THE ULTRASTRUCTURE OF *PIRICULARIA ORYZAE CAV.*[1]

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In South-East Asia, *Piricularia oryzae* is one of the most economically important organisms because it attacks rice crops everywhere and does severe damage to the same crop. Although only the asexual stage is observed in this fungus a great deal of variation is easily found in nature. Breeding of resistant varieties of rice to this pathogenic organism is obviously hindered due to the obscure information concerning the mechanism of variability of this fungus. Cytologically, much work has been done by Suzuki (1967) in the past thirty-five years and his conclusion is that the persistent heterocaryosis is the origin of variability of this fungus and it is multinucleate. Following Suzuki, Chu *et al* (1965) reported that *P. oryzae* is multinucleate in both hyphae and conidia and that each nucleus consists of four chromosomes and would divide with classical manner of mitosis. However, Yamasaki *et al* (1965) gave evidences that this organism is uniuncleate. Its economical importance and the limited resolving power of optical microscopy used as a tool in the previous studies justifies an investigation of the fine structure of this organism. This report describes the electron microscopical structure of the wild races of *P. oryzae* and the techniques involved. However, results obtained from our preliminary studies are also presented.

Material and Methods

Stocks of three different physiological races of *P. oryzae* including race no. 5, 13 and 17 were obtained from the Taiwan Agricultural Research Institute. Different races were separately inoculated on barley medium which were incubated in culture room at 28°C for twelve days then the conidia were harvested by washing the inoculated barley with liquid Tanaka medium (TCM). The suspension obtained were filtered through a thin layer of cotton and then subjected to centrifugation. The pellet was resuspended in liquid TCM and kept in the culture room for intervals of 0, 4 and 24 hours. Centrifugation

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and resuspension were applied repeatedly in handling the tiny samples in harvesting, fixing, washing and dehydration. The cultured conidia with or without germ tubes or branching mycelia were fixed in 5% glutaraldehyde in phosphate buffer for one hour at room temperature and washed with Millonig's phosphate buffer for four or five time in a period of two hours and then washed with distilled water. The second fixative, Dolton's chrome osmiun tetroxide was used. The operation was conducted at 0°C for the first ten minutes and then at room temperature for next two hours. Before carrying out dehydration quickly in a series of alcohol, samples were washed once in distilled water. An improved epoxy resin embedding method (Luft, 1961) had been refered. In addition, open-face method (Elio sparvoli *et al*, 1965) and sheet mica technique (Persijn *et al*, 1965) were included in embedding samples with Epon 812. Sections around 1,000 A thick were cut with a glass knife on a Sorvall MT-2 microtome and stained with Reynold's lead citrate (Reynold, 1963). Micrographs were obtained with Hitachi 11-A electron microscope.

