J. Hort. Sci.
Vol. 2 (2): 94-98, 2007

Effects of growth regulators and explant-type on *agrobacterium*-mediated transformation in brinjal (*Solanum melongena* L.) cv. Manjarigota

D. P. Prakash, B. S. Deepali, R. Asokan, Y. L. Ramachandra[1], Lalitha Anand and Vageeshbabu S. Hanur
Division of Biotechnology
Indian Institute of Horticultural Research
Hesaraghatta Lake Post, Bangalore - 560 089, India
E-mail: vageesh@iihr.ernet.in

ABSTRACT

Effects of growth regulators and type of explants on transformation and *in vitro* morphogenetic responses of brinjal cv. Manjarigota were studied. Both hypocotyl and cotyledonary explants showed marked influence on *in vitro* morphogenetic responses after *Agrobacterium* co-cultivation. Hypocotyl explants showed callus initiation and regeneration responses earlier than cotyledonary leaves. Hypocotyl explants were found to be better than cotyledonary leaf explants in regenerating shoots after *Agrobacterium* co-cultivation. There was delay and reduction in both callus and regeneration responses in *Agrobacterium* co-cultivated explants. Hypocotyl explants showed the highest regeneration response on MS medium containing 2 µM BAP and 0.05 µM NAA while cotyledonary leaves did not show regeneration response after *Agrobacterium* co-cultivation. However, they showed green buds on MS medium containing 10 µM BAP and 1 µM NAA, which could not differentiate into shoots. Overall, hypocotyl explants were found better in regenerating shoots after *Agrobacterium* co-cultivation.

Key words: Growth regulators, explant, brinjal, transformation

INTRODUCTION

Agrobacterium-mediated transformation in plant species is the most widely used transfer system in plants which has been applied to transform and regenerate a few species with commonly used procedures (Van Wordragen and Dons, 1992). Brinjal is one of the crop plants in which in vitro plant regeneration was achieved on media supplemented with various growth regulators. (Sharma and Rajam, 1995; Gleddie *et al*, 1983; Magioli *et al*, 1998). The nature and concentration of growth regulators, in association with specific genotype, explant type and culture medium can cause significant differences in morphogenetic response of brinjal (Sharma and Rajam, 1995; Magioli *et al*, 1998; Magioli *et al*, 2000; Allichio *et al*, 1982; Gleddie *et al*., 1983). Usually, high-frequency regeneration protocols are employed in transformation studies. The adventitious shoot regeneration capacity of cells or tissues to be used in transformation studies significantly affects success in gene transformation (Yildiz *et al*, 2002). However, highly efficient protocols resulted in low transformation frequency and efficiency of less than 0.1 % in brinjal (Chen *et al*, 1995). Hence, it is necessary to analyze the effect of growth regulators and plant-related factors influencing *Agrobacterium* co-cultivation. 'Manjarigota' is the most preferred south Indian round type brinjal cultivar. Hence, we have made an attempt to study the effects of a basic operational step, growth regulators and explants on transformation and *in vitro* morphogenetic response in brinjal cv. Manjarigota during *Agrobacterium*-mediated transformation.

MATERIAL AND METHODS

Plant material

Genuine breeder-seed material of brinjal cv. Manjarigota was obtained from the Division of Vegetable Crops, IIHR. Seeds were soaked in gibberellic acid (100ppm) for three hours, dipped for 1 minute in 70 % ethanol, washed once in sterile distilled water, followed by sterilization for 8-10 minutes in sodium hypochlorite (approximately 4% available chlorine) solution and rinsed five times in sterile distilled water. These were germinated in culture tubes on half-strength MS (Murashige and Skoog, 1962) basal medium containing 3 % sucrose (w/v); pH was adjusted to 5.8 and the medium was gelled with 0.8 % agar. pH was adjusted to 5.8 before autoclaving.

[1]Department of Biotechnology, Kuvempu University, Shankaraghatta, Shimoga, India

Media sterilization and culture conditions

Culture medium and instruments were sterilized by autoclaving at 121°C, 15 psi pressure for 20 minutes. Cultures were incubated in culture racks provided with white fluorescent tubes with a light intensity of 30-40µE m^{-2} s^{-1} under a 16 hr photoperiod in a culture room maintained at 25°C ± 2°C.

Explants

Fifteen to twenty day old-aseptically-grown seedlings, hypocotyl segments obtained after removing apical meristem and basal root stub (1cm long) and cotyledonary leaves separated from stalk and tip were used as explants.

