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ISOLATION AND PARTIAL PURIFICATION OF TOXIN FROM *COLLETOTRICHUM FALCATUM*: THE CAUSAL AGENT OF RED ROT IN SUGARCANE

AMBREEN KHADIJA ALVI* AND JAVED IQBAL

School of Biological Sciences, University of the Punjab, Lahore, Pakistan
**Corresponding author: botanist_sbs@yahoo.com*

Abstract

The red rot fungus (*Colletotrichum falcatum*) spores were isolated from infected stalk pieces of sugarcane and grown on Czapek Dox agar medium to obtain pure culture. Spores were obtained from the pure culture and grown in Czapec Dox liquid medium for further studies. It was observed that the fresh and dry weights of fungal mycellial mat significantly increased with time. In addition, fungal growth resulted in a significant change in the pH of the Czapec Dox growth medium over a period of 35 days and gradually increased from 6.5 in the control medium to 8.4. The fungal toxin was extracted from the growth medium and used to treat cell suspension of a red rot resistant sugarcane cultivar HSF-240. The maximum toxin was produced after 28 days of fungal growth in the growth medium which was ascertained by the dry weight of residue obtained from the ethyl actate fractions, as well as percent mortality of sugarcane cells in suspension. The LD_{50} value (50% cell mortality) of toxin in ethyl acetate (ETA) fraction was 22.03 days while in water it was 30 days. The fungal toxin extracted in ETA fractions was more effective than the one extracted in water. It was mainly due to high solubility and potency of the toxin in ETA than in water. In general, the maximum toxicity was noted at the 4th week (28 days) which decreased at 5th and 6th week of mycelial growth.

Introduction

The red rot disease of sugarcane is caused by the fungal pathogen *Colletotrichum falcatum* Went. It is one of the oldest diseases of sugarcane and is of great economic importance and a serious threat to the sugar industry particularly in sub- tropical countries (Alexander & Viswanathan, 2002; Viswanathan & Samiyappan, 2002). The fungus produces spores both by asexual (conidia) as well as sexual (perithecium) means (Alexander & Viswanathan, 1996). Like other pathogens, it infects the host plant by appressoria and haustaria by penetrating inside the epidermis of host plant (Guerber & Correll, 2001; Armstrong-Choa & Banniza, 2006).

Sugarcane is one of the most economically important crops, being cultivated mainly for sugar for many centuries. Now it is also used to produce ethanol and bio-fuel. However, in the recent years, its production has deteriorated due to various stresses and diseases such as red rot. The worldwide loss in cane yield and sugar recovery is approximately 5-10 % per annum (Viswanathan & Samiyappan, 2002). During the recent years, the greatest loss to sugarcane industry in Pakistan was also due to red rot. The loss in cane weight was recorded to be about 29.07 % and resulted in 30.8 % loss in sugar recovery (Hussnain & Afghan, 2006). Besides resulting in loss of yield and quality, the disease is also responsible for eliminating several important sugarcane cultivars from cultivation and continues to be a problem in other countries such as USA, Bangladesh, India, Australia and Thailand (Viswanathan & Samiyappan, 2002).

The toxin produced by growing fungus is involved in triggering red rot disease in sugarcane. The presence of such toxin was suggested as early as 1929 by Lee. Therefore, the most rationale approach to select disease resistant cultivars is either to isolate the mycotoxin from fungal culture filtrates or purify toxin produced by the pathogen and use it to induce resistance at cellular level in *In vitro* conditions (Mohanraj *et al.*, 1995; Mohanraj, 1996; Mohanraj *et al.*, 2003; 2004). A number of previous studies have shown a positive correlation between toxin resistance at cellular level and plant pathogen at the whole plant level (Rines & Luke, 1995; Jayashankar *et al.*, 2000; Parsad & Naik, 2000). Thus, the information so generated has been used to develop many red-rot tolerant lines/cultivars of sugarcane by selecting resistant cells and regeneration of resistant plants by tissue culture (Mohanraj *et al.*, 2003; Sengar *et al.*, 2009).

Therefore, this study was conducted to study the growth of the red rot pathogen on Czapec Dox liquid medium. In addition, the fungal toxin produced by the *C. falcatum* mycelium was isolated from the growth medium, partially purified and its effects were studied on cell mortality of a red red-rot resistant sugarcane genotype (HSF-240). This information was used to calculate the LD_{50} values for fungal toxin isolated in ethyl acetate and water fractions.

