METHODS OF SEED HEALTH TESTING TO DETECT SEED-BORNE PATHOGENS OF SORGHUM, PEARL MILLET, PIGEONPEA, CHICKPEA AND GROUNDNUT

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In exchange of germplasm seed examination is very important in plant quarantine since they are likely to carry diseases. For detection of infection physical examination of seed is done first under dry condition with the aid of a magnifying lens. This is followed by biological examination in detail under the microscope. For identification of bacteria and viruses special techniques are used which are mentioned here.

DRY SEED EXAMINATION

Seeds can be examined with the naked eye, under magnifying lens or low power stereoscopic microscope with magnification upto 50 to 60 times in good light. The abnormalities observed on seeds can identify the casual organisms(pathogens) to a certain degree of accuracy as mentioned below:

Sorghum

Sl. No.	Type of abnormality	Description	Pathogens associated with seed
1	Ergotted grain	Presence of slightly curved hard cream to light brown sclerotia 10-12 long and thick.	Sphacelia sorghi Syn. Claviceps sp.
	Smut sori	Oval or conical dirty grey sac measuring 5-15 mm long and 3-5 mm broad with sorus skin intact	Sphacelothica sorghi (Grain t).

Contd.....2/-

Sl. No.	Type of abnormality	Description	Pathogens associated with seed
		Sorus sacs cylindrical, elongate, and usually curved covered with whitish membrane upto 4 cm in length and 6-8 mm in width.	*Tolyposporium ehrenbergii* (Long smut)
		Sori solid, long black, often curved and pointed measuring 3 to 18 mm in length.	*Sphacelotheca cruenta* (Loose kernel smut)
3	Smutted grain	Normal grain coated with brown-black powder (smut spores).	*Sphacelotheca reiliana* (Head smut) *S. sorghi* (Grain smut) or *Sphacelotheca cruenta* (Loose kernel smut)
4	Discoloured seed.	Blackening of seed	*Alternaria* spp. *Curvularia* spp. *Drechslera* spp.
		Pink or red discolouration of seeds.	*Fusarium* spp.
		Black spots (acervuli) on the surface of the seed.	*Colletotrichum graminicola*
5	Pycnidia	Presence of black erumpent pycnidia on glumes and seed.	*Ascochyta sorghi* *Macrophomina phaseoli* and *Phoma* spp.
6	Sclerotia	Round blackish sclerotia on the surface of the seed.	*Rhizoctonia bataticola*
		Several black, ovoid or ellipsoid sclerotia immersed in the seed coat.	*Gloeocercospora sorghi*

Contd....3/-

Pearl millet

Sl. No.	Type of abnormality	Description	Pathogens associated with seed.
1	Ergotted grain	Dark brown sclerotia slightly longer and harder than the normal grain. Length 3-5 mm and width 2.5 - 3 mm.	*Claviceps fusiformis*
	Smut sori	The sori are larger than normal grain, 3-4 mm long and 2-3 mm broad, oval or 'P' shaped and dark brown.	*Tolyposporium penicillariae*
	Discoloured seed	Blackening of seed	*Alternaria* spp. *Curvularia* spp. and *Drechslera* spp.

Chickpea

1	Pycnidia	Pycnidia on seeds having concentric brown deep lesions.	*Ascochyta rabiei*
2	Discoloured seed	Seeds are lighter with wrinkled surface, and dull in colour as compared to healthy grains.	*Fusarium oxysporum* f. sp.*ciceri*
		Yellowish or white slime on seed.	Bacteria

Groundnut

1	Discoloured seed	Presence of bluish grey or bluish black patches on seed coat.	*Sclerotium rolfsii*

Contd....4/-

Sl. No.	Type of abnormality	Description	Pathogens associated with seed
		Stippling with small, light brown spots on seed coat.	*Cylindrocladium crotalariae*
		Small size seed with discoloured seed coat.	Peanut mottle virus
		Deformed seeds, reduced in size, wrinkled, and seed coat discoloured with round necrotic areas.	Tomato spotted wilt virus
		Small seeds, deformed, often with split pericarps.	Peanut stunt virus
2	Sclerotia	Minute black sclerotia on seeds.	*Macrophomina phaseoli*
		Flattened black irregular shaped sclerotia on pods and seeds.	*Sclerotinia* spp.
3	Bacteria	Blackened seeds	*Pseudomonas solanacearum*
4	Spotting on pods	Pods with brown necrotic lesions/ spots.	*Pratylenchus brachyurus* (Nematode)
5	Galls on pods	Large galls on groundnut pods	*Meloidogyne arenaria* (Nematode)

MICROSCOPIC EXAMINATION

Washing and sedimentation:

To detect the smut spores and downy mildew oospores of sorghum and pearl millet and fungi adhering on the surface of the seed such as conidia of *Curvularia* spp., *Drechslera* spp., and *Fusarium* spp. etc., the following technique is employed:

Contd....5/-

A fixed number of seeds (100) are shaken vigorously in a given quantity of water (25 ml) to which some detergent is added or in alcohol for 10 minutes. The suspension thus obtained may be examined or the suspended spores may be concentrated by centrifuging, evaporating or filtering the fluid. The concentrated fluid may be examined under compound microscope after diluting with fluid.

(The diagrams , of different species of *Curvularia*, *Drechslera* and *Fusarium* are given on page [illegible].

Embryo extraction method:

Pearl millet seeds (5g) are soaked for 20 minutes in 100 ml of 10% sodium hydroxide solution containing 0.05 g of trypan blue. The temperature is maintained at 75°C. After the incubation for one hour at 55 to 60°C, the seeds are tipped in a plastic sieve (0.5 mm mesh size) and washed for three minutes in running tap water. A fine brush is used for continuous stirring during washing of these seeds. The washed seeds are boiled in lactophenol for clearance. Blue stained *Sclerospora* type mycelium is clearly visible if present, in the form of a net around the scutellar region.

This technique is very rapid. In this method, seed is ready for observation within 90 minutes. Due to the non-fragmentation of the seed, we can know the exact location of mycelium within the seed.

INCUBATION TEST

Blotter method:

The seeds are sown on three layers of moist blotters placed in sterile petri plates of 9 cm diameter. Number of seeds per petri plate may vary from 10 to 25, depending upon the size of the seed. The seeds

Contd.....6/-

are incubated for 7 days at 20°C ± 2°C near ultra-violet light with 12 hours light followed by 12 hours darkness. After incubation, the seeds are examined under stereo-binocular microscope (at 50 or 60 X magnification) for the presence of fungi.

