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In vitro shoot regeneration from cotyledonary leaf explant in chilli and bio-hardening of plantlets

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ABSTRACT

A protocol for direct shoot regeneration from cotyledonary leaf explant in chilli was developed and method for bio-hardening of *in vitro* regenerated plantlets using *Glomus mosseae, Gigaspora margarita* and mixed arbuscular mycorrhizal fungi (AMF) strains were standardized. The experiment was undertaken with four chilli cultivars namely KtPL-19, Pusa Sadabahar, ArCH-001 and Salem. Explants were excised from 21-day-old *in vitro* raised seedlings. Direct shoot organogenesis was observed on cotyledonary explant with slight callusing. Number of shoot buds per explant was maximum (5.73) and days taken for shoot bud induction was minimum on MS medium supplemented with 1.0 mg l^{-1} of TDZ in all the cultivars. However, response of Pusa Sadabahar was better in terms of number of shoot buds than other cultivars under study. Fifteen to 20 days were required for shoot bud induction in all the cultivars except Salem which took 25-30 days. Among the different treatments tested for shoot multiplication the best treatment was MS + 6.0 mg l^{-1} BAP + 1.0 mg l^{-1} kinetin + 0.5 mg l^{-1} GA_3. The length of the shoot increased with increase in BAP and GA_3 levels. The *in vitro* regenerated shoots were inoculated on to half-strength MS medium supplemented with 1.0 mg l^{-1} IBA where more than 90 per cent rooting was observed. When *in vitro* raised plantlets were treated with AMF high plant survival was observed. The maximum survival (97.08 %) was recorded with mixed strain of *Glomus mosseae* and *Gigaspora margarita.* The root and shoot length was also maximum when plantlets were treated with mixed AMF strains. The developed protocol may be used for mass multiplication of elite chilli genotypes as well as regeneration of genetically transformed cell/tissue.

Key words: *Capsicum annuum,* cotyledonary leaf, *in vitro* regeneration, bio-hardening.

INTRODUCTION

Application of cell and molecular biology techniques for genetic improvement can serve as an efficient tool for further improvement of chilli (*Capsicum annuum* L.), the important vegetable cum spice crop in the world. Availability of a repeatable *in vitro* regeneration system is pre-requisite for application of this technique. Unlike other Solanaceous species, chilli is recalcitrant to *in vitro* regeneration especially for the shoot elongation. Although some reports on regeneration from various explants through organogenesis and somatic embryogenesis are available, however, the inter-varietal differences in regeneration from various explants and species are pronounced (Christopher and Rajam, 3). Moreover, the capability of chilli plant to regenerate *in vitro* from different tissues or cell culture would permit asexual propagation of elite or difficult-to-isolate stocks, cell selection for useful variants and recovery of transformed plants from genetically engineered cells. Furthermore, there are also inter-varietal differences in chilli explants responding to various levels and combinations of plant growth regulators (Fortunato and Tudisco, 6). Since *in vitro* plantlets are raised under controlled environment, their direct outdoor transplantation without prior and proper hardening often results in poor field survival (Varma and Schuepp, 17). This seems to be one of the major bottlenecks in commercial exploitation of this technology. Arbuscular mycorrhizal fungi (AMF) can be used to reduce the impacts of *ex vitro* stressful environment on the plantlets. Keeping these points in view, regeneration and bio-hardening studies were conducted on four chilli cultivars.

MATERIALS AND METHODS

Chilli cultivars namely, KtPL-19 (V_1), Pusa Sadabahar (V_2), ArCH-001 (V_3) and Salem (V_4) were taken for studies at the Central Tissue Culture Laboratory and Division of Vegetable Science, IARI, New Delhi. The dried seeds (obtained from National Seed Corporation of India, New Delhi and Division of Vegetable Science, IARI, New Delhi) were soaking in sterile distilled water for 12 h prior to *in vitro* culture. Surface sterilization was carried out with 0.1 % $HgCl_2$ with agitation for two minutes followed by three rinses in sterile double-distilled water. Fifteen seeds of each cultivar were then inoculated in 250 ml conical flasks, each containing 50 ml MS (Murashige and Skoog, 10) medium with 30 g l^{-1} sucrose, gelled with 7 g l^{-1} agar

