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Factors affecting *in vitro* shoot regeneration in hypocotyls of brinjal (*Solanum melongena* L.) in the early steps of *Agrobacterium*-mediated transformation

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ABSTRACT

An attempt was made to assess the effect of size, age and position of the explant, pre-culture and high cytokinin concentration in the pre-culture medium on shoot regeneration in brinjal hypocotyls co-cultivated with *Agrobacterium*. The study was carried out using hypocotyl explants of brinjal cv. Manjarigota, *Agrobacterium* strain A208 and shoot regeneration medium (full-strength basal MS medium, 2µM BAP + 0.05µM NAA, 3% sucrose and 0.8% agar) containing Cefotaxime (250-500mg l^{-1}) and Kanamycin (100mg l^{-1}). Hypocotyl explants showed callus initiation and shoot regeneration response after 10-12 and 20-22 days of culture, respectively. Five-day-old explants did not survive *Agrobacterium* infection, and ten-day-old explants showed higher shoot regeneration (29±1.91%) than older explants. Explants of medium size (1cm long; 32±2.62%) from the apical region (38.57±2.61%) showed better shoot-regeneration ability than explants of any other size or region. A period of four days of pre-culture (33.33±3.76) was optimal best for best shoot-regeneration in hypocotyl explants. No regeneration was seen in hypocotyl explants at shorter or longer pre-culture period. High cytokinin (10µM) in shoot regeneration medium during pre-culture enhanced shoot regeneration response (47.27±2.98%) in explants co-cultivated with *Agrobacterium*. Effects of various factors documented in this study will be useful in developing an efficient *Agrobacterium*-mediated transformation protocol in brinjal cv. Manjarigota.

Key words: Eggplant, pre-culture on high BAP, explant characters, PCR

INTRODUCTION

Brinjal (eggplant, aubergine, *Solanum melongena* L.) is a principal vegetable crop grown in many geographical parts of the world. India is the second largest producer of brinjal in the world after China. Brinjal is as nutritious as any other solanaceous crop, hardy and suitable for growing in varied agroclimatic conditions. It is mainly cultivated in small-family farms and is a source of income for the resource-poor farmer. A vast scope exists for increasing profits from brinjal cultivation (Balappa and Hugar, 2002), achievable by developing varieties resistant to biotic and abiotic stresses hampering production. Of late, plant transformation technology has gained popularity for crop improvement. *Agrobacterium* is a natural genetic engineer, and, is still preferred for plant transformation due to the simplicity of this transformation system and for precise integration of the transgene (Veluthambi *et al*, 2003). Brinjal is highly responsive to *in vitro* culture *via* organogenic or embryogenic pathways (Collonier *et al*, 2001). *Agrobacterium*-mediated transformation has been successfully used for producing transgenic plants. Also, efforts have been made to work out *Agrobacterium*-- and culture medium-related factors on *in vitro* response (Magioli and Mansur, 2005). However, poor survival of explants, drastic reduction and high variation in shoot regeneration response in *Agrobacterium* co-cultivated explants, are major drawbacks in the existing transformation protocols. In some cases, highly efficient *in vitro* regeneration protocols greatly reduced or failed to regenerate shoots after *Agrobacterium* co-cultivation (Magioli *et al*, 2000; Billings *et al*, 1997; Chen *et al*, 1995). This may be due to unfavourable conditions at various steps in plant transformation such as explant character, high concentration of antibiotics, prolonged *Agrobacterium* co-cultivation, etc., leading to necrotic response in explants. Improving shoot regeneration frequency of transformation, therefore, is one of the major challenges in brinjal transformation. Sanyal *et al* (2005) reported that a combination of physical and physiological conditions during agro-inoculation, co-cultivation and selection on Kanamycin medium were critical determinants,

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resulting in an altered competence for regeneration, co-transformation frequency and elimination of escapes in transformation of a species or genotype. Hence, it appears that a comprehensive effort could uncover factors needed for successful transformation, *in vitro* response of explants along with *Agrobacterium* infection in a cultivar. This would help standardize a transformation protocol favoring high plant recovery from rapid and efficient *in vitro* shoot regeneration. 'Manjarigota' is a round-stripe, productive cultivar and is the most preferred in India because of its agreeable taste. We have used this variety as a model plant to study the effects of explant and growth regulators (Hanur *et al*, 2006; Prakash *et al*, 2007a; Prakash *et al*, 2008), antibiotics and gelling agents (Prakash *et al*, 2007b) on *in vitro* response in hypocotyl explants co-cultivated with *Agrobacterium*. The scope of this study was to investigate the effect of explant characters, pre-culture period and high cytokinin concentration in the pre-culture medium on *in vitro* response in hypocotyl explants co-cultivated with *Agrobacterium* in brinjal cv. Manjarigota.

