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Impact of Agrochemicals on *Peronospora sparsa* and Phenolic Profiles in Three *Rubus arcticus* Cultivars

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The main arctic bramble (*Rubus arcticus*) cultivars are susceptible to downy mildew (*Peronospora sparsa*), which seriously threatens the cultivation. The efficiency of Aliette, Euparen M, phosphite-containing Phosfik, Phostrol, Farm-Fos-44, and Kaliumfosfiet, as well as Bion was evaluated in the greenhouse. Fewer symptoms and less *Peronospora* DNA were found in plants treated with Euparen M and Bion, whereas Aliette, Phosfik, and Phostrol gave moderate protection. Three arctic bramble cultivars showed varying susceptibility to *P. sparsa*. An inexpensive and fast in vitro plate test gave results parallel with those obtained in the greenhouse. Quantitative differences were found in the phenolic profiles of the leaves of different cultivars and in different treatments. Several phenolic compounds were tentatively identified in arctic bramble for the first time, for example, monomeric and oligomeric ellagitannins and galloylglucoses. Negative correlation was found between the amount of *P. sparsa* DNA and flavonol glycosides and some ellagitannins in the leaves 8 days after inoculation, suggesting a possible role for these phenolics in the defense.

KEYWORDS: Actigard; Aliette; arctic bramble; BTH; ellagitannin; Euparen M; Farm-Fos-44; flavonol; HPLC; Kaliumfosfiet; mass spectrometry; Phosfik; phosphite; Phostrol; real-time PCR

INTRODUCTION

Downy mildew diseases in plants are caused by oomycetes, which are specialized biotrophs often capable of damaging all above-ground plant parts. In *Rubus* species, downy mildew (*Peronospora sparsa*) appears as reddish, angular lesions on the leaves, where sporangiophores extend from the abaxial surface of the leaf (*1–3*). However, the most severe consequence of downy mildew is the "dryberry" disease, which may cause total yield loss, particularly in rainy seasons (*2–5*). Arctic bramble (*Rubus arcticus* ssp. *arcticus*) is a northern *Rubus* plant, which produces deep red fruits with unique aroma. The fruits are used by the liqueur industry as well as for delicate cuisine. Downy mildew has strongly hampered the cultivation of arctic bramble in Finland (*3, 4*) and, therefore, resistant cultivars and efficient plant protection methods are currently being explored. Quantitative analysis of resistance has not been done on the present cultivars, but preliminary experiments and experience in the field indicate that cv. Pima is especially susceptible, whereas another main cultivar, Mespi, shows some resistance to the disease (*6*). Fungicides Euparen M (tolylfluanid) and Aliette (fosetyl-Al) are the only products registered in Finland against downy mildew in arctic bramble and the problem, particularly with Euparen M, is the long withholding period.

Salts of phosphorous acid (H_3PO_3), that is, phosphites, have been widely used against oomycete plant pathogens, including *Peronospora* species (*5, 7, 8*). In contrast to phosphates ($-PO_4^{3-}$), plants cannot use phosphites as a phosphorus source until phosphites are slowly converted to phosphates by soil microbes, although some phosphite-containing products are labeled as fertilizers (*9*). Phosphites have a direct antimicrobial effect on oomycetes (*10, 11*), but they also act indirectly by reinforcing defense reactions of plants. Potassium phosphonate has been found to induce localized cell death and accumulation of phenolic material at the infection site in *Arabidopsis*, which was observed to inhibit the growth of *Phytophthora palmivora* (*12*). Localized induction of pathogenesis-related (PR) proteins is also suggested to take place in K_2HPO_3-treated cauliflower, whereas a systemic induction of PR proteins and phytoalexins has been found in fosetyl-Al-treated potato (*8, 13*).

Bion [marketed as Actigard in the United States; the active ingredient benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester is often referred to as BTH] is a widely studied model compound for plant activators, and it has been shown to induce resistance to a wide variety of pathogens by acting as a functional analogue of salicylic acid in plants (*14, 15*). Efficacy has also been found against downy mildews of *Arabidopsis*,

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cauliflower, and tobacco caused by *Peronospora* species (*16–18*). Similarly to phosphites, Bion may induce the local or systemic synthesis of PR proteins and secondary metabolites, for example, phenylpropanoids (phenolics) (*16, 17, 19*). Phenolic compounds have been suggested to participate in defense against oomycetes, because their accumulation has been observed at the onset of infection by oomycetes, the response often being faster and/or stronger in resistant genotypes (*20–22*). However, no information is available about phenolics and their role in *Rubus*−*Peronospora* pathosystems.

The aims of this study were (i) to evaluate the resistance of three genotypes of arctic bramble, cvs. Pima and Mespi and newly selected clone 12B14, to *P. sparsa* under controlled environment in a greenhouse, (ii) to measure *P. sparsa* DNA as a marker for pathogen growth in the host, (iii) to analyze phenolic profiles of leaves qualitatively and quantitatively in different cultivars and treatments, and (iv) to evaluate disease control by different types of agrochemicals and, specifically, their impact on growth (pathogen DNA and symptoms) and propagation (sporulation) of the pathogen and on the composition of phenolic compounds.

MATERIALS AND METHODS

Plant and *Peronospora sparsa* Material. Arctic bramble cvs. Pima, Mespi (*23*), and a new clone (12B14) were propagated vegetatively for the experiments in the greenhouse of the University of Kuopio. The clone 12B14, which was obtained from open-pollination of cv. Elpee (*24*), was selected because it showed the fewest symptoms of downy mildew on field tests at the University of Kuopio between 2002 and 2005. All plants were kept dormant below 4 °C until the start of the experiment. Plants were grown in 2 L pots in peat−sand−vermiculite (14:3:3) under the following conditions: relative humidity, 70%; daylight, 18 h; and temperature, 13–18 °C (night−day). Isolation and maintenance of *P. sparsa* have been described previously (*6*). Fresh spores were produced for the inoculations.

