INOCULATION METHODS AND DISEASE RATING SCALES FOR MAIZE DISEASES

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PREFACE

The maize improvement programme heavily depends on identifying genotypes with resistance to biotic and abiotic stresses. The successful phenotyping for diseases resistance is dependent on screening maize genotypes under artificially inoculated condition, following scientifically accepted methodology. The present publication provides step by step methodology for artificial inoculation and disease rating scales for the major maize diseases of India. This will be of immense use to research workers, students and maize scientists interested in identifying resistant genotypes to various diseases.

I congratulate the authors for bringing out this publication.

The support and encouragement received from Indian Council of Agricultural Research is gratefully acknowledged.

29/3/12

(R. Sai Kumar)
Project Director

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INOCULATION TECHNIQUES AND DISEASE RATING SCALES FOR MAJOR DISEASES OF MAIZE

In India Maize is prone to a number of biotic stresses like foliar diseases, ear rot, and stalk rots caused by fungi and bacteria. Under favorable environmental conditions, these pathogens are capable of causing severe losses and deteriorate the quality of the produce. Though, Chemical control measures for some of the diseases are effective in reducing losses yet their use is limited by the high cost involved and residual toxicity they leave in the food chain. However, for minimizing the losses due to diseases and simultaneously increasing the production to meet the burgeoning demand, it is necessary to introgress an adequate level of genetic resistance against maize diseases of economic importance. Thus, the evaluation of maize germplasm against various important maize diseases is an integral part of all maize breeding programs, where selection for resistance to these diseases will lead to yield stability. Locations with high levels of natural infection are often referred to as “hotspots”, and can be used effectively to identify disease resistance and promising germplasm in a breeding program. These hot spot locations have been identified for important maize diseases. However, natural infection will be variable from year to year under the influence of climatic conditions and crop management practices. The use of artificial inoculation methods can provide more uniformity in the evaluation process and allow for the elimination of highly susceptible genotypes from the breeding program. These methods can be utilized at all stages of the selection process, and can effectively assist in the selection of more resistant genotypes frequently before flowering.

The techniques for preparing of inoculums for various maize diseases and field inoculation methods/techniques and disease rating scales are described here for maize workers, which can be carried out by anyone who are interested in selection for resistance. Breeders and pathologists can join their hands to run the program successfully.

1. Foliar Diseases

1.1 Maydis Leaf Blight (MLB) *Helminthsporium maydis* (*Cochilobolus heterostrophus)* and Turcicum leaf blight (TLB) *Exserohilum turcicum* (*Setosphaeria turcica)*

1.1.1 Distribution of Maydis Leaf Blight (MLB) *Helminthsporium maydis* (*Cochilobolus heterostrophus)* - Jammu & Kashmir, HP, Sikkim, Meghalaya, Punjab, Haryana, Rajasthan, Delhi, UP, Bihar, MP, Gujrat, Maharashtra, Andhra Pradesh, Karnataka, Tamil Nadu. Disease is prevalent in warm humid temperate to tropical regions where the temperature ranges from 20-30^{0}C during cropping period. (Fig. 1)

1.1.2 Distribution of Turcicum leaf blight (TLB) *Exserohilum turcicum* (*Setosphaeria turcica)* - Jammu & Kashmir, H.P., Sikkim, W.B., Meghalaya, Tripura, Assam, U.P,

Fig. 1 Plant susceptible to MLB

Uttara khand,Bihar, M.P., Gujrat, Karnataka, Tamil Nadu**.** Prevalent in areas where cool (18-27^{0}C) /humid condition prevails and maize is planted in temperate and high lands. (Fig. 2)

Fig. 2 Plant susceptible to TLB

1.1.3 Techniques for inoclulum preparation and field inoculation to create artificial epiphytotic condition to screen maize germplasm for (TLB and MLB)

1.1.3.1 Inoculation procedures: Standard resistant and susceptible checks need to be included in all evaluations so that the level of infection can be compared with previous inoculations.

Method-1

The pathogens are isolated by collecting diseased leaf lesions and placing in a moist chamber. After two-three days newly formed spores on the surface of the lesions are picked up with the help of fine flattened needle under a dissecting microscope, placed in a droplet of sterile water and streaked across the surface hardened, acidified water agar in Petri plates. After few hours the spores (Fig.3) start to germinate and they are cut out of the agar and transferred to hard, acidified PDA.

