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Response of Glucosinolate and Flavonoid Contents and Composition of *Brassica rapa* ssp. *chinensis* (L.) Hanelt to Silica Formulations Used as Insecticides

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Silica-based substances have increased in popularity in recent years as alternative insecticides in horticultural crop protection. However, no research has been conducted into the influence of silica on plant biochemistry. Formulations (Fossil Shield 90.0s, AE R974, AL-06-109, Surround) were applied electrostatically on pak choi. Plants were harvested on two dates to measure immediate (first) and decelerated (second) influence, as well as recovery following the removal of silica formulations. The predominant individual glucosinolate (GS) in pak choi Black Behi was 1-methoxy-3-indolylmethyl GS. A significant increase in total glucosinolate contents in all treatments was measured from the first to second harvest. During the first harvest, no changes in glucosinolate levels in plants were found in any of the treatments. After a 48 h recovery period, two substances showed decreased amounts for indole GS compared to the control. Flavonoids (kaempferol and isorhamnetin) decreased from the first to second harvest. Kaempferol in particular decreased in treated plants from the first to second harvest date. The shading of leaves by silica mainly caused the decrease in secondary metabolites in treated plants. Treatments with silica formulations as an alternative insecticide cause shifts in the composition and contents of bioactive secondary metabolites in *Brassica rapa* spp. *chinensis* plants and should, therefore, be used with care to control insects.

KEYWORDS: Electrostatic application; Fossil Shield; Aerosil; AL-06; powder; greenhouse; indole; aliphatic

INTRODUCTION

Pak choi (*Brassica rapa* ssp. *chinensis* (L.) Hanelt) belongs to the large plant family of Brassicaceae and is widely consumed all over the world (*1*). Brassicaceae plants are attacked by a wide range of insect pests (*2*). To prevent severe crop loss and to be able to sell insect-free and high-quality food, plants require protection against insect pests (*3*). In the past, this was generally performed using chemical plant protection agents, many of which have since been removed from the market due to rigorous laws throughout the world. In addition to legal requirements, an increasing number of consumers now seek residue-free and healthy food for healthy nutrition. This has led to the reconsideration of diatomaceous earth (DE) and other silica-based products, which have been in use since the early 1900s to protect stored produce. In their trials with stored product pests, Mewis and Ulrichs (*4*) demonstrated the mode of action of silica formulations, which act through desiccation of insects. Their findings were transferred and successfully established in horticulture production systems against different pests (*5*, *6*). Although these results now offer an alternative plant protection method, the physiological effects silica treatments have on plants have not yet been examined in further detail.

In addition to their use as popular food products, Brassicaceae are known to have valuable anticancer and antioxidant properties, due to their typical secondary metabolites such as glucosinolates (GS) and phenolics (*7*, *8*). Total glucosinolates are generally divided into aliphatic (alkenyl), aromatic, and indole GS, on the basis of their side chains, relating to their biosynthesis pathway (*9*). In studies of *B. chinensis*, the following main glucosinolates were identified: 3-indolylmethyl GS, 1-methoxy-3-indolylmethyl GS, 4-pentenyl GS, and 3-butenyl GS (*10*). Many authors have reported anticarcinogenic, cholesterol-reducing, and other pharmacological preventive effects against diseases by hydrolysis products of certain glucosinolates, such as 4-methylsulfinyl isothiocyanate and allyl isothiocyanate (*11*, *12*).

In several studies, descriptions have been given of changes in antioxidant components and content in Brassicaceae plants and varieties due to different climate conditions, use of fertilizers, and/or herbivore attacks (*9*, *13*−*17*). Plant-wounding processes by herbivore attacks can change the GS content of plants (*18*). It has also been documented that direct applications of fly ash lead to alterations in the secondary metabolite content and composition, as established in experiments conducted by Anderson et al. (*19*).

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Plants have to be covered completely to ensure insecticidal effectiveness when using silica formulations, and electrostatic dust application can be used for plant protection (*20*). On the basis of the described physical mode of action of silica, the plant cuticle may also be damaged in addition to the insect cuticle. These incidents could change not only the photosynthesis of plants but also their resistance to UV stress. Moreover, wounding stress may activate signaling cascades in the plant that, according to Jahangir et al. (*21*) and Halkier and Gershenzon (*22*), cause modifications in metabolite composition. The authors reported that plants exposed to abiotic and biotic stress factors can respond with changes in GS or phenolics upon defense system activation. Ulrichs et al. (*23*) proved in their experiments a reduction of the photosynthetic rate by the use of silica formulations as insecticides. They determined the photosynthetic reduction is due to the shading effect of the silica. In a study conducted by Schonhof et al. (*24*) with increased amounts of radiation, the quantity of GS decreased as a consequence. They have also proven that the contents of individual alkenyl GS, such as 3-methylsulfinylpropyl GS and 4-methylsulfinylbutyl GS, can be influenced by radiation as well as some of the indole GS.

Flavonoids represent another group of important and essential antioxidants in *B. chinensis* plants. Therefore, their biochemistry could be potentially influenced by silica applications as well. Numerous authors have discovered that kaempferol was the main flavonoid in *B. chinensis*, alongside smaller amounts of isorhamnetin and quercitin (*25*−*27*). In their experiments, the effect of light on flavonoids was also examined. In a review by Jahangir et al. (*21*), a reduction in total flavonoids due to reduced radiation was also mentioned. Not only flavonoids but also hydroxycinnmic acids and phenolics accumulate upon UV radiation in the vacuoles of the epidermal cells to supply plants with a layer of protection from the sun.

