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Full Length Research Paper

An improved *Agrobacterium* mediated transformation in tomato using hygromycin as a selective agent

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Bacterial wilt is a devastating disease of tomato crop throughout the world. This disease is very dangerous in hot and humid regions, where it spreads with the irrigation water to whole field within days, which resulted in severe decline in yield. Two varieties of tomato were used for developing bacterial wilt resistance. Riogrande responded more efficiently as compared to Roma. Regeneration frequency of 90.6% was achieved when leaf discs were used and 82.5% when hypocotyls were used as explant for tomato cv. Riogrande. While 65.4% regeneration was achieved from hypocotyls and 72.6% regeneration was obtained when leaf discs were used as explant for cv. Roma. Explants were co-cultivated with *Agrobacterium* strain EHA101 containing a binary vector pTCL5, having hygromycin phosphotransferase (HPT) gene which confers resistance to hygromycin and β-glucuronidase (GUS) gene in addition to Xa21 gene. Hygromycin (25 mg/l) was used as selectable marker while GUS is a reporter gene. Acetosyringone (50 μM) enhanced transformation effeciency. Preselection period of 7 days was found to be indispensable for successful transformation of tomato crop. Transformation efficiency of 24% was observed for Riogrande and 8% for Roma. Molecular analysis of transgenic plants produced was carried out for hygromycin resistance gene. Transgenic plants contained the expected band of 670 bp. PCR analysis confirmed the foreign DNA in to the plant genome.

Key words: Tomato (*Lycopersicon esculentum*), hygromycin, disease resistance, Xa 21 gene.

INTRODUCTION

Tomato crop faces many biotic and abiotic problems. Fungal, bacterial and viral diseases cause severe damage to this crop. Bacterial wilt causes extensive losses to tomato crop every year. Tomato production in Pakistan is declining and the major cause is “Bacterial wilt”. Bacterial wilt or Southern bacterial blight is a serious disease caused by a bacterium *Ralstonia solanacearum* in tropical and subtropical regions (Ruben, 1999).

Bacterial Wilt causes serious yield reduction in tomato within no time and there is no chemical control due to its soil borne nature. This bacterium survives in the soil for extended periods and enters the roots through wounds made by transplanting, cultivation or insects and through natural wounds where secondary roots emerge (Ray, 2004). Disease development is favored by high temperatures and high moisture. The bacteria multiply rapidly inside the water-conducting tissue of the plant, filling it with slime. This results in a rapid wilt of the plant, while the leaves stay green. If an infected stem is cut crosswise, it will look brown and tiny drops of yellowish ooze may be visible. Control of bacterial wilt of plants grown in infested soil is difficult. Rotation with resistant plants, such as corn, beans and cabbage, for at least three years provides some control.

Agrobacterium mediated transformation is an effective and widely used approach to introduce foreign DNA into dicotyledons plants. In different plant species different gene transfer protocol are applicable. *Xa-21* gene has a wide host range. It is primarily used for the production of resistance against bacterial blight in rice (Rashid et al., 1996) and is also for bacterial canker resistance in citrus

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Abbreviations: **AA**, Amino acid medium; **GUS**, β-glucuronidase; **cv**, cultivar; **PCR**, polymerase chain reaction.

Table 1. Transformation media compositions used in different stages of transformation of tomato cvs. Riogrande and Roma.

Stage	Media code	Composition
Co-cultivation	CIM + As	MS+ 0.5 mg/l Kin + 0.5 mg/l IAA with 200µM Acetosyringone, pH 5.7-5.8.
	AA + As	AA media (Toriyama and Hinata 1985),+ 200 μM acetosyringone, pH 5.7-5.8.
Pre-Selection	CIM-Cf.	CIM with 500 mg/l Cefotaxime
Selection	CIM + Hyg + Cf	RIM+ 10 mg/l Hyg + 500 mg/l Cf, pH 5.7-5.8.
	CIM + Hyg + Cf	CIM + 50 mg/l Hyg + 500 mg/l Cf + 4g/l gelrite, pH 5.7-5.8.
Regeneration	RM_1 + Hyg +Cf	MS + 3% sucrose + 4g/l gelrite + 25 mg/l Hyg + 500 mg/l Cf, pH 5.7-5.8.

Figure 1. Partial diagram of binary vector.

(Guo and Grosser, 2002). The plasmid DNA of a potential canker resistance gene (pC822) from the *Xa21* gene family of rice (which provides broad spectrum *Xanthomonas* resistance in rice) was PEG-mediated co-transformed into protoplasts.

Tomato is a model plant and has been transformed with different genes, such as anti-ripening (Picton et al., 1993; Reed et al., 1996), insect (Fischhoff et al., 1987) and herbicide tolerance (Fillatti et al., 1987b), virus resistance (Kim et al., 1994; Raj et al., 2005) and salt tolerance (Gidbert et al., 2000; Jia et al., 2002; Roy et al., 2006). *Agrobacterium* mediated transformation in tomato has been reported by various researchers (Romero et al., 2001; Rui, et al., 2000; Costa et al., 2000; Saiqun et al., 1999). The present study was carried out to introduce the *Xa- 21* gene into two tomato cultivars Riogrande and Roma for bacterial wilt resistance using *Agrobacterium*-mediated transformation.

MATERIALS AND METHODS

All the experimental work was performed at Agricultural Biotechnology Programme (ABP) of the National Agricultural Research Center (NARC), Islamabad. Seeds of tomato Cv. Roma and Riogrande were obtained from the Horticultural Research Institute (HRI), National Agricultural Research Center (NARC), Islamabad.

Media and physical conditions for plant transformation

Table 1 shows the different media compositions used in the transformation. Media was poured in Petri plates of 9 cm diameter and was sealed with parafilm later on. Gubis et al. (2003) and Raj et al. (2005) used half strength MS media for *in vitro* germination.

