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A PHYSIOLOGICAL AND ULTRASTRUCTURAL STUDY OF CELLULASE IN TOBACCO ABSCISSION CELLS

by

STEVEN J. LIEBERMAN

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.

1984

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

A PHYSIOLOGICAL AND ULTRASTRUCTURAL STUDY OF CELLULASE IN TOBACCO ABSCISSION CELLS

by

Steven J. Lieberman

Advisor: Professor Jack G. Valdovinos

A study was conducted to determine if cellulase is involved in cell wall degradative processes leading to abscission in tobacco flower pedicels. A cytochemical method was utilized for ultrastructural localization of cellulase. Deposition of a copper precipitate was utilized to detect cellulase activity in the cells. Copper precipitate was observed principally in the middle lamella region of the cell wall during advanced stages of abscission where pollination was prevented, and in ethylene-treated tissue. In these studies, break-strengths of pedicels decreased prior to increases in cellulase activity. Morphometric analysis was utilized to measure quantitative ultrastructural changes during abscission. An increase in surface area of the rough ER was found with ethylene treatment. Cellulase enzymes were also characterized by a viscometric assay following biochemical extraction procedures. The most significant increases in buffer-soluble cellulase activity were observed during advanced stages of abscission where pollination was prevented. Flower pedicels initially broke before enzyme levels had increased dramatically. No significant increase in salt-soluble cellulase activity was observed. Both exo- and endocellular fractions were assayed to differentiate between cellulase activity found within the cellular free space and in the cell. The most dramatic increase in exocellulase activity was found in capped tissue harvested at 5 days after anthesis. Pedicels initially broke before the exocellulase activity had increased to a high level. The most significant increase in endocellulase activity was observed in capped tissue harvested at 4 days after anthesis. Upon electrophoresis of the exocellular fraction, a protein band was isolated (band 4) which contained exocellulase activity and was observed only in gels of capped tissue harvested at 5 and 6 days after anthesis. Band 4-exocellulase activity was found at a relatively high level in tissue harvested at these stages of the time-course. Pedicel break-strengths were decreasing when the increase in enzyme activity was first observed. This study suggests that cellulase may have an important role during abscission, after initial break-strength decreases have already begun. Results of a study for localizing peroxidases in gels, indicated that they were secreted into the wall during abscission.

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This dissertation is dedicated to the memory of my mother Elaine Ruth Lieberman. Her contributions were infinite in terms of my achieving this goal.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Table I.

Table II.

Table III.

Figure

Introduction

Abscission is a process whereby fruits, petals, seeds, leaves, pedicels and other organs are shed by plants. Experimentation on abscission is believed to have started with the work of Molisch (1880; see Addicott, 1970), more than a century ago. However, man's knowledge of leaf abscission and his awareness of the environmental factors that may affect it dates back to 285 B.C. At that time, Theophrastus (285 B.C.; see Addicott, 1970), commenting on deciduous trees stated that good soil "and a moist situation conduce to keeping the leaves late; for those which grow in dry places, and in general where the soil is light, shed their leaves earlier, and the older trees earlier than young ones."

The environmental factors that influence abscission include water, light, temperature, gases, soil conditions, mineral elements and parasitic organisms (Addicott, 1968).

Both drought and flooding conditions can initiate abscission in field-grown plants. However, abscission will not occur in explant tissue if there is a deficiency of water, or if the tissue is submerged in water (Addicott, 1968; Carns et al., 1951; Pfeiffer, 1928). Abscission can also result from inadequate illumination of leaves. This is common when plants are shaded by nearby foliage (Addicott, 1968). Sudden changes in temperature (i.e. a light frost) will initiate abscission. For abscission to occur, physiological temperatures must be maintained at a moderate level. Gases such as ethylene and ammonia can induce and accelerate abscission (Addicott, 1968). Oxygen is required for abscission to occur (Carns et al., 1951), while carbon dioxide has an inhibitory effect on the process. Mineral soil factors also influence leaf abscission. Large amounts of nitrogenous substances tend to delay abscission (Addicott, 1965), while a lack of nitrogen, calcium, magnesium, boron, iron, potassium, sulfur or zinc will accelerate abscission (Hambridge, 1941). Finally, several species of parasitic insects and fungi have the ability to cause abscission (Addicott, 1968).

Environmental factors influence internal physiological conditions or processes, in that they may cause the retardation or acceleration of abscission (Addicott, 1968). The internal conditions or processes include (a) sugar, pectin, cellulose and other carbohydrates; (b) energy-yielding respiration; (c) enzymic reactions; (d) amino acids, purines and other nitrogenous substances; (e) levels of plant hormones and (f) the molecular biological pathway (Addicott, 1968).

Temperature changes will affect metabolism and respiration so that abscission is promoted (Miller, 1938). Light changes during photosynthesis will cause substrates and cell walls to promote abscission (Biggs and Leopold, 1957; Brown and Addicott, 1950). High humidity will cause solubilization of cell walls which in turn will promote abscission (Pfeiffer, 1928). Oxygen and carbon dioxide will affect respiration so that abscission is promoted and retarded respectively (Carns, 1951). Ethylene will cause changes in respiration and induce enzyme synthesis which results in an acceleration of abscission (Abeles and Holm, 1967).

One of the primary internal physiological processes which may in part be responsible for abscission, is the degradation of cell wall substrates by hydrolytic enzymes. Several reports have indicated that pectic enzymes are important during cell separation processes (Yager, 1960; Valdovinos and Muir, 1965; Morré, 1968; Rasmussen and Bukovac, 1969; Stosser, 1970; Riov, 1974; Greenberg, Goren and Riov, 1975). Cellulases have also been correlated with the cell wall degradative processes leading to abscission (Horton and Osborne, 1967; Abeles, 1969; Lewis and Varner, 1970). Additionally, they may have important regulatory roles in cell growth and differentiation (Fan and MacLachlan, 1966; Ridge and Osborne, 1969), pollen tube elongation (Roggen and Stanley, 1969) and fruit ripening (Hobson, 1968; Pharr and Dickinson, 1973).

In studies of bean, cotton and Coleus explants, Abeles (1969) has shown that an increase in cellulase activity occurs during abscission. Other investigators have reported similar findings in bean plants (Horton and Osborne, 1967; Lewis and Varner, 1970; Reid et al., 1974), and citrus fruits (Ratner et al., 1969). In a more recent study in bean plants, antibodies against a specific cellulase isozyme were found to inhibit the cell wall breakdown processes that result in abscission (Sexton et al., 1980).

In contrast to those studies implicating cellulase as the critical enzyme in abscission, studies with Begonia flower pedicels (Hänisch Ten Cate et al., 1975) and Shamouti oranges (Huberman and Goren, 1979) have suggested that this may not be the case. In Begonia flower pedicels cellulase activity did not increase until abscission was essentially complete (Hanisch Ten Cate et al., 1975) while in orange explant studies a rise in cellulase activity was not always followed by abscission

Huberman and Goren, 1979).

In view of the research which questioned the overall importance of cellulase in abscission, a further study of the relationship between cellulase and abscission was warranted. Previous studies of tobacco flower pedicels have shown that both the middle lamella and primary cell wall regions undergo breakdown during abscission (Valdovinos and Jensen, 1968). Since hydrolytic enzymes (such as cellulase and pectinase) may act on these cell walls, tobacco flower pedicels were utilized as a model system for the study.

LITERATURE REVIEW

The term "abscission zone"was first introduced by Von Mohl (1860) to describe a group of cells found at the base of a leaf in many plants (Sexton and Roberts, 1982). He found that these cells were smaller and more meristematic than those in adjacent regions of the zone. Von Mohl (1860) suggested that the cells in the distal region (toward the blade) were important for the separation of the leaf from the stem while those located in the proximal region (toward the stem) were concerned with protection of the exposed cells after leaf fall (Sexton and Roberts, 1982).

The major focus of abscission research has been on the separation processes occurring in cells of the distal region. Therefore, this review will emphasize cell separation changes that result in the eventual abscising of various plant organs. Most of the early work on abscission was conducted with leaf tissue. However, in recent years the importance of flower pedicel and fruit abscission studies has been recognized.

The number and position of abscission zones on a particular plant organ varies considerably from specie to specie. In many plants containing simple leaves, such as Coleus, one abscission zone is found at the base of the petiole (Sampson, 1918; Myers, 1940). In Phaseolus vulgaris two abscission zones are present in the primary leaves. One is found at the base of the lamina while the second one is located at the base of the petiole (Webster, 1973). In contrast to these plants, in species of the genus Nicotiana, no structurally distinguishable abscission zone develops in the leaves (Webster, 1973) and abscission never occurs (Gawadi and Avery, 1950). However, in *Nicotiana tabacum* flower pedicels one abscission zone is present at the base of the organ. At the other end of the spectrum, the leaves of *Acacia decurrens*, which are about 15 cm long, contain approximately 4,000 abscission zones (Webster, 1973).

The region containing the abscission zone in tobacco flower pedicels is defined by an indentation or invagination of tissue which can be observed with a light microscope. The indentation is composed of epidermal tissue and can be observed to a limited extent with the naked eye as a v-shaped invagination of the tissue. Ultrastructural studies of abscission zone tissue in *Nicotiana tabacum* and *Lycopersicon esculentum* flower pedicels has indicated that the indentation contains branches which follow the middle lamella between walls of the cells (Jensen and Valdovinos, 1967).

The abscission zone is composed of several tiers of cells that are characteristically smaller than cells of adjacent regions. When compared with other cells, abscission cells are more densely protoplasmic (Facey, 1950; Webster, 1973; Yampolksy, 1934), contain large deposits of starch (Gilliland et al., 1976; MacKenzie, 1979), and have smaller intercellular spaces (Addicott, 1965; Marynick and Addicott, 1976; Osborne and Sargent, 1976).

Although the abscission zone has been defined as a structurally weak region of tissue, this may in fact be a misnomer. The reduction of structural elements such as lignin within the abscission zone tissue may be compensated for by the presence of well-developed collenchyma tissue (Sexton and Roberts, 1982). Since lignin is very resistant to enzyme hydrolysis (Vance et al., 1980), its presence within abscission cell walls might inhibit cell separation processes. In some plants such as bean and tobacco, the abscission zone tissue is initially as strong as tissue found in adjacent regions of the zone. Only after cell separation processes have begun can a decrease in break-strength be detected at the abscission zone (de la Fuente and Leopold, 1968; Morré, 1968; Valdovinos et al., 1972). As a result of time-course studies where the break-strengths of abscission zone tissue have been measured, the abscission process has been divided into a "lag" and "separation" phase (Sexton and Roberts, 1982).

The initial structural change that is commonly associated with the lag phase of abscission is an increased rate of cell division (Sexton and Roberts, 1982). This usually occurs within 2 or 3 rows of cells near the separation layer located in the distal portion of the abscission zone (Brown and Addicott, 1950; Carns, 1966; Halliday and Wangermann, 1972; Lloyd, 1927; Webster, 1968; Webster, 1973). Cell division may be controlled by the level of ethylene in the tissue (Webster, 1970). It has been suggested that in Phaseolus explant tissue the initial decrease in ethylene levels (6 h after excision) may cause cell division to begin (Webster, 1973). In some plants, hormones such as auxin, cytokinin and gibberellic acid may participate in cell division within the abscission zone (Webster, 1973). In spite of the data implicating cell division as being essential for abscission, there is strong evidence suggesting that the contrary may be true. This is due to the fact that ethylene acceleration of abscission may inhibit cell division (Brown and Addicott, 1950; Gawadi and Avery, 1950; Poovaiah and Rasmussen, 1973; Rubinstein and Leopold, 1964), abscission is not stopped by inhibitors of DNA synthesis (Ratner et al., 1969) and cell division does not occur in many plants (Gawadi and Avery, 1950; Jaffe and Goren, 1979; Morris, 1964).

A second structural change that occurs within the abscission zone both before and after abscission is cell enlargement (Webster, 1973). As a result of the enlargement of cells that are located in the abscission zone and proximal to the separation region, shear forces may develop across the cell walls of the zone (Leopold, 1967). The cells which remain on the stem after abscission has occurred may show tendencies for continued enlargement. This is suggested by the fact that they appear swollen, indicating the tissue may have been forced off the plant by enlargement of several cell layers (Webster, 1973). The cell enlargement which occurs in Acer leaf abscission regions may be correlated with the development of stress forces within the tissue (Moline and Bostrack, 1972). This may lead to a rupturing of epidermal tissue prior to leaf abscission (Moline and Bostrack, 1972). In Capsicum plants cell enlargement causes the cell walls of vessel elements to rupture during abscission (Gawadi and Avery, 1950). The possibility may exist that where cell growth or elongation occurs within the abscission zone region, it may reflect a mechanical stress that can lead to the breakage of tissue (Webster, 1973).

Ethylene treatment of Phaseolus leaf tissue effects cell enlargement by stimulating the lateral expansion of abscission cells (Webster, 1970). This may involve a reorientation of cell wall microfibrils as has been observed in Pisum sativum stem tissue (Apelbaum and Burg, 1971). If this situation existed in abscission zone tissue, the reorientation of the microfibrils could lead to the development of mechanical stresses across the cell walls (Webster, 1973).

During the lag phase of abscission, cells of the separation layer become increasingly distinguishable as they accumulate cytoplasm and organelles (Addicott, 1965; Bornman et al., 1966; Valdovinos et al., 1972). This may consist of an increase in rough ER (Iwahori and Van Steveninck, 1976; Sexton et al., 1977; Valdovinos et al, 1972), dictyosomes (Gilliland et al., 1976; Iwahori and Van Steveninck, 1976; Sexton et al., 1977) and mitochondria (Gilliland et al., 1976) within abscission cells during the latter stages of the lag period. Associated with these changes is an increase in protein synthesis (Abeles, 1968; Abeles and Holm, 1966; Abeles et al., 1971; Lewis and Bakhshi, 1968), which is mainly confined to the separation layer of the abscission zone (Osborne, 1968; Stosser, 1971; Webster, 1968). These proteins may play an important part in cell separation processes. Finally, there is a large increase in the respiratory rate within the separation layer (Addicott, 1965; Marynick, 1977; Poovaiah and Rasmussen, 1973). This is essential for abscission since low oxygen levels delay the process considerably (Addicott, 1965; Marynick and Addicott, 1976).

Cell separation occurs within a 1 to 5 cell wide area of the abscission zone called the separation layer [e.g. Tison, 1900; Hodgson, 1918; Kendall, 1918; (Facey, 1950; Gawadi and Avery, 1950; Halliday and Wangermann, 1972)] In Nicotiana tabacum L. "Little Turkish" 4 to 6 tiers of cells are involved in cell separation (Yager, 1957) during natural abscission while in ethylene-induced abscission separation occurs in only 2 to 3 tiers of cells (Valdovinos and Jensen, 1974). In the distal region of the abscission zone, cell walls undergo various chemical alterations during the separation phase of abscission. These modifications cause a weakening of the abscission zone tissue which ultimately results in the abscising of a particular plant organ.

Cell separation is initiated in various types of separation layer tissue depending upon the nature of a specific plant. For example in Nicotiana plants separation begins on the adaxial (upper) side of the flower pedicel. The cortical cells which are located to the distal side of the indentation begin to separate and this spreads to the abaxial (lower) side of the pedicel (Yager, 1957). Prior to separation a swelling of the epidermal tissue seems to occur. At the same time dissolution within the pith may begin (Yager, 1957).

In contrast to this, in Coleus plants cell separation begins on the abaxial (lower) side of the leaf petiole. It then spreads to the epidermal cells and cortex until the petiole is attached to the plant only by the upper cortex and the xylem elements (Myers, 1940). In intact Phaseolus plants and in explant tissue, cell separation is initiated in the pith cells of the leaf petiole (Webster, 1970). It then continues through the vascular tissue to the cortical cells (Webster, 1973).

Several factors may effect the position of initial cell separation within the tissue. In Phaseolus leaves, separation will commence inside the pulvinar tissue if it is supplied with a considerable amount of water or if it is treated with ethylene. Under conditions where the water level supplied to the pulvinus is low, cell separation is initiated on the external surface of the tissue (Brown and Addicott, 1950). The treatment of cotton explants with abscisic acid will cause cell separation to begin either on the adaxial or the abaxial side of the tissue. However, in untreated and auxin treated explants separation begins adaxially (Bornman et al., 1967).

While cell separation is occurring, several changes may take place in the proximal region of the abscission zone. These changes, as first suggested by Von Mohl (1860) are primarily concerned with protection of the exposed cells after abscission occurs (Sexton and Roberts, 1982). Development of the protective region involves the deposition of lignin, suberin and cutin within the walls of several tiers of cells. Additionally, tyloses and tannins as well as other cellular inclusions may accumulate during this period (Webster, 1973). As a result of these modifications, the cells form a protective layer over the exposed tissue, after abscission has taken place (Webster, 1973). This protective region continues to undergo change, especially beneath the surface of the tissue where meristematic activity occurs in the cells. As a result of this activity, older protective cells are eventually replaced by new cells near the surface of the tissue (Webster, 1973).

With the use of cytochemical staining techniques, ultrastructural methods and biochemical extraction procedures, much information has been obtained regarding the cell wall changes that precede cell separation. In many plants these changes involve dissolution of the middle lamella region and breakdown of the primary region within the cell wall. This in turn may be followed by mechanical disruption of the separation zone tissue.

Accompanying the cell wall breakdown is an increase in RER and in the activity and number of dictyosomes (Gilliland et al., 1976; Iwahori and Van Steveninck, 1976; Osborne and Sargent, 1976; Valdovinos et al., 1970). This may suggest that these organelles are involved in the secretion of cell wall degrading enzymes (Gilliland et al., 1976; Osborne and Sargent, 1976; Sexton et al., 1977). Although plasmodesmata have been suggested as possible sites for enzyme secretion in the wall (Osborne and Sargent, 1976), recent studies have been unable to substantiate this hypothesis (MacKenzie, 1979; Sexton et al., 1977).

The importance of the middle lamella region of the wall in cell separation processes has been a major focus of abscission studies. The pectic nature of this region was first discovered by Magnin in 1888 (Webster, 1973). Lee (1911) observed that the middle lamella region of the wall swells during abscission (Webster, 1973). The specific chemical changes associated with the pectic substances of this region were first observed by Facey (1950). She used both pectin stains and solubility studies to conclude that during cell separation the pectins in the cell walls are converted from calcium pectate to pectic acids and then to water-soluble methylated pectins. Facey (1950) also showed that methylation is required for cell separation to occur. The methylation of the free pectic acids may cause a disruption of the middle lamella region of the cell wall (Valdovinos and Muir, 1965; Sexton and Roberts, 1982). This is supported by in vitro studies on apple fruit tissue which suggest that methylation of cell walls causes cell separation (Knee, 1978). The results of the study conducted by Facey (1950) have been substantiated with the help of two techniques which are specific for the localization of pectins. These include the hydroxylamine-ferric chloride histochemical technique (Reeve, 1959) and a ruthenium red staining technique designed for plant material (Colombo and Rascio, 1977). With the use of these techniques as well as others in ultrastructural studies, it has been demonstrated that methylated pectins are removed from the separation zone during abscission (Bornman, 1967; Davenport and Marinos, 1971; Stosser et al., 1969). This is further supported by the fact that the middle lamella swells and eventually disappears during the course of cell separation (Bornman, 1967; Iwahori and Van Steveninck, 1976; Osborne and Sargent, 1976; Sexton, 1976; Sexton et al. 1977; Valdovinos and Jensen, 1974; Valdovinos et al., 1972).

As their solubility increases and more pectins are removed from the separation zone during abscission, there is a concomitant loss of calcium from the cell wall (Stosser et al., 1969; Poovaiah and Rasmussen, 1973). The importance of calcium as a stabilizing ion in the middle lamella region of the wall was originally recognized by Sampson (1918). Although several studies have examined the possible role of calcium ions in cell separation, it is still difficult to verify whether the release of Ca^{2+} from the cell wall leads to a loss of pectins or whether the reverse situation actually occurs (Sexton and Roberts, 1982).

Both pectin methyl esterase (PME) and polygalacturonase (PG) have been implicated in abscission processes of various plants. In tobacco flower pedicel tissue Yager (1957) found that PME activity increases under conditions which promote abscission. Valdovinos and Muir (1965) demonstrated in Coleus plants that the enzyme system responsible for esterification of pectins is highly active during cell separation processes. However, in spite of the evidence suggesting a role for PME during abscission, several other studies have been unable to demonstrate a consistent correlation between the enzymes activity and abscission (Abeles et al., 1971; Hänisch Ten Cate et al., 1975; Moline and Bostrack, 1972; Ratner et al., 1969).

Morré (1968) utilized break-strength measurements and a cucumber pericarp bioassay to show that the level of pectinase (containing PG) increases prior to cell separation in Phaseolus leaves. His results are supported by a study on orange fruit abscission, where a similar bioassay was utilized to detect pectinase activity in the tissue (Rogers and Hurley, 1971). Alternatively, in a different variety of orange, Rasmussen (1973) used a viscometric assay and found no increase in pectinase activity during abscission.

An increase in an exopolygalacturonase has been reported during citrus leaf abscission (Riov, 1974). Ethylene treatment of citrus fruits causes a similar increase in PG activity over the course of abscission (Greenberg et al., 1975). However, during bean leaf abscission no increase in PG activity is observed (Berger and Reid, 1979). Furthermore, the levels of PG activity remained relatively constant throughout the course of abscission in both ethylene treated and air treated explants (Berger and Reid, 1979). Despite this evidence, the enzyme may still play an important role in cell separation processes, since high levels of PG are found in the abscission zone as compared to the petiole in bean leaf tissue (Berger and Reid, 1979).

In some species of plants including Coleus, Gossypium and Psoralea the dissolution of the middle lamella may in itself lead to cell separation (Myers, 1940; Becker, 1968; Bornman et al., 1969). However, in other groups of plants pectin dissolution alone may be insufficient to cause actual separation of the leaf from the plant (Morré, 1968). In *Phaseolus* plants cell separation does not occur until long after initial break-strength measurements are recorded (Morré, 1968). By this time areas of the primary cell wall have been broken down in addition to the dissolution of the middle lamella region. This implies that in these plants breakdown of other cell wall components, such as cellulose, may be necessary to insure that cell separation and abscission will take place.

In addition to the cell wall changes associated with the middle lamella during abscission, Facey (1950) also observed various modifications within the primary wall region. She found that in *Fraxinus* leaf tissue the cellulosic component of the primary wall swelled as abscission progressed and the wall became very weak and distorted looking. This was supported by ultrastructural studies which show that the primary wall swells and undergoes a disorientation of its microfibril components during abscission (Bornman, 1967; Gilliland et al., 1976; Iwahori and Van Steveninck, 1976; MacKenzie, 1979; Osborne and Sargent, 1976; Valdovinos et al., 1972). In *Phaseolus* plants, Rasmussen (1965) found that cellulose and other primary wall constituents were broken down during abscission. This data is supported by ultrastructural studies which show that the primary wall disintegrated during this process (Bornman, 1967; Valdovinos and Jensen, 1968). In various plants cell wall breakdown is sometimes so extensive that it may result in total cell collapse (Bornman, 1967; Valdovinos and Jensen, 1968).

A significant amount of research has been done which suggests that cellulase (endo-β 1:4-glucan 4-glucanohydrolase "β1:4 glucanase") may function as a cell wall degrading enzyme during the course of abscission. Although no direct evidence exists in terms of what cellulase degrades within separation zone cell walls (Sexton and Roberts, 1982), cellulose has been suggested as a possible substrate for the enzyme (Lewis at al., 1974).

The term cellulase has generally been used to describe a group of enzymes whose function is to degrade cellulose (Reese, 1963; Gould, 1969). This group consists of a C_1 enzyme whose function may be to degrade insoluble cellulose, and a C_x enzyme (β 1:4 glucanase and β-glucosidases) which degrades solubilized cellulose (King and Vessal, 1969; Pavlista, 1977). The β 1:4 glucanase can degrade cellulose that has had its hydroxy groups (OH) replaced with carboxymethyl groups to varying degrees of substitution (DS) (Halliwell, 1963: Klop and Kooiman, 1965; Wirick, 1968; Selby and Maitland, 1967; Pavlista, 1977). This C_x-type of cellulase has been shown to attack the cellulose molecule between two unsubstituted anhydrous glucose units and only at sequences of two or more unsubstituted anhydrous molecules (Hercules Inc., 1971; Klop and Kooiman, 1965; Wirick, 1968; Isherwood, 1970; Pavlista, 1977). Since the β 1:4 glucanase attacks solubilized cellulose, there has been some controversy as to whether it is indeed a true cellulase (Pavlista, 1977). However, there is evidence suggesting that this enzyme acts in conjunction with C_1 enzymes (Pavlista, 1977). Furthermore, under conditions of a long incubation time these enzymes will attack insoluble cellulose (Selby and Maitland, 1967; Hartley et al., 1973; Sobotka and Stelzig, 1974; Vose, 1974; Lewis et al., 1974; Fincher et al., 1976).

In many experimental studies on abscission, carboxymethylcellulose has been utilized as a substrate for cellulase (endo- β 1:4 glucanase). In conjunction with this substrate, a viscometric assay specific for the determination of cellulase activity, has also been widely used. While employing this assay method, Horton and Osborne (1967) reported an increase in cellulase activity during the course of bean leaf abscission. This result was later confirmed by Abeles (1969) in abscission studies of bean, cotton and *Coleus* explants. Furthermore, other investigations have demonstrated a close correlation between cellulase and abscission. With the use of break-strength measurements it has been found that some cellulase activity is evident prior to initial break-strength decreases (Craker and Abeles, 1969a; Craker and Abeles, 1969b; Durbin et al., 1981). Additionally, high levels of cellulase activity have been observed before actual abscission takes place (Craker and Abeles, 1969a; Goren et al., 1973; Lewis and Varner, 1970; Osborne, 1968; Pollard and Biggs, 1970; Rasmussen, 1973; Rasmussen, 1974; Ratner et al., 1969).

Horton and Osborne (1967) also found that the senescent changes which occurred in the distal region of the abscission zone were under hormonal control and were responsible for the increase in cellulase activity within the separation zone. In a similar study Abeles (1969) also showed that ethylene regulated the production of cellulase which was essential for cell separation. He found that inhibitors of RNA and protein synthesis (actinomycin D and cycloheximide) blocked the induction of cellulase activity in ethylene-treated explants. This was also reported in inhibitor studies of citrus fruit (Pollard and Biggs, 1970) and citrus leaf (Ratner et al., 1969) abscission.

In addition to its role as a regulator of cellulase synthesis, ethylene has been shown to affect this enzyme in another important way. Dela Fuente and Leopold (1969) found that when bean petiole explants were exposed to ethylene an increase in the break-strength of the tissue occurred. Upon removal of the ethylene, further reduction of the break-strength was prevented (Dela Fuente and Leopold, 1969) eventhough the level of cellulase remained relatively constant (Abeles and Leather, 1971). This paradox was clarified by Abeles and Leather (1971) who showed that ethylene was also required to control the secretion of cellulase from inside the cell to the cell wall. Therefore the cellulase activity measured after ethylene removal may have represented enzyme that was previously synthesized but failed to reach the cell wall (Abeles and Leather, 1971; Rasmussen, 1973).

In a study of leaf abscission Abeles and Leather (1971) isolated 2 different cellulase fractions from bean explants. One particular fraction which they referred to as an exocellular cellulase was extracted from the intercellular space by means of a vacuum infiltration technique. The exocellular cellulase activity only increased in response to ethylene treatment and Abeles and Leather (1971) associated this enzyme fraction with cell wall degradation. The other cellulase fraction was then extracted by homogenizing the tissue explants and it was referred to as an endocellular cellulase. In control (aged) explants the endocellular cellulase activity increased at a slow rate with time. However, when ethylene was applied to the tissue the enzyme activity rapidly increased and this was correlated with a decrease in break-strength (Abeles and Leather,1971). These results were later confirmed by Rasmussen (1973) in a study of citrus fruit abscission. He used the same extraction procedure and found that ethylene caused a similar increase in both exo- and endocellulase activity.

