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Induction of defense genes and secondary metabolites in saskatoons (*Amelanchier alnifolia* Nutt.) in response to *Entomosporium mespili* using jasmonic acid and Canada milkvetch extracts

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ABSTRACT

Entomosporium leaf and berry spot represent the most important disease of saskatoons (*Amelanchier alnifolia* Nutt.). This disease, caused by the ascomycete *Entomosporium mespili* (D.C.) Sacc., is difficult to control *via* conventional methods and no known saskatoon cultivar carries resistance to this pathogen. The aim of the present study was to enhance existing and induced defense responses of saskatoons using two host defense inducers, namely jasmonic acid and an extract from Canada milkvetch on two saskatoon cultivars, Smoky and Martin. Both inducers exhibited an ability to differentially induce the synthesis/accumulation of defense-related genes including those encoding for PR-1, PR-2, PR-5, LOX and PAL. Pre-treatment of saskatoon leaves with these inducers reduced the disease levels especially when applied to the moderately susceptible cultivar Martin. An accumulation of various hydroxycinnamic acid and proanthocyanidin derivatives also correlated with the disease levels recorded on both cultivars. Results are discussed on the basis of the induced pathways in an attempt to lay the ground for a better understanding of this host–pathogen interaction. In the meantime, we have shown that the use of defense enhancers may be useful in integrated management strategies to control entomosporium leaf and berry spot in saskatoons.

1. Introduction

Amelanchier alnifolia Nutt. is a native fruit shrub that is important to the economy of several Canadian provinces especially Alberta, Saskatchewan, and Manitoba (Holtslag et al., 2003; Lange, 1998). It produces berry fruits called saskatoons (also called serviceberries or juneberries), which are very rich in antioxidants. Their production is hampered by several abiotic and biotic stresses among which entomosporium leaf and berry spot (ELBS) is the main one. This disease is caused by the ascomycete *Entomosporium mespili* (D.C.) Sacc. (Lange and Bains, 1994). This fungus is a non-host specific pathogen that causes necrotic spots on leaves, fruits, and succulent stems of over 60 species in the *Rosaceae* family worldwide, especially the *Pomoidae* sub-family (Sinclair et al., 1987). In saskatoons, *E. mespili* is primarily spread through asexual conidia produced in acervuli, in which they are able to overwinter. These fungal structures (Fig. 1) are found in the center of the lesions observed on young branches and infected leaves or fruits (Holtslag et al., 2003; Horie and Kobayashi, 1979). Disease symptoms first appear on leaves as small brown spots that develop yellow halos. The spots may eventually coalesce and cause the entire leaf to become yellow and prematurely fall (Bains, 2000).

Besides the direct damaging effect that *E. mespili* lesions have on fruit quality, diseased plants show a decrease in the photosynthetic rate due to leaf areas lost to lesions and the premature defoliation under heavy infection conditions (Holtslag et al., 2003; Horie and Kobayashi, 1979; St-Pierre, 1997). ELBS was of minor importance until 1990, when it caused an important economical loss in the

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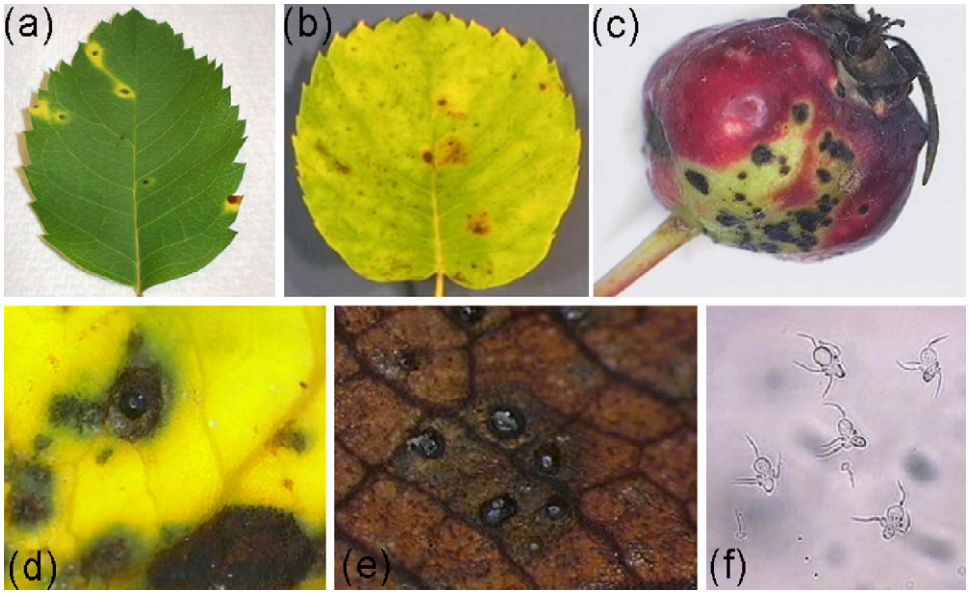

Fig. 1. Symptoms (a–e) caused by *Entomosporium mespili* (f) on saskatoon leaves at early (a) and late (b) stages and on the berries (c) under field conditions; (d and e) microscopic view of the brown to grey irregular necrotic lesions on leaves; (f) insect-like conidia typical of *E. mespili*.

central and north-central areas of Alberta, Canada (Davidson et al., 1991; Pesic-van Esbroeck et al., 1991). By 1994, the prevalence of the disease in these areas had increased to destroy most of the fruit production that year (Lange, 1998; Lange and Bains, 1994). Consequently, saskatoon industry began searching for means to effectively control this disease.

Plants possess both pre-formed and inducible structural and biochemical mechanisms to resist invasion by pathogens. Pre-formed secondary metabolites (i.e., phytoanticipins) can primarily restrict pathogen infection but their increase and the activation of a panel of defense-related compounds (i.e., phytoalexins, PR-proteins) can further limit the pathogen's activity, growth, and spread (Daayf et al., 2003b; van Loon et al., 2006). Some defenses can also help reinforce plant cell walls (Slusarenko et al., 2000) and other tissues (Moreira et al., 2009). Such defenses can be induced using a range of biotic and abiotic elicitors (Daayf et al., 2003a; Moreira et al., 2009). Among the inducible mechanisms, there is an extremely wide range of plant phenolics, terpenoids and nitrogen-based molecules with fungitoxic proprieties (Harborne, 1999; Daayf and Lattanzio, 2008).

