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BIOLOGICAL CONTROL OF LEAF SPOT CAUSING FUNGAL PATHOGENS IN RED EDGE DRACAENA AND SOW THISTLE

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Leaf necrosis is a common fungal problem of plants. During a survey of Lahore, Dracaena Red Edge (*Dracaena mariginata* Lam.) and Sow thistle (*Sonchus oleraceus* L.) were infected with leaf spots. Pathogens isolation from the infected leaves of both plants was done followed by confirmation of Koch's pathogenicity postulates. Based on morphological and genetic characterization, *Alternaria arborescens* Simmons. and *Phyllosticta aristolochiicola* R.G. Shivas. were isolated from *D. mariginata* and *S. oleraceus*, respectively. In addition to isolation and identification, biological control of both pathogens was conducted using methanol extract of Cinnamon (*Cinnamomum verum* J. Presl.). All concentrations of extract suppressed the growth of both pathogens to variable extent. In case of *A. arborescens*, 0.5% concentration induced approximately 90% suppression in fungal growth. Contrastingly, *P. aristolochiicola* was least affected at lower concentrations of extract as only 4-10% arrest in fungal biomass production was recorded at 0.5-1.5% concentrations. However, 2.0% or more of the extract was most potent as fungal pathogens failed to grow at these concentrations. Therefore, Cinnamon extract was considered more effective in controlling *A. arborescens* than *P. aristolochiicola*. Further, studies are required to identify active antifungal compounds in Cinnamon crude extract against the target pathogens.

Keywords: *Alternaria arborescens*, biological control, *Cinnamomum verum*, Methanolic extract, pathogenicity, *Phyllosticta aristolochiicola*

INTRODUCTION

Leaf spot diseases are very drastic problem for the growers of fruits, vegetables and ornamental plants which are mostly caused by a variety of fungal pathogens, but some are instigated by bacteria. Many pathogens e.g. *Alternaria* and *Colletotrichum* affect bedding plants, cut flowers and cut foliage, tropical foliage plants and woody crops. Fungal leaf spots rarely kill a crop but cause massive losses. Mostly leaf spot diseases develop as small, scattered, circular to oval dead areas in the leaves; usually tan, dark brown, yellow, gray, purple or black. Some spots are raised, shiny, and coal black, others may dropout leaving ragged holes, some are marked with light and dark concentric zones and merge into large, angular to irregular dead areas. The spots vary in size and color depending on the plant affected, the specific organism involved, and the stage of development and environmental conditions. Concentric rings of darkish margins also appear (Barnett and Hunter, 1987; Woong *et al*., 1996).

Two plants are identified as the host of leaf spot fungi in the current study i.e., Dracaena Red Edge (*Dracaena mariginata*) and Sow thistle (*Sonchus oleraceus*). Dracaena is a genus of about 40 species of trees and succulent shrubs. It belongs to family Asparagaceae (formerly the family Ruscaceae). Majority of the species are native to Africa, few in Southern Asia and one in tropical Central America (Chase *et al*., 2009). Sow thistle is a weed that belongs to genus of flowering plants. These are also called hare thistle or hare thistle lettuces. Commonly known as Sow thistle, Annual sow thistle, Milk thistle, Milk weed, Thalaak (Mullin *et al.*, 1965). It belongs to Astereacea family. It is native to Africa and temperate regions. Inflorescence is yellowish and white in color. These are commonly found along the road sides.

By the thorough survey of literature, it comes into view that fungal leaf spot diseases are the major problems for ornamental growers. Most common fungal pathogens involved in causing fungal necrotic spot disease are *Alternaria and Phyllosticta.* Due to the hazards and damages, control of pathogenic fungi has become an obligatory requirement. The control can be done by many methods. Pathologists suggest pathogen-free seeds, cuttings and plugs and use of resistant cultivars. Chemical treatment is the last resort for disease management of fungal leaf spots for many ornamentals. More recent fungicides include fludioxinil, propiconazole, myclobutanil, azoxystrobin and trifloxystrobin. Continuous usage of chemicals makes the pathogen resistant so alternate fungicides should be used. The main disadvantage of using fungicides is the risk of poisoning humans or animals, contamination of livestock and it is not environment friendly. Biologically based and environmentally safe methods are effective to control disease and are the best alternative of chemical treatment. Natural yields or plants derived compounds subsidize a great extent in fight against pathogenic microorganisms (Vyvyan, 2002).