Mature seeds of Roma and Riogrande were sterilized by the protocol described by Chaudhry et al. (2004). The overnight soaked seeds of Riogrande and Roma were surface sterilized with clorox (Sodium hypochlorite 5.25%) for 8 min and rinsed 3 times with autoclaved distilled water with each wash for 5 min. Seeds were continuously shaken during treatment with clorox.

The dried sterilized seeds were aseptically inoculated on MS media (Murashige and Skoog, 1962) containing 30 g l^{-1} sucrose and 6 g l^{-1} agar as solidifying agent. The pH of the media was adjusted to 5.8 before autoclaving at 121°C for 20 min. These inoculated seeds were placed in the growth room for three weeks for production of *in vitro* seedlings. After three weeks, hypocotyls (5 mm) and leaf discs (1-2 mm in diameter) were used for transformation experiments.

Regeneration media (RM_1) comprised MS + 0.5 mg/l IAA, 0.5 mg/l GA_3 and 1.5 mg/l Kin (Table 1). Antibiotics were filtered sterilized and added to autoclaved media. Hypocotyls segments and leaf discs excised from aseptically germinated plants were placed on different regeneration media, kept in a growth room at 25°C and 16 h photoperiod with 48 umol $m^{-2}S^{-1}$ light intensity to determine the regeneration potential of the two cultivars.

Agrobacterium-mediated transformation of cvs. Riogrande and Roma

Hypocotyls (0.5 cm) and leaf discs (2 mm) explants of Cv. Riogrande and Roma were excised from 21 days old *in-vitro* raised seedlings. Transformation was done by direct infection method.

Agrobacterium strain *EHA101* (Hood et al., 1986) containing *p*TCL5 (Baisakh et al., 1999) plasmid (Figure 1) was used in the present study. pTCL5 is a binary vector, which contains hygromycin resistance genes, *GUS* (reporter) gene and Xa-21 gene (Ohta et al., 1990). This intron-*GUS* gene does not express *GUS* activity in *Agrobacterium tumefaciens* cells but when transferred in plant cells then it gives blue color due to β-glucuronidase activity.

Bacterial culture (10 µl) was cultured into 50 ml liquid YEP (Yeast Extract Peptone) medium (An et al.,1988) containing 100 mgl^{-1} kanamycin and 50 mgl^{-1} hygromycin in a conical flask and placed at 100-110 rpm for two days in the dark at 28°C.

Hypocotyls and leaf discs were pre-cultured on RM_1 medium (Table 1) for one day. Two days bacterial culture having 0.6 OD at 600 nm was poured into small petri plates. The plant material was immersed in bacterial suspension for 2 min with constant shaking. Hypocotyls (3 cm) and leaf discs (2 cm) were removed and blotted dried on sterile tissue paper to remove excess bacteria and placed on filter paper on the callus induction media along with 50 µM

Table 2. Hypocotyls and Leaf disc derived Regeneration Percentage of Two Tomato Cultivars.

Hypocotyls derived Regeneration					
Varieties	**RM1**	**RM2**	**RM3**	**RM4**	**RM5**
Riograndae	82.4	72.9	51.7	16.23	58.03
Roma	65.43	55.06	45.5	13.367	37.37
Average	73.92	63.98	48.6	14.8	47.7
Leaf disc derived regeneration					
Varieties	**RM1**	**RM2**	**RM3**	**RM4**	**RM5**
Riograndae	90.66	79.4	68.23	25.06	65.53
Roma	72.63	68.07	57.4	18.2	45.17
Average	81.65	73.73	62.81	21.63	55.35

RM1 = IAA(0.5 mg/l) + Kinetin (1.5) + GA_3(0.5 mg/l)
RM2 = NAA (0.5 mg/l) + Kinetin (2.0 mg/l)
RM3 = NAA(0.4 mg/l) + BAP (2.5 mg/l)
RM4 = 2,4-D (2.5 mg/l) + BAP (3.0 mg/l)
RM5 = IAA (0.5 mg/l) + BAP (2.5 mg/l)

acetosyringone. Before that, 1 ml of AA media + acetosyringone was also poured over the CIM. Infected explants were placed on those Petri-plates, which were sealed with parafilm and placed in incubator at 28°C for 2 days.

Selection and regeneration of the transformed

The infected cultures were washed with liquid MS medium containing 500 mgl^{-1} cefotaxime and then transferred to RM_1 + Cf medium (Table 2) for pre-selection. Pre-selection medium consisted of (MS salts +vitamins +IAA (0.5 mg/l) + kinetin (0.5 mg/l) + 500 mg/l of cefotaxime). After pre-selection period of 7 days, the explants were transferred to RM_1 containing 25 mgl^{-1} hygromycin for selecting transformants. Callus tissues with regenerating shoots were removed from the hypocotyls and transferred on rooting medium (MS salts + vitamins, 3% sucrose, 0.5 µM IBA, 25 mgl^{-1} hygromycin and 4 gl^{-1} gelrite) for complete plant development. After 2 weeks of selection, hypocotyls and leaf discs were transferred to regeneration medium (Table 1). After shoot regeneration, plantlets were transferred to rooting media for root induction.

Gus assay

GUS assay was carried out essentially as described by Jefferson et al. (1987). Plant leaves and roots parts were placed in X-Glue solution (1 ml/l 5-Bromo 4-Chloro 3-indoyl-B-D-glucuronide, 0.5% triton X-100, 20% methanol, 50 mM phosphate buffer) for overnight at 37°C and were then examined under microscope for blue spots.

DNA extraction and polymerase chain reaction (PCR) analysis

Genomic DNA was extracted from leaf tissues of transgenic and control plants according to CTAB method (Doyle and Doyle, 1990). Two specific primers sequences for the hygromycin coding region were designed to amplify a 670 bp fragment of this gene from genomic DNA. Hygromycin forward primer, 20 mer with '5 GCTCCATACAAGCCAACCAC-3' forward sequence and reverse primer with 5'- CGAAAAGTTCGACAGCGTCTC-3' sequence was used for hygromycin amplification. PCR reaction 10 µl was performed using 10 ng of the template DNA, containing 2 µl of 10 x PCR buffer, 0.6 µl of 0.1 M $MgCl_2$, 1 µl of 10 mM dNTP mixture, 0.5 µl of each 5 µM forward and reverse primer, 0.3 µl of Taq polymerase and 4.1 µl of nano-pure water.

