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Full Length Research Paper

Comparison of transgenic plant production for bacterial blight resistance in Pakistani local rice (*Oryza sativa* L.) cultivars

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The study was carried out to improve bacterial leaf blight resistance in three rice cultivars (Basmati - 370, DR - 82 and IR - 6) by *Agrobacterium* mediated transformation system. Three week-old scutellum derived calli were infected with *Agrobacterium* strain EHA101, containing binary vector pTCL5 which has Xa 21 gene. Different levels of acetosyringone were tested to enhance transformation efficiency. Acetosyringone at 300 µM showed 56.6% *GUS* expression with 100 and 200 µM acetosyringone showing 13.3 and 30.0% *GUS* expression, respectively. Maximum transformation efficiency was obtained using DR - 82 with calli exposed to 300 µM acetosyringone for 2 min. Direct hygromycin selection with 48 h of co-cultivation was superior to pre-selection in all three cultivars. Transient *GUS* expression was 51.4% while stable *GUS* expression in calli was 18.8%. PCR analysis confirmed the presence of the Xa 21 gene in transformed regenerated plants. Stable varietal transformation efficiency was DR - 82 > Basmati-370 > IR - 6. Resistance of transgenic plants against *Xanthomonas oryzae pathovar oryzae* was evaluated with various strains/isolates at the seedling stage. All PCR positive transgenic plants of DR - 82 and Basmati - 370 were resistant with lesion areas less than 5% of the inoculated leaf area. The tested transgenic plants were resistant to all the indigenous and exotic strains tested due to the broad spectrum protection provided by the Xa 21 gene.

Key words: Transformation efficiency, selection, regeneration, resistance evaluation.

INTRODUCTION

Rice (*Oryza sativa* L) occupies a very important place among the food crops cultivated all over the world (Mahadevappa, 2004). At the global level it is the most important food crop, ranking second to wheat in area cultivated and first as the staple food in most countries. So the rate of growth in rice production is to be increased by 50% by year 2025 (Khush and Virk, 2000).

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Abbreviations: As, acetosyringone; **AAM,** amino acid medium; **CIM,** callus induction medium; **Cf,** cefotaxime.

Rice is an important cereal crop in Pakistan for both, domestic consumption and export. Various diseases seriously affect rice production in Pakistan among them bacterial leaf blight, blast, narrow brown spot, stack burn, seedling blight and leaf spot are major (Wang and Leung, 1999). Bacterial leaf blight (BLB) disease, caused by *Xanthomonas oryzae pathovar oryzae (Xoo),* is one of the most serious diseases in most rice growing countries due to its high epidemic potential and destructiveness to high-yielding cultivars in both temperate and tropical regions (Sujatha and Sonti, 2005). In the kaller belt of Pakistan, famous for producing high quality rice, the incidence of BLB is increasing in recent years. A survey of rice growing areas of Punjab in the 1997 and 1998, BLB

was found in all northern districts. In 1997, in Muridke, Narang and adjoining areas, BLB was observed in patches showing 5 to 10% disease (Khan et al., 2000).

The deployment of resistant cultivars through genetic engineering appears most effective and economical method of controlling BLB. Developing long-term resistance in rice cultivars with specific genes against pathogens shows that there is need not only for resistant cultivars, but also for cultivars differing in resistance (Mew and Khush, 1981). A dominant gene designated as Xa 21, known to confer broad spectrum resistance to BLB, was transferred from a wild species, *Oryza longistaminata* to different rice cultivars (Tu et al., 2000a) but no report is available for using this gene to transform the Basmati - 370, DR - 82 and IR - 6 cultivars. The Xa 21 locus consists of a small multigene family and that transgenic lines expressing a single member of the Xa 21 gene family confer resistance to Xoo race 6 (Song et al., 1995). Unlike all other cloned plant disease resistance genes, the deduced amino acid sequence of Xa 21 encodes a glycosylated leucine rich repeat (LRR) extracellular domain, a single pass transmebrane domain and a serine threonine kinase intracellular domain. The sequence of the predicted protein of Xa 21 is strongly suggestive of a role in cell surface recognition of a peptide ligand and subsequent activation of an intracellular defense response (Wang et al., 1995).

Transformation of both *japonica* and *indica* rice (*O. sativa* L) has been reported by several laboratories. Rice transformation/improvement has been carried out by sexual hybridization (Repellin et al., 2001), electroporation and *Agrobacterium* (Marchand et al., 2007), particle gun bombardment (Baisakh et al., 2001), protoplast and *Agrobacterium* (Martino et al., 2007) transformation methods). Rice genetic transformation has taken rapid strides since the first transgenic rice plant was produced few years ago (Raineri et al., 1990; Baba et al., 1986). During the last 10 years, tremendous progress has been made to develop a high frequency, routine and reproducible genetic transformation protocol for rice; however, *indica* cultivars are difficult to transform (Rashid et al., 1996).

This study was carried out to develop methods to enhance transgenic plant production and to develop disease resistance in Basmati - 370, DR - 82 and IR - 6 through *Agrobacterium* mediated transformation. Our ultimate goal was to enhance productivity, durability and broad-spectrum resistance to the BLB disease through genetic engineering of three commercial cultivars.

MATERIALS AND METHODS

Genetic constructs

Transformation of rice calli was carried out using *Agrobacterium* strain EHA101 (Hood et al., 1986) containing the pTCL5 plasmid (Figure 3). The pTCL5 plasmid is a binary vector, containing a resistant gene for hygromycin, the GUS (reporter) gene from pCAMBIA1301 in the T-DNA region and the Xa 21 gene isolated from pB822 (Ohta et al., 1990). The GUS gene has an intron in the middle of the coding region and is driven by the 35S promoter from cauliflower mosaic virus. This intron-GUS gene does not express GUS activity in *A. tumefaciens* cells, but when transferred into plant cells it gives blue color due to *β-glucuronidase* activity. pTCL5 binary vector was constructed at IRRI by Baisakh et al., (2000) and provided by Co-Author Dr. Hamid Rashid.

Rice cultivars and culture media

Three rice (*Oryza sativa* L.) cultivars, including the basmati rice cultivar, Basmati - 370 and two long-grain cultivars DR - 82 and IR - 6 were used as recipients of transferred genes. Seeds of all cultivars were provided by IABGR, NARC Islamabad. Media used for callogenesis; organogenesis and transformation are listed in Table 1.

