

# Ethylene and Not Embolism Is Required for Wound-Induced Tylose Development in Stems of Grapevines<sup>1</sup>[C][OA]

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The pruning of actively growing grapevines (*Vitis vinifera*) resulted in xylem vessel embolisms and a stimulation of tylose formation in the vessels below the pruning wound. Pruning was also followed by a 10-fold increase in the concentration of ethylene at the cut surface. When the pruning cut was made under water and maintained in water, embolisms were prevented, but there was no reduction in the formation of tyloses or the accumulation of ethylene. Treatment of the stems with inhibitors of ethylene biosynthesis (aminoethoxyvinylglycine) and/or action (silver thiosulfate) delayed and greatly reduced the formation of tyloses in xylem tissue and the size and number of those that formed in individual vessels. Our data are consistent with the hypotheses that wound ethylene production is the cause of tylose formation and that embolisms in vessels are not directly required for wound-induced tylosis in pruned grapevines. The possible role of ethylene in the formation of tyloses in response to other stresses and during development, maturation, and senescence is discussed.

Tyloses, outgrowths of xylem parenchyma cells into the lumen of adjoined vessels via vessel-parenchyma pits, occur widely among plant species (Saitoh et al., 1993) and are induced by environmental stimuli such as wounding and pathogen infection (Biggs, 1987; Pearce, 1990). Tyloses may impair xylem function by blocking vessels, but they are also a component in wound healing and may inhibit the intrusion and spread of pathogens (Bonsen and Kučera, 1990; Pearce, 1991; Salleo et al., 2002). Several hypotheses have been advanced to explain tylose initiation (Talboys, 1958; Sequeira, 1965; Wallis and Truter, 1978; VanderMolen et al., 1987). One persistent idea is that tyloses form in response to the presence of air embolisms in xylem vessels (Klein, 1923; Zimmermann, 1978; Canny, 1997), although most of the pertinent literature is old. Other hypotheses to explain tylose initiation include a role of indole-3-acetic acid (IAA; Buddenhagen and Kelman, 1964; Sequeira, 1965), unidentified substance(s) derived from pathogens (Talboys, 1958; Beckman, 1966),

and changed metabolic pathways in xylem parenchyma cells due to the presence of pathogens (Wallis and Truter, 1978). Although these hypotheses offer explanations for tylose development under some specific environmental conditions, they do not provide a general model to explain tylosis under diverse environmental conditions and in response to different agents and stressors.

Induction of ethylene biosynthesis is a common response of plants to many of the same biotic and abiotic factors that induce tylose formation (Abeles et al., 1992). In addition to wounding and pathogen infection, natural senescence (Dute et al., 1999), heartwood formation (Chattaway, 1949; Parameswaran et al., 1985), frost (Cochard and Tyree, 1990), and flooding (Davison and Tay, 1985) are conditions stimulating tylose development. All of these stimuli are also known to increase ethylene production. Ethylene production in response to wounding has been demonstrated in a wide range of species (Nilsen and Orcutt, 1996). Infection by pathogens often leads to an increased ethylene production by host plants (e.g. Pegg, 1976; Boller, 1991), and some pathogens have themselves been found to produce ethylene (Fukuda et al., 1984; Arshad and Frankenberger, 1991). Ethylene production is elevated in the above-ground portions of flooded plants (Hunt et al., 1981; Voesenek et al., 1990; Rijnders et al., 1992). Higher internal ethylene concentrations have been measured in senescing plant tissues (Abeles et al., 1992), and Ingemarsson et al. (1991) also measured high ethylene concentrations in the heartwood of *Pinus sylvestris*. Clearly, conditions that trigger tylosis are also conditions that stimulate ethylene biosynthesis, leading us to suppose that the role of

