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Agrobacterium-Mediated Fungal Resistance Gene Transfer Studies Pertaining to Antibiotic Sensitivity on Cultured Tissues of Lettuce (*Lactuca Sativa* L. cv. Solan kriti)

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A B S T R A C T

Development of an efficient protocol for genetic transformation in plants requires effective shoot regeneration and antibiotic selection systems. Genetically engineered disarmed *Agrobacterium tumefaciens* strain containing binary vector pCambia with *chiII* (fungal resistance gene) and *hpt* (hygromycin resistance) genes was used for genetic transformation studies. Hygromycin and cefotaxime sensitivity studies were conducted using leaf and petiole explants of lettuce (*Lactuca sativa* cv.Solan Kriti) to explore the aptness of hygromycin resistance as a selectable marker and cefotaxime in controlling excessive bacterial growth during genetic transformation studies. Explants (leaf and petiole) showed decrease in fresh weight as concentration of the hygromycin increased resulting in full or partial inhibition of shoot regeneration. A negative correlation was observed between the concentration of hygromycin and fresh weight of the explants at different intervals of time. Effect of different concentrations of cefotaxime was studied on the regeneration potential in leaf and petiole explants of lettuce. PCR analysis of genomic DNA using specific designed primers was done to detect the presence of *chiII* and *hpt* genes in hygromycin resistant plantlets of lettuce. Out of five randomly selected putative transgenic shoots, four shoots were found positive for the presence/integration of *chiII* and *hpt* genes during T-DNA transfer and integration into the plant genome. The results indicate that hygromycin and cefotaxime act as an effective selective agent during genetic transformation studies.

Introduction

Lettuce (*Lactuca sativa* L.) is a widely used leafy vegetable belonging to the family Asteraceae (2n = 18). It is nutritionally rich with medicinal property and well known for its high vitamin A content, and minerals like calcium and iron. This is the only crop which is rich in Lactupicrin and act as an anticancerous as well as, suitable candidate for the production and delivery of therapeutic proteins (Resh, 2001; Ryder, 2002; Mohebodini *et al.*, 2011). However, this crop is severely affected by a number of biotic and abiotic stresses which causes enormous crop yield losses during commercial cultivation of lettuce. *Agrobacterium tumefaciens* mediated genetic transformation is most common and feasible method to transfer gene of interest into different crop plants and is a widely used method for developing resistance against various diseases (Srivastava, 2003). When

gene transfer is attempted, efficient selection system is required whereby transformed cells can be separated from untransformed cells. Several studies support the concept that most of the foreign genes introduced by *Agrobacterium* are normally transmitted to the progeny (Gelvin, 1998). Many groups have reported the transformation ranging from 8% to 20% transformation efficiency in lettuce (Dias *et al.,* 2005; Ziarani *et al.,* 2014). Ideal transformants can be found with difficulty, depending upon the plant material to be transformed and to some extent on the nature and the transgene complexity. The establishment of a transformation procedure requires the use of a selectable marker gene; which allows the preferential growth of transformed cells in the presence of a selective agent. Selection efficiency depends on the size of exposed tissue, developmental stage of the plant cells, regeneration response and concentration of the selective agent. In transformation of lettuce, two most popular aminoglycosides antibiotic resistance marker genes are *npt-II* (neomycin phosphotransferase-II) for kanamycin resistance and *hpt* (hygromycin phosphotransferase) for hygromycin-B resistance.

Hygromycin B is an aminoglycoside antibiotic for selection which is inactivated by *hptIV* gene isolated from soil bacterium *Streptomyces hygroscopicus* and *E. coli*. Aminoglycoside antibiotic could combine with ribosme 70S subunit in the chloroplast and mitochondria and interferes with protein translocation by causing mistranslation, finally render etiolation and death of plant. The enzyme hygromycin phosphotransferase produced by *hpt* gene phosphorylates hygromycin-B, and therefore inactivates it. This requires as a first step knowledge of relative tolerance of the plant cells to antibiotics for which resistance marker exist. The sensitivity of plant cells to the selection agents depends upon the genotype, the explants type, the developmental stage, and the tissue culture conditions and should, therefore be determined under actual conditions of the genetic transformation and regeneration processes (Koronfel, 1998).

