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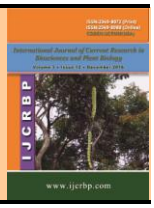

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Resourceful and High Efficiency *Agrobacterium* Mediated Transformation of Maize (*Zea mays* L.) Using Coleoptilar Nodal Explants

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Abstract

Coleoptilar nodal explants, which are independent of season, were used to develop high efficiency transformation method in maize. Conditions which affect *Agrobacterium*-mediated genetic transformation have been standardized by using these explants on hygromycin selection regime. Hyper virulent *Agrobacterium* strain EHA105 containing gateway vector pMDC99 with *Ascorbate - Glutathione* pathway coding genes harboring with *hygromycin phosphotransferase* (*hpt II*) and *bar* as plant selectable marker genes was used. The survival frequency of calli at three different stages *viz*., at the end of I sub-culture on normal medium and II selection as well as on regeneration media supplemented with hygromycin were taken into consideration for the assessment of optimal conditions after checking with PCR. Of the different parameters used with different conditions, one day pre-conditioning of explants, 0.8 optical density of bacterial culture in plain MS liquid infection medium having 5.8 pH along with 200 µM acetosyrengone, 15 minutes infection time for the explants after treating with 0.1% Macerozyme, application of vacuum infiltration for 10 minutes, three days of co-cultivation period were found to be optimal for getting high frequency of transformation through *Agrobacterium*-mediated genetic transformation in maize. Transformation frequency of 3.25% was obtained on average with these optimal conditions, for which the explants were co-cultivated with these conditions and inoculated on callus induction medium having hygromycin at 10 mg l^{-1} for selection. The selected calli were transferred onto regeneration medium supplemented with BAP and Kinetin, at a concentration of 1 mg l^{-1} each for plantlet development. Putative transformants were screened by using *hpt II* gene specific primers and confirmed after performing southern analysis.

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Abbreviations: 2, 4-D - 2, 4-Dichlorophenoxyacetic acid; *hpt II* - Hygromycin phosphotransferase; MS medium - Murashige and Skoog medium; YEP -Yeast Extract Peptone.

Introduction

Gramineae, one of the largest monocotyledonous families which include more than 10,000 species including rice, maize and wheat. Next to rice and wheat maize is the staple food crop for human consumption, animal feed and bioethanol production as well as model plant for cereal research (Vega et al., 2001) throughout the world. Mainly this crop is affected by biotic as well as abiotic stresses which control the crop productivity. Hence, several approaches through conventional breeding were adopted to conquer these limitations. Even though these methods are fruitful, when considering the biotechnological methods, crop improvement programme through genetic engineering takes place in less time than conventional one. Particle bombardment method is expensive and has some limitations. Monocot cells were not susceptible for *Agrobacterium* and not the natural hosts of *Agrobacterium tumefaciens* (De Cleene and De Ley, 1976), but few hyper virulent strains were used for generating transgenic maize (De Cleene, 1985; Reyes et al., 2010). Recovery of fertile transgenic plants was first reported by Gordon-Kamm et al. (1990). This method was used to transform various maize target tissues, including immature zygotic embryos from inbred lines (Fromm et al., 1990; Gordon-Kamm et al., 1990; Frame et al., 2002; Ishida et al., 1996 and 2007).

Few critical parameters play the vital role in efficient recovery of transgenic maize through *Agrobacterium* mediated genetic transformation. Immature zygotic embryos and its derived calli were more suitable target explants for transformation in maize (Brettschneider et al., 1997; Songstad et al., 1996), but the availability of immature embryos throughout the year is difficult, laborious and seasonal dependant. Hence, alternative amenable seasonal independent explant from mature seed derived embryo (coleoptilar node) was chosen for this study. Zhang et al. (2002) and Huang and Wei (2004) used multiple shoots developed from the mature embryos. Cao et al. (2014) used multiple shoot developed from the coleoptilar node. The main objective of this study is to find out the suitable explant which is independent of season in giving reliable and consistent frequency of regeneration as well as transformation with *Agrobacterium* mediated genetic transformation on hygromycin selection regime. Some of the critical parameters *viz*., pre-conditioning of explants, density of bacterial population, strength and pH of the infection medium, acetosyrengone concentration, explant treatment with Macerozyme, vacuum application, infection time of explant with bacterial culture and co-cultivation period which show significant effect on survival frequencies on selection media, regeneration and transformation frequencies were studied to establish the conditions for high throughput results.