Alternative electrostatic silica plant protection had to be examined with regard to the influence on secondary metabolite production in *Brassica* plants. Not only are these compounds highly important in plant defense against insect pests, they are also valuable due to their health-promoting effects. For this reason, the aim of the present work was to study the impact of silica-based formulations used in alternative plant protection measures on the plant biochemistry of *B. chinensis*.

MATERIALS AND METHODS

Plant Material. The trials were carried out in a greenhouse at Humboldt-Universität zu Berlin (Germany). *B. chinensis* seeds 'Black Behi' were obtained from the East−West Seed Co., The Philippines. Ten days after seeding, the plants were singly planted into 10 cm diameter plastic pots in the greenhouse at 20−25 °C in standard potting soil (Gramoflor (5.2−6.0 pH; N: 90−210 g L^{-1}; P: 140−230 g L^{-1}; K: 190−310 g L^{-1}, and 0.7−1.2 g L^{-1} salt). Light was provided with 10 klx intensity exposure for 12 h day^{-1}. The relative humidity (RH) ranged between 60 and 70%. Demand-driven irrigation was applied throughout the entire experiment.

Silica Formulations. The silica materials were obtained from the Fossil Shield Co. (Eiterfeld, Germany). The commercial product FS90.0s is a natural amorphous silica formulation based on diatomaceous earth (DE), with a particle size of 1−15 μm in diameter. These substances consist of 60−80% amorphous silicates, 12−16% aluminum oxide, and minor amounts of other oxides with a surface area of approximately 300 $m^2 g^{-1}$. The second group of silica are Aerosils (AE). These are synthetically produced in amorphous forms in flame hydrolysis processes or pyrogenetically in an oxyhydrogen flame, starting from fluid chlorine silane. The average size of primary particles of the formulation AE R974 is 12 nm with surface areas below 100 $m^2 g^{-1}$ (Degussa/Evonik, 2009). Furthermore, our group developed a new product called "AL-06-109", which is based on natural silica. With an extensive pore system, it contains a high specific surface area of approximately 800 $m^2 g^{-1}$ with small hollow spherules. AL-06-109 is amorphous and contains approximately 86% silicon dioxide.

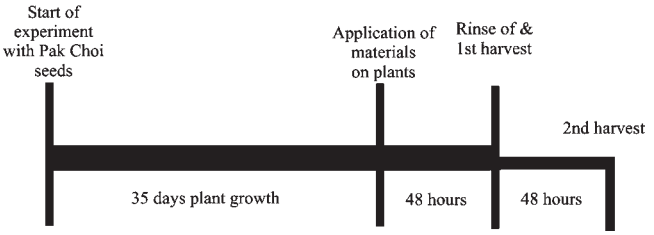

Figure 1. Experimental set of differently treated *Brassica chinensis* plants with diverse formulations (19−22 °C, 55−70% RH).

AL-06-109, AE R974, and FS90.0s were all highly hydrophobic substances. Surround (Engelhard) consists of kaolin and a spread-sticker made of natural minerals. Surround is composed of Al_2O_3, SiO_2, and traces of Na_2O, TiO_2, and Fe_2O_3. The average particle sizes ranged from 2 to 100 μm with low surface areas.

Experimental Design. *B. chinensis* plants were raised to the age of 35 days, after which time silica formulations were electrostatically applied onto plants as demonstrated by Mucha-Pelzer et al. (*20*). In the experiment presented in **Figure 1** we tested whether plants are affected by silica particulate matter. For this reason, we analyzed secondary metabolites in plants after being covered for 48 h with silica. The silica was then immediately rinsed off, and the plants were harvested after a drying time of 10 min (first harvest). It could be that plants recover from treatment, which is why some of the rinsed plants were left another 48 h after the first batch and only then harvested and analyzed as described above (second harvest). Plants from the first and second harvests were cut at the base and shock frozen in liquid nitrogen. Undusted plants served as control at the two harvest points. Finally, all samples were freeze-dried and ground. The experiment was repeated twice with a replicate number of 10 for each variation.

Extraction: Glucosinolates. All solvents and the Millipore water (ultrapure) used were of HPLC grade. The GS content in differently treated *B. chinensis* plants was analyzed by extraction, followed by HPLC analysis. According to the procedures of Mewis et al. (*28*), 40 mg of fine powder was used for the extraction. The lyophilized plant material was extracted three times (1 × 750 μL and 2 × 500 μL) using aqueous methanol (70%, 80 °C) for 5 min. As an internal standard, 60 μL of 4-hydroxybenzyl GS (sinalbin, purified from *Sinapsis alba* seeds as potassium salt) was added to the tubes for the first boiling step. The extracts were centrifuged at 4500 rpm for 5 min after each extraction step. The collected supernatants were combined, dried to approximately 300 μL, and filled with water to a final volume of 2 mL. The extracts obtained were desulfated using 75 μL of aryl sulfatase solution (H-1 from *Helix pomatia*, Sigma Aldrich) on DEAE Sephadex A-25 (Sigma Aldrich) columns, preconditioned with imidazole formic acid solution (6 mol/L in 30% formic acid) and sodium acetate buffer (trihydrous, 0.02 mol/L, pH 4.0), according to the method of Mewis et al. (*28*). Desulfated GS were eluted using 1 mL of Millipore water after about 12 h. The extract was then filtered through a 0.22 μm PTFE filter (Spin-X, Costar) to eliminate dust particles.

