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Microbial oligosaccharides differentially induce volatiles and signalling components in *Medicago truncatula*

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ABSTRACT

Plants perceive biotic stimuli by recognising a multitude of different signalling compounds originating from the interacting organisms. Some of these substances represent pathogen-associated molecular patterns, which act as general elicitors of defence reactions. But also beneficial microorganisms like rhizobia take advantage of compounds structurally related to certain elicitors, i.e. Nod-factors, to communicate their presence to the host plant. In a bioassay-based study we aimed to determine to what extent distinct oligosaccharidic signals are able to elicit overlapping responses, including the emission of volatile organic compounds which is mainly considered a typical mode of inducible indirect defence against herbivores. The model legume *Medicago truncatula* Gaertn. was challenged with pathogen elicitors (β-(1,3)-β-(1,6)-glucans and *N,N′,N″,N‴*-tetraacetylchitotetraose) and two Nod-factors, with one of them being able to induce a nodulation response in *M. truncatula*. Single oligosaccharidic elicitors caused the emission of volatile organic compounds, mainly sesquiterpenoids. The volatile blends detected were quite characteristic for the applied compounds, which could be pinpointed by multivariate statistical methods. As potential mediators of this response, the levels of jasmonic acid and salicylic acid were determined. Strikingly, neither of these phytohormones exhibited changing levels correlating with enhanced volatile emission. All stimuli tested caused an overproduction of reactive oxygen species, whereas nitric oxide accumulation was only effected by elicitors that were equally able to induce volatile emission. Thus, all signalling compounds tested elicited distinct reaction patterns. However, similarities between defence reactions induced by herbivory and pathogen-derived elicitors could be ascertained; but also Nod-factors were able to trigger defence-related reactions.

1. Introduction

Plants are able to recognise a multitude of chemical signals from their environment and to react appropriately to diverse challenges. Some of these signalling compounds act as general elicitors of defence reactions (Ebel and Cosio, 1994; Boller, 1995). They are perceived at low concentrations and comprise diverse structures, including carbohydrates, (glyco-) proteins, lipids, and sterols (Ebel and Cosio, 1994; Boller, 1995; Nürnberger et al., 2004). The relevant compounds are in general evolutionarily conserved amongst microorganisms (not only pathogens), not present in the potential host plant, and important for the fitness of the microbe; thus, they parallel pathogen-associated molecular patterns (PAMPs) important for non-self recognition in the animal immune system (Gomez-Gomez, 2004; Nürnberger et al., 2004; Zipfel and Felix, 2005). Elicitor-induced defence responses include the oxidative burst, the strengthening of cell walls, the hypersensitive reaction, and the activation of genes encoding for pathogenesis-related (PR) proteins and enzymes of phytoalexin synthesis (Ebel and Mithöfer, 1998). In legumes, beneficial microorganisms like rhizobia that might primarily be perceived as intruders, take advantage of compounds structurally related to certain elicitors, i.e. nodulation factors (Nod-factors), to communicate their presence to the plant. In fact, while Nod-factors induce root hair deformations, cortical cell divisions, and in some cases even complete nodule-like structures in their host plants, they are able to cause reactions that occur in the context of pathogen defence in non-host plants (Staehelin et al., 1994; Baier et al., 1999; Bueno et al., 2001) and in host plant cell cultures (Savouré et al., 1997).

Although plants' reactions to pathogen attack are mostly seen as distinct from plant to herbivore interactions, the induced defence reactions against both arthropods and pathogens intersect considerably. For instance, plants' defences against certain

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sedentary herbivores (e.g. galling insects or mites) include reactions that are typical for the response to pathogen attack, such as the hypersensitive response and the accumulation of phenolic compounds (Fernandes, 1990; Fernandes and Negreiros, 2001; Ollerstam et al., 2002). These similarities might be due to the minor tissue damage those pests inflict on the plant (Walling, 2000). But even lepidopteran larvae induce defence reactions that so far have only been reported to occur after pathogen attack or the application of pathogen-derived elicitors, such as the local accumulation of defensive compounds at the wounding site or hydrogen peroxide production (Leitner et al., 2005; Maffei et al., 2006).

Amongst the great variety of inducible defence mechanisms in plants, the emission of volatile organic compounds (VOCs) is one example of indirect defence directed against herbivores. However, volatile emission has also been reported after infection by different strains of the bacterial pathogen *Pseudomonas syringae* van Hall in tobacco (*Nicotiana tabacum* L.) or bean (*Phaseolus vulgaris* L.) (Croft et al., 1993; Huang et al., 2003). In peanut plants (*Arachis hypogaea* L.), the white mould (*Sclerotium rolfsii* Sacc.) has been reported to induce VOC release upon infection (Cardoza et al., 2002). These results give rise to the question, what kinds of elicitors caused this reaction.

The accumulation of phytohormones is generally separated into pathogen- and herbivore-induced responses as well. The jasmonic acid (JA) pathway is thought to be involved in the activation of defences against herbivores and necrotrophic pathogens, whereas the salicylic acid (SA)-mediated pathway contributes to the defence against biotrophic pathogens (Gatehouse, 2002; Kessler and Baldwin, 2002; Glazebrook, 2005). If rhizobia are regarded simplistically as intruders of the plant, the question arises how stress-associated phytohormone levels change during the establishment of symbiosis. Indeed, previous studies showed that for successful nodulation to occur, it is crucial to suppress the activation of signalling cascades involving the SA and the JA pathways. In contrast to interaction with wild type rhizobia, SA accumulates in the roots of *Medicago sativa* L. inoculated with mutants of *Sinorhizobium meliloti* that are incapable of synthesising Nod-factors, while at the same time nodulation is clearly reduced (Martínez-Abarca et al., 1998). Furthermore, the exogenous application of SA or JA inhibits nodulation (Martínez-Abarca et al., 1998; Sun et al., 2006).

In view of these partially overlapping responses to diverse biotic stimuli it was intriguing to search for further potential parallels in the defences against pathogens and herbivores. Given the structural similarity of Nod-factors to certain pathogen-derived elicitors (i.e. chitin fragments), these compounds might also induce defence-related responses under certain circumstances.

Thus, in order to gain deeper insight into general and specific reactions of a plant to biotic signals, several microbial oligosaccharidic signalling compounds (Fig. 1) were used in this study and their impacts on defensive traits of the plant were compared. Besides pathogen-derived elicitors (β-(1,3)-β-(1,6)-glucans and *N,N′,N″,N‴*-tetraaectylchitotetraose), *Medicago truncatula* Gaertn. cv. Jemalong A17 was challenged with symbiotic signalling substances (Nod-factors). Considering that those substances act on different plant tissues in nature, aboveground, belowground and both, a direct comparison of the induced responses under physiological conditions are not possible. As a consequence, a bioassay was performed, applying the respective signal compounds to the aboveground part of cut plants.

Out of a multitude of possible reactions, the emission of VOCs as well as changes in the levels of the phytohormones JA and SA were chosen for comparison. Up to now, no clear relationship between these parameters has been demonstrated for pathogen-derived elicitors. In addition, results on the involvement of the well known signalling compounds reactive oxygen species (ROS) and nitric oxide (NO) in signal transduction are presented, in an attempt to link the induced stress responses observed to the level of ROS- and NO-mediated reactions.

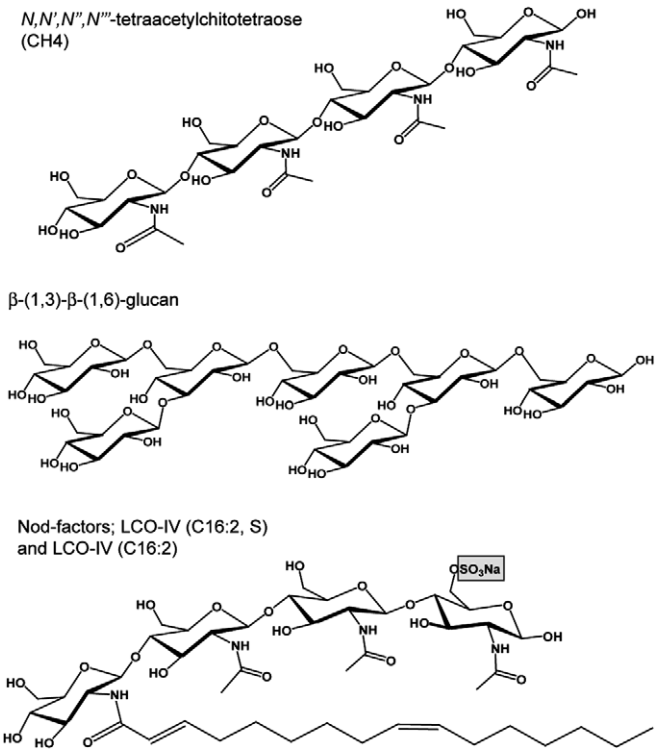

Fig. 1. Chemical structures of the signalling compounds used in this study. *N,N′, N″,N‴*-tetraacetylchitotetraose (CH4); β-(1,3)-β-(1,6)-glucan from the oomycete *Phytophthora sojae* cell wall as an example for an elicitor structure that is active in legumes; LCO-IV (C16:2, S) and LCO-IV (C16:2); Nod-factors. Both molecules differ only in the presence or absence of the sulphate group (marked grey) that provides for host specificity of LCO-IV (C16:2, S) in *Medicago truncatula*.

2. Results

2.1. VOC emission

Certain oligosaccharides are well-known signalling compounds of microbial origin, which are often involved in plant-microbe interactions. Out of these structurally diverse elicitors, branched β-(1,3)-β-(1,6)-glucans from the phytopathogenic oomycete *Phytophthora sojae* as well as *N,N′,N″,N‴*-tetraacetylchitotetraose (CH4) were used in this study to simulate pathogen attack (Fig. 1).

