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Optimization of *Agrobacterium* Mediated Genetic Transformation in Himalayan polar (*Populus ciliata* wall.)

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ABSTRACT

Populus species are important resources for industry and in scientific study on biological and agricultural systems. Our objective was to enhance the frequency of plant regeneration in Himalayan poplar (*Populus ciliata* wall. ex Royle). *Agrobacterium*-mediated plant genetic transformation requires a two step process for its success, selection and regeneration of transformed tissues and the elimination of the transformation vector *Agrobacterium*. Antibiotics used for suppressing *Agrobacterium* in plant transformation might have negative effects on plant tissues and regeneration. The effects of antibiotics on growth suppression of *Agrobacterium* and plant regeneration were investigated for enhancing *Agrobacterium*-mediated transformation. This study uses cefotaxime with combination of kanamycin to eliminate *Agrobacterium tumefaciens* LBA4404 and selection of transgenic plant. First the effect of antibiotic cefotaxime was evaluated during in vitro shoot morphogenesis in *Populus ciliata* L. Later on effect of different concentrations of cefotaxime and kanamycin (50 mg/l) were studied on the growth of agrobacterial cells and regeneration potential of leaf and petiole tissues after co-cultivation. The maximum per cent (75%) and (78%) shoot regeneration were obtained on MS regeneration medium with 300mg/l cefotaxime in leaf and petiole explants, respectively. In leaf and petiole explants the growth of agrobacterial cells were controlled at concentration of 400 mg/l cefotaxime and maximum per cent shoot regeneration 33.00% and 48.00% was obtained on MS medium supplemented with 400 mg/l cefotaxime, respectively. A protocol for high frequency transformation of insect resistance gene in Himalayan poplar have been standardized.

Keywords: *Agrobacterium tumefaciens*; Antibiotics; Himalayan poplar; Genetic transformation; cefotaxime; *in vitro* shoot regeneration

INTRODUCTION

Agrobacterium-mediated transformation is a well-established procedure for introducing foreign DNA into plant cells. It has several advantages such as high transformation efficiency compared to protoplast transfer (Rao et al. 2009) the ability to transfer large pieces of DNA, minimal re-arrangement of transferred DNA, and characteristic insertion into the recipient genome of a discrete segment of DNA at a low copy number (Kumar et al. 2005; Tyagi et al. 2007) compared to particle bombardment approach (Shrawat et al. 2006; Rao et al. 2009). The successful *Agrobacterium*-mediated transformation method has been established in several crops such as rice (Hiei et al. 1997; Dong et al. 1996), maize (Ishida et al. 1996), and barley (Tingay et al. 1997; Wu et al. 1998), wheat (Cheng et al. 1997; Weir et al. 2001; Wu et al. 2003) polar (Thakur et al. 2012)

A successful *Agrobacterium*-mediated plant transformation requires efficient procedures for suppressing bacteria following co-cultivation and subsequently for selecting transformed cells. As the plant tissue is affected by various components in culture media during plant transformation, antibiotics used for suppressing Agrobacteria might have negative effects on plant tissues and regeneration. The ideal antibiotics should be stable, soluble, unaffected by pH and media components, lack side effects, as well as being non-toxic to plant cells, and inexpensive (Falkiner 1988; 1990). Carbenicillin and cefotaxime have commonly been used as effective antibiotics for suppression of *Agrobacterium* cells (Mathias and Boyd 1986; Tang et al. 2000; Alsheikh et al. 2002). Both carbenicillin and cefotaxime, belonging to β-lactam group, have minimal toxicity on most plant tissues and thus have been widely accepted in *Agrobacterium*-mediated transformation (Pollock et al. 1983; Okkels and Pedersen 1988).

Poplars are serving as a backbone to the rural economy of India. It is the most widely distributed, indigenous poplar of India at a height of 1500-3000 m above mean sea level covering the areas of Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Uttar Pradesh and some of the North-eastern states. It is native of Himalayas from Kashmir to Arunachal Pradesh. *Populus ciliata* have got multipurpose uses as they are considered ideal trees for successful inter cultivation with agricultural crops. They possess high timber and fiber value for industrial applications. Timber from poplar is light in weight and easy to saw and work (Chaturvedi 1982). Their high productivity levels make them ideal for plantation forestry and they constitute a veritable ‘saving bank’ when harvested. Both *Populus deltoides* and *P. ciliata* grow fast and are easy to propagate vegetativly and can be adapted to various soil types of different regions from boreal to sub-tropical and from mountains to riparian. They also play a significant role in environmental protection, especially in preventing soil erosion and potentially in phytoremediation of contaminated soils and ground water. *P. ciliata* have proved suitable for the manufacture of wood cement composites by incorporating calcium chloride as an accelerator and are also found suitable for making compressed wood. *P. ciliata* hold an excellent promise as a source of fibre for various grades of paper, fine paper, packing paper and newsprint. Their leaves are used as fodder.

Poplar plant is affected by the various biotic stresses, seriously damaged by lepidopteron and coleopteran pests that cause defoliation resulting in remarkably slower growth rate (Kulman 1971)) and high lignin content. Due to these problems several hybrid *Populus* has yielded with improved quantitative and qualitative genes through conventional breeding. But the progress in tree improvement through conventional breeding is relatively slow. The technique of genetic engineering has provided tools to accelerate and diversify the genetic manipulation of *Populus* by directly adding novel gene from heterogeneous sources to poplar genome. *Agrobacterium*-mediated transformation has been most widely used for yielding hybrid poplar with novel gene. Several proteins such as proteinase inhibitors, lectins and crystal toxins of *Bacillus thuringenesis* have insecticidal properties against diverse insects with different mode of action (Hofte and Whiteley 1989; Gatehous et al. 1994; Sudhakar et al. 1998) and number of genes (COMT, 4CL, CCoAOMT, F5H and OMT genes) have been targeted in efforts aiming at altering lignin composition in a number of *Populus* sp. through transgenics development (Hu WJ et al. 1999; Meyermans H et al. 2000; Huntley SK et al. 2003; Wi SG et al. 2004; Thakur et al. 2012).

In the present study we evaluate the effect of cefotaxime on the in vitro regeneration potential of leaf and petiole explants of *Populus ciliata* Wall. and on the elimination of *Agrobacterium* in transformed poplar tissues to improve transformation efficiency and identify the suitable explants with high transformation efficiency.