HPLC Analysis of Glucosinolates. HPLC analysis of 40 μL of desulfo GS extracts was carried out on a Dionex HPLC (Dionex P680A), equipped with a diode array detector. Separation was performed on a narrow-bore column (Acclaim 120, 250-2.1, 5 μm, RP18, Dionex) at 20−25 °C. Millipore water (A) and acetonitrile (B, 40%) were used as gradients at a flow rate of 0.4 mL/min. Gradient elution started at 0.5% B (1 min.), 0.5−20% B (7 min), 20% B (2 min), 20−50% B (9 min), 50% B (3 min), 50−99% B (6 min), a 5 min hold at 99% B, 99−0.5% B (3 min), and a 7 min final hold at 0.5% B. Compounds were monitored by photodiode array detection between 190 and 360 nm. Desulfo GS peaks were identified at 229 nm using retention time and UV spectra. Calculations of GS concentrations in micromoles per gram of dry weight (DW) were carried out relative to the peak area of the internal standard. The sum of all detected GS referred to the total amount of GS.

Extraction: Flavonoids. The flavonol aglycone concentration in differently treated *B. chinensis* plants was analyzed using acid hydrolysis and HPLC analysis. Acid hydrolysis was performed on the basis of the methods of Harbaum et al. (*26*) and Schmidt et al. (*29*). Lyophilized plant material (20 mg) was hydrolyzed with 50% aqueous methanol and 1.6 M HCl (1 mL of 100% methanol and 1 mL of 3.2 M HCl) in duplicate. After

incubation at 90 °C for 2 h, the extract was cooled to room temperature, adjusted to 2 mL with 50% aqueous methanol and sonicated (Bandelin Sonorex) for 5 min. The extract was then filtered through a 0.22 μm PTFE filter by centrifugation. Because the comparable data for flavonoids in pak choi in the literature are mostly in milligrams of flavonoid, concentrations of samples were calculated in milligrams per gram of DW, whereas the GS data are commonly calculated in micromoles per gram of DW.

HPLC Analysis of Flavonoids. According to Krumbein et al. (*30*), flavonoids were determined as aglycones using a modified HPLC-DAD-ESI-MS^2 method. Flavonoid aglycones were qualified and quantified using a HPLC series 1100 by Agilent (Waldbronn, Germany), equipped with a photodiode array detector. Extracts were separated on a Prodigy ODS 3 column (150 × 3.0 mm, 5 μm, 100 Å, Phenomenex, Aschaffenburg, Germany) at a temperature of 25 °C using a water/acetonitrile gradient. Solvent A consisted of 99.5% water (Milli-Q) and 0.5% acetic acid, whereas solvent B contained 100% acetonitrile. The gradient running was the following: 30−35% B (5 min), 35−39% B (12 min), 39−90% B (5 min), 90% B isocratic (5 min), 90−30% B (5 min), and 30% B isocratic (5 min). The flow was 0.3 mL/min, and the detector wavelength was 370 nm. The external calibration curve was achieved using the standards of kaempferol and isorhamnetin (Carl Roth GmbH, Karlsruhe, Germany). Concentrations between 0.1 and 10 mg/100 mL were estimated. The sum of the concentrations of the flavonoids' aglycones of kaempferol and isorhamnetin was considered to be the total concentration of flavonoids. The flavonoid glycosides of kaempferol and isorhamnetin were identified as deprotonated molecular ions and characteristic mass fragment ions by HPLC-DAD/ESI-MS^2 using an Agilent series 1100 ion trap mass spectrometer in negative ionization mode. An Agilent series 1100 MSD equipped with an ion trap mass spectrometer was used. Nitrogen was used as the drying gas (12 L min^{-1}, 350 °C) in addition to nebulizer gas (40 psi) with a capillary voltage of ~3500 V. Helium was used as the collision gas in the ion trap. Mass optimization was performed for quercetin [MH] m/z 301. Also, arbitrary m/z 1000 was used as target mass in the auto mode to include higher mass fragments. The MS^n experiments were performed in auto or manual mode until MS^4 in a scan from m/z 200 to 2000.

Statistical Analyses. The data were subjected to one-way ANOVA and Fisher LSD for comparisons between formulations with $p < 0.05$. To compare harvest dates, a repeated measurement ANOVA was conducted using the Proc MIXED procedure from SAS (Fisher LSD, $p < 0.05$).

RESULTS

Glucosinolates. The following individual GS were detected in pak choi plants: 2-hydroxy-3-butenyl GS (progoitrin), allyl GS (sinigrin), 4-methylsulfinylbutyl GS (glucoraphanin), 5-methylsulfinylpentyl GS, 3-butenyl GS (gluconapin), 4-hydroxybutyl GS, 4-pentenyl GS (glucobrassicanapin), 4-hydroxy-3-indolylmethyl GS (4-hydroxyglucobrassicin), 3-indolylmethyl GS (glucobrassicin), 4-methoxy-3-indolylmethyl GS (4-methoxyglucobrassicin), and 1-methoxy-3-indolylmethyl GS (neoglucobrassicin) (**Table 1**; **Figure 2**). The aromatic 2-phenylethyl GS was most of the time below the detection level in the plant samples, and therefore, it was excluded from the quantitative analysis. In our study, the individual GS with the highest amounts in pak choi were identified as 1-methoxy-3-indolylmethyl GS at the first and second harvests (**Figure 2**).

GS increased significantly in silica-dusted and control plants from the first to the second harvest (repeated measurement ANOVA, **Figure 3**). A significant increase was found for total GS (df = 1, $F = 35.35$, $p < 0.0001$), aliphatic GS (df = 1, $F = 27.91$, $p < 0.0001$), and indole GS (df = 1, $F = 18.39$, $P = 0.0001$). The constitutive amount of total GS increased significantly from the first harvest, after the plants had been dusted, rinsed, and harvested after 48 h of recovery time, as with the undusted plants (**Figure 3**). The total GS contents for formulations FS90.0s, AE R974, and Surround after the recovery time at the second harvest increased significantly. In the recovery time, plants treated with AL-06-109 increased in total GS content by 2.8-fold from the first to the second harvest, which turned out to be an extremely significant result ($p < 0.001$, **Figure 3**). Within these results for AL-06-109, both aliphatic ($p < 0.001$) and indole ($p < 0.01$) contents increased from the first to the second harvest (not included in the figure). For FS90.0s and AE R974, the increases from the first to the second harvest were found only in the aliphatic GS ($p < 0.05$) content; there was no significance in the indole GS. The indole GS increase was significant ($p < 0.01$) in plants treated with Surround and controls, whereas the aliphatic GS content did not increase after the dust was removed from the plants.

