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Synergistic use of hypocotyl explants and high BAP preconditioning for enhanced transformation frequency in brinjal (*Solanum melongena* L.)

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ABSTRACT

Poor regeneration is one of the limiting factors in the development of transgenic crops since *Agrobacterium* as a plant pathogen can disturb the fragile *in vitro* conditions with wounding and infection regimes. We have tried to optimize the transformation system in two important varieties of brinjal after *Agrobacterium* infection to the explants. The effect of explant was studied and hypocotyls were found to be better than cotyledonary leaves. High BAP during the preconditioning period was found to further enhance the regeneration rate. Therefore, use of hypocotyls and high BAP during preconditioning can improve the regeneration of transformed cells and recovery of transformants in vegetables especially brinjal.

Key words: *Solanum melongena*, transformation, hypocotyl, BAP, preconditioning.

INTRODUCTION

Brinjal (eggplant, *Solanum melongena* L.) is one of the most important vegetable crops in India and Southeast Asia. Crop improvement through breeding has substantially addressed many problems associated with yield and quality in brinjal. The most important factor that is responsible for severe reduction in yield and marketable quality of the crop which needs to be addressed is the damage caused by the brinjal shoot and fruit borer (*Leucinodes orbonalis* Guenee) (Pyralidae: Lepidoptera). No source of stable genetic resistance is available in brinjal germplasm and this has necessitated the use of Bt transgenic technology (Kumar *et al*, 1998**)**. Successful development of transgenics in brinjal depends on the availability of standardized *in vitro* regeneration and *Agrobacterium* mediated genetic transformation systems (Collonnier *et al*, 2001; Rotino and Gleddie, 1990; Kumar and Rajam, 2005).

The success of *Agrobacterium* mediated genetic transformation of plants generally depends on several plant, bacterial and *in vitro* factors such as genotype, explant type, physiological state and age of donor plant, *Agrobacterium* virulence, conditions for inoculation of the tissue with *Agrobacterium*, growth of *Agrobacterium* with respect to the transformed plant cells and plant tissue necrosis caused by *Agrobacterium* (Sharma and Rajam, 1995; Lin *et al*, 1994). A major problem with any transgenic development is the severe reduction in the transformation frequencies even after optimizing the regeneration conditions. This is because *Agrobacterium* is basically a plant pathogen and upon wounding and cocultivation with the explant, the bacterium may elicit defense responses. *Agrobacterium*-induced responses may be similar to abiotic responses, nonpathogenic bacteria and/or unique to *Agrobacterium* only (Robinette and Matthysse, 1990; Ditt *et al*, 2001; Hanur, 2004). Recent studies have unraveled the roles of some of the host factors in *Agrobacterium*-host plant interactions (Ditt *et al*, 2001; Gelvin, 2000). Deliberate infection and cocultivation of the explant with virulent *Agrobacterium* during routine transformation experiments thus affects the established redifferentiation and morphogenetic pathways in the cultured tissues, causing break down of the *in vitro* conditions standardized before transformation.

One efficient way of increasing the regeneration of transformed cells is to optimize the regeneration protocol during and after *Agrobacterium* challenge and under selection pressure. This method not only takes into consideration routine requirements of optimum regeneration conditions but also helps in identifying factors that can be used to regenerate transformed cells *vis-à-vis* untransformed

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cells in the cultured tissue. Moreover, unlike in the case of routine regeneration protocols where non-transgenic regenerants are generated, using this protocol, the regenerants are (mostly) transformants that are useful. We report here a simple way of increasing frequency of transformed regenerants by manipulation of two parameters, choice of explant type and preconditioning with higher levels of the cytokinin hormone, 6-benzylaminopurine (BAP) in two popular varieties of brinjal, Arka Keshav (purple long type)and Manjarigota (synonym Manjarikota) (striped round type).

