Organogenesis in the meristem cultures of cymbidiums

V. Anatomical and histochemical studies on phagocytosis in the mycorrhizome of *Cymbidium goeringii* REICHB. f. (*C. virescens* LINDL.)

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Summary

Anatomical and histochemical studies on phagocytosis were carried out with the juvenile portion (digestion was in an early stage) and the mature portion (digestion was in a late stage) of the wild rhizome of *Cymbidium goeringii* REICHB. f. Using PAS reaction and ruthenium red method, it was detected that the positive substance with PAS reaction in fungal clot was mainly pectin-like substances. The results with ninhydrin-Schiff's reaction and azure B method suggested that protein and RNA of fungi were gradually decomposed in the course of digestion. Remarkable increases in size and DNA content in host- and digestion-cells were indicated by the result with Feulgen method. The significance of phagocytosis in the transference of nitrogenous compounds was discussed in connection with the organogenesis of this orchid.

Introduction

It has been demonstrated in the previous papers of this series[10,11,12,13] that the terrestrial Cymbidium such as *Cymbidium goeringii* REICHB. f. is somewhat different from the semi-epiphytic Cymbidium in requirement for shoot formation. Rhizome-tips of *C. goeringii* cultured on Knudson C medium with Nitsch's microelement, develop into the rhizomes which show no tendency to make shoot. However, the addition of 10 mg/*l* kinetin to the medium induced shoots in most of samples in the dark.

In the wild condition, after the seeds of *C. goeringii* germinate, they form the rhizomes which grow into the ground. The rhizomes, in which abundant mycorrhizal fungi are always observed, produce shoots after a period of underground life.

The possibility is considered that the necessary substances for shoot formation are supplied to the orchid through the digestion of fungi.

Although phagocytosis in mycorrhizae and mycorrhizomes of orchids has been investigated by BERNARD[1], BURGEFF[2] and many other workers, comparatively little are known of the chemical changes of the fungal matter in the course of digestion.

In the present paper, anatomical and histochemical studies on phagocytosis in the mycorrhizome of *C. goeringii* are described and the significance of the result is discussed in connection with the organogenesis of this orchid.

Materials and Methods

The rhizomes in a state of nature of *Cymbidium goeringii* REICHB. f. were collected monthly from September 1968 to August 1969 in Sanageyama, Aichi Prefecture. Anatomical and

