Interaction between Plants and Bacteria: Glucosinolates and Phyllospheric Colonization of Cruciferous Vegetables by Enterobacter radicincitans DSM 16656

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Abstract
For determining interactive plant-bacterial effects between glucosinolates and phyllospheric colonization by a plant growth-promoting strain, Enterobacter radicincitans DSM 16656, in cruciferous vegetables, the extent of bacterial colonization was assessed in 5 cruciferous vegetables (Brassica juncea, Brassica campestris, Brassica oleracea var. capitata, Brassica rapa var. alboglabra, Nasturtium officinale) using a species-specific TaqMan™ probe and quantitative real-time PCR. Colonization ability of inoculated E. radicincitans in the phyllosphere of these species varied from inability to colonize B. rapa up to a very good colonization rate of B. campestris. In addition to morphological factors and other plant compounds, the colonization rate was affected by different individual aromatic and aliphatic glucosinolates and their concentration, revealing that both plant pathogens and plant growth-promoting bacteria were affected by glucosinolates in their colonization behavior. In contrast, after E. radicincitans inoculation neither the total nor the individual glucosinolate concentrations in the phyllosphere of the 5 cruciferous species were affected, indicating that the non-pathogenic E. radicincitans might cause only poor cell damage by metabolizing plant cell components and does not induce a plant defense response and thus subsequently an increased glucosinolate concentration in the phyllosphere. Moreover, E. radicincitans induced no stimulation of indole glucosinolate biosynthesis by additional bacterial auxin supply.

Introduction
Members of the family Brassicaceae are horticulturally important crop plants and highly consumed vegetables [Behr, 2006; Monteiro and Lunn, 1999]. These cruciferous vegetables are characterized by glucosinolates, a group of phytochemicals found exclusively in plants of the order Brassicales, including the family Brassicaceae [Halkier and Du, 1997] and in the genus Drypetes belonging to the Euphorbiaceae [Rodman et al., 1998]. Plant species strongly influences bacterial colonization, leading to pronounced differences in community composition [Janczik et al., 2006; Lindow et al., 2002]. In Brassicaceae, this effect can be explained by the genotypically different formation of microbially affected secondary plant metabolites, mainly glucosinolates, and hence varying concentrations and composition of these...
phytochemicals. It was demonstrated by Ruppel et al. [2008] that the bacterial population density was positively correlated to the alkenyl glucosinolates 2-propenyl, 3-butenyl and 4-pentenyl whereas the aromatic glucosinolate 2-phenylethyl showed a negative correlation to the phyllospheric bacterial population size. Moreover, in plant-bacterial interaction, glucosinolates are involved in the plant defense response against microbial pathogen attack [Mikkelsen et al., 2003] resulting in reduced bacterial colonization and growth [Brader et al., 2001, 2006; O’Callaghan et al., 2000; Tierens et al., 2001]. In the process of chemical defense, the synthesis of structurally different aliphatic, aromatic, and indole glucosinolates (table 1) changes as a response to a particular pathogen [Brader et al., 2006].

While there are a few reports on glucosinolate-affect ed synthesis due to specific bacteria in the rhizosphere [e.g. Bending and Lincoln, 2000; O’Callaghan et al., 2000], little is known about the exact interaction between individual glucosinolate concentration and colonization of the phyllosphere by plant growth-promoting bacteria (PGPB).

Enterobacter radicincitans DSM 16656 (formerly Pantoea agglomerans) is a deeply investigated and established PGPB strain characterized by its ability to fix atmospheric nitrogen and produce phytohormones such as auxins.

