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Research Article

SCREENING AND UTILIZATION OF FUSARIC ACID (FA) FROM *FUSARIUM OXYSPORUM*

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ABSTRACT
Fusarium oxysporum strains are ubiquitous soil inhabitants that have the ability to exist as saprophytes, and degrade lignin and complex carbohydrates associated with soil debris. Fusarium toxins are produced by over 50 species of *Fusarium* and have a history of infecting the grain of developing cereals such as wheat and maize. They include a range of mycotoxins, such as: fusaric acid; the fumonisins, beauvercin and enniatins, butenolide, equisetin, and fusarins. Rapid growth rate of fungi and increased fungal biomass was obtained in Potato dextrose agar medium where the mixed populations of microbes exist from the natural soil sample. Discrete colonies of various fungal species were obtained. Fusarium oxysporum was identified microscopically by slide preparation and its visual spore structure. This fungus was individually isolated in PDA slants and stored in refrigerated conditions. The fungus was scraped and dried and its mycotoxins fusaric acid was analyzed by HPLC equipped with a reverse phase column packed with nucleosil 120 -5 C18 and set at 50'C. The samples were eluted with linear methanol. Fusaric acid can be scientifically applied in agricultural aspects. At present its positive role has dominated the agricultural biotechnology and breeding programmers. The wide scope and applications of fusaric acid is a wonderful route for improvement of plants and agriculture as a whole.

Key Word: *Fusarium Oxysporum, Fusaric Acid, Agriculture*

INTRODUCTION
Fusarium oxysporum is a remarkably diverse and adaptable fungi have been found in soils ranging from the Desert, to tropical and temperate forests, grasslands and soils of even the tundra *Fusarium oxysporum* strains are ubiquitous soil inhabitants that have the ability to exist as saprophytes, and degrade lignin and complex carbohydrates associated with soil debris. They are also pervasive plant endophytes that can colonize plant roots and may even protect plants or be the basis of disease suppression. Although the predominant role of these fungi in native soils may be as harmless or even beneficial plant endophytes or soil saprophytes, many strains within the *Fusarium oxysporum* complex are pathogenic to plants, especially in agricultural settings.
Pathogenic strains of *Fusarium oxysporum* have been studied for more than 100 years. Fusarium toxins are produced by over 50 species of *Fusarium* and have a history of infecting the grain of developing cereals such as wheat and maize. They include a range of mycotoxins, such as: fusaric acid (Rani *et al.*, 2009); the fumonisins, which affect the nervous systems of horses and may cause cancer in rodents; the trichothecenes (Miller *et al.*, 1991), which are most strongly associated with chronic and fatal toxic effects in animals and humans; and zearalenone, which is not correlated to any fatal toxic effects in animals or humans. Some of the other major types of *Fusarium* toxins include: beauvercin and enniatins, butenolide, equisetin, and fusarins.

MATERIALS AND METHODS
Sample Collection
Soil samples were collected from a garden area in Jaipur city, Rajasthan, India. The equipments needed for collection of samples should cause least disturbance to the soil during collection viz. sterilized polythene bags, scoop, spade or shovel. Collection was done in sterilized polythene bags and labeled according to area. These fresh samples were inoculated soon after collection.

Media Type, Preparation* and *Sterilization

Growth of fungi requires nutrients and environmental conditions. Nutrient preparations made in laboratory used for growth of the organism are called culture medium. In the present investigation, solid Potato Dextrose Agar (PDA) medium has been used for the surface growth of fungi in order to (1) observe colony appearance (2) identify fungal species from mixed consortium/polyculture in petriplate (3) lot storage of cultures, and (4) to observe biochemical reactions.

PDA (Potato Dextrose Agar) medium was prepared and autoclaved by standard laboratory methods. This is a General-purpose media used for the culturing fungi. Sterilizing of the PDA medium to above 121^0Celcius for 15 minutes in an autoclave destroys nearly all living cells and spores. Container used was conical flask (500 ml) which was capable of holding intended volume of medium and allow expansion during sterilization.