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and kept in dark at 25±1^{0}C temperature. After germination, cultures were exposed to light for 16 h d^{-1}. The cotyledonary leaf segment excised from 3-week-old seedlings were used as explants for direct shoot bud regeneration. Explants were inoculated on MS medium containing 30 g l^{-1} sucrose, 7 g l^{-1} agar and different plant growth regulators alone or in combination for organogenesis. Fifteen explants in each treatment were inoculated in separate test tube. The media were autoclaved at 120^0 C for 20 minutes for sterilization. The heat-labile phyto-hormones like IAA, thidiazuron (TDZ), GA_3, *etc.* were added to the medium after autoclaving using 0.20 µm size microfilter (Millipore® USA). The regenerated shoots were then sub-cultured onto proliferation medium supplemented with different growth regulators. Proliferating micro-shoots were sub-cultured and transferred to fresh proliferation medium after an interval of three to four weeks. A total of 15 cultures were maintained in each treatment and only proliferating clumps were maintained in one flask. Observations on average number of shoots per culture and mean shoot length were recorded after four weeks of culture. Shoots derived on multiplication medium were separated and transferred onto earlier standardized rooting medium *i.e.,* half-strength MS medium supplemented with 1.0 mg l^{-1} Indole-3-butyric acid (IBA) (Ranjan *et al.,* 11). The culture room was maintained at 25 ± 2°C temperature, 16/8 h of light and dark cycle by cool-white fluorescent tubes (47 µmol m^{-2}s^{-1}).

After rooting of plantlets, they were transferred in small plastic pots filled with peat and Soilrite® (1:1) for hardening. The plantlets were covered with inverted glass beaker to create favourable micro-climate. The plantlets, after primary hardening for 20 days, were then shifted to glasshouse wherein they were subjected to different AMF inoculation to enhance *ex vitro* survival. For this, plantlets were transplanted in plastic pots (15 cm) filled with sterile soil, sand and peat (2 : 1 : 1). Twenty gram soil-based cultures of different AMF, *viz., Glomus mosseae, Gigaspora margarita* and mixed strain obtained from Division of Microbiology, IARI, New Delhi were added in each pot at the base of roots. After transplanting, the plants were immediately irrigated with sterile tap water and maintained in glasshouse (25 ± 2°C). After 30 days, growth of hardened plantlets was studied.

The experiments were laid out in factorial completely randomized design (CRD) with three replications. Percentage data were subjected to arc sin transformation before analysis. The analysis of data was done SPSS 10.0.

RESULTS AND DISCUSSION

Direct shoot organogenesis was observed on cotyledonary leaf explant (Table 1). Although slight callus growth developed at the cut surface of cotyledonary explant, it appeared that the shoot buds regenerated directly (microscopic observation) from the explant tissue without intervention of callus growth. More than 85% per cent explants showed response for shoot bud induction on MS medium supplemented with 1.0 mg l^{-1} TDZ (Fig. 1). The same neo-formation event with histological evidence was earlier reported by Agrawal *et al.* (1). With increase in concentration of BAP and thidiazuron (TDZ), the number of shoot buds increased significantly however, at the highest concentration combination (10.0 mg l^{-1} BAP or 2.0 mg l^{-1} TDZ), there was a sharp decline. Maximum number of shoot buds was recorded in treatment with 1.0 mg l^{-1} of TDZ (T7). Benzyl aminipurine at 8.0 mg l^{-1} was found to be most responsive. Significant difference was also observed among the cultivars. Maximum number of shoot buds was recorded in cv. Pusa Sadabahar, which was significantly higher compared to other cultivars. However, cv. Salem was least responsive. Interaction between cultivar and treatment showed that the highest number of shoot buds (6.92) could be achieved in cultivar Pusa Sadabahar on medium containing 1.0 mg l^{-1} TDZ (T7) which also gave the highest regeneration frequency. Among the three cytokinins tested, TDZ was found to be most effective for direct shoot bud regeneration on cotyledonary explant. It has been reported that TDZ has strong cytokinin-like activity in various bio-assays and is efficient in stimulating cytokinin dependent shoot regeneration in wide varieties of plants (Malik and Saxena, 9). Earlier, Szasz *et al.* (15) also reported TDZ to be most effective substituted phenyl-urea compound for direct shoot regeneration in chilli as it has profound effect on cell division and differentiation. Most of the published report on pepper used combination of auxin and cytokinin for shoot regeneration from hypocotyl and cotyledonary explants. However, Sripichitt *et al.* (14) found that cytokinin (BA) alone was more effective than combinations of auxin and cytokinin.

