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Identification of a hydroxy substituted calamenene—a sesquiterpene associated with wound reactions in non-infected xylem of *Tilia* spp.

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Abstract

Xylem of lime trees (*Tilia* spp.) with wound reactions was structurally investigated by scanning (SEM) and transmission electron microscopy (TEM) as well as chemically analyzed by direct thermal desorption-gas chromatography–mass spectrometry (DTD-GC–MS). Wound reactions in the outer xylem lead to distinct discolourations around the wound. Within a 4-week response no fungal infection occurred in discoloured xylem. At the fine structural level, wound reactions become primarily visible as the secretion of dark-staining substances from parenchyma cells into lumens of vessels and fibres. With increasing reaction time vessels aggregate large amounts of secretion products, whereas in fibres wall-associated linings are formed and the inner secondary wall appears incrusted. After 2–3 months a narrow, greenish-brown boundary developed at the transition between the discoloured outer and the unchanged inner xylem. This green-brown boundary layer remained non-infected also in older wounds. DTD-GC–MS analyses revealed that the sesquiterpene Hydroxycalamenene represents a key substance of wound reactions in non-infected lime trees. Other substances such as fatty acids or their esters and coniferyl aldehydes or their derivatives were also found. TEM investigations of the samples after DTD-GC–MS showed less pronounced cell wall-attached linings in fibres as well as reduced incrustation of inner secondary walls. The massive deposits in the vessel lumens remained unchanged. The role of these wound reaction products and their ways of synthesis are discussed.

Keywords: *Tilia* spp.; Xylem; Wound reactions; Electron microscopy; Direct thermal desorption-gas chromatography–mass spectrometry (DTD-GC–MS); Sesquiterpene; Hydroxycalamenene

1. Introduction

Trees respond to mechanical injuries by initiating wound reactions in the living cells of the affected tissues. In the outer xylem of hardwoods, parenchyma cells in close vicinity to a wound are responsible for initiating reactions which result primarily in the occlusion of water conducting vessels, preventing air embolism, water loss, and penetration of micro-organisms (Blanchette and Biggs, 1992; Pearce, 1996). In this regard two main strategies developed during the evolution of hardwoods: certain species such as oak and black locust form tyloses, others like birch and lime achieve vessel blockage by the synthesis of fibrillar/granular substances in parenchyma cells and their secretion through pits into the lumen of neighbouring vessels (Bonsen, 1991; Schmitt and Liese, 1990). The latter strategy was observed also in fibres although at reduced intensity (e.g. Duchesne et al., 1992; Pearce, 2000).

Wound reactions have been thoroughly investigated at the fine structural level (e.g. Biggs, 1987; Blanchette and Biggs, 1992; Schmitt and Liese, 1992; Liese and Dujesiefken, 1996; Pearce, 1990, 1996), however, knowledge of the chemical nature of the substances deposited in vessels and fibres is still rudimentary. Pearce (1996) provided a summary of some substances isolated and identified from the sapwood of trees infected with fungi. They possess antimicrobial activity and are therefore grouped into the phytoalexin-like compounds. Chemically, they belong to different groups of substances, e.g. stilbenes, lignans, flavonoids, terpenes and also phenylpropanoids. The present study on early wound reactions in non-infected xylem of *Tilia* spp. focused on the cellular localization and chemical identification of wound-associated substances and on a semi-quantitative determination of their concentration in relation to the duration of the wound response.

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2. Results and discussion

Wound reactions in the xylem of lime trees become macroscopically visible as discolourations in the affected tissues close to a wound. Within the first 4 weeks after wounding a dark-brown discolouration developed up to a depth of a few millimetres from the wound surface. After 1–2 months a narrow zone along the transition between dark-brown discoloured outer and unaltered inner xylem turned to greenish-brown. This so-called boundary layer or reaction zone is regularly formed in older wounds and acts as a barrier against pathogens (Pearce, 2000). Numerous vessels in this area contained large amounts of granular deposits; most vessels were completely occluded (Fig. 1). Transmission electron microscopy revealed responses within the first weeks as mostly granular deposits in vessels (Fig. 2) or broader linings along their walls (Fig. 3) and narrow, cell wall-attached linings in fibres (Figs. 3 and 4). In both cases, these substances were synthesized in parenchyma cells and secreted through the pits into the lumens of adjacent vessels and fibres (Fig. 2). In addition, the inner secondary wall of the fibres with linings mostly appeared dark-stained in contrast to the outer secondary wall regions, which were less stained (Figs. 3 and 4). This fine structural feature points to a certain wall incrustation as suggested by Pearce (2000) from light microscopic observations. No fungal hyphae occurred in the discoloured zone of these young wounds as well as in the boundary layer of older wounds.