Greenhouse Experiment. The agrochemicals used in the experiment were distilled water (positive control for infection), 0.15% (w/v) Euparen M 50WG (negative control for infection, tolylfluanid fungicide, Bayer), 0.3% (w/v) Aliette 80WG (the second negative control for infection, fosetyl-Al/fungicide, Bayer), 0.4% (v/v) Phostrol (potassium, sodium, and ammonium salts of phosphorous acid/fungicide, Nufarm Americas Inc.), 0.5% (v/v) Phosfik (undefined salts of phosphorous acid, Kemira Growhow Oyj.), 0.5% (v/v) Farm-Fos-44 (potassium salt of phosphorous acid, Farm-Fos Ltd.), 0.4% (v/v) Kaliumfosfiet (potassium salt of phosphorous acid, van Iperen), and 0.025 and 0.2 g/L active ingredient (ai) of Bion 50WG [benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester, Syngenta]. Euparen M and Aliette were selected as negative controls for infection because they are the registered products against downy mildew of arctic bramble in Finland.

The cv. Pima was treated with all of the chemicals because it is known to be highly susceptible to the disease, enabling the evaluation of disease control by the chemicals. The water-treated cv. Mespi and clone 12B14 were included in the study mainly to evaluate their basal resistance to *P. sparsa* and compare it to that of cv. Pima. However, to obtain information about the chemicals (their efficacy and impact on arctic bramble) in more than one cultivar, the clone 12B14 was also treated with Aliette (negative control for infection), Phosfik (representative of phosphite-based compounds), and 0.2 g/L ai of Bion.

Five plants were selected randomly for each treatment and numbered. The chemicals (ca. 5 mL/plant) were sprayed on the plants just before the beginning of flowering. The concentrations used were those recommended by the manufacturers, or lower if preliminary experiments indicated injury of foliage. Bion was used at two different concentrations to test if a lower concentration of 0.025 g/L (ai) is sufficient for inducing resistance to downy mildew equally well compared to the 0.2–0.3 g/L concentration used in preliminary experiments.

Four days after the chemical treatments, all of the plants were inoculated by spraying with downy mildew spores suspended in sterile water (7500 spores/mL, ca. 2 mL/plant). Silwet Gold surfactant (0.025%, Chemtura) was added in the suspension to facilitate even distribution of the suspension on the leaves. Relative humidity was raised to 95% after inoculation to facilitate germination of the spores. Symptoms of downy mildew were evaluated twice a week in each plant by counting the number of symptomatic leaves per plant (five plants per treatment). Reddish angular lesions, usually bearing visible sporangiophores on the abaxial side of the leaf, were considered to be the symptoms.

All plants were sampled 4 and 12 days after the chemical treatments, that is, 0 and 8 days after inoculation. Each sample consisted of two young, full-size leaves collected from one plant. Samples were stored at −80 °C.

Plate Experiment. Before the inoculation in the greenhouse, three leaves from each plant were collected and placed abaxial leaf surface up on 0.8% water agar plates. The detached leaves were inoculated with the same spore suspension as the whole plants by applying four drops (5 μL each) on each leaflet. Sealed plates were stored at room temperature.

One of the three leaves in each plate was sampled for real-time PCR 2, 4, and 6 days after inoculation. At each time point, one of the three leaflets of each leaf was detached and stored at −20 °C. From the other two leaves remaining on the plates, sporangiophores grown on the abaxial side of the leaflets were counted under a stereomicroscope 4, 6, 8, 10, and 12 days after the inoculation. The following scale was used: 0, 0; 1, 1–5; 2, 6–10; 3, 11–20; and 4, >20 sporangiophores per leaflet.

Analysis of Phenolic Compounds. Phenolic compounds were extracted separately from each plant (two leaves per plant) sampled 0 and 8 days after inoculation. From the first sampling time, all collected samples, except those from 12B14 treated with Phosfik and Aliette, were analyzed. From the second sampling time, cv. Pima treated with water (control), 0.2 and 0.025 g/L of Bion or Phosfik, clone 12B14 treated with water or 0.2 g/L Bion and cv. Mespi treated with water were analyzed.

Frozen leaves were weighed (g of fw) and extracted twice with 5 mL and once with 2 mL of 70% acetone in water by homogenization with an Ultra-Turrax for 1 min. After centrifugation at 5000*g* for 5 min, the supernatants were combined and stored at −20 °C. Prior to HPLC, acetone was evaporated in a vacuum centrifuge at room temperature and the concentrate was adjusted to 4 mL with water. Pellets remaining from the extraction were also centrifuged under vacuum for 30 min to evaporate acetone, after which the pellets were further stored at −20 °C for DNA extraction.

Total phenolic content of the phenolic extracts was determined with the Folin−Ciocalteu method (*25*). Results are expressed as milligrams of gallic acid equivalents per gram of fresh leaves. Individual phenolic compounds were analyzed by HPLC, the Hewlett-Packard 1090 series apparatus (Waldbronn, Germany) consisting of two pumps, an autosampler, a column oven, and a diode array detector coupled to HP Chemstation data handling software. Samples (15 μL) were analyzed at 35 °C with a flow rate of 2 mL/min in a 60 × 4.6 i.d., 3 μm ODS Hypersil column (Agilent Technologies). The following linear gradient of acetonitrile (A; % v/v) in 1% formic acid (B) was used to separate the phenolic compounds: 0–15 min, 0–8% A; 15–30 min, 8–13% A; 30–35 min, 13–25% A; 35–40 min, 25–100% A; 40–41 min, 100% A; 41–48 min, 100–0% A; 48–55 min, 0% A. The following standard compounds were used in the quantification: ellagic acid for ellagitannins, rutin and kaempferol for flavonols, gallic acid for galloylglucoses and other compounds with absorbance around 280 nm, and chlorogenic acid and *p*-coumaric acid for hydroxycinnamic acids. Compounds **1**, **2**, **4**, **5**, **24**, **28b**, **29b**, and **38b** were not quantified due to their low concentration or coelution with other compounds in HPLC.

Identification of Phenolic Compounds. The peaks in the phenolic profiles were tentatively identified by a Finnigan LTQ linear ion trap mass spectrometer (San Jose, CA) as described previously (*19*). The HPLC apparatus consisted of HP 1100 series pumps, an autosampler, and a diode array detector. The HPLC conditions, column, and gradient were as described above. Data were collected in negative ionization mode at *m*/*z* 150–2000.

