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Pith Autolysis in Plants: IV. The Activity of Polygalacturonase and Cellulase during Drought Stress Induced Pith Autolysis[1]

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The water potential, amount of pith autolysis and activities of apoplastic cellulase and polygalacturonase of tomato stems were measured during 24 h of drought stress (DS) and for 24 h following reirrigation. During DS the water potential dropped from -5.5 to -10.4 bars and rose to -8.3 bars soon after reirrigation. Drought stress induced considerable pith autolysis, more of which occurred after reirrigation. Pretreatment with mechanical perturbation (MP) of the stems or application of exogenous ethephon on the buds hardened the tomato plants against DS-induced pith autolysis. Drought stress caused an increase in apoplastic polygalacturonase and an even greater increase in apoplastic cellulase. Reirrigation caused a large transient increase in the former and a decrease in the latter. The apoplastic reducing sugar content (as galacturonic acid) of the stem rose in parallel with the activity of the enzymes. Both DS and MP caused an increase in ethylene evolution, although the former was significantly greater than the latter. However, when MP preceded DS, the amount of ethylene produced was significantly less than DS alone induced. Pretreatment with either MP or exogenous ethephon inhibited the increase in apoplastic cellulytic enzymes.

It is concluded that DS induces ethylene evolution from the tomato stem, causing an increase in the stem apoplastic cellulytic enzymes, which in turn start the autolysis of the pith cell walls. Pretreatment with MP or ethephon, each of which induces ethylene evolution, hardens the stem so that it does not produce more ethylene during DS, and thus becomes resistant to DS-induced pith autolysis.

Key words: Cellulase — Ethylene — Mechanical Perturbation — Pithiness — Thigmomorphogenesis — Water stress.

Susceptible cultivars of tomato react by undergoing whitening of the pith (caused by replacement of cellular water by air) followed by autolysis of the pith, when they are subjected to drought stress (DS) (Aloni and Pressman 1981, Pressman et al. 1983). This syndrome is termed pithiness or pith autolysis. The susceptible cultivar Hossen produces increased amounts of ethylene during drought stress as well as following reirrigation. If the plants are induced to produce ethylene prior to DS by mechanical perturbation (MP) or by exogenous ethephon, they become hardened against DS-induced pith autolysis (Pressman et al. 1983).

We can divide the syndrome of pith autolysis into the early "air pocket" phase and the later cellulytic phase, in which the cell walls as well as the protoplasts disappear. This communication addresses the second stage. Since the cell walls are lysed, it seemed reasonable to look directly for the presence of cell wall degrading enzymes (in the apoplastic spaces) during the time course of the development of pith autolysis. We also studied the effects of the pretreatment with MP or ethephon on the activity of such enzymes during DS and following reirrigation.

Materials and Methods

Plant material—Tomato plants (*Lycopersicon esculen-*

Abbreviations: DS, drought stress; MP, mechanical perturbation; PG, polygalacturonase.

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tum, cv. Hossen) were grown in the greenhouse, one to a pot, in coarse vermiculite. Day and night temperatures (±2°C) were 27°C and 22°C, respectively. All pots were subirrigated by flooding the benches containing the pots 3 out of 4 days with running water and every fourth day with a dissolved fertilizer (5% solution of 20 : 20 : 20, N : P : K).

When the plants had 6–7 leaves (ca. 3.5 months) 480 plants were divided into 4 groups of 120 plants each. Two groups were not subjected to MP, and the other two groups were mechanically perturbed each day for 5 days by stroking all the internodes up and down 10 times. Another 288 plants were also divided into 4 groups of 72 plants each. Two groups were not subjected to ethephon, while the other 2 groups were treated with ethephon by adding a 100 nl drop of 300 ppm ethephon (including 0.025% tween-80) to each bud daily for 5 days.

