



Research Article

***Agrobacterium*-mediated transformation of brinjal (*Solanum melongena* L.) using fungal resistant gene**

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Abstract

The investigation standardized the protocol for *Agrobacterium*-mediated transformation system in Brinjal (*Solanum melongena* L), cultivar 'Arka Neelkanth'. Initially the regeneration system was standardized with regeneration medium (MS basal medium supplemented with 8.88 μ M BAP + 1.14 μ M IAA) by using 10-12 days old cotyledon explants. Transformation of the *hevein*, a gene responsible for fungal resistance, into cotyledon explants was carried out with *Agrobacterium tumefaciens* strain LBA4404 harbouring binary plasmid pGPTV/*hevein* (Plasmid Gus Plant Transformation Vector) with marker gene neomycin phosphotransferase (*npt-II*). Transformation efficiency showed a higher percentage in one day pre-cultured explants, infected with bacterial suspension of optical density 0.4 at OD₆₀₀ nm with 1:1 dilution for 10min and 48hrs co-cultivation on regeneration medium in the dark. Transformed explants were cultured on selection medium. The PCR molecular confirmation of amplified *npt-II* gene from the genomic DNA of the transformed plant indicates the integration of the *hevein* gene in the brinjal plant.

Key words

Pre-culture, *Agrobacterium* transformation, co-cultivation, selection, regeneration

INTRODUCTION

Eggplant is a vegetable crop widely cultivated in the Mediterranean area (France, Spain, Italy, Greece and North African countries), India and China. The *Leucinodes orbitalis* is a shoot and fruit borer insect under the family Lepidopteran, which specifically damages Brinjal. The farmers use high doses of toxic and carcinogenic fungicides and pesticides to protect the brinjal plant from this insect/pest (Meah, 2003). As a result, fungicide and pesticide residues enter the human food chain and may cause serious health hazards. These fungicides and pesticides are costly and also harm our environment. So use of insect/pest resistant varieties of brinjal are better alternatives to chemical control. Genetic engineering techniques like transformation can accelerate the development of brinjal varieties, which is not possible through traditional breeding and tissue culture alone. *A. tumefaciens*-mediated transformation is an effective and widely used approach to introduce desirable genes into plants. Guri and Sink (1988) first reported the *Agrobacterium*-mediated transformation of brinjal, and others also quickly followed (Rotino and Gleddie 1990; Filippone and Lurquin 1989; Kumar *et al.*, 1998;

Hanyu *et al.*, 1999; Fari *et al.*, 1995). *Hevein* is a chitin-binding protein that presents in laticifers of the rubber tree (*Hevea brasiliensis*). A cDNA clone (*HEV1*) encoding the *hevein* (strong antifungal protein against several fungi) isolated by using the polymerase chain reaction. *HEV1* consists of 1018 base pairs long that includes 204 amino acids as an open reading frame. The deduced amino acid sequence consists of a putative signal sequence of 17 amino acid residues followed by a 187 amino acid polypeptide. This polypeptide chain has two features. The amino-terminal region of 43 amino acids is identical to *hevein* and it shows homology to several chitin-binding proteins and to the amino termini of wound inducible proteins in potato and poplar (Broekaert *et al.*, 1990). Stable transformation of brinjal with different *Agrobacterium*-vectors has been achieved using various explants of *in vitro* plantlets (Guri and Sink, 1988; Filippone and Lurquin, 1989; Franklin and Sita, 2003). This study describes a protocol for genetic transformation in brinjal (*Solanum melongena* L.) using cotyledon explants and expression of *hevein* and *npt-II* gene in the transformed plant using *A. tumefaciens* LBA4404 strain carrying binary vector pGPTV/*hevein*.

MATERIALS AND METHODS

Plant material: Seeds of brinjal cultivar *Arka Neelkanth* were obtained from the Indian Institute of Horticulture Research (IIHR- ICAR) Bangalore. Seeds were sown on coir-pith containing 96 well pro-tray and sprinkled with water once in a day and it germinated as small plantlets. The 10-12 days old cotyledons were used as explants for *Agrobacterium*-mediated transformation.

Culture media: MS medium (Murashige and Skoog, 1962) was prepared adjusted to pH 5.8 and sterilized by autoclaving at 121°C/15psi for 15min. For regeneration, MS basal medium with growth hormones and for selection, MS basal medium with growth hormones and antibiotics were used. Hormone-free MS medium with antibiotics was used for root induction and LB broth was used for *Agrobacterium* culturing (Table 1).