Materials and Methods

Isolation, growth and preservation of fungal (*Colletotrichum falcatum*) colony: The infected cane pieces (1-1.5 cm) were obtained from the Shakarganj Sugarcane Research Institute (SSRI), Jhang and placed in Petri plates containing 2% autoclaved agar medium. The fungal colony was allowed to grow for 7-8 days in dark at 25°C in an incubator. It was then identified under microscope and purified by inoculating on Czapek Dox agar medium (Czapek, 1902-1903; Dox, 1910). This procedure was repeated until pure fungal colony was obtained. The pure fungal cultures were further verified by Koch's postulates (1893). The purified fungal colonies were preserved at 4°C in a refrigerator on Czapec Dox agar medium.

Collection of spores in water suspension: For the extraction of fungal spores, fresh fungal colonies were prepared on Czapec Dox agar medium. After sufficient growth of fungus, autoclaved distilled water was poured into Petri-plates containing pure colonies and swirled gently. The distilled water containing the spore suspension was then collected in a sterilized test tube.

Spore count using haemocytometer: Fungal spores were identified and counted under microscope. The number of viable spores mL^{-1} of suspension were counted using a haemocytometer under a microscope (Model Zeiss). The spores were first counted under low magnification (10x) then counted under high magnification (40x). For this purpose, the number of spores in the smallest cubes was considered. Spores in four of the 16 cubes (per set) were counted and total number of spores per mL was calculated by the following formula:

$$\text{Number of spores in four cubes} = 4 \times 16 \times 10^4 \text{ spores mL}^{-1}$$

Preparation of extract containing fungal toxin: The Czapec Dox liquid medium was prepared and pH was adjusted to 6.4. One hundred ml of the medium and one ml of filtered fungal suspension containing 3.072×10^6 spores/ml of *Colletotrichum falcatum* was added to each of 28 jars. The jars were incubated at 25^oC in the dark and kept flat to provide maximum surface area for cultures which were harvested after every seven days in quardiplicate. The fungus was removed by filtration through cheese cloth (0.05 mm mesh) and squeezed to obtain maximum yield of filtrate. The pH of the culture filtrate was recorded. A control culture medium without fungal suspension was also collected to serve as blank. The toxin activity, pH of the culture filtrate, fresh and dry weight of the fungus and spores in medium were recorded after every harvest.

Isolation of toxin from culture filtrate: Frozen samples of culture filtrate were thawed and the pH was adjusted to 3.0 by the addition of 1 M H_2SO_4. The acidified solution was partitioned three times against equal volumes of ethylacetate (EtoAc). The EtoAc fraction was then combined and dried over anhydrous Na_2SO_4 before evaporating to dryness on a film evaporator at 40^oC. The aqueous phase was also film evaporated to dryness. The residues from both phases were weighed and then dissolved in 100 ml holding buffer (Citric acid monohydrate (10.5 g), Glucose (100 g), $MgSO_4.7H_2O$ (1 m*M*), KH_2PO_4 (1 m*M*), $CaCl_2.2H_2O$ (5 m*M*) and NaOH (6.20 g) in 1 L at 6.1 pH). This was the final concentration used in cell bio-assay.

Cell isolation: The callus of a red rot resistant sugarcane genotype (HSF-240) was induced from shoot apical meristem on MS basal medium (Murashige & Skoog, 1962). Then the plantlets of sugarcane were regenerated *In vitro* on BAP (1 mg L^{-1}) and NAA (1 mg L^{-1}) supplemented medium. One g leaves of *In vitro* grown young plantlets were cut into small pieces (1 cm) and put in a falcon tube containing 10 ml enzyme solution [Macerozyme R_{10} (0.3%), Cellulase R_{10} (2%), Pectolyase Y_{23} (0.07%) prepared in 100 ml at 5.55 pH]. The leaves were vacuum-filtered with enzyme solution until most of the air spaces were removed and the tissue appeared dark. The mixture was incubated at 25^oC in the dark overnight and then vortexed at slow speed for 15 min. until the solution became turbid. The cells released were filtered through two layers of autoclaved cheese cloth and centrifuged at 1500 rpm at 25^oC for approximately 5 min. The enzyme solution was removed and replaced with holding buffer. The pelleted cells were again dispersed in the holding buffer. The cells were washed three times with holding buffer and centrifuged. The concentration of cells was made up to 10^5/ml. Viability of the cells was tested with pheno-safranine (0.1% in holding buffer at pH 6.1) (Widholm, 1972)