Fig. 3 Conidiospores of *E. turcicum*

After two weeks of incubation at 20-25^{0}C, these culture may be transferred to fresh plates of acidified PDA for multiplication. When the fungus growth covers the surface of Petri plate fully, the cultures are ready for use (Fig. 4). About 20 Petri dishes of full grown cultures are macerated in a warring blender for 15-30 seconds, strained through a layer of cheese or muslin cloth and made up to four-five liters of suspension. This stock suspension is taken to the field and diluted in a compressed air sprayer (which is not ever used for pesticidal spray) @ 1 litre/12 liters of water. Spray should be done into the whorls of the plants where it will be retained for longer period/enough to permit the spore germination. If inoculation is sprayed over the leaves it evaporates before germination. Inoculation should be made twice a week for three weeks, when plants are 30-45 cm high, 120 Petri dishes of pure culture will be enough for 1000 plants.

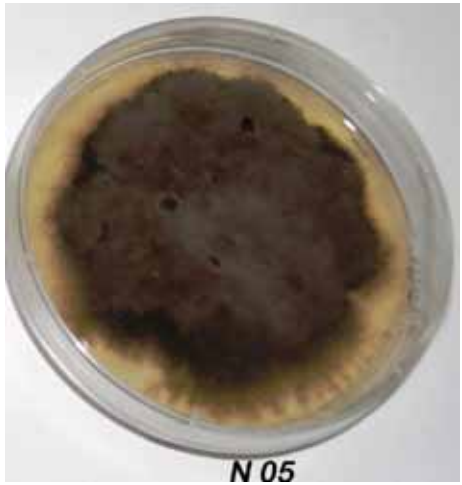

Fig. 4 Sporulating culture of *Exserohilum turcicum*

Method-II

This is an easiest method to prepare inoculum by collecting heavily infected leaves collected in the previous year. This should be done before leaves become fully mature. Infected leaves should be stored in large gunny bags in dry conditions protected from moisture and rodents. To prepare inoculum, the dry leaves are ground into a meal about the coarseness of wheat bran.

Inoculation is done by placing a pinch of leaf meal into whorl of each plant, when plant attains the height of 30-45 cm. A second inoculation may be made five to ten days later. This method of inoculation will be ineffective if dry weather prevails following application of the leaf meal. To overcome this situation, 10-12 ml of water can be applied in the whorls by means of sprayer. High humid weather is congenial for inoculation and disease spread. In case of rain soon after inoculation, water spray is not at all required.

Method-III

About an inch layer of sorghum grains (nearly 40 to 45 g) is dispense in a conical flask (500 ml), soaked in water for about 3-4 hours and excess water is drained off. The flask containing sorghum grains is autoclaved twice, seeded with fungus under aseptic condition and kept for incubation at 25-27 ^{0}C. The flasks are shaken once in 2-3 days to facilitate uniform growth on grains. After incubation of about a fortnight the material is ready for inoculation (Fig. 5).

The above impregnated sorghum grains are allowed for drying by spreading them on a clean paper sheet in shade at room temp. After drying, prepare a fine powder (Fig. 6) of these grains with the help of mixer- grinder and put a pinch of this powder in the leaf whorl. The inoculum should be directed

Fig. 5 Inoculum multiplication on Sorghum grains

Fig. 6 Inoculum in powder form

into the whorl (Fig. 7) of the plant followed by spraying 10 -12 ml of water in the whorls by means of sprayer (Fig. 8) so as to maintain adequate moisture for longer period to permit spore germination. Spraying the inoculum over the leaves is not recommended because the water evaporates before the spores germinate. Inoculation should be done in late afternoon (3-6

Fig. 7 Magnifying view of inoculation through leaf wourl technique

Fig. 8 creation of artificial inoculation

PM) to avoid the maximum day temperature during incubation period. Second inoculation can be followed if the symptoms do not appear even after a week of first inoculation.

1.1.4 Evaluation and recording of disease reaction of MLB and TLB

The scale consists of five broad categories designated by numerals from 1 to 5. Intermediate ratings between two numerals (1.5, 2.5, 3.5 etc.) have also been given, thereby providing for a total of nine classes or categories. Wherever possible, observations on lesion types can also be made, such as large sporulating wilt type or small chlorotic, non-sporulating type.

Data can be recorded 30- 35 days after inoculation on the disease rating scale is as follows:

1.0 - Very slight to slight infection, one or two to few scattered lesions on lower leaves.

2.0 - Light infection, moderate number of lesions on lower leaves only

3.0 - Moderate infection, abundant lesions are on lower leaves, few on middle leaves.