No significant differences were found between volatile blends of control *M. truncatula* plants and those treated with CH4, except for an elevated emission of *n*-tetradecane (Fig. 2b). Treating plants with the β-glucan elicitor strongly induced the emission of VOCs compared to control plants that were cut and placed in tap water (Fig. 2a and c). Thirteen sesquiterpenoids, and the homoterpenes 3*E*,7*E*-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) and 3,8-dimethyl-1,3,7-nonatriene (DMNT) were found to be induced. The VOC blend emitted was similar to that found after herbivore feeding in *M. truncatula* (Leitner et al., 2005) regarding the potential to induce sesquiterpenoid emission. However, the overall induced VOC patterns differed. In contrast to the substantial induction of volatiles by β-glucans, CH4 was inactive in this respect.

Furthermore, two Nod-factors that are involved in *Sinorhizobium meliloti* – *M. truncatula* symbiosis were tested for their ability to induce the emission of VOCs. These molecules share the chito-

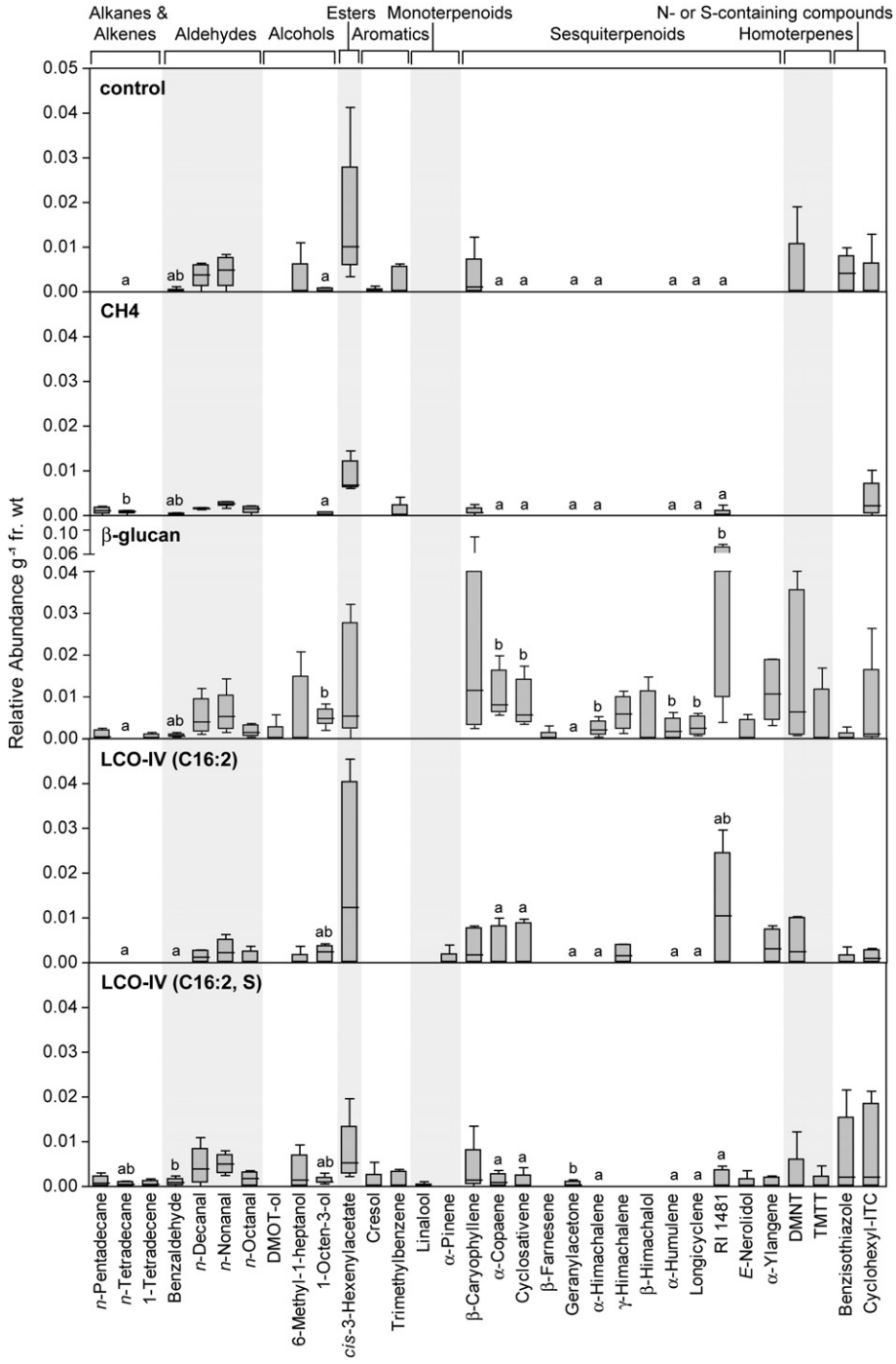

Fig. 2. Box-plots representing the relative quantification of volatiles induced by treatment with microbial oligosaccharides. Control (detached plant placed in tap water); 100 μM CH4; 200 μg ml^{-1} β-glucan elicitor; 10 μM LCO-IV (C16:2); 10 μM LCO-IV (C16:2, S). $n = 6$ for control and Nod-factors; $n = 5$ for β-glucans and CH4. Small letters indicate significant differences as determined by ANOVA and Newman–Keuls *post hoc* test. Abbreviations: DMOT-ol, 2,6-dimethyl-3,5,7-octatrien-2-ol; RI 1481, unidentified sesquiterpene with retention index 1481; Cyclohexyl-ITC, cyclohexylisothiocyanate.

tetraose backbone with a fatty acid chain (C16:2) attached to it by an amide bond. But while the lipo-chitooligosaccharide LCO-IV (C16:2, S) carries a sulphate group, providing for activity and host specificity, LCO-IV (C16:2), which lacks this substituent, is unable to induce nodulation in *M. truncatula* (Fig. 1c) (D'Haeze and Holsters, 2002). Strikingly, both compounds induced volatile release by *M. truncatula* (Fig. 2d and e). Though the quantity of VOCs emitted varied highly within each treatment group, the blends were qualitatively clearly distinct from the control. However, due to the above-mentioned variability of VOC emission, hardly any of those differences were statistically significant using analysis by ANOVA and Newman–Keuls *post hoc* test. Still, there was a trend towards the emission of certain sesquiterpenoids in higher abundance, namely α-copaene, cyclosativene, α-ylangene, and the unidentified sesquiterpene (RI 1481) for both Nod-factors, γ-himachalene after treatment with LCO-IV (C16:2), and *E*-nerolidol after treatment with LCO-IV (C16:2, S). Furthermore, some of the changes in the emission pattern seemed to be rather characteristic, as for example the emission of geranylacetone that could be found only after treatment with LCO-IV (C16:2, S). In contrast, the emis-

sion patterns observed after treatment with LCO-IV (C16:2) seemed to be concentrated between control and β-glucan treatment. Additionally, in contrast to the induction with pathogen-derived elicitors, small amounts of monoterpenoids were detected: α-pinene in the case of LCO-IV (C16:2) treatment and linalool upon LCO-IV (C16:2, S) treatment.

For the description of complex volatile patterns, multivariate statistical methods might be superior to univariate ones. Furthermore, considering the low number of samples, methods depicting single samples might be advantageous. Hence, non-metric multidimensional scaling (NMDS) was used to visualise multivariate patterns. In the first line of analysis, square root transformed data and Euclidean distance as a dissimilarity measure were used (Fig. 3). With a stress of 9.04 the fit of the model was fairly good. The results were largely in line with those of ANOVA and *post hoc* test. Treatment with β-glucans resulted in the emission of clearly distinct VOCs, such that this group could easily be distinguished from the others. However, there was a large overlap between the effects of the active Nod-factor, LCO-IV (C16:2, S), and the control, though a part of the analysed samples displayed fairly distinct patterns. The inactivity of CH4 with respect to its impact on VOC emission is depicted very clearly; in comparison to all other treatments, including the control, the variation between single samples was the smallest by far. Finally, treatment with the inactive Nod-factor, LCO-IV (C16:2), resulted in strongly varying emission patterns, but roughly spanning the area between control and β-glucan treatment. Strikingly, in terms of the qualitative composition of the VOC blends detected, the overlap between effects produced by β-glucans and the active Nod-factor was bigger than between β-glucans and the inactive Nod-factor (Table 1). As a consequence, a binomial variant of NMDS was calculated as completion, based on a probabilistic distance measure, hence disregarding relations between concentrations of certain compounds, but emphasising qualitative traits of volatile blends. The results of this analysis were clearly distinct from those gained using continuous variables, and improved grouping of treatments could be depicted in ordination diagram models in contrast to originally scaled measurements (Fig. 4), as evaluated by a slightly lower stress of 8.26. To give but one example, the formerly rather broad group of β-glucan treatments got more pronounced and well shaped. The remaining groups were configured more distinct, with resolved overlaps. This could be interpreted that only regarding qualitative traits of volatile patterns results in good separability of the different treat-

Table 1
Comparison of qualitative differences in volatile blends induced by microbial oligosaccharides. Any substance that was detected in controls was excluded from the list

	β-glucans	LCO-IV (C16:2, S)	LCO-IV (C16:2)	CH4
Longicyclene	x			
α-Himachalene	x			
α-Humulene	x			
1-Tetradecene	x	x		
Geranylacetone	x	x		
E-Nerolidol	x	x		
TMTT	x	x		
γ-Himachalene	x		x	
α-Ylangene	x	x	x	
Cyclosativene	x	x	x	
α-Copaene	x	x	x	
$C_{15}H_{24}$ (RI 1481)	x	x	x	x
n-Octanal	x	x	x	x
n-Tetradecane	x	x	x	x