### Table 1. Structural formulae of glucosinolates assessed in this study

<table>
<thead>
<tr>
<th>Glucosinolate group</th>
<th>Trivial name</th>
<th>Semisystematic name</th>
<th>Structure of R group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliphatic GS</td>
<td>glucobrassicin</td>
<td>3-indolylmethyl</td>
<td><img src="attachment" alt="Structure of R group for glucobrassicin" /></td>
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<tr>
<td>Alkenyl GS</td>
<td>sinigrin</td>
<td>2-propenyl</td>
<td><img src="attachment" alt="Structure of R group for sinigrin" /></td>
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<tr>
<td>Hydroxy-Alkenyl GS</td>
<td>progoitrin</td>
<td>2-hydroxy-3-butenyl</td>
<td><img src="attachment" alt="Structure of R group for progoitrin" /></td>
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<tr>
<td></td>
<td>gluconapoleiferin</td>
<td>2-hydroxy-4-pentenyl</td>
<td><img src="attachment" alt="Structure of R group for gluconapoleiferin" /></td>
</tr>
<tr>
<td>Aromatic GS</td>
<td>gluotropaeolin</td>
<td>benzyl</td>
<td><img src="attachment" alt="Structure of R group for gluotropaeolin" /></td>
</tr>
<tr>
<td></td>
<td>gluconasturtiin</td>
<td>2-phenylethyl</td>
<td><img src="attachment" alt="Structure of R group for gluconasturtiin" /></td>
</tr>
<tr>
<td>Indole GS</td>
<td>glucobrassicin</td>
<td>3-indolylmethyl</td>
<td><img src="attachment" alt="Structure of R group for glucobrassicin" /></td>
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<tr>
<td></td>
<td>neoglucobrassicin</td>
<td>N-methoxy-3-indolylmethyl</td>
<td><img src="attachment" alt="Structure of R group for neoglucobrassicin" /></td>
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<tr>
<td></td>
<td>4-methoxy-glucobrassicin</td>
<td>4-methoxy-3-indolylmethyl</td>
<td><img src="attachment" alt="Structure of R group for 4-methoxy-glucobrassicin" /></td>
</tr>
<tr>
<td></td>
<td>4-hydroxy-glucobrassicin</td>
<td>4-hydroxy-3-indolylmethyl</td>
<td><img src="attachment" alt="Structure of R group for 4-hydroxy-glucobrassicin" /></td>
</tr>
</tbody>
</table>

GS = Glucosinolates.
and cytokinins [Kämpfer et al., 2005; Scholz-Seidel and Ruppel, 1992]. *E. radicincitans* is able to improve root and shoot growth and the yield of winter wheat, barley, maize, pea [Höflich and Ruppel, 1994; Ruppel, 2000] and *Brassica* species [Ruppel and Wernitz, 2004; Ruppel et al., 2006]. This bacterial strain was shown to colonize the endophyllosphere of wheat without inducing plant defense reactions [Ruppel et al., 1992]. *Brassica* species could probably respond differently to colonization by *E. radicincitans*: first, glucosinolate concentration could increase in the colonized phyllosphere due to the plant’s defense response; second, indole glucosinolate concentration might increase as a consequence of additional auxin supply from *E. radicincitans* since the additive auxin might induce predominant metabolization of indole-3-acetaldoxime to indole glucosinolates as auxin and indole glucosinolates are both derived from the key metabolite indole-3-acetaldoxime [Bak et al., 2001].

Increasing glucosinolates in the plant’s tissue have generated considerable pharmacological interest due to their human health-promoting effects, particularly anticarcinogenic properties [Talalay and Fahrey, 2001]. Applications of biological elicitors could be effective in increasing desired phytochemicals [Schreiner and Huyskens-Keil, 2006].

Therefore, the aim of the present study was to examine whether the colonization ability of the PGPB strain *E. radicincitans* is uniform in 5 different cruciferous vegetables. We further tested whether the colonization ability is related to different glucosinolate concentrations and composition, especially to the alkenyl and aromatic glucosinolates, which were shown to affect the native bacterial phyllospheric population [Ruppel et al., 2008] in the phyllosphere of these plants and probably modified by other plant compounds potentially affecting the colonization ability of *E. radicincitans*. In addition, we wanted to determine whether in this specific plant-bacterial interaction, i.e. cruciferous vegetables – *E. radicincitans*, the glucosinolate concentration could be promoted by *E. radicincitans* colonization, thereby suggesting phytochemical stimulation by additional bacterial auxin production or enhanced plant defense response induced by *E. radicincitans*.

To verify the results of in vivo plant experiments, plant extracts and glucosinolate standards were monitored for *E. radicincitans* growth responses in pure culture experiments. The colonization ability of *E. radicincitans* in the phyllosphere was assessed using a species-specific primer TaqMan™ probe and quantitative real-time PCR. To our knowledge, this is the first published study using quantitative real-time PCR analysis to quantify targets of *E. radicincitans* in the phyllosphere of plants performed in conjunction with phytochemical analysis to examine the interaction between glucosinolates and bacterial colonization of the plant phyllosphere as well as the phytochemical impact of *E. radicincitans*.

