Biocontrol Treatments Confer Protection Against *Verticillium dahliae* Infection of Potato by Inducing Antimicrobial Metabolites

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Verticillium wilt, caused by *Verticillium dahliae* Kleb., is a serious potato (*Solanum tuberosum* L.) disease worldwide, and biocontrol represents a promising eco-friendly strategy to reduce its impact. We used extracts from Canada milk vetch (CMV) and a set of four *V. dahliae*–antagonistic bacterial strains to coat potato seeds at planting and examined the degree of protection provided against *V. dahliae* as well as accumulation of soluble phenolics as markers for induced resistance. All tested treatments were effective in reducing disease severity, and CMV showed the highest level of protection. In this treatment, flavonol-glycoside rutin was a highly abundant compound induced in potato tissues, with levels two to three times higher than those detected in non-inoculated controls and *V. dahliae*–inoculated plants. We investigated dose-dependent effects of rutin on *V. dahliae* growth and sporulation in vitro and in planta. The effect of rutin on mycelial growth was inconsistent between disk assay and amended medium experiments. On the other hand, significant reduction of *V. dahliae* sporulation in vitro was consistently observed starting at 300 and 100 µM for isolates Vd-9 and Vd-21, respectively. We successfully detected 2-protocatechuoylphloroglucinolcarboxylic acid (2-PCPGCA) using ultra-performance liquid chromatography tandem mass spectrometry, indicating that *V. dahliae* dioxygenally oxidizes quercetin. Quercetin, as an aglycone, is freed from the sugar moiety by glucosidases and rhamnosidases produced by the fungus and is a substrate for quercetinases. The occurrence of quercetinases in *V. dahliae* provides a background to formulate a hypothesis about how by-product 2-PCPGCA may be interfering with potato defenses.

Verticillium dahliae Kleb. is a serious soilborne fungal pathogen causing disease in many crops worldwide (Alkher et al. 2009) and increasingly emerging as a threat to potato production. In the absence of resistant cultivars, control strategies are limited to cultural practices such as crop rotation (Subbarao et al. 2007). However, the multiyear survival of *V. dahliae* resting structures (microsclerotia) in the soil and its ability to infect multiple hosts reduce the effectiveness of this practice. Alternative control methods include the use of fumigants (Fravel and Larkin 2000), green manures (Davis et al. 1996; El Hadrami et al. 2009a; Ochiai et al. 2007), amendments (Bailey and

Lazarovits 2003), and biological control agents (Mercado-Blanco et al. 2004; Paulin et al. 2009; Tjamos et al. 2004; Uppal et al. 2008). Fumigation is costly and can affect human health and the environment negatively. Green manures and organic amendments seem promising but have not provided consistent results. Nevertheless, these and other biological methods may reduce the impact of this disease if incorporated into an integrated pest management (IPM) strategy (Bailey and Lazarovits 2003). Implementation of an IPM strategy requires screening biocontrol agents and understanding their mechanisms of action (Uppal et al. 2007, 2008). Nonhost plant extracts can be a source of natural chemicals with a potential role in fungitoxicity or induction of plant defenses (Daayf et al. 1997; Uppal et al. 2008), for which secondary metabolites are suitable markers (Arfaoui et al. 2007; Daayf et al. 2003).

Higher plants accumulate a wide range of secondary metabolites, such as phenolics, saponins, and terpenes (Bennet and Wallsgrove 1994; Daayf et al. 2006; Osbourn 1996). Phenolic compounds and their glycosides are widely distributed in the plant kingdom and play important roles in cell protection (Daayf and Lattanzio 2008; Harborne and Williams 2000; Osbourn 1996). They can serve either as physical barriers between the stressed cells and their environment or as biochemical defenses against herbivores, pathogens, and ultraviolet radiation (Dixon and Paiva 1995; El Hadrami and Daayf 2009; El Hadrami et al. 2010; Ramos et al. 1997; Treutter 2006). Secondary metabolites with a flavonol or flavanone structure (i.e., rutin, quercetin, kaempferol and naringenin) (Supplementary Fig. 1) are naturally occurring antioxidants that accumulate in different plant tissues and play an important role in preventing cell oxidative injuries during plant defenses (Andersen and Markham 2006; Báidez et al. 2007; Treutter 2006). They can inhibit pathogen growth or oxidize into quinones, which can restrict microbial growth and development (Barry et al. 2002; Bennett and Wallsgrove 1994). Rutin (quercetin-3-rutinoside) is a flavonol glycoside that is widely present in fruit and vegetable crops and one of the first flavonoid signals released by host plant roots toward rhizobia (Phillips 1993) and mycorrhizal fungi (Lagrange et al. 2001) and during plant pruning and other processes that result in wounding. In their coevolution with specific or alternative hosts, pathogens have evolved mechanisms to degrade, detoxify, or tolerate such molecules. Degradation of flavonols such as quercetin is well-documented in bacteria (Braune et al. 2001). In fungi, such a phenomenon has been described, so far, in ascomycetes, i.e., *Aspergillus* spp. (Bartz 1971; Merckens et al. 2007; Steiner et al. 2002; Tranchimand et al. 2008; Westlake et al. 1961) and basidiomycetes (Sariaslani and Dalton 1989) but not in *V. dahliae*. For instance, *Aspergillus flavus* is known to activate glucosidases