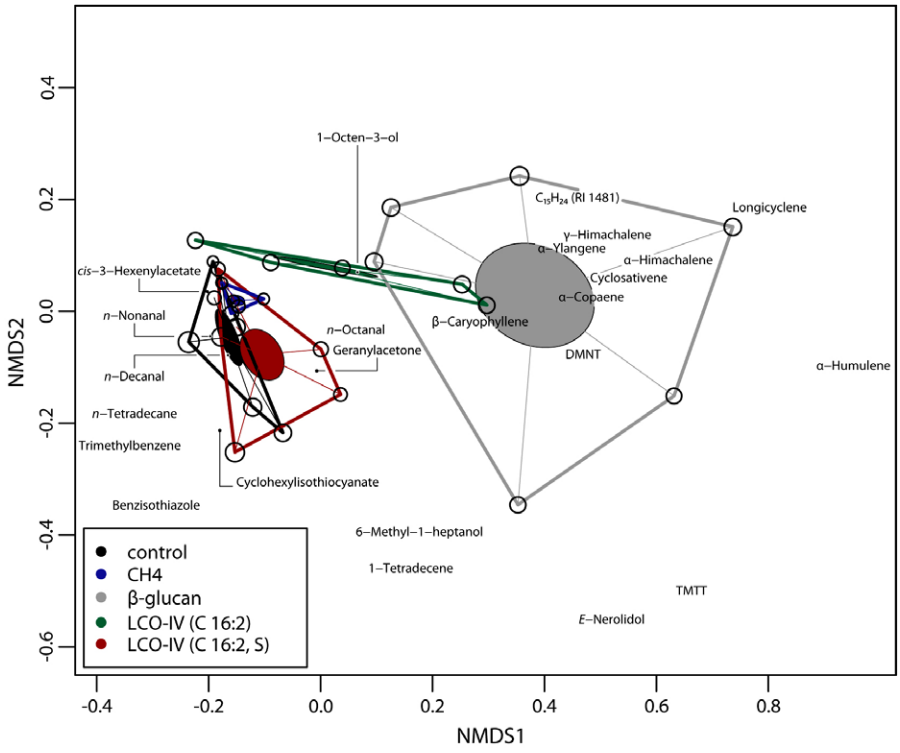

Fig. 3. Non-metric multidimensional scaling (NMDS) plots of VOC patterns in response to microbial oligosaccharides using square root transformed data and Euclidean distance as a dissimilarity measure. Stress, 9.04. Open circles indicate the relative location of single volatile samples in ordination space. The size of the circles represents the goodness of the fit into the model for each particular sample. The centroids are given by the intersection of the spiderweb-like lines within each treatment group; the groups' standard errors are given by filled ellipses. The size of the ellipse can be interpreted as a measure of consistency for the respective group. The relative distances between any sample or substance shown represent similarities, and positive (low distances) or negative (high distances) correlations, respectively. Variables (VOCs) are plotted according to their weighted averages of ordination scores (samples) in which they occur.

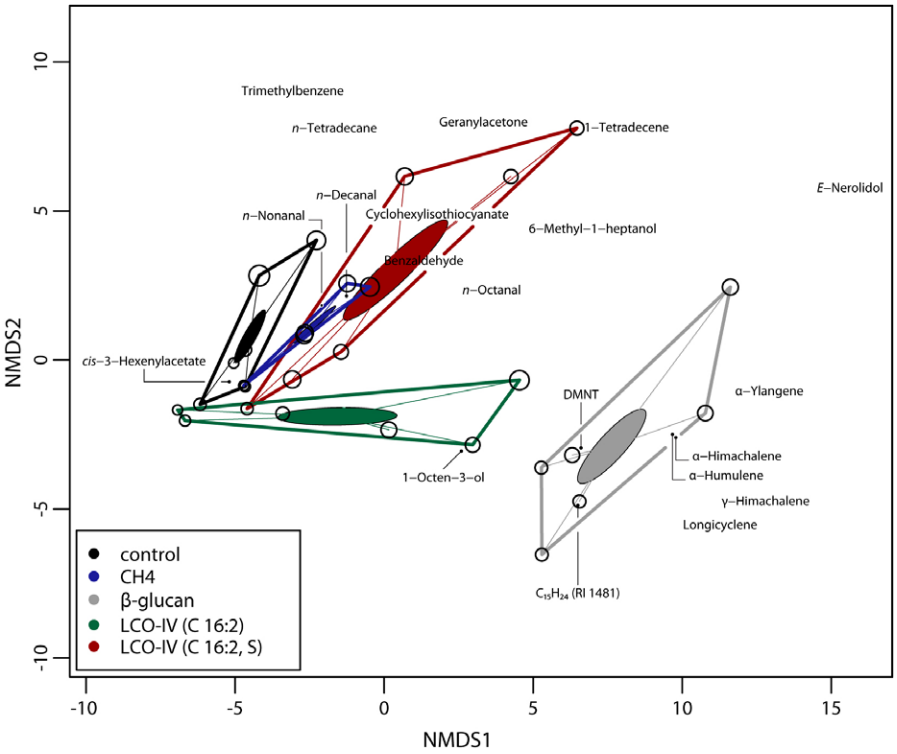

Fig. 4. Non-metric multidimensional scaling (NMDS) plots of VOC patterns in response to microbial oligosaccharides using square root transformed data and binomial distance as a dissimilarity measure. Stress, 8.26. Open circles indicate the relative location of single volatile samples in ordination space. The size of the circles represents the goodness of the fit into the model for each particular sample. The centroids are given by the intersection of the spiderweb-like lines within each treatment group; the groups' standard errors are given by filled ellipses. The size of the ellipse can be interpreted as a measure of consistency for the respective group. The relative distances between any sample or substance shown represent similarities, and positive (low distances) or negative (high distances) correlations, respectively. Variables are plotted according to their weighted averages.

ments. It also showed that treatment with both Nod-factors produced rather variable effects: about half of the plants tested responded to the treatment, whereas the other half remained largely unaffected. In conclusion, qualitative and quantitative aspects of volatile blends emitted gave different pictures and it remains to be answered whether quantity or quality matters more in the biological context.

2.2. Phytohormone levels

Two phytohormones involved in plants' defence responses are SA and JA: Conventionally, SA is seen to be mainly linked to defence reactions upon pathogen attack, whereas JA is usually implicated in defence against herbivores (Bostock et al., 2001). Here, the levels of both hormones were determined in response to elicitation with microbial oligosaccharides. Hardly any significant differences in SA levels between any of the treatments and the control were detected within the first 24 h after elicitation (Fig. 5a and b). Only two samples deviated from control values. These were taken 8 h after CH4 treatment and 2 h after treatment with LCO-IV (C16:2). These variations might be attributed to a Type I error due to the small sample number, as the rest of the data fit perfectly in the control curve. The accumulation of SA can clearly be attributed to cutting of the stem. None of the elicitors tested was able to further increase SA levels.

In contrast, JA levels rose markedly though transiently after treatment with both β-glucans and CH4, showing the highest content within the first 60 minutes, whereas neither of the Nod-factors caused the JA levels to rise above those found in the control (Fig. 5 c and d). Astoundingly, the accumulation of JA in *M. truncatula* was drastically higher (up to about 8-fold) after treatment with pathogen-derived elicitors than after herbivore damage (cf. Leitner et al., 2005).

2.3. Accumulation of reactive oxygen species and nitric oxide

As enhanced VOC emission is usually associated with accumulation of JA, the fact that VOC emission and phytohormone levels could not be correlated was fairly puzzling. In order to find a link between the primary stimulus and VOC emission, the accumulation of ROS and NO were assessed using microscopic techniques.

The excessive production of ROS and NO is a trait that has been described in the plant's reaction to pathogen attack and elicitation with pathogen-derived elicitors (Lamb and Dixon, 1997; Durner and Klessig, 1999). In preliminary studies for NO detection using elicited leaves, hardly any reaction could be observed. Only in a few cases, where vascular bundles were uncovered by smashing the leaf, could the staining of this tissue after the treatment with β-glucans be seen (data not shown). This led to the assumption that there might be strong tissue specificity of NO production. Thus, in all following experiments, cross-sections of the stem were used for microscopic analysis. On the one hand, effects concerning vascular bundles could easily be visualised using this experimental setup, and on the other hand this procedure better fit the results presented above, as the site of action of the elicitors was kept comparatively constant. However, morphological studies on cross-sections showed that the cut tissue treated with elicitors in the experiments on VOC emission and phytohormone levels still belonged to the root (stems were cut directly at the soil surface). This means that remaining root tissue as well as aboveground tissue came in contact with the particular elicitors and might contribute to the responses. In contrast, the plant parts used to assess ROS and

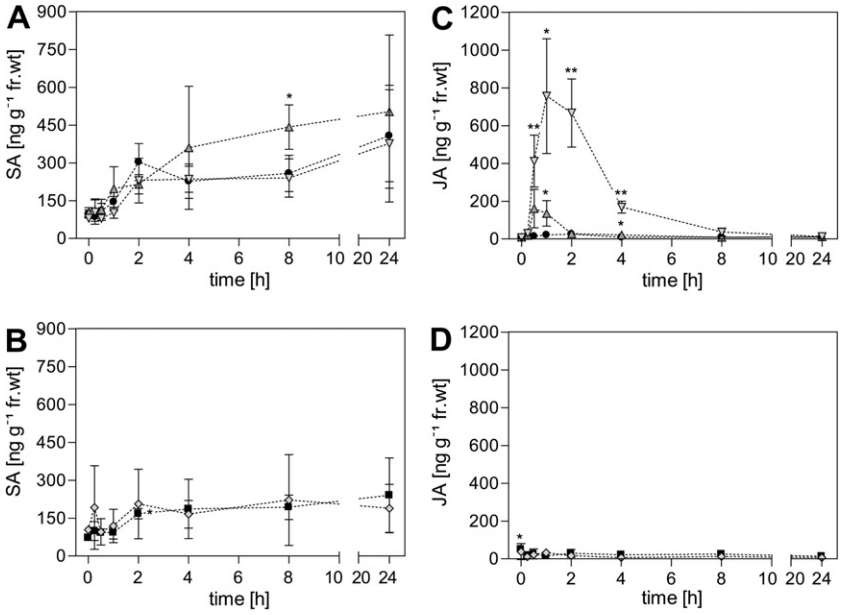

Fig. 5. Salicylic acid (SA) and jasmonic acid (JA) levels after treatment with microbial oligosaccharides. (A) SA levels in control plants (cut and placed into tap water, ●), plants treated with 100 μM CH4 (▲), and plants treated with 200 μg ml^{-1} β-glucan (▼). (B) SA levels of plants treated with 10 μM LCO-IV (C16:2) (inactive Nod-factor, ■), and 10 μM LCO-IV (C16:2, S) (active Nod-factor, ◇). C, JA levels in control plants (cut and placed into tap water, ●), plants treated with 100 μM CH4 (▼), and plants treated with 200 μg ml^{-1} β-glucan (▼). D, JA levels of plants treated with 10 μM LCO-IV (C16:2) (inactive Nod-factor, ■), and 10 μM LCO-IV (C16:2, S) (active Nod-factor, ◇). The results shown are the mean ± s.d. of three independent experiments. Asterisks indicate statistically significant differences as determined by two-tailed *t*-test. $*p < 0.05$; $**p < 0.01$.