**Results**

**Specific Bacterial Strain Responses on Glucosinolate Standards and Plant Extracts**

The in vitro experiment showed a positive growth response of *E. radicincitans* on all added substrates in minimal medium (fig. 1). All plant extracts and both tested
glucosinolate standards (2-propenyl and benzyl) supported the growth of *E. radicincitans* in minimal medium compared to the control without carbon. As the growth of *E. radicincitans* was higher at 2-propenyl compared to the glucose + sucrose mixture, this aliphatic 2-propenyl glucosinolate seems to be a preferred carbon source under limited nutrition conditions, whereas the aromatic benzyl glucosinolate led to the lowest bacterial growth. Comparing the growth response of *E. radicincitans* to the glucose + sucrose mixture, growth on 2-propenyl standard and on plant extracts containing a high 2-propenyl concentration (*Brassica juncea* cv. Green in Snow, *B. juncea* cv. Red Giant, *B. oleracea* var. *capitata* cv. Türkis) was increased by 50 and 290% (fig. 1, table 2). Plant extracts characterized by an increased 3-butenyl concentration (*Brassica campestris*, *Brassica rapa* ssp. *chinensis*) and 4-pentenyl concentration (*B. rapa*) also revealed strong bacterial growth (fig. 1, table 2). In con-

<table>
<thead>
<tr>
<th>Table 2. Individual glucosinolate concentration (mg 100 g⁻¹ fm) in the leaf extract of 11 cruciferous plant species (in vitro experiment)</th>
</tr>
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<tbody>
<tr>
<td>Plant species</td>
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<td>---------------------------------------------------------------</td>
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<tr>
<td><em>Brassica rapa</em> var. <em>alboglabra</em></td>
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<tr>
<td><em>Brassica rapa</em></td>
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<tr>
<td><em>Brassica rapa</em> ssp. <em>rapifera</em></td>
</tr>
<tr>
<td><em>Brassica</em> <em>rapa</em> ssp. <em>chinensis</em></td>
</tr>
<tr>
<td><em>Brassica oleracea</em> var. <em>capitata</em></td>
</tr>
<tr>
<td><em>Brassica oleracea</em> var. <em>italica</em></td>
</tr>
<tr>
<td><em>Brassica oleracea</em> var. <em>botrytis</em></td>
</tr>
<tr>
<td><em>Brassica juncea</em> cv. <em>Green in Snow</em></td>
</tr>
<tr>
<td><em>Brassica campestris</em></td>
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<tr>
<td><em>Nasturtium officinale</em></td>
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</tbody>
</table>

GS = Glucosinolates; fm = fresh matter; 2P = 2-propenyl; 3B = 3-butenyl; 4P = 4-pentenyl; 2H3B = 2-hydroxy-3-butenyl; 2H4P = 2-hydroxy-4-pentenyl; 3MSP = 3-methylsulfinylpropyl; 4MSB = 4-methylsulfinylbutyl; 5MSP = 5-methylsulfinylpentyl; 2PE = 2-phenylethyl; n.d. = not detectable. Each value represents the mean of 9 samples. Mean values are compared for each individual glucosinolate. Values followed by the same superscript are not significantly different.

<table>
<thead>
<tr>
<th>Table 3. Total glucosinolate concentration, major aliphatic, aromatic and indole glucosinolates in 5 cruciferous plant species (plant pot experiment)</th>
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<tr>
<td>Plant species</td>
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<td>---------------------------------------------------------------</td>
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<tr>
<td><em>Brassica rapa</em> var. <em>alboglabra</em></td>
</tr>
<tr>
<td><em>Brassica juncea</em></td>
</tr>
<tr>
<td><em>Nasturtium officinale</em></td>
</tr>
<tr>
<td><em>Brassica oleracea</em> var. <em>capitata</em></td>
</tr>
<tr>
<td><em>Brassica campestris</em></td>
</tr>
</tbody>
</table>