Quantitative Real-Time PCR. To quantify *P. sparsa* DNA, real-time PCR analysis was performed on the samples collected from the greenhouse experiment 8 days after inoculation and from the plate experiment 2, 4, and 6 days after inoculation. From the greenhouse samples, the pellets remaining after the extraction of phenolics were used for DNA extraction without further homogenization. Leaflets collected from the plates were thawed and homogenized without buffer in FastPrep FP120 (Savant Instruments Inc.) for 25 s. DNA was extracted using E.ZNA SP Plant DNA Miniprep kit from Omega Bio-Tek according to the manufacturer's instructions. DNA concentration was measured with a Nanodrop ND-1000 UV−vis spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Real-time PCR was performed using the protocol and equipment described previously (*6*). As a modification to the method, 0.8 μL of enzyme blend was used. Each sample was analyzed as two replicates. Results are expressed as percentage of *P. sparsa* DNA relative to plant DNA.

Statistical Analyses. Statistical analyses were performed with SPSS 14.0 for Windows (SPSS Inc.). Means between Pima control and other treatments/cultivars were compared with the analysis of variance using Tukey's post hoc test or with the nonparametric Mann–Whitney *U* test if conditions for the analysis of variance were not fulfilled (symptom evaluations and PCR results). The means were derived from five parallel analyses, each from a different plant. Possible correlations between the concentrations of phenolics (0 and 8 days after inoculation) and the amount of *P. sparsa* DNA (8 days after inoculation, greenhouse) were analyzed by calculating the Pearson correlation coefficient.

Principal component analysis (PCA) was used to analyze the variation of phenolic profiles among different cultivars or treatments of arctic bramble. Principal components (PC) were derived from the concentrations of 34 individual phenolics measured from the greenhouse samples 8 days after inoculation (5 plants/treatment or cultivar). The first four PCs (1–4), which explained 78% of the total variation among treatments/cultivars, were included in detailed analysis. The PCA groups compounds that follow a similar trend among the different treatments/cultivars under one PC. This grouping is illustrated by factor loadings, which describe the contribution of individual phenolics to each PC. The higher the factor loading, the higher is the contribution of that compound in the specified component. Finally, the regression factor score of each variable (sample) in each PC is calculated on the basis of the original concentrations relative to their factor loading in the specific PC. The significance of the variation was evaluated by performing the analysis of variance (Tukey's post hoc test) between factor scores of different treatments in each of the four PCs.

RESULTS AND DISCUSSION

Efficacy of the Agrochemicals in the Greenhouse. A controlled environment for inoculation was established in the greenhouse to test different agrochemicals against downy mildew of arctic bramble. High relative humidity, controlled temperature, and the use of a surfactant were found to be crucial for successful inoculation. **Figure 1** displays the development of symptoms in different treatments of cv. Pima and clone 12B14 as a percentage of symptoms observed in Pima control, which was used as a positive control for infection. No phytotoxicity related to the agrochemical treatments was observed in the plants.

The first visible symptoms of downy mildew emerged in the control plants of cv. Pima 7 days after inoculation and continued spreading until 3 weeks after inoculation, when the foliage began to senesce. The fewest symptoms in cv. Pima throughout the experiment were observed in the plants treated with Euparen M (negative control for infection) or with the higher concentration of Bion (**Figure 1A**). Aliette, the other negative control for infection, gave only moderate control of downy mildew in this experiment, similarly to other phosphite-based products (Phosfik, Phostrol, Farm-Fos-44), suggesting a similar mode of action. On the other hand, Kaliumfosfiet, which also contains

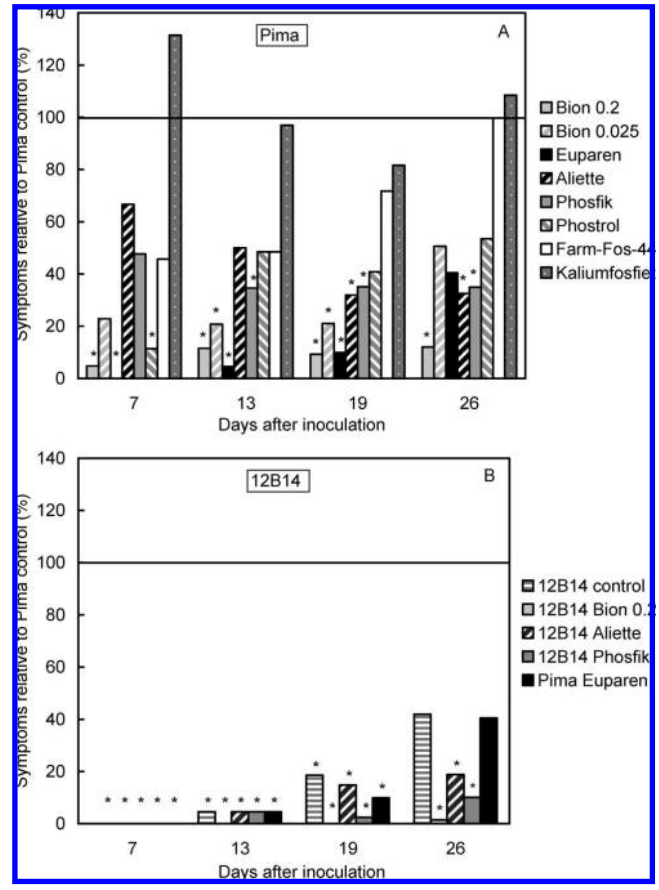

Figure 1. Development of downy mildew symptoms in arctic bramble (**A**) cv. Pima and (**B**) clone 12B14 treated with various agrochemicals. The symptoms are expressed as percentage of those in untreated cv. Pima (100%), where 3.5, 8.7, 16.2, and 13.8 symptomatic leaves per plant were found 7, 13, 19, and 26 days after inoculation, respectively. Results from cv. Pima treated with fungicide Euparen M are marked in **B** as the reference to successful protection against downy mildew. Statistically significant differences ($P < 0.05$; Mann–Whitney *U* test) from cv. Pima control within the same sampling date are marked with asterisks.

potassium phosphite, had no efficacy against *P. sparsa*. However, phosphite-containing compounds such as Phosfik might be an option for the protection of arctic bramble. The benefit from using potassium phosphites is their reduced or eliminated withholding period between the application and harvest, which could make them particularly desirable for the secondary and tertiary sprayings later in the season to complement the effect of an initially applied fungicide. Prolonged use of phosphites alone might not be a viable option because insensitivity to fosetyl-Al or phosphites has been found among different isolates of *Bremia lactucae*, *Phytophthora cinnamoni*, and *Phytophthora infestans* (*26–28*). In 12B14 relative efficacies similar to those in cv. Pima were found between the three agrochemicals, Aliette, Bion (0.2 g/L), and Phosfik, selected for testing (**Figure 1B**).

