In vitro evaluation of red rot toxin influence on sugarcane (*Saccharum officinarum* L.) var. CoC 671

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Red rot of sugarcane caused by *Colletotrichum falcatum* Went has become a threatening disease for the popular cultivar CoC 671. The work on isolation of red rot resistant lines using cellular selection is in progress. During present work an improved technique is developed for isolation of red rot toxic metabolite in higher quantity using sugarcane host extract medium (1 g/10 l of culture filtrate). The toxin was partially purified and incorporated into the MS medium supporting the growth of sugarcane callus of var. CoC 671. The lower levels (0.05%) did not inhibit the growth, however, the delay in callus proliferation was seen beyond 0.1% toxin level. The growth of callus was completely inhibited with 0.5% toxin concentration in the basal medium. The results confirm the pathogenicity of the isolated metabolite and limits of toxicity *in vitro* condition.

SUGARCANE var. CoC 671, which is showing a great promise as an early maturing variety, has become susceptible to red rot disease caused by *Colletotrichum falcatum* Went[1]. There is a need to select red rot resistant lines using this variety. The efforts to induce red rot resistance through mutation[2] and similar approaches[3] have not yielded desirable results. Plant cell and tissue culture technology has helped in selecting the disease-resistant lines in sugarcane[4–7]. However, these reports do not contain the studies on resistance to red rot. It is necessary to isolate the fungal toxin produced by *C. falcatum* strain growing on this local variety and use for screening the tolerance of the varietal cells *in vitro* condition. Methodology on screening for red rot susceptibility in the initial stages of field multiplication has been reported[8]. During the present work, the *in vitro* screening is carried out for finding the limits of tolerance to the toxic metabolite produced by the *C. falcatum.* We present the modified method of isolation of toxic metabolites from the pathogen.

Diseased stalks of CoC 671 were collected from Aland sugar factory area (Karnataka, India) from five-month-old sugarcane field. They were cut into small pieces, i.e. 0.2 to 0.3 cm and washed thoroughly with the sterile distilled water. The stem pieces were disinfected with 70% alcohol for 30 sec followed by washing with sterile distilled water to remove the traces of alcohol. The diseased materials were again transferred to 0.1% $HgCl_2$ for 3 min and washed thrice with sterile distilled water. After surface sterilizaton, the diseased materials were gently placed on oats meal agar in petri plates. The petri plates were kept at room temperature (25 ± 3°C) until the fungal growth almost covered the surface of the medium.

The identity of this pathogen was confirmed using cultural, morphological and pathogenicity tests. The morphological characters of pure pathogen were observed daily after incubation. The fungus showed the cottony growth of mycelium which turned grey with the age. The conidial masses were dark grey at first and then changed into pink masses.

The microscopic characters of the fungus were also observed. The hyphae were thin, branched, hyaline, septate and contain oil droplets. Numerous black setae were found around stroma and many falcate sickle shaped conidia were found at the tip of short conidiophores.

In vitro production of toxic metabolites by *C. falcatum* Went has been demonstrated[9] which causes symptoms similar to those caused by the pathogen. The Czapeck Dox medium and Host extract medium were used for toxin production. The host extract medium containing 250 g of sugarcane var. CoC 671 extract, $NaNO_3$ – 2 g, K_2HPO_4 – 1 g, $MgSO_47H_2O$ – 0.5 g, KCl – 0.5 g and $FeSO_4$ $7H_2O$ – 0.01 g were dissolved in 1000 ml of distilled water and pH of the medium was adjusted to 7.0. 250 ml of each were dispensed in 500 ml conical flasks and autoclaved. The flasks were each inoculated with 6 mycelial disks (8 mm diameter) cut from the margin of young growing colonies of pathogen. The flasks were incubated at room temperature in still condition for 15 days.

