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INFECTION AND TRANSMISSION OF *CURVULARIA LUNATA* FROM SEED TO SEEDLING IN PEARL MILLET (*PENNISETUM TYPHOIDES*)

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ABSTRACT

Pearl millet (Pennisetum typhoides), also known as Bajra, is one of the four most important cereals (rice, maize, sorghum and millets) grown in tropical semi-arid regions of the world primarily in Africa and Asia. Our aim is to study the infection and transmission of Curvularia lunata (C. lunata) in pearl millet. The seed of pearl millet (Pennisetum typhoides) naturally infected with Curvularia lunata were black discolored. Out of 134 seed samples of local cultivar collected from 21 districts of Rajasthan ,106 carried 0.5% to 92.50% incidence of infected seeds which on incubation yielded 1-85% incidence of C. lunata. Histopathology of symptomatic seeds revealed infection of C. lunata confined to pericarp and aleurone layer near hilar end in seeds showing black discoloration near hilar region only and to all parts including endosperm and embryo in heavily blackened seeds. Asymptomatic (healthy looking) seeds also carried infection in pericarp and endosperm. In growth test, the hyphal inoculum produce in seed spread to seedling and caused loss in germination. Thus, we find that the embryonic infection in seed of pearl millet infected with Curvularia lunata caused pre- and post-emergence mortality and the pathogen is both externally and internally seed-borne. The internal inoculum affects the seed germination.

Keywords: *Pearl Millet, Curvularia Lunata, Seed Infection, Transmission.*

Introduction

Pearl millet (bulrush) commonly known as bajra *Pennisetum typhoides* (Burm.f.) Stapf and Hubb.Syn.*P.glaucum* (L.) R. Br.*P.americanum* (L.) K.Shum. (Ferraris 1973) is a member of family Gramineae, sub family Panicoideae. It is a major staple food crop for millions of people living in the arid and semi-arid tropics regions of the world primarily in Africa and Asia. It is well adapted to production systems characterized by low rainfall (200-600 mm), low soil fertility, and high temperature, and thus can be grown in areas where other cereal crops, such as wheat or maize, would not survive. The crop suffers from many serious fungal diseases which cause heavy losses. The major diseases observed in pearl millet were rust, ergot and smut (Girgi et al. 2006).

Seeds of pearl millet are of great economic interest and also contribute a major part of diet, they play a vital role in associating microorganism, which prove hazardous for the seed or the new plant created from it, so, any infections agent which is associated with seeds having potential of causing a disease in a seedling or plant, is seemed as seed- borne pathogen (Agarwal 1996). Seeds are regarded as highly effective means for transporting plant pathogens over long distances.

Seed-borne diseases have been found to affect the growth and productivity of crop plants. A seed-borne pathogen present externally or internally or associated with the seed as contaminant may cause seed rot, seed necrosis, reduction or elimination of germination capacity as well as seedling damage resulting in development of disease at later stages of plant growth.

Seed-borne mycoflora of pearl millet reported from different parts of the world include *Puccinia penniseti, Claviceps fusiformis, Pyricularia grisea, Cercospora fusimaculans, Sclerospora graminicola*, *Aspergillus flavus, A. fumigatus,*

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A. niger, Cladosporium spp., *Fusarium moniliforme, F. oxysporum, Drechslera tetramera, Rhizopus* spp. Seed-borne diseases have been found to affect the growth and productivity of crop plants.

Fungi form a major group of pathogens that can be seed-borne or transmitted through seeds. Commercially, discolored pearl millet seeds caused by fungi are of poor quality reducing their acceptability and thus, low market value of the produce. *Curvularia lunata* is a seed-borne pathogen (Richardson 1990) and caused various diseases as leaf spot, leaf blight, seed rot and ear blight (Gan and Dostaler 1990).On leaves it forms small yellowish brown spots at margin which later become necrotic. Similar dark brown spots which later become brownish black on rice were observed by Kamaluddeen (2013) and Akinbode (2010). Initially these spots were similar to the ones on the leaf but later covered the whole leaf Sheath. *Curvularia penniseti* also causes leaf spot disease (Chahal et al. 1994).