NO accumulation were actually stems with no root tissue remaining (data not shown).

For all experiments only examples of vascular bundles are presented, as all observable effects were restricted to this tissue and its near surroundings. The accumulation of ROS was assessed with nitroblue tetrazolium (NBT), producing purple to blue precipitate in the presence of superoxide anions (Fig. 6). Regarding controls, some precipitates could be observed in all samples. Slight staining of the xylem in all samples can however, be regarded as "background" due to lignification in that region; but the phloem also exhibited faint staining. The responses observed to the treatments with all oligosaccharides tested were considerably stronger. All substances applied induced the pronounced accumulation of ROS. Phloem and cortical parenchyma were particularly affected; in some cases the staining also extended to the inner parenchyma. However, no pronounced or consistent differences between the different elicitors could be observed.

The detection of NO gave a much more distinct picture of effects induced by the different oligosaccharides (Fig. 7). Wounding and treatment with CH4 did not bring about any pronounced production of NO, though in some cases, slight staining of the phloem and parenchyma tissue could be observed. Considerably stronger effects could be detected after elicitation with β-glucans. After 60 min, distinct staining became visible in phloem and meristematic tissue; the signal increased with time, yielding strong staining after 180 min, again concerning phloem and cambium, and in some cases also parts of the cortical parenchyma.

Also, both Nod-factors induced NO accumulation. Though the temporal development of the reaction in response to the inactive Nod-factor, LCO-IV (C16:2), was highly variable, NO accumulation could consistently be shown after 180 min, affecting the phloem and cambium, comparable to β-glucan treatment, though the staining was of somewhat lower intensity. An entirely different pattern was observed for the active Nod-factor. In this case, staining was observed as early as 15 min after application, mostly in the cortical parenchyma, and thereafter it was drastically decreased. However, again after 180 min, pronounced staining was visible in the phloem, cambium, and cortical parenchyma, in several cases as pronounced as the staining shown for β-glucans. Altogether, the intensity of the effects observed varied greatly after all treatments. This was particularly prominent for the two Nod-factors. However, this variation is consistent with the high variability of the effects observed on induced VOC emission. The pictures shown in Fig. 7 represent examples of strong reactions observed.

Sections for the detection of ROS and NO were done not only directly at the contact site between the wounded stem and the elicitor solution, but also 1 cm above. Results were basically the same within this distance. That is, the observed effects can be considered not to be strictly localised to the wounding site and independent of the immediate contact with the elicitor. In summary, the combined results on ROS and NO accumulation in reaction to different oligosaccharides gave three distinct patterns: for the control, no increased production, of ROS or of NO, could be detected. Although ROS accumulated in response to CH4 treatment, this elicitor failed to induce excessive NO production. Finally, β-glucans as well as both Nod-factors induced overproduction of ROS and NO, though the latter accumulated in differing temporal and spatial patterns.

3. Discussion

Using *Medicago truncatula* as a model plant, reactions to diverse biotic stimuli have been recorded. Together with a previous study (Leitner et al., 2005), certain overlaps and distinctions in plants' defence responses can be summarised (Table 2). Conspicuous resemblance was found in the plants' reactions to different forms of herbivory and elicitation with the pathogen-derived β-glucans. Though all responses monitored were traceable after these stimuli, the quantitative as well as the spatio-temporal patterns differed considerably. Regarding the other elicitors of biotic origin, more distinctions became clear. Overproduction of ROS seems to be a general response to biotic stimuli, but not to mechanical damage in this species. Interestingly, several other species produce hydrogen peroxide in reaction to wounding, except for some legumes (Orozco-Cardenas and Ryan, 1999). The emission of VOCs and NO

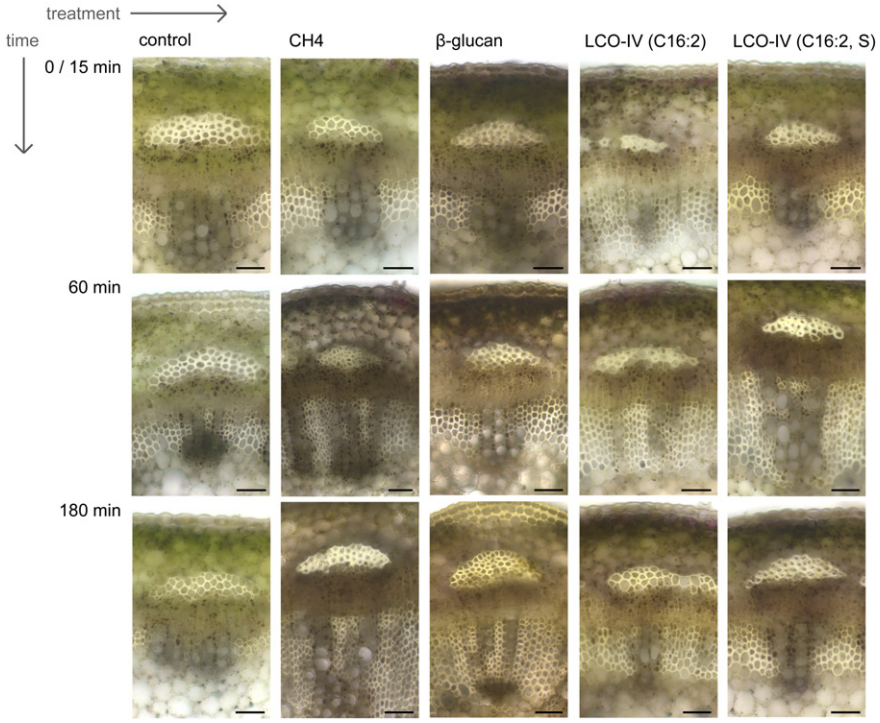

Fig. 6. Detection of reactive oxygen species (ROS) in cross-sections of *Medicago truncatula* stems in reaction to microbial oligosaccharides using nitroblue tetrazolium (NBT, purple–blue staining). Each column shows the reaction to a certain elicitor; each row depicts one point in the temporal development of ROS production. Control, plants cut and placed in tap water; CH4, treatment with 100 μM CH4; β-glucan, elicitation with 200 μg ml^{-1} β-glucans; LCO-IV (C16:2), treatment with 10 μM inactive Nod-factor; LCO-IV (C16:2, S), treatment with 10 μM active Nod-factor. Scaling bars represent 50 μm. Only samples of the control were taken immediately after wounding; the first samples of any other treatment were analysed 15 min after elicitation. There was no difference between 0 and 15 min.

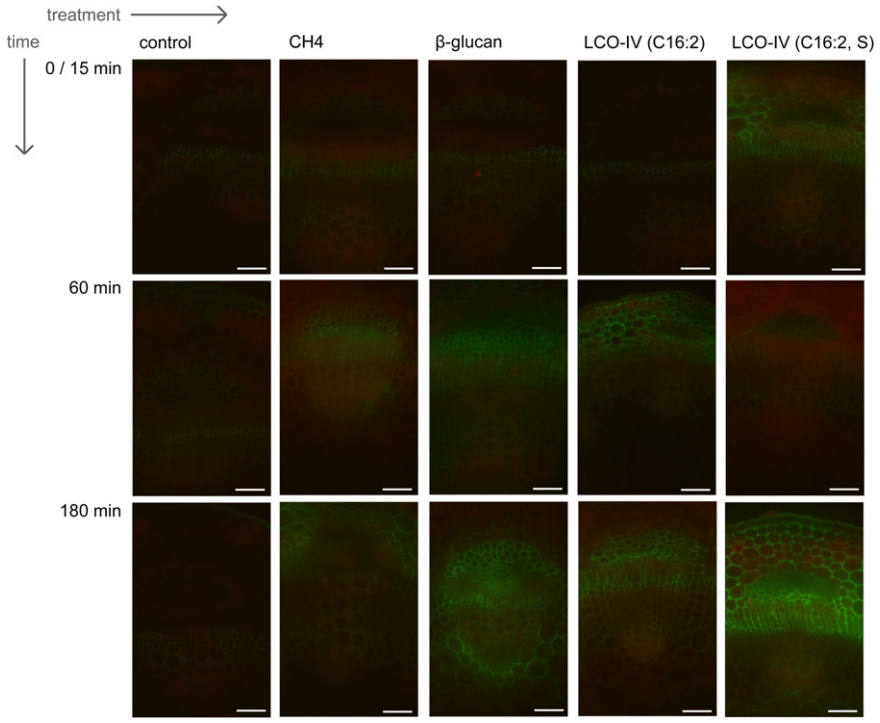

Fig. 7. Detection of nitric oxide (NO) in cross-sections of *Medicago truncatula* stems in reaction to microbial oligosaccharides using 4,5-diaminofluorescein diacetate (DAF-2 DA, green fluorescence). Pictures are overlays of the fluorescence signals produced by DAF-2 DA and chlorophyll autofluorescence. Each column shows the reaction to a certain elicitor; each row depicts one point in the temporal development of NO production. Control, plants cut and placed in tap water; CH4, treatment with 100 μM CH4; β-glucan, elicitation with 200 μg ml^{-1} β-glucans; LCO-IV (C16:2), treatment with 10 μM inactive Nod-factor; LCO-IV (C16:2, S), treatment with 10 μM active Nod-factor. Scaling bars represent 50 μm. Only samples of the control were taken immediately after wounding; the first samples of any other treatment were analysed 15 min after elicitation. There was no difference between 0 and 15 min.

