Priming of Antimicrobial Phenolics During Induced Resistance Response Towards *Pectobacterium carotovorum* in the Ornamental Monocot Calla Lily

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Calla lilies are herbaceous monocotyledonous plants that are highly sensitive to *Pectobacterium carotovorum*, the causal agent of soft-rot disease. Results demonstrate that, in response to elicitation using plant defense activators, the calla lily produces elevated levels of antimicrobial phenolics and that these compounds contribute to increased resistance against *P. carotovorum*, as shown by reduced bacterial proliferation in elicited leaves. The polyphenolic nature of the induced compounds was supported by autofluorescence, absorbance spectra, and reaction with Folin−Ciocalteu reagent. Two plant defense activators, Bion and methyl jasmonate, differed in both their capacity to induce accumulation of polyphenols and their resistance against the pathogen. Methyl jasmonate elicitation brought about higher accumulation of free phenolics relative to Bion, suggesting priming of bioactive polyphenols as a principal factor in the calla lily defense against *P. carotovorum*. To further characterize the nature of induced compounds, two major compounds were collected and identified as swertisin and isovitexin by mass and nuclear magnetic resonance spectroscopies.

INTRODUCTION

Calla lilies *(Zantedeschia* spp.) are herbaceous bulbous plants of high ornamental value that are confined to the African continent, extending from the Cape Province to the eastern coastal belt of southern Africa. The genus belongs to the monocotyledonous Araceae family (*1*) comprising eight species, all of which are common hosts of *Pectobacterium carotovorum* (previously *Erwinia carotovora* subsp. *carotovora*), the causal agent of soft-rot disease (*2*). Soft-rot enterobacteria are Gram-negative necrotrophs that cause plant tissue maceration, often resulting in loss of the whole plant (*3*). Despite their economical significance, especially in warm climates, these crops sustain substantial yearly losses because of affliction by the soft-rot pathogen *P. carotovorum*, with no effective control measures currently available (*2–4*).

The plant defense elicitor benzothiadiazole (BTH-Bion), known to act through the salicylic acid pathway and methyl jasmonate, acting through the jasmonic acid pathway was recently shown to elicit a defense response against *P. carotovorum* in the white calla lily (*5*). The results suggested that the defense response of the calla lily against *P. carotovorum* employs the jasmonate/ethylene signaling pathway for a durable protection. Jasmonate has been implicated as a signal molecule in wound response against feeding insects as well as in resistance against necrotrophic pathogens, including *P. carotovorum* (*6*). A recent review (*7*) clearly distinguishes between defense against biotrophic (feeding on live tissues) and necrotrophic pathogens. In the latter case, programmed cell death in the host tissue, regulated by salicylic acid-dependent pathways, is unlikely to limit the pathogen growth. The hypersensitive response (HR) leading to cell death, a common mechanism underlying plant defense response, is therefore hardly expected to overcome the necrotrophic *P. carotovorum*. This pathogen is known for its capability to survive in microaerobic or anaerobic environments, to progress intercellularly, and to produce high levels of exoenzymes, including pectinases, cellulases, and proteases (*3*). Although biotrophic pathogens may be halted by common defense mechanisms associated with cell death, such as oxidative burst, changes in cell wall composition, salicylic acid dependent synthesis of pathogenesis-related-proteins (PR), and local production of phytoalexins (*8, 9*), necrotrophs such as *P. carotovorum* or the fungus *Botrytis cinerea*, in fact, benefit from programmed cell death (*7, 10, 11*). Thus, such pathogens are restricted mainly by resistance mechanisms involving the jasmonate/ethylene signaling path-

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way. The latter often mediates changes in the production of plant bioactive secondary metabolites (*12–15*). Such compounds, characterized by antimicrobial activity in response to a pathogen or elicited by abiotic agents, fall into the definition of phytoalexins (*16, 17*). Although phytoalexins are highly diversified chemical compounds, plants of a given family generally use similar phytoalexins for defense (*17*). Plants of the Leguminosae, for example, produce isoflavonoid derivatives whereas members of the Solanaceae produce sesquiterpenes. The defense response of *Arabidopsis* against *P. carotovorum* involves mainly the jasmonic acid pathway, leading to production of phytoalexins from the biosynthetic pathway of tryptophan and, subsequently, indole glucosinolates, compounds shown to play a central role in the defense response (*6*).

The identified number of secondary metabolites induced via the jasmonate pathway in monocots is limited. Some compounds were identified in *Allium cepa* and *Iris pseudacorus*. More data is available on members of the Poaceae family such as *Avena sativa*, *Oryza sativa*, and *Zea mays*. The physiological condition in which plants are able to better or more rapidly mount defense responses such as the production of phytoalexins and/or other inducible genes (i.e. priming) has been reported mostly in dicotyledonous plants and only rarely in monocotyledons and gymnosperms (*18, 19*). Hence, a recent study showing different patterns of priming and eliciting activities during the defense response of monocots versus dicots is of particular significance (*20*). Taken together, current knowledge about the involvement and function of secondary metabolites or other mechanisms comprising the defense response of monocots other than grasses is rather limited (*21*). Therefore, we aimed to unravel the role of polyphenols and priming in the defense response of the ornamental monocot calla lily against the necrotrophic pathogen *P. carotovorum*. We show that methyl jasmonate elicitation, brought about higher accumulation of free phenolics relative to Bion treatment, suggests priming of bioactive polyphenols as a key factor in calla lily defense against *P. carotovorum*.

MATERIALS AND METHODS

Materials. HPLC grade solvents were purchased from BDH (Poole, UK). Bacterial growth mediums Luria–Bertani (LB) and nutrient-broth (NB) were purchased from Difco (Sparks, MD, USA). Other chemicals, including methyl jasmonate, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bion was kindly provided by Syngenta (Basel, Switzerland).

Bacterial Strains. *Pectobacterium carotovorum* was isolated from *Z. aethiopica* on crystal violet polypectate (CVP) at room temperature. The Pectolytic strain was restreaked onto LB medium, and single colonies were cultured and stored in 20% (v/v) glycerol at −80 °C.

