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Agrobacterium-Mediated Transformation of *hevein* gene to *Lycopersicum esculentum* Mill. Cultivar Arka Abha

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***Abstract*: A simplified and efficient transformation method was developed to produce a transgenic *Lycopersicum esculentum* plant. Primarily the regeneration system was standardized with 10-12 days old cotyledon explants inoculated on Regeneration Transfer (RT) medium (RT = MS basal medium supplemented with 8.88µM of BAP and 1.14µM of IAA) for callus, shoot induction and MS basal hormone-free medium for root induction. Transformation work was carried out with *Agrobacterium tumefaciens* strain LBA4404 harbouring binary plasmid *PGPTV/hevein* (Plasmid Gus Plant Transformation Vector) with kanamycin resistance marker gene -neomycin phosphotransferase (*npt-II)*. Transformation efficiency of cotyledon explants showed higher percentage on 24hrs pre-culture on petri-dish containing RT medium, infection with bacterial suspension having optical density 0.4 at OD_{600}nm on with 1:1 dilution for 10min and 48hrs co-cultivation period on petri-dish containing RT medium in the dark. Putative transformed explants were cultured on Selection Transfer (ST) medium (ST medium = RT medium containing antibiotics Augmentin - 250mg/l + Cefatoxin - 200mg/l + Kanamycin - 050mg/l). The Polymerase Chain Reaction (PCR) molecular confirmation of amplified *npt-II* gene from genomic DNA of the transformed tomato plant was an indication of the integration of *the hevein* gene in the tomato plant. The antifungal activity was confirmed by treating the transformed plant with *Fusarium oxysporum* and had resistance against the fungal disease**.

***Keywords*: Regeneration, Pre-culture, Co-cultivation, *hevein* gene, *Agrobacterium*.**

1. INTRODUCTION

Tomato (*Lycopersicum esculentum* Mill. under the family Solanaceae) an important vegetable crop widely cultivated throughout the world and rich in vitamins A and C, carotenoids and lycopene [01]. Effective and sustained control of fungal pathogens is an essential issue in agricultural systems and the global losses caused by pathogens are estimated to be about 12% of the potential crop production. A lot of chemical fungicides used in the agricultural system; many fungi are continually becoming resistant to existing fungicides and also it causes an environmental issue. *Agrobacterium*-mediated transformation remains the most successful gene transfer method in plants because of its advantages associated with natural gene transfer, low copy number, defined and preferential integration of the transgene into transcriptionally active regions on the chromosome and production of fertile transformed plants [02],[03],[04]. *L. esculentum* is one of the most important vegetable crops and a genetic model for improving other dicotyledonous crop plants. The first report of tomato transformation was by McCormick [05]. Since then there have been numerous publications of transformation in various cultivars of tomato [06],[07],[08],[09],[10],[11],[12],[13]. An improvement of transformation efficiency, mainly due to the optimization of kanamycin concentration during shoot initiation, elongation and rooting of tomato regenerates also reported [14].

The *hevein* is a chitin-binding protein and present in laticifers of the rubber tree (*Hevea brasilensis*). A cDNA clone (HEV1) encoding of *hevein* isolated by using the polymerase chain reaction. HEV1 has 1018 base pairs long and includes an open reading frame (ORF) of 204 amino acids. The observed amino acid sequence contains a signal sequence of 17 amino acid residues followed by a 187 amino acid polypeptide. The amino terminal region (43 amino acids) is identical to *hevein* and shows homology to several chitin binding proteins and the amino termini of wound-inducible proteins in potato. The carboxyl-terminal portion of the polypeptide has 144 amino acids and it is 74-79% homologous to the carboxyl terminal region of wound-inducible genes of potato [15]. Plants produce a vast number of antifungal compounds as part of pre-existing and developmentally regulated defence barriers and also as components of the defence response induced upon pathogen infection. The best studied among them are several classes of proteins having anti-microbial properties which include chitinases [16],[17]. To obtain maximum efficiency of transformation using *Agrobacterium* factors like bacterial inoculum density, explants age, inoculation period, pre-incubation period and co-cultivation duration are acting a significant role in this process.

2. MATERIALS AND METHODS

Germination of Seedlings

Seeds of tomato (*L. esculentum)* cultivar Arka Abha were obtained from Indian Institute of Horticulture Research (IIHR-ICAR) Bangalore. Seeds were sown on coir-pith containing 96 well pro-tray and sprinkled with water once a day and it germinated as small plantlets.

Culture Media preparation

Murashige and Skoog (MS) medium prepared and adjusted to pH 5.8, then sterilized by autoclave at 121°C for 15 min [18]. Here, MS basal medium (MS) = Hormone free MS medium, Regenerative Transfer (RT) = MS basal medium + growth hormones and the Selection medium (ST) = MS basal medium + growth hormones + antibiotics were used. Petri-dish containing RT medium used for pre-culture and co-cultivation; culture bottles containing ST medium used for regeneration and selection; test tubes containing MS hormone-free media used for root induction. The test tube was containing LB broth with antibiotics used for *Agrobacterium* culture (Table-1).

