

WOUND-INDUCED VASCULAR OCCLUSIONS IN *VITIS VINIFERA* (VITACEAE): TYLOSES IN SUMMER AND GELS IN WINTER¹

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Vascular occlusion in xylem conduits is a common response to environmental stresses, and plant species are recognized as primarily tylose-forming or gel-forming. These stresses occur throughout the year, but there is little information on the wound responses throughout the year and in growing and dormant tissues. Wound-induced vascular occlusions were evaluated by type (tylose or gel), temporal progress, and spatial distribution for grape stems pruned in four seasons through an entire year. Tyloses were formed predominantly in summer and gels in winter. Cytohistological analyses indicated that wound-induced gels were pectin-rich. Both gel formation and tylose development were complete within 7 d and 10 mm from the cut regardless of the season of the wounding. Most vessels were affected by wounding, but a higher fraction of vessels developed occlusions in summer and autumn (over 80%) than in winter and spring (about 60%). The study is the first to show a single species is capable of producing primarily either tyloses or gels and that the type of wound-induced occlusion is dependent upon the season in which wounding occurs. Winter conditions limit the wound response to reversible gel formation that may contribute to refilling of embolized vessels in the spring.

Key words: parenchyma; pathogen; tyloses; vascular occlusions; vessel; Vitaceae; *Vitis vinifera*; water transport; xylem.

The xylem network of conduits is comprised of cylindrical cell wall remnants in which water, nutrients, and other solutes such as growth regulators are transported. Although the concentration of solutes in xylem sap is generally quite low (Enns et al., 2000), sundry microbes including pathogenic fungi and bacteria inhabit the xylem if given the opportunity. For most pathogens, this opportunity is created when wounds, such as during herbivory, expose the xylem vasculature. In response to both pathogens and wounding, vascular occlusions develop in the conduit lumen. It is generally believed that vascular occlusions that occur during leaf abscission and in response to wounding are required in many species for wound sealing and reducing the risk of pathogen intrusion (Bonsen and Kučera, 1990; Dute et al., 1999; Saitoh et al., 1993). The vascular occlusions that develop in response to infection are often thought to be involved in the compartmentalization of pathogens for disease defense, but this interpretation has been questioned (Pearce, 1996; Moersbacher and Mendgen, 2000; Sun et al., 2006).

Although xylem conduits are primarily dead cells, they are in contact and communication, especially via pits, with living paratracheal parenchyma cells that surround vessels. Paratracheal parenchyma are active in regulating xylem conduit contents. Tyloses and gels (also called gums in many earlier publications, see Rioux et al., 1998) are the two main types of vascular occlusions, and both originate from paratracheal parenchyma. Tyloses are outgrowths of parenchyma cells into the vessel lumen (Esau, 1977), and gels are amorphous substances secreted into the vessel lumen from the parenchyma cells (Harling and Taylor, 1985; Rioux and Ouellette, 1989). During tylose formation, ultrastructural changes in parenchyma cells and cell walls (Foster, 1967; Pearce and Holloway, 1984; Barnett et al., 1993) may include the accumulation of phenolic

compounds (Schmitt and Liese, 1993; Rioux et al., 1998) and crystals (Ranjani and Krishnamurthy, 1988) in the cytoplasm, and even cell divisions inside tyloses (Schmitt and Liese, 1994). Wound- and pathogen-induced gels are pectin-rich substances (VanderMolen et al., 1977; Shah and Babu, 1986) that can later become crosslinked by proteins (Soukup and Vitrubová, 2005). Woody angiosperm species typically react to insult by producing tyloses or by secreting gels (e.g., Schmitt and Liese, 1993).

Grapevines produce tyloses in response to the pathogenic xylem bacteria *Xylella fastidiosa* (Stevenson et al., 2004) and in response to wounding (pruning) in summer (Sun et al., 2006). However, environmental stimuli such as wounds from insect or vertebrate feeding can occur at any time throughout the growing season. In viticulture, shoots are pruned in summer and in winter, and it is in winter that pruning wounds lead to invasion of wood rot fungi (Munkvold and Marois, 1995). Yet, little is known about the formation of vascular occlusions in different seasons in grapevine or other systems. This study was conducted to investigate whether there is a seasonal component to the wound response of vascular occlusion in grape stems.

