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DETECTION AND ISOLATION OF A NOVEL TOXIC METABOLITE FROM A PLANT PATHOGENIC FUNGUS, *LASIODIPLODIA THEOBROMAE*

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ABSTRACT

Secondary metabolites like antibiotics and toxins produced by fungi are very useful in the fields of pharmacy, medicine and biotechnological industries. *Lasiodiplodia theobromae* is a ubiquitous Deuteromyceteous fungus, found in the tropics and subtropics, which is known to produce many important secondary metabolites. In the present work a novel toxic metabolite has been isolated and partially characterized. It has an interesting effect on *Schizosachharomyces pombe* cells. A cell free culture filtrate of *L. theobromae* was evaluated for toxicity against the *S. pombe* cells. The toxin metabolite has been purified from the concentrated eight days old cell free culture filtrate using preparatory TLC and bioassay directed screening. Ninety one percent of death of *S. pombe* cells was observed after 48 hrs of incubation with purified toxin. The purified mycotoxin was partially characterized using UV and IR spectroscopic techniques.

Key words: Lasiodiplodia theobromae, mycotoxin, Schizosachharomyces pombe, cell death

INTRODUCTION

Fungi constitute a highly versatile group of eukaryotic heterotrophic microorganisms that have occupied most natural habitats. Secondary metabolites like antibiotics and toxins produced by fungi have proved to be very useful in the fields of pharmacy, medicine and biotechnological industries. Millions of secondary metabolites produced by fungi have not been studied, as revealed by many fungal genome sequence analysis [1].

Lasiodiplodia theobromae is a ubiquitous Deuteromycete fungus, found in the tropics and subtropics. It is an opportunistic plant pathogen but also known to produce a number of biologically active metabolites. Some of these metabolites are jasmonic acid (JA), lasiodiplodin and its derivatives, theobroxides, mellin, 2-Octeno- δ lactone, ethyl hydrogen funarate, 3-formyl indole, phenyl-2-nonanol and indole-3-carboxylic acid [2; 3; 4]. Except 2-Octeno- δ lactone, which is an aromatic compound, most of these substances have potato micro tuber inducing activity. Theobroxide is useful to induce flowering in plants [5]. JA is the plant growth regulator [6]. *L. theobromae* is also known to cause mycotoxicoses in humans [7].

In the present work, a novel mycotoxin was detected in the culture filtrate of *L. theobromae* (MTCC 3068) and its effect was studied on *S. pombe* cells. The purity of the mycotoxin was assessed and partially characterized using UV and IR spectroscopic techniques. The purified toxic metabolite showed 6-7 % more toxicity than that of culture filtrate.

MATERIALS AND METHODS

Fungus

Lasiodiplodia theobromae (MTCC 3068) was procured from IMTECH Chandigarh, India and maintained by sub culturing every month at ambient temperature in basal medium having composition g/l: sucrose, 50; $NaNO_3$, 7.5; KH_2PO_4, 2.0; KCl, 0.3; $MgSO_4$. $7H_2O$, 0.6; $FeSO_4$. $7H_2O$, 0.6; $ZnSO_4$ $7H_2O$, 0.03; $MnSO_4.7H_2O$, 0.003; $CuSO_4$ $7H_2O$, 0.003; Na_2MoO_4 $2H_2O$, 0.003; yeast extract, 1.0; pH 5.5 [8; 9].

Schizosaccharomyces pombe

S. pombe Var. Paul Linder 3360 was also obtained from IMTECH, Chandigarh. It was maintained on Yeast Extract Glucose medium having composition g/l: glucose, 30; yeast extract, 5.

Preparation of cell free culture filtrate (CFCF) from fungus

8mm mycelial mat was cut with sterile cork borer from two days old *L. theobromae*, grown on basal salt agar plate and transferred to 250 ml Erlenmeyer flask containing 100 ml of basal salt broth. Multiple numbers of such flasks were incubated for 8 days at 30 ± 1 ^{0}C in static submerged condition. Under aseptic condition the metabolized growth medium was filtered through a pre-weighed Whatman No.1 filter paper to obtain the final CFCF. This CFCF was used as a crude mycotoxin to study its effect on *S. pombe* cells. To study effect of salicylic acid and ascorbic acid on toxin metabolite production, 10 µg of each of the chemical were added in 100 ml of medium.

Fractionation of CFCF to separate the toxin metabolite

Ten liters of 8th day old CFCF was lyophilized to 10 ml and fractionated with equal volume of ethyl acetate. Organic phase was separated using micropipette, evaporated to dryness and dissolved in 1 ml of water. Both the fractions were assessed for toxicity.