MATERIAL AND METHODS

Shoot Regeneration Medium [SRM: full-strength basal MS medium (MS salts, organics and vitamins; Murashige and Skoog, 1962) containing 2µM BAP (BAP – 6-benzyleaminopurine), 0.05µM NAA (NAA - naphthalene acetic acid), 3% sucrose and 0.8% agar] was used as the culture medium. pH was adjusted to 5.75±0.05 with NaOH (Sodium hydroxide) or HCl (Hydrochloric acid) (0.1N) prior to autoclaving. Sterilization of the culture medium and instruments to be used was done by autoclaving at 121°C and 15 pounds per square inch (psi) pressure for 20 minutes. The medium was cooled to under 45°C, antibiotics were added (wherever necessary) and poured into petriplates under a laminar airflow chamber (Kirloskar, India). After solidification, the medium was used in the experiments. Cultures were incubated at a light intensity (white, fluorescent tubes) of 30-40µE m^{-2} s^{-1} under 16h photoperiod in a culture room maintained at 25± 2°C. Genuine breeder-seeds of brinjal cv. Manjarigota (a predominant, year-round bearing, locally well-adapted and preferred brinjal type in India) were obtained from Division of Vegetable Crops, ICAR-IIHR (Indian Institute of Horticultural Research), Bengaluru. After removing the apical meristem and basal stubs of the aseptically-germinated seedlings, hypocotyls (Prakash *et al*, 2007a) were used as the source of explants.

Experiments on assessing the effect of various factors on *in vitro* response were carried out using a procedure representing the conditions in transformation studies. Single-colony of *Agrobacterium tumefaciens* A208 strain (Source: NRC on Plant Biotechnology, New Delhi) was inoculated onto Yeast Extract Mannitol Broth (YEMB-mannitol 10gl^{-1}, yeast extract 0.4gl^{-1}, Nacl 0.1 gl^{-1}, $MgSo_4$. $7H_2O$ 0.2gl^{-1} and K_2HPO_4 0.5gl^{-1}) containing Kanamycin (Sigma, USA; 50mgl^{-1}), incubated in a shaker at 180rpm (rotations per minute) under 28±1°C, *Agrobacterium* culture grown overnight spun at 5000rpm for 5 minutes, the supernatant discarded, bacterial pellet dissolved in sterile, liquid half-strength MS medium, cell concentration measured at optical density of 600 nanometer (OD_{600}) using a spectrophotometer (BioRad, USA); 0.3-0.5 OD_{600} culture was prepared using sterile, liquid half-strength MS medium, for further use. Fifteen to twenty day-old, medium sized (1cm) hypocotyl explants were placed on SRM in *petri* plates for pre-culture for two days. Explants were collected in a sterile *petri* plate and immersed for 10-15 minutes in *Agrobacterium* culture (0.3-0.5 OD_{600}) for infection. The explants were then blotted onto sterile tissue-paper towels to remove excess *Agrobacterium* placed back onto the medium used for pre-culture, and incubated for co-cultivation for two days. The explants were then transferred onto a fresh culture medium containing Cefotaxime (Taxim, India; 500mgl^{-1}) for the next two days. Finally, these were transferred onto a culture medium containing Cefotaxime (250mgl^{-1}) and Kanamycin (100mgl^{-1}).

To study the effect of age of the explant, hypocotyl explants 5, 10, 15, 20, 25 and 30 days old were cultured; to study the effect of size of the explant, hypocotyl explants of small size (0.5cm), medium size (1cm), large size (1.5cm) and very large size (2cm) were cultured; to study the effect of position of the explant, the entire hypocotyl (stem) was divided into three segments, namely, apical, middle and basal region, and cultured; to study the effect of pre-culture, the hypocotyl explants were cultured on SRM for 0, 1, 2, 3, 4, 5, 6, 7 or 8 days prior to infection with *Agrobacterium*; to study the effect of high cytokinin concentration in the pre-culture medium, hypocotyl explants were pre-cultured on SRM without BAP (0µM), on basal SRM, and on SRM containing high BAP concentrations (10, 20, 30 or 40µM). Effect of all these factors was assessed by the above-mentioned experimental procedure. In addition, hypocotyl explants were cultured on SRM without *Agrobacterium* co-cultivation as a Control, along with all the other experiments.