PCR reactions were run for 35 cycles with an initial denatured step for 3 min at 94°C, annealing temperature of 51°C for 2 min and 72°C for 3 min as extension temperature. The cycles consisted of 94°C for 20 s, 51°C for 20 s and 72°C for 45 s. Amplified DNA was detected on 2% agarose gel containing ethidium bromide (10 $mgml^{-1}$) solution. The resolved PCR products were visualized by placing the gel on the UV transilluminator and photographed by gel documentation system.

RESULTS AND DISCUSSION

Regeneration from hypocotyls and leaf discs explants of tomato cultivars

The highest regeneration was observed on RM1 (Table 1) medium. Highest regeneration frequency of 90.66% was recorded for leaf discs of cv. Riogrande (Table 2), followed by 82.4% regeneration from hypocotyls of the same cultivar. Similarly optimum regeneration of 72.63% was observed for leaf discs while 65.43% regeneration for the hypocotyls of cv. Roma. Minimum regeneration was observed on RM4 containing 1 mg/l 2, 4-D and 1.5 mg/l BAP. A total of 16.23% was observed for cv. Riogrande and 13.3% for cv. Roma from hypocotyls. Riogrande showed 68.2 and 51.7% for leaf disc and hypocotyls respectively and Roma showed 57.4 and 45.5% of regeneration on leaf discs and hypocotyls respectively on RM3 (Table 2).

Factors affecting transformation efficiency

A highly efficient *Agrobacterium* mediated transformation protocol was established to introduce gene for resistance against bacterial wilt in two important cultivars of tomato that is Riogrande and Roma. Factors affecting trans-

Table 3. Effect of seedlings age, Acetosyringone concentration and days of pre-selection on transformation efficiency and GUS expression for hypocotyls and leaf discs of tomato (*Lycopersicon esculentum* M.) cv. Riograndae and Roma.

Effect of seedlings age on transformation efficiency					
Varieties	**10 days**	**20 days**	**24 days**	**28 days**	
Riograndae	22.23	55.16	75.0	20.0	
Roma	13.5	50.0	72.06	11.0	
Average	17.86	52.58	73.53	15.5	
Effect of Acetosyringone concentration on transformation efficiency					
	0 µM	**50 µM**	**100 µM**	**200 µM**	
Riograndae	0.0	0.0	57.38	85.23	
Roma	0.0	0.0	74.91	87.16	
Average	0.0	0.0	80.0	79.16	
Effect of days of pre-selection on transformation efficiency					
	0 day	**3 days**	**5 days**	**7 days**	**10 days**
Riograndae	0.0	0.0	57.38	85.23	22.26
Roma	0.0	0.0	74.91	87.16	25.03
Average	0.0	0.0	6.14	6.20	23.65

Table 4. Transformation efficiency of two tomato cultivars (*Lycopersicon esculentum* M.) cv. Riograndae and Roma using hygromycin as selective marker

Treatments	**Varieties**	**Hygromycin resistant calli**	**Regenerated calli**	**GUS +ive explants**	**Transformation Efficiency**
T1	V1	73.47	34.09	12.14	11.13
	V2	42.07	25.30	6.03	4.90
	Average	57.77	29.69	9.08	8.01
T2	V1	52.14	22.61	14.92	11.06
	V2	33.99	13.95	11.64	8.80
	Average	43.06	18.28	13.28	9.93
T3	V1	12.47	0.00	0.0	0.00
	V2	13.33	0.00	0.00	0.00
	Average	12.90	0.00	0.00	0.00

V1 = Riograndae, V2 = Roma
T1 = 10 mg/l, T2 = 25 mg/l, T3 = 50 mg/l

formation efficiency included seedling age, days to pre-selection, acetosyringone concentration and hygromycin concentration in selection of transgenic plants

Type and quality of starting material

Type and quality of starting material is one of the important factors for successful transformation. Takashina et al. (1998) reported that the explants nature affected callus induction and regeneration. Two types of explants, leaf discs and hypocotyls, were used in the present research. Many workers reported the hypocotyls (Jatoi et al., 1995; Park et al., 2003) and leaf disc (Soniya et al., 2001; Raj et al., 2005; Roy et al., 2006) as explants source. Gubis et al. (2003) used 6 different types of explants (hypocotyls, cotyledon, epicotyls, leaf petiole and internodes of 13 cultivars of tomato). JaeBok et al. (2001) reported the results that germination rate depends upon the genetic basis of the variety (Figure 5 and 6).

Seedlings' age

The *GUS* expression for seedlings of different age was significantly different (Table 5) for both cultivars which ranged from 44.34 to 50.05 for Riogrande and Roma, respectively (Table 3). The different age of seedlings showed highly significant effect on transformation efficiency (Table 3), where 24 days age showed maximum *GUS* expression of 73.5% followed by 20 days with 52.5% (Table 4b). The interaction effect of both cultivars and different days of seedlings was also highly significantly different (Table 3). For cv. Riogrande a

maximum of 75 and minimum of 20 values was recorded for 24 and 20 days, respectively. Similarly for cv Roma 73.5 was maximum value at 24 days while 15.5 was minimum at 28 days. The above results clearly showed that 24 days seedlings were ideal for transformation with maximum *GUS* expression. However with the increase in the age of seedling that is, more than 24 days, percentage of *GUS* expression was decreased. Similarly 20 days seedlings also showed less *GUS* expression. Ling et al. (1998) reported that with increase in age of seedlings, transformation efficiency decreased. Raj et al. (2005) used two week old seedlings and Chaudhry et al. (2004) used three weeks old seedlings for callus induction and regeneration. From these results it can be concluded that young explants are more effective, as compared to matured explants (Table 3).