Callogenesis

Mature seeds were dehulled, washed in distilled water and surface sterilized with 70% Clorox^{TM} for 20 min. The seeds were further washed three times with sterile deionized water and cultured on MI medium (Table 1) for callus induction. The cultures were incubated in the dark at 25°C for 2 weeks and then shifted to 16/8 h light/dark cycle for 1 week. After 3 weeks, the proliferated calli derived from the scutella were separated with a flamed scalpel. Compact calli were selected for transformation.

Agrobacterium mediated transformation

A. tumefaciens strain EHA101, containing binary vector pTCL5, was cultured into 50 ml liquid Yeast Extract Peptone (YEP) medium (An et al., 1988) in 250 ml flasks containing 50 mg/l kanamycin, 50 mg/l hygromycin and shaken at 100 - 110 rpm for 2 days in the dark at 28°C. Each culture was centrifuged at 3000 rpm for 15 min and the pellet was resuspended in M2 medium and vibrated on a vortex mixture. Embryogenic compact calli were placed in bacterial suspension for 1 - 2 min. To remove excess bacteria, calli were blotted dry on sterile blotting paper. The co-cultivation plates were prepared by spreading 1-2ml of liquid M3 medium on the filter paper placed over the top of co-cultivation M4 medium. The infected calli were transferred to the co-cultivation plates. These plates were sealed with parafilm and placed in the incubator at 28°C for 2 -3 days.

Selection and regeneration

The study was carried out through optimizing/performing tissue culture and transformation parameters such as callus induction, solidifying agent (agar/gelrite), calli selection, control regeneration (data not shown), age of explant, co-cultivation time period, acetosyringone concentration, hygromycin dose selection, cefotoxime concentration.

Lethal dose selection

Hygromycin was used as a selectable marker to differentiate between transformed and non-transformed calli. Different concentrations of hygromycin (20, 35, 50, 65 and 80 mg/l) were tested to determine the lethal dose. For this purpose, the explants were transferred to selection medium M6 without use of cefotoxime (Table 1) to screen calli.

Table 1. Media used for tissue culture and transformation protocol in rice cultivars.

Stage	Media code	Composition
Callogenesis	M1 = (CIM)	N6 salts and vitamins (Chu et al., 1975) + 30 g/l sucrose + 2.0 mg/l 2,4-D + 4g/l gelritte, pH 5.7-5.8
Co- cultivation	M2	N6 salts and vitamins + 30 g/l sucrose + 2.0 mg/l 2,4-D + 100 µM acetosyringone, pH 5.7-5.8
	M3 = (AA + As)	AA media (Toriyama and Hinata 1985) + 200 μM acetosyringone, pH 5.7-5.8
	M4 = (CIM + As)	N6 salts and vitamins + 30 g/l sucrose + 2.0 mg/l 2,4-D + 4g/l gelrite + 100 µM acetosyringone, pH 5.7-5.8
Pre-Selection	M5 = (CIM + Cf)	M1 medium + 1000 mg/l cefotaxime
Selection	M6 = (CIM + Hyg + Cf)	M1 medium + 50 mg/l Hyg + 1000 mg/l cefotaxime
Washing	M7	MS salts and vitamins + 30 g/l sucrose + 2.0 mg/L 2,4-D + 1000 mg/l cefotaxime, pH 5.78-5.80
Regeneration	M8 = (RM + Hyg + Cf)	MS salts and vitamins (Murasgige & Skoog. 1962) + 3% sucrose + 3% sorbitol + 2g/L casine hydrolysate + 1.0 mg/l NAA + 5.0 mg/l BAP + 0.5 mg/l GA3 + 4g/l gelrite + 50 mg/l Hyg + 1000 mg/l Cf , pH 5.7-5.8

Antibiotic selection

After co-cultivation the infected calli were washed with M7 medium (Table1). Cefotoxime was added to kill *Agrobacterium* cells still attached to the surface of calli. These calli were blotted dry on sterile filter paper and transferred to M5 medium (Table 1) for pre-selection. After one week on pre-selection medium, these calli were transferred to M6 medium (Table 1) for selection of transformants. Another method of selection, which has shown to be effective, was also tested. In that case calli after co-cultivation and washing were directly transferred to M6 without performing pre-selection step on M5 medium. Selection was done for two weeks.

Regeneration

Transformed calli after two weeks of selection period were transferred to regeneration medium (M8 medium) for shoot regeneration and root development. Regeneration medium, M8 contained MS salts and vitamins, 3% sucrose, 3% sorbitol, 2 g/L casine hydrolysate, 1.0 mg/l NAA, 5.0 mg/l BAP, 0.5 mg/l GA3, 4 g/l gelrite, 50 mg/l Hyg and 1000 mg/l Cefotoxime (Table 1). Transformation efficiencies were calculated based on plant number (TEP).

$$\text{TEP (\%)} = \frac{\text{No. of Plants obtained}}{\text{No. of calli inoculated}} \times 100$$

GUS assay

GUS assay was carried out essentially as described by Jefferson et al., (1987). Plant leaves and calli were placed in X - Gluc solution (1 mg/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.

Confirmation of transgenic plants by PCR analysis

Genomic DNA was extracted from leaf tissues of transgenic and control plants according to CTAB method (Doyle and Doyle, 1990). Plasmid (pTCL5) was isolated from *A. tumefaciens* strain EHA101 by miniprep method (Chaudhary, 1991).

Primer sequence for amplification of Xa 21 gene were F5'ATAGCA ACTCATTGCTTGG3' and R3'CGATCGCTATAACAGCAAAAC5'.

PCR was optimized for $MgCl_2$, primers concentration and annealing temperature. Pre-PCR denaturing at 94°C for 5 min, annealing at 50, 57 and 52°C for 2 min followed by extension at 72°C for three min followed by 35 cycles of denaturing at 94°C for I min, annealing at 52°C for 1 min and extension at 72°C for 2 min with a final extension cycle of 15 min at 72°C.

PCR products were analyzed on 1.0% agarose gel prepared in TE buffer and visualized by UV illuminator after staining with ethidium bromide. Bromophenol blue was used as loading/tracking dye. DNA bands were compared with 1 Kb ladder to locate the position of bands.

Resistance identification of transgenic plants

To find resistance of transgenic lines, transformed plants were infected with eight bacterial blight strains/isolates of *Xanthomonas oryzae* pv. *oryzae (Xoo)* that were PXO 61, PXO 280, PXO 86, T 42, Xo 145, XO 128, XO 136 and X0 146. Inoculation was done at the seedling stage following the clipping method. The inoculum was grown on modified Wakimoto's medium at 28°C and 48 h and adjusted to a concentration of about 10^9 bacteria cells per ml. Two weeks after inoculation when the susceptible check's lesion became obvious and stable, individual plants were scored for disease resistance.

