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Mycomolecules against *Alternaria solani* causing Early blight of tomato

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Abstract
Mycomolecules isolated from mushroom possess antimicrobial properties which forms bioactive compounds of high therapeutic and pharmacological value. Antimicrobial principles from macro basidiomycetes against plant pathogens was not yet well explored. In this view, a study was proposed to screen mushroom fungi *viz., Lentinus edodes, Volvariella volvaceae, Ganoderma lucidum and Auricularia polytricha* against tomato early blight pathogen *Alternaria solani*. Methanol extracted mycomolecules from the cell free culture of mushroom was used in different experiments of the study. Results from dual culture technique revealed that *Ganoderma lucidum* showed maximum antifungal activity by inhibiting the mycelial growth of *A. solani* (67%). Among the various mushroom fungi, *G. lucidum* cell free culture filtrates exhibited maximum inhibition of spore germination of *A. solani* (53%) at 24 hours. The methanol extracted metabolite fractions of *G. lucidum* at 0.2% concentration inhibited maximum mycelial growth of *A. solani* (69%). Results indicates that methanol extracted cell free culture fractions of *G. lucidum* possess antifungal activities against the growth of *A. solani* and these mycomolecules could be further explored for the development of fungicides against the pathogen.

Keywords: Mycomolecules, *Ganoderma lucidum*, basidiomycetes, *Alternaria solani*

Introduction
Tomato (*Solanum lycopersicum* L.) is an important vegetable crop grown in varied agro climatic conditions, its production is being affected by many fungal, bacterial and viral diseases. Among the fungal diseases of tomato, early blight caused by *Alternaria solani* (Ellis and Martin) Jones and Grout is an important disease-causing production losses varying from 10 - 80 per cent (Datar and Mayee, 1986) [5]. Major insect pests of tomato include Aphids, Tomato Fruit worms & Horn worms, Leaf-footed Bugs & Stink Bugs, Flea Beetles, Whiteflies, Thrips, Spider Mites and Cutworms. (Fouche *et al.*, 2000) [8]. Often tomato is also affected by several nematodes including *Meloidogyne* spp., *Nacobbus aberrans, Ditylenchus dipsaci, Globoderaro. stochiensis,. G. pallida, Pratylenchus* spp., *Paratrichodorus* spp., *Tylenchorhynchus* spp., *Xiphinema facolum, Rotylenchulus reniformis* and *Dolychodorus heterocephalus* (Greco and Vito, 2011) [10]. Disease management in tomato is widely practiced using chemicals (Singh *et al*., 2001) [22]. Indiscriminate use of chemicals led to development of fungicidal resistance and environmental pollution (Rai *et al*., 2000) [16]. Extensive use of chemical fungicides has resulted in the accumulation of toxic compounds potentially hazardous to humans and the environment, and also results in the build-up of resistance of the pathogens. As tomato an important edible crop, large quantity of pesticides was being used, there is a growing demand for chemical pesticide free organic tomato. Current research is focused on search of antimicrobials agents from green channels such as plants, fungi and bacteria in order to identify their biopesticidal compounds. Mushroom fungi are important as natural sources of medicines and possess number of bioactive compounds *viz.*, antibacterial, antifungal, antioxidant, antiviral (Reis *et al.,* 2011; Rouhana-Toubi *et al.,* 2015) [17, 18]. Ecofriendly approaches for plant disease management includes mushroom fungi as promising source of antimicrobials against plant pathogens as evidenced by the antimicrobial activity of the culture filtrates of *Ophiocordeyceps sinensis* against soil borne pathogens of *Fusarium oxysporum* f. sp. *lycopersici* (Sangeetha *et al.*, 2015) [20], *Coprinus comatus* against *Fusarium oxysporum* f. sp. *lycopersici* (Jeeva and Krishnamoorhy, 2018) [11], ethanolic extracts of

Leucopaxillus gignatea against *Fusarium solani*, *Collectotrichum graminicolum* and *Bacillus subtilis* (Feleke and Anila Doshi, 2017) [7]. The present investigation was made with an aim to identify a potential mushroom fungus with antimicrobial activity against *Alternaria solani*, the tomato early blight pathogen.