Up-to-date, studies on saskatoon–*E. mespili* interactions have mainly focused on describing the progress of epidemics in the field, testing certain fungicides and assessing cultivars' responses to natural infections (Holtslag et al., 2003; Lange, 1998; Ronald et al., 2001). Very little is known about the plant biochemical or molecular processes that are affected during this interaction. *E. mespili* being an obligate parasite also adds to the difficulty studying this interaction.

Ronald et al. (2001) showed that no known saskatoon cultivar carries immunity/resistance to the pathogen *E. mespili*. We hypothesize that both pre-existing and pathogen-induced defenses in saskatoons are not successful in stopping this pathogen. In this context, we hypothesize that boosting pre-existing and pathogen-induced defenses and possibly inducing new ones in saskatoon plants, using defense inducers, may be useful as part of an integrated disease management strategy.

The objectives of the present study were to: (i) follow the disease progress in a field situation, (ii) develop an *in vitro* test with detached leaves to study the interaction saskatoon-*E. mespili*, (iii) evaluate the ability of selected treatments (Jasmonic acid, and Canada milkvetch extract) to induce defense responses in saskatoon plants against *E. mespili*, and (iv) assess the expression of selected defense-related genes in response to the tested treatments and, where possible, their final products (i.e., phenolics).

2. Materials and methods

2.1. Plant material

Two Saskatoon cultivars Smoky and Martin were used in this study. Smoky is a highly susceptible cultivar to ELBS and Martin is moderately susceptible (Ronald et al., 2001; Zatylny and St-Pierre, 2003). These two cultivars were selected among other cultivars grown in an orchard at 'The point', an experimental field station at the University of Manitoba. Selected shrubs were labeled and the branches to receive a treatment were tagged.

2.2. Fungal material, growth conditions and inoculum production

A set of *Entomosporium mespili* strains was isolated from naturally infected leaves and berries displaying ELBS symptoms and collected from the field in July 2008. The infected tissues were examined under the microscope especially areas with brown to grey irregular spots 2–5 mm in diameter (Holtslag et al., 2003) (Fig. 1). These areas prominently harbored acervuli in the center of the lesions. Incubating the acervuli under high humidity yielded insect-like conidia very typical of *E. mespili* (Fig. 1). A single conidium per examined lesion was transferred onto Petri dishes containing various growth media (PDA, V8-Agar, V8-PDA, Rye A, or a saskatoon-based medium). Twenty days after transfer and incubation at 20 °C, black mycelia developed on the plates containing the saskatoon-based medium. Examining these mycelia under the microscope showed the presence of fungal structures resembling the ones described by Sivanesan and Gibson (1976). These structures were cycindrical, sort-stalked, 8-spored grouped in tick-walled asci with hyaline and smooth ascospores, thus confirming the presence of *E. mespili* (Fig. 1). Single ascopores were then transferred back onto the saskatoon-based medium and used in further experiments. Conidial suspensions obtained from infected leaves were calibrated at 10^4 conidia per milliliter using a hemacytometer and sprayed onto the leaves.

2.3. Field experiments

Selected and tagged plants of cultivars Smoky and Martin in the orchard were sprayed with water (Control), 10 μM of Jasmonic acid (JA) or a 1/10 dilution in water of Canada Milkvetch extract (El Hadrami et al., 2008). Six, 24 and 48 h post-treatments (h.p.t.), the leaves were detached and quickly frozen in liquid nitrogen and transferred to −80 °C until they were used for RNA or secondary metabolites isolation.

2.4. Artificial inoculations and disease evaluation

Since the leaves used in the field trial may have been naturally infected, another set of leaves was detached, surface-sterilized and placed in Petri dishes under sterile conditions and sprayed as described above. Twenty-four hours later, the leaves were sprayed with a conidial suspension of *E. mespili*, and then samples were harvested at 6, 24 and 48 h.p.t. Leaves treated with water either inoculated, or not, with *E. mespili*, were used as controls.

Disease severity was also assessed on these leaves 7 days post-inoculation according to an arbitrary scale where: 1 = no lesions, 2 = with lesions, less than 10 necrotic spots, 3 = more than 10 necrotic spot lesions, and 4 = with coalescing lesions and chlorosis in all the leaf. Where applicable, the protection percentage (PP) was calculated as follows: $PP\ (\%) = 100(1 - (x/y))$; where x and y are disease severity values recorded for treated and control plants, respectively.

2.5. Preparation of total RNA

RNA was extracted from saskatoon leaves collected at 0, 6, 24 and 48 h.p.t. The collected leaves were reduced to a fine powder in a mortar and a pestle pre-cooled in liquid nitrogen. The total RNA was isolated from 300 mg fresh weight and purified according to the protocol described by Komjanc et al. (1999). The quality of the RNA was checked on an agarose gel and the concentrations and purity were determined using a spectrophotometer (Ultrospec 3100, Biochrom Ltd., Cambridge, UK), based on absorbance at 260 nm and the ratio A_{260}/A_{280}.

2.6. Reverse transcription and cDNA synthesis

Messenger RNA was isolated from each extracted sample and converted to cDNA using the M-MLV reverse transcriptase kit (Invitrogen Inc.) according to the manufacturer's recommendations. The synthesized complementary DNAs were further used for the assessment of several defense-related genes (*PAL*, *LOX*, *PR-1*, *PR-2*, and *PR-5*). The constitutively expressed 18S ribosomal RNA gene was used as a reference control.

2.7. Primers design and PCR conditions

Primer pairs for each targeted defense-related gene were designed using Primer 3 software (Rozen and Skaletsky, 2000). Since there was no available sequence in the GenBank for these genes in saskatoons, we used sequences from a relative species, *Malus domestica*, as a template (Table 1). PCR was then performed for each targeted defense-related gene using 1 μM of each designed primer in a final reaction volume of 25 μl containing 1 μl cDNA, 1× PCR buffer, 1 mM dNTPs mix, 5 mM $MgCl_2$, 0.1 U Taq DNA polymerase and H_2O. PCR amplification was carried out in a programmabale thermocycler (Biorad, USA) with an initial denaturation at 94 °C for 5 min followed by 35 reaction cycles consisting of 30 s denaturation at 94 °C, 30 s annealing at different annealing temperatures depending on the tested primers set and 1 min elongation at 72 °C, followed by a final extension for 10 min at 72 °C. PCR products were analyzed by electrophoresis using a 1.5% agarose gel containing 0.01% ethidium bromide at 105 V for 30 min. The gels were visualized using an AlphaImager HP version 6 UV-translaminator (Alpha Ease FC software, Alpha Innotech, San Leandro, USA) equipped with a 16 bit CCD camera and P90 U thermoprinter (Mitsubishi Inc., Japan).

