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# RESEARCH ARTICLE

## OBSERVATION OF HISTOPATHOLOGICAL CHANGES IN LEAVES OF *PENNISETUM GLAUCUM* (PEARL MILLET) INFECTED WITH *PUCCINIA SUBSTRIATA* VAR. *PENNICILARIAE*

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## ABSTRACT

My work is to study the life cycle of rust fungi in pearl millet. Histopathology is the study of the structural manifestations of diseases at the light microscopic level and it is the microscopic examination of biological tissues to observe the appearance of diseased cells and tissues in very fine detail. The study describes an improved method for fixation of sampling of fungal infected plant parts, staining and observation of fungal infections in plant tissue for histopathological visualization. During the study it was observed that in particular technologies such as stereo microscopy and compound microscopy have enhanced our ability to visualize hyphae in the plant tissue. We have done germination test to determine the percent germination and vigor index of seeds. Hemocytometer count has been done to determine the number of spores per ml which is used to spray on the young plantlets by brush method. Micrometry is also done to measure the dimensions of the rust spores. In order to study the host and pathogen interactions I have done maceration technique, and observed the entry of pathogen by the formation of haustoria.And also we noticed that a single species of rust fungi may be able infect two different host plants in different stages of its life cycle, and may produce up to five morphologically and cytologically distinct spore producing structures.

***Key words:*** *Pennisetum glaucum,* Histopathology, Haustoria, Puccinia substriata, Germination test.



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## INTRODUCTION

*Puccinia*, the largest genus, consists of over 4,000 species and is characterized by the production of pedicellate, bi-celled teliospores. An estimated 168 rust genera and approximately 7000 species, more than half of which belong to the genus *Puccinia*, are currently accepted. Rust fungi are highly specialized plant pathogens with several unique features. Taken as a group, rust fungi are diverse and affect many kinds of plants. However, each species has a very narrow range of hosts and cannot be transmitted to non-host plants. In addition, most rust fungi cannot easily be grown in pure culture. *Pucciniasubstriata var. penicillariae* is characterized by appearance of small reddish-brown orange, round to elliptical uredinia on the foliage with increased rust infection; leaf tissue gets dried and becomes necrotic from the leaf apex to base. In later stages, uredinia are replaced by telia which are black elliptical and sub-epidermal. Gold and Mendgen, 1991 cleared that how the haustoria will produce by the spore to enter the host tissues. Haustoria may be produced by mono or dikaryotic mycelium of rust fungi. Monokaryotichaustoria merely appear as intracellular extensions of intercellular hyphae with no significant morphological specialization.

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Heath and Skalamera,1997, these two Pathologists confirmed that always dikaryotichaustoria develop from external haustorial mother cells (HMC) with a slender neck that penetrates into the host cell and a haustorial body that forms distally to the neck. The HMC therefore functionally resembles an appressorium.Rust fungi has been divided into three categories depending upon the "Number of spores states in their life cycle'', Macrocyclic, demi cyclic, and micro cyclic (Cummins and Hiratsuka 2003). A small green to yellow slightly raised spots develop over the leaf surface, with more on the topside than the outer side. As the spots grow larger, up to 2mm, and rise further from the surface of the leaf, they develop into reddish-orange pustules, round to elliptical, with yellow halos. Masses of spores (Urediniospores) form in the pustules and spread the rust within and between crops. Later, as the pustules age they become darker as another spore type (teliospores) appears; the leaves start to die from the tips towards the base. If the disease is severe, spots occur on the stems and the plants fall over (lodge). The teliospores have thick walls and this helps them survive in the soil.

## MATERIALS AND METHODS

**Collection of seeds:-**I brought *Pennisetumglaucum*cobs of variety-1(NHN-9) from the field and separated into seeds, from biotechnology department, Manasagangotri campus,

University of Mysore. One more variety that is variety-2(BNH-3) bought from Devraj market, Mysore. (Fig 1.0: a and b).

