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Effect of Phytotoxin of *Colletotrichum falcatum* Went. *(Physalospora tucumanensis)* on Sugarcane in Tissue Culture

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A phytotoxin produced by the sugarcane red rot fungus, *Colletotrichum falcatum* Went. was partially purified and its effects studied on sugarcane callus development, plantlet differentiation and shoot growth in tissue culture. The findings indicate that all the above attributes were markedly reduced by the fungal toxin. Potential for the possible use of the toxin to produce red rot resistant sugarcane genotypes by pathogen toxin induced somaclonal variation is examined.

Keywords: Phytotoxins, red rot disease, sugarcane, tissue culture.

The red rot disease of sugarcane *(Saccharum officinarum)* caused by the fungal pathogen *Colletotrichum falcatum* Went (Perfect stage: *Physalospora tucumanensis* Speg. Arx and Muller) results in very high loss in yield and quality of the crop in the Indian subcontinent (Alexander and Viswanathan, 1996). Deployment of disease resistant/tolerant cultivars is the major management measure against the disease. The traditional method to obtain red rot resistant cultivars is by hybridization and selection of resistant progenies with good yield and quality attributes as determined by disease reaction in response to pathogen inoculation. However this is a long drawn process and is resource intensive in terms of land, labour and cost. Hence attempts to generate red rot resistant genotypes using biotechnological methods are considered useful. Use of tissue culture and the resulting somaclonal variation for sugarcane improvement is well known (Heinz et al., 1977; Sreenivasan and Jalaja, 1995). Induction of directed variability to produce disease resistant somaclones using metabolites of the pathogen in tissue culture has been attempted in some host pathogen systems (Nyange et al., 1995; Jaisankar et al., 1999). *In vitro* techniques and the potential of using phytotoxins of plant pathogens as agents to select for disease resistance and the problems involved in such procedures have been reviewed by Daub (1986).

In sugarcane, production of phytotoxin by the red rot pathogen *(Colletotrichum falcatum)* and its role in pathogenesis is known (Olufolaji and Bomgboye, 1986; Mohanraj et al., 1995). Rameshsundar et al. (1999) have suggested the use of red rot toxin as a molecular marker for red rot resistance in sugarcane. Hence the effects of the red rot toxin on sugarcane in tissue culture which may have the potential to generate toxin/disease resistant somaclones were investigated.

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Materials and Methods

Fractionation of red rot phytotoxin

The sugarcane red rot pathogen *Colletotrichum falcatum* was isolated by plating disease affected tissues of the red rot susceptible sugarcane variety CoC 671 on oatmeal agar. Subsequently the fungus was multiplied on Czapek's liquid medium for 10 days in a rotary shaker. The liquid culture was homogenized in a blender, filtered through Whatman No1 filter paper and the filtrate used as source of pathogen toxin.

The phytotoxin from the culture filtrate was fractionated using the solvent extraction method of Nair and Ramakrishnan (1973) and partially purified by thin layer chromatography (TLC) using the procedure of Olufolaji and Bomgboye (1986). The physical and chemical properties of the toxin as well as its biological activity were ascertained as described by Mohanraj et al. (1995).

Production of healthy sugarcane callus

Two sugarcane varieties highly susceptible to red rot disease viz. CoC 671 and CoC 92061 were used for the studies. Sugarcane callus was raised using the innermost leaf sheath as the explant. The explants were surface sterilized for 2 minutes in 0.1% $HgCl_2$ and then subsequently in 10% sodium hypochlorite for 10 minutes. Three washes were given using sterile distilled water. For callus generation MS tissue culture medium (Murashige and Skoog, 1962) supplemented with 0.5 mg/l each of nicotinic acid, pyridoxine HCl, thiamine HCl, 2 mg/l of glycine and 3 mg/l 2–4D was used. Surface sterilized very small tissue samples of the above two sugarcane varieties were plated on the medium and incubated at 20–25 ºC. After 20 days of growth the embryogenic calli developed from the tissues were subsequently multiplied and used for all toxin related studies.

Survival of calli in toxin incorporated medium

MS tissue culture medium with the required supplements was prepared, the partially purified red rot toxin incorporated in it, at concentrations of 100, 500, 1000, 1500, 2000 and 2500 µg/ml and sterilized. Very small fragments of viable embryogenic calli growing in toxin free MS medium were transferred to the MS callus medium containing the above concentrations of the partially purified red rot toxin. The cultures were incubated at 20 ºC for 25 days and the percentage of callus clumps which survived and continued to regenerate and proliferate was recorded. The per cent calli that failed to regenerate at different toxin concentrations is presented in *Fig 1*. Five replications with 25 callus clumps each were maintained.

