Volume-4, Issue-2, Feb-2015 • ISSN No 2277 - 8160

Research Paper | Medical Science

SCREENING AND CHARACTERIZED THE ANTAGONISTIC FUNGUS OF Trichoderma koningii AGAINST Colletotrichum falcatum BY FOOD POISONING TECHNIQUE

Prince. L Prist University, Thanjavur, Tamilnadu, India. *Corresponding author

ABSTRACT

Biocontrol is environmentally safe and in some cases is the only option available to protect plants against pathogens (Cook, 1993). The present study were screened the presence of antagonistic potentials of selected fungal isolates against Colletotrichum falcatum by food poisoning technique and characterized the antagonistic fungus of Trichoderma koningii. Colletotrichum falcatum Went causing Red rot disease in sugarcane was isolated from the infected sett in sugarcane field in Tamilnadu, India. The antagonistic fungus were screening of by dual culture experiments and food poisoning method. The maximum percentage inhibition of growth of C. falcatum was found with Trichoderma koningii (75%) followed by Botrytis cinerea (73.8%), T. glaucum (72.3%), T. viride (70%), Penicillium chrysogenum (69.2%), Gliocladium virens (56.9%), T. harzianum (53.8%) and P. citrinum (23.1%). The potential biocontrol of Trichoderma koningii was molecular characterized. The phylogenetic analysis revealed that 598 bps sequence of ITS region of Hypocrea koningii PPTK was similar (99%) to the existing species of Hypocrea koningii CY059 (GenBank Accession Number HQ607942).

KEYWORDS : Biocontrol, Colletotrichum falcatum, Trichoderma koningii, Dual Culture and Food Poisoning Method.

INTRODUCTION

In India, most of the newly released cultivars to replace the susceptible ones succumbed to the pathogen almost as soon as they become popular due to the frequent emergence of new variants of the pathogen. The sugar industry in India suffers losses of more than 500 million dollars (US) every year due to red rot disease (Padmanaban *et al.*, 1996; Viswanathan, 1997). Sandhu *et al.* (1969) have been estimated the reduction in the sucrose contents and weight of the cane due to red rot disease. Red rot disease caused by the fungal pathogen *Colletotrichum falcatum* Went (Perfect state: *Glomerella tucumanensis* (Speg.) Arx & Muller is a threatening disease of sugarcane, causing severe yield loss in most of the sugarcane-growing states in India (Alexander and Viswanathan, 1996). Plant diseases cause major production and economic losses in agriculture and forestry. The bacterial, fungal and viral infections, along with infestations by insects result in plant diseases and damage. **Biological control** of plant diseases has been considered a viable alternative method to manage plant diseases. Biological control is the inhibition of growth, infection or reproduction of one organism using another organism (Cook, 1993). The present study were screened the presence of antagonistic potentials of selected fungal isolates against *Colletotrichum falcatum* by food poisoning technique and characterized the antagonistic fungus of *Trichoderma koningii.*

MATERIALS AND METHODS

Isolation of pathogen

Colletotrichum falcatum Went causing Red rot disease in sugarcane was isolated from the infected sett in sugarcane field showing the typical symptoms of the disease like lack of germination or poorly grown or wilting seedlings. The standard tissue isolation procedure was followed to isolate the pathogen.

Isolation of soil mycoflora (Dilution Plating Method)

Dilution plating method described by Warcup (1950) was used to isolate the fungi.

