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Cellulase Activity and Localization During Induced Abscission of *Coleus blumei*

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Abstract. Treatment with dimethipin (2,3-dihydro-5,6-dimethyl-1,4-dithiin 1,1,4,4 tetroxide) inhibited the increase in cellulase activity and decrease in breakstrength associated with the normal course of abscission in *Coleus*. Application of the surfactant UBI-1126 (Emery OAL 20 in isopropyl alcohol) increased cellulase activity and accelerated the process of abscission in *Coleus* explants within 24 h of application. Cellulase activity was localized histochemically at the electron microscopic level in surfactant-treated tissue. The enzyme activity was localized primarily in the cell wall, middle lamella, and paramural bodies of abscission zone cells.

An increase in the activity of cellulase and other hydrolytic enzymes has been associated with foliar and floral abscission in several plants (Addicott 1970, 1982, del Campillo et al. 1988, 1990, Horton and Osborne 1967). Various chemicals which have been demonstrated to promote or delay the abscission process have also been shown to have a concomitant effect on cellulase activity (Addicott 1982, Fischer and Bennett 1991).

Dimethipin (2,3-dihydro-5,6-dimethyl-1,4-dithiin 1,1,4,4 tetroxide, also called UBI-N252 or Harvade) and the surfactant UBI-1126 (Emery OAL 20 in isopropyl alcohol) have been reported to have either stimulatory or inhibitory effects on abscission in explant test systems (Baird et al. 1978, Reid 1976, 1985). This study examines the effect of dimethipin and UBI-1126 on petiole breakstrength and cellulase activity in the *Coleus* petiole abscission system. It also examines the ultrastructural localization of cellulase in *Coleus* abscission zones.

Materials and Methods

Plant Material

Plant material was obtained from a clonal stock of *Coleus blumei* maintained in the greenhouse with a minimum night temperature of 20°C. To prepare explants used in the treatments, comparable stems with five nodes were cut from the plants and delaminated. The apical meristem was also removed.

Defoliant

A stock solution of dimethipin (Uniroyal Chemical Co., Bethany, Connecticut, USA) was prepared by dissolving 100 mg of chemical in 100 ml warm distilled water. After appropriate dilution, the spray adjuvant UBI-1126 was added at 1% (vol/vol). Solutions containing only the surfactant were prepared by mixing 1% UBI-1126 in distilled water. Explants were sprayed to run-off with a glass atomizer nozzle and placed in beakers of distilled water. Control explants were sprayed with distilled water. Explants were placed in an environmental growth chamber at 25°C with 500 $\mu M\ m^{-2}\ s^{-1}$ light supplied by fluorescent and incandescent bulbs for a 16-h photoperiod. Tissue was harvested at 24, 48, and 72 h after treatment.

Cellulase Extraction

Cellulase (β-1,4-glucan-glucanohydrolase, E.C. 3.2.1.4) was extracted by harvesting nodes 3 and 4 and the corresponding petioles for five replicate samples. Tissue was ground in 20 ml of 0.02 mol/L sodium phosphate buffer fortified with 0.5 mol/L NaCl (pH 6.1), with a mortar and pestle and a small amount of purified sea sand. The extract was centrifuged at 20,000 *g* for 15 min at 4°C. The supernatant volume was measured and the extract stored on ice prior to assay.

Cellulase Assay

Cellulase activity was measured viscometrically (Durbin and Lewis 1988) using a 1.2% wt/vol carboxymethyl cellulose (CMC) substrate (CMC type 7HF, Hercules Powder Co., Wilmington, DE, USA) in 0.02 M sodium phosphate buffer (pH 6.1) with 0.5%

toluene added as a preservative. The assay mixture contained 0.4 ml substrate and 0.2 ml enzyme extract and was maintained at 30°C in a water bath during the reaction procedure. Viscosity was measured after a 15-min reaction time. Viscosity data were converted to units of activity (ΔB/ml/h) as described by Durbin and Lewis (1988). Cellulase activity for all treatments was then expressed as unit/g fresh weight of tissue.