Effect of partially purified toxin on shoot initiation and plantlet development of sugarcane calli

After 25 days of growth in MS callus medium with different concentrations of the red rot toxin very small fragments of embryogenic calli grown with toxin concentrations of 0, 1500, 2000 and 2500 µg/ml were subcultured onto MS differentiating medium (with

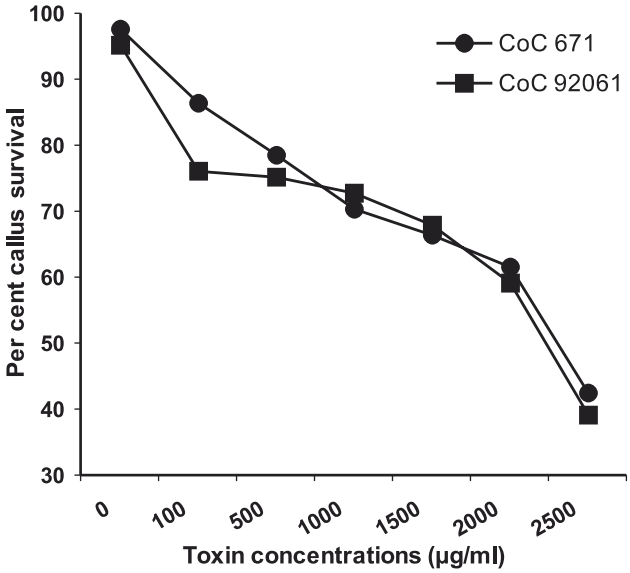

Fig. 1. Effect of partially purified red rot toxin on initial survival of sugarcane calli

3 mg of NAA/l, 2 mg of kinetin/l) containing the same concentrations of the phytotoxin and incubated at 20 ºC. After 20 days of incubation the per cent callus explants, which showed shoot differentiation *(Table 1)* was recorded. Five replications with 25 callus clumps each were maintained.

Table 1

Effect of red rot toxin on shoot differentiation and plantlet development of sugarcane calli

Toxin concentration (µg/ml)	Shoot differentiation (%) Varieties	
	CoC 671	CoC 92061
00	100.0	100.0
1500	78.4	70.4
2000	64.0	56.8
2500	42.2	43.2

Effect of red rot toxin on shoot growth of differentiating plantlets

From the calli showing shoot differentiation (shoot initials) in MS differentiating medium with 0, 1500, 2000 and 2500 µg/ml concentrations of the red rot phytotoxin very small fragments of uniform size and growth were selected. They were freshly transferred to MS differentiating medium containing the same concentrations of red rot toxin and

incubated for 1 month at 20 ºC. The cultures were subject to fluorescent lighting (2000 lux) and darkness for 16 and 8 h, respectively. After 30 days of growth the growing plantlets were examined for differences in shoot height (mm) and the per cent reduction in growth between plantlets grown on toxin free medium and those on toxin incorporated medium was recorded *(Table 2).* Three replications with 15 shoots each selected randomly were maintained.

Table 2

Effect of red rot toxin on shoot differentiation and plantlet development of sugarcane calli

Toxin concentration (µg/ml)	Per cent inhibition of shoot growth over control Varieties					
	CoC 671			CoC 92061		
	30 days	60 days	90 days	30 days	60 days	90 days
1500	28.8	31.1	40.0	31.1	33.3	42.2
2000	44.4	51.1	57.7	46.6	55.5	62.2
2500	66.6	77.7	86.6	73.3	82.2	91.1

Results

The findings of the study presented in *Fig. 1* clearly indicate the marked inhibitory effect of the pathogen toxin on regeneration and development of calli of the two red rot susceptible sugarcane varieties. At a concentration of 2500 µg/ml the toxin reduced the viability of callus clumps by more than half. The callus clumps, which did not survive long initially showed discoloration and subsequently dark brown necrosis and death of the tissues *(Fig. 2. A* and *B).*

When the surviving calli at different toxin concentrations were transferred to MS differentiating medium containing the respective concentrations of the phytotoxin plantlet differentiation and shoot initiation were markedly reduced *(Fig. 2. C* and *D).* In a toxin free medium all the callus clumps produced shoot differentiation while at a toxin concentration of 2500 µg/ml only less than 50% of the calli produced differentiating shoots. Similarly the pathogen toxin severely inhibited shoot growth as determined by measurement of shoot height. By 30 days after subculturing there was a reduction of 28.8 and 31.1 per cent in shoot growth of the two sugarcane varieties with a toxin concentration of 1500 µg/ml. During the same period reduction in shoot height of the varieties CoC 671 and CoC 92061 were 66.6 and 73.3 per cent, respectively, at a toxin concentration of 2500 µg/ml.