Leaf and petiole explants of lettuce were subjected to increasing doses of hygromycin to identify lowest concentration required to completely inhibit callus growth and adventitious shoot differentiation. If the growth in presence of a normal inhibitory concentration is taken as an indicator of antibiotic activity then that would represent the lowest concentration appropriate for selection of resistant tissue/ callus/ shoots in *Agrobacterium* co-cultivated explants. One, another most commonly used antibiotic in genetic transformation is cefotaxime, which is required after co-cultivation experiment. Cefotaxime have a broad

spectrum of activity against both gram positive and gram negative bacteria where it block the cell wall mucopeptide biosynthesis by inhibiting the cross linking of peptidoglycan by binding and inactivating transpeptidases thus inhibiting cell wall biosynthesis. Thus it requires an efficient knowledge about the relative tolerance of the plant material to antibiotics for which a resistance marker exists. However, it depends on many factors like genotype of the explants, explants type, the development stage and regeneration ability of explants and also the impact of antibiotics used during transformation to eliminate *A. tumefaciens*.

The objective of the present investigation was to study the effect of hygromycin and cefotaxime on cultured tissues of lettuce and based on these results, an efficient *in vitro* selection system for the further transformation was developed.

Materials and Methods

Plant material and culture medium

The certified seeds of lettuce (*Lactuca sativa* cv.Solan Kriti) were procured from the Department of Vegetable Science, Dr Y. S. Parmar University of Horticulture and Forstery, Nauni, Solan. The leaf and petiole explants were obtained from the fifteen to twenty days old glass house grown seedlings. The explants were washed thoroughly under running tap water for half an hour and then treated with 0.2% bavistin solution for 2 minutes and 1 % $HgCl_2$ for 1 minute and then washed with sterilized distilled water for 3-4 times to remove any traces of mercuric chloride.

Agrobacterium strain and plasmid vector

Genetically engineered *Agrobacterium tumefaciens* strain harbouring plasmid pCAMBIA bar- ubi-chi 11, which contained *hpt* as a plant selectable marker, and the gene of interest *chi 11* (rice chitinase gene), obtained from Dr S. Muthukrishnan, Kansas State University, USA, was utilized for *Agrobacterium*-mediated gene transfer studies. In this construct, the expression of *chi 11* gene was driven by Ubi promoter and *hpt* gene was under the control of CaMV 35S promoter. The *hpt* gene was located close to the T-DNA left border and downstream of the *chi 11* gene (Fig. 1).

Effect of hygromycin on the growth of callus and shoot regeneration

The leaf and petiole explants excised from 15-20 days old glasshouse grown seedlings were cut into small pieces, weighed for their fresh weights on mettler balance under aseptic conditions in laminar air flow cabinet and cultured on MS shoot regeneration medium without (control) and with different concentrations of hygromycin in different Petriplates. The initial fresh weight of the explants was recorded. The selective medium for hygromycin sensitivity studies was prepared by adding different concentrations of hygromycin into pre-sterilized molten MS basal medium (Murashige and Skoog, 1962) containing 0.25 mg/l BAP and 0.10 mg/l NAA for leaf explants and MS basal medium containing 0.75 mg/l Kn and 0.10 mg/l NAA for petiole explants under aseptic conditions by filter sterilization through a 0.22µm pore size Millipore membrane filter. Different concentrations (2.5, 5, 7.5, 10, 12.5 and 15 mg/l) of hygromycin were added to study the effect of antibiotic on the relative growth (fresh weight) of the cultured explants. The cultured leaf and petiole explants were observed for callus formation/adventitious shoot regeneration and eventual changes in fresh weights. Morphological changes were observed in these tissues from 0 to 35 days in culture. Relative growth (fresh weight) of explants was calculated at the interval of seven days. Each treatment consisted of six replications, each with five leaf and petiole explants.