2.8. Soluble phenolics isolation and analysis

Nine apparently healthy leaves of each treatment were collected from the field. A 1-g portion was reduced into a fine powder using a mortar and a pestle pre-cooled in liquid nitrogen. The powder was then mixed with 1 ml of 80% methanol and subjected to vigorous agitation. The homogenates were further centrifuged at 10,000 rpm for 5 min. This procedure was repeated three times after mixing the residue with fresh 80% methanol. The combined methanolic fractions were further evaporated under a nitrogen stream down to 300 μl. Purification of the extracts was carried out using petroleum ether and ethyl acetate. The final ethyl acetate extracts were evaporated to dryness and the residue was dissolved in 300 μl of HPLC-grade methanol.

Soluble phenolics were analyzed by reverse-phase high pressure liquid chromatography (HPLC) using a Waters 2695 separation module coupled with a Water 996 photodiode array detector. Forty microliters of each phenolic extract were injected onto an RP-18 (5 μm) lichrospher 100 column, and eluted at a flow rate of with a gradient of acetonitrile/0.1% H_3PO_4 in water, as follows: time in min/% acetonitrile: 0/0, 5/5, 10/5, 14/10, 20/20, 23/20, 30/35, 35/35, 43/50, 48/75, 55/100, 60/100, 62/0, 65/0, 90/0. Data were analyzed using the Empower 2.0 (Waters, Ville-Saint-Laurent PQ, Canada). Compounds were identified based on their retention time, their characteristic UV spectra in reference to our database and through co-elution with commercial standards.

2.9. Data analysis

All trials were conducted following a randomized complete blocks design and the quantitative data were statistically analyzed using general linear model (GLM) using SAS Package (Statistical Analysis Systems Institute Inc., Cary, NC, USA) or the ANOVA module of STATISTICA (Statsoft, 1999). Five to 10 leaves were included as repetitions for each treatment × replicate. The means were further compared according to Newmann–Keuls or the least significant differences (LSD) tests at $P < 0.05$.

Table 1
Primer pairs used to amplify saskatoon defense-related genes.

Designation	Function	GenBank Acc No.	Forward and reverse primers (5′–3′)	Size (bp)
18S	18S ribosomal RNA gene	cDNA, *Malus domestica* DQ341382	F: AAACGGCTACCACATCCAAG R: TCATTACTCCGATCCCGAAG	460
PR-1	Anti-microbial activity	cDNA, *Malus domestica* AF507974	F: CTTGACGTGGGATGACAATG R: TACGCCAAACCACCTGTGTA	243
PR-2	β-1,3-Glucanase	cDNA, *Malus domestica* AY548364	F: TTCCAGCCCTCAATGATGCT R: GGCCACAAGAGTTGTGTCAA	233
PR-5	Osmotin-like protein	cDNA, *Malus domestica* AY548366	F: ATTCATGGTCTGGTCGCTTC R: ATTCATGGTCTGGTCGCTTC	224
PAL	Phenyl-alanine ammonia lyase	cDNA, *Malus domestica* AF494403	F: GGCATTTGGAGGAGAATTGA R: TCAACAAGCACTTGCCTCAG	219
LOX	Lipoxygenase	cDNA, *Malus domestica* AY742295	F: ACCCTGACAAGGCATTCTTGA R: CGAACCGGTTTTTCAATCTC	243

All primers were designed based on available cDNA sequences from *Malus domestica* and used in PCR at an annealing temperature of 50 °C.

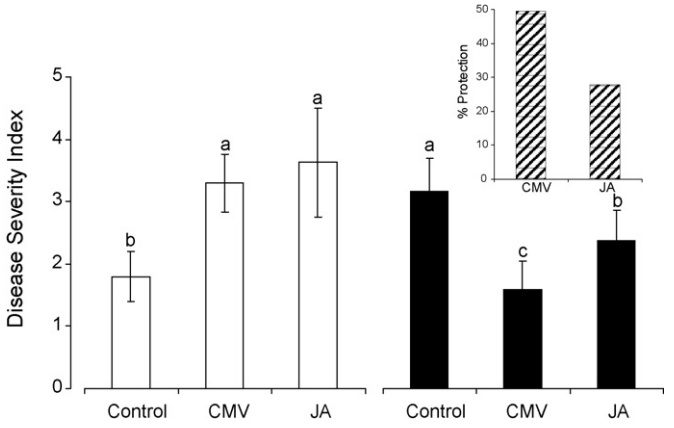

Fig. 2. Disease severity of entomosporium leaf spots in field saskatoons' leaves of cultivar Smoky (highly susceptible, □) and Martin (moderately susceptible, ■) treated with JA or CMV: (▨) represents the percent protection provided by each treatment as compared to the control. The bars represent the mean of 5 replicates ±SD, with 10 leaves per replicate. Values with the same letter do not differ significantly at $P < 0.05$.

3. Results

3.1. Field experiments

3.1.1. Disease assessment

Saskatoon shrubs of cultivars Smoky and Martin were selected in the field and the treated branches were labeled. Two series of treatment with water (control), JA or CMV were carried out. In each series, treated leaves, including the ones with obvious natural infection, were detached at 24 and 48 h after treatment to be further inoculated with *E. mespili* to guarantee a sufficient amount of inoculum. Disease severity (Fig. 2) was recorded on the treated and control plants 7 days after inoculation (d.a.i.). The two cultivars responded differentially to the treatments. While disease severity significantly increased in cultivar Smoky, as compared to the non-treated control, after treatment with either JA or CMV, it significantly decreased in cultivar Martin, where CMV and JA provided 50% and 28% disease reduction, respectively (Fig. 2).

3.1.2. Differential expression of defense-related genes (Fig. 3)

The transient expression of *PR-1*, *PR-2*, *LOX* and *PAL* was assessed in Saskatoon leaves treated with either JA or CMV, and in controls, at 0, 6, 24 and 48 h.p.t. A baseline expression of all selected defense-related genes was recorded in all analyzed samples at 0 h.p.t.

