Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(4): 3164-3168
Received: 13-05-2019
Accepted: 15-06-2019

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Testing the efficacy of biocontrol agents against *Alternaria porri* under *in vitro* conditions

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Abstract
The Present investigation was carried out during 2015-16 in the Laboratory of the Department of Plant Pathology J. N. K. V. V. Jabalpur at Completely Randomized Design (CRD). The experiment on effect of biocontrol agent against *Alternaria porri* causing purple blotch of onion five biocontrol agents. *Alternaria* sp. is a major leaf spot and blight causing pathogen on various plant species including onion purple blotch caused by *Alternaria porri*. The pathogen identified as *Alternaria porri* attacked all above ground plant parts. Initially small, whitish sunken lesions appear on the succulent leaves. Spots are elliptical to irregular in shape, which enlarge rapidly with the advancement of the disease and coalesce to form larger dead patches resulting in the blight. *Alternaria* porri was found pathogenic to the onion. The pathogenicity of the fungus was established by artificial inoculation of onion leaves with the conidial suspension of the pathogen. Among the Four bioagent tested, *Trichoderma viride* followed by *Trichoderma harzianum* was most effective against *A. porri* under *in vitro* condition.

Keywords: Biocontrol agents, *Alternaria porri*, *in vitro* conditions

1. Introduction
There are a number of biological factors that are considered to cause deterioration of onion bulbs for instance respiration and pathogen which attacks and make the bulbs unfit for marketing [1]. The productivity of the onion in India is very low as compared to many other countries. Damage due to diseases is one of the prime factors responsible for limiting the production and productivity of onion. Purple blotch caused by *Alternaria porri* is one of the major disease of onion. The name purple blotch for this disease was proposed by [2]. He named the causal organism as *Alternaria alli* which was later amended to *Alternaria porri*. [3] made first report on leaf spot and blight disease on onion in Bombay and attributed it to *Alternaria* spp. It is estimated to cause yield losses up to 25 per cent loss in Rabi and 59 per cent in Kharif season [4]. Three species of *Alternaria* viz. *A. palandui* (Ayyangar), *A. porri* (Ellis) Ciferri, and *A. cepulae*, have been recorded on leaves of onion causing purple blotch disease [5]. The disease was first reported as leaf spot or blight of onion from New Jersey in 1879 [6]. In India, the disease was first reported from Maharashtra by [3]**.** In Madhya Pradesh, *A. palandui* has been reported from Gwalior [7]. Besides *Allium cepa* L. and *Allium sativum* L. has been recorded as an additional host [8]. *A. cepulae* caused severe damage to onion at Dharwar, Karnataka [9].
The losses about 50-100 per cent due to purple blotch of onion have been reported by [10]. The pathogen *Alternaria porri* destructs the leaf tissue which hinders the stimulus for bulb initiation and delay in bulbing and maturation. Sever attack on flowering, results in to complete girdling of flower stalks with necrotic tissue, causing their collapse and total loss of seed production capacity [11]. Germination as well as seedling growth (radical and plumule elongation) were adversely effected up to (80 per cent) when the seed were treated with fungal culture filtrate [12].
This disease can be reduced by preventing the leaves from wetness. Another preventive measure can be removal of plants debris from the field and proper irrigation. Management through biological method is also used for managing the disease because natural enemies are best to control and not harmful for environment [13]. The present study was planned to assess the yield losses due to purple blotch disease and to study the response of different biocontrol agents against disease in order to manage the purple blotch disease.

2. Material and Methods
The present investigation on purple blotch (*Alternaria porri*) of onion was undertaken effect biocontrol agents in minimizing the disease under *in vitro* condition. The materials and methods used have been described below.

2.1 Materials

1) Materials Required

The materials used, included ingredient of media, disease samples, cleaning solution, antibiotics, absorbent cotton, hand sprayer, bucket, copper utensils, muslin cloth, etc.

2) Glasswares

Standard 'Borosil' make glassware's like petridishes, beakers, funnels, pipettes, Erlenmeyer flasks, volumetric flask, culture tubes, measuring cylinder, etc. were used during the course of study.

