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In vitro selection at cellular level for red rot resistance in sugarcane (*Saccharum* sp.)

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Abstract In the present study, in vitro selection technique using pathogen culture filtrate of *Colletotrichum falcatum* Went was employed with the aim to identify associations (if any), between selection at the cellular and plant level for red rot resistance in sugarcane (*Saccharum* sp.). Five to eight months old sugarcane calli of genotypes CoJ 88 and CoJ 64 were screened in vitro against pathogen culture filtrate for two selection cycles. Effect of pathogen culture filtrate on callus survival and/or proliferation was observed to be directly related to its concentration in the selection media. Calli survived and exhibited further proliferation at 5, 10 and 15% v/v pathogen culture filtrate concentrations whereas, at higher concentrations (20 and 25% v/v) proliferation was completely inhibited. Shoot regeneration percent was higher in calli selected on 5% pathogen culture filtrate concentration than those selected on 10 and 15% concentrations. In vivo screening of field transferred somaclones against two pathtypes (Cf 03 and Cf 08) showed considerable variation for red rot resistance. Somaclones regenerated from resistant and/or tolerant calli exhibited better resistance than the parental genotypes. The results indicated that in vitro selection for red rot resistance was effective and expressed when somaclones were screened in the field. This indicated a positive association between in vitro and in vivo methods of selection for disease resistance in sugarcane.

Keywords Callus · Pathogen culture filtrate · In vitro · Red rot · Sugarcane

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Introduction

Sugarcane is an economic and highly productive crop used as the chief source of sugar for centuries and recently to produce ethanol, a renewable energy source of bio fuel. Red rot caused by *Colletotrichum falcatum* went is a devastating fungal disease of sugarcane. It reduces yield and causes a deterioration in juice quality, leading to economic losses for both growers and millers. It occurs in many cane growing countries but a continuous threat for subtropical countries. Most commercial varieties in India are susceptible to red rot to varying degrees. Besides causing losses in yield and quality, it is also responsible for varietal failure in this crop. Thus, a number of elite varieties like BO 70, BO 74, Co 213, -312, -453, Co 1148 and CoS 510 have been wiped out due to susceptibility to this disease (Rafay 1950; Chona and Srivastava 1960; Kirtikar et al. 1972; Singh et al. 1985).

Use of resistant varieties is the most satisfactory, long lasting and economical means of fighting to this disease. Resistance of a variety breaks down and become susceptible with time due to the emergence of new highly virulent pathotypes of *C. falcatum*. Selection and development of genotypes resistant to red rot, coupled with high yield and improved quality traits, requires a long time in this vegetatively propagated crop through conventional breeding programme. In vitro selection approach appears a new tool to rectify specific defects and/or to improve a specific desirable trait of highly adapted sugarcane cultivars without involving a sexual cycle. This technique is considered particularly useful in improving vegetatively propagated crops, as the mutated character can be maintained through asexual propagation. Selection of resistant cells and callus cultures in vitro followed by regeneration of resistant plants has been utilized as a direct application of the cell

culture approach to crop improvement. The most common approach to select disease resistant lines in culture has been to use phytotoxic fungal culture filtrate or purified toxins produced by the pathogen (Behnke 1979; Strobel 1982; Wenzel 1985; Daub 1986; Chawla et al. 1987; Mohanraj et al. 1995, 2003, 2004). A positive correlation has been found between toxin resistance in vitro at the cellular level and resistance to the pathogen at the plant level (Thanutong et al. 1983; Mitchell 1984; Nyange et al. 1995; Rines and Luke 1995; Jayasankar et al. 2000; Prasad and Naik 2000). In vitro selection in sugarcane has been used for selection of eye spot resistant lines (Heinz et al. 1977; Larkin and Scowcroft 1983; Prasad and Naik 2000), red rot resistance (Mohanraj et al. 2003), salt stress (Gandonou et al. 2004) and drought stress (Errabii et al. 2006). In present study the phytotoxic effects of pathogen culture filtrate (PCF) on 5–8 months old sugarcane calli and association between selection at callus and plant levels were studied for red rot resistance.