Screening of Antagonistic Fungus

Dual Culture Experiments (Skidmore and Dickinson, 1976)

The sterilized potato dextrose agar medium was poured into the petriplates and allowed to solidify. After solidification, colony interaction between the test pathogen *C. falcatum* and some soil fungi were studied in *in vitro* dual culture experiments. The test pathogen *C. falcatum* and the soil fungi such as *Botrytis cinerea, Gliocladium virens, Penicillium chrysogenum, P. citrinum, Trichoderma glaucum, T. koningii, T. harzianum* and *T. viride* the fungal and plant pathogen were grown separately on PDA medium. Then agar blocks cut from the actively growing margin of the individual species of plant fungi and test organism were inoculated just opposite to each other approximately 3 cm apart on potato dextrose agar medium in petriplates. Three replicates for each set were maintained. Controls were set in single and dual inoculated culture of the fungus. The position of the colony margin on the back of the disc was recorded daily. The measurement was taken on the fifth day. Assessments were made when the fungi has achieved an equilibrium after which there was no further alteration in the growth. Since both of the organisms were mutually inhibited, the assessment was made for both organisms.

The percentage inhibition of growth was calculated as follows.

$$= \frac{r - r^1}{r} \times 100$$

r = growth of the fungus was measured from the centre of the colony towards the centre of the plate in the absence of antagonistic fungus.

r^1= growth of the fungus was measured from the centre of the colony towards the antagonistic fungus.

The colony interaction between the test pathogen and the soil fungi were assessed following the model proposed by Porter (1924) and Dickinson and Broadman (1971). Five type of interactions grade as proposed by Skidmore and Dickinson (1976) have been used.

Types are as follow

1. Mutual intermingling without any macroscopic sights of interaction – Grade 1.
2. Mutual intermingling growth where the growth of the fungus is ceased and being over growth by the opposed fungus – Grade 2.
3. Intermingling growth where the fungus under observation is growing in to the opposed fungus either above (or) below – Grade 3.
4. Sight inhibition of both the interacting fungi with narrow demarcation line (1-2) – Grade 4.
5. Mutual inhibition of growth at a distance of >2mm – Grade 5. Based on the biocontrol potentials three fungal organisms were selected for further investigation.

Food poisoning method

Agar blocks of equal size (5 mm dia) cut from the actively growing margin of the pathogenic fungus *C. falcatum* and the biocontrols *Botrytis cinerea, Trichoderma glaucum* and *T. koningii* were inoculated separately into 250 ml conical flasks containing 100 ml of sterilized potato dextrose broth medium. The flasks were incubated at 25±2°C for 15 days. After 15 days of incubation, the staling substances were filtered first through Whatman No.1 filter paper and then

through Seitz filter (GS). The filtrates were transferred aseptically into sterile conical flask, condensed and stored at 4°C for further use.

The culture filtrates prepared in such a way were added separately to the cooled potato dextrose agar medium to give the concentrations of 5, 10, 15 and 20 per cent and allowed to solidify. After solidification 5 mm agar blocks cut from the actively growing margin of the test fungus (*C. falcatum*) was inoculated at the center of each plate. The plates were incubated at 25 ± 2°C for five days. The radial growth was measured periodically and the mean growth rate was calculated. Control was also maintained. The percentage of inhibition of growth was calculated as follows:

$$\text{Percentage of inhibition of growth} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

Molecular characterization of potential biocontrol

Isolation of Genomic DNA (Murray and Thompson, 1980)

The potential biocontrol fungal cultures were inoculated in PD broth at 28°C for 5 to 7 days, the fungi were centrifuged at 5000g for five minutes and ground using a mortar containing 600 µL CTAB lysis buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris, 20 mM EDTA, 1% PVP) at 65°C. The mycelial mixture was transferred into a 1.5-mL eppendorf tube and heated to 65°C for 30 minutes, extracted twice with an equal volume of phenol/ chloroform/isoamyl alcohol (25:24:1) and washed with chloroform / isoamyl alcohol (24:1). After centrifugation at 10,000×g for 5 minutes, the supernatant was transferred to a new microtube and precipitated by adding equal volume of isopropanol at −20°C for one hour. Finally, the DNA pellets were collected by centrifugation (12000g, 15 minutes), washed with 75% ethanol twice and re-suspended in 40 µL TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0). RNA was removed by adding 2 µl of RNase A (10 mg/ml; Invitrogen) at 60°C for 10 minutes.

