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Research Article

BIOLOGICAL CONTROL OF ONION BLACK MOLD BY INDIAN CULINARY SPICES UNDER IN VITRO CONDITIONS

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ABSTRACT

Black mold disease caused by *Aspergillus niger* is the predominant infection occurs in onions (*Allium cepa* L.). Use of synthetic fungicides to control plant diseases is a common practice but their toxicity and development of pathogen resistance needs to find alternative sources for disease management. Spices have been known to have potential antimicrobial properties and this study has been focused to control onion black mold using Indian culinary spices under in vitro conditions. Various parts of fourteen Indian culinary spices have been selected and phytochemical analysis was performed from the methanolic extracts. *Aspergillus nig*er was isolated from infected onion bulbs and antifungal assay was performed under in vitro conditions. Phytochemical tests revealed that glycosides, steroids and terpenoids were the major phytochemicals universally present in the samples followed by flavonoids, tannins and phenols. Preliminary antifungal screening revealed that among fourteen spices tested, cardamom, cinnamon, clove, pepper, star anise and stone flower were exhibited inhibitory activity against the black mold. Various concentrations of the extracts ranging from 15 to 120 mg/ml were prepared and minimum inhibitory concentration (MIC) values were determined. Significant inhibitory activity was found at 15mg/ml concentration for cinnamon and clove. Stone flower at 30mg/ml was able to inhibit the pathogen and moderate inhibition was found in cardamom.

Keywords: *Aspergillus niger*, biological control, onion black mold, postharvest, spices

INTRODUCTION

Fungal diseases are of major concern in postharvest decay of fruits and vegetables which reduces shelf life and market value [1]. Contamination by storage fungi and their mycotoxins reduce the quality of food products. India is the second largest producer of onions (dry) after China with 1.6 million MT annually [2]. Post harvest diseases of onions include black mold, blue mold, neck rot, brown rot, soft rot and smudge among which, black mold and blue mold are the predominant ones [3]. *Aspergillus niger* is an saprophytic and filamentous fungi found in soil, forage, organic debris and food products causing black mold disease on the onion (*Allium cepa* L.) bulbs [4]. The fungus requires an optimum temperature of 28°C-34°C for growth and warm, moisture conditions favor the disease development [5]. The infection starts early at germination stage and continue throughout storage [6, 7]. The pathogen is transmitted by contaminated soil or seed and the infected bulbs show discoloration on the neck, streaks of black mycelia and spores beneath the outer dry scales [8, 9, 10]. Black mold disease can be effectively controlled by chemical treatment [11, 12] but is undesirable due to the potential health hazards. There is a growing need to develop alternative methods for effectively controlling black mold disease of onions.

Control of plant diseases using chemical fungicides is commonly practiced around the world but their toxicity and non-biodegradable nature restricts their usage. Further, increasing public concern of pesticide residues and proliferation of resistance development among pathogen populations are the two major obstacles of using chemical fungicides [13, 14]. Natural products research might substitute synthetic fungicides and contribute to the development of new products for plant disease management. Antimicrobial agents from indigenous plant origin are quite effective and spices occupy a considerable attention as they have a great potential to be used as antimicrobial agents [15]. Spices have been recognized for their value of preserving foods and medicinal values for centuries due to their antimicrobial potential and are considered as alternatives to conventional antimicrobial agents [16]. In this study, we aimed to explore the antifungal activity of Indian culinary spices against the black mold disease of onions under in vitro conditions thereby to reduce the dependency of synthetic pesticides.

MATERIALS AND METHOD

Isolation of pathogen

Onion bulbs showing black mold symptom were inoculated into potato dextrose agar and incubated at 30°C for 5 days. A spore suspension containing 1 x 10^7 spores/ml was prepared from the fungal culture and used as inoculums.

Spices

Fourteen samples of spices viz., *Laurus nobilis* (Bay leaf), *Capparis spinosa* (Caper), *Carum carvi* (Caraway), *Elettaria cardamomum* (Cardamom), *Cinnamomum verum* (Cinnamon), *Syzygium aromaticum* (Clove), *Coriandrum sativum* (Coriander), *Cuminum cyminum* (Cumin), *Foeniculum vulgare* (Fennel), *Myristica fragrans* (Mace) *Piper nigrum* (Pepper), *Papaver somniferum* (Poppy), *Illicium verum* (Star anise), *Parmelia perlata* (Stone flower) were used in this study.

Phytochemical analysis

Samples of spices were pulverized and extracted in methanol (1:10 w/v) at room temperature for 48 hrs and filtered. The filtrates were concentrated to dryness under reduced conditions at room temperature and phytochemical analysis was carried out following standard procedures [17, 18, 19].

