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INDUCTION AND REGENERATION OF HYPOCOTYL DERIVED CALLI IN HOT CHILLI (*CAPSICUM FRUTESCENS* L.) VARIETIES

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Abstract

An efficient and reliable *In vitro* protocol was established for induction and regeneration of calli in varieties of hot chilli (*Capsicum frutescens* L.) var. Nepali and NARC-IV. Early initiation and induction with sustainable calli growth in both varieties was achieved on Murashige & Skoog medium supplemented with 1.5 mg l^{-1} of 2,4-D. Better performance in both varieties regarding shoot initiation, regeneration rate (%) and number of lateral shoots per regenerants were achieved on medium containing 3.0 mg l^{-1} BA. Rooting was achieved on half strength MS basal medium containing 1.0 mg l^{-1} IBA. Plantlets exhibiting normal morphology with extensive rooting were acclimatized in greenhouse.

Introduction

Chilli is an economically important vegetable crop belonging to the family Solanaceae. Seventy percent of this crop is produced in Asia (Venkataiah *et al*., 2003). In Pakistan, chilli is cultivated on a large scale covering an area of about 48.7 thousand hectares with an annual production of 90.5 thousand tonnes and average yield of 1.9 tonnes per hectare (Anon., 2005). The popularity and demand for chilli are providing a boost to the chilli industry but its production is constrained by vulnerability of the high yielding varieties and hybrids to a multitude of pathogens (fungus, virus, bacteria and nematodes) restricting their potential yield (Venkataiah *et al*., 2006). In Pakistan, chillies have a higher incidence of bacterial wilt (*Ralstonia solanacearum*) ranging from 0.5% to 25% in field and up to 40% in tunnel cultivation (Burney, 1997). Globally, the most important aspect of chilli breeding is to incorporate resistance to viral, fungal, bacterial diseases and nematode infestations, while retaining high yield capacity (Venkataiah *et al*., 2003). Plant genetic transformation has become an important tool for functional genomics and an adjunct to conventional breeding programmes. Although excellent progress has been made in obtaining transgenic plant from Solanaceae family, but chilli has lagged behind, most likely due to formation of ill defined buds or shoot-like structures either resisting elongation or producing rosettes of distorted leaves which generally do not produce normal shoots (Ochoa-Alejo & Ramirez-Malagon, 2001). In the present study, a reliable and reproducible method for *In vitro* regeneration of Nepali and NARC-IV varieties of hot chilli (*Capsicum frutescens* L.) has been established for rapid multiplication of normal fertile plants using different concentrations of 2,4-D and BA.

Materials and Methods

The certified seeds of hot chilli (*Capsicum frutescens* L.) varieties Nepali and NARC-IV were imbibed in sterile distilled water for 24 hours and then surface sterilized with different concentrations (5, 10 and 15 % v/v) of Sodium hypochlorite (Commercial

Clorox, NaOCl, 5.25%) for 10 minutes. This was followed by washing with sterilized distilled water to avoid the toxic effect of the Sodium hypochlorite. The sterilized seeds were germinated aseptically on Murashige & Skoog, (1962) basal medium (MS macro, micro elements, vitamins, 30 g l^{-1} sucrose and 6 g l^{-1} agar). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 15 minutes. Seeds which germinated in dark at 26±1°C after 1 week were shifted to light condition of 16 hours photoperiod with light intensity of 1600 lux. Observation on mortality, infection and survival percentage of cultures were recorded 2-3 weeks after the inoculation.

Explants consisting of hypocotyl 0.5-1.0 cm in length were cultured on callus induction media (CIM) based on MS medium supplemented with various concentrations of 2,4-D (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg l^{-1}) in combination with Kinetin (0.5 mg l^{-1}). Explants were cultured in 25 ml test tubes containing about 8 ml of culture medium. Data was recorded on callus initiation period, callus induction and callus growth. Shoot regeneration was tested on regeneration media (RM) based on MS medium (macro, micro elements, vitamins plus 30 g l^{-1} sucrose and 6 g l^{-1} agar) supplemented with various concentrations of BA (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mg l^{-1}) in combination with IAA (1.0 mg l^{-1}). Observations were recorded on initiation period, regeneration rate and number of lateral shoots per regenerants. Rooting of regenerants was achieved on rooting media consisting of half strength MS (macro and micro elements), vitamins, 15 g l^{-1} sucrose and 6 g l^{-1} agar with various concentrations of IBA (0.5, 1.0, 1.5, 2.0 and 2.5 mg l^{-1}). Initiation period and number of shoots with roots was recorded as rooting parameters after 7 days of inoculation. Treatments for each experiment were replicated thrice and arranged according to Completely Randomized Design (CRD). All the collected data were subjected to Analysis of Variance (ANOVA) followed by Duncan's Multiple Range (DMR) test for separation of means.