The biological inhibitions by different natural substances, such as essential oils and plant extracts have been investigated widely against fungal activities (Bajwa and Iftikhar, 2005; Shafique *et al*., 2006, 2012; Bajwa *et al.*, 2008). Dubey *et al*. (2000) tested *Ocimum gratissimum*, *Zingiber cassumunar*, *Cymbopogon citratus*, and *Caesulia axilliaris* against *Aspergillus flavus*. To control *Alternaria* spp. and *Phyllosticta* sp., Cinnamon powder was used. It belongs to family Laurceae. It has antibiotic and antifungal activity. The plant is fabricated by several natural substances having antagonistic activities against several microorganisms (Tung *et al.*, 2008).

MATERIALS AND METHODS

***Survey and collection of infected leaves*:** A survey was conducted to study the diseases of ornamental plants of Lahore. Besides many other infected plants, Dracaena Red Edge (*Dracaena mariginata*) and Sow thistle (*Sonchus oleraceus*) were found to be infected with necrotic leaf spots. Both of these plants were selected for the study of causal organisms. Size, color, shape and appearance of necrotic spots were noted and photographs of infected plants as well as leaves were also taken. For pathogen identification, five leaves per plant were selected randomly. Samples were stored at 4°C until processed for pathogen study. Fungal growth medium, Malt Extract Agar (MEA) (2 %, pH 6.5) was prepared for pathogen isolation and purification (Dhingra and Sinclair, 1993).

***Isolation and purification of fungal pathogens*:** For the isolation of endophytic fungal pathogens, at least 3-4 necrotic spots from each infected leaf were cut into 3 mm^2 small pieces (also contain some of the healthy leaf portion) and were dipped in 1% sodium hypochlorite solution for 5 min to kill epiphytic fungal flora. Five of these leaf pieces were inoculated aseptically onto MEA and inoculated petriplates were incubated at 25±2°C. Inoculated leaf samples were observed daily for the emerging fungal mycelium which was then transferred aseptically onto the fresh MEA petriplates and allowed to grow at 25±2°C for the purification of fungus cultures. Pure cultures were stored at 4°C.

***Identification of Fungi using Morphological Characters*:** Morphological observations were made on 7 d old pure cultures grown on MEA at 25±2°C. The colony characters included color of culture and reverse, number of growth zones, diameter of colony (cm), presence of aerial and submerged mycelium, type of conidial chains and abundance of conidia. Microscopic characteristics i.e., color, shape, number and position of septa of conidia and their attachment with the conidiophores; ornamentation of conidial walls; presence, size and shape of conidial beak and presence of apical or basal pores were recorded. Photographs were taken to record the macro - and micro - morphological characters. Species were key out by comparing their description with published authentic literature (Ellis 1971, 1976; Domsch *et al*., 1980; Simmons 1999; Barnett and Hunter, 1998; Leslie and Summerell, 2006; Crous *et al*., 2012).

***Identification of fungi on the basis of rDNA sequence analysis*:** The Internal Transcribe Spacer sequences of rDNA from both pathogens were amplified using the universal primer pair ITS1 (Forward) and ITS4 (reverse) using total fungal genomic DNA as template Akhtar *et al.* (2014a). Amplified PCR products were sent for nucleotide sequencing and resulting DNA sequences were analyzed by nucleotide BLAST. Homology of sequences was recorded and used for identification of fungal strains.

***Pathogenicity test*:** To make fungal spore inoculum, the protocol of French and Hebert (1982) was followed for both the target pathogens. Under aseptic conditions, spores from the surface of 7 days old pure fungus culture were scratched and suspended in 10 ml of sterilized Saline Tween 80 (0.9% NaCl, 0.1% Tween 80). Such stock of spore suspension was serially diluted to prepare the suspension of 5 x 10^5 spores ml^{-1} using a haemocytometer.