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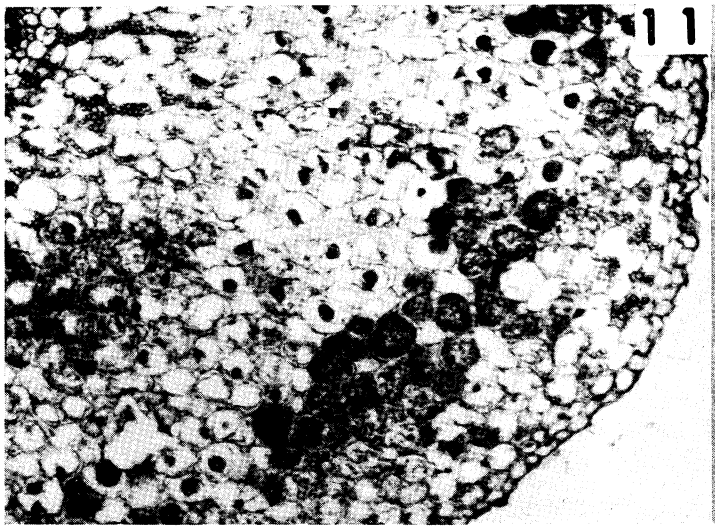
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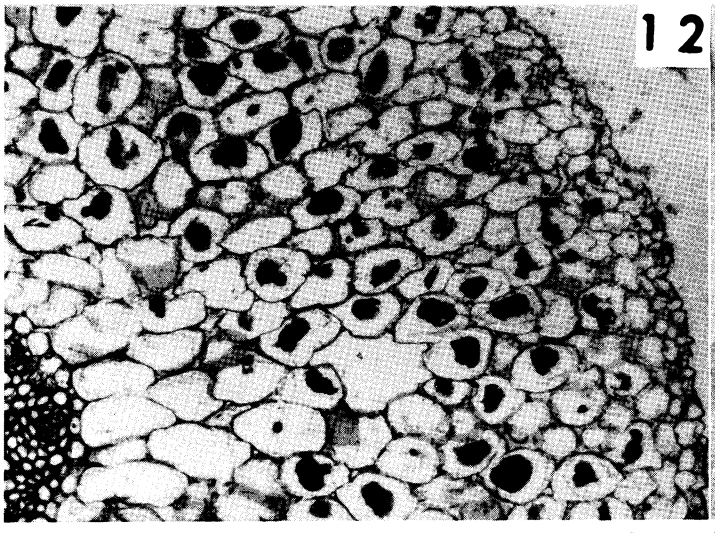
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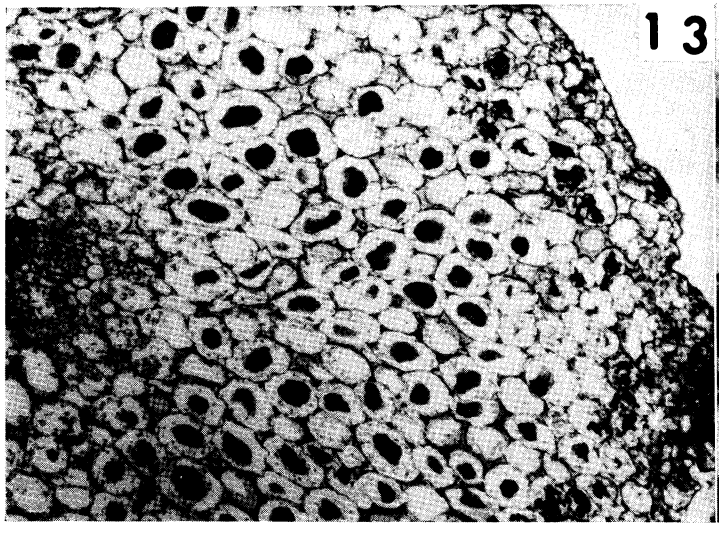
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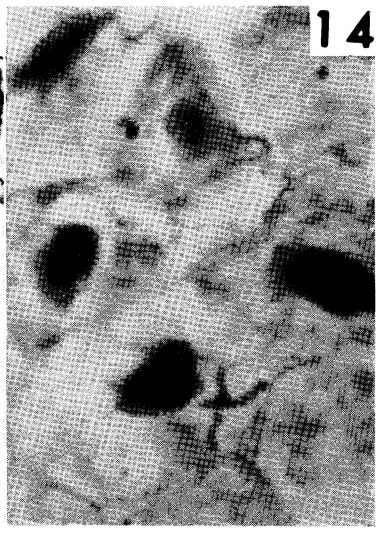
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histochemical studies were carried out with the juvenile portion (white-coloured) and the mature one (brownish yellow-coloured) of the rhizome.

The rhizomes to be used for anatomical and histochemical sutdies were fixed in FAA (formalin-alcohol-acetic acid). After getting dehydrated and paraffin embeded, the tissue was cut into transverse sections at 10 μ. For the lipid test rhizomes were cut on a freezing microtome at 30~40 μ.

Staining. For anatomical study the sections were stained with safranin. PAS reaction[4] was used for detection of total carbohydrates of insoluble polysaccharides. Starch was stained with potassium iodide-iodine[4] and pectic substances were stained with ruthenium red (1 : 5000)[4]. Ninhydrin-Schiff's reaction[4] was employed for detection of total protein. DNA and RNA were detected by using both azure B method and methyl green-pyronin method[4]. Controls were run through differential extraction with 0.1% ribonuclease at pH 6.8 for 1 hour at 40°C[4]. Feulgen method[4] was also employed for detection of DNA. The unfixed sections of the rhizome cut for lipid determination were stained with Sudan III[4].