Results and Discussion

Effect of NaOCl on disinfestation and survival percentage of seeds: From the data presented in Table 1, it is evident that 10% NaOCl solution significantly increased the survival percentage of Nepali (66.0%) and NARC-IV (58.0%). Higher concentration had an inhibitory effect which resulted in mortality of seeds in both varieties. Low concentration (T_1) of NaOCl solution failed to provide effective control of contamination in Nepali (67.0%) and NARC-IV (72.0%). For the establishment of *In vitro* culture of any plant species, it is of prime importance to find out the safe sterilization agent that can remove the fungus and bacteria from the explant tissues (Ahmad *et al*., 2003). Increasing concentration of NaOCl has a tendency to reduce contamination but leads to increase mortality of seeds with reduced survival percentage. Similarly, at low concentration of NaOCl mortality is reduced but does not bring about the desired control of contamination. Thus, it can be concluded from the results that T_2 (10% NaOCl) was the best treatment for effective control of contamination in both varieties which resulted into reduced seed mortality with increased survival percentage.

Table 1. Effect of NaOCl on disinfestation and survival percentage of Nepali and NARC-IV seeds.

	NaOCl solution (% v/v)	Infection (%)		Mortality (%)		Survival (%)	
		Nepali	NARC-IV	Nepali	NARC-IV	Nepali	NARC-IV
T_1	5	67	72	4.86	5.55	33.00	28.00
T_2	10	29	34	5.53	9.02	66.00	58.00
T_3	15	17	19	16.16	18.75	29.00	20.00

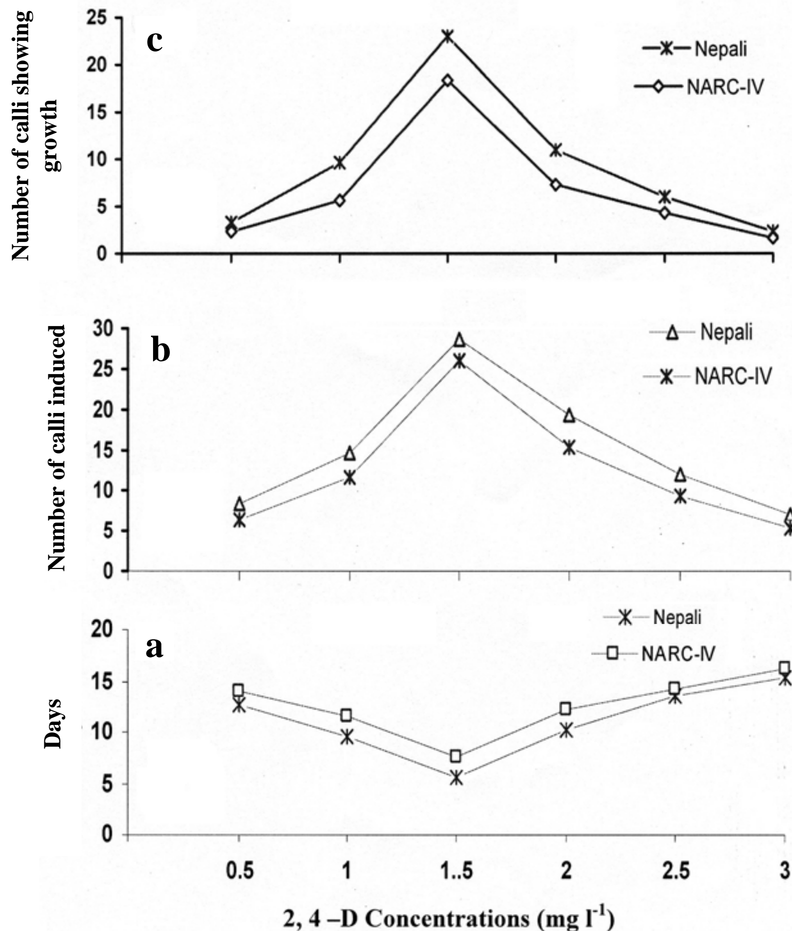

Fig. 1. Effect of different 2, 4-D concentrations on the initiation (a), induction (b) and growth (c) of callus in chilli varieties: Nepali and NARC-IV.