3.1.2.1. PR-1. In cultivar Smoky, noticeable accumulation of *PR-1* transcripts was recorded among all tested treatments at 6 h.p.t. A transient expression of this defense-related gene was recorded in the non-treated control 24 h.p.t. and increased at 48 h.p.t. No such activation was observed in response to the JA treatment either at 24 or 48 h.p.t. CMV treatment, on the other hand, induced a strong accumulation of *PR-1* transcripts at 24 h.p.t., which then was undetectable at 48 h.p.t.

A different pattern was recorded with cultivar Martin. As compared to the non-treated control, which showed a weaker induction of the accumulation of *PR-1* transcripts than cultivar Smoky both at 24 and 48 h.p.t., CMV and JA treatments increasingly induced the accumulation of these transcripts over time. Response to JA was the strongest, especially at 48 h.p.t.

3.1.2.2. PR-2. In cultivar Smoky, accumulation of *PR-2* transcripts was similar to that of *PR-1*. In cultivar Martin, the only noticeable up-regulation was recorded in response to JA treatment 48 h.p.t.

3.1.2.3. PR-5. In cultivar Smoky, *PR-5* transcripts increased in response to CMV treatment at 24 h.p.t., and in the non-treated control at 48 h.p.t. Cultivar Martin showed an up-regulation of *PR-5* in response to both JA and CMV treatments at 48 h.p.t.

3.1.2.4. LOX. The over time expression of *LOX* was similar to the one described for *PR-5* and *PR-2*, although the baseline expression due to detaching the leaves relate more to *PR-2* than to *PR-5* in both cultivars.

3.1.2.5. PAL. In cultivar Smoky, there was a transient increase in the accumulation of *PAL* transcripts in the healthy control detached leaves at 48 h.p.t. in response to JA and CMV at 24 h.p.t. In cultivar Martin, no significant increase in *PAL* transcripts was recorded amongst the treatments over time.

3.2. In vitro experiments

3.2.1. Disease assessment

None of the tested treatment showed a significant reduction of disease severity in cultivar Smoky (Fig. 4). However, in cultivar Martin, both JA and CMV resulted into a significant decrease in disease severity by over 20% of the control (Fig. 4). There was less chlorosis on the treated than the non-treated leaves (Fig. 4).

3.2.2. Differential expression of defense-related genes (Fig. 5)

3.2.2.1. PR-1. In cultivar Smoky, an over time increase in the transient expression of *PR-1* was recorded in the non-treated samples, with the higher accumulation at 48 h.p.t. A similar activation was observed in response to JA. CMV induced the highest accumulation of *PR-1* transcripts at 24 h.p.t., while the levels detected at 48 h.p.t. were comparable to the non-treated control. In cultivar Martin, both JA and CMV induced an increase in *PR-1* transcripts as early as 6 h.p.t. and continued thereafter, with the highest levels recorded at 48 h.p.t.

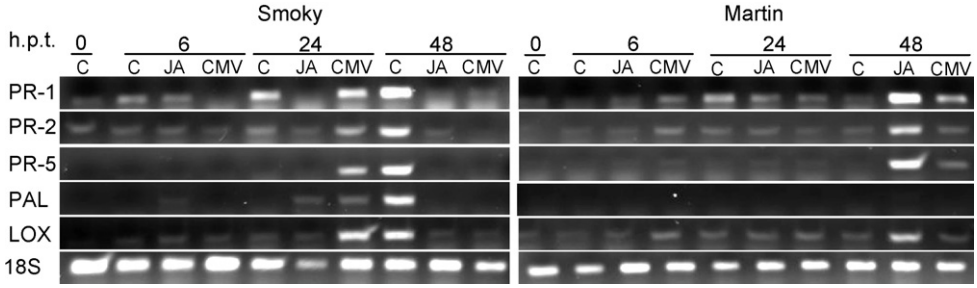

Fig. 3. Over time expression of selected defense-related genes in the leaves of two saskatoon cultivars, Smoky (highly susceptible) and Martin (moderately susceptible), in response to pre-treatment with water (control C), 10 μM jasmonic acid (JA) or 1/10 dilution in water of Canada milkvetch extract (CMV) under field conditions. The 18S ribosomal RNA gene was used as internal control. Results are representative of one of three independent replicates. h.p.t.: time after treatment.

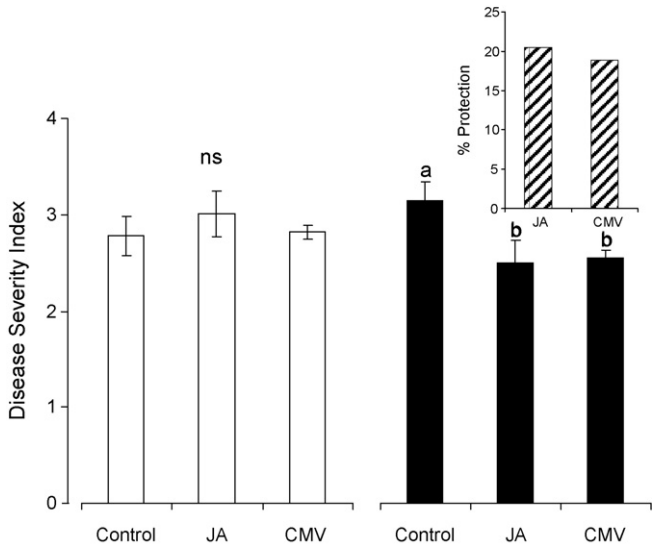

Fig. 4. Effect of pre-treatment with JA and CMV on disease severity caused by *E. mespili* on saskatoons leaves of two cultivars Smoky (highly susceptible, □) and Martin (moderately susceptible, ■) maintained *in vitro*: (▨) represents the percent protection provided by each treatment as compared to the control. The bars ±SD represent the mean of two independent experiments with 3 replicates per treatment in each. Values with the same letter do not differ significantly at $P < 0.05$.

3.2.2.2. PR-2. The accumulation of *PR-2* transcripts was similar to that of *PR-1*, except for a weaker induction of *PR-2* in cultivar Smoky in response to JA at 48 h.p.t.

3.2.2.3. LOX. *LOX* expressed similarly to *PR-2*, but its baseline expression was lower over time in both tested cultivars.