Characteristic differences in the chromatograms were obtained by DTD-GC–MS between the control and discoloured, non-infected xylem with 1, 2 and 4 weeks of response (Fig. 5). A qualitative comparison of these chromatograms showed additional signals (signal No. 1–3) in discoloured xylem, which were not found in the control. Signal No. 1 represents a key substance because increasing duration of the wound reaction resulted in an extraordinary increase in its intensity. A search in the NIST database spectral library indicated that this substance might very likely be "2,5-dimethyl-8-isopropyl-5,6,7,8-tetrahydronaphthalen-1-ol", a sesquiterpene. The identified compound is also known under the synonym 5-hydroxy-calamenene (Rimpler et al., 1970). Consequently, the mass spectrum contains all typical fragments of the calamenene skeleton like $m/z = 175$ (basis peak) or $m/z = 147$ (fragment of the Retro–Diels–Alder-reaction), which can be obtained

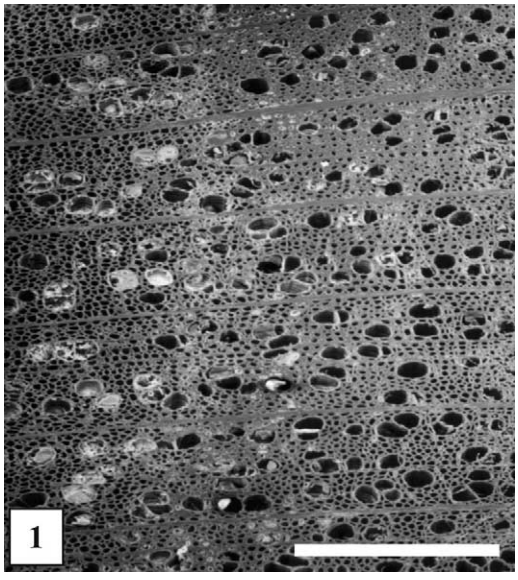

Fig. 1. *Tilia* sp., 11 months old wound, greenish-brown boundary layer with numerous occluded vessels (left) and the dark-brown outer xylem close to the wound (right). SEM, transverse surface. Bar = 0.5 μm.

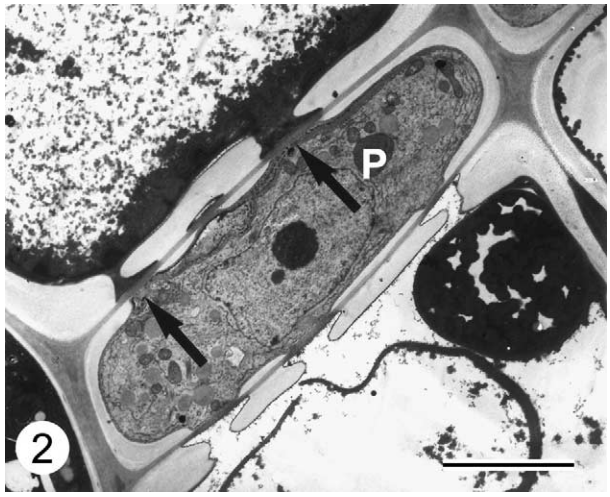

Fig. 2. *Tilia americana* L., wound adjacent xylem 4 weeks after wounding. Distinct secretion of dark-staining substances from parenchyma cells (P) through pits into vessel lumens (arrows). TEM, transverse section. Bar = 5 μm.