The above method provides excellent conditions for development of mycelial growth and for sporulation of a wide range of fungi viz, *Alternaria*, *Cercospora*, *Colletotrichum*, *Curvularia*, *Drechslera*, *Aspergillus*, *Fusarium*, *Phoma*, *Gloeocercospora*, etc.

Deep freez method:

This is the modified blotter method. In this method the seeds are sown on three layers of moistened blotters placed in sterile petri dishes. These plates are incubated at 10°C for three days to induce germination, and further at 20°C for two days. The seedlings are then frozen at -20°C overnight. Then the plates are incubated at 20°C near ultra violet, 12 hours light and 12 hours darkness for 5 to 7 days. The dead seedlings serve as media for growth of fungal pathogens. The bacterial antagonisms are eliminated by adding a few drops of terramycin to the blotters.

This method is useful for detection of *Fusarium* spp. and *Septoria* spp. in cereals.

Agar plate method:

When compared to the blotter method, this method is time consuming and also costlier. The seeds treated with 1% chlorine for 5 minutes are plated immediately (without washing) on suitable agar medium. The seeds are spaced as per their size, mostly 10 seeds per petri dish. The agar medium used is either malt extract agar or potato dextrose agar. The seeds are incubated at a fixed temperature 20°C ± 2°C for a period of 5 to 8 days (7 days commonly).

Contd....7/-

The results are recorded by examination of fungal colonies. An experienced analyst familiar with the colony characters of fungi present in certain seeds can identify and count colonies by naked eye. For specific identification, the pathogens should be examined microscopically.

Selective method

Fusarium oxysporum f. sp. ciceri

Czapex Dox Agar method:

The seeds are pre-treated with 2.5% sodium hypochlorite solution for 2 minutes. The pre-treated seeds are sown on Czapax Dox agar media in petri plates. The media contains sodium nitrate 2g, potassium dihydrogen phosphate 1g, magnesium sulphate 0.5 g, potassium chloride 0.5 g, ferrous sulphate 0.01 g, sucrose 30 g, agar 20 g, distilled water 1 litre, 750 mg dicrysticin-S, 500 mg penta chloro nitrobenzene, 25 mg malchite green and 2g yeast extract. The plates are incubated at 20°C for 8 days near ultra violet light alternated by 12 hours light and 12 hours darkness.

This method is useful for detection of Fusarium oxysporum f. sp. ciceri in chickpea.

SEEDLING SYMPTOM TEST

Hiltner's brick stone method:

A sterile brick stone granules with a maximum size of 3-4 mm is used as medium. Hundred kernels of cereals are sown in each container and covered with 3 cm of the medium. The medium is moistened with water so that watering is not needed later. The containers are placed in darkness at room temperature and the results are recorded after two weeks. Seedling attack especially by Fusarium should be recorded after three weeks. At the time of recording the results, all the seeds and seedlings are removed from the medium to enable close examination of the roots and coleoptiles for

Contd.....8/-

signs of disease.

This method is useful for detection of cereal seedling pathogens.

Standard soil method:

A uniform soil mixture is used as medium. The medium for the test is prepared by mixing 3 parts of 'P' soil (4 parts of clay and 6 parts of peat), 1 part of sterilized sand with 0.6 parts of water(by volume) for 5 minutes to get uniformity. Seeds are sown in plastic multipot trays. In each pot only one seed is sown and covered with 3 to 4 cm of the medium. These multipot trays are covered with polythylene bags to keep the moisture throughout the test period. The results are recorded after 2 to 4 weeks of incubation depending on the kind of the seed and temperature. Cereals are incubated at 10°C for two weeks for development of *Fusarium* spp. and subsequently one week at 20°C for development of *Septoria* and *Drechslera* spp At the time of recording the results all the seeds and seedlings are removed from the medium to enable close examination of the roots and coleoptiles for signs of disease.

This method is useful for detection of cereal seedling pathogens.

Test tube agar method:

Test tubes of 16 mm diameter, containing 10 ml of water agar (2.5%) are taken. In each tube one seed is sown on the agar medium. The tubes are incubated at 20°C or at suitable temperature depending on the nature of the pathogen and the host under artificial day light tubes of 12 hours light and 12 hours darkness. To retain moisture the tubes are covered in groups by aluminium foils which is removed when the seedlings have reached the cover.

Contd.....9/-

The period of incubation depends on the time needed for the development of seedlings and symptoms which generally vary from 10 to 14 days.

This procedure is more efficient than the blotter test as it allows better development of seedlings and symptoms. The symptoms can be easily studied on roots and as well as on the green parts.

Water agar plate method for detection of bacteria

Seeds are soaked in an antibiotic solution such as streptomycin at a concentration of 200 ppm for three to four hours. The seeds are plated out on water agar (1.5%) after draining off the excess antibiotic solution. In each petri dish, 25 seeds are placed and incubated at 20°C in darkness. After 12 days the diseased seedlings show delayed germination and stunted growth. From infected parts the yellowish bacterial ooze can be seen under the microscope. Further studies for identification of bacteria can be done by transferring bacterial ooze to a medium specific for a particular type of bacteria. For Xanthomonas D_5 medium is required.

This method is successfully used for the detection of Xanthomonas campestris and other species of bacteria in cereals and pulse crops.

Phage-plaque method

This method involves the use of bacteriophages specific for a known species.

For multiplication of bacteria seeds are treated with 2% sodium hypochlorite for 10 minutes, macerated and incubated for 24 hours. Sample of this material is transferred into a sterile flask and a standard suspension of phage particles is added. Samples of this mixture are plated immediately and after 6-12 hours, bacterial growth is compared

Contd. 10/-

with the indicator bacterium. Presence of homologus bacteria is indicated by significant increase in number of phage particles in the second plating.

Serological methods

In this method different tests and procedures are followed, such as slide agglutination test, tube precipitin test, microprecipitin test, latex flocculation test, agar gel double diffusion test, and immuno. fluorescence method. An antiserum must be provided for each test. Depending upon the antiserum these tests may be used for any pathogen.

INDICATOR TEST

Hypodermic injection method

This method is based on the hyper sensitive reaction of plants. With the help of a fine hypodermic needle the bacterial suspensions are injected into the inter-cellular spaces of the tobacco leaf or stem. The necrotic area developed over the leaf surface indicates the pathogenicity of the bacteria. Since only plant pathogenic bacteria are able to induce necrosis or disease in tobacco, this method can be used for rapid selection of the pathogenic isolates.

DETECTION OF GROUNDNUT VIRUSES

Mechanical transmission

Grafting:

On a normal seedling, a scion from infected seedling is wedge grafted. The grafted plant is maintained at high level of humidity to ensure grafting union and virus transmission.

