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Efficacy, stability and persistence of Ganosol, a *Ganoderma* based fungicide against plant pathogens

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A B S T R A C T

Ganoderma which was identified to have antifungal potential by dual culture technique against sheath blight pathogen of rice was developed as an emulsifiable concentrate. The formulation of 10EC at 0.25% was standardized as the best emulsifiable concentration for use against the pathogen. The pre inoculation spray of the formulation was observed to produce 63.62% reduction in lesion height caused by the sheath blight pathogen. Two isoforms of poly phenol oxidase, PPO 1 and PPO 3 were observed to be induced only in Ganosol 10EC sprayed plants and were absent in control. The formulation was tested to have good emulsion stability. The antifungal activity of the formulation persisted up to seven days after spraying the formulation on rice plants. The formulation retained its 100% antifungal efficacy up to three months of storage. Surprisingly, spraying cowpea plants with the formulation as simultaneous spray reduced the number of lesions produced by *Groundnut Bud Necrosis virus* in cowpea by 88.39% when compared to the inoculated control, suggesting the antiviral potential of the formulation.

Keywords: Ganoderma**,** sheath blight, Groundnut Bud Necrosis virus, Ganosol, dual culture

Introduction

Biologically active substances from various mushrooms have been proved to possess antifungal, antibacterial and antiviral properties and they can also be used as insecticidal and nematicidal agents (Wasser 2002). A lectin named AAL from *Agrocybe aegerita* (Brigantini) Singer has been found to inhibit *Tobacco mosaic virus* infection (Sun *et al*. 2003). Studies on the toxicity of 14 mushrooms on several insects revealed that proteins, such as lectins and haemolysins play a major role as good insecticidal candidates (Wang *et al.* 2002). Several commercial formulations from mushrooms are presently available in the market. PSK, a polysaccharide from *T. versicolor* has been shown to induce potent antimicrobial and antitumor activity (Wasser 2002). Strobilurin from *Strobilurus tenacellus* (Pers. ex. Fr.) Singer (Anke *et al*. 1977) and Oudemansin from *Oudemansiella mucida* (Schrader: Fries) Hohnel (Musilek *et al*. 1969) are being used as agricultural fungicides. Another compound, azoxystrobin has high potential use in the control of downy and powdery mildew pathogens (Sendhilvel 2003). Kresoxim -methyl (Ammermann *et al*. 1992), another fungicidal compound is more effective than azoxystrobin against cereal powdery mildew. Both the compounds are having low mammalian toxicity and get rapidly degraded in soil making them environmentally benign. Thus, the evidence on the medicinal and therapeutical values as well as the variety of compounds with biological activity from mushrooms pave us the way to use them in the field of management of plant pathogens as well. The present investigation was a novel approach to develop a pesticidal formulation based on the metabolites identified from *Ganoderma* mushroom against sheath blight of rice.

Materials and Methods

Eighteen mushrooms collected from different parts of Tamil Nadu were screened for their antifungal activity by dual culture technique (Dennis & Webster 1971). Six solvents *viz*., ethyl acetate, diethyl ether, hexane, chloroform, acetone and petroleum ether were tested for their efficacy in extracting the antifungal metabolites from the mycelial mass as well as the culture filtrate of mushrooms. The diethyl ether extract was used to develop an emulsifiable concentrate (EC). The formulation as EC was developed using the recommended quantities of emulsifying agents, stabilizing agent and solvent. Emulsion stability was tested by measuring the volume of creamed matter at the top or the sediment at the bottom of the formulation in standard hard water. The *Ganoderma* formulation (Ganosol 10EC at 0.25 %) which was identified as effective in controlling the pathogen growth *in vitro* was further tested under glasshouse conditions as pre inoculation, simultaneous and post inoculation spray. Native PAGE analysis was done to assess the synthesis of isoforms of PPO after spraying the formulation in rice. The persistence of the antifungal activity was assayed by spraying Ganosol 10 EC at 0.25 % on rice plants under glasshouse condition. The formulation was stored at different temperature levels and bioassays on the inhibition of mycelial growth of the pathogen were carried out by poisoned food technique. The antiviral activity of the formulation was also tested against *Groundnut bud necrosis virus* in cowpea local lesion host C-152.