Materials and methods

Plant material

This study was conducted on two commercial sugarcane genotypes namely CoJ 88 and CoJ 64 classified as moderately and highly susceptible to red rot, respectively. Healthy spindles of these varieties were obtained from sugarcane research station, Ladhowal, Punjab Agricultural University, Ludhiana (India).

Callus initiation and induction of somatic embryogenesis

The explants used for callus initiation comprised of apical spindle tips. Explants were prepared for surface sterilization with Teepol^{TM} (0.5% v/v) for 5–10 min under tap water. They were further treated with Bavistin (0.25% w/v) solution and placed in a rotary shaker for 10 min of rigorous shaking. Finally, the explants were treated with $HgCl_2$ (0.1% w/v) for 10 min under aseptic conditions in laminar air flow system followed by rinsing thrice with autoclaved distilled water. These sterilized spindles were used as explants for callus induction on callus initiation MS medium (Murashige and Skoog 1962) supplemented with 2,4-dichlorophenoxyacetic acid (2-4-D) (4 mg l^{-1}) + kinetin (0.5 mg l^{-1}) + inositol (560 mg l^{-1}) + proline (100 mg l^{-1}) + sucrose (30 g l^{-1}). Initiated callus was excised and subsequently subcultured on MS medium containing 2,4-D (2.5 mg l^{-1}) + kinetin (0.5 mg l^{-1}) + inositol (560 mg l^{-1}) + proline (100 mg l^{-1}) + sucrose (30 g l^{-1}) after 5 weeks of primary culture. The medium was solidified with 0.8% agar and pH adjusted at 5.8 using 0.1N HCl/0.1N NaOH prior to autoclaving at 121.6°C and 108.0 kPa for 20.0 min. The cultures were maintained at 25 ± 2°C in culture tubes under dark conditions. Embryogenic calli, identified by their nodular appearance, were selectively excised after 4 weeks of growth and transferred to fresh MS medium. The process was continued for 18–36 weeks for proliferation and induction of somaclonal variation. All chemicals used were of analytical grade (Sigma Chemicals Co. USA, Merck, Hi-Media and Qualigens India Ltd.).

Isolation, maintenance and multiplication of the red rot pathogen

Red rot pathogen was isolated from naturally infected canes of the genotypes CoJ 84 and CoJ 64. Setts were surface sterilized with 90% ethanol and longitudinally split open with sharp knife. The bits taken from diseased tissue were cultured on oat meal agar medium [Champion white oat (30 g l^{-1}) (Hindustan Vegetable Oils Co. Ltd, India) + agar (20 g l^{-1})] slants. Single spore isolates were maintained on oat meal agar slants at 4 ± 2°C and renewed periodically. Fungal isolates were multiplied on oat meal agar medium in Petri dishes (9 × 15 mm) at 25 ± 2°C.

Fractionation of the pathogen culture filtrate

Three mycelial discs (10 mm) of 4-week old fungal culture were inoculated in 100 ml oat meal liquid medium in 250 ml Erlenmeyer flasks. The flasks were incubated in a rotary shaker set at, 75 rpm, 25 ± 2°C for 20 days. The culture filtrate containing the phytotoxic compounds was filtered through several layers of sterilized cheesecloth. The culture filtrate and crushed spores were pooled, homogenized and further filtered through Seitz Filter (0.45 µm) to remove mycelium and spores. The pH of culture filtrate was adjusted to 5.8 by addition of 1N NaCl/NaOH. Finally, the pathogen culture filtrate was filter sterilized through syringe-driven, disposable membrane filters (Millipore^{TM}, Millipore Corporation, Bedford, MA, USA) having 0.5 µm pore size and stored at 4°C.

In vitro selection and plant regeneration

Selection medium was prepared by adding filter-sterilized volumes of PCF to the autoclaved molten MS medium incorporated with 2,4-D (2.5 mg l^{-1}) + kinetin (0.5 mg l^{-1}) + inositol (560 mg l^{-1}) + proline (100 mg l^{-1}) + sucrose (30 g l^{-1}) to provide concentrations of (5, 10, 15, 20 and 25% v/v). Calli of differing ages (5, 6, 7 and 8 month old) were cultured on the toxic media in test tubes. The cultures were incubated at 25 ± 2°C in the dark for two selection cycles (each selection cycle of 5 weeks).