Effect of cefotaxime on the regeneration potential

The leaf and petiole explants were excised and cultured on selective shoot regeneration medium that was prepared by adding different concentrations of cefotaxime (100, 200, 300, 400 and 500 mg/l) into pre-sterilized molten shoot regeneration medium for leaf (MS basal medium containing 0.25 mg/l BAP and 0.10 mg/l NAA) and petiole (MS basal medium containing 0.75 mg/l Kn and 0.25 mg/l NAA) explants under aseptic conditions by filter sterilization through a 0.22µm pore size Millipore membrane filter to study its effect on the regeneration potential of the cultured explants. Morphological changes were observed in these explants for callus formation and adventitious shoot regeneration.

Agrobacterium tumefaciens-mediated genetic transformation and callus induction

Prior to infection of explants with *Agrobacterium*, four fresh colonies of *A. tumefaciens* harbouring plasmid pCAMBIA bar- ubi-chi 11 on YMB medium plate (1% Mannitol, 0.04%, Yeast extract, 0.01% NaCl, 0.02% $MgSO4.7H_2O$, 0.05% K_2HPO4, 1.5% Agar agar, pH 7.0) were inoculated in liquid YMB medium supplemented with 50 mg/l kanamycin and 25 mg/l streptomycin and incubated overnight at 28^0C with continuous shaking at 250 rpm. *Agrobacterium* cells were harvested by centrifugation at 10,000 rpm for 10 minutes and resuspended in liquid MS basal medium containing 30g/l sucrose to a final density of 10^8cells/ml had an OD 0.521 at 540nm, which was fixed for genetic transformation experiment. The explants were pre-cultured on shoot regeneration medium for 72 hours and then infected by immersing in *Agrobacterium* suspension for 1 minute with gentle shaking three to five times during the infection process. Subsequently, the infected explants were dried on a sterile Whatmann filter paper and transferred onto same pre-culturing medium for 72 hours for co-cultivation at 26±2 ^{0}C. Following co-cultivation, the infected explants were transferred onto selective shoot regeneration medium containing 300 mg/l cefotaxime and 7.5mg/l hygromycin and kept at 26±2 ^{0}C under the 16-h light, 8-h dark cycle in the culture room for callus induction. To avoid non transformed callus escaping from the hygromycin selection, the infected explants were subjected to hygromycin selection.

DNA isolation and PCR analysis

Genomic DNA was isolated from non-transformed shoots (control) and hygromycin resistant shoots/plantlets, according to modified cetyl- trimethyl ammonium bromide method (Doyle and Doyle 1990) with minor modifications. Plasmid DNA was isolated using Plasmid Isolation Kit. PCR analysis were carried out to detect the presence of the *hpt* and chitinase genes respectively, using primers HPT-F 5'ATGAAAAAGCCTGAACT CACCGCGA3' and HPT-R 5'TCCATCACAGTTTGCCA GTGATACA 3' and CHI-F 5'GGACGCAGTCTCCTT CAAGA 3' and CHI-R 5'ATGTCGCAGTAGCGCTTGTA3'. Linkage analysis of these two genes was conducted using PCR amplification.

Results and Discussion

Lettuce (*Lactuca sativa* L. Solan Kriti) is an agronomically important leafy vegetable that can be grown worldwide. The production of lettuce is challenged by many stresses; these stresses either alone or together or in combination with abiotic stresses cause heavy losses. Plant diseases seriously affect the quality and yield of lettuce. The conventional methods for the control of post-harvest fungal diseases are mainly dependent on the intensive and extensive use of chemical fungicides, which have drawbacks such as damage to the ecological system and residual poisoning to human and animals. Therefore, it is desirable to develop fungal resistant plants through plant genetic engineering. Among fungicidal genes, chitinase genes have been proven effective in controlling fungal pathogens such as *Pythium* spp. *Pythium ultimum*, *Bremialactucae*, *Sclerotinia Sclerotium* in many crop plants. Genetically engineered disarmed *Agrobacterium tumefaciens* strain containing binary vector pCAMBIA bar- ubi with *chi* (fungal resistance gene) and *hpt* (hygromycin phosphotransferase) genes was used for genetic transformation studies. All transformation systems for creating transgenic