Materials and methods

Plant materials

NM81A, a proprietary maize inbred line of Nuziveedu Seeds Limited, Hyderabad was used as a source material for the explants.

Explant preparation

Seeds were soaked in 2% Bavistin for one hour and subsequently washed with sterile distilled water for 3-4 times. Then the seeds were treated with 0.1% $HgCl_2$ and few drops of surfactant, Tween-20, for 15 minutes followed by thorough washing with sterile distilled water. Sterilized seeds were kept for germination on wet sterile cotton spread in the bottles. These bottles were kept in dark for three days at 27±1ºC, until the sprouts attain half inch length. Then the plumules were longitudinally sliced at coleoptile ring and coleoptilar nodal explants were separated from plumules and scutellae (Fig. 1A).

Callus induction and regeneration

MS medium (Murashige and Skoog, 1962) supplemented with 2, 4-D and Kinetin at a concentration of 5 and 1 mg l^{-1} respectively was used for callus induction. MS medium with BAP and Kinetin, each at 1 mg l^{-1} concentration was used for regeneration and plain MS medium without any hormones for rooting.

Agrobacterium strain and construct

Agrobacterium hyper virulent strain, EHA105, was used with gateway vector pMDC99 containing *Ascorbate-Glutathione pathway* coding genes tailored in single vector under stress inducible promoter *rd29* driven by *T7 RNA polymerase* for high expression of transgenes with *hpt II* and *bar* as plant selectable markers.

Transformation

Selection of transformed callus

Medium used for callus induction was supplemented with filter sterilized hygromycin at a concentration of 10 mg l^{-1} and used for selection purpose. After first

subculture, explants were transferred onto selection medium for further selection. After two subcultures on selection media, putative transformed calli (Hygromycin resistant) were transferred onto fresh regeneration medium for shoot regeneration (Fig. 1C and 1D).

Plant regeneration and acclimatization

Hygromycin selected calli were transferred onto regeneration medium (Murashige and Skoog, 1962) having BAP and Kinetin each at 1 mg l^{-1} and incubated in light for 15 days and regenerated shoots were transferred to plain MS medium for further plant elongation and rooting. Profusely rooted shoots were washed in running tap water and transferred into polythene disposable cups having a mixture of cocopeat, perlite and soil in a ratio of 1:1:1 and covered with transparent polythene cover to maintain proper humidity. After one week, the plants were transferred into pots containing a mixture of soil, farmyard manure and sand in a ratio of 4:4:1 until maturation and seed setting (Fig. 1E).