Breakstrength Determination

Breakstrength was measured as the force necessary to cause the petiole to separate from the stem across the abscission zone. This measurement was used as an indicator of the progressive weakening of the tissue in this area. Petioles from nodes 3 and 4 were clamped to a strain gauge (Fisher Scientific, Pittsburgh, Pennsylvania, USA) and force was applied mechanically to the stem. The force at which separation occurred was recorded as the breakstrength. Petioles from five replicate samples were used per experiment. Breakstrength values obtained were used to compute an average breakstrength per node. Each experiment was repeated four times.

Electron Microscopic Localization of Cellulase Activity

Cellulase activity in the abscission zone was localized by modification of the method described by Bal (1974) as outlined below. At 0 and 48 h after excision, abscission zones from nodes 3 and 4 of *Coleus* explants were identified through the use of freehand sections. The tissue was immersed in fixative (4°C) consisting of 3% glutaraldehyde in 0.05 mol/L phosphate buffer; a razor blade was used to produce specimens of the abscission zone which were 1–2 mm in size. The tissue was transferred to fresh fixative for 30 min. After washing for 15–20 min in cold buffer, the segments were incubated in 0.02% CMC at 30°C for 10 min. After incubation, the segments were transferred to hot (70°C) Benedict's solution for 5 min, washed briefly in distilled water, post-fixed in 1% OsO_4 for 1 h, and then dehydrated and embedded in Spurr's resin as described previously (Baird et al. 1978). Sections were cut on a Sorvall MT-2 ultramicrotome with a diamond knife, mounted on uncoated copper grids, and either stained with uranyl acetate or left unstained. Material was examined with a JEOL 100CS electron microscope at 80 kV. Reaction controls were prepared by (1) omitting treatment with the substrate and (2) by boiling the sections in buffer for 10 min prior to fixation.

Results

No significant increase in cellulase activity was measured for the first 24 h after excision in control (treated with distilled water) *Coleus* explants. Activity increased rapidly, however, during the next 48 h. Average breakstrength decreased from 225 g/node to approximately 15 g/node during the 24–72 h time period; separation of all petioles from the stem was virtually complete by 72 h after excision (Fig. 1).

Explants treated with only a 1% solution of the surfactant UBI-1126 (Fig. 2) yielded higher levels of cellulase activity than measured in control (Fig. 1)

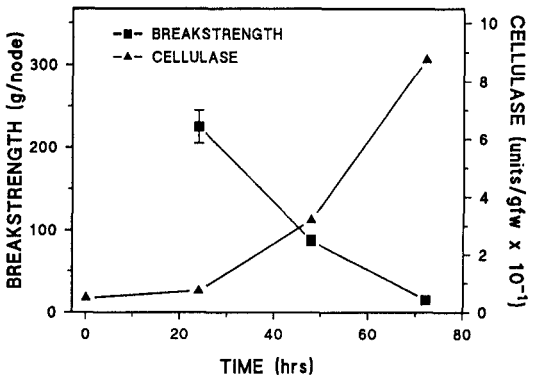


Fig. 1. Time course measurement of average breakstrength (■) and cellulase activity (▲) in control (treated with distilled water) *Coleus* explants. Vertical lines represent 95% confidence intervals.

or defoliant-treated explants (Fig. 3) at all time intervals. The average breakstrength per node declined about 50% more rapidly in surfactant-treated plants than in untreated controls or defoliant-treated plants. Separation at all nodes was nearly complete in 48 h as opposed to the 72 h measured in the controls.

Treatment of *Coleus* explants with dimethipin (Fig. 3) delayed the decrease in petiole breakstrength and inhibited the increase in cellulase activity, which normally accompany abscission. Twenty-four hours after treatment with dimethipin the breakstrength values for treated plants were approximately 40% higher than that of control plants. Cellulase levels were comparable. By 48 h after treatment, average breakstrength in treated plants was 40% greater than in controls and cellulase levels were 30% less. By 72 h after excision, cellulase levels in dimethipin-treated explants were ~10% those measured in control explants.