Performing one-way ANOVA separately for the first and second harvests revealed no significant results for total GS (df = 4, $F = 31.59$, $p = 0.8153$; df = 4, $F = 70.02$, $p = 0.2308$) or aliphatic GS (df = 4, $F_{1st} = 14.02$, $p_{1st} = 0.7593$, $F_{2nd} = 34.05$, $p = 0.4830$). Indole GS were not significant in the first harvest (df = 4, $F = 13.02$, $p = 0.6076$), whereas significant differences between substances (df = 4, $F = 121.86$, $p = 0.0001$) were found in the second harvest. After a 48 h recovery time at the second harvest, the plants did not show any significant differences in aliphatic or total GS contents within formulations compared to the control. However, AL-06-109 and Surround showed significantly higher amounts of indole GS at the second harvest than plants treated with FS90.0s and AE R974. In the untreated control at the second harvest, the amounts of indole GS detected were twice the contents of AL-06-109- and Surround-dusted *Brassica*.

Table 1. Individual, Aliphatic, and Indole Glucosinolate Contents of *Brassica chinensis* Plants after Treatment with Different Formulations (19−22°C, 55−70% RH)

	glucosinolate[a] (μmol/g of DW)				
glucosinolate type	AL-06-109	FS90.0s	AE R974	Surround	control
First Harvest: 48 h Dust Application					
2-hydroxy-3-butenyl GS	2.19 ns	2.81 ns	3.39 ns	2.75 ns	2.09 ns
allyl GS	0.06 ns	0.21 ns	0.33 ns	0.09 ns	0.55 ns
4-methylsulfinylbutyl GS	0.00 ns	0.02 ns	0.00 ns	0.03 ns	0.00 ns
5-methylsulfinylpentyl GS	0.71 ns	1.24 ns	1.22 ns	1.20 ns	1.31 ns
3-butenyl GS	0.93 ns	0.96 ns	2.27 ns	1.53 ns	1.70 ns
4-hydroxybutyl GS	0.87 ns	1.69 ns	1.25 ns	2.25 ns	1.52 ns
4-pentenyl GS	0.75 ns	1.02 ns	1.43 ns	1.18 ns	1.63 ns
aliphatic GS	**5.51 ns**	**7.93 ns**	**9.89 ns**	**9.03 ns**	**8.80 ns**
4-hydroxy-3-indolylmethyl GS	0.36 ns	0.14 ns	0.32 ns	0.44 ns	0.36 ns
3-indolylmethyl GS	0.33 a	0.24 a	0.28 a	0.51 ab	0.65 b
4-methoxy-3-indolylmethyl GS	0.77 ns	0.52 ns	0.61 ns	0.86 ns	0.54 ns
1-methoxy-3-indolylmethyl GS	4.68 ns	5.92 ns	3.99 ns	6.74 ns	7.48 ns
indole GS	**6.13 ns**	**6.82 ns**	**5.21 ns**	**8.55 ns**	**9.03 ns**
total GS	**11.64 ns**	**14.75 ns**	**15.10 ns**	**17.58 ns**	**17.83 ns**[b]
Second Harvest: 48 h Recovery Time					
2-hydroxy-3-butenyl GS	7.25 ns	5.32 ns	5.72 ns	5.57 ns	5.78 ns
allyl GS	0.27 ns	0.24 ns	0.27 ns	0.25 ns	0.25 ns
4-methylsulfinylbutyl GS	0.00 ns	0.00 ns	0.00 ns	0.00 ns	0.05 ns
5-methylsulfinylpentyl GS	3.53 b	2.04 a	2.25 ab	1.52 a	2.19 ab
3-butenyl GS	2.85 ab	3.45 b	4.27 b	2.27 ab	1.07 a
4-hydroxybutyl GS	2.38 ab	2.50 ab	2.26 b	2.72 ab	2.80 a
4-pentenyl GS	3.76 ns	3.53 ns	4.36 ns	2.47 ns	1.81 ns
aliphatic GS	**20.04 ns**	**17.07 ns**	**19.12 ns**	**14.81 ns**	**13.94 ns**
4-hydroxy-3-indolylmethyl GS	0.26 a	0.32 ab	0.34 ab	0.21 a	0.60 b
3-indolylmethyl GS	0.64 ab	0.49 a	0.39 a	0.92 bc	1.06 c
4-methoxy-3-indolylmethyl GS	0.85 a	0.88 a	0.60 a	0.85 a	1.27 b
1-methoxy-3-indolylmethyl GS	11.20 a	6.56 b	3.97 a	13.35 b	14.23 b
indole GS	**12.95 b**	**8.24 a**	**5.31 a**	**15.33 b**	**17.18 b**
total GS	**32.99 ns**	**25.32 ns**	**24.42 ns**	**30.14 ns**	**31.12 ns**

[a] One-way ANOVA, Fisher LSD ($p < 0.05$); different letters (a, b) indicate significant differences within the individual as well as aliphatic, indole, and total GS among treatments; ns stands for no significance detected. [b] Equals 1.30 μmol/g of fresh wt (FW).