Response to toxin: A two-fold dilution series of toxin solutions were prepared in duplicate in holding buffer in a 96 well flat-bottom micro-test plate in two replicates (50 µl/well). Then fifty µl cell suspension was added in each well and the plates were incubated at 25^oC for 3 h. Control wells contained only the holding buffer without any toxin. After incubation, the cells in each well were stained with fifty µl phenosafranine (0.1% prepared in holding buffer). After 1 minute, 10 µl was drawn from the bottom of the well and dispensed onto a microtest slide or haemocytometer. 100 cells were counted in each sample. The proportion of the dead cells was counted in each well and corrected against the dead cells of control. The values were expressed as a percentage according to the following formula:

$$(C\text{-}T \times 100) / C$$

where, C is the number of live cells in control wells containing holding buffer only and T is the number of live cells in test wells containing toxin preparation.

Data analysis: The percentage cell death was plotted against the time (days) on a scatter plot using Micosoft Excel for Windows (version 2003). A trend line was then computed and the LD_{50} values were calculated from the regression equation.

Results

Change in the pH of fungal growth medium: The pH of the fungal growth medium significantly increased during 42 days of growth in Czapec Dox liquid medium. The pH at 0 days was 6.5 which increased to a maximum of 8.4 after 35 days. However, there was a dip in pH (8.2) after 42 days of fungal growth (Fig. 1).

Fresh and dry weight of fungal mat: The fresh and dry weights of fungal mycelium grown on Czapec Dox liquid medium also registered an increase up to 42 days of growth. During the first two weeks of growth, there was a rapid increase in growth rate in terms of fresh (0.445 g) and dry (0.073 g) weights. However, the growth rate was a bit slow during the third and fourth weeks. After the 4th week, the fungal growth rate once again increased rapidly and the maximum fresh (1.519g) and dry weights (0.314g) of the fungal mycellial mat were recorded at 42 days (Fig. 2).

Dry weight of residue in ETA and water fractions: The dry weight of residue obtained from the water fractions was recorded to be significantly greater than that obtained from the ETA fractions. Moreover, the dry weight obtained from the water fraction showed a continuous decrease from the control amount (3.42 g) over the 42 day time period and reached a minimum of 2.89 g. However, the dry weight of residue obtained from the ETA fractions showed a different trend and was initially observed to increase upto 28 days reaching a maximum amount of 0.34 g over the control value of 0.10 g. After this the dry weight of residue decreased and 0.25 and 0.20 g of dry residue was obtained at 35 and 42 days respectively (Fig. 3).

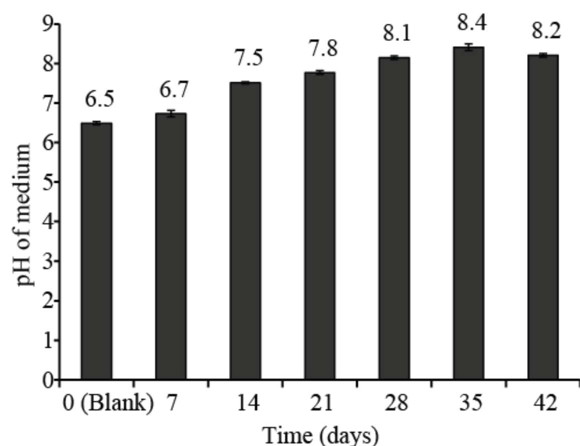

Fig. 1. Changes in the pH of the Czapec Dox liquid medium due to growth of *Colletotrichum falcatum* fungal mycelium at various time intervals (days). The blank represents the pH at 0 days (blank sample which was not inoculated with fungal spores). The mean pH of the Czapec Dox medium is written above the respective bars.