GS = Glucosinolates; DM = dry matter; H = high, M = medium, L = low concentrations of total glucosinolates; 2P = 2-propenyl; 3B = 3-butenyl; 4MSB = 4-methylsulfinylbutyl; 2PE = 2-phenylethyl; 3IM = 3-indolymethyl; n.d. = not detectable. Each value represents the mean of 9 samples. Mean values are compared for each individual glucosinolate and glucosinolate group, respectively. Values followed by the same superscript are not significantly different.
Contrast, the growth on benzyl and plant extracts containing high concentrations of 2-phenylethyl (*Nasturtium officinale*) was about 60% lower (fig. 1, table 2).

### Concentration and Composition of Glucosinolates and Other Carbon Sources in Cruciferous Plants

The 5 investigated cruciferous species varied in their total and individual glucosinolate concentrations resulting in low, medium, or high glucosinolate levels (table 3). Moreover, these species differed in their composition, especially in a particular major aliphatic (2-propenyl, 3-butenyl, 4-methylsulfinylbutyl) or aromatic (benzyl) glucosinolate combined with varying indole glucosinolate concentrations (0.04–0.20 mg g⁻¹ dry matter, DM) and percentages (0.3–8.5%) (table 3). The concentration of the predominant aliphatic or aromatic glucosinolate ranged from 0.37 to 6.63 mg g⁻¹ DM which translates to between 20 and 94% of the total glucosinolate content (table 3).

The investigated cruciferous species also differed in their concentrations of other carbon sources (table 4). *Brassica rapa var. alboglabra* showed the highest concentration in phyllospheric fructose, sucrose and total carbon whereas glucose was the predominant monosaccharide in *B. campestris* compared to all other investigated cruciferous species. The lowest concentration in mono- and disaccharides as well as in total carbon was measured in *N. officinale*, resulting in the lowest C/N ratio. The highest C/N ratio was shown by *B. oleracea var. capitata* and *B. campestris*.

### Concentrations of Flavonoids and Carotenoids in Cruciferous Plants

The major flavonoids in the investigated cruciferous species were quercetin, kaempferol, and isorhamnetin as well as lutein and β-carotene were the predominant carotenoids. The cruciferous species exhibited different flavonoid concentrations with the highest quercetin concentration in *N. officinale*, and highest kaemperol concentrations in *B. rapa var. alboglabra* and *B. oleracea var. capitata* (table 5). Additionally, isorhamnetin occurred in *B. juncea* and *B. campestris*. With respect to the carotenoids, *N. officinale* and *B. campestris* were characterized...
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by increased lutein concentrations (table 5). In addition, B. campestris also comprised the highest /H9252-carotene concentration. B. oleracea var. capitata showed the lowest carotenoid concentrations (table 5).

Colonization by Inoculated E. radicincitans Cells of the Phyllosphere of Cruciferous Plants

The inoculated E. radicincitans cells were able to colonize the phyllosphere of B. campestris in high concentrations even after a plant growth period of about 6 weeks (fig. 2). In B. oleracea var. capitata leaves, only weak colonization was found; however, the introduced bacterial cells did not significantly colonize the plant phyllosphere of the other three cruciferous plants (B. juncea, B. rapa var. alboglabra, N. officinale).

Effect of E. radicincitans Inoculation on Glucosinolate Concentration and Other Plant Compounds in Cruciferous Plants

Neither the total glucosinolate concentration nor the individual glucosinolates in the phyllosphere of the investigated 5 cruciferous species were affected by E. radicincitans inoculation (data not shown). Overall, E. radicincitans inoculation did not alter glucosinolate concentration or composition whether the phyllosphere was strongly colonized or not.

Additionally, the phyllospheric concentration of the compounds used as further carbon sources by E. radicincitans (sugars, total carbon) and other phytochemicals potentially affecting E. radicincitans growth (flavonoids, carotenoids) was not altered either after E. radicincitans inoculation (data not shown).

