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# Benzothiadiazole Induces the Accumulation of Phenolics and Improves Resistance to Powdery Mildew in Strawberries

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Benzothiadiazole (BTH) enhanced the accumulation of soluble and cell-wall-bound phenolics in strawberry leaves and also improved the resistance to powdery mildew infection under greenhouse conditions. The most pronounced change was seen in the levels of ellagitannins, which increased up to 2- to 6-fold 4 days after the BTH application, but persisted only in the inoculated plants. The induction of phenolic metabolism by BTH was also reflected in the fruits, several compounds being increased in inoculated, BTH-treated plants. Basal salicylic acid (SA) content was high in strawberry leaves, but increased in a similar fashion to other phenolics after the treatments. Several phenolic compounds were identified in strawberries for the first time. For example, ellagic acid deoxyhexose, three agrimoniin-like ellagitannins, sanguiin H-10- and lambertianin C-like ellagitannins in the leaves, ellagic acid, *p*-coumaric acid, gallic acid, and kaempferol hexose in the cell-wall-bound fraction of the leaves, and kaempferol malonylglucoside in the fruits. The findings show that BTH can enhance the accumulation of phenolics in strawberry plants which may then be involved in the BTH-induced resistance to powdery mildew.

**KEYWORDS: Cell-wall-bound; *Fragaria* × *ananassa*; ellagitannin; fruit; leaves; salicylic acid; *Sphaerotheca macularis***



## INTRODUCTION

Phenylpropanoids, generally termed phenolics for simplicity, constitute an interesting group of plant secondary metabolites due to their importance not only on plant but also on human health. Phenolics are suggested to be a group of bioactive components in fruits and vegetables, the consumption of which is connected to reduced risk of major diseases such as heart and coronary disease and cancers (*1*, *2*). From the perspective of plant defense against pathogens, phenolics may act as soluble antimicrobial or antifeeding compounds or they may cross-link with callose, proteins, and polysaccharides into the cell walls, thus inhibiting fungal penetration and the absorption of nutrients by the invading fungus (*3*−*6*). This has been found critical, for example, in the resistance of barley and *Arabidopsis* to powdery mildew disease (*6*, *7*).

Enhancement of phenolic metabolism of plants has been often connected with the induced resistance phenomenon. Exploitation of induced resistance in plant protection has become a promising approach against several bacterial, fungal, and viral plant diseases during the past decade as the application of certain nontoxic chemicals may activate the endogenous defense mechanisms of plants, providing long-lasting, wide-spectrum resistance (see refs *8* and *9* for review). What ultimately causes the improved resistance varies, since a different set of secondary metabolites and pathogenesis-related (PR) proteins may be synthesized depending on the plant family and species. However, the accumulation of soluble or cell-wall-bound phenolics has been observed along with enhanced resistance to pathogens in many plants (e.g., *4*, *6*, *10*). Benzothiadiazole (BTH), the functional analogue of salicylic acid, has been found to strongly induce defense reactions and production of phenolics (*6*, *10*, *11*).

During the past 10 years, strawberry (*Fragaria* × *ananassa* Duch.) production has increasingly been transferred from the fields into covered systems. Today about 50% of strawberries are produced under glass in many European countries, which has also led to persistent powdery mildew (*Sphaerotheca macularis* Wallr. ex Fr.) problems, since the humid greenhouse environment is highly favorable to the pathogen (*12*), overriding resistance observed in the field. The control of powdery mildew

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with fungicides is problematic since only a limited number of appropriate fungicides is available and powdery mildew fungi have a high tendency of developing fungicide-tolerant strains as observed with other crops (e.g., *13*, *14*). In the strawberry, BTH has been used to control gray mold and *Phytophthora* (*15*, *16*) but not powdery mildew despite the importance of the disease and the efficacy of BTH found against powdery mildews in other plant species (*17*, *18*).

Fruits of rosaceous plants are known as rich sources of phenolics (*19*, *20*), but little is known about the phenolic composition of their leaves, which may have more impact on health of the whole plant and disease resistance than do the fruit phenolics. Furthermore, few studies have been conducted on the changes of secondary metabolites in edible plant parts in any crop species after BTH treatment, although it might have an influence on the nutritional quality of the crop (*21*, *22*).

The aims of this study were (i) to characterize soluble and cell wall-bound phenolic compounds present in the leaves and fruits of strawberries cv. Jonsok, (ii) to follow their concentration after BTH treatment and/or powdery mildew infection, and (iii) to evaluate the efficacy of BTH treatment to control powdery mildew in greenhouse conditions.

## MATERIALS AND METHODS

**Plant Material.** Micropropagated plants of strawberries cv. Jonsok were used in the main experiment (experiment 1) performed in the greenhouse of the Research Garden of the University of Kuopio in 2004. Frigo plants of cv. Jonsok (bare-root, cold-stored), which give crops within 6−8 weeks after planting, were used in the second experiment (experiment 2) in 2005, which was established to obtain fruits for the analysis of phenolic compounds after BTH treatment. Both types of plants were grown in 12-cm pots in a peat-sand mixture (3:1) and were fertilized weekly with Superex-9 fertilizer (N 19%, P 5%, K 20%) supplied with micronutrients (Kekkilä, Finland). The following growth conditions were used: relative humidity 50−70% (night-day), daylight 20 h, and temperature 17−22 °C (night-day). In experiment 2, the daylight period was 24 h. Plants were grown until they had at least three fully developed, normal size leaves before starting the experiments.

**Preparation of Inoculum.** The powdery mildew population used in the inoculations originated from strawberries cv. Jonsok previously grown in the same greenhouse and was maintained on young, susceptible plants of cv. Jonsok. No uncontrolled powdery mildew infections occurred in the greenhouse during the experiments. Inoculations were performed by shaking fresh, spore-containing leaves above the plants. Spore density was evaluated microscopically by examining glass plates placed among the plants.

**Experimental Design.** The plants from experiment 1 were numbered and divided randomly into four different groups (100 plants in each group), which were placed on separate tables to prevent spreading of contamination from the other treatments. Two groups were sprayed with distilled water and the other two groups with 0.4 g $L^{-1}$ of active BTH (Bion 50WG, Syngenta) in distilled water until runoff (ca. 5 mL per plant). After 2 days, one group of water- and BTH-treated plants was inoculated with powdery mildew, whereas the other groups were left untreated. The BTH concentration of 0.4 g $L^{-1}$ was selected on the basis of preliminary experiments where concentrations above 0.3 g $L^{-1}$ gave better protection against powdery mildew than lower concentrations (data not shown). Strawberry plants have also shown tolerance to at least 2 g $L^{-1}$ of active BTH in other studies (*15*).