4.0 - Heavy infection, lesions are abundant on lower and middle leaves, extending to upper leaves.

5.0 - Very heavy infection, lesions abundant on almost all leaves, plants prematurely dry or killed by the disease.

1.2 Brown spot (*Physoderma maydis*)

1.2.1 Distribution - Jammu & Kashmir, H.P., Sikkim, W.B., Punjab, Rajasthan, M.P., Karnataka. This disease mainly occurs in subtropical areas with abundant rainfall with high temperature (Fig. 9).

Fig. 9 Plant susceptible to Brown spot

1.2.2 Mass multiplication of inoculum- For preparation of inoculum, the infected leaves (fresh or stored for 1 year in brown paper bags) are taken and crushed into small pieces. These crushed mass are put in water for thorough moistening and then blended in a blender in/with tap water. The mixture is filtered through muslin cloth. The filtrate is diluted to bring the concentration of sporangia up to 5000/ml of water. This inoculum is filled in small dropper bottles and the derived plants at 30-35 days old are inoculated by putting 2-3 drops of inoculum into the whorl. The disease appears after/ in 10-12 days.

1.2.3 The disease rating scale is followed as similar to in case of TLB and MLB.

1.3 Banded leaf and sheath blight (BLSB) *(Rhizoctonia solani* f.sp. *sasakii = Thanatephorus cucumeris)*

1.3.1 Distribution - Jammu & Kashmir, HP, Sikkim, Meghalya, Assam, Punjab, Haryana, Rajasthan, MP, Delhi, Uttarakhand, Bihar. This disease, mainly occurs in tropical; and subtropical areas with moist condition and mild temperature.

1.3.2 Mass multiplication of inoculum- Soak barley grains in water for 24 hours and dispense 40 g in 250 ml conical flask after removing excess water and autoclave at a pressure of 1.05 kg/sq cm (15 lb/sq.cm) for 30 minutes. In case of 100 ml flasks only 10 gm of material may be used. Fresh culture should be prepared in petriplates from last year's infected leaves (Fig. 10) and pathogen start growing on culture plate. Purify the

Fig. 10 Plant susceptible to BLSB

culture by single hyphal tip method. 2-3 days old pure culture is suspended in distilled sterile water, to make suspension and seed 5 ml of the suspension in each flask. Incubate them at 27^0C for 10 days. The impregnated grains can later on be used for inoculation or stored after drying at 15ºC for subsequent use.

1.3.2 Inoculation Technique- Inoculation should be made during the rainy days when moist condition is prevailed and crop is 30-40 days old. Impregnated barley grains should be placed at junction of sheath and leaf (Fig. 12) can create optimum level of disease and do not fall away

Fig. 12 Inserting inoculum (Sorghum grain) in leaf & sheath

with strong wind or heavy rain. Two to four grains should be inserted between stalk and sheath on second or third internodes level from soil for better inoculation.

1.3.3 Evaluation and recording of disease reaction - Disease is recoded after 30-35 days of inoculations on basis of following rating scale.

1.0 - Infection is on one leaf sheath, lesions are one or few, non-coalescent (Fig. 13)

Fig. 13 Photograph showing disease scale of BLSB

2.0 - Infection is on two to three leaf sheaths, lesions are few and non-coalescent on third leaf sheath from ground level.

3.0 - Infection is not up to the ear shoot but on more than two leaf-sheaths

4.0 - Infection is on all leaf sheaths up to the ear shoot but shank is not infected

5.0 - Infection presents beyond the ear shoot; reduced ear size, husk leaves bleached and caked with or without

sclerotial development (Fig. 14); kernel formation absent or rudimentary

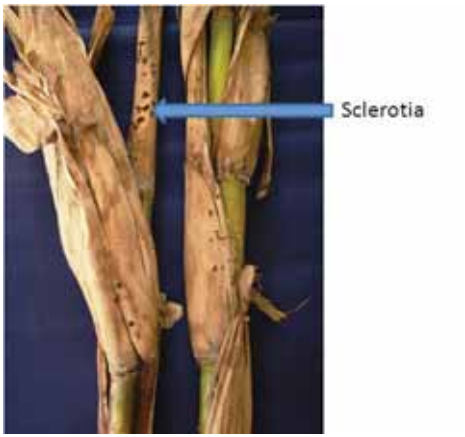

Fig. 14 Plant showing sclerotia of *Rhizoctonia soloni* f. sp. *sasakii*

1.4 Rusts

1.4.1 Common rust (*Puccinia sorghi*) and polysora rust (*P. polysora)*

1.4.1.1 Distribution– Common rust found in Jammu & Kashmir, H.P., Sikkim, W.B., Punjab (*rabi*), Haryana (*rabi*), Rajasthan, U.P., Bihar (*rabi*), M.P., Maharashtra, A.P., Karnataka, Tamil Nadu. The disease is prevalent in cool temperature (16-23ºC) and high relative humidity (100%).

polysora rust found in Andhra Pradesh., Karnataka, Tamil Nadu. The disease is favoured by high temperature (27º C) and high relative humidity.