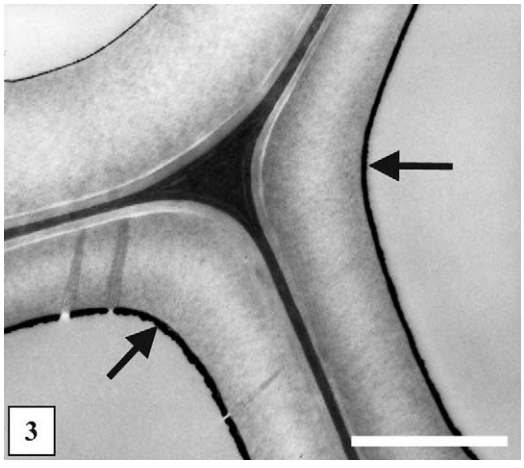

Fig. 3. *Tilia americana* L., wound adjacent xylem 3 weeks after wounding. Fibre walls with narrow dark-stained aggregations of wound reaction products along the secondary wall (arrows). Note also the slightly darker stained inner secondary wall layer pointing to a certain incrustation. TEM, transverse section. Bar = 2.5 μm.

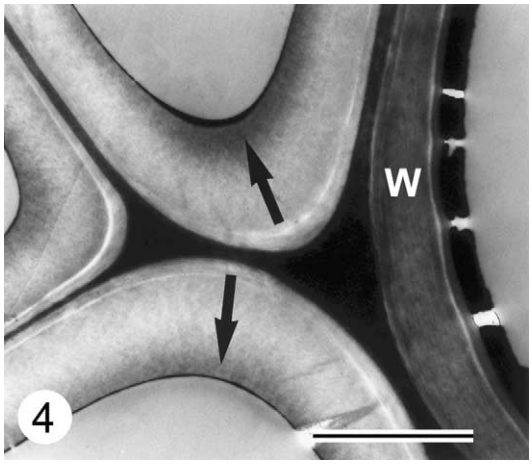

Fig. 4. *Tilia americana* L., wound adjacent xylem four weeks after wounding. Vessel wall (W) with distinct aggregations of dark-stained wound reactions products. Fibre walls show intense dark staining of their inner secondary wall (arrows). TEM, transverse section. Bar = 2.5 μm.

according to usual disintegration rules (McLafferty and Tureček, 1995). 5-Hydroxycalamenene can be metabolized from acetyl-CoA via 3-hydroxy-3-methylglutaric acid, mevalonic acid and farnesyl-diphosphates (Hauptmann, 1985). In *Tilia europaea* a similar compound, 7-hydroxycalamenene, has been isolated and identified in the discoloured reaction zone between fungus-infected outer and healthy inner xylem (Burden and Kemp, 1983). A definition statement regarding the position of the hydroxygroup in the calamenene skeleton of the detected compound, however, requires an extended analytical investigation using pure substances. Substituted derivatives of a hydroxy-calamenene isolated from *Heterotheca subaxillaris* (Lam.) were also investigated by Rugutt et al. (1996) using ^{1}H and ^{13}C NMR. The authors pointed out that calamenenes in general can be understood as A-ring-aromatized cadinanes, which are common in higher plants and are characterised by a toxic efficiency against different organisms. This might be an explanation for an increasing content of this sesquiterpene with time as an early response to restrict a possible penetration of fungi, independent of their presence.

In contrast to the signal intensity of hydroxycalamenene, the intensity of the signals No. 2 and 3 (Fig. 5B–D) steadily decreased with increasing reaction time. After 4 weeks it was only approximately 1/10 of hydroxycalamenene. Based on the suggestion of the spectra library search, these signals probably correspond to fatty acids or their esters. It is known that terpenes can also be formed as a result of the decomposition of lipids followed by a transformation of the fatty acids (Hauptmann, 1985). The observed decline in the concentration of these compounds might indicate the formation of calamenene via compounds derived from the degradation and transformation of lipids.

Furthermore, the chromatograms show two other substances (signals No. 4 and 5 in Fig. 5B–D) with a slight increase in the signal intensity with progressing duration of wound reaction. These substances can be assigned as coniferyl aldehyde (No. 4) or their derivatives (No. 5), which are one of the monolignol elements of lignin. This assumption is also in agreement with investigations by Lavola (1998) who described an increasing synthesis of substances similar to the derivatives of coniferyl alcohol or flavonoids as a result of wounding. As there were no fungal hyphae in this part of the xylem, it can be assumed that the formation of these derivatives may constitute a second step of active tree reactions. In this context, the detection of the lignin precursors might also be an indication of the so-called "secondary lignification" of cell walls as discussed by Fink (1999).

