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# Research Article
# *In vitro* Evaluation of Fungicides and Plant Extracts to Control Purple Blotch Disease of Onion in Pakistan

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## Abstract

**Background and Objective:** Purple blotch caused by *A. porri* (Ellis) Cif. is a devastating disease of onion (*Allium cepa* L., 2n =16) throughout the world. This study compared effectiveness of the most frequently used fungicides and different plant extracts as control measures for the purple blotch disease. **Materials and Methods:** The fungal inhibition potential of leaf extracts was evaluated from 7 plants, indigenous to District Swat as well as the commonly available fungicides under laboratory conditions. Multivariate analysis and the ANOVA results revealed a direct relationship for the plant extracts/fungicides application and mycelial inhibition. **Results:** The extracts of *C. sativa* (1 and 2 ppm) was found the most effective and caused over 91.89 and 91.66% inhibition of mycelial growth. The order of efficacy of the 1 ppm plant extract was followed by *D. mucronata* (79.00%), *D. viscosa* (78.99%), *C. procera* (77%), *J. adhatoda* (60.65%) and *A. altissima* (57.98%), while in 2 ppm 83.14% was followed by 82.09, 82.02, 71.33, 55.05 and 39.32%, respectively. Among the 5 fungicides applied, DuPont™ Curzate® M8 in both concentrations were the most effective and caused over 89.88% inhibition. Potential synergy of the of leaf extracts of *C. sativa*+*J. adhatoda* was highly effective, resulted in 80 and 84.28% mycelial inhibition. **Conclusion:** The results highlight the critical role of finding the right proportion of appropriate compounds to perform as a potential pesticide. These results indicate to the importance of phyto extracts as promising alternatives to synthetic fungicides for being cost effective, eco-friendly and sustainable means of controlling the purple blotch of onion.

Key words: Onion, *A. porri*, *in vitro* culture, microscopic isolation, synergism, fungicides control

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Onion (*Allium cepa* L., 2n =16) is a biennial herb and attains a height of about 15-45 cm but under human niches it is usually grown as an annual and harvested in its first growing season[1,2]. Onions are among the top 5 most important fresh vegetables crop consumed worldwide[3,4]. It is valued for their distinct flavor and the crop is grown for a variety of purposes i.e., from kitchen to factory made products. Both mature and immature onions bulbs are employed in flavoring and seasoning food ingredients as well as in the preparation of soups, ketchups, salad and pickles[5,6].

Onion bulbs contains a number of minerals and are used for treating many disease conditions and is applied as lachrymatic agent, antimicrobial agent (bacterial and fungal agents), lowers cholesterol, anti-cancer and antioxidant agent due to the presence of quercetin. The total world production of onion was about 86.34 million tones and Pakistan ranked 5th with 2.25% share in global onion production from about 127.8 thousand ha, with in annual production of 1.7 million t onion produced/each hectare of land[5,6]. Several pathogens particularly fungi cause diseases in onion and limits its productivity, among them important fungal diseases of onion are leaf blight, purple blotch, basal rot and downy mildew. These diseases are mostly controlled with synthetic fungicides[7-9].

*Aternaria porri* (Ellis) causes the purple blotch of onion (*Allium cepa* L.) and result in heavy losses in almost all onion-growing areas of the world[3,4,10]. During humid conditions the spores start infecting leaves by forming appressoria before it enters through stomata and epidermal cells[4,10]. Spores of *A. porri* are club-shaped and are produced singly or in the form of many long chains with green, black or gray colours, spores are wind dispersed when the plants debris becomes dry[11,12]. Literature survey reveals plants may be used for controlling the growth of diseases causing pathogens of onion[13-20]. Currently practiced procedures for controlling purple blotches by the use of fungicides such as mancozeb and difenoconazole etc. In addition to time consuming, costly (fungicide treatments estimated to cost farmers additional $1.2 billion in the world),[21,22] are environmentally hazardous and results in. accumulation of toxic residues in the crops and ecosystem[11,23,24]. Besides, the misuse of pesticides has not only has led to environmental pollutions but also several medical conditions such as cancers leukemia are associated with the use of pesticides[25]. Since, fungi as a group, causes serious losses to crops yield, quality and ultimately interfere the livelihood of farmers, therefore, sustainable measures of fungal disease control are direly warranted[26,27].

The most important techniques available for the control of pest and different groups of fungi is by biological control strategies that include the application of beneficial microbes, their metabolic derivatives, plant extracts, essential oils or any organic-based material to suppress the disease orits causal pathogens[2]. The control of the plant pathogens by the resistance sources from allied species have been characterized. A partially dominant gene (Bs1) from *Allium roylei* which confers resistance to progenies of *A. cepa*× *A. roylei*[28], gene Hm1 was the first resistance gene which was noted in the different varieties of maize plants genotypes. This gene encode NADPH that in activate the HC toxin produced by *Cochliobolus carbonum.* The other defense-related responses to infection with *A. solani* involve elevated expression of the PR-1B gene following exogenous application of SA on tomato roots , PR-1 like protein after leaf treatment of tomato with arachidonic acid and sequential expression of 2 ACC synthase genes (ST-ACS4 and ST-ACS5) in potato[17,28]. Reports have indicated that in addition to their suppression of plant pathogens, some natural plant products increased oxidative enzymes in plants that can play an important role in the resistance[29-33].

The current research work focuses on the isolation and identification of *A. porri* form infected onion plants and applying extracts of different plant species and available fungicides tested for their anti *A. porri* properties. To see the best synergistic/combination effects of different plants extracts were combined and fungicides were likewise applied. To the best of our knowledge this it the first report using plant extracts against *A. porri.*

## MATERIALS AND METHODS

**Collection, isolation and identification of the pathogen:** The plant material was collected from different regions of district Swat, Pakistan. The plants were identified and voucher specimens deposited at the herbarium Department of Botany, Hazara University, Mansehra. The experimental work was conducted in Department of Pathology, Agriculture Research institute (ARI), Mingora Swat, KP, Pakistan during the year 2013-2014. Table 1 represent location and disease severity and the infected plants samples. Infected samples were collected in sterile bags and stored in refrigerator at 4°C soon after collection and identified with the help of standard photographs in published papers[34].

