We investigated the role of polyamines (PAs) in lima bean (Phaseolus lunatus) leaves on the production of herbivorous mite (Tetranychus urticae)-induced plant volatiles that attract carnivorous natural enemies of the herbivores. To do this, we focused on the effects of the exogenous PAs [cadaverine, putrescine, spermidine and spermine (Spm)] on the production of volatiles, H$_2$O$_2$ and jasmonic acid (JA) and the levels of defensive genes, cytosolic calcium and reactive oxygen species (ROS). Among the tested PAs, Spm was the most active in inducing the production of volatile terpenoids known to be induced by T. urticae. An increase in JA levels was also found after Spm treatment, indicating that Spm induces the biosynthesis of JA, which has been shown elsewhere to regulate the production of some volatile terpenoids. Further, treatment with JA and Spm together resulted in greater volatile emission than that with JA alone. In a Y-tube olfactometer, leaves treated with Spm + JA attracted more predatory mites (Phytoseiulus persimilis) than those treated with JA alone. After treatment with Spm + JA, no effects were found on the enzyme activity of polyamine oxidase and copper amine oxidase. However, induction of calcium influx and ROS production, and increased enzyme activities and gene expression for NADPH oxidase complex, superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase and glutathione peroxidase were found after treatment with Spm + JA. These results indicate that Spm plays an important role in the production of T. urticae-induced lima bean leaf volatiles.

**Keywords:** Calcium and ROS signaling • Herbivore-induced volatile organic compounds • Jasmonic acid • Lima bean • Polyamines • Quantitative gene expression.

**Abbreviations:** ADH, alcohol dehydrogenase; ANOVA, analysis of variance; APX, ascorbate peroxidase; CAT, catalase; Cad, cadaverine; CLSM, confocal laser scanning microscope; DMNT, (E,E)-4,8-dimethyl-1,3,7-nonatriene; FPS, farnesyl pyrophosphate synthase; GC-MS, gas chromatography–mass spectrometry; GPX, glutathione peroxidase; GR, glutathione reductase; HIPPV, herbivore-induced plant volatile; HR, hypersensitive response; JA, jasmonic acid; LOX, lipoxygenase; MANOVA, multivariate analysis of variance; MAPK, mitogen-activated protein kinase; MRM, multiple reaction monitoring; NBT, nitroblue tetrazolium; OS, oecimene synthase; PCA, perchloric acid; PAO, polyamine oxidase; Put, putrescine; ROS, reactive oxygen species; Rboh, respiratory burst oxidase homolog; SA, salicylic acid; SOD, superoxide dimutase; Spd, spermidine; Spm, spermine; TMTT, (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene; VOC, volatile organic compound.

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**Exogenous Polyamines Elicit Herbivore-Induced Volatiles in Lima Bean Leaves: Involvement of Calcium, H$_2$O$_2$ and Jasmonic Acid**

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Introduction

In response to herbivory, plants emit so-called ‘herbivore-induced plant volatiles’ (HIPVs), a subset of volatile organic compounds (VOCs), that attract carnivorous natural enemies of herbivores (Arimura et al. 2005, Sabelis et al. 2007, Frost et al. 2008). The composition of typical induced volatile blends has been reviewed for many plant systems (Dudareva et al. 2004, Dudareva et al. 2006) and their biological functions have been discussed (Kessler and Baldwin 2001, Vancanneyt et al. 2001, Van Poecke and Dicke 2004, Shiojiri et al. 2006, Browse and Howe 2008, Howe and Jander 2008). However, plants are continuously interacting with the external environment, and an intriguing question is how plants can emit a specific blend of volatiles in response to different stress factors. The treatment of lima bean plantlets with heavy metals (Hg²⁺, Cu²⁺ and Fe³⁺) resulted in a characteristic blend of volatiles that was observed otherwise only after treatment of plants with the channel-forming peptide alamethicin (Engelberth et al. 2001). When coronal, a synthetic 6-ethyl indanyl isoleucine conjugate, was applied exogenously to economically important crops such as tobacco, tomato, soybean, barley and lima bean it resulted in the induction of defense-related secondary metabolite accumulation in both cell cultures and plant tissues, as well as specific abiotic and biotic stress-related gene expression (Schuler et al. 2004). These results clearly show the outstanding importance of the ‘cross-talk’ between the network of signaling pathways for an integrated and adjusted response that is ultimately reflected in the composition of volatile blends or other phytochemicals (Maffei et al. 2007a, Maffei et al. 2007b).

Polyamines are basic low molecular weight aliphatic amines that are found at concentrations from micromoles to millimoles in almost all living cells. These amines have been thoroughly investigated due to their role in a variety of cellular processes, such as chromatin organization, mRNA translation, ribosome biogenesis, cell proliferation and programmed cell death, as well as in adaptive roles in response to both abiotic and biotic stresses (Kusano et al. 2007). For example, spermine (Spm) plays a role as a mediator in defense signaling against pathogens (Takahashi et al. 2003, Takahashi et al. 2004), and resistance to virus infection was enhanced by polyamines (Yamakawa et al. 1998). Whether polyamines are involved in the defense against herbivores has been scantily tested (Winz and Baldwin 2001). S-Adenosylmethionine decarboxylase (SAMDC), a key enzyme in the biosynthesis of Spm and spermidine (Spd), was found to be induced in lima bean plants in response to spider mite (Tetranychus urticae) damage (Arimura et al. 2002). However, endogenous Spm and Spd were not elevated in infested leaves throughout the duration of infestation (Arimura et al. 2002).

Yamakawa et al. (1998) reported that Spm accumulated outside of cells after lesion formation, inducing both acidic PR (pathogenesis-related) proteins and resistance against tobacco mosaic virus via an SA (salicylic acid)-independent signaling pathway in tobacco plants. To better understand the mechanism(s) of HIPV production in lima bean leaves, it is important to determine whether extracellular accumulation of Spm and Spd increases during T. urticae infestation. Although we could not detect extracellular Spm and Spd in whole leaves of infested lima bean (R. Ozawa et al. unpublished data), we cannot exclude the possibility that some polyamines which concentrate around infested sites are involved in the induced responses of lima bean leaves infested by T. urticae. In this report, we supplied polyamines exogenously to infested lima bean leaves to detect their possible effects.

The coordination of internal processes in plants and their balance with the environment are connected with the excitability of plant cells. Ion channels and signaling roles are the major focus of plant polyamine research (Kusano et al. 2007). From the physicochemical point of view, polyamines are polycations at physiological pH. It might not be a surprise, therefore, that polyamines have been found to affect certain cationic channels in both animal and plant cells (Bruggemann et al. 1998, Dobrovinskaya et al. 1999). Spm stimulates the activity of (i) two important mitogen-activated protein kinases (MAPKs) involved in plant defense, wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK); and (ii) defense gene expression (Takahashi et al. 2003, Takahashi et al. 2004). Reactive oxygen species (ROS) and Ca²⁺ influx have been found to be upstream of polyamine action on MAPK (Tun et al. 2006), whereas exogenous Spd has been shown to alter the activity of ROS-scavenging systems (Kubis 2008). Amine oxidases catalyze the oxidative de-amination of polyamines and contribute to important physiological processes through their reaction products [i.e. aminoaldehydes, 1,3-diaminopropane and hydrogen peroxide (H₂O₂)] (Cona et al. 2006). Polyamine oxidases (PAOs) are involved in plant defense responses and can increase response to wounding (Angelini et al. 2008).

