GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

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EVALUATION OF TOMATO TRANSGENICS CO-TRANSFORMED WITH *ech42* and *bgn* AGAINST FUNGAL DISEASE RESISTANCE

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ABSTRACT
Attempts were made to analyse southern hybridization positive transgenic tomato line (CG2-17-6) carrying both ech42 and bgn under single T-DNA region in T_3 generation. Sixteen progenies were analyzed for the presence of the transgene using ech42 and *bgn* specific primers. Eleven plants showed presence of both ech42 and *bgn*. Biochemical analysis confirmed the expression of ech42 and *bgn*. Expression level of endochitinase and endoglucanase revealed 4.09 and 5.17 fold higher activity, over non-transgenic control plant. In vitro screening of transgenic tomato lines against fungal pathogens *Sclerotium rolfsii*, *Alternaria solani* and *Rhizoctonia solani* using plate bioassay and detached leaf assay against *Alternaria solani* showed direct relationship between the chitinolytic activity and inhibition of pathogen growth.

KEYWORDS: Co-transformed, Transgenes, Endochitinase, Endoglucanase, Tomato, southern hybridization.

INTRODUCTION

Tomato (*Solanum lycopersicum* Mill.) is the second most important vegetable crop throughout the world, amenable to plant tissue culture and genetic transformation processes. It is a major horticulture and second most important vegetable crop after potato in the world. Tomato has been an excellent model system for both basic and applied plant research, related to fruit quality, disease and pest resistance and stress tolerance, due to ease of culture under a wide range of environment, short life cycle, photoperiod insensitivity, great reproductive potential and diploid species with small genome (Arumuganathan and Earle, 1991; Peterson *et al.*, 1998). A major goal of plant biotechnology is to develop effective and lasting means to reduce crop losses due to biotic and abiotic stresses. Plant diseases are the major constraints in plant growth and development, resulting in greater crop losses. Fungal diseases in particular, severely limit the production of major agriculture and horticulture crops. In Indian context, fungal diseases are rated as one of the most important factors contributing to an average of 30-40% yield losses (Punja, 2006). Conventional methods to control pathogen utilize the phenomenon of induction of natural resistance into cultivars through breeding programmes and wide spread use of chemical fungicides. Although these strategies have been successful, generally they suffer from limitations, such as lack of proper resistance source, evolution of new pathogen races and barriers to introduction of resistance genes and degradation of environment. In this scenario, introduction of specific genes encoding disease resistance through transgenic technology seems to be a viable alternative to protect crop plants against pests and diseases (Veluthambi *et al.*, 2003; Bhargava *et al.*, 2007; Afroz *et al.*, 2010). In this study, Attempts were made to analyse southern hybridization positive transgenic tomato line (CG2-17-6) carrying both ech42 and *bgn* under single T-DNA region in T_3 generation.

MATERIALS & METHODS

Raising of plants

Forty seeds from *ech42* and *bgn* positive T_2 generation CG2-17-6 (pRAGS2 -17-6), (Sharad, 2013) were sown in nursery trays having sand: soil: FYM in the ratio of 3:4:1. After 20 days of sowing, survived seedlings were further transferred to bigger pots having soil, sand and FYM. DNA was isolated from individual plants by modified C-TAB method (Mace *et al.*, 2003).

PCR screening of T_3 generation plants

DNA isolated from individual T_3 generation tomato plants was screened for the presence of *ech*42 and *bgn* using the following primers-

ech42	Chit Hyb F	Forward primer	5’ GGCAAGCACCATGTCACCCTT 3’	Kumari *et al.* (2011)
	Chit Hyb R	Reverse primer	5’TGGGGGAGCTCAGCAGGTTCT 3’	
bgn	Mod glu 5 F	Forward primer	5’ TTTGCGTGGCTGCCCCAAGAC 3’	Gaurav (2009)
	Mod glu 5 R	Reverse primer	5’ GTGAAGGCGGTCCTGCTGCTGAC 3’	

Analysis of expression of *ech42* and *bgn* in tomato

Total protein was extracted from *ech42* and *bgn* positive T_3 generation PCR positive tomato transformants by following the procedure described by Velaquez and Hammerschmidt (2004) with slight modification. Non transformed plant was used as control. 1g of leaf tissues was ground with the help of mortar and pestle using liquid nitrogen and homogenized in 0.01 M sodium acetate (pH 5.0) in a ratio of 3 ml of buffer for 1 g of fresh tissue. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C and the supernatant was filtered using 0.22µ membrane filter. The total protein was estimated using

Lowry's method (Lowry *et al.*, 1951) and equal quantity of protein was used for further assays.