Growth regulators

Hypocotyl explants were cultured on MS basal medium containing 3 % sucrose (w/v), 1, 2 or 3µM BAP 0.05 and 0.1 µM NAA and gelled with 0.8 % agar. Cotyledonary leaves were cultured on MS basal medium containing 3 per cent sucrose (w/v), 10 and 12.5 µM BAP in combination with 1, 2 or 3 µM NAA and gelled with 0.8 % agar.

Plant transformation

Agrobacterium strain A208 harboring the plasmid pBinBt-01 (Kumar *et al*, 1998) was used for plant transformation. The *npt*II gene conferring kanamycin resistance served as a selectable marker. Explants were precultured for two days on MS medium containing various hormones, depending on the explant-type. These were collected into a sterile petriplate, infected with *Agrobacterium* culture for 20-25 minutes, and placed back onto the parent medium. Explants were co-cultivated for two days, transferred onto culture media containing cefotaxime (500 mg/l) for two days and were then transferred onto medium containing cefotaxime (500 mg/l) and kanamycin (100 mg/l). Hypocotyl explants and cotyledonary leaf explants were cultured without *Agrobacterium* co-cultivation on MS medium containing hormones as specified for these explants, as control.

Data analysis

Observations on *Agrobacterium* overgrowth and health of explants were recorded every week for upto 4 weeks. Observations were further recorded after 4 weeks of culture on callus initiation and regeneration response. All treatments had six replications. Analysis of variance (ANOVA) was carried out to test statistical significance of the results observed. Fischers's Least Significant Difference (LSD) was used to determine statistical significance among means.

RESULTS AND DISCUSSION

Effect of growth regulators on transformation and morphogenetic response in brinjal cv. Manjarigota

In the present study, hypocotyl explants showed first signs of callus initiation and regeneration response at 8-10 days and 18 to 20 days of culture initiation, respectively. All the explants cultured showed callus initiation response. Growth regulator combinations significantly affected regeneration response in hypocotyl explants upon *Agrobacterium* co-cultivation (Table 1, Plate 1). Regeneration was highest (30.85 %) on hypocotyl explants grown in the presence of 2 mM BAP and 0.05 mM NAA, and lowest (18.27%) on 2 µM BAP and 0.1µM NAA. Inclusion of 0.05 mM NAA with BAP showed better regeneration response than 0.01 µM NAA.

Cotyledonary leaf explants showed the first signs of callus initiation and shoot bud initiation at 13-15 days, and four-six weeks of culture initiation, respectively. Cotyledonary leaf explants produced a profuse callus with adventitious roots. Highest number of explants showing green buds was recorded in cotyledonary leaf explants cultured on MS medium containing 10 mM BAP and 1 µM NAA (21%, with an average of 5.98 buds per explant) and

Table 1. Effect of Growth regulator concentration on transformation and morphogenetic response of hypocotyl explant in brinjal cv. Manjarigota

BAP µM	NAA µM response (%)	Callus initiation	Regeneration response (%)
1	0.05	100	25.55[ab]
1	0.1	100	21.31[ab]
2	0.05	100	30.85[a]
2	0.1	100	18.47[b]
3	0.05	100	26.96[ab]
3	0.1	100	18.27[b]

Fractions were converted into percentages; percentage data was subjected to angular transformation; CD= 11.74, SEm=2.060; differences are significant at 1 %; values followed by the same letter are not significantly different.

Table 2. Effect of growth regulators on transformation and morphogenetic response of cotyledonary leaf explant in brinjal cv. Manjarigota

BAP µM	NAA µM	Callus initiation (%) explant±SE	No. of green buds per buds (%) ±SE	Explants showing green
10	1	100	5.98±0.15	21 ± 3.41
10	3	100	3.46±0.20	13 ± 1.91
10	5	100	0.00±0.00	0 ± 0.00
12.5	1	100	4.71±0.16	17 ± 1.914
12.5	3	100	3.24±0.12	5 ± 1.000
12.5	5	100	0.00±0.00	0 ± 0.000

lowest from cotyledonary leaf explants cultured on MS medium containing 12.5 µ MBAP and 1 mM NAA (17 % of explants showed 4.71 green buds). Cotyledonary leaf explants did not show regeneration from shoot buds upon *Agrobacterium* co-cultivation (Table 2). Irrespective of the BAP level, explants cultured on MS medium containing 5 µM NAA did not respond.