The chromatograms of thermodesorbed substances obtained from different zones in 11 months-old wounds differ in the intensity of the characteristic signal No. 1 (Fig. 6). It is obvious that the highest concentration of hydroxycalamenene was found in the two outer zones (Fig. 6A and B) corresponding to the dark-brown discoloured xylem between the wound surface and the boundary layer. This region no longer contained living parenchyma cells and thus could be infected with fungi. In this region, parenchyma cells probably degenerated during the first months after wounding without building an efficient barrier against microorganisms. The chromatogram of Fig. 6C is related to tissue taken from the distinctly discoloured greenish-brown boundary layer, where nearly all the cells were heavily filled with wound reaction products (compare Fig. 1). Fungi were not present here. In this boundary layer, a relatively small amount of hydroxycalamenene was detected. Possibly, hydroxy-calamenene is here less thermodesorbable because of strong interactions with other substances deposited mainly in vessels due to wound reactions. Furthermore, it can be deduced that wound reactions are structurally and chemically negligible in the xylem a few millimeters behind the region of discolouration (Fig. 6D), because TEM did not show wound reaction products, and the chromatograms are comparable to those obtained from the control tissues (compare Figs. 5A and 6D).

TEM of samples with a 4-week response and after DTD-GC–MS treatment showed an inhomogeneous staining of the S2 layer in fibre walls, especially a less pronounced staining of the inner S2 (Figs. 7 and 8). This part of the wall is apparently altered, but all other wall regions appear normal. The regularly observed wall-attached linings of dark-stained material in the fibres of samples with wound reactions was now less pronounced and in many cases fibres did not show these structures. In the vessels, the massive deposits of the granular material were visibly unaltered due to DTD-GC–MS