Acetosyringone concentration

The addition of different concentrations of acetosyringone showed highly significantly different (Table 3) effect on explant proliferation and callus growth after co-cultivation with *Agrobacterium*. The highest value 80 was recorded for 100 µM acetosyringone while the lowest one of 37.3 was recorded for control (with out acetosyringone) (Table 5b). The varietal and interaction effects were both non significant for different concentrations of acetosyringone (Table 5).

Hiei et al. (1994) and Rashid et al. (1996) have reported the addition of 50 µM acetosyringone in AA medium, to be effective for the transformation of rice by *Agrobacterium*. It has also been reported that acetosyringone increases the transformation in Arabidopsis and in soybean (Chang and Chan, 1991). Expression of *GUS* gene was enhanced in the hypocotyls and leaf discs after the inclusion of acetosyringone in the medium. These results are in line with Raj et al. (2005) who reported 200 µM of acetosyringone in co- cultivation of tomato cv. Pusa Ruby with *Agrobacterium* strain LBA4404. Reda et al. (2004) used 100 µM of acetosyringone for *Agrobacterium rhizogene* strain DCAR-2 containing pBI-121 binary vector.

Pre-selection period

After co-cultivation, the pre-selection period is very important. Pre-selection for 3, 5, 7 and 10 days was carried out. Shoot regeneration from hypocotyls was optimum when infected explants were left on CIM-Cf medium for 7 days before hygromycin selection. The varietals effects for transformation efficiency were highly significant (Table 5). This ranged from 32.9 for cv. Riogrande to 37.4 for cv. Roma. The different days' interval was also significantly different (Table 5). The highest value (23.6) was observed for 10 days treatment while no value was observed for control and 3 days pre-selection. The interaction effect of both treatment and cultivars was also significant (Table 5). A maximum of 87.1 was recorded for cv. Roma with 7 days pre selection while 85.2 for cv Riogrande on the same pre selection period.

Different concentrations of cefotaxime in pre-selection

In pre-selection of tomato cv. Riogrande and Money maker different concentrations of cefotaxime that is, 250, 500, 750 and 1000 mg/1 were used to optimize the standard dose which can control the overgrowth of bacteria but could not inhibit the callus formation and regeneration. At 250 mg/l the transformation efficiency was low because at this dose bacterial growth was not controlled and most of the explants became dead. This result is contradictory with Hu and Phillips (2001). They reported that 250 mg/l cefotaxime completely control the overgrowth of bacteria. Cefotaxime (500 mg/l) was optimized to control the overgrowth of bacteria and also not affect the callus growth and regeneration. These results are in confirmatory with Roy et al. (2006); they also used 500 mg/l of cefotaxime to control bacteria in tomato cv. Pusa Ruby for drought tolerance.

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. But antibiotics, which are commonly used to eliminate *A. tumefaciens* from plant tissues, have also been shown to influence morphogenesis either positively or negatively (Ling et al., 1988).

The higher doses of cefotaxime that is, 750 and 1000 mg/l showed necrosis and no callus formation and regeneration were observed. Yepes and Aldwinckle (1994) reported inhibition of callus formation and shoot regeneration, when high doses of cefotaxime and carbenicillin were used.

Selection of transformants

Hygromycin at 25 mg/l was optimized as a lethal dose for selection of transformed explants. When no selective agent applied in medium, then mostly callus produced shoots and many escapes were formed.

GUS expression was observed in explants transferred after 7 days of pre-selection on selection medium containing 25 mg/l of hygromycin as shown in Figure 2 (a, b, c, d, e and f). The cultivars means for hygromycin resistant calli were significantly different (Table 5). These ranged from 29.7 for cv. Roma to 40.0 for cv. Riogrande (Table 5). The effects for different concentrations of hygromycin were also significantly different (Table 5) with the highest value of 57.7 for 10 mg/l while minimum value of 12.9 was recorded for 50 mg/l hygromycin. The

Table 5. Analysis of variance for different parameters effecting transformation efficiency of hypocotyls and leaf discs of tomato (*Lycopersicon esculentum* M.) cv. Riograndae and Roma.

ANNOVA for seedlings age		
Source	**df**	**Mean Square for treatments**
Varieties	1	146.20*
Treatments	2	5181.94**
Varieties × Treatments	2	14.12**
Error	12	19.51
Total	17	
ANNOVA for Acetosyringone concentration		
Varieties	1	100.04ns
Treatments	3	2426.15**
Varieties × Treatments	3	76.26ns
Error	16	26.86
Total	23	
ANNOVA for days of pre-selection		
Varieties	1	148.34**
Treatments	4	9255.42**
Varieties × Treatments	4	82.51**
Error	20	15.12
Total	29	
ANNOVA for Hygromycin resistant calli		
Varieties	1	1185.35**
Treatments	2	3139.23**
Varieties × Treatments	2	394.47**
Error	12	26.00
Total	17	
ANNOVA for Regenerated calli		
Varieties	1	152.30**
Treatments	2	1346.59**
Varieties × Treatments	2	38.08*
Error	12	9.78
Total	17	
ANNOVA for GUS +ive explants		
Varieties	1	44.08**
Treatments	2	276.64**
Varieties × Treatments	2	13.99*
Error	12	3.16
Total	17	
ANNOVA for Transformation Efficiency		
Varieties	1	36.12**
Treatments	2	166.61**
Varieties × Treatments	2	14.93*
Error	12	2.45
Total	17	

**significant at 1% level
*significant at 5% level
ns non-significant

interaction effects also showed significantly different values; these ranged from 12.4 at 50 mg/l to 73.4 at 10 mg/l hygromycin for cv. Riogrande. While 13.3 at 50 mg/l to 42.07 at 10 mg/l hygromycin was observed for cv. Roma. The cultivars means for regeneration were significantly different. These ranged from 13.0 for cv. Roma to 18.9 for cv. Riogrande. The effects for different concentrations of hygromycin on regeneration were also significantly different with the highest value of 29.6 for 10 mg/l while 0 value was recorded for 50 mg/l hygromycin. The interaction effects also showed significantly different values; these ranged from 0 at 50 mg/l to 34.0 at 10 mg/l

