SCREENING OF ANTIFUNGAL MICROBIAL SECONDARY METABOLITES AGAINST PHYTOPATHOGENS

SHABNAM KHAN, BELA NABAR

Abstract: *Colletotrichum* spp., *Rhizoctonia solani* and *Sclerotium rolfsii* are fungal plant pathogens which lead to huge economic losses. In the present study, a total of 96 isolates (49 actinomycetes spp., 31 yeast spp. and 16 bacterial spp.) were isolated from Waldhuni nallah area. The results of primary screening showed that out of 96 isolates, six isolates showed antifungal activity against the phytopathogens. Extraction of the secondary metabolites was carried out using ethyl acetate and characterized by using thin layer chromatography (TLC) and the isolate Y-98 was found to contain leucine. MIC of the secondary metabolites against

Colletotrichum spp., *Rhizoctonia solani* and *Sclerotium rolfsii* was found to be at 1:100 dilution, 1:10 dilution and 1:10 dilution respectively. MIC of the positive control (Carbendazim) was found to be at 1% against the three pathogens. Using bioautography method, the isolate Y-98 showed maximum activity against the three pathogens. The isolate Y-98 was found to produce 0.96 grams of secondary metabolite per 100 ml of the cell free supernatant. The isolate A-02 was identified as *Nocardioides luteus* and the isolates Y-124, Y-98, Y-92, Y-50 and Y-65 were found to belong to *Schizosaccharomyces* spp.,

Tricosporon spp., *Saccharomyces* spp., *Geotrichum* spp. and *Kriegeriales* spp. respectively.

Keywords: Antifungal Secondary metabolites, *Colletotrichum* spp., *Rhizoctonia solani* and *Sclerotium rolfsii*.

Introduction: Agricultural crop is a volunteered or cultivated plant whose product is harvested by humans at some point of its growth stage. Generally, in agricultural fields crops are infected by variety of pathogens including ants, aphids, etc. Phytophagous nematodes can be serious pests, and some micro-organisms, bacteria and actinomycetes can also cause plant diseases. However, most damage is caused by fungi, which account for most soil-borne crop diseases [1]. This study focuses on three fungal plant pathogens i.e.

Colletotrichum spp., *Rhizoctonia solani* and *Sclerotium rolfsii*.

The genus *Colletotrichum includes* a number of plant pathogens of major importance, causing diseases of a wide variety of woody and herbaceous plants. Chilli (*Capsicum* spp.), an important economic crop worldwide, is severely infected by anthracnose disease which may cause loss in yield up to 50%. The genus was recently voted the eighth most important group of plant pathogenic fungi in the world, based on perceived scientific and economic importance [2]. As plant pathogens, *Colletotrichum* species are primarily described as causing anthracnose diseases, although other maladies are also reported such as red rot of sugar cane,

coffee berry disease, crown rot of strawberry and banana, and brown blotch of cowpea. [3]

Rhizoctonia solani is a plant pathogenic fungus with a wide host range, which leads to major yield losses.

Rhizoctonia solani cause various plant diseases such as collar rot, brown patch (a turfgrass disease), damping off in seedlings, as well as black scurf of potatoes, bare patch of cereals, root rot of sugar beet, belly rot of cucumber, sheath blight of rice etc. [4]

Sclerotium rolfsii is a polyphagous soil borne pathogen infecting over 500 plant species worldwide. Southern blight, crown rot and white mold are the diseases caused by the fungus *Sclerotium rolfsii*. There are various methods in controlling the diseases caused by these pathogens, but the two common ones are use of chemical pesticides and biological control. In commercial agriculture, crop protection against phytopathogens relies heavily on chemical pesticides. [5]. Infection caused by *Colletotrichum* spp. is usually controlled by spray treatment of azoxystrobin, famoxadone, iprodione, procymidone, tolyfluanid and carbendazim. Pentachloronitrobenzene (PCNB) is also used in agricultural fields to control the infections caused by *Colletotrichum spp.*,

Rhizoctonia solani and *Sclerotium rolfsii*. Quradis fungicide and mancozeb fungicides are used in agricultural fields to control the infections caused by *Rhizoctonia solani*. Chemicals like metam-sodium, ammonium bicarbonate, azoxystrobin, flutolanil, tebuconazole, methlybromide are used in agricultural fields to control the infections caused by *Sclerotium rolfsii* [6].