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and esterases, such as rutinases when grown on rutin (quercetin 3-*O*-rhamnoglucoside)-rich media as the sole carbon source, and also an inducible dioxygenase called quercetinase. The latter is able to oxygenolytically cleave two carbon-carbon bonds from the heterocyclic ring of the rutin aglycone (quercetin) into carbon monoxide and 2-protocatechuoylphloroglucinol-carboxylic acid (2-PCPGCA) (Supplementary Fig. 2). The same reaction is thought to take place in *Pullularia* spp. (Oka and Simpson 1971), *Fusarium* spp. (Bartz 1971), and other *Aspergillus* spp. (Oka et al. 1971). This formation of carbon monoxide is peculiar to fungi, since most bacteria reduce flavonols instead of oxidizing them, with the exception of three prokaryotes that possess dioxygenases and do, in fact, release CO in the same way as fungal quercetinases (Wray and Abeles 1993).

In recent studies, we tested the efficacy of several biological treatments (beneficial bacteria and plant extracts) in reducing potato verticillium wilt in vitro, under growth-room conditions, and in the field for two seasons at various locations (Uppal et al. 2007, 2008). These studies allowed us to test various treatment application methods and to categorize them based on their mode of action (i.e., antagonism, antibiosis, enhancement of host defenses). The objective of the present study was to investigate the mechanisms by which Canada milk vetch (CMV) extracts induced potato defense responses against *V. dahliae*. Bearing in mind the ability of fungi to detoxify plant defense secondary metabolites and to interfere, pre- or post-transcriptionally, with host defense pathways (El Hadrami et al. 2009b), we investigated the response of *V. dahliae* to rutin and related metabolites produced by potato in response to this fungus. In order to elucidate potential mechanisms of degradation, we subsequently analyzed catabolic processes through which *V. dahliae* responds to these secondary metabolites.

RESULTS

Potato protection against verticillium wilt using biological treatments.

Disease severity was reduced in all inoculated plants pretreated with selected bacterial strains or CMV extract as compared with the inoculated control. CMV treatment showed the most significant effect in reducing verticillium wilt (Fig. 1). The observed effects correlated with the frequency of colonization of the tissues by *V. dahliae* detected by polymerase chain reaction (PCR) (Fig. 1).

Soluble phenolics analysis in potato tissues after biological pretreatment, *V. dahliae* inoculation, or both.

As most selected biocontrol treatments were expected to act through induction of host resistance, soluble phenolics were examined as markers of such an induction. Flavonoids and hydroxycinnamates were the main families detected by high-performance liquid chromatography (HPLC), ultra-performance liquid chromatography (UPLC), and thin-layer chromatography (TLC) (Fig. 2) in potato tissues in response to infection by *V. dahliae* or treatment with different biological agents, especially CMV. The flavonol-glycoside rutin accumulated greatly in plants treated with CMV and then inoculated with *V. dahliae* (Fig. 2). Complementary analysis involving spiking and coelution with commercial rutin standard on TLC and HPLC along with UPLC-tandem mass spectrometry (MS/MS) analyses (Fig. 2C) confirmed the accumulation of rutin in potato plants treated with the tested biocontrol agents, especially CMV. Other flavonoids (apigenin, kaempferol, quercetin, and umbelliferone derivatives) were also detected but to a lower extent than rutin. Induced and accumulated hydroxycinnamates (Fig. 2A) included derivatives of caffeic, chlorogenic, and *p*-coumaric acids.

Both treatment with CMV and inoculation with *V. dahliae* have led to an accumulation of rutin twice the level of the healthy control (Fig. 2B). However, combining CMV treatment and inoculation resulted in an accumulation three times higher than that in the CMV-treated, noninoculated plants, almost four and two times the level of the healthy and inoculated controls, respectively (Fig. 2B). The other biological treatments did not show a significant increase in rutin accumulation (Fig. 2B). Rutin was not detectable in crude CMV extracts.