After 15 days of incubation, the cultures were filtered and the mycelial mat was homogenized with five volumes of (w/v) sterile distilled water with pestle and mortar. The homogenate was centrifuged at 1000 *g* for 15 min and the pellets were discarded. The supernatant was reduced to one tenth of the original volume by evaporating at 100°C on water bath. The reduced volume was combined with equal volume of methanol, stirred well and kept at 5°C overnight and then filtered. The methanol was removed by distillation and the left out filtrate was taken into a separating funnel and the pH was adjusted to 3.5 with dilute 0.1N HCl. An equal volume of diethyl ether was added and shaken well. The ether phase was separated and mixed well with equal volume of 5% aqueous solution of sodium carbonate, separated again and the aqueous phase was discarded. The ether solution was evaporated to dryness at 40°C on water bath. It was then weighed and kept in a desiccator for further use. The concentrated toxic metabolite from mycelium was distinguished as endotoxin and that of culture filtrate as exotoxin.

The isolate of *C. falcatum* used in this study has produced the toxic metabolites in both Czapek Dox medium and sugarcane host extract medium. It was relatively

Table 1. Effect of toxic metabolite isolated from *C. falcatum* on callus growth* of sugarcane var. CoC 671

Toxin con. % in MS basal medium	Days after subculture 15	30	45	60
Control	0.19 (± 0.026)	0.53 (± 0.033)	1.41 (± 0.003)	1.56 (± 0.056)
0.05	0.18 (± 0.022)	0.63 (± 0.013)	1.00 (± 0.13)	1.30 (± 0.019)
0.1	0.34 (± 0.002)	0.65 (± 0.014)	1.17 (± 0.048)	1.35 (± 0.013)
0.2	0.11 (± 0.006)	0.28 (± 0.006)	0.47 (± 0.003)	0.55 (± 0.004)
0.5	0.10 (± 0.006)	0.12 (± 0.003	0.31 (± 0.015)	0.46 (± 0.005)

*Fresh weight in grams.