The induction of HIPVs by biotic stress can be crucial in crop systems, allowing plants to attract carnivorous natural enemies of pests and representing a highly sustainable tool in the struggle to survive and reproduce. The objective of this study was to clarify whether polyamines are involved in the production of HIPVs, by evaluating early and late responses. Early responses were assessed by evaluating changes in jasmonic acid (JA), Ca²⁺ homeostasis and production of H₂O₂; whereas late events were studied by gas chromatography–mass spectrometry (GC-MS) analyses of emitted HIPVs, as well as enzyme activity and gene expression of the main ROS-scavenging enzymes.
Effects of exogenous polyamines on volatile organic compounds (VOCs) and JA production in lima bean leaves

Polyamines are ubiquitous molecules in the plant kingdom and are required for normal development of prokaryotes and eukaryotes. Changes in polyamine concentration occur upon abiotic and biotic stress and, although the physiological rationale for such alterations is still far from clear, these variations imply stomatal regulation and the triggering of cascade responses in the signal transduction pathway (Walters 2003, Alcazar et al. 2006, Cona et al. 2006, Kusano et al. 2003, An et al. 2008). When 1 mM polyamines was applied to lima bean leaves, a differential response in VOCs emitted resulted in increased amounts of three homoterpenes [(E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) and an isomer of TMTT]. We then tested the combined effect of polyamines and JA addition in all polyamines on VOCs emission from lima bean leaves (Table 2). Hopke et al. (1994) found that lima bean leaves treated with 0.1 mM JA barely emitted VOCs. However, by comparing the profile of volatiles emitted from leaves treated with a polyamine with those emitted from leaves treated with the same polyamine+JA (Tables 1, 2), we observed a significant effect of JA addition on the changes in the volatile profile in all polyamines tested [P = 0.0003 for Cad, P = 0.0013 for Put, P = 0.044 for Spd and P = 0.0015 for Spm; multivariate analysis of variance (MANOVA)].

### Results

**Effects of exogenous polyamines on volatile organic compounds (VOCs) and JA production in lima bean leaves**

Polyamines are ubiquitous molecules in the plant kingdom and are required for normal development of prokaryotes and eukaryotes. Changes in polyamine concentration occur upon abiotic and biotic stress and, although the physiological rationale for such alterations is still far from clear, these variations imply stomatal regulation and the triggering of cascade responses in the signal transduction pathway (Walters 2003, Alcazar et al. 2006, Cona et al. 2006, Kusano et al. 2003, An et al. 2008). When 1 mM polyamines was applied to lima bean leaves, a differential response in VOCs emitted resulted in increased amounts of three homoterpenes [(E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) and an isomer of TMTT]. We then tested the combined effect of polyamines and low JA concentration (0.1 mM) on volatile emission by lima bean leaves (Table 2). Hopke et al. (1994) found that lima bean leaves treated with 0.1 mM JA barely emitted VOCs. However, by comparing the profile of volatiles emitted from leaves treated with a polyamine with those emitted from leaves treated with the same polyamine+JA (Tables 1, 2), we observed a significant effect of JA addition on the changes in the volatile profile in all polyamines tested [P = 0.0003 for Cad, P = 0.0013 for Put, P = 0.044 for Spd and P = 0.0015 for Spm; multivariate analysis of variance (MANOVA)]. However, when JA treatments were compared with the control (buffer), the volatile profiles were not significantly different (P = 0.20, MANOVA). We then compared the amount of each volatile compound emitted from the polyamine+JA-treated leaves with that from JA-treated leaves (Table 2). When Spm and JA were applied together, the amounts of (Z)-3-hexenyl acetate, (Z)-β-ocimene, (E)-β-ocimene, an unknown terpenoid (I), DMNT, α-copaene, TMTT and an isomer of TMTT increased significantly (P < 0.05, Dunnet’s test). Spm+JA treatment resulted in

### Table 1 Effects of exogenous polyamines on volatile emissions in lima bean leaves

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Treatments</th>
<th>Cadaverine</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene</td>
<td>MES buffer</td>
<td>ND</td>
<td>2,396 ± 432*</td>
<td>2,030 ± 302*</td>
<td>1,605 ± 144*</td>
</tr>
<tr>
<td>(Z)-3-Hexenyl acetate</td>
<td>ND</td>
<td>1,498 ± 1,498</td>
<td>ND</td>
<td>ND</td>
<td>35,318 ± 25,359</td>
</tr>
<tr>
<td>(Z)-β-Ocimene</td>
<td>ND</td>
<td>ND</td>
<td>632 ± 376</td>
<td>206 ± 206</td>
<td>4,526 ± 1,513</td>
</tr>
<tr>
<td>Unknown terpenoid (I)</td>
<td>516 ± 516</td>
<td>880 ± 880</td>
<td>ND</td>
<td>4,710 ± 1,781*</td>
<td>ND</td>
</tr>
<tr>
<td>(E)-4,8-Dimethyl-1,3,7-nonatriene (DMNT)</td>
<td>61,183 ± 28,968</td>
<td>20,098 ± 9,737</td>
<td>7,805 ± 4,229</td>
<td>32,015 ± 3,473</td>
<td>212,429 ± 53,801*</td>
</tr>
<tr>
<td>Menthol</td>
<td>ND</td>
<td>ND</td>
<td>1,768 ± 1,040</td>
<td>905 ± 675</td>
<td>4,086 ± 2,105</td>
</tr>
<tr>
<td>Methyl salicylate</td>
<td>5,821 ± 2,707</td>
<td>1,768 ± 1,040</td>
<td>905 ± 675</td>
<td>4,086 ± 2,105</td>
<td>10,847 ± 2,096</td>
</tr>
<tr>
<td>α-Copaene</td>
<td>ND</td>
<td>2,715 ± 1,568</td>
<td>1,480 ± 1,480</td>
<td>3,859 ± 1,327</td>
<td>ND</td>
</tr>
<tr>
<td>Unknown terpenoid (II)</td>
<td>ND</td>
<td>1,710 ± 587*</td>
<td>2,138 ± 105*</td>
<td>2,458 ± 273*</td>
<td>1,001 ± 1,342</td>
</tr>
<tr>
<td>(E)-β-Caryophyllene</td>
<td>ND</td>
<td>ND</td>
<td>781 ± 455</td>
<td>762 ± 442</td>
<td>ND</td>
</tr>
<tr>
<td>Unknown terpenoid (III)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>967 ± 967</td>
<td>ND</td>
</tr>
<tr>
<td>Unknown terpenoid (IV)</td>
<td>ND</td>
<td>711 ± 711</td>
<td>1,487 ± 527</td>
<td>1,642 ± 555</td>
<td>ND</td>
</tr>
<tr>
<td>Unknown terpenoid (V)</td>
<td>ND</td>
<td>433 ± 433</td>
<td>1,375 ± 487</td>
<td>1,476 ± 741</td>
<td>ND</td>
</tr>
<tr>
<td>Isomer of TMTT</td>
<td>ND</td>
<td>451 ± 451</td>
<td>ND</td>
<td>514 ± 514</td>
<td>6,388 ± 2,546*</td>
</tr>
<tr>
<td>(E,E)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (TMTT)</td>
<td>1,963 ± 1,018</td>
<td>2,017 ± 906</td>
<td>641 ± 289</td>
<td>2,068 ± 1,357</td>
<td>34,540 ± 13,360*</td>
</tr>
</tbody>
</table>

The headspace volatiles of lima bean leaves were collected 24 h after treatment. The amounts of each compound are represented with peak areas of ion intensities (×10³) detected by GC-MS. Data are expressed as mean ± SE.