**Calculate the germination percentage and vigor index of *Pennisetumglaucum*(L.):** The germination percentage and vigor index of *Pennisetumglaucum* was done by paper towel method (Abdul Baki *et al*., 1973) (Fig.1.1: a). *Pennisetumglaucum* cobs of variety-1(NHN-9) collected from the field of biotechnology department, University of Mysore, Manasagangotri and variety 2(BNH-3) from Devaraj market Mysore were placed on wet germination sheets to study the germination percentage and vigor of the seeds. Both were calculated using the formula,

$$\text{Germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds plated}} \times 100$$

**Collection of infected plant material:** Collection of plant material, leaves showing symptoms of rust disease were collected from the filed directly. From the same field in the campus I collected the already rust disease infected leaves in a polythene bag and kept for storage in refrigerator. (Fig 2.0: a and b)

**Micrometry experiment**: -By this, I measured the dimensions of the rust spores using the technique using microscope (Fig 2.1: a and b). In order to measure the dimensions of common microorganisms by calibration and standardization of microscope using stage micrometer and ocular micrometer. At 40X, one ocular division gives 2. 5micrometer.After calculating calibration factor, the length and breadth of teliospores calculated that is 28.75micrometer and 26.25micrometer respectively. Similarly, dimensions of Aeciospores also calculated and observed that the length and breadth of spores that is 41.5micrometer and 21.25micrometer. Total number of spores calculated by the following formula: -

**Spore count =spores per ml= (n) X $10^4$**

Where, n – Average cell count per square of 4 corners square counted.

$$= 159 \times 10^4$$

Therefore, spore count = 15,90,000 per ml.

**Isolation and identification of Pathogen from *Pennisetumglaucum* samples:** Leaves showing symptoms of rust disease were collected from Pearl millet field Biotechnology department, University of Mysore. The pearl millet seeds of variety MP-4010 were collected from public and private seed agencies of Mysore district. Screening Isolation and identification of the Pathogen *Pucciniasubstriata* var. *penicilariae* of field looking diseased irrespective of plant growth stages were collected from pearl millet field from the campus itself. The infected leaves were thoroughly washed with running tap water and then, immediately examined under a compound microscope for preliminary investigation of the pathogen. Morphological characters of the fungus were studied by observing under compound microscope. The morphological characters of uredinia, urediniospores, telia, teliospores, spermogonia, spermatia (pycniospores) and aecia, aeciospores etc. On the basis of morphological characters, the causal fungus was identified as *Puccinia substrata* var. *penicilariae* (Fig 3.0: b). The pathogen was isolated from the infected *Pennisetumglaucum* sample by standard blotter method. The collected plant material was cut into small pieces and washed in distilled water for 2 to 3 times and then soaked in 2% sodium hypochlorite solution for two minutes for surface sterilization. After sterilization the materials were washed in distilled water 2 to 3 times repeatedly. The washed materials were placed in 9cm diameter plastic Petri plates on three layers of moist blotters and incubated at 24$^o$C for 12h in alternating cycle of light and darkness for 7 days (ISTA 1996).

**Maintaining of pathogen:** Incubation for seven days at 22 ± 2° C. After incubation, the plates were thoroughly examined under stereo-binocular microscope and screened for the incidence of *Pucciniasubstriata* var. *penicilarae* and were identified based on the morphological, conidial, fruiting bodies and culture characters based on the standard procedures. The pathogen was not seen in the media as it is an obligate parasite. We didn't get any pathogen in media (Fig 3.0: a)

**SPORE GERMINATION ASSAY: (Fig 4.0: a and b):** Spore germination study was carried out by "Hanging Drop Technique". Hanging drop or wet preparations permit examination of fungi in normal living conditions. The suspension of exudates and fungal spores (200–300) from 10-day old cultures were prepared to study the germination of fungal spores. All the tested fungi were individually picked up with a sterile inoculation needle and mixed in the exudates. For the each experiment the suspension of fungal spores was placed on a cover slip, and then inverted over the concave depression of the slide to produce a hanging drop. The slides were then placed in moist chambers prepared by placing two moist filter papers in the inner surfaces of a Petri plate. Pimpalgaonkar and Chandel,2014 took three replications which were made for each suspension fungal spores. Spores mixed in distilled water without leaf exudates served as the control. The slides were incubated at 25+2°C for 24 hours and then fixed with cotton blue in lactophenol. Germination was observed under a light microscope. All the experiments were conducted in triplicate for each test fungus. In order to observe the spore germination in normal water and 0.1sucrose solution. I prepared 0.1M sucrose, simply mix 0.1 moles of sucrose, which is equivalent to 34.2 grams, with enough deionized water to make 1 liter of solution. With a bit of preparation, you can prepare the solution using proper laboratory methods. For the above experiment we prepared, 0.1M sucrose solution by dissolving 0.342 grams of sucrose in 10 ml of deionized water.