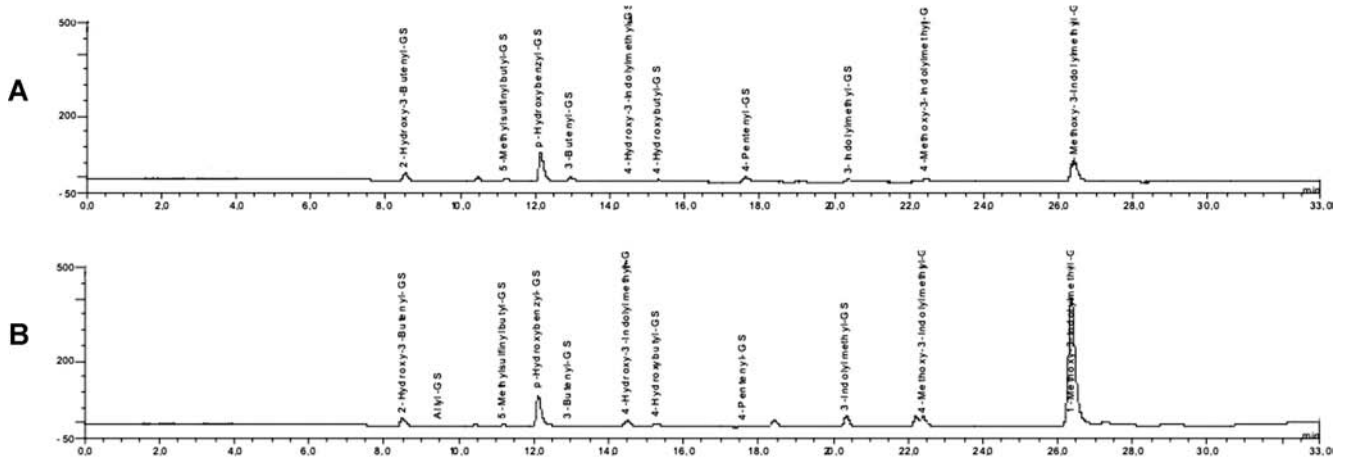

Figure 2. Glucosinolate profiles of *Brassica chinensis* plants after treatment with different formulations: (**A**) profile for treatment with AE R974; (**B**) control profile. Glucosinolates were recorded at 229 nm.

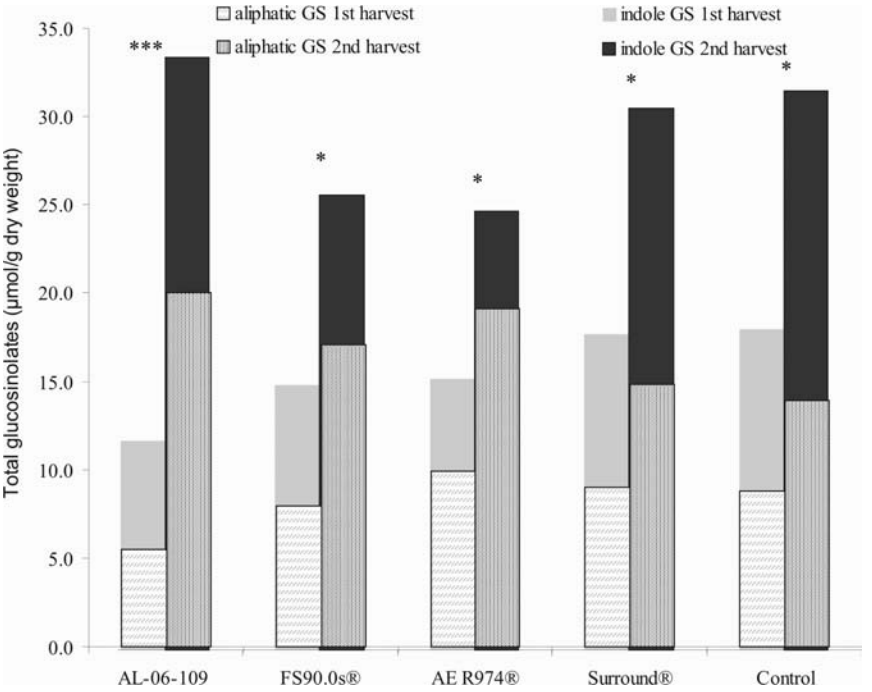

Figure 3. Total glucosinolate (GS) content of *Brassica chinensis* plants after treatment with different formulations under greenhouse conditions (19−22 °C, 55−70% RH). The graph shows the comparison of different formulations for total GS amounts for substances and harvest dates (divided into the aliphatic and indole GS parts: first harvest, aliphatic in white/gray, indole in light gray; second harvest, aliphatic in gray/black, indole in black) (first = 48 h dust application, second = another 48 h later, after recovery). Asterisks indicate differences in the total GS content between the substances for the two harvest dates (repeated measurement ANOVA: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$).

With regard to the individual GS in **Table 1**, the dusting treatment of plants influenced only certain GS. Although the concentrations in treated plants at the first harvest were slightly lower for 1-methoxy-3-indolylmethyl GS than in control plants, no significant difference was determined. The only individual GS with significant differences between substance treatments at the first harvest date turned out to be 3-indolylmethyl GS, whereas the amounts in plants treated with AL-06-109, FS90.0s, and AE R974 were less than those in the control plants; Surround plants were unaltered.

At the second harvest, three aliphatic individual GS were significantly raised during the recovery period. 5-methylsulfinylpentyl GS in AL-06-109-treated plants, 3-butenyl GS in FS90.0s- and AE R974-treated plants, and 4-hydroxybutyl GS in AE R974-treated plants were higher than the control. All indole GS decreased during the recovery period up to the second harvest date. 4-methoxy-3-indolylmethyl GS were significantly reduced for all substances, whereas 1-methoxy-3-indolylmethyl GS decreased during the 48 h recovery only in plants treated with AL-06-109 and AE R974. The 3-indolylmethyl GS content decreased in plants previously dusted with FS90.0s and AE R974. 4-hydroxy-3-indolylmethyl GS decreased in recovered plants from treatment with AL-06-109 and Surround at the second harvest date.