Regenerated shoots were transferred onto shoot elongation medium (SEM: SRM containing 100mgl^{-1}

Cefotaxime and 50mgl^{-1} kanamycin) in culture tubes. Shoots with a well-developed primary meristem were transferred onto root induction medium (RIM: MS medium supplemented with 5µM IBA + 0.1µM BAP, 3% sucrose, 0.8% agar, 50mgl^{-1} Cefotaxime and 25mgl^{-1} kanamycin in culture tubes.

Adequate number of explants and replications were used in all the experiments. Each experiment was repeated at least thrice. Observations were recorded on callus-initiation (bulge near the cut-end) and shoot regeneration response at four weeks after culture. Frequency (%) of callus-initiation response (number of explants showing callus-initiation response/ number of explants cultured X 100) and frequency (%) of shoot regeneration response (number of explants showing regeneration response/ number of explants cultured X 100) was calculated. Percentage data was subjected to angular transformation and, then, subjected to Analysis of Variance (ANOVA) to test the significance of results obtained. Comparison between mean values of treatment was made by Least Significant Difference (LSD) method to identify the best treatment. Frequency (%) of elongated shoots and root-induction was calculated.

Molecular analysis of the transformants was carried out using PCR for confirmation for presence of the marker transgene. Good quality DNA was isolated from 10 putative transformants using CTAB method, and PCR was carried out with antibiotic-specific forward primer (*npt*-F) 5' GATGGATTGCACGCAGG 3' and reverse primer (*npt*-R) 3' GAAGGCGATAGAAGGCG 5' to confirm the presence of the transgene. PCRs were performed in a total volume of 25ml, comprising 2.5µl of 10X Reaction Buffer containing 15mM $MgCl_2$ (Genetix), 0.25µl of 10 mM dNTPs mixture (Genetix), 1µl of each primer (10µM, MWG), 0.33µl of *Taq* DNA polymerase (Genetix), 2.0µl (100-200 ng) of DNA sample and 17.92µl of sterile water. As a Positive Control, 2.0µl of *pBinBt-01* DNA was used. Non-transformed plant DNA and sterilized water samples were used as the Negative Control. PCR was carried out in MWG^R PCR system (MWG, Germany). DNA was denatured at 94^{o}C for 5 min, followed by 35 amplification cycles. Each cycle was programmed with three different thermal periods: at 94°C for 1 min to denature DNA, at 56^{o}C for 40 sec to anneal the primers, and at 72^{o}C for 1 min for the extension of DNA by *Taq* DNA polymerase. The final extension lasted for 5 min at 72^{o}C. The amplified product was mixed with 6X loading buffer (30% sucrose, 0.05% xylene cyanol and 0.05% bromophenol blue), and loaded along with 500bp (base pair)/0.5 kb (kilo base pair) ladder (Genei, Bangalore) onto 1.2% agarose gel containing ethidium bromide (0.001%). Electrophoresis was conducted at 50 volts for five hours, and the gel was photographed under UV light using Alpha Digi Doc system (Herolab, Germany). Gels were scored for presence or absence of the expected size of the amplified product.

RESULTS AND DISCUSSION

In the present study, hypocotyl explants cultured without co-cultivation with *Agrobacterium* on SRM showed callus-initiation and shoot regeneration response at 4-5 and 10-12 days, respectively, of culture initiation. These showed 70-80% shoot-regeneration response. *Agrobacterium* co-cultivated hypocotyl explants showed callus initiation and shoot regeneration response at 10-12 days and 20-22 days, respectively, of culture. Explants gave rise to shoots at one end and adventitious roots at the other, indicating polarity. As expected, there was a delay and drastic reduction in shoot-regeneration response (Fig. 1A, 1B and 2) in *Agrobacterium* co-cultivated explants. Magioli *et al* (2000) observed similar results in earlier studies. However, we made a few interesting observations on causes for poor survival of explants, on delay and drastic reduction in shoot