Standard tissue isolation procedures were followed to isolate the pathogen from the infected leaves[7]. The infected leaves were surface sterilized with 1:1000 mercuric chloride ($HgCl_2$) solution and repeatedly washed with sterilized distilled water to remove the traces of mercury (if any) and then

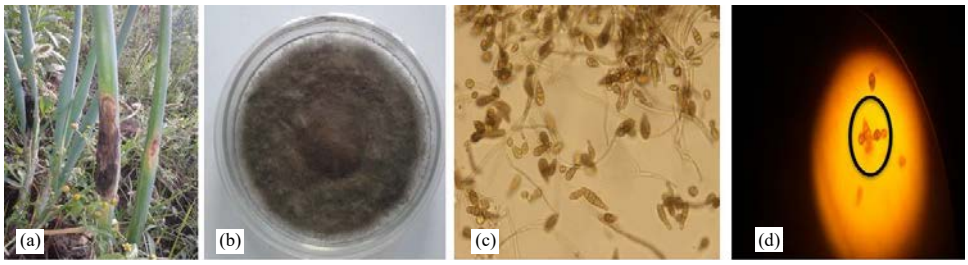


Fig. 1(a-d): (a) Representative infected leaf, (b) Pure culture of *A. porri*, (c) Conidiophores of *A. porri* and (d) Single spores of *A. porri*

transferred to sterilized petri dish containing potato dextrose agar (PDA) and incubated at room temperature (27±1°C).

**Single spore isolation:** Ten milliliter of sterilized 20% potato dextrose agar (PDA, Difco Laboratories, USA.) was poured into petri plates. Dilution of the spore suspension was prepared using sterile distilled water from 12 days old culture. From each culture, 1 mL of such suspension was spread uniformly on agar plates which were incubated at 27±1°C for 8 h. These plates were examined under microscope to locate germinated conidia. Germinated conidia were then marked under the microscopic field (biological digital microscope) with ink on the surface of the plate. These marked agar areas were cut and transferred to PDA slants with the help of sterile cork borer under aseptic conditions and incubated at a temperature of 27±1°C. Fungus studied in slants and morphological characters recorded. In cases where single spore isolated were identical, then they were allowed to multiply further. Pure cultures derived from such slants were used for further analysis (Fig. 1a-d).

**Culture maintenance and conidia quantification:** The pathogens were sub-cultured on PDA slants and allowed to grow at 27±1°C for 12 days. Representative samples of such slants were preserved in a refrigerator at 5°C and renewed after 30 days interval. Further, the isolated fungal pathogen was confirmed after examining one hundred conidia under biological digital microscope (Olympus-DP21-Color camera) under 20x/0.040 Ph1 and 40x/0.65 Ph2 objectives from mature pure culture of the fungus. The morphological traits were compared to the available literature. Stage and ocular micrometer (mm), were used to measure the length, breadth, beak length and number of septa of the fungus. The average length and breadth of the conidial body, beak and septal numbers were recorded. These observations were compared with those of the standard measurements[34] for identification of the pathogen (Fig. 1a-d).

**Extracts preparation and *in vitro* evaluation:** Shortly after collection leaves of *C. sativa*, *C. procera*, *D. vascosa*, *D. mucronata*, *J. adhatoda*, *A. altissima* and *M. azedarch* were dried under shade at room temperature and chopped to pieces with the help of mortar and pestle and then grounded into coarse powder form with an electric blender at Department of Pharmacy university of Malakand, KP, Pakistan. The powdered fractions were transferred into separate closed containers for soaking. 200 g of powdered was add 80% methanol (analytic grade) and genteelly kept on electronic shaker for 5 days, extracts were filtered using Whatman filter paper (No. 1) the filtrate was dried through rotary evaporator. The dried extracts were weighed and dissolved in sterile double distilled water to prepare 25 and 50 mg $mL^{-1}$ of extract which were kept refrigerated unless they were used for the experiment[35]. Chemicals are effective means of controlling the purple blotch of onion and therefore, the present study was designed to find out new suitable chemical, for the control of this deadly onion disease. The efficacy of 5 different fungicides viz., Curzate (cymoxanil 8%, Mancozeb 64%), Acrobat MZ (Dimethomorph 9%, Mancozeb 60%), Topsin-M (Thiophanate Methyl 70%), Ridomil Gold (Metalaxyl M 4%, Mancozeb 64%), Cabrio Top (Pyraclostrobin 5%, Metiram 55%) were evaluated at 2 different concentration viz., 2 and 4 g m $L^{-1}$ (0.2 and 0.4%). For *in vitro* evaluation the above 2 concentrations were applied by poison Food technique using 3 replicates and the radial growth of the fungal colony was recorded on 12-19 days and compared with the untreated control plates. The inhibition of fungal growth over control was calculated as mentioned in literature[6]:

$$I = \frac{C - T}{C} \times 100$$

Where:

I = Inhibition of (%) mycelial growth
C = Radial growth of fungus in control
T = Radial growth of fungus in treatment

**Potential synergism of plants extracts and fungicides:** To see potential synergism of the extracts and available fungicides, the best performing extracts as well as the available fungicides were combined in one treatment using the same concentration and applied in exactly the same conditions as applied for individual plates.

**Statistical analysis:** The data was analyzed using SPSS, Statistic, Origin 75 softwares, means were compared using LSD (Least significance difference test at 5% probability level) and ANOVA analysis.