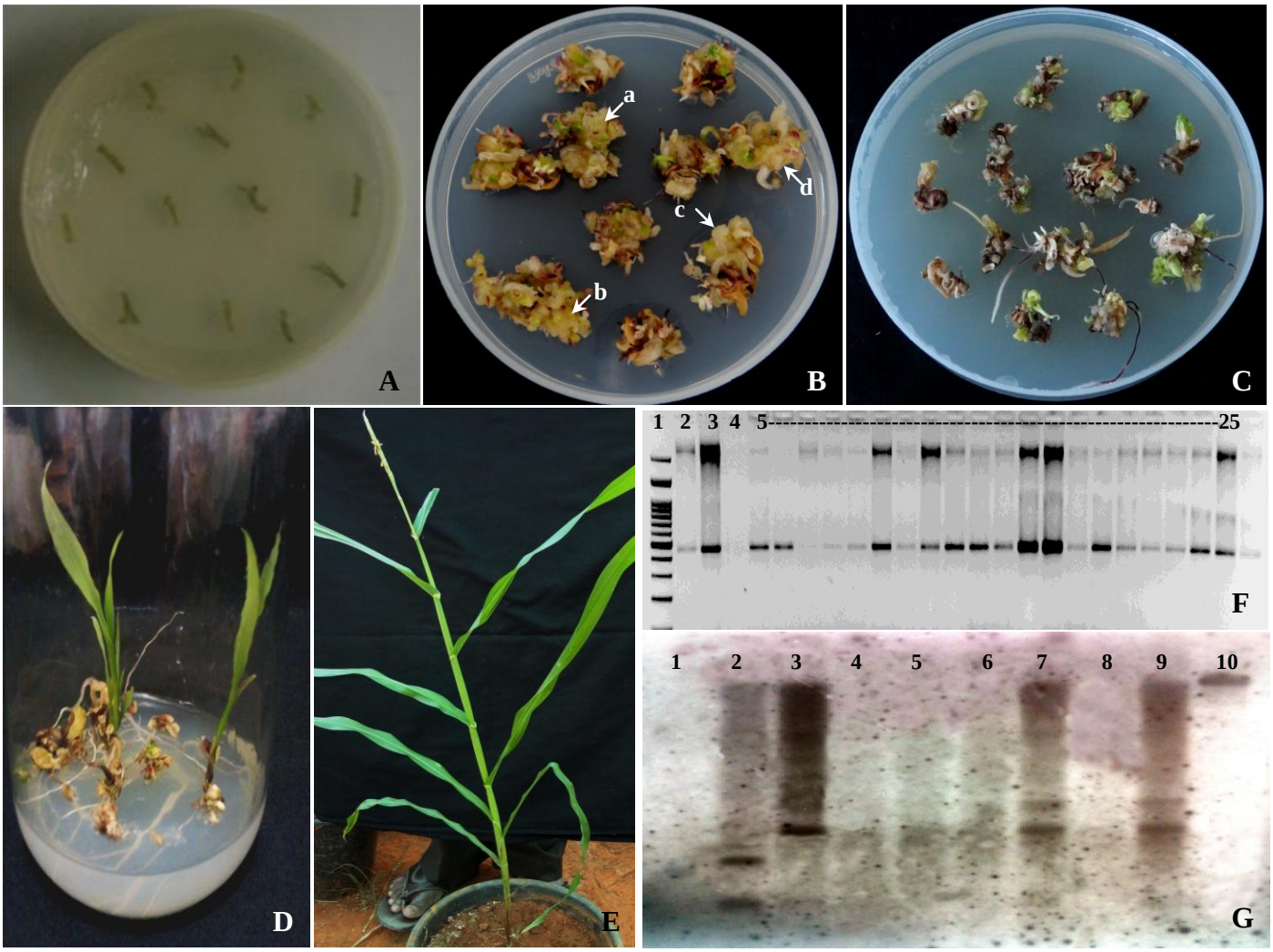

Fig. 1: *Agrobacterium* mediated genetic transformation in maize using coleoptilar nodal explants (**A**); showing callus proliferation from the explants (**B**) with embryogenic (**a & b**) and non-embryogenic calli (**c & d**); Callus proliferation on selection medium supplemented with hygromycin (**C**); Regeneration and rooting of shoots (**D**); Hardened putative transgenic maize plant in polyhouse (**E**); Molecular screening of putative transgenic plants through PCR using *hpt II* primers resulted in 480bp amplicon (**F,** 5-25); 100bp ladder (1), plasmid DNAs (2&3), control wild type genomic DNA (4) also seen on the gel; Integration of genes in transgenic plants were confirmed through southern blotting using *hpt II* probe (**G**) showing control wild type plant DNA (1), different putative transgenic lines (2-9) and undigested transgenic DNA (10) also seen on blot.

PCR analysis

Genomic DNAs were extracted by C-TAB method (Doyle and Doyle, 1990) from both transformed as well as untransformed (control) plants. Putative transgenic plants which were selected through Hygromycin selection were subjected to PCR analysis using *hpt II* gene specific primers *viz*., Forward 5′ CACAATCCCACTATCCTTCGC 3′ and Reverse 5′ GCAGTTCGGTTTCAGGCAGGT 3′ which result in 480bp amplicon (Fig. 1F). The amplified product was visualized on 1% Agarose gel.