Statistical analysis

All resulting scales of total callus browning, callus differentiation, production of green tissue or spots and plantlet formation among the three rice cultivars with different treatments along with three replicates on the MS medium were statistically analyzed using analysis of variance technique with a computer program MSTAT-C. Treatment means were compared using Factor CRD (b) and Anova-1. Variation was noted at 5% probability level ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Establishment of a high-efficiency *Agrobacterium* mediated

Table 2. Effect of rice cultivars and different ages of calli on GUS expression.

Varieties	Age of calli (Days)						Variety means
	17	20	23	28	34	40	
Basmati-370	30.00 e	60.00 b	80.00 a	45.00 c	20.00 f	10.00 g	40.83
DR-82	35.00 de	55.00 b	75.00 a	40.00 cd	20.00 f	0.00 h	37.50
IR-6	20.00 f	30.00 e	45.00 c	20.00 f	10.00 g	0.00 h	20.83
Treatment Means	28.33 C	48.33 B	66.66 A	35.00 C	16.66 D	3.33 E	---

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability level that is $\alpha = 0.05$

** In data, capital alphabets and small alphabets denote treatment/variety means and individual values as average of the three replicates.

transformation system has greatly facilitated the widespread application of transformation in *japonica* rice and also to some extent in *indica* rice. However, the reports of transformation in *indica* rice revealed either low transformation efficiency (Aldemita and Hodges, 1996) or success only with very specific genotypes (Rashid et al., 1996). Limited research has been undertaken on the improvement of local rice cultivars by genetic modifications. In present study, different factors were optimized to enhance transgenic plant production, in addition; transgenic plants produced were evaluated for bacterial blight resistance against various strains of *Xanthomonas oryzae* pv *oryzae*.

Factors affecting transformation efficiency

Various factors, that is choice and age of explant, infection and co-cultivation period, acetosyringone concentration, cefotoxime dose, selection procedure and hygromycin concentration were studied to enhance transgenic plant production in three rice cultivars that is Basmati - 370, DR - 82 and IR - 6.

Age of explant as starting material

Scutellum derived calli are frequently used as starting material for rice transformation (Aldemita and Hodges, 1996). Scutellum derived calli of different ages (17, 20, 23, 28, 34 and 40 days) of three rice cultivars (Basmati - 370, DR -82 and IR - 6) were infected with *Agrobacterium* strain EHA101 containing pTCL5 vector. After two days of co-cultivation period with *A. tumefaciens* strain EHA 101, GUS expression was noted to determine the more responsive calli. It was observed that Basmati - 370 showed maximum (40.8%) GUS expression, while DR - 82 and IR - 6 showed 37.5 and 20.8% GUS expression, respectively (Table 2). It was also observed that varietal effect was significant ($\alpha = 0.05$) for *GUS* expression.

GUS expression from different ages of calli was found significant ($P < 0.05$). Calli of 20-23 days exhibited maximum GUS expression that is 48.3 to 66.6% followed by 35.0% GUS expression from 28 days old calli. GUS expression obtained from 17, 34 and 40 days old calli was 28.3, 16.6 and 3.3% respectively. Hashizume et al. (1999) obtained high frequency of transformation that is 54.0 and 57.0% from 19 and 27 days old calli respectively. Calli of 20-23 days seemed to be more reliable as they performed well in the sense of morphology, compactness, regeneration and plantlet formation capacity (data not shown).

Effect of three rice cultivars and different ages of calli as an interaction on GUS expression was observed significant at 5% probability level. Overall maximum GUS expression (80.0%) was obtained from 23 days old calli in Basmati - 370, whereas 75.0 and 45.0% in DR - 82 and IR - 6 (Table 2). Similar results were obtained by Khan et al. (2007) who reported that older calli of over four weeks were less efficient whereas 22-25 days old calli were found to be highly efficient in transformation in IR -6 while Asghar et al. (2007) obtained maximum transformation efficiency from 21-24 days old calli in Basmati- 385.

Effect of different co-cultivation time period

In order to get high efficiency transformation in three rice cultivars viz. Basmati - 370, DR - 82 and IR-6 for 21 days old calli were co-cultivated with *A. tumefaciens* strain EHA101*/pTCL5* (Figure 4b) for 1, 2, 3 and 4 days. Maximum GUS expression (41.2%) was noted in cultivar, DR-82 upon different co-cultivation periods, while Basmati -370 and IR-6 showed 30.0 and 15.0% calli growth respectively. So it was recorded that varietal effect on different co-cultivation periods was significant ($\alpha < 0.05$).

Effect of different co-cultivation periods on calli GUS expression was found significant ($\alpha = 0.05$). Two days co-cultivation period showed maximum GUS expression that is 51.6%, whereas lowest GUS expression (6.6%) was obtained from four days co-cultivation period and 18.3 and 38.3% GUS value was obtained after one and three days co-cultivation periods respectively (Table 3). It was obvious that in one-day *Agrobacterium* activity was less but after 2 days, the cause of less bacterial growth may

Table 3. Effect of rice cultivars and different co-cultivation periods to GUS expression.

Varieties	Co-cultivation period (Days)				Variety means
	1	2	3	4	
Basmati-370	20.00 ef	55.00 b	40.00 c	5.00 hi	30.00 B
DR-82	25.00 de	70.00 a	55.00 b	15.00 fg	41.25 A
IR-6	10.00 gh	30.00 d	20.00 ef	0.00 i	15.00 C
Treatment means	18.33 C	51.66 A	38.33 B	6.66 D	---

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability level that is $\alpha = 0.05$

** In data, capital alphabets and small alphabets denote treatment/variety means and individual values as average of the three replicates.

be the start of death phase of bacteria (Figure 6a). Rashid et al. (1996) and Asghar et al. (2007) also stated 2 days as best co-cultivation period in rice to get highest transformation percentage. Saharan et al. (2004) described that maximum transient GUS expression was obtained after 3 days of co-cultivation. Yookongkaew et al. (2006) reported two categories, <50% and >50% GUS-expressing areas in seedlings after 3 days of co-cultivation. The reason for this difference might be due to the difference in the bacterial strain and genotype of rice studied.

