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ORIGINAL ARTICLE

Effect of *Agrobacterium* Induced Necrosis, Antibiotic Induced Phytotoxicity and Other Factors in Successful Plant Transformation

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Effects of *Agrobacterium* infection, antibiotic and NaOCl washes on *Agrobacterium* removal and both explants physiological changes during transformation experimental procedures were studied. At the end of the experiments explants survival efficiency of *Ammi majus* and pearl millet were 8% and 5% respectively. Biotic and abiotic stress factors responsible for lower efficiency were investigated with various other factors and strategies were discussed which are need to be considered for higher transformation events and target tissue survival.

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Agrobacterium mediated genetic transformation is a natural and simple way to do genomic changes in plant characters. Fundamentally this transformation process is highly tailored gene shifting and integration method which has the potential to change the functionality of plant cell through stable genetic changes. In the present study two important plants were considered namely, *Ammi majus* and Pearl millet (*Pennisetum glaucum* L.) for study the biotic and abiotic stress factor on plant tissue during transformation process. A successful

Agrobacterium-mediated plant transformation requires efficient procedures for suppressing bacteria following co-cultivation and a comprehensive approach to reduce suppressing effects of antibiotics on plant propagation. Highly efficient *Agrobacterium* mediated plant transformation has not yet been well-established; however, applications of *in vitro* culturing, antibiotic and chemical washing biochemically damage plant tissue and these stress factors were responsible for unsuccessful transformation experiments.

Ammi majus (L.), also known as bishop's weed from Apiaceae family is one of the wild pharmacopoeial plant species. The seed contains furanocoumarins (Hamerski and Matern, 1998), which stimulate pigment production in the skin that is exposed to bright sunlight (Bown, 1995; Chevallier, 1996). In recent study, leaf, stem and calli of *Ammi majus* were used for efficient genomic DNA isolation (Magdum, 2013). Modification of plant metabolic pathway for higher production of medically important secondary metabolite or byproduct requires basic changes in the genomic DNA of *Ammi majus*, which could be achieved by *Agrobacterium* mediated transformation (AMT).

Pearl millet is widely grown as a multi-purpose cereal grain crop principally for food, feed, fodder, fuel, and much on more than 26 million hectares, primarily in arid and semi-arid regions of India and Africa. It is a high yielding, drought tolerant summer crop and can be grown in low rainfall areas where other crops such as maize and sorghum are not profitable (FAO, 2004). Due to lack of *Agrobacterium attachment site* in monocots, like pearl millet, the absence of wound response and the associated activation of virulence genes would be the reasons of absence of crown gall tumor formation and recalcitrance. But there actively dividing, embryogenic cells, which are co-cultivated with *Agrobacterium* in the presence of acetosyringone, which is a potent inducer of virulence genes (Vasil, 2005) and the possibility of gene transfer exists in monocots. A number of researchers reported recently about AMT in monocots (Assem et al., 2009; Ceasar and Ignacimuthu, 2011; Jha et al., 2011; Karthikeyan et al., 2011; Duan et al., 2012) but it was observed that no recovery of regenerates has been possible. Although the communication between plant cell and *Agrobacterium* via chemical signaling and transport is not yet fully understood, many studies of *Agrobacterium*-mediated transformation have reported necrosis and a poor survival rate of target plant tissues (Hansen, 2000; Olhoft et al., 2001; Chakrabarty et al., 2002; Das et al., 2002; Toldi et al., 2002; Dan et al., 2004; Zheng et al., 2005; Assem et al., 2009; Ceasar and Ignacimuthu, 2011; Jha et al., 2011; Karthikeyan et al., 2011).

Hansen and Durham, (2000) have also reported that co-cultivation of wheat and maize tissues with *Agrobacterium* resulted in necrosis (Fig.1) due to programmed cell death, Some factors may be the result of, or linked to, hypersensitive defense reaction in plants to *Agrobacterium* infection, which may involve the recognition of specific signal from the *Agrobacterium* that triggers the burst of reactive oxygen species at the infection site.

Antibiotics in the regeneration medium also affects on regeneration efficiency (Nauerby et al., 1997; Ling et al., 1998; Ieamkhang and Chatchawankanphanich, 2005). Some researchers have used timentin to inhibit systemic bacteria in tissue culture and to suppress *Agrobacterium* in genetic transformation (Cheng et al., 1998; Ieamkhang and Chatchawankanphanich, 2005).