***Detached leaf method*:** For this method, sterilized petriplates lined with 2 filter papers were taken and moistened with 2 ml of doubled distilled sterilized water. The detached leaves from the healthy plants were placed separately in the petriplates in such a way that their petiole ends touched the moisten filter papers. After that approximately 10^3 spores were inoculated on the leaf surface with the help of micropipette under aseptic conditions, plates were incubated at 25±2°C and observed regularly for the emergence of disease symptoms. After the onset of disease, re-isolation of pathogen from the infected leaves was carried out to fulfill the Koch's pathogenicity postulates. Disease rating scale was made on the basis of disease incidence and disease severity. Disease incidence was observed as the symptoms appeared on the plant and disease severity was calculated with the help of following formula:

$$\text{Disease Severity} = \frac{\text{Area of plant part affected}}{\text{Total leaf area}} \times 100$$

***Biological control*:** Cinnamon was used to control the selected pathogens. Spice (Cinnamon) was purchased from the local market, oven dried at 40-45°C for 24 hrs and grinded to make fine powder (approximately 50 g). The soluble ingredients were then extracted by solubilization in methanol solution for 48 hrs at room temperature. After that, materials were filtered through muslin cloth followed by filter paper. The filtrates were evaporated in hot air oven at 45°C to yield 4.82 g of crude methanolic extract of Cinnamon. Then the methanolic extracts were dissolved in specific amount of sterilized distilled water to prepare stock solution of plant material.

Methanol residues were first dissolved in dimethyl sulfoxide (DMSO) and then sterilized distilled water was added to obtain the final concentration of 0.6 g/ml. To check the bioactivity of extract, four different concentrations viz., 0.5, 1.0, 1.5, 2.0, 2.5% of the organic solvent residue solution were prepared. In 100 ml flasks, 55 ml malt extract broth was

sterilized by autoclaving at 121°C and 15 lb/inch^2 for 15 min. Four concentrations 0.5, 1.0, 1.5, 2.0, 2.5 g/100 ml were prepared by adding 0.5, 1.0, 1.5, 2.0 % stock solutions, and 4.5, 4.0, 3.5, 3.0,2.5 ml sterilized distilled water respectively to each flask to make the total volume up to 60 ml, while control received 5 ml of distilled water. The 60 ml medium of each treatment was divided into four equal parts in 100 ml conical flasks to serve as replicates. Approximately 10^3 spores were inoculated into each respective flask under aseptic conditions to avoid contamination and incubated at 28+2°C for 7 d. After 7 d, fungal biomass from all the 4 replicates of each treatment was collected on pre-weighted filter papers and dried biomass was determined after oven drying overnight at 55-60°C. The rate and percentage of fungal biomass increase or decrease was determined from the oven dried biomass to evaluate the relative effects of various concentrations of Cinnamon methanolic extracts on target pathogens by applying the following formula:

$$\text{Growth increase/decrease (\%)} = \frac{\text{Growth in control - Growth in treatment}}{\text{Growth in control}} \times 100$$

RESULTS

The symptoms of the infected leaves of Red edge Dracaena and Sow thistle were studied. Symptoms observed on Red edge Dracaena were 1 mm circular dark brown spots with yellow margins (Fig. 1). The necrotic spots found on Sow thistle were 2-3 mm in size and dark brown in color (Fig. 2).

Figure 1. Red edge Dracaena. A: Infected plant and B: characteristic symptoms of necrosis on leaf.

***Identification of red edge dracaena leaf spot pathogen*:** Fungal pathogen was rapid growing on MEA 25±2°C, attaining the diameter of 4.2 cm in 7 d. The colony texture was fluffy to downy. The dorsal side of colony was dark greenish to light brown while the reversed side was light brown in color. Conidia were produced in short chains with only transverse septa which were 1-4 in number and 15-40 µm × 8-12 µm in size (Fig. 3). Based on morphology, pathogen was identified as *Alternaria arborescens.*

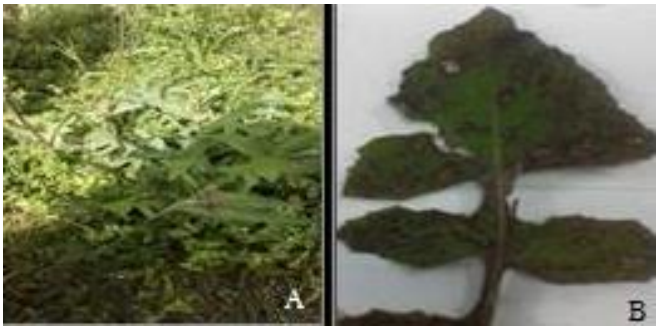

Figure 2. Sow thistle weed A: Infected plants and B: characteristic symptoms of necrotic leaf spots.