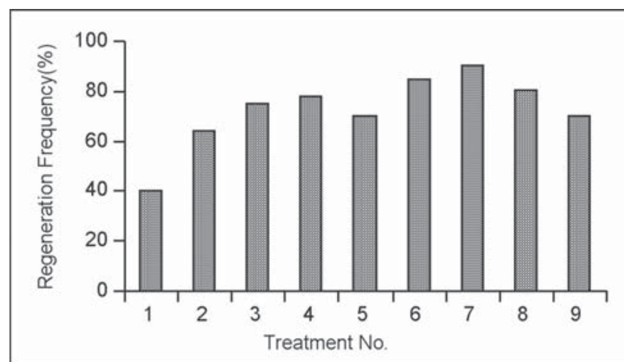

Fig. 1. Regeneration frequency of cotyledonary explant (pooled of all the cultivars).

Table 1. Effect of different growth regulators on direct organogenesis from cotyledonary leaf explant in chilli.

Treatment No.	Medium + Growth regulator (mg l^{-1})	No. shoot buds/ explant					No. days taken for shoot bud induction			
		V_1	V_2	V_3	V_4	Mean	V_1	V_2	V_3	V_4
T_1	MS + 2.0 BAP + 1.0 IAA	1.89	1.90	1.00	0.75	1.38	+	+	+	+
T_2	MS + 4.0 BAP + 1.0 IAA	2.25	2.10	1.95	1.09	1.85	+	+	+	+
T_3	MS + 6.0 BAP + 1.0 IAA	4.25	4.10	3.05	2.90	3.57	++	++	++	+
T_4	MS + 8.0 BAP + 1.0 IAA	5.10	5.75	4.72	3.25	4.71	+++	+++	+++	++
T_5	MS + 10.0 BAP + 1.0 IAA	4.25	4.85	3.20	2.05	3.59	+++	+++	+++	++
T_6	MS + 0.5 TDZ	2.00	2.50	2.10	1.10	1.92	++	++	++	++
T_7	MS + 1.0 TDZ	6.25	6.92	5.23	4.50	5.73	+++	+++	+++	++
T_8	MS + 1.5 TDZ	5.25	5.20	4.20	2.50	4.29	+++	+++	+++	++
T_9	MS + 2.0 TDZ	4.25	3.90	2.15	1.20	2.88	++	++	++	+
	Mean	3.94	4.14	3.07	2.15					

CD ($p \leq 0.05$) – Treatment: 0.10; Cultivar: 0.07; Interaction: 0.20. + = 30-35 days; ++ = 25-30 days; +++ = 15-20 days.

Table 2. Effect of different growth regulators on in *vitro* shoot multiplication in chilli.