## RESULTS

**Identification of *A. porri*:** In field, onion blight/purple botch symptoms progress starts from tip of the older leaves as small, whitish, sunken, oval shaped lesions which later on became elliptical or oblong, brown to purple at the center and bordered by a light brown area. Further, these lesions coalesce and spread rapidly on leaf blade and affected leaves show drying from tip downward. The leaves break at the affected area and hang down. Concentric light and dark rings were observed on the infected leaves. In older spots, the conidia were produced abundantly on bunch of conidiophores. Brown lesions with reddish-purple margins resembling bull's eye were also noticed. Lesions usually girdle leaves, causing them to fall over or hang down. In severe cases, blotches were increased up to 3-4 inches long and covered with conidia. The symptoms noticed are exactly as reported previously for onion blight (purple blotch) disease and confirm the diseases as purple blotch of onion.

**Confirmation of *A. porri*:** The pathogen was isolated from infected leaves of onion and cultured on PDA media. Single spore isolation was carried out for detail analysis and *in vitro* evaluation. The young hyphae were hyaline, slender, radiating and septet. The conidiophores are may be single or in groups, the conidia are erect, simple, cylindrical, septet. While the body of conidium is ellipsoidal tapering to the beak and having 7-9 transverse septa and 1-3 longitudinal septa were observed. The above characteristics of the pathogen are exactly as mentioned in respectively and also the pathogen was reconfirmed as *A. porri* (Fig. 2).

***In vitro* evaluation:** In the current study antifungal activity of the methanolic extracts of seven plant viz. *C. sativa* L., *A. altissima* Mill., *M. azedarch* L., *D. vascosa* L., *C. procera* R. Br., *J. adhatoda* L., *D. mucronata* Royle (Table 1), was assayed in 2 concentrations. The data indicated that all plants except *J. adhatoda* significantly reduced fungal growth in both concentrations. The data was recorded for 10 consecutive days and radial growth recorded. The mean inhibitory effects of 7 aqueous plant extracts against *A. porri* are summarized in (Fig. 3). Day second (2 days post inoculation, 2 DPI) till 10 DPI the fungal radial growth was compared to control and overall there was no significant increase in the radial growth of fungal mycelium. The current results indicated that both 1 and 2 ppm of *C. sativa* application was very effective and inhibited fungal growth. The 1 ppm inhibited fungal growth by (38.10%) at 2DPI when while 2 ppm at 2DPI inhibited fungal growth by 38.46% when it was compared to the control, Similarly, the reading of present mycelial growth inhibition at 10 DPI was 91.8% with 1 ppm and was more or less similar to 2 ppm concentration of 91.66% when compared to the control (Table 2). The grand mean (GM) of present fungal inhibition on day 10 was 3.3875, while Co-efficient of variation (CV) was 6.71 and least significant difference (LSD) was 0.1856 with 1 ppm. While the GM of present fungal inhibition on day 10 was 3.3583, CV was 1.88 and LSD was 0.516 with 2ppm (Table 2).

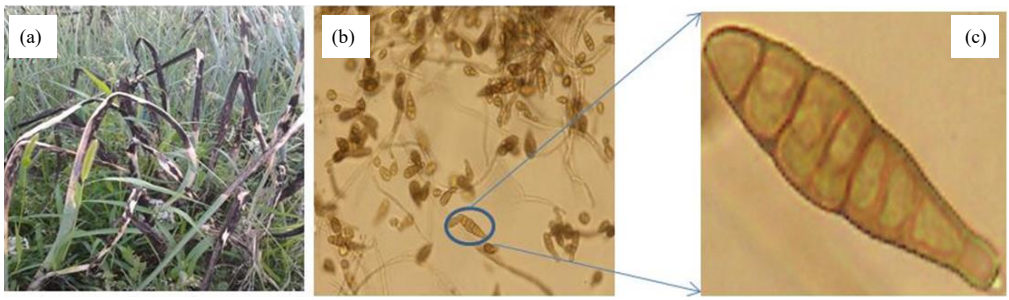


Fig. 2(a-c): Microscopic characterization of *A. porri* with mycelia and conidiophores morphology, (a) Diseases severity, (b) Mycelia for *A. porri* and (c) Single spore