Samples were collected 0, 2, 4, and 7 days after inoculation, i.e., 2, 4, 6, and 9 days after water/BTH treatment. For each laboratory analysis, five different plants per treatment were randomly selected at every sampling time and one young, full-size leaf per plant was collected separately in liquid nitrogen. Each plant was sampled only once to avoid an eventual cutting-effect. The samples were stored at −80 °C. All results presented in this paper are from experiment 1, except for those done on fruits.

Powdery mildew infection was evaluated from 30 control and 30 BTH plants 8, 11, 15, 20, 25, and 27 days after inoculation. From each plant, the total number of infected and healthy leaves was counted, and the number of powdery mildew patches and diseased areas were determined from two leaves selected prior to inoculation. Diseased area was estimated as $mm^2$ using a piece of graph paper.

Experiment 2 was carried out similarly to the first one with a few exceptions. The frigo plants were treated with water and BTH twice, i.e., at the beginning of flowering and 12 days after the first treatment. Spore density on the leaves of the inoculated plants was ca. 90 per $cm^2$. Since insect pollinators were not present in the greenhouse, flowers were hand-pollinated with a small brush. Ripe fruits were harvested and weighed separately from each plant and stored at −20 °C. After cropping, leaves were also cut off and weighed (fresh weight). The weight of fruits and foliage was determined from 18 and 35 plants per treatment, respectively.

**Extraction of Phenolic Compounds.** For the analysis of soluble phenolics, frozen leaves were weighed and extracted three times with 10 mL of 70% acetone in water by homogenizing with an Ultra-Turrax for 1 min. After centrifugation at 5000*g* for 5 min, the supernatants were combined and acetone was evaporated in a vacuum centrifuge at room temperature. The concentrate was made up to 10 mL with distilled water, and 2.5 mL of methanol was added to obtain a final methanol concentration of 20%. The extracts were stored at −20 °C.

For the analysis of NaOH-hydrolyzable cell-wall-bound phenolics, the remaining pellet from the extractions was washed once with 70% acetone, 50% methanol, and 100% methanol to remove traces of residual soluble phenolics. The hydrolysis was carried out as previously described (*6*) with following modifications: 1 mL of 1 M NaOH was added per 20 mg of pellet, and the hydrolysis was carried out at 70 °C for 1 h. The acidified hydrolysate was extracted twice with an equal volume of ethyl acetate by shaking vigorously for 10 min. Dried samples were stored at −20 °C prior to HPLC (high-performance liquid chromatography) analysis. Cell-wall-bound phenolics, particularly ether-linked compounds, are not easily released from the cell wall material, and strong alkali hydrolyzes are used for the extraction (*5*, *23*, *24*). This work focused on phenolics bound with ester bonds to polysaccharides in the cell walls and not on the tightly bound polymers such as lignin. Hydrolysis time and conditions were adjusted to maximize the yield of total phenolics. The yield increased rapidly during the first hour of hydrolysis, clearly smaller amounts being gradually released for at least 24 h, after which the test was stopped. The compounds released after the first hour were considered to derive from ether-linked material or polymers (*23*, *24*). Recovery of the standard compounds gallic acid, *p*-coumaric acid, ferulic acid, hydroxybenzoic acid, and rutin was analyzed by adding 0, 20, and 40 $\mu$g of each compound together with NaOH into tubes containing 10 mg of dried, homogeneous pellet and calculating the yield after the treatment with NaOH and ethyl acetate extraction. Recoveries above 90% were achieved for *p*-coumaric (91%), ferulic (91%), and hydroxybenzoic acid (98%) in contrast to gallic acid, rutin, and catechin, which degraded rapidly during the hydrolysis or were not extracted into ethyl acetate. However, gallic acid and kaempferol derivatives were detected in the samples, indicating that at least some of the compounds remained intact. The gallic acid detected may also have been released from a derivative, which is more resistant to alkali. Either way, similar degradation was assumed to occur in all samples.

Phenolics and soluble solids (as % Brix) from the fruits were extracted as previously described (*25*). The homogenate consisted of fruits from one plant, of which a subsample of 5 g was weighed. Fruits from eight plants per treatment were analyzed. An aliquot (12 mL) of the total acetone extract (50 mL) was concentrated to less than 4 mL by vacuum evaporation, after which 1 mL of methanol was added (20% final concentration) and the extract was made up to 5 mL with distilled water. The extracts were analyzed by HPLC without further storage.

**Analysis of Phenolic Compounds.** Total phenolic content of all phenolic extracts was determined with the Folin−Ciocalteu method (*26*) and measured from the HPLC samples, except for the NaOH-hydrolyzed total phenolics, which were analyzed from the acidified hydrolysate before ethyl acetate extraction. Results are expressed as milligrams of gallic acid equivalents per gram of fresh leaves or fruits. Individual

