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Molecular and pathogenic variability of multilocus isolates of *Alternaria lini* Dey cause Alternaria blight in linseed (*Linum usitatissimum* L.)

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Abstract
The pathogen of Alternaria blight of linseed was isolated from infected leaf, buds and seeds collected from eleven locations of India and the isolates were designated as Nj, Kr, Ji, Fd, Rr, Hr, Pr, Ri, Nr, Plr and Ra. All the isolates showed variability in term of colony colour, conidial shape, size, septation and growth behavior at different pH, temperature and media. Three out of 11 isolates show grey (Nj, Kr, Fd), 6 grayish black (Ji, Rr, Ra, Ri, Nr, Hr) and two black (Plr, Pr) colour with circular colony pattern. Conidial size of all isolates ranged from 20.32 to 28.25µm length with 3.76 to 7.55µm width. Nj isolate have maximum conidial size whereas, Fd isolate have minimum size. The conidia also showed dark brown (Nj, Kr, Plr), and light brown colour. Three out of eleven isolates shown obclevate shape (Nj, Rr and Pr), three flask shaped (Kr, Ji and Plr) and rest of the isolates (Fd, Hr, Ri, Nr, Ra) were oval in shape. Transverse septation were ranged from 7-2 and longitudinal from 4-1 septaion with 7 transverse septa were found in conidia of Plr isolate. Similarly, maximum with four longitudinal septa were recorded in Plr isolate. Pathogenic behavior of the isolates revealed that Nj isolates is more pathogenic indicating maximum disease severity as compare to other. Profiling of soluble protein of multilocus isolates showed that number of protein bands varies from 9-14 with 14 maximum number of bands was found in Nj, Hr and Nr isolates, whereas, the minimum with 09 bands were found Fd and Ri isolates indicating variability exist among the isolates.

Keywords: Molecular, pathogenic variability, multilocus isolates, *Alternaria lini*

Introduction
Linseed (*Linum usitatissimum* L.) is known as founding crop (Zohary and Holf, 1993) [35] which is being evaluated as a crop platform for the production of bio-industrial and nutraceutical products (Jhala *et al.*, 2008) [16]. The genus *Linum* was suggested by Hutchinson (1948) [15] which is the member of family Linaceae (Gill, 1987) [9]. More than 230 species are belonging to the genus *Linum* (Tutin *et al.* 1968 and Heywood, 1993) [32, 12] are distributed worldwide and this genus divided into five sections *i.e.*, *Linum*, *Linastrum*, *Cathartolinum*, *Dasylinum*, and *Syllinum* (Gill, 1987) [9]. *Linum* section contains the cultivated species, *L. usitatissimum* L. (Tammes, 1928; Tutin *et al*., 1980) [30], two ornamentals *L. grandiflorum* and *L. Perenne* (Nag *et al.*, 2015) [23] and *L. angustifolium* and *L. bienne*. Linseed is one of the few crops which is cultivated in all continents of the world. It is the sixth largest oilseed crop in the world and is one of the oldest cultivated plants (Bhatty and Rowland, 1990) [3]. Flaxseed is grown as either oil seed crop or a fiber crop (Diederichsen *et al*., 2003) [8]. In India, linseed is popularly known as Alsi (Hindi, Gujrati and Punjabi), Tisi (Bangali) and Jawas (Marathi).
Linseed seeds have good Nutritive and medicinal values. It is one of the richest sources of linolenic fatty acids (Katare *et al.*, 2012) [17]. Flax seed consumption reduces the risk of cardio vascular disease (Hurteau, 2004) [14], total cholesterol (Bierenbaum *et al.*, 1993) [4] as well as platelet aggregation (Allman *et al.*, 1995). Linatine antibiotic can also be obtained from its seed (Gill, 1987) [9].
Linseed plant is suffered by various diseases among which Alternaria blight is a major disease, causes heavy loss in terms of quality and quantity of fiber and seed of linseed. The disease was first reported by Dey (1933) [7] from flower bud (Kolte and Fitt, 1997) [18], Kanpur, Uttar Pradesh. Later Siddiqui (1963) [27] reported the occurrence of Alternaria blight on linseed cultures at IARI, New Delhi and other parts of the country. The fungus was named as *Alternaria lini* after the first report of this disease in 1933 (Dey, 1933) [7]. Arya and Prasad (1952) [2] recorded a severe outbreak of the disease at Delhi in 1949 and reported that the pathogen was identical with *Alternaria brassicae* (Berk) Sacc. var. *macrospora* (Broun) in morphology, pathogenicity and physiology. The disease appears on all the aerial parts of the plant.