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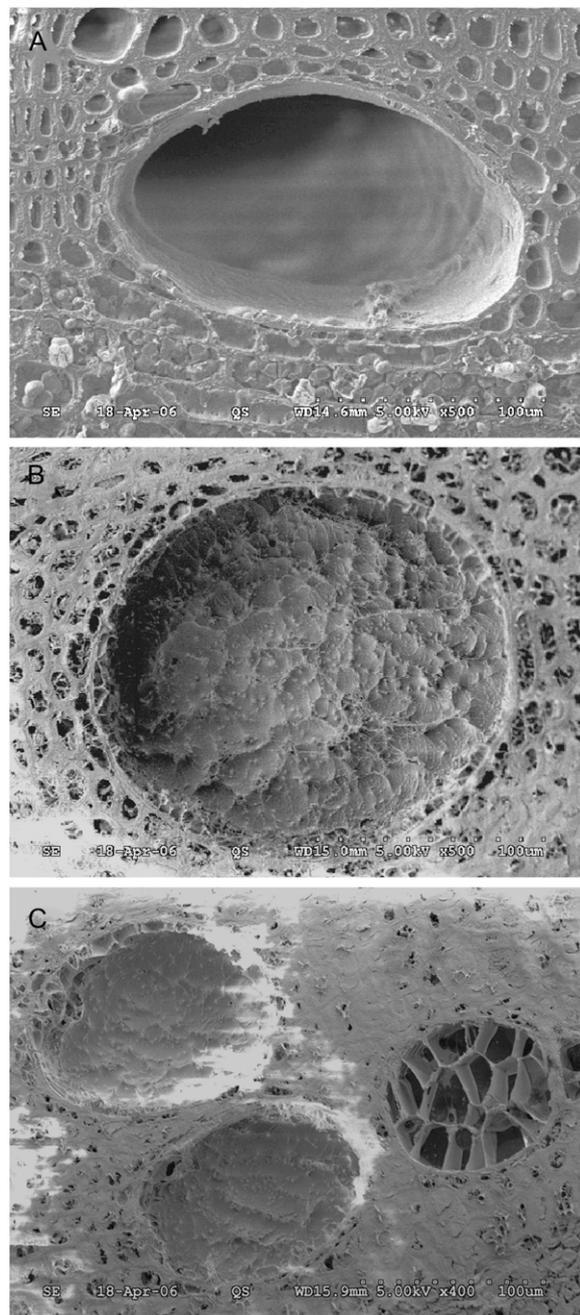
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ethylene in tylosis formation might be quite general. Ethylene is known to regulate diverse physiological and metabolic activities in plants and to be involved in many aspects of growth and development of cells and tissues (Mattoo and Suttle, 1991; Reid, 1995), including xylem development (Parkhurst et al., 1972). This suggested to us that ethylene could be a coordinating factor in the development of tyloses.

Little has so far been revealed about role of ethylene in tylose development, although some research suggested its involvement. Scott et al. (1967) observed tylose development in abscission layers of bean (*Phaseolus vulgaris*) petioles during natural senescence and more and faster tylose development in the petioles with an accelerated abscission process that was stimulated by exogenous ethylene or auxin. When Pegg (1976) treated *Verticillium*-infected tomato (*Solanum tuberosum*) plants with ethylene, he found a significant increase in tyloses in a resistant cultivar. Van Doorn et al. (1991) reported that tylose development was reduced in the cut stem of lilac (*Syringa vulgaris*) flowers treated with ethylene inhibitors and suggested that tylose development may be regulated by ethylene. However, there were no measurements of tylosis, ethylene production, or its suppression by ethylene inhibitors. In our recent work with tyloses in actively growing stems of grapevines (*Vitis vinifera*), we found that there were almost no tyloses in intact stems but that they formed rapidly in response to pruning (Sun et al., 2006). Tyloses developed simultaneously from several parenchyma cells within a single vessel but separated in time among vessels. Tylose development was faster in the basal and middle regions of the stems than in the apical region. All tyloses formed within approximately 1 cm and 7 d from the cut, but about one-half of the vessels did not become completely occluded. We report here a study, using this well-



**Figure 1.** Images of experimental treatments. A, A rubber tube traps the cut end of stem at one end and is connected at the other end to a 3-mL syringe that collects gas evolved from the cut end. B, The stem is cut in degassed water, and the cut end is capped with a water-filled rubber bulb to avoid exposure of the cut surface to air in experimental periods. Scale bar = 4 cm. [See online article for color version of this figure.]



**Figure 2.** Cryo-SEM of vessels 4 mm under the cut surface in samples at day 6 after cutting. A, Gas-filled vessel in stem cut in air and capped with an air-filled rubber pipette bulb. B, Ice-filled vessel in stem cut in water and capped in a water-filled rubber pipette bulb. The textured substance in the vessel is ice. Images in A and B are representative of 40 to 60 vessels observed in each of two or three replications of stem sections per treatment. Occasionally, gas-filled vessels were observed in stem sections cut under water (less than 10%) and only very rarely was an ice-filled vessel observed in stem sections cut in air. C, Water (ice)-filled vessels (two vessels at the left) and tyloses formed in a water-filled vessel (the vessel at the right) in stems cut in water and capped in a water-filled rubber pipette bulb. Surface ice was evaporated to reveal the tylose structure.

characterized system, in which we test the role of embolisms and ethylene in tylose formation.