Table 1: Mycomolecules and their mushroom source against major pathogens

Mycomolecule	Mushroom Source	Target pathogen
Ganodermin	*Ganoderma lucidum*	*Botytis cinerea*
Pleurostrin	*Pleurotus ostreatus*	*Botryosphaeria berengeriana*
Eryngin	*Pleurotus eryngii*	*Mycosphaerella arachidicola*
Lyophyllin	*Lyophyllum shimeji*	*Physalospora piricola*
Grifoline	*Albatrellus dispansus*	*Alternaria alternate*
Hypsin	*Hypsizigus marmoreus*	*Botytis cinerea*
Rufuslactone	*Lactarius rufus*	*Alternaria brassicae*
Cordymin	*Cordyceps militaris*	*Rizoctonia solani*
Cinnamic acids	*Ganoderma lucidum*	*Pencillium ochrochloron*
Chrysotrione	*Hygrophorus chrysodon*	*Fusarium verticillioides*
Agrocybin	*Agrocybe cylindracea*	*Mycosphaerella arachidicola*
Lentin	*Lentinus edodes*	*Mycosphaerella arachidicola*
Hydroxypyrene	*Cordyceps militaris*	*Fusarium oxysporum*
Phellinsin	*Phellinus sp*	*Pyricularia grisea*

*(Sivanandhan *et al.*, 2017) [23]

Materials and Methods

The tomato early blight pathogen *Alternaria solani* and the mushroom fungal cultures *viz.*, *Lentinus edodes*, *Volvariella volvaceae*, *Ganoderma lucidum and Auricularia polytricha* obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore were used for the studies.

In vitro screening of mushroom fungi against *A. solani*

Mushroom fungi *viz. Lentinus edodes*, *Volvariella volvaceae*, *Ganoderma lucidum and Auricularia polytricha* were tested for its antagonistic activity against *A. solani* by following dual culture technique (Dennis and Webster, 1971) [6]. A 9 mm mycelial disc of mushroom fungi was placed at the edge of the Petri plates containing PDA medium on one side. Similarly, on the opposite side a 9 mm mycelial disc of *A. solani* was placed. The dual culture plates were incubated at 28±2ºC for 7 days. Three replications were maintained for each treatment. Plates with *A. solani* only and respective mushroom fungi served as control. The plates were examined periodically and measurements on the radial mycelial growth of *A. solani* and mushroom fungi were recorded till the control plate attained full growth (90mm). The percent inhibition of mycelial growth of *A. solani* was calculated by using the formula proposed by Vincent (1947) [27].

Percent inhibition of growth (PI) = C-T/C×100

Where, C is the growth of pathogen in control (mm) and T is the growth of pathogen in treatment (mm).

Solvent extraction of metabolites from mushroom fungi

Mycelial discs (measuring 9 mm dia.) was cut from margin of a 10 day old culture of *Lentinus edodes*, *Volvariella volvaceae*, *Ganoderma lucidum and Auricularia polytricha* grown in PDA medium in petridishes and inoculated in 250 ml conical flasks containing 100 ml of sterilized PD broth. The flasks were placed on a rotary shaker maintained at 120 rpm and incubated at 25ºC for 20 days. After incubation, the culture filtrate and the mycelial mat were separated by filtration through Whatman No. 40 filter paper and the filtrate was centrifuged at 10,000 rpm and the Cell Free Culture filtrate (CFC) was extracted separately with methanol as solvent. Liquid-liquid extraction was carried out three to four times with methanol. The extracts from CFC of mushrooms were evaporated separately under reduced pressure using a rotary evaporator to obtain the residues. The condensate or residue so obtained from solvent was dried and dissolved in methanol (1mg/ml) and filtered with membrane filter (0.48 µm), stored at 4ºC used for further studies.

Effect of methanol extracted metabolites on *A. solani* spore germination test

The methanol extracted metabolites of various mushrooms were tested separately against spore germination of *A. solani* using cavity slides. A drop of extracted metabolites of mushrooms were placed separately in a cavity slide and a drop of spore suspension (1×10^6 spores/ml) of *A. solani* prepared in sterile distilled water was added to extracted metabolite and thoroughly mixed. The cavity slide was placed in the Petri dish moistened with cotton and incubated at room temperature (28 ± 2ºC). Three replications were maintained for each treatment. The spore suspension in sterile water alone served as control. The spore germination was observed and recorded after 6, 12 and 24 hours under phase contrast microscope and the percent inhibition of spore germination was calculated using the formula (Akhter *et al.*, 2006) [1].

