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Microbial Colonization of the Aquatic Duckweed, *Spirodela polyrhiza*, during Development

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수생식물 개구리밥 (*Spirodela polyrhiza*)과 미생물

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ABSTRACT

Fresh specimens of the aquatic macrophyte, *Spirodela polyrhiza*, have been examined employing scanning and transmission electron microscopy. Observations revealed the occurrence of microbial colonization during development. Submerged parts of the small, free floating *S. polyrhiza* body exhibited a variety of microorganisms such as bacteria, cyanobacteria, and diatoms throughout their development. However, immature and/or young plants normally demonstrated much less microbial colonization compared to mature plants. During the study, heavy colonization by the microorganisms was routinely encountered at maturity, especially in the fully developed abaxial fronds and root caps. The mucilaginous layer was shown along the root caps, and the microorganisms appeared to be either clustered or attached to this layer. In contrast, only moderate degrees of colonization were observed in the root, and little to no colonization was observable in the adaxial frond surface. Transmission electron microscopy clearly demonstrated the microbial colonization to be external in the *S. polyrhiza* specimen examined in the current study. The association between the microorganisms and *S. polyrhiza* has been considered non harmful, as no frond senescence and almost no mechanical penetration of the plant by the microorganisms were noticed during the study.

Key words : Colonization, Microorganism, Non harmful relationship, *Spirodela polyrhiza*

INTRODUCTION

Colonization of the hydrophyte by various microorganisms is a well-known phenomenon observed in aquatic macrophytes. The submersed regions of free-floating small aquatic macrophytes are often colonized by large populations of bacteria. This occurrence has been documented in some fresh water species (Baker & Orr, 1986; Rimes & Goulder, 1986; Underwood &

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Baker, 1991). Duckweeds, including *Spirodela polyrhiza*, are members of the smallest aquatic flowering plant family, Lemnaceae (Landolt, 1998; Lemon & Posluszny, 2000). These duckweeds are readily found in ponds, streams, lakes, rice paddies, and in some fresh water marshes (Logsdon, 1989). Members of duckweeds are also known to have colonies of various microorganisms in their submerged parts (Hossel & Baker, 1979; Zuber, 1984; Duong & Tiedje, 1985; Underwood & Baker, 1991). For the duckweed-microorganism association, various degrees of both deleterious and beneficial effects have been recorded. Some of the bacteria may have harmful effects on the host macrophyte, exhibiting symptoms of either overall senescence (Underwood & Baker, 1991) or frond senescence with pits on the surface or invasion to the frond tissue (Rogers & Breen, 1981; Zuberer, 1982). However, a beneficial outcome to the host plant, whereby growth factors probably produced by epiphytic bacteria are utilized to the plants advantage, has been also reported by Duong & Tiedje (1985), whose group investigated the duckweed-cyanobacteria association among *Spirodela*, *Lemna*, and *Wolffia*. They revealed a constructive relationship between the two in which the host provided a more favorable environment for the cyanobacteria for improved nitrogen fixation. Interestingly, no significant effect on the final population density was shown in a host inoculated by cultured bacteria, whereas significantly higher levels of senescence were found in *Lemna* plants inoculated with a natural population of the bacteria (Underwood & Baker, 1991).

The present study reports the results of experiments that examine whether various epiphytic bacteria have benzeficial or unfavorable effects on the free-floating giant duckweeds, *Spirodela polyrhiza* during their growth period. The entire plant body, including the fronds, connective stalks, roots, root caps, and reproductive pockets of young and mature duckweeds were examined by using both scanning and transmission electron microscopy. The study focuses on the relationship between the giant duckweed and microbial colonization within their environment. The work presented has been carried out as part of a larger study on the structural aspects of the giant duckweed in regards to the structural and cellular aspects of *S. polyrhiza*. The structures of root system, fronds, and connective stalks will be the subject of following papers.