Bioassay

In vitro bioassay was done using *S. pombe* cells. *S. pombe* cells were grown in liquid yeast extract media in 250 ml Erlenmeyer flask containing 100 ml of YE media. Flask was incubated at 30 ^{0}C on shaker at 150 rpm till the exponential growth of *S. pombe* obtained (24 to 30hrs). Then the cell culture was treated with the CFCF or fractions (water and ethyl acetate) of mycotoxins or purified mycotoxin and further allowed to grow for 24hrs. Next day, 20 µl of yeast culture and 20 µl of 0.4 % trypan blue prepared in PBS were mixed and cells were observed in a compound microscope. The dye could enter the dead cell only so they appeared blue whereas live cells resisted the entry of dye. The number of dead cells and number of live cells were counted in one field. Cell counting was repeated in two more of the microscopic fields and an

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average percentage of cells died due to toxin metabolite were calculated.

TLC

Aluminium backed silica gel 60 F_{254} TLC foils (20 x 10 cm) of 0.25 mm thickness (Merck, Darmstadt, Germany) were used. The TLC foils were pre-conditioned by a pre-run with methanol as mobile phase and dried in oven at 120 ^{0}C for 20 mins before sample loading.

Purification of toxin metabolite

Fractions showing toxicity in bioassay was loaded on TLC foils manually and run with different mobile phases; methanol, ethyl acetate (EtoAc), chloroform, acetone, toluene and EtoAc: CHCl3 (1:1). The separated bands were visualized under UV illuminator (DESAGA, SARSTEDT-GRUPPE, CabUVIS) at 254 nm and R*f* of each band was calculated. The separated bands on silica plate were marked, scooped out and then each band was dissolved in D/W and centrifuged. The supernatant obtained were then used to study the mycotoxicity on *S. pombe* cells.

Checking purity of mycotoxin

The major mycotoxic compound separated using EtoAc: CHCl3 mobile phase in TLC was checked for purity by eluting it and subjecting to re-TLC analysis using three different mobile phases, Toluene: EtoAc: Formic acid (6:3:1 v/v/v), Water: Acetone: Chloroform (1.5:12:88 v/v/v) and EtoAc: CHCl3 (1:1 v/v).

Characterization of the major mycotoxin metabolite

The major mycotoxin metabolite was obtained in enough amounts and characterized using UV and IR spectroscopic techniques.

Characterization by UV spectrophotometric analysis

The toxic metabolite showing major toxicity in bioassay was dissolved in water and scanned in the entire UV range in UV spectrophotometer (Shimadzu UV-1800). The same metabolite was also dissolved in two other solvent systems, methanol, acetic acid and shift in absorption maxima was studied.

Characterization by IR spectroscopy

The purified toxic metabolite eluted from TLC was dissolved in chloroform and kept overnight to evaporate the chloroform. The final sample obtained in the form of crystals, was used as sample for IR spectroscopy analysis in range of 200 nm to 1200 nm (IR spectrometer model MB 3000 from ABB).

RESULTS

Detection of toxin metabolite using bioassay

CFCF obtained from three to eight day old culture of *L. theobromae* showed toxicity against *S. pombe* cells. The maximum toxicity was obtained on eighth day. Few cells of *S. pombe* died after 4hrs of incubation. After 48hrs, most of the *S. pombe* cells were killed due to toxic metabolite. The toxicity of the CFCF was found to increase with increasing its volume. *S. pombe* cells incubated with 0.5 ml of filtrate (8 day old) showed more cell death (about 85.5 %) than cells incubated with 0.25 ml of filtrate (about 74.6%) (Fig 1).

Isolation of toxin metabolite

Addition of ethyl acetate to CFCF created two fractions. A bioassay of both the fractions showed that water fraction was comparatively more toxic than ethyl acetate fraction (Fig 2).

Purification of toxic metabolite

The water fraction containing major toxic metabolite was lyophilized and subjected to TLC analysis using five different mobile phases, which showed results as shown in Table 1. As maximum number of bands were obtained in ethyl acetate and chloroform mobile phases but they did not properly resolute, another mobile phase of ethyl acetate: chloroform (1:1) was tried in which the water fraction of CF was separated into 6 different bands (Fig 3). Bioassay studies showed that one of the five metabolites, obtained at the R*f* value 0.432 was found to be a major mycotoxin. In presence of this metabolite 91.16% of *S. pombe* cells were died after 48 hrs as compared to 85% cell death observed in presence of crude water fraction at similar conditions (Fig 4).