MATERIALS AND METHODS

Pruning treatment of current-year shoots—Current-year shoots of six-year-old *Vitis vinifera* var. Chardonnay plants grown in the UC Davis experimental vineyard were subjected to pruning treatments during four seasons: summer (30 June), autumn (25 August) in 2004, and winter (25 January) and spring (28 March) in 2005. Pruning cuts were imposed as previously described (Sun et al., 2006) at 10–20 cm from the shoot base on three replicate shoots for each experiment, one shoot on each of three vines. In each season, all experimental shoots were treated and samples collected on the same day. Samples for day 0 were collected from the cut end of each detached shoot and fixed immediately in formalin-acetic acid-alcohol (FAA) (Ruzin, 1999). Subsequently, samples were taken from the cut end of the attached stem at various days after pruning. At each sampling, three samples consisting of a 4-cm long stem segment including the cut end were taken and fixed in FAA immediately for at least 48 h. For analysis, each sample was hydrated to water via an ethanol series of 50%, 40%, 30%, 20%, and 10% for 30 min each. Transverse freehand sections were made at depths of 2–10 mm from the cut, then observed under a microscope (Olympus

¹Manuscript received 16 February 2008; revision accepted 11 September 2008.

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Vanox AHBT, Olympus Optical, Tokyo, Japan) equipped with a digital camera (Pixera Pro 600ES, Pixera Co., Los Gatos, California, USA).

Cytochemical tests of major components of gels—Two winter samples (days 9 and 14) and two spring samples (days 11 and 14) were analyzed for gel composition. Some areas were selected and photographed before treatment. The same areas were also observed and compared after any staining or treatment to detect any major chemical components of gels. In preparation to test for lipid components of gels, sections were dehydrated to 70% ethanol through an ethanol series with 10 min at each step, and stained with saturated Sudan IV in 70% ethanol for 10 min. The sections were then temporarily mounted with 70% ethanol to check whether the gels in vessel lumens stained red. The ninhydrin-Schiff's reaction method and aniline blue black staining were used to test for protein (O'Brien and McCully, 1981; Ruzin, 1999). For detecting any pectin components of gels, sections hydrated to water were treated with pectin-staining dyes (coriphosphine O and ruthenium red), periodic acid-Schiff's (PAS) reagents, and pectinase, (Jensen, 1962; Webster, 1973; Ueda and Yoshioka, 1976). When treated with coriphosphine O, the sections were stained in 0.03% coriphosphine O in distilled water for 15 min, followed by a rinse in distilled water, and then temporarily mounted in 65% sucrose solution. The stained sections were observed and examined for the distribution of orange fluorescence in xylem tissue after excitation with violet (excitation filter 405 nm, barrier filter 455 nm) or blue (excitation filter 490 nm, barrier filter 515 nm) light. When treated with ruthenium red, sections hydrated to water were stained in freshly prepared 0.005% ruthenium red in distilled water for 10 min, rinsed in distilled water, and then temporarily mounted in 10% glycerin solution for observations. Some sections were also treated with PAS reagents as described in Ruzin (1999). To avoid a false positive result possibly from the aldehyde fixation, some sections were treated with Schiff's reagents without a previous treatment with periodic acid as a control of the PAS treatment. When treated with pectinase, sections were put in a 10-ml vial with 2–3 ml of undiluted pectinase solution at pH 4.0 (polygalacturonase solution from *Aspergillus niger*, Sigma-Aldrich, St. Louis, Missouri, USA). The vial was capped and incubated in a water bath at 50°C for 28 h. After cooling to room temperature, the sections were rinsed in distilled water two times and temporarily mounted in water with or without staining in ruthenium red to examine the presence or absence of the gels present previously. Control sections were incubated under the same conditions but with distilled water instead of the pectinase.

Light microscopy and quantitative analysis of gels and tyloses—The sections were temporarily mounted with a cover slip in water for light microscopy. Five areas, each containing 4050 vessels and including some consecutive xylem sectors bounded by rays, were chosen randomly for analysis. The analysis of each replication (cut stem) included 200–250 vessels. All the vessels in each area were counted into the following categories: vessels without occlusions, vessels with gels, vessels completely occluded by gels, vessels with tyloses, vessels completely occluded by tyloses, vessels with gels or tyloses, and vessels completely occluded. Partial and complete occlusions were determined by visual inspection at low magnification.

RESULTS

Winter pruning wound responses—There were generally no vascular occlusions in stems of grapevines in unpruned controls in winter (Fig. 1A). In response to pruning, gels were observed as the major occlusion type in vessel lumens of secondary xylem (Fig. 1B). At the early stage of gel secretion to vessels (observed as early as day 3), gels were translucent, amorphous, uniform in texture, and deposited only at the vessel lateral walls where axial parenchyma cells were present.