MATERIAL AND METHODS

Seeds of brinjal cvs. Arka Keshav and Manjarigota were surface sterilized using 70% ethanol for 45-60 sec followed by two washes with sterile distilled water and treatment with sodium hypochlorite (approximately 4% available chlorine) for 5 min. The seeds were germinated aseptically on 0.5X Murashige Skoog (MS) (Murashige and Skoog, 1962) basal medium containing 3% sucrose and 0.8% agar. Cotyledonary leaf explants and single hypocotyl explants excised from 7-10 day old seedlings were used as explants. For regeneration experiments, the MS medium was supplemented with different levels of BAP and a-naphthalene acetic acid (NAA) (shoot induction medium, SIM). In the experiment involving hypocotyl explants, the SIM treatments included 1, 2 or 3 mM BAP and 0.05 or 0.1 mM NAA while for cotyledonary leaf explants, the SIM included 10 or 12.5 mM BAP and 1, 3 or 5 mM NAA. For the experiment involving different levels of BAP, the level of NAA was kept constant at 0.05 mM. The pH of the medium was adjusted to 5.7-5.8 prior to autoclaving at 121°C for 20 min. Aseptic seed germination and culture incubation were carried out at 26±1°C under 16 h photoperiod. A minimum of 8-10 explants was inoculated onto the 100 mm sterile glass petri plates containing respective media. For preconditioning, the explants were maintained on the SIM for 2 days. For high BAP preconditioning experiment, the cultures were placed on the SIM with different (0, 2, 10, 20, 30 and 40 mM) BAP levels (and 0.05 mM NAA) for 2 days and later shifted to the SIM with 2 mM BAP and 0.05 mM NAA during cocultivation and subculture. For the experiment involving explant selection, the total numbers of explants were 658 for cotyledonary leaves and 1061 for hypocotyls (three replications) and for the experiment involving challenging hypocotyls with *Agrobacterium*, the total number of explants was 879 (three replications), using both varieties. For the experiment involving varying levels of BAP in Arka Keshav, the total number of explants was 470 (four replications). After every 8-10 days, subculturing was done onto fresh SIM or shoot elongation medium (SEM, MS medium supplemented with only 2 mM BAP). Kanamycin (Kancin^{Ò}, 100 mg l^{-1}) was used for selection in the medium after cocultivation and during subsequent subcultures of the explants. Cefotaxime (Taxim^{Ò}, 500 mg.l^{-1} for the first subculture and 250 mg l^{-1} for subsequent cultures) was used in the medium to arrest *Agrobacterium* growth.

A. tumefaciens A208 host cells containing pBinBt modified binary vector (Kumar *et al*, 1998a, b) with a cassette containing CaMV35S promoter, synthetic *cry1Ab* coding region, pAOCS terminator and *nptII* selectable marker, were used for challenging hypocotyl explants in the experiments using bacterial treatment and different levels of BAP. *Agrobacterium* was grown on *Agrobacterium* minimal medium (ABMM) broth containing kanamycin (50-100 mg l^{-1}) at 28°C for 6-8 h. Actively growing mid-log phase culture was centrifuged, washed with fresh ABMM broth and concentration adjusted to an O.D. of 0.1-0.3. Hypocotyl explants were cocultivated with this *Agrobacterium* suspension for 10 min and then transferred to the SIM plates. After 2 days, the cultures were transferred to the SIM with kanamycin. Elongated and well-differentiated shoots were excised from callus mass after 15-25 days and transferred onto the SIM, SEM or rooting induction medium (MS medium supplemented with 5 mM NAA).

Table 1. Regeneration response in Arka Keshav and Manjarigota cultivars of brinjal using different explants and *Agrobacterium* treatment

Explant	Cultivar	Mean no. of explants cultured±SE	Mean no. of explants showing regeneration±SE	Regeneration response (%)
Cotyledonary leaves	Arka Keshav	129.3±8.5 (388)*	5.32±4.1 (16)	4.1
	Manjarigota	90.0±0.0 (270)	12.6±3.7 (38)	14.1
Hypocotyls	Arka Keshav	250.1±32.5 (752)	185.6±16.2 (557)	74.1
	Manjarigota	103.0±33.8 (309)	48.3±21.6 (145)	46.9
Hypocotyls treated with *Agrobacterium*	Arka Keshav	133±14.9 (399)	29.6±13.7 (89)	22.3
	Manjarigota	160.0±28.3 (480)	39.0±9.1 (117)	24.4

*Numbers in parantheses indicate total no. of explants

RESULTS AND DISCUSSION

The present study showed that hypocotyl explants were better for regeneration response compared with cotyledonary leaf explants, which are routinely considered as the explant of choice in many crops including tomato and other vegetables (Allichio *et al*, 1982; Magioli *et al*, 1998; Magioli and Mansur, 2005). The use of hypocotyls as explants has not been much investigated (Magioli *et al*, 1998**)**. Our initial observations indicated that hypocotyls were better (with an average of 61%) than cotyledonary leaves (with an average of 9%) in both varieties of brinjal (Table 1) with respect to regeneration *per se*. While hypocotyl explants produced more shoots, leaf explants only showed more callus and rooting (Plate 1). Arka Keshav gave a better regeneration response (74%) than Manjarigota (47%) when hypocotyls were used as explants.