Checking purity of the toxic metabolite by TLC

The major toxic metabolite obtained at R*f* value 0.432 was eluted and re-run in TLC using three different mobile phases- toluene: ethyl acetate: formic acid (6:3:1 v/v/v), water: acetone: chloroform (1.5:12:88 v/v/v) and ethyl acetate: chloroform (1:1 v/v), which showed single band at 0.46, 0.53 and 0.43 R*f* values respectively.

Characterization of the purified mycotoxin using spectroscopic techniques

U.V. Spectroscopic analysis

The purified toxin metabolite constituted in three solvents separately showed different absorption maxima (Table 2).

IR spectroscopic analysis

In the IR analysis peaks due to alcohol (1072), alkenes (964), amines (795), alkyl halides (640), acid chlorides (555) functional groups were observed (Fig 5).

Exogenous addition of Salicylic acid and Ascorbic acid inhibits production of toxin metabolite:

The amount of pure toxin obtained was too less in quantity to characterize it further. Salicylic acid and ascorbic acid were exogenously added to find whether the toxin metabolite is inducible or not. Exogenous addition of 10 µg of salicylic acid reduced the toxicity of CFCF. Addition of 10 µg of ascorbic acid also reduced the activity of CFCF.

DISCUSSION

L. theobromae is a pathogenic fungus that infects a large variety of plants all over the world [10]. It is also known to produce many biologically important secondary metabolites [11]. It causes human infections, albeit rarely [7].

In the present study, *S. pombe* has been used to study effect of toxic metabolite of *L. theobromae* at a cellular level. *S. pombe* has become an important tool to study cell biology because it is eukaryotic and it is fairly big in size. Cell death caused by toxin metabolite could be easily monitored by vital staining (Fig 1). Although culture filtrate of *L. theobromae* was able to show toxicity after the age of three days onwards, maximum toxicity was observed on eighth day, therefore eighth day old CFCF was used to assess the toxicity and to isolate the toxin metabolite. The *S. pombe* cells responded within 4hours and maximum dead cells were seen after 48hrs (Fig 2).

The earlier reported phytotoxic substance from this fungus, JA was extracted using ethyl acetate [2]. The novel toxin metabolite studied in the present work was different as it was isolated from the residual water fraction after extraction of CFCF with ethyl acetate. JA is rather a plant hormone, however, why it is produced by this fungus is unexplored [12]. The novel toxic metabolite studied in the present work was produced in very meager amount by *L. theobromae.* Induction of this toxin metabolite was tried by exogenous addition of salicylic acid and ascorbic acid in the culture medium; however, both of these substances reduced the toxicity of the CFCF even further. Salicylic acid was used because it is known to induce antimicrobial compounds in plants and ascorbic acid was used because it is an antioxidant, which can influence the metabolism, also ascorbic acid is reported to modulate bacteriocidal activity [13].

The purified toxin metabolite, when dissolved in water it gave absorption maxima of 190 nm, however when dissolved in acetic acid, it showed a shift in absorption to 246 nm. The data obtained in UV spectrophotometric analysis is not useful for absolute identification of unknown compound but will be useful for comparative analysis at the time of structural elucidation. The IR pattern of the compound was obtained, however it awaits enough amounts of production and complete characterization. Salicylic acid was used to induce production of mycotoxic metabolite because it is a multifaceted hormone known to modulate production of other toxic metabolite such as coronatine in Pseudomonae syringeae [14]. Ascorbic acid was also used as it is a potent antioxidant and may modulate mycotoxin production in fungus by controlling reactive oxygen species. Further experiments on the induction of this toxic metabolite would be needed to isolate this compound in enough amounts needed for characterization. The preliminary characterization done in the present work surely proves that it is a novel metabolite with a potential application in biotechnology industry. Bioassay based separation protocol developed in the present work could be useful to completely characterize this toxin metabolite.

CONCLUSION

The present study has identified a novel cytotoxic metabolite causing cell death in *S. pombe. L. theobromae* is producing this secondary metabolite after eight days of growth in very meager amount. This metabolite is not inducible by salicylic acid or ascorbic acid; it may be inducible by some bacteria present in its natural habitat. This toxic compound has been isolated and partially characterized.

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REFERENCES:

[1] Nutzmann H-W., Dominguez Y. R., Scherlach K., Schroeckh V., Horn F., Gacek A., Schumann J., Hertweck C., Strauzz J. and Brakhage A.A. (2011), Bacteria –induced natural product formation in the fungus Aspergillus nidulans requires Saga/Ada-mediated histone acetylation *PNAS*, 108 (34):14282 – 14287.