When the observations were continued up to 90 days it was observed that reduction in shoot growth continued to occur through out the duration of the studies with all the concentrations of the phytotoxin used. However the reduction in shoot growth observed between 30–60 and 60–90 days was much less than that observed initially at 30 days. Ninety days after subculturing the variety CoC 92061 showed 91.1% reduction in shoot growth compared to the growth in a toxin free substrate. In general the phytotoxin was slightly more inhibitory to the calli of the sugarcane variety CoC 92061 than CoC 671.

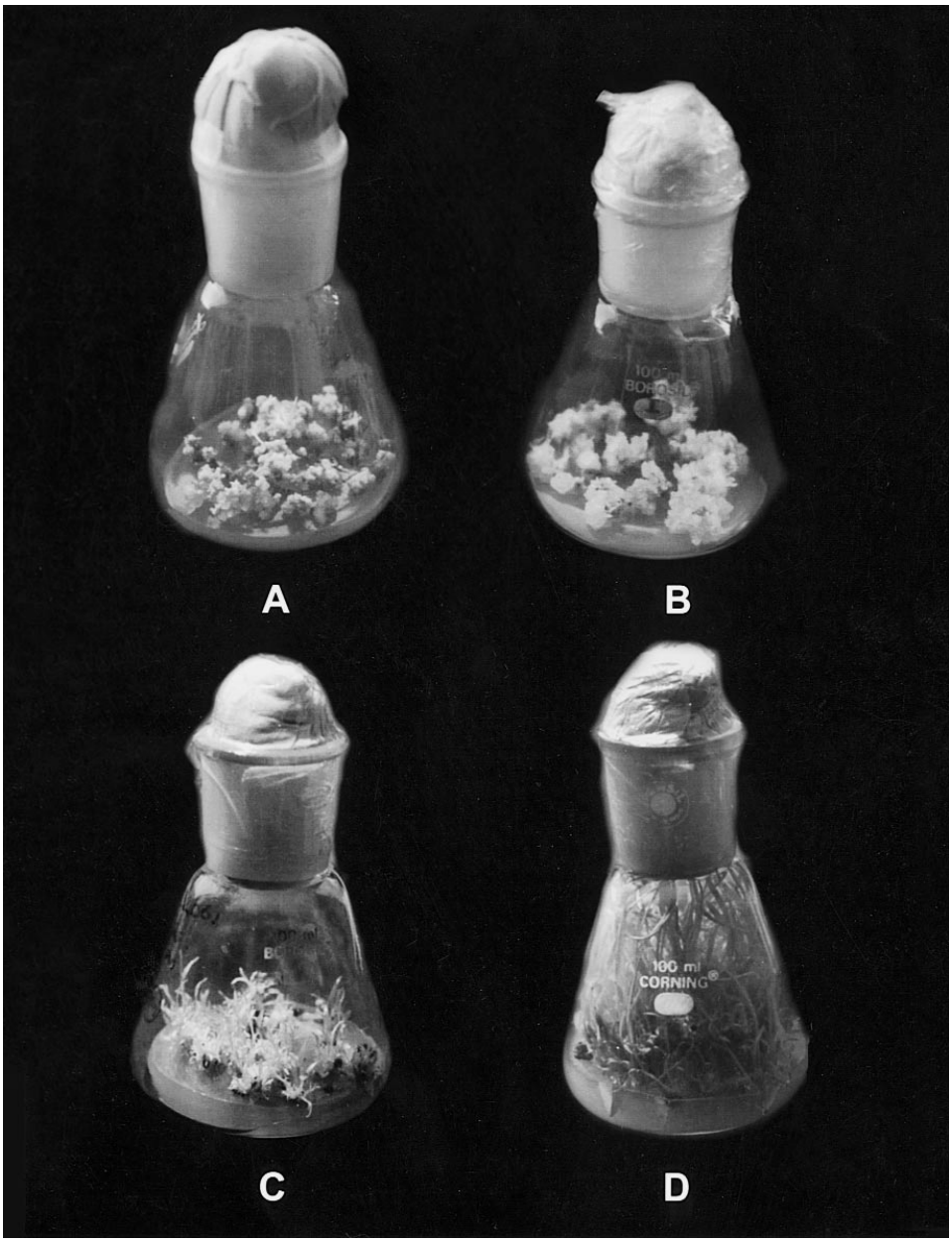

Fig. 2. Effect of red rot phytotoxin on callus regeneration and shoot differentiation.
A) Calli in toxin incorporated medium;
B) Calli in toxin free medium;
C) Inhibition of shoot differentiation in toxin amended differentiating medium;
D) Profuse differentiation and shoot growth in toxin free medium