Treatment No.	Medium + Growth regulator (mg l^{-1})	No. shoots / culture					Mean shoot length (cm)				
		V_1	V_2	V_3	V_4	Mean	V_1	V_2	V_3	V_4	Mean
T_1	MS + 1.0 BAP + 1.0 KIN	2.45	3.10	2.85	2.80	2.80	1.50	1.75	1.35	1.25	1.46
T_2	MS + 2.0 BAP + 1.0 KIN	3.10	3.25	3.50	2.70	3.14	1.65	1.82	1.39	1.31	1.54
T_3	MS + 3.0 BAP + 1.0 KIN	3.75	4.05	3.90	3.05	3.69	2.05	2.10	1.75	1.45	1.84
T_4	MS + 4.0 BAP + 1.0 KIN	3.85	5.50	4.05	3.50	4.23	2.30	2.55	1.95	1.70	2.13
T_5	MS + 5.0 BAP + 1.0 KIN + 0.5 GA_3	4.20	6.25	5.25	3.80	4.88	3.90	3.95	2.80	2.75	3.35
T_6	MS + 6.0 BAP + 1.0 KIN + 0.5 GA_3	5.00	6.80	5.50	4.23	5.38	4.35	4.60	3.25	3.05	3.81
T_7	MS + 7.0 BAP + 1.0 KIN + 1.0 GA_3	5.50	5.50	4.75	4.50	5.06	5.40	5.75	4.50	3.90	4.89
T_8	MS + 8.0 BAP + 1.0 KIN + 1.0 GA_3	4.20	5.00	4.05	3.10	4.09	5.62	6.00	4.60	4.00	5.06
	Mean	4.01	4.93	4.23	3.46		3.35	3.56	2.70	2.43	

CD ($p \leq 0.05$) – Treatment: 0.12; Cultivar: 0.08; Interaction: 0.25. CD at ($p \leq 0.05$) – Treatment: 0.12; Cultivars: 0.08; Interaction: 0.25.

Number of days for shoot bud induction was also significant in different treatments and cultivars (Table 1). Fifteen to 20 days were required for shoot bud induction in all the cultivars in treatments T_4, T_5, T_7 and T_8 except Salem, which required 25 to 30 days. At lower concentration the effect was most delayed (30-35 days). The time required for shoot bud induction was in general, higher in medium containing low concentrations of cytokinin and *vice-versa*. This might be due to the fact that higher cytokinin concentration has high regeneration ability by early induction of meristematic activity (Szasz *et al.*, 15). Since callus induction on cotyledonary explant are less totipotent than most of other solanaceous crops are generally programmed towards vegetative growth, hence, they require higher concentration of cytokinin for early shoot bud induction (Kaparakis and Alderson, 8).

Significant differences were found among the treatments with regard to number of shoots per culture (Table 2). It was observed that maximum number of shoots (6.80) developed on medium containing 6.0 mg l^{-1} BAP, 1.0 mg l^{-1} kinetin and 0.5 mg l^{-1} GA_3 (T6) and minimum in the medium containing 1.0 mg l^{-1} BAP and 1.0 mg l^{-1} kinetin. It was also observed that with the increase in cytokinin level up to 6.0 mg l^{-1}, the number of shoots increased significantly and decreased thereafter. Among the cultivars, the maximum number of shoots was recorded in cultivar Pusa Sadabahar.

Mean shoot length increased significantly with increase in BAP and GA_3 levels (Table 2). The maximum shoot length was recorded in the medium supplemented with 8.0 mg l^{-1} BAP, 1.0 mg l^{-1} kinetin and 1.0 mg l^{-1} GA_3. Cultivars also showed significant differences with respect to me an shoot length. Irrespective of the growth regulator treatments, the maximum shoot length was recorded in cv. Pusa Sadabahar and minimum was in Salem. This might be due to difference in genetic capability among the cultivars. There was significant interaction between treatment and cultivar with regard to mean shoot length. With the increase in cytokinin (BAP) level, the number of shoots increased. Addition of GA_3 also increased number of shoots. This might be due to elongation of small buds which were not able to elongate in medium devoid of GA_3. Increase in BAP concentration beyond 6.0 mg l^{-1} decreased the number of shoots significantly. It was also observed that with the increase in cytokinin concentration, shoot elongation was not much affected as it was only induced with addition of GA_3. The shoot length was better with 1.0 mg l^{-1} compared to 0.5 mg l^{-1} GA_3. Increase in shoot length with application of GA_3 is a well known fact as it promotes internodal elongation in wide range of species. After shoot multiplication, the *in vitro* regenerated shoots were inoculated on to ½ MS medium supplemented with 1.0 mg l^{-1} indole-3-butyric acid (IBA) where more than 95 per cent rooting was observed (Ranjan *et al.* 11).