Initiation of gel secretion was nearly simultaneous in different parenchyma cells around a vessel. Gels appeared patchy (Fig. 1C) when few parenchyma cells were present around the vessel, then merged to form a layer attached to the inner lateral wall of the vessels (Fig. 1D), and usually had a loosely wavy surface toward the center of the lumen (Fig. 1E). The number of wave crests was frequently similar to the number of surrounding parenchyma cells. Continued secretion of gels finally sealed

some vessel lumens as early as day 5 after wounding. Gels completely sealing a vessel lumen were transparent, uniform in texture, and colorless at the beginning, and then turned dense due to light-refractive particles inside, occasionally turning yellowish in unstained sections (Fig. 1F) and finally dark brown. Tylosis occurred in very few vessels, usually beginning after day 6. Tyloses can be easily distinguished from gels even though they might be of similar size, because tyloses contain cytoplasm and are not uniform in texture. In a vessel with tyloses, only a few of the axial parenchyma cells surrounding a vessel were involved in tylose development, and the number of tyloses in transverse sections was usually fewer than six. These tyloses appeared as round or oval balls with a diameter or a wide dimension of 5–30 μm , rarely over 40 μm (Fig. 1G). Most tyloses turned yellow or brown from their usually colorless original status. In a few vessels, gels and tyloses appeared together (Fig. 1H). In these cases, tyloses failed to increase in number and size, while gel deposition continued until the vessel lumen was completely sealed. Complete occlusion of vessels by enlarged tyloses was very rare.

Cytochemical properties of vascular occlusions—Several histochemical tests were used to evaluate the components of pruning-induced gels (Table 1). Gels emitted orange fluorescence in specimens treated with coriphosphine O (Fig. 2A, B) and were stained red by ruthenium red (Fig. 2C), indicating that wound-induced gels were pectin-rich. Negative results with Sudan IV, the ninhydrin-Schiff's reaction, and aniline blue black indicate that gels contained little or no lipids or proteins. However, these tests may well have not detected small but significant quantities of either, and some lipids may have been extracted during sample preparation. Gels in vessel lumens did stain red after treatment with the periodic acid-Schiff's reaction (Fig. 2D), indicating the presence of aldehydes, which were probably derived from the oxidation of saccharides by periodic acid. When sections with prominent gels in vessel lumens (Fig. 2E) were treated with pectinase at 50°C for 28 h, the gels disappeared (Fig. 2F), and ruthenium red no longer produced red components in the vessel lumens. The control sections (treated with water only) showed a continued presence of the gels in vessel lumens and stained with ruthenium red. Gels formed after wounding in spring yielded the same histochemical reactions.

In secondary xylem of grapevine stems, there are three structure elements in addition to vessels: xylem fibers, axial parenchyma, and ray parenchyma. All axial parenchyma are paratracheal, and some fiber cells and ray parenchyma cells have direct contact with vessels. In winter, starch grains were observed in xylem fibers and most ray parenchyma cells, but not in axial parenchyma cells or ray parenchyma cells with direct lateral wall contact with vessels. Pectin-rich components were detected in the axial parenchyma cells and those ray parenchyma cells that were in contact with pectin-containing vessels (Fig. 2A, B). No positive pectin reactions were detected in ray parenchyma cells not in direct contact with vessels or in xylem fibers (Fig. 2B). These same observations were made in the samples pruned in spring.

Wound-induced gels and tyloses in various seasons—Upon wounding in winter, the percentage of vessels with gels (PVG) generally increased at all depths until saturating at about day 11 (Fig. 3A). The PVG rapidly increased at 2 mm and 4 mm from day 3 to day 9, but increased slowly, particularly until day 7, at