Sap inoculation:

The young leaves with symptoms are macerated in a pestle and mortar

Contd.....11/-

with addition of an equal amount of distilled water (weight/volume basis). Whenever found necessary phosphate buffer (PH 7.8) and a reducing agent (sodium bisulphite) for avoiding viral inactivation due to the oxidation of the sap, are added. The extract thus obtained is squeezed through the layers of the absorbent cotton wool. For inoculation, the leaves are first dusted with an abrasive (carborundom or celite) and then rubbed with absorbent cotton wool dipped into the extract followed by gentle washing of the leaves after a few minutes. An insect proof glass house is used for keeping inoculated plants. Regular observations are made for local lesions or systemic symptoms. French bean (*Phaseolus vulgaris* L.) is used as an indicator plant for peanut mottle virus of groundnut.

Serological methods

Passive hemoglutination test:

This is the most sensitive serological technique. Guteral dehyde fixed red blood cells are coated with antiserum after treatment with tannic acid. Antibody sensitized red blood cells are added to different dilutions of test solutions. The test is conducted in lucite plates containing "U" shaped wells. In a positive reaction, red cells agglutinate, forming smooth mat with a serrated margin on the bottom of the well. In negative reaction red cells form a discrete red ring at the periphery of the well.

This technique is used to detect the viruses in crude plant extracts. This test is also employed for the detection of Tomato spotted wilt virus, peanut mottle virus and peanut clump virus antigens of infected groundnut plants.

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Ouchterlony's agar gel double diffusion method:

In this method wells are made on plates containing noble agar media. Antiserum is added into the central well and different antigen preparations are added into the peripheral wells. Here diffusion takes place through the pores of agar. A positive reaction results in the appearance of a thin white band where antigen and antibody coalesce.

This method can be used to test several antigens at the sametime, with a slight modification by adding 3 5 diiodosalycilic acid into the agar for dissociating long rod shaped viruses. It can also be applied successfully to detect peanut mottle virus and cowpea mild mottle virus.

Enzyme linked immunosorbent assay method(ELISA)

This test is employed to detect PMV in seed. It is more sensitive test and specific serological technique is now available for detection of plant viruses. The γ - globulins extracted from antisera is added to the wells of special microtiter plate and kept for incubation for 3 hours at 37°C. Then test samples including crude plant extracts or purified viruses or extracts from seed are added to the γ - globulins coated wells. The sample containing specific viral antigens are bound to the γ - globulins inside the wells. The test samples are washed and enzyme-conjugated γ - globulins are added to the wells. The labelled antibodies bind to the viral antigen already bound to the γ - globulins coated on the plastic surface. Then substrate (P-nitrophenyle phosphate) is added to the wells. Look for the development of the yellow colour - the colour change in the substrate is proportional to the viral antigen concentration.

Using ELISA technique, it is possible to test nearly 2500 groundnut kernels for the presence of PMV in a day. This technique facilitates the identification of non-seed transmitting genotypes and provides a useful tool for plant quarantine programs.

Contd.....13/-

ELISA PROCEDURE

Coat wells with γ - globulins (coating antibodies)

↓

Incubate 3 hours at 37°C

↓

Wash with PBS + Tween-20 (7.4 pH)

↓

Add sample extracts to the coated wells

↓

Incubate overnight at 4°C

↓

Wash with PBS + Tween-20 (7.4 pH)

↓

Add γ - globulins conjugated with the enzyme alkaline phosphatase (detecting antibodies)

↓

Incubate 3 hours at 37°C

↓

Wash with PBS + Tween-20 (7.4 pH)