The increasing use of chemical inputs causes several negative effects i.e.,

development of pathogen resistance to the applied agents and their non-target environmental impacts. Scientific efforts for several decades now have been focused on developing alternative approaches for managing plant diseases. Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture. Biological control simply means that pathogens are antagonized by the presence, activities or products of other similar or different organisms that they encounter in the plant's rhizosphere or phyllosphere **[7]**

Certain antagonistic bacteria are considered ideal biological control agents owing to their rapid growth, easy handling and aggressive colonization of the rhizosphere. These bacteria may mediate biocontrol by one or more of the several mechanisms of disease suppression. A primary mechanism of pathogen inhibition is by the production of secondary metabolites. Secondary metabolites are defined as substances with a low molecular weight, which are not the products of the primary metabolic pathway of the producing organism. [8]

Materials and Methods:

15 soil samples were collected from highly polluted Waldhuni River. Collected soil samples were sealed in sterile polyethylene bags. Isolation of the organisms was carried using sterile nutrient agar plates, sterile potato dextrose agar plates and sterile actinomycetes agar plates. All the three phytopathogens (*Colletotrichum* spp., *Rhizoctonia solani* and *Sclerotium rolfsii*) were procured from University of Agricultural science, Dharwad. *In-vitro* antifungal activity of the microbial secondary metabolites was determined against the phytopathogens by Using the cell free supernatant by agar well diffusion method. Carbendazim was used as the positive control [9].

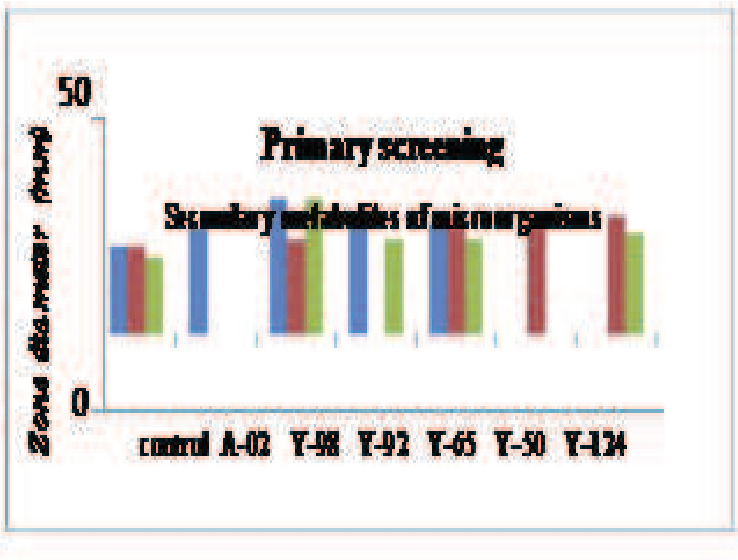
Ethyl acetate was used to extract the secondary metabolites. Then the activity of the upper layer and the lower layer was tested against the phytopathogens by agar well diffusion method [10]. TLC was used to characterize the secondary

metabolites. MIC of secondary metabolites and positive control was determined by agar dilution method [11]. Bioautography was also performed to determine the antifungal activity of the secondary metabolites [12]. The cell free supernatant of the effective isolate Y- 98 was used to determine the quantity of the secondary metabolite produced by it. Gram staining and slide culture technique were carried out for actinomycetes spp. [13]. For further identification, biochemical tests were performed as per Bergey's Manual of

Determinative Bacteriology (9th edition). Identification of yeast spp. was done by microscopic examination using lactophenol cotton blue [14].