3.2.3. Soluble phenolics analysis

Soluble phenolics in saskatoon leaves detached at 0, 24 and 48 h.p.t. after treatment with sterile distilled water (SDW), JA or CMV were analyzed by HPLC (Figs. 6–8). Selected accumulated compounds have been tentatively identified (Table 2) based on their retention time, typical UV spectra, and comparison with commercial standards (hydroxycinnamic acids, flavanols and flavonols along with various derivatives and conjugates) separated under the same running conditions and included in our database. Co-elution of the samples with commercial standards was also conducted when necessary to confirm putative identifications. Both qualitative and quantitative differences were recorded between the two tested cultivars in response to the applied treatments in terms of flavan-3-ols, flavan-3,4-diols and polymerized proanthocyanidins (4.8% of the total; Fig. 6), flavonols (31.5% of the total; Fig. 7) as well as hydroxycinnamates (63.6% of the total; Fig. 8). For instance, proanthocyanidins P3 and P6 tentatively identified as epigallocatechin and procyanidin derivatives, respectively, showed differential accumulation patterns in cultivars Smoky and Martin in response JA and CMV, as compared to the non-treated controls (Fig. 6). The total content of proanthocyanidins (PAs) was similar between the two tested cultivars and only minor differences were recorded among treatments. In cultivar Smoky, increase in the accumulation of these compounds seemed to be similar in response to wounding and to the treatment with JA and CMV (Fig. 6). However, the overall trend was an increase in the accumulation of PAs in response to the treatments, especially 48 h.p.t. Flavonols' accumulation, on the other hand, was more responsive to the treatments specifically JA, at 24 and 48 h.p.t. in Smoky and Martin, respectively (Fig. 7). The major flavonols detected in the treatments include rutin (F3; Fig. 7), and several 3-*O*-oside derivatives of quercetin with sugar moieties being either xyloside, glucoside, or rutinoside (F1, 2, 4, 5, 6; Fig. 7) and kaempferol (F7, 9, 10; Fig. 7).

Hydroxycinnamates' accumulation varied among the two tested cultivars with higher amounts detected in Smoky (Fig. 8). Three major peaks H3, H8 and H12, tentatively identified as chlorogenic acid, caftaric acid and a non-identified hydroxycinnamic derivative exhibiting a λ_{max} at 327.8 nm, respectively, showed a differential pattern of accumulation. H3 and H12 highly accumulated in Smoky while H8 was highly induced in Martin (Fig. 8). Overall, hydroxycinnamates' content increased over time in response to the treatments as well as to wounding.

4. Discussion

The present study reports on several biochemical and molecular changes in saskatoon leaves in response to JA and CMV used as potential biological inducers of saskatoon's defenses against *E. mespili*. Due to the qualitative and quantitative variability of the inoculum in the field, we applied the treatments in the field and collected the leaves to further inoculate them *in vitro* (field experiments) and separately did both pre-treatment and inoculation on detached leaves in another set of experiments (*in vitro* experiments). In both cases, the wounding generated by cutting the leaves off the shrubs induced transcript accumulation of some of the tested defense genes, namely *PR-1* and *PR-2*, whereas it did not seem to affect *LOX* and *PR-5*. Our findings corroborate what was reported in other plant species in that *LOX* was induced in response to JA and to infection (Creelman and Mullet, 1997; Bate and Rothstein, 1998; Porta et al., 1999; Porta and Rocha-Sosa, 2002). However, *LOX* was not induced in response to wounding.

We also showed an activation of *LOX* 6 h.p.t. in response to CMV, suggesting that this plant's extracts trigger common pathways induced by JA. Therefore, it is likely that this defense-related gene is involved in saskatoon's defense responses against *E. mespili*, given its role in controlling the synthesis of a number of signaling (i.e., JA) and anti-microbial molecules (i.e., phytoalexins) and in the development of the hypersensitive response in many pathosystems (Creelman and Mullet, 1997; Bate and Rothstein, 1998; Weber et al., 1999; Rustérucci et al., 1999).

PR-1, *PR-2* and *PR-5* were responsive in saskatoon leaves to exogenous application of either JA or CMV, as early as 6 h.p.t.

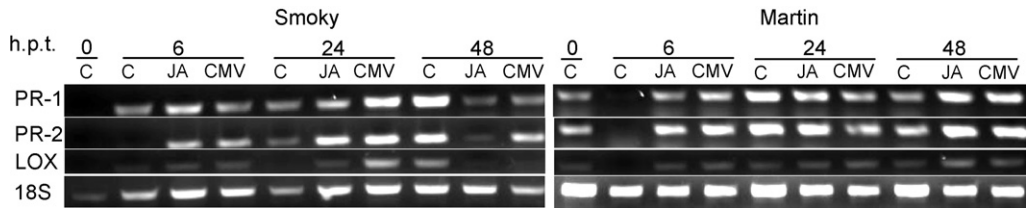

Fig. 5. Over time expression of several defense-related genes in two Saskatoon cultivars Smoky (highly susceptible) and Martin (moderately susceptible) in response to pre-treatment with water (control C), 10 μM jasmonic acid (JA) or 1/10 dilution in water of Canada milkvetch extract (CMV) under controlled conditions. The 18S ribosomal RNA gene was used as internal control. Results are representative of one of three independent replicates. h.p.t.: time after treatment.