Bacterial strain and vector: *A. tumefaciens* strain LBA4404 harbouring binary plasmid pGPTV/*hevein* was used for transformation. It contains selectable marker

gene *npt-II* encoding the enzyme neomycin phosphotransferase conferring kanamycin resistance.

Explant sterilization: The 10-12 days old cotyledons were used as explants for *Agrobacterium*-mediated genetic transformation. Explants surface sterilization was done by 0.5% bavistin for 30min to avoid the fungal infection. Then the explants were washed with 2% (v/v) of detergent Tween-20 for 10min and then with 2% sodium hypochlorite for 10min and after explants sterilized with 70% ethanol for 30 seconds followed by continuous shaking 0.1% mercuric chloride (HgCl₂) for 90 seconds then finally rinsed three times with sterilized distilled water. Then the explants edge portion was cut and inoculated on petri-dish containing regeneration media.

Pre-culture: After surface sterilization, the cotyledon explants were inoculated on petri-dish containing regeneration media and kept 24hrs for pre-culture. Per petri-dish, around 20 explants were inoculated.

Table 1. Different types of media used for *Agrobacterium* transformation in brinjal

Name of medium	Basal medium	Growth hormones	Antibiotics
MS medium	MS basal medium	-	-
Regeneration medium	MS basal medium	BAP - 8.88 µM IAA - 1.14 µM	-
Selection medium	MS basal medium	BAP - 8.88 µM IAA - 1.14 µM	Augmentin - 250 mg/l Cefatoxin - 200 mg/l Kanamycin - 050 mg/l Kanamycin - 050 mg/l Rifampicin - 010 mg/l Streptomycin -100 mg/l
Luria- Bertani (LB) broth medium	LB Medium	-	-

The methods standardized in this investigation include *Agrobacterium* density and infection time and cocultivation period,

Agrobacterium density and infection: A single colony of *Agrobacterium* harbouring gene pGPTV/*hevein* was taken from the master plate and inoculated to 10ml of LB broth containing antibiotics. The cultures were kept at 28°C in orbital shaker incubator overnight for 18 hours. After growth, the bacterial suspension has to be maintained at optical density of 0.2, 0.4 and 0.6 OD₆₀₀ nm, then it was diluted 1:1 dilution with sterile distilled water. The pre-cultured cotyledon explants were infected with diluted *Agrobacterium* by immersion method for the different intervals for 05, 10 and 15 seconds and were blotted dry on pre-sterilized filter paper.

Co-cultivation: The infected explants were inoculated on petri-dish containing regeneration medium and allow to grow in different intervals of 24, 48 and 72 hours in the dark for co-cultivation. In each petri-dish, around ten explants were inoculated.

Selection media transfer: After co-cultivation, the explants transferred to the selection medium containing different antibiotics (Augmentin-250 mg/l, Cefatoxin-200 mg/l and Kanamycin-50 mg/l) for callus, shoot induction and shoot elongation. The shoot regeneration was recorded and the effect of *Agrobacterium* concentration during infection, infection time and co-cultivation period were studied with respect to transformation frequency. The shoots obtained were transferred to the rooting medium to obtain complete plantlets.

Rooting and hardening: The elongated shoots were transferred to the test tube containing rooting medium for root induction. The rooted plants were transferred to poly-cup containing sterilized coco-peat and 1% neem cake for 15days under the poly-tunnel. Subsequently, plantlets transferred to a greenhouse for acclimatization. Then the hardened plants were transferred to poly-bags containing a sterilized mixture of garden soil: sand: farmyard organic manure (2:2:1) ratio under the greenhouse for further development.

Molecular confirmation by PCR: The presence of the *npt-II* gene was confirmed by the polymerase chain reaction (PCR) using *npt-II* gene-specific primer pair Forward primer (5'-CTG AAT GAT CTG CAG GAC GAG G-3') and Reversed primer (5'-GCC AAC GCT ATG TCC CGA TAG C-3'). Plant genomic DNA isolation and subsequent PCR analysis was carried out according to Edwards *et al.* (1991). Plant genomic DNA was isolated using fresh leaves of both putatively transformed as well as non-transformed (control) plants using the CTAB (Cetyl Trimethyl Ammonium Bromide) method

Antifungal activity of transformed plant: The transformed brinjal plant treated with *Fusarium oxysporum* to check antifungal activity. A loopfull of culture was taken from potato dextrose agar plate and diluted with 10ml sterile distilled water in the test tube in aseptic condition. Then the diluted culture poured near the root zone of the transformed, non-transformed plant and the control plant was not treated with the culture but simply maintained for comparison (Koo *et al.*, 1998). The antifungal activity of the transformed and non-transformed plants were tested, according to Lee *et al.* (2003).