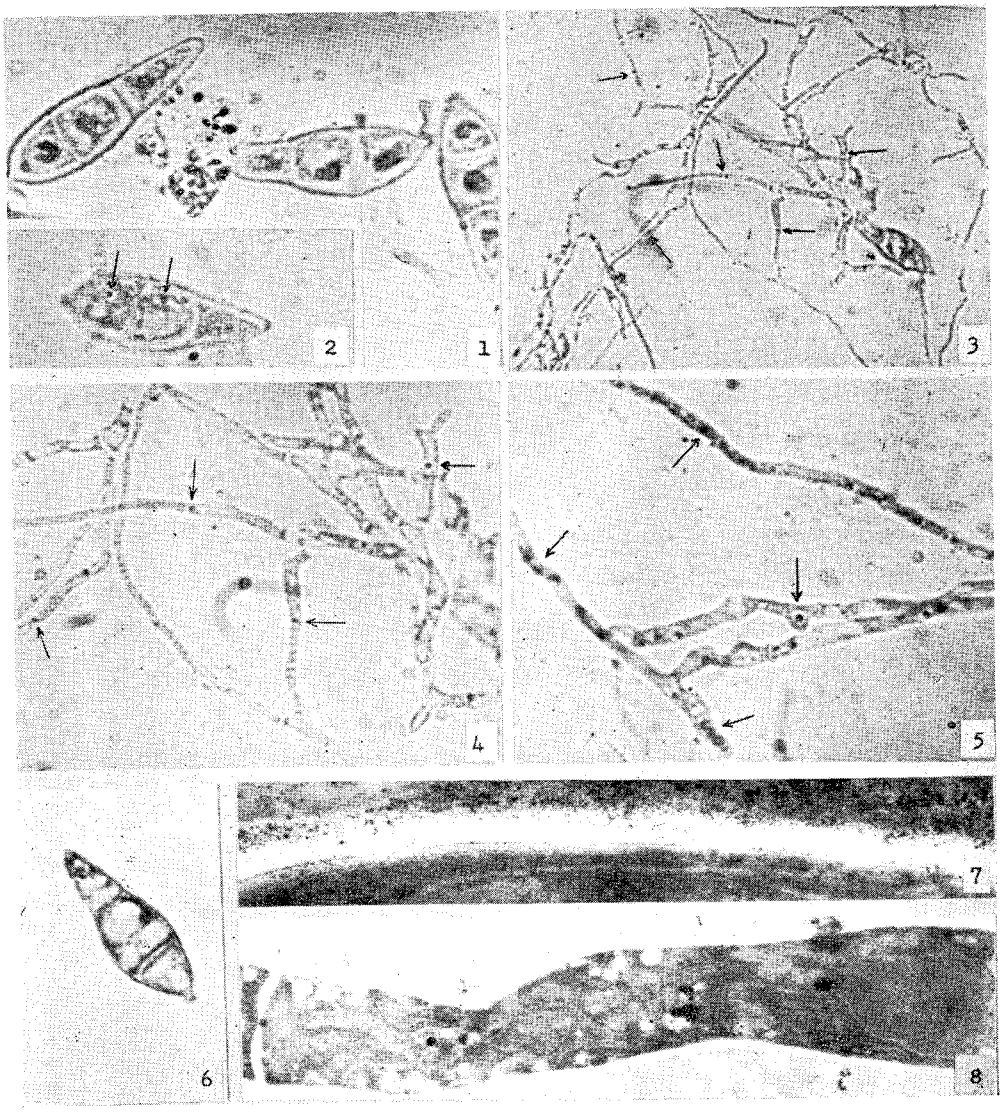
In preliminary studies race no. 13 was studied for its growth with micro-cuiture technique (Vasil & Hilderbrandt, 1965), phase-constrast microscopy and 8 mm cinamatography. Samples drawn from cultures of different intervals were fixed in Fleming strong solution or 10% formalin and stained by Feulgen and phospholipid technique respectively (Merchant *et al*, 1960).

Observations

The growth of *P. oryzae* was associated with cytoplasmic streaming toward an advancing mycelial tip and so was acropetal. New septum was formed after the tip proceeded to a length of normal mycelial cell. When temperature fixed at 28°C, it took about two hours for its growth from one cell to another. Budding could be seen from older cells and ultimately it became a new branch