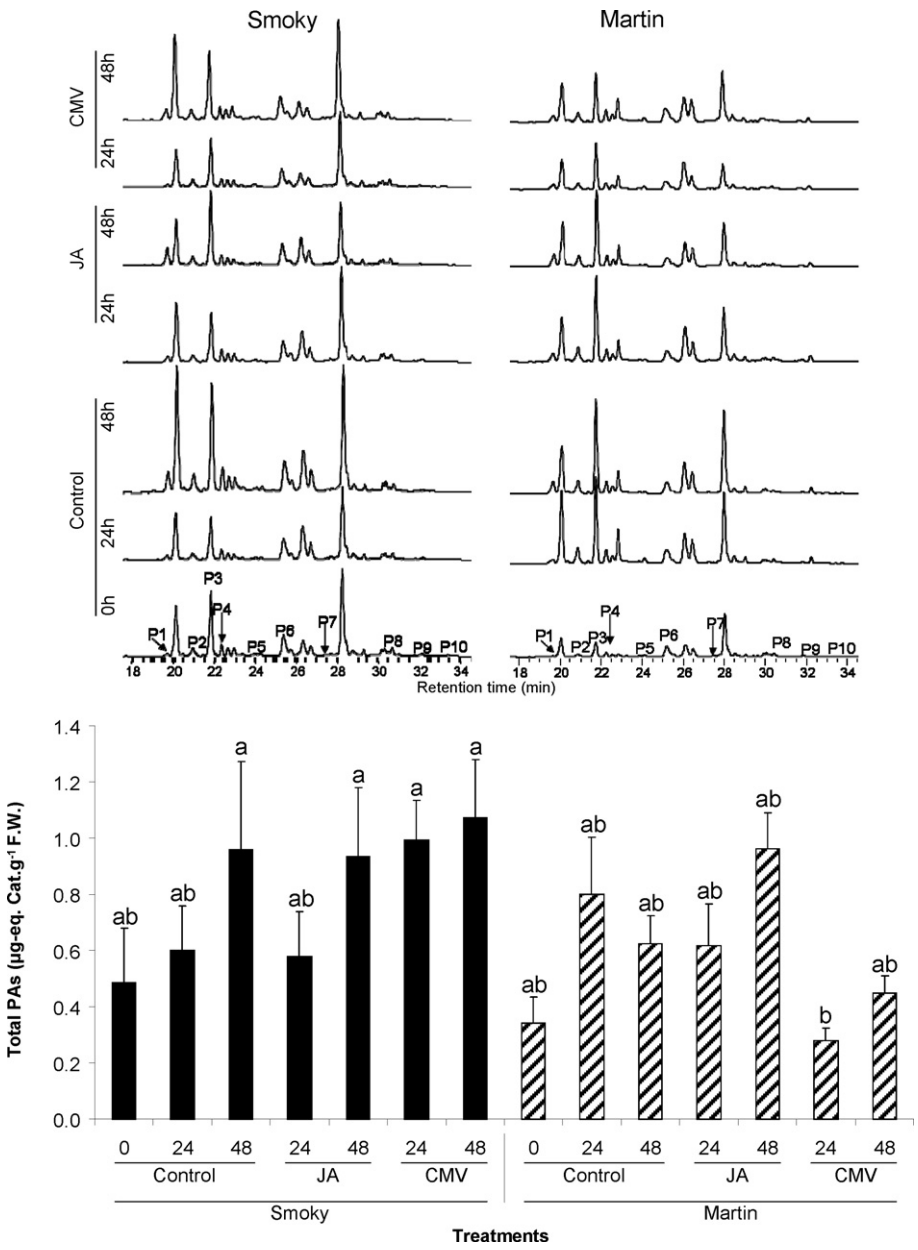

Fig. 6. HPLC chromatograms and histograms showing the levels of flavan-3-ols, flavan-3,4-diols and polymerized proanthocyanidins detected in saskatoon leaves, from field shrubs, of cultivars Smoky (highly susceptible) and Martin (moderately susceptible) after pre-treatment with water (control C), 10 μM jasmonic acid (JA) or 1/10 dilution in water of Canada milkvetch extract (CMV). Contents were calculated based on three independent replicates. Bars represent the standard deviation and do not differ significantly if assigned the same letter at $P < 0.05$.

for *PR-1* and *PR-2* and 24 h.p.t. for *PR-5*. These findings are in accordance with these PR genes being commonly activated in response to necrotrophic pathogens such as *E. mespili* (Kunkel and Brooks, 2002). They also concur with some of other findings in the potato-*Phytophthora infestans* pathosystem, where we showed a differential activation of these PR-proteins in two potato cultivars in response to two pathotypes of the oomycete (Wang et al., 2005, 2006; El Hadrami et al., 2006). JA and SA signaling pathways often show complex interactions involving either positive or negative interplay (Kunkel and Brooks, 2002), which may explain the late activation of *PR-1* in some of the tested treatments, especially the ones with JA.

The analysis of phenolics in saskatoon leaves grown in the field revealed qualitative and quantitative differences, between cultivars and among treatments, in response to JA, CMV, and infection by *E. mespili*. Like for the PR genes, part of the induction of these compounds was due to wounding. However, the majority of the compounds differentially accumulated in response to either JA or CMV. An effect of environmental conditions was also apparent, which may have minimized some of the differences that could have been ascribed to the treatments. Over 60% of the soluble phenolics' pool was deemed to be hydroxycinnamic derivatives including caffeic, caftaric and *p*-coumaric acids. Hydroxycinnamates are well known to be precursors of lignin synthesis (Dixon and Paiva, 1995; Lewis and Sarkanen, 1998; Hatfield et al., 2008) especially after infection with necrotrophs such as *E. mespili*. These findings confirm some of our earlier results on various pathosystems (Daayf et al., 1997; Ramos et al., 1997; El Hassni et al., 2007; Wang et al., 2008; El Hadrami et al., 2009; El Hadrami and Daayf, 2009). The remaining 40% of the soluble phenolics pool accounted for