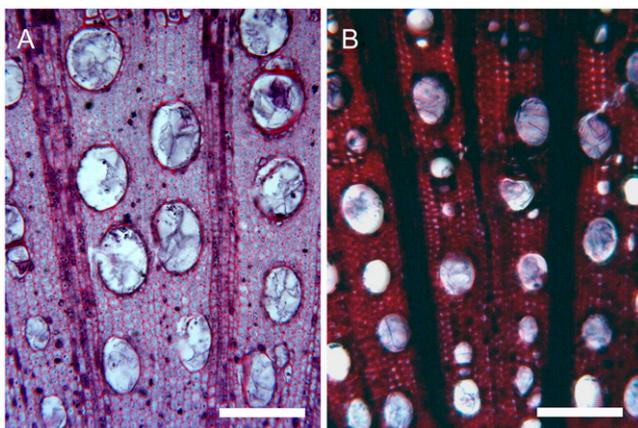
## RESULTS

### Embolisms Do Not Trigger Wound-Induced Tylose Development

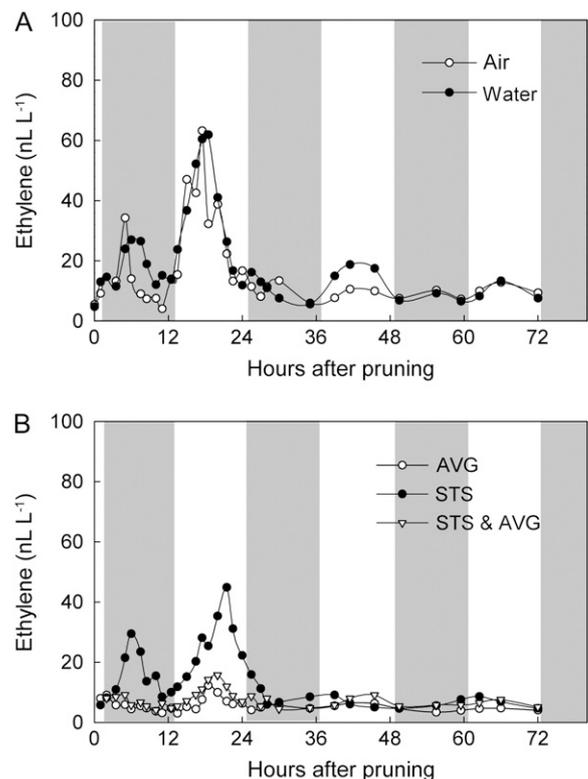
To test the role of air embolisms in tylose development, stems cut in degassed water and capped with a water-filled pipette bulb were compared to stems cut in air and capped with an air-filled pipette bulb (Fig. 1B). Cutting stems in air led to embolisms in vessels (Fig. 2A), and those cut in water remained full of water (Fig. 2B). Tylose development was observed in the vessels without (Figs. 2C and 3B) and with (Fig. 3A) embolisms. The vessels involved in tylose development in secondary xylem were similar in stems under both treatments. At day 4, 26% and 21% of vessels contained tyloses in the stems cut in air and in water, respectively. By day 8, these values had increased to 63% and 61%, respectively, but there was no difference between treatments. Vessels with tyloses were analyzed for the extent of occlusion in transverse section. Tyloses did not show obvious differences in morphology, size, or number between stems wounded in air or water (with and without embolisms, respectively) on day 4 and at day 8 (data not shown; Fig. 3, A and B). These observations show that in these experiments, wound-induced tylose development was not related to the presence or absence of air embolisms.

### Wounding Induces Enhanced Ethylene Production

Whether shoots were pruned in air or in water, production of ethylene (determined as the accumulation of ethylene in air in a 3-mL syringe attached to the cut end) increased in response to wounding. Ethylene production increased in a biphasic manner with peaks



**Figure 3.** Tylose development in stems 4 mm under the cut surface at day 8. In stems cut in air (A) and in stems cut under water (B), most vessels contained tyloses. Scale bar = 200  $\mu\text{m}$ .



**Figure 4.** Pattern of ethylene concentration evolved from the cut end of grape stem. Data are for samples of gas collected for 1 h and are normalized to 7.2-mm-diameter shoot. A, Stem cut and remained in air (white circle) or cut in water and then exposed to air after 2 h (black circle), both without the treatment of an ethylene inhibitor. B, Stem cut in AVG (white circle), STS (black circle), or STS followed by AVG (gray circle) solutions and then exposed to air after 2 h. Data are from one replication and are representative of three or four replications for each treatment, although the timing of the peaks did not coincide exactly in each experiment. Vertical shaded and white bars indicate approximate nighttime and daytime, respectively.

at about 6 and 18 h after pruning and an intervening decline to the initial level at 11 to 14 h (Fig. 4A). The second peak was about 2-times greater than the first peak and 10-times greater than the initial concentration (Table I). By 30 h after pruning, the ethylene concentration was again at the initial level where it remained with a slight diurnal oscillation.