Beta-lactum group antibiotics known as minimal toxicity to plant tissue, like Cefotaxime and carbenicillin, have been widely accepted and commonly been used as effective treatment for suppression of *Agrobacterium* cells (Okkels and Pedersen, 1988; Tang et al., 2000; Alsheikh et al., 2002).

The effects of various antibiotics on *Agrobacterium* suppression and plant regeneration were studied in *Arabidopsis* (Lin et al., 1995), papaya (Yu et al., 2001), pine (Tang et al., 2004; Tereso et al., 2006), tobacco (Nauerby et al., 1997; Cheng et al., 1998), tomato (Ieamkhang and Chatchawankanphanich, 2005) and wheat (Han et al., 2007). Alsheikh et al., (2002) also reported that no single antibiotic was effective in controlling all of the strains. Simple AMT procedure contains steps like *Agrobacterium* co-cultivation with plant tissue and antibiotic washing to disinfect *Agrobacterium* for normal growth of plants, but experiments ends with lower number of live transformants due necrosis and phytotoxicity by biotic stress of *Agrobacterium* and abiotic stress of antibiotic use respectively (Fig.1).

In this study, we observed the effect of *Agrobacterium* infection, antibiotic and chemical agent washing on plant tissue physiology, like browning/necrosis and response towards the growth of transforming tissues. Factors need to be considered for increasing efficiency of successful transformation was addressed.

MATERIALS AND METHODS

Explant preparation

Standardization of plant tissue culture protocols for both plants, *Ammi majus* and Pearl millet, were done in a separate study. *Ammi majus* leaf explants were used for successful callus induction on *Ammi majus* callus induction medium (AMCIM) contains MS salts (Murashige and Skoog, 1962) with 3% sucrose and 0.8% agar, pH 5.8, with 2 mg L^{-1} IAA, 2 mg L^{-1} Kn and 1000 mg L^{-1} CH. *Ammi majus* shoot induction medium (AMSIM) contains only contains Glutamine 50mg L^{-1} in addition to AMCIM.

Immature embryos of pearl millet were used as explants for its callus induction on MS salts with 3% sucrose and 0.8% agar, pH 5.8, were used in Pearl millet Callus Induction Medium (PMCIM), which was supplemented with 3 mg L^{-1} 2, 4-D and Pearl millet Shoot Induction Media (PMSIM) were supplemented with 3 mg L^{-1} BAP. The previously cultured and healthy calli were taken for both plant studies for *Agrobacterium* infection and transformation. Calli was cut into same size 3 mm to 4 mm pieces of tissues by using a sterile scalpel and blade and used as explants in further study.

Agrobacterium Culture Preparation

Glycerol stocks of the strains (LBA4404 and EHA105) of *Agrobacterium tumefaciens* with respective plasmids were inoculated to 10 ml LB broth (Bacto-tryptone 10 gm L^{-1},Yeast Extract 5 gm L^{-1}, NaCl 5 gm L^{-1}, pH 7.2) with the suitable antibiotics (Kanamycin and Rifampicin) concentration and grown overnight in incubator shaker at 220 RPM and 28 C. This mother culture was re-grown in 50 ml LB broth with respective antibiotic concentration, till the optical density reached 0.6 to 0.8 for LBA4404 and 0.2 to 0.4 for EHA105 at 600nm.

LBA infection to *Ammi majus*

In vitro cultured calli of *Ammi majus*, which was having good, healthy growth, was taken for transformation experiment. Transfer calli explants, immediately after cutting, to the bacterial suspension in the petri dish, and co cultivate with

LBA4404 for about 3 min. Blot the explants dry over a sterile blotting paper and placed on co-cultivation media (CCM) with composition of MS salts + 3% sucrose + 0.8% agar + Acetosyringone 200 µM, (pH 5.8).

EHA 105 infection to embryogenic calli of Pearl millet

In vitro grown embryogenic calli and active EHA105 *Agrobacterium* culture were incubated in a conical flask for 10 min with gentle shaking. The explants were then blotted dry on an autoclaved blotting paper and placed on CCM. All plates wrapped with aluminum foil and Placed in BOD chamber for two days of co-cultivation at 24°C in dark condition.

Washing of co-cultivated *Ammi majus* explants with Cefotaxime

After tow days, *Agrobacterium* infected tissues were transferred to sterile conical flask and washed them with distilled water for the 3 times. Add 25 ml ½ MS liquid media with 500 mg L^{-1} Cefotaxime. Put conical flask in the shaker incubator for 1 Hr at 120 RPM at 24°C. Wash the tissues again with distilled water in the sterile conical flask. Blot them on sterile blotting paper and put them on the culturing plates by using new sterile forceps. Repetition of the antibiotic washing procedure followed, when re-growth of *Agrobacterium* on tissues were observed. Every 3 weeks, explants are sub cultured on fresh culturing medium containing the Cefotaxime (500 mg L^{-1}).

