Cellulysin from the plant parasitic fungus *Trichoderma viride* elicits volatile biosynthesis in higher plants via the octadecanoid signalling cascade

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Abstract Cellulysin, a crude cellulase from the plant parasitic fungus *Trichoderma viride*, induces the biosynthesis of volatiles in higher plants (*Nicotiana plumbaginifolia*, *Phaseolus lunatus*, and *Zea mays*) when applied to cut petioles by the transpiration stream. The pattern of the emitted volatiles largely resembles that from a herbivore damage or treatment of the plants with jasmonic acid (JA) indicating that cellulysin acts via activation of the octadecanoid signalling pathway. The treatment with cellulysin raises the level of endogenous JA after 30 min and is followed by a transient emission of ethylene after 2–3 h. Volatile production becomes significant after 12–24 h. Inhibitors of the JA pathway effectively block the cellulysin-dependent volatile biosynthesis.

Key words: Induced volatile biosynthesis; Cellulysin; Jasmonic acid; Ethylene; *Phaseolus lunatus*; *Nicotiana plumbaginifolia*; *Zea mays*

1. Introduction

The plant pathogen or plant herbivore interactions are accompanied by the rapid development of multicomponent defense responses [1]. The individual components include the build up of chemical defenses such as antimicrobial phytoalexins, the biosynthesis of proteinaceous antifungals (e.g. the pathotoxic thionins [2] as well as structural defensive barriers such as lignin and hydroxyproline-rich cell wall proteins [3]). Plants under attack by insect herbivores often show up a comparable defensive scenario. An interesting recent discovery is the emission of volatiles from herbivore-attacked plants to attract the natural enemies of the herbivore [4,5]. Signals for the activation of the various defenses are often hydrolytic enzymes of the pathogen or herbivore [1], but low molecular elicitors [6] and peptides such as systemin [7] have been identified as well. Particular effective is the phytotoxin coronatine, an amino acid conjugate produced by some pathovars of *Pseudomonas syringae* (e.g. *tomato*, *glycinea*, *atropurpurea*) [8,9], which elicits volatile emission in many higher plants when applied to cut petioles through the transpiration stream [10]. In a similar fashion JA and several amino acid conjugates of JA are able to induce volatile biosynthesis which largely resembles the pattern of compounds emitted after herbivore damage [10,11].

A prominent example is the almost identical release of a blend of terpenoids, fatty acid fragments and indole from corn seedlings upon feeding by *Spodoptera* caterpillars or after treatment with JA [11,12], the end product of the octadecanoid signalling pathway. The latter appears to be an early and highly conservative development in evolution, since JA induces volatile biosynthesis in *Pteridophyta*, *Gymnospermae* and *Angiospermae* as well [10]. Other highly effective elicitors of volatile biosynthesis in plants are the isoleucine conjugates of 1-oxo-indane-4-carboxylic acid [13,14] and the only recently identified volicitin from the regurgitate of *Spodoptera* caterpillars which is a conjugate of L-glutamine with 17-hydroxylinolenic acid [15]. On the other hand, volatile biosynthesis in plants is also stimulated by the action of hydrolytic enzymes like for example a β-glucosidase from bitter almonds [16] when applied to leaves of *Phaseolus lunatus*. A β-glucosidase activity from the salivary secretions of the caterpillars of the large cabbage white butterfly *Pieris brassicae* was suggested to be the active component triggering volatile emission from damaged cabbage leaves [17].

Considering that the octadecanoid signalling pathway plays, indeed, a pivotal role in the signal transduction between the early events of cell wall damage and the subsequent build up of chemical defenses, one should expect that also a number of hydrolytic enzymes from invading fungals will trigger volatile biosynthesis in plants. Although this specific question concerning individual fungal proteins as elicitors for volatile biosynthesis has been, as yet, not systematically addressed, there are, of course, a number of reports indicating that fungal cell wall fragments evoke plant defense reactions through the octadecanoid signalling pathway [18,19]. Low molecular elicitors that themselves are products of cell wall damage by lytic enzymes (endogalacturonidases, chitinases), like, for example, oligouronides and chitosan fragments [20] or acetylchitoheptaose [21] have been shown to enhance the endogenous JA level in various plants.