Because symptom expression does not always correlate to disease severity, the growth of *P. sparsa* was determined by quantitative PCR using selected samples collected in the greenhouse 8 days after inoculation. Percentages of *P. sparsa* DNA (**Figure 2A**) were generally consistent with the development of symptoms (**Figure 1A**) in the different treatments of cv. Pima. The highest percentage of *P. sparsa* DNA was found in the controls, followed by Phosfik-treated plants. Unexpectedly, the 0.2 g/L Bion treated plants, which showed very few symptoms in all evaluations (**Figure 1A**), had more pathogen DNA than did the plants treated with the lower concentration

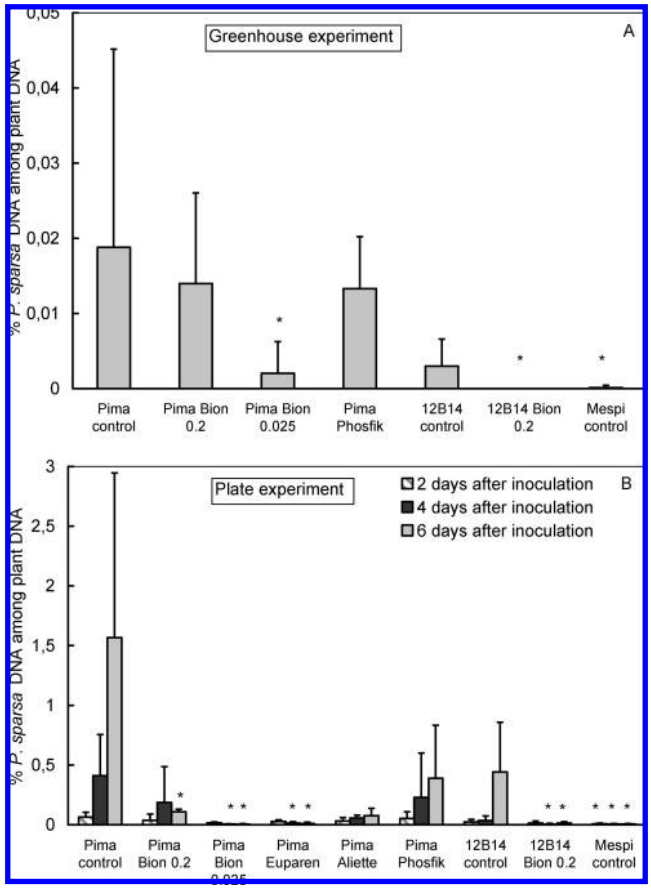

Figure 2. Percentage of *Peronospora sparsa* DNA relative to plant DNA measured with real-time PCR (**A**) 8 days after inoculation in the greenhouse and (**B**) 2, 4, and 6 days after inoculation on water agar plates. The bars represent standard deviation of mean derived from three to five plants per treatment. Statistically significant differences ($P < 0.05$; Mann–Whitney *U* test) from cv. Pima control within the sampling date are marked with asterisks.

of Bion (0.025 g/L) (**Figure 2A**). Thus, better protection might actually be obtained with the lower rather than with the high concentration of Bion, at least during the early days of infection. Similar observations about Bion were made on rice by Schweizer et al. (*29*).

Efficacy of the Agrochemicals in the in Vitro Plate Test. Genotypic differences in the resistance to *P. sparsa* have been found to be expressed even in detached leaves of micropropagated arctic bramble (*6*). In the present experiment, the in vitro model was used to measure the growth of sporangiophores and the amount of *P. sparsa* DNA after chemical treatments of cv. Pima and 12B14. The leaves were detached and inoculated on plates after treatment of the whole plants with the agrochemicals in the greenhouse experiment described above.

The degree of sporulation in vitro showed a tendency similar to the incidence of symptoms in the corresponding treatments in the greenhouse (**Table 1** and **Figure 1**). The most intense and earliest sporulation was found on the leaves of cv. Pima control, whereas no sporangiophores grew on Bion- and Phostrol-treated leaves. In the other treatments, sporangiophores developed later than in the controls. In 12B14, Bion treatment prevented sporulation, whereas Aliette or Phosfik treatments had no significant protective effect in 12B14 (**Table 1**). In general, 12B14 showed higher susceptibility to *P. sparsa* on the plates than in the greenhouse when compared with the efficacy of the corresponding treatments on cv. Pima.

Table 1. Degree of Sporulation of *Peronospora sparsa* on Detached Leaves of Arctic Bramble on Water Agar Plates 6–12 Days after Inoculation

		degree of sporulation[a]			
cultivar	treatment[b]	6 days	8 days	10 days	12 days
Pima	control	1	4	4	4
	Bion 0.2	0	0*	0*	0*
	Bion 0.025	0	0*	0*	0*
	Euparen	0	0*	0*	1
	Aliette	0	0*	0.5	2.5
	Phosfik	0	0*	0*	0.5*
	Phostrol	0	0*	0*	0*
	Farm-Fos-44	0	0*	0.5	2
	Kaliumfosfiet	0	1	4	4
12B14	control	0	3	4	4
	Bion 0.2	0	0*	0*	0*
	Aliette	0	1	2.5	4
	Phosfik	0	0.5	2.5	3.5
Mespi	control	0	0*	0*	0*

[a] Sporulation was evaluated using the following scale: 0, 0; 1, 1–5; 2, 6–10; 3, 11–20; 4, >20 sporangiophores per leaflet. Statistically significant differences ($P < 0.05$; Mann−Whitney *U*) between cv. Pima control and other treatments/cultivars within the column are marked with asterisks. Means were derived from 10 leaves per treatment. [b] Plants were treated with the chemicals 4 days before the leaves were detached, after which the leaves were inoculated on water agar plates.