Discussion

Bacterial Growth Responses to Glucosinolate Concentration and Composition

The effect of changing carbon substrate composition on E. radicincitans indicates the strong interrelation between this enterobacterial strain and glucosinolate composition under nutrient-limited conditions. The distinct preference of E. radicincitans for alkenyl glucosinolates (exemplified by 2-propenyl and plant extracts containing a high 2-propenyl, 3-butenyl and 4-pentenyl concentration) in comparison with lower utilization of aromatic glucosinolates (exemplified by benzyl standard and plant extracts containing high 2-phenylethyl concentrations) might be due to the relatively easy cleavage of the double-bonded carbon in respect to carbon which is integrated in benzene ring systems. This glucosinolate-specific effect on bacterial growth in vitro was also found in vivo by Ruppel et al. [2008] as they demonstrated that the bacterial population density in the plant phyllosphere was positively correlated to the alkenyl glucosinolates 2-propenyl, 3-butenyl and 4-pentenyl whereas the aromatic glucosinolate 2-phenylethyl showed a negative correlation to the phyllospheric bacterial population size. This glucosinolate specificity is underlined by calculating the effect of the main glucosinolates 2-propenyl, 3-butenyl, 4-pentenyl and 2-phenylethyl on the colonization ability of E. radicincitans by means of multiple regression analy-

Table 6. Multiple regression analysis to determine the effect of aromatic 2-phenylethyl glucosinolate and alkenyl glucosinolates (2-propenyl, 3-butenyl and 4-pentenyl) in 11 cruciferous plant extracts (in vitro experiment) on colonization ability of E. radicincitans (n = 33), r = 0.830

<table>
<thead>
<tr>
<th>Variables</th>
<th>Standardized regression coefficients (β)</th>
<th>p level¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Propenyl</td>
<td>0.83</td>
<td>0.000</td>
</tr>
<tr>
<td>3-Butenyl</td>
<td>0.24</td>
<td>0.008</td>
</tr>
<tr>
<td>4-Pentenyl</td>
<td>−0.03</td>
<td>0.718</td>
</tr>
<tr>
<td>2-Phenylethyl</td>
<td>−2.21</td>
<td>0.021</td>
</tr>
</tbody>
</table>

¹ Significant at p ≤ 0.05.
sis. It was clearly shown that 2-propenyl and 3-butenyl positively affected the colonization ability of the introduced bacterial strain and that 2-phenylethyl had a significant negative effect, while 4-pentenyl had no significant impact (table 6).

Moreover, no direct inhibition of bacterial growth occurred in nutrient solution by adding pure glucosinolates to the medium, which indicates that the glucosinolates themselves are nontoxic [Manici et al., 1997; Mithen et al., 1996]. Glucosinolates can be released from the vacuoles in the surrounding tissue [Bones and Rossiter, 1995; Mari et al., 1993; Tierens et al., 2002] which probably prevented the adhesion of the bacterial strain and that 2-phenylethyl had a significant negative effect, while 4-pentenyl had no significant impact (table 6).

Colonization of Introduced E. radicincitans Cells in the Phyllosphere

That E. radicincitans could not colonize the phyllosphere of N. officinale might be due to the high concentration of aromatic 2-phenylethyl as well as its breakdown products. Major degradation products – catalyzed by the endogenous thioglucosidase enzyme (myrosinase) – include isothiocyanates, thiocyanates, and nitriles [Mikkelsen et al., 2002; Rask et al., 2000], which are all known to have antimicrobial effects [Brader et al., 2001; Manici et al., 1997; Mari et al., 1993; Tierens et al., 2001], while glucosinolates themselves do not have bactericidal effects as shown in our pure culture experiments with aliphatic 2-propenyl, aromatic benzyl glucosinolates and cruciferous plant extracts. Myrosinase is stored in idioblastic myrosin cells, while glucosinolates are located in the cell vacuoles in the surrounding tissue [Bones and Rossiter, 1995; Chen and Andreasson, 2001]. Thus, the glucosinolate-myrosinase system is a physically separated two-component system and glucosinolate breakdown products are only generated by cell disruption occurring by wounding, chewing, or maceration by herbivores and microbes [Tierens et al., 2001]. As phyllospheric microorganisms can cause glucosinolate breakdown by metabolizing plant cell components, it was found that glucosinolate degradation products differ in their toxicity. Aromatic isothiocyanates are more toxic than aliphatic ones, resulting in higher antifungal activity [Manici et al., 1997; Sarwar et al., 1998]. The generally higher toxicity of aromatic isothiocyanates could also cause enhanced antibacterial activity, leading to the inability of E. radicincitans to colonize the phyllosphere of N. officinale that contains very high concentrations of the aromatic 2-phenylethyl glucosinolate compared to all other cruciferous species investigated in this study. Finally, Brader et al. [2006] demonstrated a 10-fold reduction in bacterial growth by Pseudomonas syringae in transgenic Arabidopsis thaliana having an enhanced aromatic glucosinolate concentration. Moreover, the low colonization in the phyllosphere of N. officinale might also be caused by the relatively low levels of further carbon sources such as phyllospheric sugars and phyllospheric total carbon (table 4). The relatively low β-carotene concentration in the leaves (table 5) could also induce this low colonization effect as Ruppel et al. [2008] found a positive correlation between β-carotene concentration and microbial phyllospheric population since β-carotene, a precursor of vitamin A, mainly acts as microbial growth factor compared with other carotenoids. The distinct growth restriction of E. radicincitans in N. officinale might also be due to the markedly high level of quercetin (table 5) as quercetin was found to be a potential antibacterial [Li and Xu, 2008] and antifungal agent [Skadhauge et al., 1997].