Statistical analysis: Each experiment consisted of nine treatments. In each treatment, 30 explants were inoculated (9x30=270 explants inoculated). Statistical analysis was done using the Graphpad InStat Statistics Version 3.1.

RESULTS AND DISCUSSION

Brinjal regeneration: The objective of the present study was to transform the *hevein* gene into brinjal through *Agrobacterium*, but before doing this plant regeneration system was standardized using cotyledon explant. The majority of reports suggest using cotyledons from 10-12 days old seedlings for transformation (Frary and Earle,

1996; Park *et al.*, 2003; Sun *et al.*, 2006; Kaur and Bansal, 2010; Rai *et al.*, 2012). The necrotic effect occurred from more than 15-days old seedlings. The 10-12 days old cotyledon explants were inoculated on the regeneration medium (MS basal medium fortified with BAP 8.88 μ M and IAA 1.14 μ M) and were found to respond well for callus induction, shoot development and elongation (Plate 1 A). After shoot induction, shoot elongation occurs in the same culture bottle of regeneration medium. MS hormone-free medium used for root induction, then the rooted plants kept hardening under the greenhouse.

Agrobacterium transformation: The cotyledon explants of cultivar Arka Neelkanth were infected with *A. tumefaciens* strain LBA4404 harbouring pGPTV with *hevein* gene. *Agrobacterium* culture was grown in LB broth medium with kanamycin antibiotic at 28°C in orbital shake incubator for 18hrs as the plasmid has kanamycin resistant *npt-II* gene. The 24hrs pre-culture cotyledon explants respond well for infection and transformation. Factors influencing successful transformation, such as optical density of bacterial suspension, infection time and co-cultivation period were optimized during this investigation.

Effect of Agrobacterium density 0.2, 0.4 and 0.6 at OD 600nm with other variables: The transformation frequency depends on the optical density of *Agrobacterium* suspension, duration of infection, and co-cultivation. But at the same time, more growth of *Agrobacterium* inhibits the growth of cotyledons and leads to the death of the explants. Also, less growth of *Agrobacterium* causes improper infection on explants leads to chimeras and escapes. Later on, the non-transformed explants die on the selection medium. In this study, the maximum 13.00 \pm 1.00 explants induced callus, 32.00 \pm 1.00 shoots produced from the callus,