Material and Methods

Explant Preparation and Effect of cefotaxime on shoot regeneration

The plant material (young, tender leaf and petioles) were procured from the nursery of *P. ciliata* established in the glasshouse of the department of Biotechnology, Dr. Y S Parmar University of Horticulture and Forestry, Nauni-Solan. The leaf and petiole explants were surface sterilized and cut into small pieces of 0.5–1.0 cm size and inoculated on the selective shoot regeneration media medium (MS medium + 0.024mg/l TDZ + 79.7 mg/l adenine) for leaf explants (Table 1) and (MS medium + 0.044mg/l TDZ + 79.7 mg/l adenine) for petiole explants (Table 2) containing different concentrations of cefotaxime (100, 200, 300, 400 and 500 mg/l). The growth and differentiation of the explants were recorded to study the effect of cefotaxime on the regeneration potential of explants. A high frequency plant regeneration protocol in Himalayan poplar from leaf (Gaurav et al. 2012) and petiole (Gaurav et al. 2015) explants had already been standardized in our laboratory. These cultures were incubated at 25 ± 2^0C temperature under 16 h photoperiod of 125 lm $m^{-2}s^{-1}$ irradiance provided by cool-white fluorescent tubes.

Bacteriostatic effect of antibiotic cefotaxime on *Agrobacterium tumefaciens*

Agrobacterium Strain and Plasmid

Agrobacterium tumefaciens strain containing *binary vector pBin-1Aa* harboring *cry1Aa* (insect resistance gene) along with kanamycin resistance gene (*npt-II*) for selection of transformed cells was used. A single colony of *A. tumefaciens* strain was suspended into 10 ml liquid YEB medium containing 50 mg/l kanamycin at 28^0C temperature overnight with an agitation of 150 rpm. After 24 h, the cells were collected by centrifugation at 5,000 rpm for 10

min, re-suspended into liquid MS medium to adjust the final O.D. of 0.562 to get a final density of 5.9 X 10^8 cells/ml for co-cultivation.

Effect of cefotaxime on the suppression of Agobacterial cell growth.

Pre-cultured leaf (MS medium + 0.024mg/l TDZ + 79.7 mg/l adenine) and petiole (MS medium + 0.044mg/l TDZ + 79.7 mg/l adenine) explants were immersed in bacterial suspension for 15-20 seconds and blotted on pre-sterilized filter paper and then inoculated on the same shoot regeneration medium for co-cultivation. The leaf and petiole explants were co-cultivated for 48 hrs. and 72 hrs., respectively. After co-cultivation the leaf and petiole explants were transferred to the fresh selective shoot regeneration medium, respectively containing Kanamycin 50 mg/l and different concentrations of cefotaxime (100, 200, 300, 400 and 500 mg/l) for selection of transformed cells and inhibition of further agrobacterial growth (Table 3 & 4). These cultures were incubated at 25 ± 2^0C temperature under 16 h photoperiod of 125 lm $m^{-2}s^{-1}$ irradiance provided by cool-white fluorescent tubes.

PCR analysis of Putative transgenic shoots

Genomic DNA was isolated from young leaves of putative transformed and control (non-transformed) plants by modified CTAB method (Doyle and Doyle 1990). PCR analysis was performed to detect the coding region of Cry I Aa gene using two set of the designed (forward and reverse) primers: 5'GGGATGGCTAACAACCCAAAC 3' and 5'GGCAAACTCTGGTCCAGAGAGAAACC 3', respectively. Polymerase chain reaction was carried out at 94^0 C for 4 min, followed by 32 cycles each at 94^0 C for 1 min., at 50^0 C for 1 min. and at 72^0 C for 2 min and final extension for 72^0 C for 4 min. The analyzed PCR products were visualized after electrophoresis on a 1.2 % agarose gel stained with ethidium bromide under UV irradiation.

Results:

Effect of different concentrations of cefotaxime on the leaf and petiole explants:

Effect of different concentrations of cefotaxime (100, 200, 300, 400 and 500 mg/l) on the regeneration potential of leaf and petiole explants was studied. In the leaf explants with increase in the concentrations of cefotaxime, percent shoot regeneration was also increased upto 300 mg/l cefotaxime, later on the percent shoot regeneration declined and only callus formation was observed at 500 mg/l cefotaxime concentration (Fig. 1). The maximum per cent (75%) shoot regeneration with average number of shoots (2.0) were observed on MS shoot regeneration medium with 300mg/l cefotaxime (Table 1).

Similarly in petiole explants, with the increase in the concentrations of cefotaxime, percent shoot regeneration was also increased upto 300 mg/l cefotaxime then the percent shoot regeneration starts decreasing and no callus formation was observed upto 500 mg/l cefotaxime concentration in petiole explants. The maximum per cent (78%) shoot regeneration with average number of shoots (3.0) were observed on MS shoot regeneration medium with 300mg/l cefotaxime (Table 2). (Fig. 2)

All the observations were recorded after 45 days of inoculation of explants.

Effect of different conc. of cefotaxime and kanamycin (with 50mg/l) on the suppression of Agrobacterial cell growth (after co-cultivation) and selection of transformed cell

The effect of varying concentrations of cefotaxime (100, 200, 300, 400 and 500 mg/l) with same concentrations of kanamycin (50 mg/l) was studied on the regeneration efficiency and their capability to control the overgrowth of agrobacterial cells after co-cultivation. In leaf and petiole explants at lower concentrations (0, 100, 200, mg/l) of cefotaxime showed overgrowth of agrobacterial cells and at higher concentrations of cefotaxime, the agrobacterial cells growth was completely inhibited (Fig. 3 & 4). In leaf explants per cent shoot regeneration (33.00%) and average number of shoots (2.0) per plant was found maximum in 400 mg/l cefotaxime with 50 mg/l kanamycin (Table 3). Whereas in petiole explants per cent shoot regeneration (48.00%) and average number of shoots (2.3) per plant was found maximum in same conc. of cefotaxime and kanamycin as in leaf explants (Table 4). At lower concentrations of cefotaxime, all the explants died due to uncontrolled growth of agrobacterial cells. At the concentration of 300 mg/l cefotaxime the density of agrobacterial cells started decreasing and it was controlled only by 3 to 4 subcultures of explants on the fresh selective medium (Fig. 3 & 4).