Based on regression analysis between rutin concentrations and vascular discoloration scores, variation of rutin concentration explains about 60% of the vascular discoloration variability observed. Considering only the inoculated treatments in the analysis showed a similar result, with 66% of the vascular discoloration variability explained by rutin content ($y = 1.0754x + 1.1889$; $R^2 = 0.6635$).

Inhibition of *V. dahliae* growth and sporulation by rutin.

Since rutin was the major soluble phenolic accumulated in either pretreated or inoculated plants or both, we tested its direct or indirect effect on *V. dahliae* grown in vitro, using a commercial standard. In addition, to determine the sporulation inhibition threshold, we assayed the growth of *V. dahliae* mycelia on solid media containing rutin concentrations of 0, 100, 300, 600, 900, and 1,500 μ M. Analysis of the effect of rutin on mycelial growth using paper disks showed IC_{50} values (concentration causing 50% inhibition) of 1.28 ± 0.1 and 1.00 ± 0.2 mM for Vd-21 and Vd-9, respectively, but the variability and low differences in growth inhibition did not allow calculation of IC_{50} values when rutin was directly incorporated into the culture medium. On the other hand, significant reduction of *V. dahliae* sporulation in vitro was observed starting at 300 and 100 μ M for isolates Vd-9 and Vd-21, respectively (Fig. 3). We further examined the interaction between *V. dahliae* and rutin in planta. Complementary experiments conducted on potato leaves, stem sections, and roots infiltrated with 75 μ g of rutin per milliliter and further inoculated with *V. dahliae* revealed an ability of the fungus to induce necrosis and grow in infiltrated tissues. Analysis of soluble phenolics from these tissues showed

Fig. 1. Six-week postinoculation disease severity (ratio to the wounded noninoculated control) on potato plants pretreated, by 24-h presowing seed coating, with Canada milk vetch plant extract (CMV) or different biocontrol agents, *Bacillus pumilus* (M1), *Pseudomonas fluorescens* biotype F (DF37), *Bacillus* sp. (S1), and inoculated (+VD) or not with *Verticillium dahliae*. As the same results were observed for both tested isolates, only data for Vd9 isolate is shown. Bars represent the standard error. Histograms within the same chart having the same letter do not differ significantly according to the Newman-Keuls test ($P < 0.05$). The numbers above the inoculated treatments represent the frequency of tissue colonization by *V. dahliae* according to polymerase chain reaction assays.

a degradation of rutin and the appearance of by-products with characteristics recalling phloroglucinol derivatives (Fig. 4).

Degradation of rutin and its derivatives by *V. dahliae* in vitro.

After determining that *V. dahliae* was able to grow in vitro in the presence of rutin and that this flavonol-glycoside is degraded in its presence in planta, we ran a filter-paper disk assay and examined whether *V. dahliae* uses the whole molecule as a carbon source or only the sugar moiety. In either case, *V. dahliae* is left in the presence of a potentially toxic flavonol aglycone (quercetin). We then included rutin, its aglycone quercetin, a related flavanone, and phloroglucinol, given the nature of the by-product derivative detected in planta. The degradation of these compounds by *V. dahliae* was examined in sterile distilled water (SDW) or liquid media composed only of inorganic salts, 'minimum media', or Czapeck-Dox. In SDW and minimum media, *V. dahliae* isolates were unable to grow

properly unless supplemented with either glucose, rutin, quercetin or naringenin, confirming the ability of both tested isolates to use these flavonols and flavanones as a carbon source. Czapeck-Dox, on the other hand, was more favorable for growth and development, and showed a decrease over time in the concentration of these flavonoids (up to four weeks after transfer) (Fig. 5). UPLC-MS/MS analysis revealed the presence of phloroglucinol derivatives such as 2-PCPGCA (Supplementary Fig. 3). Including phloroglucinol in liquid media did not affect the growth or development of the two tested *V. dahliae* isolate (Fig. 5).

Confirmation of the *V. dahliae*-derived quercetinase activity in vitro.

After predicting a number of parameters about the putatively secreted quercetinase protein, we wanted to confirm its enzymatic activity in vitro, and therefore, we proceeded with protein isolation and enzymatic assays. Examination of quercetinase