Results

The eighteen mushrooms collected were screened for their antifungal effect against four pathogens *viz*., *Rhizoctonia solani* Kühn, *Macrophomina phaseolina* (Tassi.) Goid., *Pyricularia grisea* Cavara and *Sarocladium oryzae* (Sawada) Gams and Hawksworth by dual plate technique. Among the fungi tested, the maximum reduction of the mycelial growth was exhibited by *Ganoderma* against *R. solani*. Among the six solvents tested, diethyl ether was found to be efficient in extracting the antifungal metabolites from the culture filtrate of *Ganoderma* against *R. solani* (Table 1). Different emulsifiable concentrations of Ganosol were prepared and tested for their antifungal activity against *R. solani.* 10, 20, 30, 40 and 50 emulsifiable concentrates (EC) at 0.1%, 0.25%, 0.5%, 0.75% and 1.0% were tested for their antifungal efficiency. Among them, 10 EC at 0.25 percent was found as the best emulsifiable concentration for use against *R. solani*. The formulation, Ganosol 10EC was also standardized. Emulsion stability of the formulation was tested with standard hard water (Table 2). For all the formulations with its different concentrations tested, the sedimentation or creaming level did not exceed the critical limit of 2 ml, indicating that the formulation was having good emulsion stability. A glass house experiment was conducted which revealed that the pre inoculation spray of the formulation produced 63.62 percent reduction of lesion height caused by the disease (Table 3). One day after the spraying of the formulation and challenge inoculation with the pathogen, there was a reduction of

56.24 percent in the lesion height over control and the reduction persisted up to seven days (Table 4). Ganosol 10 EC at 0.25 per cent was tested for its stability at 4, 11 and 28 ± 2ºC (room temperature) for different durations. The formulation retained its 100 per cent antifungal efficacy up to three months against the sheath blight pathogen. Four isoforms of PPO (PPO 1, PPO 2, PPO 3 and PPO 4) were induced in rice plants sprayed with *Ganoderma* formulation as pre, simultaneous and post sprays and challenge inoculated with *R. solani*. PPO 4 isoform was induced in all the treatments including healthy and inoculated controls. The isoforms PPO 1 and PPO 3 were induced only in Ganosol 10 EC sprayed plants and were absent in control plants. In healthy and inoculated controls alone, PPO 2 isoform was induced. Spraying of cowpea plants with the formulation reduced the number of lesions of *Groundnut bud necrosis virus* to a significant level when compared to the inoculated control. Simultaneous spray of the formulation and the virus inoculation was found to be the best treatment, which produced 88.39 per cent reduction of the number of lesions followed by pre inoculation spray (63.67%) compared to the control, indicating the antiviral potential of the formulation (Table 5).

Table 1.

Solvent extraction of the antifungal metabolites from *Ganoderma* sp. collected from the place known as Veppankulam in Tamil Nadu

Solvent	Nature of the solvent	Antifungal activity of *Ganoderma* extract against pathogens			
		R. solani	***P. oryzae***	***S. oryzae***	***M. phaseolina***
Ethyl acetate	Intermediate	-	-	-	-
Diethyl ether	Intermediate	+	-	-	+
Hexane	Non polar	-	-	-	-
Chloroform	Polar	-	-	-	-
Acetone	Polar	-	-	-	-
Petroleum ether	Non polar	-	-	-	-

- Absence of antifungal activity; + Presence of antifungal activity

Table 2.

