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In vitro Evaluation of Some Fungicides and Tea Extract Against *Pestalotia* sp. and *Colletotrichum* sp., The Causal Agents of Leaf Spot and Anthracnose of Azalea

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Abstract

The effect of five fungicides and tea extracts was tested against anthracnose disease and leaf spot of *Azalea* during summer (2015) at the laboratory in Rasht Branch, Islamic Azad University. Commercially formulated fungicides at 1000, 2000 and 3000 ppm concentrations and the regression of normalized growth rate were used to determine the EC50. The application of different fungicides on *Pestalotia* sp., Bavistin 50WP, Dithane M-45, Aliette, Benlate 50 WP and Topsin M 70 WP gave significant reduction in colony growth, i.e. 0.5, 0.5, 0.50, 3.38 and 2.56 mm, respectively as compared to control (88.00 mm) in 2000 ppm at recommended doses. The application of different fungicides on *Colletotrichum* sp. Resulted in significant reduction in colony growth. The fungicides were tested against *Colletotrichum* sp. at 1000, 2000 and 3000 ppm concentrations, and no growth was observed in Mancozeb, Thiophanate-methyl and Fosetyl aluminum at any concentrations. Three of the tested fungicides suppressed mycelial pathogen growth effectively. However, there were significant differences in the sensitivities exhibited by the examined pathogen isolates. Concentrations of extracts from fresh tea leaves used to control the disease agents could not prevent the growth of fungal colony.

Keywords: Chemical fungicides, Fungal colony, Guilan, *Rhododendron* spp., Tea extracts.

INTRODUCTION

In Guilan province, azalea (*Rhododendron* L.) leaves during the spring for propagation may be infected with *Pestalotia* sp. and *Colletotrichum* sp. In a number of specimens during the vegetation season on leaves are visible blotch and necrosis, leading to their premature falling. Multiple methods were evaluated in a series of laboratory experiments for the purpose of eliminating *Pestalotia* and *Coletotricum* from leaves of azalea to prevent spread of azalea leaf spot during the propagation phase of production (Kita and Mazurek, 2003; Kowalik and Muras, 2007; Kowalik *et al.* 2010). This study was to investigate the effect of five fungicides and tea extracts against anthracnose disease and leaf spot on azalea in culture plates at the laboratory during spring (2015).

Rhododendrons (*Rhododendron* spp.) are popular and economically important ornamental plants in the Ericaceae family and are widely grown in the Guilan region of northern Iran. In the Lake Maggiore area, the market for acid-loving plants is valued at 13 million euros per year. Azalea is the most popular species grown, accounting for 50% of total production. More than one million plants are sold each year (Rabbogliatti, 2004), with a portion of the production grown for export (Bertetti *et al.*, 2009).

Azaleas are susceptible to several foliar diseases (Benson and Williams- Woodward, 2001) including anthracnose, a fungal disease that causes leaf spots and defoliation. The fungi *Phyllosticta, Septoria, Pestalotia*, and *Colletotrichum* are common causal agents of this malady. Unfortunately, diverse types of discolorations, spots and necrosis are frequently observed on the surface of leaf blades, which could be caused by fungi inhabiting leaf tissue. The symptoms mentioned above are accompanied with deformations and premature fall of leaves (Kowalik *et al*., 2006; Kowalik, 2008, 2009) (Fig. 1).

Fig.1. Leaf spot of azalea as the symptoms of the disease.

Anthracnose, which is caused by the *Colletotrichum* sp., is a widespread and destructive disease on container-grown azalea. Anthracnose is a hot, wet weather disease. While Kurume-type azaleas are considered to be most susceptible to attack by causal *Colletotrichum* sp., extensive damage was also seen on more resistant Indica-type azaleas this summer.

Typically, softwood cuttings taken from diseased azaleas in production blocks are the most likely source of anthracnose in Alabama nurseries. The risk of the spread of this disease among crops can be greatly reduced by taking cuttings from fungicide-protected disease-free stock. When rooting azalea cuttings in a mist bed, a recommended fungicide should be applied to suppress disease spread through the block of liners. Flats of diseased liners should be discarded. Sanitation practices such as cleaning propagation areas of debris between crops and cleaning pruning tools with a surface disinfectant such as rubbing alcohol will also help slow disease spread.