Callus induction

Effect of different 2,4-D concentrations on callus initiation period (days) of chilli varieties: Nepali and NARC-IV: Fig. 1a illustrates the time required by both varieties to initiate calli when supplemented with different 2,4-D concentrations. Significant response was exhibited by both varieties when treated with 1.5 mg l^{-1} 2,4-D requiring minimum days (6-8 days) for callus initiation. Time required for callus initiation was drastically increased (13-16 days) in both varieties at sub-optimum (<1.5 mg l^{-1} 2,4-D) and supra-optimal (>2.0 mg l^{-1} 2,4-D) concentrations than the optimum. These results indicate that 1.5 mg l^{-1} 2,4-D was the most suitable treatment for callus initiation. This might be linked with the availability of auxin in optimum concentration for activation of expansin enzyme for explant cell wall loosening and extensibility leading to increase in initial growth of explant. Preece & Read (2005) found that optimum concentration of auxin caused

acidification of explant cell wall by activation of expansin enzyme which brought about the callus initial growth. However, slight difference in response of both varieties towards same concentration of 2,4-D (1.5 mg l^{-1}) might be due to delayed biochemical conversion of 2,4-D to functional form by the varieties leading to response variation within species of the same genus (Gandonou *et al*., 2005). Delayed callus initiation at low concentrations of 2,4-D (< 1.5 mg l^{-1}) might be due to reduced enzyme activity of RNA polymerase involved in growth processes whereas higher concentrations (> 2.0 mg l^{-1} 2,4-D) may lead to extra and abnormal growth of explant by preventing conversion of immature cytoplasm to mature cytoplasm (Taiz & Zeiger, 2002).

Effect of different 2,4-D concentrations on calli induction in chilli varieties Nepali and NARC-IV: Owing to preceding results, promotive effect of 1.5 mg l^{-1} 2,4-D was noticed for the highest number of calli induced as observed previously for callus initiation by both varieties (Fig. 1b). Kumar *et al*., (2005) reported that optimal concentration of 2,4-D maintained maximum growth (26-29 calli) due to increased DNA and RNA synthesis with accelerated growth. Further, Davletova *et al*., (2001) reported that 2,4-D exerted the primary control on endogenous synthesis and metabolism of IAA and cytokinin in cells which played significant role in the process of callus induction. Slight difference for callus induction was depicted by both varieties. The initiation of callus and subsequent callus morphogenesis was specific to genotype as confirmed by Gandonou *et al*., (2005) who reported that genotypic constitution greatly affected callus induction.

Proportional decrease in number of induced calli (5-8 calli) in both varieties was observed with increase and decrease in 2,4-D concentrations. Salisbury & Ross (2005) reported that too low auxin (<1.5 mg l^{-1} 2,4-D) would not stimulate enzyme activity for nucleotide incorporation in RNA which is necessary for continued growth whereas at higher concentrations than optimum it will act as effective herbicide resulting in saturation of cells with auxin and upsets the fluctuations that are required for normal growth and differentiation.

Effect of different 2,4-D concentrations on calli growth in chilli varieties Nepali and NARC-IV: Observation made for callus initiation and induction showed synergism with callus growth on same concentration of 2,4-D (1.5 mg l^{-1}) with Nepali consistently exhibiting slightly better response relative to NARC-IV (Fig. 1c). The improvement in callus growth (18-23 calli) at 1.5 mg l^{-1} 2,4-D might be linked to early initiation and induction of callus on this concentration as seen for previous characters. Taiz & Zeiger, (2002) reported that activation of enzyme caused by optimum concentration of auxin resulted into increase in DNA, RNA and protein content. This further increased transcription of rRNA and enzyme activity with increase in rate of RNA chain initiation and chain propagation by RNA polymerase accelerating callus growth. Varied response of auxin leading to decreased calli growth (1-3 calli) at suboptimal concentrations (< 1.5 mg l^{-1} 2,4-D) might be due to reduced availability of endogenous IAA which is required to activate enzymes involved in transcription of RNA whereas at supra-optimal concentrations than optimum, the growth inhibition of protein synthesis is generally attributed to auxin-induced ethylene biosynthesis. Difference in capacity of Nepali and NARC-IV to produce callus might be linked to the presence of competent cells which respond to external signals produced by hormone application to enter a specific