Five days after starting, plants of groups 2, 4, 6 and 8 were taken out of their pots and exposed to DS for 0–24 h. At the 24th hour the plants were put back in their pots and reirrigated with running water and by flooding the trays in which the pots were standing. Groups 1, 3, 5 and 7 served as controls of the MP and ethephon treatments without DS. Three groups of 4 plants each (12 plants) were used as one treatment for each required time interval (0, 3, 6, 12, 30, 36, 48 and 72 h).

The water potential of each leaf of each plant was measured with a pressure bomb, just before estimating the degree of pith autolysis and eluting the stem-internode tissue for apoplastic enzyme assays. The latter observations were made separately of each internode from the top (internode IV) to the fourth internode from the top (internode I). For the estimation of pith autolysis, each internode was cut longitudinally. The rating system used was: 0=not pithy, 1=slight to moderate whitening of the tissue, 2= complete whitening of the pith, 3=the beginning of a hole in the pith, 4=a moderate hole in the pith, and 5=all of the pith disintegrated to form a hollow internode. From the same internode of 4 plants, a piece of 1.5–2.0 cm was excised and each of these pieces were cut to four 3.75–5.00 mm sections for the elution of apoplastic enzymes.

Extraction of apoplastic cellulase, polygalacturonase and reducing sugars—The extraction of apoplastic cellulase and polygalacturonase (PG) was accomplished by the diffusion method of Hanish Ten Cate et al. (1975) as modified by Huberman and Goren (1979). In their anatomical observations, Aloni and Pressman (1981), could not distinguish any breakage or disruption of membranous structure, although the experiments they performed were long term experiments. Our apoplastic enzyme extraction procedure was performed on internode sections from tomato stems which were not pithy or in the first phase of pith autolysis development (ratings 0–3). The internode sections were incubated for 18 h in 8 ml of Na,K-phosphate buffer (50 mM; pH 7) containing 0.05% L-cysteine (to protect the cellulase), at 4±1°C, while shaking in the dark. At the end of the elution, the buffer containing the apoplastic enzyme fraction of the internodes was decanted and saved for analyses. The cellulase determinations are reported as the percent difference from the viscosity of water, and hence cannot be reported as a function of extracted protein. In addition, a significant amount of the pith tissue was found to be no longer present as pith autolysis progressed. Since autolysis is complete, the proteins in the autolysed tissue also must be broken down to their constituent amino acids. Because of this, neither cellulase nor polygalacturonase activity were presented on the basis of measureable protein. If they had been, enzyme activity levels would have been artifactually high when divided by the low protein levels due to pith autolysis. Therefore, precipitation of the protein extract was omitted since it was unnecessary for satisfactory testing of enzymic activity.

The buffer containing the apoplastic enzymes was centrifuged (20,000×*g* for 10 min in an IEC Ultracentrifuge model BO35, rotor A-147), and a 0.2 ml aliquot was taken for the assay of reducing sugars by the method of Riov (1974). The solution was dialysed overnight against the same buffer diluted 10 times. All operations were performed at 4°C.

Assays of cellulase and PG—Cellulase activity was assayed viscometrically by adding 10 ml of 1.1% of the sodium salt of carboxymethyl cellulose in Na,K-phosphate buffer 0.05 M pH 6.0 as substrate to 0.5 ml of the processed eluate. One milliliter of the reaction mixture was allowed to drop through a viscometer pipette held at 37°C and the drop time recorded. The time was compared to 0.5 ml of diluted buffer which served as a control and the percent change calculated. Thus, cellulase activity was expressed as percentage changes in viscosity ($\%$-$\Delta\eta$), as defined by Huberman and Goren (1979). This method is quite precise, and has been in use at least since 1967 (Almin and Eriksson 1967, Almin et al. 1967).