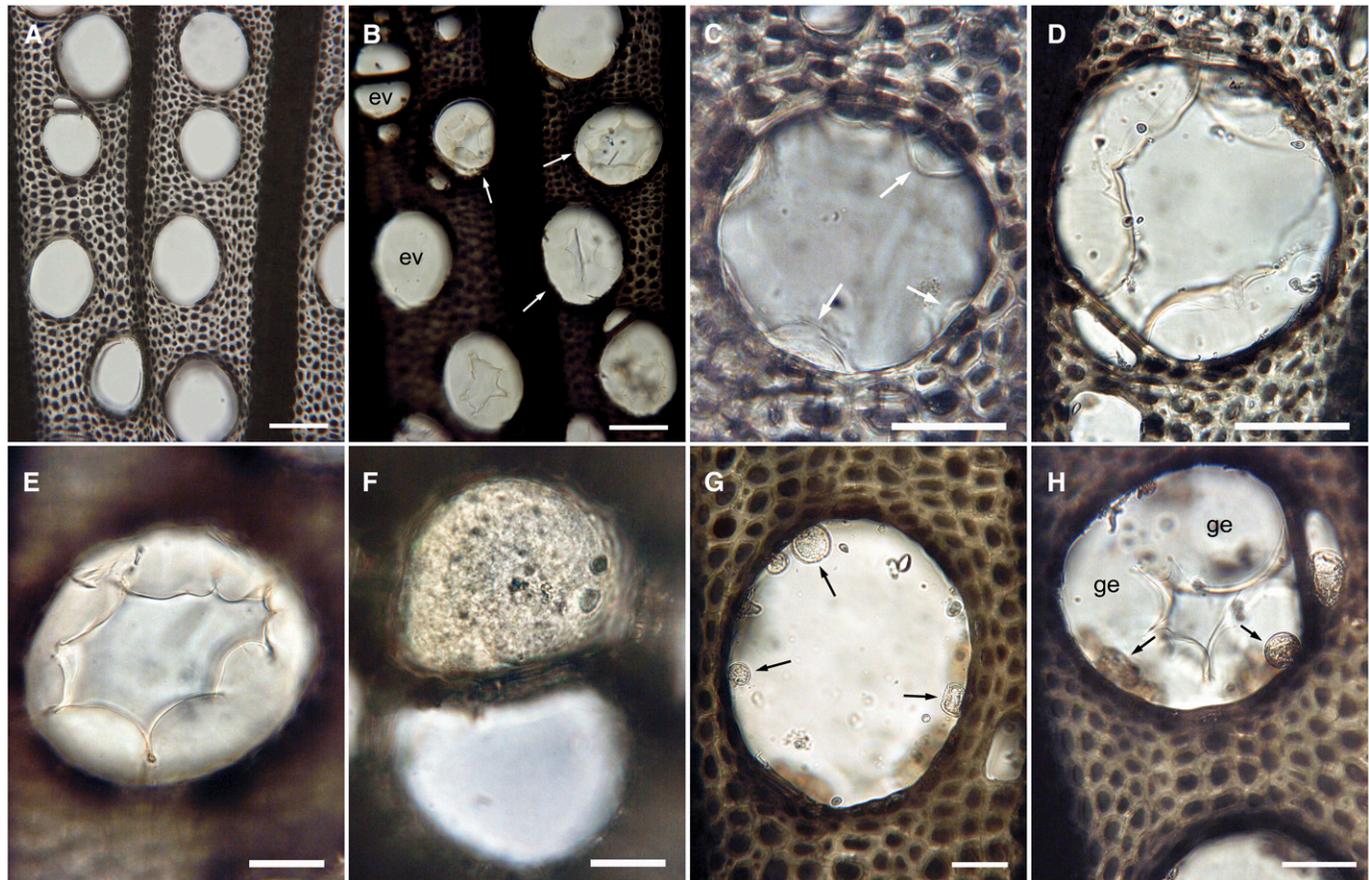


Fig. 1. Wound-induced vessel occlusions in winter. (A) Day 0, no occlusions. (B) Day 11, translucent occlusions present in most vessels (arrows); empty vessel (ev). (C) Initial appearance of translucent gels from several places along vessel lateral walls (arrows). (D) Translucent gels present in a thin layer with some discontinuities around vessel lateral walls. (E) Continuous layer of gels with wavy edge toward the vessel lumen. (F) The lumen in the vessel is completely occluded by yellow gels with small particles. (G) Vessel lumen with small tyloses (arrows). (H) Vessel lumen occluded by gels (ge) and small tyloses (arrows). Bar equals 100 μ m in A and B, 40 μ m in C–H.

the other depths. The PVG at saturation was an inverse function of the depth from the wound, ranging from about 60% at 2 mm to about 30% at 10 mm. The percentage of vessels with tyloses (PVT) exhibited similar patterns over time and depth as shown by the PVG, although the tyloses appeared to saturate after 14 d instead of 11 d, and the maximum values were considerably lower (Fig. 3B). Maximum PVT was about 10% at 2 mm and less than 3% at 10 mm (Fig. 3B).

Gel formation in vessels was highly dependent upon the season in which stems were pruned. The PVG in stems pruned in

summer did not reach a measurable level, and the PVG in stems pruned in autumn was not over 8% even at day 20 after pruning (Fig. 3C). In contrast, the PVG reached 60% in stems pruned in winter before decreasing again to about 35% in spring. The spatial distribution (depth) of wound gel formation was unaffected by season of wounding. The primary seasonal difference in tylose formation was also in the quantity of affected vessels. PVT reached at least 70% in summer and 90% in autumn, decreased to less than 10% in winter, and increased to 45% in spring (Fig. 3D).

Consequences of vascular occlusions for xylem transport are dependent on the fraction of affected vessels regardless of type—gels or tyloses. Both the percentage of vessels with occlusions and the percentage of vessels fully occluded were lowest (64% and 51%, respectively) in winter, greater in spring (73% and 61%, respectively), and highest in summer (89% and 51% at day 7, respectively) and autumn (88% and 74%, respectively) (Fig. 4).

DISCUSSION

Novel alternation of wound-induced vascular occlusion types in grape stems—The results of this study demonstrate

TABLE 1. Histochemical tests of wound-induced gels in stems of *Vitis vinifera*.