Percent callus survival was calculated for each treatment to study the effect of PCF on callus survival and/or proliferation. The overall means were expressed as arc sine transformations as per statistical methods (Gomez and Gomez 1976). Factorial CRD was calculated as per the statistical programme CPCS 1 (Cheema and Singh 1993).

After two selection cycles on PCF incorporated media, calli showing resistance and/or tolerance were selected in vitro and incubated on regeneration medium [MS + 6-benzyl amino purine (BAP) 1.0 mg l^{-1} + indole-3-acetic acid (IAA) 0.2 mg l^{-1} + inositol 560 mg l^{-1} + proline 100 mg l^{-1} + sucrose 30 g l^{-1}]. Regenerated plants were elongated on ½ MS medium devoid of hormones. Rooting was initiated on medium containing MS + indole-3-butyric acid (IBA) 2 mg l^{-1} + naphthalene acetic acid (NAA) 3 mg l^{-1} + inositol (560 mg l^{-1}) + proline (100 mg l^{-1}) + sucrose (60 g l^{-1}). All cultures were incubated in 16 h light/8 h dark photoperiod under light intensity of 50 μE m^2 s^{-1} provided by cool white fluorescent light. Plantlets with developed roots were hardened at 25 ± 2°C with 55% relative humidity in incubation room for 4–5 days. Plantlets were shifted to polybags containing field soil with farmyard manure and kept in a glass house for 10–12 weeks at 30 ± 5°C and 70–80% humidity. Thereafter, plantlets were transferred to the field and planted in a completely randomized design (CRD).

In vivo screening of somaclones for red rot resistance

Somaclones transferred to the field were evaluated for red rot resistance against two pathoypes, Cf 08 (isolated from sugarcane genotype CoJ 84) and Cf 03 (isolated from sugarcane genotype CoJ 64) of *C. falcatum*, at 180 days after transplanting through artificial inoculation (spore suspension 1 × 106 per ml) by plug method (Srinivasan and Bhatt 1961). The inoculations were done on the middle of third exposed internode from bottom. Bore holes were made with Indian institute of sugarcane research (IISR), cane inoculator and 1 ml of spore suspension was injected into bore holes of each cane. Holes were immediately sealed with plasticine to avoid aerial contamination. After a further 60 days, the inoculated canes were split open longitudinally and graded as resistant (R), intermediate (I) and susceptible (S) to red rot based on 0–9 scale developed at Sugarcane Breeding Institute, Coimbatore (India).

Results

Effect of pathogen culture filtrate on callus growth

A differential effect was observed on callus survival and/or proliferation when 5–8 months old sugarcane calli were treated with different concentrations of PCF (Table 1). Pathogen culture filtrate inhibited callus growth and degree of inhibition was observed to be directly related to the concentration in the selection medium. Callus survival and subsequent proliferation was observed at 5, 10 and 15% v/v PCF concentrations in the selection media whereas, proliferation was completely inhibited followed by 100% cell death at higher concentrations (20 and 25% v/v) (Fig. 1a). In first selection cycle, the percent callus survival for both the genotypes was low with increasing concentrations of PCF of either pathotype. However, in the second selection cycle the survival percent increased up to 72 and 50% in genotypes CoJ 88 and CoJ 64, respectively. Callus clumps showing discoloration and subsequent dark brown necrosis, did not survive. The browning of the calli was considered as an indication of cell death because most of the brown calli did not proliferate further, even after an incubation period of longer than 5 weeks. Percent survival of calli in the genotype CoJ 88 with respect to age and concentration of the culture filtrate was higher than that of CoJ 64 (Table 1). There was little effect of age of PCF on the survival and/or proliferation of the calli. Calli of both the genotypes cultured on toxin free medium exhibited 100% survival and proliferation in both the selection cycles.