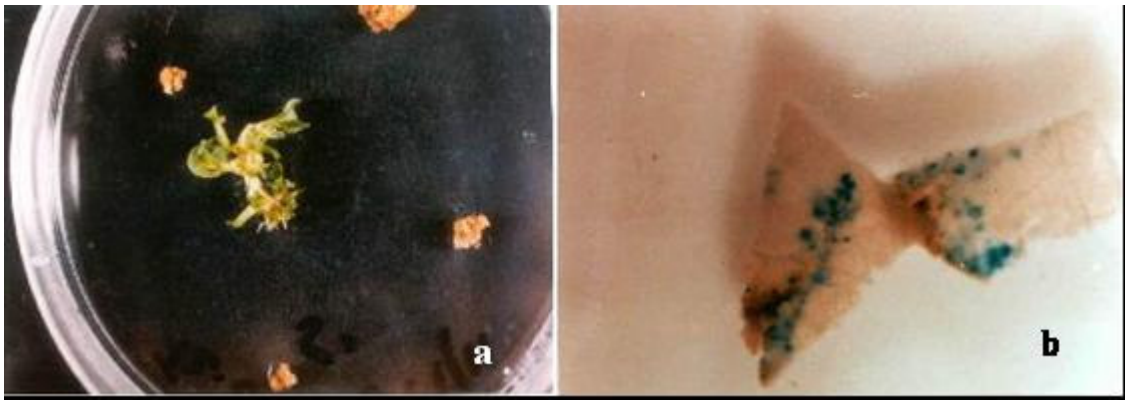

Figure 2. (a) Transgenic shoot formation from hypocotyls. **(b)** Transient GUS expression in hypocotyl.

hygromycin for cv. Riogrande. While 0 at 50 mg/l to 25.3 at 10 mg/l hygromycin was observed for cv. Roma.

The cultivars means for *GUS* expression were significantly different. These ranged from 5.89 for cv. Roma to 9.0 for cv. Riogrande. The effects for different concentrations of hygromycin on *GUS* expression were also significantly different with the highest value of 13.2 for 25 mg/l while 0 value was recorded for 50 mg/l hygromycin. The interaction effects also showed significantly different values; these ranged from 0 at 50 mg/l to 14.9 at 25 mg/l hygromycin for cv. Riogrande, while 0 at 50 mg/l to 11.64 at 25 mg/l hygromycin was observed for cv. Roma.

The cultivars means for *GUS* expression were significantly different; these ranged from 4.56 for cv. Roma to 7.4 for cv. Riogrande. The effects for different concentrations of hygromycin on transformation efficiency were also significantly different with the highest value of 9.9 for 25 mg/l while 0 value was recorded for 50 mg/l hygromycin. The interaction effects also showed significantly different values; these ranged from 0 at 50 mg/l to 11 at both 25 mg/l and 10 mg/l hygromycin for cv. Riogrande, while 0 at 50 mg/l to 8.8 at 25 mg/l hygromycin was observed for cv. Roma. These findings are contradictory to Arillaga et al. (2000), who reported that the root formation from the transgenic shoots from cotyledons explants occurred kanamycin. Roy et al. (2006) used 40 mg/l of hygromycin as a selective marker for drought tolerance in tomato cv. Pusa Ruby. At this dose all the explants turned black in case of control explants (Table 5).

Histochemical localization of *GUS* in transgenic tomato plant

To localize the cellular expression pattern of *GUS* gene driven by 35S promoter, leaf tissues of the transgenic plants surviving in pots were sectioned and subjected to histochemical staining. Blue staining of leaf appeared after overnight incubation in the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc). The results showed that 35S promoter was active in veins and cut surfaces of leaf section. No histochemical staining was detected in the leaf pieces of control plants as shown in Figure 3.

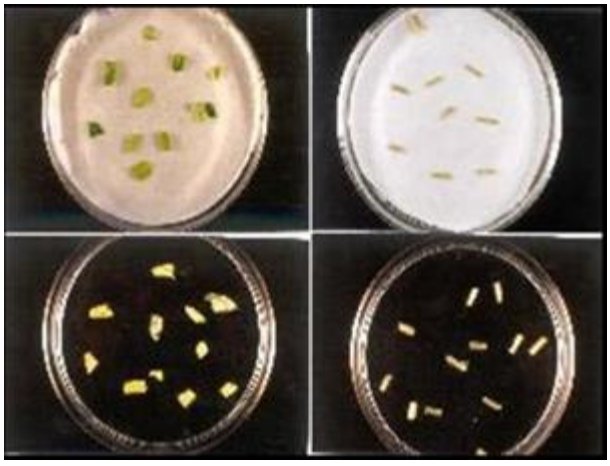

Figure 3. Photographic presentation of transformation in tomato cv. Riograndae and Roma, Leaf discs and hypocotyls of Riogarande present on the co-cultivation plate and on pre-selection medium.

PCR analysis for hygromycin resistance gene

Genomic DNA from 4 independently obtained transgenic tomato plants and a control (non-transgenic) plant was subjected to PCR analysis for presence of transgene. The samples (lane 2-4) from transgenic lines gave the predicted DNA fragment band of 670 bp for the hygromycin resistance gene (Figure 3). No DNA amplification was detected in the samples from the Control plants. Presence of T-DNA was confirmed by PCR analysis (Figure 4).