Fig. 2. Accumulation of rutin in response to biological treatments and inoculation with *Verticillium dahliae*. **A**, High-performance liquid chromatography (HPLC) (maxplot output) showing the soluble phenolics profile from (---) potato plants control and (—) plants treated with Canada milk vetch (CMV) extract and then inoculated with *V. dahliae* (isolate Vd9). The samples were from upper stems harvested 6 weeks after inoculation. Labeled peaks have been tentatively identified based on their retention time, characteristic UV spectra, coelution with commercial standards, and complementary thin-layer chromatography analysis. 1 and 8: methoxycinnamic acid derivatives; 2: aeginin derivative; 3: unidentified; 4: chlorogenic acid derivative; 5: caffeic acid derivative; 6: *m*-coumaric acid derivative; 7: *p*-coumaric acid derivative; 9: benzoic acid derivative; 10: rutin; 11, 12, 13, and 15: unidentified flavonols; 14: kaempferol derivative; 16: 4-methyl-umbelliferyl- β -glucuronide; 17, 19, and 20: 4-methyl-umbelliferone derivatives; 18: quercetin derivative; 21: naringenin derivative. On the right side, four HPLC chromatograms illustrate the variation of rutin (peak 10) in treatments involving CMV. **B**, Bars show rutin accumulation (peak 10, measured in micrograms per gram of fresh weight [F.W.]) in tissues from non-treated (control) and biocontrol-treated potato plants in the presence or in the absence of *V. dahliae* isolate Vd9 (VD). Bars represent the standard deviations to the means. Histograms with the same letter do not differ significantly according to the Newman-Keuls test ($P < 0.05$). Right, the UV spectrum of rutin. **C**, Ultra-performance liquid chromatography and mass spectrometry profiles of a commercial standard (rutin) and the major induced compound in potato plants upon treatment with CMV extract and inoculation with *V. dahliae* isolate Vd9 (CMV+Vd). m/z : 611 represents rutin and the 303 its aglycone deprived of its sugars moiety (quercetin).

Fig. 3. Reduction of spore production by *Verticillium dahliae* in response to increasing concentrations of rutin.

activity revealed an enzymatic activity averaging 160 international units (IU) (1 IU = 1 μ mol quercetin per hour cleaved under standard conditions). The specific activity average was 2,516 μ mol quercetin per min per milligram of protein per gram of mycelium fresh weight (FW). This activity was abolished in the presence of either kojic acid or prohexadione-Ca, both known to be dioxygenase inhibitors.

DISCUSSION

An earlier study focusing on the effectiveness of several bio-control agents in controlling verticillium wilt, when applied to potato seed-pieces at planting, revealed lower levels of disease in response to treatment with CMV extract, under both controlled and field conditions in various locations and growing seasons (Uppal et al. 2008). In the present study, we show this protection to occur along with a substantial accumulation of the flavonol glycoside rutin in treated potato tissues, whereas potato's inability to fight verticillium wilt in the other treatments, which involved beneficial bacteria, was associated with lower accumulation of rutin. Thus, rutin seemed to play a differential role, in a dose-response manner, in the response of potato to *V. dahliae* infection. However, defense-related sesquiterpenes such as rishitin and solavetivone have probably been induced by the tested treatments but have not been considered in this study.

Extracts from potato plants that were treated with certain biological agents prior to inoculation inhibited the growth of *V. dahliae*. Commercial rutin clearly reduced the rate of sporulation of both isolates. Previous papers had also clearly shown the inhibitory effects of rutin and quercetin on the growth of *V. dahliae* (Baidez et al. 2007). Our findings confirmed a direct

Fig. 5. Over time, variation of rutin (RUT), quercetin (QUE), naringenin (NAR), and phloroglucinol (PHL) content added to the Czapeck-Dox liquid media (commercial standard solutions of the compounds calibrated at 100 μ g/ml), relative to noninoculated control media, in the presence of two *Verticillium dahliae* isolates (Vd9 and Vd21). Every datapoint represents an average of three replicates, and statistical significance as compared with the initial time is shown with asterisks (* indicates <0.05; ** indicates <0.1; and *** indicates <0.01).

Fig. 4. **A**, High-performance liquid chromatography showing the soluble phenolic profile of potato leaves noninfiltrated or **B**, infiltrated with rutin and further inoculated with *Verticillium dahliae* isolates. **C**, shows the UV spectra of a commercial phloroglucinol standard (—) along with the phloroglucinol-related compound detected in potato leaves infiltrated with rutin after inoculation with *V. dahliae* (-.-.-). Similar results were shown on stem sections. **D**, Thin-layer chromatography (TLC) showing the degradation of rutin (RUT) (initially present in potato leaves (noninfiltrated) or supplemented (infiltrated)) by two isolates of *V. dahliae* (Vd9 and Vd21) and the accumulation of a by-product related to phloroglucinol (PHL) (i.e., 2-protocatechoylphloroglucinolcarboxylic acid). QUE = quercetin. R_f (retardation factor) values of the detected compounds on TLC are to the right. The arrows indicate the by-products that increased in intensity after inoculation or infiltration of rutin into the tissues.