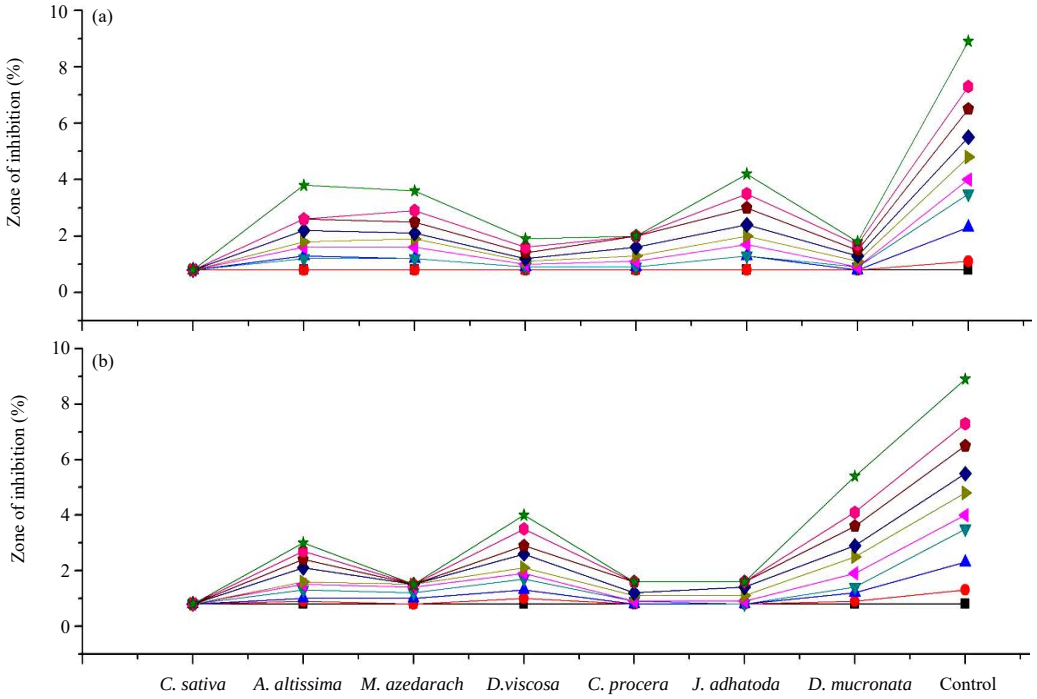


Fig. 3(a-b): Mean radial growth of *A. porri* mycelium with (a) 1 and (b) 2 ppm concentration of seven plants, maximum inhibition was observed with *C. sativa* while least inhibition was recorded in *J. adhatoda* and *D. mucronata* compared with untreated control, maximum inhibition was observed with Curzate while no inhibition was observed in Topsin-M
Different color dots represent mean value of collected samples, data collection dates and time

Table 1: List of plant species with their local names, families and fungicides their concentration used against *A. porri*

| Botanical names | Local names | Family | Fungicides | Composition | Concentration (1 and 2 mg $L^{-1}$) | |
|---|---|---|---|---|---|---|
| *Calotar pisprocera* R.Br. | Splmai | Apocynaceae | DuPont™ Curzate® M8 | Cymoxanil 8%, Mancozeb 64% | 1 ppm | 2 ppm |
| *Cannabis sativa* L. | Bangh | Cannabaceae | Acrobat MZ 90-600 | Dimethomorph 9%, Mancozeb 60% | 1 ppm | 2 ppm |
| *Dodonaea vascosa* L. | Ghowlasky | Sapindaceae | Topsin-M 70W | Thiophanate Methyl 70% | 1 ppm | 2 ppm |
| *Daphne mucronata* Royle | Leeghona | Thymelaeaceae | Ridomil Gold | Metalaxyle M 4%, Mancozeb 64% | 1 ppm | 2 ppm |
| *Justicia adhatoda* L. | Bhaikan | Acanthaceae | Cabrio top | Pyraclostrobin 5%, Metiram 55% | 1 ppm | 2 ppm |
| *Ailanthus altissima* Mill | Asela Bekana | Simaroubaceae | | | 1 ppm | 2 ppm |
| *Melia azedarch* L. | Shandai Bekana | Meliaceae | | | 1 ppm | 2 ppm |

Table 2: Inhibition (%) of *A. porri* mycelium using extracts of *C. sativa* L. *D. mucronata* Royle, *D. vascosa* L., *C. procera* R. Br., *J. adhatoda* L., *M. azedarch* L. and *A. altissima* Mil

| | Concentration | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 ppm | | | | | | | | | | 2 ppm | | | | | | | | | |
| Treatments | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *C. sativa* | 0 | 38.10 | 65.10 | 77.00 | 80.13 | 83.12 | 85.34 | 97.17 | 89.23 | 91.89 | 0 | 38.46 | 65.89 | 77.00 | 80.00 | 83.45 | 85.23 | 87.00 | 89.00 | 91.66 |
| *A. altissima* | 0 | 27.12 | 43.23 | 60.89 | 62.32 | 62.00 | 60.47 | 60.23 | 64.31 | 57.98 | 0 | 38.46 | 56.52 | 62.33 | 66.00 | 67.00 | 68.80 | 68.99 | 70.00 | 71.33 |
| *M. azedarach* | 0 | 27.12 | 55.53 | 55.00 | 60.45 | 60.00 | 61.13 | 61.56 | 60.54 | 60.00 | 0 | 38.46 | 56.52 | 71.86 | 65.00 | 68.75 | 72.72 | 76.92 | 97.00 | 83.14 |
| *D. viscosa* | 0 | 27.12 | 60.76 | 70.67 | 72.00 | 77.12 | 78.00 | 78.43 | 78.23 | 78.99 | 0 | 23.07 | 43.47 | 51.42 | 52.51 | 56.25 | 52.72 | 55.38 | 55.20 | 55.05 |
| *C. procera* | 0 | 27.12 | 60.98 | 70.67 | 72.09 | 70.98 | 70.99 | 71.00 | 72.98 | 77.00 | 0 | 38.00 | 68.00 | 74.50 | 77.32 | 78.00 | 78.08 | 75.09 | 78.02 | 82.02 |
| *J. adhatoda* | 0 | 27.87 | 43.78 | 54.00 | 57.98 | 58.00 | 58.90 | 59.54 | 59.00 | 60.65 | 0 | 38.40 | 68.00 | 77.07 | 77.50 | 77.80 | 74.45 | 75.38 | 78.80 | 82.09 |
| *D. mucronata* | 0 | 27.87 | 60.00 | 74.34 | 77.00 | 77.00 | 76.01 | 76.00 | 76.00 | 79.00 | 0 | 30.00 | 47.83 | 60.00 | 52.56 | 47.27 | 47.99 | 44.61 | 43.38 | 39.32 |

The easily available 5 fungicides viz. DuPont™ Curzate® M8 fungicide (WP), Acrobat MZ 90-600, Topsin-M 70W, Ridomil Gold, Cabrio Top were also applied and the data of fungal mycelial growth consecutively recorded in 19 days. Mean redial growth of *A. porri* mycelium on 2 and 4 gm $mL^{-1}$ data was summarized in (Fig. 4). The fungicide DuPont™ Curzate® M8 in both concentrations was the most effective and caused over 81.23% inhibition of mycelial growth. The efficacy of 2 g $mL^{-1}$ fungicides was Cabrio Top (82.92%) followed by DuPont™ Curzate® M8 (81.23%), Acrobat MZ