In support of earlier studies (Horton and Osborne, 1967; Abeles, 1969), Lewis and Varner (1970) found that cellulase activity increased during abscission of debladed bean seedlings (intact plant with leaf blade removed) and bean petiole explants. However, unlike the results reported previously (Horton and Osborne, 1967; Abeles, 1969), they observed the increase in enzyme activity only when cellulase was extracted in a high salt buffer (Lewis and Varner, 1970). They found that two different forms of cellulase existed in the abscission zone tissue. One form was soluble in a phosphate buffer and its activity did not vary consistently with abscission. The other form of the enzyme was soluble in a salt-enriched buffer (as previously mentioned) and its activity consistently increased during abscission while tissue break-strength decreased (Lewis and Varner, 1970). Additionally, Lewis and Varner (1970) reported that this increase in salt-soluble or "residual cellulase" activity as they referred to it, was due to the *de novo* synthesis of the enzyme. They concluded that the salt-soluble form of cellulase was more closely correlated with abscission than was the buffer soluble form of the enzyme.

With the use of isoelectric focusing techniques, Lewis et al. (1970) isolated two molecularly different forms of cellulase from the abscission zone of bean. The isozyme which they referred to as cellulase 4.5 (∿ isoelectric point) was mainly found in the phosphate buffer and its activity did not vary considerably during ethylene-promoted abscission. However, cellulase 9.5 (∿ isoelectric point) was primarily detected in the high salt buffer and it was not present nor did it accumulate within the zone until the abscission process had begun (Lewis et al., 1970).

In a study of bean leaf abscission, Reid et al. (1974) followed the activities of these two different forms of cellulase. The methods they utilized to isolate cellulase were based on modifications of both the Lewis and Varner (1970) and Abeles and Leather (1971) techniques. They reported that the cellulase 4.5 form of the enzyme was found in both abscising and non-abscising tissue and that its activity did not change appreciably during abscission. Furthermore, they indicated that grinding of the tissue was necessary for extraction of cellulase 4.5. Alternatively, Reid et al. (1974) observed that extraction of the cellulase 9.5 form of the enzyme was primarily accomplished by vacuum infiltration. Although they found that cellulase 9.5 could only be detected under conditions where abscission had been induced, its activity was not pronounced until the tissue break-strength had decreased to virtually zero. Based on these results, Reid et al. (1974) suggested that cellulase 9.5 does not initiate abscission of bean leaf explants, but rather it may play an important role during the advanced stages of the cell separation process. Furthermore, they presented evidence suggesting that cellulase 4.5 was primarily an endocellular enzyme while cellulase 9.5 was an extracellular enzyme (found in the cell wall and intercellular spaces). Their findings may suggest that these 2 molecularly different forms of cellulase are respectively similar to the endocellular and exocellular (intercellular) cellulase fractions previously isolated during bean leaf and citrus fruit abscission studies (Abeles and Leather, 1971; Rasmussen, 1973).

In a recent study, the cellulase 9.5 isozyme found in bean leaf abscission zone tissue, was purified and monospecific antibodies were raised against it (Koehler et al., 1980). The cellulase 9.5 antibodies did not cross-react with other cellulase isozymes (Koehler et al., 1980). Therefore, these antibodies were used to measure the quantitative changes in both cellulase 9.5 and cellulase 4.5 during bean leaf abscission (Durbin et al., 1981; Sexton et al., 1981). Additionally, they were utilized in determining the enzyme's distribution within the tissue (Sexton et al., 1980). With the use of a radioimmune assay (Chard, 1978), Sexton et al. (1980) found that cellulase 9.5 was first detected within the tissue just prior to initial break-strength decreases. Furthermore, they observed that the rise in cellulase 9.5 paralleled the decrease in tissue break-strength. Both immunocytochemistry and microdissection techniques were used to localize the cellulase 9.5 within the abscission zone. The results indicated that high cellulase 9.5 activity was primarily restricted to the separation layer, and that the highest level of activity was found in the stele (Sexton et al, 1980; Sexton et al., 1981).

To further clarify the role of cellulase 9.5 in abscission Sexton et al. (1980) injected rabbit serum containing cellulase 9.5 antibodies directly into bean leaf abscission zone tissue. When break-strength measurements were taken, they found that 90% of the abscission zones injected with the antibodies did not break under an applied pressure of 200 g. This contrasted with control tissue injected with normal rabbit serum, where the average break-strength was approximately 49g. From these results Sexton et al. (1980) concluded that the injection of cellulase 9.5 antibodies during abscission virtually prevented any cell wall degradation. Furthermore, since the cellulase 9.5 anti-serum stopped the abscission process in bean plants, they suggested that the isoenzyme had a pivotal role in abscission.

The role of cellulase in abscission has also been studied extensively in citrus plants. During citrus fruit development abscission can occur at two different sites. Initially, young fruits will abscise at the shoot-peduncle abscission zone (zone "A") while mature fruits can only abscise at the peduncle-fruit abscission zone (zone "C")(Goren and Huberman, 1976; Greenberg et al., 1975). During abscission of both young and mature Shamouti orange fruits, Goren and Huberman (1976) found that ethylene promoted and 2,4-D delayed the formation of several cellulase isoenzymes. Additionally, they detected an increase in these cellulase isoenzymes after excision of non-abscising mature fruits (from zone "A"). This increase was also enhanced and delayed, respectively, with ethylene and 2,4-D treatment of the tissue.

More recently, Huberman and Goren (1979) extracted the total enzyme activity from exo- and endocellular fractions of fruits treated with ethylene or 2,4-D. They utilized a viscometric assay to determine the cellulase activity in these two cellular fractions. They found that ethylene promoted and 2,4-D delayed cellulase activity in both exo- and endocellular fractions. While using an electrophoretic assay Huberman and Goren (1979) observed that the activity of almost all the cellulase isoenzymes was similar in both exo- and endocellular fractions treated with ethylene and 2,4-D. Furthermore, they found no specific isoenzyme which was unique to either cellular fraction and all isoenzymes found in the cell were secreted into the wall. Their results were similar for both abscising fruits and excised non-abscising mature fruits (abscission zone "A"). Based on these findings they suggested that the rise in cellulase activity (and other hydrolytic enzymes) is not the only process necessary for abscission to occur. Furthermore, they reaffirmed an earlier proposal (Goren and Huberman, 1976) that whenever an increase in cellulase activity is not followed by abscission, the substrate is either immune to the enzyme or it does not reach its site of action.

Both exo- and endocellular cellulase activities have also been determined during abscission of Begonia flower pedicel explants (Hänisch Ten Cate et al., 1975). Although Hänisch Ten Cate et al. (1975) found that the activities of both of these cellulase fractions increased, the increases were not observed until abscission was essentially completed. They concluded that cellulase was not the cause of cell separation in Begonia pedicels but rather that its activity increased as a result of abscission.

In a recent study of abscission in tobacco flower pedicels, Lieberman et al. (1982) utilized a cytochemical technique to localize cellulase at the ultrastructural level. They found that the site of cellulase activity (as indicated by Cu precipitate) was mainly confined to the middle lamella region of the cell wall where breakdown was evident. Additionally, they observed smaller amounts of the enzyme in the primary and secondary regions of the cell wall. In time-course studies of both ethylene-treated tissue and tissue where pollination was prevented, Lieberman et al. (1982) showed that cellulase activity was at its highest level during advanced stages of abscission. They also found that the break-strengths of the abscission zone decreased prior to the increase in cellulase activity for both ethylene-treated tissue and tissue where pollination was prevented. From these results they concluded that in tobacco flower pedicels, abscission is not initiated by cellulase. Instead, they suggested that cellulase plays an important role during the advanced stages of abscission, after initial cell wall breakdown has begun.

In addition to the increases in pectinase and cellulase activity that have been observed during abscission, an increase in peroxidase activity has also been reported during this period (Duygu, 1976; Gaspar et al., 1978; Henry and Jensen, 1973; Henry et al., 1974; Poovaiah and Rasmussen, 1973; Poovaiah et al., 1973). In an ultrastructural study, Henry and Jensen (1973) localized peroxidase activity during ethylene-induced abscission of tobacco flower pedicels. They found that an increase in intensity of peroxidase staining occurred within the middle lamellar and adjacent regions of the cell walls after 2 h of ethylene treatment. The staining intensity continued to increase through 5 h of ethylene exposure at which point abscission occurred. With the use of a spectrophotometric assay Henry et al. (1974) found that the peroxidase activity increased substantially in pedicel tissue treated with ethylene for 3 to 4 h. However, during the 4 to 5 h interval after ethylene exposure they observed a decrease in peroxidase levels within the tissue. When they compared the pedicel break-strength with changes in peroxidase activity they found that initial break-strength decreases occurred simultaneously with increases in peroxidase levels (3 h). From their results Henry et al. (1974) concluded that the close correlation between cell wall breakdown and increases in peroxidase activity may suggest an important role for the enzyme during abscission. Furthermore, they suggested that the increase in peroxidase activity may be related to the modification of cell wall proteins involved in cell wall extension.

In addition to the middle lamellar and cell wall breakdown which precedes cell separation, anatomical studies have shown that in order for abscission to be complete mechanical forces are required to rupture xylem vessels within the tissue (Sexton and Roberts, 1982). It has long been suggested that these forces are generated within the abscission zone tissue itself. In bean plants Leopold (1967) found that the proximal tissue of the separation layer expanded during abscission while the distal tissue contracted. It has been demonstrated that the expansion of proximal tissue is caused by an ethylene-promoted enlargement of separation zone cells (Webster, 1973; Wright and Osborne, 1974). The cells of the distal region have been shown to contract in response to a loss in turgor which may result from senescence and tylose-induced water deficit (Sexton and Roberts, 1982).

Recently, Sexton and Redshaw (1981; see Sexton and Roberts, 1982) proposed a different mechanism to explain mechanical separation. They suggested that as cell walls are degraded the tensions across them are released and this in turn increases the tension in adjacent intact tissues. This leads to the separation of collenchyma cells and the stretching of xylem elements. Simultaneously, the cortical cells absorb water and expand. They found that as a result of this, the tension is maintained within the stele and the xylem elements will eventually break under these conditions. The evidence in support of this hypothesis includes the fact that the expansion of separation zone cells during abscission is a widespread phenomenon (Halliday and Wangermann, 1972; Sexton and Redshaw, 1981; Wright and Osborne, 1974). Finally as a result of the lignification which has been found to occur distal to the expanding cells, a region containing more rigid tissue is established. It has been suggested that the expanding separation zone cells exert pressure against this lignified tissue region (Samuel, 1927) and this may contribute in part to the mechanical separation of the tissue (Sexton and Roberts, 1982).

In some of the earlier papers on abscission it was reported that both illuminating gas from street lamps and tobacco smoke could cause the defoliation of various plants (see Abeles, 1973 and Sexton and Roberts, 1982; [Kny, 1871; Stone, 1913; Wehmer, 1900, Fitting, 1911; Molisch, 1911; Kendall, 1918; Hodgson, 1918]). The fact that ethylene was found to be the component responsible for defoliation was shown by Harvey (1913, see Abeles, 1973) and the confirmed by Doubt (1917, see Abeles, 1973).

A significant amount of evidence implicating a role for ethylene in abscission has come from studies with bean explants. It has been reported that excision of abscission zone tissue will induce a wound response causing the release of ethylene from explants at a relatively high rate (Rubinstein and Abeles, 1965; Jackson and Osborne, 1970). The initial rate of release of "wound" ethylene has been found to decrease to a low level within 12 h after excision and it remains at this level until abscission occurs (82h) (Jackson and Osborne, 1970). Immediately after abscission a second rise in ethylene release has been found to occur from the distal tissue. This has been shown to be followed by a smaller rise in ethylene from the petiolar tissue (Jackson and Osborne, 1970). From these results Jackson and Osborne (1970) suggested that ethylene is produced within the tissue after an aging or senescing period has occurred. Additionally, they indicate that ethylene initiates the events which ultimately lead to abscission. Their conclusions were similar to those reported in other studies involving bean explant tissue (Rubinstein and Abeles, 1965; Abeles, 1967).

When ethylene is exposed to bean explants for various periods of time, two intervals (stages) with differing sensitivities to the hormone have been found (Addicott, 1970). Several papers have reported that during the Stage I interval the explants were relatively insensitive to applied ethylene (Abeles, 1968; Jackson et al., 1973; Jackson and Osborne, 1970). However, in a more recent study, Abeles et al. (1971) showed that low levels of ethylene actually accelerated the aging process associated with Stage I and in fact may be required for its passage. During Stage I various senescent changes have been observed in the distal region of explants. These alterations have been found to include changes in auxin metabolism (Abeles, 1967).

During Stage II, ethylene treatment of explant tissue has been shown to substantially accelerate the separation process (Abeles, 1968; Abeles and Rubinstein, 1964; de la Fuente and Leopold, 1968; Jackson and Osborne, 1970). However, it has been reported that if ethylene is withdrawn from the tissue during this interval the rate of abscission will drop to that of the controls (de la Fuente and

Leopold, 1969). Ethylene has also been shown to induce both RNA and protein synthesis during this period (Abeles and Holm, 1966). Based on the fact that simultaneous applications of actinomycin D and ethylene prevented abscission and ethylene rapidly stimulated RNA synthesis, Abeles (1968) concluded that ethylene was an inducer of abscission. In addition to its role in promoting aging (stage I), Abeles et al. (1971) later concluded that ethylene induces the enzymes necessary for cell separation.

A considerable amount of evidence has suggested that ethylene may regulate the aging process associated with abscission (stage I) through its effects on auxin metabolism. When intact plants have been treated with ethylene, the amount of diffusible auxin recovered has been found to be considerably reduced (Michener, 1938; Valdovinos et al., 1967). This effect of ethylene has been demonstrated in several ways. To begin with, ethylene has been found to inhibit the polar transport of auxin in vivo (Burg and Burg, 1967; Morgan et al., 1967). Additionally, the application of ethylene has been shown to increase auxin destruction. In cotton as well as other plants Morgan et al. (1967) showed that an ethylene fumigation of the tissue enhanced IAA oxidase activity. It has been suggested that in cotton plants, the ethylene-induced IAA oxidase activity may mediate ethylene stimulated abscission (Schwertner and Morgan, 1966). However, in pea and Coleus plants Valdovinos et al. (1967) found that ethylene did not enhance the decarboxylation of auxin. Based on this they had proposed that the ethylene effect on transport cannot be explained by means of increased auxin destruction. They suggested that if the ethylene effect was at transport sites for auxin in the tissue, a buildup of auxin would occur where synthesis was taking place. Their results indicated that this does not happen in Coleus plants (Valdovinos et al., 1970). As an alternative hypothesis they reported that ethylene inhibits auxin synthesis. In their study involving cell free preparations derived from light grown pea and Coleus plants, Valdovinos et al. (1967) found that the conversion of TTP to IAA was reduced considerably if the plants were treated with 25 PPM ethylene for 18 h prior to harvesting.

The role of ethylene-mediated reduction of auxin transport in both ethylene-induced and natural leaf abscission was examined by Beyer and Morgan (1971) in the cotyledons of cotton plants. It has been suggested that since ethylene can reduce basipetal auxin transport capacity and accelerate abscission, it may therefore regulate abscission in part by effecting basipetal auxin transport (Beyer and Morgan, 1971; Burg and Burg, 1967; Morgan and Gausman, 1966). In the studies with cotton plants Beyer and Morgan (1971) found that basipetal auxin transport capacity of cotyledons decreased with increasing age and there was a simultaneous increase in the rate of ethylene production. They suggested that in order for the inhibition of auxin transport by ethylene to be a regulating step in abscission the inhibition must occur before abscission occurs. Furthermore, they suggested that the 12 h lag period which precedes abscission in cotton plants is a sufficient time interval for ethylene to exert its effect on auxin transport.

In a later study Beyer (1973) obtained more conclusive evidence to suggest that ethylene regulated abscission in part through its ability to inhibit auxin transport. He found that (a) ethylene-mediated auxin transport inhibition and abscission were both reversible processes, (b) both effects of ethylene exhibited a similar sensitivity to temperature, and (c) the effectiveness of ethylene in both processes is reduced by auxin pretreatment. From these results Beyer (1973) concluded that the ethylene control of auxin transport as well as the effect of ethylene on auxin synthesis (Valdovinos et al., 1967) and metabolism (Hall and Morgan, 1964) must be given important consideration when the role of ethylene in abscission is examined.

Although there is strong evidence suggesting that ethylene is the regulating hormone in abscission (Jackson and Osborne, 1970), its specific role in the process is still somewhat unclear. The fact that ethylene effects auxin transport, synthesis and metabolism while causing an increase in RNA and protein synthesis suggests that the hormone has a multi-functional role in abscission. The difficulty in determining the role of ethylene in abscission as well as other plant processes is complicated by the fact that the natural ethylene-forming system is highly structured and very delicately poised in the intact cell (Lieberman, 1979). If the localization site(s) of the ethylene-forming system could be found in the cell, it might provide more information concerning the role of ethylene in various processes including abscission. Thus far there has been limited success in isolating the ethylene synthesizing system, since homogenization of the tissue has been found to destroy the system (Lieberman, 1979). However, there is some recent evidence which suggests that the ethylene synthesizing system is localized on the surface of the plasma membrane (Lieberman, 1979).

MATERIALS AND METHODS

Plant Growth Conditions

Tobacco plants (Nicotiana tabacum L. "Little Turkish") were grown throughout the year in both the greenhouse and in large environmental chambers until they flowered. A 14 h photoperiod at minimum temperatures of 25° C during the day and 20° C at night was observed in the greenhouse. In environmental chambers the plants were exposed to 14 h per day of light from cool-white fluorescent and incandescent lamps at an intensity of 20,000 lux, and temperatures of 25 ± 2° C during the day cycle and 22 ± 2° C during the night cycle.

Preparation of Tissue for Cytochemical Localization of Cellulase

For ethylene-treated tissue, the upper 25-30 cm portions of stems with unopened, mature flower pedicels were severed at anthesis and placed in plastic beakers containing water. The beakers were placed in environmental chambers (Sherer Gillette Model CEL 25-7HL) under constant incandescent and fluorescent lighting, giving an intensity of 20,000 lx, 50% ± 5% relative humidity, and a temperature of 25° ± 2° C. An airflow of ca. 60 liter-min was passed into the chambers continuously. The air first passed through an ethylene scrubber (Valdovinos, Jensen and Sicko, 1972). During experiments where the plant material was exposed to ethylene, the gas was added to the flow of air to provide a concentration of 5 ul/liter. Tissue was harvested at 1, 2, 3, 4 and 4.5 h after ethylene treatment. Abscission occurred at ca. 4.5 h after ethylene treatment.

For abscission studies where pollination was prevented ("natural abscission"), a small aluminum foil cup or a gelatin capsule was placed over the stigma (Figure 1; Figure 2) at anthesis (Yager and Muir, 1958). Tissue was harvested from the greenhouse at 2, 4, 6, and 8 days after anthesis. Abscission occurred at 8 days under these conditions.

The pedicels of both ethylene-treated tissue and tissue where pollination was prevented were harvested so that the tissue was ca. 2 mm long and contained the abscission zone. The tissue segments were fixed immediately in vials of 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h at 4° C. Following a fixation, the tissue was rinsed in phosphate buffer (pH 7.2) five or six times over a period of 24 h. Each segment was then cut in half (see Appendix A, Schedule I).

At this point a modification of the cytochemical technique in Bal et al. (1976) was utilized. The tissue was incubated in a 0.02% sodium carboxymethylcellulose (CMC) (Cellulose Gum CMC-7HSP, Fisher Scientific) solution containing 0.1M phosphate buffer (pH 7.2) for 20-25 min at 25° C. This was followed by posttreatment of the tissue in hot Benedict's solution at 80° C for 10-15 min. The tissue was washed in distilled water for 15-30 min, treated with 1% osmium tetroxide in 0.1 M PO_4 buffer (pH 7.2) for 1-2 h, dehydrated in an ethanol-propylene oxide series, and embedded in Epon 812 (Luft, 1961) (see Appendix A, Schedule I).

Control tissue was harvested at the time intervals noted above and then subjected to the following conditions: (1) Benedict's solution but no CMC substrate, (2) no CMC substrate and no Benedict's solution, (3) CMC substrate without Benedict's solution, and (4) boiled tissue with or without CMC and with Benedict's solution.

Sections were cut at various depths through the cortical tissue with an LKB ultramicrotome and a Dupont diamond knife. The ultrathin sections were collected on 300-mesh grids and stained with uranyl salts.

A Hitachi Hu 11E electron microscope, operating at 75 kV, was used for examining the sections (see Appendix A, Schedule I).

Flower Pedicel Break-Strength Determination for Cytochemical Study

Tissue break-strength was determined in both natural and ethylene-induced abscission time-course experiments. During abscission where pollination was prevented, the pedicels of greenhouse grown tobacco plants were prepared as previously described and harvested at 2, 4, 5, 6, and 8 days after anthesis. When ethylene-treated tissue was utilized, the plants were prepared as previously discussed and the pedicel tissue was harvested at 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 h after treatment. In both time-course experiments the individual flower pedicels were excised 5 mm proximal to the separation layer (Henry, 1972). At each time interval 10 pedicels were collected and the break-strengths of the pedicels were determined, using the method of Dela Fuente and Leopold (1968). They defined the term break-strength as the amount of force (in grams) necessary to cause complete separation of the cell layers comprising the abscission zone. The break-strength was determined by holding the proximal portion of the flower pedicel against the pan of a top-loading Mettler balance (precision ± 0.01g) and pressing the distal end of the pedicel against the balance pan at a 45° angle (Henry, 1972). The force required to break the pedicel at the abscission zone was expressed in grams. Each measurement represents an average of 10 different pedicel break-strengths at each time interval. The standard error for the break-strength measurements was also determined.

Preparation of Tissue for Morphometric Study

Tobacco plants requiring an ethylene treatment were given an air-ethylene mixture while the control plants were treated with air alone. Pedicel tissue containing the abscission zone was harvested at 4.5 to 5 h after ethylene treatment at which point abscission was imminent. The tissue segments were then fixed in glutaraldehyde and osmium tetroxide, embedded in epon, sectioned, and were examined in the transmission electron microscope.

Randomizing Procedure and Methods of Analysis for Morphometric Study

To insure randomness each abscission zone was cut into 4 pieces before embedding in epon and blocks containing these tissue pieces were randomly selected for sectioning for electron microscopy. Several tissue blocks were selected for each sample. Only one thin section containing separation layer cells (isodiametric cortical cells) was photographed from each block. Then a new block was selected or the previously used area of a block was cut away with a razor blade, the block was retrimmed and then sectioned. All cells in each section were photographed at 5,100X. A montage of each cell in the section was prepared so the entire cell could be analyzed at 13,260X. A transparent array of points and lines, containing 1.0 cm square spacings was randomly superimposed on the montage of each cell that was morphometrically analysed. The surface area of the rough ER, smooth ER, Golgi cisternae and Golgi vesicles were then estimated from the surface density by the line intercept method (Weibel and Bolender, 1973). The relative cell volumes of the nuclei, nucleoli, chloroplasts, mitochondria, vacuoles, cell walls, microbodies as well as the relative volume of the cytoplasm (excluding vacuoles) were estimated from the volume density by the point counting method (Weibel and Bolender, 1973). In this study, 30 control cells (montages) and 30 ethylene-treated cells (montages) were analysed. This is a sufficient number of cells to statistically verify the significance of the data. The mean and standard deviation were determined for each sample and the standard t-distribution test was used to evaluate the significance of differences in the mean values.

Preparation of Tissue for Viscometric and Electrophoretic Assays

Tobacco plants were grown in environmental chambers as previously described and a time-course study of abscission where pollination was prevented, was carried out. The abscission zone tissue was harvested at anthesis as well as 2, 4, and 6 days after anthesis. Under these conditions abscission consistently occurred at 6 days. As a control, a time-course study of non-capped, pollinating flower pedicels (non-abscising pedicels) was carried out.

Flower Pedicel Break-Strength Determination for Viscometric Assays

The tissue break-strength was determined throughout a time-course of abscission where pollination was prevented. At day 0 as well as 2, 4, 5, and 6 days after anthesis, the flower pedicels were harvested as previously described for break-strength determination studies. The method of Dela Fuente and Leopold (1968) was again utilized to measure the break-strengths. The standard error for the break-strength measurements (10 at each time interval) was also determined.

Extraction of Total Buffer-Soluble and Salt-Soluble Cellulase

Buffer-soluble cellulase was extracted from abscission zone tissue by a modification of the procedure utilized by Abeles (1969). In initial experiments the abscission zone tissue sections (ca. 2 mm) were surface sterilized with a 30 sec wash of 2% NaOCl followed by 2 rinses of sterile distilled water. However, this procedure was eliminated since differences in cellulase activity were not observed whether or not the tissue was surface sterilized. Homogenization of the abscission zone tissue sections was accomplished with a pre-chilled mortar and pestle. Twenty-five tissue sections were ground up in 2.5 ml of a 0.05 M potassium phosphate buffer (pH 6.1) containing 0.25% polyvinylpyrrolidine (PVP). The homogenates were then filtered through Miracloth (Calbiochem Corp.) and centrifuged at 10,000 g for 10 min. The supernatant fraction contained the buffer-soluble cellulase.

The pellet and the Miracloth-containing residue (buffer-insoluble) were then combined and washed twice with homogenizing buffer. After the buffer was decanted the buffer-insoluble residue was resuspended in 2.5 ml of a 0.05 M potassium phosphate buffer (pH 6.1) containing PVP and 1 M NaCl. The residue was stirred periodically for 10 to 20 min in an ice bath and then centrifuged at 37,000 g for 10 min. The supernatant contained the salt-soluble cellulase (Ferrari and Arnison, 1974).

Extraction of Exo- and Endocellular Cellulase

The exocellular cellulase (exocellulase) was extracted from the tissue by first weighing out 25 abscission zone sections and placing them in 2.5 ml of a potassium phosphate buffer (pH 6.1) containing 0.25% polyvinyl pyrrolidone (PVP). When electrophoresis was utilized prior to the viscometric assay a volume of 1.0 ml of buffer was alternately used. The sections were incubated in the buffer solution for 24 h at 0° C. The solution was then decanted an utilized for determination of exocellulase activity located in the cell free space

(Hänisch Ten Cate et al., 1975).

The endocellular cellulase (endocellulase) was extracted after the abscission zone sections were washed with buffer to remove residual exocellulase (Hänisch Ten Cate et al., 1975). The sections were then homogenized, filtered and centrifuged as previously discussed for the preparation of buffer-soluble cellulase.

Preparation of Substrate for Viscometric Assay

A 1.14% sodium carboxymethylcellulose (CMC) (Cellulase Gum CMC-7HSP, Fisher Scientific) solution was prepared by adding CMC to a 0.05M potassium phosphate buffer (pH 6.1) containing 0.05 M NaF. In order to prevent clumping within the substrate, the buffer was continuously stirred with a magnetic stirrer while the CMC was slowly added to it. The CMC was dissolved in approximately 3 h under these conditions. For studies involving electrophoretic purification of proteins prior to viscometric assay a 0.58% CMC solution was utilized.

Viscometric Assay for Cellulases

For determination of buffer-soluble and salt-soluble cellulase activity as well as exo- and endocellulase activity, equal volumes (2.5 ml) of both the enzyme preparation and the 1.14% CMC solution were initially combined in a test tube. After carefully sealing the test tube with Parafilm, they were gently inverted several times to insure proper mixing of the assay medium. The enzyme-substrate solutions were then placed in a water bath maintained at 37° C. A buffer + substrate mixture served as a control in the assay.

When the exo- and endocellular fractions were purified by means of polyacrylamide gel electrophoresis (PAGE) the exocellulase activity was determined by the method of Ferrari and Arnison (1974). Gel slices (2 mm) containing protein were added to 0.3 ml phosphate buffer (pH 6.1) and incubated at 37° C for 60 min with shaking. The gel slices were then removed and the enzyme preparation was combined with 4.0 ml 0.58% CMC solution. The enzyme-substrate mixture was placed in a water bath at 37° C. A buffer + substrate mixture was again used as a control.

Cellulase activity was determined by measuring the loss in viscosity of the CMC-enzyme solution with a model LVT Wells-Brookfield Micro (cone-plate) Viscometer (Brookfield Engineering Laboratories, Stoughton, Massachusetts). A 1.0 ml aliquot of either the CMC-enzyme mixture or control solutions (containing no enzyme) was placed in the viscometer and the viscosity (centipoise [cps]) of the solution was determined after allowing for equilibration of the mixture in the sample cup (several minutes). After the initial viscosities of the enzyme-substrate mixtures and control solutions were measured, the preparations were incubated in a water bath for approximately 4 h at 37° C. During the incubation period subsequent changes in viscosity were measured at 2 and 4 h intervals. Similar to the method of Abeles (1969) the cellulase activity was expressed as the percent change in viscosity of the enzyme-substrate solution compared to a blank without enzyme (% Δ n).

