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Screening somaclones of ginger (*Zingiber officinale* Rosc.) derived through *in vitro* mutagenesis to soft rot and bacterial wilt diseases

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Seventy five somaclones of ginger cvs. Maran and Rio-de-Janeiro regenerated through *in vitro* mutagenesis were subjected to preliminary screening against soft rot and bacterial wilt diseases using toxic metabolites of *P. aphanidermatum* and *R. solanacearum* by electrolyte leakage method. Twenty five per cent somaclones exhibited low leakage of electrolytes to toxic metabolite(s) of *P. aphanidermatum.* Eighteen per cent somaclones recorded low leakage of electrolytes to toxic metabolite(s) of *R. solanacearum.* Somaclones of cv. Rio-de-Janeiro exhibited low leakage of electrolytes to toxic metabolite(s) of *P. aphanidermatum* while somaclones of cv. Maran exhibited low leakage of electrolytes to toxic metabolite(s) of *R. solanacearum.*

Key words: Electrolyte leakage, bacterial wilt, soft rot, ginger

INTRODUCTION

Soft rot disease caused by *Pythium aphanidermatum* and bacterial wilt disease caused by *Ralstonia solanacearum* are the major constraints in production of ginger (*Zingiber officinale* Rosc.). Attempts to isolate resistant clones using conventional breeding techniques were not successful in ginger as genetic variability available for disease resistance/ tolerance is low and all the available cvs./ varieties are susceptible to the diseases. Studies were conducted to manage soft rot and bacterial wilt diseases in ginger using cultural, chemical and biological methods by several workers and none of the methods gave absolute control for the two diseases (Kumar and Hayward, 2005). Breeding through selection and hybridization is not possible in ginger due to lack of variability and absence of natural seed set. Broadening the genetic base through *in vitro* techniques like *in vitro* mutagenesis, indirect organogenesis, indirect embryogenesis etc. are of great significance in crop improvement programmes in ginger. Somaclonal variation often arises in tissue culture as a manifestation of changes in the genome of differentiating vegetative cells induced by tissue culture conditions or epigenetic influence (Larkin and Scowroft, 1981). Any genetic change induced by *in vitro* conditions of tissue culture is expected to generate stable plants carrying interesting heritable traits. Hence, investigations are undertaken at College of Horticulture, Kerala Agricultural University, to screen somaclones of ginger derived through *in vitro* mutagenesis to soft rot and bacterial wilt diseases by electrolyte leakage method.

MATERIALS AND METHODS

Calli were induced from shoot tip explants of cvs. Maran and Rio-de-Janeiro in half strength MS medium (Murashige and Skoog, 1962) supplemented with 2,4-D 1.00 mg l^{-1}. The induced calli were then subcultured to half strength MS medium supplemented with BAP 3.00 mg l^{-1} for shoot morphogenesis. Morphogenic cultures derived from calli of two cvs. with shoot tips less than 5.00 mm length were subjected to γ irradiation at 10 Gy. A ^{60}Co source (Gamma chamber 900 of BARC, Mumbai) with a dose rate of 306.8 Gy / h was used for irradiation. After the irradiation, shoot tips were immediately transferred to half strength MS medium supplemented with 3.00 mg l^{-1} BAP. The elongated shoots were rooted in the same medium. Well rooted plantlets were washed free off the medium and planted in mud pots of size 11 x 8 cm filled with sterile sand, kept under shade and watered daily. After two

weeks, the plantlets were transferred to big poly bags of size 12" x 8" filled with potting mixture in the proportion 1:1:1 sand, soil and cow dung. The plantlets were maintained in shaded net house under natural photoperiod. Forty somaclones of Maran and 35 somaclones of Rio-de-Janeiro produced through *in vitro* mutagenesis were subjected to preliminary screening against soft rot and bacterial wilt diseases by electrolyte leakage method.

The predominant species of *Pythium* causing soft rot of ginger in Kerala is *P. aphanidermatum*. The pathogen was isolated from naturally infected rhizomes by following standard isolation procedures and its identification was confirmed (Ricker and Ricker, 1936). Pathogenicity of the isolated culture was tested by inoculating with seven day old culture of the fungus on healthy surface sterilized ginger rhizomes. Inoculated rhizomes were kept in aseptic moist chamber and incubated at room temperature till rotting of the rhizomes was observed. Toxic metabolite(s) of *P. aphanidermatum* were produced as per the procedure reported by Paul and Shylaja (2009). Five mm culture discs of 7-days-old culture of the fungus was inoculated to asparagine or synthetic mucor medium (Hesseltine, 1954). The cultures were incubated for 15 days under shaking condition at 27°C with a shaking speed of 100 rpm. The culture filtrate was collected after filtering successively through a muslin cloth and Whatman No.1 filter paper. The filtrates were concentrated to one-tenth of its volume using a hot plate maintained at 100° C to produce concentrated culture filtrate (CCF) which was then diluted to 10 per cent (v/v) for electrolyte leakage studies.

