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Development of fruit and shoot borer resistant transgenic brinjal with *Cry1Aabc* gene: An assessment of factors influencing transformation efficiency

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Abstract
Brinjal is popular vegetable crop grown in the tropics and subtropics. Fruit and shoot borer (FSB) *Leucinode orbonalis* is most serious and destructive pest causing heavy yield losses. *Agrobacterium* mediated genetic transformation was performed in two brinjal cultivars *viz* RHRB-35 and Manjarigota with *Cry1Aabc* gene. Several factors influencing transformation efficiency such as type of explants, *Agrobacterium* cell density, infection time, pre-culture duration, co-cultivation duration, overgrowth control antibiotics have been evaluated. Combination of cefotaxime and carbenicillin at 250 mg/l concentration effectively control *Agrobacterium* overgrowth. Two week old Cotyledon and three week old hypocotyls explants precultured for 2 days and then infected with *Agrobacterium* suspension (OD_{600}= 0.2 to 0.4) for 1 min showed maximum transformation efficiency. Among the two brinjal cultivars, RHRB-35 exhibited maximum transformation efficiency (8.44 %). The transgene integration into putatively transformed brinjal eggplant genome was confirmed by PCR analysis using gene specific primers.

Keywords: *Solanum melongena*; explant; overgrowth control antibiotics; transformation efficiency

Introduction
Brinjal (*Solanum melongena* L.) is commonly known as eggplant, aubergine, melongena and guinea squash. It is an often cross pollinated plant with chromosome number 2n = 24. Brinjal is low in calories and high in nutrition. The vegetable has high water content and is good source of fiber, calcium, phosphorus, folate and vitamin B and C. It is also used in ayurvedic medicine for curing diabetes, hypertension and obesity. In popular medicine, it is indicated for the treatment of several diseases, including diabetes, arthritis, asthma, and bronchitis (Kashyap *et al.*, 2003) [6]. It is commonly used as a model crop for its physiological, cellular, biochemical, molecular and genetic diversity (Magioli and Mansur, 2005) [9].

Brinjal is prone to attack from insects, pest and diseases, the most serious and destructive of which is fruit and shoot borer (FSB) *Leucinode orbonalis*. FSB larvae bore into tender shoots and fruits, retarding plant growth, making the fruit unsuitable for the market and unfit for human consumption. Yield losses of 60%–70% even after repeated insecticidal sprays have been reported (Shelton 2010) [18]. Heavy utilisation of pesticides has negative effect on surrounding environment and also creates serious risk to consumer's health and safety.

Development of resistant cultivars through traditional breeding has many limitations like undesirable linkage, time consuming crossing programme, progeny selection and incompatibility among different species (Bhat *et al*. 2013) [2]. Transgenic approach can accelerate the development of FSB resistance in brinjal. Genetic transformation in brinjal *via Agrobacterium* was first reported by Guri and Sink (1988) [4]. Since then there have been many attempts of brinjal transformation for a variety of purposes including insect resistance (Kumar *et al*., 1998), virus resistance (Pratap *et al*., 2011) [3], parthenocarpy development (Rotino *et al*., 1997) [14], abiotic stress tolerance (Prabhavathi *et al*., 2002; Sagare and Mohanty, 2012; Xiao *et al*., 2017) [12, 17, 19].

A wide range of factors, such as genotype, age of explants and type, pH of media, regeneration and co-cultivation conditions, temperature, *Agrobacterium* cell density, gene construct, cell competence after wounding and control of *Agrobacterium* overgrowth influence transformation efficiency (Pawar *et al*. 2013) [11]. In the present study parameters like influence of plant growth regulators, optimization of kanamycin in selection media, selection of explants and age of explants, preculture, co-cultivation of explants, control of *Agrobacterium* overgrowth, rooting, effects of explants and genotype on transformation efficiency were studied for the establishment of an efficient transformation protocol for development of transgenic brinjal expressing *Cry1Aabc* gene.