3) Equipments

Equipments used during the course of investigation including hot air oven, autoclave, BOD incubator, laminar air flow, refrigerator, electric balance, research compound microscope, LPG gas burner, water bath and Inoculation needle, wash bath, forceps, enamel tray, scalpel, puller, knife, sauce pan, glass micro slides, cover slips, scissor, brush, dropper, teasing needle, spirit lamp were used during experimentation.

4) Collection of diseased plants

The plants showing typical symptoms on leaf were collected from Horticulture field J.N.K.V.V. Jabalpur as well as the adjoining areas of Jabalpur. The infected leaves were carefully removed from the plant, the samples were kept in clean polythene bag and brought to the laboratory for isolation. The samples were washed with tap water and dried with the help of blotter paper to remove traces of water before isolation.

5) Source of bioagents

The bioagents, *Trichoderma viride*, *Chaetomium globosum*, *Psudomonas fluorescens* and *Bacillus subtilis*, were tested against purple blotch causing pathogen.

6) Chemicals

i) Cleaning solution: Composition of cleaning solution: Potassium dichromate, Concentrated sulphuric acid and Distilled water. This solution was used to clean the glassware's like Petri dishes, Erlenmeyer flasks, Pipettes, Funnels, Beaker, Measuring cylinders and Culture tubes.

ii) Sterilization: A 1: 1000 solution of mercuric chloride was prepared and used for surface sterilization of leaf tissues. Alcohol was used for sterilization of hands, working surface during isolation of the pathogen.

Mounting medium: Lectophenol was used as a mounting medium for test fungus.

7) Culture Medium

Potato dextrose agar medium (PDA) amended with streptomycin sulphate was used during the course of investigation. The contents of PDA were as follows:

A general composition of Potato Dextrose Agar (PDA)

Constituents	**Quantity**
Potato	200gm
Dextrose	20gm
Agar agar	20gm
Distilled water	1000ml

2.2 Methods

1) Preparation of medium

Peeled potatoes were cut into small pieces and boiled in $^1/_2$ liter water in a sauce pan for few minutes. In other pan, agar-agar was boiled in $^1/_2$ liter water, separately so that it completely dissolved in water. Potato extract was collected and added to dissolved agar-agar along with dextrose. The medium was poured in conical flasks 250 ml, plugged with cotton and autoclaved at 121.6^0C (15 psi) for 25 minutes. Streptomycin sulphate @ 0.5 gm/l of the medium was added before pouring of medium into Petri dishes to avoid bacterial contamination.

2) Cleaning and sterilization

Prior to use, the glassware were cleaned with chromic acid solution followed by washing with liquid detergent and finally rinsed with tap or distilled water and dried. The dried glasswares were sterilized in hot air oven at 180 ± 20^0C for 2 hrs. The media and water were sterilized in autoclave at 15 psi for 20 minutes. The inoculation needle, cork borer and other metallic instruments were sterilized by dipping them in alcohol and heating red over the flame of a spirit lamp or Bunsen burner. Surface sterilization of diseased plant parts was done by dipping them in 0.1 per cent mercuric chloride for one minute, followed by three changes in sterile water.

3) Symptomatology

Symptoms of the disease were examined critically to study their course of development in the field.

2.3 Isolation, purification, morphological and cultural studies of pathogen

1) Isolation

The pathogen was isolated from the diseased leaves of onion exhibiting typical symptoms of *Alternaria porri*. Small portion of affected leaf was surface sterilized with mercuric chloride solution (0.1%) for one minute followed by three change of sterile distilled water. The diseased pieces were dried by placing between two sterile blotting papers, and placed on potato dextrose agar in Petri plates and incubated at 25 ± 1^0C in a BOD incubator. Fungal growth was observed for seven days. The appearing fungus was transferred to PDA slants.

2) Purification and maintenance of fungal culture

Culture was purified by following hyphal tip method [14] and culture obtained was maintained on potato dextrose agar (PDA) medium slants at room temperature by adopting subsequent sub culturing at periodical, regular intervals. Seven days old culture was used for further studies.

The pathogens were sub-cultured on PDA slants and allowed to grow at 25 ± 1°C for ten days and such slants were preserved in a refrigerator at 5°C and renewed once in 30 days by sub culturing.