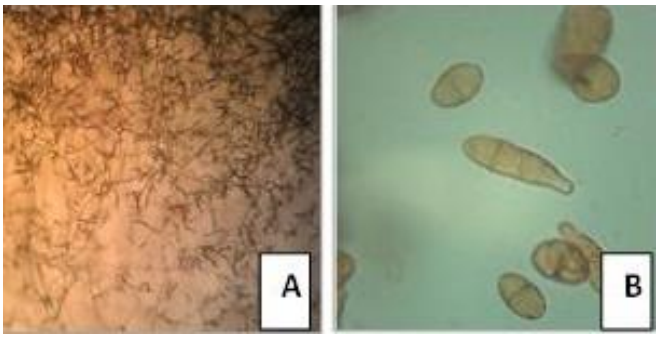

Figure 3. *Alternaria arborescens,* A: Conidial attachment with condiophores under stereoscope B: Conidia under microscope.

***Sow thistle leaf spot pathogen*:** The isolated pathogen was slow growing at 25±2°C. Colony color from both front and back side was black with irregular white margins. No sporulation was observed (Fig. 4). Based on morphology, as only sterile mycelium was present, pathogen *Phyllosticta aristolochiicola* was identified and colony diameter was 3.7 cm.

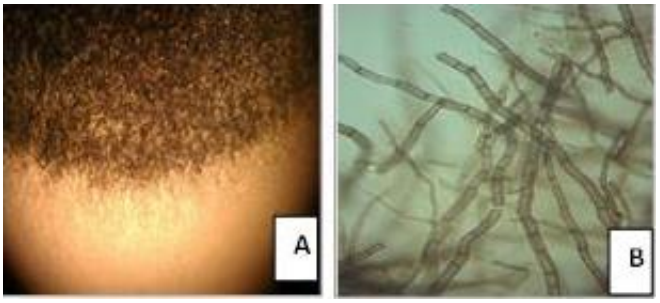

Figure 4. *Phyllosticta aristolochiicola.* Pure culture of *P.* aristolochiicola. A: under stereoscope. B: Sterile mycelium under microscope.

***Genetic Characterization*:** Morphology based identification of both pathogens were confirmed by nucleotide sequence analysis of Internal Transcribed Spacer Sequence (ITS) of rDNA. For this purpose, a DNA fragment of approximately

650 bp was amplified using universal primer pairs ITS1 and ITS4 from fungal genomic DNA (Fig. 5).

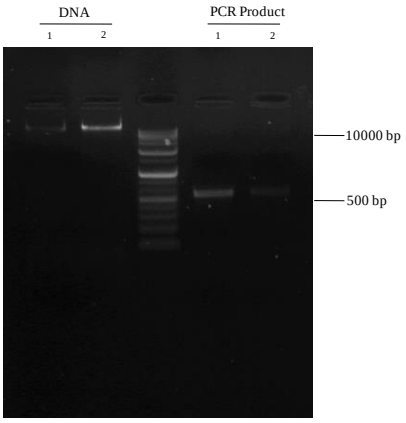

Figure 5. Agarose gel electrophoresis of total fungal genomic DNA and PCR amplified ITS region of rDNA. (1) *Alternaria arboresens* and (2) *Phyllosticta aristolochiicola.*

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GL6273         ATATGAAGGCGGGCTGGAACCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTC 71
STE-U4244      ------------------------------------CCTTGCTGAATTATTCACCCTTGTC 25
CICR1          ATATGAAGGCGGGCTGGAATCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTC 66
                                                    ***************************

Query          TTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTT 102
CC06-63-4      TTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTT 180
GL6273         TTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTT 131
STE-U4244      TTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTT 85
CICR1          TTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTT 126
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CICR1          ACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAG--CGGAGGAAAA 544
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Figure 6. Multiple sequence alignment of different isolates of *Alternaria arborescens*. Query sequence is the ITS nucleotide sequence of strain used for present study.