Dose–response data (Fig. 4) indicate that increasing the dosage of defoliant over a range of 0–250 ppm prevented the normal decrease in petiole breakstrength 48 h after treatment with the saturating dose approximately 125 ppm (6×10^{-4} mol/L). The effects of dimethipin on both breakstrength and cellulase activity could be detected at concentrations as low as 75 ppm.

The stimulation of cellulase activity by surfactant treatment was utilized to investigate the localization of cellulase activity at the ultrastructural level.

Localization of Cellulase Activity

Examination of light microscope sections from explants sprayed only with distilled water (controls) indicated that at the time of excision the *Coleus*

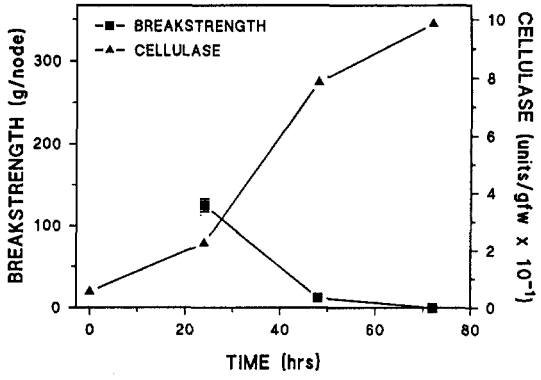


Fig. 2. Time course of average breakstrength (■) and cellulase activity (▲) from *Coleus* explants treated with 1% UBI-1126 surfactant.

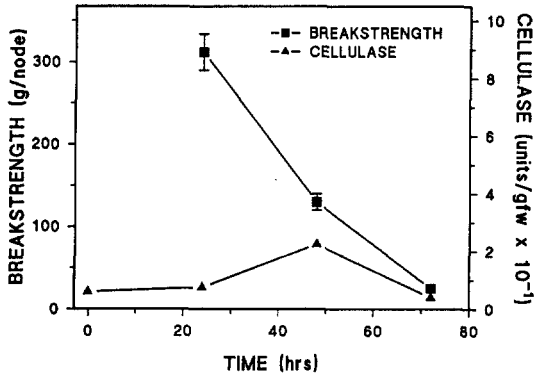


Fig. 3. Time course of average breakstrength (■) and cellulase activity (▲) from *Coleus* explants treated with a saturating dose of dimethipin (125 ppm). Vertical lines represent 95% confidence intervals.

abscission zone consisted of four to five layers of elongate, highly vacuolate cells located at the base of the petiole. Examination with the transmission electron microscope (TEM) concentrated on changes in the cell wall and middle lamellar region of these cells and indicated that in these explants all cell walls and middle lamellae were intact and stained uniformly. Time 0 reaction controls, not incubated with CMC prior to treatment with Benedict's solution, showed only sparse deposition of copper precipitate in the cell wall (Fig. 5). When similar explants were incubated with CMC, treated with Benedict's solution and examined with the TEM, little precipitate was visible in the cell wall or middle lamella (Fig. 6). Samples from explants 48 h after excision showed minor cellular disruption and some longitudinal and radial separation in the cortical cells adjacent to the vascular bundles. When