Pouring the PDA Plate

PDA plates were prepared by pouring of media in laminar air flow hood under sterilized conditions. Pouring was done immediately into a sterile, dry petri plate while holding the top carefully above the petri plate bottom in order to avoid contamination. This was done utilizing aseptic techniques in a sterile cabinet (laminar air flow cabinet). The top was replaced, allowing the agar to cool and harden, and storing petriplate in laminar air flow. Solidification of media was attained after 30 minutes. Stacking of the plate's right side up was done.

Serial Dilution of Sample

The soil sample was diluted by serial dilution technique. As, for instance the number and size of fungal colonies that grow on an agar plate in a given time is concentration-dependent, and since many other diagnostic techniques involve physically counting the number of micro-organisms or cells on specials printed with grids (for comparing concentrations of two organisms or cell types in the sample) or wells of a given volume (for absolute concentrations), dilution has been be useful for getting more manageable results. Serial dilution is also a cheaper and simpler method for preparing cultures than optical tweezers and micromanipulators. Serial dilution was done here to isolate propagule of micro fungi occurring inactively in the soil.

The first step in making a serial dilution was to take a known weight (1gm) of soil called stock and place it into a known volume of distilled water (10 ml). This produced 1/10 dilution of soil sample. This 1/10 sample was utilized for further dilution. Its 1ml was transferred to 9 ml sterilized water making dilution of 1/100. Further 1ml of 1/100 was added to 9 ml sterilized water making 1/1000. Thus, a dilution of 10^{-3} was prepared.

Inoculation by Spread Plate Method

Spreading was done using a bent glass rod. 1mL of diluted soil suspension was placed in the center of the plate using a sterile pipet. The glass rod spreader was sterilized by first dipping it into a 70% alcohol solution and then passing it quickly through the Bunsen burner flame. The burning alcohol sterilizes the rod at a cooler temperature than holding the rod in the burner flame. When all the alcohol was burned off and the rod was air-cooled, the rod was moved back and forth across the plate working up and down several times. The plate was turned 90 degrees and the side to side, up and down spreading was repeated. The plate was turned 45 degrees and spread a third time. The glass rod was not sterilized between plate turnings. The plate was covered.

1ml of 10^{-3} diluted sample was transferred to PDA plate. The fungal spores were then distributed evenly over the surface. The sample was uniformly spread on the PDA surface and incubation was done at 30°C. This method was utilized for rapid growth rate of fungi and increased fungal biomass.

This method is often used when the mixed populations of microbes exist in a natural sample (i.e. soil) and to obtain discrete colonies.

Slide Preparation* and *Morphological Identification of Fungi

The mixed fungal colonies initiated after 3-4 days of incubation at 30^0C, which contain varied fungal species. These were incubated further for 10 days, for colony development and maturation. From these

mixed colonies, single fungal species could be identified microscopically based on its morphological (size/shape/color) differences etc. Adhesive tape impressions for microscopic examination were taken from colonies on petridish. Tape was slightly pressed onto suspected fungal growth surfaces and mounted onto microscope slides. The stain solution cotton blue + lacto phenol were applied directly to the tape mounted on the slide, and a cover slip was placed. This is a direct staining procedure followed by microscopic visualization of spore morphology.

Isolation of Fusarium Oxysporum from Fungal Consortium

PDA Slant Preparation:

The slants were prepared by pouring liquefied and autoclaved PDA in test tubes. These tubes were kept in slanting position utilizing a base. After solidification they were used for inoculation.

Isolation Process:

The fusarium oxysporum colony after identification was touched by inoculating loop and streaked on PDA slant medium. This was incubated for 10-15 days at 30°C and obtained as pure culture which was not contaminated by any other species.

Collection of Fungal Biomass from Pure Culture Slant:

The fungal pure culture layer is scraped out using scooper and added into sterilized water. This was taken in test tubes and centrifugation was done for 15 minutes. The supernatant was filtered and stored. In addition, the supernatant was acidified to pH 2 and shaken vigorously for 1 minute. This was dried in incubator at 30°C.

HPLC Technique:

The dry residue after 1 hour was dissolved in 1ml of methanol and analyzed by HPLC equipped with a reverse phase column packed with nucleosil 120 -5 C18 and set at 50'C. The samples were eluted with linear methanol. The fraction containing the FA standard from the HPLC was collected, and the presence of FA was qualitatively and quantitatively confirmed by Mass spectroscopy on a TSQ 3000 triple quadrupole mass spectrometer with electro spray ionization.