developmental pathway (Venkataiah *et al*., 2006). Furthermore, such response could be linked with ability of former variety (NARC-IV) to reach its latent period (period of no increase in rate) as earlier than Nepali showing that some step in the chain of processes connecting transcription with cell growth might become limiting in NARC-IV and hence caused different response towards growth.

Callus regeneration

Effect of different BA concentrations on shoot initiation period (days) from chilli varieties Nepali and NARC-IV calli: Effective concentration of BA leading to best response for shoot initiation from both varieties (6-8 days) was 3.0 mg l^{-1} BA (Fig. 2a). Shoot initiation was remarkably improved at this concentration while proportional increase was observed in days to shoot initiation from calli relative to BA concentration (low and high regime). Better performance at 3.0 mg l^{-1} BA might be due to inhibition of tRNA- cytokinin oxidase complex formation which was protected by cytokinin thus resulting in rapid growth of shoot primordia (Sowa *et al*., 2002). The variation in response (15-19 days) at low and high BA concentration could be attributed to inability of suboptimal cytokinin (< 3.0 mg l^{-1} BA) to incorporate amino acids into proteins preventing shoot primordia formation whereas, owing to its higher concentrations (> 3.5 mg l^{-1} BA) internal level of cytokinin may increase to a point where it caused increased production of RNA or protein synthesis inhibitors inhibiting shoot meristamoids formation (Salisbury & Ross, 2005). Slight varietal difference might be linked to genotypic constitutions as confirmed by the earlier observation made by Ramage & Leung (1996) that cultivar response to cytokinin may depend on the activity of enzyme in the metabolism of these phytohormones.

Effect of different BA concentrations on regeneration rate (%) of chilli varieties Nepali and NARC-IV calli: Similar pattern existed for regeneration rate as seen earlier for shoot initiation period (Fig. 2b). The result showed that 3.0 mg l^{-1} BA was the most suitable concentration for regeneration (41-49%) in both varieties. This response could be attributed to the BA uptake and metabolism which was subsequently converted to isopently adenine (iP) and isopently adenosine (iPR) inhibiting the activity of cytokinin oxidase on cytokinin action in the early stages of shoot development (Auer *et al*., 1999). The results are in agreement with those of Gupta *et al*., (1998) who found that optimal concentration of cytokinin to auxin lead to the formation of shoot primordia by reducing the effect of endogenous auxin concentration. Salisbury & Ross, (2005) also reported that specific level of BA is required for breakdown of tRNA bound cytokinin to functional cytokinin. Increasing or decreasing BA concentration than optimum (3.0 mg l^{-1} BA) did not improve the regeneration percentage (5-9%) but affected it negatively. At low concentrations of cytokinin (< 3.0 mg l^{-1} BA), undifferentiation of callus occurred rather than proliferation of shoot. However, at low concentration increased cytokinin oxidase gene might result in reduction of endogenous cytokinin level with subsequent retardation of shoot growth due to reduced cell proliferation. While, at high concentrations of cytokinin (> 3.5 mg l^{-1} BA), reduction in regeneration rate might be attributed to inhibition of synthesis of cytokinin which disturbed the mitosis in cells of shoot primordia. Further, Christopher & Rajam (1996) reported that these changes at cellular level caused changes in chromosomes number, delayed chromosomes and anaphase bridges.