Table-1: Different types of media used for Agrobacterium transformation on Tomato.

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

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 load of microbe. Then the explants were washed with 2% (v/v) of detergent Tween-20 for 10min and then with 10% sodium hypochlorite for 10min and after explants sterilized with 70% ethanol for 30 seconds followed by continuous shaking 0.1% mercuric chloride ($HgCl_2$) for 90 seconds then finally rinsed 3 times with sterilized distilled water. Then the explants edge portion was cut and inoculated on Petri-dish containing RT media. The cotyledons explants from 10-12 day old seedling suggested a better response for transformation [13],[19].

Pre-culture

After surface sterilization, the cotyledon explants edge portion dissected 0.5cm x 1cm then inoculated on petri dish containing RT media and kept 24hrs pre-culture, 20 explants inoculated per petri dish. The 24hrs pre-culture cotyledon explants provide a better response for infection and transformation [20].

Agrobacterium density and infection

A single colony of *Agrobacterium* containing PGPTV/*hevein* was taken from (AB-KRS) master plate and inoculated to 10ml of LB broth containing kanamycin (50mg/l), rifampicin (10mg/l) and streptomycin (100mg/l). The cultures were kept at 28^0C for 18 hours in orbital shaker incubator under overnight. After growth, the bacterial suspension having optical density maintained on 0.2, 0.4 and 0.6 at OD_{600}nm then it was diluted 1:1 with Autoclaved Filtered Double Distilled Water (AFDDW). The pre-cultured cotyledon explants were infected with diluted *Agrobacterium* swirled for different interval l5, 10 and 15 minutes then blotted dry on pre-sterilized filter paper. In the present study *Agrobacterium tumefaciens* strain LBA4404 harbouring with binary plasmid PGPTV/*hevein* used for transformation. It contains selectable marker gene *npt-II* encoding the enzyme neomycin phosphotransferase conferring kanamycin resistance. The transformation frequency found to be increased with the increasing optical density of the *Agrobacterium* suspension [21].

Co-cultivation

The infected explants inoculated on petri-dish containing RT medium for different interval 24, 48 and 72 hours in the dark for co-cultivation. Per petri-dish around 10 explants were inoculated, per treatment 100 explants (10 x 10 = 100) were inoculated. During that time the virulence genes in the bacterium would have been induced. The bacteria would be bound to the plant cells around the wounded edge of the explants and the gene transfer process would have occurred. The two days co-cultivation period was found most suitable optimum condition for transformation, the similar duration was adopted by several researchers [19],[22].

Selection media transfer

After the transformation had occurred, the leaf discs were washed 3-4 times with Augmentin- 250mg/l and final wash were given with double sterile distilled water. The explants were transferred to Selection Transfer (ST) media containing different antibiotics (Augmentin - 250mg/l, Cefotaxime- 200mg/l and Kanamycin - 050 mg/l) for callus, shoot induction and shoot elongation. The percentage of shoot regeneration observed and the effect of *Agrobacterium* concentration during infection, infection time and co-cultivation period studied concerning transformation frequency.

Callus, shoot induction and shoot elongation

The cotyledon discs were cultured on ST media supplemented with BAP in 8.88µM and IAA 1.14µM. In each culture bottle around 3 pieces of cotyledon was inoculated and the cultures were incubated at 25 ± 2°C under 16/8 hours (day/night) photoperiod for callus induction, shoot induction and shoot elongation. In tomato, the callus induction and regeneration depends on genotype and the right explants selection [19].

Root induction and hardening

The 2-3cm elongated shoots obtained from induced shoots were transferred to test tubes containing rooting medium (MS hormone-free medium) for root induction [23]. The rooted plants transferred to poly-cups containing sterilized coco-peat and 1% neem cake for 10 days under the poly-tunnel. Subsequently, plantlets transferred to 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 the further development.

Molecular confirmation by PCR

Plant genomic DNA isolated from fresh leaves of transformed and non-transformed (control) plants using the CTAB (Cetyl Tri-methyl Ammonium Bromide) method. PCR amplification of genomic DNA from transgenic lines revealed for the presence of *npt-II* gene using 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') [24].

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Antifungal Activity of Transformed Plant

The transformed tomato plant treated with *Fusarium oxysporum* to check antifungal activity. The culture was taken from potato dextrose agar plate and diluted with 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 simply maintained for comparison [25].

Statistical analysis

Each experiment 9 treatment conducted, per treatment 100 explants were inoculated (per experiment 9x100=900 explants inoculated) and each experiment was repeated thrice, mean value taken from each independent experiments. Tissue culture data were subjected to analysis of variance by One-Way ANOVA to detect the significance of differences among treatment means using Duncan's Multiple Range Test at $P < 0.05$.

III. RESULTS AND DISCUSSION

Regeneration of tomato

Plant regeneration system standardized with cotyledon explants primarily for the transformation of the *hevein* gene into a tomato plant through *Agrobacterium*. The majority of reports for transformation suggest using cotyledons from 10-12 day old seedlings for transformation [13],[19],[26], [27], [28].