Alternaria blight disease was previously designated as minor disease has now become a major problem in different parts of the country (Chauhan and Shrivastava, 1975) [5].

A. lini showed variability in term of cultural, morphological, pathogenic and molecular basis. Verma and Singh (2017) [34] suggested that colony colour showed whitish, light brown to dark brown; growth pattern slow, medium to fast, colony appearance cottony, fluffy, feathery to compressed and thin; margin showed wavy, rough to smooth. They also found that variable shape of conidia in term of length, width, number of septa and presence or absence of beak. Arya and Prasada (1952) [2] reported that *A. lini* was identical with *A. brassicae* (Berk) Sacco. in morphology, pathogenicity and physiology. Conidiophores of *A. lini* arise in fascicles. Conidia develop singly or in short chains. They are dark, obclavate, muriform and measured in size 120-225 μ × 15-28 μ. At least 268 metabolites from *Alternaria* fungi have been reported in the past few decades. The mainly include nitrogen-containing metabolites, steroids, terpenoids, pyranones, quinones and phenolics (Lou *et al.*, 2013) [20]. Kralova *et al.* (2006) determined three target mycotoxins *viz.*, altenuene (AE) @4µg/kg, alternariol (AOH) @69µg/kg and alternariol monomethyl ether (AME) @16µg/kg in Jupiter cultivar of linseed.

The pathogen is perpetuated in seed and also soil through infected plant debris. The management of disease can be done through cultural *i.e.*, crop rotation (Rani and Sudini, 2013), changing in sowing date (Singh and singh, 2004b, Singh *et al.*, 2008, Singh *et al.*, 2015) [1], destruction of plant debris (Rani and Sudini, 2013), soil solarization (Patel *et al.*, 2014), use of resistant cultivars (Ramakant *et al.*, 2008), chemical (Holi and Meena, 2015), biological management (Bhoye *et al.*, 2011, Biswas *et al*, 2015). Cultural and biological strategies are mostly effective at initial stage, specially at sowing time of crops and they can not manage the disease in standing crop and even after appearance of disease. Use of resistant cultivar is also reasonable and easy method for disease management but due to development of new strain among the pathogens, resistant may be break down to susceptible one.

Keep the above facts in view, the study entitled *"*Morphological variability and pathogenic behavior of multilocus isolates of *Alternaria lini* Dey cause blight disease in linseed (*Linum usitatissimum* L.)" was undertaken in the present investigation.

Material and Methods

Collection of diseased plant materials

Infected plant materials of linseed were collected from eleven multilocation *viz.* Nawabganj, Kalyanpur, Jhansi and Faizabad (Uttar Pradesh); Raipur (Chhatisgarh), Hisar (Haryana), Pantnagar (Uttrakhand), Ranchi (Jharkhand), Nagpur (Maharashtra), Palampur (Himachal Pradesh) and Rewa (Madhya Pradesh) of India. The affected plants were brought to the laboratory and critically examined for the presence of causal organism and store in BOD at 25±2 ºC. The collected diseased materials were used for isolation and identification of the pathogen.

Isolation of pathogenic fungi

Pathogen of this disease influenced to whole areal portion of linseed. In present investigation, multilocus isolates set apart from leaf, bud and seed. The affected plant parts were thoroughly washed with sterile water to remove the dust and other surface contaminants. Small pieces of diseased parts along with some healthy portion were cut into small pieces by sterilized blade. The pieces were then dipped in 0.1 per cent mercuric chloride solution for 30 seconds with the help of sterilized forceps and washed immediately in 3-4 times with sterilized distilled water to remove the last traces of mercuric chloride solution. The pieces were then transferred at the centre of Petri dishes, containing 2.0% potato dextrose agar (PDA) medium and were incubated at 25±2 ºC. As soon as the mycelial growth was visible around the pieces, a small bit of mycelium from the margin of fungal colony was transferred to 2.0% PDA medium slants in test tube.

Purification and identification

Eleven isolates set apart from seed, bud and leaves of linseed and single spore culture technique was used for their purification. Further, these isolates identified on the basis of mycelium, conidial and conidiophore characters. Isolates were designated in symbols as Nj for Nawabganj, Kr, Ji, Fd, Rr, Hr, Pr, Ri, Nr, Plr, Ra for Kalyanpur, Jhansi, Faizabad, Raipur, Hisar, Pantnagar, Ranchi, Nagpur, Palampur and Rewa isolate, respectively.