### Aminoethoxyvinylglycine But Not Silver Thiosulfate Inhibits Ethylene Production following Wounding

In the stems treated with silver thiosulfate (STS), pruning also induced a biphasic increase in ethylene production observed in the untreated controls, with similar timing and similar concentrations (Table I), although the second peak was somewhat lower than that observed in the controls (Fig. 4B). In contrast, the pattern of ethylene production from the pruned end of stems treated with aminoethoxyvinylglycine (AVG) or with STS followed by AVG was dramatically dif-

**Table 1.** Ethylene production from grapevine stems after wounding in air or in water and in the presence or absence of ethylene inhibitors

Ethylene production was determined as the hourly accumulation of ethylene in air in a 3-mL syringe attached to the cut end. Ethylene concentration was normalized to a shoot with a 7.2-mm diameter (approximate mean diameter). Controls were the stems cut in air or cut in water with the cut end in water for 2 h before exposure to air. Treatments were the stems cut in AVG or STS with the cut end in the ethylene inhibitor for 2 h before exposure to air. Each datum is presented as mean  $\pm$  SD,  $n = 3$  or 4. –, No obvious peak.

Wound Conditions	First Peak		Second Peak	
	Time after Wounding	Concentration	Time after Wounding	Concentration
	<i>h</i>	$nL L^{-1}$	<i>h</i>	$nL L^{-1}$
Control				
Air ( $n = 4$ )	5.6 $\pm$ 1.3	32.7 $\pm$ 6.9	17.9 $\pm$ 1.5	63.8 $\pm$ 8.9
Water ( $n = 3$ )	4.8 $\pm$ 1.8	29.1 $\pm$ 3.1	17.5 $\pm$ 1.4	61.2 $\pm$ 0.9
Treatment				
AVG ( $n = 3$ )	– <sup>a</sup>	–	18.3 $\pm$ 1.5	13.9 $\pm$ 2.5
STS + AVG ( $n = 3$ )	–	–	21.0 $\pm$ 0.9	14.5 $\pm$ 1.3
STS ( $n = 3$ )	6.3 $\pm$ 1.3	40.4 $\pm$ 13.2	18.7 $\pm$ 3.0	36.0 $\pm$ 7.7

ferent from the controls. These two treatments completely eliminated the first rise in ethylene concentration; the second increase was greatly reduced (Fig. 4B; Table I). Ethylene production induced by wounding was greatly suppressed in the presence of AVG and was essentially unaffected by STS.

#### Suppression of Ethylene Production and Action Resulted in Reduction and Delay of Tylose Development in Wounded Stems

Prior to pruning, the grape stems had essentially no tyloses (Fig. 5A; Sun et al., 2006). Ethylene inhibitor(s) altered the status of tylose development within single vessels. In control stems, tyloses were observed in some individual vessels at day 3, and by day 9 a large number of vessels in secondary xylem were completely or partially occluded by 3 to 10 tyloses (Fig. 5, B and C). In the partially occluded vessels, there was a similar number of small tyloses, which usually occupied most of the vessel lumen as seen in transverse section. In the stems treated with AVG, STS, or STS + AVG, tyloses were generally absent until after day 9 (Fig. 5, D–H), although a few were present at day 9 in stems treated with STS. Very few vessels developed tyloses even at day 13 (Fig. 5, E and I), and those were small in number, usually about three, and size, 5 to 35  $\mu$ m in diameter. Tyloses rarely occluded the vessel fully of stems treated with any of the ethylene inhibitors.