1.4.1.2 Preparation of inoculum- Inoculum should be collected during the previous year from naturally infected leaves showing large number of pustules. Collection of urediniospore can be done either by lightly tapping the leaves into a cup or a suitable container (Fig. 15) or with a cyclone spore collector. The spores are dried and kept in tightly sealed glass jars or

Fig. 15 Preparation of spore suspension uredinospore for rust inoculation

vials and stored at 5º C. These spores can be stored for several years in sealed glass vials under vacuum and low temperature at –20º C. If there is a sufficient quantity of inoculum collected then the inoculations can be made directly in the field the following planting season, otherwise we can multiply the inoculum before applying in the field. Three to four months before the inoculum is needed, and these spores can be multiplied by inoculating in any susceptible maize cultivars and incubated in the greenhouse or inside a plastic house with adequate ventilation. The urediniospore from these plants are then collected as specified above or directly by tapping and rubbing the heavily infected leaves between the palms of the hands in water containing 0.02 percent Tween 20. Also, urediniospore can be collected from plants in the pot by a cyclone spore collector as the season progresses and used for inoculum.

1.4.1.3 Inoculation technique- Standard resistant and susceptible checks are needed to be included in all evaluations so that the level of infection can be compared with previous inoculations. The plants are inoculated first time at around 6-8 leaf stage and inoculation can be repeated within 2 weeks. The spore (urediniospore) suspension @ 60, 000 spores/ml (Fig.

16) is applied in the whorl using a syringe, atomizer or a knapsack sprayer (same as leaf blights). During application, the spore suspension is agitated or stirred continuously because the spores (urediniospore) tend to stick or clump together on the upper surface of the water. To avoid the clumping tween is added in the solution.

Fig. 16 Uredinospore suspension

1.4.1.4 Evaluation and recording of disease reaction- Evaluations & disease recording should be done 2-3 times during the cropping season. To save on the number of pollinations, the first evaluation should be made prior to flowering to eliminate the most susceptible germplasm. Reactions are evaluated using a scale of 1-5. Intermediate ratings between two numbers can also be recorded (e.g. 1.5, 2.5, 3.5 etc.). In addition, observation on pustule type (e.g. large, sporulating or small chlorotic, non-sporulating types) may also be recorded to measure specific resistance.

Evaluation and disease rating scale

Rating is usually done after 30 days of the last inoculation on -5 scale as described below:

1	- Resistant	- Very slight-to-slight infection, one or two to few scattered pustules on lower leaves only

2 - Moderately resistant - Moderate number of pustules on ower leaves only (light infection)

3 - Moderately susceptible - Abundant pustules on lower leaves; few on middle

4 - Susceptible - Abundant pustules on lower and middle leaves, extending to upper Leaves (Fig. 17 A & B)

Fig. 17 A Susceptible plant showing uredinal pustule of *C. rust*

Fig. 17 B Susceptible plant showing uredinal pustule of *P. rust*

5 - Highly susceptible - Abundant pustules on all leaves, plant may dry prematurely or killed by the disease.

1.5 Downy mildews

1.5.1 Brown Stripe Downy Mildew (*Scleropthora rayssiae* var. *zeae* Payak and Renfro)

1.5.1.1 Distribution - Himanchal Pradesh, Sikkim, W.B., Meghalaya, Punjab, Haryana, Rajasthan, Delhi, Uttarakhand, Bihar, M.P., Gujarat. The disease influenced by the temperature and moisture. The disease is favoured by cool and moist condition.

1.5.2 **Sorghum downy mildew (*Peronosclerospora sorghi*),**

1.5.2.1 **Distribution -** Disease found in Guajrat, Maharashtra, A.P., Karnataka, Tamil Nadu. Plant less then one month old is highly susceptible to this disease. The favorable condition for the disease is mild temperature (20-25º C) in presence of free water.