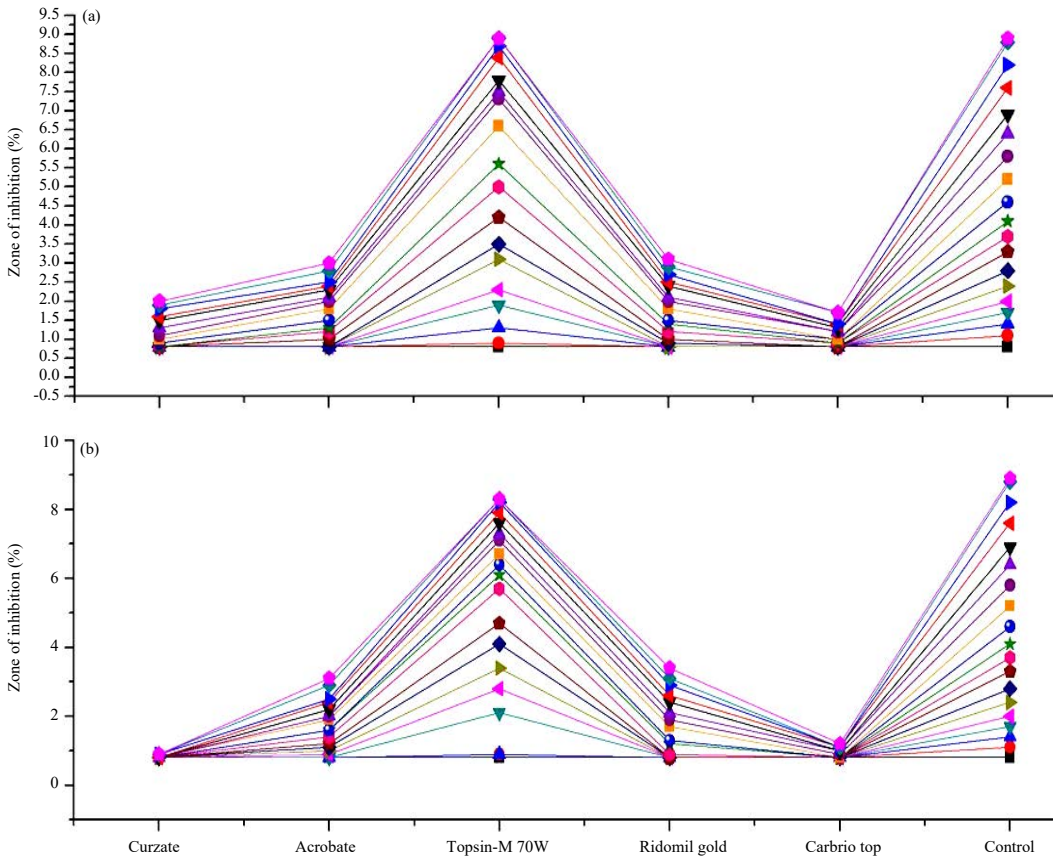


Fig. 4(a-b): Line graphs showing mean radial growth of *A. porri* mycelium with (a) 1 and (b) 2 ppm concentration of 5 fungicides, maximum inhibition was observed with Curzate while no inhibition was observed in Topsin-M
Different color dots represent mean value of collected samples, data collection dates and time

Table 3: Mean percent inhibition data of *A. porri* mycelium using 5 fungicides in 2 concentrations (0.2 and 0.4%) (DuPont™ Curzate® M8 Fungicide (WP), Acrobat MZ 90-600, Topsin-M 70W, Ridomil Gold, Cabrio Top)

| | Concentration | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatments | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
| | **1 (0.2%)** | | | | | | | | | | | | | | | | | | |
| DuPont™ Curzate® M8 | 0 | 27.27 | 42.34 | 52.94 | 60 | 66.66 | 71.34 | 75.24 | 78.39 | 80.42 | 80.56 | 80.72 | 81.23 | 79.96 | 78.85 | 78.71 | 78.58 | 78.24 | 77.80 |
| Acrobat MZ 90-600 | 0 | 27.27 | 42.34 | 52.94 | 60 | 66.66 | 71.34 | 69.72 | 67.34 | 68.67 | 67.65 | 65.38 | 65.51 | 67.10 | 66.66 | 68.42 | 69.51 | 68.18 | 77.52 |
| Topsin-M 70W | 0 | 18.18 | 7.14 | -11.76 | -15 | -29.16 | -25.00 | -27.27 | -35.13 | -36.58 | -30.43 | -26.92 | -25.86 | -17.18 | -13.04 | -10.52 | -6.09 | -1.12 | |
| Ridomil Gold | 0 | 27.27 | 42.34 | 52.94 | 60 | 66.66 | 67.85 | 69.69 | 67.56 | 65.85 | 67.39 | 65.38 | 65.51 | 67.18 | 65.21 | 67.10 | 67.07 | 67.04 | 62.92 |
| Cabrio top | 0 | 27.27 | 42.34 | 52.94 | 60 | 66.66 | 71.34 | 75.24 | 75.67 | 78.04 | 78.26 | 80.76 | 79.31 | 81.26 | 81.15 | 81.57 | 82.92 | 80.68 | 80.89 |
| | **2 (0.4%)** | | | | | | | | | | | | | | | | | | |
| DuPont™ Curzate® M8 | 0 | 27.27 | 42.34 | 52.94 | 60 | 66.66 | 71.42 | 75.75 | 78.37 | 80.48 | 82.6 | 84.61 | 86.2 | 87.5 | 88.4 | 89.47 | 89.02 | 89.77 | 89.88 |
| Acrobat MZ 90-600 | 0 | 27.27 | 42.34 | 52.94 | 55 | 58.33 | 60.71 | 63.63 | 62.16 | 60.97 | 65.21 | 63.46 | 65.51 | 68.75 | 68.11 | 68.42 | 69.51 | 67.04 | 65.16 |
| Topsin-M 70W | 0 | 18.18 | 18.18 | -23.52 | -40 | -41.66 | -46.42 | -42.42 | -54.05 | -48.78 | -39.13 | -28.84 | -22.41 | -14.06 | -10.14 | -3.94 | 0.00 | 5.68 | 6.74 |
| Ridomil Gold | 0 | 27.27 | 27.27 | 52.94 | 60 | 66.66 | 71.42 | 75.75 | 75.67 | 70.73 | 71.73 | 67.30 | 67.24 | 67.18 | 65.21 | 65.78 | 64.63 | 64.77 | 61.79 |
| Cabrio top | 0 | 27.27 | 27.27 | 52.94 | 60 | 66.66 | 71.42 | 75.75 | 78.37 | 80.48 | 82.60 | 84.61 | 84.48 | 84.37 | 85.50 | 85.52 | 86.58 | 87.50 | 86.51 |