Explanation of Figures

1. Zero-hour culture of conidia of no. 13, showing one dense, Feulgen reaction positive mass in each cell, ×1,200
2. Eight-hour culture, nucleus denoted by an arrow in each cell are degenerated by its size, ×1,200
3. Feulgen positive particle denoted by an arrow in the middle part of mycelial cells are noted in a culture of 21-hour of race no. 13, ×600
4. Same as figure 3, enlarged, ×1,200
5. Particles of various size are noted, some are out of focus, in mycelial cells treated with Suden black B, ×1,200
6. Zero-hour culture of conidia of race no. 13, showing phospholipid positive reaction particles in the upper and middle cell, ×1,200
7. Cell membrane of a conidial cell showing three dense layers enclosing two light spaces, ×26,000
8. The first mycelial cell derived from a germ tube. The septum with pore separate the conidial cell and the growing mycelium in which nucleus has resumed its normal size. Mitochondria and endoplasmic reticulum are also noted, ×4,700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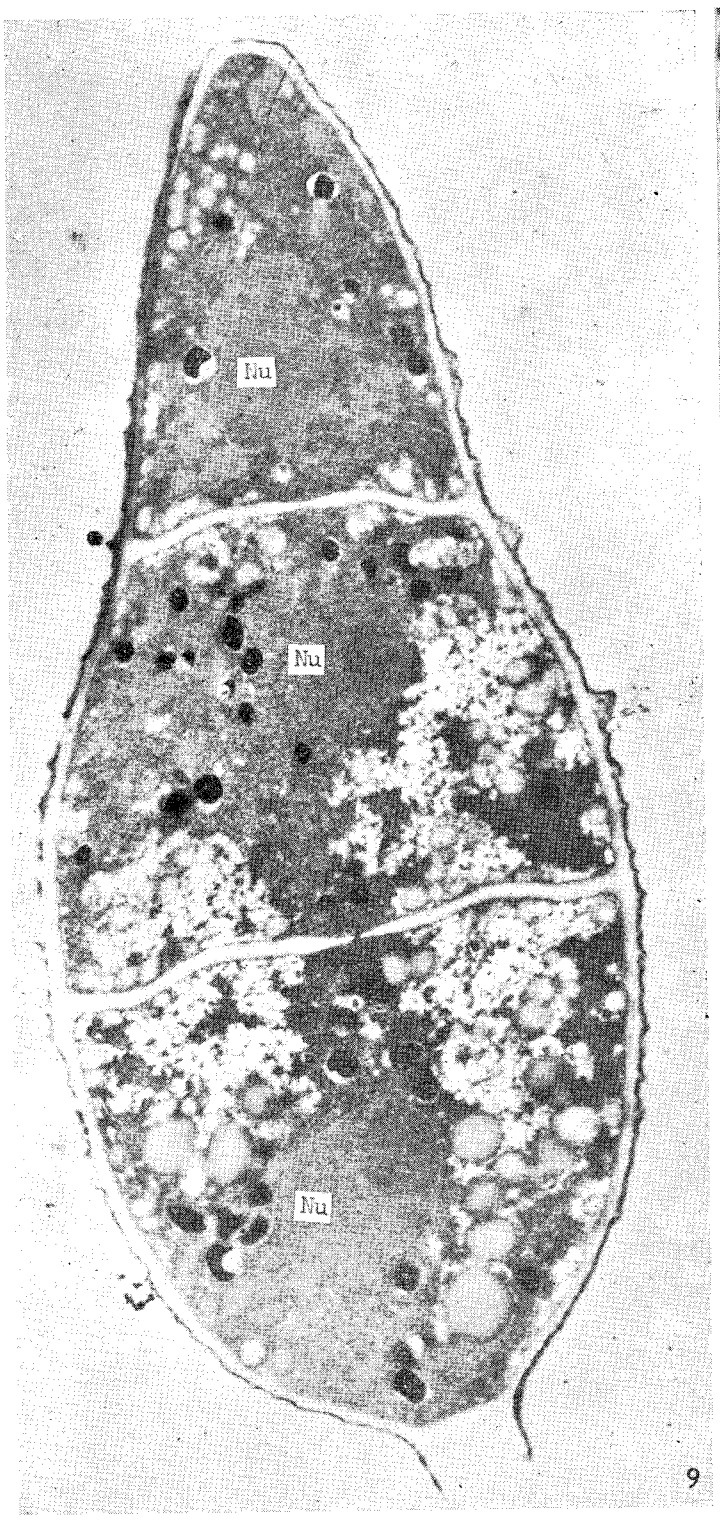
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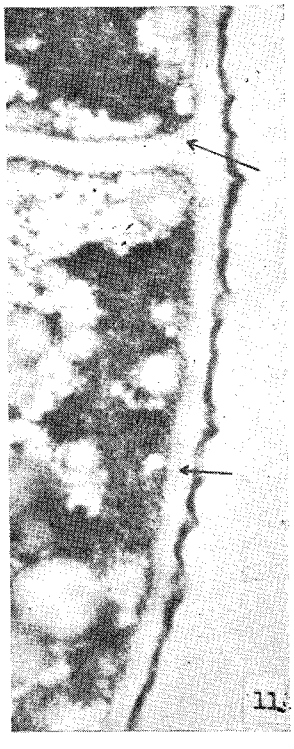
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of mycelium. During growth the cytoplasmic streaming was active both in conidial and mycelial cells and the small, black particles was busy in moving along with the stream. In germinating conidia, the center part of all three cells each had a dense, rounded mass surrounded by vigorously moving particles. This rounded dense mass was supposed to be nucleus.

