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Physiological effects of promotion and inhibition of ethylene biosynthesis on branch abscission in *Azolla*

Gillen, John Joseph, Ph.D.

City University of New York, 1993

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Physiological Effects of Promotion and Inhibition of Ethylene Biosynthesis on Branch Abscission in *Azolla*

by
John Gillen

A dissertation submitted to the Graduate Faculty in Biology
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy, The City University of New York.
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Abstract

PHYSIOLOGICAL EFFECTS OF PROMOTION AND INHIBITION OF ETHYLENE BIOSYNTHESIS ON BRANCH ABSCISSION OF *AZOLLA*

by

John Gillen

Advisor: Professor Dominick V. Basile

The phenomenon of abscission has been largely neglected in the non-flowering plants. A very small aquatic fern, *Azolla* Lam., which reproduces mainly through branch abscission was used to study anatomical and physiological aspects of abscission. Light and electron microscopy were used to establish that separation occurs between two layers of cells, beginning at the periphery and proceeding inward along the middle lamellae. Separation leaves intact cortical cell faces at both the proximal and distal sides of the abscission zone; stelar cells break mechanically. The abscission zone cells, while highly vacuolated, contain organelles found in flowering plant abscission zone cells.

The physiological effects of promotion and inhibition of ethylene biosynthesis on branch abscission were measured by adding ethylene inhibitors and/or promoters to water in which a known number of *Azolla* plants were growing, and counting the number of plants after a period of incubation followed by vigorous agitation on a mechanical shaker. 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene in higher plants, and napthaleneacetic acid (NAA), an auxin known to promote the enzymatic conversion of *S*- adenosylmethionine (SAM) to ACC, significantly increased ($p \leq 0.05$) the amount of abscission. Inhibition of branch abscission significant at $p \leq 0.05$ was observed with amino-oxyacetic acid (AOA), an inhibitor of ethylene biosynthesis, and with silver ion, an inhibitor of ethylene action. Adding NAA together with aminoethoxyvinylglycine, an inhibitor of ethylene biosynthesis, produces significantly less ($p < 0.05$) branch abscission than was observed with NAA alone. Abscission of branches of the smallest size (1.0 mm or less) was significantly increased by ACC and by AOA.

This is the first evidence that ethylene plays a role in abscission in a pteridophyte, and provides indirect evidence supporting the hypothesis that ethylene may be produced via the same biosynthetic pathway used by higher plants.

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Introduction

Ferns (Polypodiophyta) and fern allies (Lycopodiophyta) are extremely ancient groups of plants, their fossil records extending back to the Carboniferous (345 million years ago) and Devonian (395 million years ago), respectively. This latter group of plants includes the fossil species, *Cyclostigma kiltorkense*, that provides the earliest clear evidence of leaf abscission (Chaloner, 1968). Moreover, at least twelve genera of Devonian and Carboniferous lycopods, including such important ones as *Lepidodendron* and *Sigillaria*, had leaves that abscised, leaving distinctive leaf scars (Chaloner, 1967). Branch abscission among the ferns and fern allies is also abundantly documented in the fossil record (Addicott, 1982). Among some of the larger pteridophytes of the Paleozoic such as *Lepidodendron*, the scars left by branch abscission are quite similar to the branch scars of modern angiosperms and gymnosperms (Jonker, 1976).

Organ abscission, although apparently an early established process among ancient pteridophytes, seems to be relatively rare among the extant ferns and fern allies. Where it does occur, it is usually associated with vegetative reproduction. For example, a number of pteridophytes can reproduce vegetatively by production of highly modified branches or branch buds termed "gemmae" that abscise from the parent plant. In the Central American fern, *Vittaria graminifolia*, the mechanism by which this occurs remains unknown "but cryo SEM of the abscission scar suggests a shrinkage of substance previously binding the cells" (Sheffield and Farrar, 1988). In the circumboreal lycopod, *Huperzia*, gemma abscission was studied by Gillen and Basile (1988), who found that the abscission process involves the rupturing by mechanical stress of the thin walls of the abscission zone cells, rather than enzymatic degradation of either cell walls or middle lamellae. Similarly, the schizogenous process involved in the dehiscence of *Huperzia* sporangia (Gillen and Basile, 1989) appears to center on the rupture by mechanical means of cell walls along the line of dehiscence.

Abscission of what appear to be otherwise unspecialized vegetative branches, while relatively rare in most extant groups of ferns, is characteristic of the of the Salviniales. Indeed, in both genera of the Salviniales, *Azolla* and *Salvinia,* branch abscission is the chief means of reproduction, and for some sterile hybrids, the only way they can reproduce. These plants tend to form colonies of fairly uniform sized plants. One of these genera, *Azolla,* consists of small (ca. 5-20 mm in length) floating aquatic ferns and can be traced back in the fossil record as far as the Cretaceous (Jain, 1971), the era of the origin of flowering plants. The members of this genus have the property of propagating themselves asexually through branch abscission (Smith, 1938; Konar and Kapoor, 1972). The shoot of *Azolla* has a mitotically active apical cell with two cutting faces (Gifford and Polito, 1981); the branches, together with the endogenous roots (which, like the branches, have an abscission zone at their base) are traceable to segments produced on the lower side, while the leaves, which do not possess abscission zones, arise from tissues derived from the upper segments (Bierhorst, 1971).

Vegetative reproduction by means of branch abscission is often the chief or only means of reproduction, and as Mickel (1979) says of the genus, "Species identification is based on details of the glochidia, but since these plants are rarely fertile, identification is usually very difficult or impossible." Further complicating species identification is cumulative evidence from DNA "fingerprints," hybridization trials, symptomatic responses to phosphorus deficiency, and immunological work based on the responses of *Azolla's* cyanobiont, *Anabaena azollae*, to the monoclonal antibody McAb-C16, all of which suggest that the current species designations for New World *Azolla* are not valid and that the section therefore requires reorganization (Zimmerman *et al.*, 1991).

Active abscission of roots is a feature for which *Azolla* is unique in the plant kingdom (Addicott, 1982). Cell differentiation and developmental changes in the meristems of *Azolla* roots have been extensively studied (e.g., Barlow et al., 1982; Gunning et al., 1978) although the development of the abscission zones has not been investigated to date.

Anatomical Aspects of Abscission Zone Cells

There is very little information available regarding the histology of abscission in the ferns or their allies. What little has been published indicates that in fern leaf abscission zones, the protective layer may be highly lignified, with a separation layer situated between the rachis and the phyllopodium, if a phyllopodium is present (Konta, 1974; Phillips and White 1967). An early report (Bäsecke, 1908, in Addicott, 1982) suggests that the middle lamella of the separation layer cells often dissolves, and may or may not be preceded by a series of cell divisions.

Apart from this, virtually all of the information on the anatomy of abscission is based on the abscission zones of flowering plants, both herbaceous and woody. The abscission zone may be defined, after Addicott (1982), as a region at the base of most discrete organs "...where, if abscission occurs, the cellular changes of separation will take place." As summarized by Addicott (1982), the typical features include a constricted area encircling the abscission zone, with active separation often beginning close to the constriction. While vascular bundles generally pass through the abscission zone, other structures, such as fibers or resin canals, may not pass through or may be far less developed in the abscission zone. The abscission zone cells tend to be smaller in size, less vacuolated, and have much less deposition of cell-wall materials than other nearby cells, leading Addicott to term the abscission zone "...clearly a region of arrested development" (1982). An alternative explanation views the small size, isodiametric shape and nonlignification of abscission zone cells as adaptations to aid the separation process rather than as the expression of a physiological juvenile state "though it is quite conceivable that both may be essential characteristics" (Sexton and Roberts, 1982.)

The separation layer, defined by Addicott (1982) as "...a layer of cells within an abscission zone which becomes physiologically active and secretes the hydrolytic enzymes which weaken the cell walls and permit separation", tends to be quite thin, sometimes just a single layer of cells. Where cell divisions prior to separation produce several layers of cells, generally only the distal layer is actually involved in separation, while the proximal tiers constitute a protective layer. Lignification of this protective layer is common but may be much less extensive in herbaceous plants. Separation may start near the constriction and proceed inward, as in tomato and tobacco flower pedicels (Jensen and Valdovinos, 1967), or it may commence in the center and move outward, as in the leaflet abscission zone of the primary leaf of the bean (Brown and Addicott, 1950). The normal pattern of separation may be altered in chemically-induced abscission, as in cotton leaves which normally commence abscission at the abaxial surface but will begin to abscise at the adaxial surface in response to gibberellic acid and other growth regulators (Bornman *et al.*, 1967).

Separation of living cells in the abscission zone appears to proceed mainly by breakdown of the middle lamella, presumably through production of hydrolytic enzymes by the separation cells themselves (Sexton, 1976). In contrast, xylem vessels are generally broken by mechanical forces (Sexton and Redshaw, 1981), although some researchers have provided evidence for at least partial hydrolysis of xylem vessels (Bornman *et al.*, 1967; Sexton, 1979).

Where middle lamellar dissolution occurs, it is usually preceded by a swelling of the middle lamella at the corners between cells and around any intercellular spaces. After subsequent weakening and separation, most cells on either fracture surface tend to be intact and rounded (Sexton, 1976), occasionally with a few cells becoming isolated when their middle lamella is lost on all sides (Valdovinos and Jensen, 1968; Sexton, 1976). Breakdown of the primary cell wall is rare but has been recorded, notably in *Phaseolus* leaf explants treated with ethylene (Webster, 1973a). That the separation zone cells are not senescent is indicated by their ability to divide after separation (Hodgson, 1918) and by their ability to be cultured on agar (Namikawa, 1922).

Our present understanding of the ultrastructural changes that occur in abscission zone cells of flowering plants derives chiefly from the seminal contributions of Jensen and Valdovinos (1967, 1968a, b; Valdovinos and Jensen, 1968, 1974; Valdovinos et al., 1970, 1971, 1972, 1985; Henry *et al.*, 1974; Lieberman et al., 1982, 1983) and those who have followed up on their groundbreaking work, such as Sexton (1976, 1979) and his co-workers (Sexton and Hall, 1974; Sexton *et al.*, 1977), Osborne and Sargent (1976a, b), Iwahori and van Steveninck (1976), and Webster and Chiu (1975). Ultrastructural changes in abscission zone cells that occur prior to separation include increases in rough endoplasmic reticulum, indicative of increased levels of protein synthesis (Jensen and Valdovinos, 1968a; Valdovinos *et al.*, 1972; Sexton and Hall, 1974; Webster and Chiu, 1975; Osborne and Sargent, 1976a, b; Iwahori and Van Steveninck, 1976; Sexton et al., 1977), and dilation of dictyosome stack membranes, indicative of secretory processes, reported for bean (Sexton and Hall, 1974), *Coleus* and *Impatiens* (Sexton *et al.*, 1977), Hibiscus (Gilliland *et al.*, 1976), and lemon (Iwahori and Van Steveninck, 1976), but not found in flax (Wiatr, 1978). Mitochondria and chloroplasts, on the other hand, remain normal in numbers and in appearance. The primary cell wall swells but does not usually break, whereas the middle lamella dissolves (Sexton, 1976; Valdovinos and Jensen, 1968).

Valdovinos and Jensen (1974) demonstrated that ethylene-promoted abscission resembles naturally-occurring abscission to the extent that cell-wall degradation begins in the middle lamella and then spreads to other regions of the wall, although separation is more localized, usually between just two tiers of abscission zone cells in tobacco flower pedicels treated with ethylene, whereas in nature the separation zone is comprised of several tiers of cells. At the ultrastructural level, they found vesicular materials, possibly representing membranes, within the cell walls in both kinds of abscission, while two types of fibrillar components that could represent wall breakdown products were found in the cell wall middle lamella region during both kinds of abscission (S.J. Lieberman *et al.*, 1982.) Throughout the abscission process they found randomly-oriented fibrillar components ranging from 5 to 10 nm in width, but only immediately before separation did they observe fibrils oriented in tube-like pairs, each about 4 to 10 nm in width separated by a transparent area of 4 to 10 nm. Only in ethylene-treated abscission zone walls did they observe bodies of high electron density which they suggested might be protein aggregates relating to wall degradation (Valdovinos and Jensen, 1974), although they subsequently found similar bodies of somewhat lower electron density associated with cell wall fibrils in both types of abscission and likewise postulated that these were protein aggregates (S.J. Lieberman *et al.*, 1982), and also found lipid bodies in the cytoplasm near the cell wall throughout both types of abscission, but increasing in number during later stages.

Horton and Osborne (1967), working with beans, first provided evidence that ß-1:4-endoglucanhydrolase, or "cellulase" (so called because it is viscometrically assayed using water-soluble ß-1:4-carboxymethyl cellulose as its substrate, although it may not be able to hydrolyze crystalline cellulose, (Sexton and Roberts, 1982)) is the enzyme most closely correlated with abscission of leaves, by showing that cellulase activity increased at the onset of abscission, was suppressed by auxin and other abscission inhibitors, was enhanced by ethylene and other abscission accelerators, and was largely confined to the separation region. It was soon determined that the secretion of cellulase into the cell wall is under the control of ethylene (Abeles and Leather, 1971).

In bean leaf abscission zones, two forms of cellulase have been found (Lewis and Varner, 1970); an acidic form with a pI of 4.5 that is membrane bound and declines in activity during the course of abscission, whereas the basic form, with a pI of 9.5, is formed de novo during the separation process (Durbin *et al.*, 1981; Del Campillo *et al.*, 1988). The regions in which pI 9.5 cellulase were localized after exposure to ethylene were demonstrated by immunoblotting nitrocellulose tissue prints with antibodies to pI 9.5 cellulase. This form of cellulase was found in the cortical cells of the separation layer, as well as in the adjacent vascular bundles (Del Campillo *et al.*, 1990).

Tucker and Milligan (1991) have sequenced a bean leaf abscission cellulase and compared it to previously reported amino acid sequences of a cellulase for avocado fruit ripening; from genomic data they conclude that the abscission cellulase is encoded either by one gene or a few closely related genes. When an ethylene action antagonist is used to block any effects of endogenously produced ethylene in bean leaf abscission zone tissue after ethylene treatment has stopped, cellulase mRNA accumulation decreases (Tucker *et al.*, 1988).

Although most abscission zones are determined fairly early on in the development of the abscising organ, there are examples of plants which exhibit secondary abscission zones, in which the abscission zone may appear late in development, as is the case in sour cherries (Stosser *et al.*, 1969) and lemons (Iwahori and Van Steveninck, 1976), in both of which abscission occurs by means of middle lamellar dissociation without an intervening cell division phase. Other taxa may exhibit a type of secondary abscission in which the abscission zone develops in a part of the plant that does not normally abscise. This was first noted as the "shot-hole" disease of almond leaves (Samuel, 1927), in which infected cells die out followed by the lignification of surrounding cells, leading to the abscission of the disk of infected tissue when the middle lamellae of the surrounding cells break down. That wound ethylene may be the causal factor for secondary abscission is suggested by work on plants such as the bean (Webster and Leopold, 1972) and *Impatiens* (Warren-Wilson *et al.*, 1986) in which applied ethylene causes abscission zones to differentiate in mature internodal regions of stems. In the bean, secondary abscission of cortical parenchyma cells is associated with cellulase production, whereas the epidermal and pith cells appear to break mechanically without degradation by enzymes (Osborne and McManus, 1986; Osborne, 1987).

It is still not entirely clear as to what 9.5 cellulase acts upon in abscission zone cells. Lewis *et al.*, (1974) suggested cellulose, and showed that cell separation occurred in response to 9.5 cellulase plus pectinase although neither enzyme was effective by itself. However, the work of Albersheim et al., (1977) suggests that cellulases do not weaken the cellulose fibrils of cell walls, but instead are involved in the turnover of hemicellulosic materials in the wall, loosening the cell wall by permitting the fibrils to slip so as to allow cell expansion.

While other enzymes capable of hydrolyzing cell walls are known, such as polygalacturanase, there is as yet no evidence that these play any role in the abscission of leaves (Abeles *et al.*, 1992). However, some of these other enzymes may be involved in fruit abscission; contradictory reports claim that polygalacturanase levels do (Greenberg *et al.*, 1975) or do not (Rasmussen, 1973) increase at the time of citrus fruit abscission. The large increase in abscission in ethylene-treated apples is accompanied by only a small increase in cellulase activity in the abscission zone (Kondo and Takahashi, 1989). In tobacco flower pedicels, peroxidase secretion increases more than sixfold at separation (Henry *et al.*, 1974); although increased peroxidase secretion is typical of most senescent tissue (Birecka, Catalfamo, and Garroway, 1975; Birecka, Catalfamo and Urban. 1975), including the cells on the distal side of an abscission zone, Henry (1979) has shown high peroxidase localized within the walls and plasmodesmata of the cells of the separation zone itself.

For the last quarter of a century, the role of enzymatic cell-wall degradation has dominated the study of abscission, supplanting the earlier notion of increased turgor pressure as the central factor in causing abscission. In 1981, Sexton and Redshaw, using serial thick sections of *Impatiens* leaf petioles, provided evidence that at least under certain circumstances, the old "turgor mechanism" does play an important role. As the middle lamellae are enzymatically degraded, the collenchyma cells begin to separate and the xylem begins to stretch, presumably in response to the release of the tensions that had extended across the intact cell walls. The isodiametric cortical cells in the separation layer then expand as their newly-increased plasticity allows them to take up more water, producing tension along the axis of the petiole which eventually causes the stele to break. It has been suggested by Osborne (1989) that this turgor change could be driven by the soluble sugars produced by hydrolysis of the starches that often accumulate in abscission zones prior to induction of abscission.

Growth Regulators and Abscission

The only published report relating to the physiology of abscission in a fern is one by Dusek and Bonde (1965), who found that IAA at 1 mg/l inhibited the "fragmentation" of *Azolla mexicana*, while at 10 mg/l IAA increased the "fragmentation"; the addition of gibberellic acid at concentrations from 1 to 10 mg/l suppressed the effect of 50 mg/l of IAA. In unpublished work done in 1966, Ammirato (personal communication) found that NAA caused an increase of branch abscission in *Azolla*..

Apart from this, the hormones most closely associated with abscission in higher plants, namely auxin and ethylene, have been considered for a few pteridophytic taxa in relation to some of their other roles.

In the sensitive fern, *Onoclea sensibilis*, ethylene has been shown to inhibit spore germination in the dark. This can be partially overcome by exposure to light (Edwards and Miller, 1972). Inhibition of photosynthesis blocks the effect of light on ethylene-inhibited spores, and phytochrome may also be involved (Fisher and Shropshire, 1979).

Ethylene has morphogenetic effects on dark-grown *Onoclea sensibilis* gametophytes; it inhibits cell division and rhizoid elongation, and promotes filamentous elongation of the prothallus. Supra-optimal levels of IAA, which often triggers ethylene synthesis in flowering plants, inhibited elongation of filaments; auxin induction of ethylene was not detected in this case, however (Miller *et al.*, 1970).

In *Regnellidium diphyllum,* a semi-aquatic fern in the Marsiliaceae, both ethylene and auxin stimulate cell elongation in the rachis. Silver nitrate blocks the elongation response to ethylene but not to auxin (Walters and Osborne, 1979) and it is reported that methionine, an amino acid precursor of ethylene in angiosperms (M. Lieberman, 1979), does not function as a biosynthetic precursor of ethylene when applied to leaf disks of *Regnellidium*, suggesting that an alternative biosynthetic pathway may exist in this fern (Cookson and Osborne, 1978). Walters and Osborne (1979) posit a two-hormone system for control of cell elongation, in which a growth event dependent on auxin is required to precondition cells for an ethylene-dependent growth event.

Tittle (1987), using gametophytes of *Pteridium aquilinum* and sporophytes of *Matteuccia struthiopteris* and *Polystichum munitum,* obtained the first positive evidence of auxin-stimulated ethylene production in ferns and found that application of ACC, the immediate precursor of ethylene in flowering plants (Adams and Yang, 1979), greatly increased production of ethylene whereas addition of AVG or cobalt ion, which inhibit auxin-induced ethylene production (Yu and Yang, 1979) in angiosperms, significantly reduced the stimulatory effect of auxin. Tittle concluded that the ethylene biosynthetic pathway in ferns may be similar to that of angiosperms.

In marked contrast, Osborne (1989b) found no evidence of increased ethylene production after ACC was applied to nine species of ferns (including *Azolla caroliniana*), two species of lycopods, two horsetails, two liverworts, and three mosses, leading her to speculate that the lower land plants may have a non-ACC mediated pathway of ethylene biosynthesis. However, Basile and Basile (1984) have found that in the liverwort, *Plagiochila arctica* , the ACC-synthesis inhibitors AVG and AOA have essentially the same effect as silver nitrate, an ethylene action antagonist, in preventing the suppression of the ventral leaf primordia, that IAA induces ethylene in that leafy liverwort (Law, Basile, and Basile, 1985), and that supplying *P. arctica* with ACC leads to increased ethylene production (D.V. Basile, personal communication).

Thus far, however, virtually all work on the hormonal physiology of the abscission process has used the flowering plant as its model. The first work on the effects of a plant growth regulator on abscission involved the application of auxin-rich orchid pollen to debladed petioles of four species of *Coleus,* among many other genera, and strongly implicated the retardation of abscission by auxin (Mai, 1934, in Addicott, 1982). The greatest increase in time required for abscission was seen in three of the species of *Coleus*. Soon afterward this effect was repeated using the then newly-isolated IAA on debladed petioles of *Coleus blumei* and castor bean, *Ricinus communis* (La Rue, 1936). Interest in the agricultural implications of the use of auxin was stimulated by the finding that auxin slowed the abscission of ripe fruit (Gardner *et al.*, 1939).

Investigation of the diffusible auxin content of *Coleus blumei* (Wetmore and Jacobs, 1953) led to the observation that the amount of time it takes a leaf to abscise is positively correlated with the amount of auxin in the leaf, with the oldest leaves containing the least auxin. Likewise, a number of species of commercially important fruit were shown to have lower levels of endogenous auxin at the time of abscission than at earlier stages of growth (Luckwill, 1957).

Addicott and Lynch (1951) discovered that application of IAA to the proximal side of abscission zones of explants leads to the acceleration of abscission. Endogenous auxin produced in adjacent intact leaves hastens the abscission of debladed petioles of *Coleus*, especially young leaves above the debladed petiole (Jacobs, 1955). Distal application of auxin can also lead to accelerated abscission in bean leaf explants if application is delayed after the explants have been cut (Rubinstein and Leopold, 1963; Chatterjee and Leopold, 1963).

The role of ethylene as a promoter of abscission first became evident as an inadvertent consequence of a development in 19th century municipal technology: the use of coal gas as a fuel for street lighting. City dwellers noticed that trees growing close to street lamps tended to drop their leaves long before autumn. Early in this century it was established that ethylene was the culprit (see Abeles *et al.*, 1992). By the 1930's it had been shown that plants produce ethylene and that the then recently-discovered auxin promotes the production of ethylene (Abeles *et al.*, 1992), yet it was not until the 1960's and the introduction of gas chromatography as a sensitive assay for ethylene that research into the role of ethylene in the physiology of abscission began in earnest.

To describe the different abscission responses to growth regulators over time, Rubinstein and Leopold (1963) used the terms stage I and stage II. Stage I is that period during which abscission is accelerated by applied auxin and is followed by stage II, during which abscission is accelerated by applied ethylene. Stage I is a period of relative insensitivity to ethylene (Jackson and Osborne, 1972) determined in duration by the level of auxin in the abscission zone (Beyer and Morgan, 1971; Riov and Goren, 1979) and can be extended indefinitely by the application of auxin (Chatterjee and Leopold, 1965; Halliday and Wangermann, 1972). Although it was first thought that the insensitivity of stage I explants to applied ethylene was absolute (Abeles, 1968), it was later found that stage I is sensitive to low levels of ethylene and may even require ethylene to pass into stage II (Abeles *et al.*, 1971). It has been suggested by Sexton and Roberts (1982) that this could be due to ethylene's ability to diminish both the synthesis and transport of auxin in abscission (Beyer, 1975; Valdovinos *et al.*, 1967).

In stage II, the presence of ethylene is required to accelerate the cell separation process in the abscission zone (Jackson and Osborne, 1970). Sexton and Roberts (1982) see the abscission-promoting effect of auxin application at this stage as being most likely mediated by auxin-induced ethylene production (Abeles and Rubinstein, 1964; Morgan and Hall, 1964). Characteristics of abscission zone cells at stage II include:

- increased synthesis of protein and nucleic acid (Osborne, 1968),
- production of cellulases (Koehler *et al.*, 1980),

- cell expansion (Wright and Osborne, 1974),
- decreased breakstrength associated loosening of cell walls and middle lamellae (De La Fuente and Leopold, 1969; Abeles *at al.*, 1971).

It should be noted that not all flowering plants respond to ethylene with enhanced abscission; in about 40% of the flowering plant species thus far surveyed, leaf abscission occurs as a response to the addition of ethylene (Woltering, 1987; Marousky and Harbaugh, 1978) whereas the remaining species exhibit other responses, such as yellowing or wilting.