Effect of three rice cultivars and different co-cultivation periods was observed significant ($\alpha = 0.05$). Overall best results for GUS expression (70.0%) were obtained from two days co-cultivation period in DR - 82 whereas 30.0% GUS expression was obtained from two days co-cultivation period in IR - 6. Khan et al. (2007) reported 70.0% GUS expression in IR - 6. Minimum percentage of GUS expression was observed from four days co-cultivation period (Table 3). Asghar et al. (2007) also reported lowest transformation efficiency from co-cultivation period of four days. Our findings are also in accordance with Shrawat et al. (2006) who demonstrated that no GUS expression was detected in transformed embryos immediately after inoculation while levels of GUS expression was very low after 1 day of co-cultivation and extension of the co-cultivation period from 1 to 3 days significantly enhanced the frequency of transformation. Co-cultivation for more than 3 days led to decrease in transformation frequency and about 50% explants loss because of bacterial overgrowth. Our results are also in agreement with the observations of Wu et al. (2003) who also noted that co-cultivation for more than 3 days led to a decrease in transformation frequency.

Effect of acetosyringone

A. tumefaciens is widely used for genetic transformation of plants because of its natural ability to transfer foreign DNA into the host plant genome of dicots and problem in monocots has been overcome through the addition of acetosyringone (Veluthambi et al., 1989). Effect of acetosyringone on GUS expression percentage was studied among different rice cultivars (Basmati - 370, DR - 82 and IR - 6). In co-cultivation media three different doses of acetosyringone were tested that is 100 µM, 200 µM and 300 µM. Twenty days old calli of each cultivar at all acetosyringone doses were tested.

Plant specific phenolic compounds that induce the expression of *A. vir* genes are important for the gene transfer (Stachel et al., 1985). In monocots, where such compounds are not synthesized, addition of phenolic compounds such as acetosyringone during plant/bacteria interaction supports the gene transfer (Stachel et al., 1985; Wu et al., 2003). Addition of acetosyringone during co-cultivation has been used for successful transformation of callus of indica rice (Rashid et al., 1996) and *javanica* rice (Rachmawati et al., 2004). In the present study different doses of acetosyringone showed markable variation on calli showing GUS expression in all rice cultivars however; varietal effect on GUS expression was not much different. DR -82 showed maximum GUS expression leading to Basmati -370 and IR-6 respectively. Maximum dose of acetosyringone (300 µM) showed maximum GUS expression that is 56.6% while 100 and 200 µM acetosyringone showed 13.3 and 30.0% GUS expression respectively (Table 4). Saharan et al. (2004) also reported maximum GUS frequency on those calli, which were co-cultivated with co-cultivation medium containing 400 µM acetosyringone. In contrast, Yookongkaew et al. (2006) reported that acetosyringone has no significant effect on the efficiency of transient expression of rice. Statistical analysis showed that quantity of acetosyringone has significant effect on calli GUS expression at 5% probability level.

In DR - 82, 15.0 35.0 and 65.0% GUS activity was noted at 100, 200 and 300 µM acetosyringone concentration respectively. Similar results were noted for other both the cultivars (Table 4). No GUS activity was observed at medium without acetosyringone. Our results are in line with Shrawat et al. (2006) who reported that no GUS expression was observed when acetosyringone was excluded from the co-cultivation medium and demonstrated that increasing the concentration of acetosyringone from 25 to 500 µM to the co-cultivation medium

Table 4. Effect of rice cultivars and different doses of acetosyringone on calli showing GUS expression.

Varieties	Treatments (acetosyringone, µM)			Variety means
	100	200	300	
Basmati-370	15.00 e	25.00 d	55.00 b	30.00
DR-82	15.00 e	35.00 c	65.00 a	38.33
IR-6	10.00 e	30.00 cd	50.00 b	31.66
Treatment means	13.33 C	30.00 B	56.66 A	---

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability level that is $\alpha = 0.05$
** In data, capital alphabets and small alphabets denote treatment/variety means and individual values as average of the three replicates.

Table5. Effect of rice cultivars and different doses of hygromycin on calli browning, calli necrosis and calli growth.

Varieties		Different doses of hygromycin (mg/l)					Variety means
		20	35	50	65	80	
Basmati-370	CB	8.00 f	24.00 e	72.00 c	84.00 b	92.00 a	56.00 A
	CN	0.00 f	12.00 de	20.00 c	76.00 b	88.00 a	39.20 A
	CG	52.00 b	24.00 c	0.00 de	0.00 e	0.00 e	15.20 A
DR-82	CB	4.00 f	20.00 e	56.00 d	72.00 c	84.00 b	47.20 AB
	CN	0.00 f	8.00 e	16.00 cd	72.00 b	76.00 b	34.40 A
	CG	64.00 a	24.00 c	0.00 e	0.00 e	0.00 e	17.60 A
IR-6	CB	4.00 f	20.00 e	60.00 d	76.00 c	86.00 b	49.20 A
	CN	0.00 f	4.00 ef	16.00 cd	72.00 b	78.00 b	34.00 A
	CG	58.00 ab	24.00 c	0.00 d	0.00 e	0.00 e	16.40 A
Treatment Means	CB	5.33 E	21.33 D	62.66 C	77.33 B	87.33 A	----------
	CN	0.00 F	8.00 D	17.33 C	73.33 B	80.66 A	
	CG	58.00 A	24.00 D	0.00 E	0.00 E	0.00 E	

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability level that is $\alpha = 0.05$
** In data, capital alphabets and small alphabets denote treatment/variety means and individual values as average of the three replicates.

enhanced significantly the frequency of embryos expressing GUS. Matsuoka et al. (2001) and Veluthambi et al. (1989) used higher concentration of acetosyringone in co-cultivation medium for getting maximum transformation results. Manichavasagam (2004) used 50 µM acetosyringone and obtained 7.2% transformation efficiency as compared to 3.2% transformation with out acetosyringone in sugarcane.

Hygromycin selection

To select transformed calli, hygromycin was used as selectable marker (Figure 4c). Hygromycin is extensively used for rice transformation to select resistant and transformed calli. The varietal effect for calli browning, necrosis and growth was not much significant ($\alpha = 0.05$), whereas effect of different hygromycin doses as well as their interaction with the cultivars was significantly different (Table 5). Maximum calli browning and necrosis was observed at 80 mg/l dose of hygromycin in all the cultivars. The calli necrosis percentage of all cultivars increases as the dose of hygromycin increased. No calli growth was noted at 50, 65 and 80 mg/l hygromycin concentration. So 50 mg/l dose of hygromycin was considered as the lethal dose as it was the minimum concentration at which all the control calli died. The calli growth percentage of all the cultivars increased as the dose of hygromycin decreased.

