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Antifungal activity and GC-MS detection of *Argyreia nervosa* L. leaves extract against *Pythium ultimum,* a pathogen causing damping-off of Broccoli

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ABSTRACT

This study investigates the fungitoxic effects of *Argyreia nervosa* L. on *Pythium ultimum* Trow., the causal agent of broccoli damping-off. In vitro experiments utilized acetone extracts at concentrations of 5, 10, 15, and 20% resulting in suppressed fungal mycelial growth. Synergistic assessments involving *Trichoderma viride, T. harzianum,* and *Argyreia* leaf extract revealed the most significant inhibition of mycelial growth. Compatibility between the antagonists and the extract was established. Analysis of the extract identified 20 compounds, including antifungal elements like n-hexadecanoic and 9, 12-octadecadienoic acid. This study highlights *Argyreia nervosa* L. fungitoxic potential and its compatibility with biocontrol agents, suggesting its promising role in fungal disease management.

Keywords: *Argyreia nervosa* L, *Pythium ultimum*, GCMS, Damping-off

INTRODUCTION

Among the destructive pathogens, *Pythium ultimum* (Trow). is the worst pathogen that causes seedling damping-off disease in Broccoli (Dixon & Dixon, 1981). *Pythium ultimum,* which belongs to the class Oomycete, is considered a universal pathogen that causes diseases in many plants, including important crops **(**Mostowfizadeh-Ghalamfarsa, & Salmaninezhad, 2020**;** Sharma *et al*., 2020). The fungus survives in the soil as sexual oospores. These are dormant spores that can withstand both high and low temperatures as well as dehydration. The oospores can either generate sporangia or germinate directly using a germ tube. Host plants can also become infected via sporangia and oospore germ tubes. Seeds, seedlings, and roots are all affected by the fungus (Guo & Ko., 1993; Nzungize *et al*., 2012).

The Broccoli (*Brassica oleracea* var. *italica*) family of Brassicaceae is considered a significant crop worldwide among vegetables. Broccoli contains important minerals, vitamins, sugars, and the most important antioxidant, sulforaphane (Vasanthi *et al*., 2009). India ranks second in area and production of cauliflower and broccoli. World area and production are 1.21 million hectares and 20.88

million tonne, and Indian production and area are 6745 thousand tonnes and 369 thousand hectares (Annonymous, 2015b). Due to the attack of several soil-borne fungal pathogens, the yield per hectare of broccoli is badly affected.

Plant diseases caused by fungi are often controlled with chemical fungicides due to their high effectiveness. However, these fungicides have numerous disadvantages, including environmental pollution and harm to humans and animals. Additionally, the killing of beneficial insects and the development of resistance by pathogenic fungal strains to many types of these fungicides are also concerns (Kamurthy *et al*., 2016). Due to the emergence of pathogen resistance, scientists are now increasingly focused on biological control agents and their antifungal metabolites. The biological control of plant pathogens has been investigated as a possible control technique in recent years (Nega, 2014). Our objective was to identify compounds extracted using GS-MS and test their efficacy in inhibiting the mycelial growth of *Pythium ultimum* in vitro. Additionally, we aimed to explore the potential of these compounds as an alternative to synthetic fungicides for controlling plant diseases.

MATERIALS AND METHODS

Collection of plant material and extraction

Fresh leaf samples of *Argyreia nervosa* L. were collected from the forest area of Jalna, Maharashtra, India. The plant was identified following Naik, (1998). The leaves were dried, powdered, and subjected to the Soxhlet extraction process (Redfern *et al.,* 2014). The extract was subjected to a preliminary phytochemical analysis following the standard procedures described by Sofowora A., (1993) and Trease and Evans (1989).