Southern blot analysis

Gene integration and copy number was determined by using southern blot technique. Genomic DNA was isolated from both the PCR positive with *hpt II* gene and wild type control plants by C-TAB method (Doyle and Doyle, 1990). 10 µg of genomic DNA of both PCR positive and wild type control plants was digested with *Bam HI* restriction enzyme (10units/ µg) overnight at 37°C in water bath. Then the digested products were separated on 0.8% Agarose gel using 60V current and the depurinated gel was transferred to positively charged Hyband-N^+ Nylon membrane (Amersham Biosciences, UK) by upward capillary method described by Sambrook and Russel (2001). The probe DNA containing 980bp was amplified in pMDC99 plasmid by using specific primers-

HPTPRF: 5′ GCCTGAACTCACCGCGACG 3′ and
HPTPRR: 5′ CAGCCATCGGTCCAGACG 3′

Then the UV cross linked Nylon membrane was probed with non-radioactive (Alkaphos) labeled *hpt II* gene at 55°C in Hybridization oven with 60 rpm for 15 hours and finally the membrane was washed with primary-wash buffer followed by secondary-wash buffer. Detection reagent was applied to the membrane and finally exposed to X-ray film for 4 hrs. Then the film was developed in Kodak fixer and developer in dark for copy number enumeration. All the above procedures were followed as per kit instructions manual (Amersham Biosciences, UK).

Statistical analysis

Data obtained on the effects of various parameters on survival frequencies on hygromycin selection regime were subjected to statistical analysis by following Snedecor and Cochran (1968).

Results and discussion

Coleoptilar nodal explants were used for optimization of influencing parameters on recovery of hygromycin selected putative transgenic plants developed through *Agrobacterium* mediated genetic transformation. Isolated three day old explants were inoculated on MS medium (Murashige and Skoog, 1962) supplemented with 2, 4-D and Kinetin at a concentration of 5 and 1 mg l^{-1} respectively for callus induction (Fig. 1A). Cultures were incubated in dark and light for 15 and 30 days respectively at 27±1ºC by sub-culturing onto to the same medium at an interval of two weeks (Fig. 1B). Embryogenic calli developed from these cultures were transferred onto MS medium fortified with BAP and Kinetin, each at 1 mg l^{-1} concentration for regeneration.

Elongated shoots were separated and transferred onto plain MS medium without any hormones for rooting. Profusely rooted shoots were acclimatized in polyhouse for further development and seed set. These cultures were served as controls for this study. Prior to transformation, a potential concentration of hygromycin for the selection of transformed calli was determined by culturing explants on medium containing various concentrations of hygromycin *viz*., 0, 3, 5, 10, 15 and 20 mg l^{-1}. Hygromycin, at a concentration 10 mg l^{-1}, was observed as an effective one for coleoptilar nodal explants, where the browning and complete necroses of explants were observed. This concentration was adopted throughout this study for the selection of transformants. For evaluating the parameters under the different conditions, data was studied at three different stages *viz*., survival frequencies at the end of dark phase (sub-culture I) and at the end of each sub-culture on selection medium *i.e*., selection I and selection II (at the end of 15 days each) and also the regeneration frequency after transferring from the selection medium (Table 1). Pieces of calli from selection II medium and regenerated plant samples were checked with PCR for screening the putatives.

Effect of pre-conditioning of explants

Initially, embryogenic callus was subjected to co-cultivation, where the callus masses turned brown and no positive results were observed. Hence, coleoptilar nodal explants were used for co-cultivation. Prior to co-cultivation coleoptilar nodal explants were pre-conditioned for 24 and 48 hrs on callus induction medium. Explants without pre-conditioning have also been maintained to evaluate the effect. Among the three

conditions tested, 24 hrs pre-conditioning of the explants resulted in maximum survival frequency on hygromycin selection (14.9%) as well as regeneration (6.5%). Low survival frequencies were observed in other two conditions *i.e*., without pre-conditioning and 48 hrs treatment (Table 1), due to neonate and cell rigidity. Similar results were observed in wheat (Wu et al., 2003) and maize (Takavar et al., 2010).

Table 1. Effect of different parameters with different variables on survival frequency obtained on normal, selection medium supplemented with hygromycin and regeneration medium of maize cultures after co-cultivating with *Agrobacterium* cultures.

