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PATHOGENIC BEHAVIOUR OF *ALTERNARIA ALTERNATA* AND PHYTOTOXICITY OF ITS CULTURE FILTRATES ON *LEPIDIUM SATIVUM*: A MEDICINAL HERB OF IMMENSE PHARMACOLOGICAL POTENTIAL

PUSHPANJALI WAGH,* SANDEEP SINHA, HARVINDER KUMAR SINGH AND U. K. KHARE
Department of Plant Pathology, College of Agriculture,
Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur – 462 002 (M.P.)
e-mail: pushpanjalisudhir.wagh@gmailcom.

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***Corresponding author**

ABSTRACT
Alternaria alternata causing leaf spot in *Lepidium sativum* was isolated and purified from diseased leaf tissues collected from collected from the Medicinal and Aromatic plant garden, Department of Crop and Herbal Physiology, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur (M.P.); isolated and purified on Potato Dextrose Agar media. Microscopic examination of a seven days old culture revealed hyaline, septate and branched mycelia, conidiophores with 30.0-80.2 μ length and 3-6 μ width and obclavate to obpyriform conidia (23-30 x 9.2-12.7 μ) with short conical beak arranged in acropetal fashion. The isolated culture and its culture filtrates were inoculated to germinated seedlings of chandrasur and also incubated with healthy leaves in a growth chamber. Typical symptoms of *Alternaria* leaf spot was observed both in in vivo and in vitro inoculated plantlets and detached leaves respectively. Chlorosis on the hypocotyls and leaves were observed. *A. alternata* was consistently reisolated from symptomatic leaf tissues on PDA. Thus, an efficient and reliable screening method wherein the effect of the selection agent (pathogen culture, culture filtrate/phytotoxin) was demonstrated providing sound pharmacological rational in terms of micro propagation and development of *Alternaria* resistant *L. sativum*, an important medicinal herb.

INTRODUCTION

Lepidium sativum also known as common cress, garden cress, garden pepper cress, pepper grass or pepperwort (english) and chandrasur, chansur (hindi); is an annual herb, belonging to Brassicaceae family. Various parts of the plant namely; seeds, leaves and roots have been used in treating various human ailments. The plant is known to contain imidazole, lepidine, semilepidinoside A and B (Maier *et al*., 1998), β–carotenes, ascorbic acid, linoleic acid, oleic acid, palmitic acid, stearic acid (Duke, 1992), sinapic acid and sinapin (Schultz and Gmelin, 1952). *L. sativum* is reported to exhibit antihypertensive (Maghrani *et al*., 2005), diuretic (Patel *et al*., 2009), antiinflammatory, analgesic, anticoagulant (Yahya *et al*., 1994), antirheumatic (Ahsan *et al*., 1998), hypoglycemic (Patole, 1998), laxative, prokinetic (Rehman, 2011), antidiarrheal and antispasmodic properties. It has also been shown to possess antiasthmatic (Paranjape and Mehta, 2006) and bronchodilatory (Mali, 2008) potential.

However, chandrasur an important medicinal plant with significant pharmacological properties has been observed to be generally affected by many fungal pathogens in India. Among them *A. alternata* causes severe leaf spot in the northern Indian plains. *Alternaria* leaf spot disease symptoms in *L. sativum* are characterized by the appearance of brown necrotic spots on the leaf margin. The necrosis spreads towards the midrib and as a result the leaf curls up and dries, affecting the herb yield. Host Selective Toxins (HST) function as essential determinants of pathogenicity or virulence; *Alternaria* producing the most important and well known HSTs (Nishimura and Kohmoto, 1983; Huang, 2001). The HST group comprises a limited number of phytotoxins that meet the following criteria: (1) the toxin and its producer have similar host specificity; (2) the virulence of the pathogenic strains is positively correlated to their capacity to produce the toxin and (3) the toxin is able to produce, in susceptible plants, symptoms characteristic of the disease caused by the pathogen (Huang, 2001). HSTs are toxic to plant species or cultivars susceptible to the pathogens producing these toxins and there is a correlation between sensitivity to the toxin and susceptibility of the plant to the pathogen (Knogge, 1996). Investigations into the molecular and biochemical responses to these disease determinants reveal responses typically associated with host defence and incompatibility induced by avirulence determinants (Wolpert *et al*., 2002).