PG activity was assayed spectrophotometrically by measuring reduced groups liberated from Na-polypectate (Calbiochem, San Diego, CA) with the dinitrosalicylic acid reagent, using d-galacturonic acid as a standard. The 1.0 ml reaction mixture contained 0.4 ml of the processed eluate, 0.25% sodium polypectate, and 1 mM Na-hydrosulfide as an inhibitor of uronic acid oxidase (Riov 1974), in 50 mM Na-acetate buffer, pH 5.0. One unit of PG activity was defined as the release of 1 μg of d-galacturonic acid in 6 h in a 37°C shaking bath in the dark. The activity of both enzymes was calculated on fresh weight and internode bases for comparative purposes, but because of the decrease in fresh weight due to the development of pith autolysis, the data is presented on the internode basis. Each assay was run in triplicate and each experiment was performed 3 times.

Measurement of ethylene evolution—Third internodes were excised and placed 5 to a 25 ml flask which was capped with a septum closure. The flask contained a wetted pad of Whatmen #1 filter paper,(to keep the internodes from drying out), under a circle of nylon screening (to keep the internodes from touching the water directly). Also in the flask was a small vial containing saturated aqueous KOH with a paper wick, to absorb CO_2. One ml samples were taken from the flask with a syringe, and the ethylene measured by gas chromatography.

To keep the graphs from becoming intolerably cluttered, standard errors at every data point were not used. Instead, a bar representing 2 × the largest standard error was used for each graph.

Results

The time courses of pith autolysis, of the 4 internodes, together with the effects of MP, DS or MP+DS are shown in Fig. 1. A slight whitening could be seen in internodes II and III of the control plants, while there was no pith autolysis at 0 time, and hardly any pith autolysis in internodes I and IV of the control or all internodes of the MP treated plants at any time. The DS plants showed little pith autolysis in the bottom (oldest=I) or top (youngest=IV) internodes, maximum pith autolysis in the penultimate internode (III) and a moderate amount of pith autolysis in internode II. As DS lowered the water potential, the pith autolysis progressed in all internodes. At all times, internodes I, II, and III displayed less DS-induced pith autolysis when the plants from which they came were previously given MP.

In the non-treated, control plants there is an increasing basipetal water potential gradient; i.e., the lowest water potential is in the youngest, top internode (IV) and the highest water potential is in the oldest , bottom internode (I), (Data not shown). When DS was applied to the plants this gradient was accentuated, and all internodes had considerably lower water potential than the controls. After reirrigation, both the water potentials and the stem gradient returned to approximately the pre-DS level. Previous MP has little effect on the stem water potential gradient. However, when MP is given before drought stress, the resulting stem gradients were less than those of the controls, and the overall amount of water potentials were less than in the controls. After reirrigation, the water potentials of these plants followed patterns similar to the controls.

Since internode III displayed the most appreciable amount of DS-induced pith autolysis, the time courses of water potential and pith autolysis were measured (Fig. 2, a and b, respectively). The development of pith autolysis begins during the period of drought stress, but continues even after the plants are reirrigated (Fig. 2b). Both the time courses of water potential and pith autolysis varied little from the control levels following MP (Fig. 2, top and bottom, respectively). Drought stress drastically decreased the water potential of leaflets of internode III, but DS preceded by MP decreased it even more (Fig. 3 top). After reirrigation, the water potential rose in 6–12 h to near control levels in leaflets of all of the internodes of plants of both treatments (Fig 2a).