Selection and shoot regeneration from toxin resistant calli

The gradual selection of the tissues for resistance to higher toxin level was apparent as the tissues finally surviving on the toxin at the different stages resulted in a small proportion of viable shoots, which may have the potential to develop as resistant genotypes. Resistant and/or tolerant calli were selected from the treatments that were free from necrosis and proliferated vigorously. Shoot regeneration was observed after 2 weeks of culture (Fig. 1b). Plant regeneration frequency in both the genotypes, after two selection cycles, varied significantly with respect to concentration of PCF (Table 2). Regeneration frequency was lower in the selected calli of both genotypes on media supplemented with Cf 08 filtrate than on media supplemented with Cf 03 filtrate. Regeneration frequency decreased with increased PCF concentration (Table 2) in both the genotypes. The frequency of regeneration was higher in genotype CoJ 88 than CoJ 64 irrespective of PCF thereby suggesting a differential genotypic response to selection. The calli selected on media supplemented with pathotype Cf 08 also showed differential response of regeneration with respect to genotype and it was higher in CoJ 88 than CoJ 64.

Statistical analysis using factorial CRD revealed significant interactions ($P = 0.05$) between genotype, pathotype and PCF concentration (Table 3) for selection and

Table 1 Effect of pathogen culture filtrate of *Colletotrichum falcatum* Went on percentage survival of sugarcane calli

Genotype	Pathotype	PCF conc. (ml/l)	Mean callus survival (%)[a]			Overall mean (%)[b]
			Age of PCF (days)			
			0	5	10	
Ist selection cycle						
CoJ 88	Cf 08	0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00 (89.96)
		5	23.70 ± 2.44	21.60 ± 1.80	22.70 ± 2.08	22.60 ± 0.48 (28.41)
		10	17.70 ± 2.30	15.90 ± 2.02	16.40 ± 1.89	16.80 ± 0.48 (24.07)
		15	9.08 ± 2.06	7.61 ± 1.59	8.46 ± 1.48	8.38 ± 1.60 (16.81)
	Cf 03	0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00 (89.96)
		5	38.80 ± 3.38	34.40 ± 3.44	35.40 ± 3.44	35.20 ± 1.93 (36.96)
		10	29.90 ± 3.72	23.80 ± 4.07	25.30 ± 3.82	25.00 ± 2.34 (30.83)
		15	15.30 ± 2.92	13.10 ± 2.63	14.70 ± 2.61	14.40 ± 1.55 (22.25)
CoJ 64	Cf 08	0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00 (89.96)
		5	15.80 ± 2.06	10.10 ± 1.86	14.20 ± 1.66	14.30 ± 1.64 (21.35)
		10	11.40 ± 2.84	9.07 ± 2.72	9.83 ± 2.93	10.10 ± 2.39 (18.50)
		15	7.53 ± 2.56	5.37 ± 1.78	6.56 ± 1.93	6.48 ± 0.85 (14.71)
	Cf 03	0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00 (89.96)
		5	18.90 ± 1.38	16.80 ± 1.32	18.00 ± 1.12	17.90 ± 0.74 (25.01)
		10	14.60 ± 2.07	12.00 ± 1.81	13.90 ± 2.32	13.80 ± 1.85 (21.53)
		15	11.40 ± 2.57	9.40 ± 2.30	10.60 ± 2.43	10.40 ± 2.37 (18.85)
IInd selection cycle						
CoJ 88	Cf 08	0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00 (89.96)
		5	68.50 ± 8.32	70.50 ± 8.52	67.00 ± 8.52	68.60 ± 8.46 (55.94)
		10	58.30 ± 8.14	61.50 ± 7.22	55.50 ± 9.59	58.40 ± 8.31 (49.84)
		15	53.00 ± 11.09	59.90 ± 8.46	50.60 ± 9.21	54.50 ± 8.83 (47.57)
	Cf 03	0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00 (89.96)
		5	71.80 ± 6.80	73.60 ± 6.83	70.20 ± 6.97	71.90 ± 6.78 (57.95)
		10	68.60 ± 8.70	70.00 ± 8.82	70.00 ± 8.20	68.30 ± 8.72 (56.47)
		15	64.20 ± 7.85	67.10 ± 8.95	61.40 ± 7.15	64.20 ± 6.39 (53.25)
CoJ 64	Cf 08	0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00 (89.96)
		5	56.00 ± 6.50	56.90 ± 7.20	51.50 ± 8.08	71.90 ± 6.85 (47.73)
		10	49.70 ± 7.15	52.10 ± 7.41	43.60 ± 9.80	69.50 ± 8.57 (44.10)
		15	37.00 ± 10.58	43.00 ± 11.45	33.50 ± 7.28	64.20 ± 7.97 (37.92)
	Cf 03	0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00 (89.96)
		5	61.20 ± 8.20	63.90 ± 7.29	58.60 ± 8.95	61.20 ± 8.16 (51.47)
		10	56.60 ± 8.14	60.70 ± 7.76	52.00 ± 6.56	56.20 ± 7.38 (48.68)
		15	51.70 ± 9.13	56.00 ± 7.19	49.20 ± 7.03	52.30 ± 6.99 (46.30)