Effect of methanol extracted metabolites on *A. solani* mycelial growth inhibition test

The methanol extracted metabolites of various mushrooms were tested separately against mycelial growth of *A. solani* by agar well diffusion method (Stroke and Ridgway, 1980) [24]. After solidification of PDA medium in Petri dishes, four wells (5mm in diameter) were made on the plate using sterile cork borer on all four sides, giving equal distance and also by leaving one cm space from the periphery. The different concentrations *viz.*, 0.05%, 0.1% and 0.2% of various mushrooms metabolites were poured into agar wells at the rate of 100µl per well using micro pipette. Then, mycelial disc of *A. solani* (5mm diameter) taken from ten days old culture was placed at the centre of each Petri dish and incubated at 28±2ºC for seven days. Observations on the per cent inhibition of mycelial growth of *A. solani* were recorded (Vincent, 1947) [27].

Statistical analysis

The data obtained from various experiments were analysed

statistically by adopting the procedure described by Panse and Sukhatme (1985) [13] The laboratory experiments were laid out in completely randomized design (CRD) and field trials were designed in randomized block design (RBD). The data recorded on per cent values were arc-sine transformed before analysis and the critical differences (CD) were calculated at 5 per cent probability level.

Results and Discussion

Mushrooms are used as food and in pharmaceuticals since ancient times, the recent findings has proved that the mushroom fungi possess secondary metabolites of antimicrobial nature to be effective against many plant pathogens.

There is great scope for developing biopesticidal molecules from mushroom fungi that can be used for development of fungicides like Azoxystrobin in plant disease management.

Table 2: Antagonistic activity of mushroom fungi against *A. solani* by dual culture technique

Treatment	*A. solani* mycelial growth (mm)	Mushroom fungal growth (mm)	Inhibition zone (mm)	% inhibition over control
Lentinus edodes	38.29	47.17	4.58	57.46
Volvariella volvaceae	53.52	34.05	2.40	40.53
Ganoderma lucidum	29.45	53.62	6.93	67.28
Auricularia polytricha	43.54	42.75	3.71	51.62
Control	90.00	-	-	-
SEd	1.48	0.86	0.14	1.26
CD (P=0.05)	3.30	1.99	0.32	2.90

Table 3: Effect of methanolic mushroom extracts on *A. solani* spore germination

Treatment	6h		12h		24h	
	SG%	PI	SG%	PI	SG%	PI
Lentinus edodes	4.55	78.57	35.73	34.20	56.28	37.93
Volvariella volvaceae	12.62	40.56	41.01	24.48	73.81	18.59
Ganoderma lucidum	0.00	100.00	29.34	45.97	42.55	53.07
Auricularia polytricha	7.91	62.74	38.13	29.78	67.19	25.90
Control	21.23	-	54.30	-	90.67	-
SEd	0.29	1.89	1.02	0.77	1.09	0.64
CD (P=0.05)	0.65	4.35	2.28	1.79	2.42	1.47

*SG – Spore germination PI – Percent growth inhibition

Table 4: Antimicrobial activity of methanolic mushroom extracts against *A. solani* by agar well diffusion technique

Treatment	0.05%		0.10%		0.20%	
	MG	PI	MG	PI	MG	PI
Lentinus edodes	46.82	47.98	33.73	62.52	32.96	63.38
Volvariella volvaceae	56.37	37.37	45.92	48.98	45.28	49.69
Ganoderma lucidum	38.11	57.66	28.76	68.04	27.53	69.41
Auricularia polytricha	51.59	42.68	40.45	55.06	39.91	55.66
Control	90.00	-	90.00	-	90.00	-
SEd	0.61	0.68	0.95	1.52	1.20	1.29
CD (P=0.05)	1.35	1.57	2.13	3.50	2.66	2.98

*MG – Mycelial growth PI – Percent growth inhibition

In vitro screening of mushroom fungi against *A. solani* by dual culture technique

