Isolation and Determation of Molds Forming a Black Spot on the Pulp Paper*

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A black spot has often been found on a piece of pulp paper in process, in a certain paper-work manufactory in September every year. Pulp paper was cut, colored and dried into a paper-work (Fig. 1). In this manufacturing process, many black spots which seemed to have been caused by microorganisms, have appeared on paper-works.

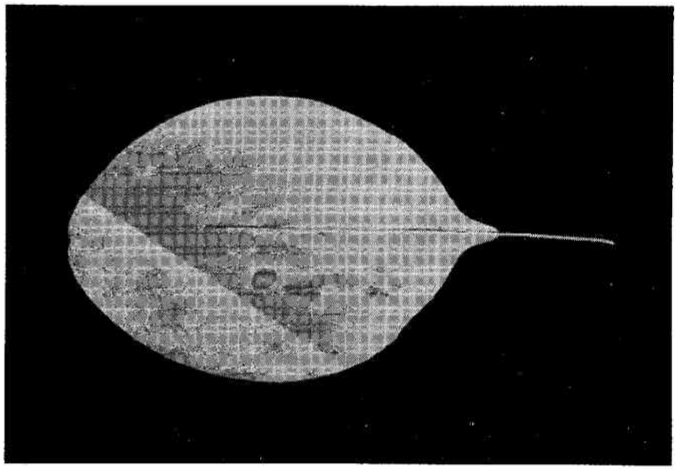

Fig. 1 A paper-work of leaf.

The present paper describes that the molds forming black spot were isolated and determined.

Isolation. Microorganisms were isolated from pieces of pulp paper, 5 by 5 mm in size and including a black spot, on the pulp-paper-tap-water-agar, on that covered with filter paper (Fig. 2) and on the potato glucose agar. Within a week at 25° C, the molds grew out of pieces of black-spot-pulp-paper, and sporulated on the pulp paper, on the filter paper and on the medium. In case of the tap water agar, cladosporia which were distinguished into two distinct groups by the colors of their colonies, grew out of all pieces of pulp paper tested. Penicillia were isolated from 6 of 12 pieces. On the po-tato glucose agar, cladosporia and penicillia were isolated

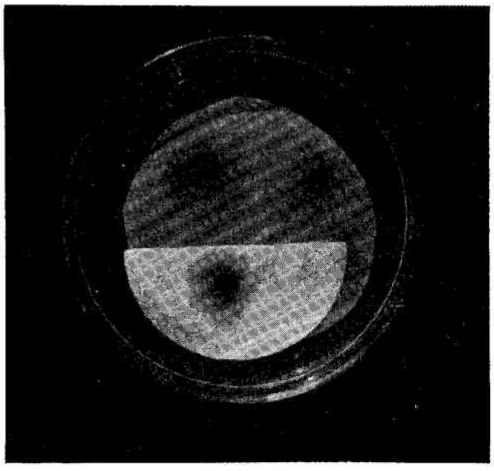

Fig. 2 Black spot forming molds, growing out of pieces of black spot pulp paper on the pulp paper tap water agar, partly covered with filter paper.

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from 25 and from 37 of 45 pieces, respectively. These isolates were distinguished into 17 cultural groups by their colony apearances. Seventeen representatives of each cultural group included 3 strains of cladosporia, 7 strains of penicillia, 2 strains of aspergilli, 1 strain of alternaria, 1 strain of mucor and 3 strains of trichodermates.

Molds forming a black spot on pulp paper. The ability of black spot formation was determined on the pulp-paper-tap-water-agar. The black-spot-forming-molds were only 3 strains of cladosporia among 17 representatives and they were developing olive-colored mycelium on the pulp-paper-tap-water-agar.