However, B. juncea, which contains a relatively high concentration of aliphatic glucosinolates such as 2-propenyl, shown to support bacterial growth in the in vitro experiment, was not colonized by E. radicincitans 6 weeks after inoculation of the plants. This inhibitory impact might be essentially due to the very hairy leaf surface of B. juncea which probably prevented the adhesion of E. radicincitans to the leaf surface and thus the colonization of the cells since bacterial adhesiveness highly depends on the surface constitution of the target [Andrews and Buck, 2002]. Moreover, the combination of medium concentrations of glucosinolates and kaempferol (table 3, 5), which is an effective antimicrobial flavonoid too [Bloor, 1995; Sousa et al., 2008], suggests an additive suppressive effect of both phytochemicals on the growth of E. radicincitans.

In contrast to B. juncea, B. oleracea var. capitata was also characterized by 2-propenyl as the major glucosinolate, but at a distinctly low concentration, and this Brassica species was slightly colonized by E. radicincitans. Tierens et al. [2001] found that in Arabidopsis thaliana, older leaves containing higher aliphatic glucosinolate concentrations than younger ones had fewer severe microbial- or fungal-induced chlorosis symptoms. The accumulation of aliphatic glucosinolates also reduced the susceptibility of A. thaliana to Erwinia carotovora [Brader et al., 2006]. As increased concentrations of aliphatic glucosinolates reduced bacterial colonization, the low concentration of aliphatic 2-propenyl in B. oleracea var. capitata leaves might allow some colonization of B. oleracea var. capitata. The growth of E. radicincitans on B. oleracea var. capitata might also be supported by relatively high concentrations of phyllospheric monosaccharides and phyllospheric total carbon (table 4) acting as additional carbon sources and compensating for the antibacterial activity of the high kaempferol concentration (table 5).
B. campestris was characterized by the highest colonization of its phyllosphere by E. radicincitans in comparison with the other species, despite the total glucosinolate concentration being nearly as high as in N. officinale. As in B. campestris the aliphatic 3-butenyl was the predominant glucosinolate, this colonization effect underlines the specific impact of individual glucosinolate breakdown products on microorganisms as aromatic isothiocyanates are more toxic than aliphatic isothiocyanates [Brader et al., 2006; Manici et al., 1997; Sarwar et al., 1998]. The high colonization of the leaves of B. campestris by E. radicincitans might also be due to other growth-promoting phytochemicals such as carotenoids [Ballows et al., 1992; Janczik et al., 2006; Müller et al., 1998]. As B. campestris was also characterized by a high carotenoid concentration – especially β-carotene – combined with moderate quercetin and kaempferol concentrations compared to all other investigated Brassica species, a distinct bacterial-colonization-supporting effect of carotenoids is suggested as demonstrated by Ruppel et al. [2008]. Isorhamnetin, the major flavonoid in B. campestris (table 5), seems to exert no suppressing effect on colonization by E. radicincitans, and no reference was found explicitly demonstrating antimicrobial activity of isorhamnetin. Moreover, its relatively high concentration in phyllospheric sugars, especially glucose and fructose, might also contribute to E. radicincitans growth in this plant phyllosphere. In B. rapa var. albolabra, two major aliphatic glucosinolates were found: 3-butenyl and methylsulfinylbutyl. Isothiocyanates of methylsulfinylbutyl are characterized by pronounced antimicrobial activity [Tiersen et al., 2001], suggesting that the strong inhibition of E. radicincitans growth in B. rapa var. albolabra could mainly be due to methylsulfinylbutyl glucosinolate. In addition, the high kaempferol concentration in B. rapa var. albolabra indicates a pronounced antimicrobial effect as E. radicincitans growth was limited as was also the case for bacterial pathogens [Pourcel et al., 2006; Sousa et al., 2008].