In 1971, rhizobitoxine, a phytotoxin produced by *Rhizobium japonicum,* a root nodule bacterium of legume, was shown to inhibit the biosynthesis of ethylene (Owens *et al.*, 1971); one of its vinylglycine analogs, aminoethoxyvinylglycine (AVG), proved to be the most effective inhibitor of pyridoxal enzymes such as ACC synthase (Amrhein and Wenker, 1979; M. Lieberman, 1979; Yu *et al.*, 1979). Pyridoxal enzymes can also be inhibited both in vivo and in vitro by the aminooxy groups of aminooxyacetic acid (AOA) (Amrhein and Wenker, 1979). The effect of AVG and AOA on inhibition of ethylene synthesis is reversible, as has been shown in tomato pericarp disks, which ripen normally when ethylene is subsequently applied (Edwards *et al.*, 1983). AVG has been proven to be effective in slowing the abscission of both leaves and fruits of apples (Greene, 1981; Kondo and Takahashi, 1989; Edgerton, 1981) and blossoms of lemons (Sipes and Einset, 1982).

Apart from inhibiting ACC synthesis, AOA and AVG have been shown to produce certain effects which are not reversible by ethylene or ACC, for example, in the direct inhibition of root initiation in mung bean (Riov and Yang, 1989) and English ivy cuttings (Geneve *et al.*, 1989), and the inhibition of somatic embryogenesis in tissue cultures of alfalfa (Meijer and Brown, 1988).

$AgNO_3$ has been shown to be an effective competitive inhibitor of ethylene action, including abscission induction (Beyer, 1976). Silver was the first substance shown to block the action of a plant hormone, and became the subject of much research by those intent upon pursuing commercial applications (Abeles *et al.*, 1992). Silver ions can cause heavy metal toxicity in plants due to noncompetitive enzyme inhibition (Veen, 1983); this can induce the production of stress ethylene in amounts up to 20-fold over controls in tomato fruits (Atta-Aly *et al.*, 1987).

Silver thiosulfate (STS) is the silver compound now most widely used as an ethylene action inhibitor and has been shown to block abscission in a variety of flowering plants such as *Coleus* (Baird *et al.*, 1984), holly and mistletoe (Joyce *et al.*, 1990) and *Zygocactus* (Cameron and Reid, 1981). STS, while less toxic and more mobile in plants than silver nitrate, can also produce heavy metal toxicity at high concentrations (Wang and Dunlap, 1990), and the thiosulfate ions themselves have caused foliar toxicity in *Pelargonium* (Cameron *et al.*, 1985).

In 1979, Adams and Yang (1979) determined that 1-aminocyclopropane-1-carboxylic acid (ACC) is the immediate precursor of ethylene in flowering plants by following a suggestion made twenty years earlier by Burg and Thimann (1959); namely, placing apple tissue that had been treated with labeled precursors into an anaerobic atmosphere, thereby allowing the ethylene precursor, which requires oxygen in order to be converted into ethylene, to accumulate. They subsequently applied ACC to plant organs from a variety of species and observed a significant increase in ethylene production, suggesting that the plants possess the enzyme that converts ACC to ethylene and that the rate-limiting step for these tissues is ACC formation (Cameron *et al.*, 1979).

To summarize the pathway of biosynthesis of ethylene in higher plants (Abeles *et al.*, 1992), methionine is converted to *S* -adenosylmethionine (SAM, or AdoMet) in a reaction catalyzed by the enzyme adenosyltransferase. ACC is then formed from SAM through the mediation of the pyridoxal enzyme, ACC transferase. In the final step, ACC is converted to ethylene by an oxygen-requiring enzyme known as ethylene-forming enzyme (EFE) or ACC oxidase.

Abscission zone cells differ in their physiology from the cells of parenchymatous tissue in that many abscission zone cells expand in response to ethylene, and are repressed by auxin, the reverse being true for most other plant cells (Osborne, 1989a). The ethylene-induced expansion of the abscission zone cells and the pressure that they exert may provide mechanical forces for the abscission process (Leopold, 1967).

The primary purpose of the present thesis research was to determine whether the physiology of branch abscission in *Azolla* is comparable with the physiology of abscission in flowering plants, to the extent that this could be ascertained by using substances known to either promote or inhibit ethylene biosynthesis in higher plants and by then assessing the effects of those substances on branch abscission in this fern.

The secondary purpose was to determine whether the histology of branch abscission in *Azolla* is comparable with that of abscission in the flowering plants, particularly as to the mechanism of schizogeny: whether the cells are broken purely mechanically, whether there is evidence for enzymatic dissolution of the middle lamella, or for degradation of the primary cell wall, or whether there might be some other, hitherto unknown mechanism.

A tertiary purpose of this research was to determine the minimum size that a branch of *Azolla* must reach before it will abscise, and whether promoters or inhibitors of ethylene biosynthesis have any effect on the incidence of abscission of the smallest branches.

Materials and Methods

Plant material.

Azolla obtained from the propagation range of the New York Botanical Garden was grown in the Herbert Lehman College greenhouse in tanks together with other aquatic plants, including *Salvinia, Lemna, Eichhornia, Pistia* and *Elodea.* Sexual reproduction has not been observed among these *Azolla* plants, hence, it has not been possible to identify these *Azolla* to the species level.

For each of 29 experiments, 15 healthy *Azolla* plants of typical size and branching pattern were selected and individually measured under the dissecting microscope by means of line drawings to scale (2X) on graph paper in order to establish the initial size of each plant and the size and pattern of its branches and abscission zones. Sketches were found to be preferable to photographs as the shadows cast by the these very small plants tend to obscure the details of the branching patterns. These four hundred and thirty-five plants were distributed into 500 ml flasks (using fifteen plants per experiment, distributed either as five plants in each of three flasks or three plants in each of five flasks) each containing 100 ml of water (which had been passed through cotton gauze to remove particulate matter) taken from the tanks that the plants had originally been growing in, to which had been added a variety of concentrations, determined by preliminary work, of:

- 1-Aminocyclopropane carboxylic acid (ACC), which in flowering plants is the immediate precursor of ethylene, at concentrations of 10^{-5}M,10^{-4}M or 10^{-3}M, prepared from a stock solution (0.101 g in 50 ml deionized water).
- Naphthaleneacetic acid (NAA), a synthetic auxin, at concentrations of 2.7 X 10^{-7}M, 2.7 X 10^{-6}M, or 2.7 X 10^{-5}M, prepared from a stock solution (0.1 g in 100 ml deionized water), used here rather than the natural auxin, IAA, which tends to break down rapidly in the culture medium.
- Aminoethoxyvinylglycine (AVG), an inhibitor of ACC synthase, at concentrations of 10^{-5}M or 10^{-4}M, prepared from a stock solution (0.03 g in 100 ml deionized water).
- NAA, at a concentration of 2.7 X 10^{-5}M plus AVG at a concentration of either 10^{-5}M or 10^{-4}M.
- Aminooxyacetic acid (AOA), also an inhibitor of ACC synthase, at concentrations of 10^{-5}M or 10^{-4}M, prepared from a stock solution (0.109 g in 100 ml deionized water).

- Silver nitrate, $AgNO_3$, a source of the silver ions that block ethylene action, at concentrations of 4 X 10^{-5}M or 4 X 10^{-4}M, prepared from a stock solution (0.68 g in 100 ml deionized water).
- Silver thiosulfate, STS, another source of silver ions, at concentrations of 4 X 10^{-5}M or 4 X 10^{-4}M. This was prepared from stock solutions of $AgNO_3$ (0.68 g in 100 ml deionized H_2O) and sodium thiosulfate (3.97 g in100 ml deionized water).
- Sodium thiosulfate, NaTS, the source of thiosulfate ions in STS, at concentrations of 1.6 X10^{-4}M or 1.6 X 10^{-3}M, corresponding to the concentrations at which the thiosulfate ion occurs in the STS in these experiments, prepared from a stock solution (3.97 g in 100 ml deionized water).
- As the controls, plants were incubated at the same times as the experimental plants under the same conditions in water from the same source, but to which nothing else was added.

The plants were then incubated for five days in a Sherer growth chamber at 22° C. daytime (16 hours) and 19 degrees night (8 hours) under cool white fluorescent lamps at 2,400 lux. At the end of the incubation period, each flask was subjected to very vigorous agitation for 60 seconds on a Yankee Kahn Shaker. Preliminary work has shown that use of this reciprocal shaker for 60 seconds does not cause mechanical damage to the plants but is sufficient to assure that all branches ready to abscise do indeed break off. This obviated the problem of branches abscising during the measuring process.

The contents were measured again and drawings (2X) were made of them individually under the dissecting microscope in order to determine how many branches had abscised, which was done by counting the number plants in each flask after treatment, and to determine the minimum size of branches that were capable of abscission. To obtain the results of the experiments reported here, a total of 3,073 plants of *Azolla* produced during a total of 29 experiments were sketched in this way and analyzed for data. It was determined beforehand that the use of 15 plants per treatment was sufficient to provide statistically significant differences. Statistical significance was determined by subjecting the data obtained to analysis of variance.

Experiments done in the fall of 1991 on the effects of ACC, NAA and AVG on *Azolla* abscission were done again in April of 1992 when growing conditions were different; an additional series of experiments on the effects of AOA and of silver ions was done in the summer of 1992. Because of considerable variation in growth rate at different months, quantitative comparisons between experiments done at different times were not justified.

Microtechnique

In order to compare the branch abscission zones of *Azolla* with the abscission zones of flowering plants, *Azolla* plants were fixed after incubation and prepared for electron microscopy by standard techniques (i.e., Mollenhauer, 1964; Hayat, 1989). The plants were placed in a glutaraldehyde solution at a concentration of 3% v/v and allowed to stand for 1 hour at room temperature. The fixed plants were rinsed with 0.1 M cacodylate buffer (pH 6.8), postfixed for 1 hour in 2% osmium tetroxide (OsO4) in 0.1 M cacodylate buffer (pH 6.8), rinsed in cacodylate buffer, and dehydrated through a graded ethanol series. For transmission electron microscopy (TEM), plants were rinsed 3X with 100% propylene oxide, and then embedded in Spurr's (Spurr, 1969) resin in flat embedment molds (Appendix I, II). For scanning electron microscopy (SEM), plants were critical point dried (CPD), using a SAMDRI-790 critical point dryer (Appendix III).

Microscopical analysis

For TEM and light microscope observation, blocks were first trimmed ca. 0.5 mm and then thick sectioned (2-4 μm) on a Reichert Jung Ultracut microtome using a glass knife. Sections were cut and regularly examined with a light microscope until the area just above a branch could be located and the block realigned. Once the desired area and proper orientation were established, the block was retrimmed to 0.25 mm and sectioned using a diamond knife (Dupont Instruments). Both thick and thin sections were cut. Thick sections were mounted on glass slides. Thin sections were mounted on thin bar grids (SPI 200 mesh HH and/or HS) and post-stained first with uranyl acetate/methanol (Stempak and Ward, 1964) and secondly with lead citrate (Reynolds, 1963).

Photography

Thick sections were photographed using an Olympus Model BHS microscope with a model PM-1035ASP automatic 35 mm camera module using phase or interference microscopy, and Kodak Panatomic-X film. Thin sections were photographed using the photosystems of an Hitachi Model HS 9 or Model H 7000 transmission electron microscope using Kodak 4869 film. Scanning electron microscope preparations were photographed using the photosystems of either a JEOL U-3 or Amray 1830 scanning electron microscope and Polaroid P/N 55 film. All negatives were printed on Kodak RC paper.

Results

Observations

Scanning electron microscopy of the plants grown in the Lehman College greenhouse shows that they are typical of *Azolla* in size and shape (Fig. 9-11). Furthermore, they are typical of *Azolla* in containing colonies of the cyanobacterium, *Anabaena azollae* Strasburger, in their leaf cavities; the trichomes of the *Anabaena* contain numerous nitrogen-fixing heterocysts (Fig. 12). In addition to *Anabaena*, the cavities contain colonies of bacteria (Fig. 13).

The roots of these plants are also typical of the genus, arising at branch points along the stem (Fig. 14). At the point where the root pushes through the stem there is a collar of epidermal cells surrounding the root (Fig. 15-16). Each root contains an abscission zone at its base, a feature unique to *Azolla* among extant plants, and the ventral surface of a plant from which roots have abscised is marked by root abscission scars approximately 0.2 mm in diameter and characterized by the persistence of the raised collar of epidermal cells (Fig. 17). Close-ups of these abscission zones also reveal plentiful diatoms (Fig. 18), which are a normal feature of *Azolla* roots (Lumpkin and Plucknett, 1982).

Plants incubated with AVG took on a reddish-purple tinge, while the plants incubated with ACC at 10-3M started to turn brown within the five days of treatment.

Branch Abscission Scars

At the base of each branch there is an abscission zone, and once abscission has occurred, scars persist, both at the base of the abscised branch (Fig. 19-20) and on the main axis of the plant from which abscission has occurred (Fig. 21-23). Most notable about these branch scars is that, except for the stelar cells, at least some of which appear to be broken, the cortical cell faces of the abscission zone appear to be intact, implying that separation has occurred between abscission zone cells. The number of cortical cells in the outermost ring of the abscission scar is approximately eight on both the distal and proximal faces of the abscission zone.

If one forces a branch to break off from a plant that has already been prepared for SEM by critical point drying, the scarring is very different, with all of the cells at the base of the branch clearly broken (Fig. 24).

An intermediate pattern was sometimes observed when abscission occurred during preparation of tissue for microscopy; here, the cells remained intact but their shape was somewhat distorted (Fig. 25-26).

Light Microscopy

Light microscopy reveals that the branch abscission zone of *Azolla* consists of two layers of cells, with separation occurring between those two layers (Fig. 27-28). The neck of the branch is quite narrow at the abscission zone; in this study the neck was found to be three cortical cells across so that in longitudinal sections to one side or the other of the stele, the abscission zone can be seen to be comprised of six cells. These abscission zone cells are somewhat flattened in shape relative to the adjacent cortical cells, but do not appear to differ otherwise, at least at the level of light microscopy, being highly vacuolated, with very little cytoplasm, in contrast to the much more cytoplasmically dense cells of the roots.

In sections made of branches that were just beginning to abscise when embedded in resin (Fig. 28), it can be seen that separation between the two layers of the abscission zone starts at the periphery of the abscission zone and proceeds inward.

Transmission Electron Microscopy (TEM)

Thin sections examined under the transmission electron microscope confirmed what had been seen in thick sections with the light microscope.

The separation process begins at the periphery and proceeds inward. The separation between the walls of the abscission zone cells is widest where it breaches the epidermis; plasmodesmata can be observed in the separated walls (Fig. 29-30). The separation zone becomes narrower toward the center (Fig. 31-32), although it does not always do so with complete uniformity (Fig. 33). The abscission zone cells are highly vacuolated, with a relatively small amount of cytoplasm mostly appressed to the area adjacent to the cell wall. That this is not artifactual is indicated by the good preservation of other nearby cells, such as those of the vascular stele, which, in addition to showing wall thickenings, also contain some cells that are much more densely cytoplasmic (Fig. 34).

Although the abscission zone cells are too highly vacuolated to make morphometric analysis practical, they do show a complement of organelles, including:

- nuclei, (Fig. 35-36), some with visible nuclear pores (Fig. 37),
- chloroplasts showing thylakoids and starch grains (Fig. 35-37),

- numerous mitochondria (Fig. 38), indicating that this is a metabolically active region,
- Golgi bodies (Fig. 39),
- endoplasmic reticulum (Fig. 40).

They also display membranous whorls that project from the cytoplasm, where they appear to be attached to the cell wall, into the vacuole (Fig. 41), and as such are strikingly similar to the membranous whorls seen by Valdovinos, Jensen, and Lieberman (1985) in tobacco flower pedicel abscission zones that had been treated with the transcription inhibitor, Actinomycin D.

Experiments

The amount of branch abscission was measured by counting the number of plants per flask after an initial number of five plants per flask (for the October experiments) or three plants per flask (for the subsequent experiments) were subjected to an incubation period of five days followed by vigorous shaking.

In order to determine what controls the minimum size branch capable of abscission, the number of abscised branches 1.0 mm or less in length was counted for each flask, as measurement of the size of abscised branches in the course of this study showed that the smallest ones were 1 mm or very slightly less in length.

The complete data are presented in Tables 1-8, and in chart format in Figures 1-8.

ACC

When ACC was added to the culture medium in October, 1991 (Table 1; Figure 1), at a concentration of 10^{-5}M or 10^{-4}M, the mean number of plants per flask after treatment was significantly higher ($p \leq 0.05$) than in the control. This was also significantly higher ($p \leq 0.05$) than with AVG at either 10^{-5}M or 10^{-4}M or with ACC at 10^{-3}M. ACC at 10^{-4}M gave a mean number that was higher but not significantly so at $p \leq 0.05$ than with ACC at 10^{-5}M. ACC at a concentration of 10^{-3}M yielded a mean number of plants that was higher than in the control or with AVG at either 10^{-5}M or 10^{-4}M but not significantly so at $p \leq 0.05$.

In April, 1992 (Table 3; Figure 3) the addition of ACC to the culture medium at a concentration of either 10^{-5}M or 10^{-4}M yielded a mean number of plants per flask significantly greater ($p \leq 0.05$) than that found in the control, with AVG alone at a concentration of either 10^{-5}M or 10^{-4}M, with NAA alone at a concentration of 2.7 X 10^{-}

^{5}M, or with NAA in combination with AVG at either 10^{-5}M or 10^{-4}M. The number obtained with ACC at a concentration of 10^{-5}M was higher than with ACC at 10^{-4}M, but not significantly so at $p \leq 0.05$.

The mean number of abscised branches 1.0 mm or less in length in October, 1991 (Table 5; Figure 5) when ACC was added to the culture medium at a concentration of 10^{-5}M or 10^{-4}M was significantly greater ($p \leq 0.05$) than in the control, with ACC at 10^{-3}M or AVG at either 10^{-5}M or 10^{-4}M. The number obtained with ACC at a concentration of 10^{-5}M was not significantly different at $p \leq 0.05$ from what was obtained with ACC at 10^{-4}M. With ACC at a concentration of 10^{-3}M, the number obtained was the same as the control and greater but not significantly so at $p \leq 0.05$ than with AVG at either 10^{-5}M or 10^{-4}M.

When ACC was added in April, 1992 (Table 7; Figure 7), at a concentration of either 10^{-5}M or 10^{-4}M, the mean number of abscised branches of minimum size was significantly higher ($p \leq 0.05$) than in the control, with AVG alone at either 10^{-5}M or 10^{-4}M, with NAA alone at 2.7 X 10^{-5}M, or with NAA at 2.7 X 10^{-5}M in combination with AVG at either 10^{-5}M or 10^{-4}M. The number obtained with ACC at 10^{-4}M was significantly greater ($p \leq 0.05$) than with ACC at 10^{-5}M.

NAA

In November, 1991, (Table 2; Figure 2) when NAA was added to the medium at a concentration of 2.7 X 10^{-7}M the increase in number of plants over the control was not statistically significant at $p \leq 0.05$, whereas at concentrations of NAA at 2.7 X 10^{-6}M and 2.7 X 10^{-5}M there were increases significant at $p \leq 0.05$. Analysis of variance showed that the increases observed at NAA 2.7 X 10^{-6}M and 2.7 X10^{-5}M were significantly greater ($p \leq 0.05$) than those observed with NAA 2.7 X 10^{-7}M, although the increase seen at NAA 2.7 X 10^{-6}M over that observed at NAA 2.7 X 10^{-5}M was not significant at $p \leq 0.05$.

When AVG at 10^{-5}M was added simultaneously with NAA at 2.7 X 10^{-5}M, the mean number of plants after treatment was not significantly ($p \leq 0.05$) greater than in the control, and when AVG at 10^{-4}M was added along with NAA at 2.7 X 10^{-5}M, the number of plants after treatment was lower than in the control, although not significantly so at $p \leq 0.05$. Analysis of variance showed that the number of plants observed with AVG 10^{-4}M plus NAA was significantly ($p \leq 0.05$) lower than that observed with AVG 10-5M plus NAA; furthermore, the numbers of plants observed with both AVG 10^{-5}M plus NAA and AVG 10^{-4}M plus NAA were significantly ($p \leq 0.05$) lower than those observed with NAA alone at either 2.7 X 10^{-6}M or 2.7 X 10^{-5}M, although not at 2.7 X 10^{-7}M.

When, in April, 1992, (Table 3; Figure 3) NAA was added alone to the culture medium at 2.7 X 10^{-5}M, the number of plants per flask after five days was significantly ($p < 0.05$) higher than in the control. Analysis of variance shows that this figure was also significantly higher at $p \leq 0.05$ than when AVG alone was added at either 10^{-5}M or 10^{-4}M, but significantly lower ($p < 0.05$) than when ACC alone was added at either 10^{-5}M or 10^{-4}M.

When AVG at 10^{-5}M was added simultaneously with NAA at 2.7 X 10^{-5}M in April, 1992, the number of plants was significantly ($p \leq 0.05$) higher than that observed in the control but lower, although not significantly so ($p \leq 0.05$) than that obtained with NAA alone at 2.7 X 10^{-5}M. This figure was also significantly ($p \leq 0.05$) higher than that obtained with AVG alone at either 10^{-5}M or 10^{-4}M, and significantly lower ($p \leq 0.05$) than that obtained with ACC alone at either 10^{-5}M or 10^{-4}M. When the AVG was added at 10^{-4}M along with NAA at 2.7 X 10^{-5}M, the number of plants observed was lower, although not significantly so at $p \leq 0.05$, than that observed with AVG at 10^{-5}M along with NAA, and higher but not significantly so ($p \leq 0.05$) than that observed with the control or with AVG alone at either 10^{-5}M or 10^{-4}M. This figure was significantly lower ($p \leq 0.05$) than that obtained with NAA alone at 2.7 X 10^{-5} or with ACC alone at either 10^{-5}M or 10^{-4}M.

In terms of the number of abscised branches of minimum length ($\leq$ 1 mm) in the November, 1991 treatments (Table 6; Figure 6), there were none at NAA 2.7 X 10^{-7}M, and a smaller number than in the control with NAA 2.7 X 10^{-6}M, with an increase over the control at NAA 2.7 X 10^{-5}M; however, none of these differences were significant at $p \leq 0.05$. When AVG at 10^{-5}M was added along with NAA at 2.7 X 10^{-5}M, the mean number of branches of minimum length was the same as in the control, and when the AVG was added at 10^{-4}M along with NAA at 2.7 X 10^{-5}M, the number observed was below that of the control, but again these figures were not significant at $p \leq 0.05$.

As to the number of abscised branches of minimum length in the April, 1992 treatments (Table 7; Figure 7), there were none with either NAA alone or in combination with either concentration of AVG. This was the same as in the control and significantly less ($p \leq 0.05$) than the number obtained with ACC alone at either 10^{-5}M or 10^{-4}M.

AVG

In addition to using AVG in treatments in combination with NAA, giving the results described above, AVG was also used alone. When AVG alone was added to the culture medium in October, 1991, (Table 1; Figure 1) at a concentration of 10^{-5}M, the number of plants per flask after treatment was lower, although not significantly so at $p \leq 0.05$, than in the control. With AVG at a concentration of 10^{-4}M, the number was higher, although not significantly so ($p \leq 0.05$) than at 10^{-5}M, and lower, although not significantly so at $p \leq 0.05$, than in the control.

Analysis of variance also showed that the number of plants obtained in October, 1991 with AVG alone at either 10^{-5}M or 10^{-4}M was significantly lower ($p \leq 0.05$) than that obtained with ACC alone at either 10^{-5}M or 10^{-4}M, and lower, but not significantly so at $p \leq 0.05$, than that obtained with ACC at a concentration of 10^{-3}M.

When AVG alone at a concentration of 10^{-5}M was added to the culture medium in April, 1992 (Table 3; Figure 3), the number of plants per flask after treatment was lower, although not significantly so at $p \leq 0.05$ than in the control. At a concentration of 10^{-4}M of AVG, the number of plants was lower than at 10^{-5}M but not significantly so at $p \leq 0.05$, nor was it significantly lower than in the control. At a concentration of AVG alone of either 10^{-5}M or 10^{-4}M, analysis of variance showed that the number of plants after treatment was significantly lower ($p \leq 0.05$), than that obtained with ACC at either 10^{-5}M or 10^{-4}M, with NAA alone at 2.7 X 10^{-5}M, or with NAA at 2.7 X 10^{-5}M added along with AVG at 10^{-5}M, and lower but not significantly so at $p \leq 0.05$ than with NAA 2.7 X 10^{-5}M added along with AVG at 10^{-4}M.

No branches of minimum size abscised when treated in October, 1991 (Table 5; Figure 5) with AVG alone at either 10^{-5}M or 10^{-4}M. This was less than the mean for the control, although not significantly so at $p \leq 0.05$. This figure is significantly lower ($p \leq 0.05$) than that obtained with ACC at either 10^{-5}M or 10^{-4}M, and lower but not significantly so at $p \leq 0.05$ than with ACC at a concentration of 10^{-3}M.