Determination. Determination was carried out mainly according to DE VRIES' methods[1], two monospore cultures of each strain. A standard inoculum for all tests was 1 loopful of 5-day culture or 1 drop of 3 ml suspension including 1 loopful of 5-day culture on potato glucose agar, and all the test media except gelatin were incubated at 28°C. Conidia and vegetative mycelium were observed on glucose agar and potato glucose agar. Conidial structures were observed with the slide cultures on potato glucose agar. Cultural characteristics were observed on the following media: glucose agar[1] Czapek's agar with glucose[1], Czapek's agar with sucrose[2], potato glucose agar[2], and oat meal agar[2]. Utilization of various inorganic nitrogen compounds was observed on Czapek's agar containing one of these compounds. The inorganic nitrogen compounds employed were sodium nitrate, sodium nitrite, and ammonium sulfate, 2 g of which were added to 1 liter of Czapek's agar. The ability to utilize various carbohydrates as a sole source of carbon was determined in Czapek's solution containing one of them. The carbohydrates employed were glucose, sucrose, maltose, mannitol, and cellulose. The amylolytic activity was investigated by the method used for *Actinomycetes* by WAKSMAN[3]. Streaked starch agar plates were flooded with an iodine solution after 7 and 12 days incubation. The proteolytic activity was determined by the liquefaction of gelatin. After inoculation, gelatin stabs were incubated during one month at 20°C. The lipolytic activity was investigated by means of ANDERSON's simple triglyceride technique. The medium employed was 1 % tributyrin suspension in glucose agar. Inoculated plates were incubated during 11 days and hydrolysis of tributyrin was shown by the formation of a hyaline zone around the giant colony. Pigment production from gallic acid was observed in the gallic acid solution, pH 4, and pH 6. Temperature range of growth was determined with the growth on potato glucose agar slants at 5°, 10°, 20°, 28°, 37°, and 50°C and the incubation periods extended over three weeks.

According to DE VRIES' system[1], the strains Pf-1, Pf-2, Pf-3, and Pf-4, and the strains Pf-5 and Pf-6 were identified with *Cladosporium cladosporioides* (Fres.) de Vries and *Cl. sphaerospermum* Penzig, respectively. This system is based on morphological and cultural properties of cladosporia, in which their physiological properties are not described.

The physiological properties of the above strains are as follows:

Cladosporium cladosporioides (Fres.) de Vries

Strains: Pf-1, Pf-2, Pf-3, and Pf-4.

Nitrate, nitrite and ammonium salts utilized, but colonies restricted on Czapek's agar including them respectively especially extremely restricted and ruffled, dark olive gray to olive black on Czapek ammonium agar. Utilize sucrose and maltose well. Glucose, mannitol and cellulose, utilized. Weakly amylolytic, proteolytic, cellulolytic and lipolysis: positive with tributyrin. Produce brown pigment from gallic acid. Optimum temperature

20° to 28℃. Temperature range for growth, 5° to 28℃. (Fig. 3).

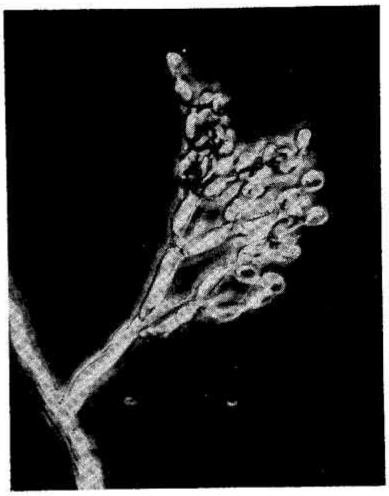

Fig. 3a and 3b Conidiophores of *Cl. cladosporioides* Pf-2 on potato glucose agar.

Fig. 3c Chlamydospores of the strain Pf-2.

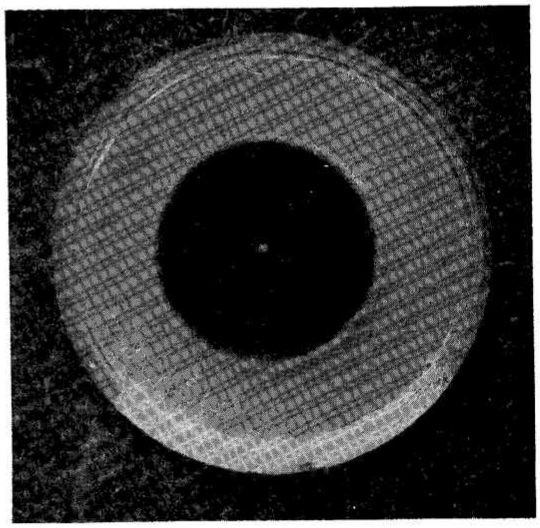

Fig. 3d Giant colony of the strain Pf-2.