Results and Discussion

Observations on the organ formation in the wilds. Observation on the organ formation of *C. goeringii* were carried out throughout a year in Sanageyama, Aichi Prefecture. The small rhizomes, in which no shoot formation occurred yet, were often found in the humus surrounding the root of the adult orchids in every season. It was observed that shoot formation occurred in some of small rhizomes in April or May and root formation was followed about a month later. The successive stages of organ formation in the wilds are shown in Fig. 17.

Anatomical observation. Juvenile rhizomes. As shown in Fig. 11, staining with safranin well differentiates fungal hyphae from fungal clots: hyphae stain pink-red but clots stain deep red. Cortex can be separated into three regions of host-cells, digestion-cells and storage cells. The host-cell region consists of 3~5 layers in which fine hyphae are distinguishable. In the middle of th cortex, there are 6~8 layers of digestion-cells in which fungi degenerate and clots are present. These aspects observed in the juvenile rhizome would represent an early stage of the digestion process. The 3~5 innermost layers of the cortex, the storage region, contain a large amount of starch but no fungal matter. It is especially interesting that a large amount of starch is present even in the juvenile rhizome without upper-ground part.

Mature rhizome. Fungal clots are found here and there in the cortex except storage region but few hyphae are observed (Figs. 12, 13). These aspects would represent a late stage of the digestion process.

The mature rhizomes collected in winter or early spring preserved a considerable amount of starch in the storage region (Fig. 13), whereas those collected in summer or autumn contained no starch (Fig. 12).

Since the growth of the orchid stops in winter, carbohydrates produced in leaves by photosynthesis may remove to the rhizomes.

Total carbohydrates of insoluble polysaccharides—Using PAS reaction, fungal hyphae, fungal

clots, cell walls and starch grains stained purplish red deeply in the juvenile rhizome (Fig. 6). The fungal clots in the mature rhizome stained as intensely as those in the juvenile rhizome (Fig. 5).

Pectic substances. With ruthenium red method for pectic substances, fungal clots and cell walls stained red strongly but fungal hyphae stained somewhat weakly in the juvenile rhizome (Figs. 7). The fungal clots in the mature rhizome stained as strongly as those in the juvenile rhizome (Fig. 8).

BURGEFF[2,15] described in his paper that fungal hyphae contained a considerable amount of glycogen. In the present study DIMEDON-PAS reaction[6] with a suitable fixation was tried for detection of glycogen, but manifest result was not obtained. Probably hyphae contain glycogen or some other polysaccharides which are digested at the beginning of the digestion process. Positive reaction with PAS in fungal clots would depend mainly on pectic substances which remain as undigested material.

Total protein. Fungal clots gave a positive reaction with ninhydrin-Schiff's reagent both of the juvenile and mature rhizome. The reaction was, however, less intense in the mature rhizome (Figs. 14, 15). The result suggests that protein of fungus is gradually decomposed in the course of digestion.

Nucleic acids. Nucleic acids (DNA and RNA) were detected by methyl green-pyronin method. Whereas nuclear DNA stained green-blue, nucleoli and fungal clots stained pink-red. As further tests to confirm existence of RNA in fungal clots, azure B method and differential extraction with ribonuclease were carried out. With azure B method nuclei stained green-blue and clots stained purple strongly in the juvenile rhizome (Fig. 1). In the mature rhizome, however, clots stained purple but intensity of staining was low (Fig. 2). In the section stained after treatment with ribonuclease, purple colour in the fungal clots disappeared (Fig. 3). The result described may indicate that a considerable amount of RNA exist in the fungal clot and are gradually decomposed in the course of digestion.