Results and Discussion:

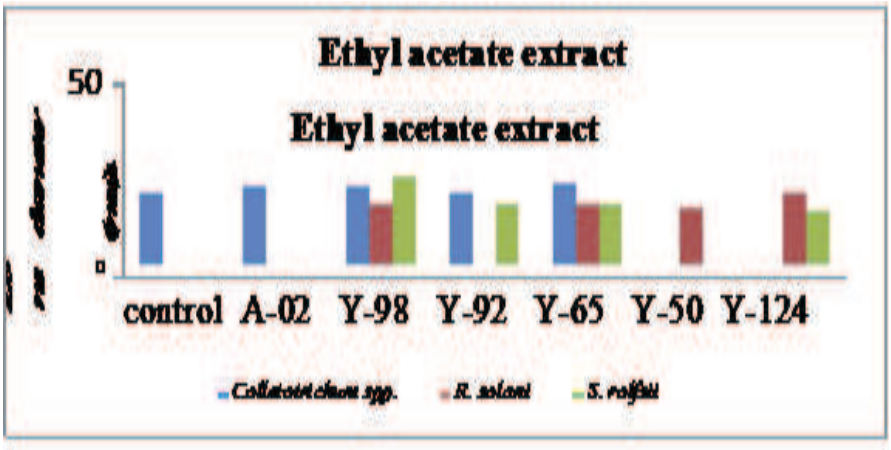
Primary Screening: - 15 soil samples were collected from Waldhuni nallah area. A total of 96 isolates were isolated from the soil samples including 49 actinomycetes spp., 31 yeast spp., and 16 bacterial spp. Primary screening of the isolates showed that out of the 96 isolates, 6 isolates showed antifungal activity out of which one isolate belonged to actinomycete spp. and the remaining five isolates belonged to yeast spp. None of the bacterial isolates showed antifungal activity. Carbendazim was used as the positive control (Graph 1).



Graph 1 :- Graph representing the antifungal activity of actinomycete spp. and yeast spp. against Colletotrichum spp., Rhizoctonia solani and Sclerotium rolfsii.

Colletotrichum spp. R. solani, S. rolfsii

Extraction using ethyl acetate: - Table 1 shows that after extraction, no much increase in the activity of the secondary metabolites was observed against all the three phytopathogens (Graph 2).



Graph 2:- Graph representing the antifungal activity of the lower layer of the ethyl acetate extract of the secondary metabolites against

***Colletotrichum* spp., *Rhizoctonia solani* and *Sclerotium rolfsii*.**

TLC :- After performing TLC, by comparing the Rf values of the standard amino acids with the Rf value of the sample, it was found that the secondary metabolite produced by isolate Y- 98 contains leucine; whereas no amino acids were found to be present in the secondary metabolites produced by isolates A- 02, Y-92, Y-65, Y- 50 and Y- 124.

Table 1:- Activity of the extracted secondary metabolites against phytopathogens:-

	Zone of inhibition		Zone of inhibition in		Zone of inhibition	
Secondary metabolites		in	mm against		in mm against	
of the effective isolates	mm against		*Rhizoctonia solani*		*Sclerotium rolfsii*	
	Colletotrichum					
	Upper	Lower	Upper	Lower	Upper	Lower
	layer	layer	layer	layer	layer	layer
A- 02	20 mm	22 mm	-	-	-	-
Y- 98	21 mm	22 mm	-	17 mm	-	25 mm
Y- 92	20 mm	20 mm	-	-	-	17 mm
Y- 65	20 mm	23 mm	-	17 mm	-	17 mm
Y-50	-	-	-	16 mm	-	-
Y-124	-	-	-	20 mm	-	15 mm
Control – Ethyl	20 mm			-		-
acetate						
Key: - A= actinomycete spp., Y= yeast spp.,			- = No zone of inhibition.			