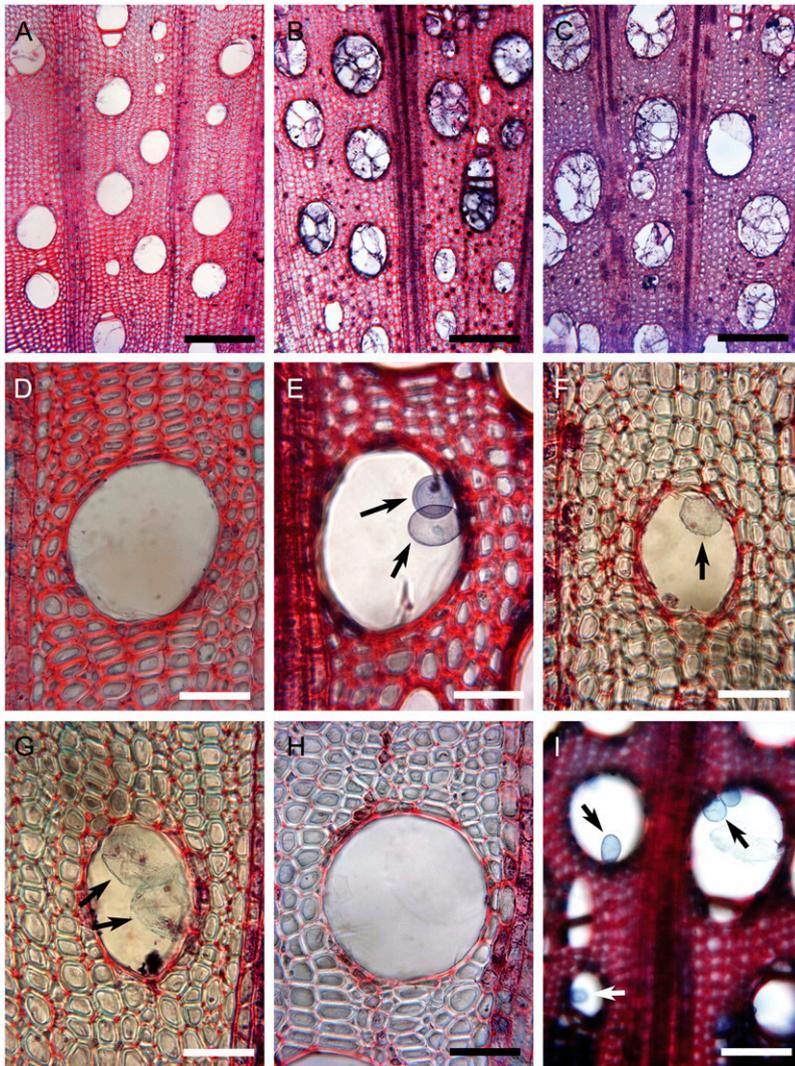
The temporal progress of tylose development was markedly affected by treatment of the stems with ethylene inhibitors. In control stems, whether pruned in water (with the cut end remaining in the water only for the first 2 h) or in air, tylosis increased rapidly from day 3 to day 9 (Fig. 6), with more than 60% of the vessels affected. Thereafter, the frequency of tyloses increased, but little, and by day 18 was 76% and 73% in stems pruned in air and in water, respectively (Fig. 6).

In stems treated with inhibitors of ethylene synthesis or action, the progress of tylose development was slower and dramatically fewer vessels were involved. With STS, the tyloses did not increase significantly until day 6, and with AVG or with STS followed by AVG (STS + AVG), tyloses generally did not develop until day 9. From day 6 or day 9, the tyloses increased very slowly and reached about 30% in the stems treated with STS or 20% in the stems treated with AVG (or STS + AVG) at day 18 (Fig. 6). These data indicate that suppression of ethylene production and action greatly reduced and delayed wound-induced tylose development.

#### DISCUSSION

The results indicate a direct relationship between the formation of tyloses in decapitated grapevine stems and ethylene synthesis and action in the wounded tissues. The wound-induced tylose development and ethylene evolution was similar in stems cut in air or water. Embolisms were present when grapevine stems were decapitated in air and were absent when the wounding was done in water and the cut end remained in water. The presence and absence of air embolisms was confirmed by cryostat scanning electron microscopy (Cryo-SEM) of vessel lumen in severed stems. The resultant tylose formation showed no obvious differences in temporal progress or morphology between these treatments. At least in grapevine, vessel embolisms are not required for wound-induced tylose development. Thus, our data do not support the long-standing notion that tyloses are induced by embolisms.