MATERIALS AND METHODS

1. Plant material : About 50~60 fresh specimens of *Spirodela polyrhiza* (L.) Schleiden were collected from Woopo Marsh, Changryoung, Kyungbook Province, during the years 2002 and 2003. After being transported from the collection sites, approximately 20 plants with at least two to three generations of offspring fronds connected to mother fronds with healthy stalks were selected for the following transmission and scanning electron microscopy.

2. Electron microscopy : For transmission electron microscopy (TEM), approximately 1~2 mm^2 tissues of the immature and mature fronds and roots were fixed in 3~6% glutaraldehyde in 0.02 M phosphate buffer for 3 hrs and post-fixed in 2% osmium tetroxide for 2~16 hrs. Following triple rinses in the same buffer, the specimens were dehydrated in a graded ethanol series (Kim & Kim, 2000; Ji & Kim, 2002), and embedded in a low-viscosity Spurr resin. About 80~90 nm ultra-thin sections were cut by Ultracut-S ultramicrotome using glass and diamond knives. These sections were mounted on 0.35% dichloro-ethane coated copper grids and stained with 1~2% aqueous uranyl acetate, followed by 1% lead citrate. The sections were examined and photographed with a Hitachi H-7100 TEM, operated at 75 kV, at the Korea Basic Science Institutes (KBSI), Daegu Branch.

For scanning electron microscopy (SEM), materials were fixed and dehydrated as they were for the above TEM procedures. The materials, were then substituted with isoamyl acetate three times and stored at 4°C. Following the substitution, the tissue samples were dried to critical point, coated with 20~30 nm platinum-palladium, and

examined using a Hitachi S-4200 SEM, operated at 15 kV, at the KBSI Daegu Branch.

RESULTS

The submerged parts of the small, free-floating *Spirodela polyrhiza* exhibited a variety of microorganisms including bacteria, cyanobacteria, and diatoms, throughout their development. During root initiation at early development, the microbial colonization began to take place where the prophyllous sheath covered the root primordia that originated from the abaxial frond. However, the fronds and roots of immature or young plants normally exhibited much less microbial colonization (Figs. 1-6) compared to those of mature plants. The least microbial colonization has been noticed in the adaxial frond surface (Fig. 1), while the abaxial surface showed sporadic colonization (Fig. 2). The root, root cap, and reproductive pocket were not quantitatively associated with various microorganisms at this immature stage (Figs. 3-6). Almost no colonization was seen at the point of root-root cap insertion, and no heavy bacterial colonies were visible in the rhizosphere of immature roots (Fig. 4). While stalk connecting fronds did not show the microbial association even at a mature stage (Figs. 7-8), extensive colonization by microorganisms was routinely encountered at maturity in a number of structures, especially in fully developed abaxial fronds (Figs. 9-11). No microorganisms, however, were detected within the epidermal or mesophyll cells of the frond (Figs. 12-13). The root caps were also associated with numerous microorganisms, and they appeared to be either clustered or attached to the mucilaginous layer (Figs. 14-15). In contrast, only moderate degrees of colonization were observed in the elongated root, and the presence of microbial colonies was sparse in the junction where the root cap inserted into the root. None of the aforementioned microorganisms were detected in the epidermal, mesophyll, or cortical cells of the fronds, stalks, roots, and root caps, except in one case where microbial invasion was observed in the outermost root cap cell (Fig. 16). The reproductive pockets that enclosed offspring generations of duckweeds demonstrated a small number of microorganisms only on the covering sheath of the pocket (Fig. 17). However, this occurrence was uncommon.

Transmission electron microscopy clearly demonstrated the microbial colonization to be external in *Spirodela polyrhiza*. No invasion of bacteria or other microorganisms was observed throughout the examination (Figs. 6, 12, 13, 14, 15), except in the one and only case mentioned earlier. Hundreds of ultra-thin sections of epidermis and mesophyll layers of the fronds, epidermal and cortical layers of connective stalks and roots, and three layers of root cap cells were carefully surveyed, yet no signs or symptoms of the microbial intrusion within the young and mature duckweeds could be detected. Although quantification of nitrogen fixation, a common measure used to prove whether a microbial association is beneficial or not, has not been attempted currently, the association between the microorganism and *S. polyrhiza* have been considered to be at least non-harmful, as the plants were healthy, without any frond or root senescence or no mechanical penetration by microorganisms, throughout development.