Isolation, maintenance and identification of pathogen

Diseased broccoli seedlings with damping-off symptoms were collected and washed in running tap water, cut into pieces (1 cm long), rinsed in sterile distilled water for 3 times, blotted dry with paper towel, and then placed on 2% water agar (Huang and Lin, 1998). After 48 h incubation at 24 0C, hyphal tips of fungi growing out from the collar region were cut and transferred onto potato dextrose agar (PDA) medium and maintained in PDA slants (Ainsworth, 1961) and stored at 24 ^{o}C in a incubator. Pathogen was identified on the basis of their morphological and cultural characteristics with the help of available literature (Mukadam and Chavan, 2006). Koch's postulates were demonstrated for the pathogen isolates.

Growth inhibition assay

Leaf extracts of *Argyreia nervosa* L. the concentrations of 5, 10 and 15 % were evaluated for their effect on *in vitro* mycelial growth of *Pythium ultimum* Trow., a fungus isolated from infected broccoli plant. The method proposed by Sánchez-Pérez *et al*. (2009) was used for this purpose. Leaf extracts of each concentration were individually added to petri dishes with PDA culture medium. Those were kept aside for 30 min for evaporation of acetone and the diffusion of the extract in PDA. A disc of 5 mm in diameter of PDA with the mycelium of *Pythium ultimum* Trow. was placed in the center of the dish, which was then incubated at 28°C. Absolute control, without leaf extract, was also maintained. The bioassay was performed in triplicate. Effect of leaf extracts (5, 10, and 15%) was determined by measuring the colony diameter at 3rd, 5th, and 7th days, and expressing it as the percentage inhibition of mycelial growth as compared to that in control, using the formula: % inhibition = [(Average mycelial growth in control - average mycelial growth in the treatment)/ (average mycelial growth in control)] x 100 (Pandey *et al*., 1982).

Compatibility of plant extract and bio agents

In the present investigation compatibility was determined for *T. viride*, *T. harzianum* and plant extracts by using PDA medium, by following methods - poisoned food technique. After sterilizing the medium, a crude water extract of *Argyreia nervosa* L. was combined aseptically to achieve necessary concentrations of 5, 10, 15, and 20 mg/ 20 ml PDA. In each sterile Petri plate, fifteen ml PDA was poured and allowed to solidify independently. A five mm actively growing culture disc of *T. viride*, *T. harzianum* was placed at the one end of the plate and *Pythium* on the other end of the plates were incubated in an inverted position at room temperature (28 ± 2°C). As a control, *Argyreia nervosa* L. extracts and *Trichoderma* sp. inoculations were placed on PDA medium without extract. Each concentration had three replications recorded. At 3, 5, and 7 days following inoculation, the radial development of the mycelium was frequently assessed (Varma & Gandhi, 2007).

Analysis of antifungal compound through gas chromatography-mass spectroscopy (GCMS)

GC-MS analysis of plant leaf extract (acetone) was done at the Sophisticated Analytical Instrument Facility (SAIF) labs, MIT CARS, Department of Agriculture Engineering Aurangabad, using the standard GC-MS model. The procedure followed for this purpose was that of Dandekar *et al.,* (2015). The analysis was performed using a GC-MS system (GC-2010 plus, Shimadzu, Agilent Technologies Inc.) equipped with an HP-5 MS capillary column (30 m x 0.25 mm, 0.25 mm, Agilent Technologies Inc.). The injection volume of each sample was 1 uL. Helium (99.999%) was used as the carrier gas at a flow rate of 1 ml/min. The temperature of the injection port was 250 °C, and the column temperature programme was as follows: 50 °C for 2 min, followed by an increase to 180 °C at a rate of 5 °C/min, an increase to 270 °C at a rate of 20 °C/min, and maintenance at 270 °C for 5 min. The MS (QP-2020) conditions included an EI ion source temperature of 230 °C, an ionisation energy of 70 eV, and a mass scan range of 40–500 amu. The major constituents were identified with the aid of a computer-driven algorithm and then by matching the mass spectrum of the analysis with that of a library (NIST) to determine its name, structure & molecular weight (Yusoff *et al*. 2017).