The present embolism or gas hypothesis is evidently based on correlative observations that tylose development occurred more frequently in the vessels close to wounds or around sites of inoculation (Biggs, 1987;

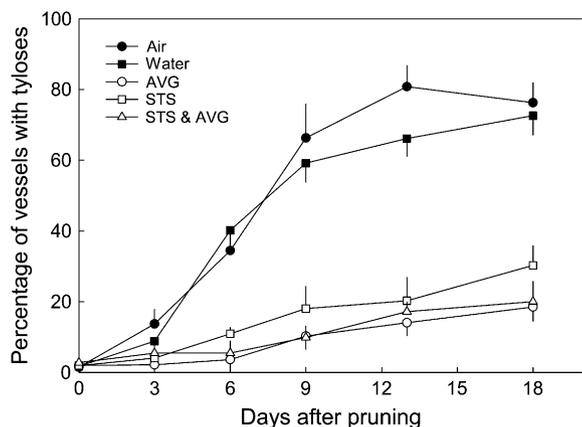


**Figure 5.** Tylose morphology 4 mm under the cut surface in stems treated without ethylene inhibitors (A–C) and in those with ethylene inhibitors (D–F). A, No tyloses were present in stems at day 0. B, In stems cut in air, most vessels were completely occluded by multiple compactly arranged tyloses at day 9. C, In stems cut in water and then exposed to air (controls for ethylene inhibitor treatments), most vessels were again completely occluded by multiple compactly arranged tyloses at day 9. D and E, In stems cut in and treated with AVG, no tyloses were present at day 9 (D), and few, small tyloses (arrows) occurred in few vessels at day 13 (E). F and G, In stems cut in and treated with STS, few small tyloses (arrow) were present in few vessels at day 9 (F), and tyloses were still few but larger in some vessels at day 13 (G). H and I, In stems treated with STS followed by AVG, tyloses did not develop in most vessels at day 9 (H) and were small and few and present in few vessels at day 13 (I). Scale bar = 200  $\mu\text{m}$  in A to C, 50  $\mu\text{m}$  in D to H, and 100  $\mu\text{m}$  in I.

Pearce, 1991) and tyloses were observed mainly in large vessels of the early wood (Cochard and Tyree, 1990) and of early metaxylem in petioles of sunflower (*Helianthus annuus*; Canny, 1997). Such vessels are vulnerable to embolism following wounding, infection, or water stress (Ellmore and Ewers, 1985; Hargrave et al., 1994; Canny, 1997; Davis et al., 1999). Although these correlations suggest a relationship between embolisms and tylose development, there has as yet been no direct test of this relationship.

The similarity of tylose development under the two wounding conditions demonstrates no relation of tylosis to embolisms. All vessels at the wound were exposed to air when the cutting was done in air. Although vessels were sap filled when observed under SEM, those images were taken after days in water. Embolisms induced by cutting may have been dissolved or displaced in that time. However, to explain the results, cutting under water would have to have created similar embolisms as cutting in air. This is highly unlikely, especially for well-watered, green-

house-grown plants. Furthermore, the distribution of tyloses did not follow the spatial distribution of gas- versus sap-filled vessels. Embolisms fill the entire vessel lumen, and in grape stems, many vessels are over 10 cm long and some extend over 100 cm (Zimmermann, 1983; Thorne et al., 2006). Thus, large numbers of pits and adjacent axial parenchyma were exposed to similar transitions from liquid to vapor on the vessel side of bordered pits upon excision in air. However, tylose development generally is restricted to the sites very close to the wound (Mace, 1978; Pearce, 1991) and in grape tylosis extends only approximately 1 cm from the wound (Sun et al., 2006). Furthermore, tyloses develop in healthy plants without mechanical injuries that would cause embolisms (Chattaway, 1949; Parameswaran et al., 1985) and in locations far from the site of pathogen inoculation in plants where vessels should not be specifically vulnerable to embolism (Fry and Milholland, 1990; Stevenson et al., 2004). Although Canny (1997; Canny et al., 2001) thinks that vessels are largely gas filled, the lack of correspond-



**Figure 6.** Frequency of tyloses at 4 mm under the cut surface of stems treated with and without ethylene inhibitors (AVG, STS, or STS followed by AVG). Each datum is presented as the mean with one SD ( $n = 3$ ).

ingly widespread tyloses argues against the gas hypothesis for tylose development, and they, too, report tyloses present in sap-filled vessels.

Other hypotheses for the formation of tyloses have focused largely on the relationships observed between tylose formation and pathogen infection by fungi or bacteria. The hypothesis that auxin induces tylose formation (Buddenhagen and Kelman, 1964; Sequeira, 1965) is supported by correlations between increased IAA concentrations in diseased plants and the formation of tyloses (Sequeira and Kelman, 1962). Moreover, tyloses formed in banana (*Musa* spp.) roots when they were treated with exogenous IAA (Mace and Solit, 1966), and cell wall expansion in developing tyloses showed a similar pattern and structural characteristics to the cell elongation stimulated by IAA (VanderMolen et al., 1987). However, Pegg and Selman (1959) detected a high level of IAA in *Verticillium*-infected tomato but saw no tylose development. A similar contrary observation was made in *Verticillium*-infected cotton (*Gossypium hirsutum*; Wiese and De Vay, 1970). Therefore, the role of IAA in tylose formation remains unclear.