DISCUSSION

It has been well established that a majority of aquatic plants are frequently colonized by a variety of microorganisms in their submerged regions. As in other aquatic species, the submerged parts of the free-floating duckweed *Spirodela polyrhiza* exhibited a variety of microorganisms including bacteria, cyanobacteria, and diatoms, throughout their development. However, it is likely that the mature duckweeds associate more with the microorganisms than do immature and young plants. Among the examined duckweed surfaces, extensive colonization occurred most frequently on the abaxial surface of the

mature fronds where roots attached. The smallest degree of microbial colonization was notable in the adaxial frond surface. The root caps were also associated with microorganisms somewhat heavily around the mucilaginous layer. However, microbial colonization in the elongated root was moderate and sparse in the junction where the root cap inserted into the root. These results contrast with those documented in the study by Zuberer (1984), who described rather heavy associations. The connective stalks and reproductive pockets showed less colonization after the adaxial fronds in this study. Depletion of sugars from the leaf surface was suspected during bacterial colonization, as a considerable variation on the capacity of bacteria to deplete leaf surface sugars probably exists among aquatic species (Mercier & Lindow, 2000). Plants with high microbial colonization probably depleted more surface nutrients than plants with low colonizations. However, the physical accessibility of residual sugars on colonized leaves may be restricted to microorganisms due to limitations in wettability and/or diffusion of nutrients in the leaf surface (Mercier & Lindow, 2000). There is some information on carbon leaked or exuded by intact duckweed plants. Approximately 2% of the carbon fixed by duckweeds is recorded to be secreted as dissolved organic carbon, in which the epiphyte plays a role as a carbon sink (Satake & Shimura, 1983). This supported the notion that some of the carbon fixed by the macrophyte is transferred to heterotrophic epiphytes (Baker & Farr, 1982). The small floating duckweeds are known to serve as hosts for a nitrogen-fixing epiphytic microflora (Zuberer, 1982, 1984).

Association of substantial microbial biomass with active duckweed populations has been known to occur in *Lemna minor* and *Spirodela oligorhiza*. A portion of the nitrogenase activity associated with well-developed duckweed mats is possibly mediated by heterotrophic bacteria (Zuberer, 1984). Unlike the findings of the present study, Zuberer (1984) showed that bacterial colonization occurred within senescent frond cells of duckweeds that were collected from different locations. The invasion of microorganisms into the plants is likely due to an easy mechanical penetration attributable to the aging process and the increased physical accessibility that results from it. Another possible explanation is that the populations are expected to vary from one site to another depending on the unique environmental characteristics of the habitat where duckweeds grew. Environmental factors such as amount of light, temperature, and nitrogen and phosphorous concentrations are expected to exert some influence on the degree of microbial colonization in duckweed species (Zuberer, 1984). According to the work of Cohen & Yamasaki (2003), when a certain bacterial strain isolated from the aquatic fern, *Azolla pinnata*, is inoculated again onto *A. pinnata* fronds, a surface-sterilant resistant density is established without causing any harm or disease.

In the submerged angiosperms, inorganic nutrients are known to be transported generally from the root to the frond (Pedersen & Sand-Jensen, 1993). Higher concentrations of diffusible ions have been found in root tip cells of *L. minor* (Echlin et al., 1979, 1980, 1981, 1982). However, the degree of root involvement in the uptake of nutrient in *S. polyrhiza* is still controversial, as effective and ineffective cases are both known. In many *Lemna* and *Spirodela* species, water absorption and nutrient uptake supposedly take place on the abaxial frond surface (Muhonen et al., 1983; Ice & Couch, 1987; Meijer & Sutton, 1987). Since a definite involvement of the fronds and roots in nutrient absorption remains to be clarified, the microbial associations in *S. polyrhiza* will be more conclusive when their nutrient uptake pattern is revealed.