The disease is well known in Indian subcontinent and the fungus was found to be predominant in Rajasthan grown pearl millet seeds (Mathur et al. 1960).

The seed-borne nature of *Curvularia lunata* has also been reported in many other crops like sorghum (Wu and Wu 1977), sudan grass (El-zayat et al.1990), rice (Lakshmanan 1992; Pandey 2000) and maize (Zhang 1998). The incidence of the leaf blight disease *Xanthium strumarium (L*.) caused by *Curvularia lunata* and *Drechslera spicifera* in Sudan, could be considered as beneficial agents for the bio-control and to establish weed management program were reported by Nayla et al. 2015. Recently, Genome sequence and virulence variation-related transcriptome profiles of *Curvularia lunata*, as an important maize pathogenic fungus were reported by Gao et al. (2014). The aim of the study is to identify and detect seed-borne fungal pathogen and their association with yield. Scanty information is available on seed infection and its significance in transmission of the disease from seed to seedling therefore a detailed investigation has been carried out.

Materials and Methods

One hundred thirty samples of local cultivars of pearl millets were collected from farmers field of 21 districts of Rajasthan during the crop reasons 1998-2002.Four hundred seeds in three replicate from each samples were studied for dry seed examination under steriobinocular microscope (40X).Incidence of *Curvularia lunata* was determined by standared blotter method and potato dextrose agar method (ISTA 1996).Two seed samples ac.nos.15 and 18 carrying 37% and 52% infection of *Curvularia lunata* were selected for further studies on location and transmission of pathogen. The seeds of each sample were categorized as Asymtomatic (bold healthy looking) and symptomatic seeds. The symptomatic seeds were further classified as weakly and heavily infected on the basis of severity of seed symptoms.

Histopathology of Naturally Infected Seeds

To find the exact location of the pathogen on the seed and its further development is examined by histological techniques. The methods employed for histopathology are component plating, cleared wholemount preparation of seed components and microtome sectioning (Johansen 1940).

Component Plating

In Component plating 25 seeds/category/sample were washed and soaked in distilled water for 4 h. and different seed components via pericarp, aleurone layer, endosperm and embryo were dissected aseptically under stereo binocular microscope with the help of sterlized forceps and needle. Each component was surface sterlized with 2% available chlorine and tested by standard Blotter Method and Potato Dextrose Agar Method. The plates were incubated for 7 days under alternating day fluorescent tubes and darkness. Observations were taken after 7 days of incubation.

Cleared Wholemount Preparation

In Cleared Wholemount Preparation 10 seeds/category/sample infected with *C.lunata* were boiled individually in aqueous solution of 10% KOH for 5 minutes to clear the tissue. Aleurone layer and cotyledons were washed and again boiled for 5 min in lactophenol containing cotton blue (1:1, v/v) and mounted in poly vinyl alcohol (Omar et al., 1979). All the seed components were pressed gently under cover slip till the cells spread uniformly (Singh et al. 1977). The slides were then kept in an oven at 60º C for drying.

Microtome Sectioning

In Microtome sectioning 5 seeds/category/sample were soaked overnight in sterlized distilled water in an oven at 60º C until they soften. The seeds were fixed in 70% alcohol for 48 h in vials. For proper infiltrations the seeds were given 2-3 fine incisions and dehydrated through tertiary butyl alcohol (TBA) series, infiltrated and embedded in paraffin wax (BDH). The embedded materials were cut into the blocks.

Wax blocks were cut to expose the tissue and emerged in 1% aqueous solutions of sodium lauryl sulphate for 12 hrs (1:1, v/v) and then transferred to acetoglycerine (mixture of acetic acid + glycerol in 1:1 ratio) for 7 days for further softening. Blocks were cut through microtome at 15-20µ thickness, deparaffinised, stained with safranine and light green combinations and mounted in DPX (Johansen 1940).