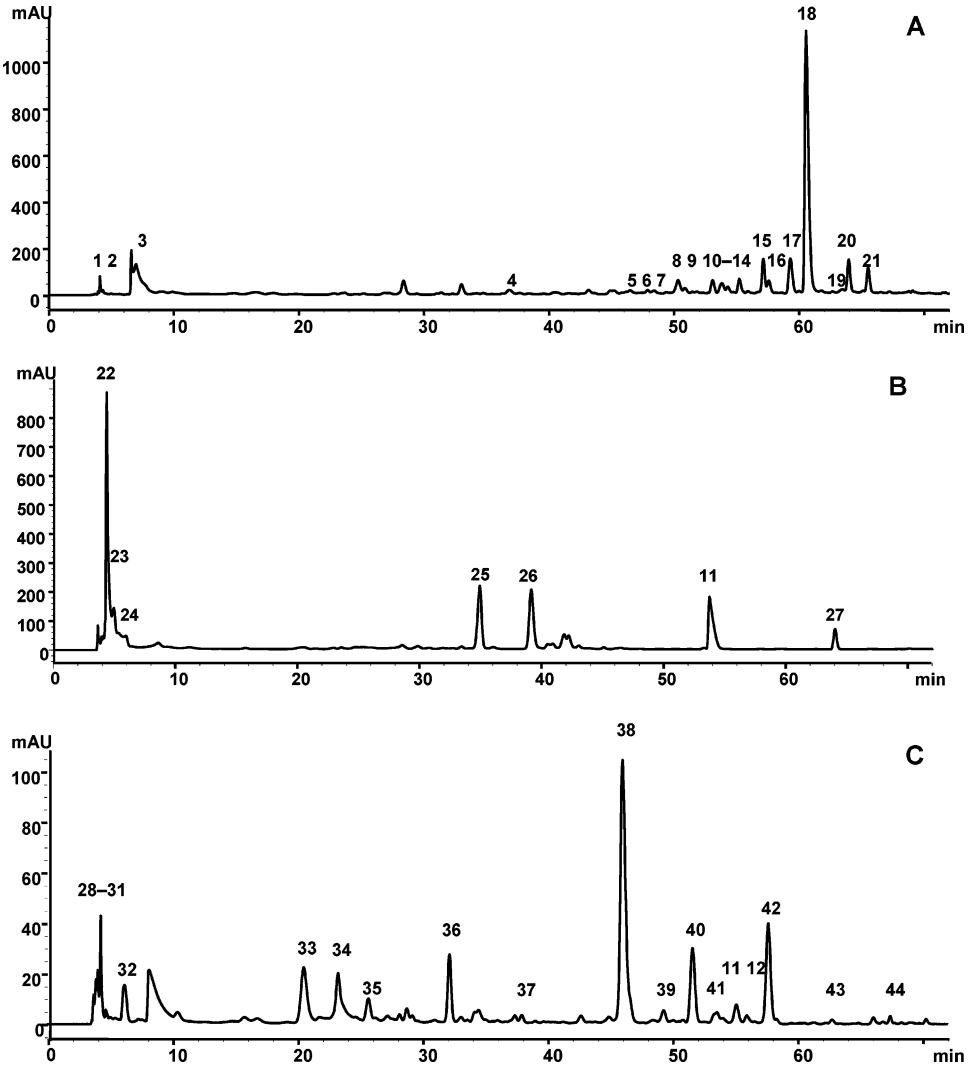


**Figure 1.** Typical HPLC chromatograms at 280 nm of (**A**) soluble leaf phenolics, (**B**) cell-wall-bound leaf phenolics, and (**C**) fruit phenolics run under similar conditions. The numbers refer to compounds listed in **Tables 2**–**4**.



phenolic compounds were analyzed by HPLC as previously described (*25*). Soluble leaf and fruit phenolics were analyzed directly from the extracts in 20% methanol. NaOH-hydrolyzed leaf phenolics were analyzed from the samples dissolved in 0.7 mL of 50% methanol. The following gradient of acetonitrile (B; % v/v) in 1% formic acid (A) was used to separate phenolic compounds: 0−10 min, 0−0% B; 10−60 min, 0−15% B; 60−70 min, 15−25% B; 70−80 min, 25−100% B; 80−83 min, 100% B; 83−90 min, 100−0% B; 90−97 min, 0% B. The following standard compounds were used in the quantification: catechin for flavan-3-ols or similarly absorbing compounds, *p*-coumaric acid for hydroxycinnamic acids, cyanidin chloride for anthocyanins, ellagic acid for ellagic acid derivatives and ellagitannins, gallic acid for phenolic acids and other compounds absorbing around 280 nm, and either kaempferol, quercetin, or rutin for flavonols. It should be recognized that the concentrations of different compounds, particularly tannins and other phenolics, cannot be compared with each other since they are relative to the specific standards used. For example, ellagic acid used in the quantification of tannins gives an underestimation of their concentration.

The main peaks in the phenolic profiles were further identified by mass spectrometry (MS). The HPLC conditions were as described above. The MS system consisted of a Finnigan LTQ linear ion trap mass spectrometer (San Jose, CA), a Finnigan Surveyor HPLC pump, and an autosampler, and a UV detector (Uvikon 735 LC, Kontron Instruments) set at 280 nm. The mass data were collected in negative ionization mode, scanning full mass spectra at *m*/*z* 150−2000 for leaf samples and at *m*/*z* 250−700 for fruit samples. Fruit samples were also analyzed in positive ionization mode. The following conditions were used: capillary temperature 280 °C, capillary voltage −10 V, and needle voltage −4.5 kV. The collision energy of 35 V was used to obtain $MS^2$ and $MS^3$ daughter ions from the most abundant parent ions.

**Determination of Salicylic Acid.** Salicylic acid was analyzed according to the method described by Meuwly and Métraux (*27*).

**Statistical Analysis.** Statistical analyses were performed with SPSS 11.5 for Windows (SPSS Inc.). Means of different treatments were compared with the analysis of variance (ANOVA) using Tukey's post hoc test when a significant difference at $P < 0.05$ or tendency (only for leaf phenolics) at $P < 0.1$ was found among the groups. Means were derived from at least five parallel analyses, each from a different plant.

## RESULTS AND DISCUSSION

**Identification of Soluble Leaf Phenolics.** Typical chromatograms from the HPLC analyses are presented in **Figure 1**. The peaks are numbered in the order of elution, and their constituent compounds were identified based on the UV spectrum, MS analysis, and available literature (for details, see Tables 1−3 in the Supporting Information). Only those compounds present in significant quantities or those that changed in the treatments were identified. In contrast to fruits, fairly limited data are available, and this study provides thus new information about the phenolic, and particularly ellagitannin, composition of strawberry leaves.

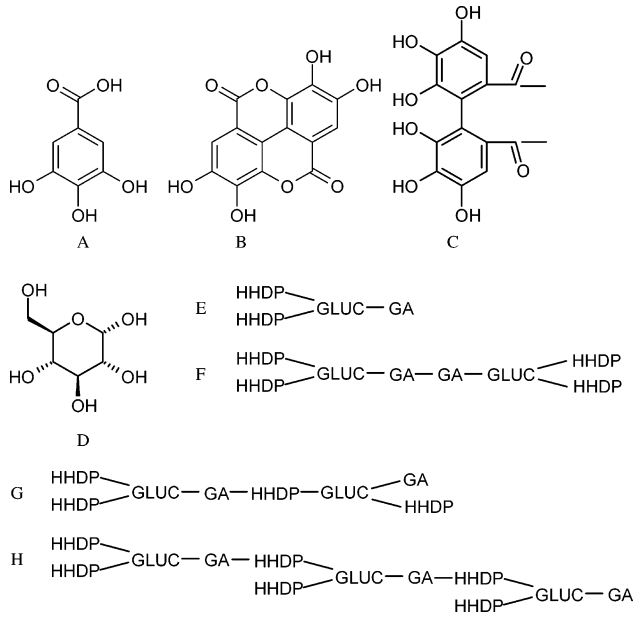


**Figure 2.** Structures of some ellagitannins and their subunits. (A) Gallic acid, (B) ellagic acid, (C) hexahydroxydiphenoyl unit, (D) D(+)-glucose, (E) casuarictin, (F) agrimoniin, (G) sanguiin H-6, and (H) lambertianin C. GA, gallic acid; Gluc, glucose; HHDP, hexahydroxydiphenoyl.