Test for Alkaloids (Mayer's test)

0.5 ml of extract was added with a drop or two of Mayer's regent by the side of test tube. Formation of white or creamy precipitate indicates positive test for alkaloids.

Test for Flavonoids (Ammonia test)

1.0 ml of the extract was taken in the test tube and ammonia solution was added (1:5) followed by the addition of concentrated sulphuric acid. Appearance of yellow color and its disappearance on standing indicates the positive test for flavonoids.

Test for Glycosides (Keller Kiliani test)

5.0 ml of each extract was added with 2.0 ml of glacial acetic acid which was followed by the addition of few drops of ferric chloride solution and 1.0 ml of concentrated sulphuric acid. Formation of brown ring at interface confirms the presence of glycosides.

Test for Phenols (Ferric chloride test)

0.5 ml of the extract was added with few drops of neutral ferric chloride (0.5%) solution. Formation of dark green color indicates the presence of the phenolic compounds.

Test for Saponins (Froth test)

1.0 ml of the extract was taken in a test tube and distilled water (2.0 ml) was added to it. The test tube was then kept in boiling water bath for boiling and existence of froth formation during warming confirms the presence of saponins.

Test for Steroids (Libermann - Burchard's test)

2.0 ml of acetic anhydride was added to 0.5ml of the extract and then added 2.0 ml of sulphuric acid slowly along the sides of the test tube. Change of colour from violet to blue or green indicates the presence of steroids.

Test for Tannins (Ferric chloride test)

1.0 ml of the extract was added with 5.0 ml of distilled water and kept for boiling in hot water bath. After boiling, sample was cooled down and to this 0.1% ferric chloride solution was added. Appearance of brownish green or blue black coloration confirms the presence of tannins.

Test for Terpenoids (Salkowski test)

5.0 ml of extract was taken in a test tube and 2.0 ml of chloroform was added to it followed by the addition of 3.0 ml of conc. sulfuric acid. Formation of reddish brown layer at the junction of two solutions confirms the presence of terpenoids.

Antifungal assay

Agar well diffusion method was followed to determine the effect of spice extracts against the black mold *Aspergillus niger* by using Saboraud's Dextrose Agar (SDA). Spore suspensions of seven day old fungal cultures were prepared in 0.5% tween 80 in sterile distilled water and adjusted to give a spore count of 10^6 spores/ml using haemocytometer. 100 µl of the spore suspension was swabbed on the surface of SDA plates and 5 mm well was created on the agar plates. 20 µl of the extracts were added to the well and the plates were kept undisturbed for 30 minutes for the pre-diffusion of the extracts. The plates were incubated at 28°C for 3-5 days and the zone of inhibition was measured using Himedia scale. The minimum inhibitory concentration (MIC) was determined as the least percentage concentration of the spice extract that shows an inhibitory effect on the mycelial growth of the test fungi using radial growth method.

Statistical analysis

Each antifungal test was carried out in 3 replications. Significant differences between values were determined by Duncan's multiple range test ($p<0.05$), following one-way ANOVA.

RESULTS AND DISCUSSION

The present study revealed the potential antifungal activity of Indian culinary spices against the onion black mold. Phytochemical analysis of the spice extracts explored that glycosides, steroids and terpenoids as the major phytochemicals universally present in the samples (Table-1). In addition to that, flavonoids, tannins, phenols were also found in most of the spices tested. Alkaloids were determined in four of the fourteen spices tested. Saponins were present only in clove extracts. Colonies successfully isolated from the infected onion bulbs were identified as *Aspergillus niger* [20, 21]. Microscopic appearance revealed that the conidia are black, spherical in chains. Conidiophores arise from long, broad, thick walled cells and phialides are borne in clusters (Fig-1). In vitro antifungal activity of 14 Indian culinary spices was investigated against black mold disease of onions. Differences in the sensitivity of fungi to spice extracts suggest its variable potency between the spices tested. Preliminary antifungal screening revealed that among the fourteen spices tested, cardamom, cinnamon, clove, pepper, star anise and stone flower were exhibited antifungal activity and MIC values (15-120 mg/ml) of those spices were determined (Fig-1). Increased antifungal activity was observed with increasing concentrations of the extracts. Among the spices tested, cinnamon has exhibited maximum inhibitory activity (33 mm) followed by clove and star anise (20 mm). MIC values obtained were 15mg/ml (cinnamon, clove, pepper) and 30 mg/ml (cardamom, star anise, stone flower) for the tested spices. Significant inhibitory activity was found at 15mg/ml concentration for cinnamon and clove. Stone flower at 30mg/ml was able to inhibit the pathogen and moderate inhibition was found in cardamom even at higher concentration.