In April, 1992, with AVG alone at either concentration (Table 7; Figure 7), there were again no abscised branches 1 mm or less in length. This was the same as in the control, and the same as with NAA alone at 2.7 X 10^{-5}M, and with NAA at 2.7 X 10^{-5}M in combination with AVG at either 10^{-5}M or 10^{-4}M. This was significantly less ($p \leq 0.05$) than the number obtained with ACC at either 10^{-5}M or 10^{-4}M.

AOA

Addition of AOA at a concentration of 10^{-5}M to the culture medium in July, 1992, (Table 4; Figure 4) was associated with significantly fewer ($p \leq 0.05$) plants per flask after treatment versus the control. The number obtained was lower but not significantly so at $p \leq 0.05$ than that obtained with NaTS at 1.6 X 10^{-4}M or with STS at 4 X 10^{-5}M, and higher but not significantly so at $p \leq 0.05$ than that obtained with $AgNO_3$ at either 4 X 10^{-5}M or 4 X 10^{-4}M, with NaTS at 1.6 x 10^{-3}M, or with STS at 4 X 10^{-4}M. When AOA was added at 10^{-4}M, the number of plants was significantly higher ($p \leq 0.05$) than with AOA at 10^{-5}M, with $AgNO_3$ at either 4 X 10^{-5}M or 4 X 10^{-4}M, with NaTS at 1.6 X 10^{-3}M, or with STS at 10^{-4}M, and higher but not significantly so at $p \leq 0.05$ than with NaTS at 1.6 X 10^{-4}M or with STS at 4 X 10^{-5}M, and lower although not significantly so at $p \leq 0.05$ than in the control.

The mean number of abscised branches of minimum size (Table 8; Figure 8) produced with AOA at a concentration of 10^{-5}M was lower but not significantly so at $p \leq 0.05$ than in the control, and higher but not significantly so at $p \leq 0.05$ than with $AgN0_3$ at either 4 X 10^{-5}M or 4 X 10^{-4}M, with NaTS at either 1.6 X 10^{-4}M or 1.6 X 10^{-3}M, or with STS at either 4 X 10^{-5}M or 4 X 10^{-4}M. However, when AOA was added at 10^{-4}M, the mean number of abscised branches was significantly higher ($p \leq 0.05$) than in the control, or with AOA at 10^{-5}M, with $AgN0_3$ at either 4 X 10^{-5}M or 4 X 10^{-4}M, with NaTS at either 1.6 X 10^{-4}M or 1.6 X 10^{-3}M, or with STS at either 4 X 10^{-5}M or 4 X 10^{-4}M.

$AgN0_3$

$AgNO_3$ added to the culture medium in July, 1992 (Table 4; Figure 4) at a concentration of 4 X 10^{-5}M yielded a mean number of plants that was significantly lower at $p \leq 0.05$ than in the control, or with AOA at 10^{-4}M, with NaTS at 1.6 X 10^{-4}M, or STS at 4 X 10^{-5}M, and lower but not significantly so at $p \leq 0.05$ than with AOA at 10^{-5}M or with NaTS at 1.6 X 10^{-3}M. This figure was higher but not significantly so at $p \leq 0.05$ than that obtained with STS at 4 X 10^{-4}M. When $AgNO_3$ was added at 4 X 10^{-4}M, the mean number of plants was higher although not significantly so at $p \leq 0.05$ than with $AgNO_3$ at 10^{-5}M, or with STS at 4 X 10^{-4}M, significantly lower ($p \leq 0.05$) than in the control or with AOA at 10^{-4}M, and lower but not significantly so at $p \leq 0.05$ than with AOA at 10^{-5}M, NaTS at either 1.6 X 10^{-4}M or 10^{-3}M, or STS at 4 X 10^{-5}M.

No branches 1 mm or less in length abscised when $AgNO_3$ was added to the medium at either 4 X 10^{-5}M or 4 X 10^{-4}M (Table 8; Figure 8). This result was the same as that obtained with STS at either 4 X 10^{-5}M or 4 X 10^{-4}M, and was significantly lower ($p \leq 0.05$) than that obtained with AOA at 10^{-4}M, and lower although not significantly so at $p \leq 0.05$ than that obtained in the control, with AOA at 10^{-5}M, or with NaTS at either 1.6 X 10^{-4}M or 1.6 X 10^{-3}M.

NaTS

When NaTS was added to the culture medium in July, 1992 (Table 4; Figure 4) at a concentration of 1.6 X 10^{-5}M, the mean number of plants per flask was significantly lower ($p \leq 0.05$) than in the control, and lower but not significantly so at $p \leq 0.05$ than with AOA at 10^{-4}M or with STS at 10^{-5}M. This number was significantly higher ($p \leq 0.05$) than with $AgNO_3$ at 4 X 10^{-5}M or with STS at 4 X 10^{-4}M, and higher although not significantly so at $p \leq 0.05$ than with AOA at 10^{-5}M or with $AgNO_3$ at 10^{-4}M. When NaTS was added at a concentration of 1.6 X 10^{-3}M, the mean number of plants was significantly lower ($p \leq 0.05$) than in the control or with AOA at 10^{-4}M, and lower but not significantly so at $p \leq 0.05$ than with NaTS at 1.6 X 10^{-4}M, with AOA at 10-5M, or with STS at 4 X 10^{-5}M. This number was higher although not significantly so at $p \leq 0.05$ than that obtained with $AgNO_3$ at either 4 X 10^{-5}M or 4 X10^{-4}M, or STS at 4 X 10^{-4}M.

The mean number of abscised branches of minimum size obtained in July, 1992 (Table 8; Figure 8), when NaTS was added to the culture medium at either 1.6 X 10^{-4}M or 1.6 X 10^{-3}M was significantly lower at $p \leq 0.05$ than that obtained with AOA at a concentration of 10^{-4}M, lower but not significantly so at $p \leq 0.05$ than that obtained in the control or with AOA at 10^{-5}M, and higher but not significantly so at $p \leq 0.05$ than with $AgNO_3$ or STS at either 4 X 10^{-5}M or 4 X 10^{-4}M. The number obtained with NaTS at 1.6 X 10^{-3}M was higher but not significantly so at $p \leq 0.05$ than with NaTS at 1.6 X 10^{-4}M.

STS

Addition of STS to the medium in July, 1992 (Table 4; Figure 4) at a concentration of 4 X 10^{-5}M yielded a mean number of plants per flask that was significantly lower ($p \leq 0.05$) than in the control, and lower although not significantly so at $p \leq 0.05$ than with AOA at 10^{-4}M. This figure was significantly higher than that obtained with $AgNO_3$ at 4 X 10^{-5}M, and higher but not significantly so at $p \leq 0.05$ than with AOA at 10^{-5}M, with $AgNO_3$ at 4 X 10^{-4}M, or with NaTS at either 4 X 10^{-5}M or 4 X 10^{-4}M. When STS was added at 4 X 10^{-4}M, the mean number obtained was significantly lower at $p \leq 0.05$ than that obtained with STS at 4 X 10^{-5}M, with the control, with AOA at 10^{-4}M, or with

NaTS at 1.6 X 10^{-4}M, and lower although not significantly so at $p \leq 0.05$ than that obtained with AOA at 10^{-5}M, with $AgNO_3$ at either 4 X 10^{-5}M or 4 X 10^{-4}M, or with NaTS at 1.6 X 10^{-3}M.

When STS was added at a concentration of either 4 X 10^{-5}M or 4 X 10^{-4}M, none of the abscised branches were 1 mm or less in length (Table 8; Figure 8). This was the same as with $AgNO_3$ at either 4 X 10^{-5}M or 4 X 10^{-4}M, significantly less ($p \leq 0.05$) than what was obtained with AOA at 10^{-4}M, and less although not significantly so at $p \leq 0.05$ than what was obtained with the control, with AOA at 10^{-5}M, or with NaTS at either 1.6 X 10^{-4}M or 1.6 X 10^{-3}M.

Discussion

These results show a significant increase ($p \leq 0.05$) in the amount of branch abscission in this aquatic fern, caused by the addition of ACC, and by the addition of NAA, and significant decrease in branch abscission in response to applied AOA, AVG, silver nitrate, silver thiosulfate and sodium thiosulfate. Auxin's ability to increase branch abscission in *Azolla* was significantly diminished when AVG, an inhibitor of ACC synthase, was added simultaneously with the NAA, providing indirect support for the hypothesis that the abscission-promoting effect of NAA seen here may be due to increased ethylene production. In angiosperms, the ability of auxin to stimulate ethylene production has been attributed to the induction of ACC synthase (Adams and Yang, 1979; Yu et al., 1979). It has recently been demonstrated that auxin does induce the biosynthesis of ethylene in sporophytes and gametophytes of several species of ferns; in one species, AVG reduced the amount of ethylene induced by exogenously-supplied auxin (Tittle, 1987). Increased production of ethylene in response to the addition of auxin has also been reported in a leafy liverwort (Law, Basile, and Basile, 1985), and in the protonema of the moss *Funaria* (Rohwer and Bopp, 1985).

ACC, which in angiosperms is the immediate precursor of ethylene, increased branch abscission in *Azolla* at two levels of concentration, 10^{-5}M and 10^{-4}M. This constitutes additional indirect evidence for ethylene-mediated control of abscission in a fern. It suggests that the role of ethylene as a mediator of abscission may have been established well back in the early days of the land plants.

Increased production of ethylene in response to both ACC and to auxin has been reported for three fern species by Tittle (1987), as well as for a leafy liverwort (Law *et al.*, 1985; D.V. Basile, personal communication) and a moss protonema (Rohwer and Bopp, 1985); the present work appears to accord with these findings, rather than with those of Osborne (1989b), who reported that ACC did not promote ethylene production in any of the eighteen species of lower plants that she tested, including *Azolla caroliniana*, although she did not present quantitative data for the amount of ethylene produced in those species. Also, she did not attempt to test the species studied by Tittle, by Law, Basile, and Basile; in the case of the moss, she only tested the sporophyte. More work needs to be done on ethylene biosynthesis in nonflowering plants to clarify these apparently contradictory findings.

At one concentration of ACC (10-4M), abscission was not confined to the bases of the branches, but also involved breakage of the main stems, suggestive of the development of secondary abscission zones. This is consistent with the finding that exposure to ethylene leads to accelerated development and abscission of secondary abscission zones in mature internodes of bean (Webster and Leopold, 1972) and *Bryophyllum* (Horton, 1976). At the highest concentration of ACC tested here (10^{-3}M), there was no promotion of abscission; the *Azolla* plants all turned brown, indicative of toxicity at this concentration. In excised carnation petals ACC at 10^{-3}M has been reported to induce premature senescence after 30 hours of incubation (Hanley *et al.*, 1989).

The situation in *Azolla* thus appears to be very different from that in the semi-aquatic fern *Regnellidium diphyllum* for which, although it does contain low levels of ACC and its conjugate MACC (Osborne, 1989b), it is reported that ACC is not converted to ethylene and does not substitute for ethylene in cell growth responses in the submerged portion nor is ethylene production blocked by AVG (Walters and Osborne, 1979; Osborne, 1989b). This could represent an adaptation to an underwater environment in which oxygen is not available for conversion of ACC to ethylene by ACC oxidase.

The present work shows that the amount of branch abscission in *Azolla* is significantly diminished with the addition of an appropriate concentration of AOA or AVG, known in angiosperms to reduce (but generally not to completely abolish) biosynthesis of ACC, ethylene's precursor (Abeles *et al.*, 1992). At the times when AVG was tested, the amount of abscission in the untreated controls was low, and the effect of adding AVG alone was not significant at $p \leq 0.05$; however, AVG added simultaneously with NAA yielded significantly less ($p \leq 0.05$) abscission than with NAA alone. In much the same way auxin has been shown to stimulate ethylene production in other species of ferns, and simultaneous addition of AVG to eliminate or significantly decrease the effect of auxin (Tittle, 1987). The auxin-counteracting effect of AVG in *Azolla* may also be compared with its effect on the flowering of *Chenopodium*, in which AVG counteracts the flowering-delaying effect of exogenously-supplied auxin (Machackova *et al.*, 1986).

That environmental factors play a strong role in determining the rate of branch abscission is shown by the great increase (approximately tenfold) in the amount of branch abscission noted in the control plants in July as against that which was noted during the previous April. Even under conditions under which abscission occurred quite readily, there was statistically significant inhibition at $p \leq 0.05$ of abscission with AOA at a low concentration (10^{-5}M), and with silver ion, particularly in the form of silver thiosulfate. Silver ion is believed to block the action of ethylene in angiosperms by competing for ethylene receptors in the plant tissue (Veen, 1985), and the silver ion also suppresses the autocatalytic production of ethylene (Veen, 1979). The ability of STS to inhibit abscission in *Azolla* is in keeping with horticultural findings over the last decade in flowering plants which show that STS is effective in preventing floral abscission in various cut flowers such as snapdragons (Farnham *et al.*, 1981), sweet peas (Mor *et al.*, 1984) and roses (Mor *et al.*, 1989), and in numerous potted plants, e.g., Christmas cactus, *Impatiens*, *Pelargonium* (Cameron and Reid, 1983), orchids (Goh *et al.*, 1985) and *Streptocarpus* (Agnew *et al.*, 1985). Furthermore, STS has been demonstrated to prevent ethylene-induced leaf abscission in holly and mistletoe (Joyce *et al.*, 1990).

The finding that abscission in *Azolla* is inhibited by silver ion may also be compared with the discovery in another nonflowering plant, in this case a leafy liverwort, *Plagiochila arctica*, that silver nitrate, as well as AVG and AOA, prevents suppression of ventral leaf primordia (Basile and Basile, 1984), suggesting that in that liverwort ethylene acts in conjunction with hydroxyproline-rich glycoproteins to suppress cellular development locally. Ethylene treatment has been shown in angiosperms to induce mRNA synthesis for a number of proteins, including bean abscission cellulase (Kelly *et al.*, 1987; Tucker *et al.*, 1988) and hydroxyproline-rich glycoproteins (Ecker and Davis, 1987). In melon seedlings, AVG and other compounds such as benzyl isothiocyanate and L-canaline were shown to block the synthesis of both ethylene and hydroxyproline-rich glycoproteins (Toppan *et al.*, 1982).

Studies in flowering plants have also shown that silver ions can block the morphogenesis-suppressing effects of ethylene, e.g., $AgNO_3$ was shown to promote the initiation of shoots from wheat callus and from a non-regenerating species of tobacco in a culture medium in which ethylene was induced by auxin (Purnhauser *et al.*, 1987).

There was also significant abscission inhibition in *Azolla* with sodium thiosulfate alone, although not as much as with silver thiosulfate at 4×10^{-4}M. Sodium thiosulfate has not been reported to inhibit abscission in flowering plants; however, the thiosulfate ion has been shown to cause leaf damage in geraniums (Cameron *et al.*, 1985) and it should be kept in mind that the thiosulfate ion, unlike the nitrate ion, is not normally encountered in terrestrial or aquatic ecosystems.

In July of 1992 the plants were proliferating while the previous fall they appeared to be entering a period of slower growth. A number of factors in the greenhouse could have contributed to this (light, temperature, nutrient status, etc.) but it should be noted that in nature *Azolla* also undergoes periods in which it produces massive "blooms," certain species doubling their biomass every three days under optimal conditions (Talley and Rains, 1980). This, together with its propensity to accumulate anthocyanin, accounts for one of *Azolla* 's more poetic Chinese names, "man jiang hong," literally, "whole river red" (Lumpkin and Plucknett, 1982).

As to what determines the minimum size at which a branch may abscise, evidence presented here suggests that ethylene may play a role, for ACC significantly promotes ($p \leq 0.05$) the abscission of branches 1.0 mm or less in length. The level of abscission of these smallest branches in the untreated control plants was so low, at no time significantly above zero at $p \leq 0.05$, that it would not have been possible to demonstrate inhibition for any treatment by comparison to the untreated plants, nor was abscission of the smallest branches high enough in the NAA-treated plants to show reduction of abscission significant at $p \leq 0.05$ with simultaneous use of NAA and AVG.

Harder to interpret is the finding that AOA at the highest concentration tested, (10^{-4}M), while it does not have a significant effect ($p \leq 0.05$) on overall branch abscission, does promote the abscission of the smallest branches. However, there is a certain amount of evidence in the literature which suggests that AOA and AVG may have physiological effects not exclusively due to their ability to inhibit ACC synthase, including the ability to inhibit somatic embryogenesis in alfalfa at levels which do not inhibit ethylene biosynthesis (Meijer and Brown, 1988), and the ability to delay floral senescence in flowers insensitive to ethylene (Wang and Baker, 1979).

An anthocyanin tinge was noted in abscission zones of plants exposed to AVG, providing more evidence that ethylene may play some as yet unexplored role in anthocyanin metabolism, as suggested by flowering plant studies that have found ethylene to inhibit light-induced anthocyanin formation in seedlings of monocots such as corn (Rengel and Kordan, 1987) and sorghum (Craker *et al.*, 1971), as well as dicots such as bean (Morgan and Powell, 1970) and mustard (Buhler *et al.*, 1978). The mechanism by which ethylene causes this inhibition has not yet been elucidated; in the case of sorghum, anthocyanin formation is increased if ethylene is applied after the light treatment is completed (Craker *et al.*, 1971).

Ethylene promotes the formation of anthocyanins in fruits such as cherries (Bukovac *et al.*, 1971), blackberries (Sapers *et al.*, 1986), cranberries (Craker, 1971), and blood oranges (Aharoni and Youck, 1982), in senescent flowers of *Lantana camara* (Mohan Ram and Mathur, 1984), and in leaves of peppermint (El-Keltawi and Croteau, 1986). Increased levels of anthocyanin in apple skins are associated with an increase of a shikimic acid pathway enzyme, phenylalanine ammonia-lyase (PAL), suggesting that ethylene induces anthocyanin by promoting PAL formation (Blankenship and Unrath, 1988; Faragher and Brohier, 1984). Use of a PAL inhibitor has been shown to inhibit synthesis of anthocyanins in ripening strawberries (Given *et al.*, 1988).

Light and electron microscopy show that the anatomy of branch abscission of *Azolla* is comparable to that of axial organs in many flowering plants in starting at the periphery and moving inward, apparently separating along the middle lamella. This is similar to what Jensen and Valdovinos (1967) found in the abscission of tobacco and tomato flowers in which the abscission zone is an indentation that extends into the pedicel.

Mechanical breakage of the vascular stele after the middle lamella has been enzymatically degraded is presumably the last step in the abscission process in *Azolla*, as it is with many flowering plants (Sexton and Roberts, 1982). The intact surfaces of the cells on both fracture faces indicate that separation has occurred by means of a hydrolytic enzyme or enzymes with middle lamellar materials, presumably pectins, as the substrate, rather than by purely mechanical forces. Ethylene has been shown to increase the activity of many enzymes, including several which have been implicated in the regulation of abscission, such as ß-1,3-glucanase (Felix and Meins, 1987) and peroxidases (Abeles *et al.*, 1988).

The presence of endoplasmic reticulum, Golgi bodies and mitochondria is suggestive of a level of metabolic activity that one might expect of cells involved in an enzymatic process such as abscission, and is in keeping with the organellar composition of other plants known to possess enzymatic abscission, such as tobacco (Lieberman *et al.*, 1983). The membranous whorls projecting into the vacuoles of the *Azolla* abscission zone are intriguing for their similarity to structures found in tobacco abscission zones (Valdovinos *et al.*, 1985) although their significance is not clear; one possibility worthy of further investigation is that they may be involved in exocytosis.

The highly vacuolated nature of the *Azolla* branch abscission zone is a feature which differs from the abscission zones of most other plants, in which the abscission zone cells tend to be more densely cytoplasmic than their neighbors (Webster, 1973b). Also, the flattened shape of *Azolla* 's abscission zone cells is in contrast to the small, isodiametric cells of the typical flowering plant abscission zone. It has been suggested that the small, isodiametric cells function by expanding and causing the rupture of xylem (Sexton and Redshaw, 1981).

Presumably, these differences relate to the specialization of *Azolla* as a delicate floating aquatic. Also distinctive is the small size of its abscission zone, only a few cells across, in keeping with the diminutive proportions of this plant and its branches. Branch abscission in this genus serves a reproductive function, rather than the function of shedding senescent plant parts.

In conclusion, the data presented here provide indirect evidence that branch abscission in *Azolla* is promoted by ethylene, as seen by the response to applied ethylene promoters ACC and auxin, and the response to ethylene inhibitors AVG, AOA and silver. That these are the same ethylene promoters and inhibitors that function in flowering plants is suggestive of the presence of a similar ethylene biosynthetic pathway in this fern.

The anatomy of branch abscission in *Azolla* is consistent with a separation process involving enzymatic hydrolysis of intercellular materials, comparable to that commonly found in angiosperms, and is compatible with the physiological findings presented here suggesting that ethylene promotes branch abscission.

Table 1. Effect of ACC and AVG on branch abscission in *Azolla* as measured by mean number of plants per flask after 5 days of treatment
(October 1991).

Initial number of plants per flask = 5
(ACC: 1-aminocyclopropane-1-carboxylic acid; AVG: aminoethoxyvinylglycine)

Treatment	Mean	Standard Error	Standard Deviation
Control	8.333	1.333	2.309
ACC 10^{-5}M	17.333	0.667	1.155
ACC 10^{-4}M	20.000	3.055	5.292
ACC 10^{-3}M	9.667	3.712	6.429
AVG 10^{-5}M	5.333	0.333	0.577
AVG 10^{-4}M	5.667	0.333	0.577

Table 2. Effect of NAA and NAA+AVG on branch abscission in *Azolla* as measured by mean number of plants per flask after 5 days of treatment (November 1991).

Initial number of plants per flask = 3
(NAA: naphthaleneacetic acid; AVG: aminoethoxyvinylglycine)

Treatment	Mean	Standard Error	Standard Deviation
Control	4.0	0.548	1.225
NAA 2.7 X 10^{-7}M	4.2	0.583	1.304
NAA 2.7 X 10^{-6}M	10.0	0.837	1.871
NAA 2.7 X 10^{-5}M	8.6	0.678	1.517
NAA 2.7 X 10^{-5}M + AVG 10^{-5}M	5.6	0.600	1.342
NAA 2.7 X 10^{-5}M + AVG 10^{-4}M	3.6	0.245	0.548

Table 3. Effect of ACC, AVG, NAA, and NAA+AVG on branch abscission in *Azolla* as measured by mean number of plants per flask after 5 days of treatment (April 1992).

Initial number of plants per flask = 3
(NAA: naphthaleneacetic acid; ACC: 1-aminocyclopropane-1-carboxylic acid; AVG: aminoethoxyvinylglycine)

Treatment	Mean	Standard Error	Standard Deviation
Control	5.6	0.510	1.140
ACC 10^{-5}M	32.6	2.159	4.827
ACC 10^{-4}M	30.4	2.379	5.320
AVG 10^{-5}M	4.4	0.245	0.548
AVG 10^{-4}M	4.0	0.316	0.707
NAA 2.7 X 10^{-5}M	20.8	5.472	12.235
NAA 2.7 X 10^{-5}M + AVG 10^{-5}M	15.8	1.463	3.271
NAA 2.7 X 10^{-5}M + AVG 10^{-4}M	10.4	0.812	1.817

Table 4. Effect of AOA and silver on branch abscission in *Azolla* as measured by mean number of plants per flask after 5 days of treatment (July 1992).

Initial number of plants per flask: 3.
(AOA: amino-oxyacetic acid; $AgNO_3$: silver nitrate; NaTS: sodium thiosulfate; STS: silver thiosulfate)

Treatment	Mean	Standard Error	Standard Deviation
Control	58.2	4.727	10.569
AOA 10^{-5}M	28.6	4.986	11.149
AOA 10^{-4}M	48.4	9.125	20.403
$AgNO_3$ 4 X 10^{-5}M	22.2	5.660	12.657
$AgNO_3$ 4 X 10^{-4}M	26.4	2.502	5.595
NaTS 1.6 X 10^{-4}M	38.8	8.393	18.767
NaTS 1.6 X 10^{-3}M	26.8	4.176	9.338
STS 4 X 10^{-5}M	40.8	3.639	8.136
STS 4 X 10^{-4}M	13.2	2.059	4.604

Table 5. Effect of ACC and AVG on number of abscised branches 1 mm or less in length after 5 days of treatment (October 1991).

Initial number of plants per flask = 5
(ACC: 1-aminocyclopropane-1-carboxylic acid; AVG: aminoethoxyvinylglycine)

Treatment	Mean	Standard Error	Standard Deviation
Control	1.333	1.333	2.309
ACC 10^{-5}M	5.000	0.577	1.000
ACC 10^{-4}M	5.000	1.000	1.732
ACC 10^{-3}M	1.333	1.333	2.309
AVG 10^{-5}M	0.000	0.000	0.000
AVG 10^{-4}M	0.000	0.000	0.000

Table 6. Effect of NAA and NAA+AVG on number of abscised branches 1 mm or less or less in length after 5 days of treatment (November 1991).