1.5.3 Rajasthan downy mildew (*P. hetropogoni)*

1.5.3.1 Distribution - The disease occurs only in Rajasthan (Fig. 18). The favorable condition for disease development is free water with slightly low temperature during night (19-26º C). The Oospore survives in alternate host i.e. *Hetropogon contortus* and *H. melanocarpus* (Fig. 19) in non cropping season.

Fig. 18 Susceptible plant to RDM

Fig. 19 *Heteropogon melonocarpus* showing Primary infection

The inoculation technique of allowed same as S.D.M.

1.5.4 Inoculation technique for BSDM

Method I

Since the pathogen is an obligate parasite and could not be

cultured artificially so the artificial disease pressure can be created by placing the powdered infected maize leaves (Fig. 20) containing oospores collected during the last season in furrows just before planting. This inoculum could also be prepared by collecting infected leaves supposed to be full of oospores from early plantings of maize of the same seasons, drying them and making powder out of the debris. Inoculum should be placed in furrows in such a manner that seeds were in proximity of inoculum.

Fig. 20 Infected leaves with Brown stripe downy mildew

Method II

Artificial epiphytotic condition can be created by putting 2-3 cm pieces of freshly infected leaves containing sporangia of the fungus in the whorls of the seedlings. This should be done during cloudy weather in the evening between 5 and 7 P.M. at 24 and 30 days after planting. In experimental plots where disease occurs year after year, only this method is adequate for creating epidemics.

Disease rating of individual maize varities can be done by evaluation all plants of the row(s) using 1-5 rating scale as described below:

1 - No infection.

2 - Light infection , a few scattered to moderate number of stripes on lower leaves.

3 - Moderate infection, abundant stripes on lower leaves and few on middle leaves.

4 - Heavey infection, stripes abundant leaves lower and middle leaves extending to upper leaves.

5 - Very heavey infection, stripes abundant on all leaves. No cob formation. Plant may killed prematurely.

1.5.2 Inoculation technique for SDM

1.5.2.1 Spraying of Inoculum

This method can be adopted when the plants are at two leaves stage. Diseased plants meant for inoculum production from which inoculum required to be drawn should be sprayed with water in the evening around 6 P.M. so that leaves will have thin film of water for good sporulation. The steps of inoculation technique to be followed are:

- By 2.30 A.M. the diseased leaves with good sporulation should be searched/collected (Fig. 21) and washed in the

Fig. 21 Searching of good infected leaves for inoculum suspension

Fig. 22 Washing of infected leaf

water @ of 15 leaves per litre of water and collected it in the buckets (Fig. 22). This is done to avoid the harsh temperature of day time, the inoculation should be done at midnight. This operation will take hardly ½ hour.

- The spore suspension collected in different buckets, should be mixed thoroughly in one bucket to make it 25 litres. A total of 25 litre inoculums suspension can be prepared from 375 diseased leaves.

- The spore suspension (inoculum) should be sprayed with the help of hand compression sprayer only and the process should be completed by 4.00 A.M (Fig.23).

Fig. 23 Inoculation of SDM by spraying method

- By 6 A.M. water spray should be given to the inoculated plot to maintain/create the required humidity artificially.
- With this method 100 per cent disease incidence in susceptible line can be created.

1.5.2.2 Spreader row technique

- Spreader rows are sown 15-20 days prior to the sowing of test entries in 2.5 meter bands at spacing of 60 cm x 30 cm. / with a row to row spacing of 60 cm and plant to plant spacing of 30 cm. Each band will be consisting of four rows surrounding the test entries from all the four directions. For this, highly susceptible variety CM-500 (Fig. 24) should be used.

Fig. 24 Susceptible and resistant plant to SDM

- Inoculation of these spreader rows can be done by following the above artificial inoculation procedure.
- Test entries should be sown as mentioned above.
- Disease incidence should be recorded at 35th day after sowing.

1.5.2.3 Rating Scale- Observations from seedling to flowering are recorded on percentage basis, i.e. diseased plants as per cent of total plant stand.

When infection is <10% of total population - resistant
10-25% disease - moderately resistant
more than 25% - susceptible to highly susceptible.

2. Stalk rots

2.1 Pre-flowering stalk rots (Erwinia Stalk rot/ ESR)

2.1.1 Distribution - Disease found in Sikkim, H.P., W.B., Punjab, Haryana, Rajasthan, Delhi, Uttarakhand, Bihar. The disease is most prevalent and destructive in the areas with high rainfall and land prone to flood condition. The disease is favoured by high temperature (30-35°C) and poor air circulation.