Materials and Methods

Plant Material

Seeds of two brinjal varieties *viz.* "RHRB-35 and Manjarigota" were collected from All India Co-ordinated Vegetable Improvement Project, Department of Horticulture, Mahatma Phule Agricultural University, Rahuri. Seeds were washed with sterile double distilled water twice and then surface sterilized for 5-6 min in 4 % sodium hypochloride (NaClO). Seeds were further washed with sterilized water thrice and then dried on blotting paper. Seeds were germinated on Murashige and Skoog (MS) inorganic salt medium (Murashige and Skoog 1962) [10]. Seedlings were grown under fluorescent light with 16 hr photoperiod and maintained at 25 ºC.

Gene construct

Agrobacterium tumefaciens strain EHA 105 harbouring recombinant binary vector pBI 121 with *Cry1Aabc* gene and NPTII gene construct was procured from National Research Centre on Plant Biotechnology (NRCPB), New Delhi. *Agrobacterium* was streaked out on Yeast Extract Mannitol (YEM) agar plates containing 10.0 mg/l rifampicin and 50.0 mg/l kanamycin and incubated for 2 days at 28°C. For infection single colony from fresh culture plate was grown and incubated at 28 ºC for 2 days on rotary incubator shaker with constant agitation of 200 rpm in YEM broth medium supplemented with appropriate antibiotics (10.0 mg/l rifampicin, 50.0 mg/l kanamycin). Bacterial suspension was centrifuged at 3000 rpm for 10 min in a 50 ml centrifuge tube. Bacteria pellet was re-suspended in YEM medium without antibiotics and diluted to desired OD (OD_{600} 0.2 to 0.4) and used for transformation or co-cultivation.

Genetic Transformation Protocol

In order to find out minimum lethal concentration of kanamycin for screening of putative transgenics and avoid escapes, cotyledons were cultured on pre-culture medium supplemented with different concentrations of kanamycin (Table 1). To evaluate effect of type and age of explant on transformation efficiency, cotyledons and hypocotyls explants of different age groups (7, 14 and 21 days old seedling) were used for transformation. In order to evaluate the effect of duration of pre-culturing and co-cultivation on transformation efficiency, explants were precultured and co-cultivated for three different durations (for 1, 2 and 3 days). Different concentrations and combinations of cefotaxime and carbenicillin were used for accessing their efficiency in controlling *Agrobacterium* overgrowth (Table 2).

Cotyledons and hypocotyls explants of different age groups were excised from seedlings careful to prevent tissue from bruising. These explants were precultured for 2 days and then infected with *Agrobacterium* suspension (OD_{600}= 0.2 to 0.4) for 1 min with gentle agitation under sterile conditions. Excess culture was blotted dry on sterile blotting paper and explants were subsequently cultured on the fresh co-cultivation media (MS supplemented with 2.0 mg/l BAP, 0.1 mg/l IAA/1.0 mg/l kinetin) for 2 days. Co-cultivated explants were transferred to selective shoot induction medium (MS supplemented with 2.0 mg/l BAP, 0.1 mg/l IAA/1.0 mg/l kinetin, 50 mg/l kanamycin, 250 mg/l cefotaxime, 250 mg/l carbenicillin). Explants were sub-cultured weekly and allowed to grow for 6-8 weeks. Those shoots greater than 4-5 cm in length were excised and transferred to rooting medium (MS supplemented with 1.0 mg/l IBA, 25 mg/l kanamycin, 250 mg/l cefotaxime, 250 mg/l carbenicillin) (Figure 1). The plantlets with well-developed root system were removed from the media. Roots were washed with sterile distilled water and transplanted to soil rite for primary hardening and irrigated with single distilled water after 5-6 days. They were covered with polythene bags for next few days.

Confirmation of transformation by PCR analysis

DNA was isolated from leaves of selected putative transgenic plants. PCR amplification was performed for confirmation of transgene with gene specific primer corresponding to *Cry1Aabc* gene construct (Forward primer: 5'ATGGACAACAACCCAAACATCAACGA3' and Reverse primer:
5'TCATTCAGCCTCGAGTGTTGCAGTAACTAA3').

Amplification reaction was composed of *Taq* DNA polymerase buffer E (M/s Bangalore Genei Pvt.Ltd.) supplemented with 1.0 mM $MgCl_2$, 250 µM of each dNTPs, 1 µM of forward and reverse primers, 20 ng template DNA and 1 unit *Taq* DNA polymerase in a reaction volume of 20 µL. Primer amplification involved an initial denaturation step of 5 minutes at 94 °C. Amplification regime consisted 40 cycles at 94 °C for 30 seconds, 55 °C for 1 minute and 72 °C for 1 minute. Final elongation was allowed for 10 minutes at 72 °C. The amplified fragments were subjected for gel electrophoresis on 1.2% (w/v) agarose gel. Plasmid DNA from *Agrobacterium* was used as control.