Hormonal balance is a key factor in regulation of morphogenesis in cultured explants (Murashige, 1974). At similar ratios, varied concentration of cytokinin (BAP) and auxin (NAA) were used in earlier studies in hypocotyl explants (Matsuako and Hinata, 1979) and cotyledonary leaf explant culture of brinjal (Sharma and Rajam, 1995; Magioli *et al*, 1998) in regeneration studies. Addition of NAA at lower concentration resulted in increased shoot regeneration rate (Makay and Kitto, 1988) and presence of NAA was found to be necessary for *in vitro* regeneration in strawberry (Barcelo *et al*, 1998). Intrinsic hormone levels in a tissue make it respond better at a particular ratio and concentration of hormones, which depends upon the genotype and explant. Usually, high frequency *in vitro* regeneration protocol is used in transformation studies. No report is available on comparison of the effect of hormones on regeneration response in explants with and without *Agrobacterium* co-cultivation. However, various reports show that the effect of growth regulators on transformation frequency depends up on the cultivar and the explant in brinjal (Magioli *et al*, 2000; Billings *et al*, 1997).

Effect of explant on transformation and morphogenetic response in brinjal cv. Manjarigota

In the present study, callus initiation was not affected by hormonal combination upon *Agrobacterium* co-cultivation, both in hypocotyl and cotyledonary leaf explants. Similarly, *Agrobacterium* infection did not affect callus initiation response (96%) in hypocotyl explants. But, it had reduced callus initiation response in cotyledonary and leaf explants to the tune of 50-60 per cent in brinjal cv. Pusa Purple Long (Kumar and Rajam, 2005). However, it reduced callusing response in hypocotyl explants (Arpaia *et al*, 1997) and cotyledonary leaf explants (Prabhavathi *et al*, 2002) upon *Agrobacterium* co-cultivation in other, earlier studies. It appears that survival and response of explants in transformation varied due perhaps to the set of conditions employed in transformation protocol. In the present study, it is clear that *Agrobacterium* co-cultivation and the set of conditions during transformation were not detrimental to the explant and were optimum for survival and response of the explants. Hypocotyl explant is more sensitive to any type of treatment after excision (Yildiz *et al*, 2002), particularly, to *Agrobacterium* infection (Chakrabarty *et al*, 2002) compared to leaf explants (Arpaia *et al*, 1997). However, variation in response may be due to the crop, genotype, nature and physical status of explant along with the set of conditions used in transformation studies.

In the present study, delay in callus initiation and regeneration response was observed in both types of explants upon *Agrobacterium* co-cultivation and 7-8 day delay in callus initiation response was delayed by 5-6 days in hypocotyl explants and 7-8 days in cotyledonary explant, respectively, as compared to the control explant. 7-8 days delay in regeneration response and 2-3 weeks delay in appearance of green buds was observed in hypocotyl explants and cotyledonary explants, respectively compared to control explants. This delay may be due to the following reasons: 1) plant cells need to withstand the shock *Agrobacterium* infection 2) process of transformation has to occur and 3) only transformed cells show response on the selection medium and these have to multiply into sufficient numbers for expression of response. Similarly, callus initiation and regeneration response were delayed in explants co-cultivated with *Agrobacterium*, as compared to the control (without *Agrobacterium* co-cultivation) explant in brinjal (Billings *et al*, 1997).

There was a drastic reduction in the regeneration

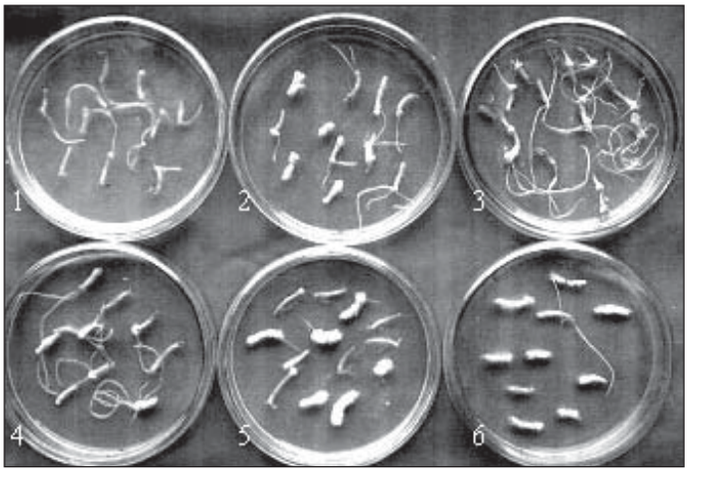

Fig 1. Regeneration response in hypocotyl explants upon *Agrobacterium* co-cultivation cultured on MS medium containing different hormone combinations: 1) 1 µM BAP+0.05 µMNAA, 2) 1 µM BAP+0.1 ìMNAA, 3) 2 µM BAP+0.05 µMNAA, 4) 2 µM BAP+0.1 µMNAA, 5) 3µM BAP+0.05 ìMNAA, 6) 3 µM BAP+0.1 µMNAA