Emulsion stability of Ganosol at different concentrations

Formulation	Creamy appearance/100ml measuring cylinder		Sedimentation/100 ml measuring cylinder	
	Below 2 ml	**Above 2 ml**	**Below 2 ml**	**Above 2 ml**
10 EC 0.1%	+	-	+	-
10 EC 0.25%	+	-	+	-
20 EC 0.1%	+	-	+	-
20 EC 0.25%	+	-	+	-

+ Presence of creamy appearance/sedimentation; - Absence of creamy appearance/sedimentation

Table 3.
Effect of Ganosol 10 EC (0.25 %) on sheath blight of rice under glasshouse

Treatment	Concentration (%)	*Lesion length (cm)	*Mean lesion height (%)	Per cent reduction over control
Healthy control	-	0.00 [a]	0.00 (1.00) [a]	-
Inoculated control	-	3.89 [h]	7.12 (15.47) [h]	-
Ganosol 10 EC pre inoculation spray	0.25	1.55 [d]	2.59 (9.27) [c]	63.62
Ganosol 10 EC simultaneous spray and inoculation	0.25	1.67 [d]	2.87 (9.75) [d]	59.69
Ganosol 10 EC post inoculation spray	0.25	1.95 [e]	3.28 (10.43)[e]	53.93
Ganoderma diethyl ether extract pre inoculation	0.25	2.17 [f]	3.79 (11.22) [f]	46.77
Ganoderma diethyl ether extract simultaneous spray	0.25	2.33 [g]	3.94 (11.44) [f]	44.66
Ganoderma diethyl ether extract post inoculation	0.25	2.39 [g]	4.32 (11.99) [g]	39.33
Carbendazim pre inoculation spray	0.25	1.00 [b]	2.42 (8.95) [bc]	66.01
Carbendazim simultaneous spray and inoculation	0.25	1.20 [c]	2.39 (8.89) [b]	66.43
Carbendazim post inoculation spray	0.25	1.25 [c]	2.40 (8.90) [b]	66.29
CD(P=0.05)		0.134	0.35	
SEm±		0.05	0.12	

* Mean of three replications; Means with in the column followed by same letter are not significantly different (P=0.05) by Duncan's Multiple Range Test; Figures in parentheses are arcsine transformed values

Table 4.
Evaluation of persistence of the antifungal activity of Ganosol 10 EC (0.25 %)

Days after spraying the formulation	*Mean lesion height (%)		Per cent reduction over control
	Pathogen inoculated	Formulation treated	
1	9.21 (17.68)[a]	4.03 (11.58) [a]	56.24
2	9.25 (17.75)[a]	4.09 (11.67) [a]	55.78
3	10.03 (18.23)[b]	4.89 (12.77) [b]	51.25
4	10.57 (18.96)[c]	5.44 (13.49) [c]	48.53
5	10.98 (19.62)[d]	5.87 (14.02) [d]	46.54
6	11.13 (20.37)[e]	6.03 (14.21) [e]	45.82
7	11.32 (20.58)[e]	6.24 (14.46) [f]	44.88
CD(P=0.05)	0.264	0.183	
SEm±	0.087	0.060	

* Mean of three replications; Means with in the column followed by same letter are not significantly different (P=0.05) by Duncan's Multiple Range Test; Figures in parentheses are arcsine transformed values

Table 5.
Antiviral activity of Ganosol 10EC (0.25 %) on groundnut bud necrosis virus

Treatment	Concent-ration (%)	Number of plants observed	*Number of lesions	*Average number of lesions	Per cent reduction over control
Healthy control	-	5	0^{a}	0^{a}	0
Inoculated control	-	5	267^{e}	53.4^{e}	0
10 EC pre inoculation spray	0.25	5	97^{c}	19.4^{c}	63.67
10 EC simultaneous spray and inoculation	0.25	5	31^{b}	6.2^{b}	88.39
10 EC post inoculation spray	0.25	5	237^{d}	47.4^{d}	10.67
CD(P=0.05)			24.70	5.43	
SEm±			7.84	1.72	

*Mean of three replications; Each replication of five plants, two leaves per plant. Means with in the column followed by same letter are not significantly different (P=0.05) by Duncan's Multiple Range Test