Table-2: Germination percentage of *Lycopersicum esculentum* cultivar Arka Abha.

Cultivar name	No of days	No of seeds showed	Percentage of Germination
Arka Abha	10-12	100	72

Seeds of tomato Arka Abha were sown on nursery pro-tray and sprinkled with water once a day and it was germinated well. The seed germination percentage 72 was observed on Arka Abha. The cotyledon explants respond well for callus, shoot induction and shoot elongation in RT media and root induction occurred MS hormone-free media (Table-2; Plate-1 A).

***Agrobacterium*-mediated transformation**

Tomato cultivar Arka Abha cotyledon explants were infected with *Agrobacterium tumefaciens* strain LBA4404 harbouring binary plasmid PGPTV with *hevein* gene. Agrobacterium culture grew well in LB broth containing kanamycin (50mg/l), rifampicin (10mg/l) and streptomycin (100mg/l) for overnight (18hrs) at 28^{o}C in an orbital shaker. The 24hrs pre-culture cotyledon explants gave good response for infection and transformation. Factors influencing successful transformation namely optical density of the bacterial suspension, infection time and co-cultivation period optimized during this investigation. Bacterial growth, virulence enhancement and dilutions for co-cultivation were carried out according to McCormick [20].

Influence of *Agrobacterium* optical density 0.2 at OD $_{600}$ nm with other variables

The transformation frequency was found to be increased with the optical density of the *Agrobacterium* suspension [21], [29]. More growth of *Agrobacterium* inhibits the growth of cotyledons and lead to the death of explants also less growth of *Agrobacterium* causes improper infection on explants *to* lead to chimeras and escapes, later on the non-transformed explants died on the selection medium. In this study during *Agrobacterium* density 0.2 at OD_{600}nm, cultivar Arka Abha shows the higher performance on infection time at 10min and co-cultivation period at 48hrs. Here 30 infected explants inoculated among the inoculants **12.00±0.00** explants was induced callus, **09.00±0.00** shoots produced from the callus, **06.00±0.00** shoots survived on the selection medium and the transformation efficiency was **20.00**% (Table-3; Graph-1; Plate-1 B-G).

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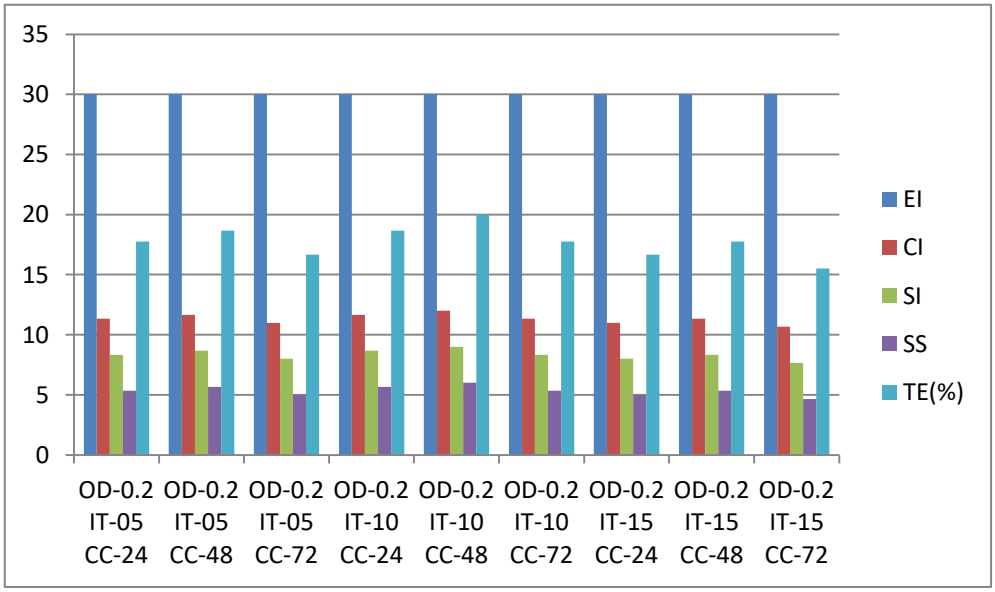
Table-3: Influence of *Agrobacterium* density 0.2 at OD_{600}nm with other variables and transformation efficiency on *hevein* positive shoots in cultivar Arka Abha.