Morphological variability

To find out morphologically variability, conidial suspention of each multilocus isolate was prepared in distilled water separately. Conidial suspension studied by stage and ocular lens to find out morphological variability based on conidial characters *i.e.*, shape, size and septation of conidia. Conidia size was measured in micrometer (µm).

Measurement of disease severity

After germination, the crop was regularly watched for first appearance of disease. The observations on leaves and bud for disease severity were recorded using by 0-5 scale (Das *et al.*, 2016) from plants of each pot. The disease severity was recorded at pre-senescence stage. These numerical ratings were used to calculate the per cent disease severity (PDS) as follows:

$$\text{Per cent disease severity (PDS)} = \frac{\text{Sum of all numerical value}}{\text{Total number of leaves examined} \times \text{maximum grade}} \times 100$$

Per cent Disease Control (PDC)

The per cent disease control was calculated by using the following formula:

$$\text{PDC} = \frac{\text{PDS in check - PDS in treatment}}{\text{PDI in check}} \times 100$$

Variability based on protein content in mycelium

All the eleven isolates of *A. lini* were grown separately on Potato Dextrose Broth for seven days. Mycelium mat was taken out washed with distilled water and dried for extraction of soluble protein. The method developed by Lowery *et al.* (1951) was used for extraction of soluble protein. The extracted soluble protein was further used for protein profiling.

Protein profiling

Profiling of soluble proteins was also done in various isolates of *Alternaria lini* cause blight disease in linseed. Analysis of total soluble proteins through sodium Do decyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) was carried out for the study of variability in different isolates of *Alternaria lini*. SDS PAGE was done to get soluble protein pattern.

Soluble proteins were electrophorsed by 10% SDS poly acrylamide gel based on method of Laemmli (1970).

Result and Discussion

Isolation, purification and identification

Two of eleven isolates set apart from bud (Fd and Ra) and four from bud (Kr, Ji, Rr and Nr) whereas rest of five isolates (Nj, Hr, Pr, Ri and Plr) set aside from leaf. All isolates were purified using by single spore culture technique (Choi *et al.*, 1999) [6] and obtained morphologically similar to *Alternaria lini* described by various scientists (Dey, 1933; Gill, 1987; Kolte and Fitt, 1997 and Rashid, 2003) [7, 9, 18, 25].

Morphological variability

Variability in conidial shape

On the basis of conidial shape, size and septation, multilocus isolates of *A. lini* were shown variability. Three out of eleven isolates shown obclevate shape (Nj, Rr and Pr) whereas, flask shaped were shown by Kr, Ji and Plr isolates and rest of the isolates (Fd, Hr, Ri, Nr, Ra) were showing oval shape of conidia. Verma and Singh (2017) [34] also obtained conidial basis variability in multilocus isolates of *A. lini.* According to them, seven out of the twelve isolates shown oval shape of conidia whereas three isolates shown obclavate and rest of the isolates were showing clavate shape.

Variability in conidial length, width and septation

Conidial length of all isolates ranged from 20.32-28.25 and width from 3.76-7.55 µm. Nj isolate have maximum conidial size representing 28.25 and 7.55 µm, respectively which was followed by Pr (203.26 µm), Rr (185.27 µm), Ra (172.66 µm), Plr (158.67 µm), Kr (155.93 µm), Nr (139.19 µm), Ri (129.72 µm), Hr (107.90 µm), Ji (98.57 µm) and Fd (76.20), respectively. Nj and Kr isolates are of same palace (Kanpur, Uttar Pradesh) also shown variation in term of conidial size.

Septation of conidia was also showed variation among multilocus isolates of *A. lini*. From the present study, it is cleared that transverse septa are more than longitudinal septa. Transverse septation ranged from 2-7 whereas 1-4 septaion were recorded in longitudinally. The maximum with7 transverse septa were found in Plr isolate followed by 6 (Fd, Pr), 5 (Ji), 4 (Kr, Ri, Nr), 3 (Hr, Ra) and 2 (Rr), respectively. The longitudinal septa are maximum with 4 were recorded in Plr isolate, followed by 2 in three isolates namely Fd, Pr and Ri. Single septum was observed in Nj, Kr, Ji, Rr, Hr, Nr and Ra isolates. Anand (2002) have also found that conidial variability among isolates of *A. alternata* (rot of chilli) in term of septation (1-6) and size (10-15×5-20µm). Verma and Singh (2017) [34] were obtained conidial variability with 23.26-45.72 µm conidial length and 6.76-17.01 µm width among twelve multilocus isolates of *A. lini* (blight of linseed). Conidial variability in term of septation was also observed by Shakthi *et al.* (2013) among isolates of *A. brassicae* (blight of mustard), Singh *et al.* (2014b) in *A. brassicae* (blight of mustard), Nikam *et al.* (2015) in *A. solani* (early blight of tomato).