Disease Transmission

The disease transmission studies were conducted using naturally infected seeds by water agar seedling symptom test and seed transplantation in Pot experiment. In Water Agar Seedling Symptom Test three replicates of 25 seeds/category/sample were sown in sterilized test tubes containing 10 ml of 1% water agar (1 seed/tube). The seeds were pretreated with 2% sodium hypochlorite for 2-3 min before sowing. The test tubes were incubated at 26±2°c under 12h of alternating cycles of light and darkness for 7 days. Observations were made at 24h interval upto 8th day. In pot experiment seeds were transplanted in 6-inch earthen pots containing sterilized soil to observe seed to seedling transmission of plant pathogenic species. In transmission studies of pathogenic *Curvularia lunata*., from seed to seedling were detected by the symptoms produced in seedlings within 2 weeks to 2 months. Percent seed germination, seed rot, seedling mortality and disease symptoms were recorded on 15th days.

Results

In dry seed examination black pin head discoloration of *C.lunata* covered the seed surface .Incubation of these symptomatic seeds yielded pure growth of the pathogen (Fig.1a, b).Seeds infected with *Curvularia lunata* revealed black discoloration, out of 134 seed samples, 106 samples contained black discoloration (Fig.1a ,b). The percentage of black discolored seeds varied from (0.5 to 92.50%). *C. lunata* was isolated in ac.nos.15 and 18 samples in untreated, pretreated seeds in SBM and PDA respectively. The incidence of fungus varied from 1-85% in untreated seeds, 0.5- 77% in pretreated seeds and 3-88% in PDA tests.

Component Plating

In component plating both asymptomatic as well as symptomatic weakly infected seeds we could easily separate various components of the pearl millet seed viz. pericarp, aleurone layer, endosperm and embryo. While various components of heavily infected seeds could not be separated easily. Similar methods were also worn by Sharfun et al. (2005) in sunflower and Nupur and Rashmi (2014) in sponge gourd (*Luffa cylindrica* l. roem) for the detection of pathogen in seeds.

In asymptomatic seeds the infection percentage of *C. lunata* was recorded in pericarp (16-20 %) and endosperm (4- 6%) of two samples ac.nos.15 and 18. Mycelia growth of fungus was not observed in aleurone layer and embryo.

In symptomatic seeds, fungal growth was recorded in all parts of seed viz. pericarp (56, 64%), aleurone layer (50, 60%), endosperm (48, 56%) and embryo (68, 72%) of weakly infected seeds (Table- 1).

In heavily infected seed all the component of seeds pericarp and embryo revealed maximum infection (Fig.1c, d). The incidence was 96-100% in pericarp, 92, 96% in aleuronic layer, 88, 90% in endosperm and 100% in embryo (Table- 1). From result mentioned in Table-1 and Figure-1 showed that *C. lunata* which is an important pathogen of pearl millet was isolated from various parts of seeds during component plating, indicating its systemic nature.

Cleared whole Mount

In cleared whole mount preparation of the seed component pericarp, aleurone layer, endosperm and embryo showed presence of conspicuous thick, dark brown, knotty and septate mycelium of *C. lunata* (Fig.1e, f).

In asymptomatic seeds of two samples mycelium was seen only in pericarp 10, 20 %, aleurone layer 0, 10% and embryo 0, 10%. In weakly infected seeds infection was occurred in all components viz. pericarp 40, 50%, aleurone layer 40, 40%, endosperm 30, 40% and embryo 30, 40% (Table-1). In heavily infected seeds, the mycelia infection in different components of seeds varied from 80-100%. Thick, dark brown, septate and branched hyphae densley ramified in the pericarp (Fig.1f, 2b) hilar region appeared dark black due to infection of (Fig. 1e) inter as well as intracellular hyphae (Fig.2a).

The cells of aleurone and endosperm were heavily colonized showing brown to black necrotic areas (Fig.2c, d). The cells of endosperm become fragile, their walls were not intact and showed signs of disintegration (Fig.2e, f). In embryo hyphae occurred in scutellum and upper few layers or tip of plumule (Fig.2g).