No of explants	OD_{600}nm	Infection Time (min)	Co-cultivation Period (hrs)	Number of explants Inoculated	Mean of explants induced callus	Mean of shoots Induced	Mean of *hevein*+ shoots survived	Percentage of Transformation efficiency (%)*
100	0.2	05	24	30	11.33±0.57^{c}	08.33±0.57^{c}	05.33±0.57^{c}	17.76
			48	30	11.66±0.57^{b}	08.66±0.57^{b}	05.66±0.57^{b}	18.66
			72	30	11.00±0.00^{d}	08.00±0.00^{d}	05.00±0.00^{d}	16.66
100	0.2	10	24	30	11.66±0.57^{b}	08.66±0.57^{b}	05.66±0.57^{b}	18.66
			48	30	**12.00±0.00^{a}**	**09.00±0.00^{a}**	**06.00±0.00^{a}**	**20.00**
			72	30	11.33±0.57^{c}	08.33±0.57^{c}	05.33±0.57^{c}	17.76
100	0.2	15	24	30	11.00±0.00^{d}	08.00±0.00^{d}	05.00±0.00^{d}	16.66
			48	30	11.33±0.57^{c}	08.33±0.57^{c}	05.33±0.57^{c}	17.76
			72	30	10.66±0.57^{e}	07.66±0.57^{e}	04.66±0.57^{e}	15.53

*Transformation efficiency = Mean of *hevein*+ shoots/ Number of explants inoculated × 100.



Graph-1: Influence of Agrobacterium density 0.2 at OD_{600}nm with other variables and transformation efficiency on *hevein* positive shoots in cultivar Arka Abha.

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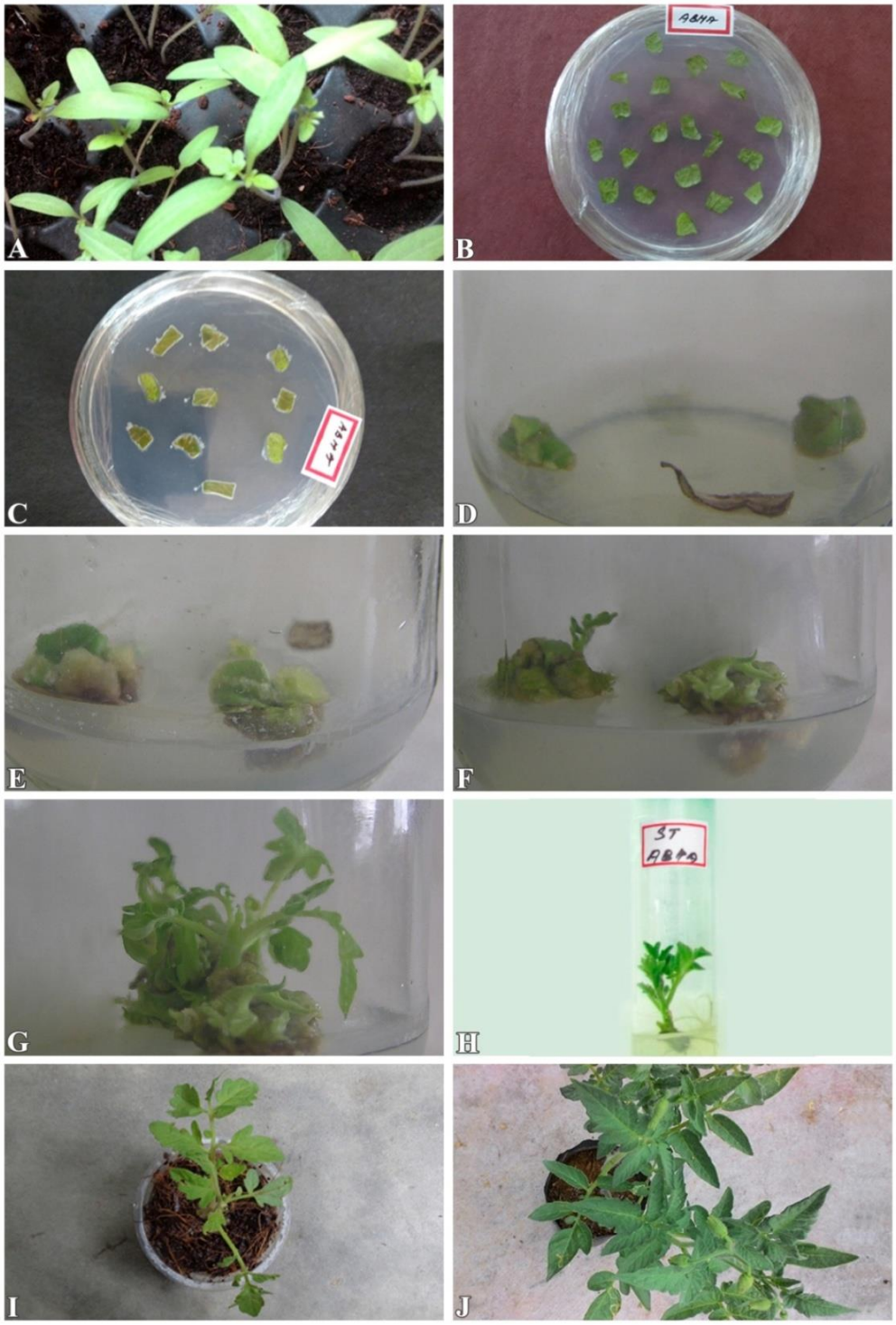

Plate-1: Different stages of *in vitro* regeneration and gene transformation of tomato cultivar Arka Abha: (A) Germinated seedlings after 10 days from seeds, (B) 24hrs pre-cultured explants on petri-dish, (C) *Agrobacterium* treated and 2 days co-cultivated explants under dark on petri-dish, (D) Explants 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 20 days from hardening and (J) Early stage of transformed tomato plant in poly-bag after 30 days from planting.