plants entail separate processes for introducing cloned DNA into living plant cells, for identifying or selecting those cells that have integrated the DNA into the appropriate plant genome (nuclear or plastid) and for regenerating or recovering fully developed plants from the transformed cell. Selectable marker genes have been pivotal to the development of plant transformation technologies because the marker genes allow scientists to identify or isolate the cells that are expressing the cloned DNA and to monitor and select for the transformed progeny. As only a very small proportion of cells are transformed in most experiments, the chances of recovering transgenic lines without selection are usually low. Since the selectable marker gene is expected to function in a range of cell types. Hygromycin resistance gene is most widely used selectable marker for plant cell transformation and sensitivity of a particular species to hygomycin is a key element in the development of any new transformation system in which a hygromycin resistance gene will be employed. The sensitivity of leaf and petiole explants for hygromycin was tested according to the fresh weight of the explant/callus and percentage of shoot regeneration. Both leaf and petiole explants showed appropriate growth *i.e.* callus initiation and shoot formation on the shoot regeneration medium devoid of hygromycin. Hygromycin sensitivity of cultured tissues of leaf and petiole explants of lettuce had shown similar results, *i.e.* both explants are highly sensitive to hygromycin even as low as 2.5 mg/l concentration of hygromycin. The non-transformed tissue did not survive on the selective medium containing hygromycin during transformation experiment. The sensitivity of leaf and petiole explants for hygromycin was tested according to the fresh weight of the explant/callus and percentage of shoot regeneration. Both leaf and petiole explants showed appropriate growth *i.e.* callus initiation and shoot formation on the shoot regeneration medium devoid of hygromycin, but on the selective media at concentration as low as (2.5 mg/l and 5.0 mg/l) of hygromycin in the culture medium, the colour of explants/tissues had changed to pale greenish yellow and finally turned brown after 35 days of culture. But increase in concentration (7.5mg/l and 10mg/l), could fasten the change in colour of explants to pale yellow and was sufficient to differentiate these from the control. At much higher concentration (12.5mg/l and 15.0mg/l), colour change and browning of the explants within 2 weeks resulted in complete necrosis of the explants. No shoot regeneration or shoot bud formation was observed from both the explants even after 5 weeks of culturing on the selective shoot regeneration medium containing different concentrations of hygromycin. In control experiment, adventitious shoot bud regeneration was observed within 35 days on the culture medium from both the leaf and petiole explants. A gradual decline in fresh weight of leaf and petiole explants were recorded with increased concentration of hygromycin (2.5 to 15 mg/l) up to 35 days. Concentration above 10.0mg/l of hygromycin inhibits shoot growth and differentiation in cultured explants of lettuce and similar results were reported by Dias *et al.,* 2006. The maximum decline in the fresh weight was observed at 15mg/l hygromycin in both the explants, whereas in case of control (without hygromycin), callus was induced from the cut edge of the both explants and a gradual increase in the fresh weights was observed. Dias *et al.,* 2006 they also used 10 mg/l hygromycin as the lethal dose for the selection of transgenic shoots containing gene against fungal tolerance in lettuce cv.Veronica. Statistical analysis of the above data have shown that there is a significant difference between the fresh weights of leaf and petiole explants/callus at different intervals in the control and six different concentrations of hygromycin (Table 1 and

2). Negative correlation coefficient was observed between different concentrations of hygromycin used versus fresh weight of explant (s)/ tissue/ callus at different intervals of time in both the explants. These results indicate that hygromycin has an inhibitory effect on the growth of cultured tissues, as it is a potent inhibitor of protein synthesis. Therefore, based on the above results, 10.0 mg/l hygromycin was used and demonstrated for the selection of transformed cells to be an effective selection agent in *Agrobacterium*-mediated genetic transformation studies in lettuce. Hygromycin has been reported for the selection of transformed and non-transformed explants with very low frequency of selection escape in lettuce (Enkhchimeg *et al.,* 2005; Dias *et al.,* 2006; Deng *et al.,* 2007) and also in many other crops like Banana (Maziah *et al.,* 2007), pigeon pea plants (Kumar *et al.,* 2004) and American ginseng plants (Chen and Punja, 2002). For successful *Agrobacterium*- mediated transformation, elimination of bacteria from culture is necessary after the co-cultivation period. This is realized by the addition of antibiotics into the culture medium; cefotaxime, an antibiotic commonly used to kill *Agrobacterium* after co-cultivation with plant material with plant material. But antibiotics, which are commonly used to eliminate *A. tumefaciens* from plant tissues, have also been shown to influence morphogenesis and regeneration potential either positively or negatively (Ling *et al.,* 1988; Ahmed *et al.,* 2007). Ahmed and his co-workers reported cefotaxime severely inhibited regeneration form *Agrobacterium* leaf explants of lettuce cultivar 'Evola' and found 50 mg/l cefotaxime to be optimum for the suppression of *Agrobacterium.* In case of lettuce (*Lactuca sativa* cv. Solan Kriti), increase in cefotaxime concentration (100mg/l to 500mg/l) showed a gradual decrease in the percent shoot regeneration in both the explants. Maximum percent (71.99%) shoot regeneration with average number of shoots per explants (0.98) were obtained on shoot regeneration medium with 100 mg/l cefotaxime concentration in leaf explants, whereas, maximum percent (59.99%) shoot regeneration with average number of shoots per explants (0.96) were obtained on shoot regeneration medium with 100 mg/l cefotaxime in petiole explants. In contrast maximum decline was observed at a higher concentration of 500mg/l cefotaxime (Table 5 and 6) showing a negative effect of the shoot regeneration potential.