Ginger plants showing symptoms of bacterial wilt disease were collected and subjected to ooze test to confirm the presence of the bacterium. Such pieces with profuse bacterial ooze were cut into small bits and surface sterilized with 0.1 % mercuric chloride solution for 1 min and then washed free off the sterilant. These bits were crushed on a sterilized glass slide with a few drops of sterile distilled water to obtain a bacterial suspension. One loopful of suspension was streaked on triphenyl tetrazolium chloride (TZC) medium to get well-isolated colonies of the bacterium (Kelman, 1954). The cell free culture filtrate of *R. solanacearum* was produced as described by Paul (1998) and Paul and Shylaja (2009). *R. solanacearum* was cultured in peptone casamino acid broth for 5 day in a shaker cum incubator maintained at 27°C with a shaking speed of 100 rpm. The bacterial broth was autoclaved at 121°C for 20 min and then filtered. Toxin was precipitated by adding acetone to the extract. The precipitate was allowed to settle overnight and then separated by centrifugation at 5600 rpm for 20 min. The precipitate was then washed with acetone and kept for evaporation. The toxic metabolite thus obtained was dissolved in distilled water to 10 per cent (v/v) for electrolyte leakage studies.

Screening by electrolyte leakage using toxic metabolites of *P. aphanidermatum* and *R. solanacearum* was conducted as per the procedures reported by Vidyasekharan *et al.* (1986), Shylaja *et al.* (1997) and Paul and Shylaja (2009). Leaves for electrolyte leakage studies were collected from three month old healthy somaclones growing in shaded net house. The medium mature leaves for electrolyte leakage studies were cut into small pieces (1 x 0.5 cm) and random samples (200 mg) were enclosed in a muslin cloth and placed in test tubes. Three milliliters of diluted toxin preparation was vacuum infiltered into leaf bits for 10 min. The leaf bits were then rinsed with 4 ml of distilled water. Conductance of ambient solution was measured in m Siemens (mS) with a high precision Systronics 20 conductivity meter. Experiments were repeated thrice.

RESULTS AND DISCUSSION

In the present investigation, CCF of *P. aphanidermatum* induced quick electrolyte leakage from leaves of somaclones. Damann *et al.* (1974) showed that an electrolyte leakage assay by *Helminthosporium victoriae* toxin is as sensitive as standard seedling root growth assay. Hence, the increase in conductivity of leachates of host tissues when treated with pathogenic toxins has been used as a sensitive assay for many *in vitro* studies of pathogenicity.

Electrolyte leakage induced by toxic metabolites of pathogens, 10 minutes after infiltration was used for comparison. Leakage of electrolytes induced by toxic metabolite(s) of *P. aphanidermatum* ranged from 15.45 to 55.25 ms in somaclones of cv. Maran and from 16.75 to 58.45 ms in somaclones of cv. Rio-de-Janeiro (Table 1). The somaclones were grouped into three based on their leakage values (Table 2). Somaclones, that came in the first two classes of frequency table exhibit-

Table 1. Preliminary screening in somaclones of ginger derived through *in vitro* mutagenesis against soft rot and bacterial wilt pathogens by electrolyte leakage method