3) Culture Characteristic and Morphological study

The observations from the seven day old culture were recorded for colony characters on PDA. The morphological features like colour, branching and septation of the hyphae, conidiophores and conidia of the pathogens were studied under compound microscope.

4) Identification of the pathogen

On the basis of morphological characteristic of the colony, mycelium, conidiophores and conidia and their comparison with the available literature, the organism was identified as *Alternaria porri.*

2.4 Efficacy of bioagents aginst *Alternaria porri* by dual culture method

The culture of test fungi i.e. *Alternaria porri* and bioagents i.e. *Trichoderma viride* and *Trichoderma harzianum* was prepared in petriplate in which autoclaved melted Potato Dextrose Agar was poured and allowed to solidify for obtaining leveled surface. The plates were inoculated with the culture of test fungi and bioagents after solidification of media by placing the disc of equal size opposite to each other and then plates were incubated at room temperature for seven days.

Bacterial bioagents, *Psudomonas fluorescens* and *Bacillus subtilis* were prepared by inoculating a loopful culture in sterilized conical flask containing hundred ml of nutrient broth. Broth culture was incubated at room temperature for three days. Autoclaved PDA poured in each of the bioagents were used for this study. Five mm disc of one week old test fungus and bioagent lawn culture was cut with the help of cork borer lifted and transferred in petriplates.

In each petriplate, one disc of bioagents and one disc of test fungus were taken at both sides. In case of *Psudomonas fluorescens* and *Bacillus subtilis*, three days old culture was streaked around the test fungus were grown in same condition on potato dextrose agar without bioagents. All these plates were incubated at room temperature for seven days. After an expiry of incubation period, the mycelial inhibition was calculated as per formula mentioned in the poisoned food method.

The details of the biocontrol agents used against pathogen *Alternaria porri* are given in the following (Table:1)

Table 1: List of bioagents

Sr. No.	Bioagents
1.	*Trichoderma viride*
2.	*Trichorema harzianum*
3.	*Psudomonus fluorescens*
4.	*Bacillus subtilis*
5.	Control

2.5 Statistical analysis

For Statistical analysis Complete Randomized Design (CRD) used.

3. Results

3.1 Collection of Disease Samples, Isolation and maintenance of fungi

The disease samples of purple blotch were collected from farmers field, Horticulture field and Vegetable Research Unit, JNKVV Jabalpur. Isolation of pathogen from samples were made on PDA by tissue isolation method. Fungi were found to be associated with the samples. The fungus was identified based on morphological characters and published literature as *Alternaria porri* and was purified by hyphal tip method. The majority of isolation yielded the fungus *Alternaria porri*. The isolated fungus culture was maintained on PDA slants for further studies.

3.2 Isolation and purification of the pathogen

The pathogen, A. porri was isolated from leaves of diseased plants and maintained on potato dextrose agar (PDA) slants. The affected portion of diseased plants were cut with the help of a sharp razor and rinsed with sterile water to remove traces of dirt. These were surface sterilized by dipping in 1:1000 mercuric chloride solution for one minute and washed twice with sterile water. These pieces were transferred aseptically on to the sterilized petri dishes containing solidified PDA under a laminar air flow. The petri dishes were incubated at 25 ± 1°C. The appearing fungus was observed after 72 hrs. The developing fungi was sub-cultured and maintained in pure form for further studies.

3.3 Symptoms of *Alternaria porri* in naturally infected field

Brown lesions with reddish-purple margins were noticed. The purple blotch symptoms development starts from the older leaves as small, whitish, sunken, oval shaped lesion which later on became elliptical or oblong brown to purple at the centre and surrounded by a light brown area. Further the lesion coalesce and spread rapidely on leaf blade and effected leaves show drying from tip downward. [11-12] observed the similar symptoms of purple blotch of onion on various parts of the plants viz., leaves, stem, seeds, flower, bulb etc.

3.4 Morphological characters

3.4.1 Colony and mycelium

The colony is dark green to black in colour, hyphae smooth, septate, profusely branched, light to dark brown in colour measuring 3.7-5.5 µm in diameter.

3.4.2 Conidiophores

The conidiophores arise singly or in groups, straight or flexuous, often geniculate, septate, pale to mid brown up to 102 µm long and 5-10 µm broad.