Dye or reaction	Target of detection	Result of test
Sudan IV	Lipids	Negative
Aniline blue black	Proteins	Negative
Ninhydrin-Schiff's reaction	Proteins	Negative
Periodic acid-Schiff's reaction	Polysaccharides and other compounds from which aldehydes are created by oxidation of periodic acid	Positive
Coriphosphine	Pectins	Positive
Ruthenium red	Pectins	Positive

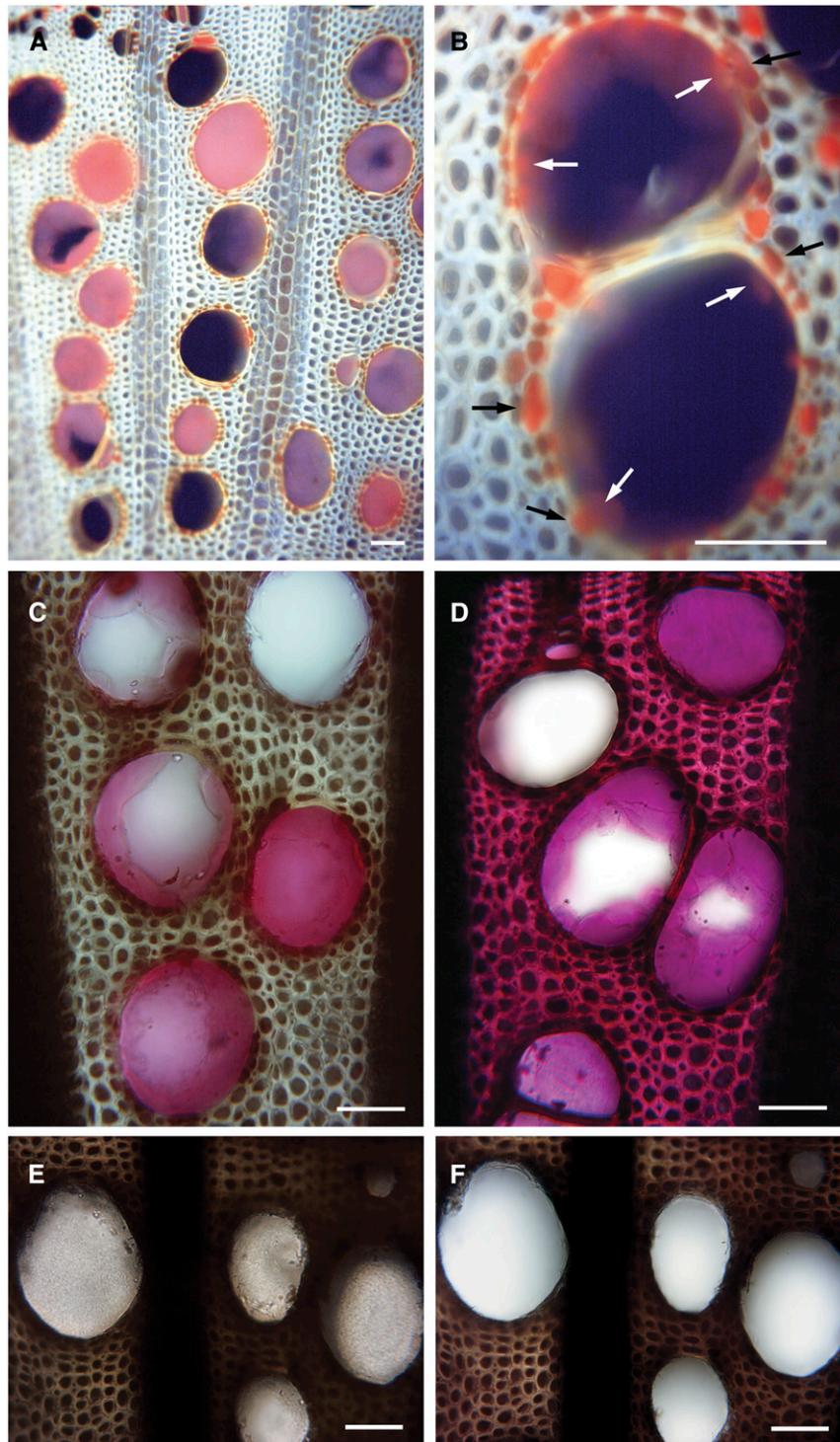


Fig. 2. Cytochemistry of vascular occlusions induced by wounding in winter. (A, B) Fluorescent micrographs of secondary xylem treated with coriphosphine O to stain for pectin. (A) Orange fluorescence from gels in vessel lumens. (B) Orange fluorescence in vessel lumens and axial parenchyma, showing origin of gels (white arrows) from paratracheal axial parenchyma cells (black arrows). (C) Secondary xylem stained with ruthenium red; gels in vessel lumens stained in red. (D) Secondary xylem stained with PAS reagents; gels stained in red. (E) Gels present in vessel lumens before pectinase treatment. (F) Absence of gels in vessel lumens after 28 h of pectinase treatment. Bar equals 60 μ m in A–D, 50 μ m in E and F.

