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Full Length Research Paper

Study on Biochemical Constituents of *Sclerotium rolfsii* -A Causal Agent of Stem Rot of Groundnut *(Arachis hypogaea L.)*

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Abstract
Sclerotium rolfsii (S. rolfsii) is a ubiquitous soil-borne fungal pathogen known to cause disease on worldwide range of agricultural and horticultural crops. In spite of economic loss caused by this pathogen, very few reports were available on this aspect, biochemical characterization of the S. rolfsii; hence the present study was under taken to study the biochemical characterization of S. rolfsii. Qualitative and quantitative analysis of free and protein-bound amino acids was carried out in mycelial mats and culture filtrate of S. rolfsii. Five amino acids were identified and were commonboth in free and protein-bound amino acids. Methionine was the most abundantly occurring amino acid in the protein-bound amino acids, whereas asparagine in case of frees amino acids. Eleven amino acids were detected in the culture filtrate, but methionine, phenylalanine and leucine/isoleucine were more abundant. Phenolic acids both in culture filtrate and mycelia mat were analyzed by 2-dimensional paper chromatography; seven were detected both in culture filtrate and mycelial mat. Gallic, ferulic, chlorogenic and cinnamic acids were common both in mycelium and culture filtrate of the S. rolfsii. The other biochemical constituents like carbohydrate fractions, phenolic compounds and nitrogen fractions present in both mycelia mat and culture filtrate were also studied.

Key words: *Amino acids, biochemical constituents, phenolic acids, Sclerotium rolfsii, stem rot,*

Introduction

Sclerotium rolfsii is a ubiquitous soil-borne fungal pathogen known to cause disease on worldwide range of agricultural and horticultural crops (Buensanteai *et al*., 2012). It infects more than 500 plant species in 100 families throughout the world (Adandonon *et al*., 2005; Ganesan *et al*., 2007). Most *S. rolfsii* diseases have been reported on dicotyledonous hosts and monocotyledonous species are also being infected, indicating the wide host range of parasitism of *S .rolfsii*. Secondary hosts are numerous, most of them are economically important like food crops and ornamental crops.

Sclerotium rolfsii is a facultative parasite that survives in the soil mainly as sclerotia which function as the main source of inoculums and remain viable for several years (Cilliers *et al*., 2000; Okereke and Wokocha 2007). *S. rolfsii* causes severe damage during any stage of crop growth (Ganesan *et al*., 2007) and attacks all parts of the plant but stem infection is the most common and serious. The first symptom is sudden wilting of the branch with the stem near the soil level is the most point of attack and a white coating of mycelium appears. Sclerotia of mustard seed size appear on the infected area at later stages. About 85% of yield loss due to the *S. rolfsii* has been reported from India. Keeping in view the losses caused by this fungus, the aim of the present investigation therefore was to evaluate the biochemical characterization of the *S. rolfsii.*

Materials and Methods

Isolation of pathogen

Cultured *S. rolfsii* strain was isolated on PDA from the plants showing stem rot or southern blight symptoms of groundnut from the area of Sadhanavaripalem, Chittoor District in Kharif season on PDA. The pure culture of the fungus was obtained and maintained on PDA for further study. The stock culture was maintained on PDA slants in a refrigerator and subcultured every two months.

Biochemical analysis of pathogen

Collection of mycelium and culture filtrate of S. rolfsii:

The fungus was grown in 1 liter Roux bottles containing 200ml of basal medium, under stationary conditions at 29±2ºC. After 10 days of incubation period, the cultures were harvested by filtration through a Whatman No 1 filter paper and fungal mat was blotted dry in four folds of a filter paper. The culture filtrate was collected separately. Both the mycelial mat and culture filtrate were analyzed for free and protein-bound amino acids.

1. Estimation of carbohydrate fractions

For analysis of various biochemical constituents in the mycelium and culture filtrate of *S. rolfsii*, the extracts were prepared according to the method of Mahadevan *et al*., (1965).

i) Estimation of reducing sugars: Reducing sugars in the ethanol extract were determined by Nelson-Somogy's method (Ram *et al*., 1979).

ii) Estimation of non-reducing sugars: Non-reducing sugars present in the ethanol extract were first hydrolyzed to reducing sugars as described by Inman, (1965). Total sugars in the hydrolyzed sample were estimated by Nelson-Somogy's method (Nelson, 1944). The quantity of reducing sugars was calculated by subtracting the reducing sugar content from total sugars and expressed as glucose equivalent.

iii) Estimation of starch: The residue left behind after ethanol extraction of the original material was used for starch extraction and estimated according to the method of Mc Cready *et al* (1959).