Flavonoids. The main flavonoid found in our experiment was isorhamnetin. Kaempferol, minor amounts of quercitin, and some phenolic acids were also detected (data not shown). Exposure of the plants to silica substances significantly influenced total flavonoid contents in repeated measurement ANOVA. They were significant

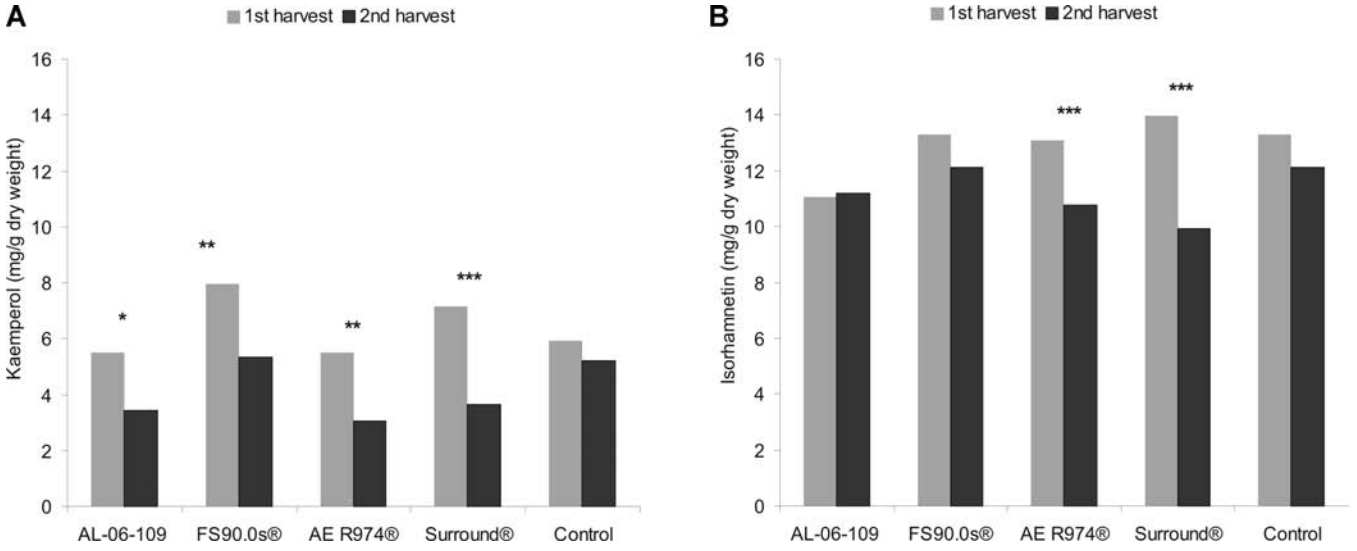

Figure 4. Kaempferol (**A**) and isorhamnetin (**B**) contents of *Brassica chinensis* plants after treatment with different formulations under greenhouse conditions (19−22 °C, 55−70% RH). First harvest (**A**) stands for plants 48 h dusted and rinsed at harvest date; second harvest stands for plants 48 h dusted, rinsed at first harvest date, and plants harvested after 48 h recovery. Graph **A** compares the two harvest dates for kaempferol contents after treatment. Graph **B** compares the first and second harvests for isorhamnetin contents after treatment. In both graphs, asterisks indicate differences in harvest dates between each formulation (repeated measurement ANOVA, Fisher LSD test; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$). Generally, if no asterisks are shown, no significance was found.

for harvest (df = 1, $F = 53.2$, $p < 0.0001$) as well as substance (df = 4, $F = 6.05$, $p < 0.0001$). For this reason, an extremely significant decrease in total flavonoid content in control, as well as in AE R974 and Surround, was found from the first to the second harvest. A significant decrease ($p = 0.0025$) was established for FS90.0s, whereas AL-06-109 showed no statistical difference in total flavonoid contents. The flavonoid kaempferol decreased in pak choi from the first to the second harvest after dust application and recovery for all used substances (AL-06-109, FS90.0s, AE R974, and Surround), whereas kaempferol in control plants remained unchanged. The highest significant decrease was detected for Surround (**Figure 4A**). Isorhamnetin amounts were reduced significantly in pak choi treated with AE R974 and Surround (**Figure 4B**).

Exposure of the plants to silica influenced the flavonoid contents in plants. One-way ANOVA comparison at the first harvest revealed significant results within substance applications for kaempferol (df = 9, $F = 11.84$, $p = 0.0323$), which were also found for the second harvest (df = 9, $F = 11.26$, $p = 0.0038$). Similarly, the isorhamnetin content of plants in the treatments was significantly different at the first (one-way ANOVA, df = 9, $F = 12.14$, $p = 0.0177$) and second harvest dates (one-way ANOVA, df = 9, $F = 8.61$, $p < 0.0001$). Among the tested substances, however, only plants treated with Surround showed a significantly decreased isorhamnetin contents at the first harvest (**Table 2**). Neither kaempferol nor total flavonoid contents of plants were significantly different within the substances and control at the first harvest. Similarly for kaempferol, no significant changes were measured at the second harvest. Nonetheless, isorhamnetin amounts in Surround-dusted and recovered plants were significantly lower than in the control. Finally, Surround-, AL-06-109-, and AE R974-treated plants produced significantly lower amounts of total flavonoids at the second harvest.

