8. AXENIC OR ARTIFICIAL CULTURE OF THE DOWNY MILDEW FUNGI OF GRAMINEOUS PLANTS

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Sclerospora

In 1969 Tiwari and Arya published the first report on axenic culture of a downy mildew, namely, *Sclerospora graminicola* (Sacc.) Schroet., the obligate pathogen causing downy mildew (green-ear) of pearl millet (*Pennisetum typhoides* Stapf. and Hubb.) has been successfully cultured for the first time on known semisynthetic medium with no evident loss of fructifications. *Sclerospora graminicola* was first grown on host callus tissue and subsequently on a modification of White's basal medium that contained casein hydrolyzate (Oxoid), 2, 4-D and kinetin. In the same time the growth of two downy mildew fungi on semisynthetic medium was announced by Shaw and Safeeulla (1969). Stem fragments of bajra (*Pennisetum typhoides*) infected with *Sclerospora graminicola*, after surface sterilization, developed callus tissue on White's medium supplemented with coconut milk, 2, 4-D, kinetin, yeast extract, indole-3-acetic acid, naphthalene acetic acid,casein hydrolysate, sucrose, agar and traces of other additives. The fungus appeared first in and later on the callus as extensive, cottony, coenocytic mycelium. Subsequently, the mycelium spread to the surface of the medium and produced numerous oogonia. Sporangiophores and sporangia were not produced. Fragment of sorghum (*Sorghum vulgare*) inflorescene and stem tips infected with *Sclerospora sorghi* also developed callus tissue and coenocytic mycelium on this medium, but neither oogonia nor sporangiophores were produced on the sorghum callus.

Sclerophthora

Srinivasan and Thirumalachar (1962) made an attempt to obtain artificial growth of *Sclerophthora cryophila* on oat meal, french bean, potato dextrose, yeast glucose and corn meal agar media. Infected leaf pieces of *Digitaria marginata* showing early symptoms were surface sterilized in chlorine water and were placed with the lower side up on the surface of the media in drops of sterile water. A faint whitish wrowth consisting of sporangia and thin walled coenocytic hyphae was observed after 6–7 days. The growth, however, even on prolonged incubation did not spread on the agar surface. Singh (1969) also attempted culturing of *Sclerophthora rayssiae* var. *zeae* on sucrose potato, sucrose oat meal and sucrose watermelon agar media but no success was obtained. He has, however, been able to assess reaction of selected maize germplasm by maintaining excised leaf pieces in solutions containing 5 percent sucrose and 20 ppm kinetin.

In Japan no serious epidemics of downy mildew has been reported of corn, at least for the last 50 years. However, the downy mildew of rice plants (yellow wilt of rice plant) caused by *Sclerophthora macrospora* (Sacc.) Thirum., Shaw et Naras.—formerly *Phytophthora macrospora* (Sacc.) Ito et Tanaka—has been one of the serious diseases in certain district of Japan, and its epidemic since 1921, especially during 1951–1968, caused a extensive loss of yields, thus igniting various types of research on the control measure of the disease and the characteristics of the pathogen. Recently a damage of corn due to *Sclerophthora macrospora* was reported in Asian countries and the descrip-

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tion of the pathogen (Sun and Ullstrup 1970, 1971) suggests that the pathogen would be identical or close to *Sclerophthora macrospora* of rice plants in Japan.

In spite of general conception that *Sclerophthora* could hardly be isolated on artificial media, several workers (Akai, Fukutomi, Katsura, Kondo, Isaka, Ono and Tokura etc.) challenged and succeeded in isolating the fungus. In 1952 the isolation of *Sclerophthora macrospora* and its cultivation on agar substrates was first described by Katsura. The isolation was made from infected rice plants onto potato sucrose, watermelon sucrose, and oatmeal sucrose agars. The fungus which was isolated was non-pathogenic on eggplant, beans, cucumbers and tomatoes. Subsequently, sporangia on sucrose potato agar and oogonia on oatmeal agar medium were observed (Katsura 1952, 1963). Akai and Tokura (1959) also reported on the physiology of the organism *in vitro*. Akai and Morinaka (1962) demonstrated the production of indoleacetic acid by *S. macrospora* in synthetic culture media. The isolation techniques and cultural difference of the fungus has been investigated by Ono and Yamamoto (1963).