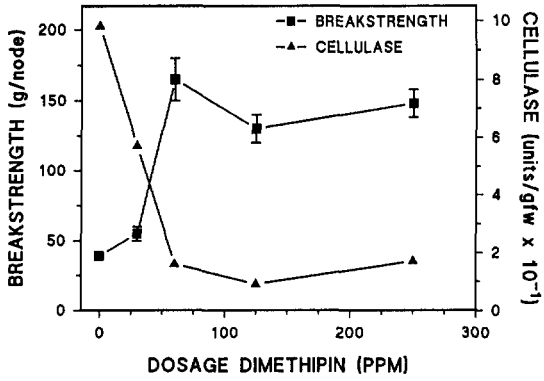
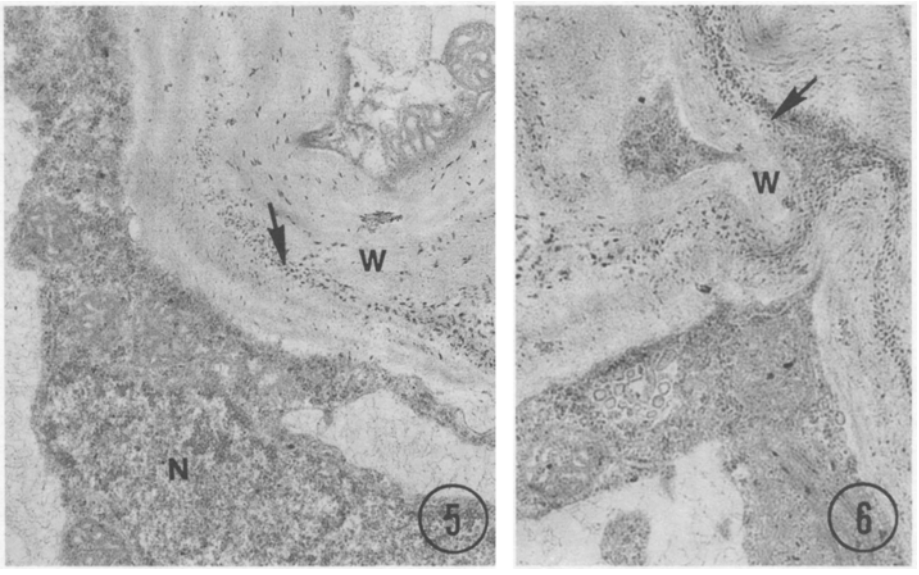


Fig. 4. Dose–response in *Coleus* explants 48 h after treatment with dimethipin.

sections were incubated with CMC and treated with Benedict's solution, copper precipitate was not observed within cortical cells, but was localized in the cell wall and occasionally in small paramural bodies (Fig. 7). In reaction control sections, precipitate was observed in the middle lamellar region of the cell wall and in adjacent areas (Fig. 8). Sections from tissue treated with the surfactant, UBI-1126, 48 h prior to harvest showed extensive cellular disruption including disruption of the plasma membrane and several paramural bodies. In tissue incubated with CMC and Benedict's solution, precipitate deposition was dense in all fields examined and was concentrated in the cell wall, middle lamella, and paramural bodies (Fig. 9). In some cases precipitate deposited in cell wall areas appeared associated with the fibrils of the cell wall. Reaction control sections not treated with cellulose substrate showed some precipitate deposition primarily in the cell wall (Fig. 10).

Discussion

Physiological data indicated that application of dimethipin to *Coleus* explants inhibited the increase in cellulase activity and decrease in breakstrength associated with the normal course of abscission in this system. Reid (1976, 1985) reported a similar effect of dimethipin application in the *Phaseolus vulgaris* explant system noting that dimethipin retarded both normal and ethylene-induced abscission. He further suggested that the chemical could be inhibiting protein synthesis. Metzger and Keng (1984) also observed an inhibition of protein synthesis in both bean and oat leaf discs following dimethipin treatment. Their study concluded that the compound acted primarily at the translational level.