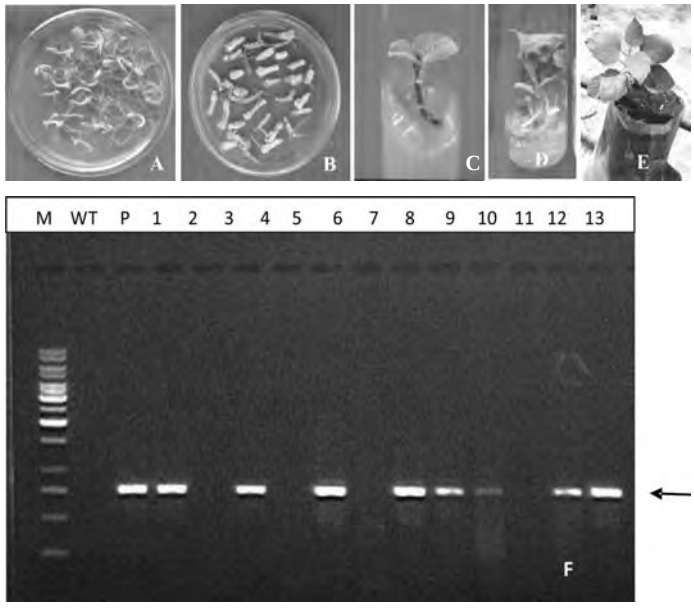

Fig. 1. A) *In vitro* shoot regeneration response without *Agrobacterium* treatment on SRM, and B) with *Agrobacterium* co-cultivation on SRM containing Kanamycin (100mgl^{-1}) and Cefotaxime (250mgl^{-1}); C) Shoot elongation on SRM containing Kanamycin (50mgl^{-1}) and Cefotaxime (100mgl^{-1}); D) Rooting on RIM containing Kanamycin (25mgl^{-1}) and Cefotaxime (50mgl^{-1}); E) Hardened plant; F) PCR amplification of ~0.9kb *nptII* gene (arrow) in transformed lines of eggplant (Lane: M- 1kb marker ladder (Genetix); WT-wild type; P-plasmid DNA; 1-12- putative transformants)

regeneration response in *Agrobacterium* co-cultivated explants, which are discussed below.

Compton (2000) suggested that measures should be taken to use explants of adequate size to ensure that sufficient number of competent cells are present to support cell division and organogenesis, especially in situations that generate cellular stress (e.g., cell culture and genetic transformation). Therefore, optimum explants-size is vital in achieving good regeneration, especially upon *Agrobacterium* co-cultivation. In our study, medium-sized (1cm) explants showed higher shoot regeneration response (32±2.62%) than the other sizes of explants tested, upon *Agrobacterium* co-cultivation (Fig. 3). Similarly, reduction in shoot-regeneration response (Frary and Earle, 1996) and variation in explants-survival

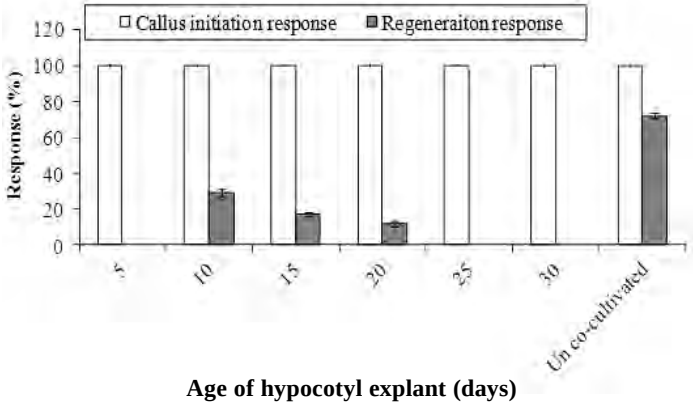

Fig. 2. Effect of age of hypcotyl explant on shoot regeneration response in brinjal cv. Manjarigota upon co-cultivation with *Agrobacterium* (*CD-0.701*; Significant at *P ≤ 0.01*)

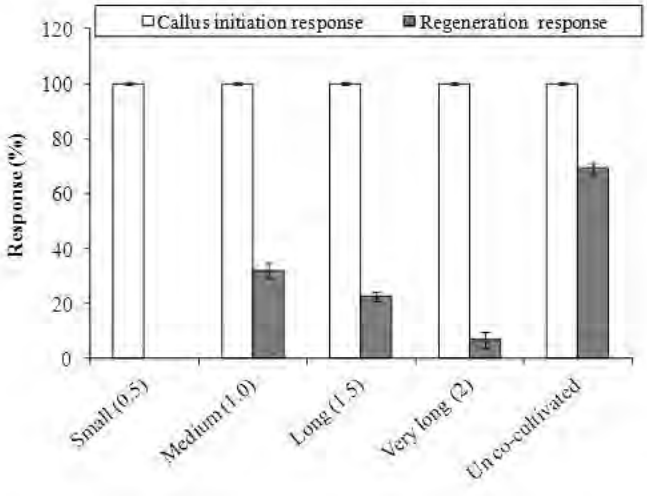

Fig. 3. Effect of size of hypocotyl explant on shoot regeneration response in brinjal cv. Manjarigota upon co-cultivation with *Agrobacterium* (*CD-1.92*; Significant at *P ≤ 0.01*)