**Effect of E. radicincitans Inoculation on Glucosinolate Concentration**

Bacterial pathogens such as E. carotovora cause considerable tissue damage and necrosis, leading to the formation of glucosinolate breakdown products [Tiersen et al., 2001]. In contrast, Pseudomonas syringae cause only slight or no cell damage during pathogenesis, and thus do not induce the release of glucosinolates by host cell vacuoles or their myrosinase-dependent conversion to antibacterial breakdown products [Tiersen et al., 2001], which also indicates that nonpathogenic Enterobacter strains might cause mild cell damage too by metabolizing plant cell components and releasing fewer glucosinolate degradation products. This slight mechanical cell disruption might be the reason why E. radicincitans inoculation did not induce a plant defense response, and thus a subsequent increase in glucosinolate concentration in the phyllosphere of the cruciferous species investigated here. However, in a study of B. rapa var. rapifera, we found a glucosinolate-stimulating influence of E. radicincitans if inoculated in combination with methyl jasmonate, which had been observed to lead to a synergistic increase in glucosinolate concentration [Sauder, 2006]. As methyl jasmonate is a signaling molecule and triggers a signal cascade that activates several defense responses including the synthesis of glucosinolates by affecting gene transcription [Mikkelsen et al., 2000], the combined inoculation of E. radicincitans and methyl jasmonate might induce an increased plant defense response.

Since glucosinolate concentration did not increase after E. radicincitans inoculation, we assume that E. radicincitans, an auxin-producing bacterium, did not stimulate glucosinolate biosynthesis via indole glucosinolates by additional bacterial auxin supply. One reason for this could be that there was insufficient bacterial auxin production to influence secondary plant metabolism or that auxin utilization by the plant mainly supported its development. Another reason could be that the inoculated bacterial cells were not able to colonize and persist in the phyllosphere although E. radicincitans grew in cruciferous plant extracts. Therefore, no bacterial-induced phytohormone supply was possible at least in 3 of the 5 plant species.

Since the investigated cruciferous species are economically important vegetables that contain various concentrations of glucosinolates, future crop management strategies designed to support plant growth and optimize glucosinolate concentrations – as interesting human health-promoting compounds – by using PGPB benefit from advances in our understanding of bacterial colonization in the cruciferous phyllosphere. The impact of aromatic and certain aliphatic glucosinolates observed in our study revealed that not only plant pathogens were affected in their growth by glucosinolates [Brader et al., 2001, 2006; Bending and Lincoln, 2000; Tiersen et al., 2001], but also the native bacterial population [Ruppel et al., 2008] and even the PGPB as exemplified by E. radicincitans in the present study. The results of this study further confirm promotion or suppression of E. radicincitans colonization and persistence and provide detailed information on how this process is dependent on the
Experimental Procedures

Two experiments were carried out: an in vitro experiment as a bacterial pure culture experiment with glucosinolate standards and Brassica plant extracts to test the glucosinolate effect on bacterial growth responses, and a second experiment with inoculation of the PGPB E. radicincitans to 5 different species of the family Brassicaceae each characterized by a particular major aliphatic or aromatic glucosinolate and by different concentrations of indole glucosinolates to determine the colonization ability of E. radicincitans with respect to the glucosinolate composition of each plant.

Plants were grown in growth cabinets (plant pot experiment) to rigorously control growth conditions since environmental factors such as radiation and temperature are frequently observed to influence the concentration of glucosinolates in the plants [Milford and Evans, 1991; O’Callaghan et al., 2000; Sarwar and Kirgegaard, 1998; Schonhof et al., 2007; Schreiner, 2005].