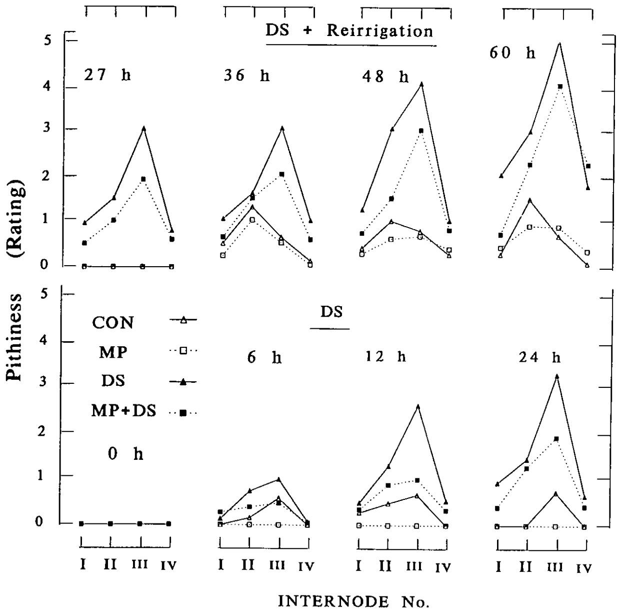

Fig. 1 Pithiness ratings (0=no pith autolysis, 5=maximally hollow stem) of internodes I to IV taken at 0, 6, 12 and 24 h after the start of DS (lower), and then at 3, 6, 12 and 24 h after reirrigation (upper). The symbols for the 4 treatments are given in the figure.

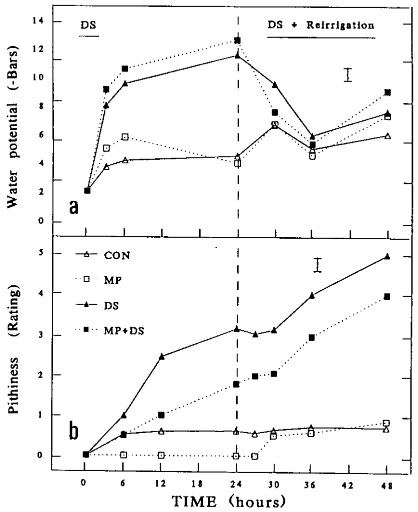

Fig. 2 Time courses of water potential (a), and pith autolysis ratings, (b) of internode III (4 internodes per replicate). The treatments are: drought stress (DS), mechanical perturbation (MP) or DS preceded by an MP pretreatment (MP+DS). The vertical bars represent 2 × the largest standard error of the graph.

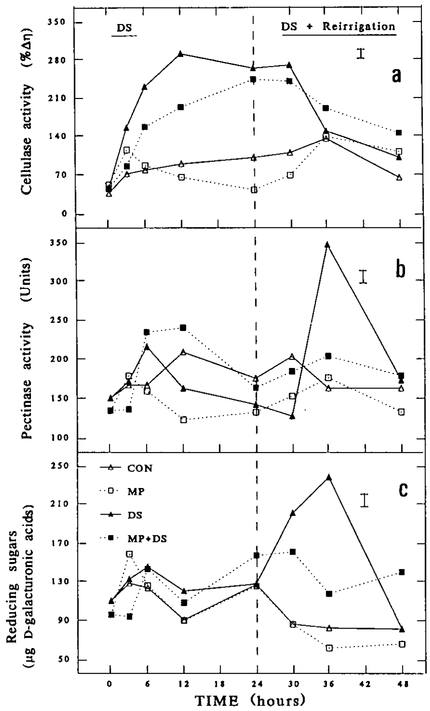

Fig. 3 Time courses of apoplastic cellulase activity (A), apoplastic polygalacturonase activity (B) and reducing sugar content of the apoplastic fraction (C) of internode III (4 internodes per replicate). The vertical bars represent 2 × the largest standard error of the graph.

Drought stress induces fairly linear pith autolysis development in the third internode during the period that water was withheld (from 0–24 h, Fig. 2 bottom). After reirrigation there was a lag in the development of pith autolysis for 6 or more h, followed by a further increase. Plants that were given MP before DS, displayed pith autolysis development that averaged 59±6% of that of the drought stressed plants without MP before, and 78±4% after reirrigation. Thus, previous MP could be said to have hardened the internodes against pith autolysis. Neither the control nor the MP treatments displayed appreciable pith autolysis during the course of the experiment.

The fact that internode III became pithy reduced its fresh weight (by an average of 10%), and this biased the computed enzyme data, when reduced to fresh weight bases. Therefore, the data was reduced to organ bases (per internode). For similar reasons, this method of computation has been successfully employed by previous authors with other systems whose mechanisms include tissue autolysis (Goren and Huberman 1976).