In order to find out lethal dose for non-transformed calli, different doses of hygromycin (20, 35, 50, 65 and 80 mg/l) were used. It was revealed that no calli growth was observed at 50, 65 and 80 mg/l doses of hygromycin, while 58.0% and 24.0% calli growth was observed at 20 and 35 mg/l hygromycin doses respectively (Table 5) but calli died later on when shifted to 50 mg/l hygromycin dose as it was selected as the lethal dose.

Yookongkaew et al. (2006) reported 50mg/l hygromycin

Table 6. Effect of rice cultivars and different doses of cefotoxime on calli browning, calli necrosis and calli growth.

Varieties	Treatments (Cefotoxime Conc.mg/l)								
	500			750			1000		
	CB	CN	CG	CB	CN	CG	CB	CN	CG
B-370	6.66	0.00 d	10.00 ef	13.33	6.66 bc	16.66 de	19.96	13.33 a	60.00 a
DR-82	10.00	3.33 cd	13.33 ef	10.00	3.30 cd	23.33 cd	13.33	6.66 bc	46.66 b
IR-6	6.70	0.00 d	6.70 f	10.00	6.66 bc	26.66 c	13.33	10.00ab	50.00 b
Treatment Means	7.7 B	1.1 B	10.0 C	11.1AB	5.5 AB	22.2 B	15.5A	10.0 A	52.2 A
Variety means									
Basmati-370	CB = 13.3 A			CN = 6.6 A			CG = 28.8 A		
DR-82	CB = 13.3 A			CN = 4.4 A			CG = 27.7 A		
IR-6	CB = 10.0 A			CN = 5.5 A			CG = 27.7 A		

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability leve, that is, $\alpha = 0.05$
** In data, capital alphabets and small alphabets denote treatment/variety means and individual values as average of the three replicates.

as the minimum concentration killing rice tissue of *indica* cv. KDML105. Similarly, in our studies hygromycin concentration of 50 mg/l was proved to be lethal as the calli died at this concentration. Same results were described for selection of calli of Super Basmati (Rashid et al., 2001), Basmati - 385 (Asghar et al., 2007), IR - 6 (Khan et al., 2007), Basmati - 370, Basmati - 385 and Basmati - 6129 (Rashid et al., 1996).

Effect of various doses of cefotoxime

The use of appropriate dose of cefotoxime is a critical step to stop the excessive growth of bacteria after two days of co-cultivation without damaging the transformed calli. Yookongkaew et al. (2006) reported 250 mg/l cefotaxime and carbenicillin as the lowest concentration that prevented bacterial growth. In present study different concentrations of cefotoxime that is 500, 750 and 1000 mg/l were used to control bacterial contamination in three rice cultivars viz. Basmati - 370, DR - 82 and IR - 6. All the cultivars tested have no significant effect on callus browning, necrosis and growth (Table 6). Cefotoxime concentration of 1000 mg/l was found optimum in pre-selection of rice calli to control contamination of excessive bacteria and at this quantity rice calli showed 52.2% growth**.** Earlier, Rashid et al. (1996) used 500 mg/l carbencillin to control bacterial growth; while same calli growth was observed by Asghar et al. (2007) using 1000 mg/l. Maximum inhibitory effect to calli growth (10.0%) was observed at 1000 mg/l dose of cefotoxime. Apart from this less browning and necrosis was obtained by testing the 500 and 750 mg/l cefotoxime concentrations but it was difficult to control the bacterial contamination on these concentrations. So, it is concluded that increase in concentration of antibiotics inhibits regeneration of the plant tissues. Nauerby et al. (1997) also reported that 500 mg/l cefotaxime and 1000 mg/l carbenicillin played inhibitory effect on regeneration of *Nicotiana tobacum*.

Lin and Zhang (2005) described that neither cefotoxime nor carbencillin had adverse effects on growth and differentiation of the rice calli. This suggests that application of the antibiotics does not necessarily account for the low efficiency of rice transformation.

Interaction of rice cultivars and different doses of cefotoxime for calli necrosis and growth was significant, whereas non significant for callus browning. Maximum necrosis was observed at 1000 mg/l, while minimum at 500mg/l cefotoxime in all rice cultivars (Table 7). Maximum calli growth was observed at 1000 mg/l cefotoxime in all cultivars but high growth (60.0%) was recorded in Basmati -370 (Table 6). Asghar et al. (2007) also reported optimal cefotoxime concentration of 1000 mg/l and observed a positive effect on control of bacterial contamination in indica rice as well enhancement in calli growth. Khan et al. (2007) used 500 mg/l cefotoxime in combination with 500 mg/l carbencillin in Basmati - 370 for selection; however, Arencibia et al. (1998) used 500mg/l cefotoxime in pre-selection process to control bacterial contamination in sugarcane.

Comparison of pre-selection and direct selection procedure for transgenic plant production

Two procedures were tested for the selection of transformed rice calli after two days of co-cultivation period. The comparison of both the procedures revealed that the varietal effect for selection was not much different whereas the effect of both the selection procedures and their interaction with rice cultivars was significant ($\alpha < 0.05$). In the selection procedure following pre selection (Figure 4c), out of total calli cultured of three rice cultivars, 41.6% were clean calli, 10.0% showed necrosis and 35.0% showed growth while, direct selection procedure (Figure 5a) showed 35.0% clean calli, 22.5% necrosis and 41.6% growth as an average of three cultivars. These results are in confirmatory observed by

Table 7. Effect of rice varieties and different selection procedures on calli resistance, calli necrosis and calli growth.

Varieties	Selection Procedures					
	Direct Selection			Selection following pre-selection		
	CR	CN	CG	CR	CN	CG
Basmati-370	45.00 b	20.00 b	40.00 d	62.50 a	10.00 cd	35.00 bc
DR-82	35.00 c	17.50 b	70.00 a	27.50 cd	7.50 d	25.00 cd
IR-6	25.00 d	30.00 a	15.00 b	35.00 c	12.50 c	45.00 b
Treatment Means	35.0 AB	22.5 A	41.6 A	41.6 A	10.0 C	35.0 AB
Variety means						
Basmati-370	CR = 53.7 A		CN = 15.0 B		CG = 37.5 AB	
DR-82	CR = 31.2 B		CN = 12.5 B		CG = 47.5 A	
IR-6	CR = 30.0 B		CN = 21.2 A		CN = 21.2 A	

*Any two means not sharing a letter common in a row or column differ significantly at 5% probability level, that is, $\alpha = 0.05$

** In data, capital alphabets and small alphabets denote treatment/variety means and individual values as average of the three replicates.