Table.1 Effect of different concentrations of hygromycin on the relative growth (fresh weight) and regeneration potential of leaf explants of lettuce (*Lactuca sativa* L. Cv.Solan kriti)

S.No.	Days	Average fresh weight of explants/callus (mg)						
		Hygromycin concentration (mg/l)						
		0	**2.5**	**5.0**	**7.5**	**10.0**	**12.5**	**15.0**
2.	7	32.97 ± 0.351	42.45 ± 0.455	55.98 ± 0.500	42.47 ± 0.450	35.66 ± 0.251	30.58 ± 0.395	38.43 ± 0.386
3.	14	34.08 ± 0.360	48.37 ± 0.480	62.09 ± 0.450	47.53 ± 0.325	37.48 ± 0.336	31.69 ± 0.323	35.53 ± 0.360
4.	21	47.45 ± 0.416	56.40 ± 0.427	64.98 ± 0.400	48.50 ± 0.417	35.43 ± 0.360	26.36 ± 0.305	32.49 ± 0.305
5.	28	52.54 ± 0.436	62.43 ± 0.480	69.33 ± 0.470	50.36 ± 0.500	30.38 ± 0.264	21.70 ± 0.251	27.65 ± 0.251
6.	35	60.14 ± 0.470	68.08 ± 0.500	71.95 ± 0.480	51.62 ± 0.321	26.23 ± 0.251	18.25 ± 0.264	24.42 ± 0.264
SE± = 0.318								
$CD_{0.05}$ =0.633								

Table.2 Coefficient of correlation between concentration of hygromycin (x) and Change in average fresh weight of leaf tissue/callus (y)

S.No.	Variables	Coefficient of correlation (r)
1.	Concentration of hygromycin and fresh weight of explants/ callus on 7 thday	-0.16
2.	Concentration of hygromycin and fresh weight of explants/ callus on 14 thday	-0.32
3.	Concentration of hygromycin and fresh weight of explants/ callus on 21stday	-0.72
4.	Concentration of hygromycin and fresh weight of explants/ callus on 28thday	-0.80
5.	Concentration of hygromycin and fresh weight of explants/ callus on 35thday	-0.85

Table.3 Effect of different concentrations of hygromycin on the relative growth (fresh weight) and regeneration potential of petiole explants of lettuce (*Lactuca sativa* L. Cv.Solan kriti)