Results and Discussion

Influence of plant growth regulators on shoot initiation of explants

In the present study the effect of different combinations of growth regulators *viz*. BAP, kinetin and IAA for *in vitro* growth of different explants was observed. Differential response of two different explants of both genotypes for different growth regulators combination for earlier establishment and higher number of shoot initiation was observed (Data not shown). RHRB-35 showed good shoot initiation on MS media with 2.0 mg/l BAP and 0.1 mg/l IAA. Whereas Manjarigota showed best shoot initiation on MS media with 2.0 mg/l BAP and 1.0 mg/l kinetin for both the explants. Bhat *et al*. (2013) [2] reported maximum regeneration efficiency on MS media with 2.0 mg/l BAP and 1.0 mg/l kinetin. Sarker *et al*. (2006) [15] observed organogenesis on MS supplemented with 1.0 mg/l BAP and 1.0 mg/l Kinetin.

Optimization of kanamycin concentration in selection media

Prior to transformation an effective concentration of kanamycin for selection of transformants was determined. The explants grew very well in control at 0 mg/l kanamycin. Explant survival was found to be decreased with increase in kanamycin concentration and duration of culture. The minimum lethal concentration to kill all non-transformed explants after three weeks was 50 mg/l. Thus 50 mg/l kanamycin was used to select putative transgenic shoots. Pratap *et al*. (2011) [3], Akhter *et al*. (2012) [1] and Jadhav *et al*. (2015) [5] also reported that 50 mg/l kanamycin concentration was optimum for regeneration and selection procedure in brinjal. Thus**,** present results are in accordance with these reports.

Effect of type of explants and age of explants:

Cotyledon was found to be better explants for transformation over hypocotyls explants. Cotyledon from 14 days old

seedlings and hypocotyls from 21 days old seedlings were found to be more suitable for transformation compared to explants obtained from other age groups. Explants at this stage were much compact, green and hardy and capable to co-cultivate. At this stage cells were in dividing phase and more competent for *Agrobacterium* mediated gene transfer with good regeneration efficiency. Explants of early stages were found to be much delicate, less responsive and in degenerative phase. Fari *et al*. (1995) [3] reported that cotyledons from 14 days old *in vitro* seedlings had a good embryogenic and organogenic potential and were superior over leaf explants in regeneration and transformation study. Sarker *et al*. (2006) [15] and Zayova *et al*. (2012) [20] also found that cotyledon explants to be more responsive for regeneration thereby exhibiting high frequency direct organogenesis of shoots.

Pre-culturing of explants

Explants were precultured for two days and those showing swelling and response during preculture were used for co-cultivation. The growth regulators included in preculture media stimulated active cell division which might result in increased transformation efficiency. Explants precultured for two days performed better over those precultured for three days. Kumari *et al*. (2013) [8] also reported that cotyledon and hypocotyl explants pre-cultured for 2 days gave high frequency shoot regeneration in brinjal. Importance of use of precultured and actively dividing cells in transformation has been highlighted by many reports (Sangwan *et al*. 1991; Jadhav *et al*. 2015) [16, 5].

Co-cultivation of explants with *Agrobacterium* strain and overgrowth control

The *Agrobacterium* concentration and duration of co-cultivation plays an important role in transformation for maximum transformation efficiency. The optimum concentration of *Agrobacterium* to be used for transformation was found to be OD_{600} 0.2 to 0.4 in which *Agrobacterium* overgrowth was under control and obtained highest transformation efficiency (8.44% in RHRB-35 and 4.36% in Manjarigota). It was observed that transformation efficiency decreased with increased concentration of *Agrobacterium* cell density due to *Agrobacterium* overgrowth on selection media. Jadhav *et al*. (2015) [5] also reported OD_{600} 0.2 as optimum cell density with which 19.8% transformation efficiency was obtained. Infection time of explants with *Agrobacterium* culture was 1 minutes in the present study. Akhter *et al*. (2012) [1] reported that infection period of 30 to 90 seconds was optimum for this purpose. Jadhav *et al.* (2015) [5] also reported that increase in infection time for 3-4 minutes results in overgrowth of *Agrobacterium*.