PCR Amplification of ITS region

The ITS region of fungal DNA was amplified using following the fungal primer set:

Forward primer: ITS-1, FWD_SEQ: TCCGTAGGTGAACCTGCGG

Reverse primer: ITS-4, REV_SEQ: TCCTCCGCTTATTGATATGC

Amplifications were performed in 100 µl PCR reaction tubes containing 5µl DNA, 10 µl 10X thermophilic buffer, 8 µl MgCl2, 4 µl dNTP, 10 µl of each primer, 52 µl double distilled water and 1 µl Taq polymerase. Amplification was done in the thermal cycler (iCycler; Bio-Rad Laboratories, CA) for 35 cycles with initial denaturation temperature 94°C for one minute 30 seconds, melt temperature 95°C for 35 seconds, annealing temperature 55°C for 55 seconds, extending temperature of 72°C for one minute, final extension temperature 72°C for ten minutes, hold temperature 4°C (Jasalavich *et al*., 2000). The ITS bands were identified by gel electrophoresis on a 2% agarose.

Purification of PCR products

Amplified PCR product (ITS region) were eluted from the gel and then melted at 55°C with 3 volumes of 6 M guanidine thiocyanate. 20 to 30 microliters of silica mixture (1.5 % agarose gel in 1 X TAE buffer (40mM Tris – acetate, 1mM EDTA)) was added and incubation was continued for 5 mins at 55°C. After cooling on ice, the silica was pelleted, washed twice in cold 80% ethanol, and dried in a heating block (55°C) for one to two minutes. The PCR fragments were eluted from the silica in 20 to 25 µl of distilled water for three minutes at 55°C.

Gene sequencing

The purified PCR products were sequenced using ABI 3500XL sequencer according to manufacturers' instructions (ABI 3500XL Genetic Analyzer). Sequences were aligned by Bioedit software.

Phylogenetic analysis

The sequence of ITS region of selected fungal strain was compared against the sequences available from GenBank using the BLASTN program and were aligned using CLUSTAL W software developed by Higgins *et al.* 1992. Phylogenetic analysis was constructed using the Neighbour Joining method Saitou and Nei (1987).

Secondary structure prediction

The secondary structure of selected fungal strain was predicted by using Genebee structure prediction software available in online (www.genebee.msu.su/service/ma2-reduced.html).

Restriction site analysis

The restriction sites in ITS regions of selected fungal culture were analyzed by using NEB cutter program version 2.0 tools in online (www.neb.com.NEBCutter2/index.php).

RESULTS

The fungal colony on potato dextrose agar consisted of abundant aerial mycelium, densely woven into compact, velvety turf in some isolates or a cottony, floccose one in another colour ranges from almost white through light ashy grey to dark grey, growing darker with age, no colour or pigmentation in reverse or in the medium. The conidia are single celled, hyaline, falcate or sickle shaped with one end rounded and the other slighted pointed. Conidia are borne either on acervuli or directly on the hyphal tips. The hyphae are hyaline, septate and contain oil globules. The acervuli consisted of a mass of short, aggregated condiophores with numerous dark brown to black setae. The pinkish spore mass was embodied in a mucilaginous matrix in the acervuli. The telemorphic stage of the fungus has been reported to occur rarely on the leaf lamina, midrib portion and leaf sheath and also in culture. The fruiting body of the perfect stage is a perithecium. The perthecia with numerous asci and paraphyses are embedded in the cane tissue but for the small protruding ostioles, which are almost in conspicuous. They are mostly found between the vascular bundles on dead or drying leaf blades, midribs and leaf sheaths.