Electrophoretic Purification of Cellulases

Exo- and endocellular fractions were subjected to electrophoresis as described by Davis (1965). The gel tubes, which contained a separating gel (7.5% Acrylamide) and a stacking gel (see Appendix A, Schedule III) were placed in a vertical polyacrylamide gel electrophoresis apparatus (Savant Instruments, Inc., Hicksville, N.Y.). The upper and lower chambers of the apparatus were then filled with Tris-Glycine buffer (pH 8.3) (see Appendix A, Schedule III). The enzyme samples (0.1-0.2 ml) containing 0.03 ml glycerol were loaded on the stacking gels and a bromphenol blue solution which served as a tracking dye was then added to the upper buffer (see Appendix A, Schedule III). The gels were run at a constant current of 3 milliamps/tube for approximately 1.5 h at 4° C in the dark. After removal of one set of gels from the tubes the gels were stained with 0.1% Coomassie blue for 1 h (see Appendix A, Schedule III). The gels were then destained overnight in a Bio-Rad Model 172A Gel Electrophoresis Diffusion Destainer (Bio-Rad Laboratories, Richmond, Ca.). The protein banding patterns that were found were used as a reference source for a duplicate set of unstained gels that were sliced and assayed viscometrically.

Localization of Peroxidases in Electrophoretic Gels

An O-dianisidine staining procedure (see Appendix A, Schedule III) was utilized to detect peroxidase bands in the electrophoretic gels (Henry et al., 1974). The staining method was applied to gels containing both exo- and endocellular proteins.

Determination of pH Optimum

The pH optimum of both the exo- and endocellulases was determined with the use of the viscometric assay for cellulase. Equivalent amounts of 0.05 M potassium phosphate buffer solutions (2 ml) and CMC substrate (2 ml) were added to test tubes containing 0.1 ml of enzyme. After gentle but extensive mixing of the solutions, the test tubes were placed in a water bath at 37° C. The initial and final viscosities

(4 h) of the different pH solutions were determined and compared with a blank control (containing no enzyme). The maximum Δ in viscosity at a particular pH was the pH optimum for the enzyme.

Determination of Temperature Optimum

The optimal assay temperature was determined for both exo- and endocellulases with the use of the viscometric techniques. The buffer, substrate and enzyme solution were combined as discussed for the pH optimum determination. After mixing, the test tubes were placed in water baths which ranged in temperature from 20° C to 60° C. The % Δ n was calculated and used to determine the optimal temperature for the cellulases.

Viscometric Assays with Commercial Cellulase

A figure showing a typical response curve to cellulase (Sigma Chemical Co., St. Louis, MO.) is included in the appendix (Schedule V). Sigma No. C-7377 CELLULASE, Practical Grade Type I from *Aspergillus niger*.

RESULTS

In tobacco plants, the process of cell separation is initiated in the cortical cells of the abscission zone region (Figure 3), slightly distal to the indentation of the zone (Figure 4; Figure 5). The cortical cells are smaller and more isodiametric when compared with those cells found on either side of the abscission zone (Figure 4; Figure 6).

Cell separation first occurs on the adaxial side of the pedicel and later spreads to the abaxial side as well (Yager, 1957). In the variety of tobacco known as Little Turkish, cell wall dissolution leading to abscission occurs in approximately 4 to 6 tiers of cells when pollination is prevented (Yager, 1957). However, during ethylene-induced abscission the separation process only extends across a 2 to 3 tier cell region of the abscission zone (Valdovinos et al., 1971). During abscission where pollination is prevented a swelling of the epidermal tissue occurs prior to separation of the tissue (Yager, 1957). When this "natural" abscission process is allowed to proceed to completion, a white ring of cells is formed at the abscission zone (Yager, 1957) approximately one day before the pedicel abscises (Figure 7). The white ring of cells may eventually cover an area of about 1 mm in width across both the distal and proximal sides of the abscission zone (Figure 8). When the pedicel breaks at the zone, the white ring of cells is also separated and some of the cells are removed with the flower portion of the pedicel while others remain attached to the stem portion of the pedicel.

Localization of Cellulase

The ultrastructural localization of cellulase was accomplished with the use of a cytochemical technique in which the deposition of copper (Cu) (a precipitate of the Benedict's solution-cellulose sugars reaction) was utilized as a marker to detect the enzyme activity. The levels of cellulase present in the tissue were determined throughout a time-course study of abscission where pollination was prevented and an ethylene-promoted abscission study. Virtually no Cu precipitate was observed in the abscission zone cells during the initial stages of both time-course experiments (Figure 9; Figure 10). This was also true for tissue harvested at approximately anthesis directly from plants grown in the greenhouse (Figure 11).

By the intermediate stage of abscission (in studies where pollination was prevented), some Cu precipitate was present in the tissue (Figure 12). At 4 days after anthesis, precipitate was sometimes observed to a small extent in the cytoplasm of the abscission cells and in association with the plasma membrane (Figure 12). Additionally, the Cu precipitate was also found in small amounts within the middle lamella region of the cell wall (Figure 12). During an equivalent stage of ethylene-induced abscission (3 h of ethylene treatment), small amounts of precipitate were also observed in the middle lamella region of the wall (Figure 13).

At 6 and 8 days after anthesis a substantially higher level of Cu precipitate was observed in the cell wall (Figure 14; Figure 15; Figure 16), as compared with earlier stages of the time-course experiment. Similar levels of precipitate were also found in the cell wall, in tissue treated with ethylene for 4 to 4.5 h (Figure 17; Figure 18). During abscission where pollination was prevented and during ethylene-promoted abscission, the Cu precipitate was found principally in the middle lamella region of the wall and, to a lesser extent, in the primary and secondary regions of the cell wall (Figure 16; Figure 18). The Cu precipitate appeared in association with fibrillar components of the cell wall (Figure 15; Figure 18).

The primary control utilized in the study, consisted of tissue which was boiled prior to treatment to destroy enzyme activity. Where tissue was harvested 6 and 8 days after anthesis, boiled, incubated in CMC substrate and post-treated with Benedict's solution, significantly less Cu precipitate was found (Figure 19; Figure 21) when compared with tissue which was not boiled (Figure 14; Figure 16). In another control where the tissue segments were harvested again at 6 and 8 days after anthesis, boiled and post-treated with Benedict's solution (without prior incubation in substrate), less Cu deposition was observed (Figure 20; Figure 22) than in the previous control (Figure 19; Figure 21).

In controls where the tissue was not boiled, the following results were found with regard to the deposition of Cu precipitate. When the tissue was treated with Benedict's solution but no CMC substrate, Cu precipitate was present in the tissue primarily during the latter stages of abscission. However, less Cu precipitate was observed (Figure 23) than in tissue treated with both CMC substrate and Benedict's solution (Figure 16). Where ethylene-treated tissue was harvested at 4.5 h, incubated in CMC substrate and not post-treated with Benedict's solution, no Cu precipitate was observed in the tissue (Figure 24). Similarly, in ethylene-treated tissue not incubated in CMC substrate nor post-treated with Benedict's solution, no

Cu precipitate was found in the abscission zone tissue (Figure 25; Figure 26).

Cytochemical-Break-Strength Correlation Study

In a tissue break-strength study, the time at which the flower pedicels will initially break rather than bend, was determined. The subsequent decreases in tissue break-strength were followed throughout a time-course of abscission. These decreases indicated the extent to which cell wall breakdown had occurred and therefore were correlated with the increases in cellulase activity, reported in the cytochemical study. During abscission where pollination was prevented, the pedicels did not break but rather bent in response to pressure through 4 days after anthesis (Figure 27). At day 5 after anthesis, an initial break-strength of 61 g was recorded and this decreased to approximately 40 g at 6 days after anthesis (Figure 27). By 8 days after anthesis, the break-strength of the pedicels had decreased to less than 5 g and abscission was occurring (Figure 27).

During ethylene-induced abscission the pedicels bent rather than broke for the first 2 h following treatment (Figure 28). After an initial break-strength reading of 43 g at 2.5 h of ethylene treatment, the pedicel break-strength decreased to approximately 19.0 g after 3 h of ethylene exposure (Figure 28). When the tissue was treated with ethylene for 4.5 h, the break-strength had decreased to less than 5 g and abscission was occurring (Figure 28).

When the break-strength data was compared with the levels of cellulase observed during the cytochemical study, the following results were found. During abscission where pollination was prevented, the break-strength decreased beginning at 5 days after anthesis, while the level of cellulase present in the cell wall did not substantially increase until 6 through 8 days after anthesis. When the pedicels were given an ethylene exposure, they did not break until 2.5 h after treatment and the level of cellulase in the wall did not essentially increase until 4 to 4.5 h after ethylene treatment.

Dense Bodies, Lipid Bodies and Membrane Configurations

Throughout the time-courses of abscission, natural and ethylene-induced processes, dense bodies were sometimes observed in association with the fibrils of the cell wall region (Figure 29; Figure 30). These bodies were not as electron dense as the Cu precipitate and were sometimes found within the "breakdown vesicle" as well as in the surrounding wall (Figure 30). They were also observed in control tissue that was boiled, incubated in CMC, and post-treated with Benedict's solution. The diameter of the dense bodies ranged from 60 to 110 nm.

Lipid bodies with a high electron dense appearance were found in both tissue where pollination was prevented (Figure 31) and ethylene-treated tissue (Figure 32). A large number of lipid bodies were observed at 6 to 8 days after anthesis and at 3 to 4.5 h after ethylene treatment. They were primarily confined to areas adjacent to the cell wall but within the cell itself. The lipid bodies had a diameter which ranged from ca. 160 to 230 nm at the narrowest point and from 280 to 306 nm at the widest point.

Different kinds of membrane configurations were observed during natural and ethylene-induced abscission processes. The predominant types of inclusion bodies found were lamellar (Figure 33-36) and tubular (Figure 34). In some cases the lamellar-type inclusions contained dense granular deposits (Figure 33). The membrane configurations varied in shape and size. A large number of membrane configurations were sometimes found in the cell wall region, where the indentation was located (Figure 36). They were observed both proximally and distally to the indentation extending several cells distance in both directions. The configurations were confined to clumped groupings within the wall and were essentially not found beyond 1 to 2 tiers of cells below the indentation. The diameter of the membrane configurations varied from 80 nm to 300 nm at the narrowest point and from 173 nm to 650 nm at the widest point.

Morphometric Analysis

The ultrastructural changes associated with abscission were studied quantitatively by the techniques of morphometric analysis. The surface area of the membranes, relative volume of the organelles and the number of organelles was determined for both ethylene-treated and control cells.

When tissue was treated with ethylene for 4.5 to 5 h, the flower pedicels were abscising and a significant increase ($p < 0.05$) was found in the surface area (um^2) of the cells' rough ER (Table I). In fact, the surface area of the rough ER more than doubled when compared with the control cells (Table I). The surface area of the smooth ER did not increase to any significant level ($p > 0.05$) with ethylene treatment of the tissue (Table I). No significant differences ($p > 0.05$) were found in the surface area of the Golgi cisternae and the

Golgi vesicles (Table I).

The relative cell volumes of nuclei, nucleoli, chloroplasts, mitochondria, microbodies, vacuoles and cell walls did not vary significantly ($p > 0.05$) with ethylene treatment of the tissue (Table II). Although the relative cell volume of the cytoplasm (excluding vacuoles) increased with ethylene treatment (Table II), when analyzed statistically the cytoplasmic volumes were not significantly different above the 0.1 probability level.

The number of chloroplasts, mitochondria and vacuoles found in the abscission zone cells (montages) did not vary markedly with ethylene treatment (Table III), and the differences were not statistically significant ($p > 0.05$). The number of microbodies, Golgi cisternae and Golgi vesicles did not change significantly ($p > 0.05$) upon exposure of the tissue to ethylene (Table III).

Viscometric-Break-Strength Correlation Study

Decreases in tissue break-strength were followed during a time-course study of abscission where pollination was prevented. These decreases were correlated with changes in cellulase activity as measured by the percent change in viscosity of an enzyme-substrate solution compared to a blank without enzyme ($\% \Delta n$) (Abeles, 1969).

When subjected to pressure the flower pedicels did not break but rather they bent until 4 days after anthesis (Figure 37). At that point an initial break-strength of 61 g was recorded (Figure 37). The break-strengths of the pedicels subsequently decreased so that by 6 days after anthesis a break-strength of 8 g was measured and abscission was occurring (Figure 37).

Buffer-Soluble and Salt-Soluble Cellulases

During a time-course study of abscission where pollination was prevented, a viscometric assay was utilized to determine buffer-soluble cellulase activity. As a control, this activity was also determined in a time-course study of non-capped, pollinated flower pedicels (non-abscising pedicels). In both time-courses, the cellulase activity was expressed as the percent change in viscosity (in 4 h) of the enzyme-substrate solution compared to a blank without enzyme.

At anthesis (day 0), the percent change in viscosity (% Δ n) was the same for both capped and non-capped flower pedicels (Figure 37). By 2 days after anthesis the % Δ n had decreased slightly, although the difference in viscosity between the capped and the control pedicels (Figure 37) was not significant.

At 4 days after anthesis there was a greater % Δ n of the capped pedicels than in the control pedicels (Figure 37), and the difference was significant. By the 5th and 6th days after anthesis, the % Δ n had increased dramatically in the abscising pedicels (Figure 37). However, in the non-abscising pedicels the % Δ n did not vary significantly during this time interval (Figure 37). In fact, the % Δ n did not fluctuate very much throughout the entire time-course in the control pedicel tissue (Figure 37). The differences in the viscosities between the capped and non-capped flower pedicels at 5 and 6 days after anthesis, was significant.

During the time-course of abscission where pollination was prevented, the tissue break-strength measurements were compared with the changes in buffer-soluble cellulase activity (Figure 37). The flower pedicels initially broke at 4 days after anthesis and the buffer-soluble cellulase activity (indicated by % Δ n) increased slightly at 4 days when compared with the control (Figure 37). However, the largest increase in buffer-soluble cellulase activity occurred after 4 days and reached a maximum at 6 days after anthesis (Figure 37). At this point the tissue break-strength had decreased to less than 10 g and the pedicels were abscising.

The salt-soluble cellulase activity was also assayed viscometrically. Beginning at anthesis (day 0) and continuing through 6 days after anthesis, the % Δ n remained virtually at the same level in both capped and non-capped pedicel tissue (Figure 38). Although the % Δ n observed in the capped and non-capped tissue was significant, it did not vary with the decreases in tissue break-strength (Figure 38).

Exo- and Endocellulases

In another series of experiments, the fraction containing exocellular cellulase (exocellulase) was isolated and then assayed viscometrically. The exocellular assay indicated the extent to which cellulase was present within the cell wall (Hänisch Ten Cate et al., 1975).

In time-course studies of abscission where pollination was prevented and a non-abscising control the % Δ n of the exocellulases were less than 5% in tissue harvested through 2 days after anthesis (Figure 39). When tissue was harvested at day 0 (anthesis) and 2 days after anthesis, the differences in the viscosities between the capped pedicels and the non-capped (control) pedicels were not significant (Figure 39). The % Δ n of the exocellulases were the same in tissue harvested at 1 day after anthesis for both capped and non-capped flower pedicels (Figure 39). When the abscission zone tissue was harvested at 3 days after anthesis the % Δ n of the enzyme-substrate mixture increased (Figure 39). However, the differences in the viscosities between the capped pedicels and the control pedicels were not statistically significant. Beginning with the tissue harvested at 4 days after anthesis, there was a significant increase in the % Δ n of the capped pedicels when compared with the controls (Figure 39). In tissue harvested at 5 days after anthesis the % Δ n of the capped pedicels increased dramatically (Figure 39), and the differences in viscosities between this tissue and the non-capped tissue was statistically significant. The % Δ n of the capped pedicel tissue had increased to its highest level in tissue harvested at 6 days after anthesis, and the pedicels were abscising (Figure 39). Additionally, at this stage the differences in viscosities between the abscising pedicel tissue and the control tissue (Figure 39) were very significant. The % Δ n of the non-capped pedicel tissue remained virtually at the same low level throughout the time-course experiment (Figure 39).

Throughout a time-course study of abscission where pollination was prevented, the decreases in tissue break-strengths (g) were compared with increases in exocellulase activity, as indicated by % Δ n.

The pedicels initially broke (in response to pressure) beginning at 4 days after anthesis (Figure 39). Additionally, a significant increase in exocellulase activity was first observed in the tissue harvested at 4 days after anthesis (Figure 39). The largest increase in exocellulase activity was found when the tissue was harvested at 5 days after anthesis, and this was paralleled by a rapid decrease in the break-strengths at this stage of abscission. The enzyme activity continued to increase, as evidenced by the tissue harvested at 6 days after anthesis, at which point the break-strength had decreased to less than 10 g and the pedicels were abscising.

The activity of an endocellular cellulase (endocellulase) was determined viscometrically during a time-course of abscission where pollination was prevented. The endocellular assay indicated the extent to which cellulase was present within the cell proper (Hänisch Ten Cate et al., 1974). As a control, a time-course of non-capped (control) flower pedicels was carried out and the endocellulase activity was determined. When the tissue was harvested at day 0 (anthesis) the % Δn (endocellulase fraction) was as much as 40% in the control tissue (Figure 40). However, the differences in viscosities between this tissue sample and the capped tissue was not statistically significant. The % Δn of both the capped and non-capped pedicel tissue did not vary significantly in tissue harvested through 2 days after anthesis (Figure 40). When the tissue was harvested at 3 days after anthesis, the % Δn of the capped pedicels increased to a level greater than that of the control pedicels (Figure 40), but the differences in viscosities were still insignificant. In tissue harvested at

4 days after anthesis the % Δ n of the capped pedicels had increased substantially (Figure 40). When this tissue was compared with the non-capped pedicel tissue, the differences in viscosities were significant. When the tissue was harvested beyond 4 days after anthesis through 6 days after anthesis, the % Δ n of the capped pedicels remained virtually at the same level as in tissue harvested at 4 days after anthesis (Figure 40). Additionally, during this time interval, the differences in viscosities between the capped and non-capped tissue were significant. The % Δ n of the non-capped flower pedicel tissue did not vary significantly throughout the time-course study (Figure 40).

Electrophoresis-Exocellulases

Exo- and endocellular fractions were extracted from the abscission zones of capped and non-capped flower pedicel tissue, and were then subjected to electrophoresis. Several protein bands were observed in gels which were run with the exocellular fractions. Where gels were loaded and run with endocellular preparations, no distinct protein bands were found. Instead, a homogenous mixture of proteins was seen throughout the gels. Therefore, only the gels containing exocellular proteins were used in the viscometric assay since individual bands could be isolated and then assayed for cellulase activity.

When the exocellular fraction extracted from capped pedicel tissue harvested at anthesis (day 0) was subjected to electrophoresis, 3 different bands of protein were observed in the gels (Figure 41). The same banding pattern was found in these gels containing exocellular proteins where the tissue was harvested at 1, 2, 3, and 4 days after anthesis (Figure 41). Throughout a time-course of non-capped flower pedicel tissue, the exocellular proteins banded in an identical pattern to that observed in gels of capped pedicel tissue harvested through 4 days after anthesis (Figure 41). When gels were loaded and run with exocellular fractions of capped tissue, harvested at 5 and 6 days after anthesis, 4 different bands of protein were found (Figure 41). The uppermost 3 bands (bands 1, 2, and 3) (Figure 41) appeared identical to those observed in non-capped pedicel tissue as well as in earlier stages of capped pedicel tissue (Figure 41). However, the 4th protein band (band 4) was only found in fractions of capped pedicel tissue harvested at 5 and 6 days after anthesis (Figure 41).

To determine which band or bands in the gels contained exocellulases, appropriate sections from unstained gels were sliced, the proteins were removed and then assayed viscometrically. When bands 1, 2, and 3 found in both capped and non-capped pedicel tissue were assayed, no significant % Δ n was observed when each band was compared with adjacent blank gel (control) slices (Table IV). However, there was a significant increase in the % Δ n of band 4 protein(s) which was only observed in capped tissue that was harvested at 5 and 6 days after anthesis (Table IV). Since exocellulase activity was only found in band 4, the viscometric studies were subsequently confined to assaying those regions of gels where this band was located.

When band 4 was assayed viscometrically, a time-course study was utilized and the % Δ n of both capped and non-capped pedicel tissue was compared. Although band 4 was not visualized in capped pedicel tissure prior to 5 days after anthesis, gel slices were still prepared for tissue harvested at day 0 through 4 days after anthesis. This procedure was followed since subtle changes in viscosity as caused by a small amount of exocellulase activity, could be readily detected in a viscometric assay. Since Coomassie blue was used as a general protein stain its affinity for cellulase would not be as specific as cellulose would in the viscometric assay. Therefore, small amounts of exocellulase might not be easily visualized in the gels when a Coomassie blue stain is utilized.

In tissue harvested at day 0 through 2 days after anthesis, there was less than a 3% Δ n when gel slices containing capped and non-capped tissue fractions were assayed (Figure 42). No significant differences in viscosities were found at these stages when both capped and non-capped tissue gel slices were compared. Then tissue was harvested at 3 days after anthesis, the % Δ n of the capped pedicel gel slices increased slightly (Figure 42). However, the difference in viscosities between this tissue and the control tissue was not significant. Although the % Δ n decreased somewhat in capped tissue harvested at 4 days after anthesis (Figure 42), the difference in viscosity was insignificant when compared with the control tissue. A very large increase in the % Δ n was found in tissue segments which were harvested at 5 days after anthesis (Figure 42). Similarly, when gel slices containing capped tissue fractions harvested at 6 days after anthesis were assayed, an increase in the % Δ n was observed (Figure 42). The differences in viscosities between both the capped and non-capped pedicel tissue in both 5 and 6 day harvested pedicels was significant.

When the break-strengths of capped flower pedicels were compared (time-wise) with increases in exocellulase activity as measured by the % Δ n, the following results were found. The pedicels initially broke in response to pressure at 4 days after anthesis (Figure 42). When the abscission zone tissue was harvested at or prior to 4 days after anthesis, no significant increase in exocellulase activity was found in the gel slices (Figure 42). In tissue harvested at 5 days after anthesis, the break-strengths of the pedicels were rapidly decreasing and the exocellulase activity of the band 4 protein was rapidly increasing (Figure 42). Finally, where abscission zone tissue was harvested at 6 days after anthesis, the exocellulase activity was at its highest level and the pedicels were abscising (Figure 42).

Optimal pH and Temperature

To determine the optimal conditions necessary for the extraction and assay of both exo- and endocellulases, both the pH's (Figure 43) and temperatures (Figure 44) at which maximum enzyme activity occurred, were recorded. The exo- and endocellular fractions were extracted from abscising pedicel tissue (harvested 6 days after anthesis), and were assayed at pH's ranging from 2.0 to 10.0. The pH optimum of the exocellulases was approximately 6.0 (Figure 43). However, since there was a minimal % Δ n between the pH range of 4.5 to 7.0 (Figure 43), in a broader sense this entire range could be considered the pH optimum of the enzyme. In terms of the endocellulase activity, the pH optimum was also observed at approximately 6.0 (Figure 43). In this case the % Δ n did not vary much between a pH of 5.5 to 6.5 and therefore this pH range could be considered optimal for the enzyme.

For determination of the optimal assay temperatures, both the exo- and endocellular fractions were assayed at temperatures ranging from 20° C to 60° C. The maximum increase in both exo- and endocellulase activity was observed at approximately 37° C (Figure 44).

Electrophoresis-Peroxidases

After learning that only one protein band found in the gels contained exocellulase activity, it seemed important to determine the origin of some of the other bands. Since Henry et al. (1974) isolated peroxidase isozymes in gels, during abscission of tobacco flower pedicels, the possibility existed that some of the protein bands found in this study were also peroxidases. A modification of the extraction and electrophoretic procedure of Henry et al. (1974) was utilized for the determination of peroxidase bands. This was necessary so that both the cellulase and peroxidase electrophoretic procedures were initially the same.

When the gels were stained with the o-dianisidine reaction mixture, the following banding patterns were observed during time-course studies of abscission where pollination was prevented and a non-capped, pollinated control. Only one peroxidase band was observed in gels containing exocellular proteins, where capped tissue was harvested through 4 days after anthesis (Figure 45). However, a second peroxidase isozyme band was observed in capped tissue harvested at both 5 and 6 days after anthesis(Figure 45). The upper or more anodic of the two bands was observed throughout the time-course experiment. However, this band became more intense in color (darker brown) when the tissue was harvested at 5 and 6 days after anthesis. In the non-capped pollinated control tissue, only one peroxidase band was observed in the exocellular gels throughout the time-course study (Figure 45). This band appeared identical to the upper or anodic band observed in the capped tissue gels (Figure 45). However, its color was less intense when compared with the capped tissue peroxidase band.

In gels containing endocellular proteins, two peroxidase isozymes were observed throughout time-course studies of abscission where pollination was prevented and a non-capped, pollinated control (Figure 46). One difference between the banding patterns observed in these studies was that both isozymes observed in the non-capped control tissue stained less intensely than the capped tissue isozymes did.

Discussion

The cytochemical technique utilized in this study has been shown to be a good method for the localization of cellulases. Although the deposition of a Cu precipitate served as a marker for cellulase activity, its presence may have also suggested the involvement of other hydrolases which could form breakdown products for the electron-dense complex. However, since substantially more Cu precipitate was observed following incubation of the tissue in CMC than in tissue not incubated in the substrate, it has been concluded that a cellulase-type enzyme is present (Lieberman et al., 1982).

In the tissue break-strength studies, the pedicels broke in response to pressure before any significant increases in Cu precipitate were observed. During abscission where pollination was prevented a large increase in Cu precipitate was first observed in the cell wall 6 days after anthesis (Figure 14). However, the pedicel break-strength initially decreased beginning at 5 days after anthesis (Figure 27). The same result was found in ethylene-induced abscission, where significant increases in Cu precipitate were not observed until 4 h after treatment (Figure 17), at which point the tissue break-strength rapidly approached 0 g (Figure 28). From these findings it has been suggested that cellulase activity, as indicated by the Cu precipitate, is involved during the advanced stages of abscission in tobacco, after initial decreases in pedicel break-strength have begun (Lieberman et al., 1982).

Similar results were reported by Reid et al. (1974) with *Phaseolus vulgaris* leaf explants. They found that a cellulase 9.5

isoenzyme could only be removed from abscission zone tissue if abscission had been induced and if the break-strength had decreased to ca. 0 g. Therefore, they suggested that abscission is not initiated by cellulase but, rather, that cellulase (primarily cellulase 9.5) may play a role in the final separation process.

In contrast to these findings, Sexton et al. (1980) reported that during abscission of bean-leaf explant tissue an increase in cellulase 9.5 was observed concomitantly with losses in tissue break-strength. Since cellulase 9.5 antibodies inhibited the loss in tissue break-strength thereby preventing abscission, they concluded that cellulase must play a major role in the abscission process.

The most significant increase in Cu precipitate was observed in tobacco abscission zone tissue where cell wall breakdown was evident (Figure 15; Figure 17). A similar result was reported in auxin treated pea epicotyls, where Cu precipitate was particularly concentrated in areas undergoing cell wall degradation (Bal et al., 1976). During the intermediate stage of abscission where pollination was prevented (ca. 4 days after anthesis), some Cu precipitate was observed in the cytoplasm and in association with the plasma membrane (Figure 12). Although the precipitate was not observed in association with specific cellular organelles, this data may still suggest that a pathway exists whereby cellulase is produced within the cytoplasm and is transported to the cell wall through the plasma membrane. The fact that this pathway may exist is supported by ultrastructural evidence indicating that an increase in rough ER during ethylene-induced abscission may reflect increased synthesis of cell wall degrading enzymes (Valdovinos et al., 1972).

It is interesting that the primary region where the Cu precipitate was found, was the middle lamella (Figure 18), which has long been considered to consist of mainly pectic substances (Albersheim et al., 1960). This result has lead to the suggestion that the middle lamella may be a point of entry for cellulases into the cell wall region. The cellulases may breakdown the cellulosic component of the wall starting from the middle lamella region. Depending upon how the cellulose bonds are broken the sugars might be released in the direction of the middle lamella region. This may explain the heavy concentration of Cu precipitate found in the middle lamella. Another possible explanation of this phenomena might be that the fibril components of the middle lamella region somehow "anchor" the precipiate so that it remains within this area of the cell wall.