RESULTS AND DISCUSSION

Macroscopic Features

Fusarium oxysporum colonies were usually fast growing on potato dextrose agar at 30°C maturing within 4 days to pale colonies and may be felty, cottony or wooly or sparse and wet-looking. The colony form was circular, irregular to filamentous, with flat or raised elevation. The margin was undulate or filiform. The color of the thallus varied from whitish to yellow, to pink shades.

Microscopic Features

Hyaline septate hyphae, conidiophores, phial ides, macro conidia, and micro conidia were observed microscopically. *Fusarium oxysporum* had septate hyphae with two types of condition: unbranched or branched conidiophores with phial ides that produce large, sickle- or canoe-shaped macro conidia and long or short simple conidiophores bearing small oval, 1 or 2 celled micro conidia singly or in clusters (Summerell *et al.,* 2003).

Fusaric acid is mycotoxins with low to moderate toxicity, which is of concern since it might be synergistic with other co-occurring mycotoxins (Bai, 1997). Fusaric acid is widespread on corn and corn-based food and feeds and is frequently found in grain, where Fusarium species are also isolated causing diseases (Bottalico, 1998).

Fusaric acid (FA) is a mycotoxins produced by the Fusarium species, among which the most high yielding was reported to be *Fusarium oxysporum* (Bacon *et al.,* 1996). It is moderately toxic to animals. It has antibiotic, insecticidal and pharmacological activity. Fusarium species are found worldwide in soil as both pathogenic and non-pathogenic strains. Large concentrations of fusaric acid reduce growth of root and root tubers. The biosynthesis of fusaric acid involves condensation reaction of polyacetate and

aspartic acid units. The assay involves HPLC, TLC, Mass spectroscopy and NMR techniques. Fusaric acid acts as an enzyme inhibitor, dopamine agent and nucleic acid synthesis inhibitor. As an orally active agent, it may have potential role in the treatment of head and neck squamous cell cancer (HNSCC). Fusaric acid (FA) is a host non-specific toxin. The high production of which has been correlated with the Virulence of plant Pathogenic strains of Fusarium species. FA (5-butylpicolinic acid) was first discovered during laboratory culture of Fusarium heterosporumnees (Rani *et al.,* 2009). It has a natural contaminant or mycotoxins accumulating during infection in corn and cereal grains are extremely toxic to animals and human beings by enhancing toxicity of other Fusariummetabolites EG-: Trichothecenes (Langseth and Rundberget, 1998).

It is not only moderately toxic to animals but also has antibiotic, insecticidal and pharmacological activity in both brain and pineal neurotransmitter and metabolites are affected it cause wilt disease symptoms in pepper, corn and used to select with resistance in plant. The most expansive producer of this toxin is *Fusarium oxysporum* or, its special forms Fusarium Species lycopersici, Fusarium *oxysporum* are ubiquitous fungi found in soil world wide as both pathogenic and non pathogenic strains (Leslie and Summerell, 1996). FA could elicit various plant defense responses at 100 nm without toxic effect. Large FA concentration reduces root and root-hair growth and induces rapid transient membranes Hyperpolarisation. It represses the production of PCN (Phenazine 1- Carboxamide) and of the quorum sensing signal Nhexanoyl-L-homoserine lactones (C6-HSL). It is toxic for Eukaryotes and Prokaryotes involved in fungal defense against Pseudomonas Spp. Biocontrol strains by repressing the production of antifungal metabolites. FA Showed higher nematicidal activity against B.xylophilers. The biosynthesis of FA involves the condensation reaction involving a polyacetate unit and Aspartic acid. The specific role of FA is Picolinic acid and involvement of ethylene in disease development is not known. Mycelial growth could not be used to measure or estimate the production of FA. FA causes rot of potato tubers. FA has a Tumoricidal activity for head and neck squamous cell cancer (HNSCC). In plant, most of the studies and FA reported toxic effect at concentration greater than 10-5 M. FA can be involved in Fungal pathogen city by decreasing cell viability. It could induce typical early defense response such as reactive O2 species. (ROS Production) FA is detected using HPLC, TLC, Mass spectroscopy; NMR (Rani *et al.,* 2009).The phototoxic pathogen city factor FA represses the production of 2, 4 diacetyl phloroglucinol (DAPG) a key factor in the antimicrobial activity of biocontrol strain Pseudomonas fluorescence. The effect of FA, a dopamine beta hydroxylase inhibiter, was determined on aggression motor activity and brain monoamines at dose of 3.2 to 60 mg/kg. FA is mycotoxins with low to moderate toxicity, which is, of concern, since it might be synergistic with other occurring mycotoxins. A FA decrease contractile response elictor with nor-epinephrine, histamine, serotonin, acetylchdine and KCl. FA does not interfere with Dopamine uptake FA represses the PCN production under different environmental condition. Moderate FA doses (50-100um) induce apoptotic features, while high FA dose (>200um) stimulates necrosis. Decarbonylation of FA gives Co (C-7) and 3-butyl pyridine, which is oxidized with $KMnO_4$ to nicotinic acid. The activity is found mainly in positions 2, 3, 5, 9 and11 of fusaric acid. The low activity at C-2 and C-3 of pyridine ring and presence of pyridine ring and presence of activity at C-4 and C-7 are consistent with the participation of 4C Krebs's cycle acid (or) its equivalent. It has been shown to produce both ethylene and ethylene like symptoms. Fusaric acid can be used as selective agent (Rani *et al.,* 2009). FA show nematicidal activity agent B.xylophilers. FA inhibits dopamine B-hydroxylane.