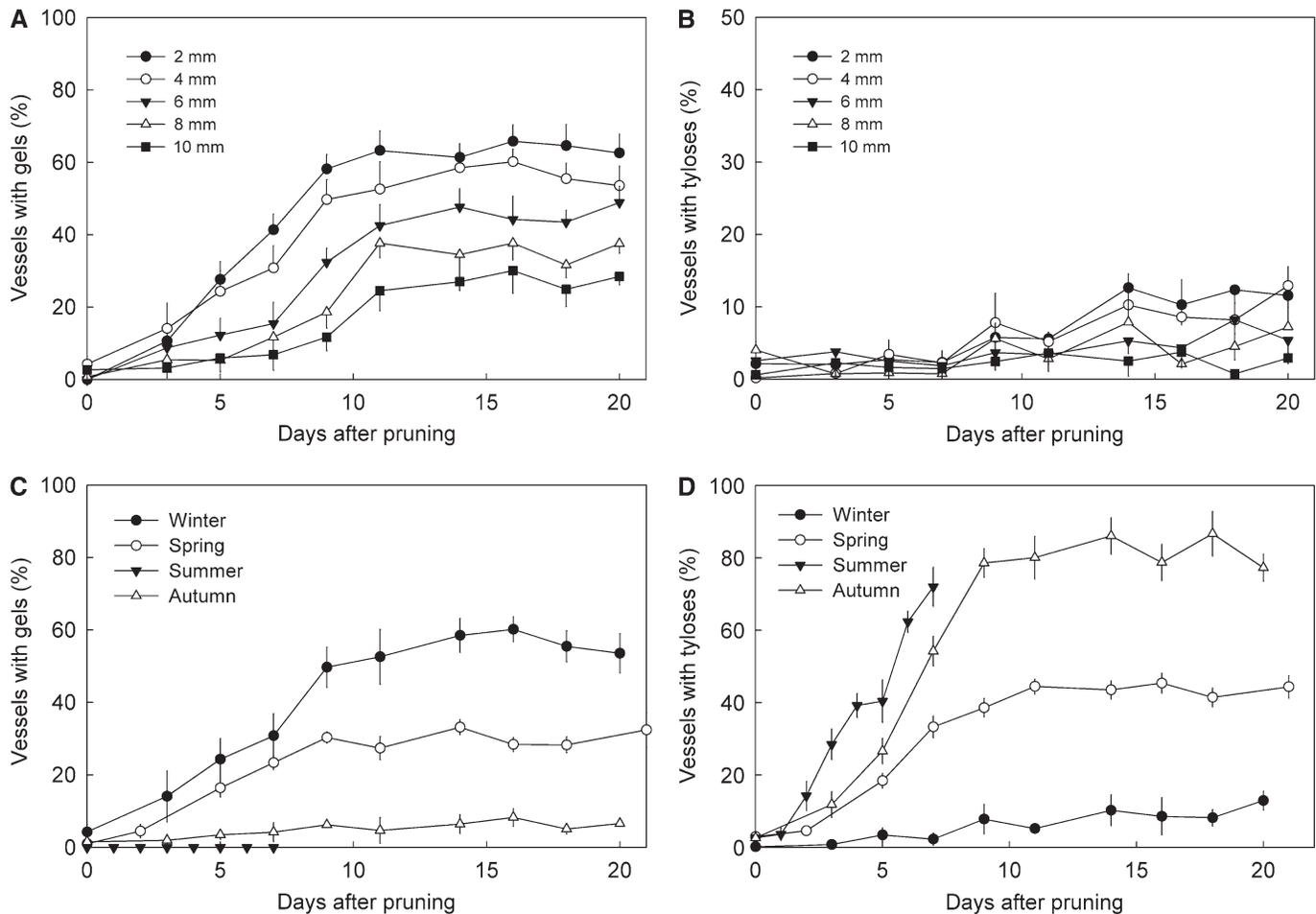


Fig. 3. Temporal progress of (A) wound-induced gels and (B) tyloses at 2, 4, 6, 8, and 10 mm from the cut surface in stems in winter and of (C) wound-induced gels and (D) tyloses at 4 mm from the cut in stems cut in various seasons. The summer assays were terminated after 9 d based upon a few preliminary assays that suggested tylosis would run its course in that time. Each datum point is a mean with one standard deviation ($N = 3$). Percentage of vessels with gels in summer was lower than measurable.