Chitinase activity assay

Preparation of colloidal chitin

Colloidal chitin was prepared by the method of Roberts and Selintrenikoff (1988) with certain modifications. Later, colloidal chitin solution (5%) was prepared and stored at 4°C for further use.

DNSA reagent

DNSA reagent was prepared by dissolving 1g of 3, 5-dinitrosalicylic acid (DNSA) in a little amount of 2 N NaOH. 30 g of sodium potassium tartarate was added and made up to 100 ml with 2 N NaOH.

Preparation of standard

Hundred mg of NAG (N-acetyl glucosamine) was dissolved in water and made up to 100 ml with water in a volumetric flask. This solution contains 1 mg of glucose per ml and was used as stock. In case of plant samples, 100 μl of crude protein, 100 μl of McILvaine buffer (McILvaine, 1920) and 100 μl of colloidal chitin was added and further steps were followed as in standard. In order to identify plants having higher chitinase activity, reducing sugars released by 100 µl of leaf extract in 30 min have been converted into pico moles of reducing sugars released per microgram of crude protein per min.

Glucanolytic activity assay

Estimation of reducing sugars by dinitro-salicylic acid (DNSA) method

Glucanase enzyme activity was assayed by using colorimetric method described by Katany *et al.* (2000). The assay mixture contained 500 μl of 5.0% (w/v) laminarin in 50 mM acetate buffer (pH = 4.8) and 50 μl of crude protein extract. The reaction mixture was incubated for 30 minutes at 45°C and reducing sugars was estimated by dinitro-salicylic acid (DNSA) method (Miller, 1959).

Screening of transgenic tomato lines for disease resistance

Bioassay against *S. rolfsii, R. solani and A. solani*

The crude protein extracted from leaves of transgenic and non-transgenic tomato plants was used to study *in vitro* inhibitory effect on the *Sclerotium rolfsii* while the plain PDA without any protein served as control. Two different concentrations of crude protein extract (750 µg, 1000 µg) were used from each transgenic and non transgenic plant. First, extract was mixed with PDA (Appendix I) and plates were prepared uniformly in three replications for each concentration of proteins and sclerotial bodies isolated from well grown fungus flasks were used for inoculation. Sclerotial bodies were taken and kept in the centre of each plate. For *R. solani and A. solani* a fungal plug from the margin of actively growing fungal plates were taken and kept in the centre of each plate. Plates were incubated overnight at 25°C and 80-90% humidity in the dark (Harighi *et al*., 2007). The inhibition of germination of sclerotial bodies, diameter of fungal growth was recorded. Per cent inhibition of fungal growth was calculated by the following formula (Rini and Sulochana, 2007).

$$\text{Per cent inhibition of growth} = \frac{\text{Colony diameter of control} - \text{Colony diameter of treatment}}{\text{Colony diameter of control}} \times 100$$

Pathogen inhibition assay in detached leaves against *A. solani*

Detached leaf assay was used for bioassay against foliar pathogen, *A. solani* according to Shah *et al.* (2010). Fresh cultures of *A. solani* were grown on PDA plates at 30 °C. When the fungal mycelia reached the edge of the plate, 5.0 mm diameter agar plug with mycelium was removed from a region closed to the edge and used for inoculations. Healthy and young leaves were collected from hardened control and transgenic tomato plants and placed on the wet Whatmann paper in petri dishes. Agar plugs containing *A. solani* were placed directly on adaxial side of the leaves and incubated in dark and moist conditions. Leaves were observed daily and percentage of leaf area with necrosis was determined.

Statistical analysis

For chitinolytic, glucatiolytic activity and bioassay against *S. rolfsii*, *R. solani* and *A. solani* experiments were conducted in three replications. Results were analyzed using Completely Randomized Design (CRD).