(77.52%), Ridomil Gold (69.69%) and Topsin-M (36.58%). Topsin-M fungicide did not inhibit growth of fungi but stimulated fungal mycelial growth. While in 4 g $mL^{-1}$ concentration DuPont™ Curzate® M8 (89.88%) was followed by Cabrio Top (87.05%), Ridomil Gold (75.75%), Acrobat MZ (69.51%) and Topsin-M(-54.05%), respectively (Table 3).

**Synergism potential of plants extracts and fungicides:** The crude extract of *C. sativa* in both concentrations 1 and 2 ppm was the most effective and caused over 91% inhibition of mycelial growth. The bioefficacy of 1 ppm extract was *C. sativa* (91.89%) followed by *D. mucronata* (79.00%), *D. viscosa* (78.99%), *C. procera* (77%), *J. adhatoda* (60.65%)

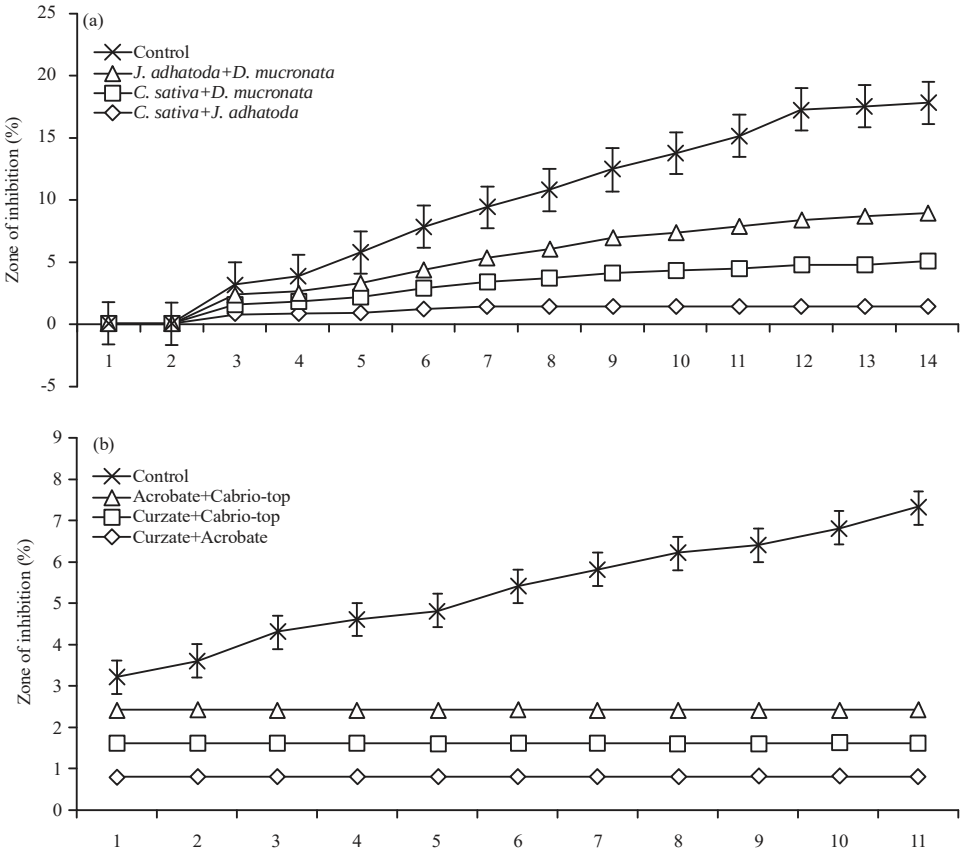


Fig. 5(a-b): (a) Inhibition (%) using 3 extracts combinations (1+1 ppm concentration) to see inhibition of *A. porri* mycelium and (b) 1+1 ppm concentration of fungicide combination is inhibit growth of *A. porri*, maximum inhibition was observed with *Cannabis sativa* while little inhibition was observed in *J. adhatoda* when compared with untreated control

and *A. altissima* (57.98%). While in 2 ppm concentration *C. sativa* (91.66%) was followed by *M. azedarach* (83.14%), *J. adhatoda* (82.09%), *C. procera* (82.02%), *A. altissima* (71.33%), *D. viscosa* (55.05%) and *D. mucronata* (39.32%). Higher concentration of plants extracts inhibited *A. porri* more as compare to low concentration because active constituents were higher. Plant extracts that inhibited the mycelial growth individually to a great extent were also applied in combinations to check their potential synergistic interactions against *A. porri.* The efficacy of both 1 and 2 ppm combinations of the *C. sativa*+*J. adhatoda,* C. *sativa* +*D. mucronata* and *J. adhatoda*+*D. mucronata* was assayed and inhibiting *A. porri* mycelial growth when compared to each treatment used alone. The combinations of *C. sativa*+*J. Adhatoda* was the most effective, resulted in 80 and 84.28% inhibition, followed by C. *sativa*+ *D. mucronata* with 73.77 and 62.90% inhibition and *J. adhatoda* +*D. mucronata* with 68 and 59.55% inhibition of the fungal mycelium in 1and 2 ppm concentration, respectively.