2.1.2 Preparation of inoculum - A virulent isolate of *Erwinia chrysanthemi* corn pathotype should be selected for inoculation. A small piece of infected internode should be removed aseptically. The infected piece should be rinsed in spirit and after that immediately dipped into mercuric chloride solution (1:1000) for 15 seconds and passed through three changes of sterile water. The pieces cut into two to three sterile water and then teased apart with the help of sterile needle. The small quantity of the resulting suspension taken out with the help of sterilized wire loop is streaked on well dried nutrient agar plate so as to get separate the cells to produce individual colonies. The characteristic colonies can be identified within 2 days of incubation at 30^0 C. This should undergo for the pathogenicity test. The culture which induced the typical symptoms of disease within 48 hours of inoculation should be used for mass multiplication and inoculation. The desired inoculum can be multiplied on nutrient broth for 48 hours at 30^0C. The inoculum was diluted 10 times with sterile water. The concentration of bacterium should be maintained approximately 1 x 10^{7-9} /ml.

2.1.3 Inoculation Technique

The appropriate time for the inoculation is when crop is at pre-silking stage. To inoculate the plants, a diagonal hole, deep upto the pith, should be made in the middle of second internode

from the ground with the help of jabber. One ml. of bacterial suspension (described above) is injected in the plant through the hole by hypodermic syringe. If necessary (if weather condition is unfavourable), one week after the second inoculation can be done in third internode from the ground. The inoculated plants are rated individually for their disease reaction using 1-5 rating scale or wilted plants and healthy plants in each plot are counted after 15 days of inoculation. The recording may be done at dry silk stage. For recoding of data the individual plants are cut from the ground in such a way that the first basal internode is intact and these plants are split open longitudinally from the first internode upward to clearly observe the spread of disease in internal tissues.

2.1.4 Disease rating scale

1 - The infection is limited to a very small spot in the pith at the site of inoculation.

2 - Disease infection spreads in half of the length of the inoculated internode in the pith and critical tissues, rind not infected.

3 - Infection covers the entire length of the inoculated internode but does not cross the nodal plates. The rind is green and the symptoms are not visible extremely, but plant shows sign of wilting.

4 - The nodal plants plates are crossed and the increasing infection also covers adjacent internode. The pith and critical tissues are degenerated. The rind of the inoculated internode is affected and the plant wilts. Ear length and the size are considerably reduced as compared to healthy plants.

5 - The disease spreads in three or more internodes. The pith, cortical tissues and vascular bundles are rotten and disorganized (Fig. 25). Rind discoloured, plant wilt and may topple down finally.

Fig. 25 Sypmtoms of Bacterial stalk rot

The plants with 1 and 2 disease rating do not show any symptoms of diseases externally and can be grouped in resistant class and plants with 3, 4 and 5 rating can be grouped as susceptible plants (Singh 1970). The average rating of all the inoculated plants may be considered for comparing the data.

For evaluation of germplasm, wilted and healthy plants should be counted 15 days after inoculation and recorded as per cent stalk rotted plants.

Genotypes population less than

5% disease	-	highly resistant,
5-10% disease	-	resistant
10-25% disease	-	moderately resistant
more than 25%	-	susceptible to highly susceptible.

2.2 Post Flowering stalk rots

2.2.1 Distribution -

Charcoal rot (*Macrophomina phaseolina*) - The disease is common in Jammu & Kashmir, W.B., Punjab, Haryana, Rajasthan, Delhi, U.P., M.P., AP, Karnataka and Tamil Nadu. Disease is prone to hot and dry weather condition. Soil

temperature above 37ºC is favorable for the disease. The organism has wide host range (Fig. 26 A & B).

Fig. 26 A Susceptible reaction to *M. phaseolina*

Fig. 26 B Susceptible plant showing microsclerotia of *M. phaseolina*

Fusarium stalk rot (*Fusarium moniliforme*) - The disease is common in Rajasthan, Uttar Pradesh, Bihar, Andhra Pradesh. Dry and warm (28-30º) early in the season followed by wet weather two to three weeks after silking favours the disease condition (Fig. 27).

Late wilt (*Caephalosporium maydis*) - The disease is common in Hyderabad, UP, Rajasthan The disease develops more rapidly in light and sandy, and heavy

Fig. 27 Susceptible reaction to F. stalk rot

Fig. 28 Susceptible plant to Late wilt

clay soils then in light loam and loam soils (Fig 28).