Shoot multiplication, elongation and root generation of in vitro developed putative transgenic shoots

In vitro developed putative transgenic shoots were further maintained and multiplied on the selective shoot regeneration medium (MS + 0.5 mg/l BAP + 0.2 mg/l IAA + 0.3 mg/l GA_3) with lower concentration of antibiotics (kanamycin 30mg/l, cefotaxime 300mg/l). The shoots elongated on the same medium. After obtaining 2-3 cm height, the shoots were transferred to the selective root regeneration medium containing 0.10mg/l IBA+ 30mg/l kanamycin + 300mg/l cefotaxime. Roots initiated after 20-22 days of inoculation on the selective medium and within 35-40 days well developed roots were observed (Fig. 5).

Amplification of cryIAa gene by polymerase chain reaction

This isolated, purified and quantified DNA (plasmid DNA and genomic DNA of putative transgenic plantlets and control plantlets) was used for amplification of transgene by polymerase chain reaction. The amplification of transgene i.e. cryIAa was carried out by polymerase chain reaction to study the presence/ integration of the target gene i.e. cryIAa into the genome of putative transgenic plantlets of Himalayan poplar. Total genomic DNA was isolated from the leaves of randomly selected 48 putative transgenic plantlets along with control plantlets by the method of Doyle and Doyle (1990). Gene specific primers were used to amplify 1.0 kb fragment of cryIAa gene by polymerase chain reaction. Out of 48 randomly selected putative transgenic plantlets, 16 have shown the amplification of cryIAa gene there by indicating the presence/ integration of cryIAa gene into the genome of transgenic Himalayan poplar (Fig. 6).

Discussion

The effect of different concentrations of cefotaxime has been studied separately on the regeneration potential of Himalayan poplar. In our experiment maximum per cent shoot regeneration was obtained on the best shoot regeneration medium with 300mg/l cefotaxime. It

has been observed that the increase in the concentration of cefotaxime upto 300mg/l, enhances the percent shoot regeneration whereas, further increase in the concentration of cefotaxime (above 300mg/l) the percent shoot regeneration declines. Cefotaxime itself did not inhibit callus growth in culture medium, but it clearly decreased shoot differentiation (Ling et al. 1998) Cefotaxime has potential to increase the growth, regeneration and embryogenesis in vitro. Cefotaxime promoted growth and morphogenesis in callus cultures of wheat and barley (Mathias and Boyd 1986; Mathias and Mukasa 1987). The addition of high concentration of cefotaxime inhibited shoot formation in many plants, e.g. *Antirrhinum majus* (Holford and Newbury 1992), *Malus* (Yepes and Aldwinckle 1994) and *Nicotiana tabacum* (Nauerby et al. 1997). Yepes and Aldwinekle, (1994) evaluated effect of Cefotaxime in morphogenesis of apple. Cefotaxime at lower dose of 250 mg/l enhanced regeneration and shoot development. Similar studies were also carried out by Humara et al. (1999) and they were observed that 250 µg/ml cefotaxime enhanced the shoot regeneration capacity. Danilova and Dolgikh (2004) reported stimulatory effect of the antibiotic cefotaxime on plant regeneration in Maize tissue culture and enhanced its morphogenesis. The highest increase in the number of regenerated plants was observed at the antibiotic concentration of 150 mg/l. Kaur et al. (2008) obtained enhanced in vitro shoot multiplication and elongation in sugarcane used at the rate of 250 and 500 mg/l cefotaxime in the medium.

Agrobacterium-mediated plant genetic transformation studies often utilize selective agents (antibiotics) for the elimination of the bacterium, allowing for the survival and regeneration of transformed cells/tissues on a negative or positive selective medium. The successful elimination of *Agrobacterium* (following sufficiently long co cultivation periods to transform cells) from the regeneration media in transformation protocol is important for the successful recovery of transgenic cells and tissues (Teixeira da Silva and Fukai 2001). In the combined effect of cefotaxime with kanamycin experiment, transformation efficiency is adversely affected by the growth of *Agrobacterium* in the medium after the co-cultivation. In order to remove the *Agrobacterium* completely after the co-cultivation from the medium, different concentrations of cefotaxime (0, 100, 200, 300, 400 and 500 mg/l) with same concentration of kanamycin (50 mg/l) were studied in the shoot regeneration medium. In the present studies, the concentration of cefotaxime 400 mg/l found to be optimum for controlling growth of agrobacterial cells and to obtain high shoot regeneration frequency after co-cultivation. Further increase in the concentration of cefotaxime (above 400 mg/l) the percent shoot regeneration declines. Thakur et al. (2005) and Thakur and Srivastava (2009) had reported 500 mg/l concentration of cefotaxime effective to control the growth of agrobacterial cells in Himalayan poplar. Cefotaxime in a concentration of 500 mg/l also worked very well to eliminate agrobacterial cells during development of transgenic cells in Lombardy poplar (*Populus nigra*) (Mohri et al. 1999) and in several economically important cottonwood hybrids (*P. trichocarpa* x *P. deltoides* and *P. deltoides* x *P. nigra*) (Han et al. 2000), whereas, 300 mg/l cefotaxime worked well in controlling agrobacterial growth in *Populus alba* transformation (Confalonieri et al. 2000). Confalonieri et al. (1997) developed transgenic plants of *P. euramiericana* and *P. deltoides* by transferring developing calli into WP medium

containing 300 mg/l carbenicillin and 300 mg/l cefotaxime, while Klopfenstein (1997) regenerated transgenic shoots from leaf explants of *P. alba* x *P. grandidentata* clone Hansen on shoot regeneration medium containing 500 µg/ml carbenicillin and 250 µg/ml cefotaxime. Igasaki et al. (2002) supplemented shoot regeneration medium with 500 mg/l carbenicillin and 500 mg/l cefotaxime to control agrobacaterial growth in transformation of *P. alba*.

Modgil and Sharma, (2008) reported that 500 mg/l cefotaxime was found to be the best concentration to control *Agrobacterium* growth after co-cultivation experiments. Mannan et al. (2009) reported that cefotaxime and kanamycin at a concentration of 500 mg/l and 20 mg/l, respectively controlled overgrowth of *Agrobacterium* with effective selection of transformed cell of *Artemisia absinthium* L. Jabeen et al. (2009) reported that the addition of cefotaxime in the medium increased the efficiency of the transformation whereas, when no cefotaxime was added in the medium excess bacterial growth was observed in the cultures leading to the death of explants.