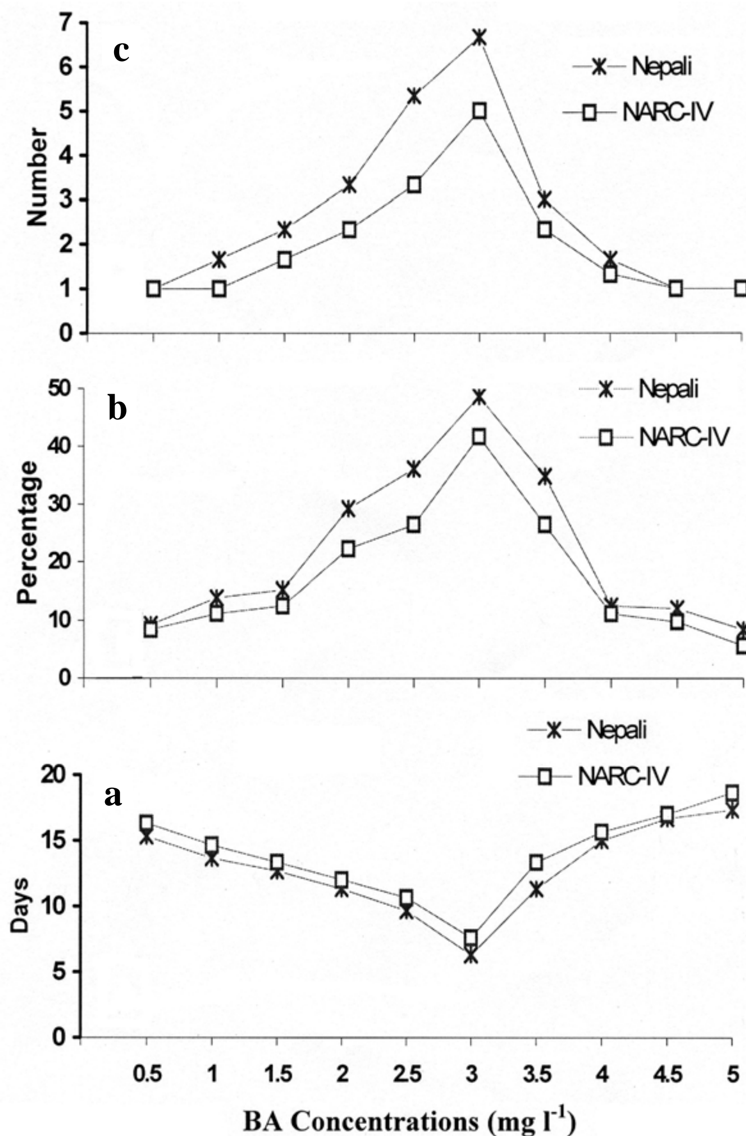

Fig. 2. Effect of different BA concentrations on the shoot initiation (a) regeneration rate (b) and number of lateral shoots per regenerants (c) in chilli varieties: Nepali and NARC-IV.

Nepali showed slight difference in regeneration rate relative to NARC-IV on same concentration of BA (3.0 mg l^{-1}). This genotypic difference in response might be linked with different inability of responsive cells of both varieties to sense the signal from endogenous cytokinin required for shoot organogenesis as proved by earlier observation of Venkataiah *et al*., (2006).

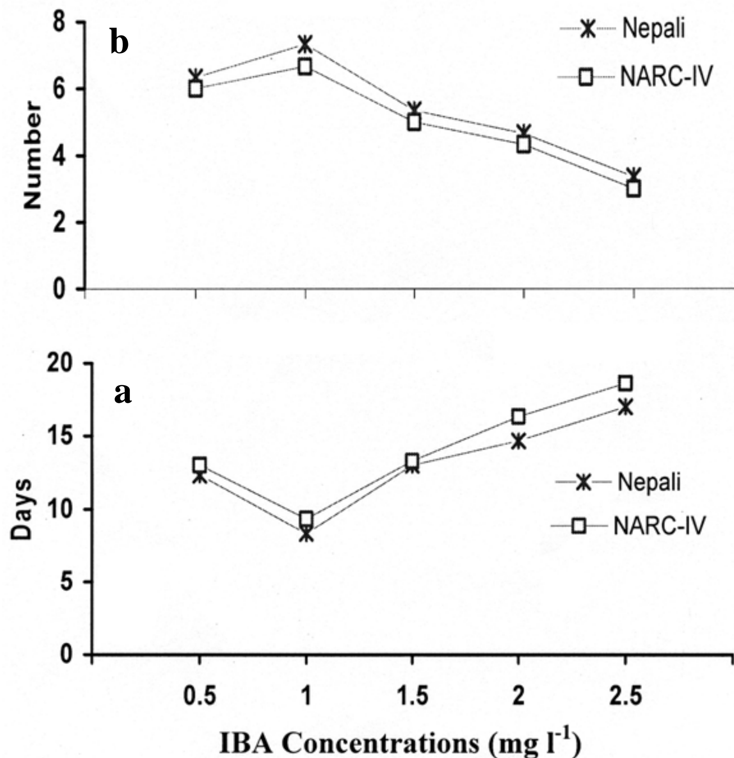

Fig. 3. Effect of different IBA concentrations on root initiation (a) and number of shoots with roots (b) in chilli varieties: Nepali and NARC-IV.