Cladosporium sphaerospermum Penzig

Strains: Pf-5 and Pf-6

Nitrate, nitrite and ammonium salts, utilzed; and colony, restricted on Czapek's agar including them respectively; especially extremely restricted and ruffled, black on Czapek ammonium agar. Utilize maltose well. Glucose, sucrose and cellulose, utilized. Not like

to utilize mannitol. Not amylolytic, proteolytic, cellulolytic and lipolysis: positive with tributyrin. Produce brown pigment from gallic acid. Optimum temperature, 28° C. Temperature range for growth, 20° to 28°C. (Fig. 4).

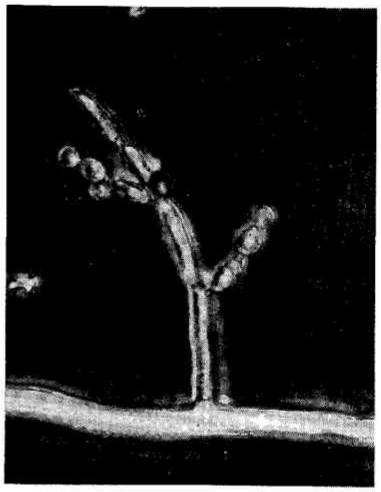

Fig. 4a and 4b Conidiophores of *Cl. sphaerospermum* Pf-6.

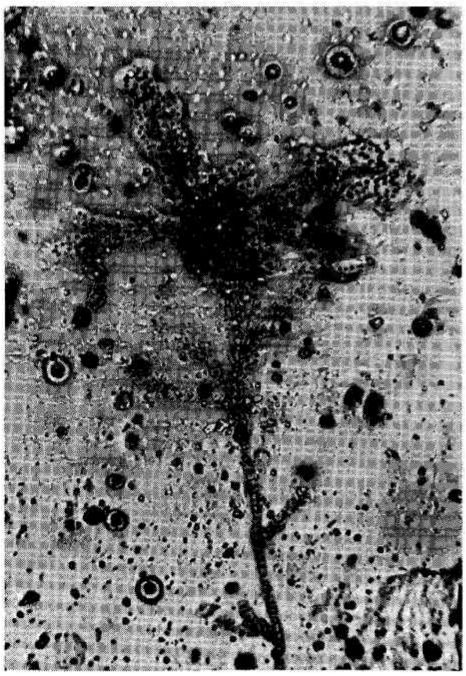

Fig. 4c Coralloid hypha of the strain Pf-6.

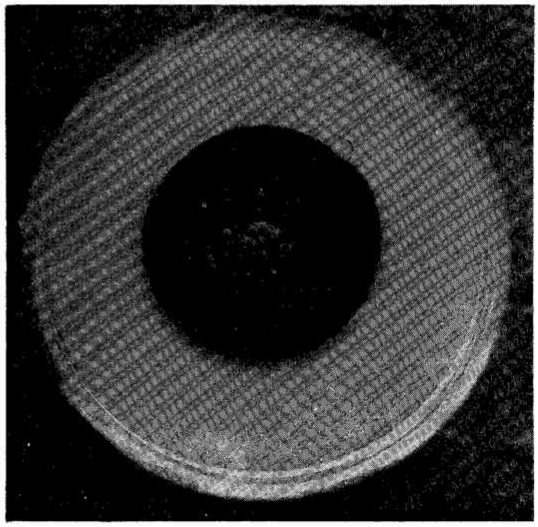

Fig. 4d Giant colony of the strain Pf-6.

The tolerance of the molds for high temperature. A loop of spores was inoculatedon a potato glucose agar slant and the inoculated slants were exposed at 37°C for 6 hr, 12 hr, 1 day, 2 days, 3 days, 5 days, or 7 days, or at 50°C for 30 min, 90 min, 150 min, 4 hr, 5 hr 12 hr, 24 hr, or 48 hr, respectively. Tolerance for high temperature was determined with

growth on each slant, incubated at 25℃ after the above treatment. Spores of the strains Pf-1, Pf-2, Pf-3 and Pf-4 no longer germinated, after exposed for 3 days at 37℃ or for 90 min at 50℃; while, spores of the strains Pf-5 and Pf-6 no longer germinated after exposed for 150 min at 50℃.