2.2.2 Mass multiplication of inoculum for Charcoal rot (*Macrophomina phaseolina*), Fusarium stalk rot (*Fusarium moniliforme*) and Late wilt (*Caephalosporium maydis*)

Isolations is done by plating surface sterilized (4 per cent sodium hydrochloride) small pieces of infected tissues on Potato Dextrose Agar (PDA) medium. Purification of cultures is made by hyphal tip method.

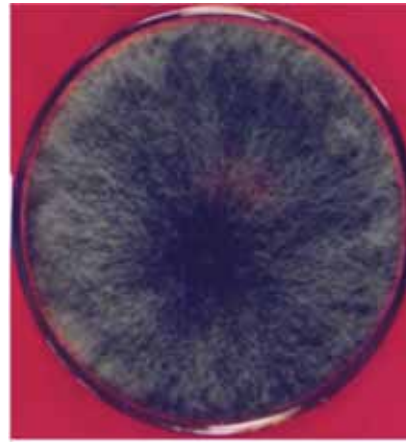

Fig. 29 Culture plate with microsclrotia of *M. phaseolina*

The fungal hyphae is aseptically transferred to culture plates containing the sterile PDA medium to get stock culture. The culture colour of *M. phaseolina* is shooty black (Fig. 29) & microsclerotia is also dark

pinhead shape (Fig. 30) when observed in under compound microscope. The culture colour of F. moniliforme is pinkish white (Fig. 31) & it can be identified by confirming the shape of microconidia (Fig. 32). The inoculum is increased on toothpicks, which are boiled several times thoroughly in water to remove resin, gum or any toxic substances that might inhibit the growth of the

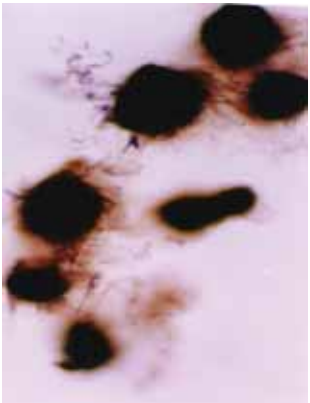

Fig. 30 Microsclerotia of *M. phaseolina*

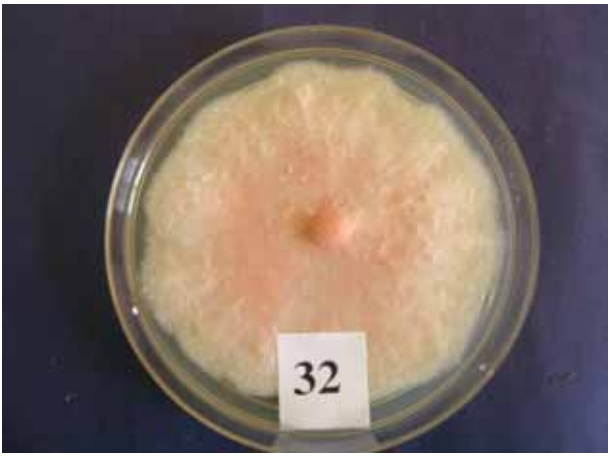

Fig. 31 Sporulating culture plate of *F. moniliforme*

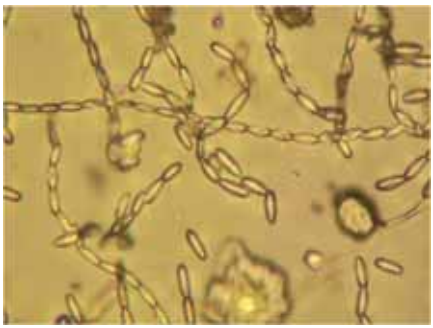

Fig. 32 Microconidia of *F. moniliforme*

fungus. After washing they are dried into sun. Keeping the tapering end upwards, the dried toothpicks is staked loosely in screw capped jars. Prior to autoclaving, potato dextrose broth is added. The level of broth is adjusted to one-third length of toothpicks after autoclaving. Subsequently, the sterilized jar was seeded with fungus and incubated 28^{0}C for one week. Abundant mycelial growth will spread on the toothpicks (Fig. 33).