The activities of 2 cell wall degrading enzymes, cellulase and polygalacturonase were tested in internodes of plants from the various treatments. In general, there was an acropetal gradient of cellulase activity, with the lowest activity in the oldest internode (I) and the highest activity in the top internode (IV), especially before reirrigation (data not shown). Polygalacturonase activity was generally higher in internodes II and III, than in internodes I and IV, regardless of treatment (data not shown).

Average apoplastic cellulase activity in internode III is shown in Fig. 3 top. After 3 h, previous MP caused a marked decrease in apoplastic cellulase activity during the first day of the experiment. Drought stress, on the other hand, caused a rapid and large increase in elutable cellulase, which remained high until 6 h after reirrigation, and then dropped to near control levels. When plants were pretreated with MP, the increase in apoplastic cellulase activity induced by drought stress (DS+MP), was significantly lower than in plants which had been given DS only. Apoplastic cellulase activity in these plants also dropped following reirrigation. Thus, during drought stress, the pattern of apoplastic cellulase activity in the internodes was similar to that of the development of pith autolysis (Fig. 2 bottom). Following reirrigation, however, there seemed to be no positive relation of the patterns of apoplastic cellulase activity (Fig. 3a) and development of pith autolysis (Fig. 2b).

The time courses of apoplastic polygalacturonase activity in the internodes showed quite a different pattern where DS was concerned, although it was similar to that of cellu-

Table 1 The effects of drought stress (DS), exogenous ethephon or ethephon pretreatment before DS, on apoplastic cellulase activity (%-$\Delta\eta$). One 100 μl drop of 300 ppm ethephon was given daily on each terminal bud for 5 days

Time (h)	Apoplastic cellulase activity per internode III			
	Control	DS	Ethephon	Ethephon+DS
0	111±22	54±10	44±22	57±13
16	161±17	171±10	34±16	97±14
40	189± 9	202±25	84±21	97±12
40+24 h[a]	109±19	200±7	165±14	117± 6

Each datum represents the average of 6 replicates of internode III±SE.
[a] +24 h after reirrigation.

Table 2 Ethylene evolution (±SE) from the third internode of plants given DS, MP or a combination of the two

Treatment	Ethylene evolution after 6 hours (pmol. per internode)
Control	150± 8
DS	310±14
MP	220±12
MP+DS	290±17

n=18.

lase activity in MP treated plants (Fig. 3b). During DS there was a 40% increase in polygalacturonase activity during the first 6 h, compared to the controls (7% increase for untreated controls, and 15% increase for stems given MP, alone). However, there was an even greater increase (74%) when DS was preceded by MP (Fig. 3b). In all treatments, the apoplastic polygalacturonase activity returned to the 0 h level by 24 h after the start of DS. However, there was a large increase in apoplastic polygalacturonase activity by 12 h after reirrigation in the DS plants (79%), but not in the MP+DS plants. In the DS plants, apoplastic polygalacturonase activity returned to near control levels by the end of the experiment. Thus, the increase in polygalacturonase activity seems to be more strongly related to the development of pith autolysis following reirrigation than to the development of pith autolysis which occurs before irrigation.

Pretreatment with MP causes a decrease in DS-induced apoplastic cellulase activity during drought stress and a decrease in DS-induced polygalacturonase activity after reirrigation. Thus, it is not surprising that the apoplastic reducing sugar content also was decreased by this treatment (Fig. 3c) since such sugars are the products of these enzymatic reactions.

When plants were pretreated with ethephon instead of MP, their stems showed a large decrease in apoplastic cellulase activity during DS and a larger increase of the enzyme activity after reirrigation (Table 1). ethephon alone caused a decrease in apoplastic cellulase activity (Table 1) as did the MP pretreatment (Fig. 3a). Ethephon+DS produced an intermediate level of cellulase activity.

If ethephon can substitute for MP in retarding the development of pith autolysis, it might be expected that MP, as well as DS can cause ethylene evolution. Table 2 shows that to be the case. When DS and MP are both given to the plant, the amount of ethylene evolution is less than the amount produced by DS alone, but more than the amount produced by MP alone.