RESULTS & DISCUSSION

Analysis of progenies of transgenic tomato with *ech42* and *bgn*

PCR analysis of T_3 generation of transgenic tomato lines

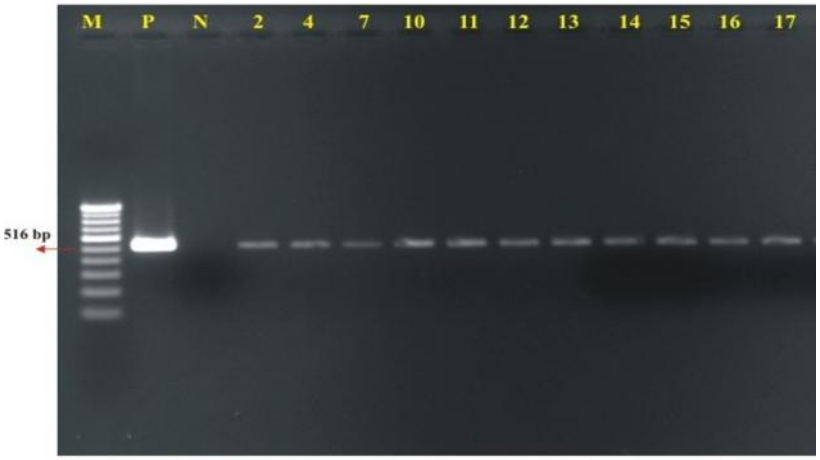
Forty seeds collected from T_2 generation southern positive *ech42* and *bgn* tomato transgenics (CG2-17-6) were sown in nursery trays. Only 16 plants survived and progenies obtained were screened for the presence of both *ech42* and *bgn*, by using gene specific primers. Eleven plants (Plate 1a & 1b) showed the presence of both the genes. These plants were further named as CG2-17-6-2,CG2-17-6-4, CG2-17-6-7, CG2-17-6-10, CG2-17-6-11, CG2-17-6-12, CG2-17-6-13, CG2-17-6-14, CG2-17-6-15, CG2-17-6-16 and CG2-17-6-17.

Expression analysis of transgenes

Expression of *ech42* in tomato transgenics with *ech42* and *bgn*

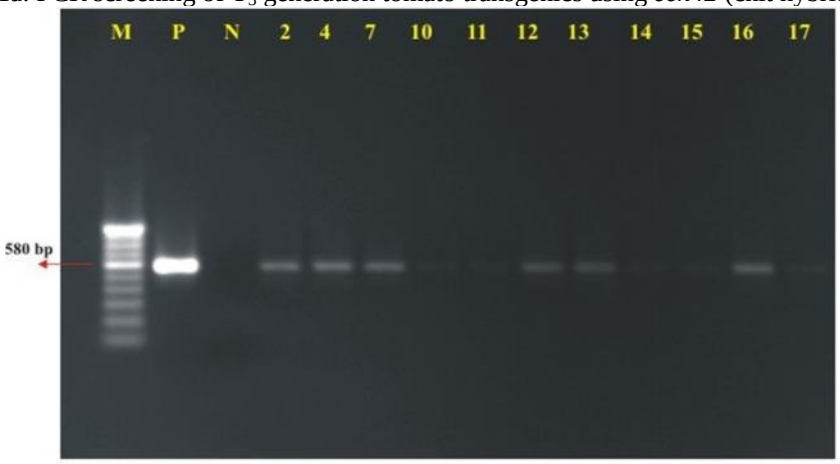
To analyse the expression of *ech42*, chitinolytic activity in transgenic and non transgenic plants was measured using crude protein extracts according to Miller (1959) with certain modifications and expressed as pmol of N-acetyl glucosamine released /μg of crude protein/min. All the PCR positive transgenic plants with *ech42* and *bgn* tested showed significantly higher level of enzyme activity compared to control. The chitinolytic activity observed in non transgenic plant was 46.605 pmol of N-acetyl glucosamine released/μg of crude protein/min and enzyme activity varied significantly among different transgenic plants (Table 1a). The amount of reducing sugars released in these plants varied between 69.615 and 258.554 pmol of reducing sugar per µg of total protein per min. Among the

eleven transgenic plants, CG2-17-6-2 showed highest chitinolytic activity (258.554 pmol/μg of protein) and CG2-17-6-7 showed lowest endochitinase activity (69.615 pmol/μg of protein).



M- 100 bp DNA ladder P- pRAGS121 plasmid with *ech*42 and *bgn*
N- DNA sample from non-transgenic plant
2,4,7,10,11,12,13,14,15,16, 17 –DNA samples from transgenic plant

PLATE 1a: PCR screening of T_3 generation tomato transgenics using *ech42* (chit hybrid) primers



M- 100 bp DNA ladder P- pRAGS121 plasmid with *ech*42 and *bgn*
N- DNA sample from non transgenic plant
2,4,7,10,11,12,13,14,15,16,17 –DNA sample from transgenic plant

PLATE 1b: PCR screening of T_3 generation tomato transgenics using Mod glu primers

TABLE 1a: Chitinolytic activity (pmol of reducing sugar / µg of total protein/min) in transgenic tomato plants carrying *ech*42 and *bgn*.