Parameter	Variable	Subculture*	Sel-I*	Sel-II*	Regeneration frequency*
Pre-conditioning of the explants	0 hrs	39.24±7.51	25.00±2.52	10.6±1.53	4.6±1.00
	24 hrs	70.00±4.47	37.00±5.51	14.9±1.16	6.5±1.16
	48 hrs	43.75±6.43	30.00±8.09	9.10±2.00	3.7±0.58
Optical Density of bacterial culture	0.6	42.50±5.00	37.5±3.52	16.67±2.52	6.9±1.53
	0.8	73.34±1.59	62.0±2.51	30.00±6.03	14.3±3.22
	1	53.34±2.00	42.67±7.64	22.67±4.51	11.7±1.16
Strength of the infection medium	Half plain MS	83.34±6.63	60.0±5.14	30.00±3.00	12.5±2.31
	Full plain MS	74.00±7.22	48.0±6.81	25.00±3.61	12.7±2.31
	Osmo. Med.	70.00±1.56	43.0±7.51	20.00±5.04	9.00±2.00
pH of the infection medium	5.2	45.46±4.17	18.19±3.61	12.73±1.00	6.20±0.58
	5.4	66.67±1.07	53.34±1.08	26.67±7.51	9.40±4.00
	5.8	76.93±6.08	57.70±7.22	30.77±7.51	12.8±2.89
Acetosyrengone concentration	0 µM	41.67±6.81	33.34±4.05	15.00±1.16	7.00±0.58
	100 µM	80.00±5.00	60.00±2.00	32.00±3.52	17.6±1.53
	200 µM	78.58±1.15	60.72±8.74	35.72±3.06	18.7±1.16
Macerozyme treatment	0.1%	63.64±2.09	45.46±5.00	23.00±5.04	12.0±2.52
	0.2%	60.00±3.06	35.00±3.06	16.00±2.09	8.70±1.16
	0.5%	26.12±3.61	21.12±2.00	12.00±2.00	6.10±1.53
Vacuum infiltration	0 min.	54.74±4.17	37.9±6.25	14.74±1.00	9.20±1.00
	10 min.	64.29±7.56	46.43±1.79	25.00±1.94	12.5±5.69
	15 min.	60.29±3.66	42.25±1.66	19.56±4.51	12.2±3.22
Infection time	10 min.	45.46±1.00	30.00±5.00	13.64±4.17	8.0±2.52
	15 min.	73.08±5.69	46.16±4.16	19.24±3.06	8.9±2.00
	30 min.	66.67±3.22	30.00±5.14	16.00±3.06	7.6±1.16
Co- cultivation period	2 days	54.55±2.65	31.82±5.14	21.82±1.00	13.2±2.09
	3 days	71.43±2.17	50.00±5.00	34.29±6.56	21.5±2.09
	4 days	58.34±2.00	25.00±2.52	20.84±1.00	11.7±2.65

*****Average value ± Std. Dev.

Effect of bacterial population (OD at 580nm)

Bacterial cell density in the culture, which is measures as optical density of the culture at 580nm, influences the transformation frequency, hence agrobacterial cells cultured overnight in liquid YEP medium containing 50 mg l^{-1} kanamycin along with 25 mg l^{-1} each of rifampicin and streptomycin at 28°C in a shaker at 180 rpm were harvested at deferent timing after attaining the OD of 0.6, 0.8 and 1.0 by spinning at 6000 rpm at 4°C for 20 minutes, then these cultures were used for co-cultivation. Explants co-cultivated with *Agrobacterium* cells harvested from the culture with 0.8 optical densities (at 580nm) resulted in maximum survival frequency in hygromycin selection (30%) and regeneration (14.3%) than the other ODs tried (Table 1). Minimum frequency of survival with 16.67% and regeneration with 6.9% were observed at 0.6 OD. At this 0.8 O.D., *Agrobacterial* cells at exponential phase of their growth were very effective for resulting high frequency of transformation (Yadav et al., 2012). Survival frequency on hygromycin selection medium was declined with increase in bacterial

cell density at 1.0 OD. Similar results were observed in sunflower (Sujatha et al., 2012) and *Populus* (Han et al., 2013).

