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Isolation, identification, pathogenicity and host range of *Aspergillus niger* causing collar rot of groundnut (*Arachis hypogaea*)

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Abstract
Groundnut (*Arachis hypogaea* L.) is an important legume crop of tropical and sub-tropical areas of the world. This crop suffers from several diseases but collar rot caused by *Aspergillus niger* van Teighem is one of the most important seed and soil borne diseases causing huge economic loss in India and abroad. In this study associated fungus was isolated on PDA medium and purified by using hyphal tip method from individual collar rot infected root of the plant collected from field showing maximum disease incidence during survey. On PDA, uniformly one type growth of fungus colony started black in colour and fungus rapidly covered the entire Petri plate within 72 hours. The hyphae were septate and hyaline more or less yellow in color. The colonies were black colored and reverse usually colorless. Conidiophores were hyaline, smooth, septate or non-septate. Conidia were globose to sub globose, dark brown to black with rough walled. Pathogenicity test of isolated and purified fungus was carried out with groundnut seeds following seed inoculation technique. On the basis of measurement and other morphological characters, the pathogen was identified as *Aspergillus niger* van. Tieghem. Thirteen domesticated crops and four weeds were artificially inoculated with highly virulent isolate (ANJP 04 isolate, isolated from Khejroli village of Jaipur district) of *A. niger* under pot house conditions during *Kharif* 2020. The result revealed that *A. niger* could produce visible symptoms on all tested plants of *Fabaceae* (cluster bean, cowpea, black gram, green gram, soybean), *Poaceae* (sorghum, maize, pearl millet), *Pedaliaceae* (sesame), *Solanaceae* (eggplant, tomato), *Malvaceae* (okra), *Amaryllidaceae* (onion) family and weeds of *Papaveraceae* (mexican prickly poppy), *Asteraceae* family (carrot grass) except plants of *Zygophyllaceae* (puncture vine weed) and *Amaranthaceae* (amaranthus) family were not produced any symptoms of collar rot disease.

Keywords: Groundnut, collar rot, *Aspergillus niger*, pathogenicity, host range

Introduction
Groundnut (*Arachis hypogaea* L.), is an important legume crop of tropical and sub-tropical areas of the world, described in 1753 by Linnaeus (Pattee and Young, 1982) [15]. It is a member of the genus *Arachis* in the sub tribe *Stylosanthinae* of tribe *Aeschynomeneae* of the family *Fabaceae*. Groundnut kernels contain about 26 per cent protein, 48 per cent edible oil, 20 per cent carbohydrates and three per cent fiber and also rich in calcium, thiamine and niacin (Haveri, 2017) [9]. As a source of edible oil, it finds its prime utility, as a consequence of it, this crop is gaining the status of "king of oil seed crops" (Reddy, 1976) [7]. Additionally, in association with symbiotic nitrogen-fixing bacteria, it fixes and enriches the soil with 80-160 kg N/ha per season (Alam *et al*. 1988). In India, the total coverage area under this crop is 39. 31 lakh hectares, production is 6.86 million tonnes with an average productivity of 1745 kg/hectare (Anonymous, 2019) [3]. Rajasthan stands second position in terms of area and production. The cultivation of groundnut is well adapted to the conditions prevailing in Rajasthan and is cultivated in about 7.34 lakh hectares with annual production 1.612 million tonnes and productivity of 2195 kg/hectare (Anonymous, 2019-20) [2]. Several abiotic and biotic factors affect the growth and development of groundnut leadingto qualitative and quantitative yield losses. Diseases are most damaging and major limiting factors that cause the largest economic losses in profitable cultivation of this crop in Rajasthan. Amongst fungal diseases, collar rot of groundnut also known as seedling light caused by *Aspergillus niger* van. Teighem is one of the important seed and soil borne diseases. Pathogen is a well known polyphagous, ubiquitous, non-target and the most destructive soil and seed inhabiting fungus (Vimalkumar and Saifulla, 2017) [23].