The strain used here, *P. carotovorum*-13, was subjected to gas chromatographic analysis of fatty-acid methyl esters (GC-FAME) in the Israeli Plant Protection and Inspection Services (Volcani Center, Bet Dagan, Israel) for identification and then was confirmed as *P. carotovorum* using intergenic transcribed spacer polymerase chain reaction (ITS-PCR) analysis as described by Toth et al. (*22*).

The green fluorescent protein (GFP) expressing strain *P. carotovorum*-13+ was generated by introducing via electroporation plasmid pPROBE-AT containing a 131-base pair *npt*II promoter fragment from Tn5 fused to *gfp* and a replicon from pBR1 carrying ampicillin resistance (*23*).

Infection Experiments. Calla lily plants were grown in pots in the greenhouse for two seasons (25–10 °C maximum/minimum, natural daylight). Fully expanded young leaves were cut off and soaked in 0.5% sodium hypochlorite (20 min) for external disinfection, followed by a double wash in sterile distilled water. Leaf discs (20 mm in diameter) were then excised and placed on Petri dishes containing 20 mL of 50% Murashige and Skoog (MS) mineral sugar-free agar medium. Leaf discs were pierced at the center with a sterile tip and

inoculated with 10 μL of a fresh culture of 10^6 colony forming units (cfu) of *P. carotovorum* as described by Luzzatto et al. (*5*). Bacterial proliferation in calla lily leaf discs (cfu/g) was quantified by plating onto LB agar containing 100 μg/mL ampicillin.

Defense Elicitation. The youngest fully spread leaf was cut at the base of the petiole and was suspended for 24 h (25 °C) in 10 μg/mL Bion water solution. Methyl jasmonate 10 mM was applied as a leaf spray 24 h before inoculation. Methyl jasmonate was diluted 1:10 with cold ethanol (4 °C, v/v), and then rediluted 1:42 with cold double-distilled water (4 °C, v/v). Leaves were sprayed to the point of runoff.

Autofluorescence. Leaf discs were infected with *P. carotovorum* 13-GFP and were viewed 24 h post challenge inoculation using the LEICA MZFLIII binocular and LEICA DC 200 camera equipped with IM 1000 software. GFP was excited by 440–520 nm light, and the emission was detected through a 520–600 GFP$_2$ filter. For autofluorescence, excitation was obtained using a 320–400 nm filter, and emission detected through a 420 UV filter.

Extraction of Phenolics. Fresh foliar material of the youngest fully spread leaf from calla lily plants was cut at the base of the petiole and was used for each of the treatments. Six treatments were applied with double-distilled water serving as control: I$^-$Pc$^-$, I$^-$Pc$^+$, B$^+$Pc$^-$, M$^+$Pc$^-$, B$^+$Pc$^+$, M$^+$Pc$^+$ (I, Inducer; B, Bion; M, methyl jasmonate; *Pc*, *P. carotovorum*). Each treatment included a free-phenolic fraction, extracted prior to hydrolysis (FII), and a glycosylated (conjugated) fraction consisting of the aglycones released after acid hydrolysis (FIII). All experiments were carried out in duplicate. A modification of the extraction method of Daayf et al. (*24*) was used to determine free and glycosidic-bound phenolics in the leaf extracts.

Foliar material was ground to a fine powder in liquid N_2 and was extracted in 80% acidified methanol (20 g [fresh weight]/100 mL). The mixture was kept overnight in the dark, and air was replaced with nitrogen to prevent oxidation. The extract was filtered through GF/C glass fiber filters (Whatman, Maidstone, England), and the filtrate was concentrated under reduced pressure at 40 °C. The aqueous residue was adjusted to pH 2.0 and partitioned against hexane to remove lipophilic compounds, e.g., chlorophylls, carotenoids, lipids, and waxes (FI). Total phenolics were concentrated to 20 mL and were sampled for later analysis. The aqueous phase containing the phenolic constituents was further partitioned against ethyl acetate (FII) and then subjected to acid hydrolysis (4N HCl, v/v) in an autoclave (20 min at 121 °C). The hydrolysate was cooled and was partitioned against ethyl acetate (FIII). The two ethyl acetate fractions obtained (FII, FIII) were dried under reduced pressure, and the residues, designated as free-phenolic fraction (FII) and glycosylated (conjugated) phenolic fraction (FIII), respectively, were resuspended in absolute methanol (2 g/mL).

Antimicrobial Assay. The antimicrobial activity of leaf-extract was assayed in liquid medium using 96-well microtiter plates. Inhibition experiments were repeated three times, and the variation coefficients were smaller than 10% in all cases. Following extraction and removal of solvents, extracts were redissolved in methanol, 20 μL or graded volumes of 5 to 40 μL aliquots were added to microtiter wells and were dried under reduced pressure prior to inoculation with 90 μl of NB and *P. carotovorum* at log-phase growth. Bacteria were inoculated at a final concentration of 10^5 cfu/ml in a volume of 10 μl. Similar aliquots of methanol alone were added to wells, and the mixtures were dried to serve as control and to represent 100% proliferation. After 24 h of incubation in a shaker at 28 °C, growth was recorded by determining absorbance at 595 nm in an ultra microplate reader ELx 808 (BIO-TEK Industries, VT, USA).

Determination of Polyphenols. Total polyphenolics were determined by the Folin–Ciocalteu colorimetric method (*25*). The appropriate dilution of extracts was oxidized with the Folin–Ciocalteu reagent. The reaction was neutralized with Na_2CO_3 solution, and the absorbance was measured at 735 nm. Results were expressed on a fresh weight basis as mg of catechin equivalents (CE) (CE /1 g of FW or tissue). Experiments were repeated twice, and samples were analyzed in triplicates.