Zhang et al. (1999). The reason of calli growth difference in both the procedures may be due to less contamination in direct selection procedure and death of non-transformed cells. Maximum calli growth (70.0%) through direct selection was observed in DR - 82 whereas maximum (45.0%) through indirect selection in IR - 6. Maximum calli resistance (62.5%) was observed through indirect selection while 45.0% through direct selection in basmati -370 (Table 7). In our study, calli growth was observed by both the procedures, however direct selection procedure proved to be better than indirect selection procedure.

Comparison of transgenic plant production efficiency

The first reports of rice transformation have been available since late 1980s (Baba et al., 1986; Raineri et al., 1990), describing that *indica* cultivars are difficult to transform, while Rashid et al. (1996) first demonstrated the stable transmission of inheritance in R1 progeny.

In order to evaluate transformation study, all the three rice cultivars (Basmati - 370, DR - 82 and IR - 6) were tested for GUS expression of hygromycin resistant calli (selected calli), hygromycin resistant plant production and GUS expression of transformed leaves and they were all significantly different among the cultivars. Maximum GUS expression from hygromycin resistant calli (61.10%) was observed in DR-82 following 42.22 and 13.33% in Basmati -370 and IR-6, respectively. Regeneration frequency in DR - 82 was found maximum that is 42.22%, whereas Basmati -370 and IR - 6 showed 27.77 and 11.11%, respectively (Figure 1). Nandakumar et al. (2007) reported that hygromycin resistant calli showed varied level of regeneration efficiency ranging from 2.0 to 7.6%. High and varied transformation efficiency like 27% (Aldemita and Hodges, 1996), 10 - 11% for Basmati - 385 (Asghar et al., 2007) and 22% for indica genotype (Rashid et al., 1996) has been reported.

Transformation efficiency was calculated as expression of *GUS* from transgenic plants produced. Histochemical analysis of GUS expression has been studied by Terada and Shimamto (1990) and Rashid et al. (1996). Maximum GUS expression was observed in DR - 82, while 17.77 and 7.77% was recorded in Basmati - 370 and IR – 6, respectively (Figure 1). Very low transgenic plant regeneration frequency (2 - 3%) in IR - 6 was observed by Khan et al. (2007) against bacterial blight. Our results in case of Basmati - 370 and DR - 82 are in contrast with Asghar et al. (2007), Nandakumar et al. (2007) as they reported low transformation efficiency, whereas transformation efficiency in IR - 6 is in accordance with them. The cause of such difference may be the variabilities in culture medium as well as strain of *Agrobacterium* used.

Molecular analysis of transgenic plants

Genomic DNA from four plants of rice cultivars independently obtained transgenic resistant plants and a control (non-transgenic) rice plant was subjected to polymerase chain reaction (PCR) analysis to check the presence of Xa 21 gene in transgenic T0 plants. PCR amplification of the Xa 21 gene was carried out by using the transgenic lines using primers F5'ATAGCAA CTCATTGCTTGG3' and Xa 21 R5'CGATCGCTATAA CAGCAAAAC5' which amplify 1.4 kb fragment specific to the Xa 21 gene. The PCR result (Figure 2) indicated that T-DNA was shown to be stably maintained in transformed rice calli and all of the samples from transgenic plants gave the predicted DNA fragment band of 1.4 kb for Xa21 gene. No DNA amplification was detected in the sample

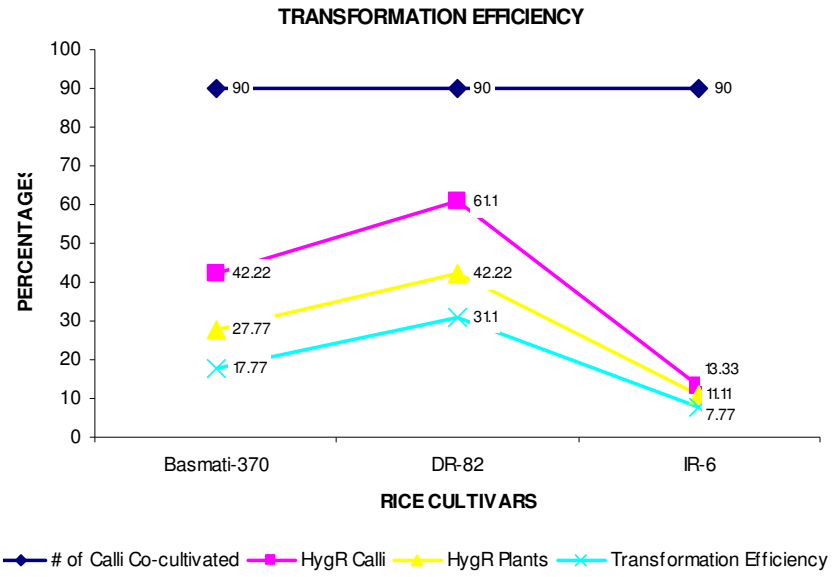

Figure 1. Comparison of three rice cultivars for transformation efficiency.

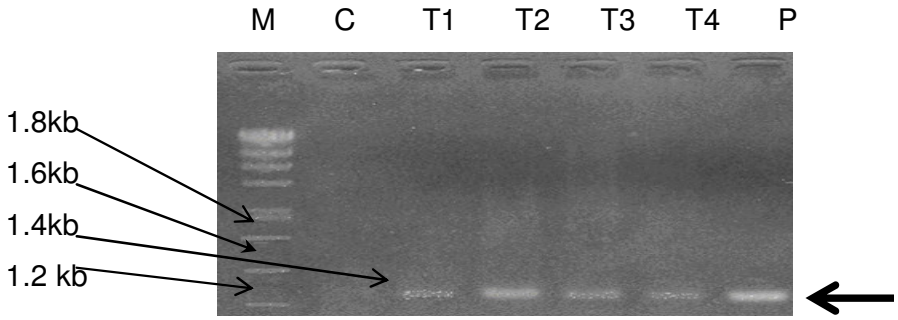

Figure 2. PCR analysis to detect the presence of Xa 21 gene in four primary transgenic plants (T1 - T4) arose from independent calli. Coulmn1: 1 kb molecular weight ladder (Marker, M), Column 2: Non-transgenic DNA sample (Control, C), Column 3 - 6: Transgenic DNA samples of Bamati - 370 (T1), DR - 82 (T2 - T3) and IR - 6 (T4), Column 7: Plasmid containing Xa 21 gene in T-DNA region (Plasmid, P).