2. Phenolic compounds

i). Estimation of ortho-dihydric phenols: *Ortho*-dihydric phenols (OD) were estimated by employing Arnow's reagent, which is specific to *ortho* groups (Johnson and Schaal, 1957).

ii). Estimation of total phenols: Total phenols were estimated by employing the Folin-Ciocalteau reagent method (Sadasivam and Manickam, 1996)**.**

iii). Phenolic acids: Phenolic acids were extracted according to the method of Bate-Smith, (1954) adopted by Das and Rao, (1964). The extract was subjected to 2-dimensional ascending paper chromatography technique on Whatman No.1 chromatography paper. The solvents employed were benzene-acetic acid-water (60:70:30) in the first direction and the sodium formate-formic acid-water (10:1:200) in the second direction (Ibrahim and Towers, 1960). The dried chromatograms were observed under ultraviolet light, first without and then with ammonia vapors, all the fluorescent spots were marked. The sheets were then sprayed with diazotized p-nitraniline (Smith, 1960) or diazotized sulphanilic acid (Ames and Mitchell, 1952) or 1% ferric chloride in order to identify the phenolic acids present in the fungal mycelium.

3. Nitrogen fractions: Total nitrogen and protein nitrogen were estimated in the mycelial mat of *S. rolfsii*.

i) Total nitrogen: It was estimated according to the method of Markham, (1942). The amount of nitrogen present in mycelium was calculated as follows: One ml 0.01 N HCl (=0.14 mg of nitrogen). The results are expressed in mg per gram fresh weight of host tissue or fungal mycelium.

ii) Protein nitrogen: Protein nitrogen was estimated by the method of Thimann and Loos, (1957).

iii) Soluble nitrogen: Soluble nitrogen fraction was calculated by subtracting insoluble nitrogen (protein nitrogen) from total nitrogen content. The results are expressed as mg of nitrogen per gram fresh weight.

iv) Total proteins: The proteins were extracted according to the method of Lowry *et al.,* (1951).

v) Amino acids

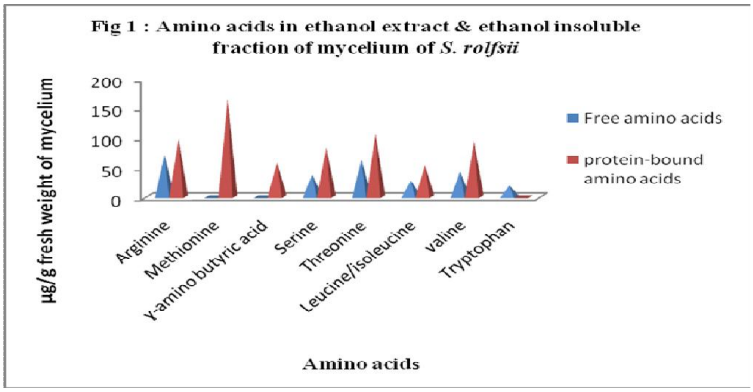
a) Free amino acids: Amino acids were extracted from mycelium and culture filtrate of *S. rolfsii* according to the method Reddy and Rao, (1975). The quantitative and qualitative determinations of amino acids were carried out by 2-dimensional ascending paper chromatographic technique using Whatman No1 paper. Solvents employed were sec-butanol-formic acid-water (75:13:12 v/v/v) and citrate buffer saturated phenol (was prepared by dissolving 6.3 g of sodium citrate and 3.7 g of potassium dehydrogenize phosphate in 100 ml of distilled water, to which 200 ml distilled phenol was added, shaken thoroughly, kept aside for a few hours and the supernatant taken up for use) as the first and second solvent systems, respectively. The dried chromatograms were sprayed with 0.2%ninhydrinand developed at 60-65^{0}C for 5 min to increase the intensity of the spots. The identity of the spots was established by co-chromatography along with the mixture of known amino acids and also comparing the R_f values of the amino acids in a known mixture with those in the extracts.

b) Protein bound amino acids: The residue left over after ethanolic extraction was hydrolyzed with 6N HCl for 30 min at 1 bar pressure in the presence of a pinch of stannous chloride (added to the residue prior to autoclaving) to prevent humic formation (Reddy and Rao, 1975). After hydrolysis, the acid was removed by evaporation. The residue of the hydrolysate was then taken in 1 ml 80% ethanol and an aliquot was stopped and analyzed for amino acids as described earlier for free amino acids.