The majority of the compounds found in soluble form in strawberry leaves were identified as ellagitannins (ET) consisting of different numbers of galloyl and hexahydroxydiphenoyl (HHDP) units esterified with glucose (**Figure 2**). The composition of ETs was determined by analyzing the fragmentation patterns where a loss of 170 or 152 ($-$ $H_2O$) mass units corresponds to galloyl, 302 to HHDP, and 180 or 162 ($-$ $H_2O$) to glucosyl units. With few exceptions, all tannins were found as doubly charged molecular ions, which is typical for electrospray ionization of tannins (*28*). For an unknown reason, isotope patterns did not strictly follow the 0.5 mass unit interval characteristic for doubly charged ions but, since further fragmentation ($MS^2$ and $MS^3$) resulted in larger ions than were found in full MS, and isotope differences were below 1.0, those molecular ions were considered as $[M - 2H]^{2-}$.

Tannins consisting of only a few subunits were identified unequivocally as galloyl-diHHDP-glucose present as two isomers, casuarictin (peak 5a) and potentillin (peak 15a), which have previously been found in strawberries by Oertel et al. (*29*) (**Figure 1A**). The molecular ion at *m*/*z* 935 fragmented to daughter ions at *m*/*z* 633 ($-$ HHDP), 463 ($-$ galloyl) and 301 ($-$ glucose; $-$ $H_2O$). The main peak in the chromatogram (peak 18) was identified as agrimoniin, consistent with earlier findings (*29*, *30*). The fragmentation also matches sanguiin H-6, which has similar subunit composition and molecular weight (**Figure 2**) (*31*), but the compound was identified as agrimoniin according to the previous identification, which was based on coelution with an authentic standard (*29*, *30*). Peaks 9 and 19 gave a similar doubly charged molecular ion at *m*/*z* 934 and fragment ions as agrimoniin, and therefore one of these two compounds may be akin to sanguiin H-6 or another tetrameric ET. The molecular weight of 1568 and fragment ions at *m*/*z* 1265, 935, 633, and 301 of peak 6 suggest that it could be similar to sanguiin H-10, identified by Mullen et al. in raspberries (*31*). Likewise, fragments from peaks 7 (molecular weight 1718) and 20 (molecular weight 2804) resembled those of nobotanin A- and lambertianin C-like compounds, respectively, found in raspberries (*31*), although the patterns were not fully identical. Peak 8 gave a $[M - 2H]^{2-}$ ion at *m*/*z* 617 (apparent molecular weight 1236) with $MS^2$ fragments at *m*/*z* 933 ($-$ HHDP), 631 ($-$ HHDP) and 301. The fragmentation suggests some structural similarity to casuarictin (peak 5a) and potentillin (peak 15a), but further work is required to fully define the structure of this ET. Peaks 4, 13, 16, and 21 were putatively identified as ETs based on their typical UV spectra and MS fragments, the spectra of compounds 16 and 21 being identical, but they could not be characterized further.

Besides the ellagitannins, three compounds with the typical UV spectrum of flavonols were found. Peak 10 was identified as quercetin hexose-deoxyhexose (not rutin) and 15b as quercetin glucuronide while peak 17 gave a weak MS signal and was not identified. Ellagic acid (EA) (peak 11) and its glycosides, namely EA hexose (peak 5b), EA pentose (peak 12), EA deoxyhexose (peak 14), and an unknown EA derivative (peak 1) were also found. Peak 2 gave a molecular ion at *m*/*z* 191 and $MS^2$ fragments at *m*/*z* 171 and 111, corresponding to quinic acid. Compound 3, and many of the more early eluting hydrophilic compounds in all extracts (fruits, leaves), could not be identified because many of them coeluted between 3 and 4 min and ionization is not easily achieved without volatile organic solvent in the mobile phase. Among the other compounds, some other ETs, flavan-3-ols, and hydroxycinnamic acids such as chlorogenic acid, were identified, but they were present at low concentration and did not alter in response to BTH or powdery mildew treatments.

**Identification of Cell-Wall-Bound Leaf Phenolics.** A few compounds were identified among NaOH-hydrolyzable phenolics extracted into ethyl acetate. Gallic acid (peak 24 in **Figure 1B**) was identified by comparison to retention time, UV spectra, and MS properties with the standard (molecular ion at *m*/*z* 169 and a fragment ion at *m*/*z* 125). Kaempferol hexose (peak 27) was also found, with a molecular ion of 447 and aglycone of 285 ($-$ 162). Similarly to soluble phenolics, EA (peak 11) was found as a dimeric ion $2[M - H]^-$ at *m*/*z* 602 with identical retention and UV spectrum to EA standard. *p*-Coumaric acid (peak 26) did not ionize but was identified by its UV spectrum and retention time compared to standard. Peaks 22, 23, and 25 were not identified. Cell wall phenolics have not been previously isolated from strawberries, but hydroxycinnamic acids, such as coumaric and ferulic acid, and flavonols have been found in cell-wall extracts of other plants (*6*, *32*). We are not aware of any previous reports on ellagic acid located in the cell walls of nonwoody plants.

**Identification of Fruit Phenolics.** Much literature is available about the phenolic compounds of strawberry fruits (e.g., *19*, *33*, *34*). Consequently, the following compounds were readily identified: two flavan-3-ols resembling catechin (peaks 33 and 35 in **Figure 1C**) and a flavan-3-ol dimer (peak 34), *p*-coumaryl glucoside (peak 36), pelargonidin 3-glucoside (peak 38), pelargonidin 3-malonylglucoside (peak 42), quercetin 3-glucuronide (peak 41), and kaempferol 3-glucuronide (peak 43) (*19*, *33*, *34*). A new compound, kaempferol malonylglucoside (peak 44), was tentatively identified on the basis of the $[M - H]^-$ molecular ion at *m*/*z* 533 with further cleavage of 44 and 204 fragments (cleavage of 248 in positive mode), which have been found typical for the fragmentation of malonylglucosides (*35*). However, peaks 28−32, 35a, 37, 39, and 40 could not be identified.