Values in parenthesis are arc sine transformed means

PCF, Pathogen culture filtrate

[a] Data are means of 4 replicates

[b] Pooled mean of calli selected on 0, 5 and 10 days old PCF

regeneration parameters studied. Interactions between all above treatments were significant in first selection cycle at selection and/or proliferation stage whereas, non significant interactions were observed in treatments (genotype × pathotype) and (genotype × pathotype × PCF concentration) in second selection cycle. At regeneration stage interactions were found to be non significant in treatments (genotype × path type), (pathotype × PCF concentration) and (genotype × pathotype × PCF concentration) (Table 3).

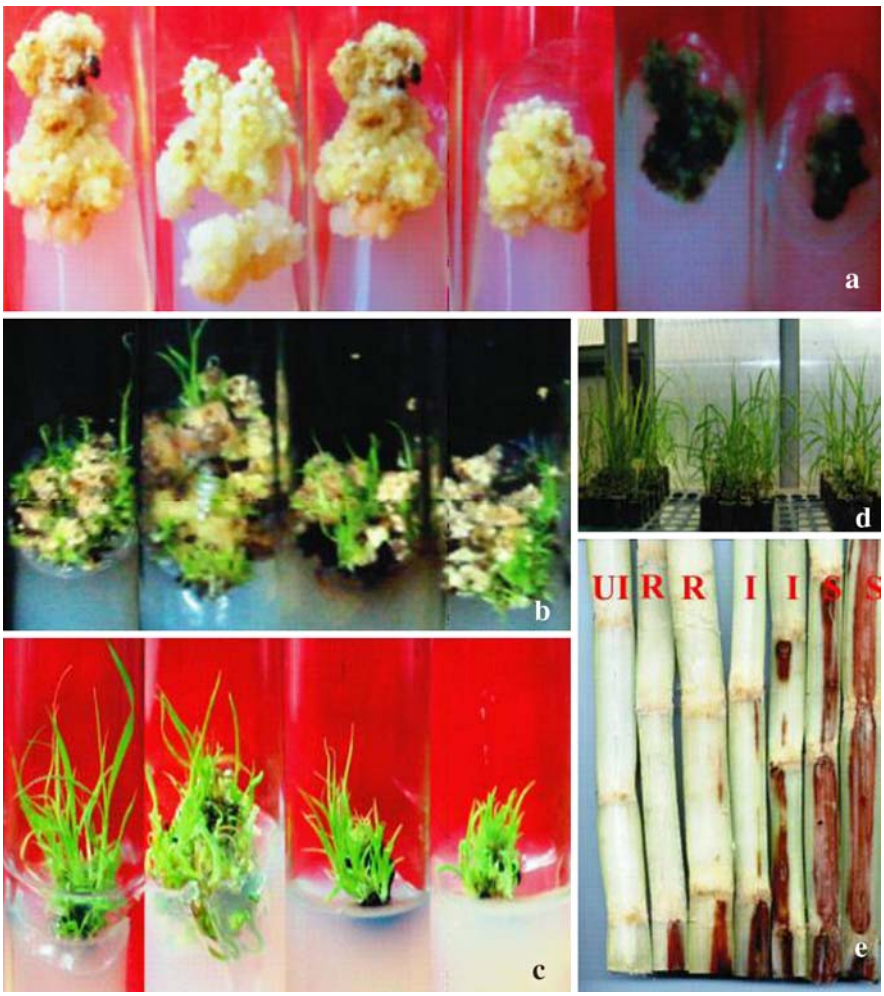

Fig. 1 **a** In vitro selection of sugarcane calli (0, 5, 10, 15, 20, and 25% v/v PCF concentrations), **b**, **c** Shoot regeneration and elongation from calli selected at 0, 5, 10 and 15% v/v PCF concentrations, **d** Hardening of the plants in the glass house, **e** field screening of the somaclones into resistant (R), Intermediate (I) and susceptible categories (S); UI: uninoculated cane