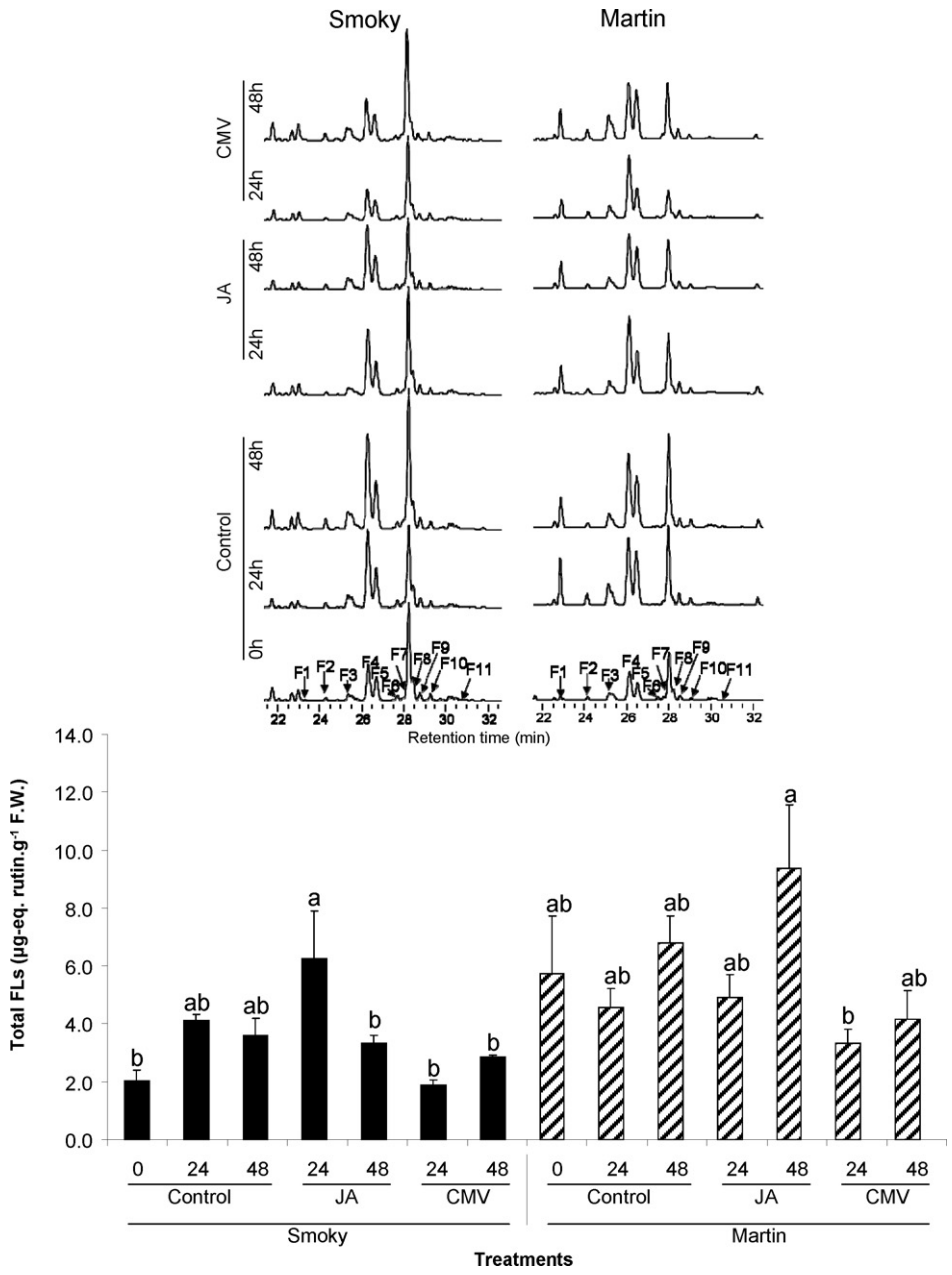

Fig. 7. HPLC chromatograms and histograms showing the levels of flavonols detected in saskatoon leaves, from field shrubs, of cultivars Smoky (highly susceptible) and Martin (moderately susceptible) after pre-treatment with water (control C), 10 μM jasmonic acid (JA) or 1/10 dilution in water of Canada milkvetch extract (CMV). Contents were calculated based on three independent replicates. Bars represent the standard deviation and do not differ significantly if assigned the same letter at $P < 0.05$.

flavonols (31.5%) and more or less polymerized proanthocyanidins/anthocyanins (4.8%). These two families of soluble phenolics are widespread in the plant kingdom and play an important role during plant pathogen interaction (Harborne, 1999; Macheïx et al., 1990, 2005). Saskatoons are phyllogenetically related to apples and the involvement of either flavonols or proanthocyanidins in the resistance to various pathogens, i.e., *Venturia inaequalis*, is well documented (Haslam, 1989; Mayr et al., 1997; Feucht and Treutter, 1999; Feucht et al., 2004; Treutter, 2005, 2006). Some of these compounds seem also to be important in the resistance of various legume species to a variety of pathogens and pests (Arfaoui et al., 2007; Dixon et al., 2005) and in potato to the soilborne pathogen *Verticillium dahliae* and the oomycete *P. infestans* (El Hadrami et al., 2006, 2008).

We used exogenous applications of JA to stimulate saskatoon leaves' defenses against *E. mespili* and showed the activation of *LOX* and *PAL*, as well as differential accumulation of soluble phenolics such as hydroxycinnamic acids and flavonols. To guarantee an infection instead of relying on the field inoculum, we used detached leaves, which did not allow us to carry the experiments over 48 h.p.t. to avoid any deterioration of the leaves ongoing a survival state. Culturing *E. mespili* to produce sufficient amounts of inoculum *in vitro* was not a straightforward task either. More studies should be devoted to producing spores of this pathogen *in vitro*. This would allow new studies on seedlings propagated under controlled conditions and inoculated with well characterized isolates of this pathogen. As hypothesized, the treatments used in the current study boosted both pre-existing and induced