The effect of different arbuscular mycorrhizal fungi (AMF) on survival, shoot length and root length were studied under glasshouse conditions. Significant difference was found between the cultivars with respect to survival per cent (Table 3). The maximum survival was recorded when plantlets were treated with mixed strain of *Glomus mosseae* and *Gigaspora margarita.* Interaction reveals that there was maximum survival in cultivar Pusa Sadabahar with mixed strain treatment and the minimum was with ArCH-001 in control.

The major bottleneck in the use of micropropagation is higher rate of mortality when the plantlets are transferred from *in vitro* to *ex vitro* conditions. Micropropagated plantlets may develop functional roots during tissue culture or may die due to the development of non-functional root systems and deficient vascular connections between the root and shoot systems.

Table 3. Effect of different AMF inoculation on survival of *in vitro* raised plantlets under glasshouse conditions.

AMF strain	Survival (%)				
	V_1	V_2	V_3	V_4	Mean
Control	69.20 (56.32)*	70.13 (56.90)*	68.25 (55.73)*	71.15 (57.54)*	69.68 (56.22)
Glomus mosseae	90.15 (71.75)	92.25 (73.78)	91.15 (72.73)	91.20 (72.78)	91.19 (72.76)
Gigaspora margarita	90.10 (71.70)	91.70 (73.26)	90.90 (72.48)	91.90 (73.50)	91.15 (72.73)
Mixed strain	98.25 (82.44)	98.90 (84.02)	95.25 (77.45)	98.3 (82.40)	97.68 (81.73)
Mean	86.93 (70.55)	88.25 (71.99)	86.39 (69.60)	88.14 (71.56)	

CD ($p \leq 0.05$) Treatment = 2.26; Cultivar = 2.31; Interaction = 4.63; *Values in parenthesis indicate transformed value.

Transferring micropropagated plants directly from culture vessels to *ex vitro* conditions is generally difficult as the transpiration rate is higher in such plants. In comparison to the *in vivo* grown plants, the *in vitro* raised plants have poor stomatal control and abnormally high cuticular water loss resulting in wilting and necrosis of leaves or even senescence of leaves and plantlets (Diaz-Perez *et al.*, 4). During *ex vitro* transfer, the plantlets are exposed to lower relative humidity and high/low temperature conditions and thus they undergo transplant shock. The lower survival percentage of non-inoculated tissue culture raised plantlets could be attributed to several anatomical and physiological abnormalities. Higher survival rates of AMF inoculated plantlets might be due to the development of strong root system (Elemeskoui *et al.*, 5), improved water uptake (Yamashita *et al.*,18), improved uptake of plant immobile nutrients (Ames *et al.*, 2). It has been reported that in *Capsicum annuum* L., mycorrhiza can enhance uptake of P (Sreenivasa,12), Zn (Sreeramulu and Bagyaraj, 13), Cu, Mn and Fe (Sreenivasa,12). Plants inoculated with mixed strain had higher survival rate than other individual strains under the investigation. It indicates that the efficiency of different fungal strains may be different even for the same host. The efficiency of AMF was possibly affected by differences in their symbiotic structures (*e.g.,* arbuscules or vesicles), which could influence the nutrient exchange processes (Hayman, 7).

Shoot length was also influenced by different mycorrhizal strains. It was maximum when plantlets were treated with mixed AMF strain (Fig. 2). Maximum shoot length was recorded in cultivar Pusa Sadabahar and minimum in Salem in control (12.25 cm). As a result, the genotypes may have different degree of host-endophyte symbiotic relations (Vaast *et al.*,16). Likewise, root length was found maximum in plants

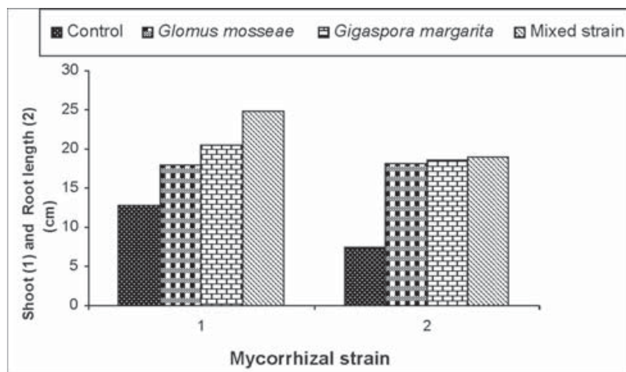

Fig. 2. Shoot and root length (pooled of all the cultivars) of *in vitro* regenerated plantlets treated with different mycorrhizal strains.