When Bal et al. (1976) first utilized this cytochemical method to localize cellulases in auxin-treated pea epicotyls, they found that the Cu precipitate was concentrated between the plasma membrane and the cell wall. However, when they treated the tissue with auxin for a period of 4 days, cell wall breakdown was apparent, and the Cu precipitate was particularly defined in the cell wall (Bal et al., 1976: Figure 1b).

Electron dense bodies were observed in cell wall regions of abscission cells which were similar to those reported by Valdovinos et al., (1972) in tobacco flower pedicel tissue. The dense bodies were observed primarily in association with the fibril components of the wall and sometimes within the "breakdown vesicle" itself (Figure 29; Figure 30). They were found throughout various stages of abscission. It was previously suggested that the electron-dense bodies (found in ethylene-treated tissue) were protein aggregates which may be related to wall degradation processes (Valdovinos et al., 1972). The dense bodies which have been described in both abscission where pollination was prevented and ethylene-induced abscission, may also represent protein aggregates.

Lipid bodies were observed in cells throughout the time-courses of both abscission processes (Figure 31; Figure 32). However, they were found in greater number in the region of the cell adjacent to the cell wall, during the advanced stages of abscission. These results seem to suggest that the lipid bodies are somehow related to the breakdown processes associated with abscission. These bodies may be released in large numbers, as a result of the senescent changes associated with flower pedicel abscission.

Membrane configurations were found in several stages of abscission, natural and ethylene-induced processes. Both lamellar and tubular inclusions (Figure 34) were found which resembled those reported by Gillespie and Hamilton (1976) in tobacco plants. They found that both lamellar and tubular inclusion bodies in the cell vacuole became more numerous with increasing severity of plant stunting. In contrast to their results, most of the lamellar and tubular inclusion bodies found in this study were observed in the cell wall area. Since these types of configurations were probably derived from the plasmalemma, the term plasmalemmasome was proposed to describe them (Marchant and Robards, 1968). Although it was suggested that membrane configurations were artifacts of fixation techniques (Olah and Rohlich, 1966), freeze-etch studies have shown that these inclusions are indeed cellular components (Griffiths, 1970; Marchant and Moore, 1973). Many different functions have been suggested for membrane configurations. Cronshaw (1965) and Esau et al. (1966) suggested that the multivesicular inclusions found in higher plants may be involved in the deposition of wall material during cell wall formation. However, Fowke and Setterfield (1969) showed that a greater number of multivesicular bodies were observed in artichoke tuber cells not depositing wall material as opposed to cells treated with auxin to stimulate wall synthesis (Gillespie and Hamilton, 1976). Since a large number of membrane inclusions were found in the cell wall region, near the indentation, it is possible that they may have a function related to cell wall metabolism.

Although the cytochemical study indicated that cellulase was found primarily in the middle lamella region of the cell wall, the site(s) of cellulase synthesis were not easily detected with this technique. Therefore, the techniques of morphometric analysis were utilized since they could measure quantitatively the ultrastructural changes observed in abscission zone cells. By determining possible changes in cell organelles it was hoped that this could provide important information concerning functional changes within the cells and possibly provide information in regard to enzyme synthesis in these cells.

The results indicated, that ethylene treatment of the tissue caused an increase in the surface area of the rough ER in abscission cells (Table I). An increase in the frequency of rough ER within abscising tissues has also been reported in several other plant species (Iwahori and Van Steveninck, 1976; Osborne and Sargent, 1976; Sexton and Hall, 1974; Sexton et al., 1977; Valdovinos et al., 1971; Valdovinos et al., 1972; Webster and Chiu, 1975). Additionally, when ethylene was exposed to undifferentiated radical cells of cotton and sugarbeet plants and expanding shoot cells of pea seedlings, a similar increase in rough ER was observed (Freytag et al., 1977; Sargent and Osborne, 1975). In a previous study, Valdovinos et al. (1972) reported an increase in the number of rough ER segments. They used randomly selected areas of electron micrographs from tobacco abscission cells exposed to an ethylene treatment (Valdovinos et al, 1972). They also suggested that this increase in rough ER may reflect increased protein synthesis for cell wall degradation during abscission. Therefore, the results presented in this study confirm that a quantitative increase in rough ER does occur, as suggested in the original study (Valdovinos et al., 1972).

Ethylene treatment of tobacco pedicel tissue did not cause significant changes in the surface area (Table I) or in the numbers per cell (Table III) of Golgi cisternae and Golgi vesicles. Several studies have shown an increase in the number of Golgi cisternae (dictyosomes) and Golgi vesicles during the course of abscission (Gilliland et al., 1976; Iwahori and Van Steveninck, 1976; Osborne and Sargent, 1976; Sexton et al., 1977). More electron dense Golgi vesicles were observed when tobacco pedicel tissue was treated with ethylene (Valdovinos et al., 1973; Valdovinos et al., 1970). However, these studies were primarily descriptive and did not employ techniques of morphometric analysis. The fact that no quantitative changes were found in Golgi cisternae and Golgi vesicles with ethylene treatment does not exclude the possibility that there is a change in Golgi activity during abscission. The change in electron density of Golgi vesicles observed during ethylene promoted abscission (Valdovinos et al., 1973; Valdovinos et al., 1970), may have indicated an alteration in Golgi activity. It has been suggested that an increase in Golgi activity may reflect increased synthesis and/or secretion of hydrolytic enzymes for use in cell wall breakdown processes (Gilliland et al., 1976; Osborne and Sargent, 1976; Sexton et al., 1977; Valdovinos et al., 1970).

During ethylene promoted abscission, no significant changes were found in the relative volumes of the cell walls (Table II). Several studies have indicated that during abscission both the middle lamella (Bornman, 1967; Iwahori and Van Steveninck, 1976; Osborne and Sargent, 1976; Sexton, 1976; Sexton et al., 1977; Valdovinos and Jensen, 1974; Valdovinos et al., 1972) and the primary wall regions (Bornman, 1967; Gilliland et al., 1976; Iwahori and Van Steveninck, 1976; MacKenzie, 1979; Osborne and Sargent, 1976; Valdovinos and Jensen, 1974; Valdovinos et al., 1972) of abscission cells undergo some swelling. However, these studies may have been primarily concerned with those areas of the cell wall where breakdown was evident. In this investigation the relative volume of the entire cell wall area surrounding each cell was determined. Therefore, measurements were obtained of cell wall areas where breakdown was evident and where it was not. Additionally, some variability in cell size of abscission zone cells was found which may account for variations in total cell wall volumes. For these reasons, an overall increase in cell wall volume may not have been measured quantitatively even though isolated areas of cell walls indicated some swelling and breakdown with exposure to ethylene.

In summary the most significant change in cell structure was an increase in the surface area of the rough ER which more than doubled with ethylene treatment of the tissue (Lieberman et al., 1983). This may further support the possibility that the rough ER may serve as a site for synthesis of cell wall degrading enzymes such as cellulase.

After completion of the cytochemical study, both viscometric assays and electrophoretic techniques were utilized in order to further characterize the role of cellulases during tobacco flower pedicel abscission. The viscometric assay has been shown to be a very sensitive method for determining cellulase and pectinase activities (Sherwood and Kelman, 1964). Furthermore, the development of a microviscometer which utilizes a cone-plate detection system, has improved upon the viscometric method since it can accurately measure small quantities of solution (1.0 ml) (Wells et al., 1961; Abeles, 1969; Abeles and Leather, 1971; Rasmussen and Jones, 1971; Rasmussen, 1973).

As opposed to the cytochemical study, the plants utilized in the viscometric assays were grown primarily in large growth chambers. Under these environmental conditions the capped flower pedicels abscised at 6 days after anthesis. When total buffer-soluble fractions extracted from both capped and non-capped pedicel tissue, were assayed for cellulase activity, the results were consistent with those found in the cytochemical study. The most significant increases in buffer-soluble cellulase activity were observed in capped tissue harvested at 5 and 6 days after anthesis (Figure 37). By these stages of abscission the pedicel break-strengths had decreased to a considerable extent (Figure 37). A small increase in enzyme activity occurred at 4 days after anthesis, when pedicels initially broke (Figure 37). However, this increase in activity was minimal when compared with that observed during the subsequent stages of the time-course experiment. These results suggest that the buffer-soluble cellulase reaches its maximum level of activity during the advanced stages of abscission, after initial decreases in pedicel break-strength have begun.

Little change in buffer-soluble cellulase activity was observed throughout a time-course of non-capped (control) pedicel tissue (Figure 37). However, a significant level of cellulase activity was found and maintained at each stage of the time-course (Figure 37). These results seem to suggest that different forms of the buffer-soluble cellulase may exist in the tissue. The level of enzyme activity found in the capped tissue prior to 4 days after anthesis, may also suggest that the tissue contains more than one form of a buffer-soluble cellulase. A similar finding was reported in differentiating tissues of the sycamore (Acer pseudoplatanaus)(Sheldrake, 1970). Both the viscometric assay and pH optima results suggested that the buffer-soluble cellulase extracted from cambial, phloem, xylem and abscission zone tissue contained several different cellulases or several forms of the same cellulase (Sheldrake, 1970). The form of the enzyme found in the non-capped tobacco pedicel tissue did not correlate with the changes in tissue break-strength. Therefore the possibility may exist that this form of cellulase does not play a direct role in abscission.

When a salt-soluble cellulase was assayed, no significant differences in activity were found between both capped and non-capped pedicel tissue (Figure 38). Throughout the time-course study a significant level of cellulase activity was found in both capped and control tissue (Figure 38). However, these levels of enzyme activity did not vary with changes in tissue break-strength. The results seem to suggest, that the salt-soluble cellulase extracted from tobacco abscission zones, does not directly participate in the processes leading to cell separation.

The viscometric results suggest that a buffer-soluble cellulase from tobacco flower pedicel tissue may play an important role during the latter stages of the abscission process. Similarly, Abeles (1969) found that the cellulase activity which increased during abscission of bean, cotton and Coleus explants, was associated with the soluble components of the cell. In an earlier investigation, Horton and Osborne (1967) showed that the buffer-soluble cellulase activity increased before abscission in bean explant tissue. They then suggested that this enzyme was important in cell separation.

Although Lewis and Varner (1970) found that cellulase activity increased during abscission of bean explants, they only observed this increase in the fraction containing a high salt buffer. They found that there was no consistent increase in cellulase activity within the buffer-soluble fraction. Therefore, they suggested that the cellulase which was soluble in a high salt buffer may have a more direct role in the abscission process. Since they used aged explant tissue in their study, this might explain in part the differing results observed in tobacco flower pedicel tissue.

While using ferritin-antibody conjugates for enzymic localization, Bal et al. (1976) showed that a buffer-soluble fraction extracted from auxin-treated pea epicotyls was localized in the distended parts of the E.R. while a buffer-insoluble fraction was localized on the inner surface of the cell wall. They suggested that the cellulase found in association with the e.r. represents a newly formed or recently formed protein being transported to the cell wall (Bal et al., 1976). The possibility may exist that cellulase is somehow changed in molecular form in route from the cell to the cell wall. To some extent this might explain the existence of different forms of the enzyme within the tissue. During abscission of bean-leaf explants, Lewis et al., (1973) have found that cellulase exists in more than one molecular form. A plasma membrane associated cellulase has been localized in kidney bean abscission zones (Koehler and Lewis, 1979). The possibility has been suggested that if these forms of cellulase are secreted into the cell wall it might first undergo an alteration in terms of its isoelectric point (Koehler and Lewis, 1979).

Both the buffer-soluble and salt-soluble fraction assays suggested that more than one form of cellulase is present in the abscission zone of tobacco flower pedicels. Furthermore, at least one form of the enzyme, a buffer-soluble cellulase can be correlated with the changes that accompany abscission. In order to further characterize the buffer-soluble cellulase, the extraction method of Hänisch Ten Cate et al. (1975) was utilized and followed by a viscometric assay.

With this extraction method the buffer-soluble cellulase was separated into two fractions. An exocellular fraction was first removed from the abscission zone sections by diffusion, with a cold temperature treatment. After homogenization of the tissue an endocellular fraction was found in the supernatant solution. The exocellular fraction contained the enzymes located in the cell wall, while the endocellular fraction contained the enzymes located within the cell.

As initially suggested by Huberman and Goren (1979), the Hänisch Ten Cate et al. (1975) procedure extracts the maximum exocellular activity in a low volume of buffer. Furthermore the specific activity of the exocellulases was high with this method (Huberman and Goren, 1979). When the volume of the extraction buffer was increased several fold, no decrease in endocellulase activity was found in tobacco pedicel tissue. This is in agreement with the results reported by Huberman and Goren (1979), that no appreciable amount of endocellulase diffused into the intercellular space during the extraction period with differing buffer volume.

A similar time-course experiment to that utilized in determining buffer-soluble and salt-soluble cellulase was also employed for the exo- and endocellular fractions. However, since the procedure for extraction of the exo- and endocellulases required that the tissue be assayed 1 day after it is harvested, the conditions were somewhat different than in the buffer-soluble and salt-soluble assays.

A significant increase in exocellulase activity was first observed in tissue harvested at 4 days after anthesis (Figure 39). At this point the pedicels initially broke in response to pressure (Figure 39). However, a dramatic increase in exocellulase activity was observed in tissue harvested at 5 days after anthesis (Figure 39). When the abscission zone tissue was harvested at 6 days after anthesis, the exocellulase activity had reached its highest level and the pedicels were abscising (Figure 39).

Considering these results it is unlikely that the level of exocellulase activity found in tissue harvested at 4 days could cause sufficient cell wall changes to account for the sudden breaking of pedicels beginning at 4 days after anthesis. The possiblity may exist that the increase in enzyme activity observed at this stage may represent in part exocellulases that are presently being sent into the wall to function at a later stage. Therefore, these results like those found for the buffer-soluble cellulase suggest that the exocellulases reach their maximal level of activity during the latter stages of abscission, after initial decreases in pedicel break-strength have begun.

The increases in exocellulase activity were greater in tissue harvested at 4 and 5 days after anthesis (Figure 39) than in buffer-soluble cellulase where the tissue was harvested at the same stages (Figure 37). This may be due to the fact that the exocellulase activity represents tissue harvested on one day and assayed 24 h later. During this incubation or extraction period additional enzyme might accumulate in the cell wall and therefore would diffuse into the exocellular fraction. If this was the case it would suggest that some of the increase in exocellulase activity found in tissue harvested at 4 days after anthesis, was due to the additional incubation period. Therefore, this might suggest that the increase in exocellulase activity at 4 days after anthesis was less significant than the results may indicate.

The results of the non-capped pedicel time-course study indicated, that virtually no exocellulase enzymes were present in the control tissue (Figure 39). Since the exocellulase activity was only found in the capped pedicel tissue after abscission had been induced (Figure 39), it is suggested that the exocellulases may function in the degradative processes leading to cell separation. A similar finding was reported by Abeles and Leather (1971) during bean-leaf abscission and later confirmed by Rasmussen (1973) in a study of citrus fruit abscission. They found that the exocellular cellulase activity only increased if the tissue was given an ethylene treatment, thereby inducing the abscission process. Furthermore, both studies associated this enzyme fraction with cell wall degradation.

The most significant increase in endocellulase activity was observed in capped pedicel tissue harvested at 4 days after anthesis (Figure 40). When the tissue was harvested after 4 days through 6 days after anthesis, no appreciable increase in endocellulase activity over that occurring at 4 days was found (Figure 40). These results seem to suggest that the increase in endocellulase activity observed in tissue harvested at 4 days after anthesis, may be due to the synthesis and/or secretion of the enzyme within the cell. Furthermore, it may provide some evidence suggesting that the endocellulases are being mobilized and perhaps even processed at this stage, for their secretion into the cell wall. The fact that there was an increase in exocellulase activity beginning with the tissue harvested at 4 days after anthesis (Figure 39), may support this hypothesis.

An increase in endocellulase activity was also reported in citrus separation zone tissue (Rasmussen, 1973). When the citrus fruits were exposed to ethylene an increase in exocellulase activity was measured first and this was followed by an increase in endocellulase activity (Rasmussen, 1973). Rasmussen (1973) suggested that the increase in exocellulase activity represented enzyme initially being secreted into the cell wall and that the latter increase in endocellulase was caused by the synthesis of new enzyme as induced by ethylene. Although an increase in endocellulase activity was also found in Begonia flower pedicel tissue, the increase did not occur until cell separation was virtually complete (Hänisch Ten Cate et al., 1975). This result suggested that cellulase was not synthesized until the abscission process was near completion. The reported differences in the results between Begonia flower and tobacco flower abscission may be due to the fact that explant tissue was utilized in the earlier study.

Since virtually the same level of endocellulase activity was found in capped tissue harvested beyond 4 days after anthesis (Figure 40), this may suggest that the enzyme is initially active at approximately 4 days after anthesis. Because the endocellulases were maintained at a constant level beyond this stage of abscission, this may indicate that the cell acts as a "sink" or "reservoir" for the enzyme prior to its release into the cell wall. As mentioned earlier the enzyme may undergo some processing during these stages for its eventual secretion into the wall.

In contrast to the exocellulase data, there was a considerably higher level of endocellulase activity found throughout the time-course of the study with non-capped flower pedicel tissue (Figure 40). Since the endocellulase activity remained relatively the same during the time-course and therefore did not vary with decreases in tissue break-strength it is possible that this form of the enzyme may not play a direct role in abscission. A similar level of this enzyme activity was observed in the capped tissue, before any increase in endocellulase activity was found (Figure 40). These results are also consistent with the buffer-soluble cellulase assay where the same level of enzyme activity was found in the non-capped pedicel tissue (Figure 37). As mentioned previously, the results may indicate that more than one form of cellulase is present within the tobacco abscission zone tissue. Several studies have shown that different forms of cellulase exist and may have varying roles during the course of abscission (Lewis and Varner, 1970; Lewis et al., 1970; Reid et al., 1974).

Once the exo- and endocellulases were isolated and assayed, it was important to further purify these fractions. Several distinct protein bands were observed when the exocellular fraction was run on the gels (Figure 41). However, no distinct bands could be seen in the gels containing endocellular proteins. This was probably due to the fact that too many proteins were released upon grinding of the tissue and they may have overlapped within the gels. Therefore, only the exocellular fractions could be used since the protein bands formed during electrophoresis could be isolated for subsequent assay.

The same banding pattern, consisting of 3 bands of protein, was observed in gels throughout a time-course of non-capped flower pedicel tissue (Figure 41) and in capped tissue harvested through 4 days after anthesis (Figure 41). A 4th protein band (band 4) was only found in gels containing fractions of capped pedicel tissue harvested at 5 and 6 days after anthesis (Figure 41). Since band 4 was only observed during the latter stages of abscission, after the pedicel break-strengths had decreased considerably, the possiblity existed that this protein band contained exocellulases.

Using the Ferrari and Arnison (1974) extraction method, the individual protein bands were isolated from unstained gels. The viscometric assay was then utilized to determine which if any of the 4 protein bands contained exocellulases. The only protein band that contained any significant cellulase activity (Table IV) was band 4 (Figure 41). During a time-course study, no increase in band 4 - exocellulase activity was observed before the capped tissue was harvested at 5 days after anthesis (Figure 42). A dramatic increase in band 4 - exocellulase activity was found in tissue harvested at 5 days after anthesis. Similarly, an increase in enzyme activity was also observed in tissue harvested at 6 days after anthesis (Figure 42).

Based on the electrophoretic and viscometric results it seems clear that the band 4 protein(s) contains exocellulases which are only found during the advanced stages of abscission, after initial decreases in pedicel break-strength have begun. Since virtually no exocellulase activity was found in band 4 when the capped pedicels initially broke, further evidence is presented suggesting that the exocellulases do not initiate the cell separation processes. However, the results do suggest that the band 4 - exocellulases may be involved during the latter stages of abscission in tobacco flower pedicels.

These results are consistent with those reported for bean-leaf abscission zone tissue (Reid et al., 1974). However, in contrast to the results reported here, Huberman and Goren (1979) found that no specific exocellulase isoenzyme was responsible for citrus explant abscission. Furthermore, their results indicated that the same cellulase isoenzymes were present in both exo- and endocellular fractions. The tobacco and citrus results may differ because 1) the citrus study utilized explant tissue and 2) the electrophoretic and assay procedures were very different. They utilized a staining method in order to localize both the exo- and endocellulases within the polyacrylamide gels.

It is possible that the differences in the reported times of occurrence of cellulase and the differences in the kinds of cellulases found, are due to the sensitivity of the assays utilized. None of the results of the cellulase studies precludes the possiblity that other enzymes participate in cell wall separation. There exists a substantial amount of evidence for the involvement of pectic enzymes in cell wall separation, as shown in tobacco flower pedicels (Yager, 1960), cotton leaf petioles (Valdovinos and Muir, 1965), bean petiole explants (Morre, 1968), debladed petioles of bean (Rasmussen and Bukovac, 1969), cherry fruit pedicels (Stosser, 1970), citrus petiole explants (Riov, 1974), and citrus fruit pedicels (Greenberg et al., 1975). Furthermore, there is reason to believe that peroxidases may also influence cell wall metabolism in tobacco flower pedicels (Henry and Jensen, 1973; Henry, Valdovinos and Jensen, 1974).

In addition to the involvement of hydrolytic enzymes in cell wall separation, other factors may contribute to abscission. In a study of orange fruit explants, Huberman and Goren (1979) showed that an increase in cellulase activity at the abscission zone was not always followed by abscission. They suggested that the increase in activity of hydrolytic enzymes(especially cellulase) was not the only process necessary for abscission. Goren and Huberman (1976) had previously proposed that when cellulase activity increased and was not followed by abscission, cellulose was apparently protected from, or unavailable to, the enzyme.

When the results of the band 4 - exocellulase assay (Figure 42) are compared with the results obtained using crude exocellulase assay (Figure 39) several factors must be considered. The overall lower levels of cellulase activity observed in the band 4 - exocellulase assay, was probably due to the fact that only 153 ug of soluble protein (as determined by the Lowry method; Appendix A Schedule IV were run on each gel. In contrast to this, 763 ug of soluble protein were utilized for each sample in the crude exocellulase assay. Another explanation for the differences in exocellulase activity might be, that all of the enzyme was not totally recovered from the gel slices. However, this was probably not the case since the band 4 - exocellulase assay was repeated several times with virtually the same changes in enzyme activity occurring each time.

Even though a small amount of soluble protein was utilized in the electrophoretic run, the band 4 - exocellulase activity was relatively high when tissue was harvested at 5 and 6 days after anthesis (Figure 42). This may suggest that the purification of the crude exocellulase fraction by electrophoresis increased the specificity of the enzyme. Although the band 4 - exocellulase results were basically consistent with the crude exocellulase results, one major difference existed. Virtually no band 4 - exocellulase activity was found in tissue harvested at 4 days after anthesis (Figure 42). However, a significant level of crude exocellulase activity was found when tissue was harvested at this same stage of the time-course (Figure 39), Since only a portion of the total exocellular fraction (two-tenths) was loaded on the gel, the difference in activity between the capped and non-capped tissue may not be obvious when the tissue was harvested at 4 days after anthesis (Figure 42). Where the total exocellular fraction was assayed, the difference in enzyme activity from capped and non-capped tissue was apparent at this stage of abscission (Figure 39).

Only the regions of gels containing protein bands (as stained by Coomossie blue), were isolated from duplicate unstained gels. Therefore, it is possible that in addition to the band 4 - exocellulase, another form of the enzyme may be present in regions where no bands were visible. This may provide another explanation for the differences in the band 4-exocellulase activity and the crude exocellulase activity observed when the tissue was harvested 4 days after anthesis.

Considering the fact that several protein bands in the gels were not characterized as cellulases, an additional electrophoretic assay was utilized to determine whether the exocellular proteins were indeed peroxidases. Although only one peroxidase band was observed when the capped tissue was harvested through 4 days after anthesis, a second peroxidase band appeared when the tissue was harvested at both 5 and

6 days after anthesis (Figure 45). However, the fact that only one of these isozyme bands (the more anodic band) appeared in gels where the tissue was harvested prior to 5 days after anthesis may have been due to the differences in the extraction procedures utilized. In the earlier study Henry et al. (1974) assayed a total enzyme fraction composed of both exo- and endocellular proteins. However, in the present study the exocellular fraction was assayed separately from the endocellular fraction. As a result of this procedure, an exocellular peroxidase band - the more cathodic band - was isolated during the final stages of abscission (Figure 45). Since this peroxidase isozyme was only observed in capped pedicel tissue, the possiblity exists that it may play an important role in the cell wall during the advanced stages of abscission. During ethylene-induced abscission Henry et al. (1974) showed that a close correlation existed between the increase in peroxidase activity and cell wall weakening. They suggested that this increase may be associated with changes in hydroxyproline-rich proteins which can then affect the extensibility of the cell walls (Henry et al., 1974).

The more anodic of the two peroxidase bands was observed in gels containing both control and capped pedicel tissue (Figure 45). However, this band stained more intensely when the capped pedicel tissue was harvested at both 5 and 6 days after anthesis. The only change in the protein banding pattern that was reported by Henry et al. (1974), was an increase in the width of the most anodic band which occurred after 3 and 5 h of ethylene treatment. They suggested that this increase in the width of the band represents an increase in peroxidase activity. Similarly, the increase in band color intensity observed with the capped pedicel tissue may also represent an increase in peroxidase activity.

In order to complete the peroxidase study, the endocellular proteins were subjected to electrophoresis and the gels were subsequently stained with o-dianizidine. The same two peroxidase bands were observed in both control and capped pedicel tissue throughout the time-course experiments (Figure 46). These studies of endocellular fractions are in agreement with the report of Henry et al. (1974) where total peroxidases were measured. The data presented in this study suggest that peroxidases are more easily secreted by cells of tissue which has been capped for 5 and 6 days after anthesis, than from non-capped tissue.

The method of extraction of exocellular enzymes consists of subjecting the capped and non-capped tissue, that has been harvested at varying time intervals, to a cold temperature treatment. The treatment involves incubating the tissue in buffer for 24 h at 0° C. This supposedly releases enzyme from the cellular free space (Hänisch Ten Cate et al., 1975). Therefore, this may suggest that such enzymes would be available under _in vivo_ conditions to act at sites within the cell wall.

Summary

The results of this study indicate that cellulase is localized in the cell wall middle lamella region, during advanced stages of abscission in tobacco plants. Furthermore, cellulase is found to a limited extent in association with the plasma membrane and within the cytoplasm. This may suggest that cellulase is secreted into the cell wall during the latter stages of the abscission process. These results are further supported by the morphometric data which suggests that an increase in rough ER, with ethylene treatment, may indicate an increase in synthesis of cell wall degrading enzymes.

The viscometric and electrophoretic results support the findings of the cytochemical study. An increase in buffer-soluble cellulase activity is found during advanced stages of abscission, primarily after decreases in tissue break-strengths have begun. A similar increase in enzyme activity is observed when exocellular cellulase fractions (cellular free space) are assayed. The most significant increase in exocellulase activity is found in tissue harvested at 5 days beyond anthesis, after initial break-strength decreases have begun. An increase in endocellulase (within the cell) is observed before substantial increases in exocellulase activity are found. These results may suggest that the endocellulases are synthesized within the cell and are eventually secreted into the cell wall during the latter stages of abscission. Upon electrophoresis of the exocellular fraction, a protein band is isolated (band 4) which contains exocellulase activity. Since this band is only observed in capped tissue which is harvested at 5 and 6 days after anthesis, the possibility exists that the band 4 - exocellulase is involved in cell wall degradative processes.

The overall results of the study suggest that cellulase is not initiating the cell separation processes that lead to abscission of tobacco flower pedicels. However, the fact that cellulase activity increases after initial break-strength decreases have begun, may suggest that the enzyme is important in the final stages of cell separation.

When electrophoretic gels containing endocellular proteins are stained for peroxidase, two different isozyme bands are isolated. However, in exocellular gels only one of these bands (the more anodic band) is observed in tissue harvested at or prior to 4 days after anthesis. The more cathodic band is observed in gels containing exocellular proteins of capped tissue harvested at 5 and 6 days after anthesis. These results suggest that peroxidases, like cellulases are secreted into the cell wall during advanced stages of abscission. Additionally, this may suggest that peroxidases play an important role in the cell wall changes which precede abscission.