S. No.	Days	Average fresh weight of explants/callus (mg) Hygromycin concentration (mg/l)						
		0	**2.5**	**5.0**	**7.5**	**10.0**	**12.5**	**15.0**
1.	0	21.45 ± 0.251	27.30 ± 0.360	30.33 ± 0.321	34.56 ± 0.360	28.50 ± 0.360	34.64 ± 0.264	44.27 ± 0.344
2.	7	35.15 ± 0.264	39.44 ± 0.455	42.28 ± 0.352	44.07 ± 0.381	37.44 ± 0.384	33.48 ± 0.336	32.03 ± 0.386
3.	14	44.63 ± 0.461	48.36 ± 0.480	51.55 ± 0.414	45.43 ± 0.404	44.59 ± 0.321	43.47 ± 0.321	42.24 ± 0.325
4.	21	52.44 ± 0.455	56.40 ± 0.427	58.45 ± 0.494	56.48 ± 0.485	48.35 ± 0.403	42.34 ± 0.386	47.54 ± 0.360
5.	28	59.49 ± 0.495	62.43 ± 0.480	64.48 ± 0.490	58.33 ± 0.493	46.52 ± 0.356	40.50 ± 0.360	38.45 ± 0.321
6.	35	64.48 ± 0.490	68.08 ± 0.500	69.39 ± 0.488	59.88 ± 0.500	42.37 ± 0.386	33.40 ± 0.325	31.37 ± 0.305
SE± =0.327								
$CD_{0.05}$ =0.652								

Table.4 Coefficient of correlation between concentration of hygromycin (x) and Change in average fresh weight of leaf tissue/callus (y)

S.No.	Variables	Coefficient of correlation (r)
1.	Concentration of hygromycin and fresh weight of explants/ callus on 7 thday	-0.50
2.	Concentration of hygromycin and fresh weight of explants/ callus on 14 thday	-0.72
3.	Concentration of hygromycin and fresh weight of explants/ callus on 21stday	-0.69
4.	Concentration of hygromycin and fresh weight of explants/ callus on 28thday	-0.89
5.	Concentration of hygromycin and fresh weight of explants/ callus on 35thday	-0.91

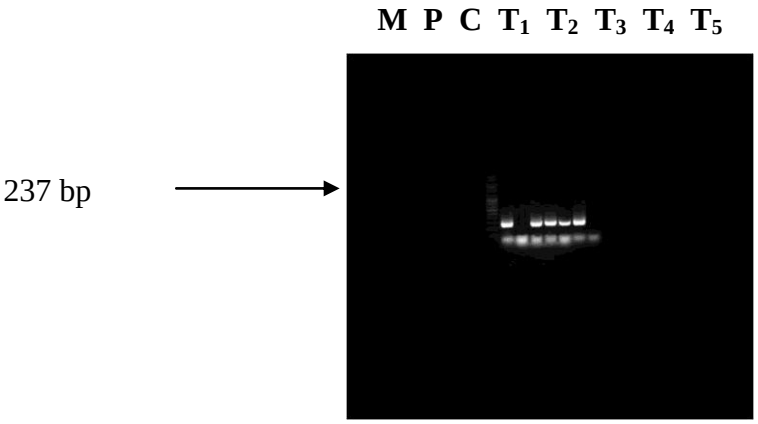
Table.5 Effect of different concentrations of cefotaxime on the relative growth of Leaf explants of lettuce (*Lactuca sativa* L.)

Sr. No.	Medium Composition	Callus formation	Shoot regeneration	Average number of shoots formed per explants	Percent shoot regeneration
1.	MS Basal + 0.25mg/l BAP + 0.10 mg/l NAA + 0 mg/l Cefotaxime	+	Shoot regeneration	1.47	73.33(59.00)
2.	MS Basal + 0.25mg/l BAP + 0.10 mg/l NAA + 100 mg/lCefotaxime	+	Shoot regeneration	0.98	71.99(58.07)
3.	MS Basal + 0.25mg/l BAP + 0.10 mg/l NAA + 200 mg/l Cefotaxime	+	Shoot regeneration	0.82	70.10(56.85)
4.	MS Basal + 0.25mg/l BAP + 0.10 mg/l NAA + 300 mg/l Cefotaxime	+	Shoot regeneration	0.73	62.99(52.52)
5.	MS Basal + 0.25mg/l BAP + 0.10 mg/l NAA + 400 mg/l Cefotaxime	+	Shoot regeneration	0.65	56.77(48.88)
6.	MS Basal + 0.25mg/l BAP + 0.10 mg/l NAA + 500 mg/l Cefotaxime	+	Shoot regeneration	0.59	54.44(47.54)
$CD_{0.05}$				0.20	8.28
SE				0.06	2.65