Washing of co-cultivated Pearl millet explants with Timentin

After two days bacterial growth was observed on infected embryogenic calli of prael millet. So explants were washed with water and then incubated with ½ MS liquid media added Timentin 300 mg L^{-1} for 1 Hr with continues shaking at 120 RPM at 24°C. The explants were blot dried on an autoclaved tissue paper. Then the explants placed on fresh culturing medium with added Timentin 300 mg L^{-1}.

Chemical washing of targeted explants

Abiotic effect of NaOCl washing techniques on the tissue for complete removal of infected *Agrobacterium* after co-cultivation has been attempted. After antibiotic wash, give three washes to agroinfected tissue with distilled water. Then tissues washed with 1% NaOCl for 2 min. Then wash the tissues for 3 times with distilled water. Dry them on blotting paper and culture them on respective growth culture media. We observed the effect of NaOCl washing on *Agrobacterium* growth and tissue of *Ammi majus* and Pearl Millet.

RESULTS AND DISCUSSION

Callus and shoot induction of *Ammi majus* and Pearl millet:

For callus and shoot induction different hormone composition were used to optimize the growth. For *Ammi majus* AMCIM, AMSIM and for pearl millet PMCIM and PMSIM were the optimized composition for callus and shoot induction respectively. In case of *Ammi majus*, leafs found best as explants for callus induction and immature embryos were found best as explants for *in vitro* growth of pearl millet. The embryos start callusing within 2-3 days in respective medium. Callus cultivation of both plants were different, as *Ammi majus* produces green calli in light at 25°C (Fig.2A) and pearl millet produces white embryogenic calli (EC) in dark at 25°C in BOD incubator (Fig.2B). For shoot induction both plants were incubated in light with their respective media composition. In pearl millet extensive rooting were observed from calli

without activated charcoal. Addition of activated charcoal (0.2 %) shows effective root inhibition.

Two days incubation in dark after transfection, good growth of *Agrobacterium* on the tissues was observed. Color of the *Ammi majus* dark green callus tissue was changed to light green (Fig.3A,B) and pearl millet white calli observed as light brown (Fig.3C,D). Pearl millet explants were showed browning due to ROS and phenolics secretion, due to this regeneration efficiency was affected. Evidences of biotic stress due to *Agrobacterium*-induced necrosis in target plant tissues and its link to reactive oxygen species were presented (Fig.3).

Effect of Antibiotic washing on calli of *Ammi majus*

In *Ammi majus* transformation experiments, 500 mg L^{-1} Cefotaxime were effectively suppressed LBA4404 than 250 mg L^{-1}. After co-cultivation period of 48 hrs 1st antibiotic wash was given to calli and they were cultured on AMCIM containing 500 mg L^{-1} Cefotaxime, no depigmentation observed and tissues were brownish green (Fig.4A). Re-growth of *Agrobacterium* was observed on surrounding of each callus after 2 days of 1st antibiotic wash due to the transient bacteriostatic activity of Cefotaxime. After 2nd antibiotic wash, calli were cultured on AMCIM containing 500 mg L^{-1} Cefotaxime and at this time depigmentation were observed on calli and tissues were brownish yellow (Fig.4B). It's showing abiotic stress and phytotoxicity of Cefotaxime on calli of *Ammi majus*. Again re-growth of *Agrobacterium* was observed after 2 days of 2nd antibiotic wash. Repeated the antibiotic wash for 3rd time and tissues were brownish white and depigmentation observed (Fig.4C). Regeneration rate of *Ammi majus* calli were affected by phytotoxicity of antibiotic wash and it slower than unwashed tissues.

Effect of antibiotic washing on EC of Pearl millet

Timentin were used in pearl millet experiment which effectively suppress EHA101 at 300 mg L^{-1} than 100 mg L^{-1}. After co-cultivation period of 48 hrs 1st antibiotic wash was given to pearl millet EC and they were cultured on PMCIM containing 300 mg L^{-1} Timentin. After 1st wash itself white embryogenic calli become yellowish white, slight browning were observed (Fig.5A). Re-growth of *Agrobacterium* was observed after 3 days due to transient bacteriostatic activity of Timentin, so 2nd antibiotic wash was carried out. EC's were blot dried and cultured on PMCIM containing 300 mg L^{-1} Timentin, tissues were brownish yellow (Fig.5B). Again re-growth of *Agrobacterium* was observed and 3rd antibiotic wash was given to agroinfected calli, tissues browning were observed (Fig.5C) with minimum growth rate.