In terms of further investigation, an immunocytochemical study may determine if the band 4-exocellulase plays a direct role in abscission. This would require that the band be further purified by isoelectric focusing techniques prior to development of cellulase antibodies. Once the question of cellulase involvement in abscission is fully clarified, further efforts should be directed toward determining the mechanism by which abscission is initiated in tobacco.

APPENDIX A

PROCEDURES

Schedule I. Transmission Electron Microscopy Solutions

1. Glutaraldehyde Fixation

   a) Phosphate buffer, pH 7.2

0.1M	KH_2PO_4	13 ml
0.1M	Na_2HPO_4	37 ml

   b) One and one-half ml of 50% glutaraldehyde solution was added to 23.5 ml of phosphate buffer, pH 7.2 to give a final solution of 3% glutaraldehyde.

   c) Plant material was harvested and placed inside modified beem capsules and immersed completely in 3% glutaraldehyde solution for 1-2 hours at 4° C.

   d) Two mm tissue sections were sliced in half and rinsed 5 times with 0.1 M phosphate buffer, pH 7.2, in order to completely remove the glutaraldehyde from the tissue.

2. Incubation Substrate and Post-Treatment

   a) A 0.02% carboxymethyl-cellulose (CMC) substrate solution in 0.1 M phosphate buffer was prepared by adding 0.1 M phosphate buffer (pH 7.2) to 0.10 grams of sodium CMC (Cellulose Gum CMC-7HSP, Fisher Scientific) until a final volume of 500 ml was reached. The solution was blended with a magnetic stirrer until fully dissolved (approximately 1-2 hours).

   b) Tissue was incubated in CMC substrate for 25 minutes.

   c) The tissue was then post-treated with Benedict's solution at 80° C for 15 minutes. The solution was prepared in the following way:

1. 86.5 g Sodium Citrate
2. 50.0 g Na_2CO_3 (anhydrous)
3. 400 ml H_2O (distilled)
4. Mix 1-3 with heating. Filter is necessary.
5. Add a second solution: 8.65 g $CuSO_4 \cdot 5H_2O$
6. 50 ml H_2O (distilled)
7. Dilute combined solutions to 500 ml

d) Then the tissue slices were washed thoroughly in distilled water for 30 minutes.

3. Osmium Fixation

a) A 1% solution of osmium tetroxide was prepared by diluting a 2% osmium tetroxide solution with one-half 0.1 M phosphate buffer, pH 7.2.

b) The tissue was placed in this solution for 2 hours at room temperature.

4. Dehydration

a) The tissue sections were removed from the 1% osmium tetroxide and subsequently incubated as follows:

Time	Incubation Medium
5 min.	50% ethanol
5 min.	70% ethanol
5 min.	95% ethanol
5 min.	100% ethanol
5 min.	100% ethanol
5 min.	100% ethanol
5 min.	Propylene oxide*

5 min.	Propylene oxide
5 min.	Propylene oxide

*Do not use near open flame.
Use when cold.

5. Epon Embedding

a) Preparation of mixture A:

62 ml of Epon 812

100 ml of dodecanyl succinic anhydride

b) Preparation of mixture B:

100 ml Epon 812

89 ml of nadic methyl anhydride

c) Mix 1 part A with 1 part B and stir slowly.

d) Add 0.2 ml of dimethylaminophenol (DMP-30) per 10 ml of Epon mixture.

e) Mix thoroughly and slowly to avoid formation of bubbles and keep covered until used.

f) Place tissue in a mixture of Propylene Oxide and Epon in a 3:1 ratio respectively for 15 minutes.

g) The tissue segments were then placed in the following mixtures:

30 min.	Propylene Oxide: Epon (1:1)
45 min.	Propylene Oxide: Epon (1:3)
overnight	Pure Epon

h) The tissue was placed in flat embeddments containing pure Epon under the following conditions:

1 day	35° C (Incandescent Lamp)
1 day	45° C
2-3 days	65° C

6. Post Staining-Uranyl Acetate

   a) 15 grams of hydrated uranyl acetate were dissolved in 50 ml of absolute, acetone-free methanol with a magnetic stirrer. The solution was then filtered into a small vessel and securely corked.

   b) The copper grids were immersed in the staining solution within a small vial inside a petri dish for 10 minutes at room temperature.

Absolute methanol	40 dips
100% ethanol	40 dips
100% ethanol	40 dips
50% ethanol	40 dips
Distilled water	40 dips

Schedule II. Scanning Electron Microscopy Solutions

1. Glutaraldehyde Fixation

   Steps a to c same as T.E.M. Solutions.

   d) Two mm tissue sections were sliced in half and rinsed 3 times with 0.1 M phosphate buffer, pH 7.2, for 5 minutes each time.

2. Dehydration

   a) After the tissue was rinsed in buffer it was placed in the following solutions for 10 minutes in the order shown. Since freon is so volatile, the capsules were only briefly blotted with a Kimwipe after each change in the ethanol and lower concentrations of freon.

10% ETOH	10.5 ml of 95% ETOH up to a final vol. of 100 ml w/d. H_2O
20% ETOH	21.1 " " " " " " " " " " " " "
30% ETOH	31.6 " " " " " " " " " " " " "
40% ETOH	42.1 " " " " " " " " " " " " "
50% ETOH	52.6 " " " " " " " " " " " " "
60% ETOH	63.2 " " " " " " " " " " " " "
70% ETOH	73.7 " " " " " " " " " " " " "
80% ETOH	84.2 " " " " " " " " " " " " "
90% ETOH	94.7 " " " " " " " " " " " " "
100% (1)	absolute (200 proof) ethanol
100% (2)	absolute (200 proof) ethanol

10% freon	113-10 ml of freon up to a final vol. of 100 ml w/100% ETOH
20% freon	" 20 " " " " " " " " " " " "
30% freon	" 30 " " " " " " " " " " " "
40% freon	" 40 " " " " " " " " " " " "
50% freon	" 50 " " " " " " " " " " " "
60% freon	" 60 " " " " " " " " " " " "
70% freon	" 70 " " " " " " " " " " " "
80% freon	" 80 " " " " " " " " " " " "
90% freon	" 90 " " " " " " " " " " " "
100% (1)	" Pure freon 113 (1,1,2-trichlorotrifluoroethane) (tf)
100% (2)	" " " " "
100% (3)	" " " " "

b) Tissue samples were stored in tightly covered vials containing 100% (3) freon 113 and were refrigerated until critical point dried.

Schedule III. Electrophoresis

Stock Solutions:

1. Solution A:

1N HCl	48	ml.
TRIS	36.6	gm.
TEMED	0.23	ml.
water to	100	ml.

(pH 8.9)

2. Solution B:

1N HCl	48	ml.
TRIS	5.98	gm.
TEMED	0.46	ml.
water to	100	ml.

(pH 6.7)

3. Solution C:

Acrylamide	48	ml.
BIS	0.46	gm.
water to	100	ml.

4. Solution D:

Acrylamide	10.0	gm.
BIS	2.5	gm.
water to	100	ml.

5. Solution E:

Riboflavin	4	mg.
water to	100	ml.

6. Solution F:

Sucrose	40 gm.
water to	100 ml.

7. Solution of ammonium persulfate:

0.14 gm of ammonium persulfate

water to 100 ml.

8. Small pore gel:

Solution A	1 part
Solution C	2 parts
Distilled water	1 part
Ammonium persulfate solution	4 parts

(pH 8.8-9.0)

9. Large pore gel:

Solution B	1 part
Solution D	2 parts
Solution E	1 part
Solution F	4 parts

(pH 6.6-6.8)

10. Tracking Dye:

5 mg. Bromphenol Blue plus distilled water to 100 ml.

11. Gel stains: Coomassie Blue

95% methanol	50 ml.
Glacial acetic acid	12 ml.
0.1 g CB to	100 ml.

0.1%

Stain 1 h.

12. Wash-solution for destaining and storing gels:

95% Methanol	100 ml.
Glacial acetic acid	50 ml.
water to	1 liter

(make up as needed)

13. Gel stains: O-dianisidine

Ortho-dianisidine (3,3'-dimethoxybenidine)	250 mg.
95% ethyl alcohol	140 ml.
Acetate buffer, pH 4.7 (0.88 M sodium acetate and 0.62 M glacial acetic acid)	20 ml.
Distilled water	36 ml.

Add 5 ml. of 3% hydrogen peroxide just before immersing gels in stain for 20 minutes. Transfer gels to distilled water.

14. Stock buffer solution:

TRIS	6.0 gm.
Glycine	28.8 gm.
water to	1 liter

(pH 8.3)

Schedule IV. Protein Determination

Stock Solutions and Methods:

1. Sample - use suitable aliquot in range of 0.1 ml (up to 1.0 ml). Adjust final volume of all samples to 1.0 ml with water.
2. Reagent A:

1% Na-K-Tartrate	1 part
0.5% $CuSO_4 \cdot 5H_2O$	1 part
(mix fresh on day of use)	
2% Na_2CO_3 in 0.1N NaOH	50 parts

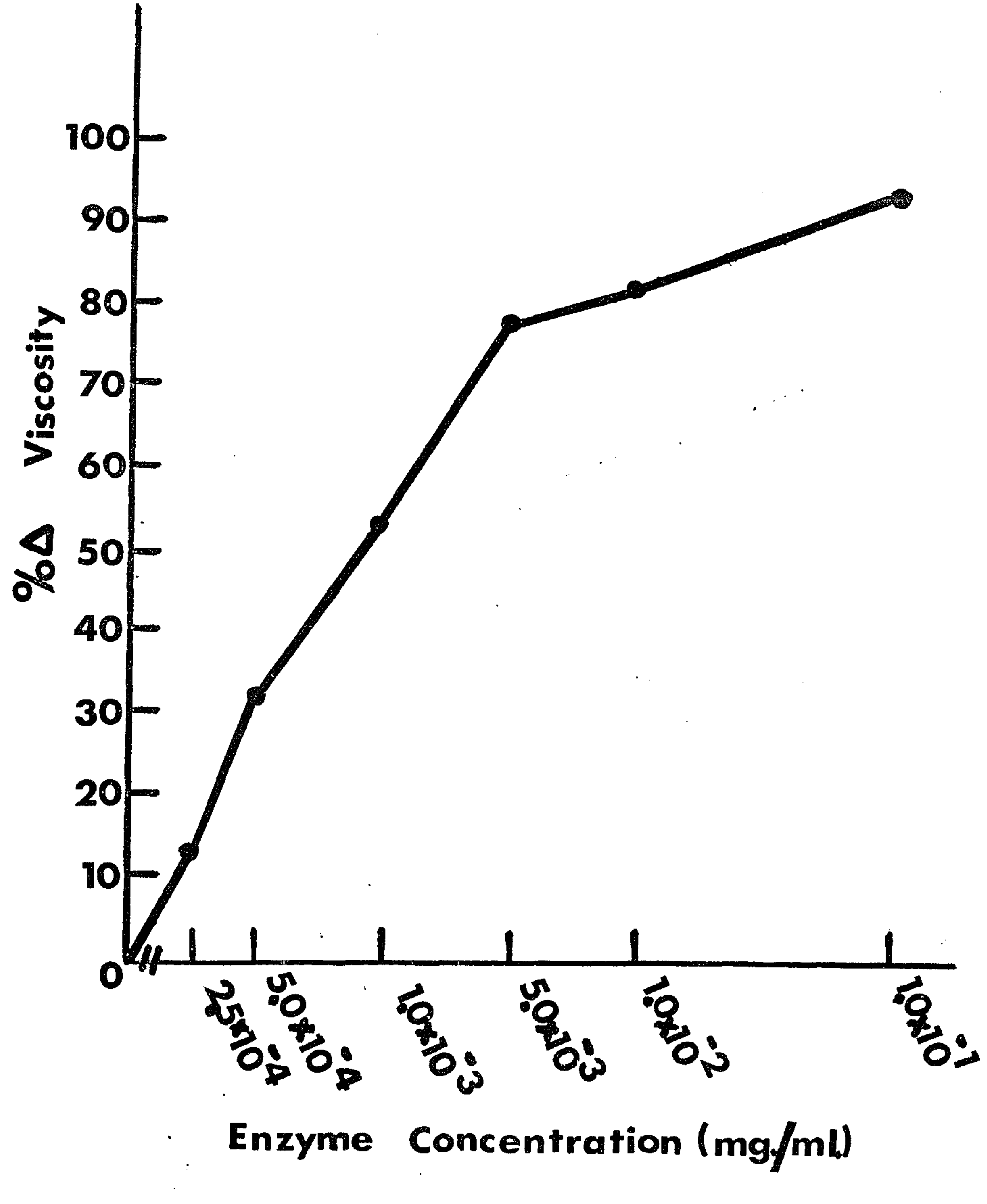
3. Reagent B: Folin Phenol reagent (2N solution) diluted 1:2 with H_2O on day of use.

   Keep stock solution closed and store in refrigerator.
4. Add 5 ml. of Reagent A to 1 ml. of sample prepared in 1. and mix with a vortex. Wait 10 min.
5. After 10 min. add 0.5 ml. of Reagent B (diluted Folin Phenol solution) to sample - carbonate solution. Immediately mix with a vortex mixer.
6. Wait 30 min. - 2 hr.
7. Read at 750 nm.
8. Run appropriate blanks of 0.1 N NaOH and/or sample buffer solution.
9. Run suitable standard curve with 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 ug bovine serum albumin (BSA) per tube.

Test samples should be assayed using procedure identical to those employed for the standard curves.

Schedule V. Commercial Cellulase Assay

Viscometric Determination of Cellulase Activity (% Δ n):



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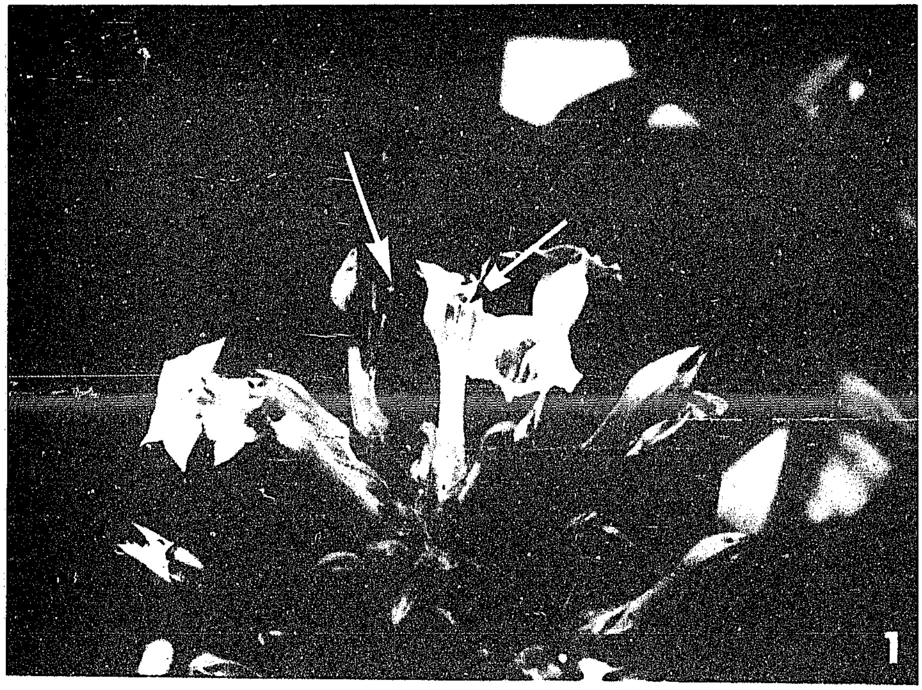
Explanation of Figures

AZ	abscission zone
CC	cortical cell
CP	chloroplast
CW	cell wall
CWD	cell wall degradation
F	fibril components
I	indentation
L	lipid body
M	mitochondria
MC	membrane configuration
MI	microbody
ML	middle lamella region
N	nucleus
NC	nucleolus
P	plasmodesmata
PM	plasma membrane
RER	rough emdoplasmic reticulum
VS	vesicular material

Fig. 1. A panicle of tobacco flowers which are capped at approximately anthesis with a gelatin capsule (arrows). This prevents pollination and therefore allows for abscission to occur.

Fig. 2. An enlarged view of the same panicle in Fig. 1 showing the gelatin capsule (arrows) covering the stigma (S) of the flower.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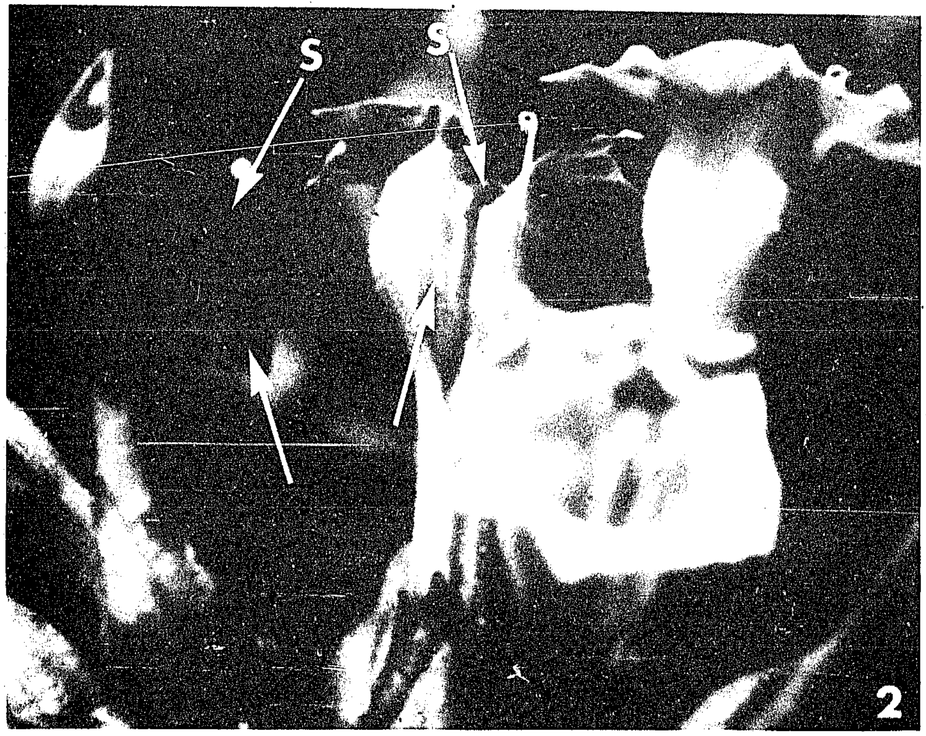

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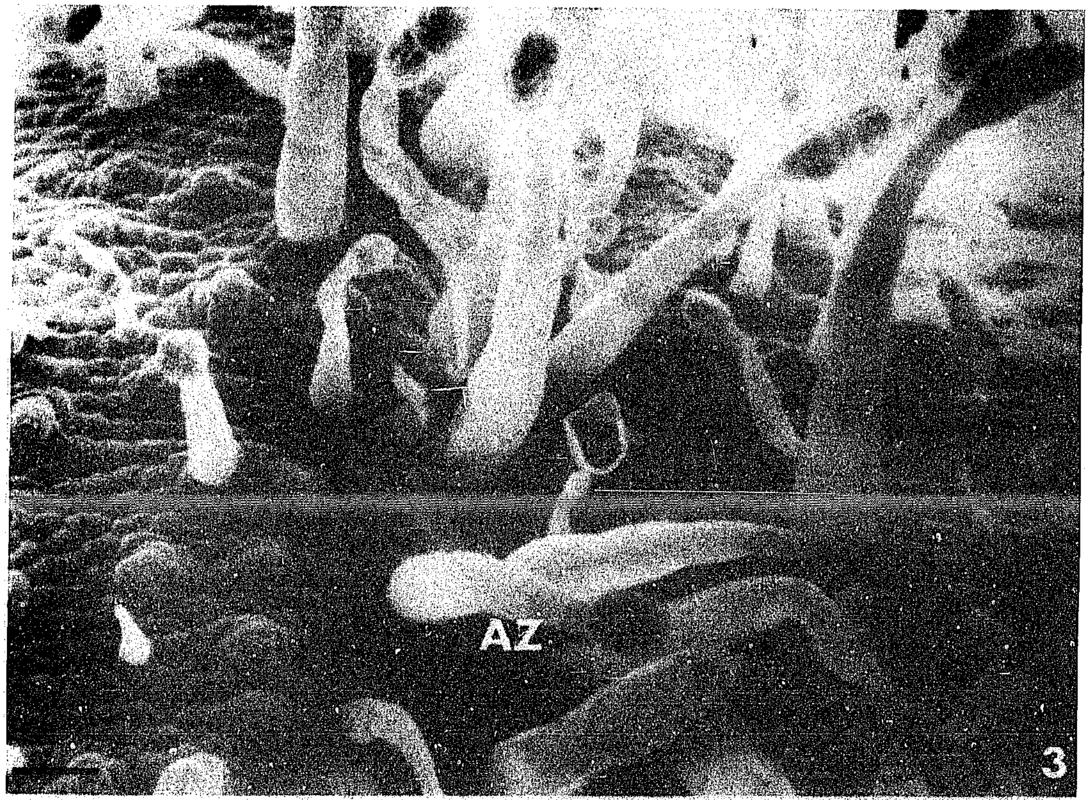
Fig. 3. A scanning electron microscope view of the surface area of the abscission region. The abscission zone (AZ) extends through the cortical cells of the tissue. Hairs and stomata are visible on the tissue surface. For tissure fixation see Appendix A, Schedule II. Approximately X700. Bar = 22 um.

Fig. 4. A scanning electron microscope view of a longitudinally cut section of the pedicel region. An indentation (I) defines the abscission zone (AZ) and is composed of epidermal tissue. The cortical cells of the abscission zone are smaller and more compact than those on either side of the zone. The arrow points toward the flower. For tissue fixation see Appendix A, Schedule II. Approximately X600. Bar = 20 um.


AZ
3

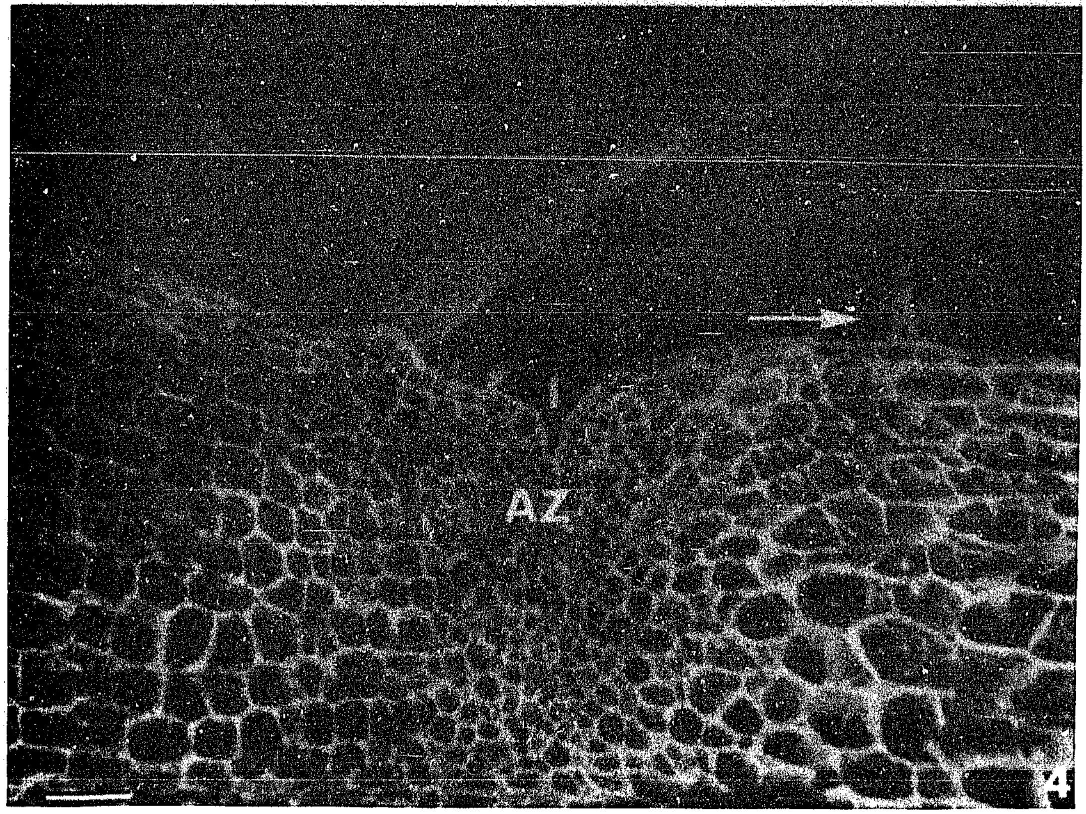
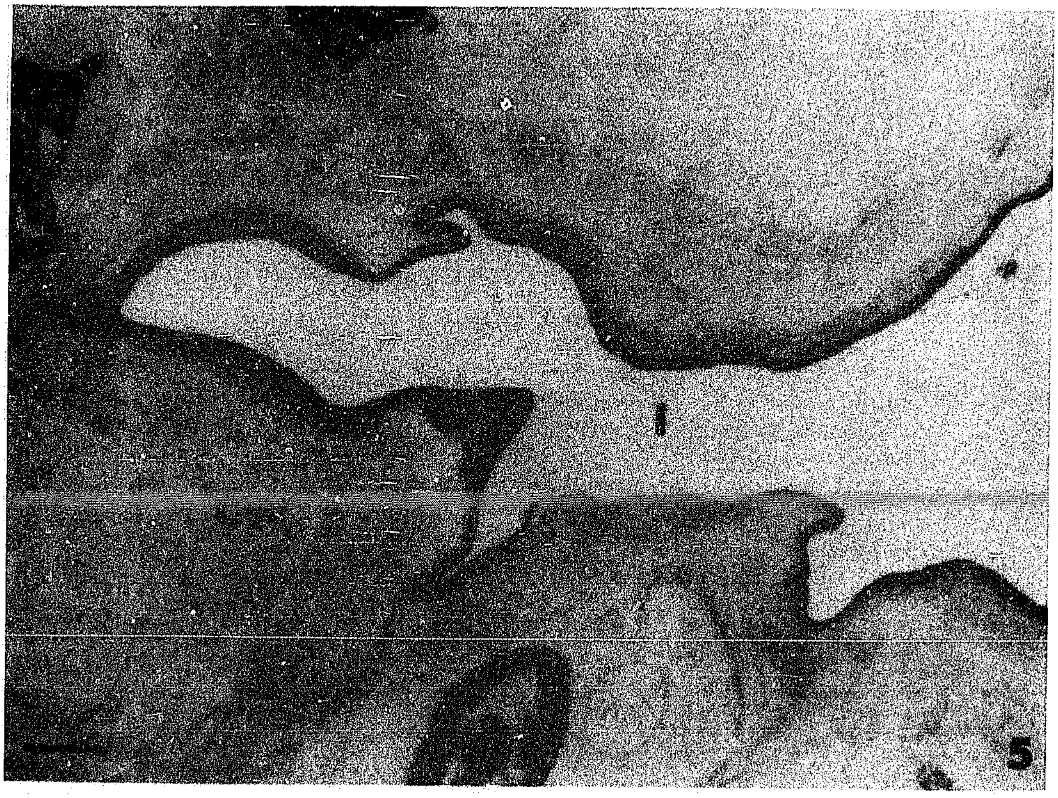

AZ
4

Fig. 5. A transmission electron microscope view of the indentation (I) found adjacent to abscission zone cells. Tissue was ethylene-treated for 4.5 h, incubated in CMC substrate and post-treated with Benedict's solution. All the tissue prepared for the TEM study was stained with glutaraldehyde and osmium tetroxide with post-staining in uranyl acetate. X10,000. Bar = 1.0 um.

Fig. 6. A transmission electron micrograph of a cortical cell within the abscission zone region of tobacco. Note the cell wall degradation (CWD), chloroplasts (CP), nucleus (N), mitochondria (M), and microbody (MI), with a crystalline core. Tissue was ethylene-treated for 4.5 h, not incubated in CMC nor post-treated with Benedict's solution. X4,000. Bar = 1.0 um.