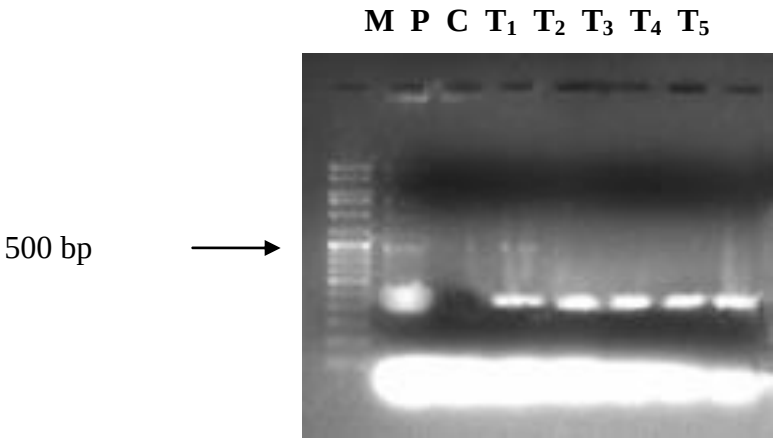
Table.6 Effect of different concentrations of cefotaxime on the relative growth of Petiole explants of lettuce (*Lactuca sativa* L.)

Sr. No.	Medium Composition	Callus formation	Shoot regeneration	Average number of shoots formed per explants	Percent shoot regeneration
1.	**MS Basal + 0.75mg/l Kin + 0.10 mg/l NAA + 0 mg/l Cefotaxime**	+	Shoot regeneration	1.09	63.22(52.65)
2.	**MS Basal + 0.75mg/l Kin + 0.10 mg/l NAA + 100 mg/l Cefotaxime**	+	Shoot regeneration	0.96	59.99(50.78)
3.	**MS Basal + 0.75mg/l Kin + 0.10 mg/l NAA + 200 mg/l Cefotaxime**	+	Shoot regeneration	0.89	56.77(48.88)
4.	**MS Basal + 0.75mg/l Kin + 0.10 mg/l NAA + 300 mg/l Cefotaxime**	+	Shoot regeneration	0.79	52.21(46.25)
5.	**MS Basal + 0.75mg/l Kin + 0.10 mg/l NAA + 400 mg/l Cefotaxime**	+	Shoot regeneration	0.69	49.99(44.98)
6.	**MS Basal + 0.75mg/l Kin + 0.10 mg/l NAA + 500 mg/l Cefotaxime**	+	Shoot regeneration	0.63	43.44(41.20)
$CD_{0.05}$				0.07	7.87
SE				0.02	2.52

Fig.1A and 1B Molecular Characterization of putative transformants of lettuce (*Lactuca sativa* L. cv.Solan kriti)



A: Confirmation of presence/integration of transgene (*chi*) into the genome of lettuce using polymerase chain reaction



B: Confirmation of presence/integration of transgene (*hpt*) into the genome of lettuce using polymerase chain reaction

M: Marker

P: Plasmid DNA

C: Non transformed DNA

T_1-T_5: DNA of putative transformed shoots

Fig.2a The detailed diagram of binary vector pCambia *bar- ubi- chi11* containing *chi 11* (Fungal resistance) gene along with *hpt* (hygromycin resistance) gene and *bar* (Phosphinothricin resistance) gene for selection in both bacteria and plant

