Evidence for a wounding-induced xylem occlusion in stems of cut chrysanthemum flowers

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Received 13 October 1999; accepted 24 December 1999

Abstract

A temperature-dependent xylem occlusion was found in cut chrysanthemum stems (Dendranthema grandiflora, cv. Viking) which were placed for 24 h in air at 5°C prior to vase life evaluation. The response was inhibited by a 5-h treatment, prior to placement in air, with aqueous solutions at low initial pH or solutions containing near-neutral antioxidants (n-propylgallate, phloroglucinol, butylated hydroxytoluene). Bacteria are known to occlude stems, but the occlusion was not related to bacterial counts in the stem ends. The number of cavitations in the xylem conduits, detected by ultrasonic acoustic emission, remained low during the storage treatment at high ambient relative humidity. The uptake of air into the stem ends ceased within 20 min whereas the occlusion developed only after several hours, showing that aspirated air was not the sole cause. A xylem blockage was also found in stems placed in water directly after cutting. In these flowers, treatments with anti-oxidants delayed the occlusion, but did not affect the number of bacteria in the stem ends. The onset of xylem cavitation occurred after the occlusion. The results suggest that the stem forms a xylem blockage both during dry storage and in stems directly placed in water. The blockage apparently involves oxidative reactions. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Wounding-induced xylem occlusion; Stems; Chrysanthemum flowers

1. Introduction

Mechanical wounding of plant tissues has been reported to increase the expression of numerous genes and to stimulate the activity of a range of enzymes, such as phenylalanine ammonia lyase (PAL; Lois et al., 1989), peroxidase (Lagrimini, 1991; Kawaoka et al., 1994), and ACC oxidase (Peck-Scott and Kende, 1999). Several of these enzymes are involved in the biosynthesis of compounds related to lignin and suberin (Espelie et al., 1986; Negrel et al., 1993; Moehs et al., 1996). These reactions to wounding primarily serve to impede the entry of micro-organisms into the opened tissue (Bucciarelli et al., 1998).

Wounding is known to result in occlusion of the xylem conduits. The occlusion may be due to deposition of materials such as gums in the lumen of xylem conduits (Chattaway, 1948; Davies et al., 1991; Kawaoka et al., 1994), and ACC oxidase (Peck-Scott and Kende, 1999). Several of these enzymes are involved in the biosynthesis of compounds related to lignin and suberin (Espelie et al., 1986; Negrel et al., 1993; Moehs et al., 1996). These reactions to wounding primarily serve to impede the entry of micro-organisms into the opened tissue (Bucciarelli et al., 1998).

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or to formation of tyloses, balloon-shaped outgrowths of cells adjacent to a conduit (Zimmermann, 1983). These occlusions also seem to prevent the entry of micro-organisms. In a single species, stem wounding may result in several types of occlusion, such as tylose formation and deposition of mucilage (Weiner and Liese, 1995).

Experiments on cut chrysanthemum by van Meeteren (1992) showed that the xylem becomes quickly occluded when the shoots are held in air. After cutting, some shoots were held for 1 h in air at 20°C. After this treatment, they had lost about 10% of fresh weight (FW), but when placed in water the rate of water uptake was high enough to compensate for shoot weight loss. Other shoots were enclosed in a plastic bag and held in air for 24 h, whereby they lost less than 2% of their weight. When placed in water their fresh weight did not recover, indicative of a serious occlusion. When air was drawn from the cut end, shoot fresh weight rapidly increased. These results suggest that the presence of emboli in the conduits is one cause of the blockage. These emboli may be due both to air that is aspirated into the conduits that are cut open and to cavitation in xylem conduits that remain unopened (Tyree and Sperry, 1989). In addition, if cut plant parts have stood in water prior to dry storage, the bacteria that are present in the xylem conduits keep on growing even during the dry storage period and may cause xylem blockage (van Doorn and de Witte, 1991). In chrysanthemum cv. Cassa a low rate of rehydration was found following a short period of desiccation. This effect was inhibited in flowers placed at 4°C rather than 24°C, prior to the period of dehydration (van Meeteren and van Gelder, 1999). This result suggested a physiological effect, but since bacteria in the stem ends were not enumerated it remained unclear whether the low temperature inhibited bacterial growth or had some effect on a reaction in the plant.