Sl. No.	Plant ID	Enzyme activity (pmol/µg/min) ±S.E.	Fold increase in chitinolytic activity
1	CG2-17-6-2	258.554^{A*}	5.54
2	CG2-17-6-4	221.351^{C}	4.74
3	CG2-17-6-7	69.615^{I}	1.49
4	CG2-17-6-10	169.705^{E}	3.64
5	CG2-17-6-11	112.400^{F}	2.41
6	CG2-17-6-12	228.483^{B}	4.90
7	CG2-17-6-13	114.378^{F}	2.45
8	CG2-17-6-14	171.491^{E}	3.67
9	CG2-17-6-15	75.037^{H}	1.61
10	CG2-17-6-16	80.767^{G}	1.73
11	CG2-17-6-17	202.093^{D}	4.33
12	Control	46.605^{J}	
	SEm+	2.16	
	CD 0.01	6.058	
	CV%	1.819	

* Values superscribed with identical letters within each column do not differ significantly

Expression of *bgn* in tomato transgenics with *ech42* and *bgn*

Similarly, the expression of *bgn* in transgenic tomato was confirmed by measuring glucanolytic activity in T_3 generation. Non transgenic plant was used as control. The PCR positive transgenic plant showed significantly higher level of enzyme activity compared to non-transgenic plant (Table 1b). The glucanolytic activity ranged from 106.334 to 385.051 pmol of reducing sugar per µg of total protein per min, among the 11 transgenic plants studied. The plant number CG2-17-6-10 showed higher (385.051) activity, where as CG2-17-6-15 showed lowest activity (106.334).The amount of reducing sugar released in non transgenic tomato was 74.356 pmol.

TABLE 1b: Glucanolytic activity (pmol of reducing sugar / µg of total protein/min) in transgenic tomato plants carrying *ech*42 and *bgn*

Sl. No.	Plant ID	Enzyme activity (pmol/µg/min) ±S.E.	Fold increase in glucanolytic activity
1	CG2-17-6-2	346.552^{B*}	4.66
2	CG2-17-6-4	306.005^{D}	4.11
3	CG2-17-6-7	140.341^{G}	1.88
4	CG2-17-6-10	385.051^{A}	5.17
5	CG2-17-6-11	190.262^{F}	2.55
6	CG2-17-6-12	227.893^{E}	3.06
7	CG2-17-6-13	197.099^{F}	2.65
8	CG2-17-6-14	306.486^{D}	4.12
9	CG2-17-6-15	106.334^{H}	1.43
10	CG2-17-6-16	117.878^{G}	1.58
11	CG2-17-6-17	319.006^{C}	4.29
12	Control	74.356^{I}	
	SEm±	3.34	
	CD 0.01	9.336	
	CV%	1.758	

* Values superscribed with identical letters within each column do not differ significantly

Bioassay of transgenic tomato plants against fungal pathogens

In vitro* pathogen inhibition assay against *S. rolfsii, A. solani* and *R. solani

Significant difference in growth inhibition of *S. rolfsii, R. solani and A. solani* was observed after 48 hrs of incubation in both concentrations of crude protein (750 *µ*g and 1000 *µ*g) taken from transgenic plants compared to non transgenic plants. Per cent growth inhibition of *S. rolfsii* ranged from 41.562 to 70.480% at 750 *µ*g and 43.376 to 72.293% at 1000 *µ*g concentration of crude protein was added to PDA (Table 2a). Against *A. solani* per cent growth inhibition ranged from 27.740 to 58.713 per cent at 750*µ*g and 29.677 to 60.640 per cent at 1000 *µ*g concentration (Table 2b). Similarly per cent growth inhibition of *R. solani* ranged from 17.045 to 44.320 at 750 *µ*g and 18.752 to 46.027% at 1000 *µ*g concentration (Table 2c). There was no significant difference in growth inhibition of pathogens between 750 g and 1000 g concentration of crude protein among eleven transgenic plants. At both concentration and against all three pathogens CG2-17-6-2 showed highest per cent growth inhibition. Among the eleven T_3 plants tested, plant CG2-17-6-2 showed highest activity.