Duration of co-cultivation has maximum influence on *Agrobacterium* overgrowth in later stages. Co-cultivation for 2 days was found optimum to control *Agrobacterium* overgrowth. Explants co-cultivated for 1 day died on selection medium indicating that 24 hrs co-cultivation period was not sufficient for obtaining transformants. It was found that extending co-cultivation beyond 3 days resulted in *Agrobacterium* overgrowth that decreased the transformation frequency. Earlier, co-cultivation for 24-48 hrs was reported in many studies (Pratap *et al*., 2011; Akhter *et al*., 2012; Kumari *et al*., 2012) [3, 1].

Use of filter paper during co-cultivation reduces the direct contact of *Agrobacterium* with that of media hence it was found to be helpful in reducing overgrowth by acting as barrier between *Agrobacterium* and media.

In the present investigation effect of antibiotics on *Agrobacterium* overgrowth after 14 and 28 days of co-cultivation was assessed. Cefotaxim and carbenicillin failed to control *Agrobacterium* overgrowth when used individually with a concentration of 250 mg/l and 500 mg/l. However, when both were used together at 250 mg/l concentration each in the media, they effectively controlled *Agrobacterium* overgrowth (Table 2). Similar results were also reported by Jadhav *et al*. (2015) [5].

Effect of explants and genotypes on transformation efficiency

Among the two explants *viz.* cotyledon and hypocotyl, the highest transformation efficiency was obtained from cotyledon explants. On an average 8.44% of transformation efficiency was obtained using cotyledon as explants in RHRB-35 while in Manjarigota it was found to be 4.36%. Hypocotyl explants were previously reported to be inferior for transformation compared to cotyledons. Results of this studies showed that cotyledon is the best explants for transformation. Transformation efficiency of 2% was obtained using hypocotyl explants of RHRB-35, while in Manjarigota it was found to be 1.03%. Genotype specific response to transformation event was observed in the present study with genotype RHRB-35 exhibiting higher transformation efficiency than Manjarigota.

Confirmation of putative transgenics by PCR

DNA isolated from putative transgenic plants of both genotypes was used for PCR analysis by using *Cry1Aabc* gene specific forward and reverse primers. The PCR analysis by gene specific primers resulted in amplification of 1800 bp in putatively regenerated plant DNA samples. Plasmid DNA was used as a positive control. This transformation protocol yielded transgenic brinjal with *Cry1Aabc* gene construct. PCR analysis performed which confirmed that the transgene was successfully integrated into two cultivars of brinjal *viz*. RHRB-35 and Manjarigota (Figure 2 and 3). This transformation protocol proved to be simple, repeatable and can be used for transformation studies in brinjal.

Table 1: Effect of kanamycin concentration on cotyledon explants survival in Manjarigota.

Treatment	Number of explants survived after 1 week	Number of explants survived after 2 week	Number of explants survived after 3 week	Number of explants survived after 4 week
MS + 2.0 mg/l BAP + 0.1 mg/l IAA + 0 mg/l Kan (Control)	50 (100%)	50 (100%)	50 (100%)	50 (100%)
MS + 2.0 mg/l BAP + 0.1 mg/l IAA + 50 mg/l Kan	12 (24%)	8 (16%)	5 (10%)	0 (0%)
MS + 2.0 mg/l BAP + 0.1 mg/l IAA + 75 mg/l Kan	8 (16%)	0 (0%)	0 (0%)	0 (0%)
MS + 2.0 mg/l BAP + 0.1 mg/l IAA + 100 mg/l Kan	6 (12%)	0 (0%)	0 (0%)	0 (0%)

Table 2: Effect of different antibiotic treatments for *Agrobacterium* overgrowth control in co cultivated cotyledon explants in Manjarigota.

Concentration of antibiotics used	Cefotaxime		Carbenicillin		Cefotaxime + Carbenicillin	
	250 mg/l	500 mg/l	250 mg/l	500 mg/l	250 mg/l	500 mg/l
Agrobacterium overgrowth (%) at 14 days	74	56	78	59	0	0
Agrobacterium overgrowth (%) at 28 days	100	80	100	84	0	0

Table 3: Transformation Efficiency in RHRB-35.