Microtome Sectioning

In asymptomatic seeds, the hyphae were only localized in pericarp (Fig.3a). In symptomatic weakly infected seeds, the hyphae was mainly seen in pericarp and endosperm. The cells of aleurone were irregularly elongated and deformed (Fig.3c). The layers of pericarp showed hyphal bits and clumps of inter-as well as intracellular mycelium (Fig.3b). The cells showed depletion in food contents (Fig.3d, e). In heavily infected seeds, mycelium was observed in all parts. Cells were filled with clump of dark brown, septate and thick mycelium (4a). The cells of endosperm become fragile. The cells contained a few or no starch grains and dark brown mycelium (4b). A Lysogenous cavity and mycelia bits around the cavity was observed in few sections (4c). The embryo was reduced in size and the aggregation of mycelium in embryo was observed in plumule and radical (4d). The hyphae probably from hilar region invaded the scutellum. The cells of epithelial layer were elongated (4e, f). The fungal infection caused necrosis and lytic cavities occupied by the dense fungal growth in the mesophyll cell of scutellum. In heavily infected seeds, endosperm and embryonal cells were densely colonized by fungal mycelium.

Transmission

Ungerminated seeds (asymptomatic and symptomatic category) showed dense growth of *C. lunata*. In heavily infected seeds the fungus caused rotting and blackening of radical base of the shoot and leaves (5a, b).

Test Tube

In water agar seedling symptom test, the germination was affected in symptomatic weak and heavily infected seeds of ac.nos.15 (78, 76%) and 18 (58, 56%) as compare to asymptomatic seeds (98, 96%). The percent incidence of pathogen was high in symptomatic seeds as compared to asymptomatic (Table-1).

In asymptomatic seeds, percentage of diseased seedling and their mortality was 4, 3% and 3,3%. In weak and heavily infected symptomatic seeds, it varied from 12-23% and 14-28% in two samples respectively (Table-1).

The pathogens may colonize the growing roots and cause rotting of germinating seeds (Fig.-5c).

Pot Experiment

Symptom started appearing on 6th day. Infected seedling showed dark brown streaks and spots on leaf, leaf sheath and stem (Fig.5d). Initially light brown eye shaped spots appeared on leaf surface which fused at maturity and formed necrotic areas (5e, f).

To know the seeds having growth of *C.lunata* were transferred into pots directly and it was observed that after 6^{th} days of seed sowing, the symptom started appearing in pearl millet. It was observed that *C.lunata* shows pathogenic reaction. Infected seedling showed dark brown streaks and spots on leaf, leaf sheath and stem. Initially light brown eye shaped spots appeared on leaf surface which fused at maturity and formed necrotic areas (5e, f).

Discussion

Pearl millet ((*Pennisetum typhoides*) (Burm.f.) stapf and Hubb is an important cereal crop cultivated throughout the world including India. A number of methods have been followed to determine seed borne mycoflora of pearl millet. Konde et al. (1980) used blotter paper and agar plate method for isolation of fungi.

Seeds of pearl millet are known to be associated with a large no. of fungi. In present study seed samples contained symptomatic seeds with various disorders like seed with brown and black discoloration and shriveled seed revealed presence of *C. lunata*.*C. lunata* is the major pathogen of the pearl millet showing 4-78% incidence. The study has proved the accurate determination of incidence and spread of inoculum in seed. Component plating and incubation were used for incidence but wholemount cleared preparation gave priliminary detection of pathogen. Detail study about location and distribution of fungal mycelium in seed can be possible only from microtome sectioning of naturally infected seed. Damages of seeds, such as seed death, seedling and plant abnormalities or decreased seed vigor caused by seed-borne pathogens are not always recognized by users. Once harmful fungi, pathogenic as well as toxigenic, have been listed, it is important to define for each of them the methods to be used for their detection and identification.