effect of the induced flavonol on the pathogen but also pointed to the induction of other defense metabolites that would complement such an inhibitory effect. They also showed the ability of *V. dahliae* to grow in presence of rutin, which suggested the ability of *V. dahliae* to metabolize this flavonol-glycoside. This ability is mainly due to this pathogen's glucosidases and rhamnosidases, which help cleave the sugar moieties from the flavonol-glycoside rutin, thereby freeing the quercetin aglycone. In addition, our results suggest that *V. dahliae* is also able to secrete, extra-cellularly, one or more quercetinases that detoxify the aglycone quercetin (*unpublished*), and additional characterization of the genes encoding these enzymes in *V. dahliae* revealed strong homology with well-characterized fungal and bacterial quercetinases. This gives *V. dahliae* the opportunity to counteract the rutin being produced by the plant in response to infection and supports earlier reports showing rutin as a quercetinase inducer in fungi (Tranchimand et al. 2008) due to its A ring.

Quercetinases, also known as extracellular 2,3- or 2,4-dioxygenases (EC 1.13.11.24 and EC 1.13.11.47, respectively), are a family of enzymes that are known to act on chromones such as the phenyl-benzopyrone skeleton, which possesses substituents like methyl or phenyl groups on carbon 2, a free hydroxyl on carbon 3, and a double bond between either carbons 2 and 3 or 2 and 4. By providing ^{14}C -radiolabeled quercetin on C_3 to these enzymes, Simpson and associates (1960) detected ^{14}CO , while providing $^{18}\text{O}_2$ showed that the oxygen does not get incorporated into the carbon monoxide but is, rather, found in the carboxyl and the ester carbonyl groups of 2-PCPGCA. In the present study, *V. dahliae* isolates were able to grow in the presence of either rutin or its aglycone quercetin. This successful growth was ascribed to the secretion of an extracellular dioxygenase that cleaved quercetin and led to the accumulation of the by-product 2-PCPGCA, which we effectively detected by UPLC-MS/MS. Although no evaluation of CO content was conducted in this study, it is expected that the cleavage of quercetin would occur as described in other fungal species (Merkens et al. 2007; Steiner et al. 2002; Tranchimand et al. 2008).

Given that rutin is highly induced in potato in response to successful biocontrol agents and only weakly to the noneffective agents and its degradation by *V. dahliae*, the success of *V. dahliae* in the rhizosphere may be enhanced by its quercetinase activity. Such activity allows it to counter host plant defenses, i.e., synthesis and accumulation of flavonols and flavonones, by turning them into by-products such as 2-PCPGCA. When released, the phloroglucinol moiety may alter the equilibrium among microbial communities in the rhizosphere, thereby helping *V. dahliae* to better colonize it over other pathogens and antagonists. The protocatechuic moiety, on the other hand, could be converted into benzoate and salicylates, which can elicit the salicylic pathway in planta after infection. This elicitation may indirectly interfere with the potato defenses activated through the jasmonic acid (JA) pathway and is in line with the negative feedback between both pathways, as described by Kunkel and Brooks (2002) and Spoel and associates (2003). In a preliminary study, we showed that CMV produces jasmonate-related compounds and may be mediating defenses that rely on the activation of the JA pathway in infected potato plants (El Hadrami et al. 2009a). Such elicitation of a given pathway (i.e., salicylic acid pathway) to interfere with the more adequate defense pathway (i.e., the JA pathway) is known in tobacco in response to *Pseudomonas syringae* (Rayapuram and Baldwin, 2007). A recent study (Thatcher et al. 2009) reported the ability of *Fusarium oxysporum* to hijack the COI1-mediated jasmonate signaling in order to promote disease development in *Arabidopsis thaliana*. The present study is in line with the occurrence of such a phenomenon in the potato-*V. dahliae*

interaction. Further studies are needed to unravel the cross-talk among these and other pathways and investigate the role of *V. dahliae* quercetinases in the phenomenon. Also, it will be of interest to study functionally, by gene knock-out and overexpression, the role of such genes in the pathogenicity of this soilborne pathogen.

In the present study, we have also seen a significant correlation between vascular browning and rutin content. Vascular discoloration can be a sign of plant defense activation through enzymatic reactions involving oxido-reductases, such as polyphenol oxidases and peroxidases. These reactions take place in the woody xylem and involve both reaction and barrier zones (Barry et al. 2002), the former in the tissue damaged by the pathogen and the latter in cambium cells (Barry et al. 2002). Barrier zones are thought to be more resistant to fungal spread than reaction zones (Bauch et al. 1980). Further investigations are needed to examine the effect of rutin on *V. dahliae* in more detail and to determine the involvement of oxido-reductases in reducing disease and its progress towards the upper parts of the plant. Comparison of the response of cultivars with differential susceptibility or tolerance to a set of isolates with varying levels of aggressiveness will be a step forward to acquire such knowledge. It would be also interesting to screen *V. dahliae* isolates for the presence of quercetinase homologs to better understand the mechanisms by which this pathogen takes advantage of the low doses of rutin produced by the host. Meanwhile, the active ingredients of CMV that induce the observed systemic acquired resistance are under investigation, and the preliminary results indicate jasmonate derivatives are potential active ingredients in CMV.