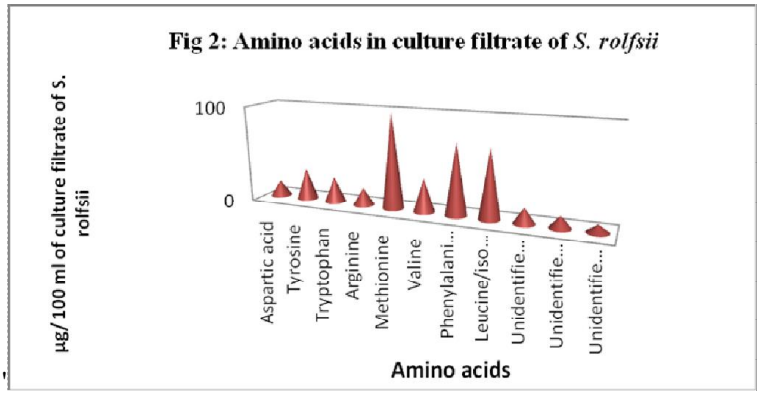
Results and Discussion

A greater knowledge of the biochemistry of fungi pathogenic to plants is expected to aid in understanding the physiological relations between the host and parasite (Mc Combs and Winstead, 1964). Several investigators have used qualitative and quantitative differences in biochemical composition of fungi to bring out taxonomic differences. Studies on the metabolic chemistry of *S. rolfsii* are quite a few (Mathur and Sarbhoy, 1977; Ram, *et al*., 1979; Sarma, *et al*., 2002; Singh., *et al*., 2002) and such studies are needed to help in understanding why this fungus is a pathogen on many host plants and also in understanding the host-pathogen interaction.

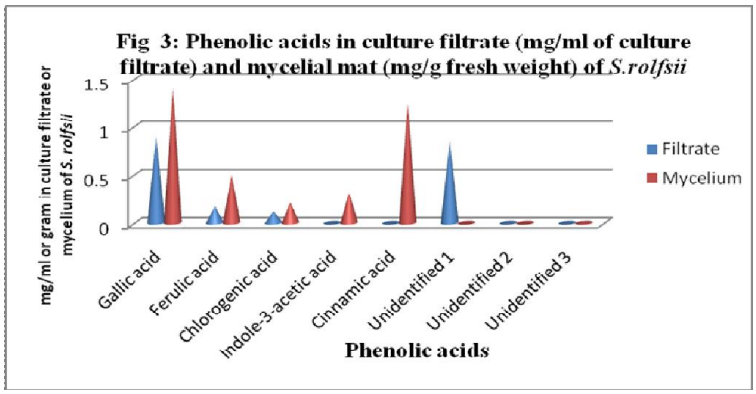
Qualitative and quantitative analysis of free and protein-bound amino acids carried out in mycelial mats as well as culture filtrates of *S. rolfsii* resulted in the detection ofsix amino acids in ethanol extract of the mycelium and seven in ethanol insoluble fraction. The amino acids asparagine, serine, threonine, leucine/isoleucine and valine were common in free and protein-bound amino acids. Methionine was the most abundantly occurring amino acid in the protein-bound amino acid, whereas asparagine in case of frees amino acids (Fig. 1).



The isolate showed variation in its ability to release amino acids into the culture medium. Eleven amino acids were present in culture filtrate, where 8 identified as aspartic acid, tyrosine, tryptophan, arginine, methionine, valine, phenylalanine, leucine/*iso*leucine and 3 were unidentified. Methionine, phenylalanine and leucine/*iso*leucine were abundant in culture filtrate. Valine, tyrosine, tryptophan were present in moderate amounts while the other amino acids present only in small quantities (Fig. 2).