Effect of chemical (NaOCl) washing

NaOCl effectively kill the *Agrobacterium*, but not complete removal of *Agrobacterium* from plant tissue at lower concentration <1% for 2 min. As we increased the washing time and concentration of NaOCl, *Ammi majus* calli were shown immediate depigmentation (Fig.6A), which was responsible for tissue death. Incase of pearl millet, NaOCl wash to agroinfected tissue were shown effect like extensive browning after culturing (Fig.6B) and this abiotic stress affected on tissue regeneration rate by killing plant cells.

Graph plotted bellow showing 92% calli of *Ammi majus* and 95% calli of pearl millet were dead because of virulence of *Agrobacterium*, antibiotic containing media and antibiotic/ chemical washing (Fig.7).

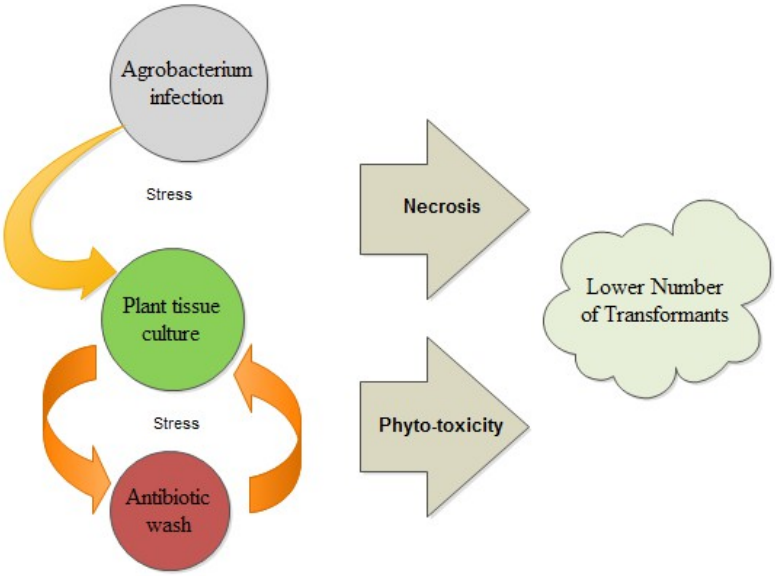

Figure 1 Effect of biotic stress of Agroinfection and abiotic stress of antibiotic wash on plant transformation process.

Table 1. Group of antioxidants used in plant transformation

Group	Antioxidants	Action
I	Ascorbic acid, Citric acid, DTT, Polyvinylpyrrolidone (PVPP) and Vitamin C	Reduce tissue browning, promote organogenesis, somatic embryogenesis and shoot growth from buds during micropropagation.
II	Cysteine, Phenoxane, 3-ter-butyl-4-hydroxyanisole and Vitamin E	Antioxidants can enhance shoot, root, and plant growth.
III	Ascorbate, Glutathione and α-tocopherol	Promote callus and shoot organogenesis but also inhibit somatic embryogenesis.

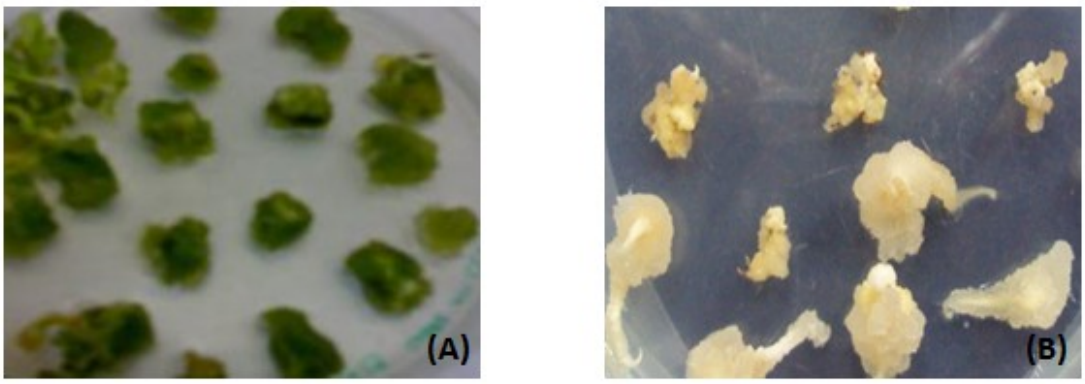

Figure 2 Callus induction of *Ammi majus* (A) and Pearl millet (B) on respective CIM.