Table 2
Comparison of defensive traits recorded in *Medicago truncatula* in reaction to biotic stimuli

	ROS	VOCs	NO	JA	SA
Herbivory[a]	+	+	+[c]	+	+[d]
CH4	+	–	–	+	n.d.[e]
β-glucans	+	+	+	+	n.d.[e]
LCO-IV (C16:2, S)	+	+/–[b]	+	–	n.d.[e]
LCO-IV (C16:2)	+	+/–[b]	+	-	n.d.[e]
Wounding/Control	–	–	–	–	+

[a] As documented in Leitner et al. (2005).
[b] Highly variable responses.
[c] Unpublished data, NO accumulates in reaction to caterpillar feeding.
[d] Depending on type of herbivory, at least slight increases detectable.
[e] SA accumulation was induced by cutting the stem. SA levels were not further influenced by the addition of any elicitors and are thus referred to as not determined (n.d.).

production could be detected after all induction treatments excluding CH4 and control (mechanical damage), and thus seem to represent common responses to biotic stimuli. Conversely, only organisms adverse to plants' health or elicitors thereof induced elevated levels of JA. Finally, the impact of oligosaccharidic elicitors on SA levels could not reliably be determined due to the substantial accumulation of SA in response to wounding. Some implications of these findings are discussed below.

Signal perception systems for pathogen-derived β-glucan elicitors, chitin fragments, and Nod-factors have been convincingly shown to be present in *Medicago* species (Côté et al., 2000; Felle et al., 2000; Cullimore et al., 2001). However, investigation of subsequent reactions predominantly concentrated on traits that were in close connection either with the activation of defence responses or with the establishment of a functional symbiosis.

So far, the possibility that plants may respond to those stimuli with the synthesis and emission of VOCs has largely escaped notice. That *M. truncatula* is in principle able to produce and emit these compounds has been shown. Gomez and co-workers (Gomez et al., 2005) described the transcript induction of two putative mono- or diterpene and two putative sesquiterpene synthases after jasmonate application and herbivory. Also the emission of a variety of VOCs in reaction to herbivory has been demonstrated (Leitner et al., 2005). Finally, with the results presented above, VOC emission could be shown after elicitation with diverse microbial oligosaccharidic signals.

NMDS represents a straightforward and robust exploratory tool for visualising volatile blends in response to diverse induction treatments in a reduced multidimensional space suitable for direct interpretation. Both qualitative and quantitative patterns can readily be evaluated using different levels of abstraction of data, i.e. originally scaled, or only with regard to the presence or absence of certain compounds. As each sample is depicted individually, the method is also applicable to small sample sizes. Treatments can be studied in direct connection to each other; mapped substances can also be interpreted in conjunction with the treatments they are most likely to correlate with, as any compound is directly projected onto the ordination. Thus, patterns could be found, which univariate methods would have been unable to capture.

The emission of volatiles induced by herbivory has often been causally linked with elevated levels of JA (Walling, 2000; Gatehouse, 2002; Ament et al., 2004). In the present study, no conclusive correlation between JA levels and VOC release could be found, as treatment with CH4 resulted not in volatile emission but in the accumulation of JA, whereas both Nod-factors induced VOC emission but not increased JA levels. Only elicitation with β-glucans gave rise to accumulation of JA and accordingly to the highest level of volatile emission. Also, increases in SA levels cannot be interpreted as responsible for volatile release, as the pattern of accumulation was the same in treated and in control plants. Cutting the plant led to an increase in SA concentration that was not further enhanced by the application of any of the elicitors tested. This is in contrast to the situation in lima bean (*Phaseolus lunatus* L.), where cutting the stem has been shown not to influence SA levels (Engelberth et al., 2001). Also responses of maize to wounding differ from those of *M. truncatula*; JA accumulates in reaction to the excision of leaves, which is not further enhanced by application of volicitin (Schmelz et al., 2003). In summary, these data seem to warn against underrating species specificity in phytohormonal action. However, we can not completely exclude an indirect role of JA; for example, other compounds might have taken over the role of JA, such as JA-conjugates or (unknown) derivatives, which have not been detected in our study.

The results of this study are, however, in line with previous reports indicating that neither of the two phytohormones is responsible of VOC emission in reaction to bacterial pathogen attack in tobacco (Huang et al., 2003). In a succeeding study, the impact of ethylene on volatile emission was assessed (Huang et al., 2005). But again, no sound evidence was found that changing levels of any of the studied phytohormones were somehow linked to alterations in volatile emission.

In the studies of Huang and co-workers (Huang et al., 2003; 2005) different strains of *Pseudomonas syringae* were used to induce VOC release. These pathogens produce several toxins, amongst them coronatine, which is known to mimic JA functionally and to induce secondary metabolism (Weiler et al., 1994); the presence of coronatine may explain volatile release independent of phytohormonal changes in this case. Still, further studies with other bacterial (Buonaurio and Servili, 1999) and fungal (Cardoza et al., 2002) pathogens strengthened the idea that volatile release may be a general reaction of plants to pathogen attack. Yet, no conclusive results on causal relationships concerning the mode of induction were found, though the study conducted by Buonaurio and Servili (1999) suggests an involvement of the lipoxygenase pathway. However, recent studies questioned the essential role of JA in the induction of secondary metabolism (Zhao et al., 2005). For example, treating *Petroselinum crispum* cell cultures with inhibitors of JA accumulation did not influence phytoalexin production or PR gene expression in response to elicitation (Hahlbrock et al., 2003); in soybean cell cultures, elicitation with β-glucans induced phytoalexin accumulation, while endogenous levels of JA, 12-oxo phytodienoic acid, and SA remained at the resting level (Fliegmann et al., 2003); treating *Hyoscyamus muticus* root cultures with either methyl jasmonate or a fungal elicitor resulted in the induction of sesquiterpenes in quantitatively and qualitatively different patterns (Singh et al., 1998).

Even though CH4 induced higher JA levels compared to herbivory, it failed to induce the release of VOCs. Conversely, both Nod-factors, the biologically active as well as the inactive, did not influence JA or SA levels but led to slightly elevated sesquiterpene emissions. These results indicate that the unexpected perception of Nod-factors in "non-target tissue" is unlikely to occur via the recognition of the chitotetraose moiety, given the clearly differing responses to the three structurally related signals. Still, in the case of β-glucan elicitation, elevated JA levels and increased VOC emission coincided, which might be regarded as incidental given the other results.

In view of the inconsistencies outlined above, an alternative hypothesis needs to be stated.

First, a multitude of other oxylipins, including a diverse range of JA conjugates, exists that is only poorly described regarding their biological activities and physiological roles (Blee, 2002; Staswick and Tiryaki, 2004; Schulze et al., 2006; Wang et al., 2007).

Furthermore, the involvement of the lipoxygenase pathway is not inevitably required for the induction of secondary metabolism (Fliegmann et al., 2003; Hahlbrock et al., 2003; Zhao et al., 2005). Yet another way to link the interactions with pathogens and symbionts to volatile emission is via the production of ROS. The oxidative burst is a well-described phenomenon in the context of the hypersensitive response (Lamb and Dixon, 1997). Also, β-glucans (Mithöfer et al., 1997) and chitin fragments (Yamaguchi et al., 2005) have been shown to induce ROS production. Still, elevated levels of ROS have not only been detected after pathogen attack or elicitor challenge, but they proved to be a rather common trait of plants' responses to other biotic threats such as herbivory or abiotic stresses (Mithöfer et al., 2004). Finally, ROS can even be detected in roots after the application of Nod-factors (Ramu et al., 2002) and in root nodules (Santos et al., 2001). As shown in Figs. 6 and 7, ROS and NO can also be detected in response to elicitation with certain microbial oligosaccharides, displaying distinct patterns of accumulation depending on the respective induction treatment.

Amongst the variety of effects produced by ROS in a cell, radical-mediated lipid peroxidation can lead to the formation of cyclic oxylipins independent of enzymatic participation. These linolenic acid-derived compounds, namely phytoprostanes, can be induced by wounding, heavy metals, and pathogen attack via the production of ROS (Imbusch and Mueller, 2000; Thoma et al., 2003). Moreover, the physiological role of NO in plants has gained increasing attention in the last few years. However, its mode of action is still largely unknown. Only recently have *S*-nitrosylated proteins been identified in *Arabidopsis thaliana*, paralleling the well-described posttranslational modifications in animals (Lindermayr et al., 2005, 2006). Furthermore, it has been demonstrated lately that in animal systems nitrated fatty acids might be important signalling compounds (Baker et al., 2005; Schopfer et al., 2005). Analogous reactions are clearly possible in plant cells and would presume the existence of another class of signal components in plants that has so far escaped notice. As the occurrence of nitrated compounds, particularly fatty acids, in plants has not yet been investigated, the analysis of such putative signalling compounds is an interesting target for further investigation. Finally, the hypothesis stated by Mithöfer et al. (2004), regarding ROS as link in mediating diverse stress responses, may also be extended by the impact of NO and to some cases of beneficial biotic interactions, too.