Aquatic plants are known to take up xenobiotic compounds from water and biotransform them in conjunction with the associated microbiota (Federle & Schwab, 1989). As observed in the present study, numerous roots of *Lemna* and *Spirodela* species showed routine colonization by a variety of microorganisms including bacteria, cyanobacteria, and diatoms (Zuberer, 1982, 1984; Duong & Tiedje, 1985). Such colonization by large

populations of epiphytic bacteria can either be deleterious or beneficial to the plant. Duong & Tiedje (1985) reported that cyanobacteria appear to benefit more than its host, the duckweed, by using the plant for physical support, protection against direct sunlight, and as a source of carbohydrates and growth factors, although commensalism has been suspected. On the other hand, significantly higher levels of senescence were shown in *Lemna* when inoculated with a natural population of bacteria (Underwood & Baker, 1991). It is possible to account for the occurrence of colonization in *S. polyrhiza* as being non-harmful, as no frond senescence or mechanical penetration of the host cell wall by cyanobacteria or bacteria were observed during the study. Since the quantification of nitrogen fixation measured by acetylene conversion to ethylene and commonly employed in the study of duckweed blooms (Duong & Tiedje, 1985) may better prove a physiological microbial association in *S. polyrhiza*, the measurement of nitrogen fixation is suggested for a categorical assessment of the duckweed-microorganism association.

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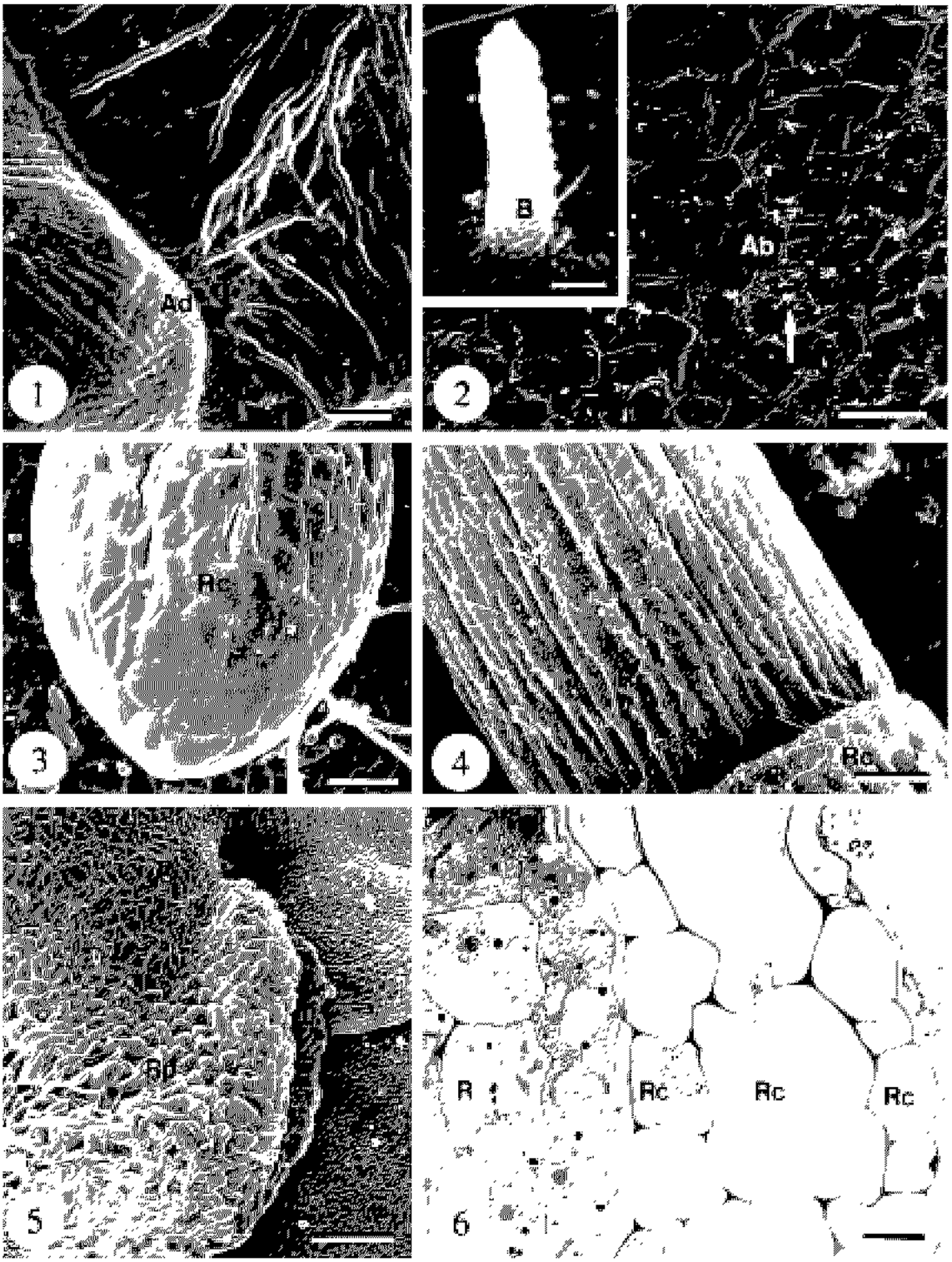
<국문초록>