I
5

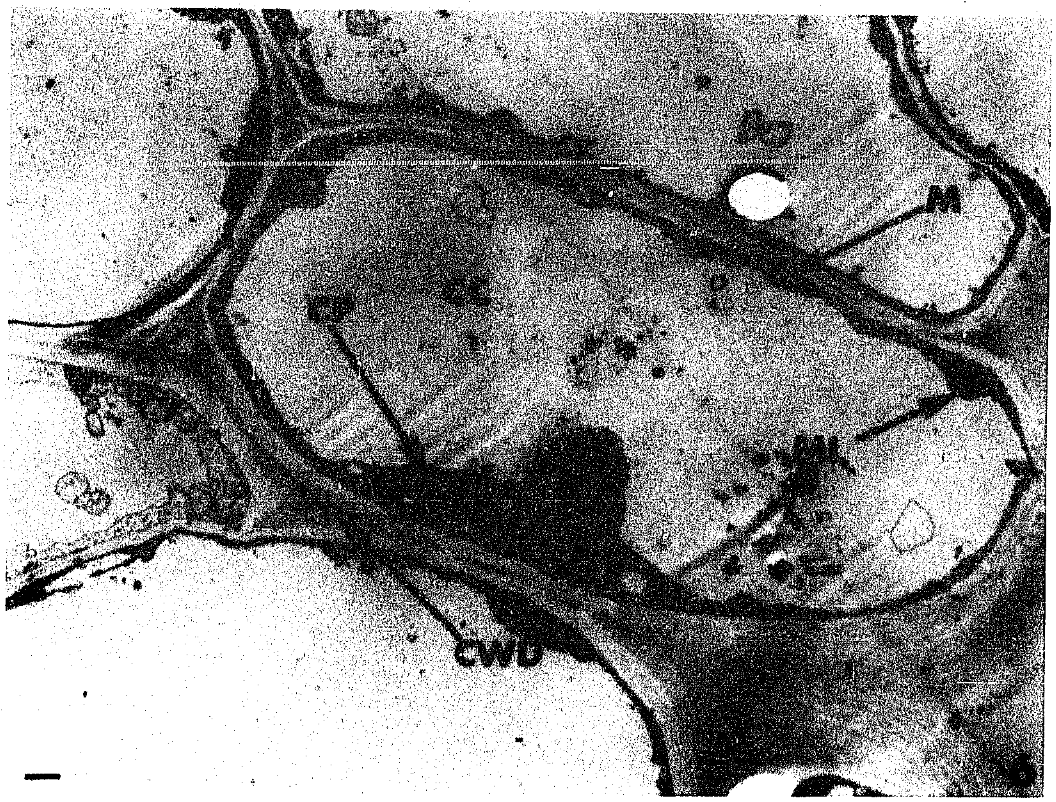

M
ML
CWD
6

Fig. 7. A panicle of tobacco flowers where one flower pedicel has been capped and where abscission is imminent. There is a white ring of cells at the base of the capped flower pedicel (arrow).

Fig. 8. An enlarged view of the capped flower pedicel in Fig. 7. The white ring of cells (arrow) which extends across both the distal and proximal regions of the abscission zone is found at the base of the pedicel.

7

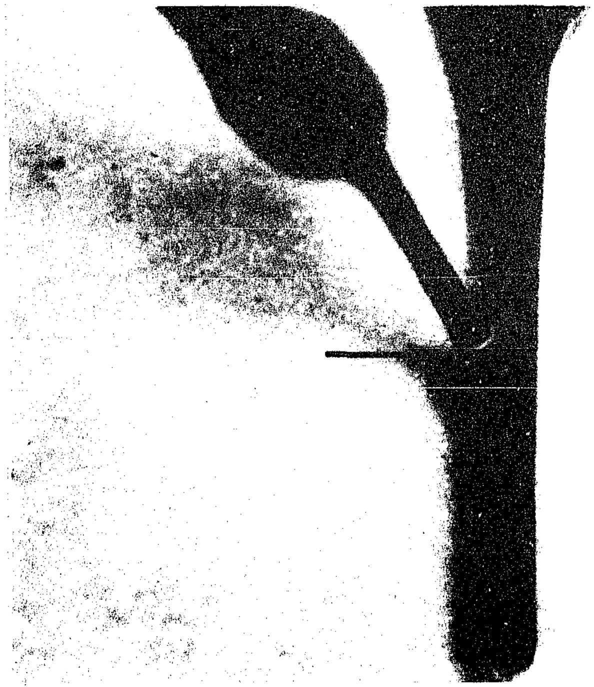


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Fig. 9. Abscission where pollination was prevented. Tissue was harvested at 2 days after anthesis, incubated in CMC and post-treated with Benedict's solution. Virtually no Cu precipitate is observed in association with the fibrils (F) of the cell wall (CW). Note the presence of a chloroplast (CP) and mitochondria (M) within the abscission cells. X10,000. Bar = 1.0 um.

Fig. 10. Ethylene-induced abscission. Tissue was harvested after 2 h of treatment, incubated in CMC and post-treated with Benedict's solution. Virtually no Cu precipitate is observed in association with the fibrils (F) of the middle lamella region (ML) of the wall (CW). X15,000. Bar = 1.0 um.

Fig. 11. Tissue was harvested directly from the greenhouse, incubated in CMC and post-treated with Benedict's solution. No Cu precipitate is observed in association with the fibrils of the middle lamella region (ML) of the cell wall (CW). Note the presence of a nucleolus (NC), nuclei (N), chloroplasts (CP), and plasmodesmata (P). X9,000. Bar = 1.0 um.


CW
9


CW
ML
10

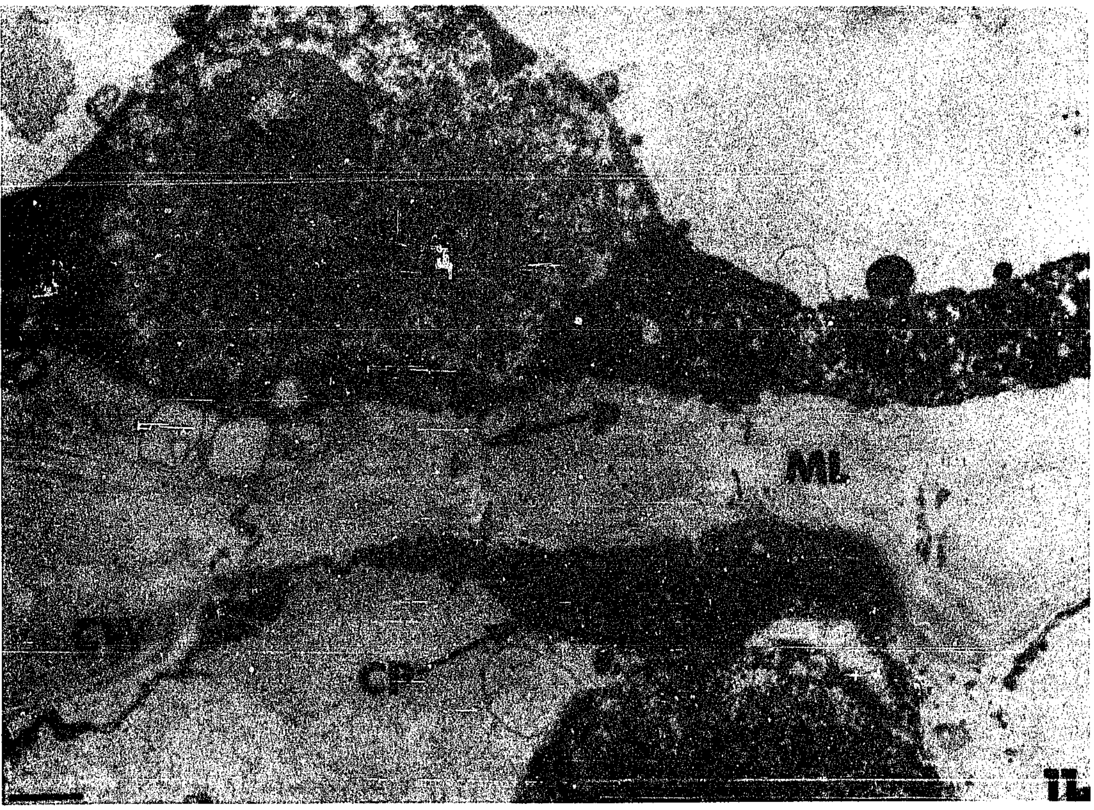
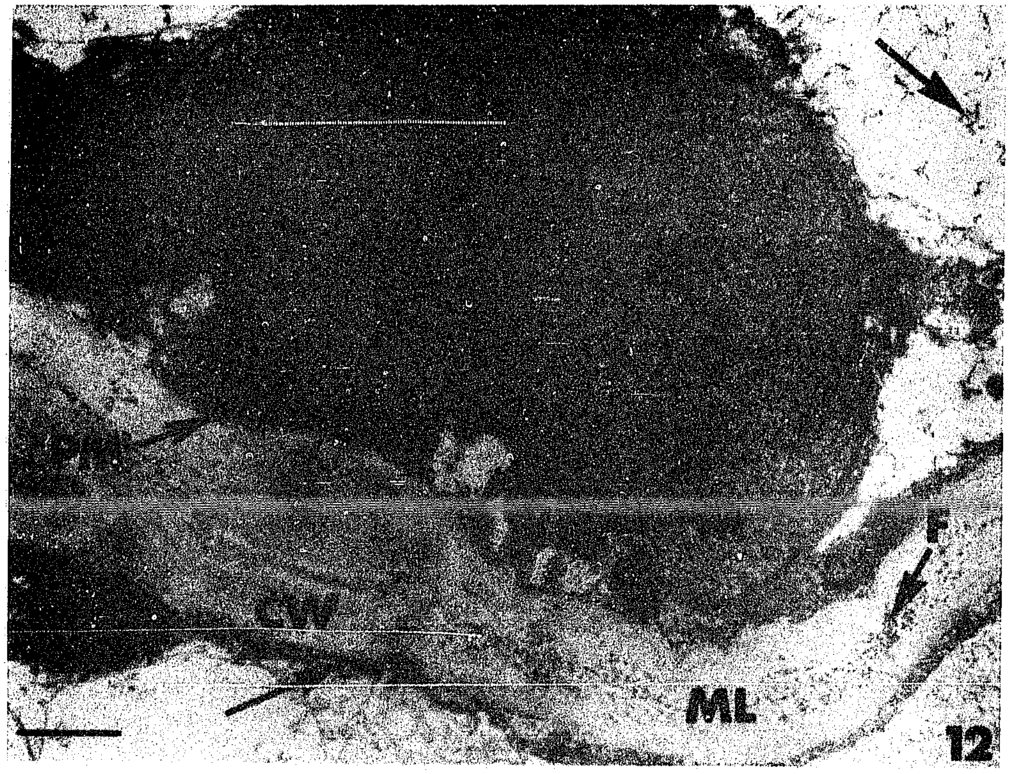

ML
CW
CP
11

Fig. 12. Abscission where pollination was prevented. Tissue was harvested at 4 days after anthesis, incubated in CMC and post-treated with Benedict's solution. Some Cu precipitate is observed in the cytoplasm (uppermost arrow), in association with the plasma membrane (PM) (lowermost arrow) and in the middle lamella (ML) region of the cell wall (CW). Note the nucleus (N), nucleolus (NC), and mitochondria (M) within the cell. X12,000. Bar = 1.0 um.

Fig. 13. Ethylene-induced abscission. Tissue was harvested after 3 h of treatment, incubated in CMC and post-treated with Benedict's solution. A small amount of Cu precipitate is observed in the middle lamella region of the cell wall (CW) in association with the fibril components (F). Note the chloroplasts (CP) within the cortical cells. X10,000. Bar = 1.0 um.


PM
CW
F
ML
12

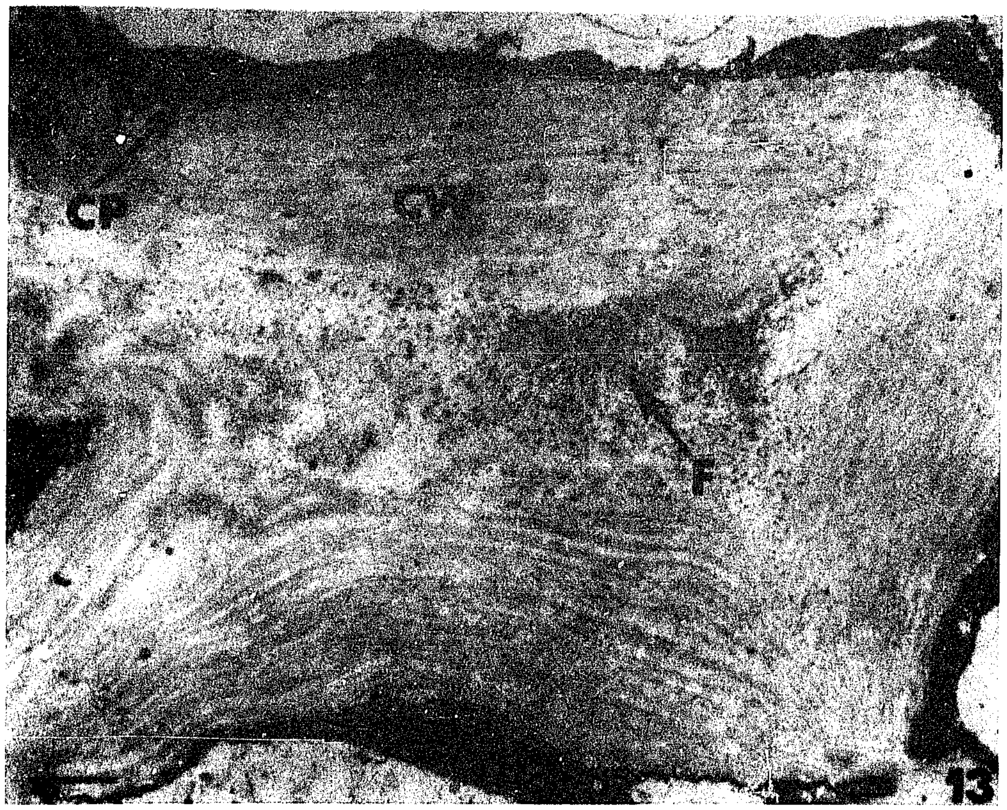
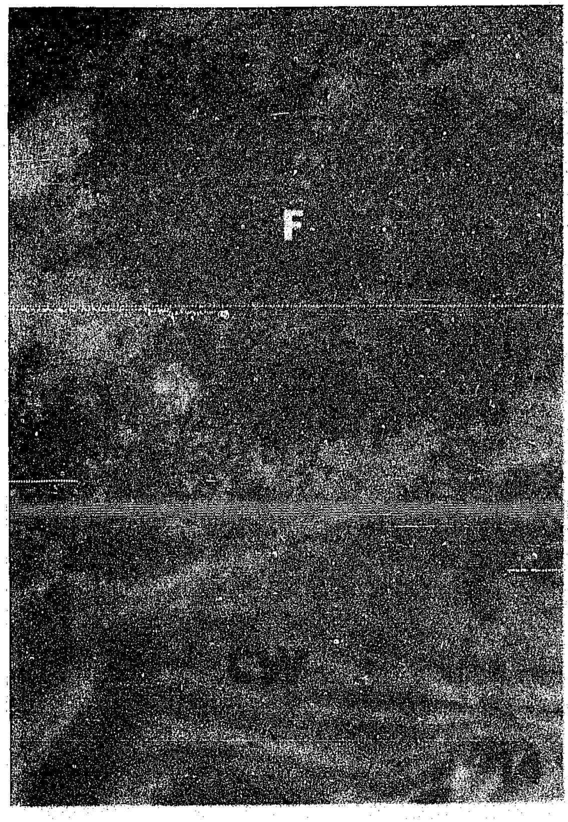
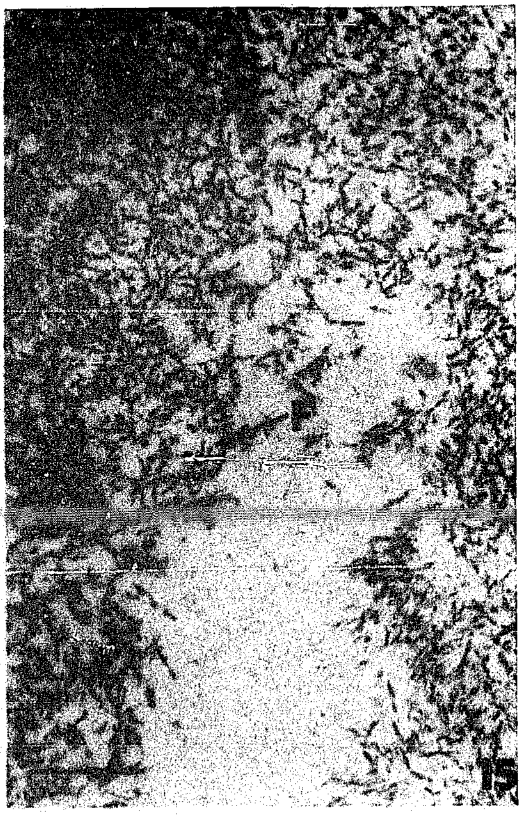

CP
CW
F
13

Fig. 14. Abscission where pollination was prevented. Tissue was harvested at 6 days after anthesis, incubated in CMC and post-treated with Benedict's solution. Note the dense Cu precipitate in association with the fibrils of the middle lamella region of the cell wall (CW). X20,000. Bar = 0.5 um.

Fig. 15. Abscission where pollination was prevented. Tissue was harvested at 8 days after anthesis, incubated in CMC and post-treated with Benedict's solution. A dense Cu precipitate is observed in association with the fibril components (F) of the wall. X29,000. Bar = 0.5 um.

Fig. 16. Abscission where pollination was prevented. Tissue was harvested at 8 days after anthesis, incubated in CMC and post-treated with Benedict's solution. Note the presence of the Cu precipitate (arrow) in the middle lamella region (ML) of the cell wall (CW). Additionally, a small amount of Cu precipitate is observed within the cell, in association with the plasma membrane. X50,000. Bar = 0.25 um.


F
CW
14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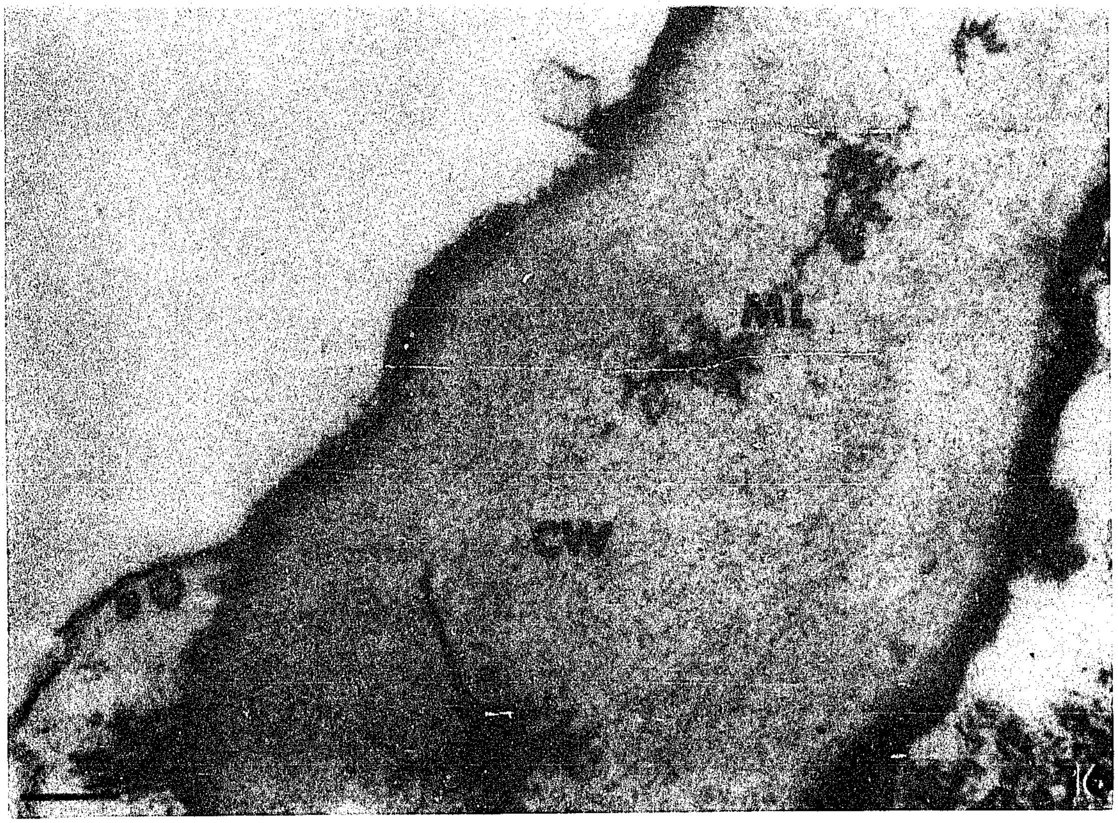
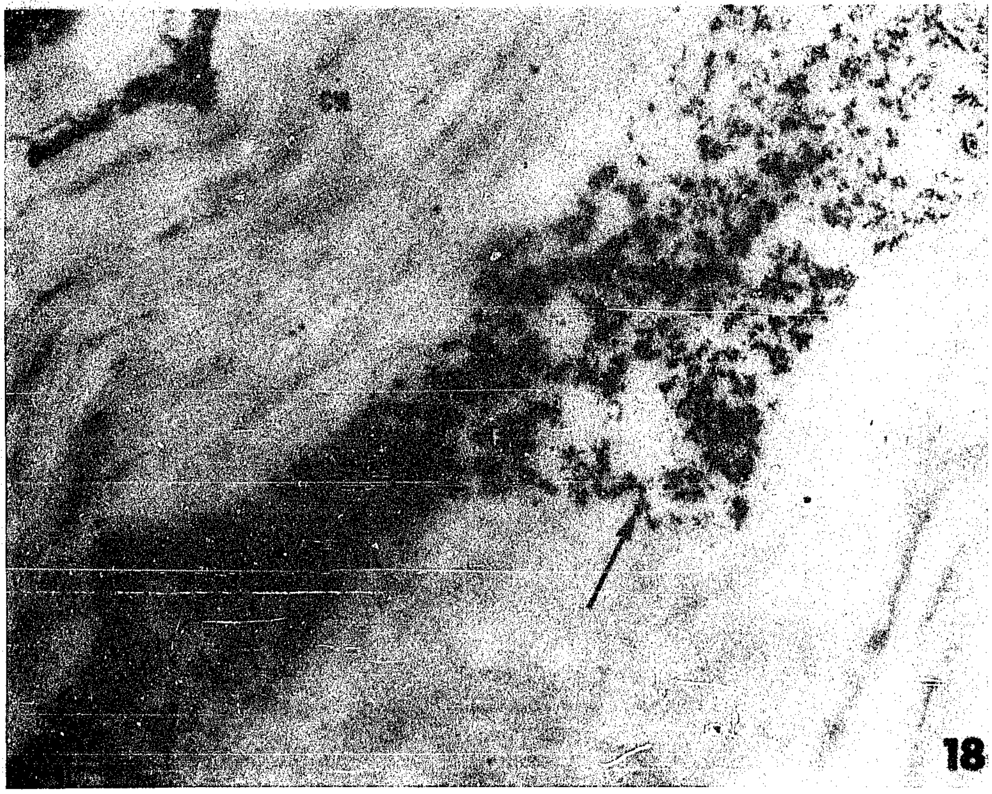

ML
CW
16

Fig. 17. Ethylene-induced abscission. Tissue was harvested after 4 h of treatment, incubated in CMC and post-treated with Benedict's solution. A dense Cu precipitate (arrow) is present in the middle lamella region where cell wall degradation (CWD) is occurring. Additionally, note the Cu precipitate in adjacent regions of the cell wall. X35,000. Bar = 0.25 um.

Fig. 18. Ethylene-induced abscission. Tissue was harvested after 4.5 h of treatment, incubated in CMC and post-treated with Benedict's solution. Note the presence of a dense Cu precipitate (arrow) primarily found within the middle lamella region of the cell wall. Additionally, some Cu precipitate is found in other regions of the wall where fibrils (F) are not found. X32,000. Bar = 0.25 um.

17



18

Fig. 19. Abscission where pollination was prevented. Tissue was harvested at 6 days after anthesis, boiled, incubated in CMC and post-treated with Benedict's solution. Very little Cu precipitate is observed within the middle lamella region (ML) of the cell wall (CW). Chloroplast (CP), fibrils (F). X7,000. Bar = 1.0 um.

Fig. 20. Abscission where pollination was prevented. Tissue was harvested at 6 days after anthesis, boiled and post-treated with Benedict's solution, without prior incubation in CMC. Virtually no Cu precipitate is observed within the cell wall (CW) middle lamella region. Chloroplast (CP), fibrils (F). X9,000. Bar = 1.0 um.

Fig. 21. Abscission where pollination was prevented. Tissue was harvested at 8 days after anthesis, boiled, incubated in CMC and post-treated with Benedict's solution. Very little Cu precipitate is present in association with the fibrils of the cell wall (CW), middle lamella region (ML). Note the lipid body (L) located within the cell. X9,000. Bar = 1.0 um.

Fig. 22. Abscission where pollination was prevented. Tissue was harvested at 8 days after anthesis, boiled and post-treated with Benedict's solution, without prior incubation in CMC. Virtually no Cu precipitate is present in association with the fibrils (F) of the cell wall (CW), middle lamella region (ML). X28,000. Bar = 0.5 um.

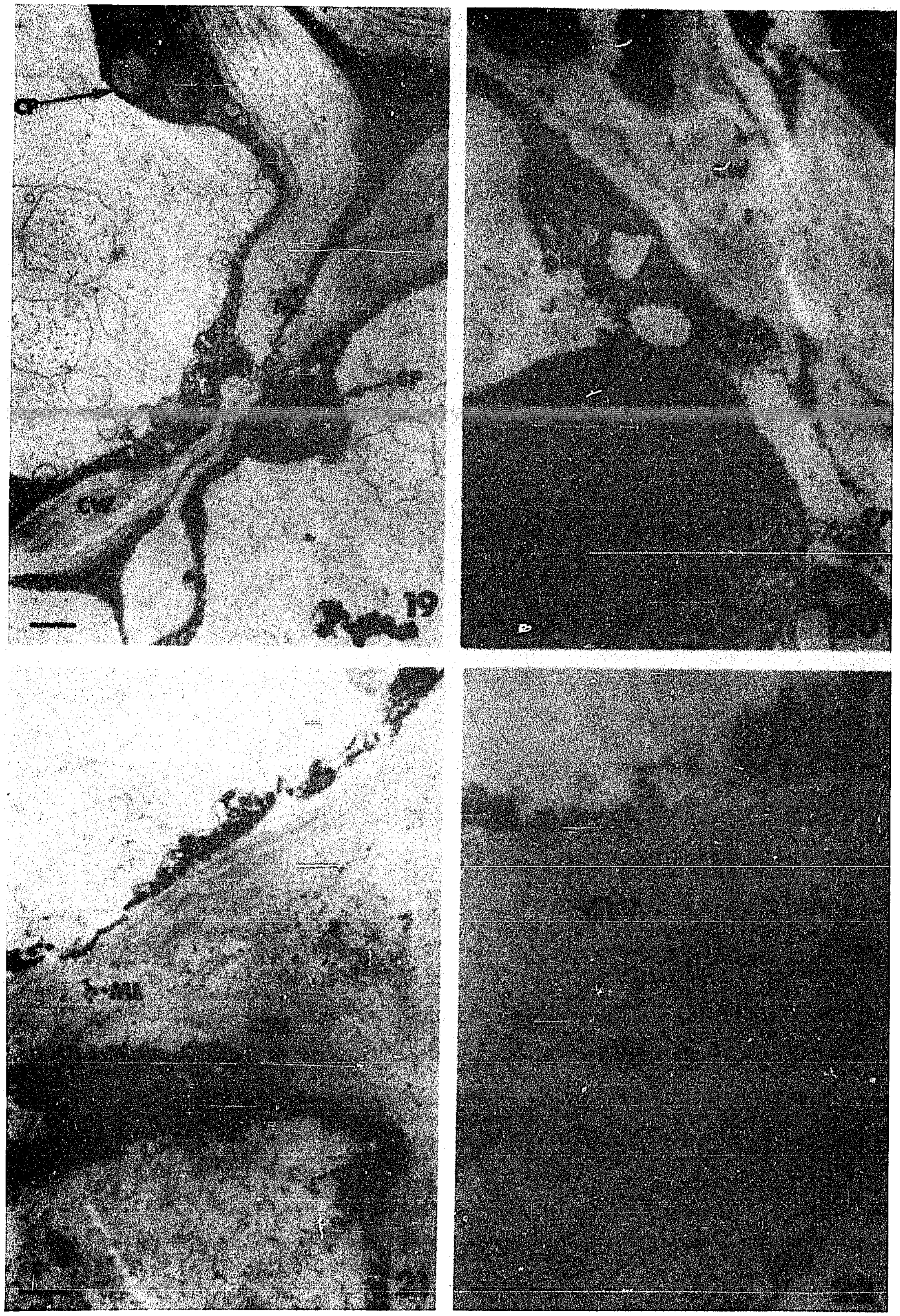


Fig. 19

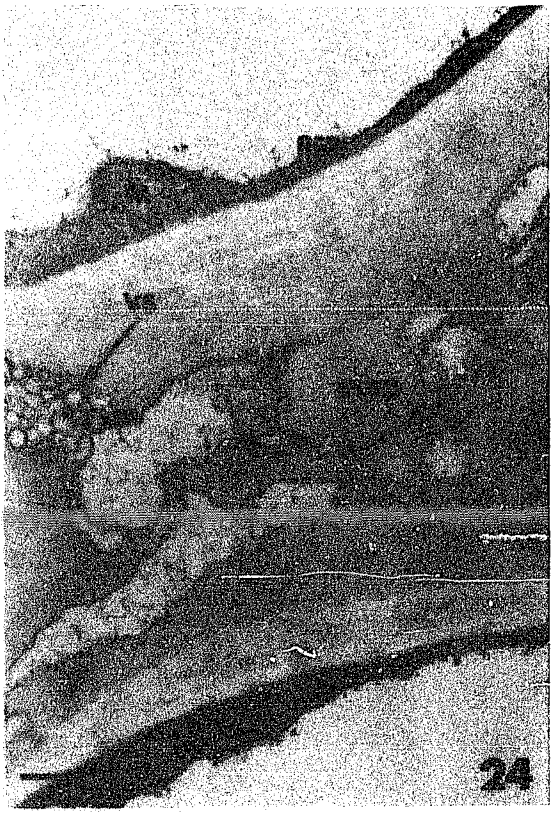
Fig. 23. Abscission where pollination was prevented. Tissue was harvested at 8 days after anthesis, and post-treated with Benedict's solution, without prior incubation in CMC. Some Cu precipitate is observed in association with the fibril components (F) of the cell wall (CW). Cell wall breakdown is occurring within the wall. Mitochondria (M). X10,000. Bar = 1.0 um.