In the present experiments on chrysanthemum stems, four possible causes of the occlusion were tested. First, the possible role of cavitation was determined by assessment of its frequency. Second, the role of aspirated air was tested by determining the time course of air aspiration in relation to the time course of the blockage. Third, the possible role of bacteria was assessed, by determining bacterial counts in the stems. Fourth, the role of a metabolic response was tested by placing the shoots at various temperatures. Thus, we report on experiments in which we attempted to separate the effects of cavitation, aspirated air, bacteria, and a blockage of the xylem due to enzymic activity in the plant.

2. Materials and methods

2.1. Plants

Flowering plants of chrysanthemum (Dendranthema grandiflora, cv. Viking) about 80 cm long were harvested in the greenhouse of a commercial grower. The plants were harvested with their roots attached. Each plant had been grown from a cutting rooted in a 4 x 4 cm package of peat moss. The cuttings grew in a loose sandy soil in the greenhouse. This method ensured that most of the roots were intact at harvest. Harvested plants were packed in cardboard boxes and arrived at the laboratory within 3 h of harvest. In most experiments the stems were cut in air, at the root-shoot junction. The leaves from the lowermost 10 cm of the stems were removed.

Cut stems were placed directly in aqueous vase solutions or were held in air and then placed in vase water. In some experiments, the stems were placed for 5 h in solutions prior to holding them in air. In other tests, they were placed in a treatment solution for 15 min prior to placement in vase water. In order to determine the time to leaf wilting, flowers were each placed in deionised water, with or without added chemicals, in individual glass vials. The vials stood in a climate-controlled room at 20°C and 60% RH. A photosynthetically active photon flux of 15 μmol m⁻² s⁻¹ (Philips TDL 36W/84 cool white fluorescent tubes) was maintained from 07:00 to 19:00 h. Leaf wilting and other visible symptoms were recorded daily.
2.2. Placement in air

In some experiments, the shoots were held in air in the climate-controlled room. In order to prevent water loss, other shoots were placed in a large, tightly closed plastic bag (van Meeteren, 1992). Ten shoots were placed in each bag. Bags were stored for various periods of time and various temperatures. The RH inside the bags was close to 100%, and the bags were held in darkness. The temperature inside the bags was recorded in one experiment, using thermocouples.

2.3. Chemicals

All chemicals were from Sigma (St Louis, MO) unless otherwise indicated. Reductants (antioxidants) used were benzoic acid, L-ascorbic acid, n-propylgallate, butylated hydroxytoluene, and phloroglucinol. Some of these were first dissolved in a minimum volume of 10 ml ethanol. Solutions of pH 3–5 were made using acetic, citric, and propionic acids.

Inhibitors of ethylene synthesis or action used were aminoethoxyvinylglycine (AVG), 1-methylcyclopropene (1-MCP; Acros, Geel, Belgium) and silver thiosulphate (STS). STS was prepared by adding an aqueous solution of sodium nitrate (Merck, Darmstadt, Germany) to an eight-fold more concentrated solution of sodium thiosulphate (Merck). Most inhibitors were applied as a 5-h pulse prior to dry storage. The pulse occurred in the light, at 20°C and 60% RH. Ethylene (3 ppm) and 1-MCP (70 ppb) were injected into 70-l stainless steel drums in which flowers were placed. The experiment took place at 20°C and the RH in the containers was close to 100%. The gas concentrations in the drum head space were checked by gas chromatography.

Some chemicals (KOH, acetone, and hypochlorite) reportedly are able to dissolve a deposit on the xylem pit membrane (Gale 1982; Wheeler 1983). A 15-min pulse treatment at 20°C, after 24 h of storage in plastic bags at 5°C, was therefore given using 0.8 N KOH, 33% (v/v) acetone, or 0.2 M sodium hypochlorite. These stems had not been treated with aqueous solutions prior to storage.

2.4. Water uptake, transpiration, and water balance

The rates of water uptake and transpiration, and the water balance of the flowers (rate of uptake minus rate of transpiration) were calculated from regular weighing of the vials and the flowers, using ten replications.