Effect of infection medium

Agrobacterium cells were suspended in various liquid infection media *viz*., plain half MS medium, plain MS medium and osmoticum medium (MS medium with 3.6% Glucose and 6% Sucrose) for the assessment of selection frequency. Among the media used, both plain MS as well as half MS media showed more or less equal regeneration frequencies (12.7% and 12.5% respectively) though the survival frequencies were 25 and 30% respectively (Table – 1) with low tissue browning than in osmoticum medium. Browning of the explants was observed upon usage the osmoticum medium and explants were dead in further subcultures with only 9% of regeneration frequency. In contrary to this, other workers reported the enhanced transformation frequency by using osmolytes in tree plants (Li et al., 2007), rye grass (Patel et al., 2013) and in sorghum (Wu et al., 2014).

pH of the infection medium

Different pH values of liquid infection medium at 5.2, 5.4 and 5.8 were tried to assess the hygromycin selection frequency. Infection medium with 5.8 pH showed maximum frequencies of selection as well as regeneration (30.77% and 12.8% respectively). In other infection media with low acidic pH of 5.2 and 5.4 resulted in low survival frequencies of 6.2% and 9.4% respectively for regeneration and 12.73% and 26.67% (Table 1) on selection media where the explants turned brown after co-cultivation. In present study, a negative correlation of survival frequencies was observed with increase in hydrogen ion concentration in the media (low pH) on both selection as well regeneration media. In other studies, it was suggested that acidic pH of the infection medium enhanced the transformation frequency (De La Riva et al., 1998; Saini and Jaiwal., 2007).

Effect of acetosyrengone concentration

Two concentrations *viz*., 100 and 200 µM of acetosyrengone were used for enhancing the survival frequency of calli on hygromycin. Of which, 200 µM showed maximum selection and regeneration frequencies (35.72% and 18.7%); minimum frequencies were observed at 100 µM concentration (32 and 17.6%) as well as in absence of acetosyrengone (15 and7%) (Table 1). Transformation studies in other crop plants also indicated that acetosyrengone at an appropriate concentration enhances efficiency of transformation frequency as in *Catharanthus* (Srivastava et al., 2009) and sunflower (Sujatha et al., 2012). In *Populus*, presence of acetosyrengone did not show any enhancement in transformation frequency (Han et al., 2013).

Effect of macerozyme treatment

Explants were treated with different concentrations (0.1, 0.2 and 0.5%) of macerozyme for 5 minutes before going to infect with bacteria. After this treatment, explants were immersed in liquid infection medium containing agrobacterial cells. Negative effect of macerozyme pre-treatment was observed on explant health after co-cultivation. In higher concentrations of macerozyme (at 0.2 and 0.5 %) explants were putrefied and hygromycin selection frequency was declined (8.7% and 6.1%) when compared with lower concentration of macerozyme (0.1%) which resulted in 12% of regeneration and 23% of selection frequencies (Table 1). In present study, no significant difference was observed in regeneration frequency with or without macerozyme treatment; whereas in sunflower Weber et al. (2003) reported enhanced transformation frequency by using macerating agents.

Vacuum infiltration for the explants

Giving suction pressure while infecting the explants with bacterial culture enhances and or facilitate bacteria to enter the host cells easily which in turn shows its effect on subsequent selection as well as regeneration frequencies. By considering this, explants were infected with *Agrobacterium* culture by applying vacuum at two different timings (10 and 15 min.) at a pressure of 75 Hg. In contrary to our consideration, application of vacuum both for 10 and 15 minutes showed more or less equal regeneration frequencies and more than without vacuum application 12.5%, 12.2% and 9.2% respectively (Table 1). No significant enhancement in survival frequency was observed by the application of vacuum in wheat (Cheng et al., 1997). Cao et al. (2014) applied vacuum for 30 minutes in maize. In contrary to this, present study revealed that the application of vacuum for 10 or 15 minutes enhanced the survival frequency on hygromycin selection medium. In particular, 10 minutes duration is suitable than 15 minutes, where the significant enhancement has been noticed not only in survival frequency on selection medium, but also the regeneration frequency too.