V. dahliae possesses the ability to respond to the rutin elicited by biocontrol agents. Knowing that rutin can be systemically transported in planta (Buer et al. 2007), there seems to be a zigzag race between the host and the pathogen, leading to either an enhanced host defense or a counter-defense by the pathogen. Biocontrol agents could be efficient in reducing verticillium wilt in potato if they are able to induce this and other metabolites at levels high enough to restrict *V. dahliae*'s development or if they act synergistically to fight the pathogen (Daayf et al. 1997). The mechanisms used by *V. dahliae* to interact with potato cultivars other than Russet Burbank remain to be investigated.

MATERIALS AND METHODS

Plant material and growth conditions.

Plants of the potato cultivar Russet Burbank, moderately susceptible to verticillium wilt, were grown from tuber seed-pieces in 7.5-cm diameter clay pots in a pasteurized soil-sand mix (1:1, vol/vol) at 20°C day and 16°C night and with a 16-h photoperiod. They were hand watered daily, were fertilized (NPK, 20:20:20), and were treated with insecticides (i.e., anti-spider mites, antiaphids) when necessary.

Inoculum preparation and inoculation procedure.

Two highly aggressive isolates (Vd1396-9 [Vd-9] and Vd1398-21 [Vd-21]) were selected from a set of single-spore *V. dahliae* isolates previously tested for pathogenicity (Alkher et al. 2009; Uppal et al. 2007). These two isolates were recovered from potato tissues grown locally. Conidial suspensions (10 ml at 10^6 conidia per milliliter) were used to inoculate each plant by root dipping (Alkher et al. 2009).

Biological treatments.

The bacteria used in this study, namely *Bacillus pumilus* (M1), *Pseudomonas fluorescens* biotype F (DF37), and a *Bacillus* sp. (S1) were previously isolated from the phyllophane

and rhizosphere of potato and canola plants (Daayf et al. 2003; Uppal et al. 2007). They were chosen on the basis of either their inhibitory effect on *V. dahliae* growth (Uppal et al. 2007), their ability to enhance potato defense mechanisms (Daayf et al. 2003), or both. Bacterial suspensions were prepared by growing bacteria in 100 ml of Difco nutrient broth (Becton Dickinson & Co., Franklin Lakes, NJ, U.S.A.) for 2 days at room temperature under shaking (200 rpm). The suspensions were then centrifuged at $3,000 \times g$ for 5 min, and the pellet was transferred into SDW and was adjusted to 10^8 CFU per ml. Extracts from CMV (*Astragalus canadensis* L.) were prepared by grinding the leaves to powder in liquid nitrogen and transferring 30 g into an Erlenmeyer flask containing 100 ml of 80% (vol/vol) ethanol. After overnight shaking at 4°C in the dark, the crude extracts were filtered through Whatman No. 1. Filtrates were diluted 10 times in SDW and were then used to coat seeds just before planting by mixing them with potato seed pieces (1 ml for 40 to 80 g of seed pieces) in Ziploc plastic bags. Control tubers were treated with SDW containing an equivalent volume of ethanol.

Disease rating.

Disease rating was conducted over time (2 to 6 weeks after inoculation), using the area under disease progress curve (AUDPC) (Campbell and Madden 1990), calculated according to the following formula:

$$AUDPC = \sum_{i=1}^{n-1} [(y_i + y_{i+1})/2](t_{i+1} - t_i)$$

where n is the total number of assessments in weeks, y_i is the disease severity at the i th assessment week, and the term $t_{i+1} - t_i$ is the time duration between two assessments. Vascular discoloration was assessed on 5-mm-thick sections from the basal part of the stem, using three replicates per treatment and five cross-sections per treatment per replicate. Healthy-looking tissues of infected plants were harvested at the end of each experiment (6 weeks after inoculation). Tissues from various parts of infected and control plants were used at the end of the experiment to determine the degree of *V. dahliae* colonization, using both classical (i.e., microsclerotia under the microscope, re-isolation) and molecular methods, using the PCR protocol described by El Hadrami and associates (2007), with a slight modification. Briefly, 200 to 500 mg of tissues from the lower, middle, and upper portions of the plant were sampled and were subjected to a DNA extraction and semiquantitative PCR, using primers for the constitutively expressed β -tubulin and 18S genes (Vd-bTubF: 5'-GTCGACCAGGTTCTCGATGT-3'; Vd-bTubR: 5'-AAGCCGACCATGAAGAAGTG-3'; Vd-18SF: 5'-CGGGGAGGTAGTGACGATAA-3'; Vd-18SR: 5'-CATTACGGCGTCCTAGAAA-3'). The amplified products were 473 bp for the β -tubulin (GenBank number VDAG_10074.1) and 407 bp for the 18S (GenBank number AY489705.1). Quantification and limits of detection were determined based on a preestablished dilution series of DNA isolated from the tested isolates, and a final DNA concentration of 10 ng/ μ l was used in all quantifications. The colonization percentages were calculated taking into account all tested samples from a given plant. Experimental replicates were analyzed separately, and an overall percentage was deduced for each treatment.