In the present paper, we report that cellulysin, a commercial, crude cellulase from the plant parasitic fungus *Trichoderma viride*, is the first example of a fungal elicitor which triggers the emission of volatiles in selected examples of mono- and dicotyledonous plants via activation of the octadecanoid signalling pathway.

2. Materials and methods

2.1. Chemicals

Inhibitors of the JA biosynthesis, phenidone, diethyldithiocarbamic acid (DIECA), indoprofen and ibuprofen as well as the cellulysin (crude powder containing approx. 50% protein) from *T. viride* were purchased from Sigma. Racemic methyl jasmonate (JAMe) was a

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generous gift of R. Kaiser, Givaudan Comp., Dübendorf, Switzerland. Free JA was prepared from the ester as described [22].

2.2. Rearing of plant material

Lima beans (*P. lunatus*, 'Ferry Morse' var. *Jackson Wonder Bush*, provided by the BASF AG, Ludwigshafen), were grown from seeds in unsterilized garden soil. Individual plants were grown in a pot (diam. = 5.5 cm) at 23°C and 80% humidity using daylight fluorescent tubes at ca. 270 μE m^2 s^{-1} and a photophase of 12 h. Experiments were conducted with 12 to 16 day-old seedlings showing two fully developed leaves. Corn plants (*Zea mays*, var. popcorn peppy, F1 hybrid) were grown as described above. Ca. 2 week-old plants carrying 2–3 clearly distinguishable leaves were used for the induction experiments. Tobacco plants were grown from seeds of *Nicotiana plumbaginifolia* in unsterilized garden soil as described for Lima beans.

2.3. Induction experiments

Petioles of *P. lunatus*, *N. plumbaginifolia* or *Z. mays* were cut and immediately transferred into vials containing a solution of the test substance in water. No other mechanical damage was inflicted to the leaf blade before or during the incubation period. In order to achieve a high concentration of the emitted volatiles in the surrounding gas phase, vials with the cut plantlets and the test solutions were enclosed in small desiccators (750 ml). The experimental set-up was kept at 25°C and was continuously illuminated (ca. 270 μE m^2 s^{-1}) during incubation. If not stated otherwise, assays with cellulysin were conducted at 50.0 μg ml^{-1}. Solutions of JA were generally applied at 1.0 mM. Inhibitor studies were performed by treatment of the plants with solutions containing the inhibitor (1.0 mM) and cellulysin (50.0 μg ml^{-1}). Ca. 1–2 ml of the aqueous solutions were consumed per leaf corresponding to a total uptake of 1–2 μmol of the low molecular test substance under standard conditions. Control experiments were made by placing freshly cut leaves into tap water, followed by volatile collection. Experiments were generally carried out in triplicate.

2.4. Collection of volatiles and identification of compounds

Plants were pre-incubated in a closed system (desiccator, 750 ml) with the test compounds for 48 h as described above (depending on the productivity of the studied plant species). Then, the emitted compounds were absorbed for 8 min onto a fused silica fibre coated with a 100 μm polydimethylsiloxane sorbent for collection of unpolar compounds (solid phase micro extraction, SPME, Supelco Inc.) [23]. Absorbed volatiles were almost instantaneously desorbed from the polymer after insertion of the fibre holder into a GC injection port (250°C, 2 min). The mixture of volatiles was separated under programmed conditions (50°C for 2 min, then at 10°C/min to 200°C) on a fused silica column (DB-1, 10 m×0.31 mm), and the individual compounds were identified by their mass spectra using reference compounds. MS: Fisons MD-800; GC interface at 260°C, scan range 35–300 Da/s.

2.5. Quantification of the endogenous jasmonate level

Petioles of *P. lunatus* were immersed into solutions of cellulysin (50.0 μg ml^{-1}) as described above. After appropriate intervals (in-between 10 min and 48 h) leaves were withdrawn from the solutions and immediately frozen in liquid nitrogen. Quantification of endogenous JA levels was then performed by an enzyme-linked immunosorbent assay [24] using an antiserum with a high specificity for (*S*)-amino acid conjugates of (-)-JA and free (-)-JAMe. The assay was performed according to the general procedure for enzyme immunoassays based on antiserum described by Weiler [25–27]. The detection limit was 0.05 pmol (-)-JAMe, with a linear range between 0.1 and 100 pmol.