Discussion

The sugarcane plant is known to possess certain specific advantages to generate genetic variability using tissue culture techniques. The commercial cultivars of the plant are hybrids and many varieties are derived as interspeifc hybrids from different species of *Saccharum.* It is reported to be highly polyploid and heterozygous in genetic constitution (Sreenivasan and Jalaja, 1982; Maretzki, 1987). The phenomenon of chromosomal mosaicism is known to be prevalent in the plant which implies that different parts of sugarcane tissue could have different genetic constitution (Sreenivasan and Jagathesan, 1975). This renders the plant more suitable to generate directed genetic variability in the form of somaclones using external agents. The potential to use phytotoxins of plant pathogens as agents for selection for disease resistance or tolerance in tissue culture systems and problems involved in such attempts have been reviewed by Daub (1986). In sugarcane itself toxin produced by the eye spot pathogen *(Helminthosporium sacchari)* has been used to develop genotypes resistant to the disease (Steiner and Byther, 1971). A preliminary step to obtain pathogen toxin resistant host somaclones is to subject the calli to the toxin and to select the tolerant ones for further development of plantlets.

The results of the study clearly show that the red rot phytotoxin markedly inhibits development of sugarcane tissues at all stages viz. callus initiation and growth, shoot differentiation and shoot growth. Mohanraj et al. (1995) have reported that relatively high concentrations of the red rot toxin were required to induce disease symptoms in the host. Similarly in tissue culture also even the high concentrations of the toxin used resulted in a limited survival of the calli and shoot differentiation, suggesting that more sensitive responses could be obtained by using further purified phytotoxin which could be effective even at low concentrations. The brownish discoloration of the calli associated with necrosis in toxin incorporated medium suggests the involvement of phenolic compounds and their oxidation products similar to those observed in sugarcane plants infected with the red rot pathogen (Chiranjivi Rao et al., 1968). The gradual selection of the tissues for higher resistance to the toxin is apparent from the observation that the tissues finally surviving the toxin at the different stages result in a small proportion of viable shoots which may have the potential to develop into disease resistant genotypes.

Many species of *Colletotrichum* are known to produce phytotoxins. The physiological activity and chemical nature of Colletotin a toxin produced by *Colletotrichum fuscum* was reported by Goodman (1959). Several reports are available on the production of phytotoxic metabolites by species of *Colletotrichum* (Goodman, 1960; Grove et al., 1966; Ballio et al., 1969; Kimura et al., 1973; Gohbara et al., 1978; Goddard et al., 1979). Production of phytotoxin by the turmeric (a monocot plant) leaf spot pathogen *Colletotrichum capsici* and its involvement in disease induction was reported by Nair and Ramakrishnan (1973). Different phytotoxin are known to have different modes of action. Kimura et al. (1973) reported that a phytotoxin produced by *Colletotrichum lagenarium* was found to function as an antiauxin. Sensitivity of sugarcane clones relating to electrolyte leakage caused by *Helminthosporium sacchari* toxin has been reported (Scheffer and Livingston, 1980).

Although there are several reports available on the use of pathogen toxins in tissue culture to evolve disease resistant genotypes of host plants, only a few reports are available on similar attempts with species of *Colletotrichum.* Narain and Das (1970) have used the phytotoxin produced by *Colletotrichum capsici* to study disease resistance in *Chillies* in tissue culture. Similarly in coffee studies have been carried out on the generation somaclones resistant to the anthracnose pathogen *Colletotrichum kahawae* in tissue culture using the pathogen toxin (Nyange et al., 1995). Responses of embryogenic mango cultures to a partially purified phytotoxin produced by *Colletotrichum gloeosporioides* have been reported to be useful criteria in the selection of disease resistant genotypes (Jaisankar et al., 1999).

The results of the present study suggest the possibility of generating somaclones resistant to red rot toxin using tissue culture techniques. With this objective, the small proportion of surviving shoots at high toxin concentration are being rooted to be conditioned and clonally multiplied for evaluation of disease reaction. In addition the partially purified red rot toxin is being further purified with a view to obtain the toxin fraction capable of inducing toxin resistant sugarcane somaclones at very low concentration.

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