Initial number of plants per flask = 3
(NAA: naphthaleneacetic acid; AVG: aminoethoxyvinylglycine)

Treatment	Mean	Standard Error	Standard Deviation
Control	0.6	0.400	0.894
NAA 2.7 X 10^{-7}M	0.0	0.000	0.000
NAA 2.7 X 10^{-6}M	0.4	0.400	0.894
NAA 2.7 X 10^{-5}M	1.2	0.583	1.304
NAA 2.7 X 10^{-5}M + AVG 10^{-5}M	0.6	0.600	1.342
NAA 2.7 X 10^{-5}M + AVG 10^{-4}M	0.2	0.200	0.447

Table 7. Effect of ACC, AVG, NAA, and NAA+AVG on number of abscised branches 1 mm or less in length after 5 days of treatment (April 1992).

Initial number of plants per flask = 3.
(NAA: naphthaleneacetic acid; ACC: 1-aminocyclopropane-1-carboxylic acid; AVG: aminoethoxyvinylglycine)

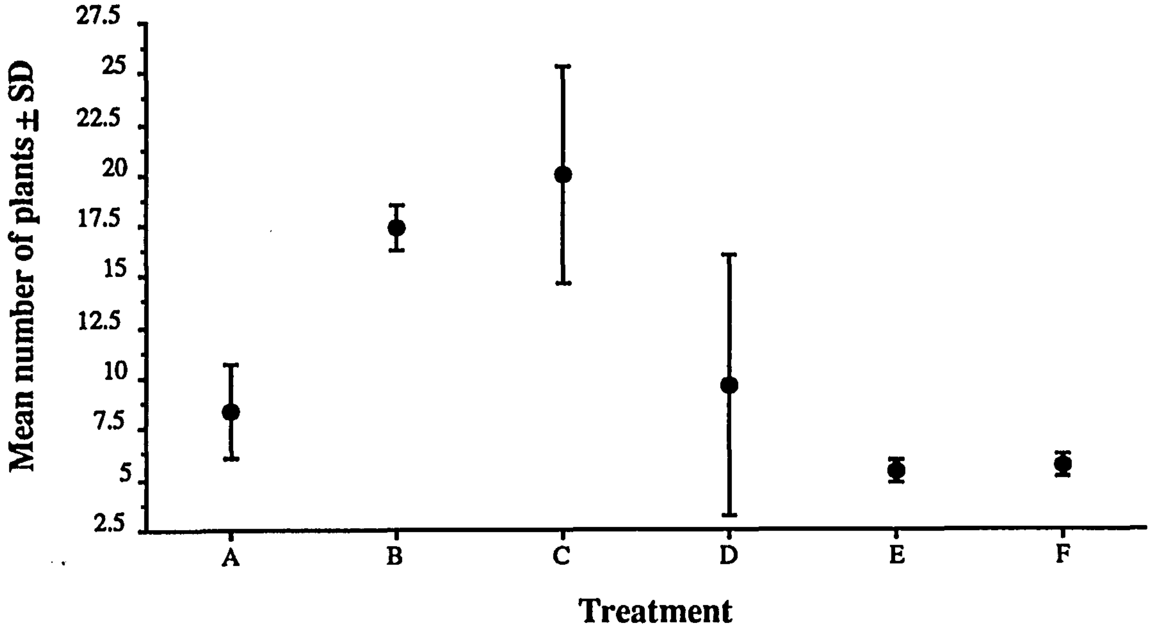
Treatment	Mean	Standard Error	Standard Deviation
Control	0.0	0.000	0.000
ACC 10^{-5}M	4.6	0.927	2.074
ACC 10^{-4}M	8.2	2.177	4.868
AVG 10^{-5}M	0.0	0.000	0.000
AVG 10^{-4}M	0.0	0.000	0.000
NAA 2.7 X 10^{-5}M	0.0	0.000	0.000
NAA 2.7 X 10^{-5}M + AVG 10^{-5}M	0.0	0.000	0.000
NAA 2.7 X 10^{-5}M + AVG 10^{-4}M	0.0	0.000	0.000

Table 8. Effect of AOA and silver on number of abscised branches per flask after 5 days of treatment (July 1992).

Initial number of plants per flask: 3
(AOA: amino-oxyacetic acid; $AgNO_3$: silver nitrate;
NaTS: sodium thiosulfate; STS: silver thiosulfate)

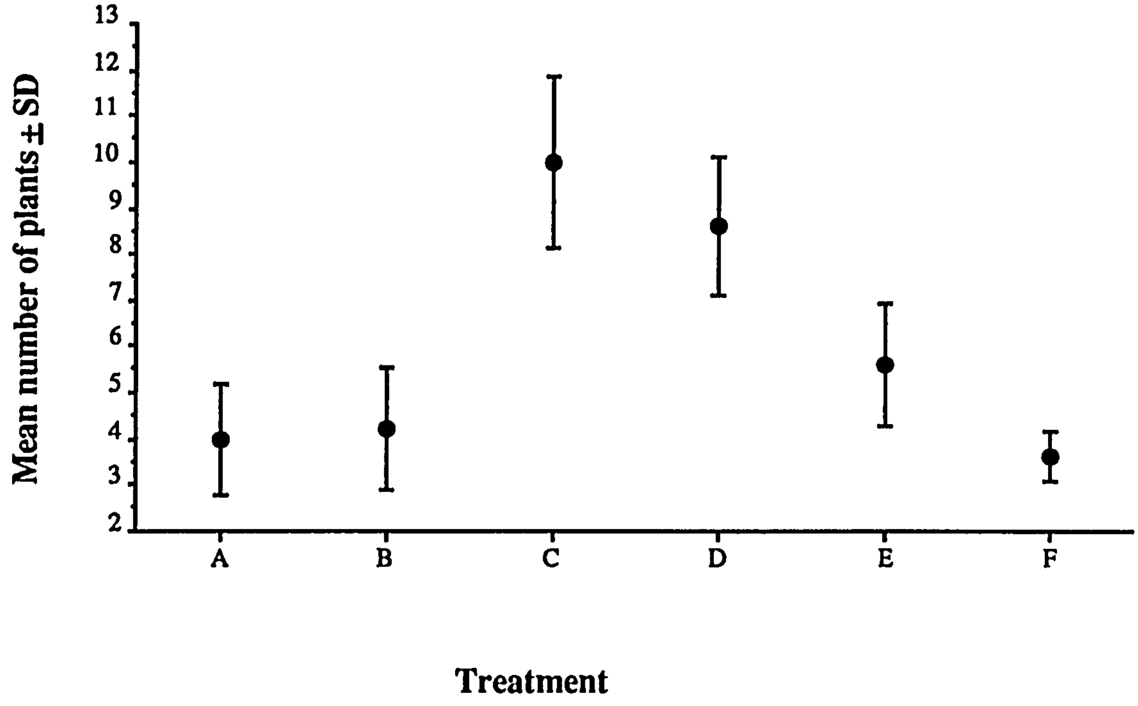
Treatment	Mean	Standard Error	Standard Deviation
Control	1.8	0.860	1.924
AOA 10-5M	1.4	0.980	2.191
AOA 10-4 M	14.4	4.654	10.407
$AgNO_3$ 4 X 10^{-5}M	0.0	0.000	0.000
$AgNO_3$ 4 X 10^{-4}M	0.0	0.000	0.000
NaTS 1.6 X 10^{-4}M	0.8	0.374	0.837
NaTS 1.6 X 10^{-3}M	1.0	0.632	1.414
STS 4 X 10^{-5}M	0.0	0.000	0.000
STS 4 X 10^{-4}M	0.0	0.000	0.000

Figure 1. Effect of ACC and AVG on branch abscission of *Azolla*, as measured by number of plants per flask after 5 days (October 1991). Initial number of plants per flask = 5.



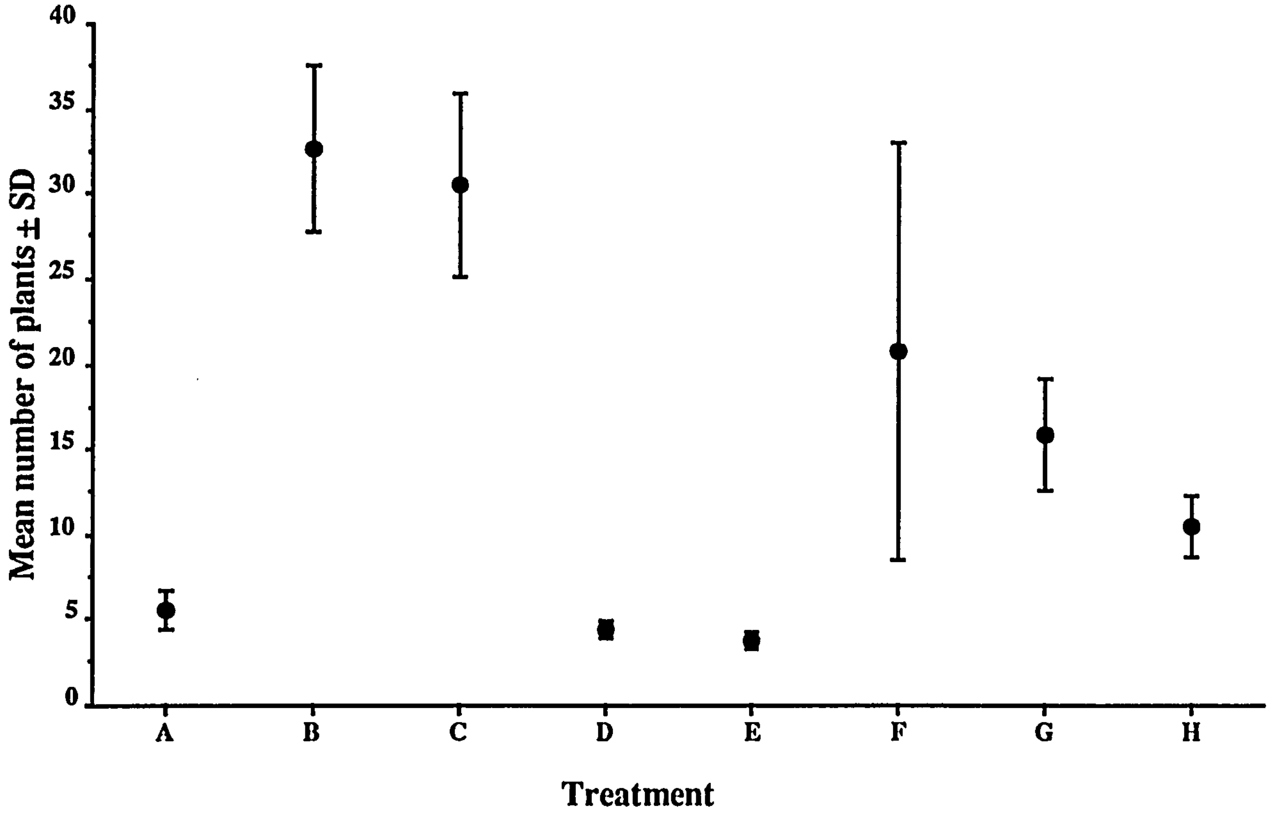
A - Control
B - ACC 10-5M
C - ACC 10-4M
D - ACC 10-3M
E - AVG 10-5M
F - AVG 10-4M

Figure 2. Effect of NAA and NAA+AVG on branch abscission in *Azolla*, as measured by mean number of plants per flask after 5 days (November, 1991). Initial number of plants per flask = 3



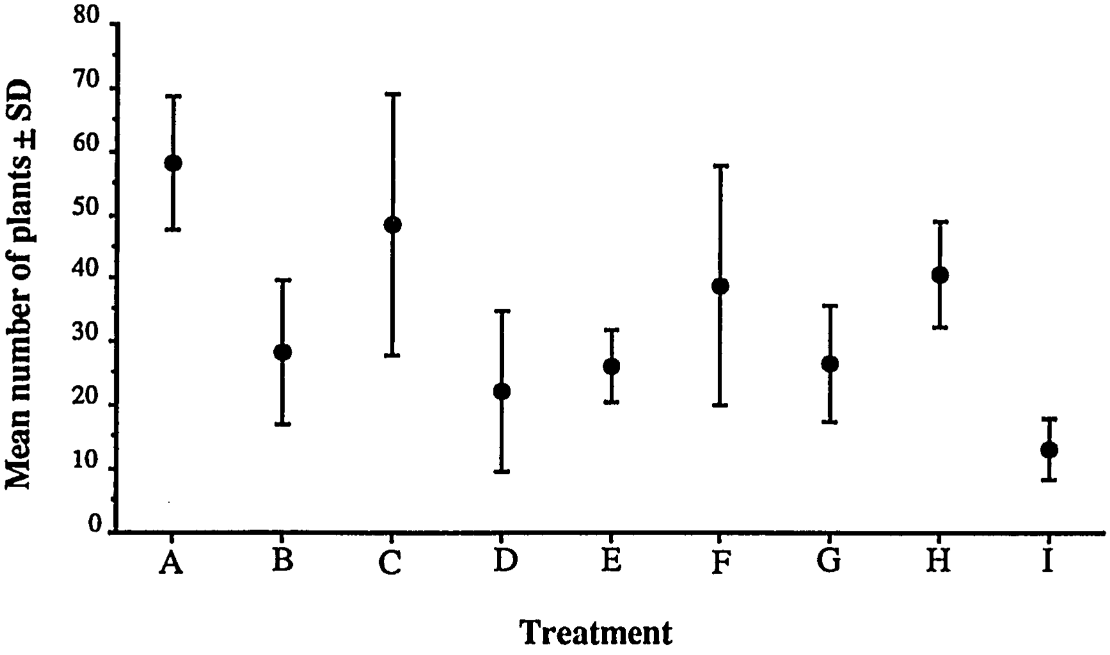
A - Control
B - NAA 2.7 X 10-7M
C - NAA 2.7 X 10-6M
D - NAA 2.7 X 10-5M
E - AVG 10-5M + NAA 2.7 X 10-5M
F - AVG 10-4M + NAA 2.7 X 10-5M

Figure 3. Effect of ACC, AVG, NAA, and NAA + AVG on branch abscission of *Azolla*, as measured by mean number of plants per flask after 5 days (April 1992). Initial number of plants per flask = 3.



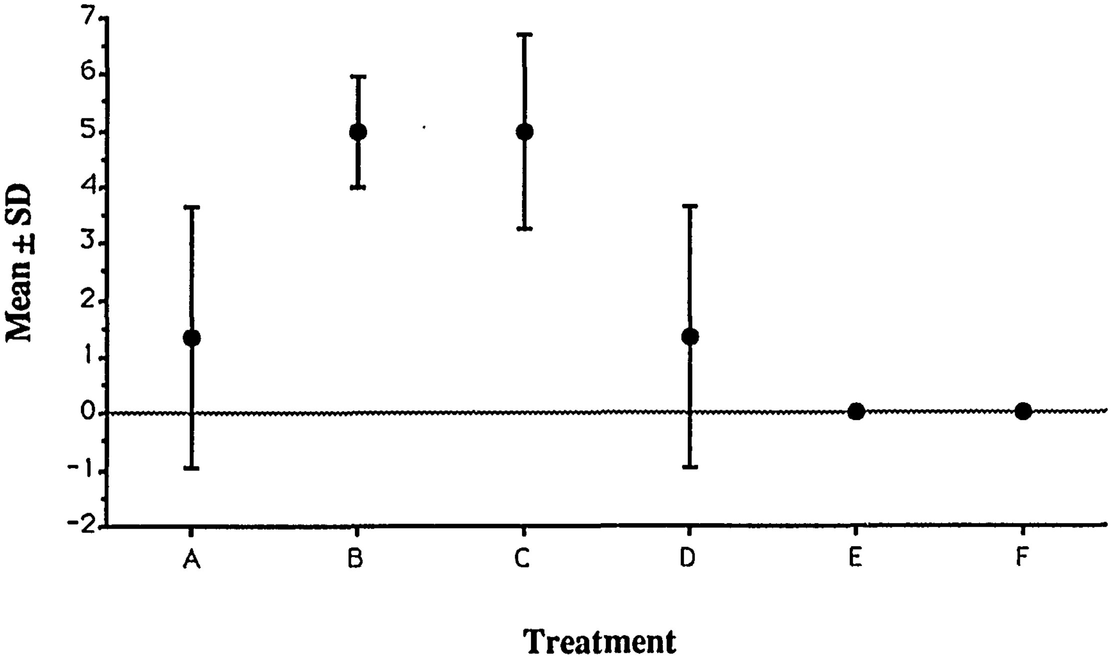
A - Control
B - ACC 10-5M
C - ACC 10-4M
D - AVG 10-5M
E - AVG 10-4M
F - NAA 2.7 X 10-5M
G - NAA 2.7 X 10-5M + AVG 10-5M
H - NAA 2.7 X 10-5M + AVG 10-4M

Figure 4. Effect of AOA and silver on branch abscission in *Azolla*, as measured by mean number of plants per flask after 5 days of treatment (July 1992). Initial number of plants per flask = 3.



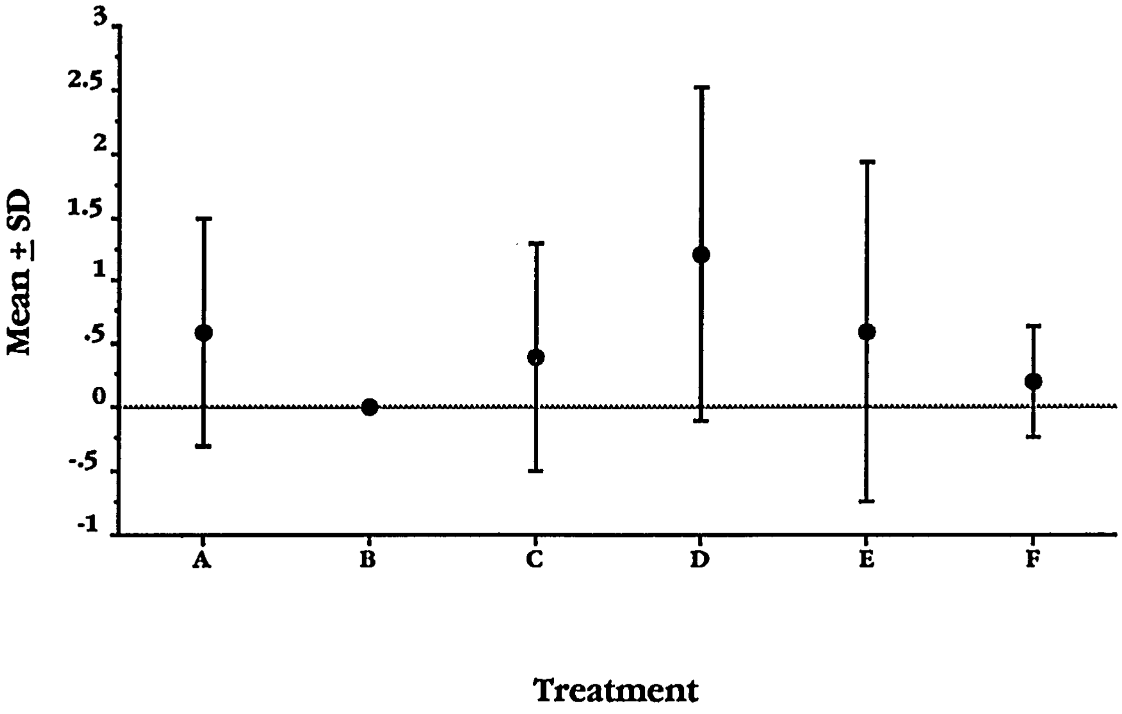
A - Control
B - AOA 10-5M
C - AOA 10-4M
D - AgNO3 4 X 10-5M
E - AgNO3 4 X 10-4M
F - NaTS 1.6 X 10-4M
G - NaTS 1.6 X 10-3M
H - STS 4 X 10-5M
I - STS 4 X 10-4M

Figure 5. Effect of ACC and AVG on mean number of abscised branches 1 mm or less in length after 5 days (October, 1991).
Initial number of plants per flask = 5.



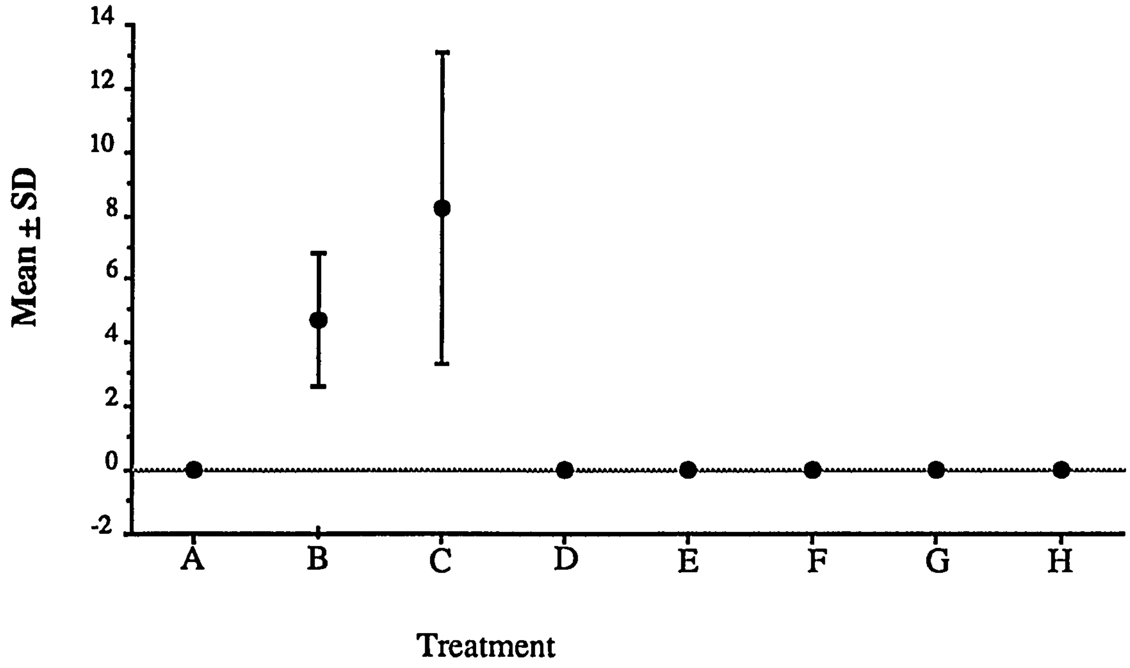
A - Control
B - ACC 10-5M
C - ACC 10-4M
D - ACC 10-3M
E - AVG 10-5M
F - AVG 10-4M

Figure 6. Effect of NAA and NAA + AVG on mean number of abscised branches 1 mm or less in length after 5 days (Nov. 1991). Initial number of plants per flask = 3



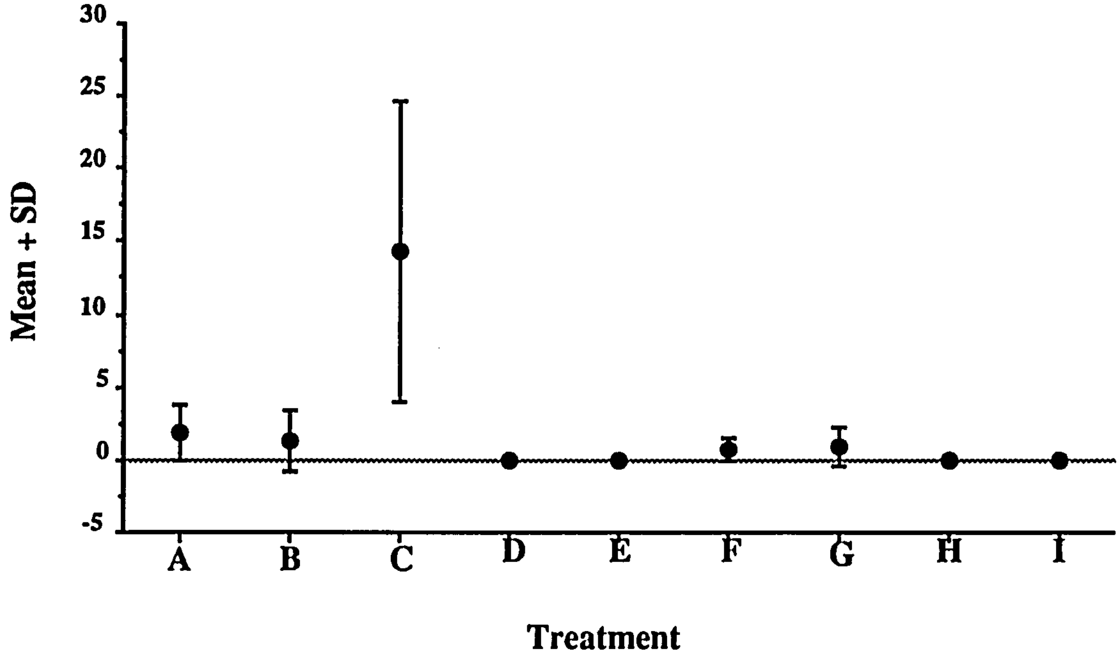
A - Control
B - NAA 2.7 X 10-7M
C - NAA 2.7 X 10-6M
D - NAA 2.7 X 10-5M
E - AVG 10-5M + NAA 2.7 X 10-5M
F - AVG 10-4M + NAA 2.7 X 10-5M

Fig. 7. Effect of ACC, AVG, NAA, and NAA + AVG on mean number of abscised branches 1 mm or less in length after 5 days (April 1992). Initial number of plants per flask = 3



A - Control
B - ACC 10-5M
C - ACC 10-4M
D - AVG 10-5M
E - AVG 10-4M
F - NAA 2.7 X 10-5M
G - NAA 2.7 X 10-5M + AVG 10-5M
H - NAA 2.7 X 10-5M + AVG 10-4M

Fig. 8. Effect of AOA and silver on mean number of abscised branches 1 mm or less in length after 5 days (July 1992). Initial number of plants per flask = 3.