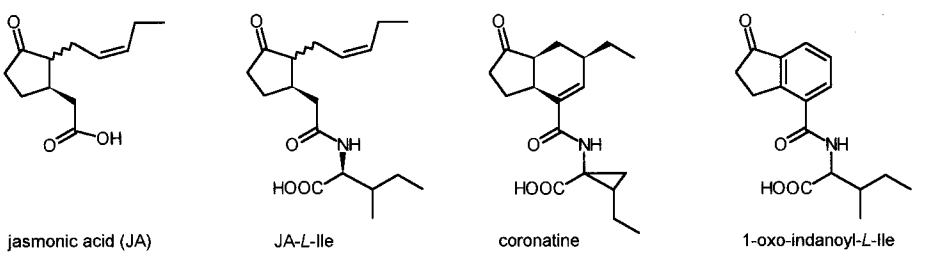
2.6. Quantification of emitted ethylene

The ethylene production was measured with a photoacoustic laser spectrometer consisting of a line-tunable infrared CO_2-laser and a resonant photoacoustic cell [32,33]. The background free detection method allows the detection of ethylene at concentrations at a pptv (volume parts per trillion) level. Standard incubations were conducted by sweeping sample gas at 1 l h^{-1} through a chamber with a Lima bean leaf blade in a vial containing the solution with the cellulase assuring a non-destructive continuous monitoring.

3. Results

If cellulysin, a cellulase cocktail from the plant parasitic fungus *T. viride*, is introduced into detached leaves of the Lima bean via the transpiration stream (aqueous solution at 50.0 μg ml^{-1}), ca. 24 h after the onset of the stimulus a massive emission of volatiles from the leaves is observed. As shown previously the mechanical damage (detachment) has virtually no inducing effect, since control experiments with detached leaves in tap water revealed, besides an unspecific background (trace amounts of *N*-alkanes, aldehydes, phenols, and sesquiterpenes) no significant production of volatiles [13]. However, 12–14 h after infiltration of the enzyme(s) the unspecific background was replaced by large amounts of (*E*)-β-ocimene (b) and 4,8-dimethyl-1,3*E*,8-dimethylnonatriene (d) [4,5,14,28]. After 24–36 h more components contributed to the headspace. A typical spectrum of induced volatiles from Lima beans is shown in Fig. 1 and comprises compounds from three major biosynthetic routes. The first type of compounds, namely (3*Z*)-hexen-1-yl acetate (a), and (3*Z*)-hexen-1-yl methylbutanoate (h) are derived from of 13-hydroperoxylinolenic acid, an early product of the octadecanoid signalling cascade. (*E*)-β-ocimene (b), linalool (c), dimethylnonatriene (d), and some trace components comprise the typical terpenoid spectrum of the Lima bean. Methyl salicylate, methyl anthranilate, and indole (g) represent aromatic volatiles emitted from many plants after herbivore or pathogen damage [29]. As shown previously, a similar pattern of volatiles is released from leaves of *P. lunatus* after treatment with JA, coronatine and certain amino acid conjugates of JA and 1-oxo-indan-4-carboxylic acid [10,11,13] (Scheme 1). Control experiments with boiled (10 min) solutions of cellulysin showed no induction of volatiles at all.

In dilution experiments the cellulysin proved to be an effective elicitor down to a concentration as low as 1.8 μg ml^{-1}. The highest tested concentration was 2 mg ml^{-1}. Within this range the type and quantity of the induced volatiles proved to be largely identical and showed no significant dependence on



jasmonic acid (JA) JA-*L*-Ile coronatine 1-oxo-indanoyl-*L*-Ile

Scheme 1.