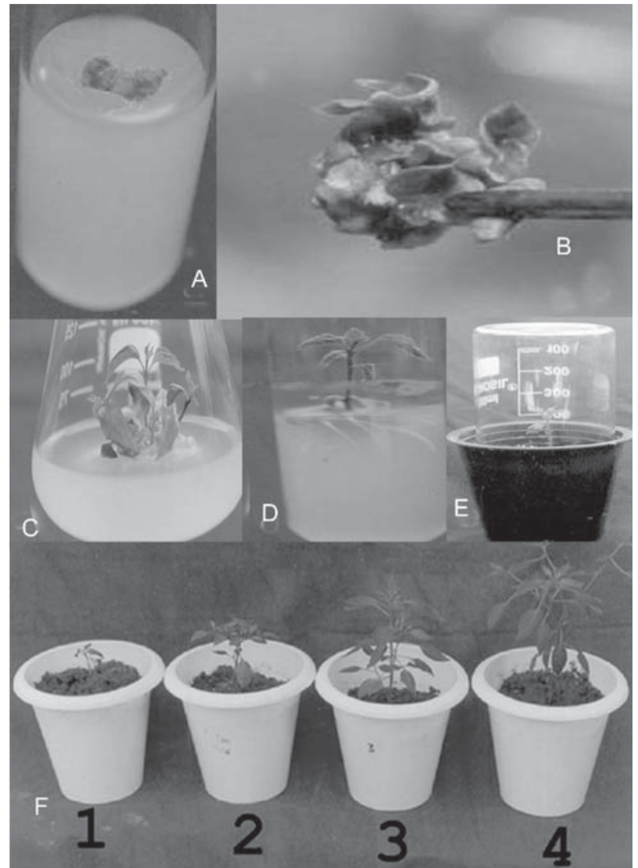

Fig. 3. Direct shoot bud induction and plantlet regeneration in chilli cv. Pusa Sadabahar (a) cultured cotyledonary explant on MS medium without growth regulators, (b) shoot bud induction on MS medium supplemnted with 1.0 mg l^{-1} TDZ, (c) shoot multiplication on MS medium with 6.0 mg l^{-1} BAP + 1.0 mg l^{-1} kinetin + 0.5 mg l^{-1} GA_3, (d) rooting of *in vitro* regenerated microshoots on ½ MS + 1.0 mg l^{-1} IBA, (e) hardening of *in vitro* rooted plantlets, (f) comparative shoot growth of plants inoculated with different strains of arbuscular mycorrhizal fungi after 30 days of acclimatization in glasshouse, 1. control, 2. *Glomus mosseae,* 3. *Gigaspora margarita,* 4. mixed strain of *Glomus mosseae* and *Gigaspora margarita.*

treated with mixed AMF strain. Significant difference was observed between *Glomus mosseae* and *Gigaspora margarita* as the root length of plants was significantly higher than control as well as other two strains (Fig. 2). The maximum root length was recorded in cultivar ArCH-001 and minimum was in Salem (Fig. 2). The AMF-inoculated plants have enhanced nutrient and water availability as reported by several workers. This could enforce the plants to develop bigger rhizosphere so that the plant may have access to the nutrients, which are made available by

direct influence of AMF. This might be the reason behind longer root growth in the mycorrhizal plantlets.

Fig. 4. Comparative root growth of tissue culture raised plants after 30 days of acclimatization in glasshouse (A) untreated plant (B) inoculated with mixed AMF strain.

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