The reliability of sporulation as a marker for *P. sparsa* growth in vitro was confirmed by quantitative PCR analysis. The leaves with no sporangiophore growth were of particular interest. Only a low amount of *P. sparsa* was measured in the leaves 2 days after inoculation, some of it likely remnants of the spores used in the inoculation, but the amount increased rapidly thereafter in the control and Phosfik-treated Pima (**Figure 2B**). Bion- and Euparen-treated plants had significantly less pathogen DNA than did the Pima controls. Again, Bion applied at the lower concentration gave better protection than did the higher Bion concentration, supporting the results obtained in the greenhouse. In the leaves on which no sporangiophores were observed (**Table 1**) the percentage of *P. sparsa* also remained close to the detection limit (**Figure 2B**), indicating that practically no growth of *P. sparsa* takes place unless visible sporangiophores also develop. The only exception was the Bion-treated (0.2 g/L) leaves of cv. Pima, but the percentage of *P. sparsa* started to decrease also in those leaves 4 days after inoculation. On the basis of these and previous results, the plate system serves as a useful, fast, and inexpensive tool for the screening of cultivars and protective agents before larger scale experiments.

Genotypic Differences in the Resistance of Arctic Bramble to *P. sparsa*. In addition to the effects of agrochemicals, preliminary observations about the degree of resistance to *P. sparsa* among arctic bramble genotypes were made in the greenhouse and plate experiment. All results obtained on water-treated plants of Pima, 12B14, and Mespi, that is, symptom development in the greenhouse (**Figure 1**), sporulation on the plates (**Table 1**), and PCR analysis from the greenhouse and plate samples (**Figure 2**), indicated that both the clone 12B14 and cv. Mespi were less susceptible to *P. sparsa* than was cv. Pima. The cv. Mespi showed no symptoms, which was confirmed by the fact that only one weakly downy mildew-positive plant was found in real-time PCR (**Figure 2A**). Promising results were also obtained with the new arctic bramble clone 12B14, which even without any protective treatments had timing and extent of downy mildew symptoms similar to those of Euparen M-treated cv. Pima (**Figure 1B**). The results are in

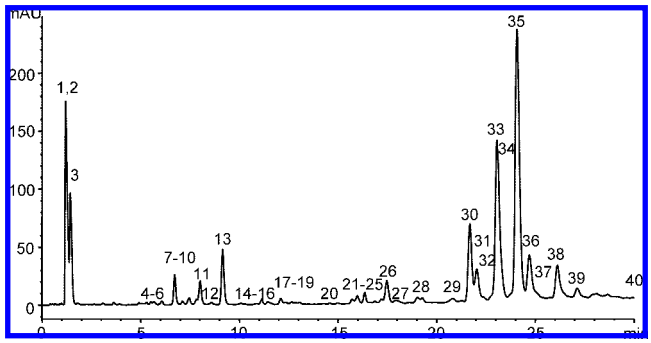

Figure 3. HPLC chromatogram of soluble leaf phenolics extracted from untreated arctic bramble cv. Pima 8 days after inoculation and monitored at 280 nm. The following compounds were tentatively identified: 1, bis(galloylglucose); 2, methyl gallate-galloylglucose; 4, digalloylglucose; 5, coumaryl quinic acid; 6, caffeoyl glucose; 7, caffeic acid derivative; 11 and 13, *p*-coumaric acid derivatives; 14, sanguiin H-5; 15, digalloylglucose; 16, roshenin B-like ellagitannin; 17, ferulic acid derivative; 18, sanguiin H-10-like ellagitannin; 19, roshenin B-like ellagitannin; 20, quercetin triglycoside; 21, unidentified ellagitannin; 22, deHHDP-lambertianin C; 23, kaempferol triglycoside and trigalloylglucose; 24, tellimagrandin I; 25, unidentified ellagitannin; 26, casuarictin or potentillin (isomers); 27, sanguiin H-10; 28, tellimagrandin I and ellagic acid glucuronide; 29, deHHDP-lambertianin C and quercetin hexose-glucuronide; 30, lambertianin A; 32, tellimagrandin II; 33, sanguiin H-6; 34, quercetin diglycoside; 35, lambertianin C; 36, unidentified ellagitannin; 37, quercetin glucuronide; 38, kaempferol hexose-glucuronide and unidentified ellagitannin; 39, kaempferol diglycoside; 40, kaempferol glucuronide.

line with the preliminary field observations made during the selection process of clone 12B14, which indicated tolerance to *P. sparsa*. However, the level of resistance of different cultivars has to be further evaluated in larger field experiments where the resistance of plants is challenged by varying weather conditions and different races of *P. sparsa*. The dry fruits occasionally found in the field, and symptomatic plants found by Lindqvist et al. (*3*), suggest that cv. Mespi is also sensitive to at least some *P. sparsa* races. Field experiments are also needed because high yield losses caused by *P. sparsa* have occurred in boysenberry despite successful protection of the leaves (*5*). Drying of fruits was not evaluated in the present experiment, because natural transmission of the disease and pollination of flowers are not easily achieved under greenhouse conditions.

Identification of Phenolic Compounds from Arctic Bramble Leaves. Phenolic compounds were tentatively identified and quantified from arctic bramble leaves to find compounds that might contribute to the defense against downy mildew. The leaves of a few *Rubus* plants grown in Asia have been studied for ellagitannins; besides that, a detailed profiling of unhydrolyzed phenolics in *Rubus* leaves is lacking. An HPLC chromatogram and representative structures of phenolic compounds from arctic bramble leaves are presented in **Figures 3** and **4**, respectively. All cultivars had qualitatively similar phenolic patterns. Altogether 44 compounds were identified and/or quantified, of which 8 compounds were classified as flavonol glycosides, 18 as ellagitannins, 5 as galloylglucosides, 6 as hydroxycinnamic acid derivatives, and 1 as ellagic acid glucuronide. The remaining 6 compounds were tentatively classified as gallic acid and hydroxycinnamic acid derivatives.

The eight flavonol compounds included four similar glycosides of quercetin and kaempferol. The nature of aglycones was confirmed by comparing the MS fragmentation with that of standard compounds. Peaks 20 and 23a were identified as quercetin and kaempferol triglycosides, respectively, consisting of two hexose (−162 fragment) and one deoxyhexose (−146) sugar moiety. Diglycosides, possibly hexose-deoxyhexoses (−308) (peaks 34 and 39), and glucuronide conjugates (−176) (peaks 37 and 40) of quercetin and kaempferol were also found. Quercetin (peak 29b) and kaempferol (peak 38b) hexose-glucuronides were found only in trace amounts coeluting with other compounds and were thus not quantified. Glucuronides and hexose-deoxyhexose diglycosides have been identified in the fruits of some *Rubus* species (*30–33*), but no previous preference was found for triglycosides and hexose-glucuronide combination.