3.4.3 Conidia

The conidia are mid golden brown in colour, smooth or minutely verrucose, having 8-12 transverse and zero to several longitudinal or oblique septa. Conidia measures 100-300 µm in length and 15-20 in width at the broadest region.

3.4.4 Identification of the pathogen

On the basis of the morphological characters as described by [15]. the causal fungus was identified as *Alternaria porri*.

A) *In vitro* Efficacy of Botanicals against *Alternaria porri*

Several workers have tested number of onion varieties for resistance to *Alternaria porri* causing purple blotch disease. In present investigation, efforts have been made to evaluate some fungicides and bioagent under *in vitro* condition to control the pathogen (*Alternaria porri*) by poison food and dual culture method.

B) Efficacy of bioagents against *Alternaria porri* (dual culture method)

The result presented in Table no. 2 that the fungal bioagent, *Trichoderma viride* recorded maximum mycelial suppression (76.55 per cent) of *Alternaria porri* followed by *Trichoderma harzianum* (70.74 per cent), *Psudomonas fluorescence* (56.27 per cent) and *Bacillus subtilis* (58.53 per cent).

Similar result were recorded by [16-17] as they reported maximum mycelial inhibition 79.35 per cent, 94.71 per cent and 53.17 per cent respectively of *A. porri* with *Trichoderma viride. Trichoderma harzianum* was best to inhibit the growth of *Alternaria porri* (73.12 per cent), as stated by [18].

Table 2: Efficacy of bioagents against *Alternaria porri* (dual culture method)

Sr. No.	Bioagents	Mean radial growth (mm)	Mycelial inhibition (%)
1	*Trichoderma viride*	20.16	76.55
2	*Trichoderma harzianum*	25.16	70.74
3	*Psudomonus fluorescens*	37.60	56.27
4	*Bacillus subtilis*	35.66	58.53
5	Control	87.00	-
	SE(m)±	0.902	-
	CD (P=0.01)	2.880	-

4. Discussion

4.1 *In vitro* evaluation of biocontrol agents against Purple blotch of onion causing pathogen

The experiment was carried out in the laboratory. In the present investigation, the biocontrol agent was used for *in vitro* evaluation against *Alternaria porri* amended with the PDA. The investigation indicated that minimum growth was recorded in treatment *Trichoderma viride* (76.55 per cent)followed by *Trichoderma harzianum* (70.74 per cent), *Psudomonas fluorescence* (56.27 per cent) and *Bacillus subtilis* (58.53 per cent) showed less radial growth of the *A. porri* among all treatments [19]. observed that *Trichoderma viride* arrested the mycelial growth (38.84 per cent) of *Alternaria porri* [16]. found the bioagents *Trichoderma harzianum* arrested the highest mycelia growth of *Alternaria porri* (79.5 per cent) followed by *Chaetomium* spp. (42.86 per cent) [20]. observed that *Trichoderma viride* (88.65 per cent) and *Trichoderma harzianum* (86.85 per cent) were highly effective in inhibiting the growth of *Alternaria porri in vitro* followed by *Trichoderma koningii* (76.58 per cent) and *Pseudomonas fluorescens* (72.55 per cent) [21]. observed that the bioagents *Psudomonas fluorescens* was effective in controlling the spread of purple blotch of onion caused by *Alternaria porri* [22]. found that among all the different bioagents evaluated (*in vitro*) against *Alternaria porri*, *Trichoderma viride* was found most effective with significant highest mycelial inhibition (94.71 per cent) followed by *Trichoderma harzianum* (91.13 per cent).

5. Conclusion

Alternaria porri attacks above ground plant parts of onion. The disease appears as small sunken, white flecks with purple colored center on leaves and flower stalks. Later, large area develops forming dead patches. The *Alternaria porri* was found pathogenic to the onion seedlings causing purple blotch/seedling blight. *Trichoderma viride* followed by *Trichoderma harzianum* was most effective against *A. porri* under *in vitro* condition.

6. Acknowledgment

The authors express thanks to Head, Department of Plant Pathology, College of Agriculture, JNKVV, Jabalpur (M. P.) for providing all the facilities to conduct work.

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