↓

Add enzyme substrate P-nitro phenyl phosphate

↓

Incubate 30 min. at room temperature

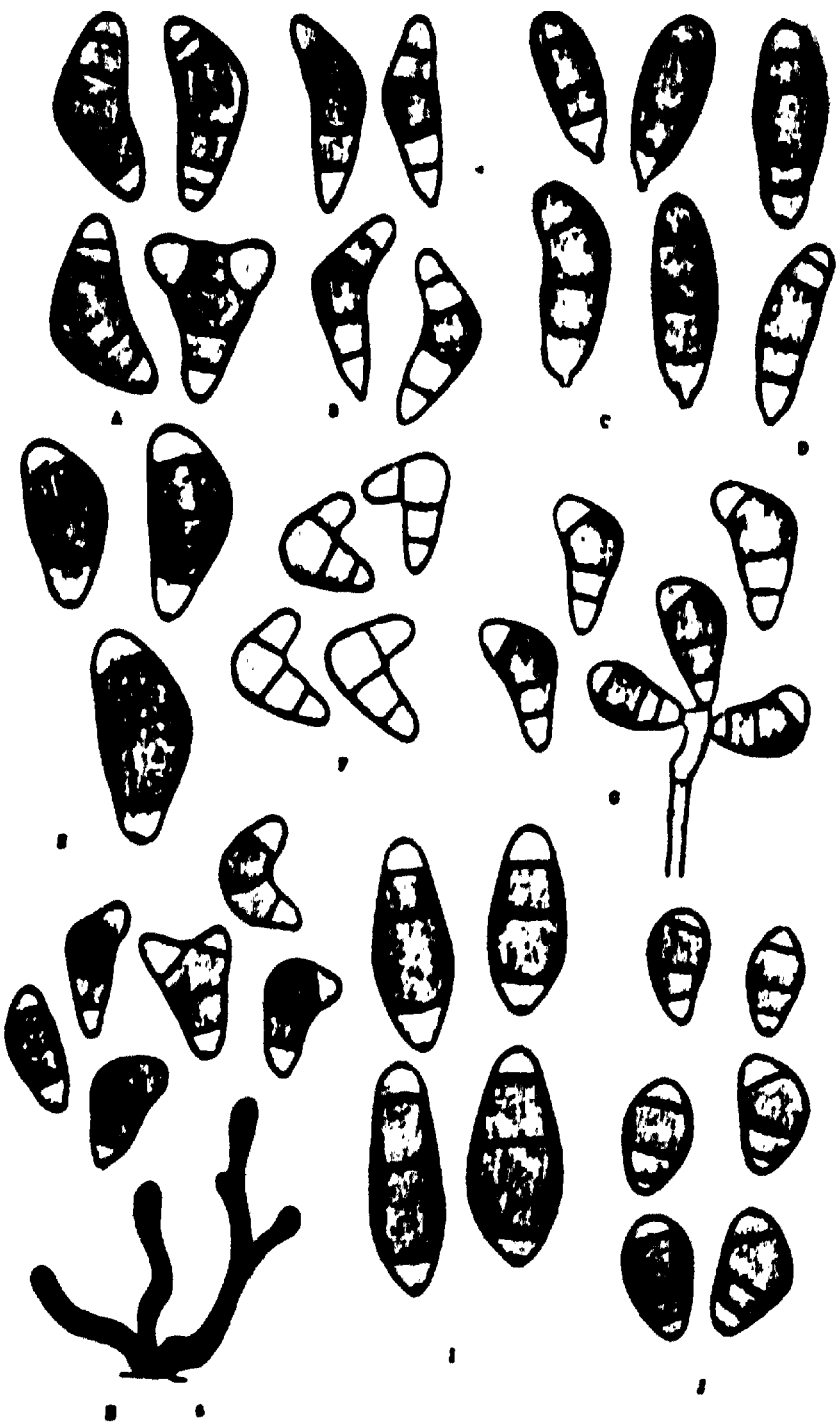
↓

Add 3N NaOH

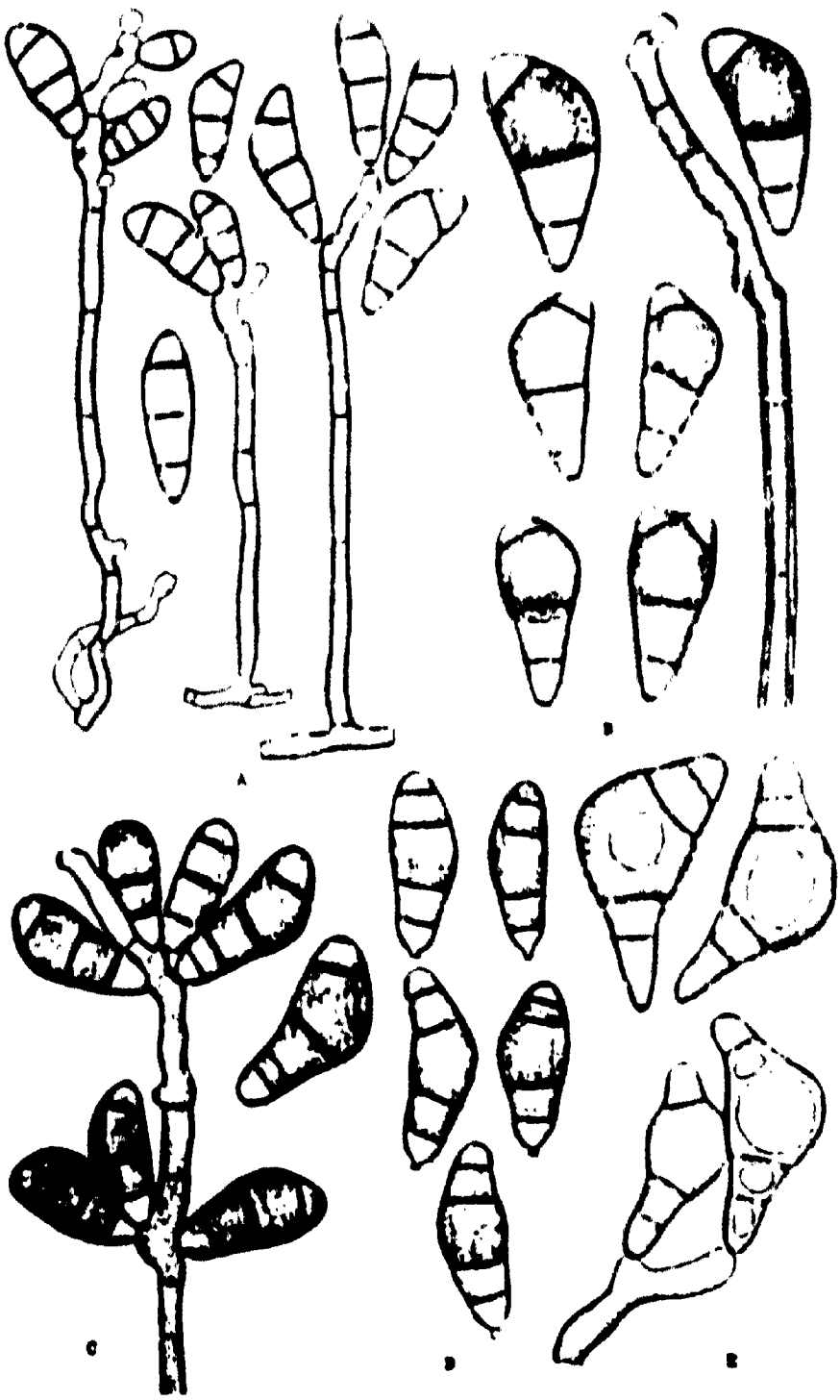
↓

Yellow color = positive reaction

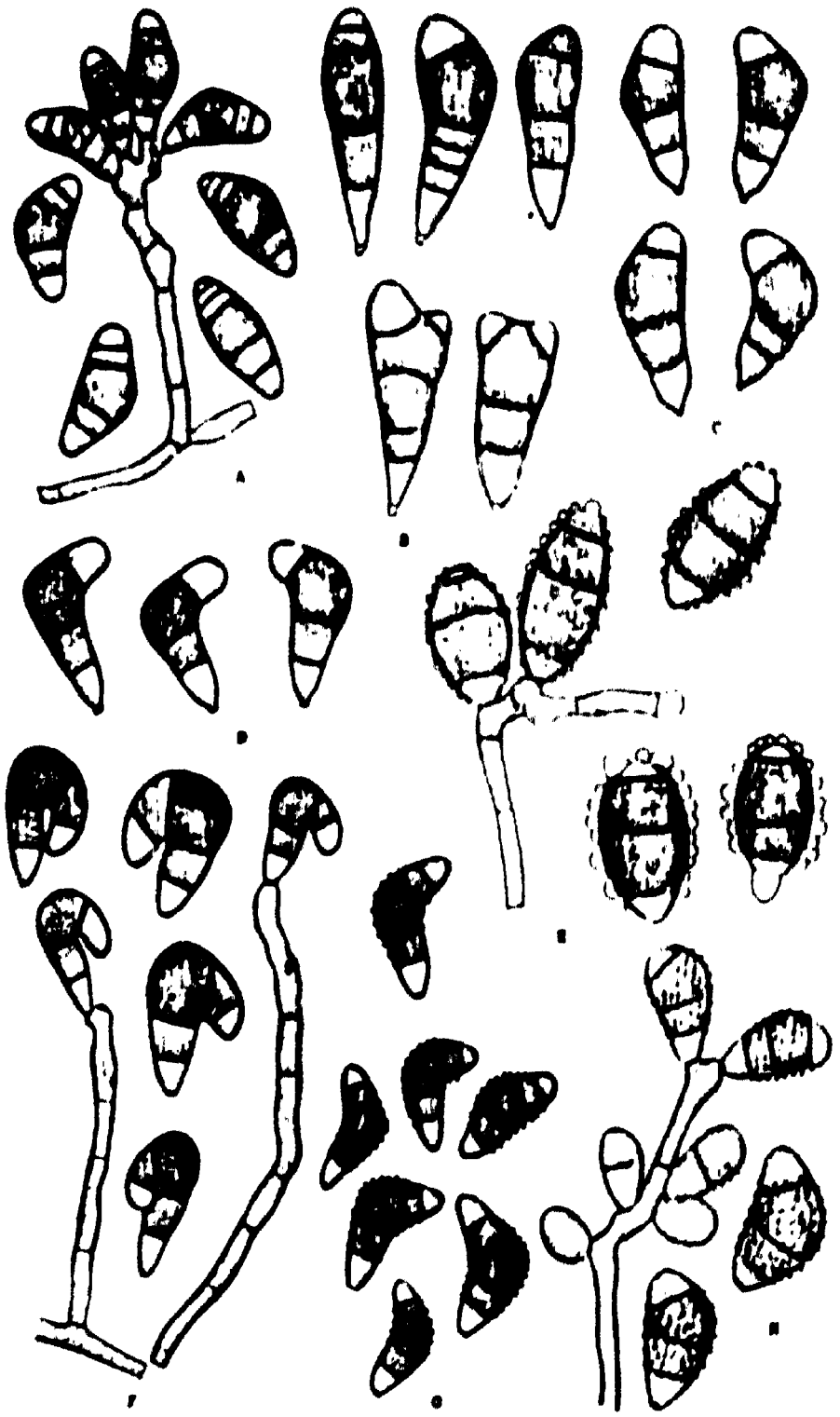
Curvularia species (1): A, *affinis*; B, *endoxyponis*; C, *lunata*; D, *brachyspora*; E, *clavata*; F, *cymbopogonis*; G, *oryzae*; H, *cymbopogonis*; I, *delicatula*; J, *aeruginosa* (× 650).



. *Curvularia* species (2): A, *fallax*; B, *geniculata*; C, *harveyi*; D, *inaequalis*; F, *intermedia*; P, *lunata*; G, *lunata*; H, *lunata* var. *aeria*; I, *oryzae*; J, *ovoidea* (A, habit