Discussion

In the present study, eighteen mushrooms, both wild as well as cultivated were collected and screened for their antifungal potential against four phytopathogens by dual plate technique. Among them, *Ganoderma* was found to be the most effective mushroom in inhibiting the mycelial growth of sheath blight pathogen, *Rhizoctonia solani*. Badalyan *et al*. (2002) established the antagonistic activity of 17 species of mushrooms against four fungi (*Cochliobolus sativus*, *Fusarium culmorum*, *Gaeumannomyces graminis* and *Rhizoctonia cerealis*) in dual culture experiments on potato dextrose agar medium. Almost all the tested mushroom species significantly inhibited the mycelial growth of the four phytopathogenic fungi, the antagonistic activity of *Pleurotus ostreatus*, *Hypholoma fasciculare*, *Ganoderma lucidum*, *Lentinus tigrinus* and *Schizophyllum commune* being particularly strong. The dual culture of *Lentinus edodes* and mycoparasitic *Trichoderma* spp. revealed that during the antagonistic interactions on solid medium, induction and inhibition of several extracellular enzymes of both the partners were observed, indicating the important role of these enzymes in the antagonistic interaction (Hatvani *et al*. 2002). Hwang *et al*. (2000) isolated the antifungal agent phellinsin A from *Phellinus* sp. capable of inhibiting the growth of fungi such as *Colletotrichum lagenarium*, *Pyricularia. grisea*, *Rhizoctonia solani*, *Aspergillus fumigatus* and *Trichophyton mentagrophytes*. Attempts were also made on the standardization of the extraction procedure of the antifungal metabolites. Among the six solvents tested, diethyl ether was found to be the most efficient in extracting antifungal metabolites from *Ganoderma*. The ethanolic extracts from fruiting bodies of *Oudeman-*

siella sp. and *Ganoderma* sp. decreased the number as well as the diameter of colonies and rates of sporulation of cucumber powdery mildew caused by *Sphaerotheca fuliginea* (Stadnik *et al*. 2003). Jonathan and Fasidi (2003) assayed *Lycoperdon pusilum* (Bat. ex.) and *Lycoperdon giganteum* (Pers.) *in vitro* for their antimicrobial activities using water, methanol and ethanol. Ethanol was found to be the best extracting solvent followed by methanol and water against *Microsporum boulardii*.

A formulated product was developed and evaluation studies were made to assess the antifungal and antiviral potential of the formulation under glasshouse condition. Natural antimicrobials are produced by a number of edible mushrooms that grow in forests on decaying wood. These natural antimicrobials help the mushrooms in competing with other fungi for nutrients. Some of these natural products including Oudemansin A and Strobilurin A were tested for the control of many plant pathogenic fungi and promising indications of disease control were observed. This led to the chemical synthesis of analogues with improved antifungal activity and optimized physical properties. These compounds have low mammalian toxicity and a benign environmental profile. One of the most significant recent advances in fungicide discovery was the introduction of synthetic analogues of Strobilurin A, a compound produced by the basidiomycete *Strobilurus tenacellus*. Azoxystrobin has high levels of activity against mildews of grape vine and vegetables, sheath blight and blast on rice and brown patch and *Pythium* blight on turf and has high potency against oomycetes, ascomycetes, basidomycetes and deuteromycetes. Its fungicidal activity was observed to be due to the inhibition of mitochondrial respiration in fungi (http://hcs.osu.edu/basicgreen/diseases/weapon.htm). For maximum effectiveness, fungicidal molecules must be formulated. This involves the addition of formulation products that alter the chemical and physical characteristics of the active ingredients. These formulated products allow compatibility with available machinery, aid in delivering the active ingredients to the site where the pathogen will be and aid in extending the residence time at that location so that economic levels of control can be achieved (Backman 1978).