A - Control
B - AOA 10-5M
C - AOA 10-4M
D - AgNO3 4 X 10-5M
E - AgNO3 4 X 10-4M
F - NaTS 1.6 X 10-4M
G - NaTS 1.6 X 10-3M
H - STS 4 X 10-5M
I - STS 4 X 10-4M

Micrographs

Figure 9. Scanning electron micrograph (SEM) of dorsal surface of *Azolla* 60X. Scale bar = 1 mm.

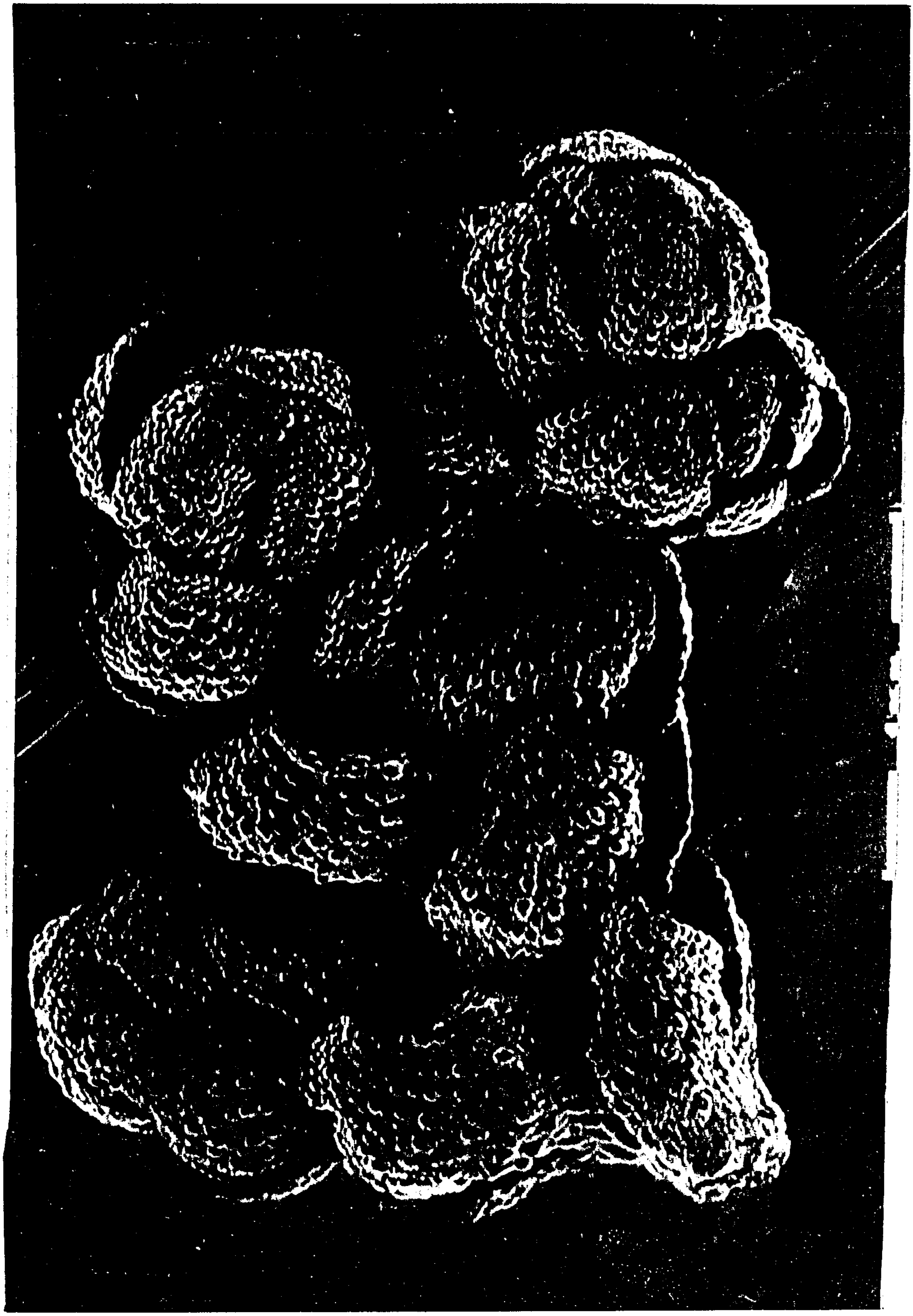


Figure 10. SEM of dorsal surface of *Azolla* showing branches (br) of various sizes. 26X. Scale bar = 1 mm.

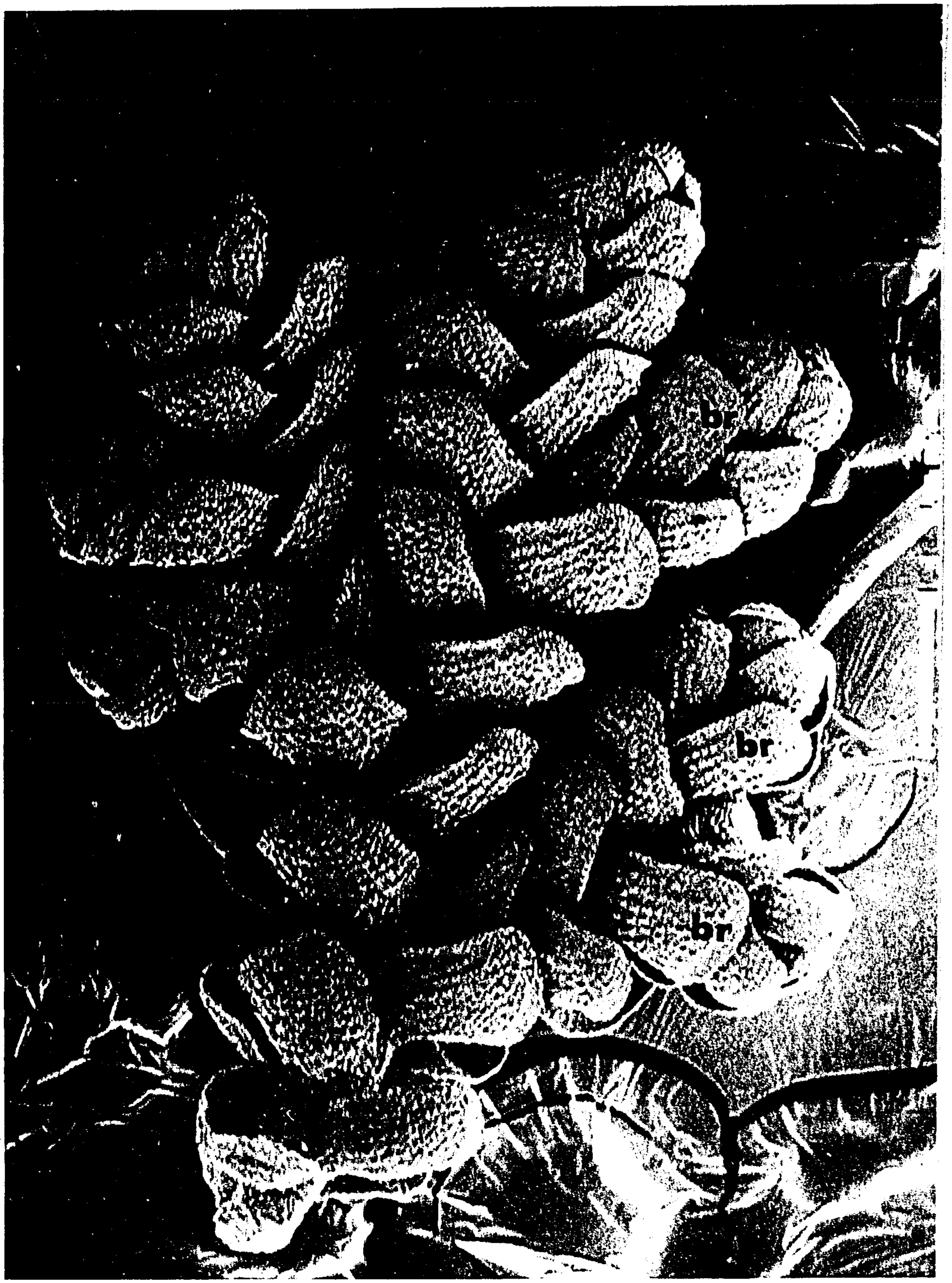

b
br
br

Figure 11. SEM of *Azolla* plant in dorsal view. 55X. Scale bar = 1 mm.

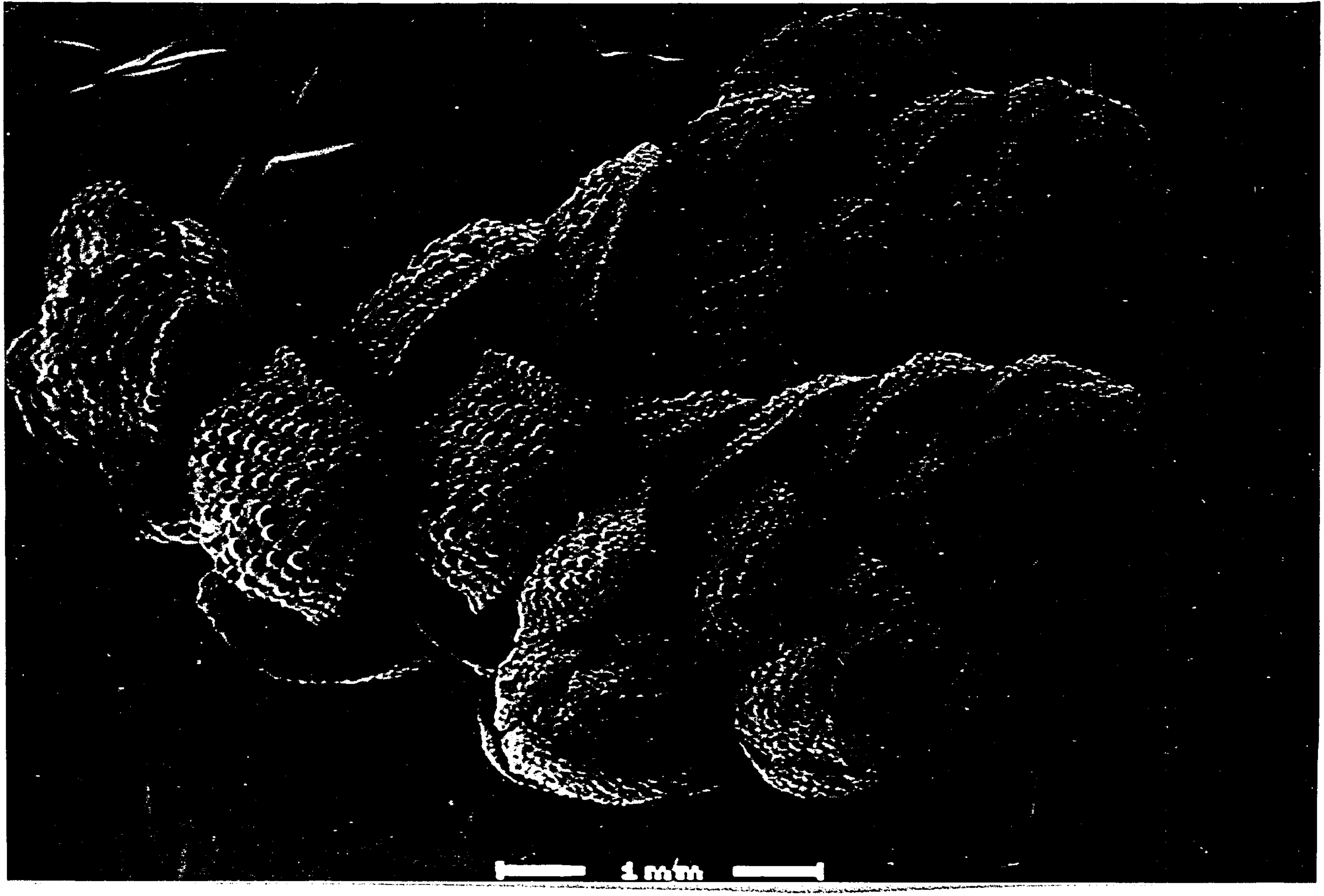

1 mm

Figure 12. SEM of *Azolla* leaf cavity sectioned to show colony of *Anabaena* (An) with heterocysts (white arrows) 596X.
Scale bar = 100 µm.

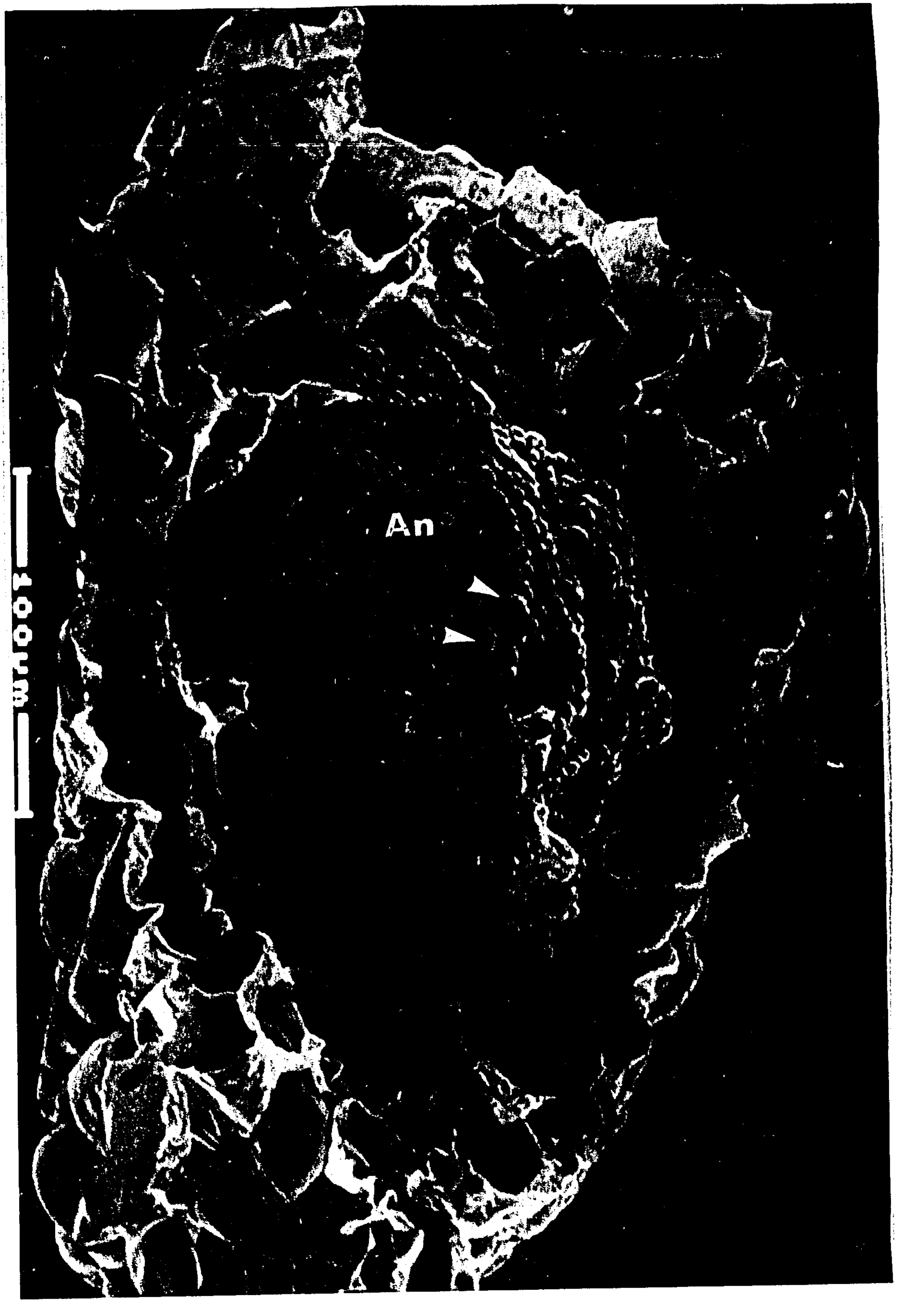

An
100 μm

Figure 13. Transmission electron micrograph (TEM) of leaf cell of *Azolla* showing bacteria (B) in gap in cell membrane (Cm). 30,000X.


Cm
B

Figure 14. SEM of ventral surface of *Azolla* showing emergent roots (arrows). 30X. Scale bar = 1 mm.

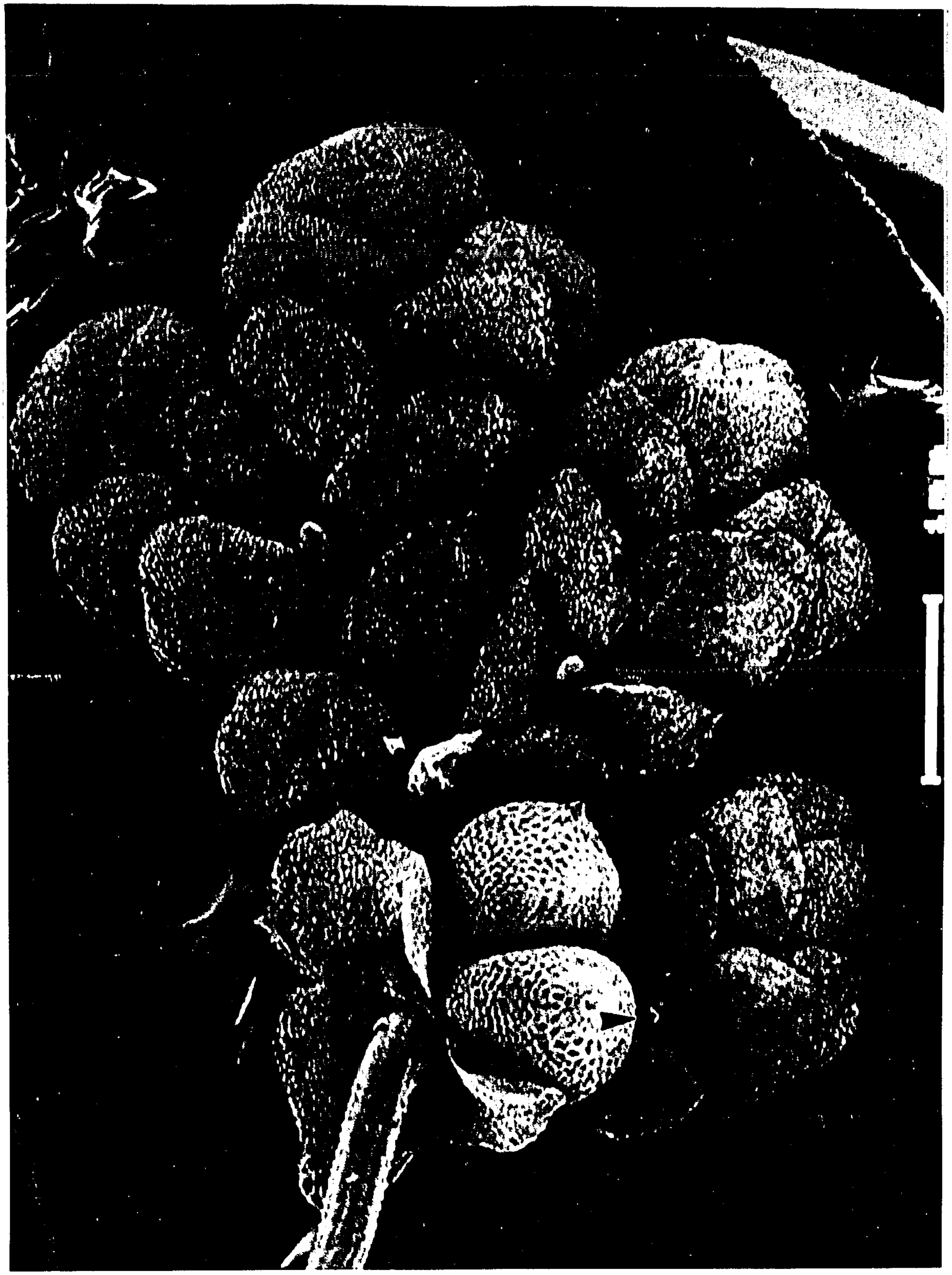


Figure 15. SEM of ventral surface of *Azolla* showing roots (R). 43X. Scale bar = 1 mm.

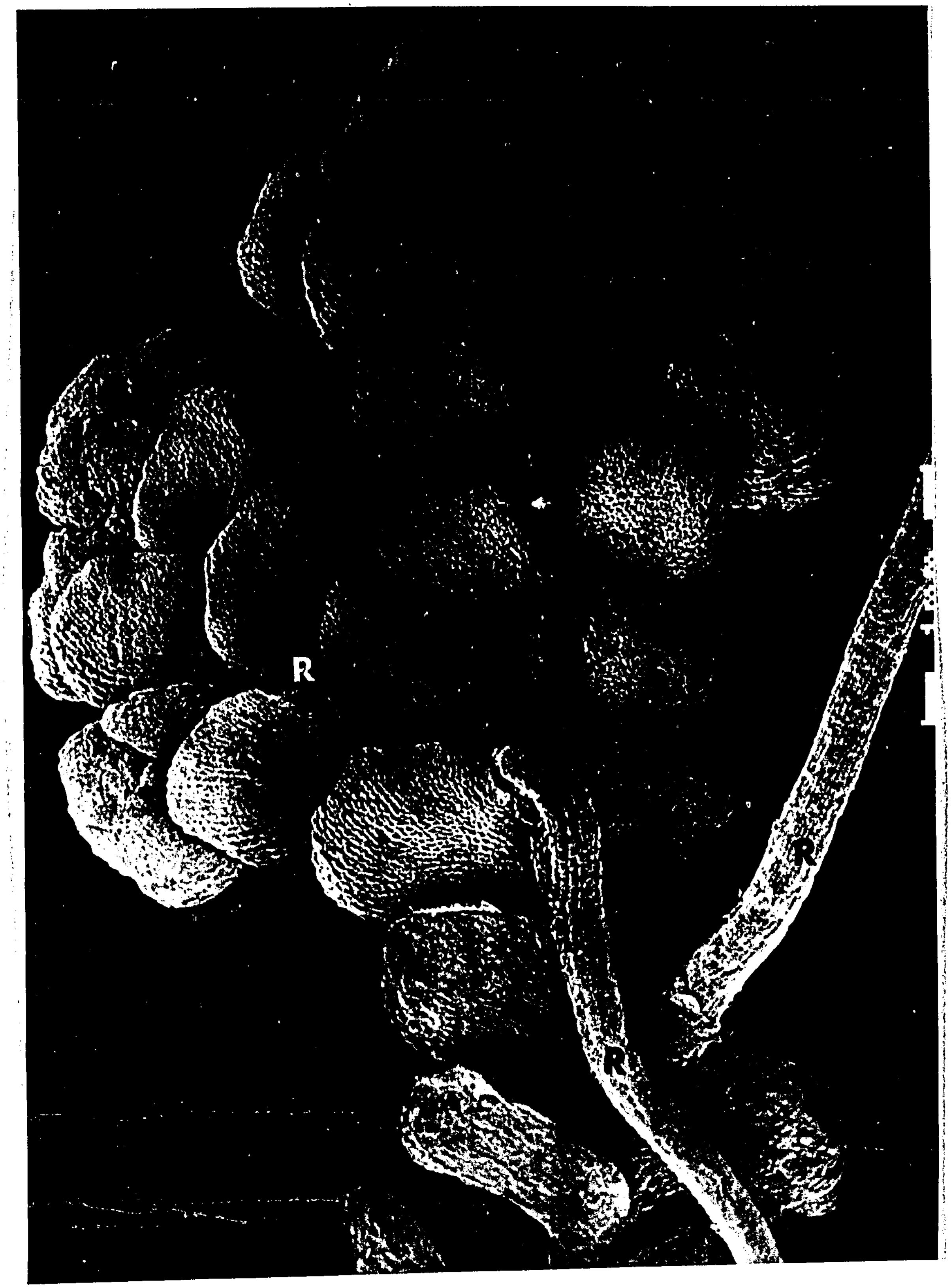

R
R
R

Figure 16. SEM of leaf base showing collar (C) of epidermal cells.
300X. Scale bar = 100 µm.