The present study has revealed that inoculum of C. lunata occurs in asymptomatic as well as symptomatic seeds of pearl millet. However, the incidence was low and infection was confined to the pericarp in asymptomatic seeds. But in symptomatic (weakly and heavily) infected seeds, pathogen was

present in all components of seed. The infection and distribution of the pathogen was correlated with degree of symptoms. Symptomatic seeds showed septate, branched, dark coloured inter as well as intracellular mycelium.

On incubation 36 fungal species belonging to 17 genera were found to be pathogenic. *C. lunata* was the most dominating fungus. Konda et a. (1980) observed 23 species of fungi belonging to 12 genera by blotter paper and agar plate method. The pretreatment seed has enabled to record the pathogenic fungi easily due to elimination of saprophytic fungi. *C.lunata*. is a seed-borne pathogen causing leaf spot and leaf blight disease.

In present study, high incidence of *C. lunata*. was observed in seed samples of pearl millet of Rajasthan. Infected seeds showed dark black discoloration. Similar discoloration of pearl millet due to *C. lunata*. was earlier reported by Bhatnagar (1971).

In component plating the mycelium of *C. lunata* was mostly confined to pericarp tissues of both asymptomatic and symptomatic seeds. The black discolored seeds of symptomatic category carried heavy infection in all parts viz. pericarp, aleuron layer, endosperm and embryo.

In component plating, the percent infection of *Curvularia lunata* ranged from 56-100% in pericarp, 50-96% in aleurone layer, 48-90%in endosperm and 68 to 100 % in embryo of symptomatic seeds of both the samples. Among all the seed components, pericarp and embryo revealed maximum infection, fungal growth and sporulation. Similar observations have also been made by Pandey et al. (2000) in rice seeds. They reported mycelium of *Cochliobolous lunatus* in all the components of rice seed. In healthy looking seeds, infection was recorded only in pericarp (16-20 %) and endosperm (4-6%) .

The seed transmission study of pathogen by component plating technique appeared helpful in detecting the deep seated seed infection. Pericarp may be removed or cleaned properly if infection is superficial. However, in case of deep-seated fungal infection especially those producing mycotoxins, seed lots must be rejected and destroyed. This technique of ISTA is also helpful in selecting healthy seed lots for raising new plants.

The cleared whole mount preparation of pericarp, aleuronic layer, endosperm and embryo showed a graded decline in invasion of hyphae from peripheral region to inner parts of seed. In asymptomatic seeds, mycelia infection was recorded mostly in pericarp, whereas in symptomatic seeds thick, dark brown, knotty, septate mycelium was seen in all the components of seed.

The cleared wholemount preparations of infected seedling showed presence of inter- and intracellular branched and septate mycelium of *C.lunata* in leaf, stem and radicle. In leaf mycelium mostly traversed along the veins. The present investigation reveals that the pathogen causes black discolouration on seed surface. Presence of inter- and intracellular mycelium suggests its internally seed-borne nature. Extent of infection varied with the degree of seed discolouration. Dwivedi (1993) reported mycelium of *C.lunata* mostly confined to seed coat and rarely in endosperm. Dense mycelia aggregation in hilar region indicates spread of pathogen from hilar region to embryo. Rastogi et al. (1990) reported extra- and intra embryonal infection of *C.lunata* in naturally infected seeds of sorghum which resulted lysis of cell wall of various seed tissues and deformation of seed parts.

All the components of heavily infected seed showed the presence of thick septate and knotty mycelium. Heavy incidence of the pathogen leads partial to complete disintegration of parenchymatous cells. Due to pressure of dense mycelium stressed parenchymatous cells either disintegrate or their lysis occurs. Mycelium was also observed in hilar region.

The microtome sections have clearly revealed various degree of mycelia infection in categorized seeds. In asymptomatic seeds, the hyphae were mostly recorded in pericarp near the hilar region. In symptomatic seeds, mycelium invaded all the components. In heavily infected seed the pathogen caused partial to complete disintegration of pericarp and the cells of aleurone. Lysogenous cavity and mycelia bits found around the lytic cavity. Similar observation has been reported by Rastogi et al. (1990). In present study, hyphal invasion through hilar region to scutellum and endosperm to scutellum confirmed, which result in necrosis of tissues.