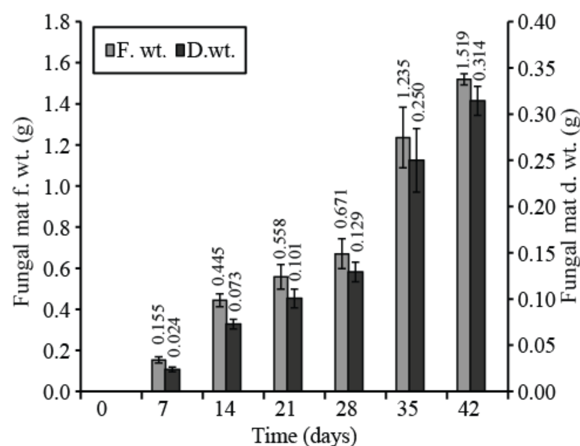

Fig. 2. Fresh and dry weights of the *Colletotrichum falcatum* fungal mycelial mat grown on Czapec Dox liquid medium. The y-axis on the left shows the fresh wt. (g), whereas the right y-axis represents the dry wt. (g) of the fungal mycelial mat. The mean values of the fresh and dry weights are written above their respective bars (n = 4).

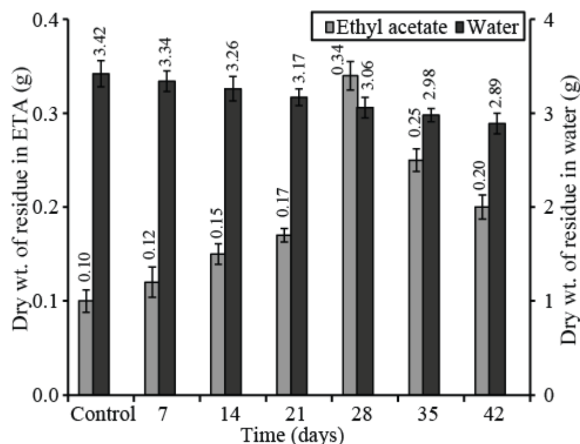

Fig. 3. Dry weights of the residue obtained from the ethyl acetate and water fractions from freeze dried culture filtrate samples of *Colletotrichum falcatum*. The y-axis on the left shows the dry weight of reside (g) in ethyl acetate fraction, whereas the right y-axis represents the dry wt. of residue (g) in water. The mean values of the fresh and dry weights are written above their respective bars (n = 4).

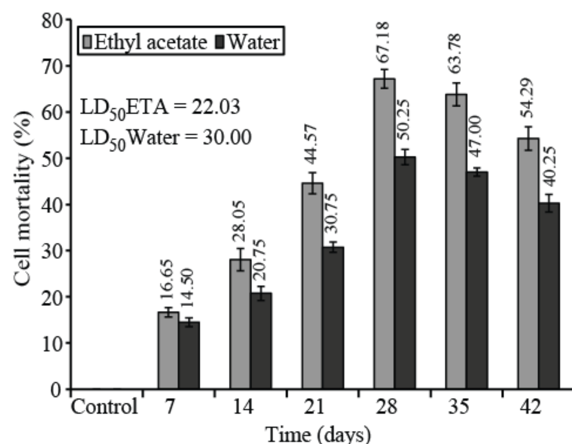

Fig. 4. Percentage cell mortality of toxin produced by *Colletotrichum falcatum* extracted in the ethyl acetate (ETA) fraction or in water at various time intervals (days). The mean percent value of cell mortality is written above each respective bar.

Toxin activity and LD50 value: Toxin was extracted either in ethyl acetate or in water at different intervals of fungal growth (0-42 days). There was a significant increase in the activity of toxin either extracted in ethyl acetate or in water over time. Overall, the toxin extracted in ethyl acetate (ETA) fractions showed greater mortality of suspension cells than that extracted in the water fractions. In both cases, cell mortality of sugarcane cell suspension increased gradually up to the 4th week (28 days) as the maximum percent cell mortality i.e., 67.18 and 50.25 % was recorded in ethyl acetate and water, respectively. Although, a slight decrease in the cell mortality was observed at 5th week (63.78 and 47 % for ethyl acetate and water, respectively), it differed non-significantly from the previous interval (28 days). At 42 days the percentage cell mortality decreased significantly as compared to both previous intervals and only 54.29 and 40.25% cells were killed by toxin extracted in ethyl acetate and water, respectively.

The LD_{50} values were calculated from regression equations calculated for ethyl acetate ($y = 2.4013x - 2.9125$) and water ($y = 1.675x - 0.25$), where x is the LD_{50} value to be calculated for 50% cell death (y). The LD_{50} values were significantly different for both ethyl acetate and water fractions i.e., 22.03 and 30.00 days, respectively. This showed that the toxin molecules in ethyl acetate were more toxic possibly due to greater solubility and stability in the ethyl acetate fraction than in water (Fig. 4).