2.5. Determination of ultrasonic acoustic emissions from stems

The method for monitoring UAEs has been described previously (van Doorn and Jones, 1994). In short, stems were placed horizontally and ultrasonic microphones (Model 8312 broad band transducer from Bruel and Kjaer, Naerum, Denmark) were clamped onto the stems at about 5 cm from the basal end using a force of about 20 kN. The signal was amplified using Bruel and Kjaer 2638 wideband conditioning amplifiers with a high pass filter (frequency 100 kHz–2 MHz). The number of UAEs was recorded using a Labtech Notebook program (version 5.03 from Laboratories Technologies Corporation, Wilmington, MA). Experiments were performed using two stems in two complete set-ups. All background spikes were eliminated by using a mains radiofrequency interference filter (150 KHz–30 Mhz, 35 dB attenuation, from RS Components Ltd, Corby, Northants., UK) and by subtracting any residual spikes that were detected by a microphone not connected to a stem.

2.6. Bacterial counts and prevention of bacterial growth in stems

Bacteria in the stems were counted using the method described by van Doorn et al. (1989). A distinction was made between bacteria on the stem surface and inside the stems, using 98% ethanol to disinfect the stem surface.

2.7. Uptake of air

The method used to determine the volume of air aspirated at the cut surface and the duration of air uptake was described previously (van Doorn,
1990). In short, chrysanthemum stems were cut under water at the shoot-root junction. Stems, with a drop of water hanging at the cut end, were then rapidly attached to the top of a pipette filled with air. The pipette was placed horizontally on the laboratory bench (in the climate-controlled room, at 20°C and 60% RH, in the light). A drop of acid fuchsin in the pipette moved with the air aspirated into the stem end. The time to the end of air uptake can be reliably determined using this method. The method may underestimate the volume of aspirated air as it takes a few seconds to attach the stem to the end of the pipette.

The rate of water uptake as related to the aspiration of air at the cut surface was determined by placing stems on the laboratory bench in the climate-controlled room for various periods of time and subsequently placing the stem ends in 10 cm of water. The rate of water uptake was determined at 1-h intervals.

2.8. Statistics

Results were compared by analysis of variance using the GENSTAT V program (Rothamsted, UK) and F-test at $P < 0.05$. All treatments included seven to ten replicate flowers. The experiments were repeated at least once.

3. Results

Vascular occlusion in the xylem was determined by assessing the rates of water uptake and transpiration and calculation of the water balance. A negative water balance, showing the presence of an occlusion in the stem, was found in all experiments. It was present from the first hour onward in stems placed in vase water following 24 h of storage at 5°C and 100% RH (Table 1). In stems directly placed in vase water the rate of water uptake on day 0–1 was more than three times higher than in stems placed in humid air at 5°C for 24 h before placement in vase water (data not presented). In stems placed in water directly after cutting, a negative water balance was found after about 3 days of vase life (Table 1). In both treatments, severe leaf wilting followed shortly after the onset of a negative water balance (Table 1) and was, therefore, taken as an indicator of the stem occlusion.

3.1. Xylem occlusion in stems placed in air following cutting

In order to prevent complications due to weight loss during dry storage the stems were placed in plastic bags that were well sealed. Under these conditions, water loss from the shoots remained below the detection limit. Leaves were fully turgid when the shoots were removed from the bags. When stored at 5°C for 24 h and subsequently placed in water, without re-cutting of the stems, the rate of water uptake was less than half that of the rate of transpiration during the first 3 days of vase life.

3.2. Effect of temperature on the occlusion in stems held in air

Shoots were stored in plastic bags for 12 and 24 h at temperatures varying from 0 to 30°C. Shoot weight loss during storage remained below the detection limit. All leaves were turgid when the shoots were removed from the bags. The stem ends were placed in water and the time to leaf wilting was recorded. It was positively correlated with temperature during storage and with the duration of storage (Fig. 1).

<table>
<thead>
<tr>
<th>Flowers placed in</th>
<th>Time to negative water balance (h)</th>
<th>Time to leaf wilting (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>humid air and then in water</td>
<td>0.0 ± 0.0</td>
<td>12.8 ± 4.3</td>
</tr>
<tr>
<td>Flowers placed directly in water</td>
<td>64.5 ± 8.2</td>
<td>74.9 ± 10.1</td>
</tr>
</tbody>
</table>

* The data refer to the period in water. Data are the means of ten replications ± S.D.
As temperature may affect bacterial growth, the number of bacteria in the stems was determined. In freshly harvested stems cut with standard sterile equipment the lowermost 5 cm of the shoots contained a relatively high number of bacteria, all located on the stem surface (Table 2). This indicates that low numbers of bacteria are present inside the xylem. When the stems were cut with non-sterile secateurs and the stem surface was sterilised with ethanol, the number of bacteria in the lowermost 5 cm was also low (Table 2). Thus, a low number or no bacteria were introduced at the cut surface. Following cutting with non-sterile secateurs and placement in humid air for 24 h at both 5 and 20°C, the number of bacteria located on the cut surface and inside the stems remained below the detection limit (Table 2).