sketch of stroma; other figures × 650).



Curvularia species (3): A, *pallescens*; B, *prasadii*; C, *prasadii*; D, *prasadii-var*; E, *robusta* (× 650).



Curvularia species (4): A, *sorghoides*; B, *inaequalis*; C, *trifolii*; D, *trifolii* f. sp. *gladioli*; E, *tuberculata*; F, *uncinata*; G, *verruciformis*; H, *verruculosa* (× 650)

GROWTH CHARACTERS OF DRECHSLERA AND THEIR CONIDIA

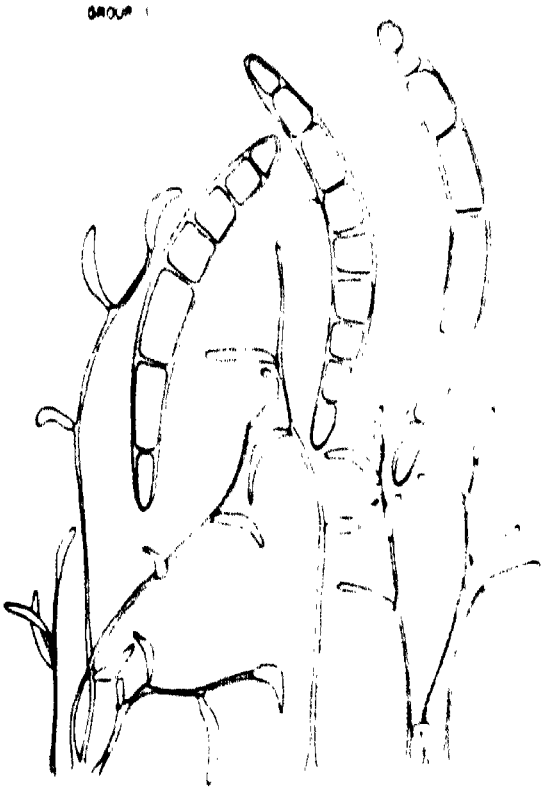


Fig 1 Drechslera maydis

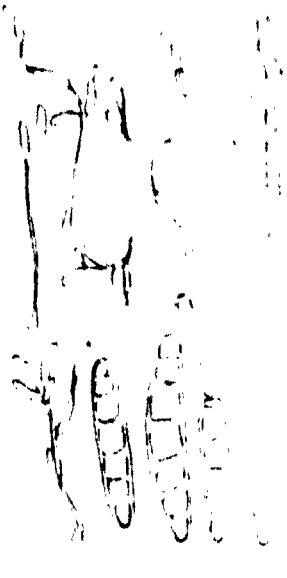


Fig 2 Drechslera

GROUP I (contd.)