Reversed Phase Analytical Chromatography. The high-performance liquid chromatography (HPLC) system (Thermo Separation Products, San Jose, CA, USA) consisted of an AS3000 autosampler, a

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100 μL injector, a 30 °C column oven, a P3000 pump, a UV6000 diode array detector, and a 25 × 4.6 mm reverse-phase Luna2 C18 column (Phenomenex, USA). A linear gradient using water and methanol at a flow rate of 1 mL/min was used, following 2 min at 40% methanol, reaching 55% methanol in 58 min, and then kept at 90% methanol for an additional 2 min. Polyphenols were monitored at 270 nm.

Optimization of Chromatographic Conditions. The advanced chromatography modeling software DryLab was used to optimize the RPLC method developed previously for the simultaneous separation of short chain organic acids and polyphenols (*26*). Earlier works in our laboratory showed that the use of carboxylic acids or buffers in the mobile phase modifies, contributes to, or even diminishes, in some cases, the antimicrobial activity. Thus, elution was performed using water and methanol at a flow rate of 1 mL/min, with the first 2 min at 20% methanol in water, followed by a linear gradient reaching 60% methanol in water in 60 min, and a column temperature of 37 °C. The optimized method allowed resolutions higher than 1.5 (Rs ≥ 1.5) for at least 17 compounds.

LC-MS and LC-MS/MS analyses. LC-MS analyses were performed using a UPLC-qTOF instrument equipped with an electrospray ionization (ESI) source (Premier, Waters-Micromass, Milford, MA). LC separations were performed on a Waters UPLC Acquity instrument equipped with an Acquity 2996 PDA detector and a 100 × 2.1 mm i.d., 1.7-μm, reverse-phase UPLC BEH C18 column (Waters Acquity). The mobile phases consisted of 0.1% formic acid in methanol/water (5:95, v/v) (phase A) and 0.1% formic acid in acetonitrile (phase B), and the following gradient program was used: 0–28% B in 22 min, 28–40% B in 0.5 min, 40–100% B in 0.5 min, followed by 100% B for 1.5 min, then returned to the initial conditions (100% A) in 0.5 min, and conditioning at 100% A (1 min); the column was kept at 30 °C and the flow rate was 0.3 mL/min. LC-MS/MS runs with ESI positive-mode settings were as follows (100–1500 m/z ions): capillary spray at 3.0 kV; cone voltage at 30 eV; the collision energy was ramped from 3 to 35 eV; argon was used as a collision gas. Leucine enkephalin was used as the lock mass. MassLynx software version 4.0 was used to control all instruments and to calculate accurate mass.

Nuclear Magnetic Resonance (NMR). A Bruker Avance DRX-500 NMR spectrometer, operating at 500 MHz for ^{1}H and at 125 MHz for ^{13}C was used for the NMR experiments; chemical shifts are expressed in δ (parts per million) referring to the solvent peaks δ_H 3.34 and δ_X 49.0 for CD$_3$OD; coupling constants, J, are in Hz. ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, and HMBC NMR experiments were carried out using the conventional pulse sequences as described in the literature.

Component A. The peak at RT = 9.98 min, identified as isovitexin, was characterized by a m/z of MH$^+$ 433.1111 (molecular formula, C$_{21}$O$_{10}$H$_{21}$) and [M − H]$^-$ m/z 431.0984 (molecular formula, C$_{21}$O$_{10}$H$_{19}$). Fragmentation of the glycone part in the MH$^+$ ion fits well with the fragmentation pattern of C-glycosides. ^{1}H- NMR parameters and splitting pattern: aglycone part, H(2′) (δ_H = 6.94, d, J(2′, 3′) = 8.5 Hz), H(3′) (δ_H = 7.86, d, J(2′, 3′) = 8.5 Hz), H(3) (δ_H = 6.63, s), H(8) (δ_H = 6.53, s); glycone part, H(1″) (δ_H = 4.89, d, J(1″, 2″) = 9.9 Hz), H(2″) (δ_H= 4.43, m), H(3″) (δ_H = 3.43, m), H(4″) (δ_H = 4.27, m), H(5″) (δ_H = 3.42, m), CH$_2$(6″) (δ_H = 3.53, m, and δ_H = 3.61). ^{13}C NMR chemical shifts: aglycone part, C(2) (δ_C = 164.8), C(3) (δ_C = 102.4), C(4) (δ_C = 182.6), C(5), (δ_C = 163.7), C(7) (δ_C = 164.8), C(6) (δ_C = 107.8), C(8) (δ_C = 93.8), and C(9) (δ_C = 157.5), C(10) (δ_C = 103.7), C(2′) (δ_C = 115.64), C(3′) (δ_C = 128.0), C(4′) (δ_C = 121.7); glycone part, C(1″) (δ_C = 73.8), C(2″) (δ_C = 70.89), C(3″) (δ_C = 79.0), C(4″) (δ_C = 62.9), C(5″) (δ_C = 81.2), C(6″) (δ_C = 61.43).

Component B. The peak at RT = 10.63 min, identified as swertisin, was characterized by a m/z of MH$^+$ 447.1271 (molecular formula, C$_{22}$O$_{10}$H$_{23}$) and [M−H]$^-$ m/z 445.1129 (molecular formula, C$_{22}$O$_{10}$H$_{21}$). Fragmentation of the glycone part in the MH$^+$ ion fits well with the fragmentation pattern of C-glycosides; the loss of different number of water molecules (from 1 to 4) is observed (*27*). ^{1}H NMR parameters and splitting patterns: aglycone part, H(2′) (δ_H = 6.95, d, J(2′, 3′) = 8.5 Hz), H(3′) (δ_H = 7.92, d, J(2′, 3′) = 8.5 Hz), H(3) (δ_H = 6.69, s), H(8) (split into 3 components with δ_H = 6.79, 6.78, 6.77 s), H(CH3) (δ_H = 3.85, s); glycone part, H(1″) (δ_H = 4.95, d, J(1″, 2″) = 9.9 Hz), H(2″) (δ_H = 4.2), H(3″) (δ_H = 3.51, m), H(4″) (δ_H = 4.02, m),
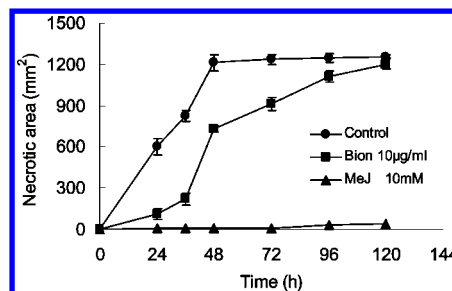

Figure 1. Time-dependent development of disease symptoms on calla lily leaf discs. Values represent average of total necrotic area (mm^2) calculated from 20 leaf discs for each time point ± standard errors.