Effect of infection time

Explants were infected with *Agrobacterium* culture with different time durations *viz*., 10, 15 and 30 minutes. Because it would cause either damage to the tissue when time exceeds or failure due to poor intrusion of bacterial cells into the explant as the exposure time is insufficient. Of the infection durations given in this study, 15 minutes exposure time resulted in maximum frequencies of selection (19.24%) as well as regeneration (8.9%) than any other durations tried. Long exposures of explants to *Agrobacterium* cells results in over growth of bacteria around the explants and browning or decay of cultures in further subcultures which causes decline in survival frequencies on selection medium. In 10 and 30 minutes infection duration 13.64 and 16% of survival frequencies and 8 and 7.6% of regeneration frequencies were observed respectively (Table 1). Similar trend of results were reported in wheat by Wu et al. (2003).

Co-cultivation period

After infection, the explants were transferred to co-cultivation medium supplemented with 200 µM acetosyrengone for different durations (Two, Three and Four days). Among them, co-cultivation for three days resulted in maximum survival frequency on hygromycin selection medium than the others tried (34.29%) and also 21.5% of regeneration frequency was observed (Table 1). In this study, co-cultivation for four days showed over growth of the bacteria and putrefaction of explants were observed even after giving cefotaxime (250 mg l^{-1}) washing to the explants. Recurrent over growth of bacteria was observed in further subcultures also. Similar results were reported by Li et al. (2004); Uranbey et al. (2005) and Sujatha et al. (2012). Hygromycin selection frequencies were declined in two and four day co-cultivation with 21.82% and 20.84% respectively.

Coleoptilar explants were inoculated in large number with many replicates to assess the transformation frequency by using all optimal conditions selected. An average of 3.25% transformation frequency was obtained and the data was given in Table 2. The putatives are screened by PCR (Fig. 1F) and the positive samples were subjected to southern analysis by using *hpt* probe, which revealed the presence of 1-3 inserts (Fig. 1G).

Table 2. Transformation frequency of maize obtained through *Agrobacterium* mediated transformation selected on hygromycin regime and subsequent molecular screening of putative transgenics through PCR using *hpt II* primers.

No. of explants Co-cultivated	Survival frequency in Selection - I	Survival frequency in Selection - II	Frequency of regeneration	PCR +ves for *hpt II*	Transformation frequency (%)
1045	61.81 (646)	34.25 (358)	22.34 (80)	34	3.25

*Total number of calli tested is given in parentheses.

Recovery of fertile transgenic plants through *Agrobacterium* mediated genetic transformation depends on several crucial parameters. Thorough optimizing and tuning those parameters with different genotypes are necessary for getting high frequencies of transformation (Cao et al., 2014), where different genotypes were used by considering the different parameters and achieved 2% transformation frequency in maize using multiple shoots developed from the coleoptilar nodal explant. In contrary to this, in present study transformation studies have been done by using callus cultures developed from the same explant and frequencies were assessed by using hygromycin selection frequency (Nyaboga et al., 2014). In the present study, it was concluded that the parameters *viz*., one day pre-conditioning of explants, 0.8 optical density of bacterial culture in plain MS liquid infection medium having 5.8 pH along with 200 µM acetosyrengone, 15 minutes infection time for the explants after treating with 0.1% Macerozyme, application of vacuum infiltration for 10 minutes, three days of co-cultivation period, play a vital role in maize transgenic development from coleoptilar nodal explant. By using above optimized parameters an average transformation frequency of 3.25% was achieved. Comparatively, explants derived from the mature embryos (Cao et al., 2014; Huang and Wei, 2005; Zhong et al., 1996) yielded less frequencies of transformation than the immature embryos (Ishida et al., 1996). All these conditions of different parameters would help us in developing a congenial high throughput method of *Agrobacterium* mediated genetic transformation in maize. Similar studies were done in Rice (Ignacimuthu and Raveendar, 2011; Sahoo and Tuteja, 2012) and Yam (Nyaboga et al., 2014). This study will help in maize genetic engineering for

agronomically demand traits into recalcitrant maize genotypes. By using these parameters fertile putative transgenic maize plants were produced.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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