Potato soluble phenolics extraction and analysis.

For extraction, 1g of FW representing a mixture of leaves, petioles, and stems was crushed in a cold mortar containing 1 ml of 80% (vol/vol) aqueous methanol solution and was analyzed by HPLC, according to the procedure described by El Hadrami and associates (2010). Identification of the major compounds was conducted based on their characteristic features,

including absorption spectra, retention times, comparison with our database standards, coelution with commercial standards as well as TLC analyses. Quantitative analyses were performed by HPLC, using the peak area with reference to a standard curve of a commercial rutin solution (Sigma-Aldrich Co., Oakville, Canada). TLC analyses were carried out using a silica gel plate and various solvent systems, i.e., chloroform/methanol/water (6:3:1, vol/vol/vol), butanol/water/acetic acid (5:4:1, vol/vol/vol). Developed TLC plates were visualized under UV light (254 to 365 nm) or by staining with specific reagents, such as the Benedict reagent (Reznik and Egger 1961). The R_f (retardation factor) of the visualized phenolics, especially rutin, were recorded in coelution and in comparison with commercial standards. Analyses were also carried out using ACQUITY UPLC coupled to a triple quadrupole mass spectrometer (Micromass Canada, Inc., Mission, BC, Canada), according to the procedure described by El Hadrami and Daayf (2009). For rutin analysis, the first and third stages of quadrupole mass analyzers were set to follow the fragmentation transition of the precursor ion at m/z 611 to the product ion at m/z 303, which was the most abundant isotopic molecular weight. Data were acquired and processed using MassLynx Software's Application Manager v. 4.1. (Waters Inc.).

Effects of rutin on growth and sporulation of *V. dahliae*.

Phenolic extracts and commercial rutin solutions were dried, the final residue was resuspended in appropriate volumes of 10% (vol/vol) ethanol and was used for in vitro assays. These assays consisted of petri plates containing water-agar media onto which plugs of two isolates of fresh *V. dahliae* (Vd-9 and Vd-21) were transferred. Four sterile 5-mm diameter filter paper disks, containing 20 μ l of either phenolic extracts, various concentrations of commercial rutin (1.6 nM to 1.6 mM with 10 $\times$ increments), 10% (vol/vol) ethanol, or water were introduced onto each plate. The diameters of *V. dahliae* colonies were measured every three days, and their variation over time was used to calculate the area under the growth progressing curve (AUGPC) as follows.

$$AUGPC = \sum_{i=1}^{n-1} [(y_i + y_{i+1})/2](t_{i+1} - t_i)$$

where y_i is the surface area of the growing colony at the time t_i . These data were used to generate a linear regression that served to identify IC_{50} values. A second experiment was set up to determine the inhibition threshold of rutin directly in the media, in which concentrations of 0 to 1.5 mM were tested on both growth and sporulation. To determine the effect of rutin on sporulation, spores produced on cultures grown on media amended with rutin concentrations of 0 to 1.5 mM were collected and counted.

Additional experiments were conducted to examine the effect of supplementation of potato tissues with rutin on further growth and development of *V. dahliae* in planta. Freshly harvested potato (cv. Russet Burbank) leaves, stem sections, and roots were washed under running distilled water, were surface-sterilized in 5% bleach, and were rinsed in SDW before being placed on wet sterile filter paper in petri dishes. These tissues were then infiltrated with a 100- μ l solution of commercial rutin dissolved in water and calibrated at 75 μ g/ml. Half the replicates were kept as controls, while the other half were further inoculated with *V. dahliae*. Control plant tissues were infiltrated with water. The inoculum was prepared from grown colonies of isolates Vd-9 and Vd-21, according to the procedure described above. Inoculations were made by spreading 100- μ l inoculum droplets around the rutin-infiltrated sites. Inoculated and control leaves and stem sections were kept at 20°C days and 16°C nights with a 16-h photoperiod, while root sections were kept

at the same temperature in darkness. At 3 and 7 days after inoculation, samples were harvested and were immediately frozen until they were extracted and analyzed. Samples harvested before the treatments were used as background controls for the analysis. Separation of the purified compounds was conducted by TLC developed in chloroform/methanol/water (6:3:1, vol/vol/vol) and compounds were further stained with 1% (wt/vol) vanillin-HCl or ninhydrin. HPLC analysis was conducted as described above to reveal both qualitative and quantitative changes in potato extracts in response to both infiltration and inoculation with *V. dahliae* isolates.