H(5″) (δ_H = 3.48, m), CH$_2$(6″) (δ_H = 3.73, m, and δ_H = 3.87). ^{13}C NMR chemical shifts: aglycone part, C(2) (δ_C = 165.0), C(3) (δ_C = 102.7), C(4) (δ_C = 182.6), C(5), (δ_C = 163.7), C(7) (δ_C = 165.0), C(6) (δ_C = 107.8), C(8) (δ_C = 89.8), and C(9) (δ_C = 157.5), C(10) (δ_C = 103.7), C(2′) (δ_C = 115.64), C(3′) (δ_C = 128.0), C(4′) (δ_C = 121.7); glycone part, C(1″) (δ_C = 73.8), C(2″) (δ_C = 70.89), C(3″) (δ_C = 79.0), C(4″) (δ_C = 62.9), C(5″) (δ_C = 81.2), C(6″) (δ_C = 61.84).

Statistical analysis. Statistical comparisons were made using one-way analysis of variance (ANOVA) by PRISM 3.02 software (Graph-Pad, San Diego, CA). Where ANOVA yielded significant results ($P <$ 0.05), post hoc analysis was performed using Tukey−Kramer's multiple comparison test. Data presented are means ± SEM. Elicitation experiments were carried out in two independent experiments, using 20 leaf discs for each elicitation treatment. The leaf discs were excised from at least four different plants.

RESULTS AND DISCUSSION

Reduction in Disease Symptoms Following Application of Plant Defense Activators. *P. carotovorum* was inoculated onto leaf discs 24 h post elicitation with either Bion (10 μg/mL) or methyl jasmonate (10 mM). Soft-rot disease, expressed as the necrotic area developed on leaf discs, was recorded 24–120 h post inoculation (**Figure 1**). With both plant defense activators, disease symptoms were reduced during the first 24 h. However, at 48 h only induction with methyl jasmonate completely inhibited bacterial development, whereas a rapid bacterial development was observed in leaf discs induced with Bion. At 120 h the disease was still halted in the methyl jasmonate treatment, whereas the effect of Bion on development of disease symptoms was lost.

The effects of elicitation and of subsequent challenge with *P. carotovorum* on the plant were also assessed, using the intrinsic fluorescence of polyphenols to evaluate the levels of polyphenols in calla lily leaves. This feature allows direct visualization under an epifluorescence microscope using a Ultraviolet (UV) filter (*28*). A large difference in tissue autoflourescence was recorded, following elicitation with either Bion or methyl jasmonate (**Figure 2**, panels **a**−**c**). Challenge inoculation with *P. carotovorum* following treatment with Bion induced the accumulation of polyphenols mainly around the infection area, with gradual decrease toward the periphery. Challenge inoculation with *P. carotovorum* following treatment with methyl jasmonate induced the accumulation of polyphenols in the whole tissue (**Figure 2**, panels **d**−**f**). The elicitation effect was dose-dependent for both plant defense activators. Jasmonate activity in plant defense has long been coupled with accumulation of secondary metabolites in plants (*6, 14, 15*). Indeed, the assessment of autoflorescence of calla lily leaf discs indicated a different mode of action for the two elicitors: Whereas Bion induced a relatively weak, localized response, methyl jasmonate induced a strong response that was further augmented following challenge with *P. carotovorum*. This suggested that methyl
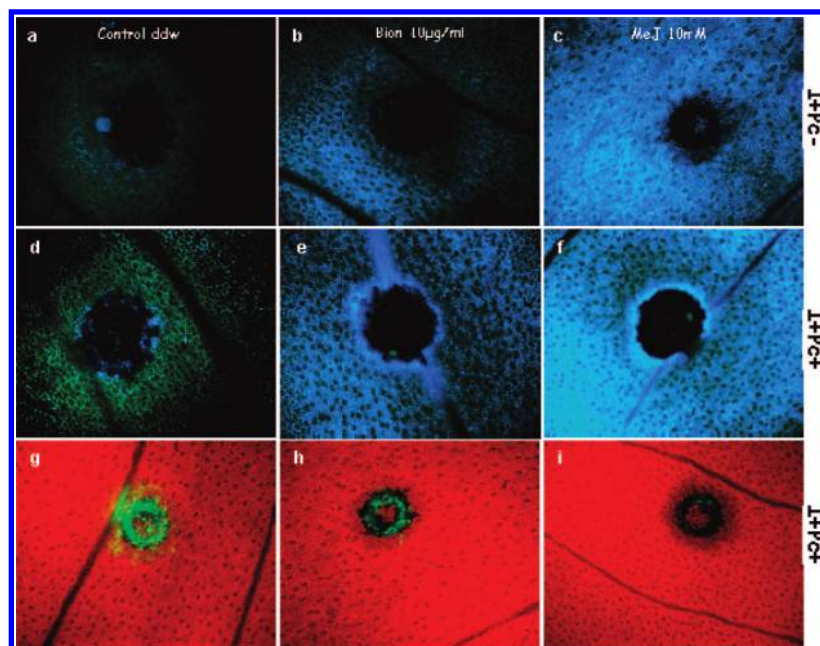
Figure 2. Autofluorescence of polyphenols under UV light of calla lily leaf discs. Elicitation with double-distilled water (control), Bion, applied as a water drench, or methyl jasmonate, applied as leaf spray, panels **a**−**c**, respectively. Fluorescence rings around the inoculation sites in leaf discs elicited as above and challenged with *Pectobacterium carotovorum* (*Pc*), 24 h post challenge, panels **d**−**f**. Disease dispersal recorded by fluorescence microscopy 24 h post challenge with *P. carotovorum*. Images show *P. carotovorum* containing pPROBE-At *gfp* plasmid spreading around the inoculation site, panels **g**−**i**.

jasmonate primes calla lily plants to respond more effectively to subsequent inoculation with *P. carotovorum*.