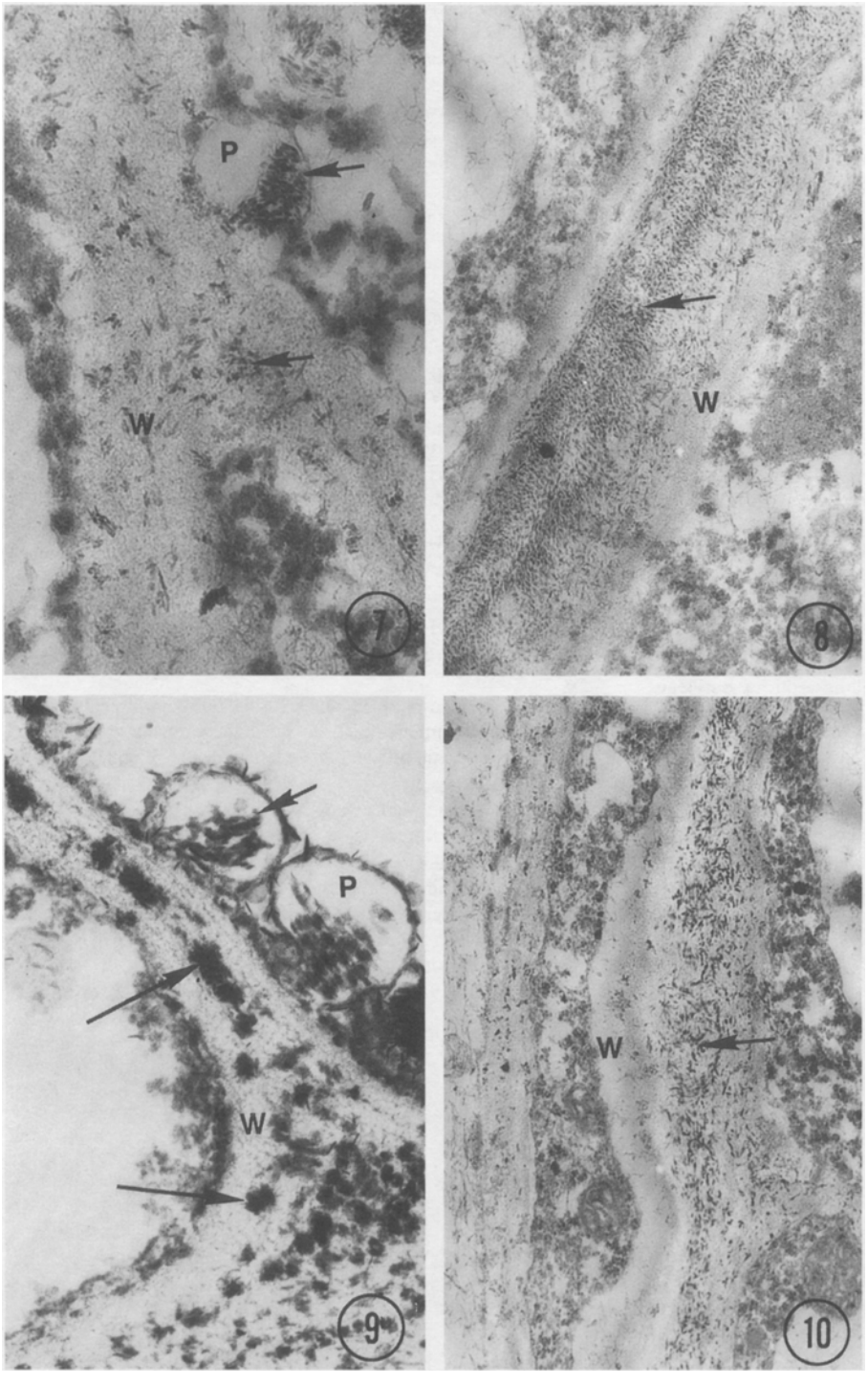


Figs. 5 and 6. Electron micrographs of cell wall regions from *Coleus* abscission zones at time 0. *Fig. 5:* Control (water treated) *Coleus* explant at time 0. Section was not incubated with substrate. Sparse copper precipitate (arrow) is visible in the intact cell wall (W). N, nucleus. ×16,000. *Fig. 6:* Control *Coleus* abscission zone at time 0, incubated with CMC (substrate) and Benedict's solution. Little precipitate (arrow) is visible in the cell wall. ×16,100.

Application of the surfactant UBI-1126 accelerated the process of abscission in *Coleus* explants. Cellulase activity increased rapidly after surfactant application with a concomitant decline in petiole breakstrength. A previous study (Baird et al. 1978) indicated that significant modifications of the plasma membrane, degeneration of the cytoplasm, dissolution of the middle lamella, and cell wall disruption were visible following surfactant treatment.