Anthracnose on azalea was reported for the first time in Florida on swamp azalea (*Rhododendron viscosum*) and the causal agent was identified as *C. azaleae* (Ellis and Everhart, 1895). Since 1954, anthracnose on azalea was observed and described in Louisiana, where the causal agent was identified as the conidial stage of Glomerella cingulata, the teleomorph of *C. gloeosporioides.*

In general, most leaf spots are not threatening to the health of the plant. Though, some defoliation may occur under severe conditions. The symptoms usually include discrete spots with

tan to brown centers surrounded by a darker border. Leaf spot is a common disease of azalea which is caused by *Pestalotia* sp. A comparison of P. sydowiana (syn. *Pestalotia sydowiana*) and *Truncatella truncata* (syn. *Pestalotia truncata*) in causing necrotic symptoms on the leaves of azalea and evergreen rhododendron leaves (Kowalik, 2013) revealed that these fungi were more often colonizing healthy leaves of pontic azalea than infected or fallen leaves. Little research has been carried out on the physiology of *Pestalotia*, an important pathogen of azalea.

Green tea is selected for the study because tea consumption has its legendary origins in China of more than 4,000 years ago. Green tea has been used as both a beverage and a medicine in most parts of Asia to help a wide range of things from controlling bleeding and helping heal wounds to regulating body temperature, blood sugar and promoting digestion (Anderson *et al*., 2005). The most abundant components in green tea are polyphenols, in particular flavonoids such as the catechins, catechingallates and proanthocyanidins (Brantner and Grein, 1994). Tea polyphenols are also known for their antifungal activity. In general, antifungal activity decreases when the extent of tea fermentation is increased, implying stronger activity in green tea than black tea (Inamdar *et al*., 2014).

Usage of biocontrol methods is the solitary alternate solution that can reduce toxic chemical substances. The searches for a harmless fungicide that do not have an ecological impact and can be involved in sustainable agriculture are a must. Several higher plants and their constituents have shown success in controlling plant disease, and are proved to be harmless and non-phytotoxic unlike chemical fungicides.

The main objectives of this research is to determine the level of sensitivity of *Colletotrichum* and *Pestalotia* to different fungicides at their recommended doses under laboratory conditions on colony growth and the effect of tea extract that may be used to manage anthracnose and leaf spot.

MATERIALS AND METHODS

Isolation of fungi

The diseased specimens for this study were collected from greenhouses of Rasht and were isolated from the diseased parts. Diseased specimens were cut into small bits and immersed in 1% sodium hypochlorite solution for two minutes and then rinsed with sterilized water in each petri dishes. The bits were then put on filter papers in sterilized petri dishes in order to absorb excessive water present on them and were then transferred to solidified potato dextrose agar (PDA) plates. To avoid bacterial contamination, streptomycin sulfate (1:10,000) was added to the medium after sterilization and before pouring. These plates were incubated at 27°C. On sporulation of the fungus, temporary mounts (glycerin water) were made and isolate was identified according to Pathak (1980). The hyphal tips were transferred onto PDA plate after growing the mycelium. The new plates were incubated at 27±1°C for acervuli production.

Identification of fungi

The fungi were then identified on the basis the morphological characteristics with the help of identifying key book (Barnett and Hunter, 1972).

Preparation of different concentrations of fungicides mixed with PDA

Different fungicides were evaluated in *in vitro* conditions against *Pestalotia* sp. and *Colletotrichum* sp. following poison food technique (Dhingra and Inclai, 1985). Commercially formulated fungicides were added to sterile PDA. Requisite quantity of individual fungicides was added to melted PDA to have concentration of 1000, 2000, and 3000 ppm. After through mixing, amended medium was autoclaved at 121°C under 1 kg/cm^2 for 20 min. Approximately 20 ml of melted PDA mixed with fungicides was poured into each 90 mm diameter Petri dish. After solidification, the inoculation (5 mm disc) from three-day-old culture of test organisms was placed at the center of the test plate. The fungicides used in the experiment are listed in Table 1. The pH of the medium was adjusted to 6.5.

Table 1. List of fungicides used in the experiment.