Ellagitannins typical of many rosaceous plants such as *Rosa*, *Rubus*, and *Fragaria* species constituted the major group of phenolics also in arctic bramble leaves. According to the literature, practically all *Rubus* species studied contain sanguiin H-6, lambertianin C, and lambertianin D as the main ellagitannins in the leaves or fruits (*32, 34*). Sanguiin H-6 (peak 33) and lambertianin C (peak 35) were also detected as doubly charged ions at *m/z* 934 and 1401, respectively, in this study. Additionally, another compound (peak 30) with a molecular weight and fragmentation similar to those of sanguiin H-6 was found but in a clearly smaller quantity and, therefore, peak 33 was regarded as sanguiin H-6 (*32, 34*). Peak 30 may be lambertianin A, which is a structural isomer of sanguiin H-6 previously found in *R. lambertianus* and *R. suavissimus* (*34, 35*). Lambertianin D was not detected, possibly due to its high molecular weight.

Several ellagitannins were characterized as either monomeric or larger fragments of sanguiin H-6 and lambertianin C. Two compounds (peaks 22 and 29a) with molecular weights of 2502, equal to lambertianin C without one hexahydroxydiphenoyl (HHDP) (−302) group, were named deHHDP-lambertianin C. Supporting the identification, fragmentation of peaks 22 and 29a was similar to that of lambertianin C. Similarly, sanguiin H-10, found in peaks 18 and 27 and previously identified from raspberry (*32*), has one HHDP unit (−302) less than sanguiin H-6. The degalloyl (−152) form of sanguiin H-6 was found as two isomers (peaks 16 and 19), which were tentatively identified as roshenin B-like ellagitannins (molecular weight of 1718), previously characterized in *Rosa henryi*, and as desgalloyl-sanguiin H-6 in *Rubus suavissimus* (*35, 36*). Ellagitannins of *Rubus* species and *Sanguisorba officinalis* are typically polymerized via sanguisorboyl coupling between gallic acid and an HHDP unit (*m/z* 469 for released sanguisorboyl unit) that links monomeric ellagitannins to oligomers (*34, 36*) (**Figure 4**); this was also found in oligomeric ellagitannins identified in arctic bramble.

Monomeric ellagitannins with molecular weights of 634 (peak 14), 786 (peaks 24 and 28a), 936 (peak 26), and 938 (peak 32) that correspond to sanguiin H-5, tellimagrandin I, casuarictin/potentillin, and tellimagrandin II, respectively, were present in arctic bramble. Casuarictin/potentillin also occurs as a dimer in sanguiin H-6 and as a trimer in lambertianin C. Casuarictin/potentillin, sanguiin H-5 and both tellimagrandins have been found previously in *Rubus* (*30, 35, 37*). Four unidentified compounds with molecular weights of 2820 (peak 21), 1902 (peak 25), 2494 (peak 36), and 3116 (peak 38a) showing UV spectra typical of ellagitannins were also found.

Five different galloyl esters of glucose with various numbers of galloyl units were also tentatively identified. Peak 1 gave an $[M - H]^-$ ion at *m/z* 663, which fragmented to 483 (−180, glucose) and 331 (−152, gallic acid), corresponding to the galloylglucose dimer. Peak 2 was tentatively identified as methyl

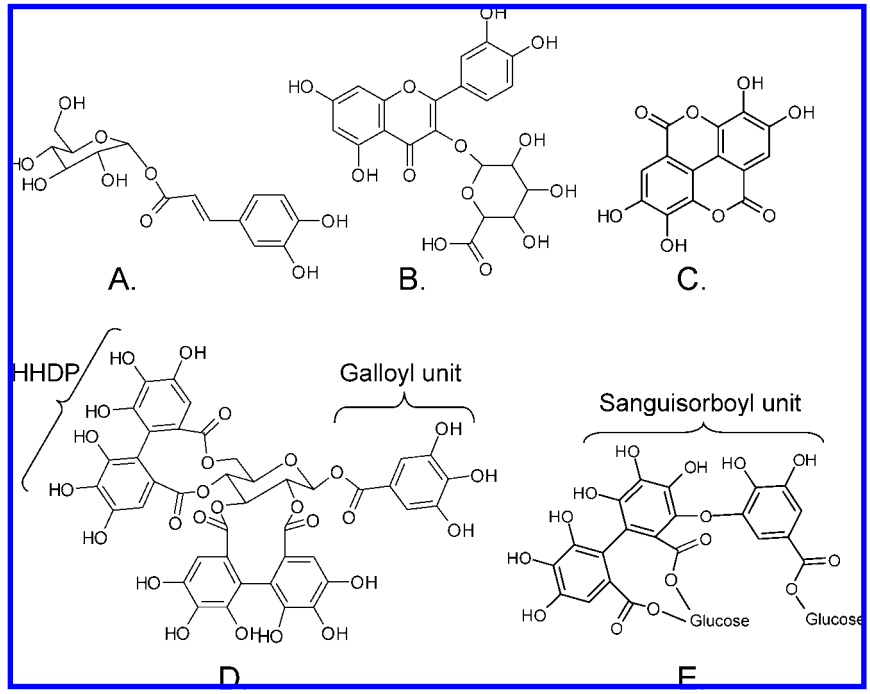

Figure 4. Representative structures of some phenolic compounds and units tentatively identified in arctic bramble leaves: (**A**) caffeoyl glucose; (**B**) quercetin-3-glucuronide; (**C**) ellagic acid; (**D**) casuarictin (galloyl-bisHHDP-glucose); (**E**) sanguisorboyl type coupling between gallic acid and HHDP unit. Released HHDP units are converted into bislactone form, ellagic acid.

gallate-galloylglucose with a molecular weight of 516 and a loss of fragment of 184 (methyl gallate). Digalloylglucose was identified in two peaks (4, 15) at *m/z* 483 and trigalloylglucose in peak 23b at *m/z* 635. Galloylglucoses have not been characterized previously in *Rubus* plants. Among hydroxycinnamic acid derivatives, coumaryl quinic acid (peak 5) and caffeoyl glucose (peak 6) were found; the latter compound was also reported by Määttä-Riihinen et al. (*30*) in arctic bramble fruits. The other hydroxycinnamic acid compounds were considered as caffeic acid (peak 7), *p*-coumaric acid (peaks 11 and 13), and ferulic acid (peak 17) derivatives, which all shared the loss of an unidentified 118 fragment. Peaks 3, 8–10, 12, and 31 could not be identified.