The type of interactions between the pathogen and the soil fungi were observed and presented in Table 1 (1 to 2 mm). The maximum percentage inhibition of growth of *C. falcatum* was found with *Trichoderma koningii* (75%) followed by *Botrytis cinerea* (73.8%), *T. glaucum* (72.3%), *T. viride* (70%), *Penicillium chrysogenum* (69.2%), *Gliocladium virens* (56.9%), *T. harzianum* (53.8%) and *P. citrinum* (23.1%) (Table 2).

The genomic DNA of *Hypocrea koningii* PPTK was isolated by the method of described by Murray and Thompson, 1980. The molecular characteristics of potential antagonist *Hypocrea koningii* were evaluated by PCR amplification of ITS region. The ITS region gene sequences of *Hypocrea koningii* PPTK obtained in this study were deposited in GenBank under the accession number JQ418547. The topology of the NJ tree inferred from the whole dataset clearly illustrates the very strong signal of ITS region of the species level in genus *Hypocrea*. There were a total of 575 positions in the final dataset. The overall tree topology suggests that the tree is divided into two main clades namely A, B. The clade A with nine taxa including the test strain *Hypocrea koningii* PPTK. The clade B had totally 12 taxa showed higher bootstrap value of 100% (Figure. 1).

The secondary structure of ITS region of *Hypocrea koningii* PPTK showed 25 stems, 14 bulge loops and 10 hairpin loops in their structure. The restriction sites of *Hypocrea koningii* PPTK were shown in Figure 2. A large number of restriction sites were found in the potential antagonist. The total restriction enzyme sites of *Hypocrea koningii* PPTK was 49. However, the cleavage sites and the nature of restriction enzymes differed from one another. The GC and AT content of *Hypocrea koningii* PPTK was found to be 55 and 45% respectively using NEB Cutter Programme V 2.0 in www.neb.com/nebcutter2/index.php.

DISCUSSION

In the present study, *Colletotrichum falcatum* were isolated from the rhizosphere of sugarcane soil. The cultural characteristic of *Colletotrichum falcatum* on potato dextrose agar observed abundant aerial mycelium, densely woven into compact, velvety turf in some isolates or a cottony, floccose one in another colour ranges from almost white through light ashy grey to dark grey, growing darker with age, no colour or pigmentation in reverse or in the medium. The morphological diversity has been found in *Colletotrichum* sp. pathogenic to various crops (Elmer *et al.,* 2001; Lardner *et al.,* 1999; Talhinhas *et al.,* 2002a,

b). Similar results have been reported for *Colletotrichum* species from chilli (Than *et al.,* 2008).

Red rot disease caused by *C. falcatum* is one of the most serious threats to sugarcane cultivation in India (Satyavir, 1976; Beniwal *et al.,* 1989; Duttamajumdar, 2008; Alexander and Viswanathan, 1996). It can reduce cane weight by up to 29% and loss in sugar recovery by 31% (Hussnain and Afghan, 2006). The disease was first described by Went in 1893 from Indonesia. Thind and Jhooty, (1990) successfully used morphological and pathological characteristics to categorize 150 isolates of *C. capsici* and *C. gloeosporoides* causing chilli anthracnose.

The investigation of colony interactions between some soil fungi and *Colletotrichum falcatum* showed maximum percentage of inhibition in *C. falcatum* with *Trichoderma koningii* (75%) and this is in agreement with the findings of Weindling (1934). *Trichoderma* as antagonist controlling wide range of microbes was well documented and demonstrated for more than seven decades ago, but their use under field conditions came much later (Chet *et al.,* 1997), and their mechanism of mycoparasitism is much more complex, involves nutrient competition, hyperparasitism, antibiosis, space and cell wall degrading enzymes. *Trichoderma* have long been recognized as agents for the control of plant disease and for their ability to increase plant growth and development. The earlier studies also revealed that antimicrobial metabolites produced by *Trichoderma* is effective against a wide range of fungal phytopathogens eg., *Fusarium oxysporum, Rshizoctonia solani, Curvularia lunata, Bipolaris sorokiniana and Colletotrichum lagenarium, Colletotrichum acutatum, Colletotrichum gloeosporioides* (Yan *et al.,* 2006, Svetlana *et al.,* 2010). *Trichoderma koningii, Botrytis cinerea* and *Trichoderma glaucum* fungal strains appeared to be most effective against the *Colletotrichum falcatum*.