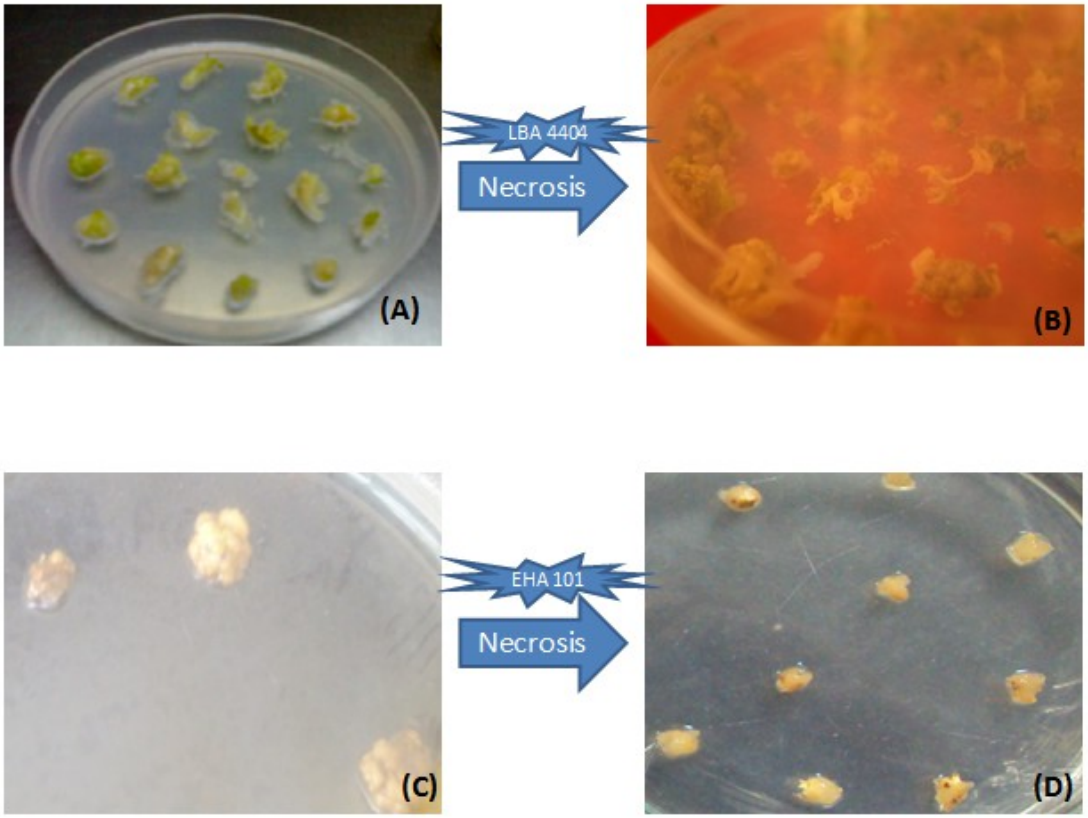

Figure 3 (A) LBA4404 infected *Ammi majus* calli **(B)** *Agrobacterium* induced biotic necrosis effect on *Ammi majus* calli **(C)** EHA105 infected Pearl millet calli **(D)** *Agrobacterium* induced biotic necrosis effect on Pearl millet calli

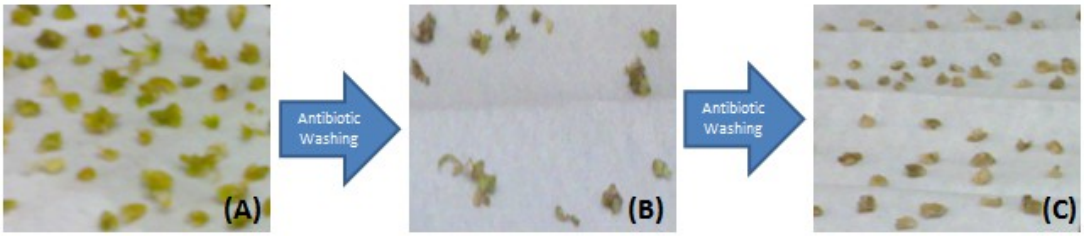

Figure 4 Effect of Cefotaxime washing on calli of *Ammi majus* (A) Calli were slight brownish green after 1st antibiotic wash (B) Browning of calli observed after 2nd antibiotic wash (C) After 3rd antibiotic wash calli were unhealthy dark brown.