nBLAST results indicated that strain of *A. arboresens* investigated for present study has 100% similarly with many other isolates of same species in GenBank for example CC06-63-4 (JN634832), GL6273 (JX241641), STE-U4244 (AF397237), CICR1 (KF747355) etc. Multiple sequence alignment (Fig. 6) of these strains was also generated by ClustalW2 program using EBI (European Bioinformatics Institute) website. The amplified ITS nucleotide sequence of *P. aristolochiicola* when analyzed by BLAST, showed 98% homology with *P. aristolochiicola* strain BRIP 53316a having the GenBank ID, NR_111791 (Fig. 7).

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Query          ACAACGCCGAAAGAACCTCTCTCACCCTTGTGTACCCACTACGTTGCTTTGGCGGGCCGA  60
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Query          GAACTTAAGCATAT  554
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BRIP 53316a    GAACTTAAGCATAT  611
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Figure 7. ITS sequence alignment of *Phyllosticta aristolochiicola.* Query sequence is the ITS nucleotide sequence of strain used for present study.

Analyses of Pathogenicity

***Detached leaf method*:** Detached leaf method was adopted to evaluate the pathogenic potential of isolated fungal pathogens on both test plants. The severity of disease was determined on the basis of disease rating scale constructed in Table 1. The characteristic symptoms observed by both the pathogens on their respective plants were studied and photographed to compare the results with the symptoms observed in their natural habitat (Figs. 8, 9). It was noticed that the symptoms started to appear within 3-15 d on Red edge Dracaena while infectious symptoms were evident after 3-21 d of inoculation on Sow thistle weed. After 24 hrs small spots were appeared

on the surface of leaves in petri plates with 10% infected area in Dracaena (Fig. 10). With the progressing time from 2 to 20 d, a slope of disease progress curve was very sharp and approximately 50% of leaf area was found to be infected in Dracaena while in case of Sow thistle weed more than 80% leaf area was found to be infected (Fig. 11). The spotted portion of leaf from the Petriplate was re-inoculated on media plates for the confirmation of the pathogen either it is same as isolated pathogen.

Table 1. Disease Rating Scale.

Infected area (%)	Score	Status
20	1-3	Highly resistant
40	3-6	Resistant
60	6-9	Moderately susceptible
80	9-12	Susceptible
100	12-15	Highly susceptible

Figure 8. Progression of symptoms in Red edge *Dracaena* in detached leaf assay from A-E.

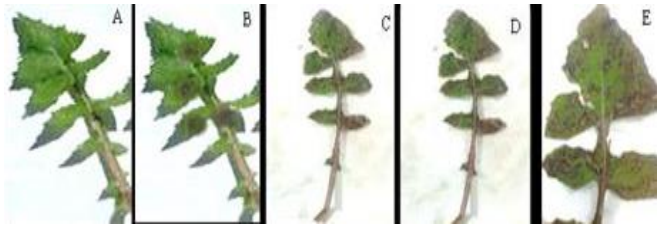

Figure 9. Progression of symptoms in Sow thistle weed in detached leaf assay from A-E.

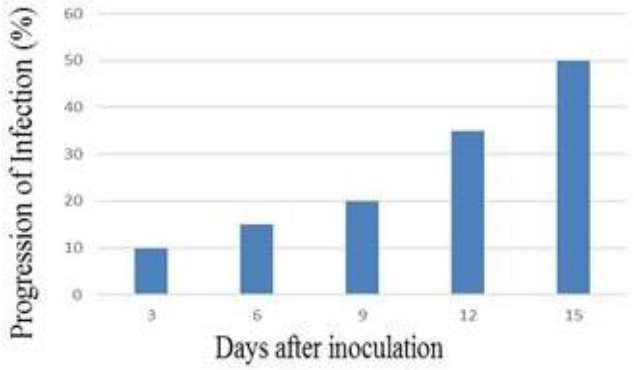

Figure 10. Disease progress curve of Red edge Dracaena by detached leaf assay.