수생식물 개구리밥(*Spirodela polyrhiza*)의 식물체 발달 과정에서 나타나는 미생물과의 상호관계를 주사전자현미경 및 세포학적으로 추적하여 연구하였다. 개구리밥은 부유성 식물로 분화 발달 초기부터 잠수부위에 여러 종류의 박테리아, 남조류, 규조류 등이 서식하였다. 미분화된 어린 식물체에는 성숙 발달한 식물체에 비해 미생물들이 낮은 빈도로 출현하였고, 성숙한 식물체에서는 특히 엽상체 하피에 가장 많은 미생물들이 서식하였으며, 그 다음으로는 점액성 물질이 층을 이루는 근관에 많이 관찰되었다. 반면, 신장 발달 중의 뿌리에는 일반적인 분포를 보였으며, 상피에는 미생물이 거의 분포하지 않는 양상을 보였다. 이들 미생물이 개구리밥 조직 내에 침입하여 식물체에 어떠한 영향을 미치는가를 조사하기 위해 엽상체, 연결사, 뿌리, 근관, 무성생식낭 등의 구조를 세포학적으로 추적한 결과, 어느 부위에서도 조직 내로 미생물이 침입하여 세포를 괴사시키거나 감염시키는 일은 거의 관찰되지 않았다. 그러므로, 개구리밥 식물체의 발달과정에 있어 미생물들은 어떠한 해를 주지 않는 것으로 추정된다. 이후 개구리밥에 서식하는 미생물의 질소고정치를 측정하여 그 기능을 식물체의 세포학적 측면과 접목시켜 연구하면 개구리밥과 미생물과의 상호관계가 공생적인지를 더 확실하게 밝힐 수 있을 것이다.

FIGURE LEGENDS

Abbreviation : Ab = abaxial frond surface, Ad = adaxial frond surface, B = bacterial epiphyte, D = diatom, E = epidermal cell, F = frond, m = microorganism, M = mesophyll cell, P = prophyllous sheath, R = root, Rc = root cap cell, Rp = Reproductive pocket, S = stalk. All figures are SEM, unless specified as TEM.