Talboys (1958) observed an inverse correlation between tylose development in hops and severity of *Verticillium* infection: The higher the number of mycelia in vessels the less the tylose development. Similar situations were also observed in banana and tomato plants infected by *Fusarium* (Beckman, 1966). These researchers suggested that the pathogens produce an unidentified substance that induces tyloses at lower concentrations but inhibits their formation at higher concentrations. An alternative suggestion is that tylose development is triggered directly by the invasion and multiplication of pathogens in axial parenchyma cells, which may greatly change their metabolic activity and stimulate the formation of tyloses (Wallis and Truter, 1978). A more general hypothesis to explain the observed relationships between pathogens and tylose development is that tylose development is a response

to diverse stimuli, including mechanical injuries and pathogens, and that slower development and fewer quantities of tyloses in susceptible host plants are due to inhibition of tylose formation by an unidentified compound that is also produced by pathogens (Elgersma, 1973).

Our study was designed to test the possible roles of ethylene in tylose initiation and development using our previously established system in which tyloses are induced by pruning in grapevines (Sun et al., 2006). Clearly, conditions that trigger tylosis are also conditions that stimulate ethylene biosynthesis, leading us to suppose that the role of ethylene in tylosis formation might be quite general. Our results indicated that treatments with ethylene inhibitors significantly inhibited but did not completely block both ethylene production and tylose development in wounded grapevine stem. When wounding was conducted in air and in water, similar ethylene production (and tylose development) was observed. Our data could not tell the rate at which STS was binding to ethylene receptors. Although STS was effective in inhibiting tylose development, the slightly greater tylose development in the STS treatment compared to the AVG treatments implies that the concentration and treatment time of AVG was appropriate to efficiently block ethylene biosynthesis. These observations, in conjunction with the similarly low tylosis in the AVG and the AVG + STS treatments, indicate that ethylene is required for wound tylosis. However, our data do not address whether other wound responses are required, i.e. whether ethylene alone is sufficient for wound-induced tylose formation.

Our conclusions are based on a specific environmental stimulus (wounding) and on experiments carried out only in grapevine. However, the hypothesis that ethylene is a general causative agent for tylose formation is particularly attractive because it provides a plausible explanation for tylose development in response not only to wounding, but also to senescence, flooding, and pathogen infection. Our findings provide an explanation for the diverse relationships that have been observed between pathogen attack and tylose development, as well as the effects of other environmental stimuli on the process. Even the reported effects of auxin may be explained, because high auxin levels are known to stimulate ethylene production (Abeles and Rubinstein, 1964; Lieberman, 1979; Yu and Yang, 1979). Our investigation needs now to be extended to test the relationships between tylose formation and ethylene in other species, under other environmental stimuli, and in response to vascular infections such as Pierce's disease in grapes.

## MATERIALS AND METHODS

### Plant Materials

Grapevine (*Vitis vinifera*) L. 'Chardonnay' plants were grown from commercial rootings in 7.6-L pots containing a mixture of soil:peat:perlite (1:1:1,

w/w/w) in a greenhouse (22°C–25°C day and 18°C–20°C night). Pots were irrigated three times daily with 400 mL of one-half Hoagland nutrient solution (Hoagland and Arnon, 1950). Two shoots per plant were trained vertically. All experiments incorporated a pruning treatment in which experimental shoots, about 3 months old, were severed with a sharp razor blade in an internode around 50 to 70 cm above the base of a 2-m-long shoot.

### Effect of Embolisms on Tylose Development

Two treatments were conducted to investigate the possible effects of air embolisms on tylose development. Some shoots were cut in air, and the pruning cut was capped with a 3-mL air-filled rubber pipette bulb; other shoots were cut in degassed distilled water, and after 2 h in the water, the pruning cut was capped with a 3-mL rubber pipette bulb full of degassed distilled water. Immediately following treatment, a 4-cm-long stem segment of each excised shoot, including the cut end, was also retained and served as the day 0 sample. Replicate shoots from each treatment were sampled 4 and 8 d after cutting. Four-centimeter-long segments were obtained from the cut end of each shoot. Samples were prepared and evaluated for tylose development as described in "Analysis of Tylose Development."