Best performing fungicides viz. DuPont™ Curzate® M8 fungicide (WP) (Cymoxanil 8%, Mancozeb 64%), Acrobat MZ 90-600 (Dimethomorph 9%, Mancozeb 60%), Cabrio Top (Pyraclostrobin 5%, Metiram 55%), were combined and applied as one treatment (under the aforementioned conditions). Further, their combined effect was investigated for possible fungicide synergism. It was interesting to see that the combination of Curzate+Acrobat MZ, Curzate+Cabrio Top and Acrobat M+Cabrio Top in concentrations (0.2+0.2%) completely inhibited fungal growth shown in (Fig. 5a, b).

ANOVA test result obtained for both concentration (1 and 2 ppm) of plant extracts and control untreated. Significant differences between fungal growth and plants extract concentration which affect the fungal growth, the p-value is higher in both concentration (2.25 and 8.84) than the $\alpha$ value is 0.05 and the F calculated value is greater than the F-critical values of both concentration shown in Table 4 and 5.

Table 4: Represented ANOVA for the measurement and variation between fungal growth and extract concentration

| | Plants extracts | | | | | | Fungicides | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plants extracts | SS | df | MS | F | p-value | F crit | SS | df | MS | F | p-value | F crit |
| Fungal growth | 80.000 | 6 | 13.3330 | 20.58 | 2.251 | 2.271 | 324.38 | 5.0 | 64.870 | 46.4640 | 1.712 | 2.31568 |
| 1 ppm | 61.154 | 9 | 6.7949 | 10.49 | 3.580 | 2.058 | 183.40 | 18.0 | 10.190 | 7.2987 | 3.971 | 1.71959 |
| Error | 34.977 | 54 | 0.6477 | | | | 125.60 | 90.0 | 1.396 | | | |
| Total | 176.13 | 69 | | | | | 633.40 | 113.0 | | | | |
| Fungal growth | 85.201 | 6 | 14.2000 | 19.00 | 8.841 | 2.271 | 352.40 | 5.0 | 70.490 | 45.9830 | 2.392 | 2.31568 |
| 2 ppm | 61.377 | 9 | 6.8197 | 9.16 | 2.920 | 2.058 | 151.70 | 18.0 | 8.428 | 5.4980 | 1.890 | 1.71959 |
| Error | 40.194 | 54 | 0.7443 | | | | 137.90 | 90.0 | 1.532 | | | |
| Total | 186.77 | 69 | | | | | 642.10 | 113.0 | | | | |

F crit: Critical value, df: Degrees of freedom in the source, SS: Sum of squares due to the source, MS: Sum of squares due to the source, F: F-statistic

Table 5: Represented synergism for the measurement and variation between fungal growth and extract concentration

| Plants extracts | SS | df | MS | F | p-value | F crit |
|---|---|---|---|---|---|---|
| Fungal growth | 107.2100 | 2 | 53.60520 | 35.23240 | 2.79070 | 3.49282 |
| 1 ppm | 50.0365 | 10 | 5.00365 | 3.28868 | 0.01126 | 2.34787 |
| Error | 30.4294 | 20 | 1.52147 | | | |
| Total | 187.6760 | 32 | | | | |
| Fungal growth | 70.8857 | 2 | 35.44280 | 35.28170 | 2.7607 | 3.49282 |
| 2 ppm | 73.3321 | 10 | 7.33321 | 7.29988 | 8.9050 | 2.34787 |
| Error | 20.0913 | 20 | 1.00456 | | | |
| Total | 164.3090 | 32 | | | | |
| Fungicides | | | | | | |
| Fungal growth | 36.69810 | 3 | 12.23270 | 27.85050 | 8.28090 | 2.92227 |
| 1 ppm | 4.392273 | 10 | 0.43922 | 1.00000 | 0.46542 | 2.16458 |
| Error | 13.17682 | 30 | 0.43922 | | | |
| Total | 54.26720 | 43 | | | | |

F crit: Critical value, df: Degrees of freedom in the source, SS: Squares due to the source, MS: Sum of squares due to the source, F: F-statistic, Source: Abd El-Khair and Haggag[36]

## DISCUSSION

In the present investigation, the bioefficacy of seven plants was *in vitro* assessed against *A. porri*. All extracts had inhibitory potential on the mycelial growth of *A. porri.* Typical symptoms of PB disease such as small, whitish, sunken, oval shaped lesions along concentric light and dark rings were observed on the leaves of infected plants and were collected from different sites of District Swat. Further, the infected leaves as well as stems in seed crop turned yellowish white and died in some instances. Similar symptoms of the disease have been described as characteristic symptoms for identification of the purple blotch of onion[37-42]. Several of these plant extracts have been previously tested and are known to have active principles with antimicrobial and antifungal activities[43-45]. However, to the best of our knowledge none of these plant extracts have been used in Pakistan to control the losses to *A. porri*. The bioefficacy of 1 ppm extract was *C. sativa* followed by *D. mucronata*, *D. viscosa*, *C. procera*, *J. adhatoda* and *A. altissima*. While in 2 ppm concentration *C. sativa* was followed by *M. azedarach*, *J. adhatoda*, *C. procera*, *A. altissima*, *D. viscosa* and *D. mucronata. C. sativa*s as the one of the most active plant species against *A. porri*.