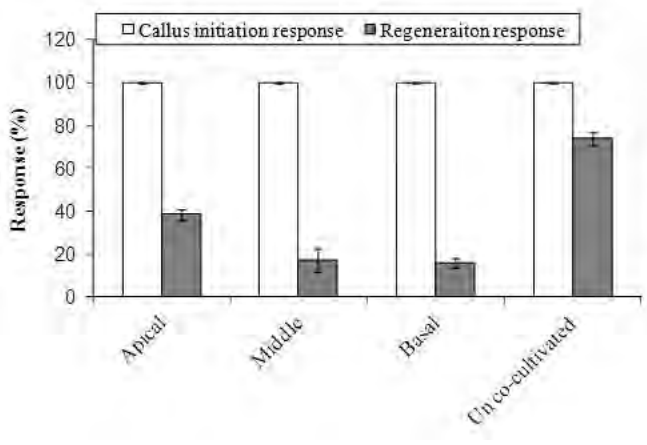

Fig. 4. Effect of position of hypocotyl explant on shoot regeneration response in brinjal cv. Manjarigota upon co-cultivation with *Agrobacterium* (*CD-2.72*; Significant at *P ≤ 0.01*)

(Lazzeri and Dunwell, 1986; Frary and Earle, 1996) was observed, with increasing or decreasing size of the explant.

In the present study, explants from the apical region showed 5-6 days earlier (14-16 days after culture) and higher (38.57±2.61%) shoot-regeneration response than explants from the middle and basal regions. Regeneration response decreased from the apical (upper) to the basal (lower) region of hypocotyl (Fig. 4). Similarly, apical ends of stem segments produced regenerated buds and shoots, while, the basal ends produced only friable callus upon *Agrobacterium* co-cultivation in a study by Billings *et al* (1997). This type of differential response from hypocotyl explants from different positions in tissue culture could be due to differences in an endogenous hormonal gradient (Sharma and Rajam, 1995). Faster and better shoot-regeneration from the proximal end of the hypocotyl explants is perhaps due to production of new shoot apical meristem (Curuk *et al*, 2002). Further, cultured explants usually exhibit polarity in cell proliferation and morphogenesis relative to the position of an organ (or piece of tissue) on the intact plant (George, 1993). However, explants from the apical region were found to show early and high shoot-regeneration response.

In the present study, explants showed no regeneration when 'no' or 'short' (0-1 day) pre-culture period was allowed. The explants gradually turned yellow at the cut-end which progressed towards the middle of the explants. Highest regeneration-response was obtained at four days (33.00± 4.04%) pre-culture (Table 1). Similarly, no regeneration was obtained in hypocotyl explants (co-cultivated without *Agrobacterium*) at short pre-culture;

Table 1. *In vitro* response in hypocotyl explants of brinjal cv. Manjarigota pre-cultured for various durations

Pre-culture period (Days)	Callus-initiation frequency (%)	Regeneration frequency (%)	Score of sensitivity of explants	Remarks (% dried shoots)
0	100	00.00 ± 0.00	B/D	-
1	100	00.00 ± 0.00	B/D	-
2	100	26.33[c] ± 3.76	H	-
3	100	28.33[b] ± 2.33	H/Y	23.07
4	100	33.00[a] ± 4.04	H/Y	46.66
5	100	08.80[d] ± 2.23	H/Y	75.00
6	100	00.00 ± 0.00	H/Y	-
7	100	00.00 ± 0.00	H/Y	-
8	100	00.00 ± 0.00	H/Y	-
Control (without co-cultivation)	100	74.46 ± 3.94	H	Nil
CD		*1.154***		

**Significant at $P \leq 0.01$; *Appearance of *Agrobacterium* overgrowth; B-Brown; D-Dead; H-Healthy; Y-Yellowing; AG+: *Agrobacterium* colonization starting around the explant; AG++: prominent *Agrobacterium* overgrowth around the explant; AG+++: *Agrobacterium* overgrowth covering the explant