CONCLUSION:

In summary, we have concluded that agro-infiltration using different explants of *Populus ciliata* Wall. along with the use of the antibiotic cefotaxime was efficient during selection for producing the transgenic plants. This combination had significant utility in poplar plant transformation towards the development of a high frequency of transgenic plantlets. PCR amplification of *cry IAa* gene finally confirmed stable transformation in Himalayan poplar.

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Table: I Effect of different concentrations of cefotaxime on the regeneration potential of leaf tissues in Himalayan poplar (Populus ciliata Wall.) (without co-cultivation)

S. No.	Medium Composition	Average number of shoots per explants	Per cent shoot regeneration
1.	MS basal medium + 0.024mg/l TDZ + 79.7mg/l adenine + 100mg/l cefotaxime	0.6	58.33 (49.82)
2.	MS basal medium + 0.024mg/l TDZ + 79.7mg/l adenine + 200mg/l cefotaxime	1.6	66.66 (54.51)
3.	MS basal medium + 0.024mg/l TDZ + 79.7mg/l adenine + 300mg/l cefotaxime	2.3	75.00 (60.06)
4.	MS basal medium + 0.024mg/l TDZ + 79.7mg/l adenine + 400mg/l cefotaxime	0.2	48.33 (43.73)
5.	MS basal medium + 0.024mg/l TDZ + 79.7mg/l adenine + 500mg/l cefotaxime	----	Callus formation
$CD_{0.05}$		0.97	5.41
SE±		0.43	2.43

(The values in the parenthesis are arc sine transformed values)

Table: 2 Effect of different concentrations of cefotaxime on the regeneration potential of petiole tissues in Himalayan poplar (Populus ciliata Wall.) (without co-cultivation)

S. No.	Medium Composition	Average number of shoots per explants	Per cent shoot regeneration
1.	MS basal medium + 0.044mg/l TDZ + 79.7mg/l adenine + 100mg/l cefotaxime	0.8	60.33 (50.92)
2.	MS basal medium + 0.044mg/l TDZ + 79.7mg/l adenine + 200mg/l cefotaxime	1.9	71.66 (57.86)
3.	MS basal medium + 0.044mg/l TDZ + 79.7mg/l adenine + 300mg/l cefotaxime	3.0	78.00 (62.31)
4.	MS basal medium + 0.044mg/l TDZ + 79.7mg/l adenine + 400mg/l cefotaxime	0.4	48.33 (44.09)
5.	MS basal medium + 0.044mg/l TDZ + 79.7mg/l adenine + 500mg/l cefotaxime	0.2	33.33(35.26)
$CD_{0.05}$		0.54	3.53
SE±		0.24	1.59

(The values in the parenthesis are arc sine transformed values)

Table: 3 Effect of various concentrations of cefotaxime with kanamycin (50 mg/l) on the suppression of Agrobacterial cell growth (after co-cultivation) and selection of transformed cell from leaf explants in Himalayan poplar (Populus ciliata Wall.)

S. No.	Medium Composition	Average number of shoots per explants	Per cent shoot regeneration
1.	MS basal medium + 0.024mg/l TDZ + 79.7mg/l adenine + 0mg/l cefotaxime + 50mg/l kanamycin	0.0	0.00 (0.00)
2.	MS basal medium + 0.024mg/l TDZ + 79.7mg/l adenine + 100mg/l cefotaxime + 50mg/l kanamycin	0.0	0.00 (0.00)
3.	MS basal medium + 0.024mg/l TDZ + 79.7mg/l adenine + 200mg/l cefotaxime + 50mg/l kanamycin	0.0	0.00 (0.00)
4.	MS basal medium + 0.024mg/l TDZ + 79.7mg/l adenine + 300mg/l cefotaxime + 50mg/l kanamycin	0.2	8.33 (3.16)
5.	MS basal medium + 0.024mg/l TDZ + 79.7mg/l adenine + 400mg/l cefotaxime + 50mg/l kanamycin	2.0	33.00 (3.16)
6.	MS basal medium + 0.024mg/l TDZ + 79.7mg/l adenine + 500mg/l cefotaxime + 50mg/l kanamycin	1.4	22.38 (3.65)
CD		0.33	3.14
SE±		0.15	1.44

(The values in the parenthesis are arc sine transformed values)

Table: 4 Effect of various concentrations of cefotaxime with kanamycin (50 mg/l) on the suppression of Agrobacterial cell growth (after co-cultivation) and selection of transformed cell from petiole explants in Himalayan poplar (Populus ciliata Wall.)

S. No.	Medium Composition	Average number of shoots per explants	Per cent shoot regeneration
1.	MS basal medium + 0.044mg/l TDZ + 79.7mg/l adenine + 0mg/l cefotaxime +	0.0	0.00 (0.00)

	50mg/l kanamycin		
2.	MS basal medium + 0.044mg/l TDZ + 79.7mg/l adenine + 100mg/l cefotaxime + 50mg/l kanamycin	0.0	0.00 (0.00)
3.	MS basal medium + 0.044mg/l TDZ + 79.7mg/l adenine + 200mg/l cefotaxime + 50mg/l kanamycin	0.0	0.00 (0.00)
4.	MS basal medium + 0.044mg/l TDZ + 79.7mg/l adenine + 300mg/l cefotaxime + 50mg/l kanamycin	0.3	10.33 (18.38)
5.	MS basal medium + 0.044mg/l TDZ + 79.7mg/l adenine + 400mg/l cefotaxime + 50mg/l kanamycin	2.3	48.00 (43.98)
6.	MS basal medium + 0.044mg/l TDZ + 79.7mg/l adenine + 500mg/l cefotaxime + 50mg/l kanamycin	1.8	23.33 (28.87)
CD		0.39	3.78
SE±		0.18	1.74

Fig.1 (A-E): Effect of cefotaxime on the regeneration potential of leaf explants in Himalayan poplar (Populus ciliate Wall) without co-cultivation

A. Leaf explants cultured on selective shoot regeneration medium (MS medium + 0.024mg/l TDZ + 79.7 mg/l adenine + 100mg/l cefotaxime) showing shoot regeneration after 4 weeks in culture.

B. Leaf explants cultured on selective shoot regeneration medium (MS medium + 0.024mg/l TDZ + 79.7 mg/l adenine + 200mg/l cefotaxime) showing shoot regeneration after 4 weeks in culture.