Taken together, several new compounds not previously detected in *Rubus* were tentatively identified and, in particular, more information was obtained on the phenolic composition of the leaves, which has not been studied previously in detail in *Rubus* species cultivated outside Asia. Although the phenolic pattern of arctic bramble leaves resembles that of *Rubus* fruits or, for example, strawberry leaves (*19, 30–32*), several distinguishable compounds were found.

Impact of Agrochemicals and Arctic Bramble Cultivar on the Phenolic Profiles. Arctic bramble genotype or the agrochemical treatments did not affect the qualitative pattern of phenolics in the greenhouse-grown leaves, the same peaks and UV or mass spectra being observed in all samples. However, large quantitative differences were observed in several compounds between the treatments and, in particular, between the cultivars. The concentrations of individual compounds and total phenolics were measured at both the day of inoculation and 8 days after the inoculation. Similar trends were observed at both time points, but the basal level of certain phenolics was increased between the sampling dates. The basal level of ellagitannins was increased 1.5-fold in cv. Pima and Mespi, whereas in 12B14 only flavonols were clearly increased between the sampling dates. These changes were reflected on the total phenolic content, which was significantly higher in 12B14, Mespi, and 0.025 g/L Bion-treated Pima than in Pima controls at the day of inoculation, whereas only a small difference was seen 8 days later. The other treatments, that is, Euparen and phosphite-based Aliette, Phosfik, Phostrol, Farm-Fos, and Kaliumfosfiet, did not affect the concentrations of total or individual phenolics. The following paragraphs describe the results obtained from the samples taken 8 days after inoculation unless indicated otherwise.

Principal component analysis (PCA) was performed to see if the treatments and cultivars were separated on the basis of their phenolic profiles and to determine which individual phenolics mainly contribute to the possible separation. **Figure 5** shows the projection of the regression factor scores of selected treatments and cultivars on PC1 versus PC3 or PC2 versus PC4 plane. In PC2 and PC4, no separate groups were formed from the regression factor scores of different treatments or cultivars. In PC1 and PC3, however, statistically significant differences in the scores between treatments/cultivars were found. In PC1, regression factor scores of cv. Mespi and 12B14 were separated from the other treatments, whereas in PC3, cv. Mespi and 12B14 were separated from each other and 0.2 g/L Bion-treated cv. Pima was separated from other treatments of the same cultivar. On the basis of the factor loadings, the compounds mostly contributing to PC1 were quercetin and kaempferol diglycosides, quercetin and kaempferol glucuronides, sanguiin H-10-like ellagitannins, roshenin B, sanguiin H-6, lambertianin C, and unidentified ellagitannins (peaks 21, 36, and 38). The compounds contributing to PC3 were quercetin and kaempferol triglycosides, tellimagrandin I, unidentified ellagitannin (peak 25), and unidentified hydroxycinnamic acids and *p*-coumaric acid derivatives (peaks 3, 10, 11, and 13). A similar pattern of variation in PCA was observed in the first sampling point, that is, on the day of inoculation (data not shown).

Agrochemicals other than Bion did not significantly affect the phenolic patterns of arctic bramble leaves. As shown in **Figure 5**, the control and Phosfik-treated cv. Pima were not separated in any of the four PCs, and no significant differences were observed in the concentrations of individual compounds.

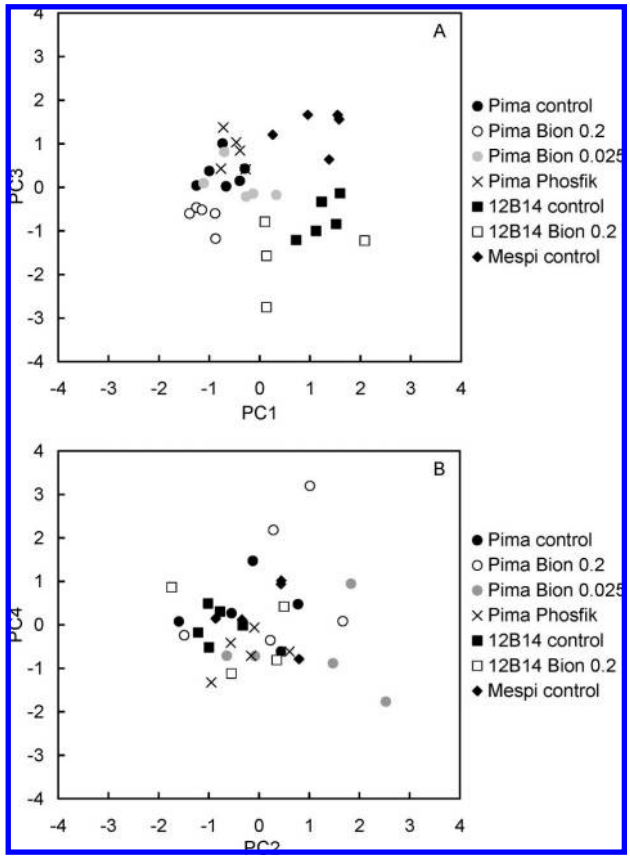

Figure 5. Scatter plot of regression factor scores of principal components (**A**) 1 and 3 and (**B**) 2 and 4. Each point presents results from one plant 8 days after inoculation.

The higher Bion dose (0.2 g/L) caused separation from Pima control in PC3, but a closer analysis of individual compounds revealed only a slight increase in quercetin diglycoside and other flavonols, whereas several compounds were decreased by Bion, namely, unidentified ellagitannins (peak 36 and 38) and hydroxycinnamic acid derivatives (peaks 3, 11, and 13). Similar trends were observed in Bion-treated 12B14 when compared with the 12B14 control. The lower concentration of Bion (0.025 g/L), however, caused the accumulation of several compounds in cv. Pima, albeit no clear separation from the controls was seen in PCA (**Figure 5**). Flavonols and ellagitannins in general were at higher levels in 0.025 g/L Bion-treated plants than in the controls, but casuarictin, both deHHDP-lambertianin Cs, sanguiin H-6-like ellagitannin, tellimagrandin II, di- and trigalloylglucoses (peaks 15 and 23), and caffeic acid derivative (peak 7) were increased in particular.