To visualize embolisms in xylem vessels, a Cryo-SEM (Hitachi S-3500 N SEM attached with Polaron Cryo-system) was used to examine the presence or absence of water (embolisms) in vessels of samples of the two treatments. Each cut end, with the rubber bulb still attached, was frozen by immersing approximately 1.5 cm of the cut end in liquid nitrogen for 5 min. This portion was then removed with shears and remained in liquid nitrogen for at least another 5 min. The frozen stem segment was transferred to a rotary cryostat microtome at –30°C to –35°C. After the top 3 to 4 mm of the stem had been removed by serial sectioning, the remaining segment was mounted in a specimen stub with the trimmed surface upwards, transferred to liquid nitrogen for 5 min, and then to the preparation chamber of Cryo-SEM. The specimen was etched for 20 to 30 min at –90°C, coated with gold in the preparation chamber, and then observed at an accelerating voltage of 5 kV. The experiments for Cryo-SEM investigation were replicated two or three times for each treatment at 3 and 6 d after cutting.

### Analysis of Tylose Development

Tylose structure and number was evaluated as previously described (Sun et al., 2006). Briefly, for tylose structure, 30- $\mu$ m-thick sections were obtained with a sliding microtome at 2, 4, 6, 8, and 10 mm from the cut end. Sections were dehydrated and cleared in an ethanol-xylene series that incorporated staining with safranin O and fast green FCF, mounted coverslips in Permount, and observed under a light microscope (Olympus Vanox AHB, Olympus Optical) equipped with a digital camera (Pixera Pro 600ES, Pixera). To determine tylose numbers, each sample was free-hand sectioned transversely at 2, 4, 6, 8, and 10 mm from the cut end, mounted in water with a coverslip, and observed by light microscopy. In each section, five areas were randomly selected for analysis. In each area, 40 to 50 vessels (occurring in 2–6 consecutive xylem sectors) were evaluated for tylose development. Vessels were considered to have tyloses when they were present, regardless of the fraction of vessel area that was occluded by tyloses in transverse section, and that fraction was estimated and recorded for each vessel as an indicator of the developmental status of tyloses in a vessel. Based on that fraction, all vessels with tyloses were then classified into six categories: <10% (vessels with <10% of vessel area in transverse section filled by tyloses), 10% to 29%, 30% to 49%, 50% to 69%, 70% to 89%, and >90%. The number of vessels in each category was determined for each area and expressed as a percentage of the total number of vessels.

### Measurements and Analyses of Ethylene Production

We estimated ethylene production in pruned stems by measuring the ethylene evolved from the cut stem end. To collect gas evolved from the cut, a 3.5-cm-long rubber tube was attached immediately after each treatment (see the following section for details of all treatments) and gas was collected in a 3-mL syringe (Fig. 1A). After 1 h, the ethylene concentration in the accumulated gas in the syringe was measured, as described by Hunter et al. (2004), using an analytical gas chromatograph (Carle Instruments) fitted with a photoionization detector (model PI-51-01, HNU Systems). Samples were collected hourly for 36 h and then at 2- to 5-h intervals for an additional 36 h. Three or four replicate shoots were used for the ethylene measurement

with each treatment and ethylene production is reported as the concentration in the 3-mL headspace. Because of slight differences in the diameter among the shoots, ethylene concentration data was normalized to a shoot with a 7.2-mm diameter (approximate mean diameter).

### Treatments with Ethylene Inhibitors

Shoots were treated, at pruning, with AVG, an inhibitor of ethylene biosynthesis, by blocking synthesis of aminocyclopropanecarboxylic acid, the precursor of ethylene (Amrhein and Wenker, 1979), and/or STS, an inhibitor of ethylene action, by saturating ethylene receptor sites (Veen and van de Geijn, 1978). Shoots were cut in a degassed solution of 0.5 mM AVG prepared from ReTain (a formulation containing 15% [w/w] AVG; Mir et al., 1999) or of freshly prepared 4 mM STS, and the cut ends remained in the inhibitor solution for 2 h before being exposed to air. In addition, some shoots were exposed to both inhibitors before being exposed to air, i.e. cut in 4 mM STS solution, and after 2 h transferred to 0.5 mM AVG solution for another 2 h. As a control for the ethylene inhibitor treatment experiment, some shoots were cut in degassed distilled water and the cut ends remained in the water for 2 h before exposure to air. Shoots pruned in air served as an additional control. Samples were collected 3, 6, 9, 13, and 18 d after cutting. Three or four 4-cm-long stem segments with the cut end included were obtained at each sampling day for each of the five treatments. A 4-cm-long stem segment of each excised shoot, including the cut end, was also retained immediately following treatment and served as the day 0 sample. All samples were fixed immediately in formalin-acetic acid-alcohol (Ruzin, 1999) for subsequent analysis of tylose development as described above.

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