that grapevines develop two distinct types of wound-induced vascular occlusions in grapevine stems that depend on the season in which wounding occurs. In response to pruning, a large fraction of vessels developed occlusions in all four seasons, but the occlusions were almost exclusively gels in winter and primarily tyloses in summer and autumn. The seasonal alternation of wound-induced gels and tyloses in the same species is apparently a novel observation. At the species level (regardless of woody or herbaceous species), plants are reported to form prominently either tyloses or gels, especially when wounded (Chattaway, 1949; Bensen and Kučera, 1990; Schmitt and Liese, 1990; Rioux et al., 1998). Tylose-producing species include many species of *Quercus* (oaks), *Ulmus* (elms, Elgersma, 1973), *Castanea* (chestnuts), *Solanum* (tomato), *Musa* (banana, Beckman and Halmos, 1962), *Gossypium* (cotton, Mace, 1978), and *Humulus* (hop, Talboys, 1958). Gel-forming species include many species of *Betula* (birch), *Pisum* (pea, Bishop and Cooper, 1984), *Chrysanthemum*, *Dianthus* (carnation, Baayen et al., 1996) and *Phragmites* (reed, Soukup and Vitrubová, 2005). Both tyloses and gels were produced in grapevine (Stevenson et al., 2004) and *Platanus* trees (Clériveret et al., 2000) infected with xylem-specific pathogens, and gels can be se-

creted directly from tyloses (Bensen and Kučera, 1990; Rioux et al., 1998). However, the gels formed in these cases were far from prominent in xylem tissue compared to gel-forming species and to the wounded stems of grape in winter.

It should be pointed out that previous data on vascular occlusions have been based mostly on samples from actively growing plants (during growing seasons or in greenhouses), so that very little information is available about possible seasonal changes of vascular occlusions. Thus, it is not clear whether the seasonal alternating wound response in grape is common or not. Schmitt and Liese (1992) found that wound-induced gel secretion in *Betula* and *Tilia* stems (two gel-forming species) was high in summer and autumn but low or nonexistent in winter. Although there were no quantitative data showing the proportion of vessels occluded by gels at the level of xylem tissue, their results suggest that after wounding few vessels became occluded in winter despite the extensive occurrence of vascular occlusions in summer. The proportion of vessels with vascular occlusions in the current study with grape stems was also higher in summer than in winter, but more than 60% of all the vessels in secondary xylem became occluded even in winter.

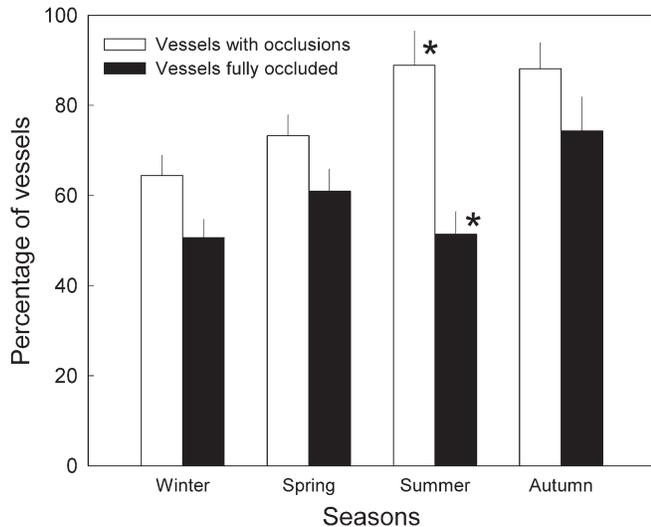


Fig. 4. Seasonal variations of wound-induced vascular occlusions in stems. Percentage of vessels with occlusions and fully occluded with gels or tyloses at 4 mm from the cut on day 14 after pruning stems in four seasons. Asterisk indicates sampling at day 7. Each datum point is a mean with one standard deviation ($N = 3$).

Regulation of wound-induced vascular occlusion types—In an early comparative study of xylem anatomy among tylose- and gel-producing species, Chattaway (1949) suggested that the type of vascular occlusion is related to the dimensions of the bordered pit aperture between vessels and parenchyma cells. Tylose development was thought to be dependent on a minimum pit aperture of 3 μm in height and 8–10 μm in width, and gels are produced in species with pits with smaller apertures. Bonsen and Kučera (1990) and Soukup and Votrubová (2005) reported observations consistent with this hypothesis. Bonsen and Kučera (1990) suggested an additional factor in more recently evolved species in which tyloses are produced when the vessel diameter is over 80 μm . In current year shoots of grapevine, mean vessel diameter varied from 61 to 68 μm , the pit aperture dimensions of vessel-ray pits were larger than the suggested minimal values, and those of vessel-parenchyma pits had a range overlapping the critical values (Sun et al., 2006). However, tyloses formed through the pit apertures smaller than the suggested minimal value in summer, and gel secretion occurred through the pit apertures larger than the suggested value in winter. Furthermore, we did not see obvious differences in tylose-forming capacity among vessels of different diameters. Thus, the proposed physical regulation of vascular occlusion types via pit aperture dimensions was not observed in grape stems.