Effect of different BA concentrations on number of lateral shoots per regenerants of chilli varieties Nepali and NARC-IV calli: Similar response was reflected here as seen previously for shoot initiation and regeneration (Fig. 2c). Supplementing medium with BA @ of 3.0 mg l^{-1} consistently showed improvement in shoot initiation, regeneration rate (%) alongwith number of lateral shoots per regenerants (5-7 shoots). This might be due to availability of BA at optimum concentration which leads to inhibition of apical dominance with subsequent increase in the number and rate of lateral shoots (Binzel *et al.*, 1996). At low concentrations of BA than optimum, production of lateral shoots might be suppressed by abundance of endogenous free IAA (Srivastava, 2005) while, higher concentrations of BA (> 3.5 mg l^{-1} BA) induced callus formation. From the above results, it is clear that moderate level of cytokinin to auxin ratio in culture medium is critical for shoot organogenesis (Venkataiah & Subhash, 2001).

The slight discrepancy in capacity of both varieties to produce maximum number of lateral shoots on 3.0 mg l^{-1} BA could be ascribed to genotypic differences. This confirms the earlier observation made by Schween & Schwenkel (2003) who reported that difference by varieties in response to same concentration of BA may result due to considerable variation in genes or blocks of genes involved in developmental pathway and metabolism of cytokinin-related compounds.

In vitro rooting

Effect of different IBA concentrations on *In vitro* rooting: Days to root initiation were significantly reduced (8-9 days) in both varieties at 1.0 mg l^{-1} IBA (Fig. 3a). Such response at this concentration resulted from early dedifferentiation of vascular cambial tissue and differentiation of xylem with subsequent development of root initials (Preece & Read, 2005). This effect may result from effect of auxin on increased transcription of mRNA with subsequent increased perception of rhizogenic signal by competent cells for root primordia formation (Taiz & Zeiger, 2002). Maximum time for root initiation (12-19 days) occurred at low and high concentration of IBA in both varieties. At low concentration of IBA than optimum, activity of free endogenous IAA may be inhibited by IAA oxidase with subsequent decrease in outgrowth of root meristamoids (Hartmann *et al*., 1997). Whereas, increasing level of IBA has tendency to increase root initiation but high concentrations (>1.5 mg l^{-1} IBA) lead to inhibition of root primordium organization (Venkataiah *et al*., 2006). However, slight varietal difference in days to root initiation at this concentration could be attributed to the propensity of competent cells of varieties to respond to hormonal signal (Cary *et al*., 2001).

A synergism existed between root initiation and number of shoots with roots. It is clear from the results that 1.0 mg l^{-1} IBA was the effective concentration for elongation of root primordial (6-7 shoots with roots) in both varieties (Fig. 3b). This effect might result from enhanced rooting *via* increased internal free IBA or may synergistically modify the action of IAA or endogenous synthesis of IAA leading to increased elongation of root primordium (Krieken *et al*., 1993). Similarly, Salisbury & Ross (2005) reported that conjugate formation stores the IBA initially and then gradually release IBA at proper level, especially during later stages of root development. Slight variation in response (3-5 rooted shoots) at low and high concentrations revealed that growth and elongation of roots is extremely sensitive to the auxin concentration; root elongation is stimulated at low concentration while at higher concentration it is inhibited due to increased synthesis of endogenous ethylene. These results corroborate with that of Ahmad *et al*., (2003) who indicated that root elongation phase was very sensitive to auxin concentrations and was inhibited by higher concentration. After 10-15 days of culture, plantlets with fully expanded leaves and well developed roots were transferred to pots containing mixture of soil and peat (1:1). The pots were covered with a plastic bag and were grown for 2-3 weeks in greenhouse. Plantlets were fully acclimated to greenhouse conditions and were growing normally.

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