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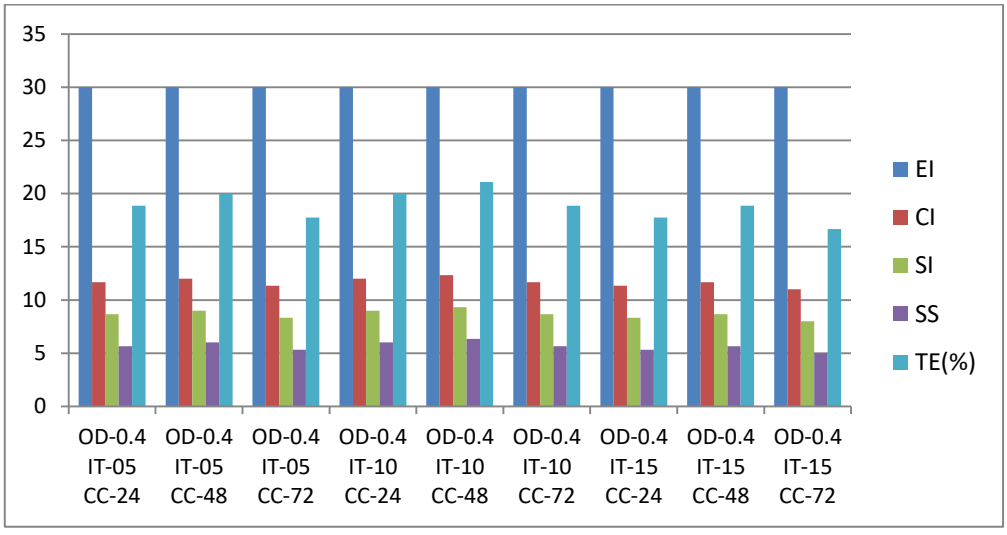
Influence of Agrobacterium density 0.4 at OD_{600} nm with other variables

To increase the infection period beyond a critical time length resulted in a decrease in transformation efficiency in all the tested varieties [21]. *Agrobacterium* infection time10min found higher transformation efficiency of the transformants. In this study, during *Agrobacterium* density 0.4 at OD_{600}nm, cultivar Arka Abha shows higher performance on infection time at 10min and co-cultivation period at 48hrs. Here 30 infected explants inoculated among the inoculants **12.33±0.57** explants was induced callus, 0**9.33±0.57** shoots produced from the callus, 0**6.33±0.57** shoots survived on the selection medium and the transformation efficiency was **21.10**% (Table-4; Graph-2; Plate-1 B-G).

Table-4: Influence of *Agrobacterium* density 0.4 at OD_{600} nm with other variables and transformation efficiency on *hevein* positive shoots in cultivar ArkaAbha.

No of explants	OD_{600}nm	Infection Time (min)	Co-cultivation Period (hrs)	Number of explants Inoculated	Mean of explants induced callus	Mean of shoots Induced	Mean of *hevein*+ shoots survived	Percentage of Transformation efficiency (%)*
100	0.4	05	24	30	11.66±0.57^{c}	08.66±0.57^{c}	05.66±0.57^{c}	18.86
			48	30	12.00±0.00^{b}	09.00±0.00^{b}	06.00±0.00^{b}	20.00
			72	30	11.33±0.57^{d}	08.33±0.57^{d}	05.33±0.57^{d}	17.76
100	0.4	10	24	30	12.00±0.00^{b}	09.00±0.00^{b}	06.00±0.00^{b}	20.00
			48	30	**12.33±0.57^{a}**	**09.33±0.57^{a}**	**06.33±0.57^{a}**	**21.10**
			72	30	11.66±0.57^{c}	08.66±0.57^{c}	05.66±0.57^{c}	18.86
100	0.4	15	24	30	11.33±0.57^{d}	08.33±0.57^{d}	05.33±0.57^{d}	17.76
			48	30	11.66±0.57^{c}	08.66±0.57^{c}	05.66±0.57^{c}	18.86
			72	30	11.00±0.00^{e}	08.00±0.00^{e}	05.00±0.00^{e}	16.66

*Transformation efficiency = Mean of *hevein*+ shoots/ Number of explants inoculated × 100.



Graph-2: Influence of *Agrobacterium* density 0.4 at OD_{600}nm with other variables and transformation efficiency on *hevein* positive shoots in cultivar Arka Abha.