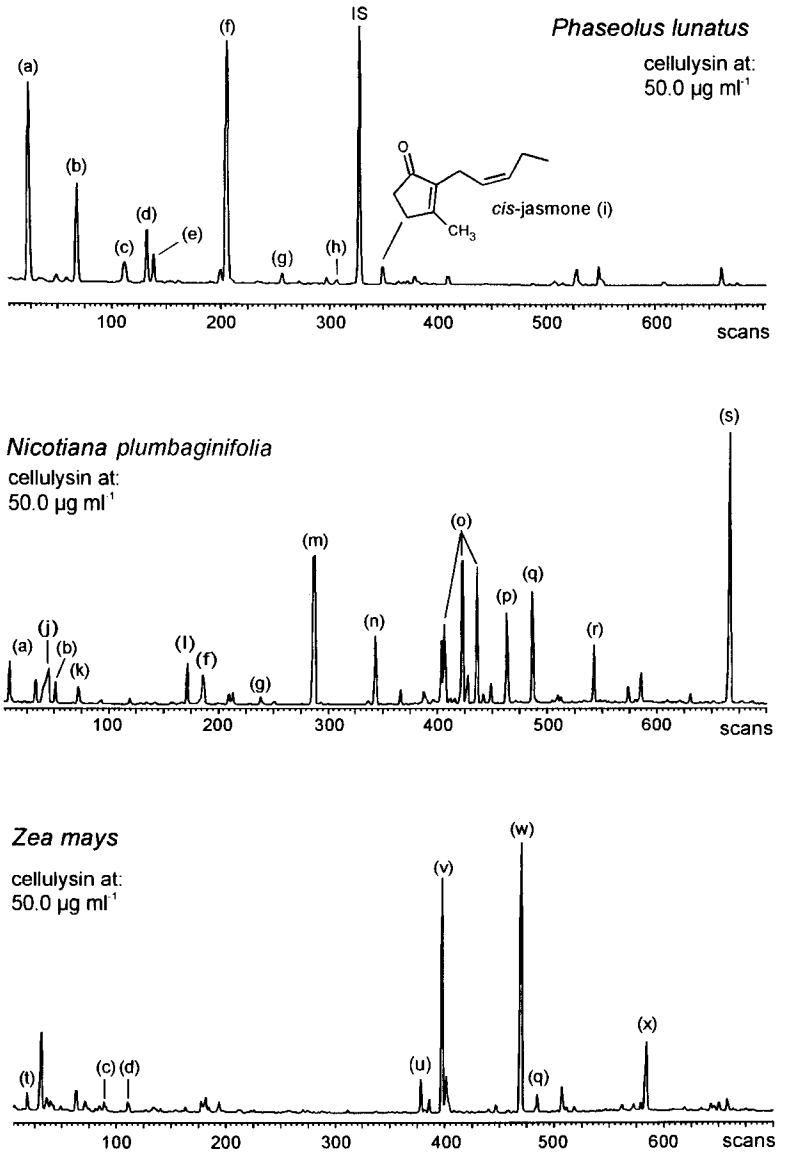


Fig. 1. Gas chromatographic profiles of the induced volatiles from *P. lunatus*, *N. plumbaginifolia* and *Z. mays* after pre-treatment of the detached leaves with cellulysin at 50.0 µg ml^{-1} for 24 h. Compounds were separated and identified by combined gas chromatography and mass spectrometry using a fused silica column DB1 (10 m×0.31 mm) under programmed conditions from 50°C (2 min) at 10°C min^{-1} to 200°C, then at 30°C min^{-1} to 280°C. Identical numbers in the two volatile profiles denominate identical compounds. Identification of compounds: (a) (3*Z*)-hexenyl acetate, (b) β-ocimene, (c) linalool, (d) 4,8-dimethylnona-1,3,7-triene, (e) $C_{10}H_{14}$, (f) $C_{10}H_{14}O$, (g) indole, (h) (3*Z*)-hexenyl methylbutanoate, (i) *cis*-jasmone, (j) methyl 2-oxohexanoate, (k) methyl benzoate, (l) (3*Z*)-hexenyl butanoate, (m) (3*Z*)-hexenyl tiglate, (n) β-elemene, (o) $C_{15}H_{24}$, (p) (3*Z*)-hexenyl benzoate, (q) 4,8,12-trimethyltrideca-1,3,7,11-tetraene, (r) $C_{15}H_{24}$, (s) neophytadiene, (t) benzylalcohol, (u) α-bergamottene, (v) β-farnesene, (w) nerolidol, (x) contamination.

the concentration of the enzyme(s). Below 1.8 µg ml^{-1} the induction of volatile biosynthesis was no longer reproducible.