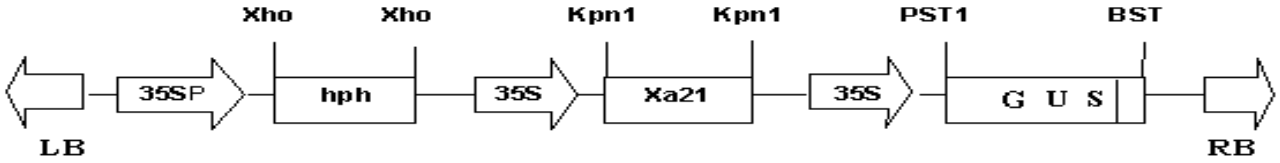

Figure 3. Schematic diagram of a part of the T-DNA region of transformation Vector pTCL5. RB: Right Border, LB: Left Border, 35S Pro: Cauliflower Mosaic Virus 35S Promoter, GUS: *ß -Glucuronidase* Coding Region, INT: First Intron of the Caster Bean Catalase Gene and HPT: Hygromycin Phosphotransferase.

from the control plant.

Evaluation of transgenic plants of rice

To evaluate resistance of transgenic plants to bacterial blight pathogen, all the transgenic plants were inoculated with eight strains/isolates at seedling stage. It was noted that all PCR positive transgenic plants of DR - 82 were resistant and the lesion area was less than 5% of the whole leaves inoculated. Usually the lesion area of non-transgenic control plants ranged from 30 - 97% (Table 8).

Figure 4. Callus induction, co-cultivation and pre-selection. (a) Scutellum derived calli of rice. (b) Calli of B - 370 on co-cultivation medium. (c) Calli on pre-selection medium.

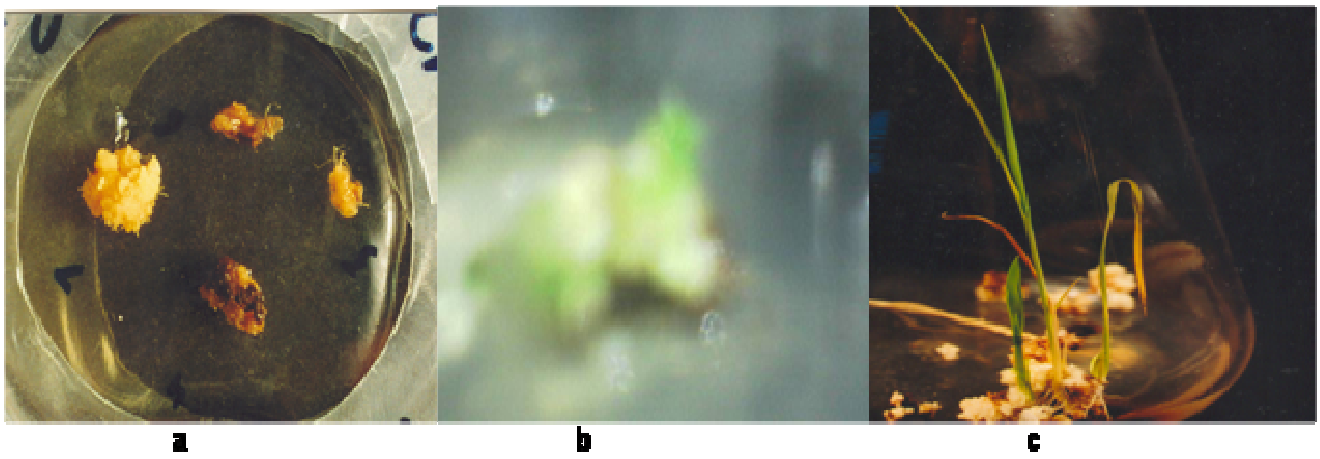

Figure 5. Selection, regeneration and transgenic plant formation. (a) Survived and died calli of rice on selection (b) Selected callus showing greening on regeneration medium (c) Transgenic plant formation from rice callus.

The tested transgenic plants were resistant to all of strains/isolates as all the transgenic plants carry Xa 21 gene, which maintain its broad-spectrum resistance to strains/isolates on Basmati - 370 and DR - 82 rice cultivars (Figure 7). The Xa 21 locus consists of a small multigene family and transgenic lines (T0) expressing a single member of the Xa 21 gene family confers resistance to Xoo race 6 (Song et al., 1995). It was also demonstrated by Ronald et al. (1992) that rice gene Xa 21 confers resistance against the bacterial pathogen *Xanthomonas* oryzae (Xoo).

Conclusion and future prospective

The deployment of resistant cultivars appears to be the most effective and economical method of controlling the diseases through genetic engineering. The transformation study approach was performed for bacterial blight resistance. For this purpose, a cloned gene, Xa 21 was transferred by marker-assisted selection into three widely used Pakistani rice cultivars. Introduction of Xa 21 gene was confirmed by PCR analysis. All transgenic plants exhibited fragment size, 1.4 kb of Xa 21 gene. Present study concludes that tissue culture studies are easy to transform for conducting functional studies of promoters and transgenic plant production for various agronomic traits that require improvement. There are number of factors that affect the transformation efficiency of rice calli. Some of the important factors studied in the present work suggest that these factors can be optimized for efficient transformation of rice. Further more, it can be concluded that tissue culture techniques and genetic transformation protocols developed in the present study will remain a method of choice to improve the production and to develop disease resistant cultivars of rice and