Discussion

The time course of the development of pith autolysis in the stem of Hossen tomato plants generally follows that of the decrease in water potential of the leaflets of each internode during DS (Figs. 1, 2). The increase in pith autolysis ceases 6–12 h following re-irrigation, but then begins again. Thus, there were 2 periods of pith autolysis development during the course of the experiment: the first during drought stress, and the second beginning several hours after reirrigation. In previous papers, it has been shown that pith autolysis develops during prolonged DS (Aloni and Pressman 1981, Pressman et al. 1983). However, this report is the first time that the short term kinetics of this process have been elucidated. This report also confirms the appearance of pith autolysis in the upper penultimate internodes, but not in the top or lower internodes (Aloni and Pressman 1981).

In a preliminary anatomical study, Aloni and Pressman (1981) showed that when tomato pith cells undergo autolysis, the protoplast is affected first, followed by softening of the cell walls and finally complete disappearance of the cell walls. A similar situation in bean stems and leaves has been documented by Webster (1970), who reported that bean stems and leaves naturally develop a hollow pith. In the abscission zone of the leaf, the first distinguishable cellular change involves the breakdown of the pith cells. This change is first manifested by a loss of pectic substances, as evidenced by histochemical observations of the inner core of the pith cells. The loss of the pectic substances of the pith cells is followed later by a breakdown of the primary cell wall and by a degradation of the cell contents. These events progress laterally through adjacent pith cells and a lysigenous cavity is gradually created within the pith (Webster 1970).

In this study we were interested in detecting the hydrolytic enzymes (cellulase and polygalacturonase), which were proved to be involved directly in cell wall softening (Linkins et al. 1973, English et al. 1972), and which we hypothesized to be also involved in the DS-induced pith autolysis in the tomato stem. By dipping sections of the treated plants in a small volume of diluted cold buffer (Hanish Ten Cate et al. 1975, Huberman and Goren 1979) we were able to get the active dissolved hydrolytic enzymes from the walls and the free space separated from the "inactive" enzymes still remaining in the live cells of the internode. Therefore, the results in this study (Tab. 1, Fig. 3) represent only the active enzymes dissolved in the walls or on their way to the walls (in the intracellular space volume). However, it is probable that at least some of the active cellulase is still attached to the cell walls as residual cellulase (Lewis et al. 1975).

We need to know the source of the eluted enzymes in order to understand the mechanism of the development of pith autolysis. Aloni and Pressman (1981) have shown that in tomato, the degradation of the cell occurs before the breakdown of the cell wall. The former undoubtedly involves the decrease in selective permeability of the plasma membrane, so that it becomes more and more leaky and so that the cell contents efflux from the dying cell. Among these contents are, we assume, the molecules of cellulase and polygalacturonase, which then lyse the cell wall matrix, producing sugars (Fig. 3) which are used in the further growth of the plant (Jaffe and Lineberry 1989).

Two primary findings emerged from this study. The first was that DS of Hossen tomato plants caused an increase in the cell wall degrading enzymes cellulase and polygalacturonase in the stem, and that the details of the time courses of these changes were related to the development of pith autolysis (Compare Fig. 2 with Fig. 3). The second finding was that pretreatment with MP on the stem or exogenous ethephon on the buds, not only hardened the stems against DS-induced pith autolysis, but also inhibited the increase in apoplastic hydrolytic enzyme activity (Table 1, Fig. 3).

The DS-induced increase in apoplastic cellulase and polygalacturonase occurs in internodes II and III, in which pith autolysis is found, and in the non-pithy top internodes. We believe that the increase in enzyme activity in the top internode is related to the fact that this is the youngest, actively growing internode (Del Campillo et al. 1988, 1990, Lewis et al. 1975, Taylor et al. 1990). Cellulase activity has been implicated in the cell wall loosening responsible for cell and tissue elongation (Linkins et al. 1973). It is interesting that in MP or ethephon pretreated plants, the longitudinal stem growth is retarded. Such a response would be consistent with the effect of these treatments on the hydrolytic enzyme activity.