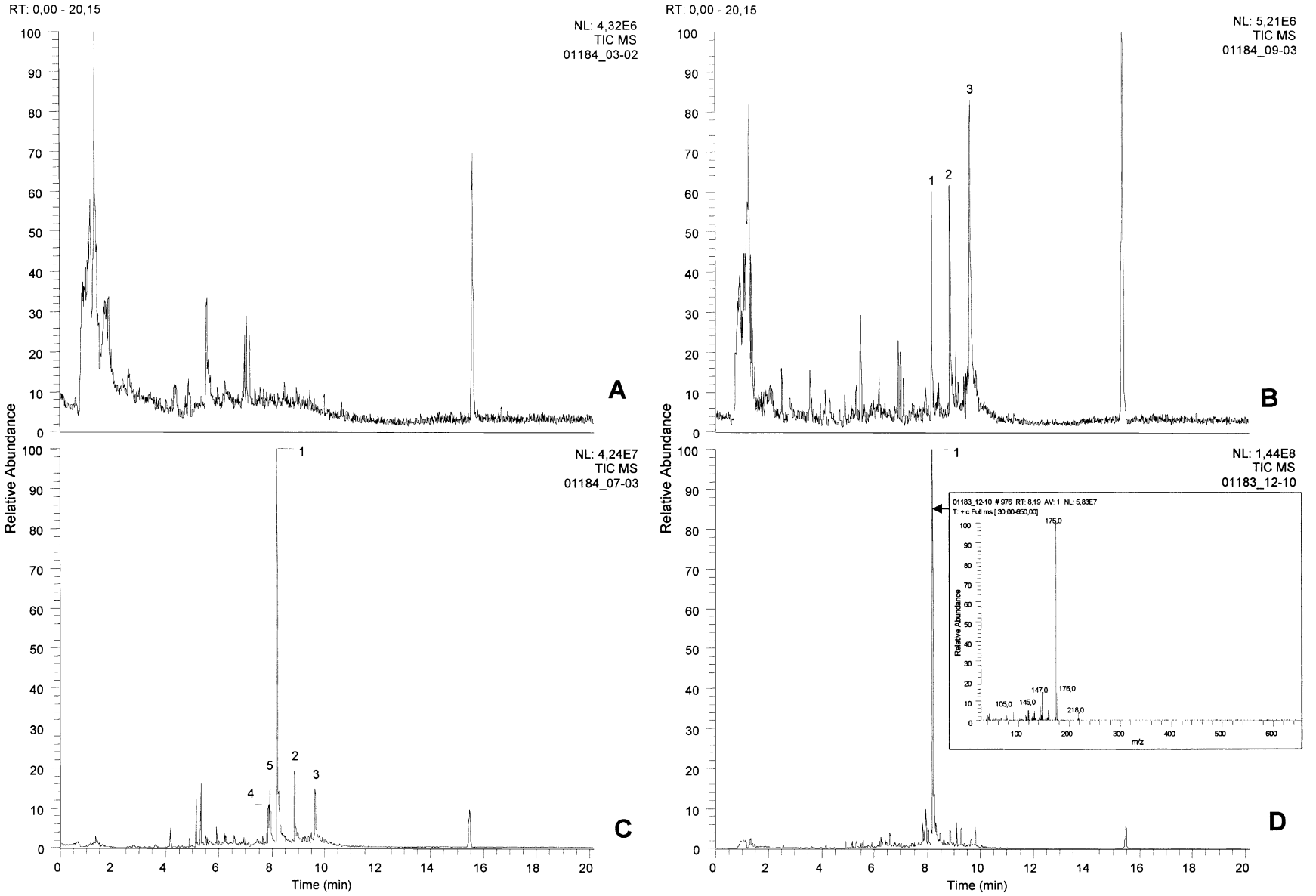

Fig. 5. *Tilia* spp., full scan chromatograms (TIC) of unaffected xylem (A) and of xylem with 1 (B), 2 (C) and 4 (D) weeks of wound reaction. Additional signals (1–5) were found in the xylem with wound reactions. Signal No. 1 represents a key substance which increases distinctly with increasing duration of the wound reaction. According to the mass spectra, this substance was identified as the sesquiterpene calamenene.