Tyloses and gels form in response to the same or similar conditions in many cases. Indeed, pathogens generally secrete hemolysins, which may evoke the same gene expression and physiological responses as herbivory. For example, wounding and the presence of xylem pathogens stimulate ethylene production (Abeles et al., 1992), and VanderMolen et al. (1983) observed gel deposition in vessel lumens in leaves of *Ricinus communis* (castor bean) when treated with exogenous ethylene. Sun et al. (2007) recently demonstrated that ethylene, and not embolism (Zimmermann, 1978), is the intrinsic factor required for wound tylose development in grapevine stems. The seasonal differences in wound gel development discussed earlier were partially but not completely attributable to temperature (Schmitt

and Liese, 1992). Although seasonal differences in wound ethylene production has evidently not been investigated, it is clear that a more complicated regulation than simply the production of wound ethylene is involved because most plants produce gels or tyloses, not both. There appears to be no information on the molecular genetic control of these wound responses, except for the possible involvement of sulfur transporters in both gels and tyloses (Cooper and Williams, 2004). Mitogen-activated protein kinase cascades are broadly implicated in the responses to disruption of cell wall integrity (Humphrey et al., 2007), but as yet these have not been tied to wound gel or wound tyloses. Wounding evidently stimulates paratracheal xylem parenchyma cells to produce pectins, which are incorporated into cell walls when tyloses are formed (Rioux et al., 1998) and secreted into the vessel lumen when gels are formed (Schmitt and Liese, 1992). Perhaps the full complement of wound response genes are available for induction in summer but only a subset are inducible during quiescent winter.

Wound-induced gel formation and function—Some ultrastructural investigations suggested that gels in the vessel lumen result from digestion of the cell walls (especially pit membrane) between vessels and parenchyma cells (VanderMolen et al., 1977), it now seems well established that the gels are pectin-rich (Koran and Yang, 1972; Rioux et al., 1998; Ouellette et al., 1999; Crews et al., 2003; Soukup and Votrubová, 2005) and derived from contiguous parenchyma (Rioux et al., 1995; Crews et al. 2003; present study). The brownish color of wound gels and surrounding tissues in grape stems should be considered to be phenolic compounds formed and secreted by paratracheal parenchyma cells (Weiner and Liese 1995; Pearce, 1996) and having some antimicrobial activity (Bostock and Stermer, 1989; Baayen et al., 1996). Similarly, accumulation of elemental sulfur in both tyloses and gels may contribute to pathogen defense (Cooper and Williams, 2004).

The functional effects of wound-induced occlusions presumably depend to some extent on the wound timing and on the proportion of affected vessels in transverse sections of a stem and may have little relation to the type of the vascular occlusions themselves. Wounds from pruning in early winter are more susceptible to infection by the devastating wood rot fungus *Eutypa lata* than those from late winter/early spring (e.g., Munkvold and Marois, 1995), but spores of other wood rot fungi are present year round (Eskalon and Gubler, 2001). The proportion of vessels with vascular occlusions was greater following pruning in summer and autumn than in winter and spring. Therefore, more pathways remain open to invasion following pruning of grapevines in winter, but at neither time was the plant completely sealed off from the external environment.

Both gels and tyloses could be involved in refilling embolized vessels (Canny, 1997; Crews et al., 2003). However, a contribution to refilling is more readily envisaged for the gel. Suberization is present with tylosis and weak or absent with gels, and the suberization makes the structures resistant to degradation, including degradation by fungi (Rioux et al., 1995; Pearce, 1996). Thus, the suberized tylose is an irreversible development that would remain as an occlusion in a refilled vessel, whereas the gel potentially is not. The low matric potential of a partially hydrated gel or the low solute potential of an enzymatically degraded gel could contribute to vessel refilling by drawing water through pits from parenchyma cells (McCully et al., 1998). Grapevines have gas-filled vessels in winter (at least in freezing climates) that must be refilled to initiate sap flow in

spring (Sperry et al., 1987), and parenchyma cells evidently supply water for refilling of embolized vessels in roots (McCully et al., 1998). Thus, gel (or solute) secretion may be a natural component of paratracheal parenchyma interaction with vessels during winter, either upon wounding or embolism. The limited testing of gel composition in this study indicated that it is mostly pectin, which would be consistent with a role in refilling, whereas a resinous or polyphenolic gel might be expected for microbial defense.

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