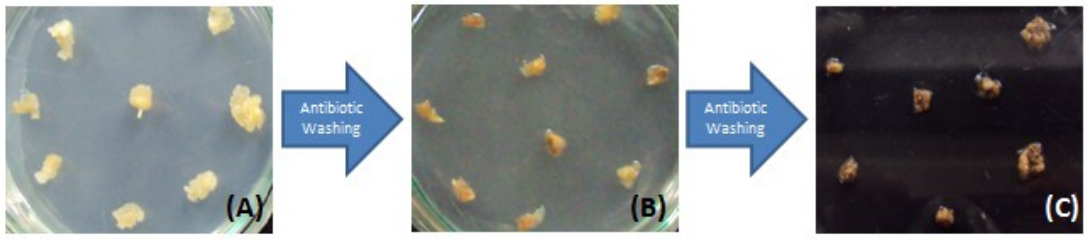

Figure 5 Effect of Timentin washing on EC of pearl millet (A) EC were slight brownish white after 1st antibiotic wash (B) Light browning of EC observed after 2nd antibiotic wash (C) After 3rd antibiotic wash EC were unhealthy dark brown.

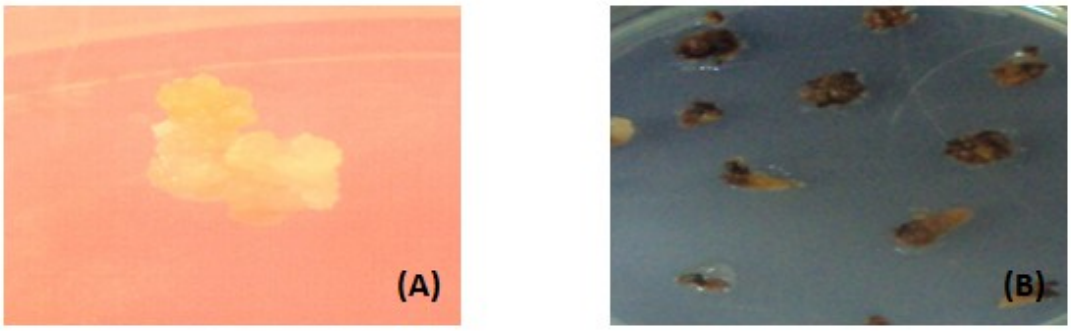

Figure 6 Effect of NaOCl washes to extensive *Agrobacterium* grown (A) calli of *Ammi majus* and (B) EC of pearl millet