ND, not detected.

*P < 0.05 (Dunnet’s test).
increased amounts of (Z)-3-hexenyl acetate, (Z)-β-ocimene and an unknown terpenoid (I) \((P < 0.05, \text{Dunnet's test})\). Cad + JA treatment and Put + JA treatment did not show any significant increase in volatile compound emission when compared with the JA-treated leaves.

In order to evaluate possible correlations between the amount of each volatile compound and the number of amino groups of the polyamines tested, a correlation table was calculated using the Pearson's product–moment correlation coefficient (Table 3). In polyamine-treated plants, three compounds, DMNT, TMTT and an isomer of TMTT, correlated significantly (Table 3). When the correlation between the amount of each compound and the number of amino groups of polyamines tested in the presence of JA was calculated, α-copae and an isomer of TMTT correlated significantly, and (Z)-3-hexenyl acetate, (E)-β-ocimene, (Z)-β-ocimene, DMNT, TMTT and an unknown terpenoid (I) showed a highly significant correlation (Table 3).

To evaluate the effect of the post-treatment duration on the production of volatiles, we performed time-course tests. Since Spm was found to induce the most significant changes, we focused on this molecule. Leaves were treated with JA at a constant concentration plus increasing concentrations of Spm (Fig. 1). After 1 and 6 h, no significant differences were found for JA plus increasing Spm concentrations with respect to JA alone. From 12 to 24 h, a significantly increasing trend was observed with 1 mM Spm + 0.1 mM JA. The data indicated that post-treatment duration and the concentration of Spm affected the profiles of the volatiles.

Since JA is required for the general stimulation of volatile biosynthesis (Engelberth et al. 2004, Browne and Howe 2008, Frost et al. 2008, Zheng and Dicke 2008), we evaluated whether exogenous polyamines can act as signals promoting JA biosynthesis. In general, with respect to MES buffer as the control, all polyamines elicited some JA production, particularly at 1 h post-treatment. However, a significant difference was only found for Spm with respect to the control at 1 h post-treatment (Fig. 2).

Table 2 Effects of exogenous polyamines and JA on volatile emissions in lima bean leaves

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Treatments</th>
<th>JA</th>
<th>JA + cadaverine</th>
<th>JA + putrescine</th>
<th>JA + spermidine</th>
<th>JA + spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene</td>
<td>ND</td>
<td>3,953 ± 2,167</td>
<td>6,068 ± 6,068</td>
<td>12,224 ± 5,769</td>
<td>132,023 ± 45,713</td>
<td>174,953 ± 18,636</td>
</tr>
<tr>
<td>(Z)-3-Hexenyl acetate</td>
<td>534 ± 314</td>
<td>5,774 ± 2,209</td>
<td>12,969 ± 3,864</td>
<td>24,512 ± 6,991</td>
<td>53,398 ± 5,420</td>
<td></td>
</tr>
<tr>
<td>(Z)-β-Ocimene</td>
<td>29,947 ± 1,942</td>
<td>119,063 ± 12,523</td>
<td>194,692 ± 36,646</td>
<td>326,479 ± 116,889</td>
<td>735,431 ± 156,276</td>
<td></td>
</tr>
<tr>
<td>Unknown terpenoid (I)</td>
<td>9,218 ± 2,065</td>
<td>25,935 ± 4,341</td>
<td>21,959 ± 5,941</td>
<td>30,441 ± 6,548</td>
<td>105,973 ± 9,972</td>
<td></td>
</tr>
<tr>
<td>(E)-β-Ocimene</td>
<td>49,920 ± 8,992</td>
<td>110,524 ± 8,919</td>
<td>106,470 ± 26,691</td>
<td>236,267 ± 97,187</td>
<td>656,305 ± 100,040</td>
<td></td>
</tr>
<tr>
<td>Menthol</td>
<td>1,277 ± 1,277</td>
<td>ND</td>
<td>1,712 ± 1,712</td>
<td>1,346 ± 1,346</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Methyl salicylate</td>
<td>11,831 ± 4,924</td>
<td>3,026 ± 2,266</td>
<td>4,882 ± 1,388</td>
<td>3,700 ± 918</td>
<td>5,942 ± 2,285</td>
<td></td>
</tr>
<tr>
<td>α-Copae</td>
<td>2,116 ± 726</td>
<td>553 ± 553</td>
<td>1,708 ± 613</td>
<td>1,660 ± 574</td>
<td>1,512 ± 874</td>
<td></td>
</tr>
<tr>
<td>Unknown terpenoid (II)</td>
<td>521 ± 521</td>
<td>ND</td>
<td>3,117 ± 3,117</td>
<td>ND</td>
<td>8,674 ± 895</td>
<td></td>
</tr>
<tr>
<td>(E)-β-Caryophyllene</td>
<td>2,168 ± 1,032</td>
<td>494 ± 494</td>
<td>ND</td>
<td>ND</td>
<td>460 ± 460</td>
<td></td>
</tr>
<tr>
<td>Unknown terpenoid (IV)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>574 ± 497</td>
<td></td>
</tr>
<tr>
<td>Isomer of TMTT</td>
<td>2,640 ± 1,567</td>
<td>883 ± 511</td>
<td>1,849 ± 1,005</td>
<td>ND</td>
<td>19,610 ± 6,611</td>
<td></td>
</tr>
<tr>
<td>(E,E)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (TMTT)</td>
<td>9,857 ± 4,362</td>
<td>3,045 ± 924</td>
<td>7,210 ± 3,176</td>
<td>8,577 ± 7,009</td>
<td>77,561 ± 24,097</td>
<td></td>
</tr>
</tbody>
</table>

The headspace volatiles of lima bean leaves were collected 24 h after treatment. The amounts of each compound are represented with peak areas of ion intensities \((10^6)\) detected by GC-MS. Data are expressed as mean ± SE.

ND, not detected.