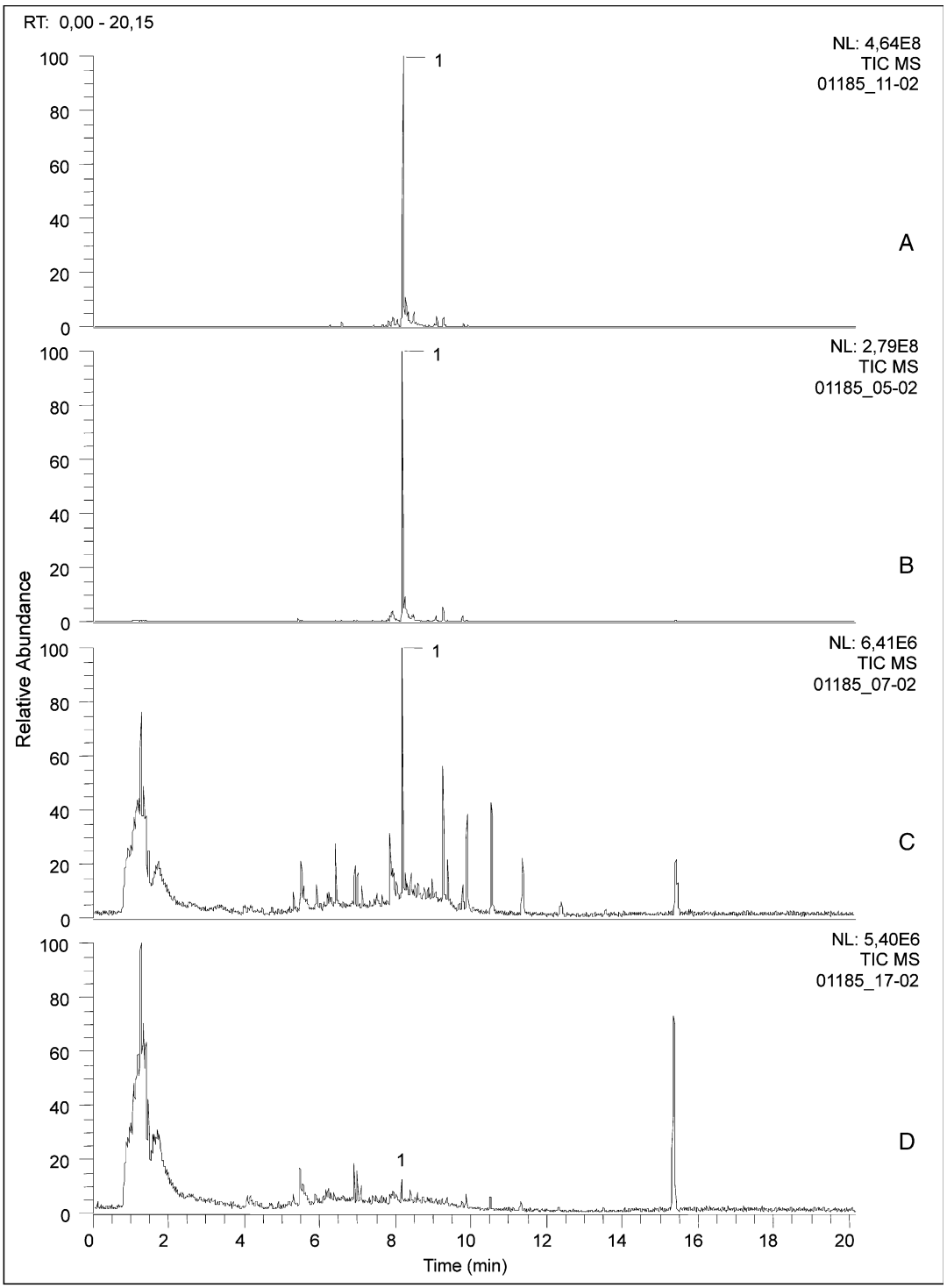

Fig. 6. *Tilia* spp., full scan chromatograms (TIC) of xylem portions with increasing radial distance from the wound surface; A = 2 mm, B = 4 mm behind the wound surface within the dark-brown discoloured xylem, C = 5 mm behind the wound surface within the green-brown boundary layer, D = control from unaffected xylem.

treatment (Fig. 8). According to these observations in the samples prior to and after DTD-GC–MS, there are additional substances in the lumens, which are not thermodesorbable with the selected parameters during DTD-GC–MS.

In conclusion, DTD-GC–MS revealed that wounding induced the formation of the sesquiterpene hydroxycalamenene in the xylem of *Tilia* spp.. Electron microscopy showed that within the first four weeks after wounding there was no fungal infection. The wound-induced

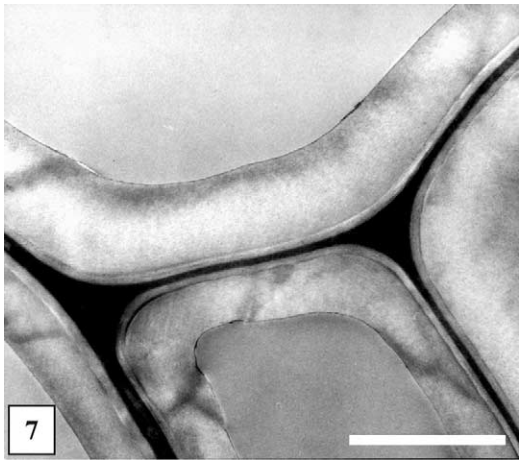

Fig. 7. *Tilia americana* L., wound adjacent xylem 4 weeks after wounding and after DTD-GC–MS. Fibre wall-attached aggregations of wound reaction substances are hardly visible and the incrustation of the inner secondary wall is less pronounced and inhomogeneous. TEM, transverse section. Bar = 2.5 μm.

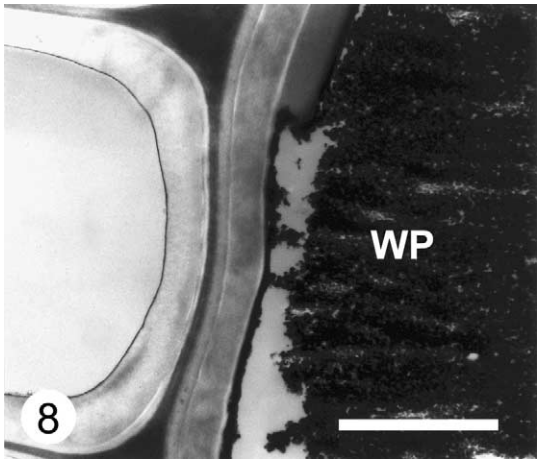

Fig. 8. *Tilia americana* L., wound adjacent xylem 4 weeks after wounding and after DTD-GC–MS. Fibres without distinct aggregations along their wall and without intense dark staining of the inner secondary wall. Wound reaction products in the vessel lumen (WP) appear structurally unchanged. TEM, transverse section. Bar = 2.5 μm.

substances were localized in cell lumens as well as in the inner parts of the cell wall. After DTD-GC–MS treatment, the wound reaction substances had disappeared in fibres from inner wall regions and outer areas of the lumen. However, the massive deposits in the cell lumen of vessels remained unchanged due to DTD-GC–MS.