Collar rot of groundnut prominently is distributed in countries with tropical and subtropical climates where high temperature prevails during the rainy season and it is present in all most all the groundnut growing areas of the world. This disease was first reported by Jochem (1926) [10] from Java. It is an important disease in the major groundnut growing states. In Rajasthan, Bakhetia (1983) [4] had reported disease incidence up to 50.00 per cent. Dighule *et al.* (2018) [6] estimated yield losses in Maharashtra from 28.00 to 50.00 per cent due to collar rot of groundnut caused by *Aspergillus niger* van. Teighem. Raper and Fennell (1965) [19] divided the genus *Aspergillus* into groups according to the colour of the conidiospores. *Aspergilli* with brown to black-shaded spores constitute the *A. niger* group. Recently, taxonomic position to *A. niger* has been assigned by Kirk *et al*. (2008) [11] to the Kingdom-*Fungi*, Phylum-*Ascomycota*, Class-*Eurotiomycetes*, Sub class- *Eurotio- mycetidae* Order- *Eurotiales*, Family-*Trichomaceae*, Genus-*Aspergillus* and Species-*niger*. Vegetative growth of *A. niger* is very rapid on culture media with submerged mycelium. The hyphae are septate and hyaline more or less yellow in color. The colonies are black coloured and reverse usually colourless. Conidiophores mostly arise directly from substratum and are smooth, septate or non-septate, varying greatly in length and diameter, *i.e.*, 200-400x7-10 µm and 20 µm, respectively. Conidial heads are fuscous, blackish-brown to purple-brown or in every shade to carbonous black, varying from small, almost columnar masses of a few conidial chains to the common globes or radiate heads, up to 300, 500 µm, or 1000 µm long. Vesicles are globose, commonly 20-50 µm up to 100 µm in diameter. Conidia are globose to sub-globose (3.5-5.0 µm in dia.), dark brown to black and rough-walled (Gilman, 2001 and Sharma, 2012) [8, 21].

The genome size of *A. niger* is about 35.5 to 38.5 Mb composed of about 13,000 genes. The DNA sequence of *A. niger* consists of approximately 33.9 million base pairs (Debets *et al.* 1990) [5].

Materials and Methods

Isolation and purification of pathogen

Isolation of pathogen was made on PDA medium from individual collar rot infected root of the plant collected from field showing maximum disease incidence during survey. Infected portion of the root was cut into small pieces (3-4 mm size) and surface sterilized by dipping in one per cent sodium hypochlorite solution for one minute followed by rinsing three times with sterilized distilled water to remove the traces of disinfectant and dried on sterilized blotter paper. These bits were transferred aseptically into Petri plates containing PDA and incubated in BOD incubator at 25 ± 1 °C. After 72 hours, the pure culture of the fungus was obtained by following the hyphal tip culture method (Riker and Riker, 1936 and Rangaswami, 1971) [18]. A disc of fungal colony, cut with the help of cork borer and placed in the middle of Petri plate containing plain agar and incubated for 2 days. Petri plate was placed under dissecting microscope and mycelial threads of pathogen was located at high magnification. The apical part of the hyphal strand (about 1 mm from end) was cut with the cork borer and removed it and transferred to another agar plate. After this, pure isolates were obtained and these were maintained for further studies. One representative isolate from each surveyed district was established from the field showing maximum disease incidence.

Identification of the pathogen

Associated fungus was identified on the basis of its morphological and colony characteristics. Hyphae of pathogen were septate and hyaline or less yellow in colour, colonies were black coloured and reverse usually dull white or colourless. Conidiophores were hyaline, smooth, septate or non-septate and varying greatly in length and diameter. Conidia were globose to sub globose dark brown to black with rough walled (Raper and Fennel, 1965) [19].

Pathogenicity test

Pathogenicity test of isolated and purified fungus was carried out with groundnut seeds following seed inoculation technique (Kataria and Grover, 1976) [12]. Apparently healthy surface sterilized groundnut seeds (RG-382 variety) were taken. The seeds were rolled, on seven days old sporulating culture of *A. niger* (ANJP-04 isolate, isolated from Khejroli village of Jaipur district) grown on PDA contained Petri-plates. Inoculated four seeds were sown at five cm depth in each earthen pot (pre-sterilized and having autoclaved soil) with three replications. The un-inoculated apparently healthy seeds served as control. These pots were kept in cage house and watered regularly as per requirement and observation on diseased and healthy plants were recorded up to 20 days of sowing.

Host range of the pathogen

Thirteen domesticated crops and four weeds (Table 1) were screened against highly virulent isolate (ANJP 04) of *Aspergillus niger* under pot conditions during *Kharif* 2020. The seeds of crops and weeds were collected during the *Kharif* 2019. The seeds were rolled on seven days old sporulating culture grown on PDA contained in Petri plates. Inoculated seeds were sown in 30 cm diameter earthen pots (pre-sterilized and having autoclaved soil) with three replications. These pots were kept in cage house and watered regularly as per requirement and observation on host reaction was recorded up to 45 days of sowing.