The most effective fungal strains such as *Trichoderma koningii, Botrytis cinerea*, and *Trichoderma glaucum* were selected for food poisoning technique. The maximum percentage of inhibitory effects was observed in *Trichoderma koningii* (80.76%) compared to *Botrytis cinerea* (76.92%) and *Trichoderma glaucum* (69.23%). It have been reported and well documented that *T.viridae* were used as potential antagonist for controlling many fungal plant pathogens such as *Colletotrichum acutatum, Colletotrichum falcatum, Fusarium oxysporum* (Porras *et al.,*2008 and Deshmukh *et al.,* 2010).

Trichoderma hypocrea is a genus of soil-borne or wood-decaying fungi containing members important to mankind as producers of industrial enzymes and biocontrol agents against plant pathogens, but also as opportunistic pathogens of immunocompromised humans. Species identification, while essential in view of the controversial properties of taxa of this genus, has been problematic by traditional methods. Although originally introduced by Persoon, (1794), the taxonomy and identification of *Trichoderma* has remained problematic until relatively recently. Until 1969, nearly all strains of *Trichoderma* were identified in literature as "*T. viride*" owing to Bisby's (1939) concept that *Trichoderma* consists of a single species. This led to the erroneous statement even in textbooks that "*T. viride* is an industrial cellulose producer". The molecular characteristics of potential antagonist *Hypocrea koningii* were evaluated by PCR amplification of ITS region. The amplified products were separated by agarose gel. The ITS region gene sequences of *Hypocrea koningii* PPTK obtained in this study were deposited in GenBank under the accession number JQ418547. The phylogenetic analysis revealed that 598 bps sequence of ITS region of *Hypocrea koningii* PPTK was similar (99%) to the existing species of *Hypocrea koningii* CY059 (GenBank Accession Number HQ607942).

The advent of molecular tools for investigations in fungal taxonomy prompted research in the mid-nineties to re-assess the morphology-based taxonomy in *Trichoderma*. The laboratories of G.J. Samuels (Beltsville, MD, USA), T. Börner (Berlin, FRG) and C.P. Kubicek (Vienna, Aust.) collaboratively pioneered a revision of Bissett's section *Longibrachiatum*. They combined the use of molecular markers (ITS1 and ITS2 sequence analysis, RAPD), physiological (isoenzyme analysis) and phenetic characters and also for the first time included an analysis of potential teleomorphs of the *Trichoderma* sp. from this section (Kuhls *et al.,* 1996; 1997; Samuels, 1996; Samuels *et al.,* 1998; Turner *et al.,* 1997). The secondary structure of ITS region of *Hypocrea koningii* PPTK showed 25 stems, 14 bulge loops and 10 hairpin loops in their structure. The free energy of ITS region of *Hypocrea koningii* PPTK secondary structure was found to be -141.8 kkal/mol. (Kuhls *et al.,* 1996; Kuhls *et al.,* 1996). The restriction sites of *Hypocrea koningii* PPTK. A large number of restriction sites were found in potential antagonist. However, the cleavage sites and the nature of restriction enzymes differed from one another. The GC and AT content of *Hypocrea koningii* PPTK was found to be 55 and 45% respectively using NEB Cutter Programme V 2.0 in www.neb.com/nebcutter2/index.php. Finally concluded that the combined use of compatible strains of biocontrol agents is the most effective and appropriate method for effective disease management in sugarcane.