Feulgen technique is perhaps the most specific staining technique available for demonstrating DNA. As conidia treated with Feulgen, the parts which show positive reaction coincided with the parts where nucleus was supposed to be located. Thus, the observed germinating (mostly from zero-hour culture, figure 1) or germinated (from four- or eight-hour culture, figure 2) conidia seemed to have one and only one prominent nucleus in each cell although nuclei of longer culture were much smaller (cf figure 1 & 2). In mycelia of twenty-one-hour culture there was still one nucleus in each cell (figure 3, 4). Obviously, the size of nucleus was smaller than that of germinating conidia but it is comparable with that in germinated conidia.

Phospholipid technique had been carried out for the demonstration of mitochondria and phospholipids. Blue-black particles of various size and number had been observed in different cells of conidia and filamentous mycelia treated with this technique. One common feature was that these particles wherever located do not occupy the location of nucleus (figure 5, 6). However, it could not be decided whether a certain particle was a mitochondrium or a phospholipid.

The fine structure of the following components: cell wall and septa, cell membrane, vacuole, nuclei, mitochondria, endoplasmic reticulum, and various cytoplasmic inclusions was studied in thin sections of *P. oryzae* conidia and mycelia.

Cell wall consisted of four layers in total; one middle lamella, two layers of wall deposit against both sides of the middle lamella and one fibriform meshwork of great density (figure 9, 11). The outmost meshwork layer was similar to that described in *Neurospora crassa* mycelia (Shatkin, 1959). Septa comprised one layer less (the outmost one) than cell walls and pores had been noted in both conidial and mycelial septa (figure 8, 9). The pores, probably

Explanation of Figures

9. Germinating conidium of race no. 17, each conidial cell has a nucleus (Nu) with nucleolus. Small pores can be seen in septa. Mitchondria is prominent around nucleus of upper conidial cell, ×10,000
10. A pore in the septum of a germinating conidium. Cytoplasmic stream seems pass through this small opening, ×20,000
11. Cell wall of a mature conidium, showing middle lamella (denoted by an arrow), deposited wall material and the out most meshwork, ×20,000

one for each septa, were obviously located at the very center of rounded septum because it could be seen only in those sections cut right through the center (figure 9, 10). It seemed that the pore was formed by less and less deposit against the middle lamella near by the central area and the failure of complete fusion of septum.

Cell membrane appeared as three dense layers enclosing two light space (figure 7). No invaginations occured in the cell membrane of conidia observed.