In order to test whether or not the previously reported xylanase-induced (minor component of crude cellulysin) ethylene biosynthesis [30,31] in leaf tissue of *N. plumbaginifolia* can be also provoked in *P. lunatus*, the gas phase of cellulysin-treated leaves was analysed by photoacoustic spectroscopy (PAS) [32]. The method is highly sensitive for several low molecular compounds (detection limit for ethylene: 0.03 µg m^{-3}) and allows a continuous monitoring of the gas phase. Thus, three leaves of *P. lunatus* incubated together within a flow cell (250 ml) with a solution of cellulysin (50 µg ml^{-1}), and the emitted volatiles were continually passed with a slow stream of air (at 1 l h^{-1}) through an intracavity, resonant photoacoustic cell of a CO_2-laser. As shown in Fig. 2, the treatment of *P. lunatus* leaves with cellulysin caused, indeed, a strong production of the plant hormone. The time course studies show that cellulysin-elicited ethylene biosynthesis starts after about 2.5 h after the onset of the stimulus and reaches a maximum after 3.5 h. This is followed by a steady decline to the level of a detached control leaf in pure tap water after about 6–7 h. The final level of ethylene emitted from the control plant after 7 h was ca. two times higher than at the beginning of the experiment, indicating that the detachment had only a marginal effect on the ethylene biosynthesis, as is the case with the biosynthesis of the other volatiles emitted later.

The phenomenon of a cellulysin-dependent induction of

volatile biosynthesis is not restricted to *P. lunatus*. Similar effects were observed after treatment of the leaves from *N. plumbaginifolia* and the monocotyledonous corn plant *Z. mays*. Thus, treatment of these plants with cellulysin (at 50.0 μg ml^{-1}) triggered a significant release of volatiles after 12–24 h. In both cases the pattern of the emitted volatiles and the time course of the induction process was identical to that resulting from treatment of the plants with JA [13,11] or, in the case of the corn plant, from damage of the leaf by a caterpillar [12].

This coincidence of the type of induced volatiles and the time course of the elicitation process strongly suggests that cellulysin may act through activation of the octadecanoid signalling cascade. This is convincingly demonstrated in Fig. 3. Plotted are the relative increase (% in total blend of volatiles) of selected compounds resulting from either JA- or cellulysin-induced volatile biosynthesis. The almost identical qualitative and quantitative composition of the headspace after elicitation with JA or cellulysin conspicuously suggests that the cellulase(s) from *T. viride* act through activation of the octadecanoid pathway and signal transduction by JA.

The crucial role of JA in the elicitation process was confirmed by two independend approaches. In the first set of experiments, petioles of *P. lunatus* were incubated with cellulysin for periods between 10 min and 48 h and, then, the endogenous levels of JA were determined by an enzyme-linked immunosorbent assay [24]. After 30 min, a maximum concentration of JA of 5.0 ± 2.8 nmol g^{-1} fresh weight was reached, corresponding to a roughly six-fold increase over the resting level of untreated plants (0.8 ± 0.05 nmol g^{-1} fresh weight). After longer incubation, the JA level decreased to about 3.5 nmol g^{-1} and remained constant around this elevated value.

In the second series of experiments, inhibitors of the JA biosynthesis were used to unravel the significance of JA for signal transduction. Salicylic acid (SA), 1-phenyl-3-pyrazolidinone (phenidone), diethyldithiocarbamic acid (DIECA), indoprofen or ibuprofen, respectively, were applied together with cellulysin to petioles of *P. lunatus*. All five substances are known inhibitors of JA biosynthesis [20,34,36]. While SA, phenidone and DIECA completely blocked cellulysin-dependent volatile production, indoprofen and ibuprofen had no influence on elicitation.