**Effect of BTH on Soluble Leaf Phenolics.** BTH induced the accumulation of soluble phenolic compounds in strawberry leaves (**Tables 1** and **2**). A statistically significant difference in the total soluble phenolic content was seen between the inoculated controls and inoculated BTH-treated plants 7 days after inoculation ($P = 0.013$). Similarly, high amounts of

**Table 1.** Soluble and NaOH-Hydrolyzable Total Phenolics in Strawberry Leaves after Treatment with BTH[a] and Powdery Mildew Conidia

| phenolic fraction | days after inoculation[a] | control | inoculated control | BTH | inoculated BTH |
|---|---|---|---|---|---|
| soluble TP | 2 | 15.6 ± 1.8[b] | 17.0 ± 2.8 | 23.1 ± 5.7 | 20.3 ± 5.8 |
| | 7 | 16.4 ± 4.0 ab[c] | 13.8 ± 5.5 a | 17.8 ± 4.4 ab | 23.5 ± 2.8 b |
| cell-wall-bound TP | 2 | 2.2 ± 0.3 | 2.6 ± 0.3 | 2.7 ± 0.7 | 2.8 ± 0.5 |
| | 7 | 3.1 ± 0.4 a | 3.4 ± 0.4 ab | 4.2 ± 0.6 bc | 4.5 ± 0.7 c |

[a] BTH was applied on the plants 2 days before the inoculation. [b] Concentrations are expressed as mg $g^{-1}$ fresh weight. [c] Means followed by different letters within each row and sampling date were significantly different at $P < 0.05$ (Tukey).

**Table 2.** Soluble Phenolics in Strawberry Leaves Quantified with HPLC after Treatment with BTH and Powdery Mildew Conidia

| peak no. | compound | days after inoculation | control | inoculated control | BTH | inoculated BTH |
|---|---|---|---|---|---|---|
| 1 | EA derivative | 2 | 7 ± 2[a,b] | 7 ± 1 | 8 ± 2 | 6 ± 1 |
| | | 7 | 8 ± 1 a* | 7 ± 1 a** | 9 ± 1 ab | 10 ± 2 b |
| 2 | quinic acid | 2 | 79 ± 14 ab | 69 ± 10 a** | 112 ± 29 b | 86 ± 25 ab |
| | | 7 | 93 ± 30 | 77 ± 34 | 90 ± 28 | 128 ± 35 |
| 3 | unknown | 2 | 1716 ± 850 | 1152 ± 229 | 1242 ± 479 | 934 ± 233 |
| | | 7 | 1246 ± 325 | 1271 ± 318 | 866 ± 177 | 902 ± 310 |
| 4 | unidentified ET | 2 | 6 ± 4 | 9 ± 10 | 20 ± 19 | 14 ± 9 |
| | | 7 | 16 ± 11 ab | 9 ± 9 a** | 10 ± 5 a* | 28 ± 15 b |
| 5 | casuarictin/EA hexose | 2 | 31 ± 3 | 32 ± 8 | 45 ± 16 | 38 ± 6 |
| | | 7 | 50 ± 13 ab | 41 ± 14 a* | 44 ± 5 ab | 61 ± 14 b |
| 6 | sanguiin H-10 | 2 | 5 ± 4 | 4 ± 3 | 10 ± 8 | 12 ± 10 |
| | | 7 | 12 ± 8 ab | 7 ± 7 a* | 6 ± 4 a* | 19 ± 8 b |
| 7 | nobotanin A-like ET | 2 | 5 ± 4 | 4 ± 3 | 14 ± 11 | 16 ± 14 |
| | | 7 | 10 ± 8 ab | 6 ± 6 ab | 5 ± 4 a* | 17 ± 10 b |
| 8 | unidentified ET | 2 | 29 ± 26 | 25 ± 14 | 88 ± 76 | 73 ± 53 |
| | | 7 | 69 ± 34 ab | 42 ± 43 a* | 37 ± 29 a* | 106 ± 52 b |
| 9 | agrimoniin-like ET | 2 | 8 ± 10 | 4 ± 4 | 38 ± 33 | 32 ± 40 |
| | | 7 | 12 ± 10 ab | 7 ± 9 ab | 4 ± 5 a* | 19 ± 11 b |
| 10 | quercetin hexose-deoxyhexose | 2 | 251 ± 92 | 354 ± 103 | 561 ± 341 | 377 ± 161 |
| | | 7 | 332 ± 144 a* | 271 ± 166 a* | 386 ± 163 ab | 562 ± 34 b |
| 11 | EA | 2 | 72 ± 36 | 82 ± 21 | 153 ± 91 | 132 ± 67 |
| | | 7 | 93 ± 39 ab | 71 ± 53 a** | 96 ± 23 ab | 150 ± 35 b |
| 12 | EA pentose | 2 | 85 ± 7 | 94 ± 15 | 101 ± 20 | 100 ± 16 |
| | | 7 | 113 ± 14 a** | 108 ± 28 a** | 125 ± 12 ab | 151 ± 22 b |
| 13 | unidentified ET | 2 | 14 ± 10 | 12 ± 10 | 32 ± 29 | 31 ± 16 |
| | | 7 | 25 ± 17 ab | 13 ± 13 a* | 14 ± 8 a* | 44 ± 30 b |
| 14 | EA deoxyhexose | 2 | 23 ± 2 | 25 ± 4 | 32 ± 10 | 35 ± 15 |
| | | 7 | 31 ± 5 a* | 29 ± 9 a** | 34 ± 4 ab | 41 ± 5 b |
| 15 | potentillin/quercetin glucuronide | 2 | 36 ± 26 | 31 ± 15 | 104 ± 89 | 88 ± 50 |
| | | 7 | 67 ± 32 ab | 44 ± 42 a* | 44 ± 32 a* | 118 ± 54 b |
| 16 | unidentified ET | 2 | 13 ± 11 | 12 ± 8 | 38 ± 31 | 36 ± 25 |
| | | 7 | 26 ± 13 ab | 19 ± 18 a* | 17 ± 13 a** | 47 ± 18 b |
| 17 | unidentified flavonol | 2 | 446 ± 191 | 743 ± 263 | 912 ± 438 | 830 ± 210 |
| | | 7 | 623 ± 284 ab | 533 ± 404 a* | 689 ± 333 ab | 1086 ± 199 b |
| 18 | agrimoniin | 2 | 304 ± 277 | 239 ± 137 | 1016 ± 879 | 872 ± 713 |
| | | 7 | 585 ± 321 ab | 366 ± 378 a* | 364 ± 306 a* | 1054 ± 543 b |
| 19 | agrimoniin-like ET | 2 | 4 ± 4 | 5 ± 4 | 13 ± 9 | 19 ± 22 |
| | | 7 | 13 ± 8 | 13 ± 13 | 11 ± 6 | 26 ± 9 |
| 20 | lambertianin C-like ET | 2 | 15 ± 11 | 13 ± 6 | 96 ± 81 | 84 ± 80 |
| | | 7 | 45 ± 35 ab | 21 ± 24 a* | 21 ± 18 a* | 90 ± 65 b |
| 21 | unidentified ET (similar to peak 16) | 2 | 14 ± 15 | 11 ± 9 | 68 ± 59 | 60 ± 53 |
| | | 7 | 41 ± 26 ab | 22 ± 23 a* | 21 ± 20 a* | 70 ± 43 b |