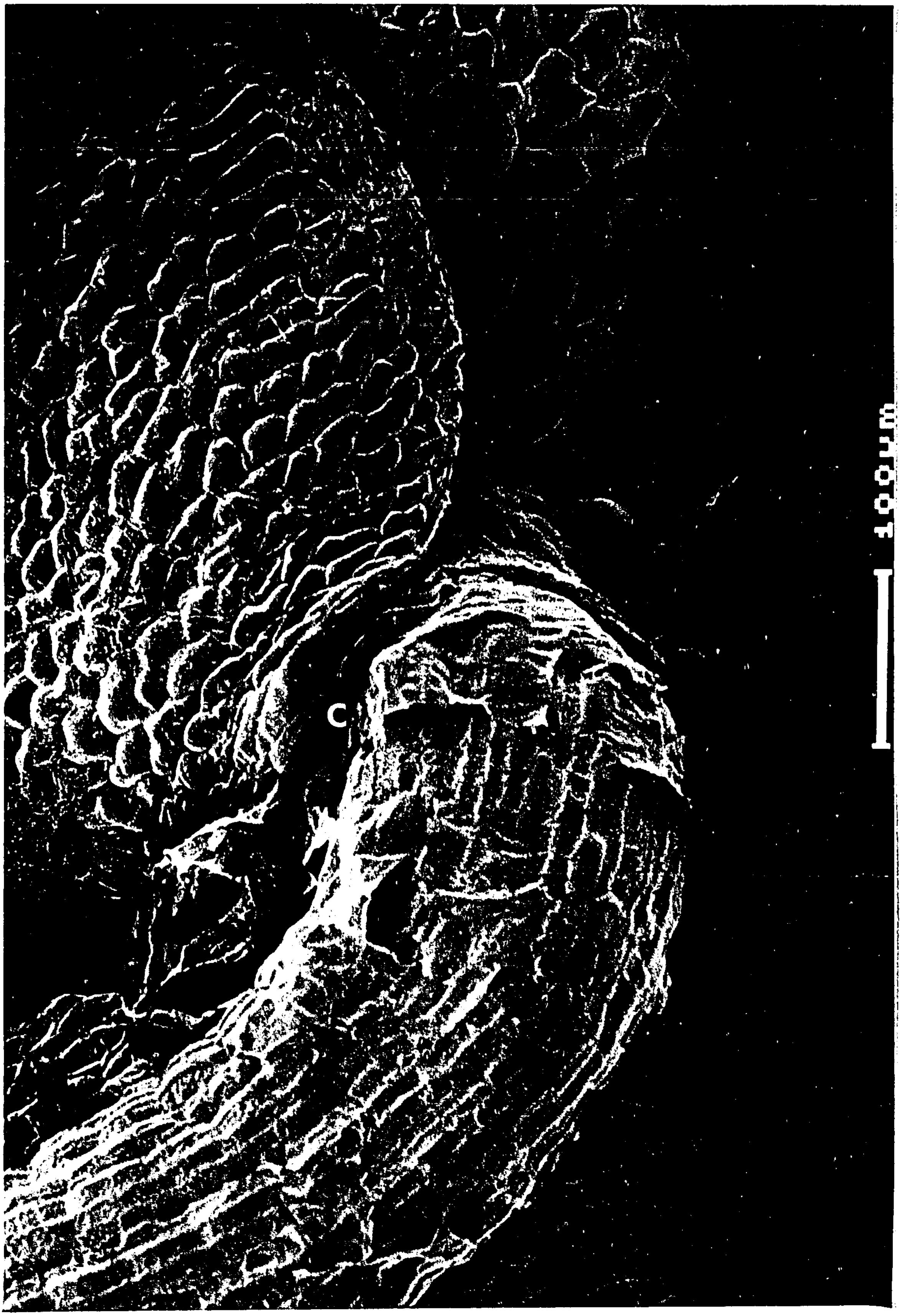
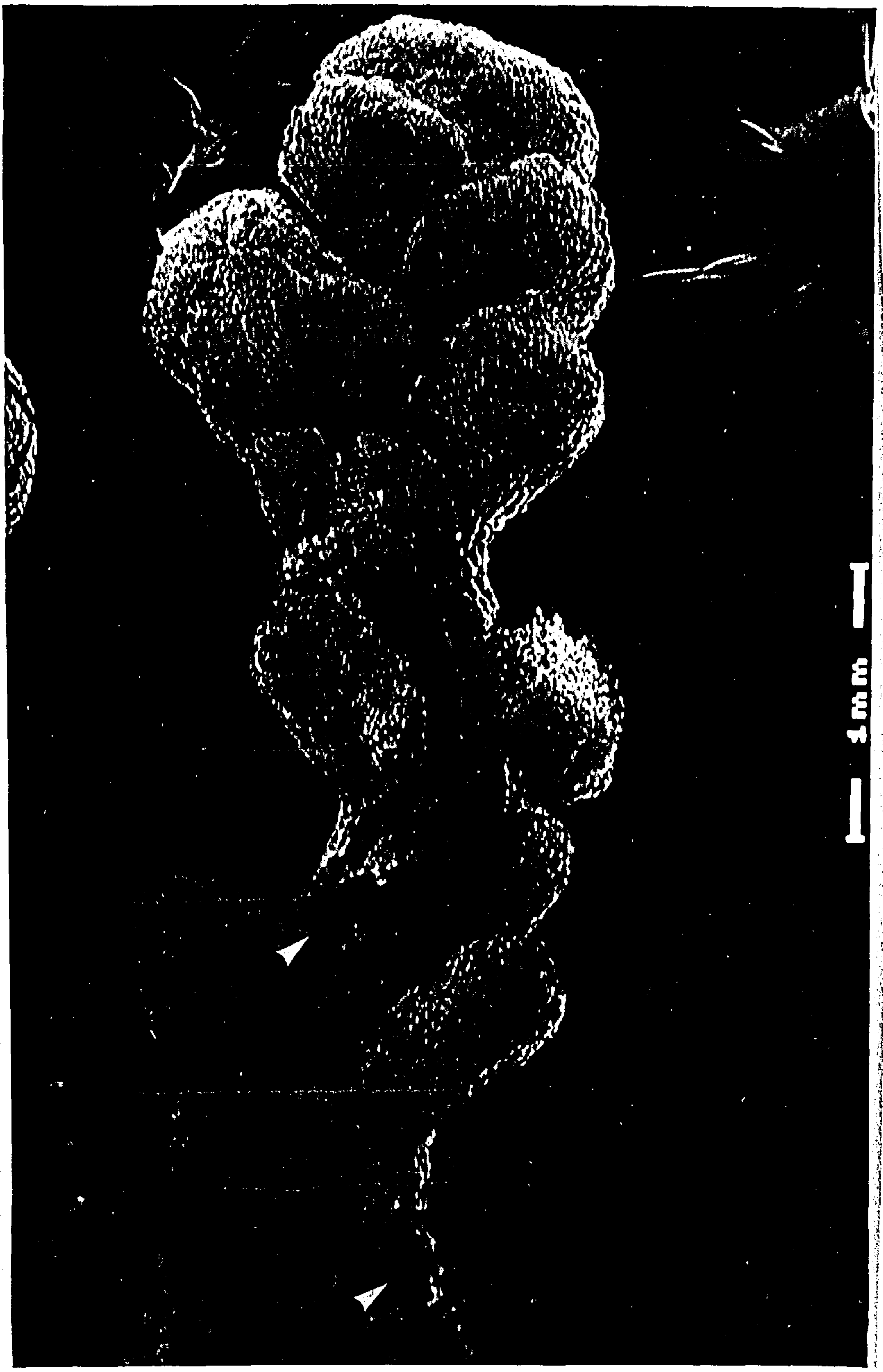

C
100µm

Figure 17. SEM of ventral surface of *Azolla* showing abscission scars (white arrows). 46X. Scale bar = 1 mm.


1mm

18. SEM of root abscission scar with diatoms (D). 300X.
Scale bar = 100 µm.

Figure 19. SEM of base of *Azolla* branch showing branch scars with intact cortical cell surfaces (C) and broken stelar cells (white arrow). 300X. Scale bar = 100 µm.

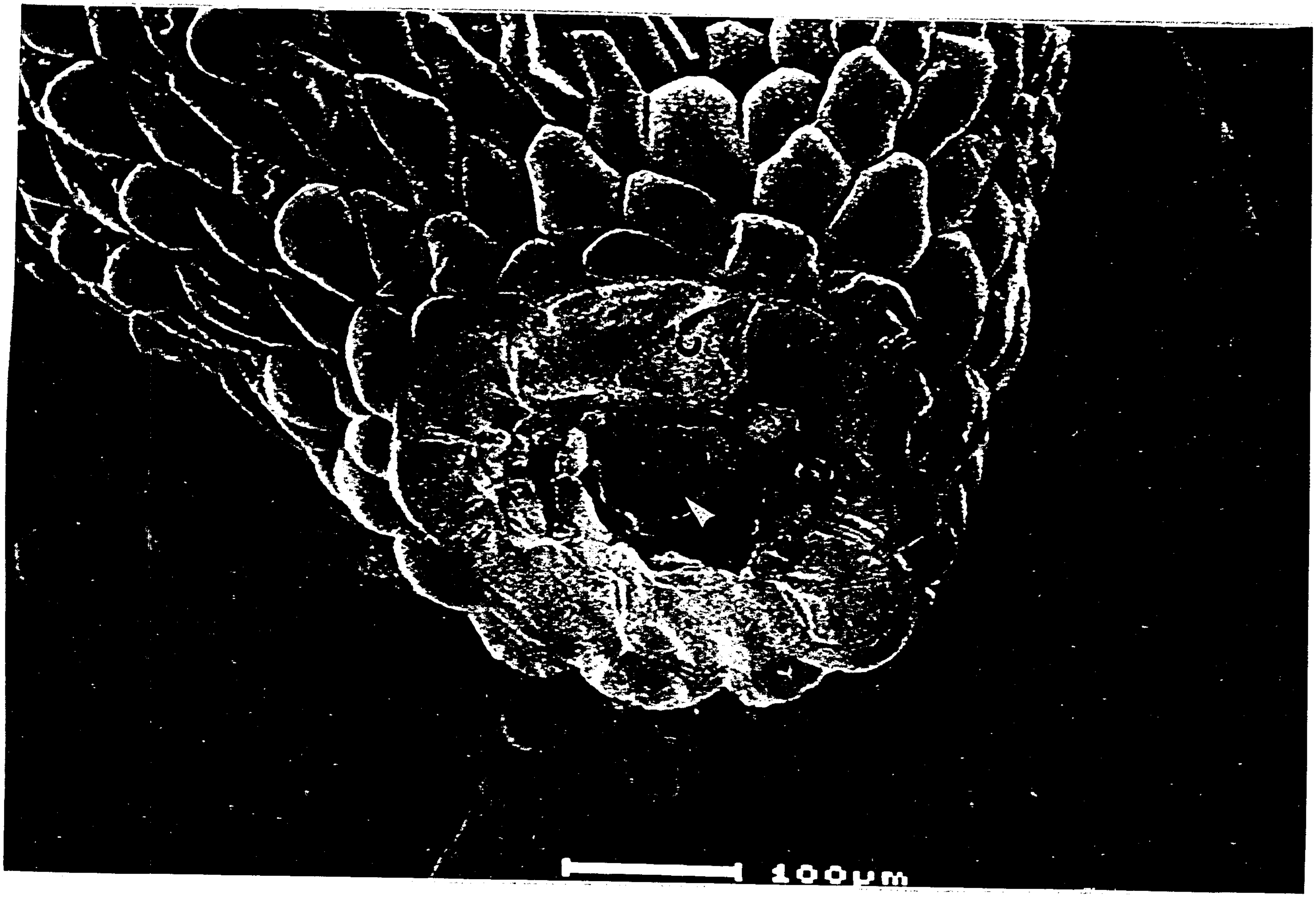

G
100µm

Figure 20. SEM of base of *Azolla* branch scar with intact cortical cell surfaces (C) and broken stelar cells (white arrow). 300X. Scale bar = 100 µm.

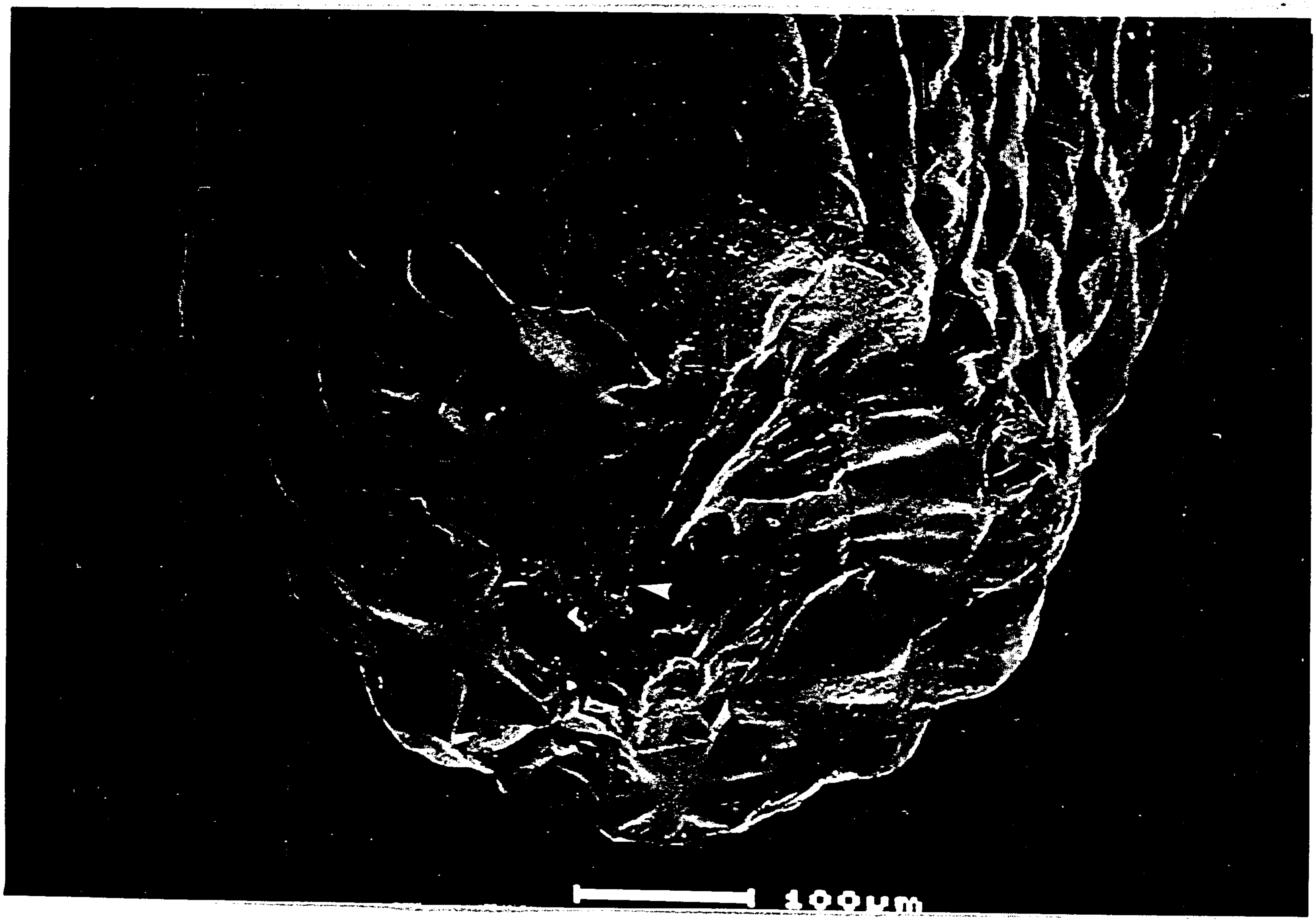

100µm

Figure 21. SEM of *Azolla* stem showing branch abscission scar (arrow) and emerging root (R). 198X. Scale bar = 100 µm.

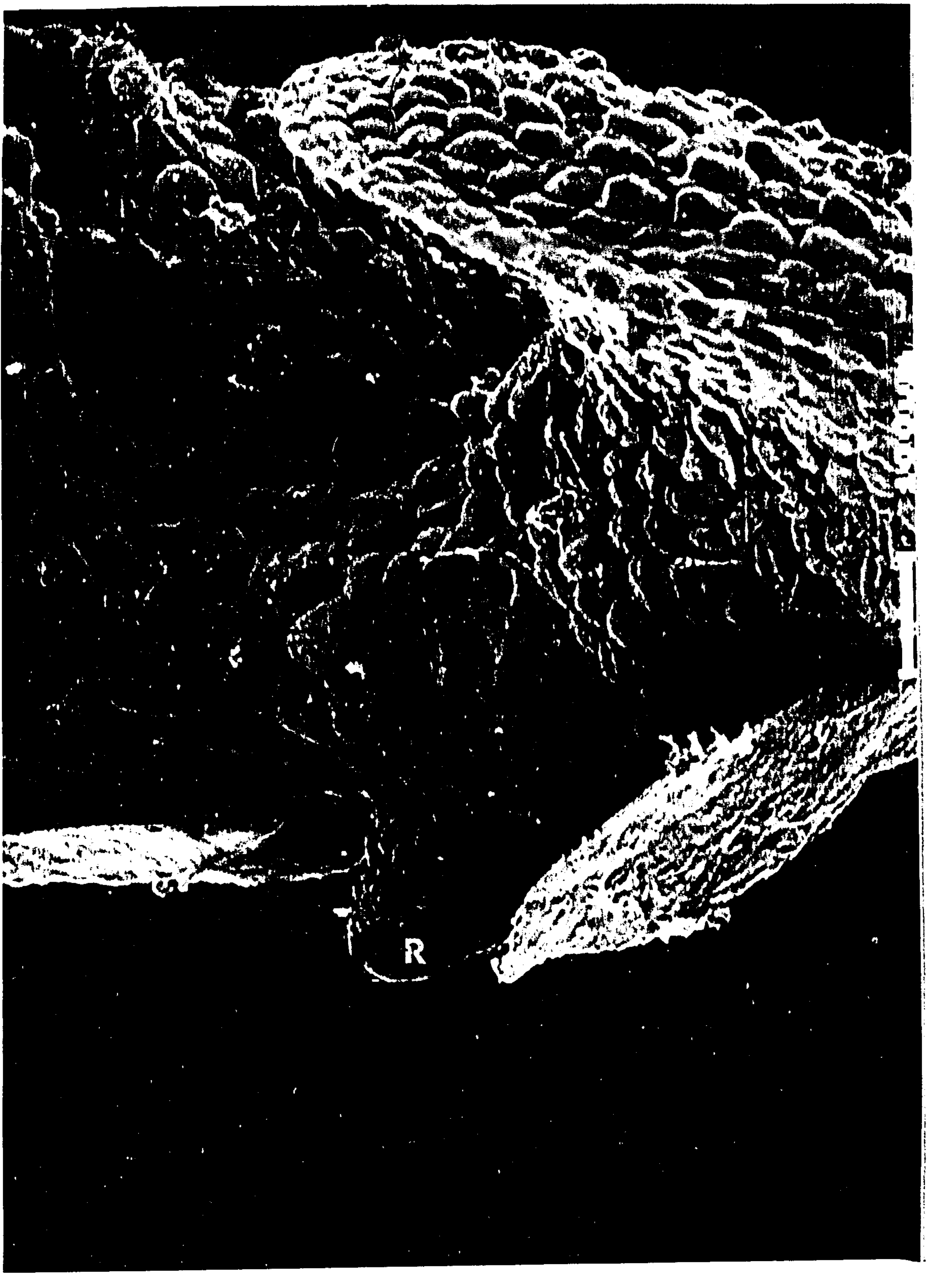

R

Figure 22. SEM of *Azolla* stem showing branch scar with intact cortical cells (C), broken stelar cells (arrow) and emergent root (R). 440X. Scale bar = 100 μm.


C
R
100µm

Figure 23. SEM close-up of branch scar on stem showing intact cortical cells (C) and broken stelar cells (white arrow). 3580X. Scale bar = 10 μm.


10 µm

Figure 24. SEM of *Azolla* branch base broken after critical point drying 202X. Scale bar = 100 µm.

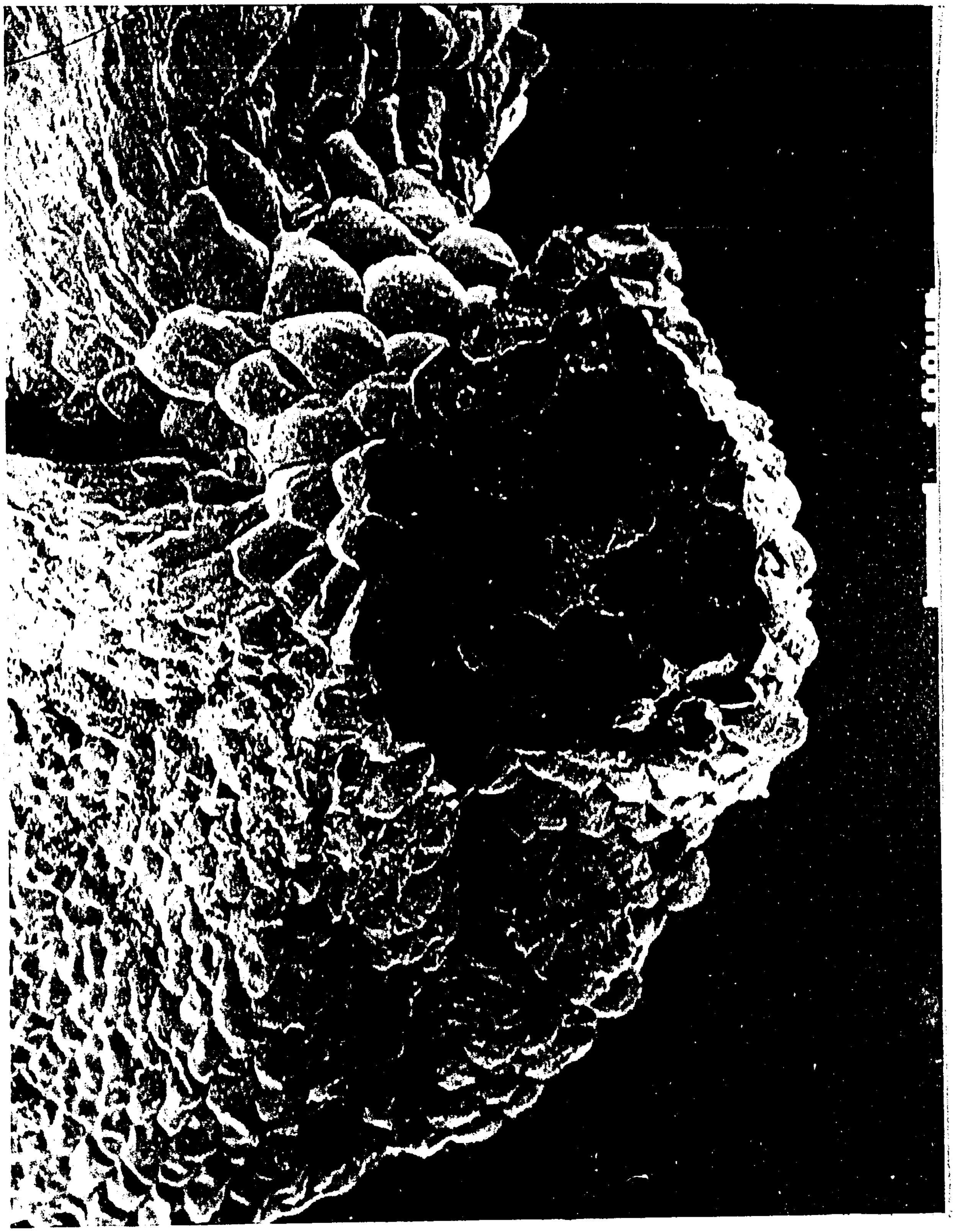

100μm

Figure 25. SEM of base of *Azolla* branch treated with 10^{-4}M ACC and forced to abscise. 304X. Scale bar = 100 μm.