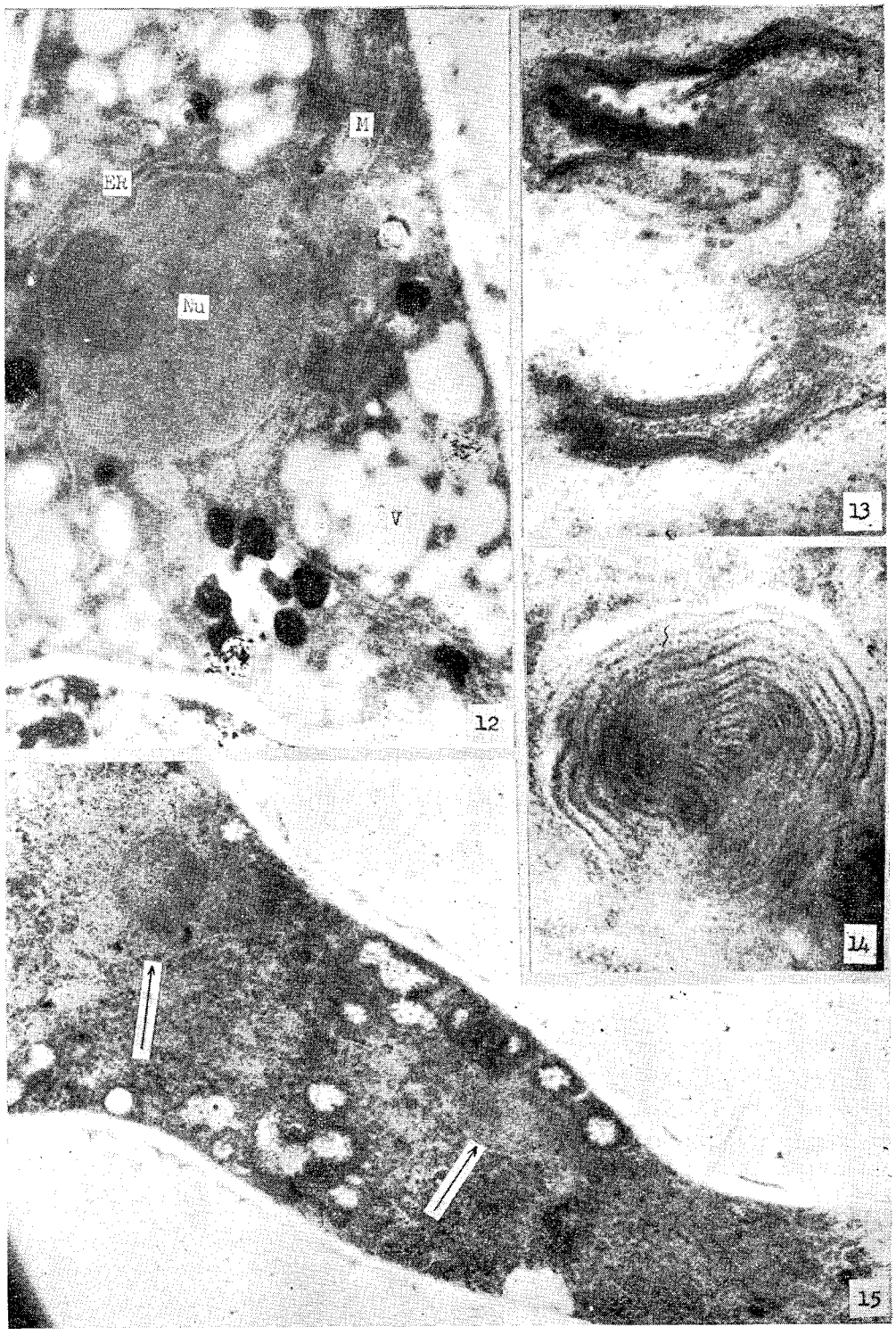
Small vacuoles were usually uniformly arranged along the inner side of cell membrane in the germinating conidia (figure 9). They became large as the germ tubes grew longer. Large vacuoles were also numerous in older mycelia.

Several membranous systems had also been found in mycelium or conidium of 4- or 24- hour culture (figure 13, 14).

The nucleus was recognizable morphologically. It was a round or oval body with a double membrane and it was comprised two kinds of granules, fine and coarse (figure 12). The coarse granules sometimes confined itself at one conner and was comparable to the nucleolus of high plant such as Vicia faba (Lafontaine *et al*, 1963). No pores had been found on the nuclear envelope. Nucleus in conidial cells was about 2μ in diameter and occupied mostly in the central part of the cell (figure 9). It was conceivable that no second nucleus existed in the same cell because if it were possible the second nucleus would have been appeared in sections of such a narrow compartment. However, we did find two daughter nuclei in a germinated conidial cell (figure 15). These nuclei were obviously smaller in size in comparison with that of undivided ones. Furthermore, one daughter nucleus had been migrated to the lower side of the cell and the other one proceeded toward the germinating tube. Nucleus in the first mycelial cell which was recovered from the germinating tube resumed its normal size as that observed in matured mycelial cells (figure 8). In *P. oryzae*, mycelial cell was long enough not to be cut through its right longitudinal phase. However, by open-face embedding we did have some matured mycelial