Table 1: List of domesticated crops and weeds used for host range studies

S. No.	Hindi/local name	English name	Scientific name
Crops			
1.	Guar	Cluster bean	*Cyamopsis tetragonoloba*
2.	Chawla	Cowpea	*Vigna unguiculata*
3.	Urd	Black gram	*Vigna mungo*
4.	Moong	Green gram	*Vigna radiata*
5.	Jowar	Sorghum	*Sorghum bicolor*
6.	Til	Sesame	*Sesamum indicum*
7.	Makka	Maize	*Zea mays*
8.	Bajra	Pearl millet	*Pennisetum glaucum*

9.	Tamaatar	Tomato	*Solanum lycopersicum*
10.	Bengan	Brinjal	*Solanum melongena*
11.	Soybean	Soybean	*Glycine max*
12.	Bhendi	Okra	*Abelmoschus esculentus*
13.	Pyaj	Onion	*Allium cepa*
Weeds			
14.	Satyanashi	Mexican prickly poppy	*Argemone Mexicana*
15.	Bhakri	Puncture vine weed	*Tribulus terrestris*
16.	Chawlai	Amaranthus	*Amaranthus viridis*
17.	Congress grass	Carrot grass	*Parthenium hysterophorus*

Results and Discussion

Isolation and identification of pathogen

Plants showing typical symptoms of collar rot disease were collected during survey from every surveyed field of eight districts *viz*., Bikaner, Jodhpur, Churu, Jalore, Jaipur, Sikar, Nagaur and Dausa districts and brought to the laboratory.

On the basis of maximum disease incidence, one sample was chosen among 25 fields of a district for isolating pathogen. Thus, selected samples of diseased plants were used for isolating causal fungus under aseptic conditions by incubating in BOD at 25+1 °C in Petri plates containing Potato Dextrose Agar (PDA) medium. Pure culture of the pathogen was obtained by hyphal tip technique. The hyphae were septate and hyaline more or less yellow in color. The colonies were black colored and reverse usually colorless. Conidiophores were hyaline, smooth, septate or non-septate. Conidia were globose to sub globose, dark brown to black with rough walled. On the basis of measurement and other morphological characters, the pathogen was identified as *Aspergillus niger* van. Tieghem. During the survey, samples were collected from surveyed fields brought to the laboratory and isolation was made from infected groundnut plants showing characteristic symptoms of collar rot. The important symptoms of the disease were dry shredded effect of wet and slimy rotting nature on seedlings. The affected portion of plants was turned dark, shrunken and shredded; later on this portion was covered with masses of spores of the pathogen that justified the name of "*Kaalijad"* (meaning 'black root'). The symptoms observed during the present survey and also noticed in the research experiments are in accordance with observations of the earlier scientists (Morwood, 1945, Suzui and Makino, 1980, Wardsworth and Melouk, 1985, Pande and Rao, 2000, Rakholia *et al*., 2012 and Divya Rani *et al.*, 2017) [13, 22, 16, 7, 24, 14]. Morwood (1945) [13] had been concluded that seeds of groundnut failed to germinate due to rotting in the soil whereas crown rot and seedling blight appeared in the initial stage. Sequentially, the symptoms appeared first on the seed and on the stem and affected plants showed wilting besides rotting of tissues just below the ground level. The rotted portion of plants turned dark and shredded with covering of black masses of fungal spores. Suzui and Makino (1980) [22] assessed that crown rot is the most serious disease of groundnut caused by *A. niger* in early stage of plant growth and newly emerged seedlings were affected at collar region causing yellowing of lower leaves, finally leading to death of collar portion of the young plants. Wardsworth and Melouk (1985) [24] have observed most typical symptom of the disease as sudden wilting of young plants. Infection mostly occurred in the cotyledon and hypocotyl regions of the newly emerged plants and disease progressed more rapidly and plants died in 30 days after sowing. If plants survived, lesions were characterized by shredded bark and generally diseased parts were covered by with dark masses of mycelium, conidiophores and conidia. Pande and Rao (2000) [14], Rakholia *et al*. (2012) [16] and Divya Rani *et al.* (2017) [7] have also been described symptoms like pre- and post-emergence mortality, rotting and discoloration of infected portion and affected plants showed wilting and rotting just below the soil surface. Infected seeds of groundnut became black and unable to germinate.