Microtome sections revealed the exact distribution of fungal mycelium in different seed components. In symptomatic seeds, the hypae mostly invaded the pericarp near the hilar region. In symptomatic weakly infected seeds, it was seen in pericarp and endosperm. The different layers of pericarp showed hyphal bits and clumps of inter-as well as intracellular mycelium. The cells of aleurone layer were irregular elongated and deformed. In endosperm, the hyphae ramified densely in peripheral cells along their walls as well as intracellularly. The cells showed depletion in food contents. In

symptomatic heavily infected seeds, mycelium was observed in all part. In pericarp, the different zones viz. epicarp, mesocarp and endocarp were indistinguishable and filled with aggregation or clump of thick dark brown and septated mycelium.

Similar observation were reported by Nupur et al. (2014) in seeds of spongy gourd (*Luffa cylindrica* L. roem).They observed the infection of *R.solani* in different seed components of (*Luffa cylindrical* L.Roem). They reported presence of thick and knotty mycelium of *Rhizoctonia solani* which leads to disintegration and undifferentiation of outer epidermal cells of cotyledons in heavily infected seeds.

The present investigation has revealed that inoculum of *C.lunata* occurs in asymptomatic as well as symptomatic seeds of pearl millet .Though the incidence was low and infection was confined to the seed coat in asymptomatic seeds. But in symptomatic infected seeds, pathogen was present in all components of seed. The infection and distribution of the pathogen was associated with degree of symptoms. The study has proved the accurate determination of incidence and spread of inoculum in seed which lead to several histological changes in the infected tissue of pearl millet.

Phytopathological Effect

The disease transmission studies showed that the seed infected with *C. lunata* resulted in failure of seed germination and the incidence of seedling mortality are correlated with the degree and nature of infection. Similar observation in Sorghum seeds were reported by Rastogi (1984).

As the incubation Period prolonged brown spots and streaks become prominent and merged to form uniform brown discoloration which progressed towards root and shoot tips. In pot experiment, the rate of seedling mortality gradually increased. Thus, the present study indicates that there is extra embryonal infection in asymptomatic seeds which is mostly confined to the outer seed coat layers. In symptomatic seeds the infection is intra embryonal and deep seated and distributed in all components of seed.

Histopathological evidence that presence of pathogen in seedling raised from naturally infected seeds under aseptic condition suggests systemic transfer of the disease at early seedling stage. During seed germination, the hyphae present in pericarp spreads inter-as well as intracellularly to coleoptiles and other emerging leaves and caused necrosis of cells. The study has proved the accurate determination of incidence and spread of inoculum from seed to seedling. Component plating and incubation were used for incidence but wholemount cleared preparation gave priliminary detection of pathogen. Detail study about location and distribution of fungal mycelium in seed can be possible only from microtome sectioning of infected seed. Disease transmission studies showed that the seed infected with *C. lunata* gave poor germination and seedling mortality.

So, from the present investigation we can conclude that the embryonic infection in seed of pearl millet infected with *Curvularia lunata* caused pre- and post emergence mortality and the pathogen is both externally and internally seed-borne. The finding include isolation of *Curvularia lunata* from pearl millet seeds and infected part of the plants for further transmission studies. To confirms its seed-borne nature and presence from seed to seedling histopathological methods were used. The work presented here is the thorough report concerning its seed- borne nature. The internal inoculum affects the seed germination and cause necrosis of cells. The presence of mycelium in pericarp to embryonal region confirms its seed-borne nature.

Further studies are needed to determine the impact of *Curvularia lunata* on pearl millet and their control measures and also the investigation into molecular identification and pathogenicity tests of specific fungi on pearl millet and their biocontrol.

Conflict of Interest: No

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