TABLE 2a: Per cent inhibition of *Sclerotium rolfsii* on PDA plate with crude protein extracts from different transgenic tomato plants carrying *ech*42 and *bgn*

Sl. No.	Plant I.D.	Concentration (µg of total protein)	
		750	1000
		Per cent inhibition	
1	CG2-17-6-2	70.480^{A*}	72.293^{A}
2	CG2-17-6-4	63.255^{C}	65.069^{C}
3	CG2-17-6-7	41.562^{I}	43.376^{I}
4	CG2-17-6-10	56.021^{E}	57.834^{E}
5	CG2-17-6-11	52.414^{F}	54.227^{F}
6	CG2-17-6-12	66.872^{B}	68.676^{B}
7	CG2-17-6-13	52.414^{F}	54.227^{F}
8	CG2-17-6-14	56.021^{E}	57.834^{E}
9	CG2-17-6-15	45.189^{H}	46.993^{H}
10	CG2-17-6-16	48.797^{G}	50.018^{G}
11	CG2-17-6-17	59.648^{D}	61.452^{D}
12	Plain PDA	00000^{K}	000000^{K}
13	Control	18.067^{J}	19.873^{J}
	SEm±	0.57	
	CD at 0.01	1.599	
	CV (%)	1.453	

* Values superscribed with identical letters within each column do not differ significantly

TABLE 2b: Per cent inhibition of *Alternaria solani* on PDA plate with crude protein extracts from different transgenic tomato plants carrying *ech*42 and *bgn*

Sl. No.	Plant I.D.	Concentration (µg of total protein)	
		750	1000
	Per cent inhibition		
1	CG2-17-6-2	58.713^{A*}	60.640^{A}
2	CG2-17-6-4	50.966^{C}	52.904^{C}
3	CG2-17-6-7	27.740^{I}	29.677^{I}
4	CG2-17-6-10	43.223^{E}	45.168^{E}
5	CG2-17-6-11	39.353^{F}	41.285^{F}
6	CG2-17-6-12	54.839^{B}	56.777^{B}
7	CG2-17-6-13	39.353^{F}	41.285^{F}
8	CG2-17-6-14	43.223^{E}	45.168^{E}
9	CG2-17-6-15	31.613^{H}	33.540^{H}
10	CG2-17-6-16	35.483^{D}	37.413^{D}
11	CG2-17-6-17	47.096^{K}	49.031^{K}
12	Plain PDA	0000	00000
13	Control	15.483^{J}	17.428^{J}
	SEm±	0.73	
	CD at 0.01	2.020	
	CV (%)	2.384	

* Values superscribed with identical letters within each column do not differ significantly

TABLE 2c: Per cent inhibition of *Rhizocotonia solani* on PDA plate with crude protein extracts from different transgenic tomato plants carrying *ech*42 and *bgn*

Sl. No.	Plant I.D.	Concentration (µg of total protein)	
		750	1000
	Per cent inhibition		
1	CG2-17-6-2	44.320^{A*}	46.027^{A}
2	CG2-17-6-4	37.504^{C}	39.200^{C}
3	CG2-17-6-7	17.045^{I}	18.752^{I}
4	CG2-17-6-10	30.688^{E}	32.384^{E}
5	CG2-17-6-11	27.274^{F}	28.971^{F}
6	CG2-17-6-12	40.917^{B}	42.614^{B}
7	CG2-17-6-13	27.827^{F}	28.971^{F}
8	CG2-17-6-14	30.688^{E}	32.384^{E}
9	CG2-17-6-15	20.458^{H}	22.165^{H}
10	CG2-17-6-16	23.861^{G}	25.568^{G}
11	CG2-17-6-17	34.091^{D}	35.797^{D}
12	Plain PDA	00000^{K}	00000^{K}
13	Control	13.632^{J}	15.349^{J}
	SEm±	0.450	
	CD at 0.01	1.248	
	CV (%)	2.045	

* Values superscribed with identical letters within each column do not differ significantly

TABLE 3: Per cent leaf area infected after 7 days of inoculation in detached leaf assay of transgenic tomato plants carrying *ech*42 and *bgn*

Sl. No.	Plant ID	Per cent leaf area infection	Fold decrease in leaf area infection
1.	CG2-17-6-2	3.399^{B*}	10.16
2.	CG2-17-6-4	4.704^{D}	7.348
3.	CG2-17-6-7	12.686^{J}	2.72
4.	CG2-17-6-10	5.955^{F}	5.80
5.	CG2-17-6-11	7.981^{G}	4.33
6.	CG2-17-6-12	4.041^{C}	8.55
7.	CG2-17-6-13	8.096^{G}	4.26
8.	CG2-17-6-14	5.898^{F}	5.86
9.	CG2-17-6-15	11.703^{I}	2.95
10.	CG2-17-6-16	10.065^{H}	3.43
11.	CG2-17-6-17	5.235^{E}	6.60
12.	Control	34.567^{K}	
13	Untreated control	0.000^{A}	
	SEm±	0.131	
	CD	0.363	
	CV	1.829	