Fig. 1. Immature adaxial frond exhibiting a very smooth surface. Bar = 3 μm.
Fig. 2. Immature abaxial frond with scattered microorganisms (arrow). Bar = 25 μm. Inset: Close-up of Fig. 2 showing an obvious microbial-frond association. Bar = 0.8 μm.
Fig. 3. Tip of the root cap at early development. Bar = 30 μm.
Fig. 4. Part of the developing root with the point of root-root cap insertion (arrows). Bar = 45 μm.
Fig. 5. Part of the reproductive pocket exhibiting inconspicuous microbial association at early growth. Bar = 75 μm.
Fig. 6. Cross-section of the root cap showing layers of cortical cells. Note the absence of the microorganisms within root and root cap cells. TEM. Bar = 5 μm.
Fig. 7. The connective stalk showing almost no microbial association. Notice the frond thickly covered with diatoms in the background. Bar = 60 μm.
Fig. 8. Cross-section of the connective stalk showing part of the epidermal cells. No microbial invasion was detected. TEM. Bar = 2.5 μm.
Fig. 9. Extensive colonization of the microorganism in fully developed abaxial fronds. A transversely dissected portion of the root that was attached to the abaxial frond is shown in the bottom left. Bar = 35 μm.
Figs. 10-11. Another extensive microorganism colony in the mature abaxial frond. Bar in Fig 10, inset, and Fig. 11 represent 90, 15, and 80 μm, respectively.
Fig. 12. Part of the epidermal and mesophyll cells of the frond. No sign of microbial invasion. TEM. Bar = 2.5 μm.
Fig. 13. SEM micrograph indicating external colonization (arrows) on the abaxial frond epidermal cells. Bar = 40 μm.
Fig. 14. Microorganisms appearing to be either clustered or attached to the mucilaginous layer (arrowheads). TEM. Bar = 2.5 μm.
Fig. 15. Root cap cells associated with numerous microorganisms (arrows). TEM. Bar = 2.5 μm.
Fig. 16. Microbial invasion (arrows) found in the outermost root cap cell. TEM. Bar = 2.5 μm.