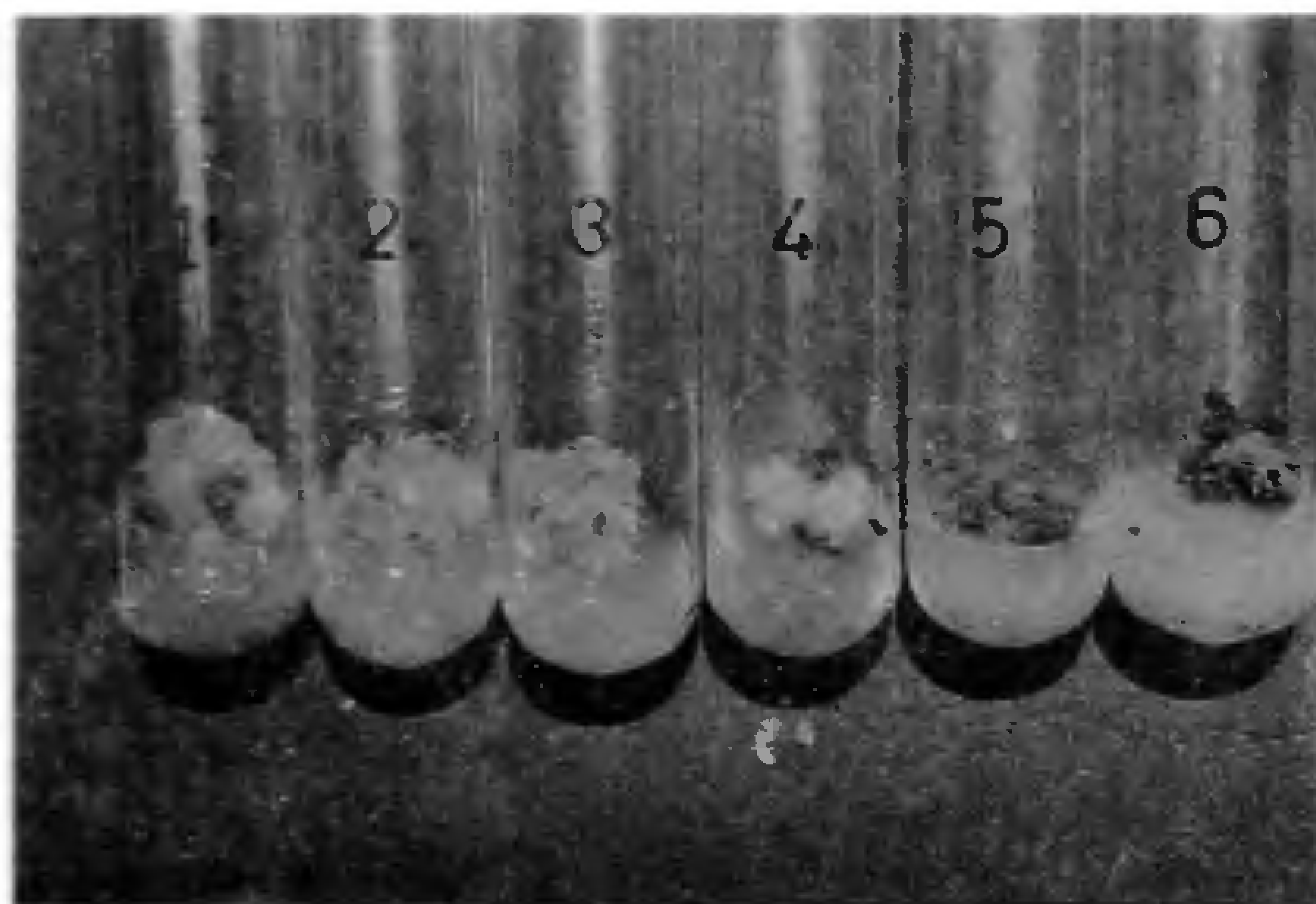

Figure 1. *In vitro* influence of red rot toxin on callus growth of sugarcane var. CoC 671. Toxin concentration: 1, control; 2, 0.05%; 3, 0.1%; 4, 0.2%; 5, 0.3%; 6, 0.5%.

very high in sugarcane host extract medium (0.8 to 1 g/10 l filtrate) as compared to Czapeks Dox medium (0.5 to 0.6 g/10 l filtrate).

There are also reports on isolation and partial purification of toxic metabolites from *Colletotrichum* species isolated from different host plants[10–12]. These reports do not contain the isolation and purification of toxin from sugarcane pathogen. The toxic metabolite production by *C. falcatum* growing on sugarcane has confirmed the pathogenicity of sugarcane red rot fungus due to the influence of phytotoxin[9]. In the present work, the toxic metabolite isolated from sugarcane host extract medium was used for bioassay using the leaves of the variety CoC 671. Typical symptoms of red rot were developed after 6 days of its administration.

Further, the studies were extended by inducing callus formation of CoC 671 sugarcane variety using tissue culture medium[13]. The methodology for callus induction is reported in our earlier communication[14]. The friable callus was used for the screening of the growth parameter with various concentrations of fungal metabolite in MS medium.

The relative growth response of the toxin treated with callus culture of sugarcane var. CoC 671 was studied by using small screw cap glass vials with MS medium. The different concentrations of the toxin (0.0, 0.05%, 0.1%, 0.2%, 0.5%, 0.8% and 1%) were incorporated into the medium. The growth responses were recorded at 15 days' interval up to 60 days. The change in the fresh weight of the callus was recorded by taking weight of the screwcap culture tubes (Table 1).

The increasing concentration of toxin in MS medium resulted in inhibition of callus growth of CoC 671. (Figure 1). The callus growing in control and 0.05% toxin level did not show any inhibitory effect, while the growth inhibition was noticed beyond 0.5% toxin level. The callus induction is slightly delayed at 0.1 and 0.2% toxin level. The callus segments subcultured on toxin level beyond 0.5% in the medium did not actively proliferate and instead turned brown, compact and finally died within ten days of subculturing.

The toxin concentration beyond 0.1% shows inhibition of the growth and beyond 0.5%, it is completely inhibited. There is a need to use this concentration for selection of red rot-resistant lines in sugarcane. The experiment also confirms the pathogenicity of the isolated toxin to susceptible variety CoC 671 under *in vitro* conditions of growth. The toxin is also being used for screening of disease resistance using serological techniques for selection of red rot resistance in sugarcane cultivar.