It is well known that inoculation with culture filtrates of some plant pathogenic fungi can produce disease-like symptoms and may also be used to select for resistance (Wenzel, 1985; Daub, 1986; Buiatti and Ingram, 1991; Crino, 1997; Svabova and Lebeda, 2005). Culture filtrates are mostly produced by fungal cultivation in liquid media and subsequent separation of the solid and liquid parts of the culture. The liquid part of the culture is used as the selection agent. This approach yields

a variety of selection agents whose composition ranges from the absence of any toxin in the filtrate (in which cases the symptoms are caused by other fungal metabolites) to filtrates that contain uncharacterised active toxins (Daub, 1986). Crude culture filtrates allow easy bioassay and screening for toxic effects on plants, cuttings, leaf discs or even cell suspension cultures of the host species (Isaac, 1991). Crude culture filtrates have been used as selective agents in numerous disease resistance studies in which they exhibit phytotoxic activity (Chen and Swart, 2002; Svabova and Lebeda, 2005).

Thus in the present study an attempt was made to characterize the uncharacterized pathogenic behaviour of *A. alternata* and also to prove the phytotoxicity of its culture filtrates on *L. sativum,* a medicinally significant herb of immense pharmacological potential.

MATERIALS AND METHODS

Collection of diseased leaf samples

L. sativum plant samples exhibiting the symptoms of *A. alternata* were collected from the Medicinal and Aromatic plant garden, Department of Crop and Herbal Physiology, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur (M.P.).

Isolation, purification, morphological and cultural studies of pathogen

The pathogen was isolated from the diseased leaves of chandrasur exhibiting typical symptoms of *A. alternata*. Small portion of affected leaf was surface sterilized with mercuric chloride solution (0.1%) for one minute followed by three changes of sterile distilled water. The diseased pieces were dried by placing them between two sterile blotting papers and thereafter on potato dextrose agar in Petri plates and incubated at 25 ± 1ºC in a BOD incubator. Fungal growth was observed for seven days. The appearing fungus was transferred to Potato dextrose agar (PDA) slants. Single spore isolation was performed and maintained on PDA slants at 25 ± 1ºC. The cultures were revived every month by fresh transfers on the PDA slants. The observations from the seven day old culture were recorded for colony characters on PDA.

Identification of the pathogen

Morphological characteristics and measurements of conidiophores and conidia were recorded. Recorded data were compared with those available in the Melkania (1980) to identify the pathogen.

Preparation of spore suspension

To establish the pathogenicity fresh conidial suspension of pathogen is required. The pathogen was grown on PDA for four to six days at 25 ± 2ºC. Circular mycelial plugs were cut from margin of the colony with the help of sterilized cork borer and transferred to the Petri plates containing 10mL of sterilized water. After 48h of incubation at 25 ± 2ºC, the mycelial plug on which abundant conidia had developed, were transferred to another Petri dishe containing 10-12mL of sterilized water collected in a sterilized clean beaker and the suspension thus obtained was immediately used for pathogenicity test.

***In vivo* inoculation of pathogen by spraying**

Spore suspension of *A. alternata* sprayed with the help of glass automizer on upper and lower surface of the leaves of chandrasur plants grown in the greenhouse. The plants were provided the optimum condition by covering them with sterilized polythene bags. Optimum humidity was maintained by irrigating the pots. These plants were then observed regularly to observed initiation of symptoms (Fig.1. 1A).

***In vivo* inoculation of pathogen by pin prick method**

Five entomological pins were mounted on a cork covering one cm^2 area and dipped in spore suspension. One gentle prick was given on each side of the leaf. The length of pin was adjusted in such a way that it does not damage the other side of the leaf. The inoculated area was immediately covered with moist sterilized cotton swab to provide humid condition. The inoculated plant was kept in a glass house and the symptoms developed were observed regularly (Fig.1.1B).

