultural characters of chickpea under *in vitro* with field conditions could be efficient. Also using growth chamber with humidity control system and temperature decreasing during plantlets acclimatization will be produced proper results.

Probable genetic changes on regenerated plantlets in order to finding and controlling of undesired factors in polyploidy phenomenon should be investigated.

ACKNOWLEDGMENTS

The authors are grateful to Department of Biotechnology and Plant Breeding for funding. We tank Plant Science Research Center and Biotechnology-Tissue Culture lab of Agriculture Faculty for consistent support during the course of this investigation.

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