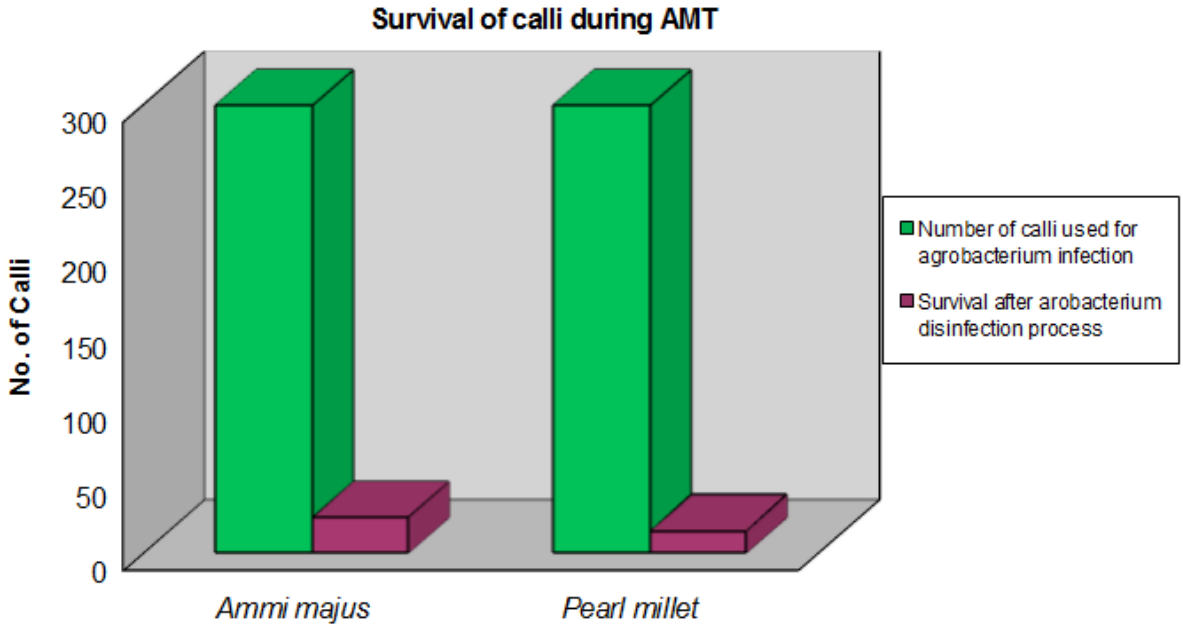

Figure 7 Graph shows 8% *Ammi majus* calli and 5% pearl millet EC were survived after *Agrobacterium* infection, antibiotic and chemical washes.

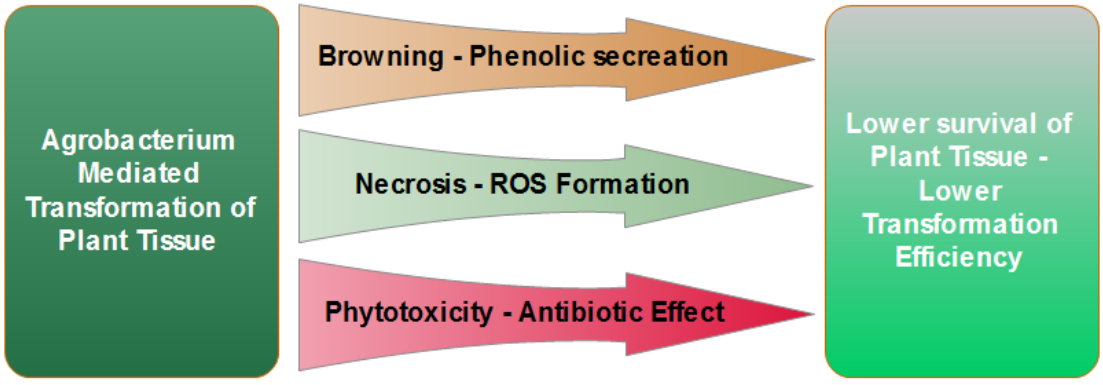

Figure 8 Reason of lower AMT efficiency

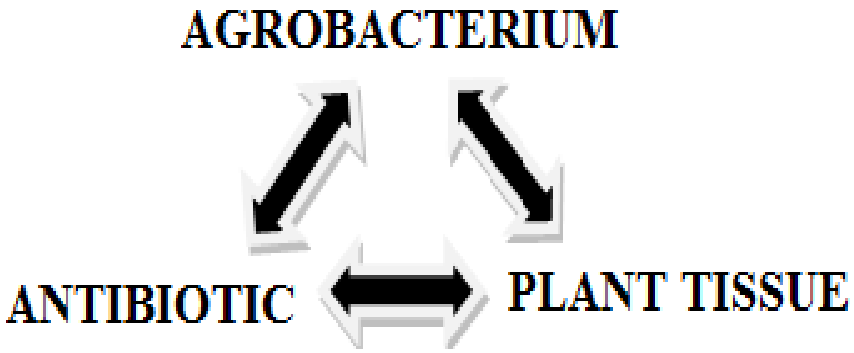

Figure 9 Key to highly efficient successful Plant transformation, Optimization the relations between (I) *Agrobacterium* virulence to plant tissue and response to antibiotic action, (II) Antibiotic bactericidal effect against *Agrobacterium* and lower phytotoxicity towards plant tissue, (III) plant tissue susceptibility for action of both *Agrobacterium* and antibiotics.

DISCUSSION

Factors for lower AMT efficiency

Antibiotic containing media and antibiotic washing affects on plant survival (Fig.8). Cefotaxime have showed a great negative impact on regeneration of transformed explants of tomato (Costa et al, 2000). The percentage of tomato explants survival was reduced gradually from 100 to 400 mg L^{-1} Cefotaxime (Mamidala and Nanna, 2009). 92% calli were dead after use of 500mg L^{-1} Cefotaxime for washing of *Ammi majus* tissue (Fig.7). Number of transformation experiments reported that Timentin a mixture of tricarcillin and clavulanic acid was effective suppress *Agrobacterium tumefaciens*, with not affecting plant tissue (Costa et al., 2000; Karthikeyan et al., 2011; Zaragozá et al., 2004; Park and Facchini, 2000; Gonzalez Padilla et al., 2003; Le et al., 2001; Liau et al., 2003). But incase of pearl millet 95% of calli were dead or non-responding after 3 washes with 300 mg L^{-1} Timentin (Fig.7). Use of chemical washing like dilute NaOCl which is a strong oxidant, it will obviously kill bacteria present on plant tissue, but simultaneously affects on plant tissue surface by oxidizing surface cell structure.