[a] Concentrations of phenolic compounds are expressed as $\mu$g $g^{-1}$ fw. Concentrations are relative to the standard compound used, and can only be compared between the treatments. [b] Means followed by different letters within each row and sampling date were significantly different at $^{**}P < 0.05$ or $^{*}P < 0.10$ (Tukey).

phenolics were found in noninoculated BTH-treated plants ($P = 0.070$) already 2 days after inoculation (i.e., 4 days after BTH application) (**Table 1**). In noninoculated BTH plants, however, the concentration of phenolics dropped to the same level as in the controls over the 7 days. Similar transient induction by BTH has been observed in cowpea (*11*). In our study, infection without BTH treatment did not affect the phenolics. The total phenolic content (ca. 2% of leaf fresh weight) was clearly higher than the concentrations measured with HPLC. The difference between the two methods may be explained by the use of standard equivalents, which may skew the concentrations, or nonphenolic compounds may have interfered with the Folin−Ciocalteu assay (*36*) and some phenolic compounds may not have been quantified by HPLC, further magnifying the difference between the two methods.

Similar but more pronounced trends were seen in the concentrations of individual soluble phenolics (**Table 2**). The strongest tendency was seen for all 13 ellagitannin compounds (compounds 4−9, 13, 15, 16, 18−21), which comprise most of the soluble phenolics in strawberry leaves. Two days after inoculation, BTH-treated plants had 2−3 times (or, in the case of late-eluting ETs such as lambertianin C-like ET, even 4−6 times) higher concentrations of these tannins than did the controls (**Table 2**). Interestingly, the high concentration was

**Table 3.** Cell-Wall-Bound Phenolics in Strawberry Leaves Quantified with HPLC after Treatment with BTH and Powdery Mildew Conidia

| peak no. | compound | days after inoculation | control | inoculated control | BTH | inoculated BTH |
|---|---|---|---|---|---|---|
| 22 | unknown | 2 | 70 ± 17 a[a,b] | 84 ± 6 ab | 108 ± 11 b | 93 ± 16 ab |
| | | 7 | 98 ± 16 a | 110 ± 15 ab | 140 ± 13 bc | 154 ± 23 c |
| 23 | unknown | 2 | 17 ± 4 a | 20 ± 3 ab | 26 ± 2 b | 22 ± 3 ab |
| | | 7 | 24 ± 4 a | 28 ± 3 ab | 36 ± 6 b | 38 ± 4 c |
| 24 | gallic acid | 2 | 14 ± 3 | 15 ± 1 | 15 ± 2 | 13 ± 2 |
| | | 7 | 15 ± 4 | 19 ± 5 | 21 ± 5 | 22 ± 4 |
| 25 | unidentified flavonol | 2 | 33 ± 10 a | 40 ± 4 ab | 54 ± 14 b | 43 ± 9 ab |
| | | 7 | 46 ± 8 | 48 ± 7 | 60 ± 14 | 56 ± 9 |
| 26 | *p*-coumaric acid | 2 | 30 ± 6 | 25 ± 6 | 30 ± 5 | 31 ± 9 |
| | | 7 | 27 ± 2 | 29 ± 2 | 29 ± 6 | 31 ± 7 |
| 11 | EA | 2 | 57 ± 14 | 65 ± 11 | 80 ± 16 | 74 ± 9 |
| | | 7 | 77 ± 10 | 86 ± 21 | 91 ± 17 | 85 ± 12 |
| 27 | kaempferol hexose | 2 | 19 ± 4 | 13 ± 3 | 17 ± 3 | 18 ± 5 |
| | | 7 | 17 ± 2 | 18 ± 2 | 17 ± 2 | 20 ± 5 |

[a] Concentrations of phenolic compounds are expressed as $\mu$g g$^{-1}$ fw. [b] Means followed by different letters within each row and sampling date were significantly different at $P < 0.05$ (Tukey).

maintained only in inoculated but not in noninoculated BTH-treated plants for 7 days, as was observed for the total phenolic content.

Other phenolic compounds followed slightly different patterns (**Table 2**). While EA aglycone, quercetin hexose-deoxyhexose, and other flavonols showed a similar pattern of accumulation to the tannins, EA derivatives (compounds 1, 5, 12, 14) did not accumulate to the same extent. There may be competition between the synthesis of ETs and other EA derivatives, with a bias toward ETs, which may explain the weaker induction of EA glycosides. The only compound that decreased after BTH treatment was compound 3, which unfortunately could not be identified. Catechin, which has previously been characterized as an antimicrobial compound in strawberries (*37*), was not present in significant quantities in the leaves of cv. Jonsok and was not increased by infection or BTH treatment. Despite the variation between plants, the trends were similar in practically all compounds at both time points, showing that BTH induces the accumulation of phenolic compounds, particularly ETs, in strawberries.

Ellagitannins are known to inhibit herbivores and act as antimicrobial barriers in woody plants (*3*, *28*), but no information is available about their influence on phytopathogens and, particularly, on biotrophs such as powdery mildews in nonwoody plants. The high total content of ETs and the subsequent induction after BTH treatment, leading to enhanced resistance, implies that ETs may be important for the resistance to powdery mildew in strawberries, the basis of which is yet mostly unknown. The induction of other soluble phenolics such as flavonols and hydroxycinnamic acid derivatives has been observed upon powdery mildew infection in other plants, which may indicate that phenolic compounds are important in the resistance to powdery mildews at least in some plant species (*4*, *5*, *38*). Soluble phenolic compounds may be toxic to microbes in several ways, including the disruption of microbial membranes, by binding to proteins or substrates or by affecting enzyme activities (*39*). However, generalizations about the roles which certain phenolic compounds or classes play in defense should be drawn with caution. Plants produce constitutively a great variety of compounds, which is yet widened upon pathogen attack, and thus sorting out the most active individual compound may be difficult.