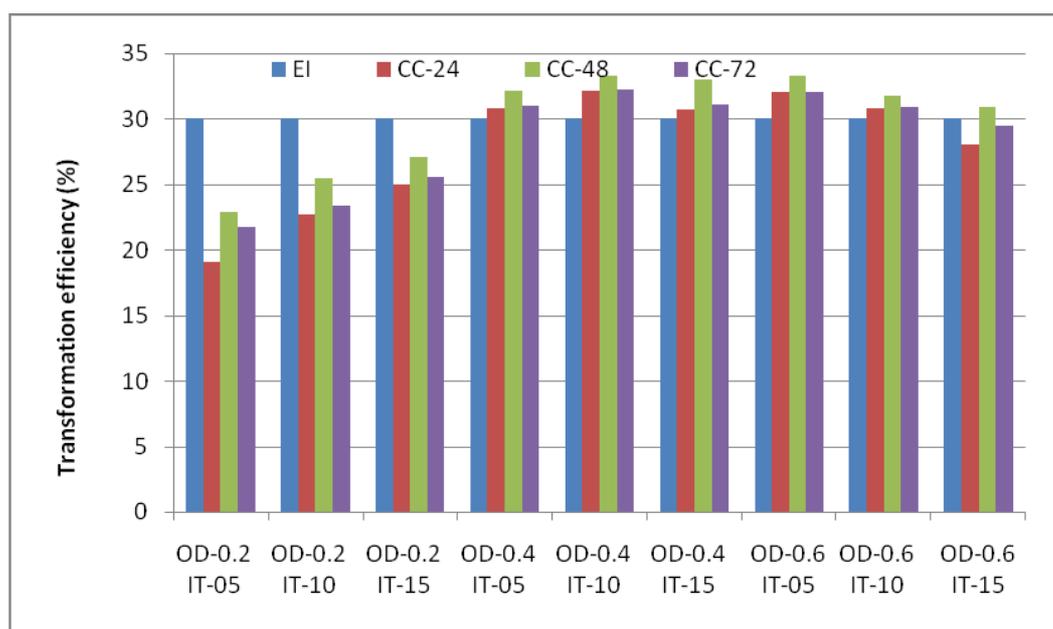


Fig. 1. Transformation efficiency on cultivar Arka Neelkanth

10.66±0.57 shoots survived on the selection medium, and the transformation efficiency was 33.33% (Table 2; Fig 1; Plate 1 B-G) in the *Agrobacterium* density 0.4 at OD 600nm, infection time 10 min, and 48 hrs co-cultivation period found higher transformation efficiency of cultivar Arka Neelkanth followed by the transformation efficiency was 32.00% in the density of *Agrobacterium* 0.6 at OD 600nm, on infection time at 5 min and co-cultivation period at 24 hrs. Consequently, the minimum 04.66±0.57 explants induced callus, 14.00±1.00 shoots produced from the callus, 02.66±1.52 shoots survived on the selection medium and the transformation efficiency was 19.04% on 10 min duration of infection and 48 hrs co-cultivation periods explants on *Agrobacterium* density of 0.2 at OD 600 nm (Table 2; Fig 1; Plate 1 B-G). The increasing the

infection time beyond a critical time length resulted in a decrease in transformation efficiency. *Agrobacterium* infection time 10 min found higher transformation efficiency of the transformants. The transformation efficiency increased with the increasing co-cultivation period. Still, during the long co-cultivation period (more than three days), bacteria were found to grow densely on the co-culture medium, which was not suitable for the growth and survival of co-cultured explants. Hence, the co-cultivation period of two days found to be the most suitable optimum condition for transformation. In this study, during *Agrobacterium* density 0.4 at OD 600nm, cultivar Arka Neelkanth shows higher performance on infection time at 10min and co-cultivation period at 48 hrs.

Table 2. Transformation efficiency of *hevein* gene into brinjal cultivar Arka Neelkanth at various concentration of *Agrobacterium* with different incubation periods

Duration of infection time (min)	Bacterial density (OD ₆₀₀)	Co – cultivation Period (hrs)	Number of explant inoculated	Number of explant responded for callus	Total number of shoots produced from responded callus	Number plants that had <i>npt-II</i> gene	Transformation efficiency (%)
5	0.2	24	30	04.66±0.57	14.00±1.00	02.66±1.52	19.04
		48	30	06.33±0.57	16.00±1.00	03.66±0.57	22.91
		72	30	06.00±1.00	15.33±0.57	03.33±0.57	21.73
10	0.2	24	30	05.66±0.57	14.66±0.57	03.33±0.57	22.72
		48	30	07.00±1.00	17.00±1.00	04.33±0.57	25.49
		72	30	06.00±1.00	15.66±0.57	03.66±0.57	23.40
15	0.2	24	30	05.33±0.57	13.33±0.57	03.33±0.57	25.00
		48	30	07.00±1.00	16.00±1.00	04.33±0.57	27.07
		72	30	06.00±1.00	14.33±0.57	03.66±0.57	25.58
5	0.4	24	30	10.00±1.00	26.00±1.00	08.00±1.00	30.76
		48	30	12.00±1.00	29.00±1.00	09.33±0.57	32.18
		72	30	11.00±1.00	28.00±1.00	08.66±0.57	30.95
10	0.4	24	30	12.00±1.00	29.00±1.00	09.33±0.57	32.18
		48	30	13.00±1.00	32.00±1.00	10.66±0.57	33.33
		72	30	12.66±0.57	31.00±1.00	10.00±1.00	32.25
15	0.4	24	30	11.00±1.00	29.33±0.57	09.00±1.00	30.68
		48	30	12.00±1.00	31.33±0.57	10.33±0.57	32.97
		72	30	12.00±1.00	30.00±1.00	09.33±0.57	31.11
5	0.6	24	30	11.00±1.00	25.00±1.00	08.00±1.00	32.00
		48	30	13.00±1.00	27.00±1.00	09.00±1.00	33.33
		72	30	12.00±1.00	26.00±1.00	08.33±0.57	32.05
10	0.6	24	30	11.00±1.00	26.00±1.00	08.00±1.00	30.76
		48	30	12.66±1.15	28.33±0.57	09.00±1.00	31.76
		72	30	12.00±1.00	27.00±1.00	08.33±0.57	30.86
15	0.6	24	30	10.66±0.57	25.00±1.00	07.00±1.00	28.00
		48	30	12.00±1.00	27.00±1.00	08.33±0.57	30.86
		72	30	11.66±0.57	26.00±1.00	07.66±0.57	29.48