In *C. sativa* different biologically active compounds have been isolated form different parts of the plant[43,46], Therefore, the extract of *C. sativa* suppressed mycelial growth of *Altenariasolani* species[45]. The extract of *C. procera* at high concentration has been reported to suppress mycelial growth and plant contains biologically active compounds[47,48]. The genetics of pathogen resistance and molecular tagging of resistant gene offers imperative support in MAS-based selection of resistant material as part of the resistance breeding program[49]. The MAPKs which control the expression of genes which were involved in the control of different fungi and control the secondary metabolism by direct phosphorylation of downstream of transcription factors and MAPK3 are founds in different higher plants which were activated through by the different stimuli such as plants regulator and different biotic and a biotic stress[50,51]. Raina *et al.*[52], have identified/isolated CrMPK3 over expressed *C. roseus* plants for defense[52,53]. The *ApR1* resistance gene detected from plants[54]. *Stb6* resistance gene was identified in wheat for the control of *Septoria tritici* blotch (STB)[55,56]. Over expression of *AtELP3* and *AtELP4* in *F. vesca* influences plant growth and development and confers resistance to anthracnose crown rot, powdery mildew and angular leaf spot caused by the obligate fungal pathogen *Podosphaera*

*aphanis*, the hemibiotrophic fungal pathogen *Colletotrichum gloeosporioides* and the hemibiotrophic bacterial pathogen *Xanthomonas fragariae*[57].

Here, *C. procera* in both concentrations suppressed the mycelial growth of *A. porri*. Extract of *D. viscosa* has been widely applied to both antimicrobial and anti-fungal assays[58,59]. Crude extracts of *M. azedarach* plays an important role in the inhibition of seed borne diseases and applied as a robust antifungal agent against various pathogenic fungi[60]. Extracts of *M. azedarach* are rich source of scopoletin, vanillin, hydroxycoumarin derivatives and the antifungal activity of *M. azedarach* is more likely to be due to the presence of these alkaloids and phenolic compounds and 5 different fungicides were applied in universally accepted proportions (1 and 2 ppm), the best performing fungicides were combined (using 1 ppm of each) to see if there exist any potential synergism (Fig. 3). These fungicides included DuPont™ Curzate® M8 Fungicide (WP), Acrobat MZ 90-600, Topsin-M 70 W, Ridomil Gold, CabrioTop the data represented in (Table 2). Our finding are supported by the similar results for other plant species and extracts[58,59].

Many of the individual constituents are acutely toxic to insects and other pathogens. However, the toxicity of these compounds can be enhanced significantly in mixtures, so that the activity of the mixture is higher than would be expected by adding up the activities of its individual constituents[60,61]. Thus, replacing one extract and using a combination of extracts could enhance the efficiency of pesticides[62,63].

In this study combinations of extracts were employed to see if there is potential synergistic interaction among the extracts. In general except for *J. adhatoda*+*D. mucronata* combination no positive synergistic interaction was seen. *J. adhatoda*+*D. mucronata* combination could inhibit fungi by 68% while their individual impact was 60.65% (Fig. 4). Rather, it has somewhat stimulatory effects when compared to the impacts of individual extract. Also doubling the concentration (2+2 ppm) did not shown positive correlation with the fungal mycelium inhibition. These results are in contradiction to most of the previous reports, where the authors using different extracts (from ours) and have drawn a direct relationship of the increase in the concentration of pesticides with the suppression of fungal mycelium[36,64,65].

Pakistan is a developing country and similar to all other underdeveloped economies of the world onion is a crop of immense socio-economic significance and the annual production of over 86.34 million tons makes Pakistan as the 5th largest producer of world oniontoday[6,7]. There are a number of different diseases caused by microorganisms which add further constraints to onion productivity[6,18]. The purple blotch of onion caused by *A. porri* is among the most significant threats to onion production in Pakistan and its neighboring countries and it is causing substantial losses to onion crops every year[6].

The conidiophores when observed under the microscope were pale to light brown in color, measured 120 mm long and 6-10 mm thick with several well defined conidial scars. Conidia were usually solitary, straight or curved or with ellipsoidal body of the conidium that tapered toward the beak. Such features have been previously described[34,42].

For the management of purple blotch disease in onion through the application of fungicides is partially effective under environmental conditions that are favorable for pathogen infection besides accumulating toxic residues in the crop and ecosystem. Therefore, it is essential to develop high yielding onion varieties with greater resistance to diseases but the fungicides uses is more serious which were highly cost and most importantly dangerous for the living life (environmental pollution[6,42]. he safe and reliable way to control this deadly disease by conventional breeding by ways of mass selection of the purple blotch-resistant genotypes through field screening does not only take long but the discrepancies in the assessment of disease reaction may also affect the introgression of PB resistance into elite high-yielding genotypes. Under these circumstances, the utilization of molecular markers in the identification and development of new onion cultivars resistant to PB disease remains the most promising option to help reduce the economically important *A. porri* infection problem. However, such facilities are limited and the high cost additional bottle necks, while the most and non-costly way for the inhibition of plant pathogen is by using potential plants and their extracts, that is a cheaper and environment friendly option[4,6,17].

## CONCLUSION

Purple blotch caused by *A. porri* is one of the most important yield limiting disease of onion bulb and seed crops. The inhibitory effects of seven plant extracts and 5 commercial fungicides were *in vitro* analysis and documented. Although, time constraint did not allowed replicating the same experiment in field trial but it offers a possible means to find out relation between *in vitro* analysis in lab and actual field application and screening for the efficacy of these cured extracts. Maximum inhibition was of *A. porri* was recorded with *C. sativa* plant and order of the efficacy of crude extract was: *C. sativa>D. mucronata> C. procera>D. viscosa>M. azedarach>A. altissima>J. adhatoda.* While, in fungicides maximum inhibition was noted in

DuPont™ Curzate® M8. Field trials are critical and if the intensity of the crude extract is equally effective in field, this study provided a baseline to use natural plant extracts as bio fungicides and greatly helpful in reducing the level of water pollution in particular which is a threat to aquatic biodiversity in district Swat, KP Pakistan.

## SIGNIFICANCE STATEMENT

This study first time reports the use of different plants extracts to control the deadly disease. The study are beneficial for the control of purple blotch caused by *A. porri* and this study help the researchers to uncover the critical areas of isolation of single conidia of *A. porri* and biological control that many researchers were not able to explore. Thus a new theory on *in vitro* evolution of single conidia and control based on biological, fungicides and their synergism may be arrived at.

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