The *gfp*-expressing *P. carotovorum* allowed a visual assessment of bacterial proliferation in leaf discs during the early stages of the infection (20 h post inoculation). Rapid proliferation and progression of bacterial cells into the leaf tissue surrounding the inoculation point was observed in control, water-treated discs. In leaf discs elicited with the plant defense activators, not only was proliferation inhibited, but bacteria were also limited to the infection site (**Figure 2**, panels **e**−**g**). In line with the above-described results, methyl jasmonate was more effective than Bion in limiting bacterial proliferation and survival, showing reduction of more than 2 orders of magnitude in counts of bacterial cells, as compared with 1 order of reduction in the Bion treatment. These results support previous reports that have demonstrated the potential of jasmonate in activating the defense response in the model plant *Arabidopsis* and in tomato. These works have shown activation of the defense response against various necrotrophs, including the fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola* and the bacterium *P. carotovorum* (*29, 30*). In *Arabidopsis*, signaling by jasmonate was shown to be specifically linked with production of its major phytoalexin, camalexin (*6, 7, 29*), and with the plant's resistance to *P. carotovorum* (*6, 31–33*).

Accumulation of Phenolics in Leaves Induced with Plant Defense Activators. Total phenolics, expressed as catechin equivalents (mg) per fresh leaf tissue (g), were analyzed in crude extract of phenolics and in fractions of "free phenolics" (aglycones), which were partitioned against ethyl acetate, and of "conjugated phenolics" (glycosylated), which were acid hydrolyzed and then partitioned against ethyl acetate (**Figure 3**). Similar levels of total phenolics were found in the control and Bion-elicited plants, whereas a 30% increase was recorded in plants treated with methyl jasmonate. When determining the free phenolics, an increase was observed following induction treatments using either Bion or methyl jasmonate as elicitors [i.e., inducers (B$^+$Pc$^-$, M$^+$Pc$^-$)]. Induction was also found in
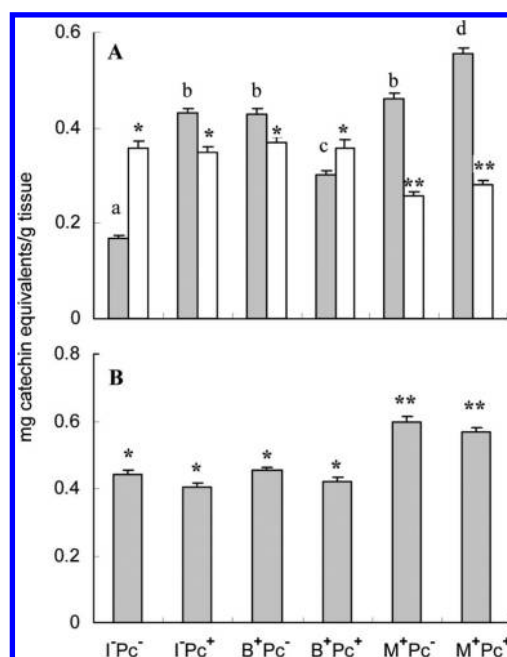

Figure 3. Free and glycosylated (acid hydrolyzed) phenolics expressed as mg of catechin equivalents per gram of leaf tissue following the indicated treatments; grey bars represent free phenolics, and white bars represent glycosylated phenolics (**A**). Total phenolic content expressed as above (**B**). Inducer (I), *Pectobacterium carotovorum* (*Pc*), Bion (B), and methyl jasmonate (M). Values represent the average of two independent assays carried out in triplicate plus standard errors. Values followed by the same letter or symbol are not significantly different at *P* < 0.01.

the levels of free phenolics following the application of the pathogen alone (I$^-$Pc$^+$) or combinations of pathogen and elicitor (B$^+$Pc$^+$ and M$^+$Pc$^+$). In the induced and challenged leaves, Bion elicitation (B$^+$ Pc$^+$) caused a 2-fold increase in free phenolics concentration, whereas methyl jasmonate elicitation (M$^+$Pc$^+$) enhanced the level of free phenolics 3.5-fold, or 75%

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more than Bion elicitation. The increase in free phenolics following methyl jasmonate elicitation was further augmented by challenge with the pathogen, unlike Bion, where the inducer or *P. carotovorum* alone elicited a stronger plant reaction than the combined Bion treatment and challenge with *P. caroto-vorum.* These results suggest priming of the plant's free phenolics by methyl jasmonate (*19*).

Glycosylated phenolics (acid hydrolyzed fraction) sustained comparable levels in control and Bion-elicited plants, whereas a 30% reduction was recorded following methyl jasmonate elicitation. These results indicate that glycosylated phenolics (aqueous fraction) may serve as a reservoir for the augmentation of free phenolics in methyl jasmonate-induced plants, at least during the first 24 h post challenge inoculation. The Folin–Ciocalteu reagent reacts with reducing phenols to form chromogens that can be detected spectrophotometrically, and all results are presented as catechin equivalents. Thus, only the quantities of phenolics in extracts that were produced identically can be compared. Indeed, total phenolics measured prior to fractionation do not add up to simple totaling of the glycosylated and free fractions.