[2] Aldridge D. C., Galt S., Giles D. and Turner W. B. (1971), Metabolites of *Lasiodiplodia theobromae J. Chem. Soc.*, C: 1623- 1627.

[3] Matsuura H., Nakamori K., Omer E.A., Hatakeyama C., Yoshihara T., and Ichihara A. (1998), Three lasiodiplodins from *Lasiodiplodia theobromae* IFO 31059. *Phytochemistry*, 49: 579 – 584.

[4] Yang Q., Asai M., Matsuura H. and Yoshihara T. (2000), Potato micro-tuber inducing hydroxyl lasiodiplodins from *Lasiodiplodia theobromae*. *Phytochemistry*, 54: 489 – 494.

[5] Yoshihara T., Ohmori F., Nakamori K., Amanuma M., Tsutsumi T., Ichhara A and Matsuura H. (2001), Induction of plant tubers and flower buds under noninducing photoperiod conditions by a natural product, Theobroxide. *J of Plant Growth Regulation*, 10: 135- 139.

[6] Creelman R. A. and Mullet J. R. (1995), Jasmonic acid distribution and action in plants: Regulation during development and response to biotic and abiotic stress *Proc. Natl. Acad. Sci.* USA, 92: 4114-4119.

[7] Maslen M.M., Collis T., and Stuart R. (1996), *Lasiodiplodia theobromae* isolated from a subcutaneous abscess in a Cambodian immigrant to Australia. *J of Medical and Veterinary Mycology*, 34: 279 – 283.

[8] Eng F., Guterrez-Rojas M., FavelaTorres E. (1998), Culture conditions for jasmonic acid and biomass production by *Botryodiplodia theobromae* in submerged fermentation. *Process Biochem*,.33 (7): 715 – 720.

[9] Dhandhukia P. C. and Thakkar V. R. (2007), Standardization of growth and fermentation criteria of *Lasiodiplodia theobromae* for production of jasmonic acid. *African J of Biotech*., 6 (6): 707-712.

[10] Thakkar V.R., Subramanian R.B. and Kothari I.L. (2004), Culture filtrate of *Lasiodiplodia theobromae* restricts the development of natural resistance in Brassica nigra plants. *Ind J of Exp Biol*., 42: 111-114.

[11] Kitaoka N., Nabeta K. and Matsuura H. (2009), Isolation and structure elucidation of a new cyclohexenone compound from *Lasiodplodia theobromae*. *Biosci. Biotechnol. Biochem.*, 73 (81): 1890 – 1892.

[12] Tsukada K., Takahashi K. and Nabeta K. (2010), Biosynthesis of jasmonic acid in a plant pathogenic fungus *Lasiodiplodia theobromae*. *Phytochemistry*, 71(17-18): 2019-2023.

[13] Goldschmidt M. C. (1991), Reduced bactericidal activity in neutrophils from scorbutic animals and the effect of ascorbic acid on these target bacteria in vivo and in vitro. *Am J Clin Nutr*., 54: 214 – 220.

[14] Corina A., Maris V., Dempsey A. and Klessig D. (2009), Salicylic Acid, a Multifaceted Hormone to Combat Disease. *Annual Review of Phytopathology* 47 (1): 177-206.

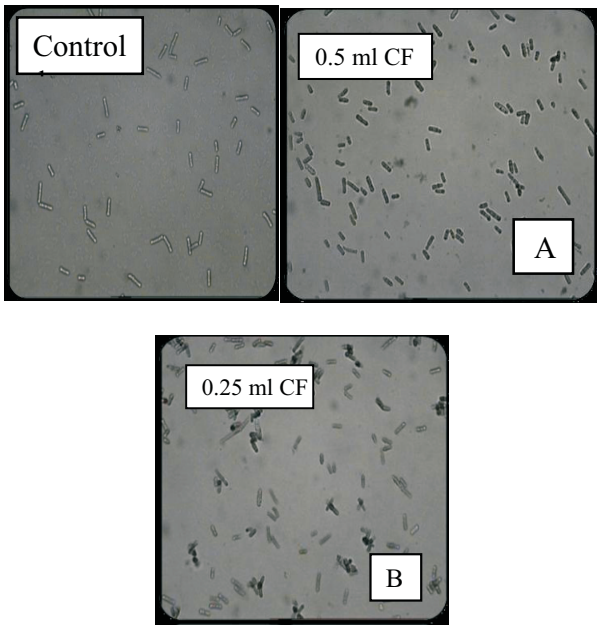

Fig 1: Effect of CFCF of *L. theobromae* on *S. pombe* cells. Dead cells are seen dark where as live cells are seen transparent. Most of the cells are dead in Fig A where as most of the cells are live in Fig B.