The phenolic compounds in the culture filtrate and mycelial mat of *S. rolfsii* were analyzed by 2-dimensional paper chromatography.Seven phenolic compounds were detected in the culture filtrate, four of which were identified as gallic, ferulic, chlorogenic and cinnamic acids and remaining three were unidentified. In the mycelial mat, seven compounds were detected, five of which were identified as gallic, ferulic, chlorogenic, indole-3-acetic acid and cinnamic acids and remaining two were unidentified (Fig. 3). Phenolic substances are commonly encountered as microbial metabolites. They are widely distributed in fungi (Miller, 1961). The synthesis of aromatic amino acids, tyrosine and phenylalanine by this pathogen may indicate that the shikimic acid pathway is operative and synthesized mostly through this pathway. These two aromatic amino acids have an important role in phenol metabolism. A great deal of information has accumulated in recent years concerning the degradation of aromatic amino acids by fungi to form phenolics (Moore and Towers, 1967; Moore *et al*., 1968; Reddy *et al*., 1975; Prasad and Reddy, 1987). The activities of phenylalanine ammonia-lyase and tyrosine ammonia-lyase were also detected in fungi. It is capable of releasing large number of phenolic compounds when grown on a simple synthetic medium. Pathogenic nature of *S. rolfsii* may partly be due to its capacity to release a large number of phenolics into the external medium, some of which may be phytotoxic and exert their effect on plants resulting in disease production.

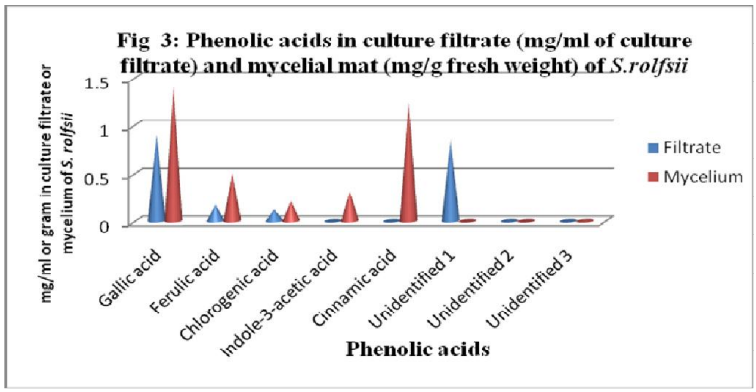


The other biochemical constituents like carbohydrate fractions, phenolic compounds, nitrogen fractions present in both mycelial mat and culture filtrate of the *S. rolfsii* are presented in Table 1.

Table 1: Biochemical constituents of the mycelium and culture filtrate (mg/g fresh weight)* of *S. rolfsii*.

S. No	Biochemical constituents	Culture filtrate	Mycelium
1	Total phenols*	0.10	**0.28**
2	*Ortho*-dihydric phenols*	0.13	**0.045**
3	Reducing sugars**	0.437	**0.372**
4	Non-reducing sugars**	2.185	**1.86**
5	Starch**	-	**45.0**
6	Total nitrogen***	-	**0.138**
7	Soluble nitrogen***	-	**0.034**
8	Protein nitrogen***	-	**0.935**
9	**Total protein****	**-**	**2.060**

**Each value is an average of three replicate samples.*
** Expressed as catechol equivalents.*
*** Expressed as glucose equivalents.*
**** Expressed as glutamic acid equivalents.*
***** Expressed as Bovine Serum Albumin equivalent*



Knowledge on major biochemical constituents of the pathogen is of immense importance in understanding the host-pathogen interaction. This may give an idea of the contribution of the pathogen in the deranged metabolism of the host due to parasitic invasion. It is well understood that the processes and mechanisms associated with disease development are a functions of both the host and pathogen and disease may be considered as the sum of their interactions. Therefore, in order to understand the host-parasite relationship one ought to know the conditions congenial for successful disease development, the biochemical nature of the pathogen, the details of host metabolites available for the parasite and the subsequent metabolic disturbances in the host due to parasitic invasion. Infected plants naturally include both the host tissues and the fungus associated with it. Hence, the interpretation of chemical changes during disease development must take into account the additive effects of the pathogen present in the host as also pointed out by Mc Combs and Winstead, (1964).

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