**Effect of BTH on Cell-Wall-Bound Phenolics.** Total cell-wall-bound phenolics followed the same pattern as soluble phenolics but with overall lower concentrations (**Table 1**). The accumulation of phenolics was detected in BTH-treated plants and, particularly, in the inoculated and BTH-treated plants, resulting in a 1.5-fold increase compared to that in the controls 7 days after inoculation. The total amount of phenolics also increased in the controls with the aging of leaves. The actual proportion of cell wall phenolics may be higher, as Röpenack et al. (*5*) observed that papilla phenolics become resistant to alkaline extraction upon powdery mildew infection in barley. Cross-linking of phenolics and other insoluble material into the cell walls may affect the penetration efficiency of fungi, as found for powdery mildew fungi in different hosts (*5*−*7*). Hence, the changes in phenolics bound to the cell walls of strawberry leaves are likely to be involved in the development of resistance.

While measuring total phenolic content gives a global picture of the accumulation of phenolics into the cell walls, analysis of individual phenolics may reveal the significance of single compounds in the resistance. Moreover, no information exists about the nature of cell-wall phenolics in strawberries. The pattern of induction of individual compounds (**Table 3**) was very similar to that observed for total soluble phenolics (**Table 1)**. Compounds 22 and 23 increased significantly on treatment with BTH, at both 2 and 7 days after inoculation (**Table 3**). EA and gallic acid were also slightly higher in BTH-treated plants after 2 days and 7 days, respectively, whereas kaempferol hexose and *p*-coumaric acid did not accumulate after the treatments. The nature and biological significance of the induced but unidentified compounds 22 and 23 remain to be discovered.

**Effect of BTH on Fruit Phenolics.** BTH application alone had no influence on the soluble solids or total phenolics in strawberry fruits, while a small increase (10%) in both was seen after inoculation (**Table 4**). Although the infection might induce the accumulation of phenolics in the fruits, the higher Brix value and also drier appearance of diseased fruits indicates that the small increase observed here likely resulted from the lower water content. However, in certain specific phenolic compounds, higher concentrations were found in inoculated and noninoculated BTH-treated plants than in the corresponding water-treated plants (**Table 4**). BTH treatment induced the accumulation of flavan-3-ols, anthocyanins, kaempferol derivatives, and several unidentified compounds (compounds 28−32, 37) whereas the concentrations of EA, EA pentose, *p*-coumaryl glucoside, and quercetin glucuronide were not affected by BTH.

A few studies have been conducted on the effects of BTH on the quality of the crops. Consistent with our results, accumulation of resveratrol, anthocyanins, and proanthocyanidins was found in grapevine after BTH treatment applied four weeks before the harvest (*10*, *21*). In grapes, the higher phenolic

**Table 4.** Soluble Solids, Total Phenolics, and Phenolic Compounds Quantified with HPLC in the Fruits of Frigo Plants after Treatment with BTH and Powdery Mildew Conidia in 2005

| peak no. | compound | control | inoculated control | BTH | inoculated BTH |
|---|---|---|---|---|---|
| - | soluble solids (% Brix) | 9.3 ± 0.5 | 10.2 ± 1.2 | 9.4 ± 1.2 | 10.5 ± 1.5 |
| - | total phenolics | 2940 ± 220[a,b] | 3150 ± 390 | 2930 ± 180 | 3190 ± 90 |
| 28 | unknown | 2.9 ± 0.4 a | 3.8 ± 1.1 ab | 3.3 ± 0.4 a | 4.3 ± 0.8 b |
| 29 | unknown | 51 ± 11 a | 64 ± 13 ab | 66 ± 7 ab | 79 ± 13 b |
| 30 | unknown | 5.0 ± 1.4 a | 7.2 ± 1.7 bc | 6.8 ± 1.1 ab | 9.0 ± 1.9 c |
| 31 | unknown | 10 ± 2 a | 15 ± 5 bc | 12 ± 3 ab | 20 ± 6 c |
| 32 | unknown | 10 ± 3 a | 16 ± 7 ab | 12 ± 4 a | 22 ± 9 b |
| 33 | catechin-like compound | 78 ± 26 | 106 ± 48 | 91 ± 40 | 125 ± 38 |
| 34 | catechin or epicatechin dimer | 94 ± 28 | 105 ± 34 | 98 ± 18 | 122 ± 21 |
| 35 | catechin-like compound | 23 ± 5 a | 27 ± 8 ab | 31 ± 8 ab | 37 ± 13 b |
| 36 | *p*-coumaryl glucoside | 30 ± 5 ab | 37 ± 7 a | 28 ± 5 b | 37 ± 6 a |
| 37 | unknown | 1.6 ± 0.5 a | 2.2 ± 0.8 a | 2.3 ± 1.6 a | 4.0 ± 1.4 b |
| 38 | pelargonidin 3-glucoside | 256 ± 29 a | 311 ± 60 b | 294 ± 17 ab | 334 ± 40 b |
| 39 | unidentified anthocyanin | 7.8 ± 1.0 a | 9.7 ± 2.5 ab | 8.7 ± 1.3 a | 11.1 ± 1.5 b |
| 40 | unknown | 35 ± 9 | 45 ± 20 | 38 ± 14 | 51 ± 10 |
| 11, 41 | EA/quercetin 3-glucuronide | 18 ± 3 | 18 ± 4 | 17 ± 2 | 18 ± 2 |
| 12 | EA pentose | 7.1 ± 1.2 | 7.7 ± 1.3 | 7.4 ± 0.9 | 8.1 ± 1.4 |
| 42 | pelargonidin 3-malonylglucoside | 103 ± 9 a | 115 ± 23 ab | 118 ± 8 ab | 126 ± 16 b |
| 43 | kaempferol 3-glucuronide | 6.3 ± 0.5 | 6.8 ± 1.1 | 6.9 ± 0.9 | 7.4 ± 0.5 |
| 44 | kaempferol malonylglucoside | 6.2 ± 0.6 a | 7.1 ± 1.4 a | 7.0 ± 0.8 a | 7.7 ± 0.3 b |

[a] Concentrations of phenolic compounds are expressed as $\mu$g g$^{-1}$ fw. [b] Means followed by different letters within each row were significantly different at $P < 0.05$ (Tukey).



content was linked to the enhanced resistance against *Botrytis cinerea* in the fruits (*10*). Preharvest BTH treatment has also given protection against postharvest gray mold in strawberries (*15*), suggesting that BTH causes long-term changes in secondary metabolism, which might not only improve resistance to pathogens but also influence the nutritional value and bioactive properties of the crop (*22*).