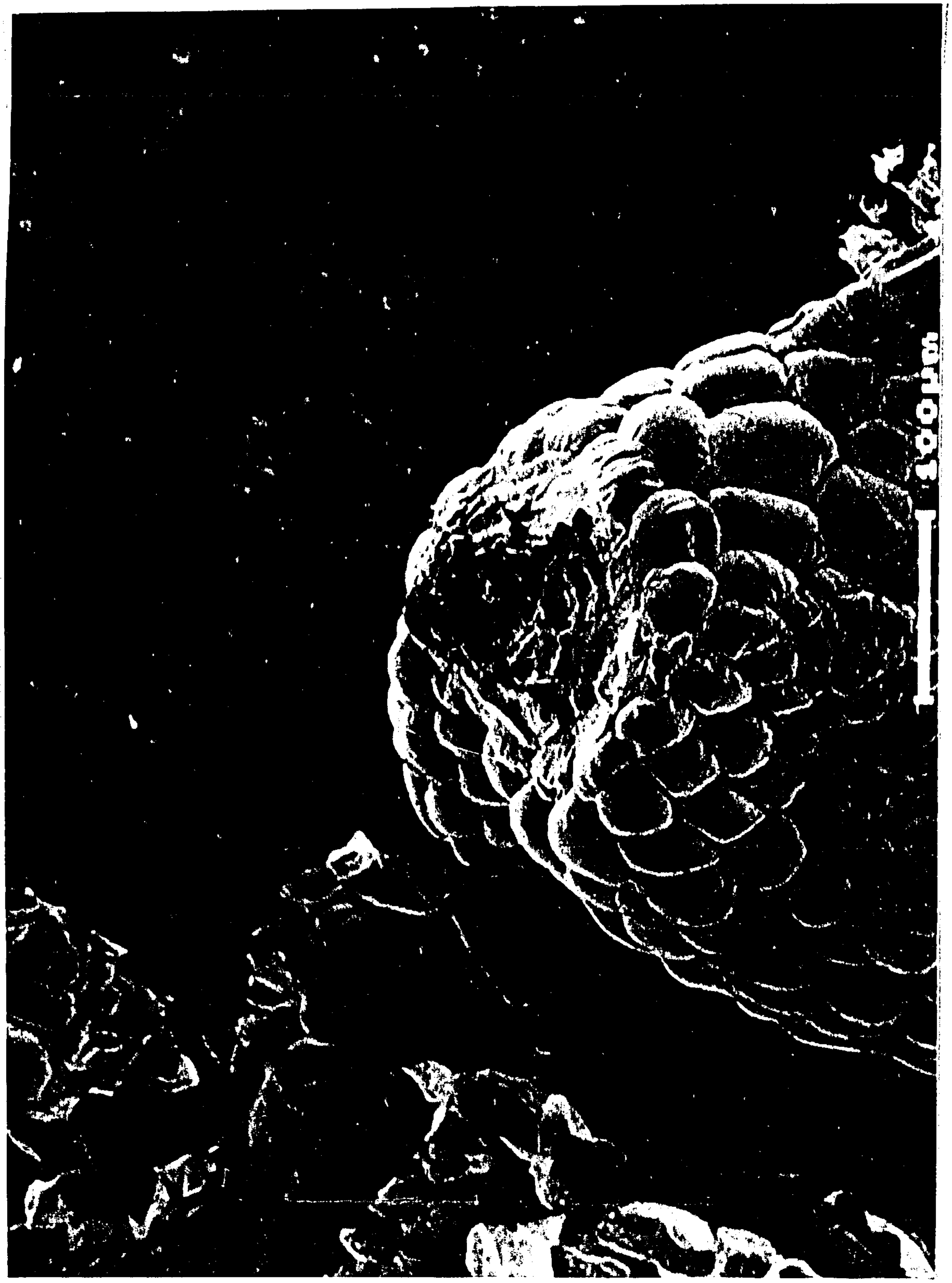

100µm

Figure 26. SEM of base of branch treated with 10^{-4}M ACC and forced to abscise, showing rough cortical cell faces (C). 302X.
Scale bar = 100 µm.


100µm
C

Figure 27. Light micrograph of *Azolla* stem section showing root (R), vascular stele (S), branch abscission zone (arrows), and *Anabaena* (An) in leaf cavity. 400X.

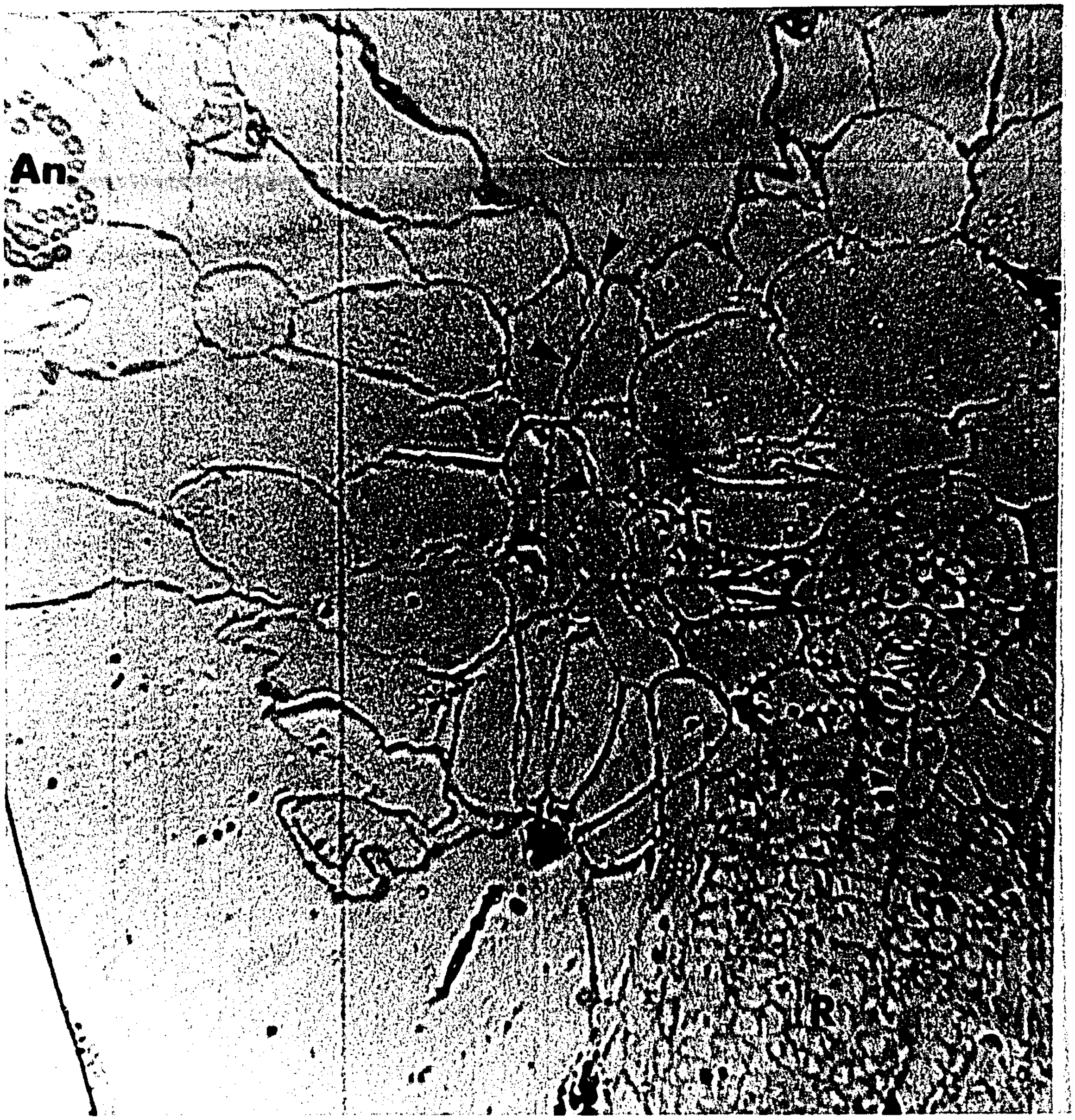

An.
R

Figure 28. Light micrograph showing abscission zone with cell separation beginning in peripheral cells (arrow). 1000X

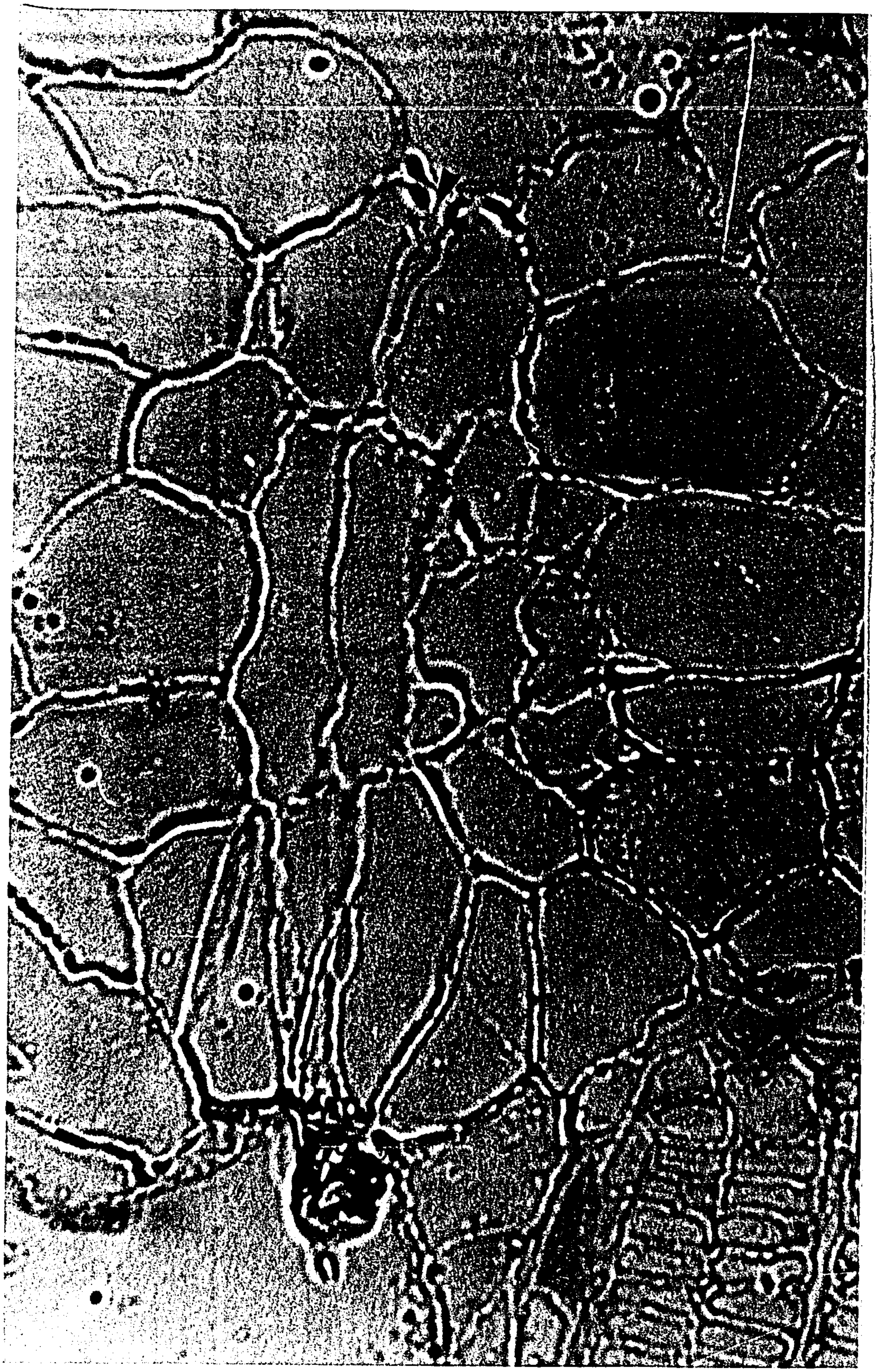


Figure 29. TEM of separating abscission zone cells at exterior of plant, showing abscission zone between separating cells (AZ), plasmodesmata (arrows), cell walls (CW), and vacuoles (V) 30,000X.

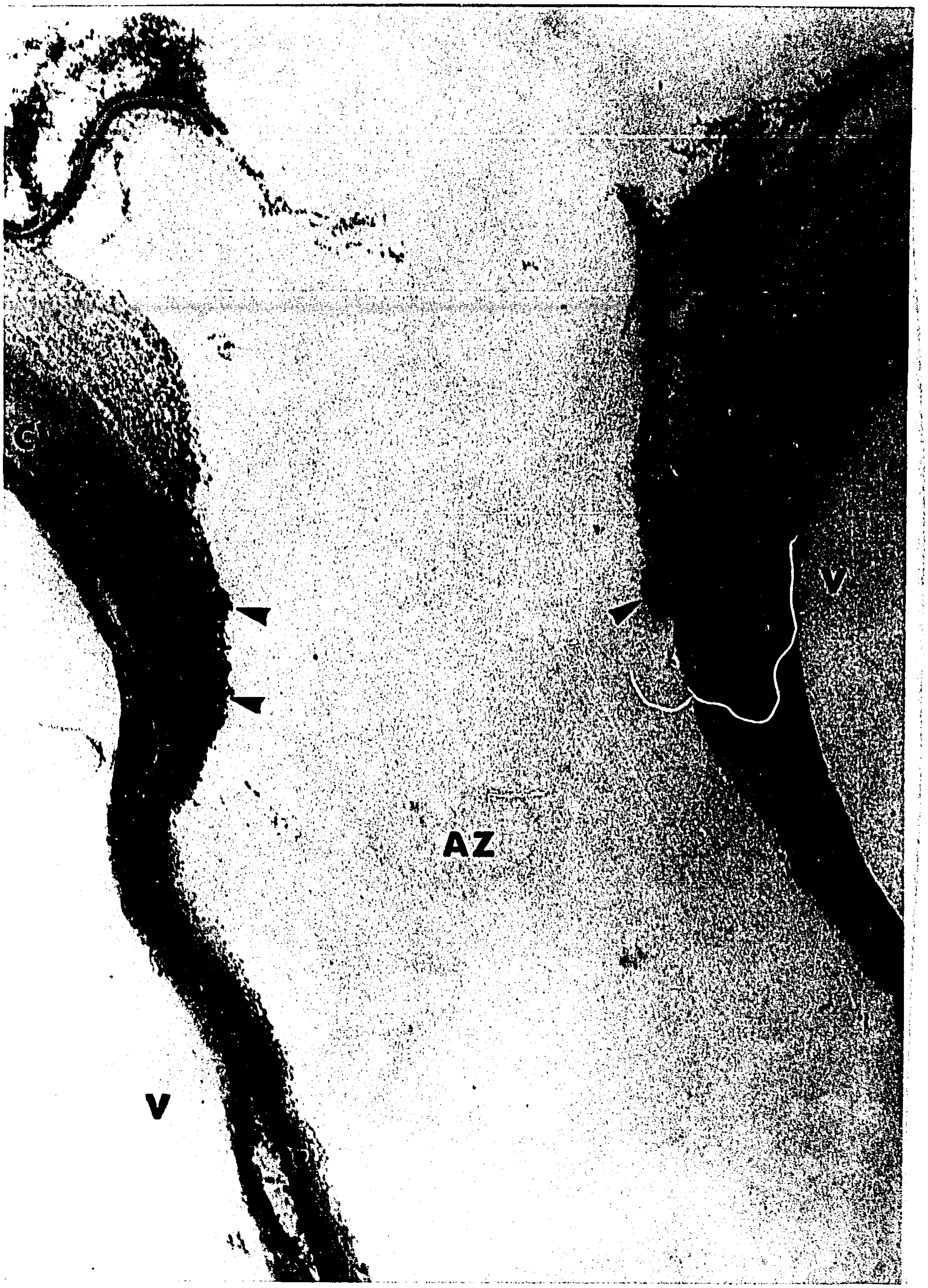

C
V
V
AZ

Figure 30. TEM of separating cells toward exterior of plant, showing abscission zone (AZ), cell walls (CW), vacuoles (V), and plasmodesmata (arrows). 10,500X.


W
CW
AZ
V
V

Figure 31. TEM of zone of separation between abscission zone cells, showing separation widest toward exterior (e) of plant and tapering inward toward interior (i). 10,500X.


e

Figure 32. TEM of abscission zone cell farther in, showing narrower separation (arrows). 10,000X.

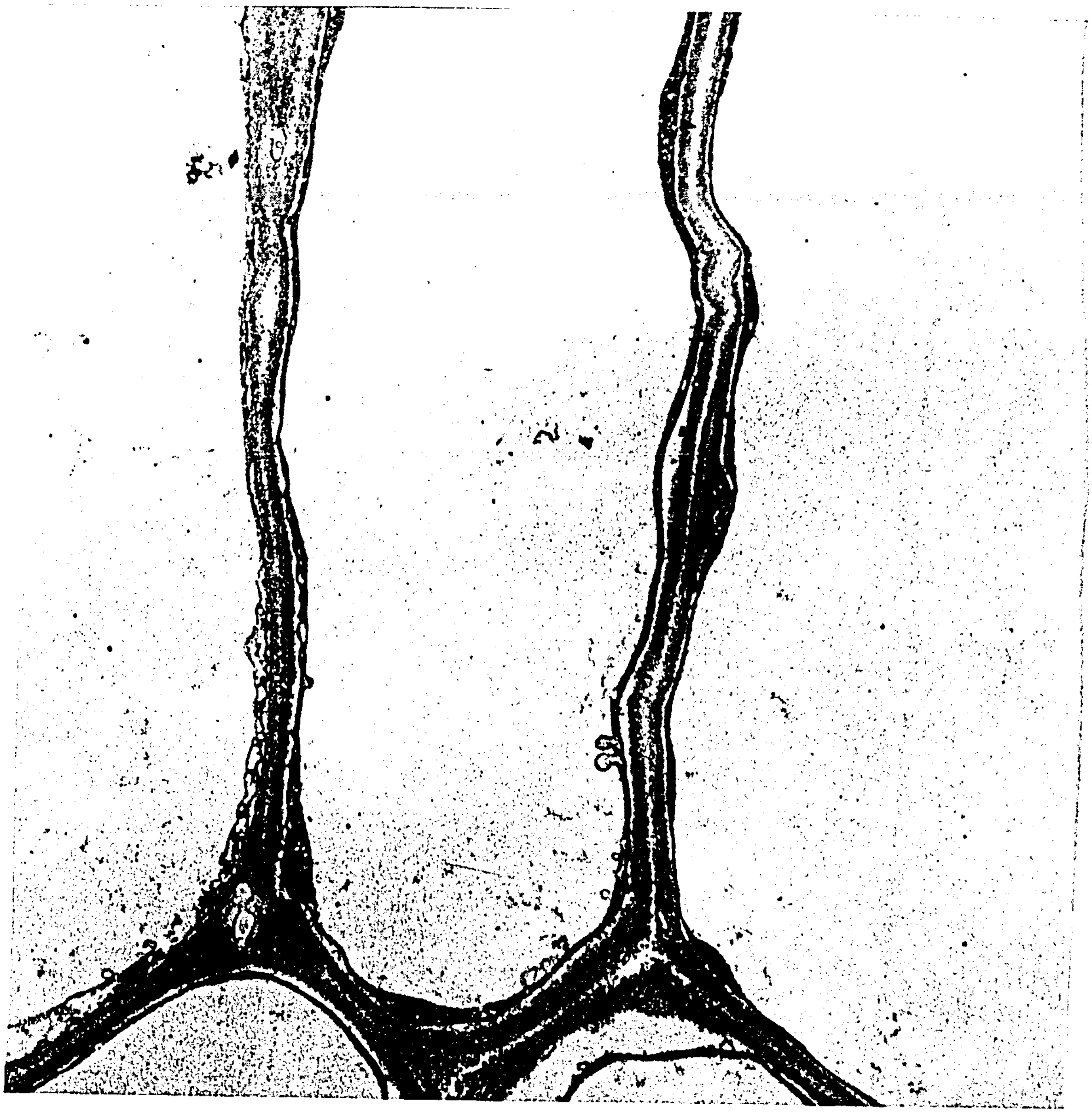


Figure 33. TEM of uneven area of separation. 30,000X

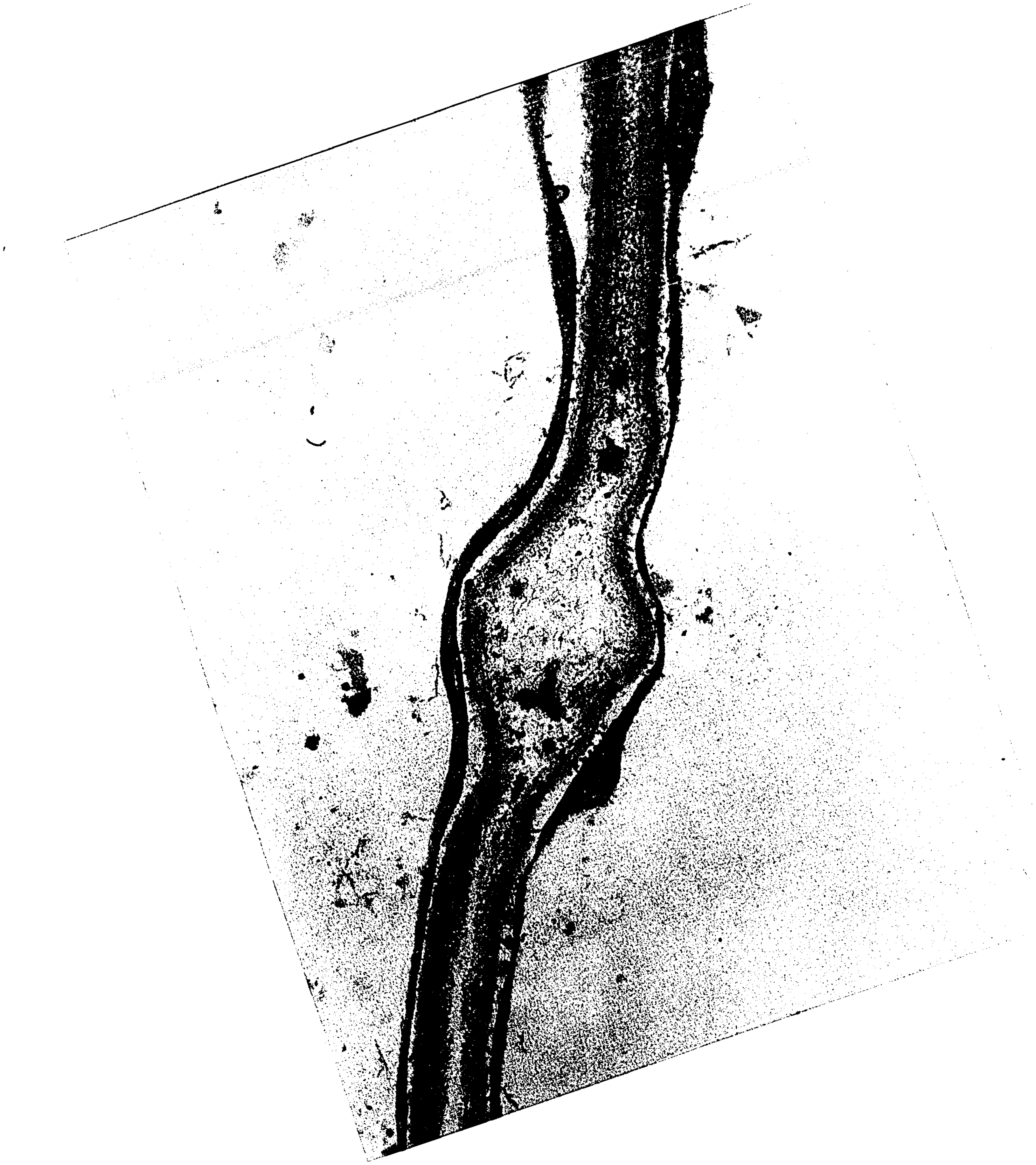


Figure 34. TEM of densely cytoplasmic stelar cell showing cell wall thickenings (cw), nucleus (n), mitochondria (m), chloroplasts (ch), and starch granules (s). 21,000X

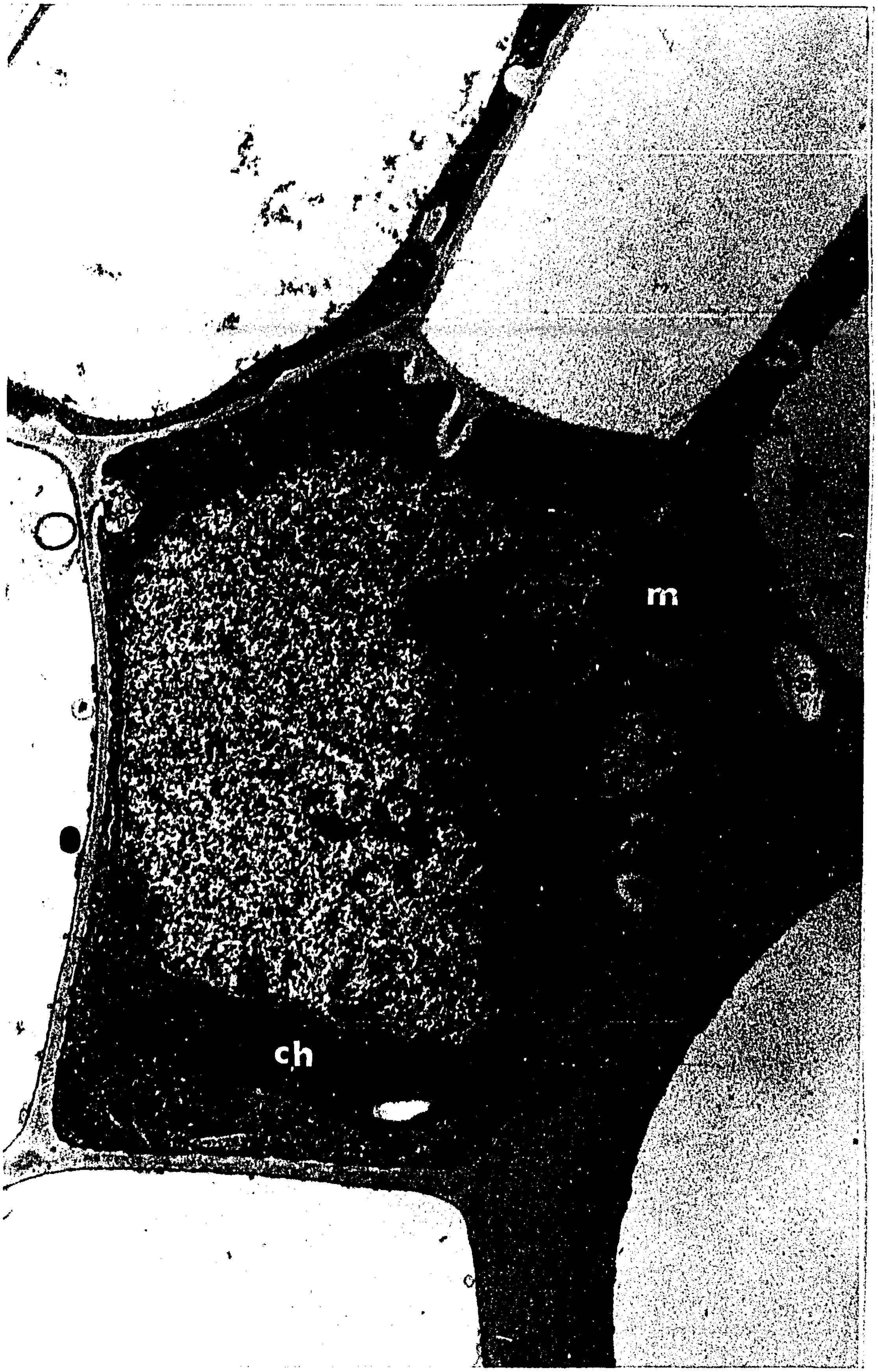

m
ch

Figure 35. TEM of abscission zone cell showing large vacuole (v) and relatively small amount of cytoplasm, including nucleus (n), chloroplasts (ch), starch granules (s), and mitochondria (m). 9,000X.


ch
n
s
v
m

Figure 36. Cytoplasmic area of abscission zone cell, showing nucleus (n), chloroplast (ch) with thylakoids (arrows) and starch granules (s), Cell wall (cw) and vacuole (v). 30,000X.


s
V
n
ch

Figure 37. TEM of abscission zone cell showing chlorplasts (ch), starch granules (s), and nucleus (n) with visible nuclear pores (white arrows). 30,000X.