***In vitro* inoculation of pathogen**

Inoculations were also performed on detached, healthy, surface-sterilized leaflets of chandrasur. Two blotter papers cut to the size of Petri plate were placed in it, covered and moistened with distilled water. Thereafter to prepare a moist chamber three to four cm glass rods were placed followed by glass slide. Gentle prick was given on each side of the leaf. The length of pin was adjusted in such a way that it does not damage the other side of the leaf. A five μL drop of conidial suspension was placed on each leaflet and eight to ten leaves per isolate were used. Leaves were incubated in a growth chamber. Control leaflets were inoculated with sterile distilled water. The symptoms of the disease, so developed, were examined critically on leaves and other parts of chandrasur plants.

Preparation of culture filtrate

For preparation of toxic culture filtrate, five mm discs of fungal mycelium were transferred to PDA medium from a 30 days old culture to a fresh PDA medium after every month for revival. and incubated at 25ºC. Then from a two weeks old *A. alternata* culture, single mycelia disc of five mm was inoculated in 50mL MS plant tissue culture medium in 250mL Erlenmeyer flasks. The cultures were incubated for 21 days. After 21 days, small mycelial mat were observed in a uniform suspension. Culture filtrate was collected by gravity through Whatman No. 1 filter paper. Culture filtrate was then subjected to centrifugation at 10,000 rpm for 20 minutes to collect supernatant. Supernatant was sterilized by passing it through a 0.22 μm nitrocellulose filter. Filtrated toxin stored at -20ºC and was used for further study (Fig.1. 2, 3, 4).

***In vitro* response of plantlet against toxic metabolites**

Mature seeds of chandrasur were washed with distilled water for 3-4 times followed by soaking the seeds in Tween 20 for five minutes. After five minutes further sterilization was carried inside the laminar airflow chamber. The seeds were washed with autoclaved distilled water for 15 minutes, then rinsed with 70 per cent ethyl alcohol for five minutes followed by treatment with 0.1 per cent $HgCl_2$ solution for five minutes. Sterilized seeds were then again washed thoroughly with autoclaved distilled water for ten times to remove $HgCl_2$ residues. Thereafter, seeds were cultured on MS basal medium

Figure 1: 1A. *In vivo* inoculation of pathogen by spraying; 1B, *In vivo* inoculation of pathogen by spraying by pin prick method; 2, 3. Preparation of culture filtrate; 4. Symptoms of *Alternaria* leaf spot on leaves; 5. Symptoms on plants at field; 6. Conidia of *Alternaria alternata*; 7. Cultural characteristics of *Alternaria alternata*; 8A. Symptoms after *in vivo* inoculation of pathogen on leaves; 8B. Symptoms after *in vivo* inoculation of pathogen on stems; 9. Symptoms after i*n vitro* inoculation of pathogen; 10. *In vitro* response of plantlet against toxic metabolites

supplemented with toxic metabolites of *A. alternata*. The culture bottles were sealed with parafilm and incubated for a week at 25 ± 2ºC under 12h photoperiod provided by white fluorescent lamps.

RESULTS

Symptomatology

A. alternata causing leaf spot in *L. sativum* was recorded in a large number of plants. The disease first appeared as small brown oval lesion which became irregular in shape gradually increasing in size and colour intensity. A light yellow zone surrounded the spot. Later, several such spots coalesced and whole leaf become yellow and ultimately defoliated. It also produced similar symptoms on stem and other plant parts (Fig.1, 4 and 5.).