**Concentration of Salicylic Acid.** The leaves of BTH-treated and untreated strawberry plants were found to contain high concentrations of salicylic acid, which was mostly in conjugated form (**Figure 3**). Conjugated SA followed a very similar trend to the other phenolic compounds, with the highest concentrations in inoculated and BTH-treated plants 7 days after inoculation (**Figure 3A**). Free SA was affected by neither BTH nor pathogen (**Figure 3B**). Free SA levels may be tightly controlled by glucosylation as found in other species (*40*, *41*) and thus a transient increase in free SA may have fallen between the sampling dates. Similarly, endogenous SA was not increased after pathogen challenge in potato and rice, which also have high basal levels of conjugated and free SA, respectively (*40*−*42*). Also, as the basal concentration of SA is already higher than the pathogen-induced concentration in *Arabidopsis* (*43*, *44*), changes observed in conjugated SA might be of minor importance in terms of signaling but rather reflect the general activation of phenolic metabolism. Nevertheless, despite a constitutively high amount of endogenous SA, BTH was capable of inducing resistance to powdery mildew, indicating that also in strawberries BTH may act independently from the accumulation of SA.

**Resistance to Powdery Mildew after BTH Treatment.** BTH improved resistance to powdery mildew in strawberries (**Table 5**). All control plants were infected after inoculation in contrast to BTH-treated plants, of which 37% had visible patches of powdery mildew mycelia on the leaves. BTH treatment particularly reduced the occurrence of patches but also significantly reduced their size. Parallel results were obtained in the experiment in 2005 (data not shown). While BTH applied prior to inoculation was clearly effective in stopping the development of infection, under constant disease pressure repetitive BTH applications would be advisable because newly developed leaves

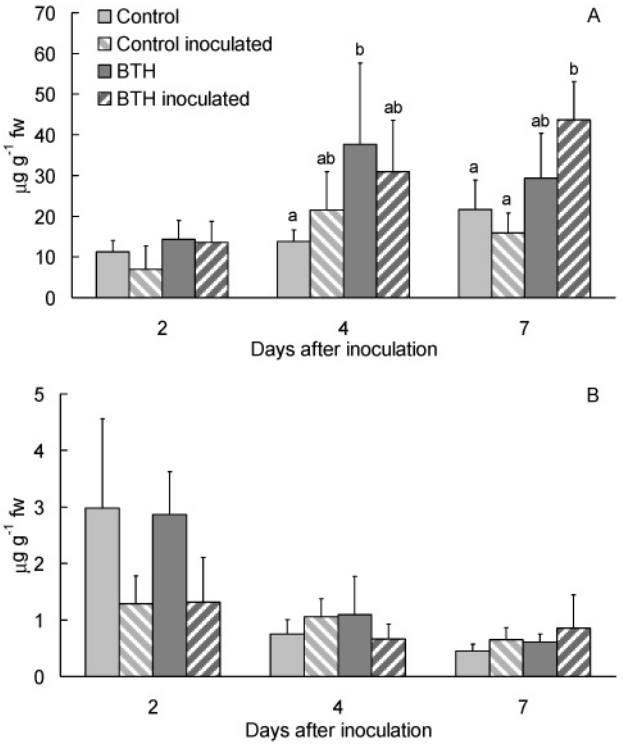


**Figure 3.** Content of (**A**) conjugated and (**B**) free salicylic acid in strawberry leaves after treatment with BTH and powdery mildew conidia. BTH was applied on the plants 2 days before the inoculation. The bars marked with different letters were significantly different at $P < 0.05$ (Tukey).

of strawberries are not only very susceptible to the disease (*12*) but also not protected by previous BTH treatment. In our experiment, the fresh weight of foliage was 17% lower in noninoculated BTH-treated plants than in untreated controls, but no other phytotoxic symptoms or reduction of yield were observed (data not shown), which is in accordance with the report by Terry and Joyce on strawberries (*15*). However, considering the effects of environment and physiological conditions on the defense induction and its costs (*45*−*47*), fitness

**Table 5.** Powdery Mildew Infection in Plants of Strawberries Cv. Jonsok Treated with Water and BTH 2 Days before Inoculation with Powdery Mildew in 2004

| days after inoculation | infected leaves per plant (%) | | powdery mildew patches per leaf[a] | | size of patches[b] ($mm^2$) | |
|---|---|---|---|---|---|---|
| | water | BTH[c] | water | BTH | water | BTH |
| 8 | 43.7 | 3.2** | 2.7 | 0.3** | 5.4 | 2.7 |
| 15 | 65.0 | 4.5** | 4.7 | 0.3** | 8.6 | 3.5* |
| 20 | 73.5 | 5.0** | 7.6 | 0.2** | 9.8 | 3.4** |
| 27 | 77.9 | 4.7** | 10.1 | 0.2** | 9.2 | 3.3** |

[a] Patches counted on both sides of leaves. [b] Only nonzero sizes have been taken for the calculation of the mean. [c] Statistically significant differences between means from 30 plants at $**P < 0.01$ or $*P < 0.05$ (*t*-test) are marked with asterisks.

costs in strawberries should be evaluated in more detail using several types of plants and conditions that occur in normal production systems.

In conclusion, BTH induced the accumulation of phenolic compounds, particularly ellagitannins, in the leaves and fruits of strawberries cv. Jonsok under greenhouse conditions while powdery mildew infection had no effect on the concentrations. Several phenolic compounds were characterized for the first time, particularly in strawberry leaves. The level of endogenous salicylic acid, mostly present in conjugated form, was very high in strawberry leaves and accumulated in a similar fashion to soluble leaf phenolics. BTH was also shown to improve resistance to powdery mildew in cv. Jonsok, which may have implications for the management of greenhouse-grown strawberries.

## ABBREVIATIONS USED

BTH, benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester; EA, ellagic acid; ET, ellagitannin; HHDP, hexahydroxydiphenoyl; SA, salicylic acid.

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**Supporting Information Available:** Tables on the detailed identification of phenolic compounds in the leaves and fruits of strawberry cv. Jonsok. This material is available free of charge via the Internet at http://pubs.acs.org.

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