Fig. 24. Ethylene-induced abscission. Tissue was harvested after 4.5 h of treatment, incubated in CMC without post-treatment in Benedict's solution. No Cu precipitate is observed within the cell wall (CW). Extensive cell wall degradation (CWD) is occurring in the wall. Note the vesicular material (VS) within the cell wall. Rough endoplasmic reticulum (RER), mitochondria (M). X20,000. Bar = 0.5 um.

Fig. 25. Ethylene-induced abscission. Tissue was harvested after 2 h of treatment, and was not incubated in CMC nor post-treated with Benedict's solution. No Cu precipitate is observed in the cell wall (CW), middle lamella region. No cell wall breakdown is occurring at this stage. Chloroplasts (CP), fibrils (F). X9,000. Bar = 1.0 um.

Fig. 26. Ethylene-induced abscission. Tissue was harvested after 4.5 h of treatment, and was not incubated in CMC nor post-treated with Benedict's solution. No Cu precipitate is present in the cell wall (CW), even though cell wall degradation (CWD) is extensive. Rough endoplasmic reticulum (RER), mitochondria (M). X10,000. Bar = 1.0 um.

23



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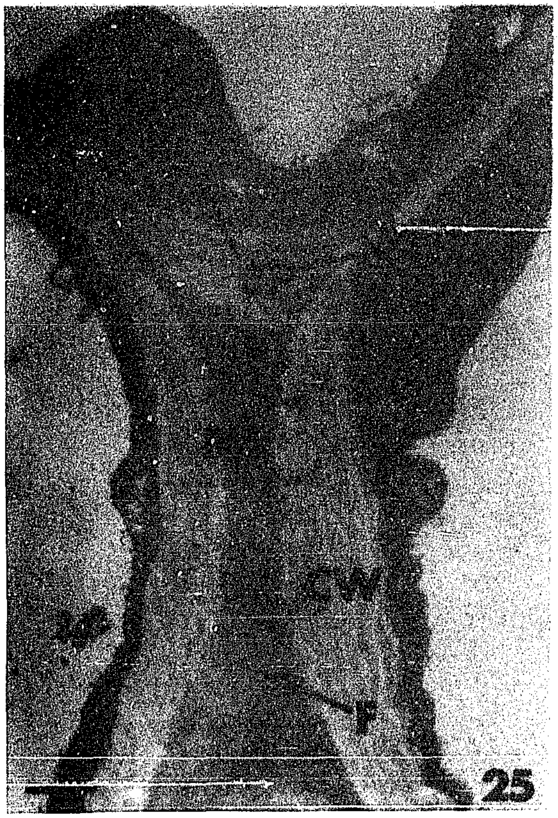

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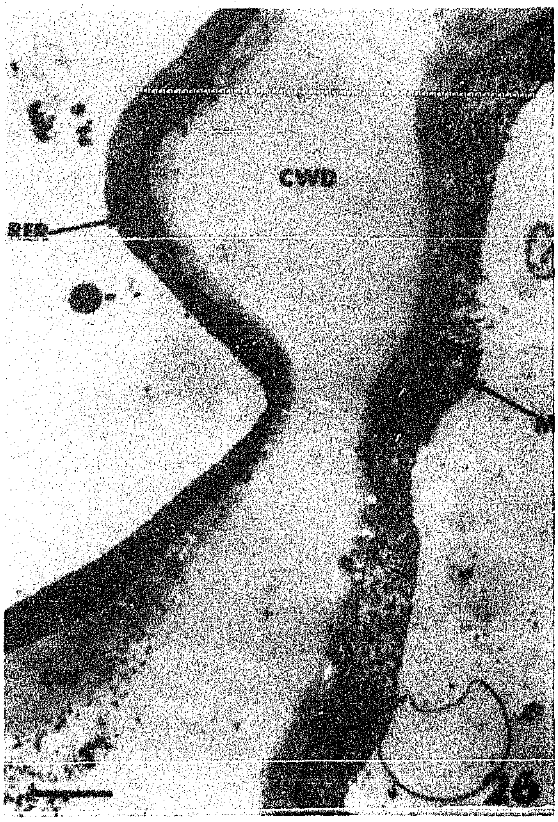

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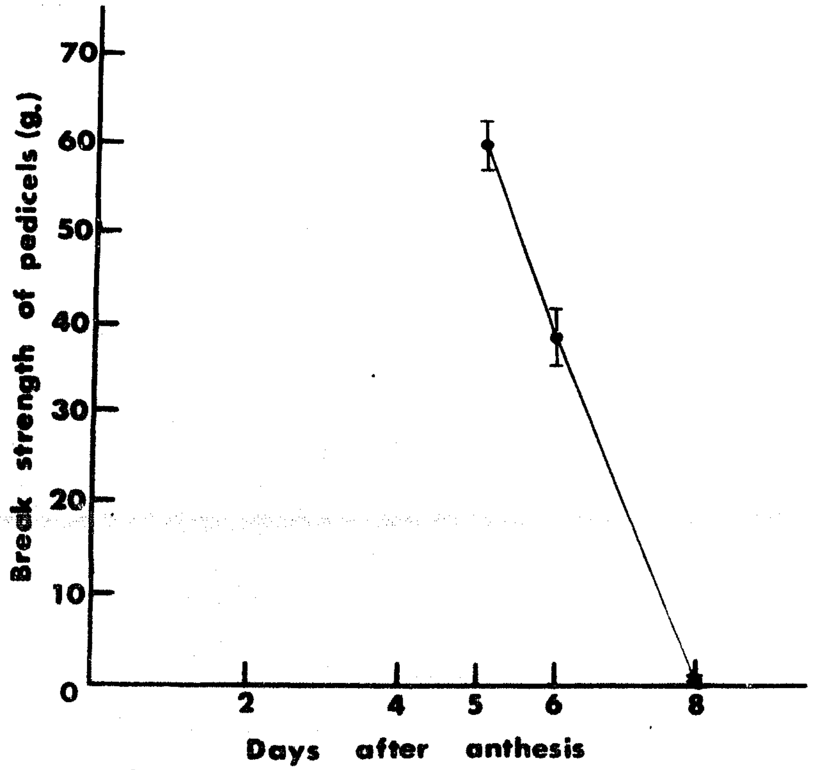
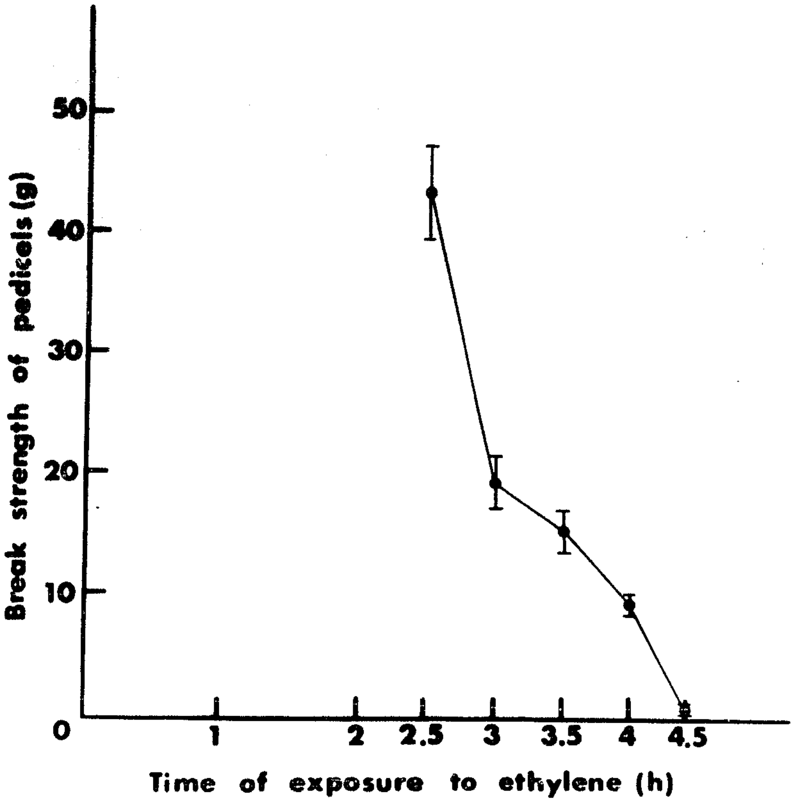
Fig. 27. Time-course study of break-strength at the abscission zone of tobacco flower pedicels in tissue where pollination was prevented. When the pedicels were subjected to pressure, they bent rather than broke during the 4 days after anthesis. Beginning at day 5 after anthesis, the pedicels initially broke and the tissue break-strengths (g) continued to decrease through 8 days after anthesis. At this stage of the time-course the pedicels were abscising.

Fig. 28. Time-course study of break-strength at the abscission zone of tobacco flower pedicels in ethylene-treated tissue. The pedicels bent rather than broke in response to pressure, through 2 h of ethylene treatment. After 2.5 h of ethylene treatment the pedicels initially broke and the tissue break-strengths (g) continued to decrease through 4.5 h of treatment. By this stage of the time-course the pedicels were abscising.


Break strength of pedicels (g.)
70
60
50
40
30
20
10
0
2
4
5
6
8
Days after anthesis

27


Break strength of pedicels (g)
50
40
30
20
10
0
1
2
2.5
3
3.5
4
4.5
Time of exposure to ethylene (h)

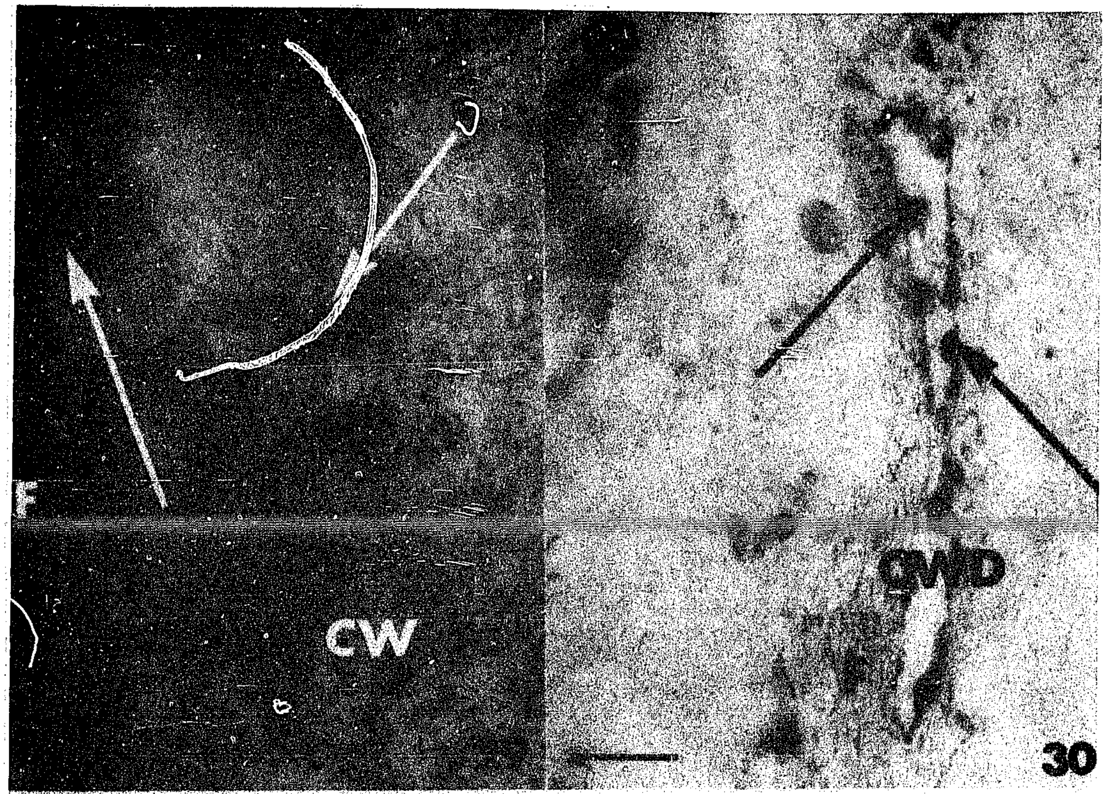
28

Fig. 29. Abscission where pollination was prevented. Tissue was harvested at 8 days after anthesis, incubated in CMC and post-treated with Benedict's solution. Note the dense bodies (arrows) found in association with the fibrils (F) of the cell wall (CW). X51,000. Bar = 0.25 um.

Fig. 30. Ethylene-induced abscission. Tissue was harvested after 4.5 h of treatment, incubated in CMC and post-treated with Benedict's solution. Dense bodies (arrows) are observed in areas of cell wall degradation (CWD) in association with the fibrils (F) of the wall. X50,000. Bar = 0.25 um.

Fig. 31. Abscission where pollination was prevented. Tissue was harvested at 8 days after anthesis, incubated in CMC and post-treated with Benedict's solution. Note the lipid bodies (arrows) found just inside the cell. X46,000. Bar = 0.25 um.

Fig. 32. Ethylene-induced abscission. Tissue was harvested after 4 h of treatment, incubated in CMC and post-treated with Benedict's solution. Lipid bodies (arrows) can be observed within the cell, near the cell wall. X28,000. Bar = 0.5 um.



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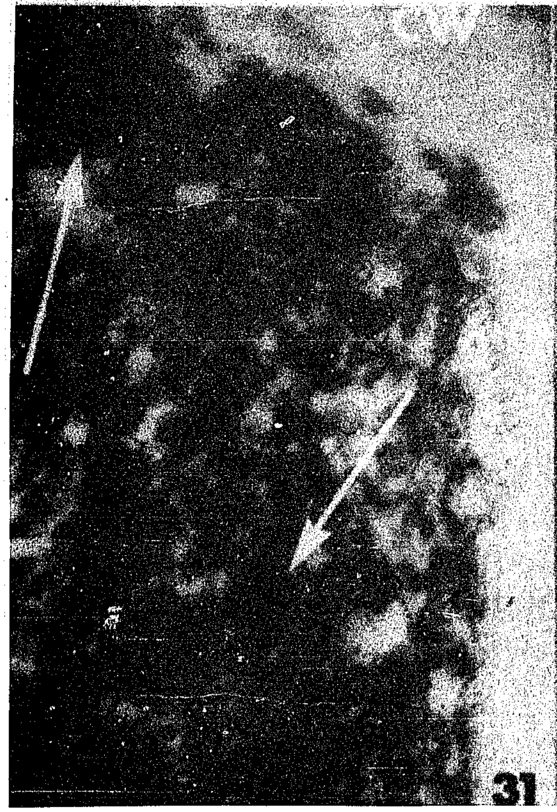

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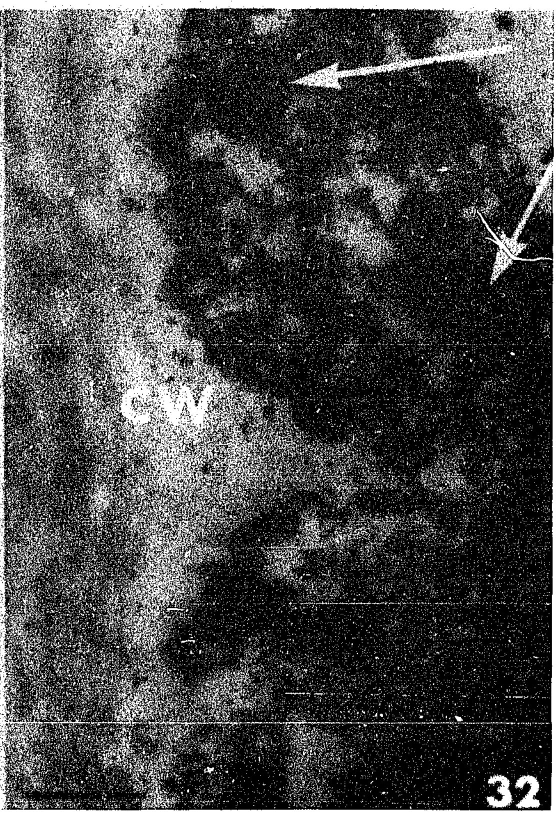

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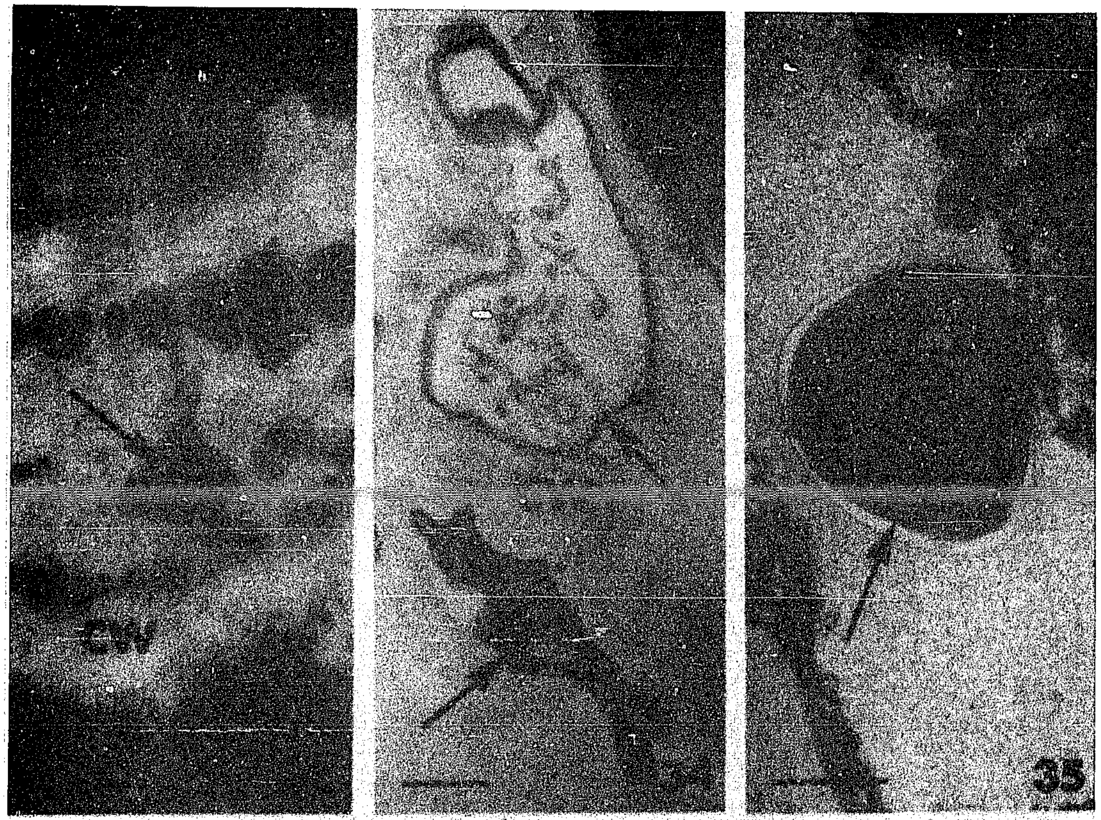
Fig. 33. Abscission where pollination was prevented. Tissue was harvested at 4 days after anthesis, incubated in CMC and post-treated with Benedict's solution. Note the dense granular deposits found within a lamellar-type of membrane configuration (arrow) in the cell wall region (CW). X55,000. Bar = 0.25 um.

Fig. 34. Ethylene-induced abscission. Tissue was harvested after 4.5 h of treatment, incubated in CMC and post-treated with Benedict's solution. Note the tubular (upper arrow) and lamellar-type inclusion (lower arrow) found in the cell wall region. X21,000. Bar = 0.5 um.

Fig. 35. Abscission where pollination was prevented. Tissue was harvested at 8 days after anthesis, incubated in CMC and post-treated with Benedict's solution. Note the lamellar type of inclusion found within the vacuole of the cell (arrow). X14,000. Bar = 1.0 um.

Fig. 36. Abscission where pollination was prevented. Tissue was harvested at 6 days after anthesis, incubated in CMC and post-treated with Benedict's solution. A large number of membrane configurations (MC) are found in the cell wall, where the indentation (I) is located. X19,000. Bar = 0.5 um.


CW
35


36

Table I. Morphometric analysis. Surface area of abscission zone cell membranes in control and ethylene-treated tissue.

Table I. Surface Area of Abscission Zone Cell Membranes

	Control	Ethylene-treated
	μm^2 [a]	
Rough ER	60.47 ± 9.20	126.41 ± 13.10
Smooth ER	0.88 ± 0.72	1.06 ± 0.78
Golgi cisternae	7.07 ± 3.07	7.07 ± 2.80
Golgi vesicles	12.02 ± 4.00	7.78 ± 3.13

[a] Means ± SE

Table II. Morphometric analysis. Relative cell volume of abscission zone cell components in control and ethylene-treated tissue.

Table II. Relative Cell Volume of Abscission Zone Cell Components

	Control	Ethylene-treated
	% cell volume[a]	
Nuclei	3.16 ± 1.23	7.84 ± 1.99
Nucleoli	0.12 ± 0.07	0.32 ± 0.11
Chloroplasts	3.88 ± 0.58	4.39 ± 0.73
Mitochondria	2.09 ± 0.23	2.84 ± 0.31
Vacuoles	75.80 ± 2.24	55.51 ± 3.18
Cell Wall	23.89 ± 1.54	22.17 ± 1.58
Microbodies	0.12 ± 0.07	0.32 ± 0.11
Cytoplasm (excluding vacuoles)	21.04 ± 2.26	36.65 ± 2.88

[a] Means ± SE

Table III. Morphometric analysis. Average number of organelles in control and ethylene-treated cells.

Table III. Average Number of Organelles in Control and Ethylene-treated Cells

	Control	Ethylene-treated
Chloroplasts	2.47 ± 0.32[a]	2.10 ± 0.27
Mitochondria	7.33 ± 0.64	6.90 ± 0.54
Vacuoles	5.46 ± 0.68	4.93 ± 0.51
Microbodies	0.17 ± 0.08	0.43 ± 0.12
Golgi cisternae	0.33 ± 0.10	0.53 ± 0.14
Golgi vesicles	3.50 ± 0.97	3.10 ± 0.89

[a] Means ± SE

Fig. 37. A time-course study of tissue where pollination was prevented (capped pedicels) and control flower-pedicel tissue (non-capped pedicels). A comparison of the tissue break-strength and buffer-soluble cellulase activity (indicated by the % Δ in viscosity) is shown over a 6 day time-course period. The control pedicels bent rather than broke throughout the time-course study. The capped pedicel tissue initially broke at 4 days after anthesis, when a small increase in enzyme activity (over the non-capped control), was observed. Most of the increase in buffer-soluble cellulase activity was found in capped tissue harvested at 5 and 6 days after anthesis. By the time these stages of the time-course had been reached, the tissue break-strengths had already decreased considerably.

The standard errors for the break-strength curve in this figure, were found to be the same in all subsequent graphs (Figs. 38, 39, and 42). Between 5 and 10 time-course experiments were run for each cellulase fraction assayed. A total of 25 abscission zone segments were used for extraction of each enzyme fraction.

FIGURE 37 BUFFER-SOLUBLE CELLULASE

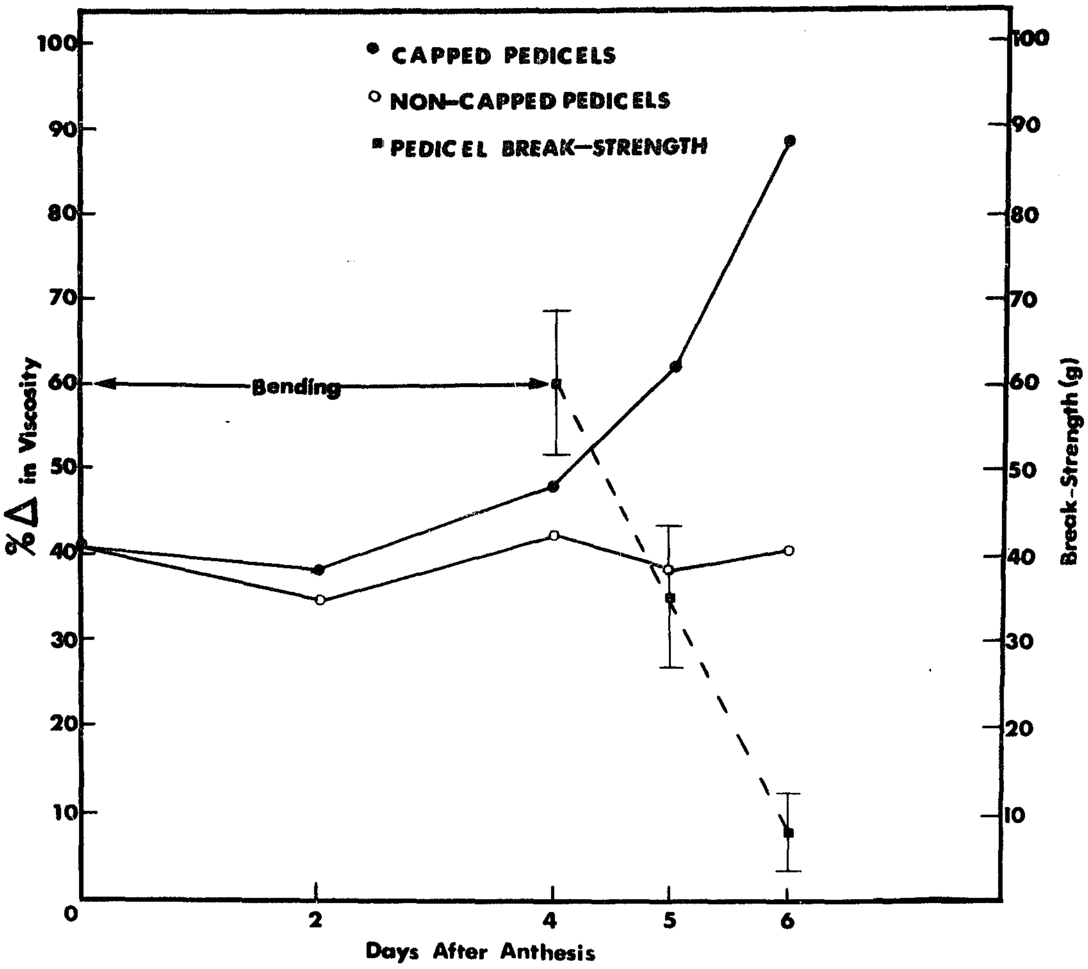


Fig. 38. Time-course study of tissue where pollination was prevented (capped pedicels) and control flower pedicel tissue (non-capped pedicels). A comparison of the tissue break-strength and salt-soluble cellulase activity (indicated by the % Δ in viscosity) is shown over a 6 day time-course period. The control pedicels bent rather than broke throughout the time course study. The capped pedicel tissue initially broke at 4 days after anthesis and the break-strengths decreased through 6 days after anthesis, when the pedicels were abscising. When compared with the non-capped pedicel tissue, no increase in salt-soluble cellulase activity was observed in the capped tissue throughout the time-course study.