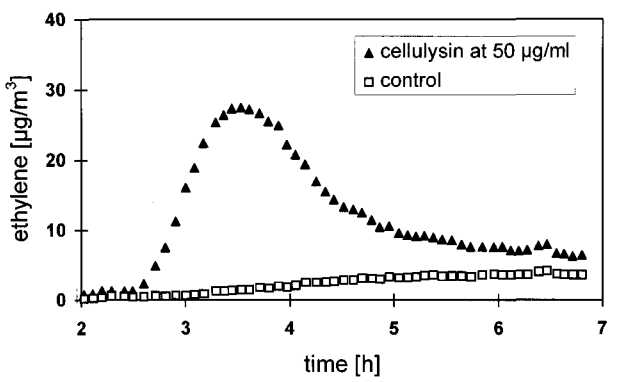


Fig. 2. Time course of the ethylene emission from a leaf of *P. lunatus* after treatment (▲) with cellulysin at 50.0 μg ml^{-1}. Data were obtained by continuous monitoring with a photoacoustic laser spectrometer [32]. A freshly detached leaf in tap water served as control (□).

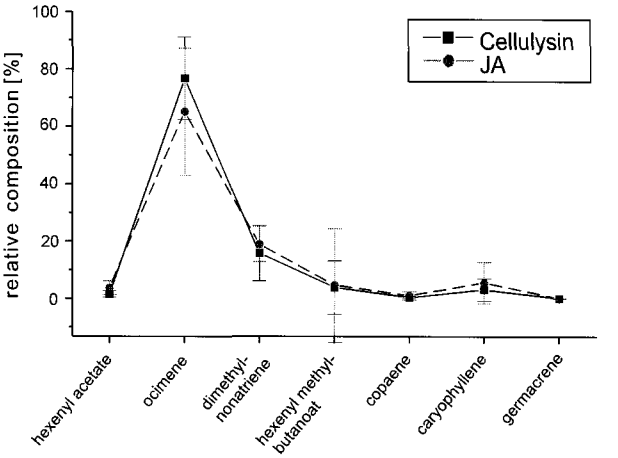


Fig. 3. Relative increase of selected, induced compounds (X-axis) common to cellulysin (●) and JA (■) treatment of a Lima bean leaf. Plotted are the relative portions (%) of individual compounds to the sum of the selected compounds (100%).

4. Discussion

Cellulysin is a complex mixture of cellulases and other proteins isolated from the plant parasitic fungus *T. viride*. When applied through the transpiration stream of freshly cut petioles of *P. lunatus*, *N. plumbaginifolia* and *Z. mays*, the enzyme cocktail elicited a massive induction of volatile biosynthesis in the tested plant species. Besides some fatty acid derived volatiles (hexenyl acetate and methylbutanoate) mostly terpenoids contributed to the blend of emitted compounds. The biosynthesis of the same type of volatiles was previously reported to be inducible by treatment of detached leaves of these and other plants with solutions of JA, the phytotoxin coronatine and amino acid conjugates of JA, as well as amino acid conjugates of 1-oxo-indane-4-carboxylic acid. As exemplified in Fig. 3, the pattern of the volatiles emitted from leaves of the Lima bean after treatment with either solutions of JA or cellulysin is identical with respect to their qualitative and quantitative composition. In the case of the Lima bean this is remarkable, since in this plant different elicitors (JA, coronatine and certain different amino acid conjugates of 1-oxo-indane-4-carboxylic acid (Krumm, T. and Boland, W., unpublished)) result in minor but highly significant differences in the blend of volatiles. Furthermore, cellulysin and JA show a similar time course of volatile induction with ocimene and 4,8-dimethylnona-1,3,7-triene appearing after about 12 h as the first compounds in the headspace. Unlike a previously reported β-glucosidase-induced volatile biosynthesis in cabbage leaves [17], the cellulysin- or JA-dependent volatile production does not require for any additional mechanical damage of the leaf blade and, hence, their mode of action appears to be more direct than that of the β-glucosidase.