Among the mushroom fungi tested, *Ganoderma lucidum* followed by *Lentinus edodes* and *Auricularia polytricha* showed reduced mycelial growth of *A. solani* (29 mm, 38 mm and 44 mm respectively) when compared to control (90 mm) with inhibition per cent of 67, 57 and 52 respectively. However, inhibition zone was maximum (6.93 mm and 4.58 mm) in *G. lucidum* and *L. edodes* respectively followed by *A. polytricha* (3.71 mm) and *V. volavceae* (2.40 mm) (Table 2) Badalyan *et al.,* (2014) [2] reported the antagonistic activity of *Pleurotus ostreatus* and *Ganoderma lucidum* by dual culture technique. Constituents of *Ganoderma* and *Agrocybe aegerita* was found to reducing local lesions of Ground nut bud necrosis virus in cowpea (Sajeena and Marimuthu, 2013) [19] and *Tobacco mosaic virus* infection (Sun *et al.,* 2003) [25]. This could be due to the effect of *Ganoderma* constituents in inhibiting the viral replication by interfering with their adsorption, viral integration, assembly and release (Gao *et al.,* 2003) [9].

Mushroom fungi metabolites against *A. solani* spore germination and mycelial growth Many of the macro fungi extracted with polar and non polar solvents contained bioactive compounds with antifungal, antibacterial and antiviral activities (Wasser, 2002) [29]. Antimicrobial compounds from 20 day old crude cell free culture filtrates of *G. lucidum, L. edodes, V. volavceae and A. polytricha.* Irrespective of mushroom species spore germination was higher with increase in duration. Among the treatments *G. lucidum* recorded higher percentage of spore germination inhibition (53.07%) followed by *L. edode* (37.93%) at 24 h. Control showed highest (90.67%) spore germination. Chen and Hyuang (2010) [4] reported that the culture filtrates of *Lentinula edodes* completely inhibited the spore germination of *Colletotrichum higginsianum.* Also, culture filtrates of *Ganoderma lucidum* inhibited spore germination of *Alternaria brassicicola* and culture filtrates of *L. edodes* suppressed the germination of *Phytophthora capsici*.(Table 3)

The agar well diffusion of methanol extracted constituents of cell free culture filtrates of *G. lucidum, L. edodes, V. volavceae* and *A. polytricha* (Table 3) showed that all the metabolites extracted exhibited significantly varied inhibition of mycelial growth of *A. solani. Ganoderma* compounds identified are mostly Triterpenes (lanostanoid-type triterpene and polyketides (Farnesyl quinone), small peptides (ganodermin) and polysaccharides with antimicrobial properties (Basnet *et al.,* 2017) [3]. Antibacterial activity of *L. edodes* against bacteria has been reported (Quereshi *et al.,* 2010) [14]. In some other studies, crude methanolic extract of *Clitocybe* sp exhibited maximum inhibition against *Colletotrichum coffaenum* (Shahid *et al.,* 2016) [21]

Testing different concentrations of methanol extracted metabolites against *A. solani*

The antimicrobial metabolites extracted using methanol was made up to different concentrations of 0.05%, 0.1% and 0.2% to test the desired concentration that could inhibit maximum mycelial growth of *A. solani*. From the results (Table 4) it is observed that all the extracted antimicrobial metabolites of all mushroom exhibited significantly varied mycelial growth inhibition of *A. solani*. *G. lucidum* inhibited maximum (69.41%) mycelial growth of *A. solani* at 0.2%. *G. lucidum* basidiocarp showed antibacterial activity against *S. typhi* and

antifungal activity against *C. albicans* (Uma Gowrie *et al*., 2014) [26]. Antimicrobial substances from *L. edodes, A. polytricha* and *V. volvaceae* showed inhibition of mycelial growth of *Alternaria solani* (Radhajeyalakshmi *et al.,* 2011) [15]. The fruiting body, mycelia and spores of *G. lucidum* contain ganoderic acid, polysaccharides, triterpenoids, fatty acids, nucleotides, protein, peptides, sterols (Kim *et al.,* 1999) [12] which account for more than 400 bioactive compounds.

Conclusion

Antimicrobial molecules from fungi, bacteria and plants to manage plant diseases can mitigate the environmental hazards and pollution by indiscriminate use of chemical fungicides. Among the mushroom fungi screened against *A. solani*, the macrofungi *Ganoderma lucidum* found to be with several high value bioactive mycomolecules needs to be identified and it has great scope for developing effective bio-fungicides against plant pathogens.

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