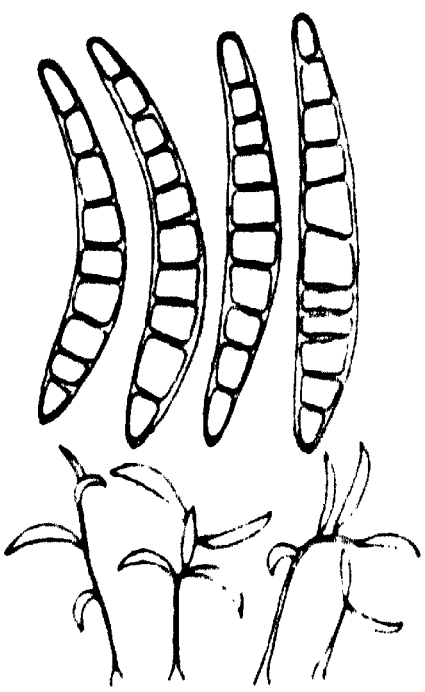


Fig 3 Drechslera oryzae

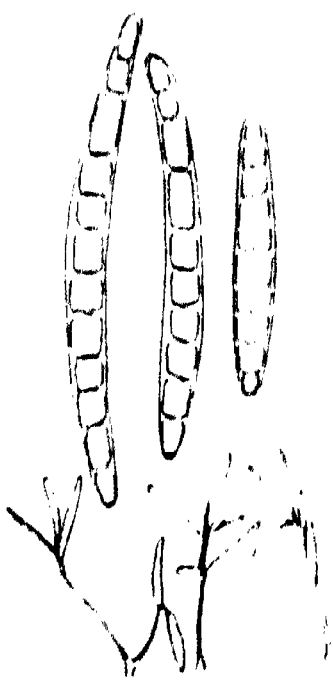


Fig 4 Drechslera sacchari

GROUP 1

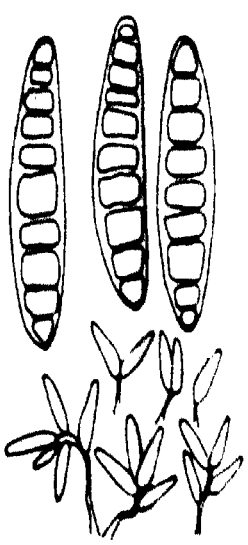


Fig. 5. Drechslera sorokiniana

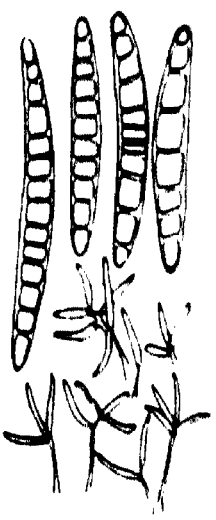


Fig 6 Drechslera victoriae

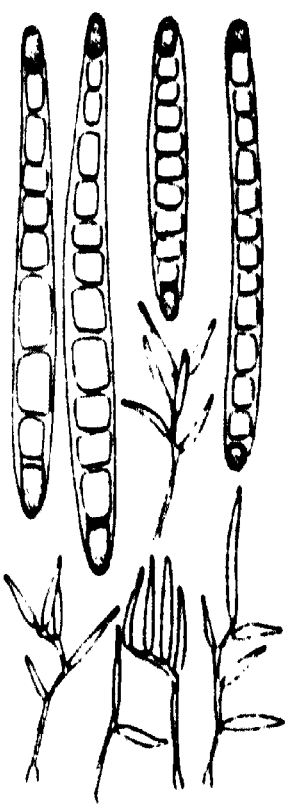


Fig 7 Drechslera teresiae

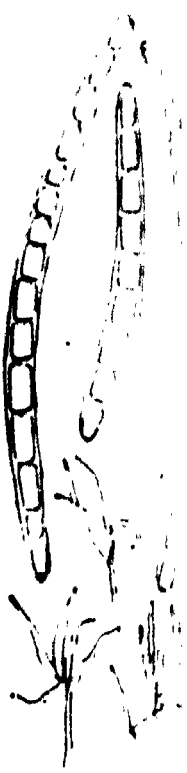


Fig 8 Drechslera [illegible]