Elongation, rooting, hardening and transfer of the plants to the soil

Regenerated shoots exhibited shoot elongation within a period of 4–6 weeks (Fig. 1c). Five to ten fibrous roots were seen which grew up to 3–4 cm in 3 weeks. Fully grown in vitro rooted plants were shifted to the glass house where, they were hardened for the period of 3 months (Fig. 1d). Plants regenerated from the calli selected at higher PCF concentrations showed lower percent survival in the glass house than those selected at lower PCF concentrations. Percent plant survival at hardening phase was 75.7, 68.7 and 55.0 in variety CoJ 88 and 71.1, 61.7 and 50.0 in variety CoJ 64 at the 5, 10 and 15% v/v toxin concentrations, respectively upon which the calli were in vitro selected. Whereas, plants regenerated from toxin free medium exhibited 78.5 and 75.0% survival in varieties CoJ 88 and CoJ 64, respectively. Survival percent at field stage varied from 80.0 to 87.3% for both the varieties.

In vivo screening of the regenerated somaclones for red rot resistance

Somaclones of both the varieties exhibited variation in red rot resistance at field level (Fig. 1e). Plants regenerated from calli, selected on higher PCF concentrations exhibited higher levels of resistance to red rot than those screened on lower PCF concentrations and control (Table 4). Further, somaclones of CoJ 88 exhibited higher levels of resistance than those of CoJ 64. The percentage of resistant plants regenerated from in vitro selected calli ranged from 20.0 to 70.0% in somaclones of CoJ 88 when concentration of PCF was increased from 0.0 to 15% against Cf 08 whereas, for Cf 03 the range was 22.9–75.0%. Resistant somaclones of CoJ 64 from calli selected on Cf 08 and Cf 03 at concentration varying from 0.0 to 15% ranged from 0.0 to 75.0% and 0.0–87.5%, respectively.

Table 2 Mean shoot regeneration from toxin insensitive sugarcane calli

Genotype	Pathotype	PCF conc. (ml/l)	Mean shoot regeneration (%)[a]			Overall mean (%)[b]
			Age of PCF (days)			
			0	5	10	
CoJ 88	Cf 08	0	40.00 ± 2.69	39.10 ± 3.40	38.20 ± 4.44	39.10 ± 3.36 (38.68)
		5	23.90 ± 5.16	26.00 ± 4.27	27.70 ± 5.14	25.90 ± 4.80 (30.54)
		10	23.40 ± 7.50	23.70 ± 5.19	24.60 ± 4.15	23.90 ± 5.57 (29.25)
		15	18.20 ± 6.24	18.90 ± 2.93	21.70 ± 4.50	19.60 ± 4.16 (26.25)
	Cf 03	0	38.80 ± 4.77	41.10 ± 4.37	39.00 ± 3.99	39.60 ± 4.12 (38.99)
		5	29.10 ± 6.22	26.60 ± 6.14	28.20 ± 6.55	28.00 ± 6.28 (31.91)
		10	27.50 ± 5.55	24.40 ± 5.40	25.60 ± 4.92	25.80 ± 5.27 (30.52)
		15	26.10 ± 6.49	20.80 ± 7.09	24.50 ± 8.39	23.80 ± 7.29 (29.16)
CoJ 64	Cf 08	0	37.10 ± 5.73	37.00 ± 3.03	36.50 ± 2.93	36.30 ± 3.88 (37.37)
		5	24.30 ± 4.53	22.50 ± 3.41	22.40 ± 4.46	23.10 ± 4.05 (28.68)
		10	22.80 ± 4.47	22.50 ± 8.22	17.70 ± 2.83	21.00 ± 5.04 (27.22)
		15	13.50 ± 13.94	11.20 ± 13.15	5.00 ± 10.00	9.90 ± 4.74 (18.00)
	Cf 03	0	36.70 ± 7.24	35.00 ± 6.14	36.00 ± 2.87	35.90 ± 4.08 (36.79)
		5	25.10 ± 7.80	22.80 ± 6.31	24.00 ± 6.24	24.00 ± 4.08 (29.29)
		10	23.80 ± 3.26	20.30 ± 3.94	22.20 ± 4.80	22.20 ± 6.77 (28.01)
		15	20.30 ± 6.35	7.30 ± 8.95	17.70 ± 2.83	15.30 ± 2.06 (22.43)