3. Experimental

For conventional transmission electron microscopy (TEM) 10 mm × 10 mm wounds were set between May and September 1999 at breast height on four about 50-year old *Tilia* spp. trees growing in a forest 40 km the east of Hamburg. Samples 2–5 mm inside of the wound surface were taken with a chisel after response periods from 1 week up to 11 months. They were then cut into small pieces with a razor blade (final size was 1 mm × 1 mm × 5 mm) and immediately fixed in Karnovsky's paraformaldehyde/glutaraldehyde solution (pH 7.2) for 24 h, followed by washing in 0.1 M cacodylate buffer (pH 7.2), and post-fixation in 2% aqueous osmium tetroxide. The samples were washed in buffer, dehydrated in a graded acetone series and embedded in Spurr's epoxy resin. A second set of samples was collected from 50 mm × 50 mm wounds set weekly during a 4-week-period in April 2001 by debarking of a 25-year old *Tilia americana* L. tree located at the arboretum of the Federal Research Centre for Forestry and Forest Products, Hamburg. The sample preparation for electron microscopy was carried out in the same way as described above. However, to avoid removement of water or acetone soluble substances, these samples were air-dried for one week and then directly embedded in Spurr's epoxy resin without fixation, washing and dehydration (Kleist and Schmitt, 2001). Ultrathin sections from the fixed material were double-stained with uranyl acetate and lead citrate, those prepared from directly embedded material were stained with potassium permanganate according to Donaldson (1992). The sections obtained from fixed samples were examined with a Philips CM 12 transmission electron microscope at an accelerating voltage of 80 kV, those samples directly embedded were examined at 40 or 60 kV to enhance the contrast. Scanning electron microscopy (SEM) was carried out with a Hitachi S-520 scanning electron microscope at 15 kV on the samples (sample size 1 cm × 1 cm × 1 cm) which had been air-dried, mounted on aluminium stubs and sputter-coated with gold.

For chemical analyses, samples were collected 1, 2 and 4 weeks after wounding at 2–5 mm behind the wound surface from slightly discoloured xylem portions of *Tilia americana* L. stems. A second set of samples was taken 11 months after wounding from *Tilia* spp. along a radial line from the wound surface beginning at a distance of around 2 mm and then 4 mm, both in the dark-brown discoloured portion. A third position in radial direction was around 5 mm behind the wound surface within the greenish-brown boundary layer. The control samples were collected from the non-discoloured xylem either near the wound or at varying distances from it. The chemical analyses were carried out using a direct thermal desorption-gas chromatography–mass spectrometry (DTD-GC–MS) system. This technique can be used for the identification of all gas chromatographically analyzable substances as well as reaction products which were formed within the injection interface during heating the sample as described by Jüngel et al. (2002). The injection interface was an Optic 2 programmable injector (ATAS Int., The Netherlands)

equipped with an DTD Automatic Liner Exchange Unit (ATAS Int.). The liners have a length of 80 mm, an I. D. of 3.4 mm, and the top of the liner has an 11 mm O. D. flange for sealing the liner with a crimp cap. Up to 1 mg of each sample was weighed into the liners sealed with magnetic crimp caps and placed into the injector, having a start temperature of 45 °C. After purging the liner with carrier gas during 30 s, the injector was heated with 10 K/s to 200 °C. The desorption time was 60 s, whereby only a part of the gas mixture was pressed onto the capillary column. The GC (GCQ™, Finnigan Corp., USA) was equipped with a BPX35 (SGE, Germany) column with a length of 30 m and an I. D. of 0.25 mm. Helium 5.0 (Messer Griesheim, Germany) was used as carrier gas for the system. The GC oven temperature started at 50 °C. After 1 min, the column was heated with 20 K/min to 250 °C with ramps at 225 °C for 3.75 min and at 250 °C for 5.75 min. The total run was 20 min. The mass spectrometer (GCQ™) recorded the mass range from 30 to 650. To reveal structural modifications during DTD-GC–MS treatment, some wood samples analyzed by DTD-GC–MS were directly embedded in Spurr's epoxy resin for TEM investigations.

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