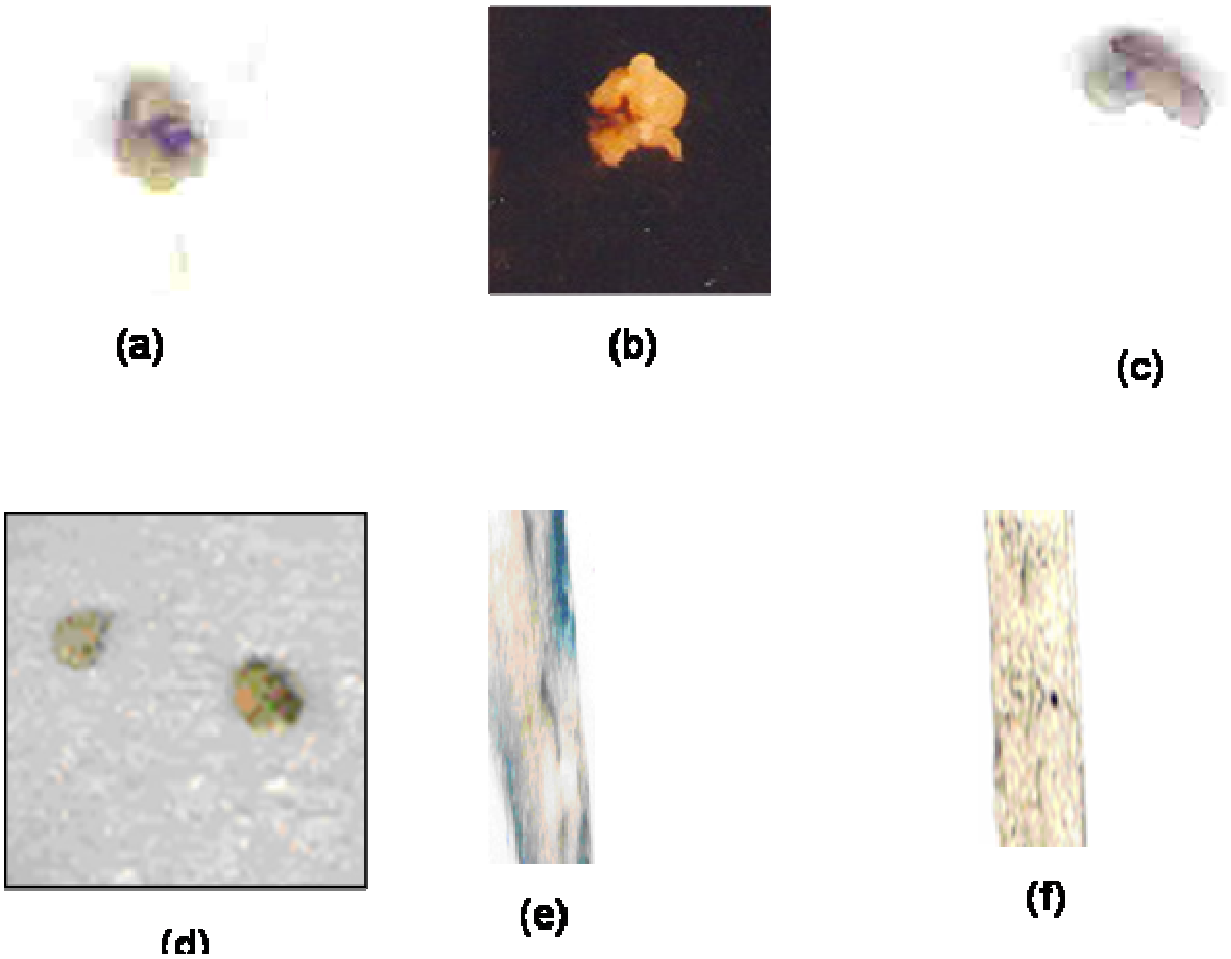

Figure 6. Transient and stable Gus at different stages. Transient *GUS* expression in rice calli at co-cultivation stage (a) in comparison with control (b). Selected callus showing *GUS* expression on greening (c) in comparison with control (d). Stable *GUS* expression obtained from transgenic leaf (e) in comparison with non-transgenic leaf /control (f).

Table 8. Reactions percentage of transgenic plants with Xa 21 gene to eight strains/isolates of *X. oryzae* pv. *oryzae (Xoo)* Basmati-370 and DR-82 rice cultivars at seedling stage.

Strains/ isolates	% disease incidence (lesion length/leaflengthratio)*			Host response	
	Basmati-370	DR-82	Avg	Basmati-370	DR-82
PXO 61	2.45	2.00	2.23	R	R
PXO 280	1.66	0.00	0.83	R	HR
PXO 86	7.90	2.10	5.00	R	R
T42	0.00	0.00	0.00	HR	HR
Xo145	7.20	0.00	3.60	R	HR
XO128	10.00	1.55	5.78	R	R
XO136	0.00	4.83	2.42	HR	R
X0 146	0.00	2.59	1.30	HR	R
Average	3.65	1.63	------	------	------

*Data was taken after 30 days of sowing.
HR = Highly resistant (infection 0%, score 0); R= resistant (infection 0 - 10%, score 1).

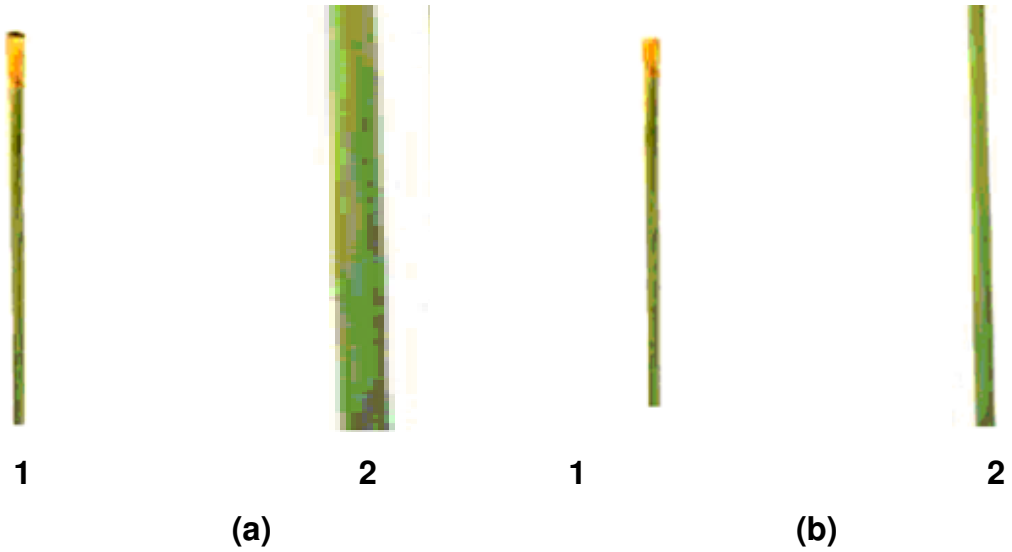

Figure 7. Resistance reactions of Xa 21 transgenic T0 plants. Control/non transgenic plants 1(a) and 1(b) of basmati - 370 and DR - 82 respectively, inoculated with isolate PXO 61. Leaves were taken two weeks after inoculation. Transgenic plants 2(a) and 2(b) of basmati - 370 and DR - 82 respectively inoculated with PXO-61. Leaves were taken two weeks after inoculation.

other cereal crops in Pakistan.

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