Influence of *Agrobacterium* density 0.6 at OD_{600}nm with other variables

In this present study, the transformational efficiency increased with the increasing co-cultivation period but during long co-cultivation period (more than three days) bacteria were found to grow densely on the co-culture medium which was not suitable for growth and survival of co-cultured explants. Hence, the co-cultivation period of two days was found to be

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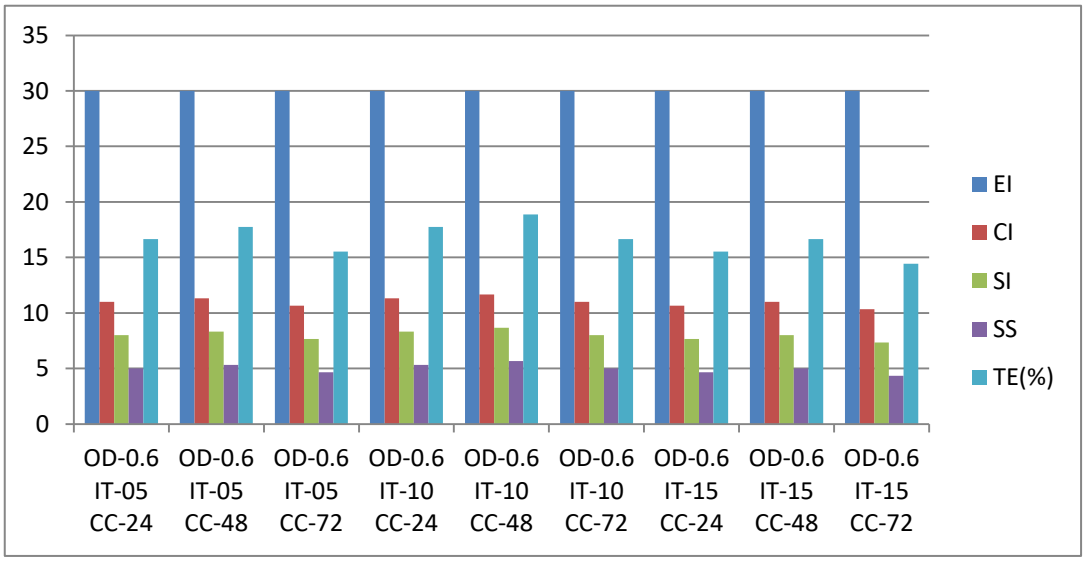
the most suitable optimum condition for transformation. The similar duration was adopted by several researchers [19],[22],[30],[31]. In this study, during *Agrobacterium* density 0.6 at OD_{600} nm, cultivar Arka Abha shows higher performance on infection time at 10min and co-cultivation period at 48hrs. Here, 30 infected explants were inoculated among the inoculants **11.66±0.57** explants induced callus, 0**8.66±0.57** shoots produced from the callus and 0**5.66±0.57** of shoots survived on the selection medium and the transformation efficiency was **18.86**% (Table-5; Graph-3; Plate-1 B-G).

Table-5: Influence of *Agrobacterium* density 0.6 at OD_{600}nm with other variables and transformation efficiency on *hevein* positive shoots in cultivar Arka Abha.

No of explants	OD_{600}nm	Infection Time (min)	Co-cultivation Period (hrs)	Number of explants Inoculated	Mean of explants induced callus	Mean of shoots Induced	Mean of *hevein*+ shoots survived	Percentage of Transformation efficiency (%)*
100	0.6	05	24	30	11.00±0.00^{c}	08.00±0.00^{c}	05.00±0.00^{c}	16.66
			48	30	11.33±0.57^{b}	08.33±0.57^{b}	05.33±0.57^{b}	17.76
			72	30	10.66±0.57^{d}	07.66±0.57^{d}	04.66±0.57^{d}	15.53
100	0.6	10	24	30	11.33±0.57^{b}	08.33±0.57^{b}	05.33±0.57^{b}	17.76
			48	30	**11.66±0.57^{a}**	**08.66±0.57^{a}**	**05.66±0.57^{a}**	**18.86**
			72	30	11.00±0.00^{c}	08.00±0.00^{c}	05.00±0.00^{c}	16.66
100	0.6	15	24	30	10.66±0.57^{d}	07.66±0.57^{d}	04.66±0.57^{d}	15.53
			48	30	11.00±0.00^{c}	08.00±0.00^{b}	05.00±0.00^{c}	16.66
			72	30	10.33±0.57^{e}	07.33±0.57^{e}	04.33±0.57^{e}	14.43

*****Transformation efficiency = Mean of *hevein*+ shoots/ Number of explants inoculated × 100.



Graph-3: Influence of *Agrobacterium* density 0.6 at OD_{600}nm with other variables and transformation efficiency on *hevein* positive shoots in cultivar Arka Abha.

Selection media and antibiotics role

The present investigation, the putative transgenic shoots selected on the selection medium containing 50mg/1 kanamycin. The non- transformed tissues were not survived on the selective medium containing 50mg/1 kanamycin. The Kanamycin sensitivity is the most widely used selectable marker for plant transformation and sensitivity of a plant species. Chimeras or escapes are a severe problem in plant transformation system. To salve of this problem, the strict and optimum concentration of selection medium needs to be employed in plant transformation in contrast the non-transformed turned necrotic.