Although consistent presence of aseptate mycelium in repeated isolation suggested that these isolates would be *Sclerophthora macrospora*, the characteristic symptoms of the disease has not so far been reproduced by inoculating these isolates to rice plants. Thus the Koch's postulates have not been fulfilled, hence these isolates could not yet be identified, in strict terms, as *Phytophthora macrospora*.

In view of the above, the present communication had to be unfortunately restricted to the isolation procedure, some characteristics on culture media and zoosporangial formation of a candidate fungus.

Since 1955 gramineous plants associated with downy mildew symptoms have been collected in various fields in the Kansai district and 16 isolates have been obtained from the infected younger plants, *Beckmannia erucaeformis, Alopecurus aequalis, Poa annua, Agropyron kamoji, Digitaria adsendens, Dactylis glomerata* and *Oryza sativa*.

The minimum, maximum and optimum temperatures for the mycelial growth were 3–5C, 36C, and 28–32C, respectively, and the optimum pH value for the growth was 5.4–6.5. The fungus cannot grow sufficiently in any synthetic medium unless thiamine is added. In test tube culture, the aerial mycelia were usually formed in 4–6 days on all over the surface of the medium, and the zoosporangia were observed on the margin of the mycelial mat of 11 isolates. The test tube culture technique was adopted to study the developmental stages of the zoosporangia.

The mycelial growth and sporulation of the fungus were discussed as a function of nitrogen and carbon sources in synthetic media. Nitrate and amino acid were well utilized as nitrogen source for mycelial growth, while ammonium-nitrogen was not. The utilization of amino acid was, in general, best demonstrated when pH of media was kept at about 6.5. Sucrose, maltose or starch found to be the best for mycelial growth.

The requirement of microelements of the fungus was discussed in terms of mycelial growth and zoosporangium formation. The mycelial growth was best respectively at 2×10^{-5} M of ferric chloride or $10^{-3}\sim10^{-5}$ M of lithium chloride, natrium chloride, potassium chloride, or magnesium chloride, or at 10^{-5} M of zinc chloride, the latter two being also favorable for zoosporangium formation. On the other hand, sliver nitrate, cadmium chloride, mercuric chloride, boric acid, aluminium chloride, potassium chromate and potassium iodide inhibited the growth of this fungus. The concomitant presence of iron, copper, manganese (10^{-5} M as chloride salt) and molybdenum (10^{-6} M as ammonium salt) in the synthetic media led to a remarkable growth of mycelium. Molybdenum ion seems to be effective for zoosporangium formation. These elements are essential for the complete growth of this fungus, suggesting that they play an important role in the metabolic system.

The media containing 0.3–0.6% KNO_3 and 1–2% glucose or sucrose were quite

favorable for the zoosporangium formation in the synthetic solution. The optimal C-N ratios for obtaining an extensive sporulation in gultamic acid media are ranged between 12 and 20, the peak of zoosporangium formation moving toward the lower side of C-N ratio with an increment of nitrogen.

The addition of phosphate buffer (pH 6.24) to a synthetic medium favors zoosprangium formation provided that the initial pH of the medium was adjusted to 6.24, and kept pH of the medium around 6.24–6.38 through incubation period. The remarkable formation of zoosporangia was obtained when KH_2PO_4 and K_2HPO_4 were added respectively to give the final concentration of 0.004 M to the basal medium containing 2% glucose. The zoosporangium formation seems to require twice as much of phosphate as the mycelial growth.

The additional presence of calcium carbonate in the synthetic or various semi-synthetic media was also favorable for the zoosporangium formation, if it was added before inoculation. The delayed addition of calcium carbonate, however, does not give any favorable effect on the formation, especially when it was made 3 days or more after the inoculation. The increase in zoosporangium formation in calcium carbonate medium seems to be attributable to stable pH of the media (6.22–6.39) due to its buffering action.