**Raising of seedlings:** Seedlings of bajra were raised in plastic pots of 10-inch diameter at rate of 5 seedlings per plot in the botanical garden, DOS in Botany, UOM.The plastic pots were filled with coco peat and soil in the ratio 1:1. (Anjum *et al*., 2013) Fig 5.0: a, b, c and d)

**Preparation of fungal inoculums:** The rust infected leaves (rust inoculum) brought from the artificially infected plant raised in green house were used to inoculate pearl millet plants. The rust urediniospore separated with the help of razor blade from the infected leaves kept under water. The infected leaves were also rubbed to separate the spores and collected in the sterilized water. The entire scrapping of leaves was thoroughly mixed in the bowl, and then drained through double layered muslin cloth. The pearl millet plants were inoculated by spraying urediniospores with the help of hand sprayer in the evening. (Fig 6.0: a, b and c)

a. Bajra cobs

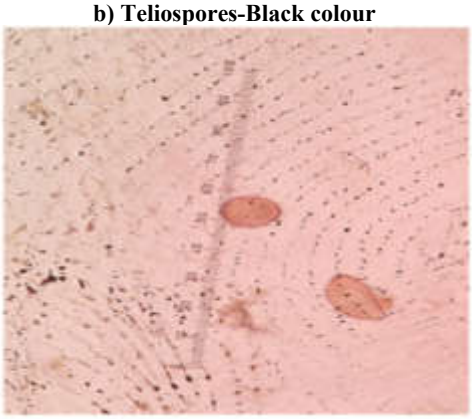
b. separated seeds from cob

Fig 1.1. a. Paper towel method, observation after 7 days

a) Rust pustules on leaf lamina (Uredospore's)

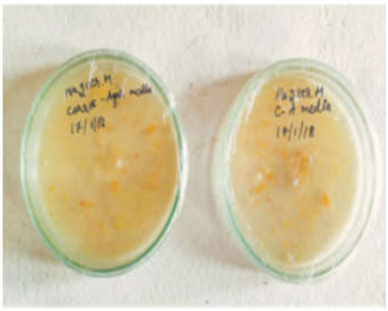
b) Teliospores-Black colour

a)Length of Uredenospores at 40X



b) Breadth of Uredenospores at 40X

Fig. 2.1:( a-b) Micrometry to study the dimensions of spores



a. Carrot agar media for pathogen culture

b. Uredospore's at 10X

**a and b Spore germination by germ tube formation at 40X**

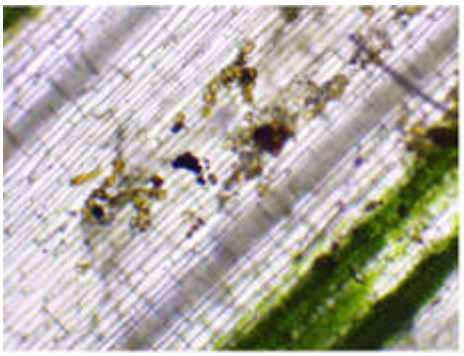
**Fig.5.0. Raising of seedlings a)4 days seedlings b) Variety-1(BNH-3) and variety-2(NHN-9) seedlings c)12days seedlings and d)30 days seedlings**

**Incubation of seedlings (Rust seen on leaves) After 12days of pathogen inoculation rust disease completely Spread on leaves and plantlets becomes wilt. a) and b) 12-day seedlings c) wilt plantlet due to rust**

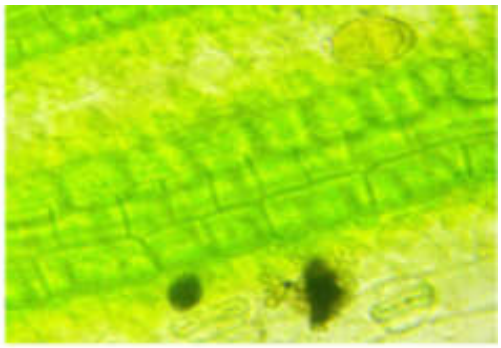
**7.0: a) Inoculation of pathogen by brush method**

**7.1: a)30 days seedlings kept for incubation**

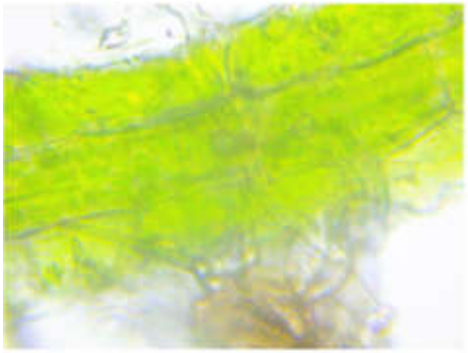
**a) Entry of spores inside leaf lamina while incubation**