Data from *in vitro* and *in vivo* animal studies indicated that *G. lucidum* and other *Ganoderma* species exhibited a broad spectrum of antifungal, antibacterial and antiviral activities. Azoxystrobin controlled the two most economically important diseases of rice *viz*., sheath blight and blast. None of the fungicides for use in rice are as effective against both the diseases (www.pested.msu.edu/Bullslidenews/pesticideNotes/ma-pn-01.pdf). Azoxystrobin and flutolanil reduced the disease severity due to *R. solani* to less than10 per cent for more than 28 days in each year of study (Burpee 1998). Kiewnick *et al*. (2001) reported that the fungicides azoxystrobin and tebuconazole reduced crown and root rot disease caused by *R. solani* by 50 - 90 percent when used at the rates of 76 to 304g a.i./ha

and 250 g a.i./ha, respectively. Glasshouse tests have confirmed that azoxystrobin has high levels of activity *in vivo* whether applied before or after pathogen inoculation. Extensive glasshouse and field tests indicated that it was safe to crops at required rates and was harmless to beneficial organisms at field application rates. It got rapidly broken down in the environment. It was reported to redistribute to emerging leaves at the time of the treatment. Root uptake has indicated high levels of systemic activity as in the case of powdery mildew when applied as soil drench.

Ganoderma constituents are studied to inhibit viral replication by interfering with their adsorption, viral integration, assembly and release (Gao *et al*. 2003). *L. edodes* is reported to produce some antimicrobial substances active against *Tobacco mosaic virus*, the causal agent of the mosaic in tobacco plants. The aqueous extracts of fruiting bodies of isolates LE 96/17 and LE 96/22 when added to the suspension of TMV reduced the number of local lesions on the leaves. This study thus demonstrated the potentiality of *L. edodes* in interfering with the multiplication of phytopathogens (Tonucci 2004). A lectin named AAL has been purified from the fruiting bodies of the edible mushroom, *Agrocybe aegerita* (Brigantini) Singer. It showed inhibition activity to the infection of *Tobacco mosaic virus* on *Nicotiana glutinosa* L. AAL at a concentration of 200 μ g/ml produced 83.43 per cent reduction in lesion number. AAL was found to attach to TMV particles thereby blocking the infection sites (Sun *et al*. 2003).

Native gel electrophoresis also revealed the presence of different isoforms of PPO. Several host coded defense proteins were also found to be induced upon treatment with the formulation. Studies with azoxystrobin revealed that the fungicide produced remarkable changes in the levels of PO, PPO and phenol. Native gel electrophoresis also confirmed the induction of isoforms of polyphenol oxidase and proteins. Induction of PPO isoforms was pronounced in challenge inoculation with azoxystrobin and not in control in response to pathogens (Sendhilvel 2003). Thus the present study implies that, several enzymes involved in various plant defense pathways have been induced in rice leaves treated with Ganosol 10EC (0.25 %) in response to the invasion of pathogens. Emulsion stability was tested with standard hard water for the formulations of *Lawsonia inermis* L., *Psoralea corylifolia* L. and *Lawsonia inermis* L. which revealed that the sedimentation levels did not exceed the critical limits of 2 ml (Bharathi 2004).

The antifungal activity of the formulation was found to be persistent up to seven days when tested in glasshouse against sheath blight of rice. The formulated product retained 100 per cent antifungal activity against the pathogen *in vitro* up to three months in the study.

The formulated product *viz*., azoxystrobin was found to be stable for a minimum of two years under ambient conditions. The residues were found up to 10 days in the leaves and 7 days in fruits after the last spray. Detected residue level was high on zeroth day after application

and there after declined. The mechanism of disappearance showed that the decrease in residues was due to photo degradation (Gareur *et al.* 2002).

Thus, the present investigation could prove the presence of antifungal as well as antiviral metabolites of *Ganoderma* against plant pathogens. Thus mushrooms can be considered as a ready source of antimicrobial metabolites which can be formulated and commercialized on a large scale. Large scale production and up scaling remain as the future challenge. These metabolites, if commercialized can protect the farming community from the risk of environmental pollution, high cost of plant protection as well as residual toxicity of chemical pesticides.

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