Explanation of Figures

12. Upper conidial cell of another germinating condidium of race no. 17. Nucleus (Nu) has its pseudopodal extensions. Vacuoles (V) large, endoplasmic reticulum (ER) can be seen near by nuclear membrane. Mitochondria (M) are classical. ×17,000
13. Myelin forms, appeared in a mycelial cell of 24-hour culture of race no. 13. ×85,000
14. The tiny membranous system appeared in a conidium of race no. 13 of 4-hour culture, note the neat arrangement of this system, ×100,000
15. One germinated upper conidial cell with two small daughter nuclei (denoted by an arrow) each with nucleolus. One daughter nuclei is migrating toward the germinating tube, ×9,500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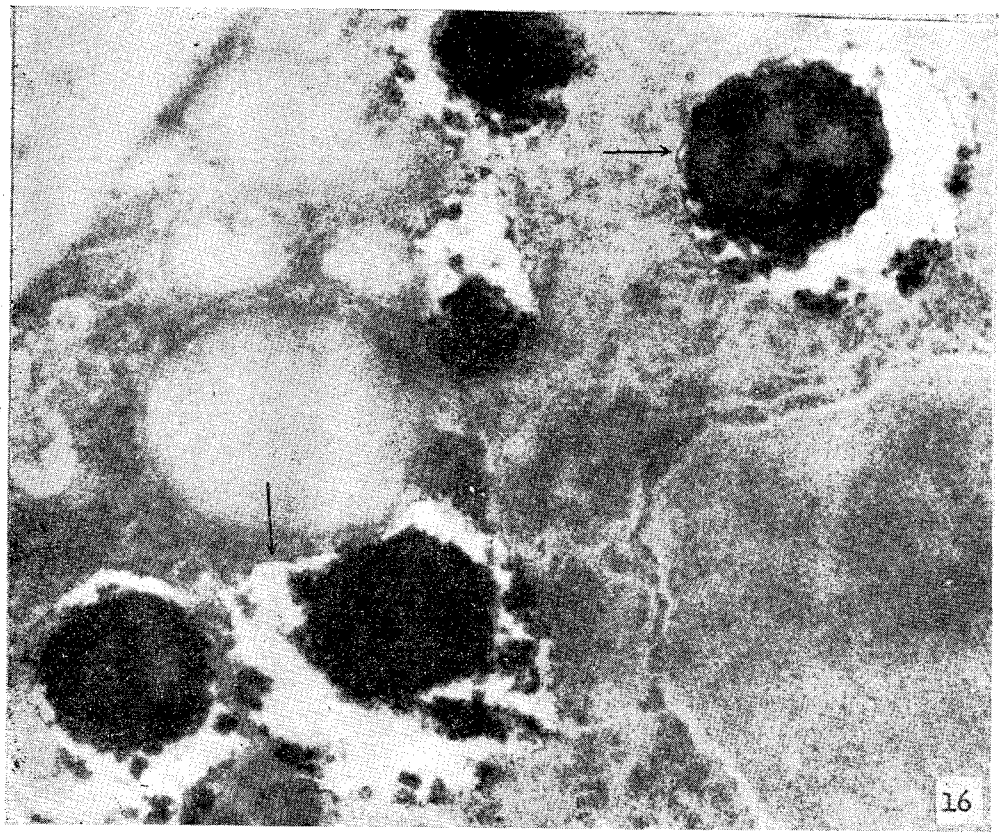