C. Leaf explants cultured on selective shoot regeneration medium (MS medium + 0.024mg/l TDZ + 79.7 mg/l adenine + 300mg/l cefotaxime) showing shoot regeneration after 4 weeks in culture.

D. Leaf explants cultured on selective shoot regeneration medium (MS medium + 0.024mg/l TDZ + 79.7 mg/l adenine + 400mg/l cefotaxime) showing shoot regeneration after 4 weeks in culture.

E. Leaf explants cultured on selective shoot regeneration medium (MS medium + 0.024mg/l TDZ + 79.7 mg/l adenine + 500mg/l cefotaxime) showing only callus formation after 4 weeks in culture.

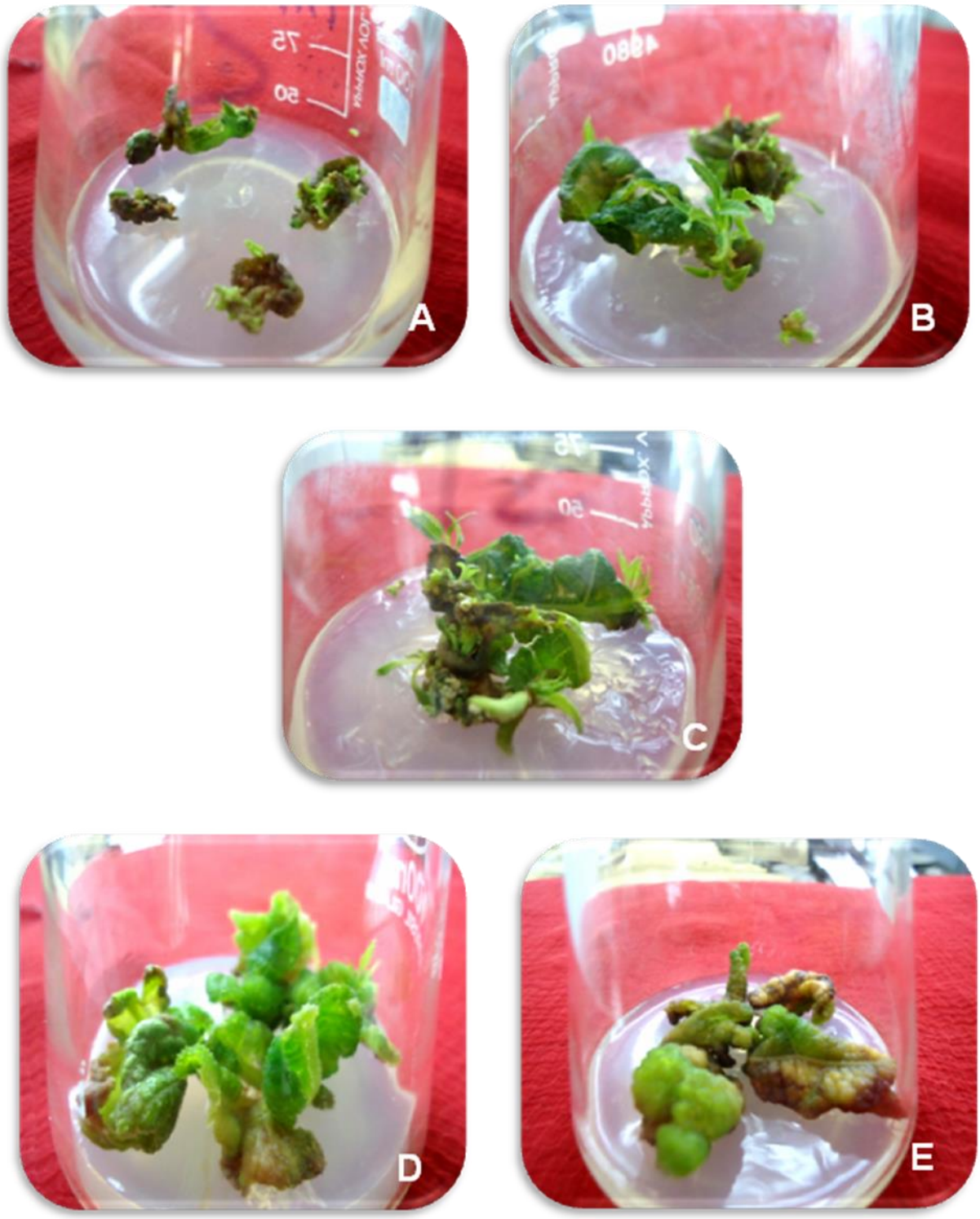

Fig 1. (A-E)

Fig.2 (A-E): Effect of cefotaxime on the regeneration potential of petiole explants in Himalayan poplar (Populus ciliate Wall) without co-cultivation

A. Petiole explants cultured on selective shoot regeneration medium (MS medium + 0.044mg/l TDZ + 79.7 mg/l adenine + 100mg/l cefotaxime) showing shoot regeneration after 4 weeks in culture.

B. Petiole explants cultured on selective shoot regeneration medium (MS medium + 0.044mg/l TDZ + 79.7 mg/l adenine + 200mg/l cefotaxime) showing shoot regeneration after 4 weeks in culture.

C. Petiole explants cultured on selective shoot regeneration medium (MS medium + 0.044mg/l TDZ + 79.7 mg/l adenine + 300mg/l cefotaxime) showing shoot regeneration after 4 weeks in culture.

D. Petiole explants cultured on selective shoot regeneration medium (MS medium + 0.044mg/l TDZ + 79.7 mg/l adenine + 400mg/l cefotaxime) showing shoot regeneration after 4 weeks in culture.

E. Petiole explants cultured on selective shoot regeneration medium (MS medium + 0.044mg/l TDZ + 79.7 mg/l adenine + 500mg/l cefotaxime) showing shoot regeneration after 4 weeks in culture.