**b) Macerated leaf tissues under 40X**



**c) Macerated leaf tissues under 40X**



**d) Hyphal growth in leaf tissues under 40X**

The constant humidity was maintained in the inoculated pearl millet pot giving irrigation. The rust was well established near greenhouse. The rust culture raised on pearl millet was utilized for creating artificial inoculation in the field trials. The rust infected leaves were collected from artificial infected rust plot near green house of Main Pearl Millet, biotechnology department, University of Mysore. These leaves were kept under muslin cloth for preventing drying. They were kept in the refrigerator at 4±1ºC till the inoculation made in the field. In the field trials, artificial inoculated condition was created by preparing fresh urediniospores suspension in water and spraying usually done during evening hours. The suspension was sprayed using "brush method" sprayer. The urediniospores used for inoculum were prepared from culture maintained near the greenhouse. The inoculum was prepared by separating the urediniospores from uredinia on leaves by scraping and suspending them in distilled sterilized water so as to have approximately 4 x $10^6$urediniospores per ml of water. The suspended urediniospores were filtered through muslin cloth. The spore concentration was adjusted by using a hemocytometer.

The plants were inoculated by spraying urediniospore inoculum with the help of an atomizer after 15 day of sowing and covered with polyethylene bags for 48 hours. The temperature was maintained at 20 to 25ºC and 70 to 80 percent relative humidity. Simultaneously the urediniospores were also inoculated on pearl millet plants sown near green house for production of teliospores. The inoculation was done in the evening.

**Inoculation of pathogen to the leaves of healthy seedlings:** Fifteen days (15) old and one-month old seedlings were dipped in spore suspension (1X106) and incubated for different intervals viz, 2, 4, 6, 8, 12, and 24 hours under darkness at 24±10 C. as the Puccinia pathogen invades through leaves to cause rust disease. After 12days of pathogen inoculation rust disease completely spreaded on leaves and plantlets becomes wilt. (Fig 7.0: a)

**Incubation of seedlings:** Pathogenicity of *P.substriata var. Penicilariae* on pearl millet. To study pathogenicity and actual symptoms produced by the pathogen *P. substriata var.*

*Penicilariae*, urediniospores were inoculated on pearl millet. Plants of pearl millet varieties like BNH-3 and NHN-9 were raised in the sterilized soil, keeping ten plants in each pot in green house. The plants were inoculated by spraying urediniospores suspension with the help of an atomizer after 30 days of sowing. The pots were covered with polyethylene bag for 48 hours (Fig 7.1: a). The temperature was maintained at 20 to 25ºC and 70 to 80 per cent relative humidity. The observations on the occurrence of the disease were taken daily after inoculation. Observations on the major symptoms produced as a result of infection on plants were recorded. The uredospores were re-examined under microscope and re-inoculated on pearl millet plant.

**Infection of plant with the inoculum:**Maceration technique for observing infected cells The inoculated seedlings were removed from the spore suspension, fixed in acetic acid (1:3) and processed further for histological observations the fixed seedlings were partially macerated in 3% (w/v) sodium hydroxide for 30 minutes at 600c and thoroughly washed in running water for 30 minutes to remove sodium hydroxide. The washed seedlings were transferred to 0.2% (w/v), warm cotton blue and stained for two hours (M.S. Sharada *et al*., 1995). (Fig 8.0: a, b, c and d)

## RESULTS

I visited Pearl millet field in biotechnology department green house, Manasagangotri, University of Mysore and collected the diseased leaf sample showing symptoms. For isolation and identification of pathogen, it was inoculated into Carrot-agar media, but as it is an obligate parasite which cannot grow without host plants so, I got negative result. Using 0.1M sucrose solution by Hanging drop method I observed the germination of spores at different time intervals, similarly using distilled water also I have observed at time intervals like 3,6,12,24,36,48,96hours and complete germination of *Puccinia* spores seen by the formation of germ tube. I calculated the percentage seed germination which is 80% and Vigor index = 938.84. The dimensions of fungal spore were calculated using Micrometry, then we got the Size of Teliospores, Length =28.75micrometer and Breadth =26. 25micrometer.Similarly, the Size of Aeciospores, Length= 41.5micrometer and Breadth=21. 25micrometer.Then, using hemocytometer the spores present in 1ml of suspension is calculated which was 15,90,000 per ml. The seedlings raised in plastic cups filled with soil and coco peat, developed leaves in 15$^{th}$ days. The leaves were used for histological studies. Then inoculums containing $10^4$ spores per ml was sprayed to the seedlings of 15 days old and incubated for different time interval 2,4,6,8,12,24,36,48 and 72 hours. The inoculated leaves of seedlings were removed at different time intervals as mentioned above and were partially macerated using the technique. Observation has been done to see the interaction of pathogen spores by germination on the host plant body.