Table 2. Individual and Total Flavonoid Contents of *Brassica chinensis* Plants after Treatment with Different Formulations (19−22 °C, 55−70% RH)

	flavonoid[a] (mg/g of DW)				
glucosinolate type	AL-06-109	FS90.0s	AE R974	Surround	control
First Harvest: 48 h Dust Application					
kaempferol	5.51 a	7.94 b	5.52 a	7.15 ab	5.90 ab
isorhamnetin	11.06 a	13.31 a	13.10 a	13.95 b	13.31 a
total flavonoids	**16.57 a**	**21.25 b**	**18.62 ab**	**21.10 b**	**19.21 ab**
Second Harvest: 48 h Recovery Time					
kaempferol	3.42 ns	5.32 ns	3.03 ns	3.62 ns	5.19 ns
isorhamnetin	11.20 ab	12.10 b	10.75 ab	9.93 a	12.11 b
total flavonoids	**14.62 a**	**17.42 b**	**13.78 a**	**10.55 a**	**17.30 b**

[a] One-way ANOVA, Fisher LSD ($p < 0.05$); different letters (a, b) indicate significant differences within the individual flavonoid among treatments; ns stands for no significance detected.

DISCUSSION

Total, Aliphatic, and Indole Glucosinolates. He et al. (*31*) stated that the GS content in different *B. chinensis* varieties ranged between 84.74 and 175.21 μmol 100 g^{-1} of fresh weight (FW). The average amount in the *B. chinensis* group was 181 μmol 100 g^{-1} of FW, which was lower than in other *Brassica* vegetables in their experiments. Shattuck and Wang (*32*) reported total GS contents between 2.53 and 4.30 μmol g^{-1} of DW; Lewis and Fenwick (*33*) measured 100 μmol 100 g^{-1} of FW. Our constitutive total GS content in control was 17.83 μmol g^{-1} of DW, which equals about 180 μmol 100 g^{-1} of FW. Our result is therefore similar to levels measured by He et al. (*31*). Several authors have proven that the cultivar and growing conditions are parameters that influence a plant's content of GS (*10*, *34*, *35*).

Chen et al. (*10*) found a GS composition of 60−65% of total aliphatic, 25−27% indole, and 8−14% aromatic GS in *B. chinensis* plants. These findings differ partially from our results. We found very low amounts of aromatic GS in our variety 'Black Behi', which was explained by Hill et al. (*36*) by the fact that some *B. chinensis* varieties do not produce any aromatic GS. Also, Mewis et al. (*37*) demonstrated that older pak choi plants (>27 days) generally produce lower amounts of aromatic GS, which is consistent with our results. Furthermore, the percentage of aliphatic GS in the total

GS content was comparably lower and the percentage indole GS content higher. Because several authors have emphasized the positive effect of indole GS (3-indolylmethyl GS), a generally higher percentage of indole GS in the total GS is positive (*10*, *13*). However, the formation of characteristic genotoxic DNA adducts in buccal mucosa and lymphocytes in rat volunteers was found following the consumption of raw broccoli and cabbage (*38*). The adduct-inducing constituent was identified as 1-methoxy-3-indolylmethyl GS; therefore, consumption of higher amounts can have a negative impact on human health. As mentioned by Wittstock and Halkier (*39*), indole GS play an important role in plant defense and would enable the plant to protect itself from the start, compared to varieties without a high proportion of indole GS. Nevertheless, Brader et al. (*40*) stated that indole GS are highly inducible by jasmonate and are involved in plant defense against bacterial pathogens.

Jahangir et al. (*21*) stated in a general review on stress application on Brassicaceae plants that abiotic and biotic factors can change the biochemistry. In a study conducted with *Brassica oleracea* by Rosen et al. (*41*), the general effects of fertilizers on change within aliphatic and indole GS in cultivars are reported. Harbaum et al. (*42*) named climatic conditions and environmental and agricultural factors as trigger elements that change the biosynthesis and concentration of polyphenols in plants. These findings are consistent with our results, in which the use of various silica formulations at different harvesting times led to changes in the amounts of glucosinolates and flavonoids. The accumulation of indole GS in plants such as *Arabidopsis thaliana* L. and *Brassica napus* L. is induced by mechanical wounding, herbivore attack, and treatment with such chemicals as methyl jasmonate (MJ) (*21*, *43*, *44*). Most studies report an increase in indole GS following exposure of plants to stress factors. Mikkelsen et al. (*45*), however, also demonstrated an increase in aliphatic GS in MJ-treated plants. Less stress occurred in the first 48 h in the first harvest, because treated plants showed a response in only one aliphatic compound (3-butenyl GS) of the 11 total detected GS. Nonetheless, the total, aliphatic, and indole GS did not differ from the control in the first harvest, in the comparison of formulations (**Table 1**). Nevertheless, once the plants had another 48 h to recover, seven substances had significantly changed within GS levels compared to the control. Higher amounts of aliphatic and indole GS were found in the second harvest in both treated and untreated plants. This led to suggestions that the removal process caused plant wounding with leftover particles and the mode of action of silicas described above. Another possibility is that, after being shaded for 48 h, the presence of light induced the production of GS (*16*). However, GS in the untreated control also increased, indicating GS ontogenic-based changes. Nevertheless, not all substances reacted at the same intensity of indole GS induction, which could be explained by the dense coverage with the white-colored dusts FS90.0s and AE R974. The dust Surround, which led to a higher indole GS accumulation in pak choi, was found to be electrostatically charged with problems; because the surface resistance was low, coverage was insufficient (*46*). In other words, sufficient sunlight was able to reach the leaf. Moreover, Surround is not very effective (*47*) as an insecticide, and therefore a comparison to regular silica substances is not possible. Although an increase in the total GS content is evident from the first to the second harvest, the GS classes are affected to different extents. The dense covers of Al-06-109, FS90.0s, and AE R974 seem to influence the aliphatic GS. Nevertheless, both Surround and AL-06-109 induced changes within indole GS in the plants from the first to the second harvest. In studies conducted by Mucha-Pelzer et al. (*20*) with the formulation AL-06-109, the plant still demonstrated reduced leaf damage by insects compared to the control after dust removal. It is, therefore, suggested that the ability of self-defense is developed for certain substances, either from leftover particles or from the induced GS amounts. However, we must still determine how many leftover silica particles are acceptable for food consumption.