Chemical name	Mode of action	Trade name	Concentration (ppm)
Mancozeb	Contact	Dithane 80 WP	1000, 2000 and 3000
Carbendazim	Systemic	Bavistin 50 WP	1000, 2000 and 3000
Fosetyl aluminum	Contact	Aliette 80 WP	1000, 2000 and 3000
Thiophanate-methyl	Systemic	Topsin M 70 WP	1000, 2000 and 3000
Benomyl	Contact	Benlate 50 WP	1000, 2000 and 3000

Measurement of radial growth and calculation of percent inhibition

Each of two isolates was transferred to five plates containing each of three concentrations of five fungicides. Plates were incubated in the dark at 20°C. Colony diameter was measured daily from day 3 to day 7, and mean growth rate (mm per day) was calculated. Growth rate was normalized as a percent of isolate growth on un-amended media (0 µg ai/ml). Normalized growth rate was regressed against log µg ai/ml fungicide concentration to determine the EC50 (µg ai/ml concentration that suppressed fungal growth to one half that of the fungus on un-amended media) for each fungicide-isolate combination.

Percentage inhibition of growth was calculated using the following formula (Naz *et al*., 2006):

Percent of inhibition = X-Y/X×100

Where,

X=Average growth of Pestalotia sp. and Colletotrichum sp. in control petri dishes;

Y=Average growth of *Pestalotia* sp. and *Colletotrichum* sp. in each fungicide treated petri dishes.

Experimental design and data analysis

The experiment was laid out in CRD with three replications. The data were analyzed statistically using SAS software package and means were compared for difference by Duncan's Test.

Plant extract preparation

Fresh leaves of tea were collected and 1 %, 2 % and 3 % (w/v) slurry were prepared in distilled water using mechanical homogenizer. The extract was further sonicated for 5 minutes to make perfect extraction. Each of this leaf slurry was mixed with the PDA medium at 1%, 2% and 3% concentrations without altering the constituent composition. Negative control included sterile distilled water instead of extracts and positive controls were prepared using various known fungicides like Mancozeb, Thiophanate-methyl, Carbendazim and Benomyl at same concentrations mentioned above. All the plates were incubated at 28 °C for 14 days until the colonies were developed (Sheema and Durai, 2014).

RESULTS AND DISCUSSION

Identification of fungus

The pathogens were identified based on morphology of reproductive structures e.g. acervuli and conidia characteristics to *Pestalotia* sp. and *Colletotrichum* sp.

Effect of fungicides against *Pestalotia* sp. and *Colletotrichum* sp.

Chemical control is the valid option for any of the disease management strategy. Being quick, cheap and easy, despite of health hazard effects, chemical control of pathogens is advocated. Five fungicides namely, Mancozeb, Thiophanate-methyl, Fosetyl aluminum, Carbendazim and Benomyl at three concentrations each were tested against *Pestalotia* sp. and *Colletotrichum* sp. The results are presented in Table 2.

Effect of various fungicides at different concentrations was found to be significant at 1 % level. Mancozeb and Aliette inhibited the growth of *Pestalotia* sp. and *Colletotrichum* sp. No growth was found at any concentration (Figs. 2, 3).

Table 2. Inhibition percentage of *Pestalotia* sp. and *Colletotrichum* sp. at different concentrations of fungicides

Fungicides	Concentration (ppm)	Inhibition of *Colletotrichum* sp. (%)	Inhibition of *Pestalotia* sp. (%)
Mancozeb	1000	100 ^{a}	100 ^{a}
	2000	100 ^{a}	100 ^{a}
	3000	100 ^{a}	100 ^{a}
Carbendazim	1000	64.70^{c}	100 ^{a}
	2000	75.67^{c}	100 ^{a}
	3000	79.53^{b}	100 ^{a}
Thiophanate-methyl	1000	100 ^{a}	54.72^{ce}
	2000	100 ^{a}	66.75^{c}
	3000	100 ^{a}	75.43^{c}
Fosetyl aluminum	1000	100 ^{a}	100 ^{a}
	2000	100 ^{a}	100 ^{a}
	3000	100 ^{a}	100 ^{a}
Benomyl	1000	15.48^{fh}	10.73^{h}
	2000	19.56^{fh}	12.96^{fh}
	3000	25.92^{f}	20.44^{f}
control	-	0^{g}	0^{g}
Level of Significance α=0.01			

*In each column, means followed by similar letter(s) were not significantly different (P < 0.05) according to Duncan's Test.