Table 1. Types of interaction of the pathogen and soil fungi

S. No	Soil fungi	Type of Interaction
1	Botrytis cinerea	Grade I
2	*T. koningii* and *T. glaucum*	Grade II
3	*Gliocladium virens* and *P. chrysogenum*	Grade III
4	*T. viride* and *T. harzianum*	Grade IV
5	P. citrinum	Grade V

Table 2. Colony interactions between *Colletotrichum falcatum* and some soil fungi in dual culture experiments

Growth response of the antagonistic and test fungi	Antagonistic Fungi Tested							
	T. v	B. c	P. ch	P. c	T. h	T. g	Glio	T. k
Colony growth of the pathogen towards antagonist (mm)	19.0	17.0	20.0	50.0	30.0	18.0	20.0	16.0
Colony growth of the pathogen away from the antagonist (mm)	21.0	18.5	24.5	55.0	37.0	22.0	24.0	19.0
% growth inhibition of the pathogen in the zone of interaction	70.0	73.8	69.2	23.1	53.8	72.3	56.9	75.0
Colony growth of the antagonist in control *i.e* growth towards the center of the plate in the absence of the pathogen (mm)	73.0	74.0	71.0	65.0	65.0	73.0	62.0	73.0
Colony growth of the antagonist towards the pathogen (mm)	63.0	66.0	63.0	27.0	51.0	65.0	50.0	67.0
Colony growth of the antagonist away from the pathogen (mm)	55.0	56.0	56.0	19.0	45.0	51.0	41.0	58.0
% growth inhibition of antagonistic in the zone of interaction	13.6	10.8	11.2	58.4	21.5	10.9	11.2	8.2

T.v - Trichoderma viride, B.c - Botrytis cinerea., P.chry - Penicillium chrysogenum, P.c - Penicillium citrinum, T.h - Trichoderma harzianum, T.g - Trichoderma glaucum, Glio - Gliocladium virens, T.k - Trichoderma koningii