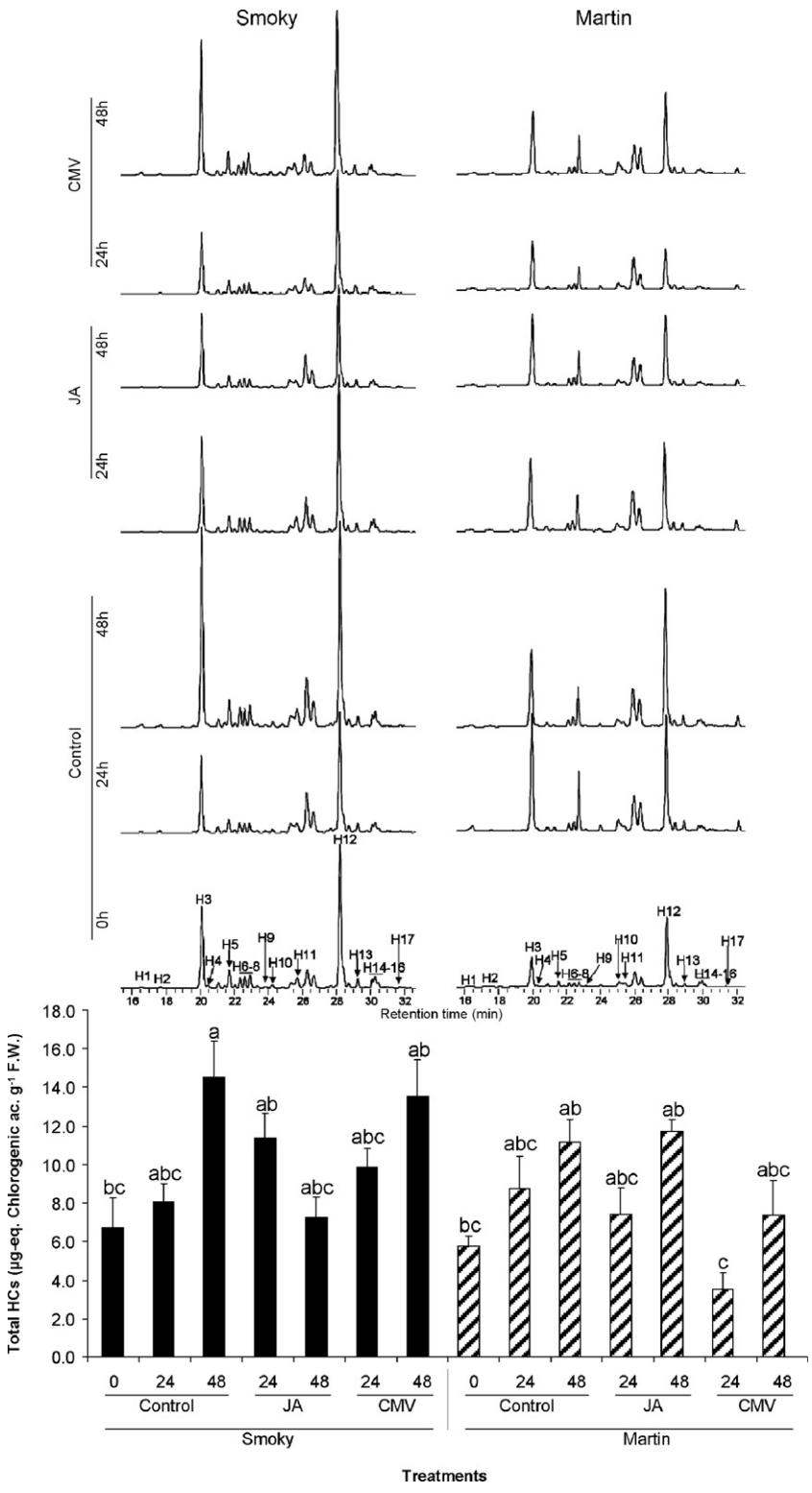

Fig. 8. HPLC chromatograms and histograms showing the levels of hydroxycinnamic acids detected in saskatoon leaves, from field shrubs, of cultivars Smoky (highly susceptible) and Martin (moderately susceptible) after pre-treatment with water (control C), 10 μM jasmonic acid (JA) or 1/10 dilution in water of Canada milkvetch extract (CMV). Contents were calculated based on three independent replicates. Bars represent the standard deviation and do not differ significantly if assigned the same letter at $P < 0.05$.

defense responses in Saskatoon tissues and therefore could be further considered as components of new integrated disease management strategies. Along with other resistance activators such as SA analogs, these treatments could be included in further analyses to advance our knowledge on induced resistance in this pathosystem and the possible cross-talks between SA and JA pathways. It will be also of interest to analyze the expression of genes encoding peroxidases and polyphenol oxidases and to follow the activities of their predominant isoforms that could play a role in the enhanced/induced resistance. A better understanding of

Table 2
List of soluble phenolics tentatively identified in saskatoon leaves of cultivars Smoky (highly susceptible) and Martin (moderately susceptible) pre-treatment with water (control C), 10 μM jasmonic acid (JA) or 1/10 dilution in water of Canada milkvetch extract (CMV).

Retention time (min)	Label	Maxima of absorbance (nm)	Family	Putative identification
19.81	P1	279.2	Flavan-3-ols, Flavan-3,4,-diols	(+)-Catechin
20.95	P2	279.2–330.2	Proanthocyanidins	(−)-Epicatechin
21.89	P3	279.2–327.8	Anthocyanins	Epigallocatechin derivative
22.41	P4	281.5		Cyanidin 3-*O*-galactoside
24.07	P5	280.4	Proanthocyanidins	Cyanidin 3-*O*-glucoside
25.35	P6	283.9–348.1		Cyanidin 3-*O*-arabinoside
27.44	P7	282.7		Polymerized proanthocyanidin 1
29.61	P8	281.5		Polymerized proanthocyanidin 2
30.66	P9	280.4		Polymerized proanthocyanidin 3
32.11	P10	276.8		Polymerized proanthocyanidin 4
32.49	P11	276.8		Polymerized proanthocyanidin 5
33.22	P12	279.2		Polymerized proanthocyanidin 6
16.59	H1	324.2	Hydroxycinnamates	3-*O*-Caffeoylquinic acid
17.69	H2	330.2		Cichoric acid
20.13	H3	325.4		Chlorogenic acid
20.46	H4	312.4		*Trans*-*p*-coumaric derivative 1
21.46	H5	314.7		*Trans*-*p*-coumaric derivative 2
22.35	H6	286.3-312.4		*Cis*-*p*-coumaric acid derivative 1
22.65	H7	327.8		n.d. hydoxycinnamic acid derivative 1
22.95	H8	329.0		Caftaric acid derivative
23.38	H9	307.6		*Trans*-*p*-coumaric acid derivative 3
24.25	H10	311.2		*Trans*-*p*-coumaric acid derivative 4
24.83	H11	331.4		Cichoric acid derivative
28.25	H12	327.8		n.d. hydoxycinnamic acid derivative 2
29.28	H13	327.8		n.d. hydoxycinnamic acid derivative 3
30.30	H14	315.9		*Trans*-*p*-coumaric derivative 5
30.99	H15	313.5		*Trans*-*p*-coumaric derivative 6
31.21	H16	319.5		*Trans*-*p*-coumaric derivative 7
31.93	H17	327.8		n.d. hydoxycinnamic acid derivative 4
23.03	F1	256.7–352.8	Flavonols	Quercetin 3-*O*-galactoside
24.20	F2	255.6–355.2		Quercetin 3-*O*-xyloside 1
25.38	F3	255.6–356.4		Rutin
26.17	F4	255.6–354.0		Quercetin 3-*O*-glucoside 1
26.55	F5	255.6–354.0		Quercetin 3-*O*-glucoside 2
27.58	F6	255.6–356.4		Quercetin 3-*O*-rutinoside
27.88	F7	265.0–356.4		Kaempferol derivative 1
28.33	F8	255.6–355.2		Quercetin 3-*O*-xyloside 2
28.66	F9	263.8–348.1		Kaempferol derivative 2
29.71	F10	265.0–348.1		Kaempferol derivative 3

the metabolic pathways involved in induced resistance as well as the interactions between the signaling networks will provide a basis for more sustainable management alternatives to control ELBS.

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