Sr. No	Observation recorded	Cotyledon	Hypocotyl
1.	Number of explants infected	150	300
2.	Number of explants showing shoot initiation	147	52
3.	Number of days required for shoot initiation	8.72	21.05
4.	Number of explants showing elongation and survival	74.66	34
5.	Number of explants showing rooting	12.66	6
6.	Number of days required for rooting	42.05	43.99
7.	Transformation efficiency	8.44%	2%

Table 4: Transformation Efficiency in Manjarigota.

Sr. No	Observation recorded	Cotyledon	Hypocotyl
1.	Number of explants infected	145	290
2.	Number of explants showing shoot initiation	139	32
3.	Number of days required for shoot initiation	13.06	26.70
4.	Number of explants showing elongation and survival	68.66	17
5.	Number of explants showing rooting	6.33	3
6.	Number of days required for rooting	49.09	50.42
7.	Transformation efficiency	4.36%	1.03%

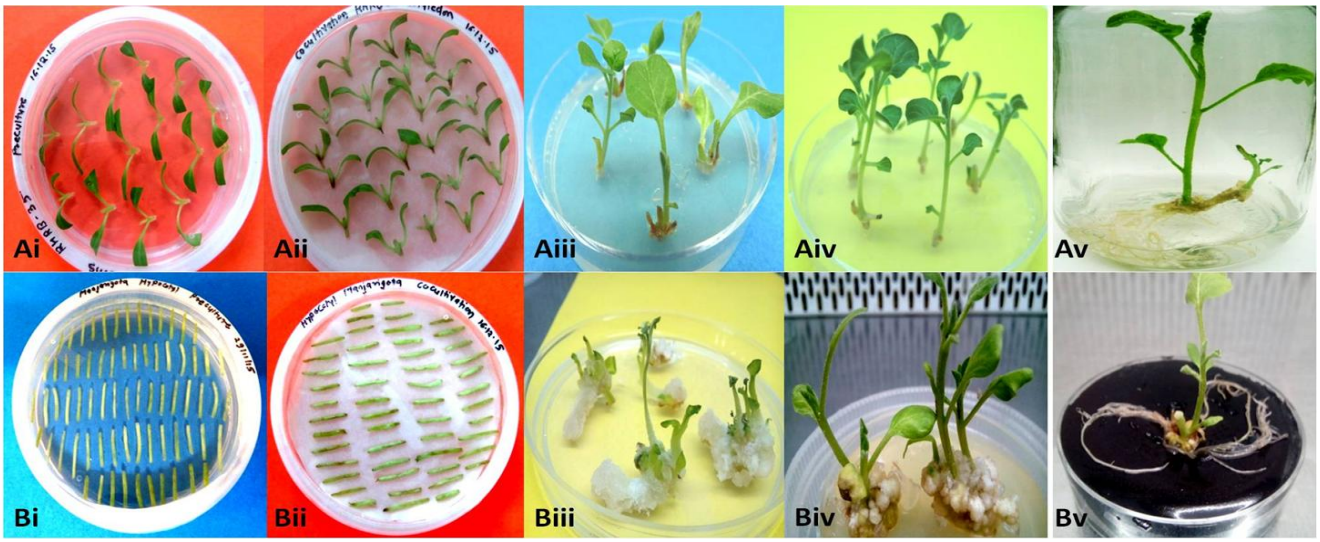

Fig 1: *Agrobacterium* mediated genetic transformation in brinjal using cotyledon and hypocotyl explant (Ai and Bi) Pre-culturing of explant on MS +2.0 mg/l BAP + 0.1 mg/l IAA; (Aii and Bii) Co cultivation of; (Aiii and B iii) Shoot initiation on MS +2.0 mg/l BAP + 0.1 mg/l IAA + 50 mg/1 kanamycin + 250 mg/1 cephotaxime + 250 mg/1 carbenicillin (Aiv and Biv) Shoot elongation (Av and Bv) Rooting of an putatively transformed elongated shoot on MS + 1.0 mg/l IBA mg/l + 25 mg/1 kanamycin + 250 mg/1 cephotaxime + 250 mg/1 carbenicillin.