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Callus, shoot induction and shoot elongation

The callus induction occurs after 15 days from the infected explants on ST medium. The transformed explants only survived on this media, non-transformed plants turned brown and died due to necrotic effect. After 30 days from the date of inoculation, the shoot induction occurred from the callus. After 45days from the date of inoculation, the shoot elongation was happened up to 2-3cm height. All the shoots not survived only a few of the *hevein*+ shoots survived and elongated the escapes and chimeras did not survive further (Plate-1 E-G).

Root Induction and Hardening

The 2-3cm elongated shoots transferred to rooting media, after 15 days from the transfer the roots occurred from the elongated shoots in a test tube containing rooting media (MS hormone-free medium) [23]. Plants with well-developed roots transferred to poly-cups containing sterilized coco-peat and 1% neem cake for 10 days under the poly-tunnel for primary hardening. Subsequently, plantlets transferred to 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 the further development. The regenerated putative transformed and non-transformed (control) plantlets were appeared normal. In this study in cultivar Arka Abha 20 numbers of shoots inoculated, 70% of shoots responded to develop roots and leaves. The average **7.26±1.15** number of leaves per plant and **3.76±0.57** numbers of root per plant observed (Table-6; Plate-1 H-J).

Table-6: Percentage and average no of leaves, roots per plant developed from the transformed plants.

Shoots inoculated	Percentage of Shoots response	Average no of leaves/plant	Average no of roots/plant
20	70	7.26±1.15	3.76±0.57

Molecular confirmation by PCR

In this study transformed cultivar Arka Abha further confirmed by the presence of the gene of interest through isolation of genomic DNA and PCR analysis (Fig-1 and 2). PCR analysis shows, lane-1: contained molecular marker (ladder DNA 100 - 1000bp); lane-2: contained the amplified plasmid DNA (positive control); lane-3: contained un-amplified non-transformed tomato plant DNA (negative control); lane-4: contained amplified transformed tomato plant DNA; lane-5: contained amplified transformed tomato plant DNA; lane-6: contained amplified transformed tomato plant DNA. The PCR amplification analysis shows that the 560bp molecular weight band found in positive control. The same band also expressed in putatively transformed plants that confirmed the integration of *npt-II* gene along with the *hevein* gene in the plant genome. But in the non-transformed (negative control) plant the band was not expressed, that indicates there was no integration of target gene [24].

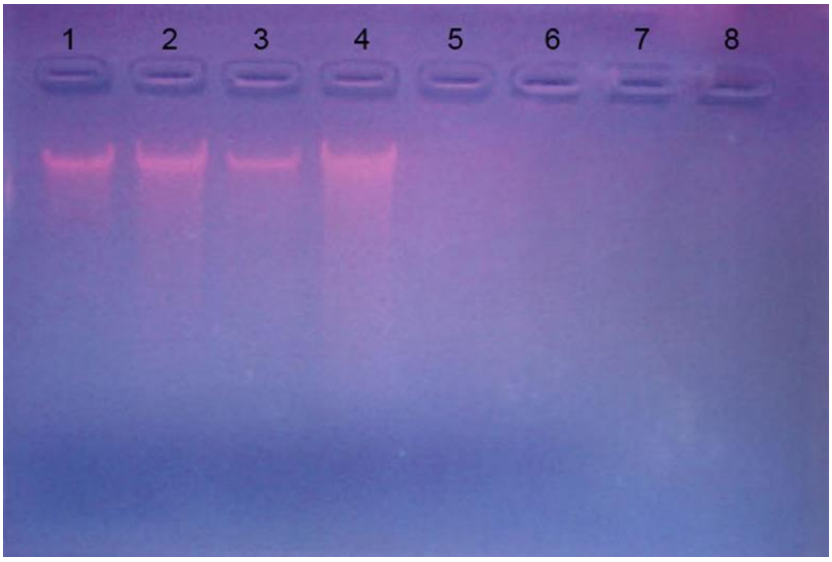

Fig-1: Isolation of DNA from Arka Abha: lanes 1, 2 and 3 show isolated DNA from transformed plants, lane-4 shows isolated DNA from the non-transformed plant.

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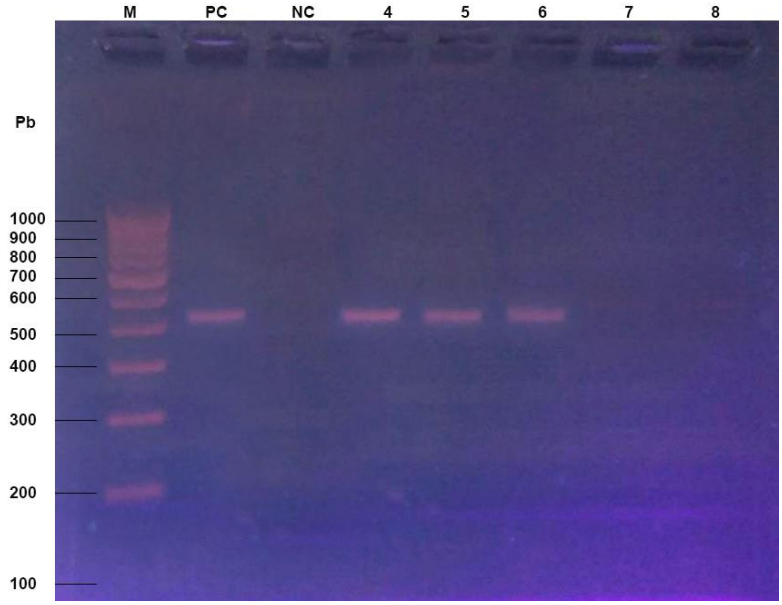