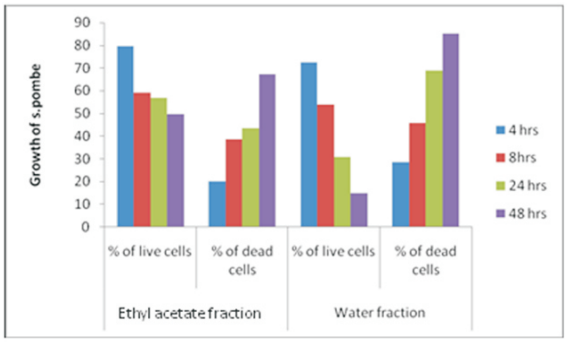

Fig 2: Effect of EtoAc and water fraction of CFCF on *S. pombe* cells after different time intervals.
The toxicity increased with increasing time of incubation and water fraction showed more toxicity than EtoAc fraction.

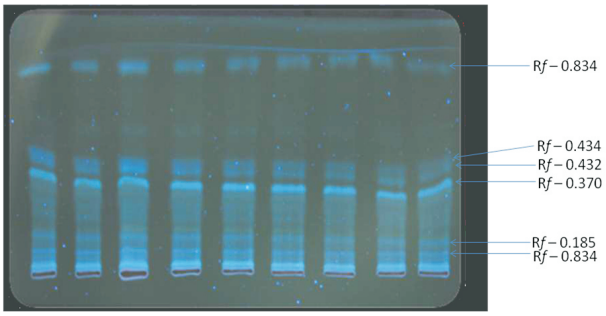

Fig 3: Separation of CFCF by TLC using EtoAc: $CHCl_3$ (1:1) mobile phase

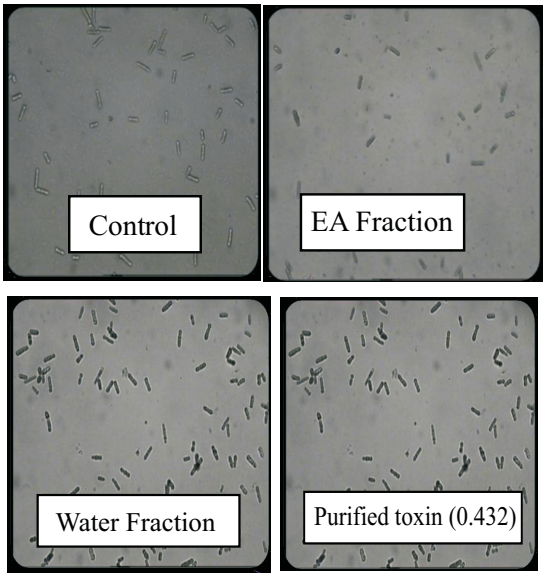

Fig 4: Figure showing effect of crude and purified toxin metabolite/s on *S. pombe* cells after 48 hrs. Water fraction shows more cell death than EtoAc fraction. The purified toxin shows maximum toxicity.

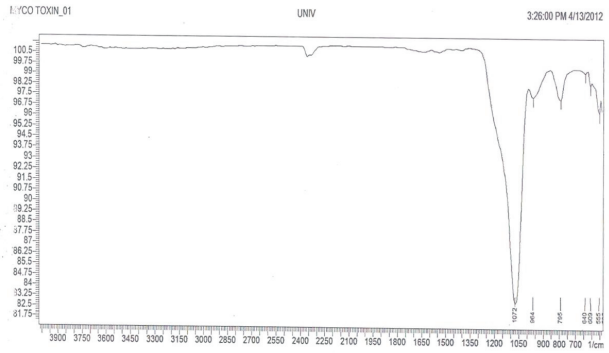

Fig 5: IR analysis of purified toxin metabolite

Sr. No.	Mobile Phase	Number of bands
1	Methanol	1
2	Chloroform	4
3	Ethyl acetate	5
4	Acetone	2
5	Toluene	nil
6	Ethyl acetate: Chloroform (1:1)	6

Table 1: Separation of water fraction of CF by TLC using six different mobile phases

Sr. No.	Solvents	λ max.
1	Water	190
2	Methanol	200
3	Acetic acid	246

Table 2: Showing λ max of the purified toxin in different solvents