The kinetics of the DS-induced increase in cell wall degrading enzyme activities suggest that the wall softening is due mostly to the action of cellulase with a small amount of increased polygalacturonase activity, as well. After reirrigation, the apoplastic cellulase activity remains high for a short while, but then decreases. The polygalacturonase activity rises to a high transient level. This suggests that the final degradation of the cell walls may be as much a function of the breakdown of pectin as of cellulose. We have not studied xyloglucan degredation during pith autolysis. Since the autolytic process is in response to an unmet need for carbon (Jaffe and Lineberry 1989), it would be interesting to examine this aspect of pith autolysis.

In several species, the activities of apoplastic cell wall degrading enzymes have been shown to increase after the tissue increases its ethylene production or after the application of exogenous ethylene. Examples of this effect have been reported to occur during the ripening of tomato fruits (Babbitt et al. 1973), abscission of beans (Abeles and Leather 1971) and citrus fruits (Huberman and Goren 1979), and senescence of morning glory flower petals (Kende and Hanson 1976, Wiemke-Gehring et al. 1974). During the development of pith autolysis in Hossen tomato stems, there is an increase in ethylene evolution after the beginning of drought stress (Table 2), and a smaller, but similar effect, following reirrigation (Data not shown.). It is interesting to note that this continued acceleration of DS-induced ethylene evolution is not to be seen in the pith autolysis-resistant variety, 172-1 (Data not shown.). Although MP also causes some ethylene production, it retards much of the DS-induced ethylene production. Thus, the DS-induced increase in cellulase and polygalacturonase activity is consistent with the hypothesis that it is mediated by the DS-induced ethylene in Hossen tomatoes.

In an earlier communication, we have reported that when tomato stems are mechanically perturbed (MP) or treated with exogenous ethephon before being given DS, these treatments harden the stems against the development of pith autolysis (Pressman et al. 1983). Further, DS plants produce more ethylene than do controls, but if DS is preceded by MP, the stems evolve apparently less ethylene than the controls (Table 2), although the difference between these data is not significant . However, when MP+DS plants are reirrigated, they produce even more ethylene than do the same plants before reirrigation. In the present report, we now extend these findings to cell wall degrading enzymes. Treatment with MP causes a decrease in the activities of both cell wall degrading enzymes, compared to untreated controls. Pretreatments of either MP or ethephon decrease the DS-induced activity of cellulase and polygalacturonase (Fig. 3, Table 1). The kinetics of this effect parallels that of the decrease in pith autolysis caused by DS and reirrigation (Fig. 2).

All of the evidence presented herein, is consistent with the conclusion that DS-induced ethylene evolution is part of the causal mechanism increasing apoplastic cellulase and polygalacturonase activities. It is reasonable to suppose that the activities of these two enzymes are responsible for the degradation of the pith cell walls during the latter stages of the development of pith autolysis, as has been reported in beans (Webster 1970). Some species, such as bean, undergo pith autolysis without any external stimulus (Jaffe and Lineberry 1989). However, others such as tomato, require an external stress such as DS to trigger pith autolysis (Pressman et al. 1983). Drought stress lowers the water potential of the plant (Fig. 2), and this in turn causes ethylene to be evolved (Table 2). We suggest that the DS-induced ethylene then causes an increase in the activities of the cell wall degrading enzymes. We have previously suggested that stress induced ethylene evolution is, at least in part, responsible for inducing pith autolysis, but that ethylene evolution which is induced by non-stress treatments, such as MP or exogenous ethephon, somehow hardens the tomato stem against pith autolysis (Pressman et al. 1983). It may now be suggested that at least one of the ways that this is accomplished is by inhibiting the increase in activity of the enzymes that break down the pith cell walls to finally produce the hollow stem.

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