Each arctic bramble cultivar had a unique phenolic profile, the differences being based on the compounds clustered in PC1 and PC3 (**Figure 5A**). The clone 12B14 and cv. Mespi were separated from cv. Pima control in both PC1 and PC3. Additionally, 12B14 and Mespi were separated from each other in PC3. The difference between Pima and 12B14/Mespi is explained by the higher concentrations of flavonols and ellagitannins in 12B14/Mespi in comparison to those of Pima, flavonols being emphasized in cv. Mespi and ellagitannins in clone 12B14. The difference between 12B14 and Mespi is explained by the higher content of the PC3-contributing compounds in cv. Mespi, namely, flavonol triglycosides, tellimagrandin I, unidentified ellagitannin and hydroxycinnamic acids, and *p*-coumaric acid derivatives (peaks 3, 10, 11, 13, and 25).

Correlation between the Concentrations of Phenolics and the Amount of Pathogen DNA. One of the most interesting aspects of the study, namely, the relationship of phenolics to the growth of *P. sparsa*, was analyzed by calculating the Pearson correlation coefficients between the amount of individual/total phenolics and the percentage of *P. sparsa* DNA. Using the same sample for the extraction of both phenolics and DNA enabled the direct analysis of correlation 8 days after inoculation. The correlations were also analyzed between the phenolic content from the day of inoculation and pathogen DNA from 8 days after inoculation to see if high amounts of preformed phenolics could be reflected as a weaker growth of the pathogen.

A significant negative correlation was found between total flavonols or ellagitannins and pathogen DNA. All individual flavonols showed negative correlation to pathogen DNA particularly 8 days after inoculation, the most significant correlation being found in quercetin ($R = -0.408$*) and kaempferol diglycosides ($R = -0.446$*) and quercetin ($R = -0.424$*) and kaempferol ($R = -0.477$*) glucuronides. Among ellagitannins, sanguiin H-6 ($R = -0.357$*), sanguiin H-10-like ellagitannins ($R = -0.285$*), lambertianin C ($R = -0.339$*), roshenin B ($R = -0.321$*), and unidentified ellagitannin (peak 21; $R = -0.389$*) had a significant negative correlation to the amount of *P. sparsa* 8 days after inoculation. Interestingly, the only ellagitannin showing negative correlation to pathogen DNA already at the day of inoculation was deHHDP-lambertianin C ($R = -0.453$*), which was at the highest level in Bion-treated plants. The total phenolic content also correlated negatively with the amount of *P. sparsa* DNA ($R = -0.322$*) at the day of inoculation but not 8 days later. Thus, the high content of specific phenolics rather than high total phenolic content might contribute to the growth inhibition of the pathogen.

Considering the proportions of phenolics in different treatments and cultivars, the high flavonol content of cv. Mespi might explain at least partly the observed resistance to the *P. sparsa* isolate used in this study. Similarly, the lower dose of Bion induced the accumulation of flavonols and enhanced resistance to *P. sparsa* at the same time. Flavonols have been suggested to act as phytoalexins or constitutive defense compounds against oomycete pathogens in other plant species. Infection by *Phytophthora megasperma* has led to the accumulation of antimicrobial flavonols in olive, whereas negative correlation was found between flavonol/phenolic contents and *Phytophthora*-induced symptoms in cacao (*38, 39*).

Ellagitannins may also play a role in the resistance of arctic bramble to downy mildew, based on the high basal concentrations of these compounds in the genotypes showing higher resistance, the slight induction by Bion, and the negative correlation to the growth of the pathogen. Casuarictin/potentillin and deHHDP-lambertianin C-like ellagitannins are particularly interesting because they were the highest in Bion-treated plants. On the other hand, 12B14 had the highest level of almost all ellagitannins already on the day of inoculation, but this could not stop the infection in the plate test. Antimicrobial and antifeeding properties of ellagitannins have been discovered (*41–43*), but most of the research related to phytopathogens has been conducted on woody plants. Although a high number of ellagitannins and gallotannins have been identified in the fruits and leaves of nonwoody plants, their role in plant pathogenesis and as phytoalexins remains obscure as does the significance of the high number of related molecular structures present in an individual plant. To confirm the activity of ellagitannins and flavonols against *P. sparsa* in arctic bramble, correlations between resistance and specific phenolic compounds should be

evaluated on a wider array of plant genotypes and pathogen races. Antimicrobial activity of isolated compounds could be tested in vitro. It would also be of interest to test whether different pathogen isolates show variable sensitivity to specific phytoalexins as has been observed with camalexin in *Arabidopsis* (*40*).

Even though nearly all agrochemicals tested in the present study provided protection against *P. sparsa*, only 0.025 g/L of Bion clearly induced the accumulation of phenolics. Thus, phosphites as well as the higher Bion concentrations are likely to induce other defense mechanisms such as pathogenesis-related proteins, which might explain the improved resistance to *P. sparsa.* Second, phosphites may also directly act on *P. sparsa*. In strawberry, a high concentration of Bion strongly induced the accumulation of phenolics and enhanced resistance to powdery mildew (*19*), contrasting with the results of the present study. Induction of phenolics by Bion has also been detected in many other plants, such as bean (*44*). However, recent studies indicate that Bion induces a wide variety of biochemical changes in plants, increased production of secondary compounds being just one means of defense activation (*45*).

In conclusion, quantitative information was obtained about the efficacy of registered and new agrochemicals, which might be suitable for the protection of arctic bramble against *P. sparsa*. The cv. Mespi showed rather good resistance to *P. sparsa* in this study, but several *P. sparsa* isolates should be tested to confirm the extent of resistance. According to DNA analyses, *P. sparsa* does not grow in arctic bramble without causing visible symptoms, which facilitates the inspection of the disease in the field. The plate test turned out to be a useful screening tool for the evaluation of the resistance of cultivars and the effect of protective chemicals. Several phenolic compounds not previously reported in arctic bramble or other *Rubus* plants were tentatively identified from the leaves. Negative correlation between the percentage of *P. sparsa* and the content of flavonols and some ellagitannins was found, providing preliminary evidence for the possible involvement of these compounds in the resistance mechanism of arctic bramble to downy mildew.

ABBREVIATIONS USED

ai, active ingredient; HHDP, hexahydroxydiphenoyl; PC(A), principal component (analysis).

Supporting Information Available: Tables showing data used for the identification of phenolic compounds, quantification and statistical data of different phenolic compounds, and factor loadings for different phenolic compounds in four principal components. This material is available free of charge via the Internet at http://pubs.acs.org.

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