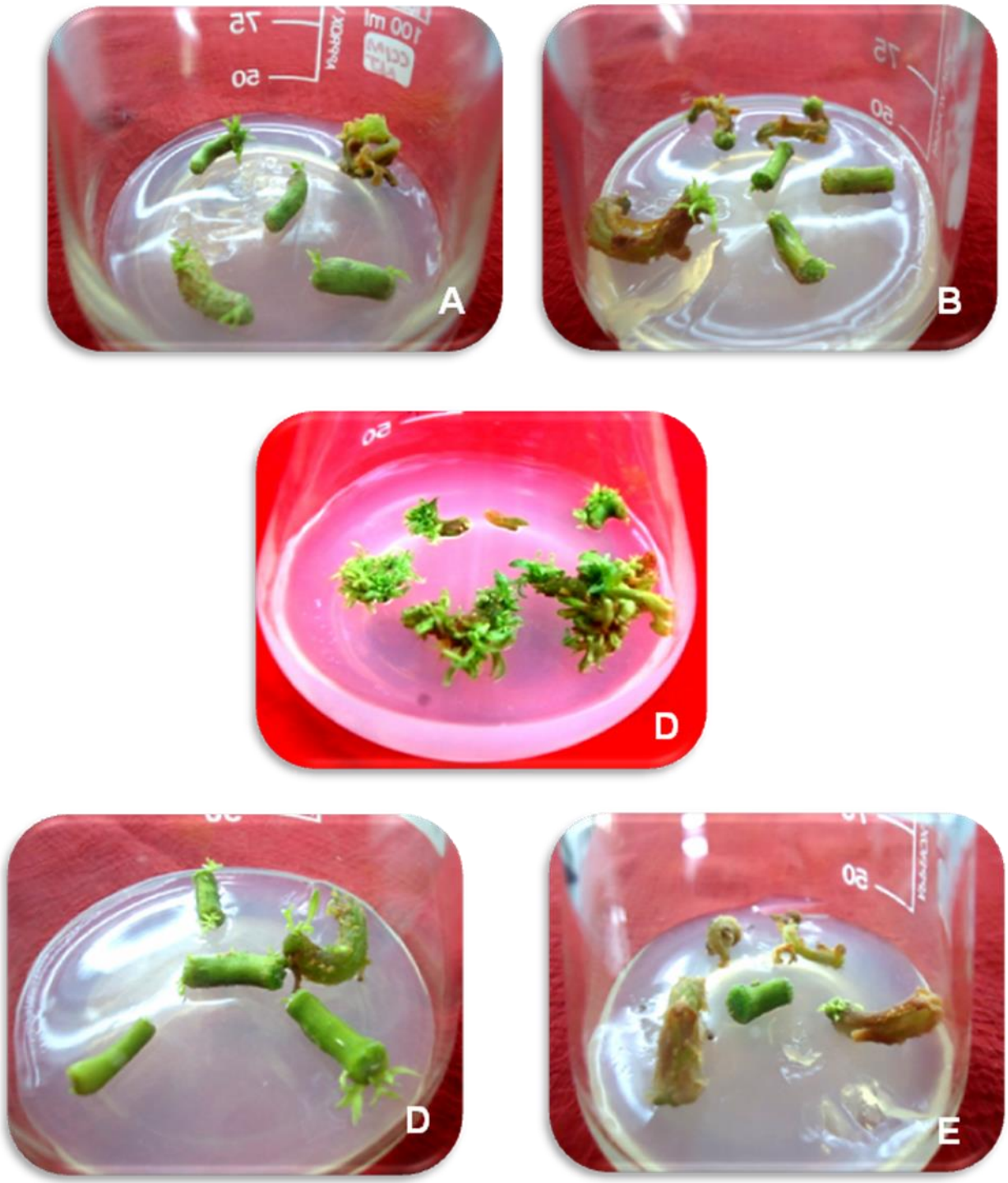

Fig 2. (A-E)

Fig.3 (A-E): Effect of cefotaxime and kanamycin (50 mg/l) on the regeneration potential of leaf explants and growth of agrobacterial cells in Himalayan poplar (Populus ciliate Wall) after co-cultivation

A. Leaf explants cultured on selective shoot regeneration medium (MS medium + 0.024mg/l TDZ + 79.7 mg/l adenine + 100mg/l cefotaxime + 50 mg/l Kanamycin) showing overgrowth of agrobacterial cells.
B. Leaf explants cultured on selective shoot regeneration medium (MS medium + 0.024mg/l TDZ + 79.7 mg/l adenine + 200mg/l cefotaxime + 50 mg/l Kanamycin) showing overgrowth of agrobacterial cells.
C. Leaf explants cultured on selective shoot regeneration medium (MS medium + 0.024mg/l TDZ + 79.7 mg/l adenine + 300mg/l cefotaxime + 50 mg/l Kanamycin) showing growth of agrobacterial cells and shoot regeneration.
D. Leaf explants cultured on selective shoot regeneration medium (MS medium + 0.024mg/l TDZ + 79.7 mg/l adenine + 400mg/l cefotaxime + 50 mg/l Kanamycin) showing shoot regeneration and no growth of agrobacterial cells.
E. Leaf explants cultured on selective shoot regeneration medium (MS medium + 0.024mg/l TDZ + 79.7 mg/l adenine + 500mg/l cefotaxime + 50 mg/l Kanamycin) showing shoot regeneration and no growth of agrobacterial cells.