The present work demonstrated that an increase in extractable cellulase activity and in cytochemical evidence of cellulase localization could be correlated with the acceleration of the abscission process. Activity was concentrated in the abscission zone, with little change in cellulase levels in the stem or distal portion of the petiole (Baird, unpublished data). Recent work in bean has not only demonstrated a similar correlation (Reid et al. 1990, Sexton et al. 1980), but has also shown that cellulase activity can be localized at the light microscopic level using cellulase 9.5 antibodies and fixed tissue sections (Sexton et al. 1980, 1981) or tissue prints on nitrocellulose membranes (del Campillo et al. 1990). In initial experiments, the bean 9.5 cellulase antibody did not react in *Coleus* abscission zones. Attempts to purify *Coleus* cellulase have met with limited success (Reid, unpublished data). We therefore chose to localize cellulase histochemically in *Coleus* abscission zones.

The cellulase localization technique used in this study relied on the formation of electron opaque cuperous oxide crystals when Benedict's solution was heated in the presence of reducing sugars (Nessler and Allen 1988). These sugars should be present in areas of high cellulase activity following treatment with exogenously applied cellulose. Other hydrolases, such as pectinase, may have also been present, especially during induced abscission, which could have formed breakdown products that would react with the Benedict's solution to yield a copper precipitate. However, the relatively low levels of copper precipitate observed in control sections at all time intervals suggested that such "background activity" was minimal. The quantity of copper precipitate observed following CMC incubation correlated well with physiological measurements of cellulase activity, breakstrength decline, and cellular disruption in *Coleus* petioles. In control tissue, little precipitate was detected in freshly harvested sections. Forty-eight hours after excision, more precipitate was localized in the cell wall and in paramural bodies of cortical cells of the abscission zone. Only scant precipitate was observed in tissues of the stem or distal portions of the petiole. This correlated well with the physiological measurements of cellulase activity detected during this time period. In surfactant-treated tissue, the rapid increase in cellulase activity noted 24–72 h after excision was evident in the amount of precipitate indicating the presence of cellulase in the cell wall, middle lamella, and paramural bodies. In this tissue, precipitate was observed throughout the ab-



Figs. 7–10. Electron micrographs of cell wall regions from *Coleus* abscission zones 48 h after excision. *Fig. 7:* Abscission zone from control (water treated) *Coleus* explant. Incubation with CMC (substrate) and Benedict's solution results in precipitate deposition (arrows) in the cell wall (W) and in some paramural bodies (P). ×17,000. *Fig. 8:* Minus substrate reaction control from abscission zone 48 h after excision. Some precipitate deposition is observed in the middle lamellar region of the cell wall (W). ×16,500. *Fig. 9:* Abscission zone section from *Coleus* explant 48 h after treatment with UBI-1126. Incubation with CMC and Benedict's solution results in heavy precipitate deposition (arrows) in the cell wall (W), middle lamella, and paramural bodies (P). ×17,000. *Fig. 10:* Reaction control (minus substrate) section from tissue 48 h after treatment with UBI-1126. Copper precipitate is evident (arrow), but not as dense as in previous figure. ×16,500.

scission zone and occasionally in cells of the petiole and stem closest to the abscission zone. A similar pattern with respect to cellulase localization in the wall and middle lamella was noted by Lieberman et al. (1981) in the abscission zones of tobacco flower pedicels. We did not, however, observe the two forms of precipitate fibril arrangement noted in tobacco (Lieberman et al. 1981); all precipitate fibrils were of the "regular fibril" type. Of particular interest was the deposition of large amounts of precipitate in the paramural bodies. Paramural bodies have been associated with abscission, senescence, or injury in a number of tissues (Jensen and Valdovinos 1967, Platt-Aloia and Thompson 1976,

Webster 1973) and treatment with UBI-1126 has been reported to increase the number of membrane invaginations and paramural bodies (Baird et al. 1978). The cytochemical localization of cellulase in some of these membrane elaborations supports suggestions made by several authors that paramural bodies constitute storage sites for materials involved in wall metabolism (Evert and Eichorn 1976, Osborne and Sargent 1976) and can be related to the process of aging and degeneration.

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