Each value is the mean ± SD of three replicates

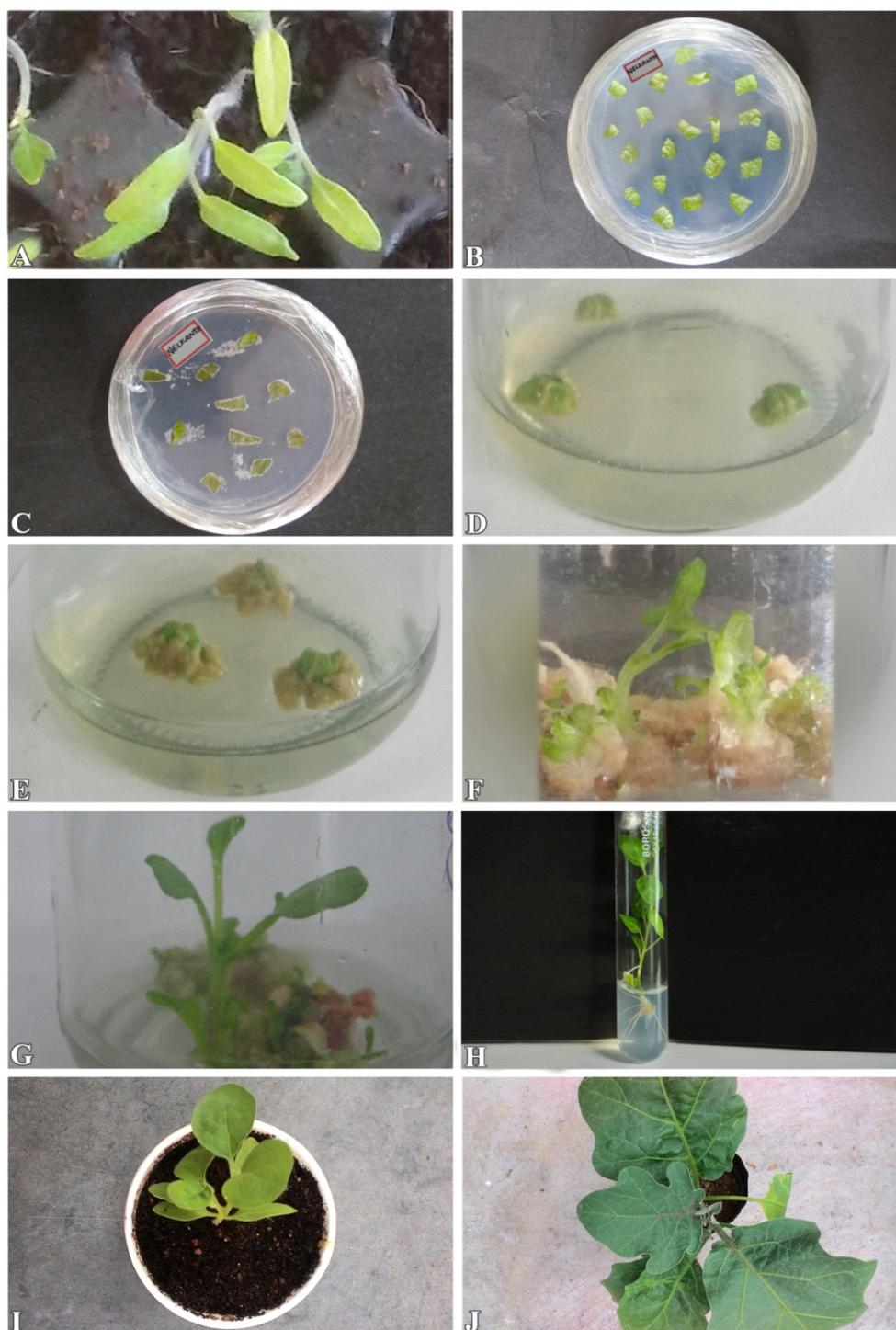


Plate 1. Cultivar Arka Neelkanth shows transformation of brinjal with different stages: (A) Germination of seeds after 10 days from seedling, (B) 24 hrs pre-cultured explants on petri-dish, (C) *Agrobacterium* treated and 2 days co-cultivated explants under dark on petri-dish, (D) Explants inoculated on culture bottles containing selection medium, (E) Callus induction after 15 days from inoculation, (F) Shoot induction after 30 days from inoculation, (G) Shoot elongation after 45 days from inoculation, (H) Root induction after 15 days from transfer to test tube containing rooting medium, (I) Hardened plant in poly-cup after 30 days from hardening, (J) Early stage of transformed brinjal plant in poly-bag after 30 days from planting.

Selection media and antibiotics role: In the present study, both callus induction and shoot regeneration achieved at the same kanamycin level in the transformed explants. In the present investigation, the putative transgenic shoots selected on the selection medium containing 50mg/l kanamycin. The non-transformed tissues did not survive on the selection medium containing 50mg/l kanamycin. The transformed shoots only survived in this medium. Rotino and Gleddie (1990) reported that the transgenic calli from leaf explants could be selected efficiently in the presence of 100 mg/l kanamycin. Chauvin *et al.* (1999) observed that by increasing the concentration of kanamycin generally resulted in full or partial inhibition of regeneration. Kanamycin sensitivity is the most widely used selectable marker for plant transformation and sensitivity of a plant species. In the present investigation, the putative transgenic shoots were selected on the selective shoot regeneration medium containing 50mg/l kanamycin. Charest *et al.* (1988) observed that only 35 mg/l of kanamycin totally inhibited shoot induction from co-cultivated thin layer cells of explants of *Brassica napus*, and 50 mg/l concentration inhibited shoot regeneration of *Brassica oleracea* (Dixit and Srivastava, 1999; Bhalla and Smith, 1998). Chimeras or escapes are a serious problem in the plant transformation system. The exact and optimum concentration of the selection medium needs to be employed in plant transformation to overcome this problem. In contrast, the non-transformed turned necrotic.