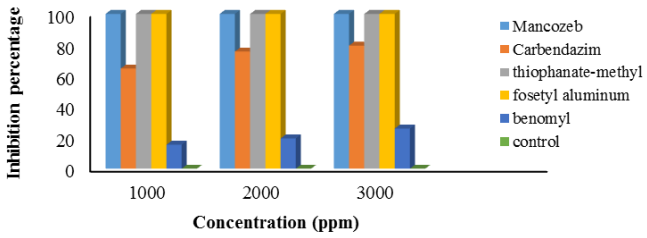

Fig. 2. Inhibition percentage of *Colletotrichum* sp. at different concentrations of fungicides

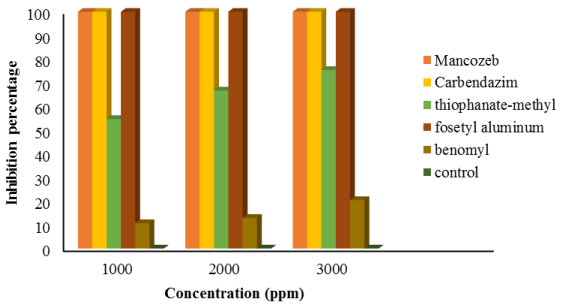

Fig. 3. Inhibition percentage of *Pestalotia* sp. at different concentrations of fungicides.

Carbendazim at 3000 ppm inhibited the growth of *Colletotrichum* sp.by 79.53% which differed from all fungicides at all concentrations. While the percentage of inhibition was 100% for all three concentrations of carbendazim on *Pestalotia* sp. . Effect of 1000 and 2000 ppm Carbendazim on *Colletotrichum* sp. and 2000 and 3000 ppm Thiophanate-methyl on *Pestalotia* sp. was statistically similar but differed from other concentrations of differed fungicides. Their inhibition percentages were 64.70 %, 75.67 %, 66.75 % and 75.43 %, respectively. Thiophanate-methyl inhibited 66.75 % which differed from other concentrations of all fungicides. All three concentrations of benomyl on both fungi had the lowest percentage of inhibition.

According to the data obtained from *in vitro* conditions, two fungicides Mancozeb and Aliette had the best effect on both fungi and it seems that the average concentrations of 2000 ppm can be used to control both fungi.

Concentrations of extracts from fresh tea leaves used to control the disease agent could not prevent the growth of fungal colony.

Rawal and Ullasa (1988) reported that Zineb, Chlorothalonil, Thiophante, methyl, prochlorza, ziram, dithionan, fosetyl aluminum, copper oxychloride and Carbendazim gave good control of canker, *Pestalotiopsis* psydii and *Glomerella psydii*. The findings of these studies are in agreement with them (Younis *et al.,* 2004).

In 1980, benomyl resulted in approximately 90% control of anthracnose on euonymus. Chlorothalonil and EBDC fungicides completely protected euonymus leaves from infection by *C. gloeosporioides*. In addition, copper and EBDC fungicides were also reported to be effective in controlling an anthracnose leaf spot of azalea. Other *Colletotrichum* species, such as *C. acutatum* and *C. fragariae*, have previously been reported to be resistant to benzimidazoles (Mahoney and Tattar, 1980).

The recognition that fungicide resistance is present in isolates of *Pestalotia* sp. and *Colletotrichum* sp. from azalea should result in reduced use of those fungicides in favor of other broad spectrum of fungicides that continue to be effective. Future management programs for control of azalea anthracnose need to be developed to control the disease and minimize fungicide resistant isolates in a population, perhaps by the use of mixing or alternating fungicides with different modes of action. These management programs need to incorporate the effective use of efficacious fungicides with nonchemical control tactics such as sanitation and environmental modification.

Use of plant products for the management of phyto-pathogenic fungi is swiftly fetching an important module of Integrated Disease Management (IDM) program. The natural plant products are bio-degradable and thus eco-friendly, and so they are growingly considered by the scientists throughout the world. Such products from higher plants contain a relatively broad spectrum and are bio-efficacious, economical and environmentally safe (Ramezani, 2006). The arbitrary usage of the common fungicides has made human being and wild life susceptible to a wide array of diseases. Plants contain various kinds of phytochemicals like saponins, alkaloids and flavonoids, etc. (commonly called secondary metabolites) that convey the antimicrobial effects. Extracts of leaves from the tea plant *Camellia sinensis* contain polyphenolic components with activity against a wide range of microbes. It is well proved that fresh leaves of tea that were used in the study have at least little effect on controlling the fungus (Inamdar *et al*., 2014).

In the present study, tea extract had no effect on disease-causing. According to data obtained, further studies should be conducted in order to obtain positive results, find different plant extracts, and apply them to the fungi.

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