**Culture, Isolation and identification of the pathogen: Fig 3.0: a and b:** The diseased plant material pathogen was inoculated onto Carrot-Agar media, but it was not grown as it is an obligate parasite which cannot grow without host plants got negative result.

**Spore germination assay: Fig 4: a and b:** After 96 hours spores started to germinate having germ tube.

**Raising of seedlings:** The seedlings raised in plastic cups filled with soil and coco peat, developed leaves in 15$^{th}$ days. The leaves were used for histological studies (Fig.5).

**Preparation of fungal inoculums: Fig 6.0: a, b and c:** The fresh inoculums of *P. substriata* var. *penicilariae* spores was obtained as per the procedure (Fig.6).

**Incubation of seedlings: Fig 7.0: a and Fig 7.1: a:** The inoculums containing $10^4$ spores per ml was sprayed to the seedlings of 15 days old and incubated for different time interval 2,4,6,8,12,24,36,48 and 72 hours.

**Maceration technique for observing infected cells: Fig:8.0: a, b, c and d:** The inoculated leaves of seedlings were removed at different time intervals as mentioned above and were partially macerated using the technique. Observation has been done to see the interaction of pathogen spores by germination on the host plant body.

## DISCUSSION

My work is to study the life cycle of rust fungi in pearl millet. For this I collected the rust spores from the infected leaves of pearl millet from the field. Then I kept in the refrigerator in order to store for the long time. While conducting the germination test of the spores at different time intervals like 1,3, 6,12,24,48,72 and 96 hours respectively in both distilled water and 0.1M of sucrose solution. Finally at 96 hours,I observed that the spores started to produce the hyphae with germtube. Many work has been done on this plant but not with the variety *Pucciniasubstriata*var. *penicillariae*instead they work on variety Indica have done germination test to determine the percent germination and vigor index of seeds. Haemocytometer count has been done to determine the number of spores per ml which is used to spray on the young plantlets by brush method. Micrometry is also done to measure the dimensions of the rust spores. In order to study the host and pathogen interactions I have done maceration technique, and observed the entry of pathogen by the formation of haustoria. Singh and Sokhi (1983) reported that rust reduced the average number of panicles per plant, grain yield per plant and 1000 grain weight in both slow rusting and fast rusting cultivars. The reductions were more in fast rusting cultivars than in slow rusting ones. Different fungal pathogens need different incubation period for the maximum spore germination. In the present studies, the germination of uredospore started after 96 hours. Response of different botanicals varied with rust of pearl millet, which might have happened due to several reasons, such as uneven distribution of inoculum in natural infections, physiological differences in their sensitivity to toxic materials and availability of less or varied number of active spores of rust. Rust infection of pearl millet forage has been reported to cause up to 51 % reduction in digestible dry matter yield (Monson *et al*., 1986). Rust caused by the fungus *Pucciniasubstriata var. indica* is one of the major diseases affecting both forage and grain production in pearl millet. Rust has been observed throughout India. All growth stages of the plant are susceptible to rust attack, and under favorable environment, plants can wither before flowering due to severe rust infection (Ramakrishnan and Sundaram 1956).

### Conclusion

Rust is one of the major foliar diseases of *Pennisitumglaucum* (L.) R.Br.(pearl millet) which is caused by a fungal pathogen

*Pucciniasubstriata var. penicillariae*in India and other parts of the world. Its impacts on grain yield reduction depends upon its severity and stages of crop growth. In fodder crops, it affects the taste and thus palatability. As pearl millet is the most important drought and heat tolerant crop, which occupies fourth place in cereals and second place among coarse cereals it must be free from the rust and other diseases. Inspite of its destructive nature not much work with respect to different aspects has been carried out. Here, I studied the histopathological changes in the leaves of bajra infected by the rust pathogen.

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