Maran			Rio-de-Janeiro		
Clone no.	Mean electrolyte leakage (ms)		Clone no.	Mean electrolyte leakage (ms)	
	P. aphani dermatum	*R. solana cearum*		*P. aphani dermatum*	*R. solana cearum*
47 MC 10 Gy	24.95	39.00	53 RC 10 Gy	19.70	40.55
48 MC 10 Gy	33.70	34.80	140 RC 10 Gy	31.40	37.25
49 MC 10 Gy	15.45	43.10	141 RC 10 Gy	23.35	40.95
51 MC 10 Gy	25.45	30.00	145 RC 10 Gy	27.90	45.35
52 MC 10 Gy	20.85	38.55	148 RC 10 Gy	16.75	38.95
95 MC 10 Gy	24.05	45.00	149 RC 10 Gy	37.40	38.95
97 MC 10 Gy	35.15	54.95	151 RC 10 Gy	47.15	38.30
99 MC 10 Gy	19.70	41.75	152 RC 10 Gy	20.70	43.40
100 MC 10 Gy	26.50	45.05	188 RC 10 Gy	22.15	27.35
101 MC 10 Gy	27.25	29.10	189 RC 10 Gy	45.30	57.75
102 MC 10 Gy	40.30	32.30	236 RC 10 Gy	28.60	44.60
103 MC 10 Gy	45.90	30.65	237 RC 10 Gy	50.50	56.20
105 MC 10 Gy	21.00	42.65	238 RC 10 Gy	38.65	40.70
106 MC 10 Gy	26.00	29.45	239 RC 10 Gy	41.35	37.70
108 MC 10 Gy	41.70	30.80	243 RC 10 Gy	45.40	58.60
109 MC 10 Gy	30.25	51.35	245 RC 10 Gy	57.80	45.85
110 MC 10 Gy	50.00	47.95	246 RC 10 Gy	29.25	36.25
156 MC 10 Gy	38.40	38.05	249 RC 10 Gy	45.40	34.55
158 MC 10 Gy	29.55	40.75	271 RC 10 Gy	21.85	37.50
159 MC 10 Gy	36.95	47.90	283 RC 10 Gy	44.35	38.95
163 MC 10 Gy	30.90	32.90	316 RC 10 Gy	48.40	37.95
165 MC 10 Gy	36.10	38.00	349 RC 10 Gy	21.05	46.90
168 MC 10 Gy	32.70	38.50	373 RC 10 Gy	25.90	41.50
169 MC 10 Gy	48.50	43.30	375 RC 10 Gy	29.90	35.15
174 MC 10 Gy	55.25	47.75	376 RC 10 Gy	47.15	58.75
178 MC 10 Gy	38.35	39.25	377 RC 10 Gy	30.75	37.80
182 MC 10 Gy	26.25	40.30	611 RC 10 Gy	21.35	55.65
190 MC 10 Gy	19.40	36.40	612 RC 10 Gy	33.15	26.95
191 MC 10 Gy	49.65	41.65	614 RC 10 Gy	43.65	37.70
192 MC 10 Gy	35.20	38.55	615 RC 10 Gy	35.30	34.90
193 MC 10 Gy	43.85	31.00	660 RC 10 Gy	58.45	37.20
194 MC 10 Gy	41.40	40.30	661 RC 10 Gy	35.45	37.90
196 MC 10 Gy	53.75	44.35	665 RC 10 Gy	45.25	45.15
197 MC 10 Gy	52.45	39.85	668 RC 10 Gy	22.10	30.25
253 MC 10 Gy	46.95	44.90	666 RC 10 Gy	31.75	39.60
254 MC 10 Gy	52.90	41.30			
255 MC 10 Gy	15.95	35.20			
257 MC 10 Gy	55.10	38.60			
260 MC 10 Gy	30.35	38.35			
265 MC 10 Gy	15.95	42.10			
S.D	11.96	6.08	S.D	11.50	8.06
C.V (%)	34.32	15.34	C.V (%)	32.90	19.55

Table 2. Frequency distribution of electrolyte leakage induced by toxic metabolites of *P. aphanidermatum* and *Ralstonia solanacearum* in somaclones of ginger

Electrolyte leakage (ms)	Frequency (%)		Electrolyte leakage (ms)	Frequency (%)	
	Maran	Rio-de-Janeiro		Maran	Rio-de-Janeiro
10.50-17.49	7.50	2.86	25.20-30.10	7.50	5.71
17.50-24.49	17.50	22.86	30.20-35.10	15.00	8.57
24.50-31.49	20.00	20.00	35.20-40.10	30.00	42.86
31.50-38.49	20.00	14.29	40.20-45.10	35.00	17.14
38.50-45.49	10.00	22.86	45.20-50.10	7.50	11.43
45.50-52.49	15.00	11.43	50.20-55.10	5.00	-
52.50-59.49	10.00	5.71	55.20-60.10	-	14.29