\(*P < 0.05\) (Dunnet’s test).
Table 3 Correlation between the amount of each emitted compound and the number of amino groups of polyamines (PAs) when lima bean leaves were treated with a PA or a PA and jasmonic acid (JA)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>PA</th>
<th>JA + PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 α-Pinene</td>
<td>0.05</td>
<td>–</td>
</tr>
<tr>
<td>2 (Z)-3-Hexenyl acetate</td>
<td>0.40</td>
<td>0.75***</td>
</tr>
<tr>
<td>3 (Z)-β-Ocimene</td>
<td>–</td>
<td>0.82***</td>
</tr>
<tr>
<td>4 (E)-β-Ocimene</td>
<td>0.38</td>
<td>0.75***</td>
</tr>
<tr>
<td>5 Unknown terpenoid (I)</td>
<td>–</td>
<td>0.77***</td>
</tr>
<tr>
<td>6 (E)-4,8-Dimethyl-1,3,7-nonatriene (DMNT)</td>
<td>0.49*</td>
<td>0.74****</td>
</tr>
<tr>
<td>7 Menthol</td>
<td>0.13</td>
<td>–0.15</td>
</tr>
<tr>
<td>8 Methyl salicylate</td>
<td>0.36</td>
<td>–0.39</td>
</tr>
<tr>
<td>9 α-Copaene</td>
<td>0.09</td>
<td>0.54*</td>
</tr>
<tr>
<td>10 Unknown terpenoid (II)</td>
<td>0.35</td>
<td>–0.12</td>
</tr>
<tr>
<td>11 (E)-β-caryophyllene</td>
<td>0.07</td>
<td>0.33</td>
</tr>
<tr>
<td>12 Unknown terpenoid (III)</td>
<td>0.13</td>
<td>0.12</td>
</tr>
<tr>
<td>13 Unknown terpenoid (IV)</td>
<td>0.07</td>
<td>–0.38</td>
</tr>
<tr>
<td>14 Unknown terpenoid (V)</td>
<td>0.07</td>
<td>0.31</td>
</tr>
<tr>
<td>15 Isomer of TMTT</td>
<td>0.56*</td>
<td>0.48*</td>
</tr>
<tr>
<td>16 (E,E)-4,8,12-Trimethyl-1,3,7,11-tridecaetraene (TMTT)</td>
<td>0.54*</td>
<td>0.54****</td>
</tr>
</tbody>
</table>

The data on the basis of which this table are prepared are shown in Table 1 and Table 2.

r, Pearson’s product–moment correlation coefficient, ***P < 0.001, **0.01 < P < 0.05.

uninfested leaves and those from Spm+JA-treated uninfested leaves were compared, *P. persimilis* showed a significant preference for the latter (Fig. 3). Since Spm+JA was found to induce a greater amount of volatiles than Spd+JA, we also compared the preference of *P. persimilis* for Spm+JA-treated leaves and Spd+JA-treated leaves in the olfactometer. The predatory mite showed a significant preference for the Spm+JA-treated leaf volatiles.

**Effects of Spm and JA on HIPV gene expression**

The emission of volatiles after biotic or abiotic stress implies gene activation and the consequent transcription of the enzyme biosynthetic machinery. The formation of (Z)-3-hexenol, (Z)-3-hexenal and (Z)-3-hexenyl acetate requires the oxidation of the precursor linolenic acid by the enzyme lipoxynase (LOX) that yields 13-hydroperoxy linolenic acid (Matsui 1998, Arimura et al. 2005). The cleavage of the latter by the action of a 13-hydroperoxy linolenic acid lyase gives rise to (Z)-3-hexenal [and 12-oxo-(Z)-9-dodecenic acid] which is reduced to (Z)-3-hexenol by the activity of alcohol dehydrogenase (ADH) (Graus et al. 2004). On the other hand, the synthesis of the homoterpene DMNT requires the formation of the sesquiterpene precursor farnesyl pyrophosphate, which is catalyzed by the enzyme farnesyl pyrophosphate synthase (FPS) (Dudareva et al. 2004, Kappers et al. 2005). Finally, one of the most common HIPVs produced by lima bean is (E)-β-ocimene, whose biosynthetic gene (*ocimene synthetase*, OS) has been recently cloned in lima bean (Arimura et al. 2008). In order to correlate the increased production of volatiles with a possible gene activation, we focused on the expression of LOX, ADH, FPS and OS, which were involved in the production of volatile compounds in plants 24 h after the treatment with either JA, Spm or Spm+JA (Fig. 4). The LOX expression level was 20-fold higher after Spm treatment, and a dramatic significant increase of almost 45-fold was observed after JA treatment. The combined treatment of Spm+JA produced a statistically significant (P < 0.05) 25-fold increase in gene expression (Fig. 4A). The expression level of ADH was slightly up-regulated by Spm and Spm+JA treatment, whereas the gene was slightly down-regulated by JA (Fig. 4B). The treatment with either Spm or JA slightly down-regulated the expression level of FPS, whereas the combined treatment with JA+Spm caused a 4-fold up-regulation of the gene (Fig. 4C). Finally, the OS gene expression level increased.
Fig. 1 Transition of volatile emission from lima bean leaves treated with JA and various concentrations of Spm. Error bars represent the SE (n=3–4). *P < 0.05 (Dunnet’s test, JA treatment as a control). Compound names: 1, α-pinene; 2, (Z)-3-hexenyl acetate; 3, (Z)-β-ocimene; 4, (E)-β-ocimene; 5, unknown terpenoid (I); 6, (E)-4,8-dimethyl-1,3,7-nonatriene; 7, menthol; 8, methyl salicylate; 9, α-copaene; 10, unknown terpenoid (II); 11, (E)-β-caryophyllene; 12, unknown terpenoid (III); 13, unknown terpenoid (IV); 14, unknown terpenoid (V); 15, isomer of (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene; 16, (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

Fig. 2 JA induction after treatment with 1 mM polyamines (Cad, Put, Spd and Spm) in lima bean leaves for up to 24 h. Error bars represent the SE. *P < 0.05 (Dunnet’s test).

Fig. 3 Response of the satiated female predatory mite (P. persimilis) in a Y-tube olfactometer when offered JA-treated leaves or JA+Spd-treated leaves vs. JA+Spm-treated leaves. A χ² test evaluated differences from a 50:50 distribution over two olfactometer arms. Predators that did not reach the end of either olfactometer arm within 10 min (NC: no choice) were excluded from statistical analyses.
about 2-fold with Spm, whereas treatment with JA resulted in a 7-fold up-regulation of the gene. The combined treatment with Spm + JA caused an almost 4-fold up-regulation of OS (Fig. 4D).

H₂O₂ and cytosolic calcium variations in lima bean leaves upon application of exogenous Spm and JA

Having assessed the effect of exogenous Spm on production of HIPVs in lima bean, we investigated the signaling pathways involved in the transduction of the chemical signals that eventually lead to volatile emission. Two signaling molecules were investigated: H₂O₂, which acts both as a signal when produced at low concentrations and as an oxidative barrier against invading pathogens, and Ca²⁺, which is a well known second messenger (reviewed in Maffei et al. 2007b).