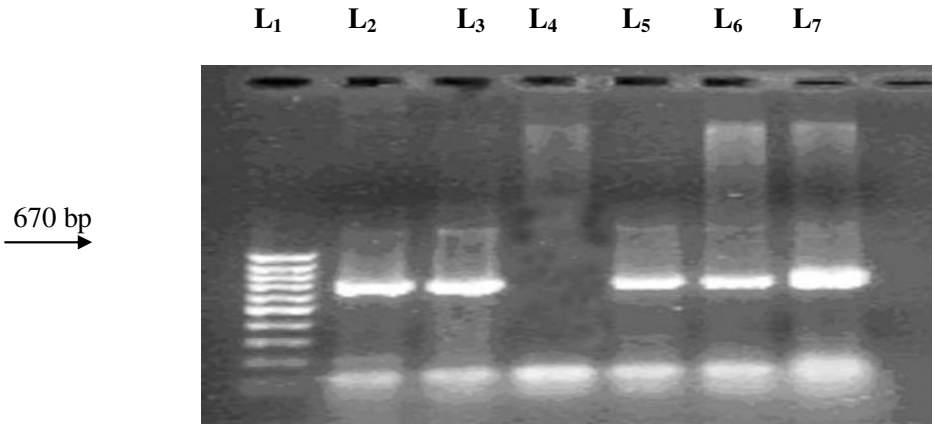

Figure 4. PCR analysis to detect the presence of hygromycin gene in transgenic tomato, (L-3, L-5 and Line-7). Lane 1 Marker ladder. Lane 2, Plasmid pTCL5. Lane 4, untransformed plant. Lane 3 and 5 cv. Riogrande, Lane 6 cv. Roma, Lane 7 cv. Money maker. Arrow indicates 670 bp fragments of hygromycin gene.

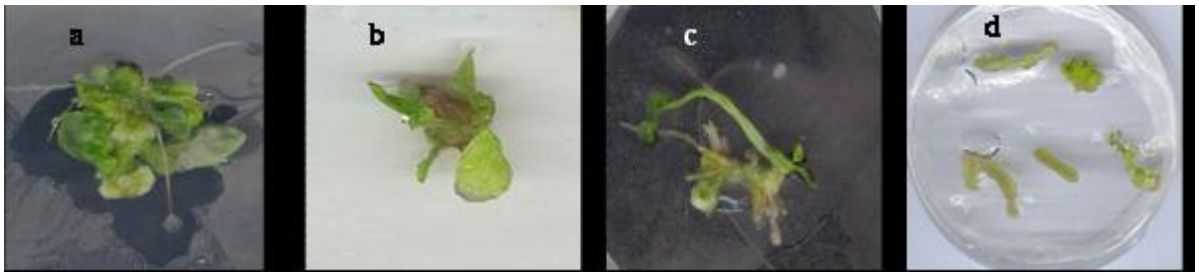

Figure 5. a. Leaf disc derived calli of cv. Riogrande on selection medium. b. Leaf disc derived calli of cv. Roma on selection medium. (c) Hypocotyls derived calli of cv. Riogrande on selection medium. (d) Hypocotyls derived calli on selection medium.

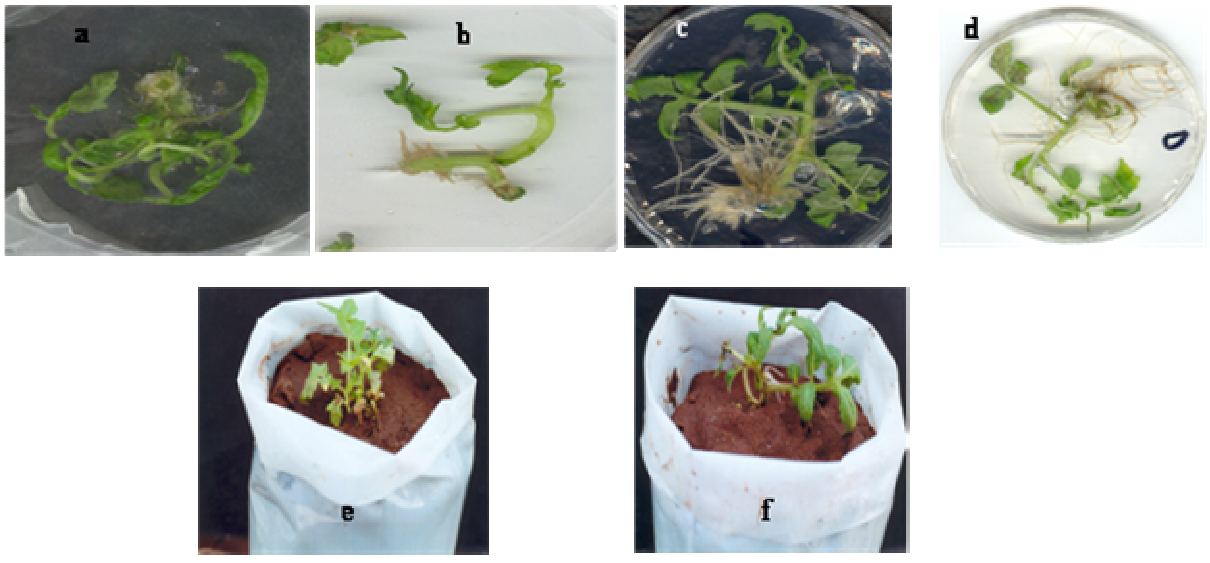

Figure 6. (a) Multiple shoot coming out from leaf disc derived calli of cv. Riogrande. (b) Shoot coming out from hypocotyls derived calli of cv. Roma. (c) Riogrande present on the rooting selection medium. (d) Roma present on rooting selection medium. (e, f) Transformed plants of Cvs. Riogrande and Roma established in soil.

DISCUSSION

The present work on regeneration of some of the *Lycopersicon esculentum* varieties showed that Riogrande has the highest regeneration frequency (92%) on RM_1, where leaf disc proved to be a better source than the hypocotyls for *in-vitro* regeneration.

Binary vector *pTCL5* was used to optimize the transformation parameters. Transient transformation frequency calculated as percentage explants showing blue colour with X-gluc. The use of acetosyringone in the media was a pre-requisite. The most important factors for stimulating the regeneration are (a) young *in-vitro* seedlings used for transformation (b) higher concentrations of acetosyringone.