Tannins possess antimicrobial properties due to its inhibition of microbial enzymes, cell envelope transport proteins, oxidative phosphorylation and complex with polysaccharides [22]. Flavonoids have the ability to inhibit spore germination of plant pathogens [23]. Saponins interact with membrane sterols and exhibit antifungal activity [24]. Presence of flavonoids, phenols, tannins, terpenoids and glycosides in spice extracts has influenced the antifungal activity against the pathogen.

Aspergillus causes spoilage of agricultural products during pre-harvest, harvest and postharvest stages [25]. *Aspergillus niger*, a soil saprophyte causes black mold disease of onions under field and storage conditions in tropic and sub tropic regions thus causing substantial economic loss [26]. Previous studies used fungal antagonists [27], plant compost leachates [28], essential oils [29] and organic amendments [30] to control onion black mold. Spices are natural antimicrobial agents used as food preservatives and additives and in this study control of onion black mold was achieved by using Indian culinary spices.

Abuse of synthetic fungicides resulted in fungicide resistance development of pathogens and toxic effects to human and environment health. Alternative measures for plant disease management which are biodegradable and low toxic are needed. Use of spices is one method for controlling onion black mold which will help to combat fungicide resistant strains and to avoid pesticides residues from the environment and commodities.

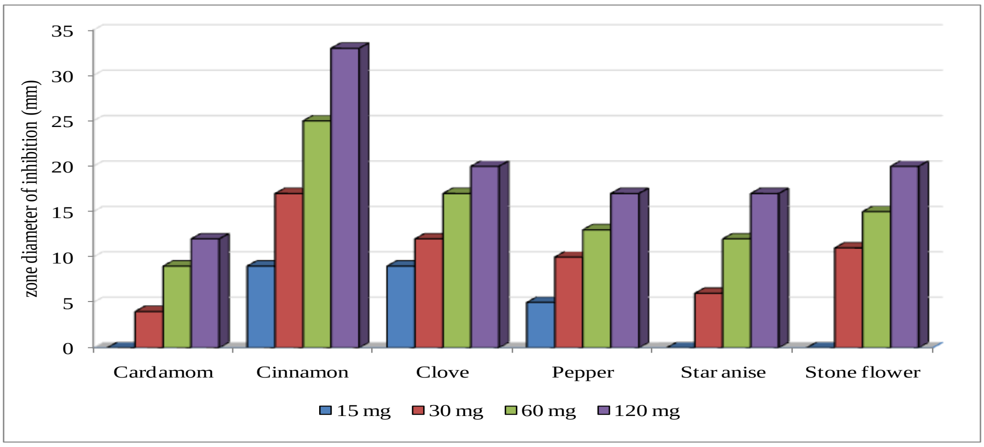
Table - 1: Phytochemical analysis of Indian culinary spices

Botanical name	Common name	Family	Part used	Phytochemical analysis							
				Alk	Fla	Gly	Phe	Sap	Ste	Tan	Ter
Laurus nobilis	Bay leaf	Lauraceae	Leaf	-	+	++	+	-	+	+	++
Capparis spinosa	Caper	Capparidaceae	Flower bud	-	+	+	++	-	+	++	++
Carum carvi	Caraway	Apiaceae	Seed	-	+	+	+	-	+	+	++
Elettaria cardamomum	Cardamom	Zingiberaceae	Seed	-	+	+	-	-	+	-	++
Cinnamomum verum	Cinnamon	Lauraceae	Bark	-	+	++	+	-	+	+	++
Syzygium aromaticum	Clove	Myrtaceae	Flower bud	+	+	+	++	+	+	++	++
Coriandrum sativum	Coriander	Apiaceae	Seed	-	+	+	-	-	+	-	++
Cuminum cyminum	Cumin	Apiaceae	Seed	-	+	++	++	-	+	++	++
Foeniculum vulgare	Fennel	Apiaceae	Seed	-	+	++	-	-	+	-	++
Myristica fragrans	Mace	Myristicaceae	Seed	+	+	++	-	-	+	+	++
Piper nigrum	Pepper	Piperaceae	Seed	+	+	++	-	-	+	-	++

Papaver somniferum	Poppy	Papeveraceae	Seed	-	-	+	-	-	+	-	++
Illicium verum	Star anise	Illiciaceae	Flower bud	-	-	+	+	-	+	+	++
Parmelia perlata	Stone flower	Parmeliaceae	Flower	+	-	+++	-	-	+	++	+

(+) presence; (-) absence

Fig-1: MIC values of spice extracts against onion black mold



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