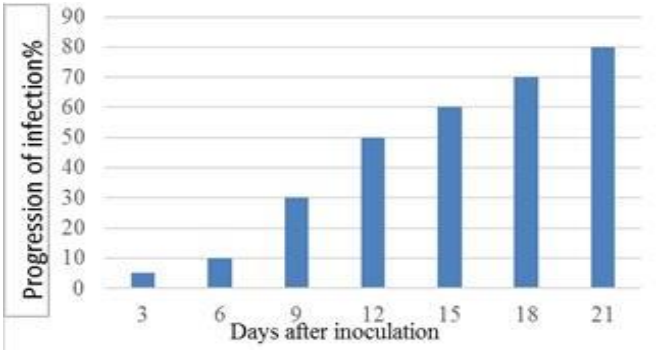

Figure 11. Disease progress curve of Sow thistle weed by detached leaf assay.

***Biological control analyses*:** Methanolic extracts of Cinnamon were used to evaluate the antifungal potential against the target pathogens. In *in vitro* conditions different concentrations of extracts exhibited different antagonistic activities against the pathogens. The species specific response of both pathogens under varied concentrations employed is presented in Figure 12. The results showed a significant inhibitory potential of all the concentrations of extract however *Alternaria arborescens* was found to be more susceptible to the extract even in its lowest concentration which caused 90% reduction in biomass production. Such results proved that Cinnamon has strong anitifungal natural compounds. In general, the results exhibited gradual suppression in pathogen growth with an increase in concentration. The maximum inhibitory potential was recorded at the highest concentrations as both of the pathogens were unable to grow when exposed to 2 and 2.5% concentrations of Cinnamon extract (Fig. 12). In other words 100% arrest in biomass production was depicted at the highest concentrations of Cinnamon extract by both target pathogens. However, the lower concentrations were (0.5-1.5%) less effective in subduing the biomass production of *P. aristolochiicola* as these concentrations induced only 4-10% decline in fungal growth (Fig. 12).

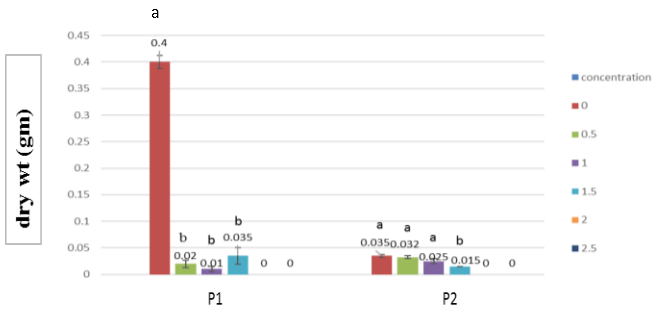

Figure 12. Effect of different concentrations of methanolic extracts of Cinnamon on the biomass production of *Alternaria arborescens* (P1) and *Phyllosticta aristolochiicola* (P2).

DISCUSSION

There are many leaf spot diseases that occur on a wide range of native and ornamental trees and shrubs and even on weeds. Many leaf spot pathogens are only able to produce symptoms in leaf tissue. Leaf spots come in a wide variety of shapes, sizes, and colors. Correct identification of the pathogen causing leaf spots on specific host plant is important for managing the disease. Many leaf spot diseases have similar biology and therefore very similar management options. There are several measures that can be taken to reduce this problem. Protective control measures are not generally warranted for most leaf spots. Therefore, biological control method using plants extracts is preferable these days. Present study thus emphasizes the identification and biological control of pathogens associated with leaf spot disease of Red Edge Dracaena (*D. mariginata*) and Sow thistle (*S. oleraceus*). Pathogen identification is mainly based on the morphology and development of conidia and conidiophores. The morphology is still considered as the most reliable method to identify pathogen at the species level, but misidentifications are also known to occur (Anderson *et al.*, 2006). Consequently, many molecular approaches have been established to assist differentiation among species i.e., analysis of ribosomal DNA (rDNA) sequences to establish molecular phylogenetic relationships within many groups of fungi (White *et al*., 1990; Mirhendi *et al*., 2007) or by using the mitochondrial small subunit (SSU) rDNA sequence method (Kretzer *et al*., 1996). Presently, *Alternaria arborescens* and *Phyllosticta aristolochiicola* was identified as leaf spot causing pathogens of Dracaena Red Edge and Sow thistle weed, respectively using microscopic study for morphological characterization as well as genetic analysis from nucleotide sequencing of amplified ITS1-5.8S-ITS4 region of rDNA. In the contemporary lines Akhtar *et al*. (2014b) isolated and identified *Alternaria arborescens* based on complete description of macro and microscopic characters followed by identification using rDNA spacer sequence and revealed that *Alternaria arborescens* was the causal agent of leaf spot of rice.