Pathogenicity of *Aspergillus niger*

The pathogenicity of isolated and purified *Aspergillus niger* was tested under pot house conditions on groundnut variety RG-382 by seed inoculation technique. Seeds were inoculated with 7 days old culture of pathogen, multiplied on PDA in Petri plates. The fungus was found highly pathogenic to cause collar rot of groundnut. Initially, seedling rotting was appeared in the cotyledon and hypocotyl regions after germination. Later on, the infected plants showed sudden wilting and plants died. In survived plants, affected crown portion turned dark, shrunken and shredded and later covered by the black masses of spores of the pathogen. The symptoms observed in artificially inoculated plants were similar to those visualized in naturally infected plants during survey. Re-isolation from the infected region of inoculated and diseased plants further yielded the fungus identical to one used for artificial inoculation of the plants. The un-inoculated plants were free from any type diseased symptoms. The pathogenicity of the isolated and purified fungus, *A. niger* was confirmed by seed inoculation technique under pot house conditions following Koch's postulates. The isolated fungus was identified as *A. niger* van. Teighem on the basis of cultural and morphological characteristics. The colonies of *A. niger* are black coloured in Petriplates and reverse usually colourless. Conidiophores generally are smooth, septate or nonseptate, varying greatly in length and diameter, i.e., 200-400x7-10 and 20 µm, respectively. Conidia are globose to sub-globose (3.5-5.0 µm in dia.), dark brown to black and rough-walled. Similarly, isolation of *A. niger* was made and pathogenicity proved by Raper and Fennell, (1965) [19] and Ramakrishna and Kolte, (1989) [17]. Cultural and morphological characteristics were also described by Gilman, (2001) [8] and Sharma, (2012) [21].

Host range of the pathogen

Thirteen domesticated crops and four weeds were artificially inoculated with highly virulent isolate (ANJP 04) of *A. niger* under pot house conditions during *Kharif* 2020. The result presented in Table 2 revealed that *A. niger* could produce visible symptoms on all tested plants of *Fabaceae* (cluster bean, cowpea, black gram, green gram, soybean*), Poaceae* (sorghum, maize, pearl millet), *Pedaliaceae* (sesame), *Solanaceae* (eggplant, tomato*), Malvaceae* (okra), *Amaryllidaceae* (onion) family and weeds of *Papaveraceae* (mexican prickly poppy), *Asteraceae* family (carrot grass)

except plants of *Zygophyllaceae* (puncture vine weed) and *Amaranthaceae* (amaranthus) family were not produced any symptoms of collar rot disease. The pathogen was re-isolated from tested plant roots and the morphological characters of the reisolated pathogen were compared with the original culture and these were similar in all respects. Hence, collar rot pathogen (*A. niger*) has wide host range. This study indicates that sensitive plant species may be collateral hosts of this pathogen which can be helpful in inoculum survival and build up and may be serious concern for coming season.

Table 2: Reaction of *A. niger* with different crops and weeds under artificial inoculation conditions (in pots)

S. No.	Hindi name	English name	Scientific name	Family	Host reaction
Crop					
1.	Guar	*Cluster bean*	*Cyamopsis tetragonoloba*	*Fabaceae*	+
2.	Chawla	*Cowpea*	*Vigna unguiculata*	*Fabaceae*	+
3.	Urd	*Black gram*	*Vigna mungo*	*Fabaceae*	+
4.	Moong	*Green gram*	Vigna radiata	*Fabaceae*	+
5.	Jowar	*Sorghum*	*Sorghum bicolor*	*Poaceae*	+
6.	Til	*Sesame*	*Sesamum indicum*	*Pedaliaceae*	+
7.	Makka	*Maize*	Zea mays	*Poaceae*	+
8.	Bajra	*Pearl millet*	Pennisetum glaucum	*Poaceae*	+
9.	Tamaatar	*Tomato*	Solanum lycopersicum	*Solanaceae*	+
10.	Bengan	*Egg plant*	Solanum melongena	*Solanaceae*	+
11.	Soybean	Soybean	Glycine max	*Fabaceae*	+
12.	Bhindi	*Okra*	Abelmoschus esculentus	*Malvaceae*	+
13.	Pyaj	Onion	Allium cepa	*Amaryllidaceae*	+
Weeds					
14.	Satyanashi	Mexican prickly poppy	*Argemone Mexicana*	*Papaveraceae*	+
15.	Bindii	Puncture vine weed	*Tribulus terrestris*	*Zygophyllaceae*	-
16.	Amaranthus	Amaranthus	*Amaranthus viridis*	*Amaranthaceae*	-
17.	Congress grass	Carrot grass	*Parthenium hysterophorus*	*Asteraceae*	+

+ = Infected, - = Uninfected

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