After 6 h treatment, Spm induced a significant increase in H₂O₂ with respect to the controls, which is shown in Fig. 5B as a green fluorescence. JA treatment produced even more H₂O₂ than Spm treatment (Fig. 5C), and the combined use of Spm + JA was synergistic and resulted in an even higher H₂O₂ burst (Fig. 5D). Fig. 5E shows the quantitative analysis of H₂O₂ production shown in Fig. 5A–D.

Intracellular calcium variations can depend on both the entry of Ca²⁺ into the cytoplasm upon release from cell organelles and entry from the apoplasm. Plant cells respond to extracellular stimuli by changing the cytosolic calcium concentration, which ultimately triggers some of the events leading to the signal transduction pathway. Fig. 6 shows the results of the experiments performed when lima bean leaves were incubated with 5 mM of the calcium indicator, Calcium Orange. Spm induced a significant increase of Ca²⁺ with respect to controls (Fig. 6B), and the presence of 0.1 mM JA resulted in a significantly higher Ca²⁺ level when compared with Spm treatment (Fig. 6C). The combined application of Spm and JA resulted in an increased Ca²⁺ level (Fig. 6D), as also demonstrated by the quantitative analysis (Fig. 6E). As observed for H₂O₂, the Ca²⁺ signature was different between Spm and JA. The JA application produced a more diffused Ca²⁺ signature that was mainly involved in conducting vessel elements (see Fig. 6C) with respect to the Ca²⁺ response to Spm, which was less diffuse (Fig. 6B).

ROS-producing and -scavenging enzyme activities and gene expression

In order to gain more insight into the sequence of the early response events of Spm- and Spm + JA-treated plants following H₂O₂ production, we analyzed the transcript accumulation and enzymatic activities of some ROS-scavenging enzymes. The analysis was performed on the enzyme activity of three ROS-producing enzymes, namely NADPH oxidase...

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Fig. 4 Effects of Spm and JA on expression of HIPV biosynthetic genes after treatment for 24 h. Quantitative gene expression of (A) lipoxygenase (LOX), (B) alcohol dehydrogenase (ADH), (C) farnesyl pyrophosphate synthase (FPS) and (D) (E)-β-ocimene synthase (OS) after Spm, JA and Spm + JA treatment. Different letters indicate significant differences (P < 0.05, Tukey–Kramer HSD). See text for description.
complex (Rboh; respiratory burst oxidase homolog), polyamine oxidase (PAO) and copper amine oxidase (CuAO), and the enzyme activity and gene transcriptional levels of five selected ROS scavenging enzymes, namely superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and glutathione reductase (GR), following Spm, JA and Spm+JA treatments with respect to controls (Fig. 7), all detected 6 h after treatment. Rboh showed increased enzyme activities after treatment with Spm and JA with respect to controls, whereas the synergistic effect of Spm+JA resulted in high levels of Rboh activity (Fig. 7A). PAO enzyme activity was always reduced by Spm and JA treatments with respect to controls, even though the combined use of Spm+JA was not statistically different from the control (Fig. 7B). CuAO activity was significantly higher than PAO activity in all treatments we tested; however, no significant differences were found between controls and treatments, with the sole exception of Spm, which showed significantly lower enzyme activities (Fig. 7C, diagonal lined boxes). SOD enzyme activity (Fig. 7C) and gene expression (Fig. 7D) always increased with treatment with Spm, JA or Spm+JA. With respect to controls, CAT enzyme activity (Fig. 7E) and gene expression (Fig. 7F) were slightly reduced by Spm treatment, whereas JA treatment significantly increased enzyme activity and gene expression; the combined use of Spm+JA still produced an increase in CAT activity and gene up-regulation, even though it was significantly lower with respect to JA treatment alone. Spm significantly reduced the enzyme activity of APX, whereas JA and Spm+JA did not show any significant difference with respect to controls (Fig. 7G). However, both JA and Spm+JA resulted in a significant up-regulation of APX gene expression (Fig. 7H). GPX and GR enzyme activities (Fig. 7I and K, respectively) and gene expression
Fig. 6 Intracellular calcium variations in lima bean leaves 1 h after application of exogenous Spm and JA. False-color image analysis reconstructions from CLSM observations and fluochemical intracellular Ca\(^{2+}\) determination and image analysis in control (A) lima bean plants, and after treatment with Spm (B), JA (C) and Spm + JA (D). A–D represent portions of the lima bean leaf blade where the green fluorescence refers to binding of Calcium Orange with Ca\(^{2+}\), whereas the chloroplasts are evidenced by a bright red color caused by chlorophyll fluorescence. (E) Quantitative analysis of the Ca\(^{2+}\) release shown in A–D. Error bars represent the SE (n = 8–10). Different letters indicate significant differences (P < 0.05, Tukey–Kramer HSD). The scale bar (100 µM) is indicated on the panels.

(Fig. 7) and (L, respectively) significantly increased only after Spm + JA treatment. Fig. 8 shows a general scheme of ROS production and scavenging following Spm and JA treatment.

**Discussion**

Polyamines are ubiquitous low-molecular weight aliphatic cations that are implicated in a large number of plant cellular processes (Walters 2003, Kusano et al. 2007, Kusano et al. 2008). One may argue that artificial stress caused by the treatment with cationic substances may have resulted in the production of induced volatiles. However, this possibility is unlikely for the following reasons. The results of the present work suggest that diamines (Cad and Put), a triamine (Spd) and a tetra-amine (Spm) induce different blends of volatiles in lima bean leaves. The difference between the diamine and triamine was the extra production of the unknown terpenoid (I) in the triamine (Spd) treatment. In contrast, the tetra-amine Spm induced a totally different blend of volatiles, suggesting that the mechanisms involved in the induction of volatiles by tetra-amine are somewhat specific to the tested polyamines.

In this study we have shown that the emission of some *T. urticae*-induced plant volatiles increased with the combined treatment with some polyamines and JA. Among the volatiles induced by the double treatment, (E)-β-ocimene and DMNT have been reported to attract *P. persimilis*, one of the carnivorous natural enemies of spider mites of the genus *Tetranychus* (Dicke et al. 1990). Thus, the increased amounts of these volatiles would explain the increased attractiveness of Spm + JA-treated leaves over JA-treated control leaves in our bioassays.