RESULT AND DISCUSSION

Growth inhibition assays

The effect of acetone extracts of *Argyreia nervosa* L. were examined on the growth of *Pythium ultimum* Trow. It showed positive antifungal activity against *Pythium* at different concentrations (5 - 15 %) at different time intervals (3 - 7 days). The leaf extract at 15 % concentration was found to be most effective against *Pythium ultimum* and caused the highest inhibition of mycelial growth after 3rd day of incubation (72.08%), followed by 5th day (75.98%) and 7th day of incubation (79.75%) (Table 1). The findings of this study are in agreement with those reported earlier by several workers (Mares, *et al.*, 2004; Muthukumar *et al*., 2010; Gholve *et al*., 2014).

In vitro inhibition by plant extract and biocontrol agents

The effects of *T. viride, T. harzianum,* and *Argyreia nervosa* L. leaf extract on *Pythium ultimum* radial growth were examined alone and in combination. All the treatments were effective in reducing the mycelial growth of the pathogen. However, combination of *T. harzianum* and leaf extract resulted in the highest inhibition of 78.4 % with least mycelial growth (13.23 mm) over control (Table 2).

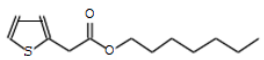
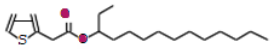
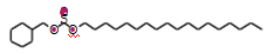
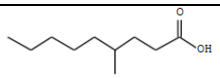
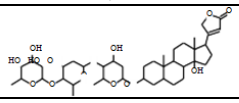
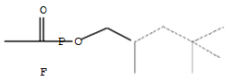
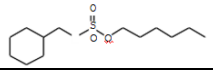
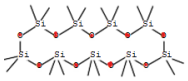
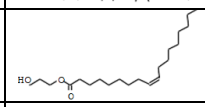
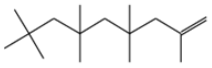
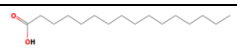
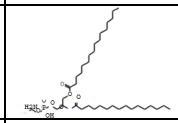
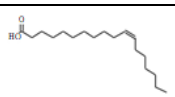
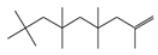
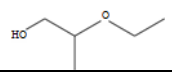
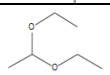
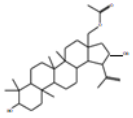
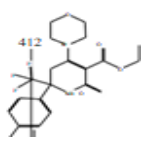
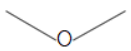
Table 1. Percent inhibition (%) in the growth of *Pythium ultimum* with extracts of *Argyreia nervosa.*

Sr. No.	Treatment	Concentration (%)	Percent inhibition (%)		
			After 3rd Day	After 5th Day	After 7th Day
1	T1	5	22.32	24.76	27.39
2	T2	10	61.09	63.87	67.86
3	T3	15	72.08	75.98	79.75
4	T4	20	69.12	72.64	76.18
5	Control	0	0	0	0

Table 2. Synergistic efficacy of *T. viride*, *T. harzianum* and *Argyreia nervosa* leaf extract on the growth of *P. ultimum*

Sr. No.	Treatment	Colony Dia. of test pathogen * (mm)	Percent Inhibition
1	*T. viride*	11.7	56.97
2	*T. harzianum*	17.21	72.52
3	*T. viride* + *T. harzianum*	21.98	75.16
4	*T. viride* + *Argyreia nervosa (leaf extract)*	10.27	66.92
5	*T. Harzianum* + *Argyreia nervosa (leaf extract)*	13.23	78.4
6	*Control*	88.7	e

Table 3:

Sr. No.	R.Time	Name	Molecular Formula	M. Weight	Area%	Structure of Compound
1	35.83	2-Thiopheneacetic acid, heptyl ester	$C_{13}H_{20}O_2S$	240	4.315	
2	34.358	2-Thiopheneacetic acid, 3-tetradecyl ester	$C_{19}H_{28}O_2S$	320.489	1.2711	
3	33.257	Sulfurous acid, cyclohexylmethyl octadecyl ester	$C_{25}H_{50}O_3$	430	2.8411	
4	28.863	4-Methylnonanoic acid	$C_{10}H_{20}O_2$	172	0.6707	
5	28.255	Digitoxin	$C_{41}H_{64}O_{13}$	764	5.3981	
6	27.367	2,4,4-Trimethyl-1-pentyl methyl phosphono fluoridate	$C_9H_{20}FO_2P$	210	1.0666	
7	26.453	Sulfurous acid, cyclohexylmethyl hexyl ester	$C_{13}H_{26}O_3S$	262	1.3284	
8	24.66	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	666	0.6433	
9	23.955	Oleic acid, 3-hydroxypropyl ester	$C_{21}H_{40}O3$	340	0.8248	
10	22.993	2,4,4,6,6,8,8-Heptamethyl-1-nonene	$C_{16}H_{32}$	224	3.1386	
11	21.051	*n*-hexadecanoic acid	$C_{16}H_{32}O_2$	256.4	1.4736	
12	20.528	Hexadecanoic acid, 1-[[[(2-aminoethoxy)hydroxyphosphinyl] oxy] methyl]-1,2-ethanediyl ester	$C_{37}H_{74}NO_8P$	692	0.8914	
13	19.071	9,12-Octadecadienoic acid	C18H32O2	280.4	6.0915	
14	18.618	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282	23.5889	
15	12.636	2,4,4,6,6,8,8-Heptamethyl-1-nonene	$C_{16}H_{32}$	224	0.768	
16	2.763	1-Propanol, 2-ethoxy-	$C_7H_{16}O_2$	132	0.6629	
17	2.555	Ethane, 1,1-diethoxy-	$C_6H_{14}O_2$	118	26.6414	
18	2.415	Lup-20(29)-ene-3,21,28-triol, 28-acetate	$C_{32}H_{52}O_4$	500	1.5799	
19	2.28	4-Morpholin-4-yl-2-oxo-6-p-tolyl-6-trifluorom	$C_{20}H_{23}F_3N_2O_4$	412	0.7171	
20	2.243	Dimethyl ether	C_2H_6O	46	11.1381	

The compatibility between the fungal antagonists *T. viride, T. harzianum* and *Argyreia* leaf extract was tested in vitro and showed that the leaf extract is compatible with both. The combined use of biocontrol agents has been shown by multiple researchers, including Muthukumar, *et al*., (2010). and Jadhav and Ambadkar (2007), to significantly improve disease management.

3.3 Preliminary phytochemical and GCMS analysis

The results obtained on phytochemical tests indicated that *Argyreia nervosa* L. plant extract contained phytochemical groups such as flavonoids, phenols, steroids, glycosides, saponins, tannins, and terpenoids. Out of these, the steroids were present in higher concentrations.

Based on the performance of the plant extract in previous in vitro experiments, *Argyreia nervosa* L. leaf extract was analyzed to ascertain the type of the chemical constituent (s) present in the extract. The findings indicated the presence of 20 chemicals in *Argyreia nervosa* leaf (Fig. 1). In the present study, Table 3 includes the molecular weight, name of the molecule, chemical formula, retention period, and peak area %. Among them, 9, 12-octadecadienoic acid and *n*-hexadecanoic acid was present, it might be responsible for retardation of fungal growth of the test fungi. Kapoor & Mishra (2014) evaluated the 9, 12-octadecadienoic acid derivatives' antifungal and antibacterial properties. In similar investigations, Asha and Yogendra (2015) reported 1, 3, 4, 5-Tetrahydroxy-Cyclohexanecarboxyl, n-Hexadecanoic acid, Phytol, and other compounds in methanolic extract of *A. nervosa*.

CONCLUSION

This study highlights the fungitoxic potential of *Argyreia nervosa* L. and its compatibility with biocontrol agents, suggesting its promising role in managing fungal diseases, particularly in the context of broccoli damping-off caused by *Pythium ultimum*. The chemical analysis of the plant extract provides insights into the potential antifungal compounds present in *Argyreia nervosa* L., which could be further explored for disease control applications.

Conflict of Interest: None of the authors have any conflicts of interest to disclose.

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