Fig-2: Polymerase Chain Reaction for Arka Abha: lane-1 shows 100 - 1000bp marker DNA (ladder), lane-2 shows plasmid DNA (positive control), lane-3 shows non-transformed plant DNA (negative control), lanes- 4, 5 and 6 shows DNA from the transformed plant.

Antifungal Activity of Transformed Plant

In the present study reveal that *Fusarium oxysporum was* cultured on the potato dextrose agar and maintained at the laboratory (Fig-3). In cultivar, Arka Abha after 15 days from treatment with *Fusarium oxysporum* the treated non-transformed tomato plant got affected by leaf wilting and brown colored stem rotting (necrosis), but the treated transformed plant was not affected by wilt disease it was survived well. When compared with the non-treated control plant, the transformed tomato plant had resistance against the fungus *Fusarium oxysporum* (Fig-4) [25].

Fig-3: Fusarium oxysporum cultured on the potato dextrose agar and maintained at the same medium by subculture.

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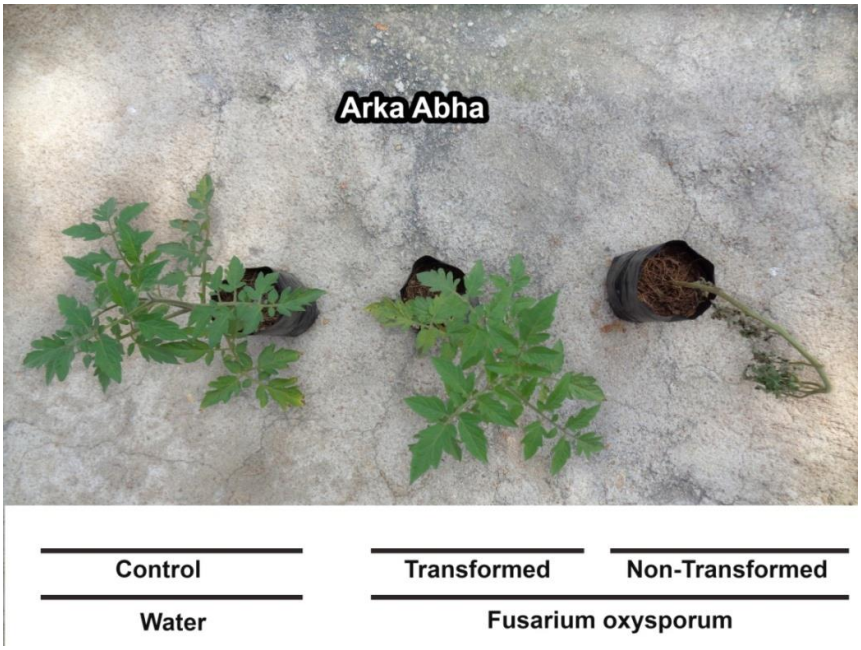

Fig-4: Cultivar Arka Abha after 15 days from treatment with Fusarium oxysporum, the non-treated control tomato plant survived, the treated transformed plant was not affected by wilt disease it survived well and the treated non-transformed plant was affected by leaf wilt and stem rot.

IV. CONCLUSION

In the present investigation, the maximum transformation efficiency was observed in the concentration of *Agrobacterium tumefaciens* density 0.4 at OD_{600}nm with 1:1 dilution with10 min infection time, pre-culture with 24 hours and co-cultivation with 48 hours in the dark. The putative transgenic shoots occurred on the ST medium containing an optimum concentration of 50 mg/l kanamycin. The callus induction, shoot induction and shoot elongation happened in the same ST media. The roots induced in hormone-free MS basal media, rooted plants transferred to the poly cup 10 days for primary hardening and subsequently, plantlets transferred to a greenhouse for acclimatization. The hardened plants were transferred to poly-bags under the same greenhouse for further development. Molecular confirmation was carried out by using the PCR method. The antifungal activity of the transformed plants was tested and confirmed by treated with *Fusarium oxysporum.* In this study, reveals that the cultivar Arka Abha shows the better mean of explants induced callus **12.33±0.57**, mean of shoots developed **09.33±0.57**, mean of *hevein*+ shoots survived **06.33±0.57** and the transformation efficiency was **21.10%** at bacterial density 0.4 at OD_{600}nm, Infection time was 10min and Co-cultivation period was 48 hrs.

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