Irrespective of the type of polyamine tested, the profiles of volatiles emitted from leaves treated with polyamine + JA were significantly different from those emitted by leaves treated with polyamine or JA alone. In fact, six major
Fig. 7 ROS-producing and -scavenging enzyme activities and gene expression in control (MES buffer) lima bean leaves and in leaves treated with either Spm, JA or Spm+JA. Enzyme activity of Rboh (A); enzyme activity of PAO and CuAO (diagonal lined bars) (B); enzyme activity (C) and gene expression (D) of SOD; enzyme activity (E) and gene expression (F) of CAT; enzyme activity (G) and gene expression (H) of APX; enzyme activity (I) and gene expression (J) of GPX; and enzyme activity (K) and gene expression (L) of GR. Error bars represent the SE. Different letters indicate significant differences (P < 0.05, Tukey–Kramer HSD).
Polyamines induce plant volatiles in lima bean

Polyamines induce plant volatiles in lima bean

compounds [(Z)-3-hexenyl acetate, DMNT, (E)-β-ocimene, (Z)-β-ocimene, TMTT, an isomer of TMTT and an unknown terpenoid (?)] and one minor compound (α-copaene) showed a similar significant correlation (Table 3). It can be argued that the addition of some polyamines may enhance the action of JA on volatile induction. The finding that volatile production increases with Spm (+JA) concentrations and with time (Fig. 1) implies the presence of a positive dose–response effect and a possible role for Spm in gene activation. Quantitative relationships exist between induced JA levels and volatile emissions (Schmelz et al. 2003a), and insect predators of spider mites are attracted by plant volatiles from lima bean leaves induced by JA (Shimoda et al. 2002). Directly and indirectly, the JA pathway controls or influences the expression of the majority of genes identified as being insect activated, and the contribution of the JA pathway to the control of gene expression can vary depending on the type of stress (Reymond et al. 2004, Balbi and Devoto 2008, Zheng and Dicke 2008). The average resting level for JA was found to be 10–40 ng g−1 FW and increased 10- to 15-fold within the first 1–2 h after damage in various combinations of plant–insect trials (Baldwin et al. 1997, Schmelz et al. 2003b, Schulze et al. 2006, Arimura et al. 2008). Our results showed that JA can be induced by Spm. Although the response (about a 2-fold increase) is far from being comparable with that of herbivore wounding, the timing of Spm-induced JA production is comparable (1–3 h). However, besides JA, other factors such as SA (Ozawa et al. 2000) and ethylene (Schmelz et al. 2003b, Arimura et al. 2002) are known to be involved in the production of the volatiles.

It is of great interest that the double treatment with JA and Spm induced the expression of LOX, ADH, OS and FPS, which are involved in the production of green leaf volatiles and volatile terpenoids (Dudareva et al. 2004, Graus et al. 2004, Arimura et al. 2005, Arimura et al. 2008). Induced expression of ADH, OS and FPS would in part explain the increased amounts of (Z)-3-hexenyl acetate and volatile terpenoids; moreover, the Spm+JA induction of LOX, with respect to the Spm effect alone, implies potentially increased lipid peroxidation with increasing JA and fatty acid volatile production (Mueller 2004). Treatment with exogenous JA has been demonstrated to alter polyamine metabolism (Walters 2003) and affect the distribution of free and conjugated polyamines (Mader 1999), suggesting the existence of a possible cross-talk between the JA signaling pathway and polyamines (Walters 2003). Little is still known about the effects of polyamines in plant defense mechanisms against herbivores and pathogens. Spm transmits a signal to activate defense pathways against pathogens and this pathway is designated the ‘Spm-signal transduction pathway’ (Kusano et al. 2007, Kusano et al. 2008). Spm was also found to induce SA-independent resistance against pathogens in tobacco plants (Yamakawa et al. 1998). Furthermore, exogenous application of Spm to tobacco leaves induces a pathway involving mitochondrial dysfunction, activation of MAPK and increased expression of hypersensitive reaction (HR) marker genes (including

Fig. 8 General schematic showing the action of Spm and JA on ROS-scavenging and -producing activities in lima bean. Arrows indicate up-regulation and/or increased enzyme activity; dotted lines indicate down-regulation and/or decreased enzyme activity. Letters correspond to those in Fig. 7.
transcription factors), and caused defense responses and HR-like cell death (reviewed by Kusano et al. 2008). All of these findings suggest that the role of Spm in plant defense is an area ripe for investigation (Walters 2003). To lead to mitochondrial malfunction, at least two events are prerequisite: activation of Ca2+ influx and the production of ROS (Takahashi et al. 2003). Apoplastic Spm can directly affect cation channel(s) and/or be catabolized by PAO. These combined reactions would result in changes in Ca2+ trafficking and the generation of H2O2, both of which can trigger the downstream reaction of the Spm signaling pathway (Kusano et al. 2007). Our data confirm that Spm is able to trigger a cytosolic calcium increase and the generation of H2O2, and that the synergistic action of JA and Spm leads to an even greater response. The production of ROS in the cell wall is achieved by different enzymatic sources, such as membrane-bound NADPH oxidases (Rboh), apoplastic oxalate oxidases, peroxidases and amine oxidases (Angelini et al. 2008, and references therein). An increasing body of evidence indicates that amine oxidases are important players in H2O2 production during both plant development and defense (Cona et al. 2006). Dissection of the molecular mechanisms controlling CuAO expression indicated that JA works as a potent inducer of basal and wounding-inducible CuAO expression (Cona et al. 2008); moreover, CuAO is present at a high level in many legume species where it has been involved in ROS production during wound healing and defense responses (Rea et al. 2002). In the present study, JA did not exert any significant effect on either PAO or CuAO.

Our results indicate a strong correlation between ROS production, calcium signaling and the induction of HIPVs upon treatment with Spm, suggesting that abiotic elicitation can result in the same pattern of responses found in plant–herbivore and plant–pathogen interactions. In fact, feeding herbivores elicit calcium signaling, JA and ROS bursts, a large transcriptional reorganization of the plant host and, after hours, a release of terpenoids (Dicke et al. 1999, Arimura et al. 2000, Ozawa et al. 2000, Schmelz et al. 2003a, Arimura et al. 2005, Maffei et al. 2007a, Browse and Howe 2008, Howe and Jander 2008). Spm induces Rboh and SOD activity and repressed PAO, CuAO and APX. In tobacco protoplasts, the effect of Spm appears to have an opposite effect on Rboh, although protoplasts respond to polyamines by increasing H2O2 accumulation (Papadakis and Roubelakis-Angelakis 2005). However, Spm has no effect on CAT, GPX and GR, as found when Spm was exogenously applied to stressed plants (Nayyar and Chander 2004). Thus, Spm appears to elicit ROS production more than ROS scavenging. JA is known to induce and elicit ROS production (Hung and Kao 2007), but our results indicate that JA also induces ROS scavenging by increasing the activity of CAT and APX. Interestingly, the combined use of Spm+JA induces a strong activation of GPX and GR enzyme activities, with a minor effect on their gene expression.

Research on polyamines in plant–herbivore interactions lags behind that of other areas of plant polyamines. However, a growing body of evidence indicates significant effects and cross-talk between polyamine metabolism and genes involved in plant responses to herbivore attacks. The present work focused on free exogenous polyamines, but polyamine conjugates may play a greater role, even through conjugation to exogenously fed polyamines. The search for environmentally friendly elicitors able to trigger plant responses to herbivore attack is of paramount importance for a sustainable use of molecular and physiological tools in crop management. In this work we showed that the use of low and suboptimal concentrations of JA combined with the use of Spm can trigger plant responses that attract predators of infesting mites. However, despite the clear correlation between Spm levels and plant response, the physiological and metabolic rationale for such an effect is still far from being clear.