Besides open questions regarding signalling cascades leading to the emission of VOCs, physiological and ecological functions remain to be discussed. In the case of pathogen attack or induction with pathogen-derived elicitors, the problem has already been addressed, and the antimicrobial properties of certain emitted compounds have been confirmed. These compounds belong to the class of lipid-derived volatiles from the lipoxygenase pathway such as (*Z*)-3-hexenol, (*E*)-2-hexenal, and (*Z*)-3-hexenyl acetate; the monoterpenoid linalool and methyl salicylate have also been shown to inhibit pathogen growth (Croft et al., 1993; Wright et al., 2000; Cardoza et al., 2002; Kishimoto et al., 2006). Many essential oils, however, exhibit broad range antimicrobial activity. Functionality of this form of defence has been demonstrated for *Phytophthora infestans*, using essential oils of various aromatic plants (Soylu et al., 2006). Interestingly, the antimicrobial impact was stronger when the oomycete was exposed to VOCs (Soylu et al., 2006).

Consequently, the emission of VOCs might represent means of direct defence in this respect. On the other hand, volatile release could contribute to the engagement of host resistance mechanisms, both systemically or in plant-to-plant communication (Farmer, 2001; Holopainen, 2004; Heil and Silva Bueno, 2007). The ability of certain VOCs (terpenoids and C6 components) to trigger the onset of resistance has already been shown in *Arabidopsis thaliana* (Bate and Rothstein, 1998; Kishimoto et al., 2005), lima bean (Arimura et al., 2000, 2001), and tomato (Farag and Pare, 2002; He et al., 2006). The situation is somewhat more complicated in the case of Nod-factors. On the one hand, it has been proposed that the establishment of a functional symbiosis could rely on the suppression of the plant's defence response (e.g. Mithöfer, 2002). This could imply the initiation of certain defence reactions before the onset of an effective suppression or an incomplete suppression of these defences. On the other hand, emitted volatiles could be employed as signals mediating the interaction between the host plant and the microbial symbiont. In a recent study, Horiuchi and co-workers (Horiuchi et al., 2005) showed that in response to plant-derived volatiles the soil nematode *Caenorhabditis elegans* transfers the nodulating bacterium *Sinorhizobium meliloti* to the rhizosphere of *M. truncatula*. Finally, elevated emission of VOCs could be a symptom of induced resistance as has been proposed to be an effect of interaction with beneficial microorganisms.

4. Concluding remarks

In summary, it was possible to demonstrate that *M. truncatula* emits a variety of VOCs in reaction to pathogenic and symbiotic oligosaccharidic signals. The distinct patterns recorded indicate a high plasticity of induced VOC emissions. From a mechanistic point of view it is intriguing that diverse, though structurally related molecules induce similar responses albeit seemingly via different signal pathways. Those signalling nets, however, remain elusive. Finally, though the compounds tested induce defence-related responses in a bioassay, the occurrence and biological relevance of such reactions in biotic interactions still needs to be thoroughly investigated and defined.

5. Experimental

5.1. Biological material

Medicago truncatula Gaertn. cv. Jemalong A17 seeds were kindly provided by Dr. J.M. Prosperi (INRA-SGAP, Montpellier, France). Seeds were allowed to germinate in the dark for four days, then the seedlings were grown in the greenhouse at 18–23 °C with a light period from 7 a.m. to 9 p.m. Humidity was kept at 60–70%.

5.2. Elicitors, induction treatments

β-Glucan elicitors were prepared from mycelia cell walls of the oomycete *Phytophthora sojae* Kaufmann & Gerdemann as described (Schmidt and Ebel, 1987) and applied in a concentration of 200 μg ml^{-1}. *N,N′,N″,N‴*-tetraacetylchitotetraose (CH4) was purchased from Sigma, Germany, and used at 100 μM. Nod-factors, the lipo-chitooligosaccharides LCO-IV (C16:2, S) and LCO-IV (C16:2), were synthesised as described (Rasmussen et al., 2004) and added at 10 μM. All substances were dissolved in water except for LCO-IV (C16:2) (stock solution in DMSO). Dilution to the respective concentration for the treatments was done with tap water. Solvent controls using DMSO did not reveal any influence on volatile emission.

In all experiments, 5 ml of the respective elicitor solution were used. Plants were cut at the soil surface and placed in the elicitor solution. Within about 24 h, these 5 ml were completely taken up by the plant, which was thereafter supplied with tap water. As controls both undamaged plants and plants cut and placed into tap water were used. Wounding plants by cutting did not induce elevated levels of volatile emission.

5.3. Analysis of VOC emission

Volatiles were collected over a period of 48 h using the closed-loop stripping method as described by Donath and Boland (1995). Desorption was done using methylene chloride (2×20 μl) containing 100 μg ml^{-1} *n*-bromodecane as internal standard; the volatiles were analysed using GC–MS (TRACE 2000 series, Finnigan, UK) equipped with an EC-5 capillary (Alltech, Unterhaching, Germany).

Compounds were identified according to their fragmentation pattern (MS) and by comparison of linear retention indices (RI). To compare mass spectra, the NIST/EPA/NIH Mass Spectral Library (Version 1998) and MassFinder (V 3.5; Dr. D. Hochmuth, Hamburg, Germany) were used. The measured retention indices were compared either with those calculated from pure reference compounds or with literature data (Adams, 2001; Linstrom and Mallard, 2005, and references therein; retention indices of the compound in question measured under comparable conditions; i.e. temperature ramp, equivalent GC column, etc.). Deviations of ±2 for reference compounds and ±5 for literature data were accepted for identical compounds in accordance with Hochmuth (2004).

Relative quantification of the compounds emitted was done by relating the respective peak areas to that of the internal standard (100 μg ml^{-1} *n*-bromodecane) and to the fresh weight of the plants.

For all statistics on VOC emission, square root transformed data was used. The first line of statistical analysis was done using one-way ANOVA combined with the Newman–Keuls *post hoc* test to compare the levels of single compounds emitted after the different treatments.

In order to achieve exploratory mappings of different treatment effects in a mathematical space as defined by the volatile blends, a Non-metric Multidimensional Scaling (NMDS) ordination was carried out (Kruskal, 1964). NMDS is a method of multivariate statistics that is employed to analyse the structure of similarity or dissimilarity of data in multidimensional feature space. NMDS represents data as distances between points in a geometric space of low dimensionality (three or less). A two dimensional solution was found to be appropriate for the data. Therefore, only the configuration of points (samples) and their *interitem* distances count, i.e. points close to each other are likely to share some intrinsic properties, whereas distant points bear little or no similarity. NMDS maps observed dissimilarities non-linearly onto ordination space and can effectively handle non-linear responses of any shape. Therefore, the method does not rely on linear relationships between variables as in Principal Component Analysis and is commonly regarded as one of the most robust ordination methods in exploratory multivariate analysis, especially in ecology where data are often noisy and/or sparse (Minchin, 1987; Legendre and Legendre, 1998; Borg and Groenen, 2005). The analysis was performed in the way recommended by Minchin (1987), as implemented in the R package VEGAN (Oksanen et al., 2006). Prior to analysis, the data were standardised using square root transformation and tested for proper transformation using the Shapiro–Wilk test of normality (Royston, 1982). In the first line of analysis by NMDS, Euclidean distance was selected as dissimilarity measure. In order to find out, if volatile blends can be separated in ordination using only binary responses (i.e. presence or absence of a particular compound in the sample), and hence disregarding any effect of concentration, data were reduced to the binomial form. A binary dissimilarity index was used in this respect.

The overall goodness of fit of the models was measured by the stress statistic, the correlation between fitted values and ordination distances (Venables and Ripley, 2002).This statistic gives the proportion of data not ideally depicted in the ordination. The reported value of stress herein is not bounded and is defined as the sum of the squared residuals from a monotonic regression of measured dissimilarities and ordination distances.

5.4. Analysis of phytohormone levels

Salicylic acid and jasmonic acid levels were determined according to the protocol for jasmonate quantification of Koch et al. (1999) with minor modifications. Briefly, plants were weighted and immediately frozen in liquid nitrogen. After the addition of 30 ml acetone:50 mM citric acid (7:3, v/v) and 150 ng [9,10-$^2H_2$]-9,10-dihydro-JA and 500 ng [3,4,5,6-$^2H_4$]-SA as internal standards plants were homogenised using an ultra-turrax. The acetone was allowed to evaporate over night at room temperature. Samples were cleared by filtration and subsequently extracted with 3×10 ml diethyl ether. The extracts were then loaded on solid-phase extraction cartridges containing 500 mg aminopropyl (Chromabond, Macherey-Nagel, Germany). After washing with 5 ml chloroform:isopropanol (2:1, v/v), bound acids were eluted using 12 ml diethyl ether:formic acid (98:2, v/v). After evaporation of the solvents the residues were methylated using excess diazomethane. The final sample volume was adjusted to 50 μl with dichloromethane and analysis was performed using GC–MS (TRACE 2000 series, Finnigan, UK) in the selective ion mode. The fragment ions were monitored at $m/z = 120$, 124, and 83 for SA, [3,4,5,6-$^2H_4$]-SA, and JA and [9,10-$^2H_2$]-9,10-dihydro-JA, respectively.

The endogenous concentrations of salicylate and jasmonate were calculated from the peak areas of the respective substance and its standard using calibration curves. [3,4,5,6-$^2H_4$]-SA and [9,10-$^2H_2$]-9,10-dihydro-JA were synthesised as described by Engelberth et al. (2001) and Koch et al. (1999), respectively, and were readily available in our Department.

Statistics were done using the Student's *t*-test. All comparisons were done between treatments and the respective controls.

5.5. Detection of reactive oxygen species

For the detection of superoxide ($O_2^{\cdot-}$) nitroblue tetrazolium (NBT; Sigma–Aldrich) was used. A droplet of NBT solution (6 mM in deionised water) was spread on a microscope slide; fresh hand-cut cross-sections of *M. truncatula* stems were put onto the liquid and covered with a cover slip. After 10–15 min of incubation the sections were rinsed by sucking water through the preparation by means of filter paper. The slides were viewed and documented using a light microscope (Axioskop, Zeiss, Germany) equipped with a digital imaging system (Spot, Visitron Systems, Germany).