A further set of experiments was conducted using potato tuber slices. Tubers from cv. Russet Burbank were washed, were surface-sterilized (0.6% [wt/vol] sodium hypochlorite solution containing 0.1% [vol/vol] Tween 20), were dried under a flow hood, and were then thinly sliced using a home chip supreme slicer (Supreme Slicer; StarFrit, Ottawa, Canada). Tuber slices (2 to 3 mm thick) were treated with SDW, 10% (vol/vol) CMV extract (0.3 g/ml in water), or 1 µg/ml of rutin, quercetin, naringenin, or phloroglucinol. Half the replicates were kept as controls and were mock-inoculated with water, while the other half were inoculated with 200 µl of a 10⁶ spore/ml conidial suspension of isolate Vd-9. Tuber slice samples were harvested at 3 and 7 days after inoculation and were analyzed by HPLC as described above.

Degradation of rutin and other related phenolics by *V. dahliae* in vitro.

To test the possible degradation of rutin and other related compounds such as quercetin, naringenin, and phloroglucinol by *V. dahliae*, the selected isolates (Vd-9 and Vd-21) were grown in 100 ml of Czapeck-Dox liquid media (Hurst et al. 2002), either nonamended or amended with 1 ml of commercial standard solutions of the tested compounds calibrated at 100 µg/ml in dimethyl sulfoxide (DMSO). Media amended only with 1 ml of DMSO were used as controls. The inoculated media as well as the controls were kept at room temperature on a shaker in the dark at 120 rpm. Samples consisting of 1-ml solutions were taken at 0, 3, 7, 15, 21, and 28 days after transfer. They were immediately analyzed by HPLC or kept at -20°C until needed. Identification of each compound or its product of degradation was based on their characteristics, including absorption spectra, retention times, comparison and coelution with commercial standards, and on TLC and HPLC. Quantitative analyses were performed by HPLC using the peak area with reference to a standard curve made for each commercial standard (Sigma-Aldrich Co.). Three replicates per treatment × isolate combination were considered for this experiment. Quantifications of the added compounds and their products of degradation were performed in triplicate and the data are reported as the recorded average values.

The products of degradation of rutin and quercetin were further characterized using an ACQUITY UPLC system (Waters Inc.) connected to a triple quadrupole mass spectrometer system Quattro Ultima (Micromass) under the same conditions described above. The first and third stages of the quadrupole mass analyzer were set to follow the fragmentation transition of the precursor ion at *m/z* 343.26 (2-PCPGCA), which was the most abundant isotopic molecular weight, to the product ions at *m/z* 155.03 and 127.03, representing protocatechuic acid and phloroglucinol, respectively. Data were acquired and processed using MassLynx Software's Application Manager v. 4.1. (Waters Inc.).

Protein extraction and quercetinase activity assay.

Proteins were isolated from 200 mg of a freshly grown mycelium of *V. dahliae* isolates Vd-9 and Vd-21 in 0.1 M potassium phosphate buffer, pH 7.0. The quercetinase activity was

assayed following the method described by Oka and associates (1971), which monitors the decrease in absorbance at 367 nm (*A*_{367nm}) as a result of quercetin breakdown ($\epsilon = 20,000$ M/cm) by a known volume of enzyme added to a mixture consisting of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.0, quercetin, and copper. Every assay was repeated five times and the data was expressed as a percentage of the control.

Experimental design and data analyses.

Experimental trials were conducted in triplicate, according to a randomized complete block design. In each trial, five individual plants were considered for disease assessment and sampling. Quantitative data were used for variance analysis using Statistica software (Statsoft Inc., Tulsa, OK, U.S.A.). Analysis of variance (ANOVA) was followed by pairwise comparison of the means, using the Newman-Keuls test at *P* < 0.05. Linear regression analysis was also conducted to determine whether rutin content explains any of the disease severity reduction assessed. In the experiments set to determine the ability of *V. dahliae* to catabolize rutin, quercetin, or naringenin, the data were expressed as a percentage of the initial amount added to the liquid media and arcsine-transformed before use in ANOVA. Rutin effects on pathogen growth and sporulation were conducted at least twice. Quercetinase activity was determined based on five independent assays performed for each treatment. Collected data points were used in a linear regression of the decrease in (*A*_{367nm}) over time to determine the enzymatic activity.

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