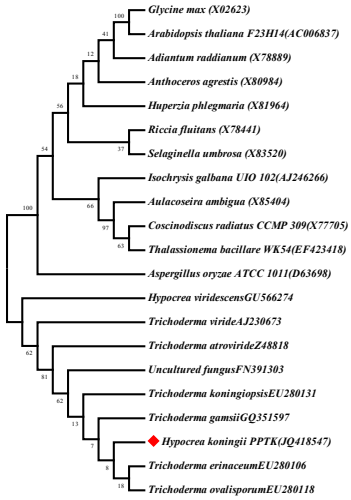

Figure 1. Phylogenetic analysis

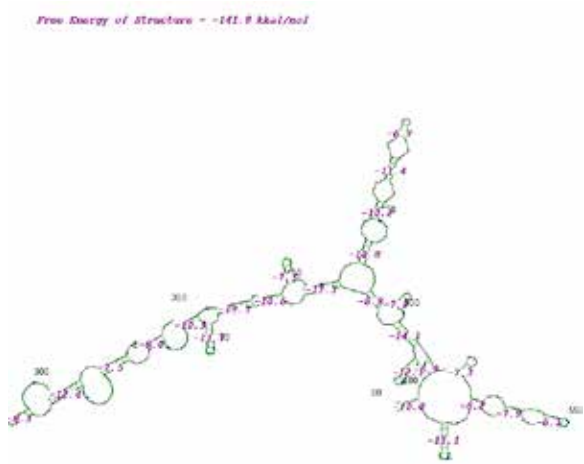

Figure 2. Secondary Structure Prediction

REFERENCES

Alexander, KC. and Viswanathan, R., 1996. Major diseases affecting sugarcane production in India and recent experiences in quarantine. In: Croft BJ, Piggin CM, Wallis ES, Hogarth DM. (eds) Sugarcane Germplasm Conservation and Exchange. Canberra, Australia, Australian Centre for International Agricultural Research, Proceedings., 67: 46–48. | Alexander, KC. and Viswanathan, R., 1996. Major diseases affecting sugarcane production in India and recent experiences in quarantine. In: Croft BJ, Piggin CM, Wallis ES, Hogarth DM. (eds) Sugarcane Germplasm Conservation and Exchange. Canberra, Australia, Australian Centre for International Agricultural Research, Proceedings.,67: 46–48. | Beniwal, M.S., Satyavir Virk, K.S., 1989. Pathogenic variability in Colletotrichum falcatum incitant of red rot of sugarcane. Indian Phytopathol 42: 95-99. | Bisby, G.R., 1939. Trichoderma viride Pers. ex Fries, and notes on Hypocrea . Trans Brit. Mycol. Soc., 23: 149-168. | Chet, I., Inbar, J. and Hadar, I., 1997. Fungal antagonists and mycoparasites. In: The Mycota IV: Environmental and Microbial Relationships. Wicklow DT and Soderstorm B, eds., 165-184. | Cook, R.J., 1993. Making greater use of introduced microorganisms for biological control of plant pathogens. Annu. Rev. Phytopathol., 31: 53-80. | Deshmukh, A.J., Mehta, B.P. and Patil, V.A., 2010. In-vitro evaluation of some known bioagents to control C. gloeosporioides Penz, and Sacc, causing Anthracnose of Indian bean. Journal of Pharma and Bio sciences., 1(2): 1-6. | Dickinson, C.H. and Broadman, F., 1971. Physiological studies of Some Fungi isolated from peat. Trans. Br. Mycol, Soc., 55: 293 -305. | Duttamajumdar, S.K., 2008. Red Rot of Sugarcane. Lucknow India, Indian Institute of Sugarcane Research, Lucknow, India, 46. | Elmer, W.H., Yang, H.A. and Sweetingham, M.W., 2001. Characterization of Collectotrichum gloeosporioides isolates from ornamental lupines in Connecticut. Pl. Dis., 85: 216-219. | Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution., 39: 783-791. | Higgins, D., Bleasby, A. and Fuchs, R., 1992. ClustalV: Improved software for multiple sequence alignment. Comput. Applic. Biosci., 8: 189-191. | Hussnain, Z., Afghan, S., 2006. Impact of Major Cane Diseases on Sugarcane Yield and Sugar Recovery. Jhang, Pakistan, Shakarganj Sugar Research Institute, Annual report. | Jasalavich, C., Ostrotsky, A. and Jellison, J., 2000. Detection and identification of decay fungi in spruce wood by restriction fragment length polymorphism analysis of amplified genes encoding rRNA. Appl. Environ. Microbiol., 66(11):4725-4734. | Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution., 16: 111-120. | Kuhls, K., Lieckfeldt, E., Samuels, G.J., Kovacs, W., Meyer, W., Petrini, O., Gams, W., Börner, T. and Kubicek, C. P., 1996. Molecular evidence that the asexual industrial fungus Trichoderma reesei is a clonal derivate of the ascomycete Hypocrea jecorina. Proc Natl. Acad. Sci. USA., 93: 7755-7760. | Kuhls, K., Lieckfeldt, E., Samuels, G.J., Meyer, W., Kubicek, C.P. and Börner, T., 1997. Revision of Trichoderma sec. Longibrachiatum including related teleomorphs based on analysis of ribosomal DNA internal transcribed spacer regions. Mycologia., 89: 442-460. | Lardner, R., Johnston, P.R., Plummer, K.M. and Pearson, M.N., 1999. Morphological and molecular analysis of Colletotrichum acutatum sensu lato. Mycol. Res. 103: 