Callus and shoot induction of transformed plant: The callus induction occurs after 15 days from explants on the selection medium. The transformed explants only survive on this media, non-transformed turned brown colour and died due to necrotic. Around 40% of the

explants only respond for callus induction. After 30 days from the date of inoculation, the shoot induction has occurred from callus. After 45 days from the date of inoculation, the shoot elongation occurred 2-3cm height; all the initiated shoots are not survived around 20% of the shoots survived and elongated from the shoot induction, the escapes and chimeras did not survive further. Various explants previously reported for brinjal transformation were less efficient in transgenic calli induction and regeneration; in general, the transformation efficiency of leaf and leaf segments was 0.76 and 0.05 plants per co-cultivated explant (Rotino and Gleddie 1990; Hanyu *et al.*, 1999).

Rooting and hardening: The roots occurred from the elongated shoots after 15 days to transfer into test tube containing hormone-free rooting medium. Plants with well-developed roots transferred to poly-cups containing sterilized coco-peat and 1% neem cake for 15 days under the poly-tunnel for primary hardening. Subsequently, plantlets transferred to a greenhouse for acclimatization. Then the plantlets transferred to poly-bags containing a mixture of garden soil: sand: farmyard organic manure (2:2:1) ratio under the greenhouse for further development. The regenerated putative transformed and non-transformed (control) plantlets were appeared normal. Magioli *et al.* (1998) reported that the 70% rooting efficiency only after exposing the cultures briefly to hormone-free media before excising the shoots. In this study, in cultivar Arka Neelkanth 20 numbers of shoots inoculated and 15.00 ± 0.00 number of shoots responded to develop roots and leaves. The average 05.33 ± 0.50 number of leaves per plant and 03.33 ± 0.50 number of roots per plant observed (Table 3; Plate 1 H-J).