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Studies on herbicidal activity of parthenin, a constituent of *Parthenium hysterophorus*, towards billgoat weed (*Ageratum conyzoides*)

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Parthenin, a constituent of *Parthenium hysterophorus*, was evaluated for herbicidal activity against *Ageratum conyzoides* (billgoat weed) under *in vitro* conditions. It inhibited/retarded germination of *A. conyzoides* at concentrations ranging from 0.02 to 0.1 mg/ml. Radicle elongation and seedling length were severely reduced. The germination of wheat, however, remained unaffected at similar concentrations. The study, therefore, paves a way for possible exploitation of parthenin as a selective herbicide.

NATURAL plant products are fast catching attention of scientists for their use as herbicides to control weeds[1]. Unlike synthetic herbicides, they do not cause pollution and undesired side effects on environment. Sesquiterpene lactone parthenin – a constituent of the noxious weed *Parthenium hysterophorus*[2,3], has been shown to be phytotoxic against some aquatic weeds[4]. A work was, therefore, initiated to explore its herbicidal activity against a terrestrial weed *Ageratum conyzoides* (billgoat weed) commonly found in agricultural fields in north-western India.

Parthenin was extracted from healthy, shade-dried leaves of *Parthenium hysterophorus* (collected locally from wildly growing stands as per the method of Saxena *et al.*[5]). The parthenin, so obtained, was dissolved in a few drops of ethanol and the final volume was made with distilled water to get solution ranging from concentrations 0.02 to 0.1 mg/ml (in increments of 0.02 mg/ml). To test the herbicidal property of parthenin, seeds of *A. conyzoides* (collected locally from wild stands) and wheat *Triticum aestivum* var. HD-2329 (procured from Punjab Agricultural University, Ludhiana, India) were subjected to germination in solutions of parthenin. Fifty seeds each of *A. conyzoides* and wheat were dipped in treatment solutions for 16 h. Treatment with distilled water served as control. The dipped seeds were then placed on the upper surface of Whatman filter paper no. 1 in a 12-cm petri plate. Below the filter paper a thin cotton pad soaked in the treatment solution was placed. The petri plates were incubated at 27°C, 16 h photoperiod and 75% relative humidity in a germination chamber. The entire experiment was arranged in a randomized block with four replications. The number of seeds germinated were counted and radicle length was measured after 72 h. After 10 days of treatment, the seedling length was measured. Mean values and the standard errors of the data were calculated and presented with respect to control. Besides, values of correlation coefficient between different concentrations and parameters were also calculated using polynomial regression analysis.

The results show that in response to different concentrations of parthenin, all the seeds of wheat germinated like that of water-treated control, whereas, seeds of *A. conyzoides* responded differently (Table 1). At the concentration of 0.02 mg/ml parthenin, the germination of *A. conyzoides* was nearly 50% whereas it was reduced to less than 20% at the concentrations ranging from 0.04 to 0.08 mg/ml. None of the seeds, however, could germinate at 0.1 mg/ml parthenin (Table 1).

Radicle length of *A. conyzoides* after 72 h was drastically reduced at all the concentrations of parthenin used and was nearly 50% of the control at the lowest concentration, i.e. 0.02 mg/ml. In contrast, a slight stimulation in radicle elongation of wheat was observed at this concentration (Figure 1). Almost similar trends were observed in case of seedling length (Figure 2).

Based on these results, a concentration of 0.04 mg/ml parthenin is recommended for further studies since at this concentration, germination of *A. conyzoides* was reduced by 90% whereas that of wheat remained unaffected.

From the present study it is clear that sesquiterpene lactone parthenin exhibits selective phytotoxicity and may, thus, find a potential use in the field of agriculture. In fact, a number of natural products such as cineole[1], 1,3,7-trimethylxanthine[6], ailanthone[7], including the sesquiterpene lactones such as artemisinin[8,9] are fast emerging as selective phytotoxins/herbicides. The greater biological activity profiles of sesquiterpene