The zoosporangia were, in general, formed abundantly on the wall of test tubes contacting with the surface of media, when the test cultures were arranged slantwise keeping the angle of 15–35°.

Characteristic of the test fungus in culture media may be summarized as follows. The mycelium was measured to be 1.2–25μ in width, and quite variable in shape. Apex of the mycelium swells, and develops in to the zoosporangium. The sporangiophores are hyaline, not distinguishable from the mycelium, and support a zoosporangium firmly. Size of the zoosporangia depends remarkably on culture conditions, such as temperature or moisture. In most cases, however, pearshaped (52.1×30.7μ), spherical (36.2×33.8μ) and spindle formed (88.5×29.9μ) were observed.

Zoosporangium formation of the fungus was observed to occur at temperatures ranging from 16 to 32C, the optimum being in 24–28C with suitable culture media. Thus the temperature for maximum sporulation in culture is higher than the optimum sporulation temperature in diseased leaves. The sporangial germination by zoospores was predominant at 16–20C in water, but was very low comparing to that of zoosporangium collected from infected plants. The number of zoospores produced in a zoosporangium was estimated to be about 4–34. The zoospores encyst after swimming, and cystospore germinates with a germ-tube.

Although the injection of mycelial suspension into the host seemed to give an diseased appearance to the injected plants, no conclusive evidence has been provided to show that the isolates is the pathogen. The infection of rice seedlings with zoospores formed on culture media has also been unsuccessful, i.e. the test plants did not show any typical symptom of this disease, while zoospores attacked on the ventral scale or epiblast of the rice seedlings. But the infected seedlings resulted an imperfect growth and fell into decay. Therefore these phenomena are distinct from natural zoospore infection. It seems that there are some physiological differences between the zoospores from culture and those natural hosts.

The above results indicated that the test organism is identifiable as *Sclerophthora macrospora* in many respects, although the fungus had a little high sporulation temperature, failed in oospore formation and produced a slightly different zoospores from natural one in terms of infection pattern (Tokura 1965).

Literature Cited

Akai, S. and R. Tokura (1959). Studies on the downy mildew of rice plant, with emphasis on the sporulation of the pathogen. Ann. Phytopath. Soc., Japan **24**: 8.

Akai, S. and T. Morinaka (1962). The production of indoleacetic acid by *Sclerophthora macrospora*. Ann. Phytopath. Soc., Japan **27**: 239–244.

Arya, H. C. and M. M. Tiwari (1969). Growth of *Sclerophthora graminicola* on callus tissue of *Pennisetum typhoides* and in culture. Indian Phytopathology **22**: 446–452.

Ono, K. and T. Yamamoto (1963). The isolation and cultural difference of a yellow wilt fungus of rice plant. Proc. Assoc. Plant Prot. Hokuriku **11**: 9–10.

Katsura, K. (1952). Some information on the downy mildew of the rice plant. Ann. Phytopath. Soc., Japan **16**: 170.

Katsura, K. (1965). Downy mildew of rice plant, infection and disease development. Ann. Phytopath. Soc., Japan, Golden Jubilee Issue **31**: 186–191.

Shaw, C. G. and K. M. Safeeulla (1969). Growth of two downy mildew fungi on semi-synthetic medium. XI Internat. Bot. Congress: 196.

Singh, J. P. (1969). Studies on histopathology and epidemiology of brown stripe downy mildew of maize (*Sclerophthora rayssiae* var. *zeae*). Ph. D. thesis, I.A.R.I., New Delhi. (in Payak et al. 1970. Diseases incited by *Sclerophthora*. Indian Phytopathology **23**: 189).

Srinivasan, M. C. and M. J. Thirumalachar (1962). *Sclerophthora cryophila* on forage grasses in India. Bull. Torrey bot. Cl. **89**: 91–96.

Sun, M. H. and A. J. Ullstrup (1970). Production and germination of sporangia of *Sclerophthora macrospora* from corn. Phytopathology **60**: 1316.