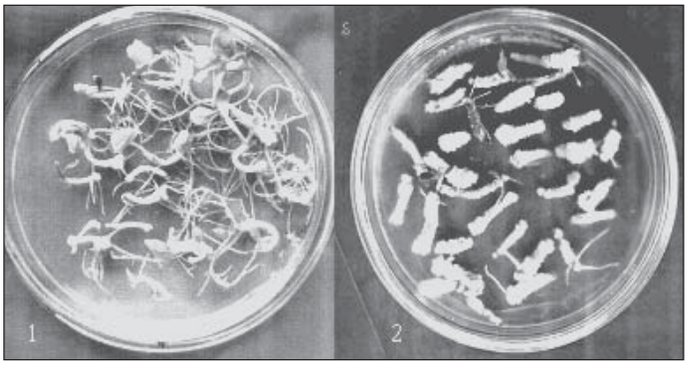

Fig 2. Comparison of regeneration response in hypocotyl explants (1) without and (2) with *Agrobacterium* co-cultivation

response of hypocotyl explants and a complete lack of response of cotyledonary leaf explants upon *Agrobacterium* co-cultivation as compared to the control explants (Plate. 2). Cotyledonary leaf explants produced green buds which could not differentiate into shoots. The occurrence of delayed and reduced regeneration response from explants upon *Agrobacterium* co-cultivation is not uncommon. Possible explanations for this phenomenon are: 1) plant cells may perceive *Agrobacterium* infection as an attack and 2) the inoculation process may influence plant regeneration negatively. Similarly, shoot bud differentiation was drastically reduced in explants subjected to *Agrobacterium* infection in cauliflower (Chakrabarty *et al*, 2002). Hypocotyl explants were most responsive upon *Agrobacterium* infection. Furthermore, early colonization of *Agrobacterium* was a major problem with cotyledonary leaf explants. It might be due to uneven surface of leaf explant, which was not completely exposed to culture media containing cefotaxime.

Arpaia *et al* (1997) reported reduced callusing response in both hypocotyl and cotyledonary leaf explants upon *Agrobacterium* co-cultivation. However, higher regeneration response was noticed in kanamycin-resistant calli obtained from hypocotyl explant as compared to that from cotyledonary explant. Kumar and Rajam (2005) reported higher callus initiation response and lower regeneration response from hypocotyl explant compared to cotyledonary leaf and leaf explants. Therefore, experimental conditions other than type of explant may be responsible for differences in response during transformation. Hypocotyl explant showed better regeneration response upon *Agrobacterium* co-cultivation in brinjal cv. Manjarigota than did cotyledonary leaf explant. Similarly, hypocotyl explant resulted in the highest transformation efficiency compared to leaf and cotyledonary leaf explants in perilla (Lee *et al*, 2005). Hypocotyl explants were successfully used in *Agrobacterium*-mediated transformation in chilli (Nianiou *et al*, 2000) and cauliflower (Chakrabarty *et al*, 2002). In conclusion, it is stated that hypocotyl explant is better as compared to cotyledonary leaf or leaf tissue for transformation studies in brinjal. The present study also vindicate that morphogenetic response varies with growth regulator and explant type in brinjal.

ACKNOWLEDGEMENT

The authors are grateful to the National Agricultural Technology Project (NATP), ICAR, for financial assistance in the form of Competitive Grants Programme (CGP) to the senior author. Thanks are due to Director, IIHR, for encouragement.