Size and DNA content of nucleus. Size and DNA content of nucleus were inspected with the sections stained with Feulgen reagent. Diameters of nuclei were 5~6 μ in the cortical cells of the apical region where no infection occurred, whereas those in the host- and digestion-cells were 20~40 μ. Although no photometric measurement was employed, a remarkable increase appears to occur not only in size but also in DNA content by infection of fungi (Figs. 9, 10). A recent report by WILLIAMSON and HADLEY[14] described the measurement of relative DNA content of nuclei in asymbiotic and symbiotic protocorm of *Orchis purpurella* by Feulgen photometry. In the cortical cells of symbiotic protocorms nuclear DNA classes

Fig. 1 (juvenile rhizome), Fig. 2 (mature rhizome) and Fig. 3 (juvenile rhizome treated with RNAase); DNA and RNA stained with azure B. ×600. Fig. 4; DNA and RNA stained with methyl green-pyronin (juvenile rhizome). ×600. N; nucleus, F; fungal clot. Fig. 5 (mature rhizome) and Fig. 6 (juvenile rhizome); insoluble polysaccharides stained with PAS reagent. ×600. Fig. 7 (juvenile rhizome) and Fig. 8 (mature rhizome); pectic substances stained with ruthenium red. ×600. Fig. 9 (nuclei in the digestion-cell region) and Fig. 10 (nuclei in the cortical cells of the apical region); DNA stained with Feulgen reagent. ×600. Fig. 11 (juvenile rhizome). Fig. 12 (mature rhizome collected in autumn) and Fig. 13 (mature rhizome collected in early spring); rhizomes stained with safranin. ×180. Fig. 14 (juvenile rhizome) and Fig. 15 (mature rhizome); total protein stained with ninhydrin-Schiff's reagent. ×600. Fig. 16; lipids stained with Sudan III (juvenile rhizome, unfixed). ×600.