5.6. Detection of nitric oxide

To detect NO by microscopic means, the method described by Foissner et al. (2000) was applied with minor modifications. Samples were stained using 4,5-diaminofluorescein diacetate (DAF-2 DA; Sigma–Aldrich). A droplet of a 10 μM solution (in 10 mM Tris/HCl, pH 7.2) was spread on a slide; fresh hand-cut cross-sections of stems were put onto the liquid, covered with a cover slip, and left for about 10 min in the dark. Objects were washed by sucking buffer through the preparation with filter paper. Staining was viewed using a fluorescence microscope (Axioskop, Zeiss, Germany), operated with two different filters. For the visualisation of the fluorescein signal, a 450–490 nm excitation filter and a 515–565 nm emission filter were used. For viewing chlorophyll autofluorescence, a band pass 546 (ex)/long pass 590 (em) filter was used. A digital imaging system was used for documentation (Spot, Visitron Systems, Germany). For the sake of comparability, exposure times were kept constant within one row of experiments. Pictures

presented are overlays of the DAF-2 DA signal and chlorophyll autofluorescence.

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References

Adams, R.P., 2001. Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectroscopy. Allured Publishing Corporation, Carol Stream, IL.

Ament, K., Kant, M.R., Sabelis, M.W., Haring, M.A., Schuurink, R.C., 2004. Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato. Plant Physiol. 135, 2025–2037.

Arimura, G., Ozawa, R., Horiuchi, J., Nishioka, T., Takabayashi, J., 2001. Plant–plant interactions mediated by volatiles emitted from plants infested by spider mites. Biochem. Syst. Ecol. 29, 1049–1061.

Arimura, G., Ozawa, R., Shimoda, T., Nishioka, T., Boland, W., Takabayashi, J., 2000. Herbivory-induced volatiles elicit defence genes in lima bean leaves. Nature 406, 512–515.

Baier, R., Schiene, K., Kohring, B., Flaschel, E., Niehaus, K., 1999. Alfalfa and tobacco cells react differently to chitin oligosaccharides and *Sinorhizobium meliloti* nodulation factors. Planta 210, 157–164.

Baker, P.R.S., Lin, Y., Schopfer, F.J., Woodcock, S.R., Groeger, A.L., Batthyany, C., Sweeney, S., Long, M.H., Iles, K.E., Baker, L.M.S., Branchaud, B.P., Chen, Y.E., Freeman, B.A., 2005. Fatty acid transduction of nitric oxide signaling: Multiple nitrated unsaturated fatty acid derivatives exist in human blood and urine and serve as endogenous peroxisome proliferator-activated receptor ligands. J. Biol. Chem. 280, 42464–42475.

Bate, N.J., Rothstein, S.J., 1998. C_6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. Plant J. 16, 561–569.

Blee, E., 2002. Impact of phyto-oxylipins in plant defense. Trends Plant Sci. 7, 315–321.

Boller, T., 1995. Chemo-perception of microbial signals in plant cells. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 189–214.

Borg, I., Groenen, P., 2005. Modern Multidimensional Scaling. Springer, New York.

Bostock, R.M., Karban, R., Thaler, J.S., Weyman, P.D., Gilchrist, D., 2001. Signal interactions in induced resistance to pathogens and insect herbivores. Eur. J. Plant Pathol. 107, 103–111.

Bueno, P., Soto, M.J., Rodríguez-Rosales, M.P., Sanjuan, J., Olivares, J., Donaire, J.P., 2001. Time-course of lipoxygenase, antioxidant enzyme activities and H_2O_2 accumulation during the early stages of *Rhizobium* -legume symbiosis. New Phytol. 152, 91–96.

Buonaurio, R., Servili, M., 1999. Involvement of lipoxygenase, lipoxygenase pathway volatiles, and lipid peroxidation during the hypersensitive reaction of pepper leaves to *Xanthomonas campestris* pv. *vesicatoria*. Physiol. Mol. Plant Pathol. 54, 155–169.

Cardoza, Y.J., Alborn, H.T., Tumlinson, J.H., 2002. *In vivo* volatile emissions from peanut plants induced by simultaneous fungal infection and insect damage. J. Chem. Ecol. 28, 161–174.

Côté, F., Roberts, A., Hahn, M.G., 2000. Identification of high-affinity binding sites for the hepta-β-glucoside elicitor in membranes of the model legumes *Medicago truncatula* and *Lotus japonicus*. Planta 211, 596–605.

Croft, K.P.C., Jüttner, F., Slusarenko, A.J., 1993. Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv. *phaseolicola*. Plant Physiol. 101, 13–24.

Cullimore, J.V., Ranjeva, R., Bono, J.-J., 2001. Perception of lipo-chitooligosaccharidic Nod factors in legumes. Trends Plant Sci. 6, 24–30.

D'Haeze, W., Holsters, M., 2002. Nod factor structures, responses, and perception during initiation of nodule development. Glycobiology 12, 79R–105R.

Donath, J., Boland, W., 1995. Biosynthesis of acyclic homoterpenes: enzyme selectivity and absolute configuration of the nerolidol precursor. Phytochemistry 39, 785–790.

Durner, J., Klessig, D.F., 1999. Nitric oxide as a signal in plants. Curr. Opin. Plant Biol. 2, 369–374.

Ebel, J., Cosio, E.G., 1994. Elicitors of plant defense responses. Int. Rev. Cytol. 148, 1–36.

Ebel, J., Mithöfer, A., 1998. Early events in the elicitation of plant defence. Planta 206, 335–348.

Engelberth, J., Koch, T., Schüler, G., Bachmann, N., Rechtenbach, J., Boland, W., 2001. Ion channel-forming alamethicin is a potent elicitor of volatile biosynthesis and tendril coiling. Cross talk between jasmonate and salicylate signaling in Lima bean. Plant Physiol. 125, 369–377.

Farag, M.A., Pare, P.W., 2002. C_6-Green leaf volatiles trigger local and systemic VOC emissions in tomato. Phytochemistry 61, 545–554.

Farmer, E.E., 2001. Surface-to-air signals. Nature 411, 854–856.

Felle, H.H., Kondorosi, É., Kondorosi, Á., Schultze, M., 2000. How alfalfa root hairs discriminate between Nod factors and oligochitin elicitors. Plant Physiol. 124, 1373–1380.

Fernandes, G.W., 1990. Hypersensitivity: a neglected plant resistance mechanism against insect herbivores. Environ. Entomol. 19, 1173–1182.

Fernandes, G.W., Negreiros, D., 2001. The occurrence and effectiveness of hypersensitive reaction against galling herbivores across host taxa. Ecol. Entomol. 26, 46–55.

Fliegmann, J., Schüler, G., Boland, W., Ebel, J., Mithöfer, A., 2003. The role of octadecanoids and functional mimics in soybean defense responses. Biol. Chem. 384, 437–446.

Foissner, I., Wendehenne, D., Langebartels, C., Durner, J., 2000. *In vivo* imaging of an elicitor-induced nitric oxide burst in tobacco. Plant J. 23, 817–824.

Gatehouse, J.A., 2002. Plant resistance towards insect herbivores: a dynamic interaction. New Phytol. 156, 145–169.

Glazebrook, J., 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. 43, 207–227.

Gomez-Gomez, L., 2004. Plant perception systems for pathogen recognition and defence. Mol. Immunol. 41, 1055–1062.

Gomez, S.K., Cox, M.M., Bede, J.C., Inoue, K., Alborn, H.T., Tumlinson, J.H., Korth, K.L., 2005. Lepidopteran herbivory and oral factors induce transcripts encoding novel terpene synthases in *Medicago truncatula*. Arch. Insect Biochem. Physiol. 58, 114–127.

Hahlbrock, K., Bednarek, P., Ciolkowski, I., Hamberger, B., Heise, A., Liedgens, H., Logemann, E., Nürnberger, T., Schmelzer, E., Somssich, I.E., Tan, J., 2003. Non-self recognition, transcriptional reprogramming, and secondary metabolite accumulation during plant/pathogen interactions. Proc. Natl. Acad. Sci. USA 100, 14569–14576.

He, P.-Q., Tian, L., Chen, K.-S., Hao, L.-H., Li, G.-Y., 2006. Induction of volatile organic compounds of *Lycopersicon esculentum* Mill. and its resistance to *Botrytis cinerea* Pers. by burdock oligosaccharide. J. Integr. Plant Biol. 48, 550–557.

Heil, M., Silva Bueno, J.C., 2007. Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. Proc. Natl. Acad. Sci. USA 104, 5467–5472.

Hochmuth, D.H., 2004. MassFinder 3, Hamburg. <http://www.massfinder.com>.

Holopainen, J.K., 2004. Multiple functions of inducible plant volatiles. Trends Plant Sci. 9, 522–529.

Horiuchi, J., Prithiviraj, B., Bais, H.P., Kimball, B.A., Vivanco, J.M., 2005. Soil nematodes mediate positive interactions between legume plants and rhizobium bacteria. Planta 222, 848–857.

Huang, J., Cardoza, Y.J., Schmelz, E.A., Raina, R., Engelberth, J., Tumlinson, J.H., 2003. Differential volatile emissions and salicylic acid levels from tobacco plants in response to different strains of *Pseudomonas syringae*. Planta 217, 767–775.

Huang, J., Schmelz, E.A., Alborn, H.T., Engelberth, J., Tumlinson, J.H., 2005. Phytohormones mediate volatile emissions during the interaction of compatible and incompatible pathogens: the role of ethylene in *Pseudomonas syringae* infected tobacco. J. Chem. Ecol. 31, 439–459.

Imbusch, R., Mueller, M.J., 2000. Analysis of oxidative stress and wound-inducible dinor isoprostanes F_1 (phytoprostanes F_1) in plants. Plant Physiol. 124, 1293–1303.