* Values superscribed with identical letters within each column do not differ significantly

Detached leaf assay against *Alternaria solani*

Transgenic plants with *ech42* and *bgn* showed more tolerance to *A. solani* compared to nontransgenic plant (Plate 2). Transgenic plant showed slight symptoms and those symptoms did not spread when incubation period was extended to 7 days. Whereas leaves from nontransgenic tomato plant showed extended chlorosis and decaying. There was significant difference in percent leaf area infected among eleven transgenics plants. Per cent leaf area (Table 3) infected in control plant was observed to be 34.567 per cent, while in transgenic plants it ranged from 3.399 to 12.686 %.

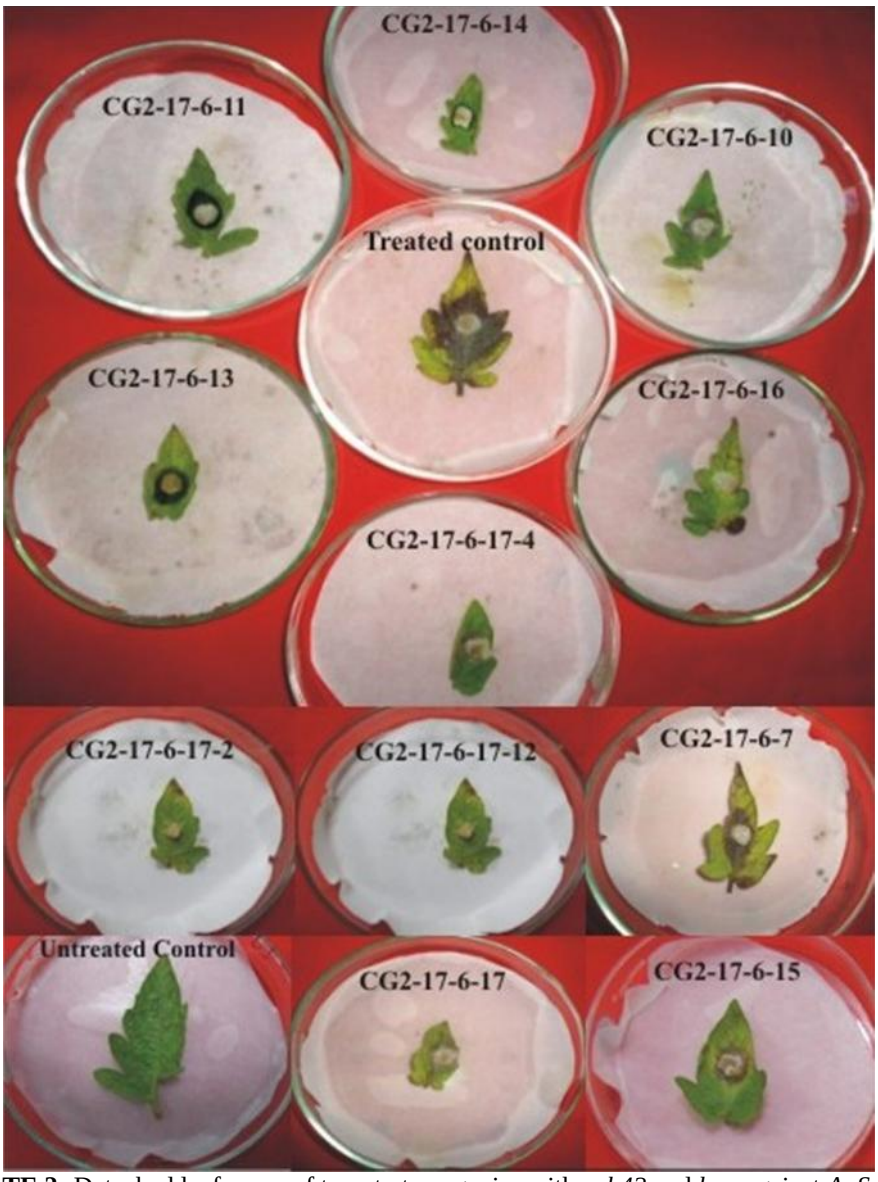


PLATE 2: Detached leaf assay of tomato transgenics with *ech42* and *bgn* against *A. Solani*

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