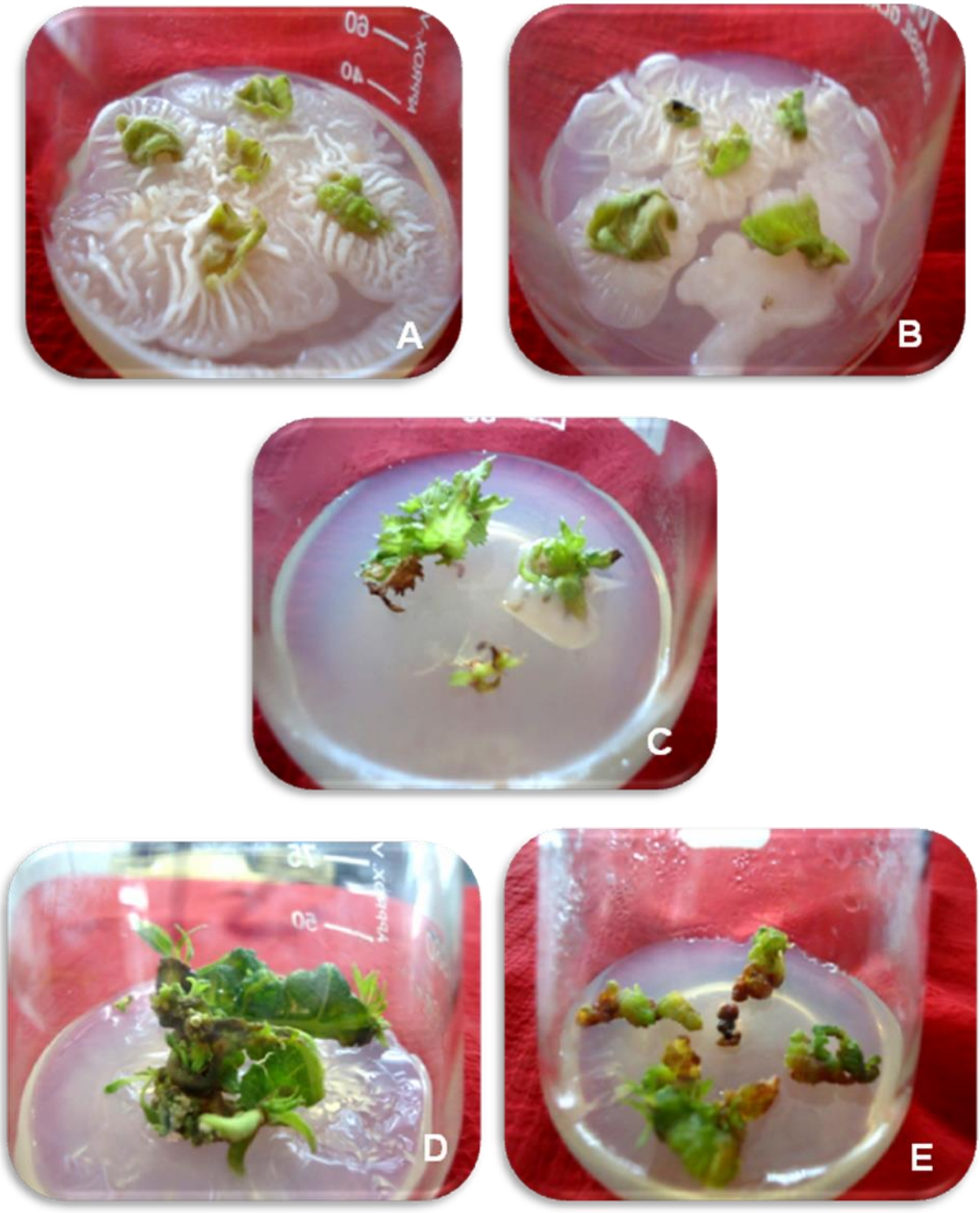

Fig 3. (A-E)

Fig.4 (A-E): Effect of cefotaxime and kanamycin (50 mg/l) on the regeneration potential of petiole explants and growth of agrobacterial cells in Himalayan poplar (Populus ciliate Wall) after co-cultivation

A. Petiole explants cultured on selective shoot regeneration medium (MS medium + 0.044mg/l TDZ + 79.7 mg/l adenine + 100mg/l cefotaxime + 50 mg/l Kanamycin) showing overgrowth of agrobacterial cells.

B. Petiole explants cultured on selective shoot regeneration medium (MS medium + 0.044mg/l TDZ + 79.7 mg/l adenine + 200mg/l cefotaxime + 50 mg/l Kanamycin) showing overgrowth of agrobacterial cells.

C. Petiole explants cultured on selective shoot regeneration medium (MS medium + 0.044mg/l TDZ + 79.7 mg/l adenine + 300mg/l cefotaxime + 50 mg/l Kanamycin) showing growth of agrobacterial cells and shoot regeneration.

D. Petiole explants cultured on selective shoot regeneration medium (MS medium + 0.044mg/l TDZ + 79.7 mg/l adenine + 400mg/l cefotaxime + 50 mg/l Kanamycin) showing shoot regeneration and no growth of agrobacterial cells.

E. Petiole explants cultured on selective shoot regeneration medium (MS medium + 0.044mg/l TDZ + 79.7 mg/l adenine + 500mg/l cefotaxime + 50 mg/l Kanamycin) showing shoot regeneration and no growth of agrobacterial cells.