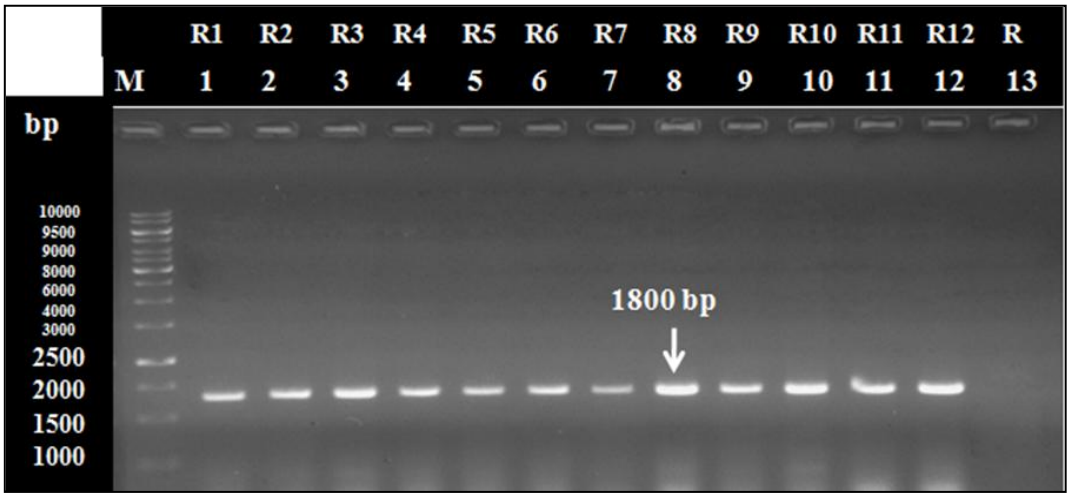

Fig 2: Molecular characterization of putative transformants of brinjal, cv. RHRB-35. Confirmation of presence of transgene using cry1Aabc gene specific primers. M = marker (1 kb ladder), R1-R12= DNA of putative transformed plants, R = non-transformed DNA (control DNA).

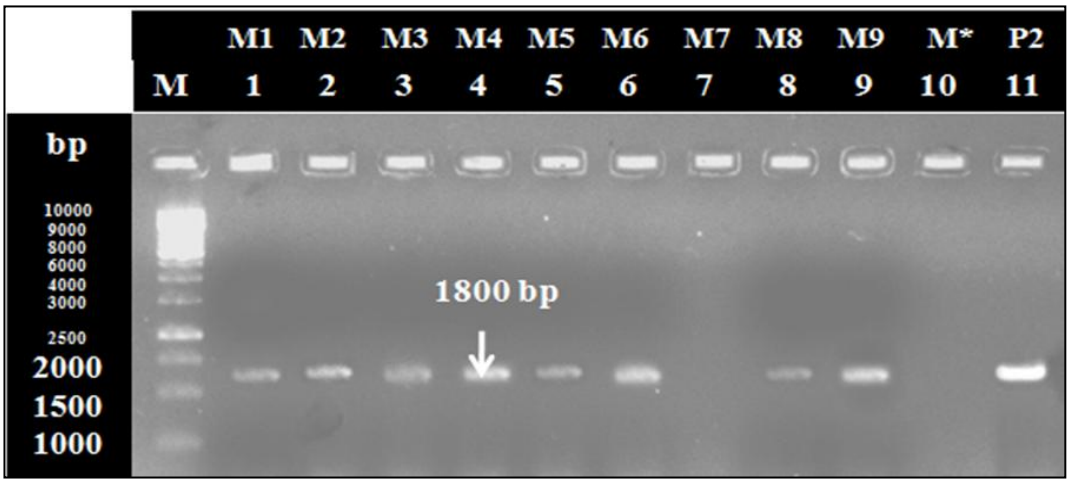

Fig 3: Molecular characterization of putative transformants of brinjal, cv. Manjarigota. Confirmation of presence of transgene using cry1Aabcgene specific primers. M = marker (1 kb ladder), M1-M9= DNA of putative transformed plants, R = non-transformed DNA (control DNA), P2=Plasmid DNA (Positive control).

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