Discussion

In this study, the fresh and dry weights of fungal mycellial mat significantly increased with time. The overall growth rate of fungal mat was significantly higher during the early growth phase but as the fungal mat grew older, the growth rate decreased. This is attributed to that during the early phases, there were plenty of nutrients and space available for fungal growth in the growth medium therefore; the fungus grew at an exponential rate. However, as the time passed, the nutrient supply was exhausted and space became limited thereby reducing the fungal growth rate (Boschke & Bley, 1998; Maheshwari, 2011). In addition, there was a significant change in the pH of the Czapec Dox growth medium over time that gradually increased from 6.5 of control to 8.4 after 35 days, showing a decrease at 42 days of fungal growth. Since the pH of the medium is an important factor for mineral availability, enzyme activity and membrane function (Robson *et al.*, 2007), an increased pH may also limit the growth rate affecting the above mentioned physiological processes. This change in pH could be because the Czapec Dox growth medium used in this study had relatively low buffering capacity as it lacked any specific buffer to maintain the pH of the fungal growth medium (Yamanaka, 2003). Also, there could be a change due to nutrient utilization by the growing fungal mycelia that would result in significant change in pH of the medium overtime (Yamanaka, 2003; Pradhan & Sukla, 2005). There is ample evidence that both nutrient/proton symport and proton-translocating ATPases occur in fungi (Jennings, 1995; Maheshwari, 2011). Such H^+ transport across membranes coupled with nutrient uptake could result in significant changes in pH as observed in this study (Hawkins *et al.*, 2000; Singh & Reddy, 2011). In addition, the actively growing fungus mat produced a toxin that was reported to be an anthroquinone compound (Malathi *et al.*, 2002; Richardson *et al.*, 2009) with two >C=O groups (Saikia *et al.*, 2004) . The synthesis of this compound could also result in a significant change in the pH of fungal growth medium as observed in this study.

A comparison of dry weight of residue obtained after evaporation showed that the dry weight obtained from the water fractions was significantly higher than those from ETA. This indicated that the solute content of the Czapec Dox liquid medium was highly soluble in water and only sparingly soluble in ETA. Moreover, the dry weight obtained from the water fraction showed a continuous decrease from the control amount (3.42 g) and reached a minimum of 2.89 g in samples collected 42 days after spore inoculum. This decrease in residue dry weight over the passage of time could be attributed to the utilization of nutrients from the medium by the growing mycelium (Boschke & Bley, 1998; Maheshwari, 2011). However, in the ETA fractions an increase in dry weight of residue was recorded up to 28 days showing a maximum value of 0.34 g after which a decrease was observed at 35 and 42 days. This trend coincides with the percentage cell mortality recorded by the ETA fractions indicating that the maximum toxin was produced after 28 days of fungal growth in the growth medium. This then gradually decreased with the passage of time. One of the reasons for reduced toxin activity after 28 days might be the increasing age of the fungal mycelium i.e., after a continuous increase until maximum potential was attained, as the fungus grew older, its ability to produce the toxin decreased (Arya & Perelló, 2010).

The LD_{50} value of the suspension cells treated with toxin produced by the red rot fungus was achieved earlier in ETA (22.03 days) than in water (30.00 days). This observation could be explained on the fact that overall, the toxin extracted in ethyl acetate (ETA) fractions was more effective than that extracted in the water fraction. This indicated that the solubility of the toxin was greater in ETA and the potency of the toxin extracted in ETA was retained for a longer time period than in water. This was confirmed by the percent cell mortality recorded by the water fractions. Previous works also indicated that most fungi propagate better and produce toxins in greater amounts in acidic medium, i.e. low pH (Saikia *et al.*, 2004; Maheshwari, 2011). ETA being an acidic solvent was a better solvent for the toxin produced by the red rot fungus as compared to water. Another reason could be that the solubility of toxin molecules was less in Czapec Dox liquid medium as it was a water based solution and had a low pH buffering capacity (Frankland *et al.*, 1995; Yamanaka, 2003). Thus with the passage of time, as the pH of the medium increased, the toxin molecules might have been degraded and lost their potential to induce cell death. These results indicated that ethyl acetate was a better solvent and could be used to partially purify the toxin produced by the *Colletotrichum falcatum* mycelium from a liquid medium.

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