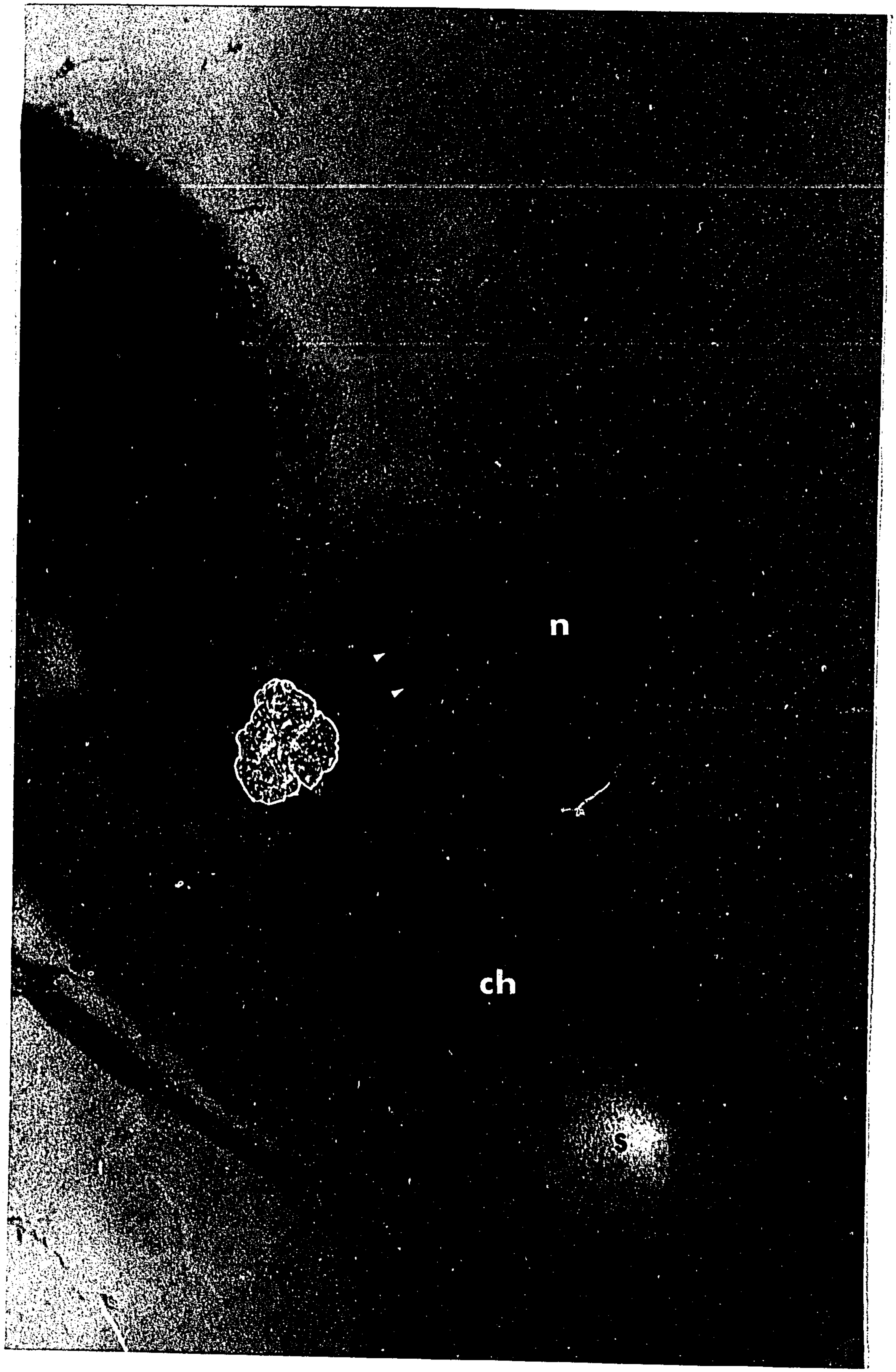

n
ch
s

Figure 38. TEM of abscission zone cells showing zone of separation between cells walls with cytoplasm closely appressed, including mitochondria (white arrows). 30,000X

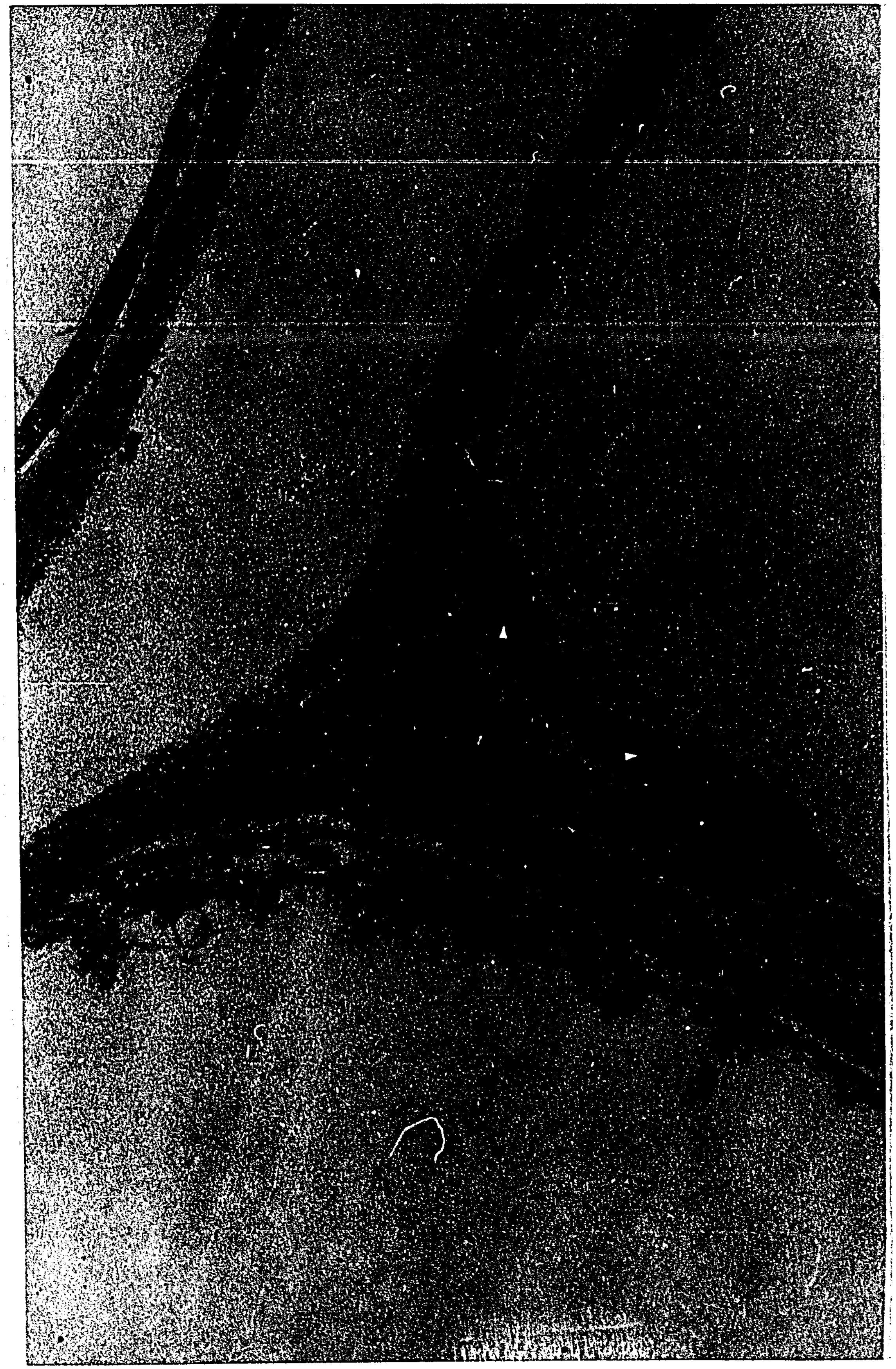


Figure 39. TEM of abscission zone cell wall with cytoplasm, showing Golgi apparatus (white arrows). 60,000X

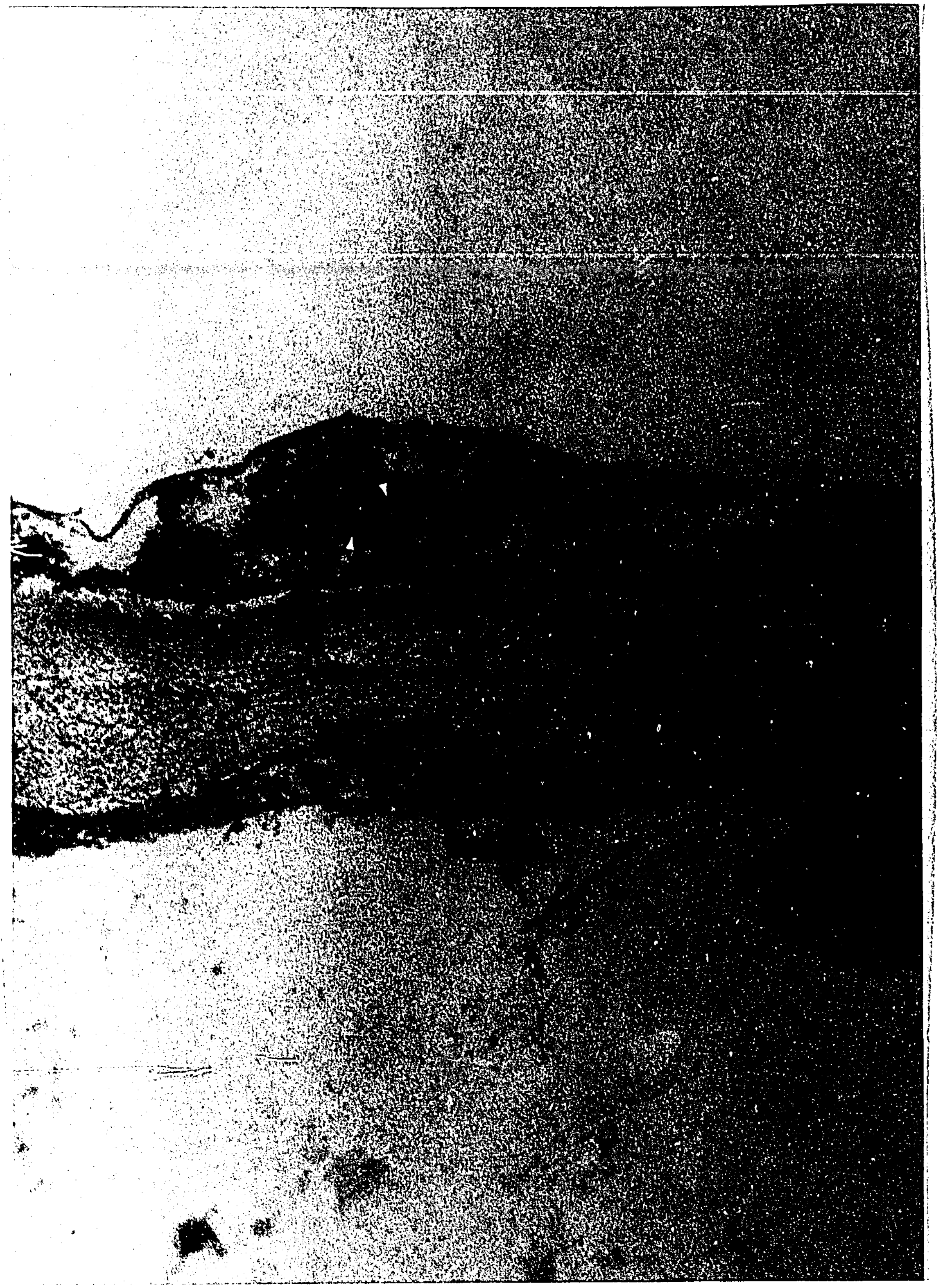
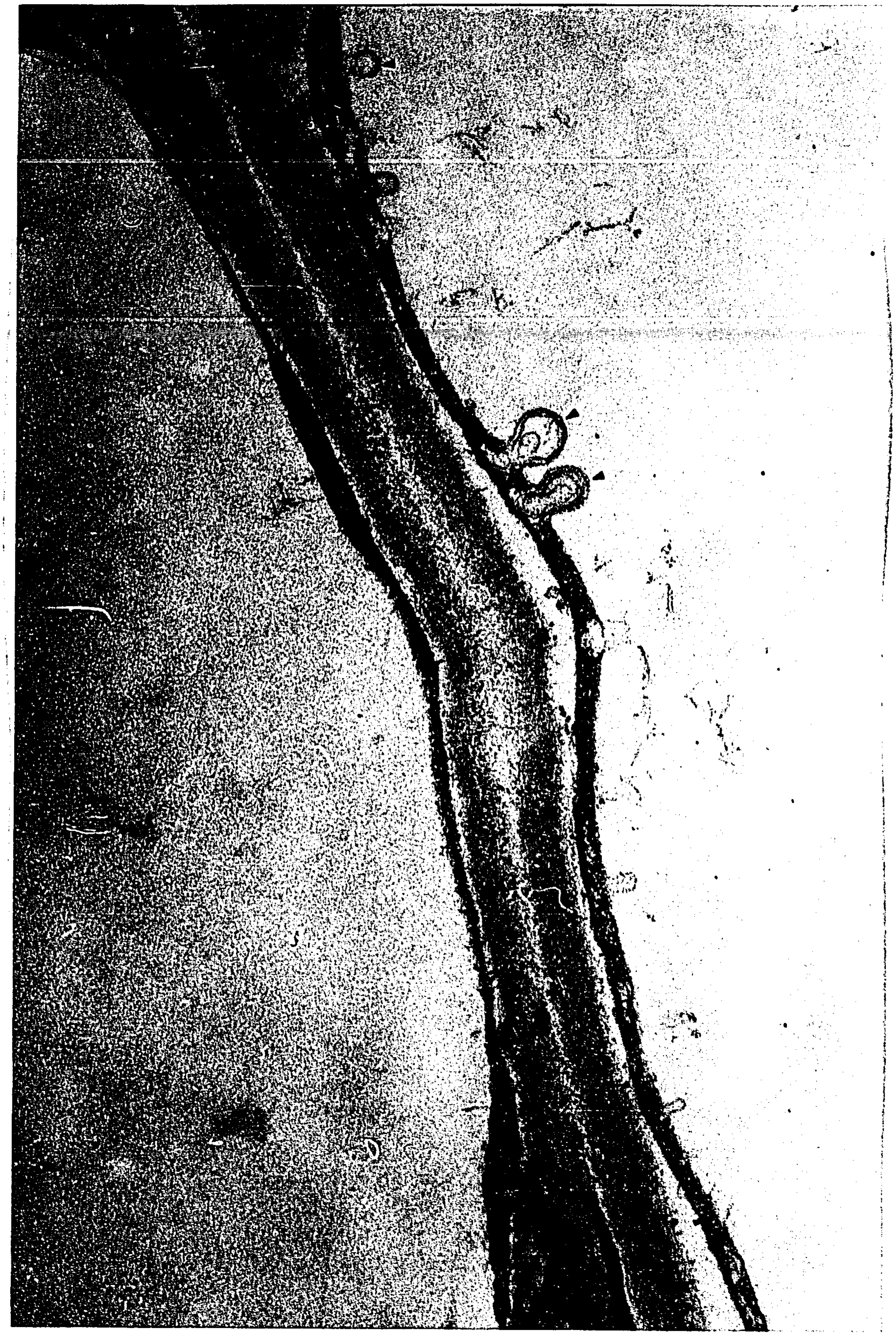


Figure 40. TEM of abscission zone cell at interior end of cell, showing chloroplasts (ch) and endoplasmic reticulum (arrows). 30,000X


ch

Figure 41. TEM of abscission zone cells with membranous whorls (arrows) protruding through cytoplasm into vacuole. 30,000X.



Appendix I

Solutions for Electron Microscopy

I. Fixatives and Buffers.

  A. Glutaraldehyde fixative.

    1. 2.6 ml of 50% glutaraldehyde solution (Fisher Scientific, Fair Lawn, NJ) was added to culture flasks containing 10 ml of culture medium to make a 3% fixation solution.

  B. Sodium cacodylate buffer.

    A 0.1 M solution (pH 6.8) of sodium cacodylate buffer was prepared by adding 10.7 gms of sodium cacodylate ($C_2H_6AsNaO_2 \cdot 3H_2O$) to 500 ml deionized water. The pH was adjusted to 6.8 with HCl.

  C. Osmium tetroxide (OsO_4) post-fixative. One gram of osmium tetroxide was dissolved in 50 ml of deionized water and kept as a stock solution of 2% at 4°C. When needed for post-fixation, the osmium tetroxide was brought to room temperature and mixed in a 1:1 ratio with cacodylate buffer to make a 1% solution.

II. Post stains.

  A. Uranyl acid in methanol (Stempak and Ward, 1964).

    Fifteen grams of hydrated uranyl acetate ($UO_2(CH_3COO)_2 \cdot 2H_2O$) were dissolved in 50 ml of absolute, acetone-free methanol with a magnetic stirrer for 8 hrs. The solution was then filtered into a small vial, closed and stored in a light-proof box (or wrapped in foil) at 4°C.

  B. Lead citrate (Reynolds 1963).

    1.33 gms of lead citrate ($Pb(NO_3)_2$) and 1.76 gms of sodium citrate ($Na_3(C_6H_5O_7) \cdot 2H_20$) were added to 30 ml deionized water, which had previously been boiled to remove CO_2, in a 50 ml volumetric flask. The mixture was shaken for one minute, then allowed to cool for one half-hour with intermittent shaking. Eight ml of 1.0 N NaOH was added and the solution was mixed gently. Previously boiled water was then decanted into small vials and stored in a dark box at 4°C. Before using it was ascertained that no precipitate had formed.

III. Embedding medium.

  Spurr Low Viscosity Embedding Medium (Polysciences, Inc., Warrington, PA).

The medium was freshly prepared as needed. The formula for a firm block was used as follows:

10.0 gm Vinylcyclohexene dioxide (VCD)
6.0 gm Diglycidyl ether of polypropylene glycol (D.E.R. 736)
26.0 gm Nonenyl succinic anhydride (NSA)

After gently mixing the first three ingredients, the catalyst was added -

0.4 gm Dimethylaminoethanol (DMAE)

The complete medium was then thoroughly but gently mixed.

Appendix II

Fixation, dehydration and embedding procedures for transmission electron microscopy (TEM).

I. Fixation

1. A 3% glutaraldehyde solution was made up in 0.1 M cacodylate buffer. Plants were removed from the culture medium and placed in a small vial containing the 3% glutaraldehyde solution in 0.1 M cacodylate buffer. Plants were fixed for 1-2 hrs at room temperature.
2. Plants were rinsed 3X in cacodylate buffer (pH 6.8) for 15-30 min each rinse.
3. Plants were post-fixed in 2% osmium tetroxide (OsO4) for 1 hr.
4. Plants were rinsed in cacodylate buffer once for 15-30 min.

II. Dehydration

1. Plants were incubated in 50% ethanol (ETOH) for 15-30 min.
2. Plants were incubated in 70% ETOH for 15-30 min.
3. Contents of the culture flasks were then poured out into a
Petri dish and examined under a dissecting microscope. A suitable number of plants were then selected and transferred to a small vial in 70% ETOHand the dehydration process was continued.
4. Tissues were incubated in 95% ETOH for 1 hr.
5. Tissues were incubated in 100% ETOH for 15-30 min.
6. Tissues were incubated in 100% ETOH for 15-30 min.
7. Tissues were incubated in 100% ETOH for 15-30 min.

Dehydration and embedding were then continued in a fume hood.

8. Tissues were incubated in propylene oxide (PO_4) which had been brought to room temperature for 15-30 min.
9. Tissues were incubated in PO_4 for 15-30 min.
10. Tissues were incubated in PO_4 for 15-30 min. Plants were then prepared for embedding in Spurr's embedding medium.

IIIa. Fixation in Spurr's. Rather than transfer the tissue directly to a Spurr's solution, part or all of the solution was removed each time and Spurr's was added to make the following concentrations:

11. 25% Spurr's for 30 min.
12. 50% Spurr's for 1 hr.
13. 75% Spurr's for 1 hr.

14. 100% Spurr's for 1-2 hrs.

IIIb. Embedding of Spurr tissue.

Flat embedment molds (Energy Beam Sciences, Agawam, MA) were then prepared and labeled.

1. A small amount of embedding medium (Spurr's) was added to each mold. The individual plant was then carefully placed at the tip end of the mold with the apex located at the top. The mold was then filled with additional Spurr's to fill the mold.
2. The molds were placed in a dessicator for 24 hrs.
3. The Spurr's embedded tissue was then polymerized in a 70°C. oven for 24 hrs. The molds were then allowed to cool to room temperature and the polymerized blocks containing the Spurr's embedded plants were removed.

Appendix III

Preparation of material for scanning electron microscopy (SEM).

I. Fixation

1. A 3% glutaraldehyde solution was made up in 0.1 M cacodylate buffer. Plants were removed from the culture medium and placed in a small vial containing the 3% glutaraldehyde solution in 0.1 M cacodylate buffer. Plants were fixed for 1-2 hrs at room temperature.
2. Plants were rinsed 3X in cacodylate buffer (pH 6.8) for 15-30 min each rinse.
3. Plants were post-fixed in 2% osmium tetroxide (OsO4) for 1 hr.
4. Plants were rinsed in cacodylate buffer once for 15-30 min.

II. Dehydration

1. Plants were incubated in 50% ethanol (ETOH) for 15-30 min.
2. Plants were incubated in 70% ETOH for 15-30 min.
3. Contents of the culture flasks were then poured out into a Petri dish and examined under a dissecting microscope. A suitable number of plants were then selected and transferred to a mesh container suitable for use in the Samdri-790 Critical Point Dryer. The container, containing the plants and a small piece of filter paper (labeled for identification and also useful to help prevent drying out of the plant material between transfers from fluid to fluid) were then transferred to a small vial of 70% ETOH and the dehydration process was continued.
4. Tissues were incubated in 95% ETOH for 15-30 min.
5. Tissues were incubated in 100% ETOH for 15-30 min.
6. Tissues were incubated in 100% ETOH for 15-30 min.
7. Tissues were incubated in 100% ETOH overnight.

A small amount of 100% ETOH was placed in the drying compartment of the Samdri-790 Critical Point Dryer. The containers of plants were added, the compartment closed, and the plants critical point dried with carbon dioxide (CO_2) according to the manufacturer's published directions.

III. Preparation of CPD plants for examination in the SEM.

1. Upon completion of critical point drying, the plants were removed, poured into a filter paper-lined Petri dish and examined under a dissecting microscope. Those that appeared to be well-fixed and critical point dried without collapse of cells were selected.
2. Copper or aluminum stubs were prepared using double stick tape.

3. Plants were attached to the stubs using a fine forceps so as not to damage the delicate tissue. They were carefully positioned on the double stick tape on the stub so that all aspects of the plant could be seen on one or another of the specimens (i.e., some with the dorsal surface facing upwards, some with the ventral surface facing upwards, and some with the apex facing upwards).
4. The stubs were mounted in a Technics Hummer II and gold coated with 200 nm gold according to the manufacturer's published directions.A small amount of silver paint was placed on the side of the stub for better contrast.
5. Plants were then observed in the SEM - either the JEOL U-3 or the Amray 1830 - and photographed using Polaroid P/N-55 film.

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