Values in parenthesis are arc sine transformed means

PCF, Pathogen culture filtrate

[a] Data are means of 4 replicates

[b] Pooled mean of calli regenerated selected on 0, 5 and 10 days old PCF

Table 3 Analysis of variance using factorial completely randomized design

Source	*df*	In vitro selection with PCF				Shoot regeneration	
		Ist selection cycle		IInd selection cycle			
		M.S.	C.D. ($P = 0.5$)	M.S.	C.D. ($P = 0.5$)	M.S.	C.D. ($P = 0.5$)
A (Genotype)	1	290.99	0.66	376.580	0.99	141.89	1.24
B (Pathotype)	1	186.98	0.66	180.55	0.99	23.16	1.24
A × B	1	18.43	0.94	1.08	NS	0.72	NS
C (PCF conc.)	3	13533.29	0.94	4944.47	1.41	405.01	1.76
A × C	3	56.31	1.33	43.06	1.99	22.02	1.49
B × C	3	21.82	1.33	29.02	1.99	7.81	NS
A × B × C	3	3.74	1.89	3.25	NS	0.95	NS
Error	32	1.29		2.88		4.50	

df degree of freedom, *MS* mean sum of squares, *CD* critical difference

Discussion

Cells and plant protoplast could be selected in cultures for resistance to a pathogenic toxins and plants with an altered response to infection by pathogen could be regenerated from these cultured cells Carlson (1973). Culture filtrate or partially purified toxin, obtained from fungal pathogen has been extensively used for in vitro selection of resistant host species (Buiatti and Ingram 1991).The preliminary and basic requirement for selection of resistant lines at cellular level is to generate genetic variability in the cells. High genetic variability induced in vitro and the correlation observed between responses of a host to a challenging pathogen in vitro and in vivo, support the use of in vitro selection techniques in resistance breeding (Van den Bulk 1991). Selection of toxin resistant cells is apparent from the observation that cells finally surviving at different screening stages results in small proportion of viable shoots,

Table 4 In vivo performance of somaclones into three reaction categories when inoculated with two pathotypes individually

Toxin conc. per cent (v/v) in MS basal medium	Pathotype	Genotype	No. of plants screened	Reaction category, number of plants/percentage in each reaction type		
				Resistant (0.0–4.0)	Intermediate (4.1–6.0)	Susceptible (>6.1)
0	Cf 08	Co J 88	35	2 (5.71)	18 (51.43)	15 (42.86)
		Co J 64	22	0 (0.00)	8 (36.36)	14 (63.64)
	Cf 03	Co J 88	35	3 (8.57)	20 (57.14)	12 (34.29)
		Co J 64	22	0 (0.00)	10 (45.45)	12 (54.55)
5	Cf 08	Co J 88	29	6 (20.69)	12 (41.38)	11 (37.93)
		Co J 64	25	3 (12.00)	12 (48.00)	10 (40.00)
	Cf 03	Co J 88	29	8 (27.59)	13 (44.83)	8 (27.59)
		Co J 64	25	4 (16.00)	13 (52.00)	8 (32.00)
10	Cf 08	Co J 88	22	9 (40.91)	8 (36.36)	5 (22.73)
		Co J 64	15	3 (20.00)	8 (53.33)	4 (26.67)
	Cf 03	Co J 88	22	11 (50.00)	9 (40.91)	2 (9.09)
		Co J 64	15	4 (26.67)	9 (60.00)	2 (13.33)
15	Cf 08	Co J 88	10	6 (60.00)	3 (30.0)	1 (10.00)
		Co J 64	8	4 (50.00)	3 (37.50)	1 (12.50)
	Cf 03	Co J 88	10	7 (70.00)	3 (30.00)	0 (0.00)
		Co J 64	8	5 (62.50)	2 (25.00)	1 (12.50)