In further trials, the pathogenic potential of *A. arborescens* and *P. aristolochiicola* was re-confirmed by applying Koch's postulate using detached leaf method. Both the pathogens induced the maximum characteristic symptoms as dark brown lesions on leaves of their respective hosts. The lesions enlarged slowly and plants started to die within few days of inoculation. The infection symptoms were evident on inoculated leaves and Dracaena Red Edge and Sow thistle weed exhibited a sharp progressive disease curve with 50 and 80% of infected area, respectively. Working in the parallel lines, Shafique *et al*. (2013) reported the use of detached leaf assay to confirm the pathogenic potency of isolated pathogen. These results were found in agreement with the work conducted by Mahmood (2010) who reported the same trend of disease development in tomato by *A. alternata*. Also, *A. arborescens* was found to cause core rot of apple in South Africa (Andersen *et al.*, 2001). *Phyllosticta* species were found to cause leaf spot of ground nut in India (Rao, 1963; Patil and Rao, 1993).

The biological inhibitions by different natural substances, such as essential oils and plant extracts have been investigated widely against fungal activities (Dubey *et al*., 2000; Manohar *et al*., 2001; Pavlou and Vakalounakis, 2005; Bajwa *et al*., 2008; Shafique *et al*., 2011). Likewise, Shafique *et al.* (2006) also reported 60% reduction in incidence of *Alternaria alternata* on wheat due to aqueous leaf extract of *Chenopodium album.* In contemporary studies, Bajwa *et al*. (2007) evaluated antifungal activity of aqueous and n-hexane shoot extracts of *Aloe vera* against few pathogenic species viz., *Alternaria alternata*, *A. citri* and *A. tenuissima*. They reported that the inhibitory effect was found to be variable with the applied concentrations and caused a significant inhibition in biomass production of the three test fungi. Presently, antifungal activity of Cinnamon was tested against two phythopathogenic fungi; *A. arborescens* and *P. aristolochiicola.* Volatile oil, which are an active constituent of most spices (and of which pepper is the most important), impart antiseptic, antibacterial, antifungal properties (Subramanyam, 1957). The bark and the leaves of *Cinnamomum* spp. are commonly used as spices in home kitchens. Some recent scientific studies have shown antimicrobial activity of essential oils of *Cinnamomum cassia* Presl., *C. osmophloeum* Kaneh. and *C. zeylanicum* Blume (Tiwari and Tiwari, 1997; De *et al*., 1999; Chang *et al*., 2001). Antifungal activity was reported for respiratory tract infecting fungi such as *Aspergillus niger* Tieghem, *A. fumigatus* Fres., *A. nidulans* (Eidam) Winter and *A. flavus* Link (Singh *et al*., 1995). Treating high moisture barley (Idler *et al*., 1996) or wheat (*Triticum aestivum* L.) grains (Scholz *et al*., 1999) with essential oil of *C. zeylanicum* protected them from deteriorating fungi and ochratoxin formation. It is reported that cinnamaldehyde is the major fungitoxic component of cinnamon bark, while other components have additive or synergistic effects on total fungitoxicity (Jham *et al*., 2005). In current research work, all the employed concentrations of methanolic Cinnamon extract suppressed the fungal growth but 2.0 and 2.5% were the most potent as these concentrations did not allow the pathogens to grow and exhibited 100% inhibition in biomass production. Furthermore, it has been suggested that higher concentrations can adversely affect the growth of the affected plant species (Reigosa *et al*., 1999).

***Conclusion*:** This study concludes that methanolic extracts of Cinnamon possess potential antifungal compounds against *A. arborescens* and *P. aristolochiicola* and can be exploited as an ideal strategy for future plant disease management programs eliminating fungal spread.

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