Table 3. Response of shoots, leaves and roots/plant from the ex-agar plants of transgenic plant

Shoots inoculated	Shoots responded	Leaves/plant	Roots/plant
20	15.00 ± 0.00	05.33 ± 0.50	03.33 ± 0.50

Each value is the mean \pm SD of three replicates

Molecular confirmation by PCR: The gel picture of the PCR amplification of *npt-II* gene (Fig 2) showed that the DNA band of 560bp size was found in positive control (plasmid DNA) and not in negative control (of non-transformed brinjal plant DNA). The same band also expressed in putatively transformed plants which confirmed the integration of *npt-II* gene along with the *hevein* gene in the plant genome.

Antifungal activity of transformed plant: *F. oxysporum* was cultured on the potato dextrose agar (Fig 3). In cultivar, Arka Neelkanth, after 15 days from treatment with *F. oxysporum* the treated non-transformed brinjal plant got affected by leaf wilting and brown colored stem rotting

(necrosis). But, the treated transformed plant was not affected by wilt disease and it survived better. Hence, the present study confirmed that the transformed brinjal plant had resistance against the fungus *F. oxysporum* (Fig 4).

In the present investigation, maximum transformation efficiency was observed at the concentration of *A.tumefaciens* density OD_{600} at 0.4 with 1:1 dilution, 10min infection time, 24 hrs pre-culture and 48 hrs co-cultivation in the dark. The putative transgenic shoots selected on the selection medium containing 50 mg/l kanamycin. The callus induction, shoot development and shoot elongation occurred in the same selection medium. The root induced

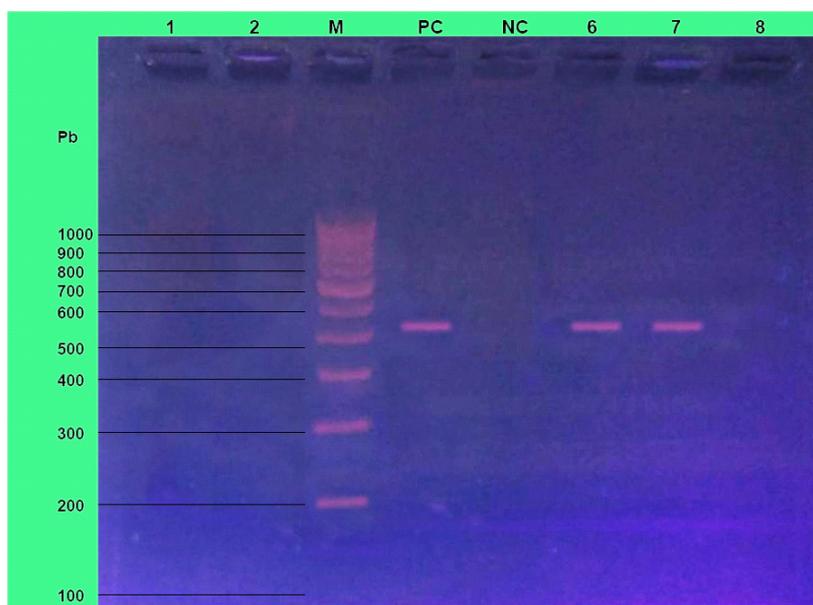


Fig. 2 Polymerase Chain Reaction of *npt-II* gene in cultivar Arka Neelkanth: lane-3 shows 100-1000bp marker DNA (ladder), lane-4 shows plasmid DNA (positive control), lane-5 shows non-transformed plant DNA (negative control), lanes-6 and 7 shows DNA from transformed plant.

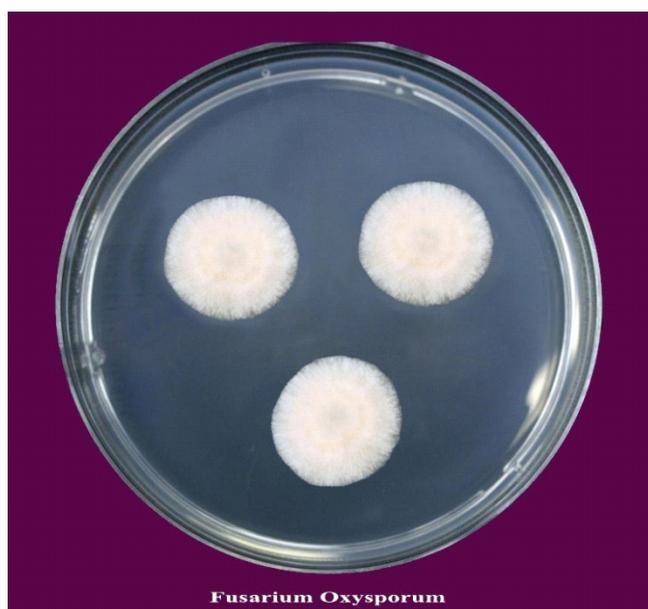


Fig. 3. *Fusarium oxysporum* cultured on the potato dextrose agar.