ing leakage value less than 24.50 ms were designated as somaclones of low leakage group, next three classes (24.50 – 45.49 ms) as of medium leakage group and last two classes (> 45.50 ms) as somaclones of high leakage group. Of the somaclones screened, 25 per cent came in the low leakage group, 54 per cent in the medium leakage group and 21 per cent in the high leakage group. The leakage of electrolytes was comparatively low in somaclones of Rio-de-Janeiro. Similarly, the number of clones of Rio-de-Janeiro were lower in the high leakage group while majority of the clones of Maran recorded leakage in the medium and high range. Twenty five per cent somaclones of Maran recorded high electrolyte leakage as compared to 17 per cent somaclones of Rio-de-Janeiro. Similar observations were made by Paul and Shylaja (2009), when they used electrolyte leakage method for bioassay of toxic metabolite(s) of *P. aphanidermatum*. They reported that cv. Maran exhibited higher leakage of electrolytes to toxic metabolite(s) of *P. aphanidermatum* than cv. Rio-de-Janeiro. Variability for the character studied was almost same in cv. Maran (34.32 %) and cv. Rio-de-Janeiro (32.90 %). Somaclones of cv. Maran which exhibited low electrolyte leakage values (< 24.50 ms) to toxic metabolite(s) of *P. aphanidermatum* were 49 MC 10 GY, 52 MC 10 GY, 95 MC 10 GY, 99 MC 10 GY, 105 MC 10 GY, 190 MC 10 GY, 255 MC 10 GY and 265 MC 10 GY. Somaclones of cv. Rio-de-Janeiro which recorded low electrolyte leakage values (< 24.50 ms) to toxic metabolite(s) of *P. aphanidermatum* were 53 RC 10 GY, 141 RC 10 GY, 148 RC 10 GY, 152 RC 10 GY, 188 RC 10 GY, 271 RC 10 GY, 349 RC 10 GY, 611 RC 10 GY and 668 RC 10 GY.

Leakage of electrolytes induced by toxin of *R. solanacearum* varied between 29.10 to 54.95 ms in somaclones of Maran and 26.95 to 58.75 ms in somaclones of cv. Rio-de-Janeiro (Table 1). Somaclones, that came in the first two classes of frequency table exhibiting leakage value of less than 35.20 ms were considered as somaclones of low leakage group, next three classes (35.20 – 50.10 ms) as somaclones of medium leakage group and last two classes (> 50.20 ms) as somaclones of high leakage group (Table 2). Of the somaclones screened, 18 per cent came in the low leakage group, 72 per cent in the medium leakage group and ten per cent in the high leakage group. The leakage of electrolytes was comparatively low in somaclones of Maran. Similarly, the number of clones of Maran were higher in the low and medium leakage group and lower in the high leakage group while majority of the clones of Rio-de-Janeiro recorded electrolyte leakage in the high range. Fourteen per cent somaclones of Rio-de-Janeiro recorded high electrolyte leakage as compared to five per cent clones of Maran. Similar observations on tolerance reaction of cv. Maran to bacterial wilt disease has been reported by Paul *et al.* (2009) when they screened adventitious bud regenerants of cv. Maran and Rio-de-Janeiro to bacterial wilt disease by three different methods viz., natural screening in sick field, screening by

electrolyte leakage method and artificial screening using *R. solanacearum*. In all the three screening methods, somaclones of cv. Maran were found tolerant to bacterial wilt disease as compared to clones of Rio-de-Janeiro. The coefficient of variation for the character studied was high in somaclones of cv. Rio-de-Janeiro (19.55 %) as compared to cv. Maran (15.34 %).

Somaclones of cv. Maran which exhibited low electrolyte leakage values (< 35.20 ms) to toxic metabolite(s) of *R. solanacearum* were 48 MC 10 GY, 51 MC 10 GY, 101 MC 10 GY, 102 MC 10 GY, 103 MC 10 GY, 106 MC 10 GY, 108 MC 10 GY, 163 MC 10 GY and 193 MC 10 GY. Somaclones of cv. Rio-de-Janeiro which recorded low electrolyte leakage values (< 35.20 ms) to toxic metabolite(s) of *R. solanacearum* were 188 RC 10 GY, 249 RC 10 GY, 375 RC 10 GY, 612 RC 10 GY, 615 RC 10 GY and 668 RC 10 GY.

Preliminary screening of 75 somaclones derived through *in vitro* mutagenesis against soft rot and bacterial wilt diseases by electrolyte leakage method revealed that 25 per cent somaclones exhibited low leakage of electrolytes to toxic metabolite(s) of *P. aphanidermatum* and 18 per cent somaclones exhibited low leakage of electrolytes to toxic metabolite(s) of *R. solanacearum*. Somaclones of cv. Rio-de-Janeiro exhibited low leakage of electrolytes to toxic metabolite(s) of *P. aphanidermatum* while somaclones of cv. Maran exhibited less leakage of electrolytes to toxic metabolite(s) of *R. solanacearum*. Preliminary screening against soft rot and bacterial diseases by electrolyte leakage method gave an indication of disease reaction of the clones. Somaclones with low leakage values identified in the present study serve as base material for further evaluation/ production programmes.

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