275-285. | Murray, HG. and Thompson, W.F., 1980. Rapid isolation of high molecular weight DNA. Nucleilc Acids Res., 8: 4321-4325. | Padmanaban, P., Mohanraj, D., Viswanathan, R., Madhusudhana Rao, M., Prakasam, N., Jothi, R. and Alexandar, K.C., 1996. Differential interaction of sugarcane varieties to pathotypes of Colletotrichum falcatum Went. Sugarcane., 4: 16-20. | Persoon, C.H., 1794. Disposito Methodica Fungorum in Classes, Ordines, Familias et Genera. In: Romer JJ, editor. Neues Magazin fur Botanik. Zurich: Ziegler and Sohne., 63-128. | Porras, M., Barrau, C. and Romero, F., 2008. Biological Control of Anthraconse with Trichoderma in Strawberry fields, ISHS Acta Horticulture: VI International Strawberry Symposium., 842. | Porter, C.L., 1924. Concerning the characters of certain fungi as exhibited by their growth in the presence of other Fungi. Am. J. Bot., 11:168-188. | Saitou, N. and Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406-425. | Samuels, G.J., 1996. Trichoderma: A review of biology and systematics of the genus. Mycol. Res., 100: 923-935. | Samuels, G.J., Petrini, O., Kuhls, K., Lieckfeldt, E. and Kubicek, C.P., 1998. The Hypocrea schweinitzii complex and Trichoderma sect. Longibrachiatum . Stud Mycol., 41: 1-54. | Sandhu, S.S., Bhatti, D.S. and Ratan, B.K., 1969. Losses in sugarcane due to red rot and smut diseases. J. Res. Punjab Univ. Ludhiana., 6(2): 341-344. | Satyavir, (1976). Red rot of sugarcane-Current scenario. Ind Phytopathol., 56: 245-254. | Skidmore, A.M. and Dickinson, C.M., 1976. Colony interactions and hyphal interferences between Septoria nodorum and phylloplane fungi. Trans. Br. Mycol, Soc., 66: 57-64. | Svetlana Zivkovic, S., Stojanovic, Z., Ivanovic, V., Gavrilovic Tatjana popovic and Jelica balaz, 2010. Screening of antagonistic activity of microorganisms against Colletotrichum acutatum and Colletotrichum gloeosporioides, Archives of Biological Science., Belgrade, 62(3): 611-623. | Talhinhas, P., Sreenivasaprasad, S., Neves-Martins, J. and Oliveira, H., 2002a. Genetic and morphological characterization of Colletotrichum acutatum causing anthracnose of lupin. Phytopathol., 92: 986-996. | Tamura, K., Dudley, J., Nei, M. and Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution., 24: 1596-1599. | Than, P.P., Jeewon, R., Hyde, K.D., Pongsupasamit, S., Mongkolporn, O. and Taylor, P.W.J., 2008. Characterization and pathogenicity of Colletotrichum species associated with anthracnose disease on chilli (Capsicum sp.) in Thailand. Pl. Pathol. 57: 562-572. | Thind, T.S. and Jhooty, J.S., (1990). Studies on variability in two Colletotrichum species Causing anthracnose and fruit rot of chillies in Punjab. Indian Phytopathol., 43: 53-58. | Turner, D., Kovacs, W., Kuhls, K., Lieckfeldt, E., Peter, B., Arisan-Atac, I., Strauss, J., Samuels, G.J., Börner, T. and Kubicek, C.P., 1997. Biogeography and phenotypic variation in Trichoderma sect. Longibrachiatum and associated Hypocrea species. Mycol. Res., 101: 449-459. | Viswanathan, R., 1997. Induction of antifungal pathogens related proteins in sugarcane. Sugarcane Breeding Institute Newsletter., 18: 1-3. | Warcup, J. H., 1950. The soil-plate method for isolation of fungi from soil. Nature (London) 146: 117. | Weinding, R., 1934. Studies on a lethal principle effective in the parasitic action of Trichoderma lignorum on Rhizoctonia solani and other soil fungi. Phytopath., 24: 1153-1179. | Yan, J., Wu, PS., Du, HZ., Zhang, QE., 2006. First report of black spot caused by Colletotrichum gloeosporioides on paper mulberry in China. Plant Disease, 95: 880. |