3.3. Effects of chemicals on the occlusion developing during storage in humid air

Experiments on the effect of various chemicals all involved placement of the shoots in plastic bags at 5°C for 24 h. In experiments with low pH, antioxidants, and anti-ethylene compounds, the stems were held in aqueous solution for 5 h prior to the period in humid air. After treatment with antioxidants, such as L-ascorbic acid (1 mM, pH 3.9) and benzoic acid (0.5 mM; pH 3.4) the time to leaf wilting was delayed (Fig. 2A). However, this may be a pH effect, as controls for low pH also delayed the time to leaf wilting. At pH 5 these solutions had no effect, but at pH 4, depending on the acid used, wilting was delayed. At pH 3 a delay of leaf wilting was found with all three organic acids tested (Fig. 2A). Antioxidants with a higher pH, such as n-propylgallate (20 mM, pH 5.3) and phloroglucinol (100 mM, pH 5.8) also delayed the time to leaf wilting (Fig. 2B). Butylated hydroxytoluene (50 mM, pH 5.7) had the same effect (Fig. 2B) despite the formation of a precipitate, as soon as the ethanol in which it was dissolved was added to water.

Pulse treatments with the anti-ethylene compounds AVG or STS for 5 h prior to placement in plastic bags at 5°C for 24 h had no effect on the time to leaf wilting. A treatment with 1-MCP during placement in humid air also did not affect the time to subsequent leaf wilting in shoots subsequently placed in water (data not shown).

The literature reports deposition of material on the conduit-to-conduit pit membranes which can be removed by KOH, acetone, or hypochlorite (Gale, 1982; Wheeler, 1983). Pulse treatments for 15 min with these compounds, directly following storage in plastic bags (at 5°C for 24 h) also considerably delayed leaf wilting (Fig. 2C).
3.4. Bacteria in stems held in humid air

Bacterial counts in the basal 5 cm of the stems were not affected by a 5-h pulse treatment, prior to holding the stems in plastic bags for 24 h at 5°C, with n-propylgallate, phloroglucinol, ascorbic acid, and benzoic acid (Fig. 3A). Bacterial counts in control stems held for 5 h in water were usually below the detection limit, after the period of storage in plastic bags (Fig. 3A). However, in one repeat experiment one out of three stems contained about 1000 cfu g FW\(^{-1}\). A 15-min pulse treatment with KOH or bleach (hypochlorite) following storage in plastic bags resulted in a low number of bacteria on day 1, but on day 4 of vase life the bacterial counts were the same as those of the controls (Fig. 3B).

3.5. Xylem occlusion in stems directly placed in water

When the roots were severed at the root-shoot junction, and the shoots immediately placed in water, the rates of water uptake and transpiration during the first day of vase life were equal. Both the rates of water uptake and transpiration dropped to progressively lower values during the following days (data not shown). The average time to leaf wilting was about 3.5 days (Fig. 4). Inclusion, in the vase water, of n-propylgallate, benzoic acid, or L-ascorbic acid considerably delayed the time to leaf wilting (Fig. 4). Inclusion of the anti-ethylene compounds AVG or STS in the water had no effect (results not shown).

The number of bacteria in the basal 5 cm of the stem, in shoots that were cut at the root-shoot junction and immediately placed in water rapidly increased. This increase occurred irrespective of the inclusion of ascorbic acid, benzoic acid, or n-propylgallate in the solution (Fig. 5).

When roots were cut under water and the shoots immediately placed in vase water, the time to leaf wilting was considerably delayed with respect to cutting in air (Fig. 4).

3.6. Ultrasonic acoustic emissions (UAE)

The frequency of UAE in stems that were cut at the root-shoot junction and then held in air at 20 or 5°C and 100% RH in darkness remained very low throughout the 24 h of the test, resulting in a low accumulated number of UAE (Fig. 6). When stems were placed in water directly after cutting, the accumulated number of UAE remained low for a few days and then increased (Fig. 6). This increase occurred after the leaves started to wilt.