Antimicrobial Activity against *P. carotovorum* of Phenolics Extracted from Induced Leaves. Previous works have demonstrated that induction of resistance in plants was followed by production of phenolics with antimicrobial activity in leaves (*24, 34*). Higher antimicrobial activity of calla lily phenolics may explain, at least in part, the enhanced protection toward *P. carotovorum*. Here, six phenolic extracts from the following treatments, I^-Pc^-, I^-Pc^+, B^+Pc^-, M^+Pc^-, B^+Pc^+, and M^+Pc^+, were obtained and then tested against fresh *P. caro-tovorum* cultures. Free phenolics extracted from Bion-treated leaves were slightly, not significantly, more active in the induced challenged leaves (B^+Pc^+) than in all control treatments (I^-Pc^-, B^+Pc^-, and I^-Pc^+). Free phenolics from leaves harvested at the same time point, but induced with methyl jasmonate, showed a minor increase in the antimicrobial activity (M^+Pc^- treatment) and a large increase in activity following challenge with the pathogen (M^+Pc^+) (**Figure 4A**).

In vitro bioassays of the conjugated phenolics (glycosylated and acid hydrolyzed) generally demonstrated higher levels of antimicrobial activities than the free phenolics fraction. However, a minor reduction (nonsignificant) in the antimicrobial activity of the glycosylated phenolics induced by methyl jasmonate was observed (not shown). These results suggest that the conjugated phenolics may have served as a source for the augmented antimicrobial activity observed in the methyl jas-monate-induced and pathogen-challenged leaves. Glycosylation of phenolics in plants may better protect them from either enzymatic or spontaneous oxidation (*35*), and it could thus serve as a reservoir of antimicrobial compounds ready for the time of attack; by contrast, free phenolics are the active form for immediate antimicrobial activity. These results are in agreement with studies showing the involvement of phytoalexins in the defense response of cucumber, where glycosylated phenolics (following hydrolysis) were considerably more active than free phenolics against fungal and bacterial pathogens (*24, 34*).

To better define the induction of the antimicrobial activity process, we assayed the effect of adding increased levels of phenolics (**Figure 4B**). Accordingly, free phenolics were extracted and fractionated from plants induced with methyl jasmonate and challenged with bacteria (I^-Pc^-, I^-Pc^+, M^+Pc^-, and M^+Pc^+). The phenolics produced were then applied on growing bacteria using four concentrations of each. The results revealed a 2-fold reduction in the minimal inhibitory concentra-
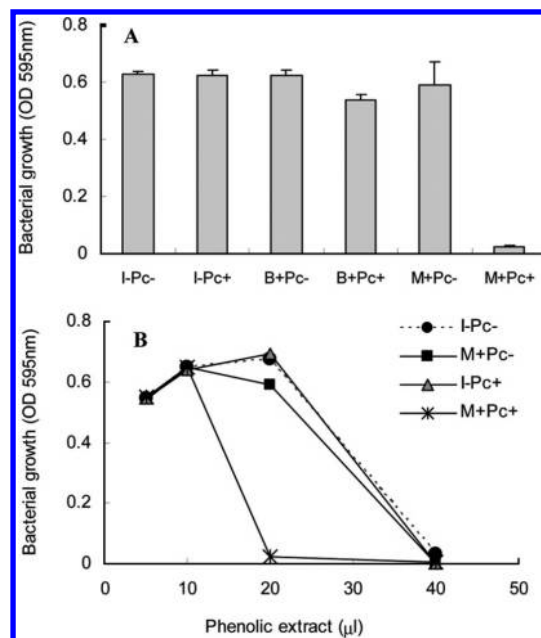

Figure 4. Antimicrobial activity of the free phenolics fraction extracted from calla lily leaves treated as indicated (**A**). Inducer (I), *Pectobacterium carotovorum* (*Pc*), Bion (B), and methyl jasmonate (M). Bars represent bacterial growth in the presence of 20 µl of free phenolics extract plus standard deviations. Bacterial growth in the presence of increasing concentrations of free phenolics extract from control (I-*Pc*-), noninduced challenged plants (I-*Pc*+), and methyl jasmonate induced (M+) and *Pc* challeged plants (*Pc*+) (**B**).

tion (MIC) required for complete inhibition of bacterial growth using the extract from methyl jasmonate-induced, *P. caroto-vorum*-challenged (M^+Pc^+) leaves. The extract from nonchal-lenged, induced leaves was only slightly more active (not significantly) than the control.

The augmented production of antimicrobial compounds found only following challenge with the pathogen is a clear manifestation of priming (*19*). The results describe potentiation of in vitro antimicrobial activity of the methyl jasmonate–challenged leaves that correlates well with the data obtained through assessment of disease symptoms and bacterial proliferation measured in vivo. The suggested conversion of the glycosylated phenolics into free active forms was further supported by the quantitative phenolics assay, demonstrating an increase in the free phenolics content simultaneously with reduction in the glycosylated forms, following methyl jasmonate treatments only. The moderate increase in total phenolics concentration in the induced challenged plants did not appear to reflect the great increase in antimicrobial activity. This may be because of limitations of the Folin–Ciocalteu method to quantify glyco-sylated phenolics in a sample that contains both free and glycosylated forms (*35*). Alternatively, antimicrobial activity may result from an increase in specific active antimicrobial compounds. Augmentation of antimicrobial activity of the free phenolics fraction (FII) was not observed in any of the other three treatments [i.e., control (I^-Pc^-), methyl jasmonate treatment (M^+Pc^-), or *P. carotovorum* inoculation (I^-Pc^+)]. The increased antimicrobial activity corresponding with 40 mg of fresh plant material (e.g., 20 µL of the crude extract) shown in the methyl jasmonate–induced challenged leaves (M^+Pc^+) may as well be associated with priming (*36*). This enhanced antimicrobial activity was not observed in any of the Bion treatments and was ten times higher than that required for antifungal activity in cucumber leaves (*24, 37*).
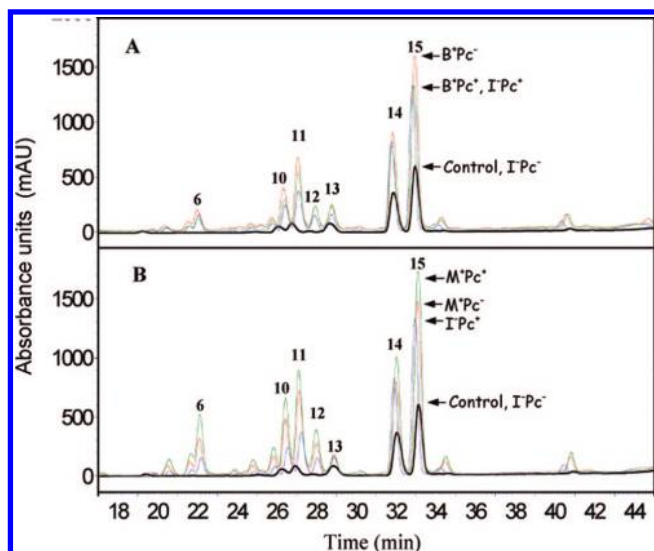
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Figure 5. Priming of free phenolics in calla lily leaves following induction with (**A**) Bion or (**B**) methyl jasmonate and challenged with *Pectobacterium carotovorum* (*Pc*). Control treatment, inducer = I, *P. carotovorum* = *Pc* (**black**, I^-Pc^-); induction treatments with Bion or methyl jasmonate, Bion = B, methyl jasmonate = M (**red**, B^+/M^+Pc^-); challenge inoculation with *P. carotovorum* (**blue**, I^-Pc^+); induction followed by challenge inoculation with *P. carotovorum* (**green**, B^+/M^+Pc^+).