Three of eleven isolates namely Nj, Kr and Plr were showing dark brown colour of conidia whereas rest of isolates were light brown in colour. Conidial colour ranged from brown to dark brown was observed in different isolates of *A. alternata* caused fruit rot in chilli (Anand, 2002).

Table 1: Conidial variability of multilocus isolates of *Alternaria lini*

Isolates	Conidia					
	Length (µm)	Width (µm)	Septa		Colour	Shape
			Trans.*	Long.**		
Nj	28.25	7.55	2	1	Dark brown	Obclevate
Kr	24.87	6.27	4	1	Dark brown	Flask shaped
Ji	22.66	4.35	5	1	Light brown	Flask shaped
Fd	20.32	3.76	6	2	Light brown	Oval shaped
Rr	27.86	6.65	2	1	Light brown	Obclevate
Hr	20.75	5.20	3	1	Light brown	Oval shaped
Pr	26.57	7.65	6	2	Light brown	Obclevate
Ri	20.46	6.34	4	2	Light brown	Oval shaped
Nr	21.25	6.55	4	1	Light brown	Flask shaped
Plr	23.86	6.65	7	4	Dark brown	Flask shaped
Ra	22.57	7.65	3	1	Light brown	Oval shaped

*Transverse septa
**Longitudinal septa

Disease severity

Eleven multilocus isolates of *A. lini* were inoculated on linseed var. Shekhar plants grown in pot and disease severity was recorded from leaves and buds separately. The maximum disease severity with 52.12% in leaves and 39.10% in bud were recorded on plant inoculated by Nj isolate indicating most virulent and Nr isolate was found least virulent showing 48.36% on leaves and 37.78% on buds. Similar results were found by Kakvan *et al.* on 35 isolates of *A. alternata* which were categorized into low, moderate and high pathogenic groups.

Table 2: Variability in pathogenic behavior of multilocus isolates of *Alternaria lini*

Multilocus Isolates	Leaf		Pooled data	Bud		Pooled data
	2014-15	2015-16		2014-15	2015-16	
Nawabganj	51.07	50.98	51.03	39.13	38.98	39.06
Kalyanpur	52.19	52.05	52.12	39.17	39.03	39.10
Jhansi	49.59	49.52	49.56	38.47	38.16	38.32
Faizabad	49.17	48.92	49.05	37.51	38.34	37.93
Raipur	50.08	49.54	49.81	38.51	38.38	38.45
Hisar	50.17	49.32	49.75	38.49	38.25	38.37

Pantnagar	50.32	49.93	50.13	38.87	38.72	38.79
Ranchi	49.95	48.96	49.46	37.77	38.52	38.15
Nagpur	48.74	47.97	48.36	37.39	38.16	37.78
Palampur	49.96	49.89	49.93	38.60	38.31	38.46
Rewa	49.83	49.63	49.73	38.41	38.27	38.34
Check	37.57	37.41	37.49	26.39	26.13	26.26
CD	02.27	02.32	2.73	1.94	01.50	01.69
SE(m)	00.77	00.79	0.93	0.66	00.51	00.57

Protein basis variability

Variability among multilocus isolates of *A. lini* was recorded on the basis of protein profiling. Number of protein band varies from 9-14 among the isolates. The maximum number of bands was found in Nj, Hr and Nr isolates, representing 14 bands. Whereas, the minimum with 09 bands were found Fd and Ri isolates. The presence or absence of the bands indicates variability among the isolates. Tiwari *et al.* (2017) [31] also found similar work on different *Alternaria* spp. encountered on various vegetable, fruits and oil yielding crops by SDS PAGE studies. Akram *et al.* (2015) [1] found variation among eight multilocus isolates of *Alternaria* cause blight disease in linseed by using polymerase chain reaction based random amplified polymorshic DNA (RAPD) marker.

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