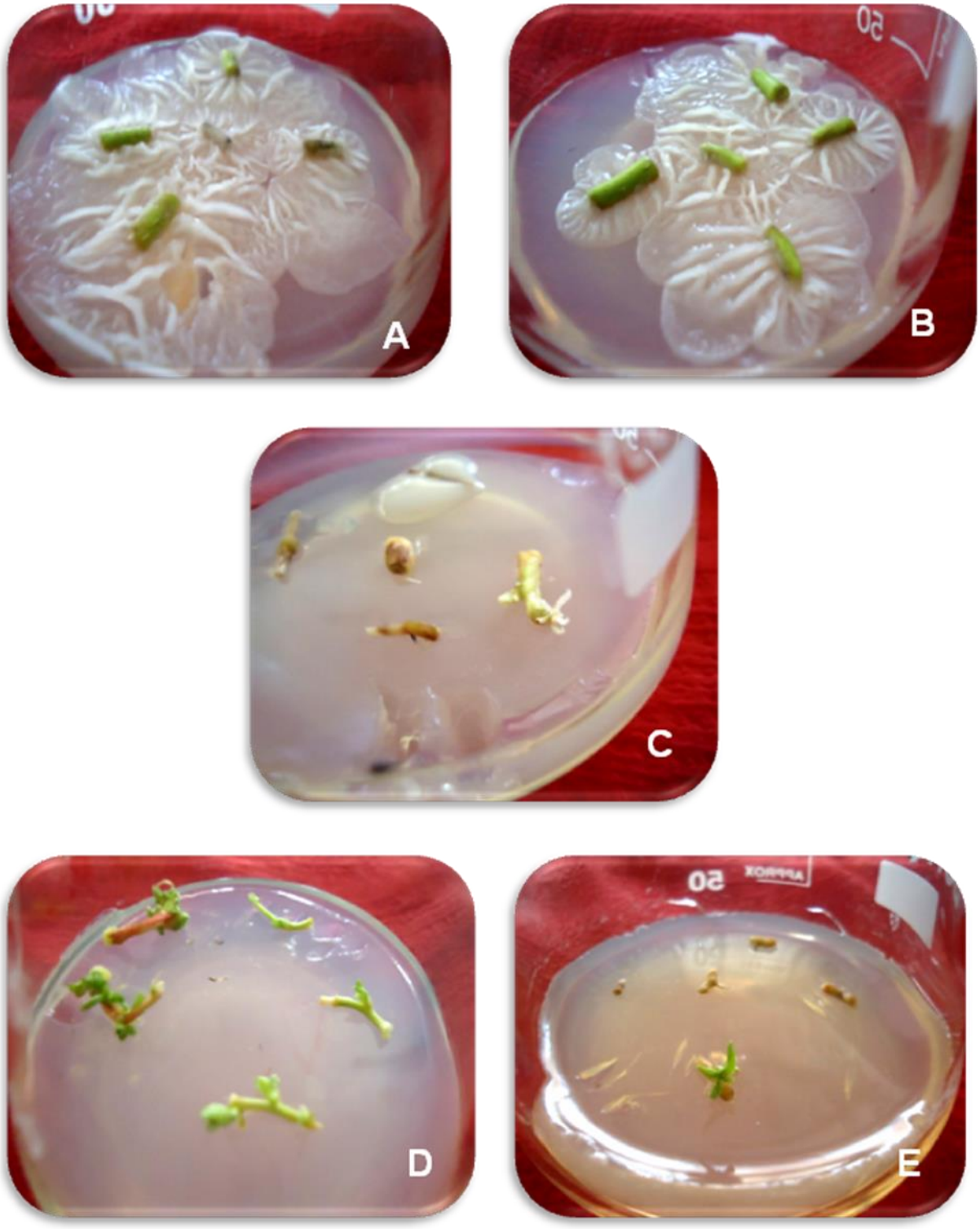

Fig 4. (A-E)

Fig.5 (A-D): Hardening of in vitro regenerated putative transgenic plantlets of Himalyan poplar (Populus ciliata Wall.)

A Root regeneration in in vitro developed shoots after 20 days on selective root regeneration medium.

B In vitro regenerated putative transgenic plantlets of Himalayan Poplar showing well developed root system

C In vitro regenerated putative transgenic plantlets of Himalayan poplar kept for hardening in pots containing mixture of sand : soil (1:1).

D Young, healthy plantlets successfully acclimatized on the planting substrate after four 35-40 days of hardening.

Fig 5. (A-D)**Fig.6: PCR analysis showing amplification of 1 Kb DNA fragment of cryIAa gene in regenerated transgenic plantlets of Himalayan Poplar (Populus ciliata Wall.).**

M: **High range DNA ruler (GENEI)**
+ve: **Positive control (Plasmid DNA of Agrobacterium Strain)**
-ve: **Negative control (Genomic DNA of control plantlets (non-transformed) of Himalayan poplar)**
T_1-T_{48}: **Putative transgenic plantlets of Himalayan Poplar**

Out of 48 randomly selected putative transgenic plantlets, 16 have shown the amplification of cryIAa gene there by indicating the presence/ integration of cryIAa gene into the genome of transgenic Himalayan poplar

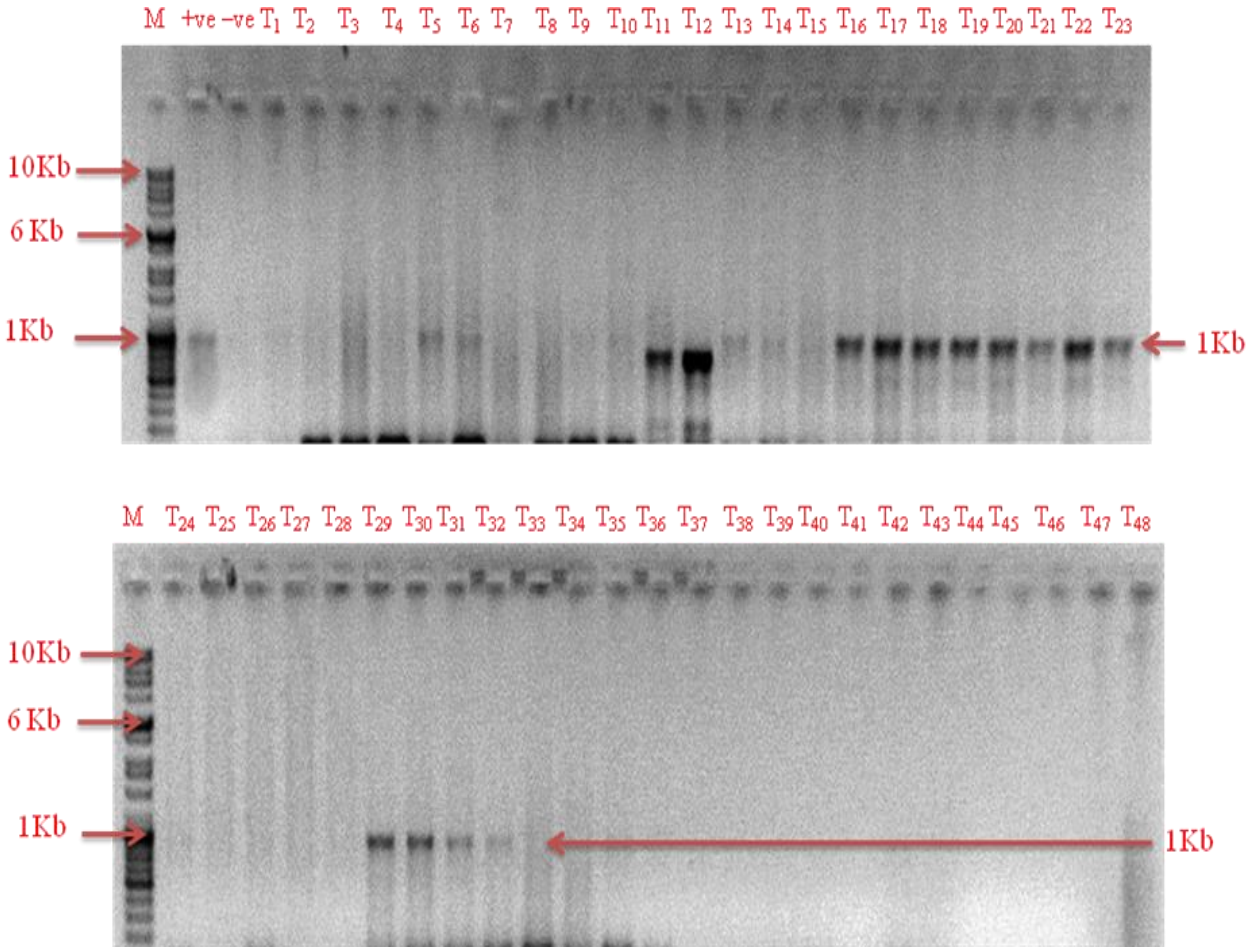

Fig 6.