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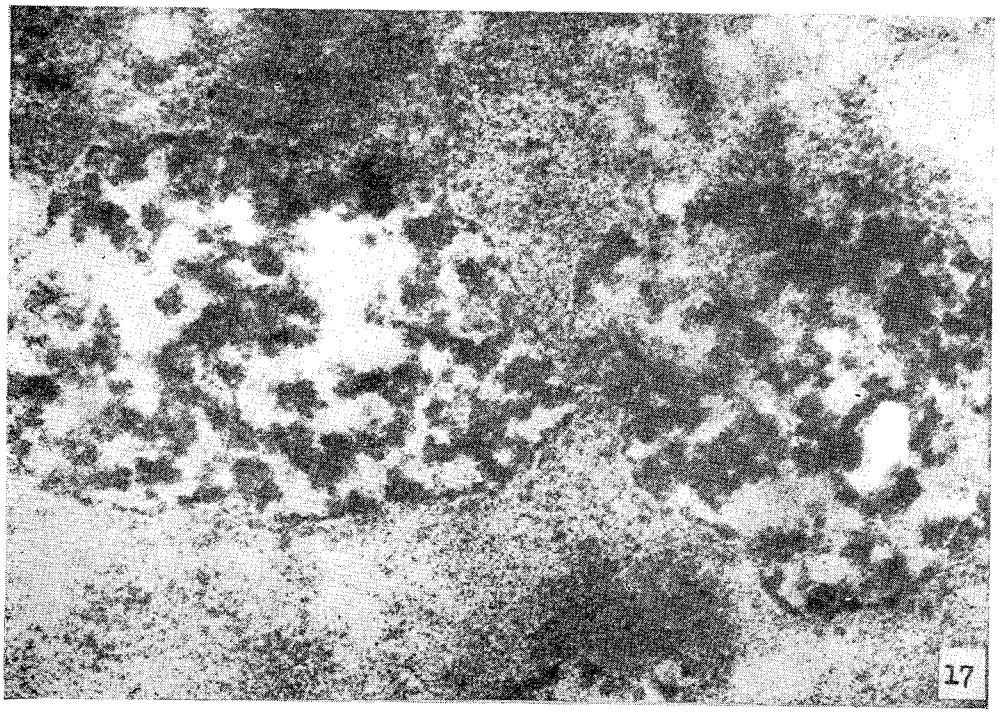

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cells cut so and examined carefully the number of nucleus within each cell. One and only one nucleus appeared in each cell. No chromosomes had been observed in nucleus of different metabolic state—resting, germinating and germinated conidial cells as well as in dividing mycelial cells. While in some cells the nuclei may have pseudopodal extensions (figure 12).

The peripheral region of the cells sometimes had long, smooth double-membrane elements, the endoplasmic reticulum (figure 7, 8 & 9). It also spreaded within the cytoplasmic interior and sometimes it connected with the nuclear envelope (figure 12).

Mitochondria in *P. oryzae* were classical. They appeared in sections elongated, circular, and irregular profiles and distributed mostly around the nucleus (figure 8, 9 & 12) and in the growing tips.

At least two kinds of dense particles had been observed. One kind of the particles had their diameter of 0.8μ. They were not sphere, often with a small electron light center and a unit membrane (figure 17). Another kind of dense particles was a sphere of 0.4 to 0.8μ in diameter. It had a dense stroma and a clear cut unit membrane. However, the dense stroma became somewhat aggregated (figure 16).

Discussion

The fine structure of *P. oryzae* had been studied by Mizusawa (1959) and his report revealed that at least three layers laminating the cell membrane. He also found lipid granules and vacuoles in conidia of 10-day and 20-day culture respectively. Although this report presents much more details we are still not sure in many points. Right now it is interesting to note the number of nucleus of this organism. Evidences given in this study by Feulgen technique and EM micrographs of cells of different races (no. 5, 13 & 17) leads us to a conclusion that *P. oryzae* is uninucleated which is discrepant to the results reported by Suzuki (1967) and Chu *et al* (1965). *P. oryzae* produces asexually, and if it is multinucleated we shall be in a state of great difficulty in studying its variability. However, the discovery of uninucleated cells of *P. oryzae* would initiate someone to study the phenomenon of heterocaryon. Heterocaryosis might be the main origin of variability in *P. oryzae* as Suzuki suggested (1967).