Some other fusarial mycotoxins (Desjardins, 2006) are trichothecene, deoxynivalenol, *F.graminearum* toxic, T-2 and beauverin. FA acts as an enzyme inhibitor, dopa Conclusion FA acts as an enzyme inhibitor, dopamine agent and nucleic acid synthesis inhibitor. FA can chelate divalent cations, esp., Zn and inactivate Zn finger proteins involved in DNA repair and protein synthesis. FA has Tumoricidal activity for head and neck squamous cell cancer (HNSCC). In vivo studies demonstrated that daily intraregional therapy for 1 month showed reduced onset of growth and overall growth of cell. Thus FA

appears to have a tumor static/Tumoricidal effect on HNSCC. As an orally active agent, it may have potential role in treatment of HNSCC. Hydrochloride beta-(N, N-diethyl amino) ethylamide of FA (DAEA) exerted an ant arrhythmic activity in adrenaline-induced arrhythmic in rats. DAEA single pretreatment in dose of 1-5 mg/kg prevented the disorder of rhythm and conductivity in most animals. A pronounced Antiarrhythmic effect was manifest at doses of 2 and 4mg/kg of DAEA. It prevents the development of arrhythmic in 50% of animals in a dose of 1.7mg/kg. The effect of FA 150-450mg daily on tardine dyskineria and mental state was studied in 15 chronic psycho geriatric patients. FA significantly relived orofacial dyskineria, tremor and rigidity and it's improved the mental state of patient (BPRS). Akineria and Anxiety were not altered. When 20mg/kg of FA was injected intraperitoneally into rabbits, rats, cats, (or) dogs. Decrease of blood pressure was observed from about 30 min to 6 hrs after injection. Increase in dose decreases the blood pressure in great level with increasing FA concentration there was marked decrease of nor-epinephrine in angiovascular system is thought to be the cause of hypertensive effect. Administration of alpha-methyl-para-tyrosine (AMPT) and FA helps the withdrawal in human's addicted to narcotics (or) amphetamines (Rani *et al.*, 2009).

Conclusion

Fusaric acid can be scientifically applied in agricultural aspects. At present its positive role has dominated the agricultural biotechnology and breeding programmed. The wide scope and applications of fusaric acid is a wonderful route for improvement of plants and agriculture as a whole.

Applications

1. FA strongly inhibits root and leaf cell function physiologically responsible for fusarium wilt of watermelon.
2. Fusaric acid induces endogenous ethylene production in tomato cutting.
3. FA is used to examine the cell death in saffron roots.
4. FA increases the mitochondrial and plasma membranes permeability.
5. FA is used for selecting resistance in barley plants.
6. FA is known as an inhibitor of metal-containing oxidative enzymes mycotoxins and antibiotics.
7. It is used as a marker compound for Fusarium contamination of grains.

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