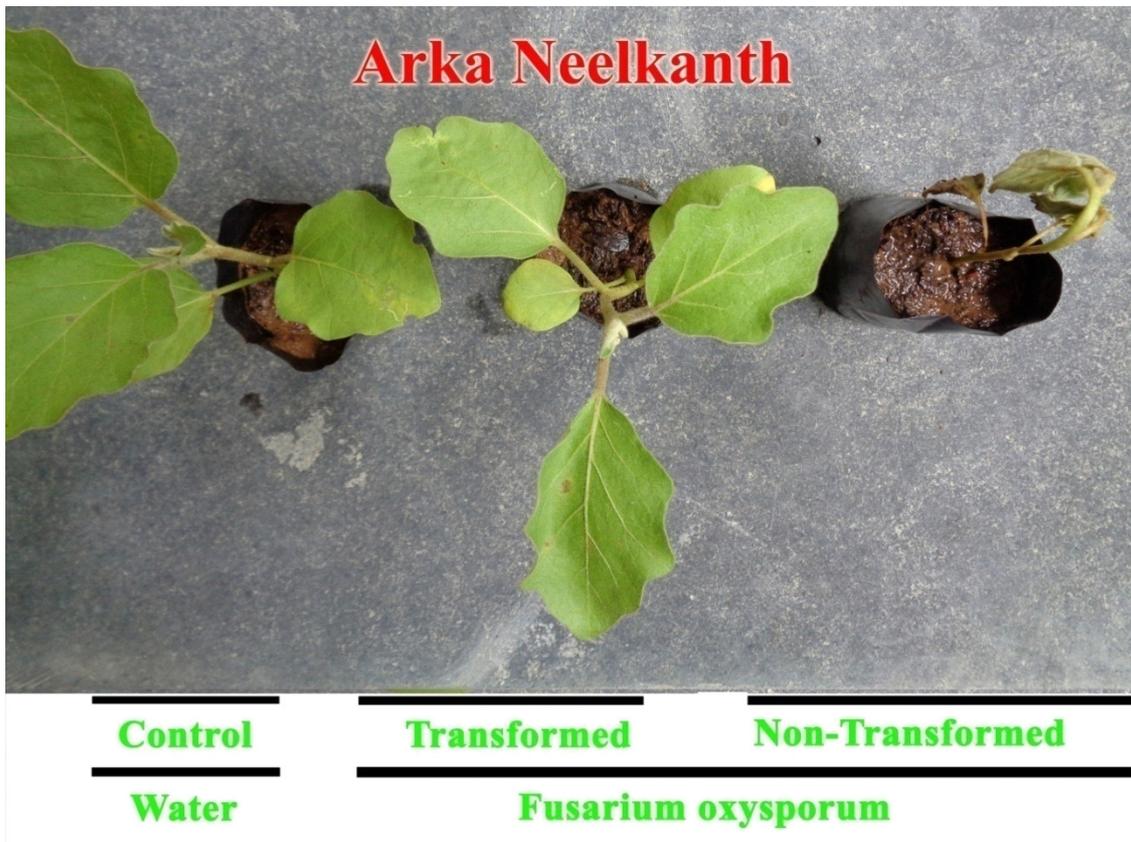


Fig. 4. Cultivar Arka Neelkanth after 15 days from treatment with *Fusarium oxysporum*, the non-treated control brinjal plant survived, the treated transformed plant was not affected by wilt disease it was survived well and the treated non-transformed plant was affected by leaf wilt and stem rotting.

on hormone-free MS medium containing antibiotics. For primary hardening, the rooted plants were transferred to the poly cup for 15 days under poly tunnel. Subsequently, the hardened plants were transferred to poly-bags under the greenhouse for further development. Molecular confirmation carried out by using the PCR method. The

antifungal activity of the transformed plants was tested and confirmed with *F. oxysporum*

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