During this course of study more concentration was focused on improvement of transformation efficiency with higher level of acetosyringone. Acetosyringone had a positive effect on transformation frequency if it was added in the Amino Acid medium during co-cultivation. Raj et al. (2005) reported 200 µM acetosyringone in co- cultivation of tomato cv. Pusa Ruby with *Agrobacterium* strain LBA 4404. Reda et al. (2004) used 100 µM of acetosyringone for *Agrobacterium rhizogene* strain DCAR-2 containing pBI-121 binary vector. These results are contradictory to those of Rashid et al. (1996) where no *GUS* expression was observed after the addition of 50 µM acetosyringone in AA medium for co-cultivation in *Moricandia arvensis*. Starzycki et al. (1997) observed strong shoot regeneration in oilseed rape after co-cultivation without acetosyringone.

Yaseen and Hameida (1998) reported that low percentage of resistant shoots obtained after the application of acetosyringone. This difference may be due to the fact that during co-cultivation, mostly workers used tobacco suspension layer on media. There are certain chemical derived from the tobacco suspension culture and this chemical in combination with acetosyringone affect the transient *GUS* expression of explants and reduces the transformation frequency. It can also be reasoned that acetosyringone is a promoter of transformation and not an absolute requirement. Initially it was thought that wounding, an absolute pre-requisite for *Agrobacterium* transformation, was required for the plant cells and bacteria to come in contact with each other. Actually wounded cells secrete low molecular weight molecules that stimulate the *vir-genes*. These molecules are acetosyringone and hydroxy-acetosyringone. These two molecules stimulate the synthesis of vir-*genes*. Further acetosyringone can act as a chemical attractant *in vitro* and may act as a chemotactic agent in nature. So acetosyringone is used to enhance the transformation procedure.

A co-cultivation period of 2-3 days was found optimal for transformation of cvs Riogrande and Money maker.

Longer co-cultivation period may result in the browning and necrosis of explant and devoid of shoot regeneration due to excessive growth of bacteria. A pre-selection period after co-cultivation was also found to be essential. No callus or shoot growth was observed, when selection was done immediately after co-cultivation. The pre-selection medium contained 500 mgl^{-1} cefotaxime, allowing cell division and callus formation prior to hygromycin selection, resulting in better regeneration of shoots from the explants. Preliminary experiments also indicated that cefotaxime has no effect on either callus formation or shoot regeneration but to prevent *A. tumefaciens* growth on the medium. Pre-selection for a prolonged period of 10 days showed a higher percentage of selected explants, but resulted in lower transformation efficiency. In this case a large number of escapes were formed. It may be due to the growth of non-transformed cells during pre-selection.

Selection of transformed tissue with lower concentration (10 mgl^{-1}) of hygromycin was insufficient and the untransformed explants regenerated, after 3 to 4 weeks. Maximum number of *GUS* expression was recorded on 25 mg/l hygromycin only when explants were transferred 7 days after co-cultivation to selection medium containing 25 mg/l hygromycin (Table 3). These results demonstrated that hygromycin is an effective selection agent for transgenic plant production in cvs Riogrande and Money maker. Lower selection pressure (10 mgl^{-1} hygromycin) resulted in a large proportion of plants (39%) and the proportion of *GUS* positive plants (15.9%) was much lower than that (23.2%) from the high selection pressure (25 mgl^{-1}). It means that an optimized concentration of a suitable selection agent can efficiently inhibit growth of non-transformed growth and increase the incidence of transgenic plant production. In order to avoid escapes, we suggest 25 mgl^{-1} hygromycin. Our results have shown that the choice of a proper selection scheme has eliminated the chance of escapes and raised the efficiency of transgenic plant production.

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REFERENCES

An G, Evert PR, Mitra A, Ha SB (1988). Binary vectors. In: Gelvin SB, Schilperoot RA (Eds) Plant Molecular Biology Manual, pp. 1-19. Kluwer Academic Publisher, Dordrecht, Netherlands

Arillaga I, Gisbert C, Sales E, Roig L, Moreno V (2000). *In vitro* plant regeneration and gene transfer in the wild tomato, *Lycopersicon chessmanii*. J. Hort. Sci. Biotech. 76(4): 413-418.

Chang H, Chan MT (1991). *Agrobacterium tumefaciens* mediated transformation of soybean promoted by Potato suspension culture. Bot. Bull. Acad. Sic. 32:171-178.

Chaudhry Z, Habib D, Rashid H, Qureshi AS (2004). Regeneration from various explants of *in-vitro* seedlings of Tomato (*Lycopersicon esculentum* L., Cv. Roma). Pak. J. Biol. Sci. 7(2): 269-272.

Costa MGC, Nogueira FTS, Otoni WC, Brommonschenkel SH (2000). *Agrobacterium tumefaciens*-mediated transformation of tomato processing cultivars. Revista Brasileira de Fisiologia Vegetal. 12(2): 107-118.

Doyle JJ, Doyle JI (1990) Isolation of plant DNA from fresh tissues. Focus 12: 13-15.

Fillatti JJ, Kiser J, Rose R, Comai L (1987b). Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Bio/Technol. 5: 726-730.

Fischho DA, Bowdish KS, Perlak FJ, Marrone PG, McCormick SM, Niedermeyer JG, Dean DA, Kusano-Kretzmer EJ, Mayer K, Rochester DE, Rogers SG, Fraley RT. (1987). Insect tolerant transgenic tomato plants. Bio/Technol. 5:807-813.

Gisbert C, Rus MA, Bolarin CM, Lo` pez CMJ, Arrillaga I, Montesinos C, Caro M, Serrano R, Morenov V (2000). The yeast HAL1 gene improves salt tolerance of transgenic tomato. Plant Physiol. 123: 393–402.