In calla lily, unlike cucumber, the potentiated state following challenge with the pathogen was demonstrated in the free phenolics fraction (*24*). This trend is similar to the pattern of *p*-coumaric acid methyl ester, shown to increase over time in the free phenolics fraction, in the cucumber model, suggesting a conversion in planta from the glycosylated to the free active form over time (*24*).

RP-LC Profiling of Polyphenolics from Calla Lily Leaves Induced with Bion or Methyl Jasmonate and Challenged with *P. carotovorum*. Increased levels of resolved polyphenolics in calla lily leaves, 48 h post induction with either Bion or methyl jasmonate and 24 h post challenge with *P. carotovorum*, were obtained in comparison to a noninduced control (**Figure 5**). The compounds were compared and analyzed by their retention time and absorbance spectra for relative peak area. The increased levels were in accord with the above-described results. **Figure 5A** demonstrates the profile of phenolics in leaves treated with Bion (10 μg/mL). All three positive treatments [i.e. Bion (I^+Pc^-), *P. carotovorum* (I^-Pc^+), or Bion followed by challenge with *P. carotovorum* (I^+Pc^+)] resulted in higher levels of phenolics as compared to control leaves treated with water (I^-Pc^-). The most significant increase in amounts of all compounds was observed following the Bion-alone treatment (I^+Pc^-). The induced and challenged leaves (I^+Pc^+) produced lower levels of phenolics than the treatments with Bion (I^+Pc^-) or *P. carotovorum* (I^-Pc^+) alone. In general, Bion, *P. carotovorum*, or Bion and *P. carotovorum* induced comparable levels of plant UV–visible compounds with phenolic nature.

An increase in the levels of phenolics was also observed following elicitation with 10 mM methyl jasmonate (**Figure 5B**). Unlike elicitation with Bion, elicitation with methyl jasmonate resulted in a different pattern of plant elicitation. All three treatments involving this elicitor (I^+Pc^-, I^-Pc^+, and I^+Pc^+) were higher than control, similarly to Bion, although the two elicitors differed distinctly. Methyl jasmonate alone (I^+Pc^-) induced higher production of UV- visible compounds as compared to the pathogen alone (I^-Pc^+), and these were further
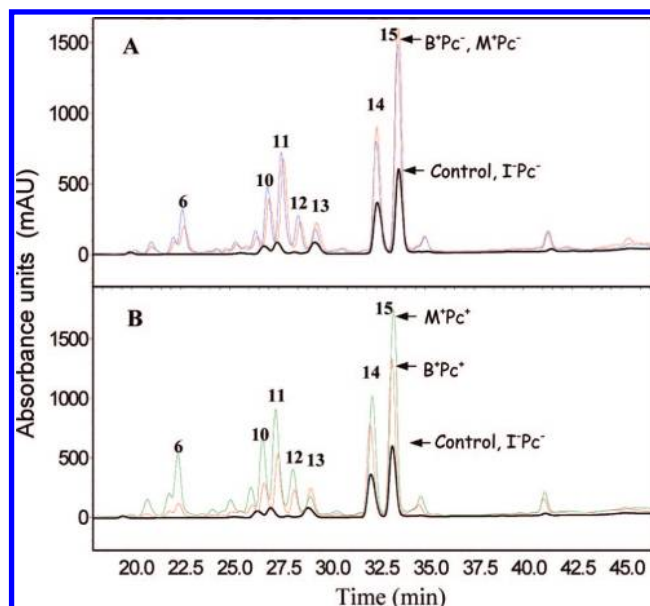

Figure 6. A comparison of free phenolics profiles induced in calla lily leaves following Bion or methyl jasmonate treatments (**A**): control treatment (black), Bion induction (red), methyl jasmonate (blue), and without challenge inoculation with *Pectobacterium carotovorum* (*Pc*). Free phenolics profiles induced in calla lily leaves following induction with Bion (red) or methyl jasmonate (green) followed by challenge inoculation with *P. carotovorum* (**B**).

augmented by challenge inoculation with the pathogen (I^+Pc^+). The *P. carotovorum* challenge increased the accumulation of phenolic compounds significantly. This augmented induction of phenolics following *P. carotovorum* challenge demonstrated priming of calla lily phenolics only in the methyl jasmonate pathway. To better characterize the differences in the mode of action of Bion versus methyl jasmonate during the defense response of calla lily, we compared the levels of leaf phenolics following induction with and without a *P. carotovorum* challenge (**Figure 6**). The comparison demonstrated similar levels of UV–visible compounds following elicitation with any of the inducers (**Figure 6A**). Subsequent to challenge with the pathogen, a significantly stronger response was recorded following methyl jasmonate treatment, indicating potentiation of phenolics associated with this pathway. This was further confirmed by peak area analysis (not shown) of all detectable peaks in the methyl jasmonate treatment versus the Bion treatment, showing an increase of more than 400% for several compounds. These results support priming as a component in the defense mechanism, which is induced by the methyl jasmonate pathway similar to induction with nonpathogenic rhizobacteria, salicylic acid, or beta-aminobutyric-acid (*19, 36*).