The toxicity of phenyl mercuric acetate (PMA) to the molds. The toxicity of PMA to the molds was determined by the growth of the molds on potato glucose agar plates including 1, 10, or 100 ppm of PMA, respectively. 0.1 ml of the spore suspension was smeared on the agar plate, and incubated at 25℃. It was 50 ml of 0.1 % agar solution, suspending 5 loops of the spores of 3 days-culture on potato glucose agar. All cladosporia tested could not grow on the medium containing 10 ppm of PMA and 1 ppm of PMA was found to have a toxic effect on the growth of the strains Pf-5 and Pf-6.

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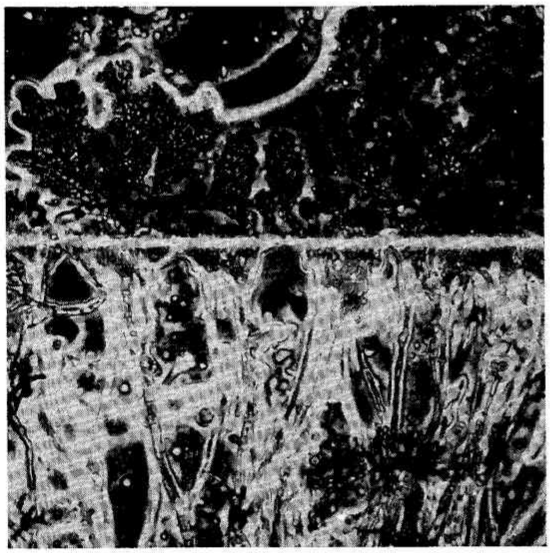

Fig. 5a Coralloid hyphae of *Cl. herbarum* IAM 10-9, in the slide culture, which served for a standard of determination.

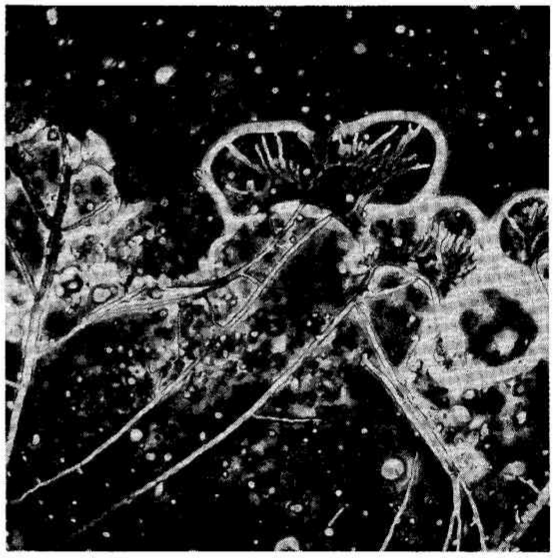

Fig. 5b Fork-like hyphae of *Cl. herbarum* IAM 10-9, in the slide culture.

SUMMARY

17 representative strains of molds were isolated from black spotted pulp paper works. It was *Cladosporium cladosporioides*, 2 strains and *Cl. sphaerospermum*, 1 strain that had the ability to form black spots on pulp paper. Their tolerances against heat and against a fungicide were investigated. Their spores no longer germinated after being exposed for 150 min at 50℃. Spores of *Cl. cladosporioides* strains no longer germinated after being exposed for 3 days even at 37℃. They lost the ability to grow on the medium, containing 10 ppm of phenyl mercuric acetate.

REFERENCES

1) DE VRIES, G. A.: *Contribution to the knowledge of the genus Cladosporium Link ex Fr. Baarm. Holland* (1952).
2) ALEXOPOULOS, CONST. J., and BENEKE, E. S.: *Laboratory Manual for Introductory, Mycology,* Burgess Publishing Co., Minn., U. S. A. (1962).
3) WAKSMAN, S. A.: *Soil Science,* **8**, 2, 86 (1919).