Individual Glucosinolates. He et al. (*31*) identified the predominant GS to be 3-butenyl GS and 1-methoxy-3-indolylmethyl GS in *B. chinensis* Choy Sum 3 and Tai Tsai, whereby different GS compositions were identified in *B. chinensis* cultivars. Shattuck and Wang (*48*) stated that 1-methoxy-3-indolylmethyl GS mainly occurred in younger plants, whereas in mature plants the amount of 2-phenylethyl GS decreased and the amount of 4-methoxy-3-indolylmethyl GS increased. 3-butenyl GS, 2-hydroxy-3-butenyl GS, 4-pentenyl GS, and 3-indolylmethyl GS were found in *B. chinensis* plants by Lewis and Fenwick (*33*). The authors stated that indole compounds were the main GS in their experiments with pak choi. However, Chen et al. (*10*) found 3-butenyl GS to be the main GS in *B. chinensis*. The GS identified in the present study for *B. chinensis* 'Black Behi' are consistent with compounds identified in the literature. Nevertheless, we found 1-methoxy-3-indolylmethyl GS to be the major constitutive compound in our cultivar. Because also 2-hydroxy-3-butenyl GS was found in control plants, the data provided by He et al. (*31*) are supported. They established that different cultivars have different GS profiles. As previously mentioned, climatic and agricultural factors play a vital role in GS composition and the amount of production, given that temperatures and radiation seem to act as a trigger for changes in biosynthetic pathways (*24*). In particular, the two substances 2-hydroxy-3-butenyl GS and 1-methoxy-3-indolylmethyl GS were found to have changed significantly from the first to second harvest date for AL-06-109 and the control.

Enhancing GS can improve plants' ability to protect themselves against enemies. However, some GS components (e.g., progoitrin) are thought to be responsible for the bitterness in *Brassica* plants and have goitrogenic effects (*49*−*51*). Other GS, such as 3-indolylmethyl GS, allyl GS, and 4-methylsulfinylbutyl GS, are of interest to humans due to their anticarcinogenic properties. Any changes in the GS composition by applying dust, therefore, should ensure that vegetables remain attractive for consumption. Fortunately, the present study reveals only slight changes in GS due to dust application.

Flavonoids. In trials conducted by Bahorun et al. (*52*), Chinese cabbage plants possessed 4.5 mg g^{-1} of DW, whereas Sakakibara et al. (*53*) found 77−222 mg 100 g^{-1} of FW. Kim et al. (*54*) established the total flavonoids (quercitin and kaempferol) of green cabbage plants to be 0.8 mg g^{-1} of FW. Vallejo et al. (*55*) reported 1−10 mg g^{-1} of DW in broccoli inflorescences. In general, broccoli plants were found to have lower flavonoid contents than *B. chinensis*. Harbaum et al. (*42*) detected different amounts of flavonoids in 10 different *B. chinensis* cultivars, depending on plant part. The amounts ranged from 15 to 38.7 mg g^{-1} of DW. The main flavonoid in 'Black Behi' is isorhamnetin, followed by kaempferol, which is consistent with the literature. Total constitutive amounts ranged between 17.21 and 19.21 mg g^{-1} of DW for 'Black Behi', which corresponds to the results of Harbaum et al. (*42*).

Numerous authors report the elicitor effect of UV light on the secondary metabolite production as a stress factor (*21*, *56*, *57*). Because in our trial the flavonoid contents from the first to the second harvest decreased in plants exposed to dust but not in untreated plants, the assumption can be made that flavonoid production was affected by dusts. With the silica coverage, ambient light is prevented from reaching the plant cuticle, thus reducing photosynthesis. Flavonoids are essential for plants to protect themselves from the damaging irradiation of sunlight (*58*−*60*). A decrease in flavonoids could cause higher risk of injury to plants, as reported in the experiment conducted by Landry et al. (*61*), in which the damaging effect of UV light on *Arabidopsis* mutants was determined. The authors found that if

flavonoid compounds were absent in mutants, the plants had greater oxidative stress and were unable to protect themselves. As a consequence, if long-term insecticidal protection measurement is necessary, the long-term effects of extensive UV light must be evaluated. Frohnmeyer and Staiger (*62*) reported potential damage in the form of a degradation of photosystem II and an impact on photosynthesis and growth. Although the common flavonoid quercetin was not produced in 'Black Behi' plants, kaempferol and isorhamnetin, which are metabolites closely related to quercetin (they differ by a hydroxyl group for kaempferol and a methoxyl group for isorhamnetin), were detected in *B. chinensis* (*63*). This does not need to be a disadvantage for healthy food production. In addition to establishing its close structural relation, Wattel et al. (*64*) also classified kaempferol as having not only a good antiradical activity but also the ability to interact with the estrogen receptor of human bone cells.

Dust application has been shown to affect plant biochemistry, even after the removal of dust and 48 h of recovery. Levels of flavonoids and partly indolyl GS were markedly reduced in the plants. The possible cause of the decrease in secondary metabolites is not clearly understood and must be explored in further detail. The long-term utilization of silica on plants also needs to be examined in greater detail to prove their usage as an insecticide without the loss of bioactive compounds in the crop plant.

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