GROUP 3

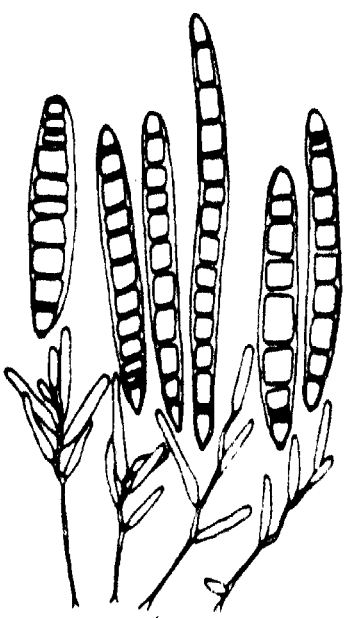


Fig 9 Drechslera halodes

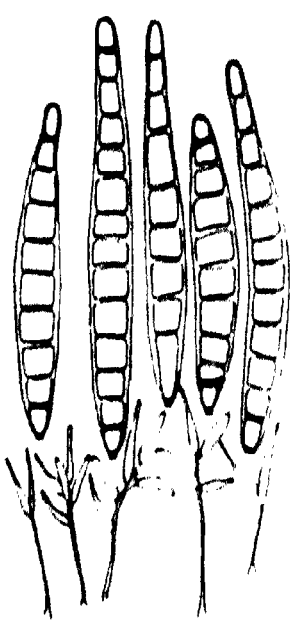


Fig 10 Drechslera rostrata

Fig 11 Drechslera longirostrata

GROUP 4

Fig. 12. Drechslera tetramera

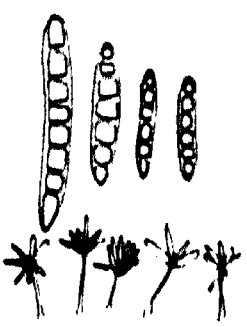


Fig 13 Drechslera hawaiiensis

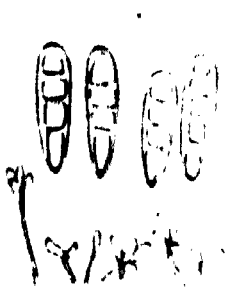


Fig 14 Drechslera dematioidea

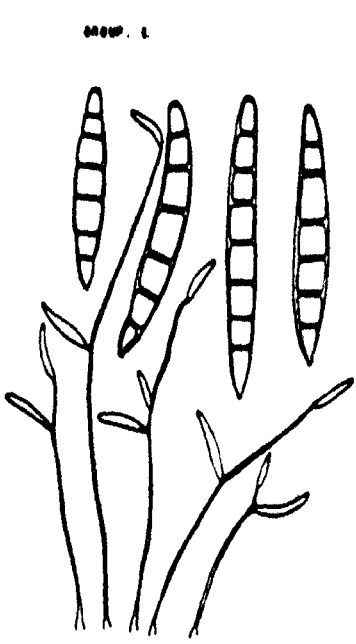


Fig. 15. Dreshslera tuberosa

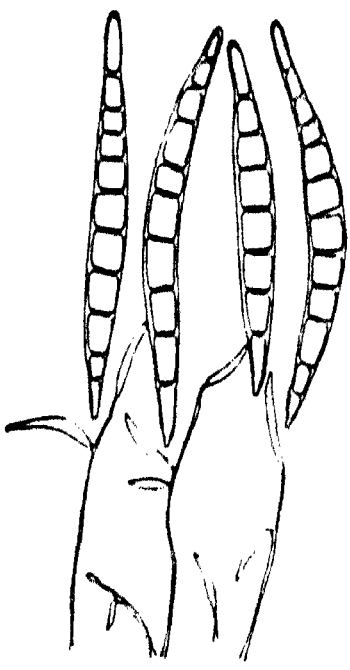


Fig 16 Dreshslera monoceras

GROUP 8

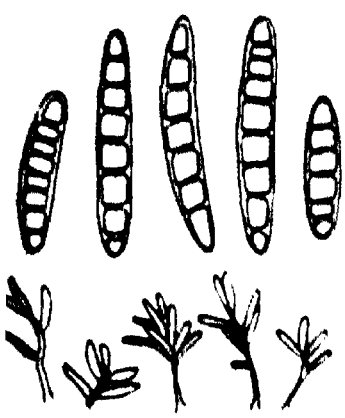


Fig. 17 Drechslera cynodontis

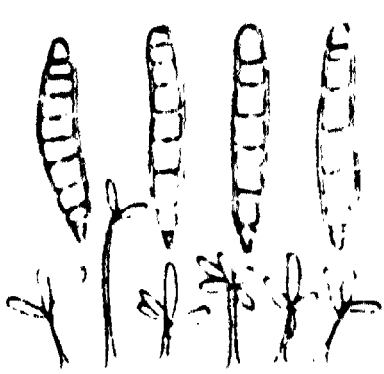


Fig 18 Drechslera micropa

GROUP 7

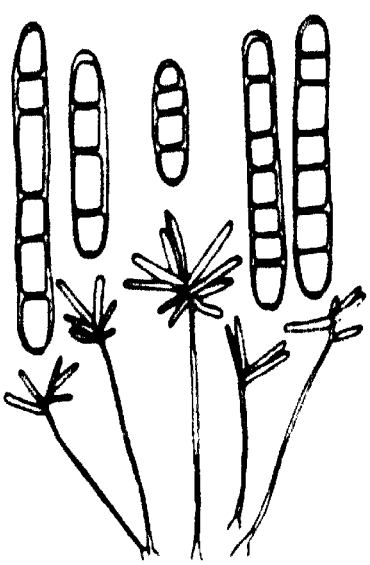


Fig. 19. Drechslera siccans

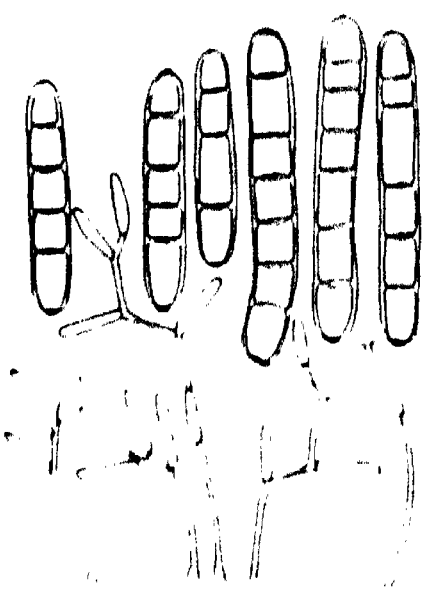


Fig 20. Drechslera [illegible]

GROUP 1 (cont'd)

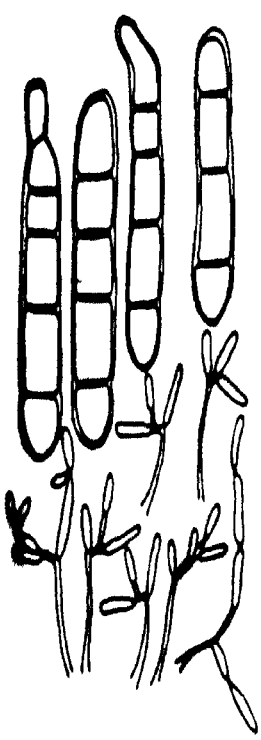


Fig. 21. Drechslera teres

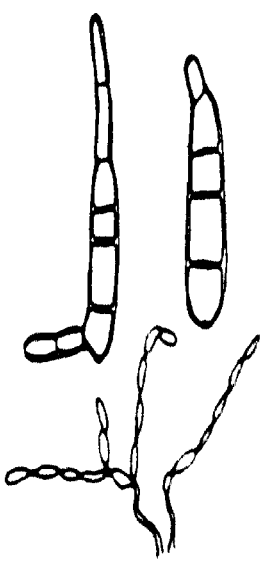


Fig. 22 Drechslera gramines

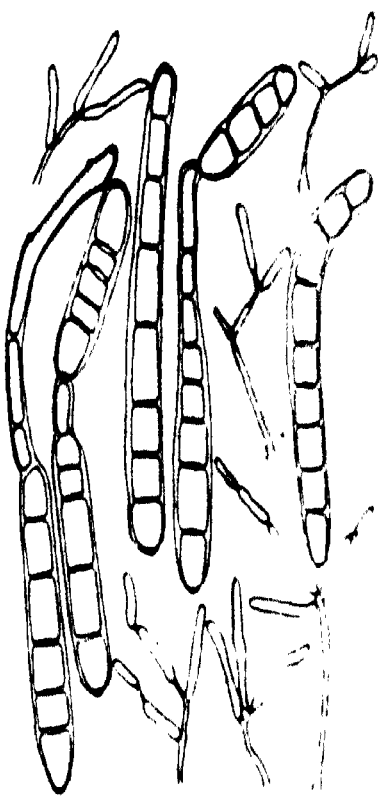


Fig 23 Drechslera tuberosa

GROUP 7 (contd.)