3.7. Uptake of air

After cutting, stems took up about 0.10 ml of
Fig. 2. Time to leaf wilting in cut chrysanthemum shoots (cv. Viking). Controls included those for ethanol, which was used as a solvent for some of the chemicals. Results are the means of ten replications ± S.D. LSD (P < 0.05) was 2.1. A and B: Shoots were cut at the shoot-root junction and placed in aqueous solution for 5 h at 20°C. They were then kept in humid air for 24 h at 5°C and subsequently placed in vase water at 20°C. The chemicals used during the 5-h treatment were given at various initial concentrations (mM) or at various initial pH (3, 4 or 5). Phlorogl., phloroglucinol. C: Shoots were cut at the shoot-root junction and directly placed in humid air for 24 h at 5°C. They were then gently shaken in treatment solution at 20°C for 15 min and subsequently placed in vase water at 20°C. Concentrations used: KOH 0.8 N, acetone 33% (v/v), and sodium hypochlorite (bleach) at 0.2 M.

4. Discussion

The results show that the xylem of cv. Viking chrysanthemum stems is rapidly occluded following cutting. This reaction occurs both in stems that are directly placed in water (Fig. 4) and in those that are first stored in humid air (Fig. 2).

In the case of shoots held in humid air prior to placement in water, the time to leaf wilting depended on the temperature during storage (Fig. 1). Such temperature dependence may be due to bacterial growth in the stems. However, in our tests bacterial numbers in the stem ends always remained below the detection limit during the

air (0.095 ± 0.023, n = 8). Highest uptake occurred during the first few minutes. Uptake ceased after 6–20 min (Fig. 7), the average duration of air uptake being 11.8 ± 5.8 min (n = 8). The rate of water uptake directly after cessation of air uptake was only a little impaired (data not shown). A clear reduction of water uptake occurred only after 2–3 h of dry storage at 20°C and after at least 12 h of dry storage at 5°C and 100% (data not shown). When stems were prepared for storage in plastic bags, they were usually held in air at 20°C for about 10–15 min. Therefore they took up most of the air prior to storage at high humidity.
period of dry storage if the stems were not placed in water prior to storage (Table 2). Bacterial growth is therefore excluded as a cause of the differences observed in Fig. 1. Uptake of air and the rate of cavitation may also be temperature dependent, but the former was restricted to the first 20 min after cutting (Fig. 7) and the latter remained very low (Fig. 6). The temperature dependence, therefore, indicates an enzymic reaction in the plant.

The hypothesis of a role of a wounding-related effect in stems that were held in humid air before placement in vase water is strengthened by the tests with antioxidants. The delay of leaf wilting by the acid antioxidants, such as ascorbic and benzoic acid, may have been due to their low pH. Treatments with acids not known as antioxidants resulted in a similar delay in wilting. However, near-neutral antioxidants, such as n-propylgallate, phloroglucinol and butylated hydroxytoluene,

![Graph](image1)

**Fig. 3.** Bacterial counts in the basal 5 cm of cut chrysanthemum flowers cv. Viking. Data are the means of three replications. A: After a 5-h pulse treatment with aqueous solutions of various chemicals (ending on day 1), holding the stems at 100% RH for 24 h at 5°C (ending on day 0), and 4 days of vase life. Statistical analysis showed no differences (at $P \leq 0.05$) on any of the days. B: After holding the stems at 100% RH for 24 h at 5°C (ending on day 0), a 15-min pulse treatment with 0.8 N KOH or 0.2 M bleach (sodium hypochlorite), and 4 days of vase life. Statistical analysis showed a difference (at $P < 0.05$) on day 1 only.

![Graph](image2)

**Fig. 4.** Time to leaf wilting in cut chrysanthemum shoots cv. Viking directly placed in vase water at 20°C following cutting in air (not indicated) or under water (indicated). Various chemicals were included in the vase water of the stems cut in air on day 0 of vase life, with initial concentrations shown in mM. Data are the means of ten replications ± S.D. LSD ($P < 0.05$) was 2.4.
also considerably delayed the time to wilting. This response indicates a role of oxidation in a wound-}

ing reaction. The effect of n-propylgallate and phloroglucinol was independent of bacterial counts in the lowermost stem segment, which indicates that they inhibit a plant process. van Meeteren (1992) also found inhibition of water uptake in chrysanthemum stems that were stored in a nitrogen atmosphere. This result indicates that some of the inhibition to water flow that develops during storage may be independent of oxidative processes.