Score on 0–9 scale

which may have the potential to develop into disease resistant lines. High concentration of red rot toxin used resulted in limited survival of calli and shoot differentiation in sugarcane Mohanraj et al. (1995). The results of present study also shows that high concentration of PCF markedly inhibits development of in vitro cultures in proliferation of sugarcane calli (Table 1). These results confirm earlier studies where differential reactions to culture filtrate were observed in sugarcane callus. In this study, high concentrations of PCF resulted in a limited survival of the calli and shoot differentiation, suggesting that more selective responses could be obtained by using further purified phytotoxin which could be effective even at low concentrations. The brownish discoloration of the calli (Fig. 1a) associated with necrosis suggests the involvement of phenolic compounds and their oxidation products similar to symptoms observed in sugarcane plants infected with the red rot pathogen (Rao et al. 1968). Similar influences of toxins on different crops have been reported by Gohbara et al. (1978) on lettuce, Jin et al. (1996) on soybean, Boonchitsirikul et al. (1997) and Narong et al. (1998) on rice and Prasad and Naik (2000) on sugarcane. Growth inhibition of sugarcane calli by the PCF provides a positive selection system in which susceptible cells presumably die and/or grow slowly, while more resistant cells proliferate faster. A second selection cycle allowed for the recovery of higher percent of resistant/tolerant callus variants.

Successful plant regeneration from in vitro selected calli and cell lines has been achieved by many workers (Gengenbach et al. 1977; Behnke 1980a, b; Thanutong et al. 1983; Daub 1986; Van den Bulk 1991). The inhibitory effect of red rot toxin on shoot regeneration in sugarcane calli was reported by Mohanraj et al. (2003, 2004). The differential regeneration response of the calli in the study reflected genotypic differences between both the genotypes with respect to pathogenicity of both the pathotypes (Table 3). CoJ 88 had a higher percentage of surviving callus with respect to PCF than CoJ 64. CoJ 64 is a highly susceptible genotype, while CoJ 88 is moderately susceptible to red rot disease under artificial field inoculations. Likewise, pathotype Cf 08 isolated from CoJ 84, proved to be more virulent than Cf 03 isolated from CoJ 64.

An important aspect of crop improvement via an in vitro selection strategy is that traits selected at the cellular level must be expressed in the regenerated plants. Our results showed that plants selected using PCF through callus and regeneration exhibited improved resistance when tested in the field (Table 4). Positive association was observed between in vitro selection at callus level and in vivo screening at plant level, for red rot resistance. The results indicated that in vitro selection for red rot resistance is effective and were confirmed further through in vivo screening by plug method. The fact that some of the somaclones tested did not differ significantly from parental genotypes may suggest that the selected calli consist of a mixture of resistant and sensitive cells probably arising from somaclonal variation. Genetic variability has been

reported in cultured plant cells (Evans et al. 1984). There are several reports on the recovery of toxin resistant regenerants from unselected cultures for *Helminthosporium maydis* resistance in maize (Brettell et al. 1980) *H. victoriae* resistance in oat (Rines and Luke 1985) and *H. sacchari* resistance in sugarcane (Heinz et al. 1977; Larkin and Scowcroft 1983). The differences in frequencies of toxin-resistant regenerants from unselected cultures among the oats, maize and sugarcane systems is probably due to the genetic nature of resistance.

The study shows that in vitro selection system for ameliorating problems like susceptibility to red rot through somaclonal variation can be successfully addressed, especially in popular genotypes like CoJ 88 and CoJ 64 which has desirable traits in all other aspects except resistance to red rot diseases. A stable selection system in vitro developed on the basis of the current study can go a long way in eliminating single trait problem while retaining others and also, shortening the time to release a variety which otherwise would entail a long period of 10–12 years by conventional means.

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