Explanation of Figures

16. The unit membrane of this dense particles is clear and its content has been aggregated. Morphologically, dense particle of this kind is similar to the lysosome appeared in other organisms, ×35,000
17. Enlargement of observed dense particles in a cell of matured conidium. The particle has a unit membrane, lightly void center and condensed amophous contents, ×68,000

In heterocaryotic stocks, there must be two haploid nuclei in each cell. Multinucleated mycelia has been reported in *Neurospora crassa* by Shatkin *et al*, (1959) and they concluded that several oval, Feulgen-positive bodies 1 to 2μ in diameter occur within each cell. However, they did not show the micrograph with several nuclei in a cell. Wells' micrograph (in Robbins *et al*, 1964) in which four haploid nuclei after meiosis existed in a basidial cell of *Schizophyllum commune* illustrates the possibility of analyzing the fine structure of multinucleated cells.

Another interesting feature is the division of nucleus. Suzuki (1967) reported that somatic nuclei of *P. oryzae* seems to be divided by typical intranuclear mitosis in essentially the same as those of higher organisms. Chu *et al* (1965) presented their result that each nucleus had 4 chromosomes and divided with classical manner of mitosis. Having reviewing these papers we expected that some chromosomes, spindle fibers, metaphase plate or so would appear in our micrograph of nucleus. As previously mentioned, these organelles have not been observed. The pseudopodal extentions of nucleus and the divided daughter nuclei in a germinated conidial cell (figure 12, 15) lead us suggest that the division is amitotic as Bakerspigel reported in Fungi imperfecti *Phyllosticta sp.* (1959) and *Scopulariopsis brevicaulis* (1960). Details are subjected to further studies.

Lysosomes are characterized by its unit membrane and the presence of one or more acid hydrolases (Gahan, 1967). Hence, it is too early to assess the dense particles of 0.4–0.8μ in diameter to be lysosome (figure 16) by morphological criterion only. Histochemical study is necessary to verify this fancy.

Some membranous system (figure 13) is comparable to the myelin forms found in human brain (Vogel, in Frey-Wyssling *et al*, 1965). Phospholipids is one of the polar lipids that give rise to so-called myelin forms (Frey-Wyssling *et al*, 1965). It is suggested that some Suden black B positive particles observed in our preliminary studies are phospholipids. In addition, Mitochondria are known to be Suden black B positive. Thus, the moving, blue-black particles in optical microscopy might coincide with mitochondria, myelin forms in the fine structure analysis of *P. oryzae*.

Technically, open-face embedding method (Elio sparvoli *et al*, 1965) combined with the mica sheet technique (Persijn *et al*, 1965) substantially help us to set the problem of orientation in electron microscopy. Therefore their suggestion deserve to be highly recommended.

Summary

The fine structure of *Piricularia oryzae* from various culture period has been studied. Other than the common structural organelles such as cell walls,

cell membrane, mitochondria, endoplasmic reticulum, vacuoles, there are two kinds of dense particles existed. The authors suggested that one of them might be morphologically lysosome.

Evidences given in this study by Feulgen technique and EM micrographs of cells of different races lead us to a conclusion that *P. oryzae* is uninucleated which is discrepant to the results reported by Suzuki (1967) and Chu *et al* (1965) but does support the conclusion drawn by Yamasaki *et al* (1965).

水稻稻熱病病原菌的細微構造

吳信淦 曹德輝

水稻稻熱病病原菌三種生理小種的胞子經不同時期培養後用電子顯微鏡技術研究其細微構造。除一般通有的細胞壁，細胞膜，粒線體，內質網，液胞等外，尙有二種組織較密的小粒，其中之一形態上極似已在其他生物體內發現的溶酶體。

從 Feulgen 氏反應及電子顯微鏡下的相片，作者發現此種病原菌胞子和菌絲細胞內均祇有一個細胞核。這與鈴木(1967)，朱等(1965)所得多核的結論相反，但支持山崎等(1965)的研究結果。

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