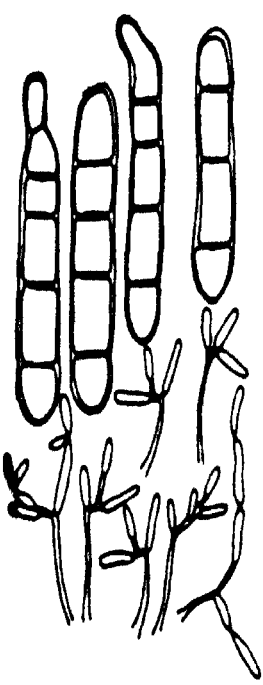


Fig. 21. Drechslera tetra

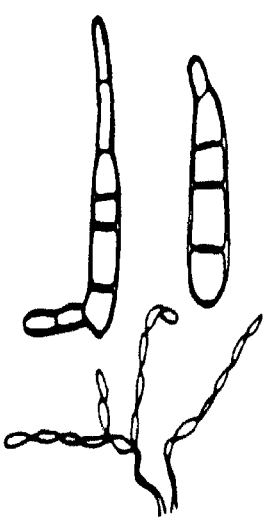


Fig. 22. Drechslera gramínea

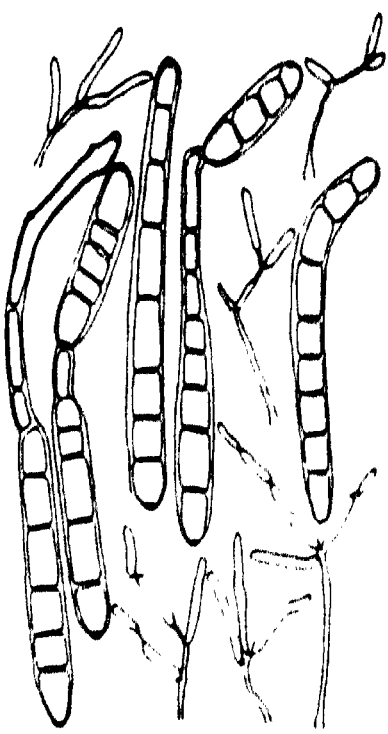


Fig 23 Drechslera tuberosa

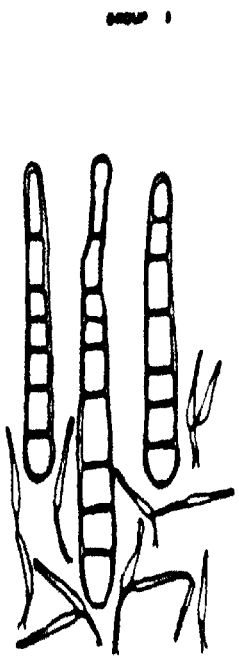


Fig. 34. Dinoflagellata dictyoides f. sp. dictyoides

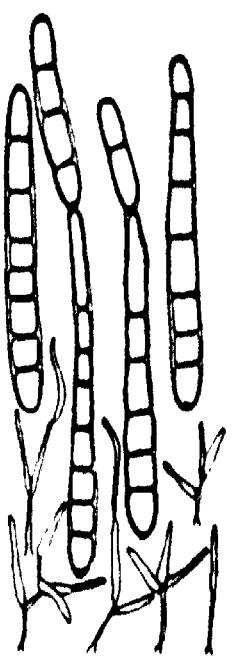


Fig. 35. Dinoflagellata dictyoides f. sp. parvonoda

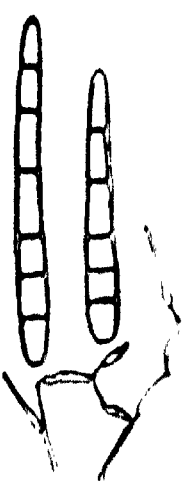
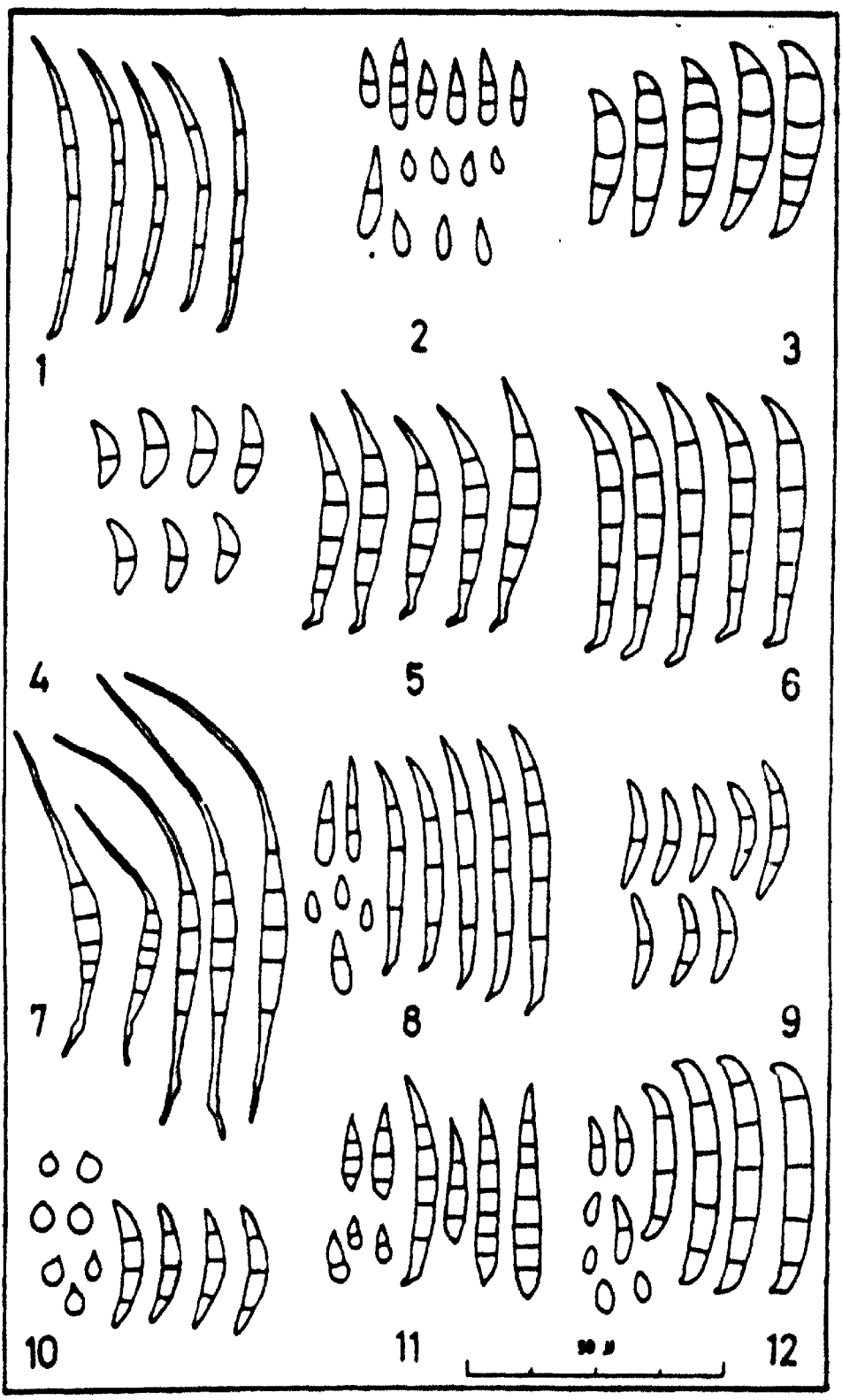


Fig. 36. Dinoflagellata [illegible]



Camera lucida drawings of conidia (800x) of various species of *Fusarium* occurring on seeds: 1. *F. avenaceum*, 2. *F. chlamydosporum*, 3. *F. culmorum*, 4. *F. dimerum*, 5. *F. equiseti*, 6. *F. graminearum*, 7. *F. longipes*, 8. *F. moniliforme*, 9. *F. nivale*, 10. *F poae*, 11. *F. semitectum*, 12. *F. solani*.

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