Fig. 33 Inoculum multiplication on tooth picks

2.2.3 Inoculation technique

Inoculations should be made with of 45-50 days old plants just after flowering stage, in the lower internodes (second) above the soil level. The toothpicks is inserted diagonally after pricking and making 2 cm hole with the help of jabber (Fig. 34)

Fig. 34 Tooth pick inoculation

in the desired internodes (Fig. 35). Disease symptoms appeared in the inoculated plants about 20-25 days after inoculation. The disease intensity and severity is recorded following 1-9 rating scale as described below:

Fig. 35 Tooth pick placed on sec. internode diagonally

2.2.4 Disease rating scale: For scoring disease severity for PFSR, 1-9 rating scale is followed:

1 - Healthy or slight discolouration at the site of inoculation (Fig. 36).

Fig. 36 slight discoloration at the site of inoculation PFSR

2 - Up to 50% of the inoculated internode is discoloured.

3 - 51-75% of the inoculated internode is discoloured.

4 - 76-100% of the inoculated internode is discoloured.

5 - Less than 50% discolouration of the adjacent internode.

6 - More than 50% discolouration of the adjacent internode.

7 - Discolouration of three internodes.

8 - Discolouration of four internodes.

9 - Discolouration of five or more internodes and premature death of plant (Fig. 37)

Fig. 37 Toppled plant susceptible to C.rot showing toothpick

3. Ear rot

The ear and cob rots are caused by species of *Fusarium*, *Cephalosporium*, *Aspergillus*, *Diplodia*, *Botryodiplodia theobromae* are rated according to the following scale:

The pathogens are isolated and identified from infected kernels. For surface sterilization put 3-6 infected kernels in 50 ml of a 1:10 dilution of commercial sodium hypochloride and water (0.3 to 0.6% final concentrations). After 2 minutes the kernels is removed the seed and rinsed in sterile water. Blot the seed dry on sterile paper and place 3 seeds separated by equal distance in a Petri dish containing potato dextrose agar (PDA). Incubate under conditions as described for the leaf blight pathogens and after three to four days the growth of the fungus should be sufficient for obtaining pure cultures of the pathogens.

Pure cultures of the suspected ear rot pathogen are prepared by transferring small sections (0.2mm^2) of the growing tip of the mycelium that show no mixture of different types of mycelium or bacterial growth. After 2-3 weeks when the fungus has covered the surface of the agar, one of the representative cultures should be observed in the microscope to assure that the correct fungus was isolated based on morphological structures. The cultures at this time should be stored in a sealed plastic bag in the refrigerator (5-10 C) to maintain good quality cultures for preparing the inoculum.

3.1.1 Preparation of Inoculum

For production of *Fusarium moniliforme* and *Aspergillus flavus* inoculum for field inoculations, add 10 to 20 ml of sterile distilled water to a Petri dish containing a pure culture of the fungus using sterile technique, and the spores and mycelia are scraped from the agar using a small laboratory spatula and this added to a jar which contains 1 liter of sterile water.

Protective rubber gloves should be used in the preparation of the inoculum since this fungus produces mycotoxins that are water soluble. The contents of the container are mixed, and the solution is poured through two layers of gauze placed in a funnel to collect the concentrated spore solution. The spore concentration obtained from a one liter jar is in the order of 2 x 10^5 spores/ml, and this solution needs to be diluted with water to arrive at the concentration for field inoculations. Use a haemocytometer to determine the number of spores/ml in the stock solution. The stock solution should be stored immediately in the refrigerator and can be used over a one week period. A spore concentration of 5x10^5 spores/ ml is prepared immediately before use (normally 5-10 ml of the stock solution added to one liter of water).

3.1.2 Inoculation procedures

Standard resistant and susceptible checks need to be included in all evaluations so that the level of infection can be compared with previous inoculations. Inoculations for *Fusarium moniliforme* (Fig. 38) and *Aspergillus flavus* (Fig. 39) ear rots are done 7-10 days after pollination using a spore suspension with 2 x 10^5 spores/ml. The period of 0-14 post female flowering is the window where the ear is most susceptible to *F. moniliforme* ear rot. For *Fusarium graminearum*, 1 ml of

Fig. 38 Susceptible reaction of F. ear rot

Fig. 39 Cob infected with *Aspergillus* ear rot

the spore suspension is injected at 7-10 days after silking, in the silk channel using a repeater syringe used for vaccinating swine.

3.1.3 Rating scale

Fig. 40 Resistant reaction of F. ear rot

1 - No infection

2 - 1 to 3 % infection (Fig. 40)

3 - 4 to 10 % infection

4 - 11 to 25 % infection

5 - 26 to 50 % infection

6 - 51 to 75 % infection

7 - > 75 % infection

NOTE

NOTE

NOTE