Another similarity between JA- and cellulysin-induced events is the early release of ethylene. Continuous monitoring of the gas phase above a cellulysin-treated Lima bean leaf by photoacoustic spectroscopy indicated an emission window of about two hours between the second and the fourth hour after the onset of the stimulus. The phenomenon perfectly matches the time course of the emission of ethylene from leaf disks of *N. tabacum* treated with a purified xylanase from cellulysin

(EIX, ethylene-inducing xylanase [37–39]). EIX, which was previously isolated from *T. viride* or commercial cellulysin also triggers ethylene production in avocado fruit disks [40]. However, preliminary attempts to purify the volatile-inducing elicitor present in cellulysin indicate that EIX and the unknown factor triggering volatile biosynthesis have to be considered as different proteins.

The involvement of JA as a signal transducer followed not only from the similarity of the time course of the induced events and the almost identical composition of the volatile blends, but could be directly proven by detecting JA levels. Thus, only 30 min after the treatment of a Lima bean leaf with cellulysin the internal level of JA began to increase reaching a maximum after about 60 min followed by a steady decline to a resting level somewhat higher than that of a control plant. Further results from inhibitor studies corroborate that the cellulysin-dependent volatile production proceeds via activation of the octadecanoid signalling pathway. Simultaneous application of cellulysin with salicylic acid, DIECA or phenidone completely suppressed volatile production. Phenidone interferes with the hydroperoxide formation from linolenic acid [35], while DIECA reduces the 13-hydroperoxylinolenic acid to the corresponding alcohol which can not be converted into JA [22]. Indoprofen and ibuprofen [36], on the other hand, were not able to inhibit cellulysin-induced volatile production. Whether or not this difference can be attributed to solubility and transport phenomena remains to be established.

The occurrence of *cis*-jasmone, cf. Fig. 1, in the headspace of cellulysin-treated Lima beans is a highly significant finding and provides another, independent piece of evidence for involvement of JA in the signal transduction pathway. Studies with Lima beans and several other plants recently showed that the emission of *cis*-jasmone from induced plants may be considered as a mode of an irreversible disposal of the stress hormone from the leaf blade to the atmosphere [41], and, consistently, *cis*-jasmone has been detected several times among the volatiles released from herbivore-damaged plants [42].

Other examples of plant responses triggered by substances obtained from *T. viride* have been published. Chappell et al. observed the induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a sesquiterpene cyclase and phenylalanine ammonia lyase in tobacco cell cultures after treatment with cellulysin [43]. Although the activation of a HMGCoA reductase and a sesquiterpene cyclase matches with the enzymatic activities required for the biosynthesis of volatile terpenoids, it is as yet, not known whether or not the same enzymes are also involved in our systems. A crude cellulase from *T. viride* was shown to trigger lignification in wheat leaves [44]. However, the inducing activity of the elicitor could not be destroyed in boiling in water (15 min) suggesting that the effect may be due to a low molecular contamination rather than to a proteinaceous compound. Further reports concerning cellulysin as an elicitor included the induction of sesquiterpenoid biosynthesis in pepper and tobacco [45]. In no case, however, the subsequent elements in the signal transduction pathway, like for example the involvement of JA, have been shown.

The pivotal role of JA in various plant defense processes is well established. While some JA-mediated defense responses elicit short-termed reactions against the attacking organism, other mechanisms lead to a longer-lasting resistance towards potential invaders. Cohen et al. showed that potato and tomato plants can be systemically protected against *Phytophthora infestans* by treatment with JA or JAMe [46]. In this context it is interesting that inoculation of plants with microorganisms as a prevention against pathogens (biocontrol) is already routinely employed in agriculture. *Trichoderma* is one of the most often used microbial agents in such strategies [47,48]. The molecular mechanisms, however, how the defense genes are activated by a prophylactic inoculation are, as yet, not known in detail. The ability of cellulysin to activate the octadecanoid pathway and subsequent defense responses as shown in this work, may represent an important clue how a prophylactic inoculation of healthy plants with *T. viride* effects local and systemic disease resistance-related responses in plants. In this context, the de novo synthesised [49,50] and emitted volatiles may function as interplant signals activating prophylactic defenses [51], or may directly serve as fungitoxic agents preventing germination of zoospores and mycelial growths [52].

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