Materials and Methods

Plants and mites
Lima bean plants (P. lunatus cv. Pole Sieva) were grown in plastic pots (diameter, 12 cm; depth, 10 cm) in a climate-controlled room (120 µmol m−2 s−1; 25±2°C, 16 h light/8 h dark). Two- to 3-week-old seedlings with fully developed primary leaves were used for the experiments. Herbivorous mites (T. urticae) and carnivorous mites (P. persimilis) were obtained from a laboratory-maintained culture. Tetanychus urticae was reared on kidney bean plants (Phaseolus vulgaris cv. Nagazuramame) and P. persimilis was reared on T. urticae living on kidney bean plants, as described above.

Applications of chemicals to lima bean leaves
For chemical treatments, we used a detached primary lima bean leaf whose petiole was placed in a glass vial (10 ml) filled with MES buffer solution (pH 5.5) containing JA (0.1 mM; Sigma-Aldrich, St Louis, MO, USA) and/or the polyamines, Cad, Put, Spd and Spm (0.1–1 mM, Wako) (Hopke et al. 1994, Ozawa et al. 2000). Experiments were conducted in the climate-controlled room under the same conditions as above and the leaves were treated for 1–24 h.

Chemical analysis of volatile compounds
Two leaves for each treatment were placed in a lidded glass bottle (2 liters) for 30 min. We collected the volatiles in the bottle using 100 mg of Tenax-TA resin (20/35 mesh, GL Sciences, Tokyo, Japan) packed in a glass tube (3.0 mm i.d.×160 mm) for 1 h (100 ml min−1). The volatiles were eluted with 2 ml of diethyl ether. n-Eicosane (0.5 µg) was used as an internal standard. The volatiles were then quantified by gas chromatography.
added to the eluate as an internal standard. The eluate was concentrated with a stream of gaseous N₂ and injected into the injection port (250°C) of a gas chromatograph–mass spectrometer (GC, Hewlett Packard 6890 with an HP-SMS capillary column: 0.25 mm i.d., 30 m length, 0.25 µm film thickness; MS, Hewlett Packard 5973 mass selective detector, ion source 70 eV). The GC oven temperature was programmed to rise from 40°C (5 min hold) to 280°C at 15°C min⁻¹. JA-induced volatiles were defined as the volatiles that were found in the headspace of treated leaves but were not found (or found in trace amounts) in that of untreated leaves. The chemical structure of each compound was elucidated by comparison of the mass spectra and the retention time with those of authentic chemical samples. 

**Determination of JA**

After 1, 2, 3, 4, 6, 17 and 24 h treatment with 1 mM polyamines, detached primary lima bean leaves were collected and were kept at −80°C until use for the following extraction. We extracted JA from leaves according to Tamogami and Kodama (1998), with minor modifications. Each treated leaf was ground in liquid nitrogen. The powder was mixed with 50 ml of acetone and 100 ng of H₂-JA (as an internal standard). After acetone evaporation, the residue was dissolved in 6.0 ml of 75% aqueous methanol. The methanol solution was passed through a Sep-Pak Vac C18 cartridge (Waters) to prepare the partially purified sample. The semi-purified solution was subjected to liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis (Ishihara et al. 2006).

Separation by HPLC was performed with an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a Mightysil RP-18 GP column (150 × 2.0 mm, 3 µm particle size, Kanto Chemical, Tokyo, Japan) at a flow rate of 200 µl min⁻¹. A linear gradient [0.1% formic acid (A) and acetonitrile (B), 40–80% B/(A+B) for 10 min] was applied. MS detection was performed on an API-3000 triple stage quadrupole mass spectrometer equipped with a Turbolon-Spray ionization source (Applied Biosystems, Foster City, CA, USA). Concentrations of JA and H₂-JA were determined by multiple reaction monitoring (MRM). The monitored mass transitions were m/z 209–59 for JA and m/z 211–59 for H₂-JA. The conditions for MS were optimized for MRM using authentic JA and H₂-JA (provided by K. Matsuda). The optimized conditions included nebulizer gas flow, curtain gas flow, ionspray voltage, TurbolonSpray temperature, collision gas pressure, declustering potential, focusing potential, entrance potential, collision energy and cell exit potential.

**Bioassays**

A Y-tube olfactometer was used to test the olfactory responses of adult female predators to plant volatiles (for details of the olfactometer, see Takabayashi and Dicke 1992). Bioassays were performed at 25 ± 2°C. In repeated experiments, we used two leaves treated for 24 h with 0.1 mM JA for 24 h and two leaves treated with a mixture of 0.1 mM JA and 0.3 mM Spm. Predators were starved for 24 h before beginning the experiments. Individuals were positioned at the starting point of an iron Y-shaped wire that was fixed at the centre of the Y-tube. When a mite reached one end of the wire arm, a 'choice' was recorded. However, when a mite did not reach an end within 10 min it was recorded as ‘no choice’. The result in each experiment was subjected to a χ² test to determine the significant difference in attraction between the results and null hypothesis (50–50 distribution of predators).

**Intracellular calcium variation determination**

Calcium Orange was diluted in 50 mM MES buffer, pH 6.0, with the addition of 0.5 mM calcium sulfate, 2.5 µM DCMU to reach a concentration of 5 µM. This resulting solution was used for an initial treatment of lima bean leaves, as previously described (Maffei et al. 2004). At 30 min after treatment with Calcium Orange, leaves of plants, treated as above, were fixed on a Leica TCS SP2 confocal scanning laser microscope (CLSM) stative. Measurements were taken on intact leaves in the presence of exogenous calcium. The microscope was operated with a krypton/argon laser at 543 and 568 nm wavelengths: the first wavelength excites the Calcium Orange, resulting in emission of green light, and the second mostly excites chloroplasts, which emit red fluorescence. Images generated by the Fluoview software were analyzed using NIH Image J software. Calibration was performed by using the Invitrogen calcium calibration kit and as described elsewhere (Mithofer et al. 2009). Digital images were processed as described elsewhere (Maffei et al. 2004).

**CLSM localization of H₂O₂ and active peroxidases using Amplex Red**

Lima bean leaves from rooted plants in pots were incubated with the dye 10-acetyl-3,7-dihydroxyphenoxazin (Amplex Red). The Molecular Probes Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (A-22188) was used and dissolved in MES-Na buffer 50 mM (pH 6.0) containing 0.5 mM calcium sulfate and 5 µM DCMU, to obtain a 50 µM solution as described in detail in Maffei et al. (2006). Leaves were...
treated, as above, and then mounted on a Leica TCS SP2 multiband CLSM stage. In the presence of peroxidase, the Amplex Red reagent reacts with H2O2, with a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin, which has excitation and emission maxima of approximately 571 and 585 nm. Scans were then recorded from 0 to 180 min using the HCX PL APO 63x/1.20 W Corr/0.17CS objective. The microscope was operated with an argon laser (458 nm/5 mW; 476 nm/5 mW; 488 nm/20 mW; 514 nm/20 mW), a 543 nm/1.2 mW HeNe laser and a 633 nm/10 mW HeNe laser. Digital images were processed as described elsewhere (Maffei et al. 2004).