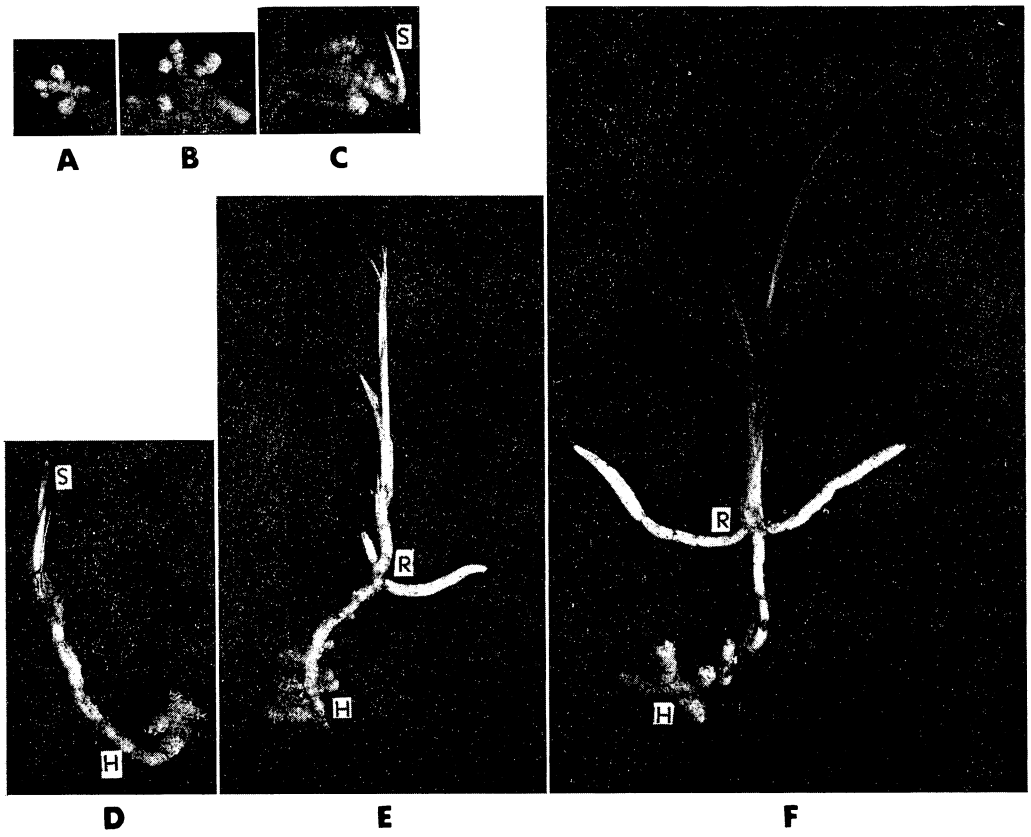

Fig. 17. Organogenesis of *Cymbidium goeringii* REICHB. f. (*C. virescens* LINDL.) in the wilds (A—F).
A and B; small rhizomes, before shoot formation.
C and D; rhizomes producing shoots.
E and F; young plants with rhizomes.
H; rhizome. R; root. S; shoot.

were between 16 c and 128 c. The appearance of new DNA classes was also reported in pea root nodules (MITCHELL[7]). The significance of this phenomenon is not yet clear.

Lipids. The unfixed sections cut on a freezing microtome for lipid determination were stained with Sudan III. Digestion-cells contained numerous lipid droplets both in the juvenile and mature rhizome (Fig. 16).

Carbohydrates and nitrogenous compounds must be supplied from mycorrhizal fungi to holosaprophytic seedlings of terrestrial orchids the same as to the permanently leafless orchids. BURGEFF[2,15] postulated that number and distribution of phagocyte cells correspond to the degree of saprophytism. The existence of numerous phagocyte cells in the rhizome of *C. goeringii* suggests that phagocytosis has a important meaning in the development of this species in the wilds.

Transference of carbohydrates from fungus to plant has been well established by many workers. However, there has been little evidence as to transference of nitrogenous compounds from fungus.

From the result of this study it is suggested that protein and RNA in the fungal matter

are decomposed in the course of digestion. The writers (unpublished) have also obtained an evidence for this by paperchromatography. The extract of symbiotic rhizome contained much more amino acids, purines, and pyrimidines than those found in the extract of asymbiotic rhizome.

Although there is no direct evidence yet, it would be conceivable that not only carbohydrates but also amino acids and decomposites of nucleic acids are offered to the orchid through the digestion of fungi and give a accerelating effect on its shoot differentiation.

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Cymbidium の生長点（茎頂）培養における器官形成（第5報）

野生シュンランの根茎における食菌現象 phagocytosis の解剖学的，組織化学的研究

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摘 要

愛知県猿投山一帯のシュンラン自生地において1年間にわたり，その生態を観察し，且毎月根茎 rhizome を採集した。根茎の若い部分（外見白色，内部は菌消化の早期の様相を示す）と成熟した部分（外見黄褐色，内部は菌消化の進んだ様相を示す）に分けて，解剖学的，組織化学的にしらべた。得られた主な知見は次の様である。

1. PAS 反応並びに ruthenium red 染色による結果から，消化の進んだ菌塊には PAS 反応に強く染まる物質が多く含まれ，これは主として菌の細胞壁に由来するペクチン様物質であろう。

2. Ninhydrin-Schiff 反応の結果から菌のタンパク質は消化の過程で除々に分解されると考えられる。

3. Azure B 染色による結果から，菌は比較的多量の RNA を含み，これは消化の過程で除々に分解されると思われる。

4. 菌糸或は菌塊の存在する皮層細胞では核は著しく大形であり，又 Feulgen 染色による核の DNA 量も著しく増加していると見られる。

5. 以上の結果から根茎における phagocytosis は炭水化物のみならず，アミノ酸，核酸分解物等を供給する可能性があり，これらの物質が根茎からの shoot 分化に促進的効果をもつのではないかと考えられる。