Hypocotyl explants in control (those that were not challenged by cocultivation with *Agrobacterium*) and *Agrobacterium* treatment differed considerably in regeneration averaging 61% and 23.3% responses, respectively. Within the varieties also the reduction was noticeable, albeit with differing magnitudes. Reduction in regeneration was from 74% to 22.3% in Arka Keshav and from 47% to 24.3% in Manjarigota. These results vindicate our view on the effect of *Agrobacterium* infection on regeneration in brinjal (Billings *et al*, 1997). In the experiment involving preconditioning of the hypocotyl explants, in Arka Keshav variety, higher levels of BAP before *Agrobacterium* cocultivation and selection (*i.e.*, during preconditioning), resulted in significant differences in the number of regenerants selected on kanamycin medium (Table 2). The frequencies ranged from 16.8% to 27.2% as compared to 19.7% in the treatment without any BAP (negative control) Preconditioning with 40 mM BAP definitively produced highest frequency of regeneration (27.2%). Previous studies have indicated that the population of competent cells increases by adequate pre-culture period facilitating improved transformation (Hamza and Chupeau, 1993; Lipp-Joao and Brown, 1993; McHughen *et al*, 1989). Extending the duration of pre-culture period increases the proportion of competent cells. Our results are in agreement with the results of earlier preconditioning experiments but with the additional information that higher BAP of 40 mM *during* preconditioning can be beneficial even to the explants challenged with *Agrobacterium*.

Table 2. Regeneration response using *Agrobacterium* treated hypocotyl explants of brinjal cv. Arka Keshav to preconditioning with different levels of BAP

BAP conc (mM) + 0.05 mM NAA	Total no. of regenerants[a] (%)	Mean no. of regenerants±SE
0	17/86[b] (19.7)	18.2±7.0
2	14/83 (16.8)	15.7±4.7
10	8/68 (11.7)	11.6±4.4
20	14/80 (17.5)	16.8±4.1
30	15/76 (19.7)	19.3±5.6
40	21/77 (27.2)	26.4±7.4

[a]Data of 4 replications; [b]No. of explants shooted/Total no. of explants inoculated; C.D *(P=0.01)* : 0.23; SE: standard error

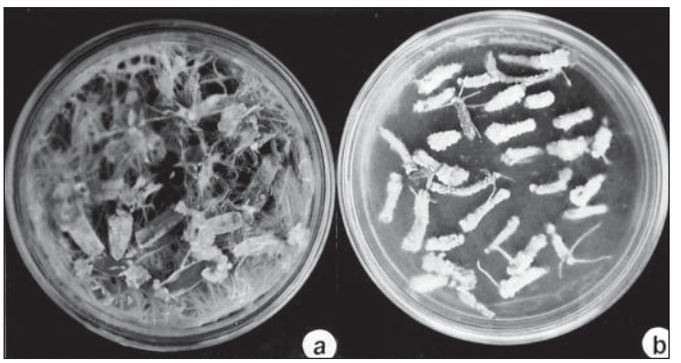

Plate 1. Typical regeneration response in brinjal using cotyledonary leaves (a) and hypocotyls (b) on shoot induction medium. Hypocotyls were better for shoot induction.

Transgenic crop production *via Agrobacterium* mediated gene transfer is dependent, among other factors, on the successful interaction between the *Agrobacterium* and specific plant tissue types, which are competent for *in vitro* regeneration along with certain treatments like preculture (Freitas *et al*, 1997). Our studies have shown that the type of explant and preconditioning treatments can considerably improve the frequency of regeneration of Bt gene transformed tissues in brinjal and this may be applicable in other crops also.

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