Enzyme activities

Leaves from five plants were treated as above, collected, pooled, frozen in liquid N2 and stored at −80°C before enzyme extraction. ROS scavenger enzymes were extracted, as described by Maffei et al. (2006). All operations were carried out at 4°C. Plant material was ground with a mortar and pestle under liquid nitrogen in cold 50 mM sodium phosphate at pH 7.5 (pH 6.5 for PAO) containing 250 mM sucrose, 1.0 mM EDTA, 1 mM MgCl2, 0.5 mM PMSF (phenylmethylsulfonyl fluoride), 0.1 mM DTT (dithiothreitol) and 1% (w/v) PVPP (polyvinylpyrrolidone) in a 1:6 proportion (w/v). The homogenate was then filtered through eight layers of cheesecloth and centrifuged at 25,000 × g for 20 min at 4°C. The supernatant was brought to 80% saturation with the addition of solid ammonium sulfate (NH4)2SO4 and stirred gently for several hours at 4°C. After centrifugation at 28,000 × g for 45 min at 4°C, pellets containing most of the enzyme activity were re-suspended in a small volume of 50 mM sodium phosphate (pH 7.5) and used directly for enzyme assays.

NADPH oxidase (Rboh; EC 1.6.3.1). The activity of Rboh was measured according to Murphy et al. (1998). A standard assay mixture contained 40 mM NADPH, 0.02% (w/v) Triton X-100, 100 mM nitroblue tetrazolium (NBT) and buffer (20 mM Tris-chloride, pH 7.5, 3 mM MgCl2) to make a total volume of 1 ml in a quartz cuvette. An additional 30 µM DPI (diphenyl iodonium) was added to the reaction mixture. Change in absorbance at 530 nm (A530) was measured spectrophotometrically. Calculation of specific activity assumed an absorption coefficient of 12.8 mM−1 cm−1.

Superoxide dismutase (SOD; EC 1.15.1.1). The activity of SOD was measured according to Krishnan et al. (2002). This method tests the ability of SOD to inhibit the reduction of NBT by the superoxide anions generated photochemically. A 1 ml assay mixture consisted of 50 mM Na-phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and enzyme extract. Riboflavin was added last, the samples were placed 30 cm below a light source (4,000 lux) and the reaction was allowed to continue for 15 min. The reaction was stopped by switching off the light. A non-irradiated reaction mixture run in parallel did not develop color and served as a control. The absorbance was read at 560 nm.

Polyamine oxidase (PAO, EC 1.5.3.11) and copper amine oxidase (CuAO, EC 1.4.3.6). PAO was extracted following the method of Li (1993) with slight modifications. The activities of PAO and CuAO were determined spectrophotometrically by a method based on the colorimetric assay of Δ1-pyrroline, using Spd or Put as substrates (Holmstead et al. 1961). In a 2.0 ml final reaction volume, 0.1 ml of extract was combined with 50 U of CAT and 0.1% o-aminobenzaldehyde, and the reaction was started with one of two different buffer and substrate combinations: 10 mM Spd in 50 mM K-phosphate buffer (pH 6.0) for PAO; 10 mM Put in 50 mM K-phosphate buffer (pH 7.0) for CuAO. The reaction was carried out at 30°C for 3 h, terminated with 2.0 ml of 10% (v/v) perchloric acid (PCA) and then centrifuged in tubes at 5,000 r.p.m. for 15 min. Formation of the Δ-pyrroline product was determined by reading the absorbance at 430 nm. Control reactions were carried out with the addition of PCA prior to adding enzyme to the reaction mixture.

Catalase (CAT; EC 1.11.1.6). CAT activities were assayed spectrophotometrically by monitoring the change in absorbance at 240 nm due to the decreased absorption of H2O2. H2O2 (ε = 39.4 mM−1 cm−1) (Zhang and Kirkham 1996). The reaction mixture in 1 ml final volume contained 50 mM Na-phosphate (pH 7.0), 15 mM H2O2 and enzyme extract. The reaction was initiated by the addition of H2O2.

Ascorbate peroxidase (APX; EC 1.11.1.11). APX activity was determined from the decrease in absorbance at 290 nm, due to the H2O2-dependent oxidation of ascorbate (ε = 2.8 mM−1 cm−1) (Zhang and Kirkham 1996). The 1 ml reaction mixture contained 50 mM Na-phosphate (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H2O2, 0.1 mM EDTA and enzyme.

Glutathione peroxidase (GPX; EC 1.11.1.9). GPX was assayed indirectly, as described by Anderson and Davis (2004). The reaction contained 125 mM K-phosphate buffer (pH 7.0), 1.2 mM cumene hydroperoxide, 1.25 mM EDTA, 1.25 mM sodium azide, 1.0 mM reduced glutathione, 0.25 mM NADPH, 0.6 IU of yeast GR (Sigma Type III) and enzyme extract in a final volume of 1 ml. After incubation for 10 min at 25°C, with all the reagents except cumene hydroperoxide, the reaction was started with cumene hydroperoxide. A decrease of NADPH at 340 nm was corrected for non-enzymatic controls.

Glutathione reductase (GR; EC 1.8.1.10). GR activity was determined at 340 nm following the oxidation of NADPH (ε = 6.2 mM−1 cm−1) at 340 nm (Zhang and Kirkham 1996). The reaction mixture contained 100 mM Na-phosphate (pH 7.5), 0.2 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and enzyme extract in a 1.0 ml final volume at 30°C. The reaction was started by adding GSSG.
Soluble protein determination
The soluble protein concentration was evaluated using the method of Bradford (1976), with bovine serum albumin as a standard.

Quantitative gene expression
Total RNA was extracted from leaves treated, as above, using the Agilent Plant RNA Isolation kit (Agilent Technologies). Three biological replicates were run for each gene. First-strand cDNA was synthesized using AffinityScript Reverse Transcriptase (Stratagene), random primers and 1 µg of total RNA at 42°C for 90 min. Primers for real-time PCR were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi) from a length of the resulting PCR product of approximately 200 bp (see below). Real-time PCR was performed on an Mx3000P Real-Time PCR system (Stratagene). The process was performed with 25 µl of reaction mixture consisting of 12.5 µl of 2 × Brilliant SYBR Green QPCR Master Mix (Stratagene), cDNA (0.5 µl from 20 µl of each reverse transcription product pool), 100 nM primers and 30 mM ROX as a reference dye. The following protocol was applied: initial polymerase activation of 10 min at 95°C and 40 cycles of 30 s at 95°C, 60 s at 55°C and 30 s at 72°C. Relative RNA levels were calibrated and normalized with the level of actin mRNA (GenBank accession No. DQ159907). Primers used for real-time PCR were as previously described (Maffei et al. 2006, Arimura et al. 2008).

Statistics
Data are expressed as mean ± SE throughout. Analysis of variance (ANOVA) and subsequent Dunnet’s test were used to determine significant differences among treatments in experiments for H₂O₂ production, Ca²⁺ release, gene expression and enzyme activity. The correlations in Table 3 were calculated using Pearson’s product–moment correlation coefficient.

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References


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