Isolation, purification, identification and pathogenicity

The diseased portions of leaf exhibiting typical symptoms of *A. alternata* were collected and the pathogen was isolated from diseased tissues of leaf on PDA. A seven days old culture was examined under microscope for identification of the pathogen. The hyphae and mycelium observed from host tissue was hyaline, septate and branched. Conidiophores were usually unbranched emerging through the stomata, arising singly or in small groups, simple, straight or flexuous, almost cylindrical, septate, rather pale brown or yellowish brown. A simple and branched both type of chains of conidia were observed. Conidiophores with 3-6μ width and 30.0-80.2μ length were examined. Conidia were found to be obclavate to obpyriform with short conical beak at the tip or beakless, generally found in chain but in some instance borne singly. Conidia borne acropetally were observed brown to dark brown and black at maturity consisting 3-4 transverse and 1-4 longitudinal or oblique septa. Size of mature conidia ranged from 23-30 x 9.2-12.7 μ (Fig.1. 7.). in culture, mycelium was abundantly produced and formed distinct concentric rings (Figure 1.7). Brown unbranched erect septate conidiphores were distributed over the colony, bearing conidia in short chain. The cultural characteristics of *A. alternata* were compared with those of described (Melkania, 1980); thereby the association of *A. alternata* was confirmed with the symptoms of chandrasur (Fig.1. 7).

In vivo inoculation of pathogen

Chlorosis followed by necrosis of leaf developed within ten days after spraying of the conidial suspension of *A. alternata*. Slow growth of the organism was noted when the plants were inoculated by the causal organism by spraying. However, in pin prick method of inoculation yellowing followed by

necrosis of the leaf was observed within eight to ten days after inoculation. Rapid growth of the organism was observed with production of abundant conidia which were rather smaller in size when compared with the conidia produced in culture. The organism re-isolated, was confirmed the presence of *A. alternata* with the symptoms observed under field condition and in poly house (Fig.1.8).

***In vitro* inoculation of pathogen**

After incubation of leaves in growth chamber, necrosis and leaf spots, similar to the original symptoms developed on all inoculated leaves within five days. *A. alternata* was consistently re-isolated from symptomatic leaf tissues on PDA (Fig.1. 9).

***In vitro* response of plantlet against toxic metabolites**

The inhibitory and phytotoxic effect of culture filtrates was observed on germinated seedlings. Chlorosis on the hypocotyl and leaves was observed in all the seedlings (Fig.1. 10).

DISCUSSION

Leaf spot disease caused by *A. alternata* (Fr.) Keisser is one of the severe diseases of chandrasur (Melkania, 1980). *Alternaria* produces the most important and well known HSTs. HSTs are toxic to plant species or cultivars susceptible to the pathogens producing these toxins and there is a correlation between sensitivity to the toxin and susceptibility of the plant to the pathogen. Fungal culture filtrates contain a spectrum of secondary metabolites, such as polysaccharides, oligosaccharides (Peros and Chagvardieff, 1987), proteins, glycoproteins, unsaturated fatty acids, stem from the cell walls, cytoplasm of the fungi, growth regulators such as auxin, kinetin and gibberellic acid (Gentile *et al*., 1992), along with toxins (host-selective and non-host-selective) that may play a role as co-determinants of pathogenicity during disease development (Buiatti and Ingram, 1991; Crino, 1997; Svabova and Lebeda, 2005). The application of filtrates to cultures *in vitro* can trigger the elicitation of various defence responses, *e.g.,* phytoalexins; activity of certain enzymes (Saindrenan *et al*., 1990; Crino, 1997; Lebeda *et al*., 2001); accumulation of phenolic acids (Cvikrova *et al*., 1992), total phenols, peroxidases and beta 1, 3-glucanase (Lebeda *et al*., 2001; Singh *et al*., 2003) and chitinase (Jayasankar and Litz, 1998). This study provide insights regarding evaluation of somaclonal variation for disease resistance in *L. sativum,* with respect to availability of an efficient and reliable screening method wherein the effect of the selection agent (pathogen culture, culture filtrate/ phytotoxin) was demonstrated over different concentration ranges. This study also provides sound pharmacological rational in terms of micro propagation and development of *Alternaria* resistant *L. sativum*, an important medicinal herb.

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