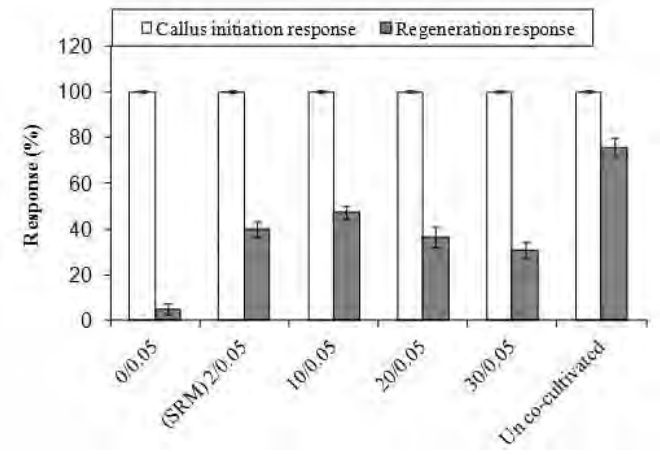

Fig. 5. Effect of high cytokinin (BAP) on shoot regeneration response in hypocotyl explants of brinjal cv. Manjarigota upon co-cultivation with *Agrobacterium* (*CD-19.05*; Significant at $P \leq 0.01$)

longer pre-culture period was also not beneficial in brinjal (Kumar and Rajam, 2005), and, two days' preculture was employed in most of the previously reported transformation studies in various genotypes. Non-competent cells could be made competent by pre-culture of explants on appropriate plant growth factors prior to *Agrobacterium* infection (Nehra *et al*, 1990). It appears that specific pre-culture conditions are required depending upon the explant type (Ling *et al*, 1998), and crop and genotype (Fari *et al*, 1995) for improved regeneration upon *Agrobacterium* co-cultivation. However, our study showed that four days (26.33±3.76) of pre-culture was optimal for best shoot-regeneration in hypocotyl explants of brinjal cv. Manjarigota.

In our study, pre-culture on high cytokinin (BAP) medium did not affect callus-initiation response (98-100%), whereas, it affected shoot-regeneration response significantly. Inclusion of BAP in the pre-culture medium enhanced shoot-regeneration response (Fig. 5). Hypocotyl explants pre-cultured on a culture medium containing 10ìM BAP showed the highest shoot-regeneration response (47.27±2.98%). Similarly, high cytokinin (kinetin) pretreatment was shown to improve shoot-regeneration efficiency in *Agrobacterium*-mediated transformation in tomato (Chy and Philips, 1987). However, in our study, further increase in BAP (>10ìM) in the pre-culture medium resulted in reduced shoot-regeneration response than in the pre-culture medium containing 2ìM BAP (SRM). Herath *et al* (2005) obtained similar results using high concentrations of BAP in culture medium in *kenaf*. Further, in this study, shoots regenerated in cultures pre-cultured on SRM containing concentrations of BAP higher than 10ìM were gigantic (thick stem with broad leaves) and leathery in nature.

In the present study, regenerated shoots in various experiments were healthy, green and showed improvement in growth through subcultures onto SRM containing Kanamycin, and these elongated (75.63% of regenerated shoots; data not shown; Fig. 1C) and rooted (80.46% of elongated shoots; data not shown; Fig. 1D) on a culture medium containing Kanamycin, which shows their resistance to Kanamycin, while Control shoots (regenerated without *Agrobacterium* treatment) showed stunted growth, and, gradually turned yellow due to chlorosis, in the culture medium. Rooted plantlets were hardened. Transformed plants were morphologically normal and fertile (Fig. 1E).

As for molecular analysis of transformants using PCR, amplification of the expected size of 750bp PCR products for *nptII* gene was observed in both the transformants (78.58%) and in the positive Control (Fig. 1F), which showed presence of the transgene. No amplification was found in the negative Controls (untransformed plant and water Control).

In conclusion, physical (size) and physiological (age and position) of the hypocotyl explant and high cytokinin (BAP) in the pre-culture medium showed a strong impact on shoot regeneration response in hypocotyl explants of brinjal co-cultivated with *Agrobacterium*. Pre-culture period was found to influence survival of explants as well shoot regeneration response, and, high cytokinin in the pre-culture

medium was found to enhance shoot regeneration response in hypocotyl explants of brinjal. Testing out effects of these factors in other genotypes and types of explant would help better understand and improve in *in vitro* shoot regeneration response in explants of brinjal co-cultivated with *Agrobacterium*.

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