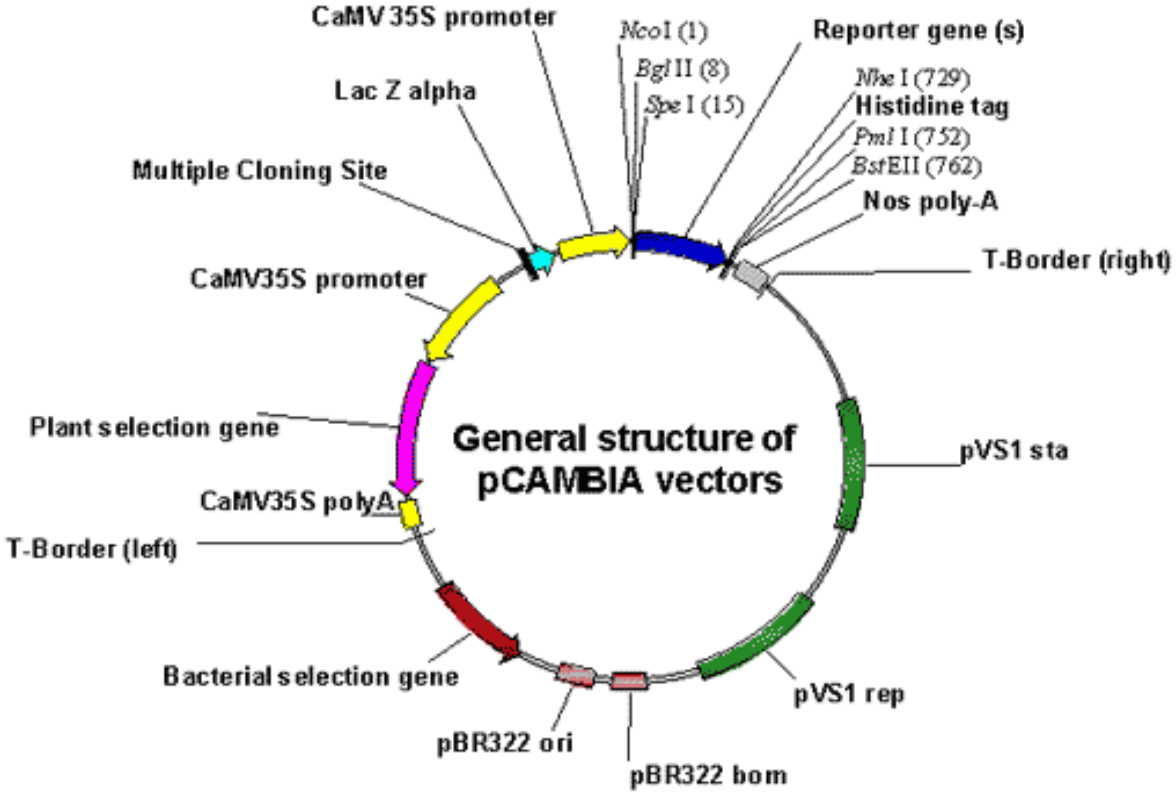
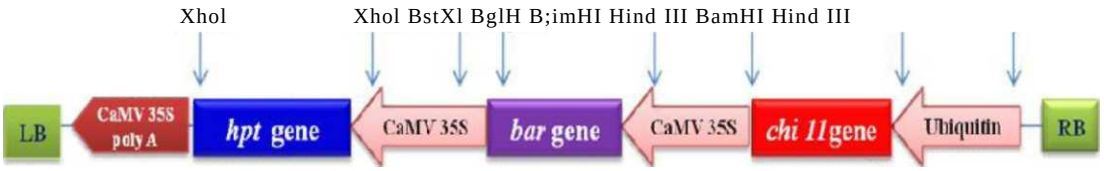


Fig.2b Schematic diagram of gene construct: T-DNA of pCambia *bar- ubi- chi11* transforming vector containing transcriptional fusion of Ubi promoter with coding region of *Chi11* gene and CaMV 35S promoter with the coding region of *hpt* gene



LB - Left border of T-DNA
RB - Right border of T-DNA
CaMV 35S - CaMV 35S promoter
Ubiquitin - Maize ubiquitin promoter
hpt - Hygromycin phosphotransferase gene
bar **-** Phosphinothricin resistance
chi11 **-** Fungal resistance chitinase gene
Poly A - CaMV 35S poly A

Five different transformed shoots along with the non-transformed shoots were analyzed by

the PCR amplification to detect the presence of *hpt* and *chi* genes. The results showed that a 500bp fragment of *hpt* gene was amplified in all the five samples of transformed DNA and was absent in nontransformed control callus (Fig 2a). However, a 237bp fragment of *chi* gene was found to be amplified in only four out of the five callus lines (Fig 2b). PCR result indicated that hygromycin is an effective selective agent and the above selection protocol applied for lettuce transformants was effective and no non-transformant escaped from hygromycin selection. Selection and identification of transformed cells and tissues are crucial steps of genetic transformation which prove to be helpful in improving the selection and transformation efficiency. This study thus reports an efficient hygromycin selection protocol for *Agrobacterium*- mediated lettuce transformation.

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