Kessler, A., Baldwin, I.T., 2002. Plant responses to insect herbivory: The emerging molecular analysis. Annu. Rev. Plant Biol. 53, 299–328.

Kishimoto, K., Matsui, K., Ozawa, R., Takabayashi, J., 2005. Volatile C6-aldehydes and allo-ocimene activate defense genes and induce resistance against *Botrytis cinerea* in *Arabidopsis thaliana*. Plant Cell Physiol. 46, 1093–1102.

Kishimoto, K., Matsui, K., Ozawa, R., Takabayashi, J., 2006. Components of C6-aldehyde-induced resistance in *Arabidopsis thaliana* against a necrotrophic fungal pathogen, *Botrytis cinerea*. Plant Sci. 170, 715–723.

Koch, T., Krumm, T., Jung, V., Engelberth, J., Boland, W., 1999. Differential induction of plant volatile biosynthesis in the Lima bean by early and late intermediates of the octadecanoid-signaling pathway. Plant Physiol. 121, 153–162.

Kruskal, J.B., 1964. Nonmetric multidimensional scaling: a numerical method. Psychometrika 29, 115–129.

Lamb, C., Dixon, R.A., 1997. The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 251–275.

Legendre, P., Legendre, L., 1998. Numerical ecology, in: S.E. Jørgensen (Ed.), Developments in Environmental Modelling, vol. 20, Elsevier, Amsterdam, pp. 1–853.

Leitner, M., Boland, W., Mithöfer, A., 2005. Direct and indirect defences induced by piercing-sucking and chewing herbivores in *Medicago truncatula*. New Phytol. 167, 597–606.

Lindermayr, C., Saalbach, G., Bahnweg, G., Durner, J., 2006. Differential inhibition of Arabidopsis methionine adenosyltransferases by protein *S*-nitrosylation. J. Biol. Chem. 281, 4285–4291.

Lindermayr, C., Saalbach, G., Durner, J., 2005. Proteomic identification of *S*-nitrosylated proteins in Arabidopsis. Plant Physiol. 137, 921–930.

Linstrom, P.J., Mallard, W.G. (Eds.), 2005. NIST Chemistry WebBook, NIST Standard Reference Database Number 69. National Institute of Standards and Technology, Gaithersburg, MD <http://webbook.nist.gov/>.

Maffei, M.E., Mithöfer, A., Arimura, G.I., Uchtenhagen, H., Bossi, S., Bertea, C.M., Cucuzza, L.S., Novero, M., Volpe, V., Quadro, S., Boland, W., 2006. Effects of feeding *Spodoptera littoralis* on Lima bean leaves. III. Membrane depolarization and involvement of hydrogen peroxide. Plant Physiol. 140, 1022–1035.

Martínez-Abarca, F., Herrera-Cervera, J.A., Bueno, P., Sanjuan, J., Bisseling, T., Olivares, J., 1998. Involvement of salicylic acid in the establishment of the *Rhizobium meliloti* -alfalfa symbiosis. Mol. Plant-Microbe Interact. 11, 153–155.

Minchin, P.R., 1987. An evaluation of the relative robustness of techniques for ecological ordination. Vegetatio 69, 89–107.

Mithöfer, A., 2002. Suppression of plant defence in rhizobia-legume symbiosis. Trends Plant Sci. 7, 440–444.

Mithöfer, A., Daxberger, A., Fromhold-Treu, D., Ebel, J., 1997. Involvement of an NAD(P)H oxidase in the elicitor-inducible oxidative burst of soybean. Phytochemistry 45, 1101–1107.

Mithöfer, A., Schulze, B., Boland, W., 2004. Biotic and heavy metal stress response in plants: evidence for common signals. FEBS Lett. 566, 1–5.

Nürnberger, T., Brunner, F., Kemmerling, B., Piater, L., 2004. Innate immunity in plants and animals: striking similarities and obvious differences. Immunol. Rev. 198, 249–266.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, R.B., 2006. Vegan: Community Ecology Package, R package version 1.8–3. <http://cran.r-project.org/>.

Ollerstam, O., Rohfritsch, O., Höglund, S., Larsson, S., 2002. A rapid hypersensitive response associated with resistance in the willow *Salix viminalis* against the gall midge *Dasineura marginemtorquens*. Entomol. Exp. Appl. 102, 153–162.

Orozco-Cardenas, M.L., Ryan, C.A., 1999. Hydrogen peroxide is generated systemically in plant leaves by wounding and systemic via the octadecanoid pathway. Proc. Natl. Acad. Sci. USA 96, 6553–6557.

Ramu, S.K., Peng, H.-M., Cook, D.R., 2002. Nod factor induction of reactive oxygen species production is correlated with expression of the early nodulin gene *rip1* in *Medicago truncatula*. Mol. Plant-Microbe Interact. 15, 522–528.

Rasmussen, M.O., Hogg, B., Bono, J.J., Samain, E., Driguez, H., 2004. New access to lipo-chitooligosaccharide nodulation factors. Org. Biomol. Chem. 2, 1908–1910.

Royston, P., 1982. Algorithm AS 181: The W test for normality. Appl. Stat. 31, 176–180.

Santos, R., Hérouart, D., Sigaud, S., Touati, D., Puppo, A., 2001. Oxidative burst in alfalfa- *Sinorhizobium meliloti* symbiotic interaction. Mol. Plant-Microbe Interact. 14, 86–89.

Savouré, A., Sallaud, C., El-Turk, J., Zuanazzi, J., Ratet, P., Schultze, M., Kondorosi, Á., Esnault, R., Kondorosi, É., 1997. Distinct response of *Medicago* suspension cultures and roots to Nod factors and chitin oligomers in the elicitation of defense-related responses. Plant J. 11, 277–287.

Schmelz, E.A., Alborn, H.T., Tumlinson, J.H., 2003. Synergistic interactions between volicitin, jasmonic acid and ethylene mediate insect-induced volatile emission in *Zea mays*. Physiol. Plant 117, 403–412.

Schmidt, W.E., Ebel, J., 1987. Specific binding of a fungal glucan phytoalexin elicitor to membrane-fractions from soybean *Glycine max*. Proc. Natl. Acad. Sci. USA 84, 4117–4121.

Schopfer, F.J., Baker, P.R.S., Giles, G., Chumley, P., Batthyany, C., Crawford, J., Patel, R.P., Hogg, N., Branchaud, B.P., Lancaster Jr., J.R., Freeman, B.A., 2005. Fatty acid transduction of nitric oxide signaling: nitrolinoleic acid is a hydrophobically stabilized nitric oxide donor. J. Biol. Chem. 280, 19289–19297.

Schulze, B., Lauchli, R., Sonwa, M.M., Schmidt, A., Boland, W., 2006. Profiling of structurally labile oxylipins in plants by in situ derivatization with pentafluorobenzyl hydroxylamine. Anal. Biochem. 348, 269–283.

Singh, G., Gavrieli, J., Oakey, J.S., Curtis, W.R., 1998. Interaction of methyl jasmonate, wounding and fungal elicitation during sesquiterpene induction in *Hyoscyamus muticus* in root cultures. Plant Cell Rep. 17, 391–395.

Soylu, E.M., Soylu, S., Kurt, S., 2006. Antimicrobial activities of the essential oils of various plants against tomato late blight disease agent *Phytophthora infestans*. Mycopathologia 161, 119–128.

Staehelin, C., Granado, J., Müller, J., Wiemken, A., Mellor, R.B., Felix, G., Regenass, M., Broughton, W.J., Boller, T., 1994. Perception of *Rhizobium* nodulation factors by tomato cells and inactivation by root chitinases. Proc. Natl. Acad. Sci. USA 91, 2196–2200.

Staswick, P.E., Tiryaki, I., 2004. The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant Cell 16, 2117–2127.

Sun, J., Cardoza, V., Mitchell, D.M., Bright, L., Oldroyd, G., Harris, J.M., 2006. Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation. Plant J. 46, 961–970.

Thoma, I., Loeffler, C., Sinha, A.K., Gupta, M., Krischke, M., Steffan, B., Roitsch, T., Mueller, M.J., 2003. Cyclopentenone isoprostanes induced by reactive oxygen species trigger defense gene activation and phytoalexin accumulation in plants. Plant J. 34, 363–375.

Venables, W.N., Ripley, B.D., 2002. Modern Applied Statistics. S. Springer, New York.

Walling, L.L., 2000. The myriad plant responses to herbivores. J. Plant Growth Regul. 19, 195–216.

Wang, L., Halitschke, R., Kang, J.-H., Berg, A., Harnisch, F., Baldwin, I.T., 2007. Independently silencing two JAR family members impairs levels of trypsin proteinase inhibitors but not nicotine. Planta 226, 159–167.

Weiler, E.W., Kutchan, T.M., Gorba, T., Brodschelm, W., Niesel, U., Bublitz, F., 1994. The Pseudomonas phytotoxin coronatine mimics octadecanoid signalling molecules of higher plants. FEBS Lett. 345, 9–13.

Wright, M.S., Greene-McDowelle, D.M., Zeringue, H.J., Bhatnagar, D., Cleveland, T.E., 2000. Effects of volatile aldehydes from *Aspergillus*-resistant varieties of corn on *Aspergillus parasiticus* growth and aflatoxin biosynthesis. Toxicon 38, 1215–1223.

Yamaguchi, T., Minami, E., Ueki, J., Shibuya, N., 2005. Elicitor-induced activation of phospholipases plays an important role for the induction of defense responses in suspension-cultured rice cells. Plant Cell Physiol. 46, 579–587.

Zhao, J., Davis, L.C., Verpoorte, R., 2005. Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol. Advances 23, 283–333.

Zipfel, C., Felix, G., 2005. Plants and animals: a different taste for microbes? Curr. Opin. Plant Biol. 8, 353–360.