Fig. 17. Reproductive pockets with a number of microorganisms on the beginning portion of the covering sheath. Bar = 30 μm.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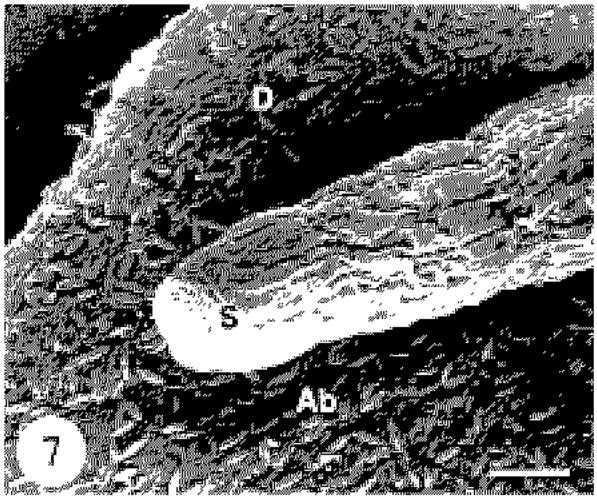
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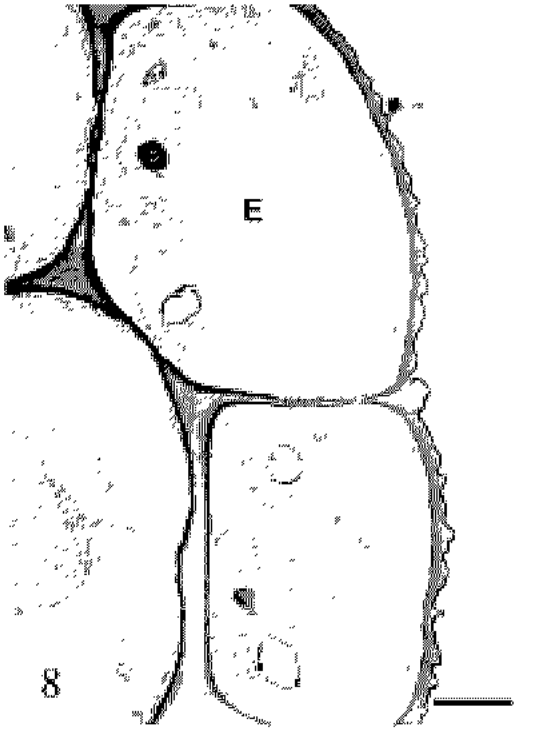
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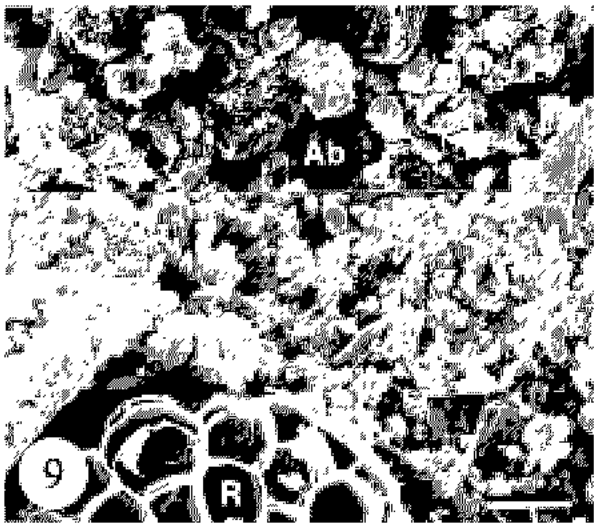
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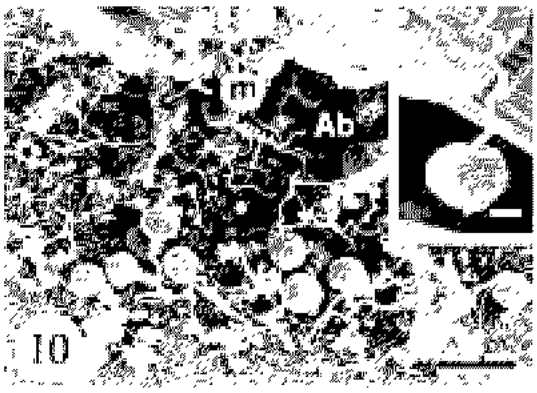
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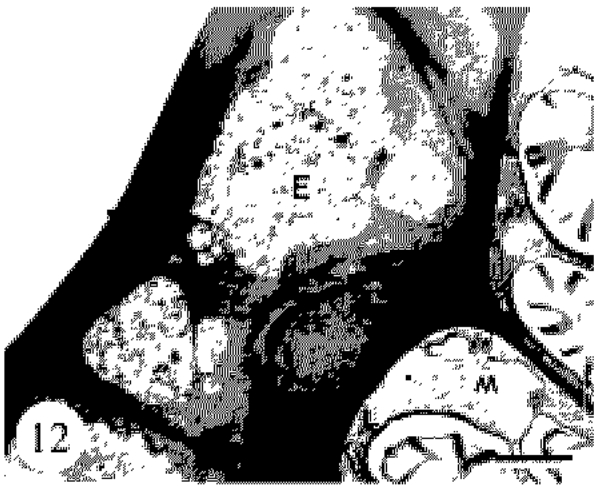
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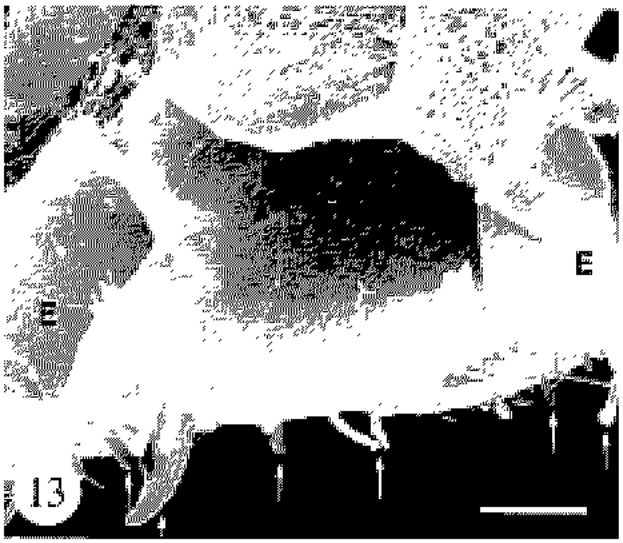
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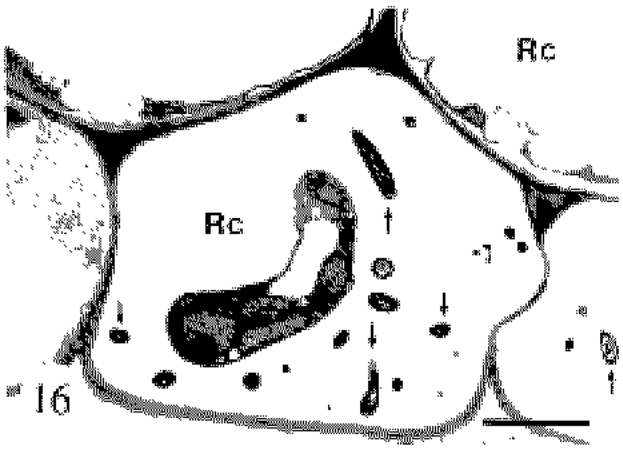
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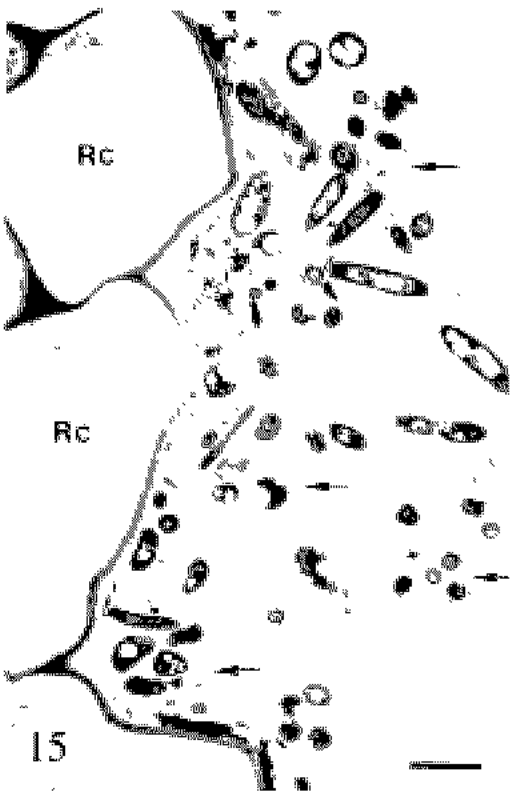
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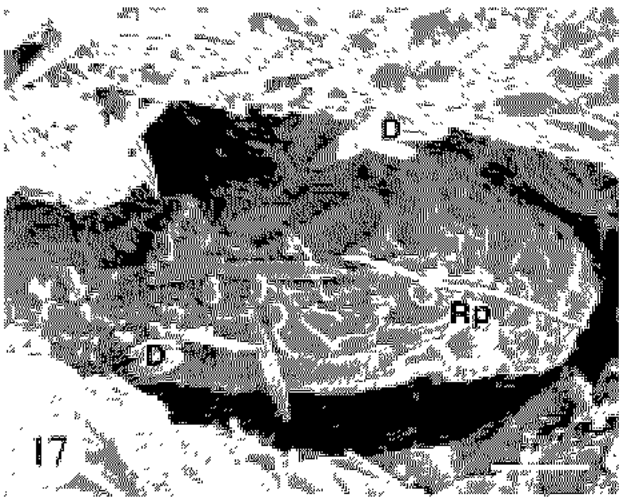
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