Gubis J, Lajchova Z, Farago J, Jurekova Z (2003). Effect of genotype and explant type on shoot regeneration in tomato (*Lycopersicon esculentum* Mill.) *in vitro*. Czech-J-of-Gene-and-Pl-Breeding. 39 (1): 9-14.

Hiei Y, Ohta S, Komari T, Abd Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6:271-282.

Hood EE, Helmer GA, Fraley RT, Chilton M (1986) .The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded ion the region of pTIBo452 outside of T-DNA. J Bact. 168: 1291-1301.

Hu W, Phillips GC (2001). A combination of overgrowth control antibiotics improves *Agrobacterium tumefaciens* mediatd transformation efficiency for cultivated tomato *(Lycopersicon. esculentum* M.). *In vitro* Cellular and Developmental Biology. Plant. 27: 12-18.

JaeBok P, Yi BY, Lee CK (2001). Effects of plant growth regulators, bud length, donor plant age, low temperature treatment and glucose concentration on callus induction and plant regeneration in anther culture of cherry tomato 'Mini-carol'. Journal of the Korean Society for Horticultural Science, 42 (1): 32-37.

Jatoi SA, Sajid GM, Quraishi A, Munir M (1995). Callogenetic and morphogenetic response of leaf explants of *in vitro* grown F_1 tomato hybrids to different levels of plant growth regulators. Pak. J. Plant Sci. 1(2): 281-287.

Jefferson RA (1987). Assaying chimeric genes in plant: the GUS gene fusion system. Plant. Mol. Biol. Rep. 5:387-405.

Jia GX, Zhu ZQ, Chang FQ, Li YX (2002). Transformation of tomato with the BADH gene from Atriplex improves salt tolerance. Plant Cell Rep. 21: 141–146.

Kim JW, Sun SSM, German TL (1994). Disease resistance in tobacco and tomato plants transformed with the tomato spotted wilt virus nucleocapsid gene. Plant Physiol. 112: 1321–1330.

Ling HQ, Kriseleit D, Gomal MW (1998). Effect of ticarcillin/Potassium clavulanate on callus growth and shoot regeneration in *Agrobacterium* mediated transformation of tomato (*Lycopersicon esculentum* M.). Plant Cell Rep. 17: 843-847.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant. 15: 472-493.

Ohta S, Mita S, Hattori T, Nakamura K (1990) Construction and expression in tobacco of a β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. Plant Cell Physiol. 31: 805-813

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Park SH, Morris JL, Park J, Hirschi KD, Smith RHM (2003). Efficient and genotype independent *Agrobacterium* mediated tomato transformation. J. Plant Physiol. 160(10): 1253-1257.

Picton S., S. Barton, M, Bouzayen, Hamilton AJ, Grierson D (1993). Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene. Plant J. 3: 469–481.

Raj SK, Singh R, Pandey SK, Singh BP (2005). *Agrobacterium* mediated tomato transformation and regeneration of transgenic lines expressing tomato leaf curl virus coat protein gene for resistance against TLCV infection. Research Communications Current science, 88(10): 1674-1679.

Rashid H, Toriyama K, Hinata K (1996). Transgenic plant production from leaf discs of *Moricandia arvensis* using *Agrobacterium tumefaciens* Plant Cell Rep. 15: 799-803.

Ray Cerkauskas (2004). Published by AVRDC – The World Vegetable Center; Visiting Scientist from Agriculture and Agri-Food Canada. Edited by Tom Kalb.

Reda E, Moghaieb A, Saneoka H, Fujita K (2004). Shoot regeneration from GUS-transformed tomato (*Lycopersicon esculentum*) hairy root. Cell Mol. Biol. Lett. 9: 439-449.

Reed AJ, Kretzmer KA, Naylor MW, Finn RF, Magin KM, Hammond BG, Leimgruber RM, Rogers SG, Fuchs RL (1996). Safety assessment of 1-aminocyclopropane-1-carboxylic acid deaminase protein expressed in delayed ripening tomatoes. J. Agric. Food Chem. 44: 388–394.

Romero JP, Houlne G, Canas L, Schantz R, Chamarno J (2001). Enhanced regeneration of tomato and pepper seedling explants for *Agrobacterium* mediated transformation. Plant Cell Tiss. Org. Cult. 67: 173-180.

Roy R, Purty RS, Agrawal V, Gupta SC (2006). Transformation of tomato cultivar 'Pusa Ruby' with bspA gene from Populus tremula for drought tolerance. Plant Cell Tiss. Org. Cul. 84: 55–67.

Ruben IV (1999). Tomatoes in the tropics. Western Press/Boulder. Colorado. p. 5.

Rui JUL, JianHua H, Yifei W, Yuefang S, RunMei Z (2000). Regeneration of transgenic tomato plants expressing rice chitinase gene via *Agrobacterium tumefaciens* mediated transformation. Acta Agricultural. Shanghai. 16(4): 18-20.

Saiqun Z, Zhibiao Y, Chang Yin W, Qifa Z (1999). Genetic transformation of isopentyl transferase gene to tomato and regeneration of transgenic plants. ActaHorticulturae. Sinica. 26(6): 376-379.

Soniya EV, Banerjee NS, Das MR (2001). Genetic analysis of somaclonal variation among callus-derived plants of tomato. Research communications. Curr. Sci. 80(9): 1213-1215.

Takashina T, Suzuki T, Engashira H, Imanishi S (1998). New molecular markers linked with high shoot regeneration capacity of wild tomato species, *Lycopersicon chilense*. Breed. Sci. 48(2): 109-113.

Yaseen YM, Hemeida A (1998). *Agrobacterium* mediated transformation of tomato from cotyledons and Hypocotyl explants. Alexandria. J. Agric. Res. 43(3): 143-149.

Yepes LM, Aldwinckle HS (1994). Factors that affect leaf regeneration efficiency in apple, and affect of antibiotics on morphogenesis. Plant Cell Tiss. Org. Cult. 37: 257-269.