REFERENCES

Allichio, R., Grosso, E. D. and Boschueri, E., 1982. Tissue cultures and plantregeneration from different explants in six cultivars of *Solanum melongena. Experientia,* **38**:449-450

Arpaia, S., Mennella, G., Onofaro, V. and Perri, E. 1997. Production of transgenic eggplant (*Solanum melongena* L.) resistant to Colorado Potato Beetle (*Leptinotarsa decemlineata* Say). *Theor. and Appl. Genet.*, **95**:329-334

Barcelo, M., Mansouri, E. I., Mercado, A. J., Quesada, A. M. and Pliego, F., 1998. Regeneration and transformation via *Agrobacterium tumefaciens* of the strawberry cultivar Chandler. *Pl. Cell Tiss. Org. Cult.*, **54**:29-36

Billings, S., Jelenkovic, G., Chin, C. K. and Eberhadt, J. 1997. The effect of growth regulation and antibiotics on eggplant transformation. *J. Amer. Soc. Hort. Sci.*, **122**:158-162

Chakrabarty, R., Viswakarma, N., Bhat, S. R., Kirti, P. B., Singh, B. D. and Chopra, V. L. 2002. *Agrobacterium*-mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower. *J. Biosci.*, **27**:495-502

Chen, Q., Jelenkovic, G., Chin, C., Billings, S., Eberhardt, J.and Goffreda, J.C.1995. Transfer and transcriptional expression of coleopteran *Cry3B* endotoxin gene of *Bacillus thuringiensis* in eggplant. *J. Amer. Soc. Hortl. Sci.*, **120**:921-927

Gleddie, S., Keller, W. and Setterfield, G., 1983. Somatic embryogenesis and plant regeneration from leaf explants and cell suspensions of *Solanum melongena* (eggplant). *Canadian J. Bot.*, **61**:656-666

Kumar, P. A., Mandaokar, A., Sreenivasu, K., Chakrabarti, S. K., Bisaria, S., Sharma, R. S., Kaur, S. and Sharma, R.P. 1998. Insect resistant transgenic brinjal plants. *Mol. Breed.,* **4**:33-37

Kumar, V. S. and Rajam, M. V. 2005. Enhanced induction of *vir* genes results in the improvement of *Agrobacterium*-mediated transformation of eggplant. *J.Biochem. Biotech.,* **14:**89-94

Lee, K. B., Yu. H. S., Kim, H. Y., Ahn, O. B., Hur, S. H., Lee, C. S., Zhang, Z. and Lee, Y. J.2005. *Agrobacterium*-mediated transformation of Perilla (*Perilla frutescens*). *Pl. Cell Tiss. Org. Cult.,* **83**:51-58

Magioli, C., Rocha, A. P. M., de Oliveira, D. E. and Mansur, E., 1998. Effcient shoot organogenesis of eggplant (*Solanum melongena* L.) induced by thidiazuron. *Pl. Cell Rep.,* **17**:661-663

Magioli, C., Rocha, A. P. M., Pinheiro, M. M., Martins, S. G. and Mansur, E. 2000. Establishment of an efficient *Agrobacterium*-mediated transformation system for eggplant and study of a potential biotechnologically useful promoter. *J. Pl. Biotech.*, **2**:43-49

Makay, W. A. and Kitto, S. L. 1998. Factors affecting *in vitro* shoot proliferation of French tarragon. *HortScience*, **113**:282-287

Murashige, 1974. Plant propagation through tissue cultures. *Annual Review of Plant Physiology*, **25**:135-166

Murashige, T. and Skoog, F. 1962. A revised method for rapid growth and bioassays with tissue cultures. *Physiol. Plant*, **15**:473-497

Nianiou, I., Karavangeli, M., Zambounis, A., Tsaftaris, A., Paroussi, G., Voyiatzis, D. and Paroussis, E. 2000. Development of pepper transgenic plants via *Agrobacterium* and biolistic transformation. *Acta Hort.,* **579**:83-87

Prabhavathi, V., Yadav, J.S., Kumar, P.A. and Rajam, M.V. 2002. Abiotic stress tolerance in transgenic eggplant (*Solanum melongena* L.) by introduction of bacterial mannitol phosphodehydrogenase gene. *Mol. Breed.* **9**:137-147

Sharma, P. and Rajam, M. V. 1995. Genotype, explant and position effects on organogenesis and embryogenesis in eggplant (*Solanum melongena* L.). *J. Exptl. Bot.*, **46**:135-141

Van Wordragen, M F. and Dons, H. J. M. 1992. *Agrobacterium*-mediated transformation of recalcitrant crops. *Pl. Mol. Biol. Reporter*, **10**:12-36

Yildiz, M., Ozacan. S. and Er, C. 2002. The effect of different explant sources on adventitious shoot regeneration in flax. *Turkey J. Biol.*, **26**:37-40

(MS Received 10 May 2006, Revised 18 August 2007)