FIGURE 38 SALT–SOLUBLE CELLULASE

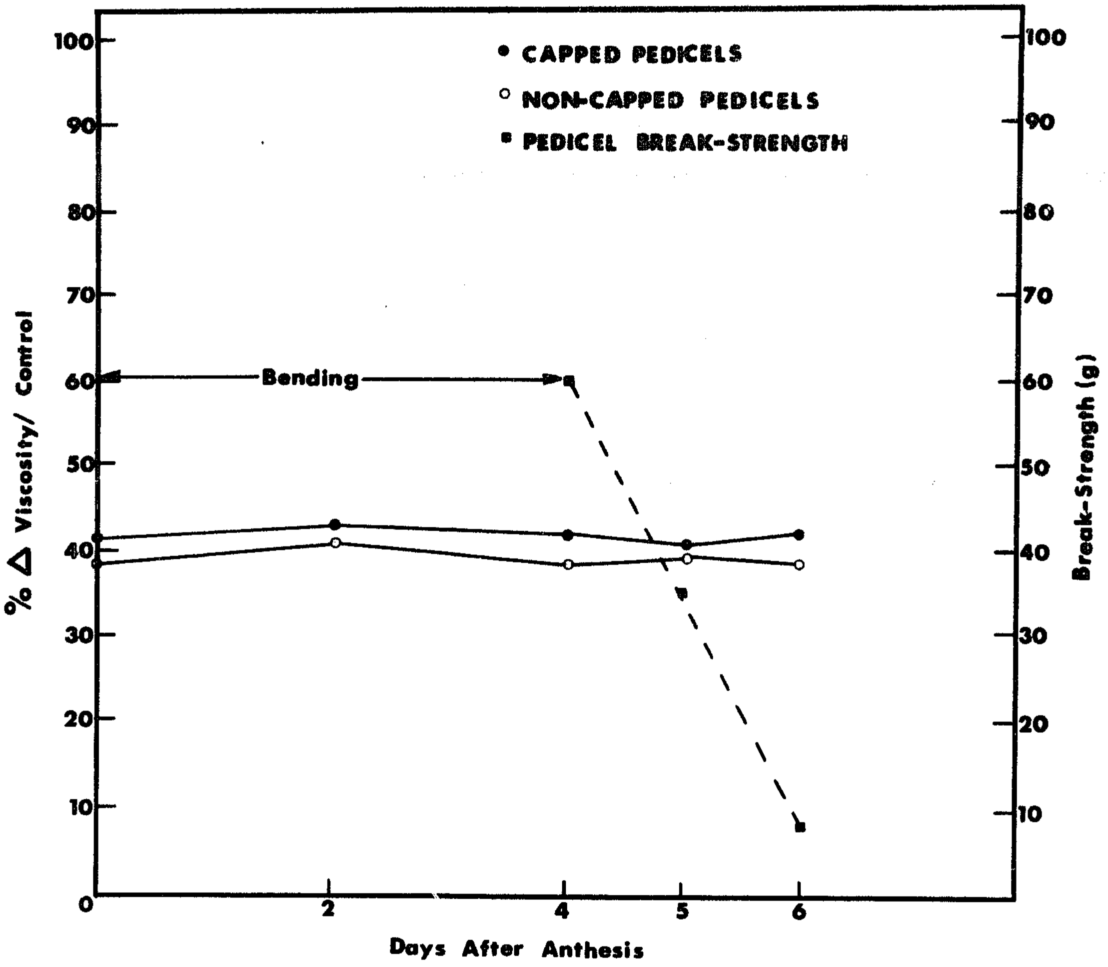


Fig. 39. Time-course study of tissue where pollination was prevented (capped pedicels) and control flower pedicel tissue (non-capped pedicels). A comparison of the tissue break-strength and exocellulase activity (indicated by the % Δ in viscosity) is shown over a 6 day time-course period. The control pedicels bent rather than broke throughout the time-course study. The capped pedicel tissue initially broke at 4 days after anthesis, when an increase in exocellulase activity (over the non-capped control) was first observed. Most of the increase in exocellulase activity was found in capped tissue which was harvested at 5 and 6 days after anthesis. By the time these stages of the time-course had been reached, the tissue break-strengths had already decreased considerably.

FIGURE 39 EXOCELLULASE

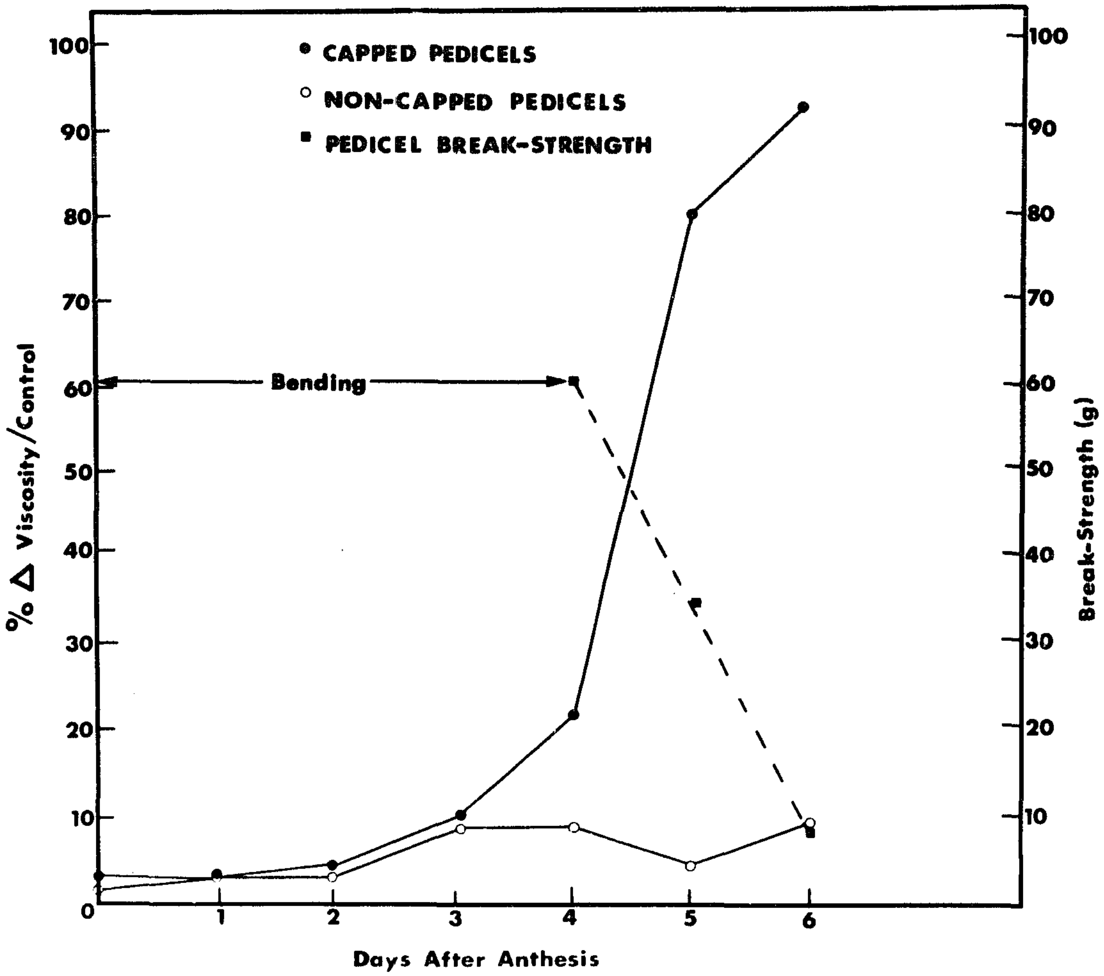


Fig. 40. Time-course study of tissue where pollination was prevented (capped pedicels) and control flower pedicel tissue (non-capped pedicels). A comparison in terms of the levels of endocellulase activity (indicated by the % Δ in viscosity) in both capped and non-capped pedicel tissue is shown over a 6 day time-course period. An increase in endocellulase activity was first observed in the capped pedicel tissue harvested at 4 days after anthesis. The enzyme activity remained at this level in the capped tissue harvested at 5 and 6 days after anthesis. There was virtually no change in the endocellulase activity of the non-capped tissue, throughout the time-course study.

FIGURE 40 **ENDOCELLULASE**

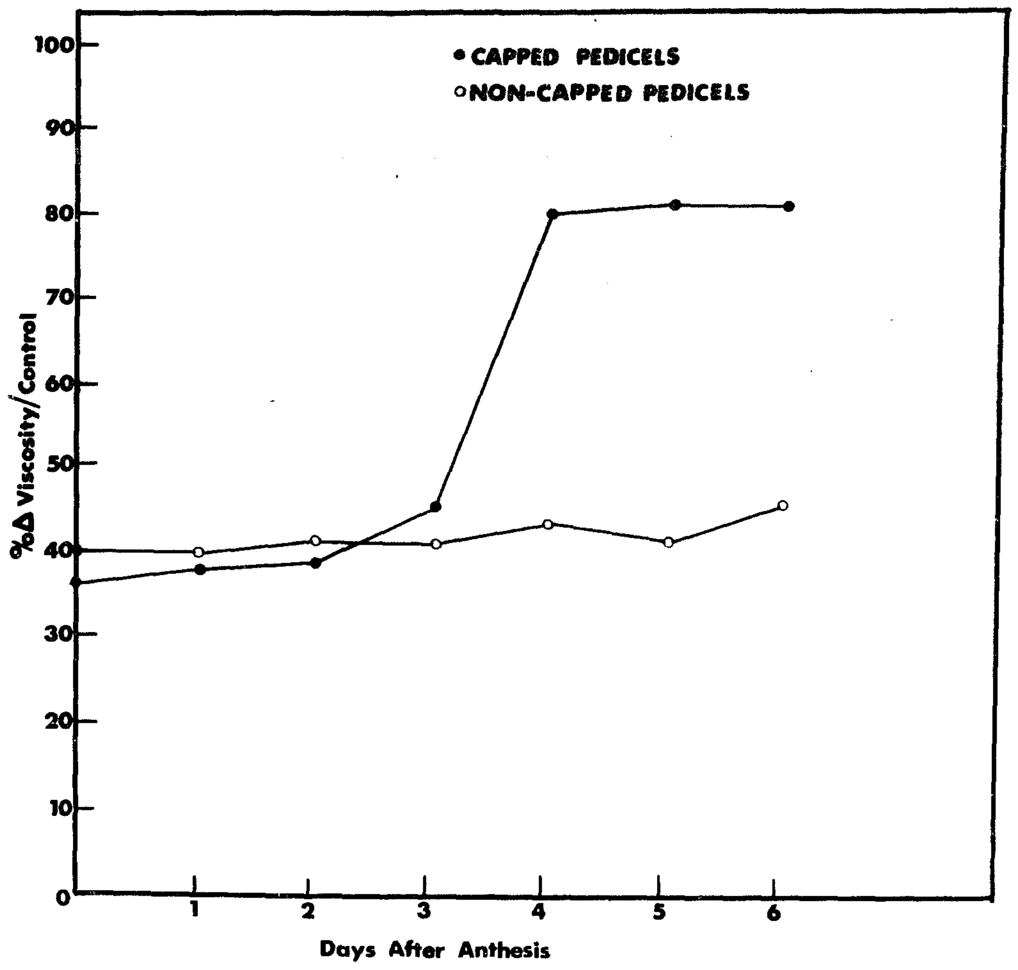


Fig. 41. Electrophoretic gel zymogram of exocellular proteins from abscission zone tissue, as stained by Coomassie blue. A comparison of both capped and non-capped pedicel tissue is shown with respect to the banding patterns that were found throughout the time-course studies.

FIGURE 41
ELECTROPHORESIS : EXOCELLULAR PROTEINS

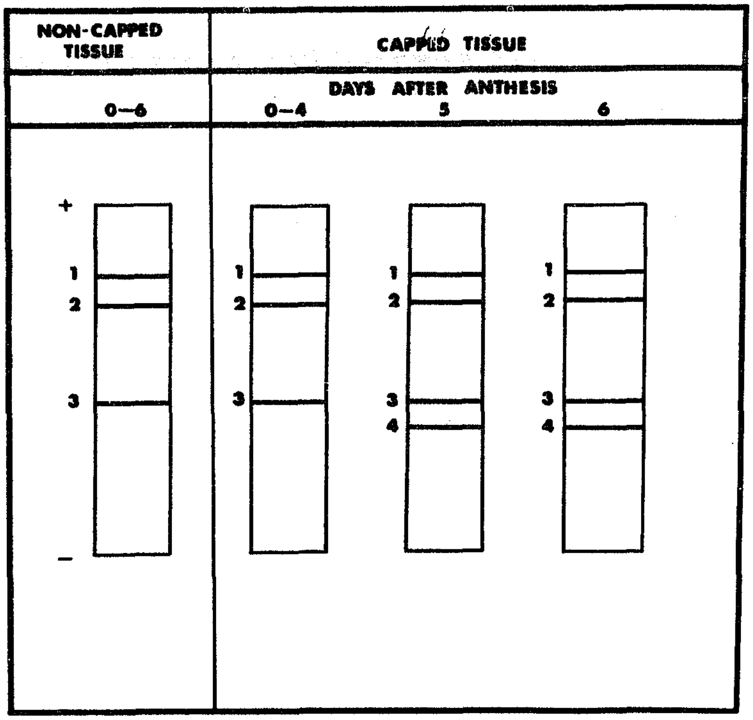


Table IV. Viscometric determination of cellulase activity in exocellular electrophoretic gels.

Table IV. Viscometric Determination of Cellulase Activity in Exocellular Electrophoretic Gels

Exocellular Protein Bands	% Δ in Viscosity/Control Tissue Harvested 6 days after Anthesis Capped Tissue	 Non-Capped Tissue
Band 1	0%	1%
Band 2	0%	2%
Band 3	1%	0%
Band 4	47.2%	-

Fig. 42. Time-course study of tissue where pollination was prevented (capped pedicels) and control flower pedicel tissue (non-capped pedicels). A comparison of the tissue break-strength and band 4-exocellulase activity (indicated by the % Δ in viscosity) is shown over a 6 day time-course period. The control pedicels bent rather than broke throughout the time-course study. The capped pedicel tissue initially broke at 4 days after anthesis. An increase in band 4-exocellulase activity was first observed in the capped pedicel tissue harvested at 5 days after anthesis. This increase in enzyme activity was found to continue in the tissue harvested at 6 days after anthesis. The pedicel break-strengths had already decreased considerably by the time the increase in band 4-exocellulase activity was observed. Virtually no band 4-exocellulase activity was found in the non-capped tissue, throughout the time-course study.

FIGURE 42 BAND 4 EXOCELLULASE

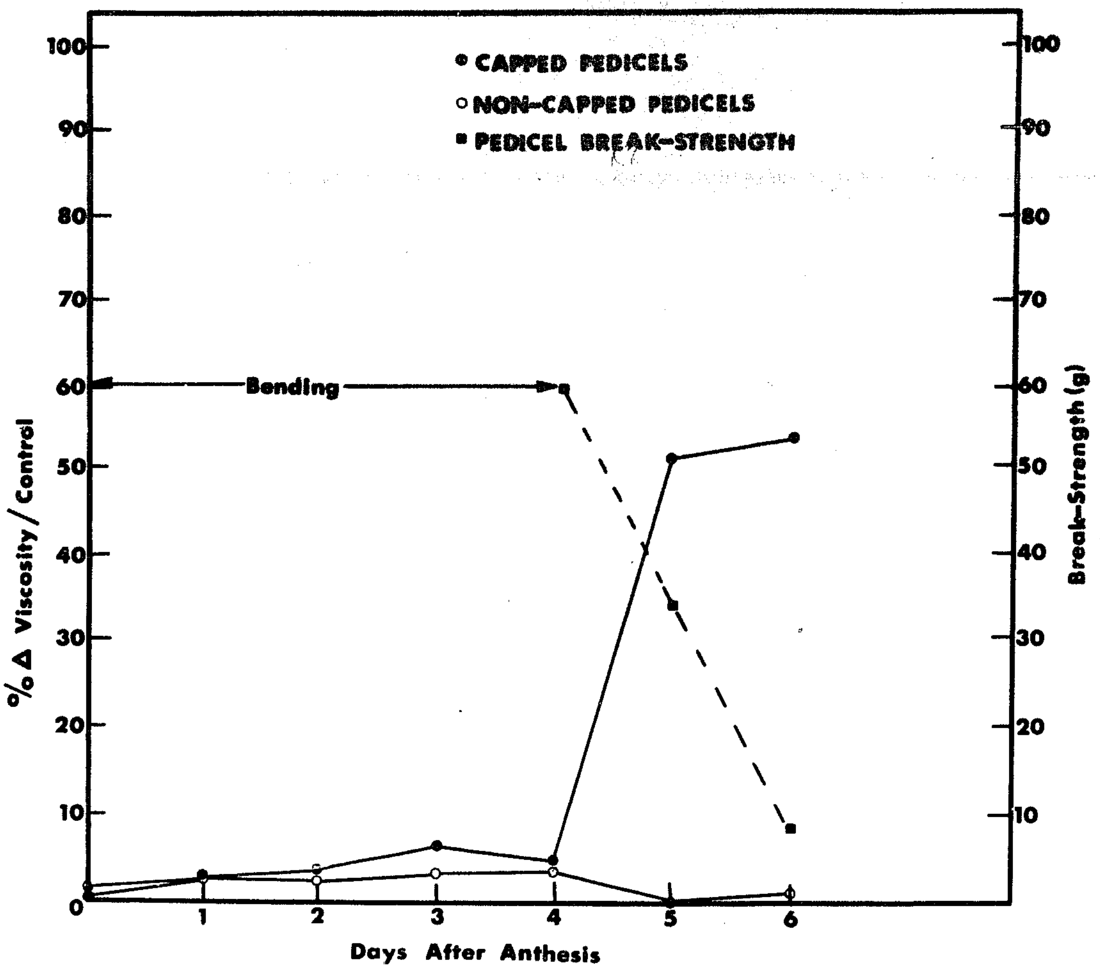


Fig. 43. Optimal extraction and assay pH for exocellulase and endocellulase activity in capped pedicel tissue harvested at 6 days after anthesis.

FIGURE 43 pH OPTIMUM

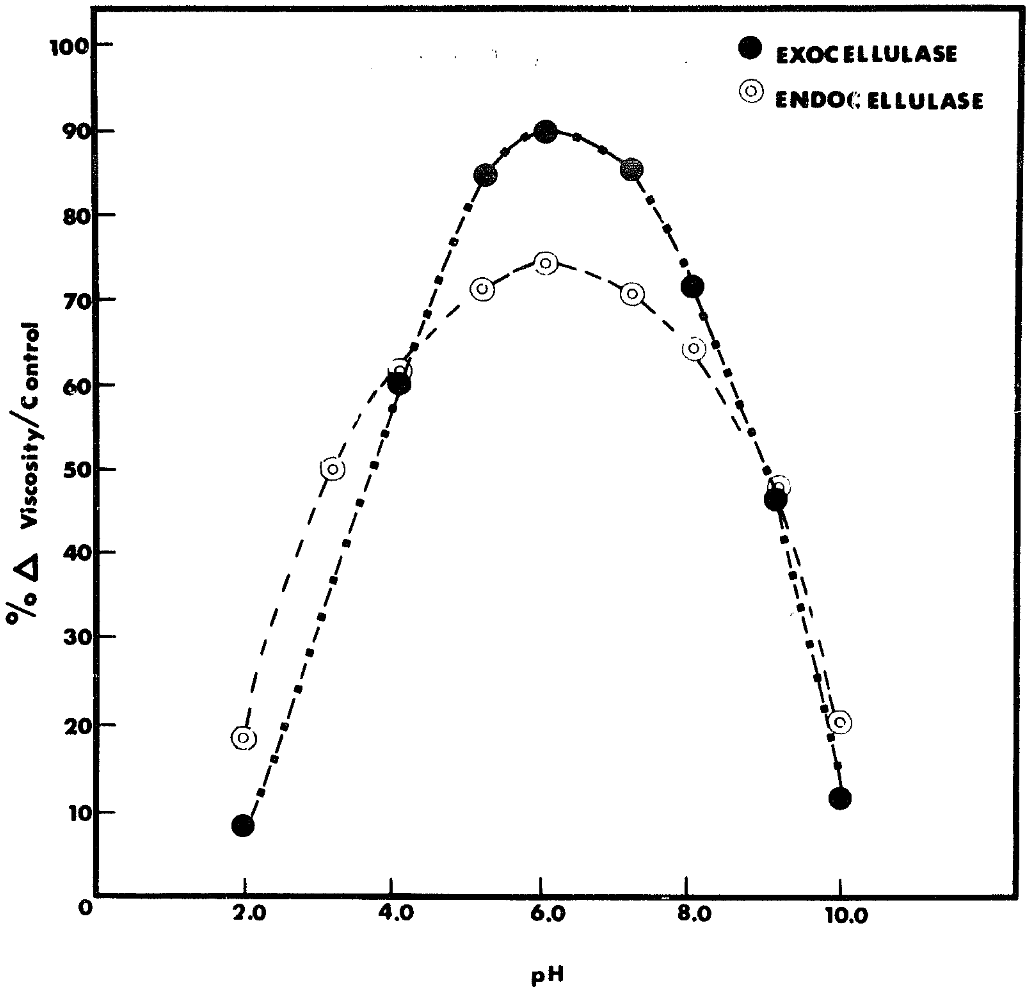


Fig. 44. Optimal assay temperature for exocellulase and endocellulase activity in capped pedicel tissue harvested at 6 days after anthesis.

FIGURE 44 TEMPERATURE OPTIMUM

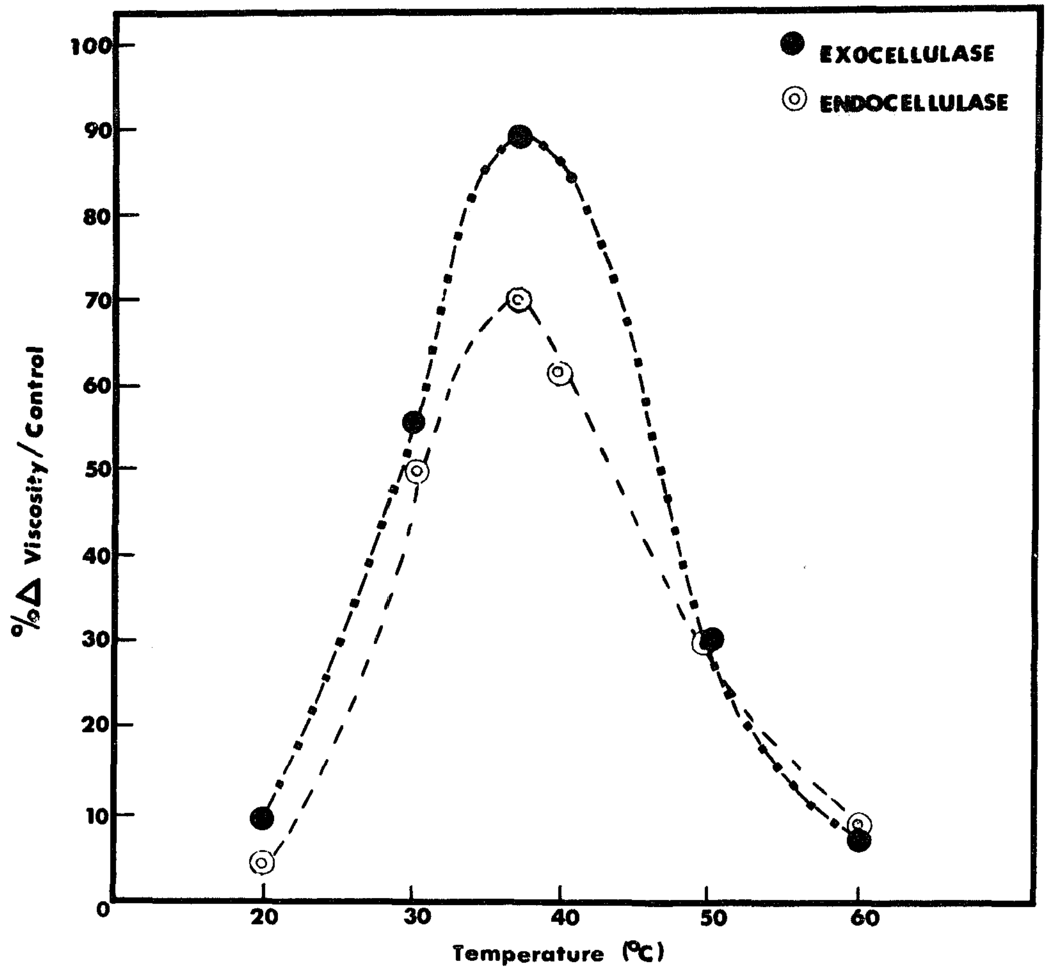


Fig. 45. Electrophoretic gel zymogram of peroxidase from the exocellular fraction of abscission zone tissue. A comparison of both capped and non-capped pedicel tissue is shown with respect to the banding patterns that were found throughout the time-course studies.

Fig. 46. Electrophoretic gel zymogram of peroxidase from the endocellular fraction of abscission zone tissue. A comparison of both capped and non-capped pedicel tissue is shown with respect to the banding patterns that were found throughout the time-course studies.

FIGURE 45

ELECTROPHORESIS: EXOCELLULAR PEROXIDASES

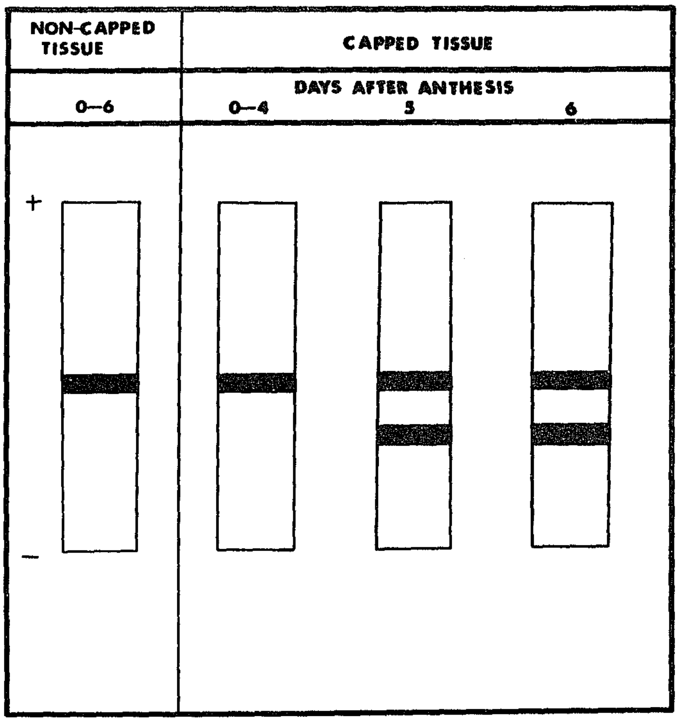


FIGURE 46

ELECTROPHORESIS: ENDOCELLULAR PEROXIDASES