Plant defense mechanism causes ROS generation for the purpose of killing the bacteria (Fig.8). This ROS production is usually followed by the hypersensitive response to pathogens leading to rapid cell death (necrosis) (Greenberg et al., 1994). Unfortunately, tissue necrosis is remarkably intensive in the shoot producing area around the cambium layer (Thomzik 1995).

Factors need to be considered for AMT efiiciency improvement

Antibiotics should be bactericidal instead of bacteriostatic avoid reoccurrence of bacteria (Leifert et al., 1992). Combinations of antibiotics may be more effective in killing contaminants (Leifert et al., 1991; Kneifel and Leonhardt, 1992). If antibiotic combinations are synergistic, the effective concentration of each antibiotic can be reduced, and the reduced concentration of each antibiotic will produce fewer toxic side effects. Leifert et al., (1992) recommend the use of short antibiotic treatments to prevent the development of antibiotic resistance in bacterial contaminants. Successful antibiotic treatment of infected plants requires determining the minimal bactericidal concentration (MBC) and its antibiotic phytotoxicity

to plant materials before treatment begins (Barrett and Cassells, 1994).

Lethal browning is the result of formation of oxidized polyphenols, so antioxidants, such as ascorbic acid and cysteine, may be used, but the results are variable (Monnier, 1990). The best concentration of antioxidants was 15 mg L^{-1} ascorbic acid and 50 mg L^{-1} L-cysteine which reduced necrosis of cotyledons by half in the two genotypes tested (Belide et al., 2011). Yinghui Dan, (2008) explains three groups of antioxidants could be used as required shown in Table 1.

Above observation and results explains, for successful and highly efficient plant transformation event, there is need of key strategies for standardization of transformation protocol. For each plant bellow showed relation of *Agrobacterium* virulence, activity of antibiotic and resistance of plant tissue to reduce necrosis and phytotoxicity (Fig.9). Then it will be easy way to use *Agrobacterium* as tool for gene transformation.

Bellow stated some of the points may responsible for increasing efficiency of AMT,

- To avoid necrosis due to biotic stress of *Agrobacterium* virulence, use the strain of with suitable virulence at optimized cell density. Cell density can be used from 0.05 to 1.2 and infection time could be 1 day to 3 days at dark condition depends on virulence of *Agrobacterium* strain.
- To avoid phytotoxicity due to abiotic stress of antibiotic wash and media combined antibiotic, criteria for selection of antibiotic or group of antibiotic should be high bactericidal against *Agrobacterium* and low phytotoxicity to desired explants. Avoid more than one antibiotic wash to agroinfected explants.
- To reduce stress and increase efficiency of transformation, suitable antioxidants need to use in optimized concentration.

Optimization of such factors proved to be of considerable importance for the establishment of successful transformation systems through AMT in any plant system. Observation of tissues color changes and healthiness could be factor of understanding plant tissues conditions and needs. Factors other than *Agrobacterium* and antibiotics, percentage of gelling agent, supporting additives like activated charcoal and its percentage, level of antioxidants, sub-culturing time, light intensity, humidity and temperature need to be considered for achieving higher yield of transformants.

CONCLUSION

In the present study, we observed that the explants of *Ammi majus* and Pearl millet were very sensitive to co-cultivation with *Agrobacterium* and turned necrotic within 48 Hrs after agro-infection. Callus induction and shoot formation rates were significantly affected due to stress stimulating actions of agroinfection and antibiotics. A recalcitrant system of *Agrobacterium tumefaciens* mediated transformation need to be managed by providing stress less environment to explants. For high efficiency transformation not only requires factors like optical density of *Agrobacterium* culture, inoculation and co-cultivation time, concentration of acetosyringone, but also other factors like use of antioxidants, selection of antibiotic washing technique which give one wash elimination of *Agrobacterium*, dryness of culture media, temperature, humidity, light intensity and light cycles and the most important, each day

observation of explant's reactions. These factors defiantly produce considerable differences in enhancing stable transformation by suppressing the *Agrobacterium*-induced necrosis and normal growth of transgenic plant. Applications of above studied factors would be results in high transformation frequency with saving time as well as experimental costs.

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