Aspiration of air may be one of the prerequisites for the occlusion in stems that were held in humid air prior to vase life. We previously found (van Doorn and D’hont, 1994) that the xylem occlusion in cut rose flowers is often due to the combined effect of a high number of bacteria and the presence of air emboli in the xylem conduits. Either of the two is often inadequate to produce early wilting. Similarly, in chrysanthemum both air aspirated at the cut surface and a wounding-induced blockage may be required to produce early leaf wilting. Indeed, van Meeteren (1992) showed good evidence for a role of air in the occlusion of stems that was held in humid air prior to placement in vase water. However, air aspirated at the cut surface seems not adequate to fully explain the occlusion, since aspiration of air ceased long before a clear reduction in the rate of water uptake was found.
We also found some evidence for a wounding response in shoots that were directly placed in vase water. The number of cavitations in these shoots was high, but the rise in the number of cavitations started only after the occlusion had developed. In these stems, however, many bacteria were found in the basal stem end within a few days. These may well add to the occlusion. The effect of compounds such as benzoic acid, ascorbic acid, and n-propylgallate (Fig. 4), however, indicates that the high number of bacteria in the stems was not adequate to fully explain the occlusion. These compounds delayed the occlusion but did not affect the number of bacteria in the stem ends. Acids may promote water flow in the xylem (Durkin, 1979) and, hence, may have an effect other than reducing a plant-induced blockage. However, the effect of n-propylgallate, having a pH closer to neutral, indicates a plant-mediated effect in stems directly placed in water (Fig. 4). The occlusion in stems directly placed in water is apparently also partly due to the presence of aspirated air in the stem ends. This is evident by comparison with treatments in which stems were cut under water and then placed in vase water. Leaf wilting in these stems occurred much later than in stems cut in air (Fig. 4).

Although the evidence for a physiological response may be adequate, at least for stems placed in humid air prior to vase life, it remains unclear which processes are involved. Ethylene production often increases after cutting of plant tissues (van Doorn et al., 1989). However, ethylene is apparently not a rate-limiting step in the present system. None of the ethylene inhibitors tested had an effect on the time to leaf wilting, either for stems that were held in humid air or those that were directly placed in water. The other chemicals used in the present tests are not specific inhibitors. Thus, they provide no clues as to the enzymes involved. For example, phloroglucinol is an inhibitor of both peroxidase and polyphenoloxidase (Srivastava and van Huystee, 1977). Propylgallate is a non-specific scavenger of activated oxygen (Roberts et al., 1991) and inhibits several enzymes, such as lipoxygenase (Macri et al., 1994) and peroxidase (Lee and Fletcher, 1992). Butylated hydroxytoluene also inhibits a range of enzymes involved in oxidative reactions, including lipoxygenase (Matsui et al., 1991).

The anatomical nature of the purported physiological occlusion is also unclear. One possibility is a deposition of materials on the pit membranes. Electron micrographs often show such a deposit in angiosperms, in particular after wounding (Gale, 1982; Wheeler, 1983; Baas, P. and Schmitt, U., personal communication). Preliminary FTIR data indicated that material deposited on pit membranes was rich in compounds related to cinnamic acid (Schmitt, U., personal communication). We now also found that a short placement of the stems in an aqueous solution of KOH, acetone, or sodium hypochlorite (see Section 2.3), after a period of storage in air, delayed leaf wilting in chrysanthemum. The treatment with KOH and bleach temporarily reduced the bacterial counts in the stems. By day 4, however, the counts were the same as in controls (Fig. 3B), but leaf wilting in stems treated with KOH or bleach did not occur until days 7–8 (Fig. 2C). This is evidence against a role of bacteria in the effect of KOH and bleach. Deposition of material on the pit membrane may therefore be a candidate for the explanation of the plant-induced blockage.

Acknowledgements

The authors are grateful for the contributions of Pieter Baas (State Herbarium, University of Leiden, The Netherlands) and Uwe Schmitt (Institut für Holzforschung, University of Hamburg, Germany).

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