MIC: MIC of secondary metabolites and positive

control was determined by agar dilution method. MIC for the secondary metabolites produced by the isolates A-02, Y- 98, Y- 92 and Y- 65 against *Colletotrichum* spp. was found to be at 10^{-2} dilution. MIC for the secondary metabolites produced by the isolates Y- 98, Y- 65, Y- 50 and Y- 124 against

Rhizoctonia solani was found to be at 10^{-1} dilution. MIC for the secondary metabolites produced by the isolates Y- 98, Y- 92, Y- 65 and Y- 124 against

Sclerotium rolfsii was found to be at 10^{-1} dilution. MIC for the positive control (carbendazim) was found to be 1% against all the three fungal plant pathogens.

Bioautography: - The isolate Y- 98 showed maximum activity against *Colletotrichum* spp., *Rhizoctonia solani* and *Sclerotium rolfsii*. While the isolates Y- 92, Y- 65 and A- 02 showed moderate activity against *Colletotrichum* spp. The isolates Y- 65, Y-124 and Y- 50 showed moderate activity against *Rhizoctonia solani*; whereas the isolates Y- 92, Y- 65 and Y- 124 showed moderate activity against *Sclerotium rolfsii*.

Quantitation: - The isolate Y- 98 was found to be effective against all the three phytopathogens. The quantity of secondary metabolite present in 100 ml of cell free supernatant of the isolate Y-98 was found to be 0.96 grams.

Identification: The microscopic appearance (Gram staining) of the isolate A- 02 showed the presence of Gram positive rods. Slide culture technique was performed which showed the presence of vegetative mycelium. All the characteristics indicate that the isolate A- 02 belongs to genus *Nocardioides*. Non-fermentation of sucrose and rhamnose sugars; and non-motility of the culture indicates that the culture is *Nocardioides luteus*. Single-celled growth and a rod-shaped cell was observed for isolate Y- 124. Hyphae and barrel-shaped arthroconidia were observed for isolate Y-98. Big oval cells and some budding cells were observed for isolate Y- 92. Hyphae and arthroconidia in chains and few individual arthroconidia from the fragmentation of the chains were observed for the isolate Y- 50 and elongated cells and few budding cells were observed for isolate Y- 65. Thus, depending upon the characteristics observed under the microscope the isolates Y-124, Y-98, Y- 92, Y- 50 and Y- 65 were found to belong to *Schizosaccharomyces* spp., *Tricosporon* spp., *Saccharomyces* spp., *Geotrichum* spp., and *Kriegeriales* spp. respectively.

Conclusion:

Hence, it can be concluded that the secondary metabolites produced by*Tricosporon* spp., *Saccharomyces* spp., and *Kriegeriales* spp. can be used against *Colletotrichum* spp. in agricultural fields. The secondary metabolites produced by *Nocardioides luteus* can also be used against *Colletotrichum* spp. in agricultural fields. The secondary metabolites produced by *Tricosporon* spp., *Geotrichum* spp., *Kriegeriales* spp. and *Schizosaccharomyces* spp. can be used against *Rhizoctonia solani* in agricultural fields. Whereas, secondary metabolites produced by *Saccharomyces* spp., *Schizosaccharomyces* spp., *Kriegeriales* spp. and *Tricosporon* spp. can be used against *Sclerotrium rolfsii* in agricultural fields. However, further studies are necessary to evaluate the effect of these potential biocontrol agents in greenhouse and field conditions and also to purify the secondary metabolites produced by these yeasts spp. and actinomycete spp. HPLC can also be carried out to determine the structure of the compounds present in the secondary metabolites.

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Shabnam Khan, Bela Nabar/Department of Microbiology/Smt C.H.M. College /

(Affiliated to Mumbai University) Dist –Thane /Maharashtra.