Characterization of Induced Molecules. To complete the identification of the induced molecules we selected the compounds 14 and 15 from the free phenolics (FII) fraction (**Figure 5**), which share similar UV absorbing spectra. Using the analytical HPLC system to separate and collect material for structural analysis, these compounds were initially pooled together. We then turned to UPLC-MS and UPLC-MS/MS to completely separate the pooled compounds. Indeed, using UPLC allowed the isolation and analysis of three compounds, by MS and MS/MS in positive and negative electrospray ionization (ESI) modes, major component A at RT 9.98 min, major component B at RT 10.63 min, and a minor component C at RT 11.27 min.
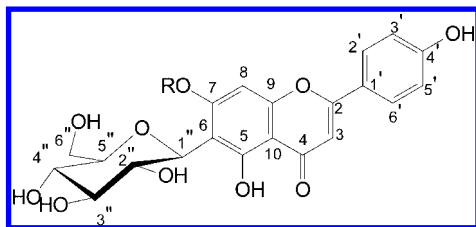
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Figure 7. Chemical structures of components A (R = H, isovitexin) and B (R = CH3, swertisin).

The product ion mass spectra of flavonoid-*O*-glycosides usually have the characteristic loss of a neutral carbohydrate moiety from protonated positive parent MH^+ ions, as well as from negative $[M - H]^-$ ions ([27]). The MS/MS spectra of some protonated flavonoid-*C*-glycosides, on the other hand, exhibit substantial fragmentation of glycone where the loss of different numbers of water molecules (from 1 to 4) is usually observed ([27]) ([38]). ESI MS and MS/MS spectra accurate mass measurements suggest the compounds are flavonoid-*C*-monoglycosides. The product ion spectra of MH^+ 433.1 (RT 9.98 min, compound A) and MH^+ 447.1 (RT 10.63 min, compound B) show extensive fragmentation of glycone. The mass difference between these two molecular ions is 14.016 Da, which corresponds to a CH_2 group (calculated 14.0156, error 0.35 mDa). The MS/MS spectrum of the MH^+ 447.1 ion (RT 11.27 min) shows very weak fragmentation of the parent ion. The absence of carbohydrate loss in the MS/MS spectrum of the MH^+ 447.1 ion (RT 11.27 min) supports our assumption that components A, B, and C are flavonoid-*C*-monoglycosides.

The pooled mixture was than analyzed by NMR spectroscopy; ^{1}H- NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, and HMBC spectra were obtained. According to the ^{1}H- NMR spectrum, there were indeed two major components (A and B) in this fraction showing a high degree of agreement in their NMR characteristics, and a minor component (C). The relative ratio of these compounds may be estimated as A/B/C = 4.3/7.4/1. The ^{1}H NMR parameters and splitting patterns, as well as ^{13}C NMR chemical shifts for the two major components A and B are presented in the experimental part. The assignment of the signals is based on the analysis of ^{1}H- NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, and HMBC spectra. Comparison of our data with the NMR parameters of mono-*C*-glycosides of different flavonoids shows that component A may be identified as the mono-6-*C*-glucoside of apigenin (isovitexin) and component B as the mono-6-*C*-glucoside of 7-*O*-methylated apigenin (swertisin) ([39–41]) (**Figure 7**). According to the literature data, swertisin exists in solutions as a mixture of rotamers ([40]). Our data support this point: the signal from H(8) is split into 3 components.

In conclusion, bacterial soft-rot caused by *P. carotovorum* is one of the most devastating diseases of colored and white-flowered calla lilies. The plant defense activators Bion and methyl jasmonate were tested for their potential to induce polyphenolic compounds as part of a defense mechanism against this necrotroph bacterium in the plant. Results provided the first conclusive evidence that a monocot geophyte plant produces elevated levels of phytoalexins in response to elicitation treatment and that these compounds contribute to increased resistance against *P. carotovorum*. An increase in antimicrobial phenolics was demonstrated concomitantly with the development of resistance, as evaluated by reduced disease symptoms and bacterial proliferation in elicited leaves.

The plant reaction to elicitation by the two elicitors, Bion, acting through the salicylic acid pathway, and methyl jasmonate,

involving the jasmonate-dependent signaling pathway, differed in the level of induced resistance against *P. carotovorum* and in the induced accumulation of phenolics. First, whereas Bion was efficient only for a short term, methyl jasmonate afforded several days of protection against the bacteria. Second, Bion enhanced polyphenolics accumulation, but unlike methyl jasmonate, this enhancement was not further augmented following challenge with *P. carotovorum*. Finally, increased antimicrobial activity following challenge with *P. carotovorum* was evident only in response to methyl jasmonate elicitation.

In line with these findings, data presented here depict a role for jasmonate elicitation in calla lily defense against *P. carotovorum*, simultaneous with increased production of phenolics and antimicrobial activity. Priming as a constituent of defense response in monocot plants has been shown only recently in potentiation of suspension-cultures of wheat and rice by application of exopolysaccharides derived from *Pantoea agglomerans* ([20, 42]). Here, we demonstrate that priming also plays a role in the defense response of a non-Poaceae monocot plant, the ornamental calla lily. Taken together, priming of bioactive flavonoids polyphenols elicited via the jasmonic acid pathway is suggested as a major constituent of the defense response of calla lily against *P. carotovorum*.

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