Plant Material and Experimental Design

In vitro Experiment
A microplate bacterial pure culture experiment was conducted using a bacterial pure culture of E. radicincitans strain DSM 16656 [Kämpfer et al., 2005]. The experiment was conducted to analyze the ability of the bacteria to use the aliphatic glucosinolate 2-propenyl (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and the aromatic benzylglucosinolate (AppliChem GmbH, Darmstadt, Germany) as carbon source in comparison to glucose and to a control without carbon in minimal medium.

The experiment was carried out in block design with 3 replicates in growth cabinets (type V81014, Vötsch Industrietechnik GmbH, Balingen-Frommern, Germany) under controlled climatic conditions of a 12:12 h light/dark photoperiod, corresponding to a photosynthetic photon flux density of 400 μmol m⁻² s⁻¹, 70–80% relative humidity, and 15/10°C day/night temperature. Five plants each of B. juncea, B. campestris, B. oleracea var. capitata, and B. rapa var. alboflagla were grown in pots with 600 g substrate. For N. officinale, 25 seeds were placed in one 600-gram pot. A total of 225 mg nitrogen per pot was added. For each species, 6 pots were used for each replicate. Water was supplied according to the requirements of the plants. Plants were harvested at the 6-leaf stage.

Bacterial cells of E. radicincitans were grown in standard nutrient solution (Merck KGaA, Darmstadt, Germany) at 29°C in a rotary incubator at 100 rpm for 48 h. To remove all additional nutrients, bacterial cells were washed twice in sterile tap water by centrifugation at 8,000 g for 20 min before inoculation. Plants were inoculated with 10⁸ cells per plant by spraying the bacterial suspension onto the plants leaves when two leaves had emerged. Control plants were inoculated with sterile tap water only.

Quantification of E. radicincitans using a Species-Specific TaqMan Probe and the Plant Housekeeping TEF Gene

16S rDNA (E. radicincitans-specific) and housekeeping TEF (plant-specific) gene copy numbers were determined for the same plant sample using two different real-time PCR procedures. E. radicincitans gene copy number was determined using a TaqMan™ assay and a species-specific probe, while the TEF gene copy numbers were measured using the SybrGreen I approach. Primer probe design and PCR conditions were as described earlier [Ruppel et al., 2006], DNA was extracted from 10 mg freeze-dried leaf material using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA concentration was measured photometrically at 260 nm (Nanodrop ND1000, Technology Inc., Wilmington, Del., USA) and quality was checked photometrically by the A₂₆₀/A₃₂₀ ratio calculation to be between 1.9 and 2.1 and the A₂₆₀ measurement was nearly 0. The colonization of inoculated E. radicincitans cells was calculated using the ΔΔCt method, shown as x-fold increase of E. radicincitans cells in the inoculated treatment samples relative to the noninoculated control sample and normalized to the reference gene (housekeeping TEF gene) [Livak and Schmittgen, 2001].
Sample Preparation
A mixed sample of 30 plants of each cultivar of the leafy *Brassica* vegetables and 300 plants of *N. officinale* was used from each replication (n = 3). 15 g of fresh matter of leaves was used for each carotenoid and sugar analyses. For glucosinolate and flavonoid determination, about 200 g fresh matter of leaves was immediately deep frozen (–40°C), freeze-dried, and then the dry matter was finely ground.

Glucosinolate Analysis
The HPLC method reported by Krumbein et al. [2005] was used for glucosinolate determination. Duplicates of freeze-dried sample material (0.5 g) were heated to and incubated at 75°C for 1 min, extracted with 4 ml of a methanol/water mixture (v/v = 7:3, at 70°C), and then, after adding 1 ml 0.4 M barium acetate, centrifuged at 4,000 rpm for 10 min. For an internal standard, 200 μl of a 5 mM stock solution of sinigrin in methanol was added to one of the duplicates just before the first extraction. The residue was extracted twice more with 3 ml of the methanol/water mixture (v/v = 7:3, 70°C). The supernatants were pooled and made up to 10 ml with the methanol/water mixture (v/v = 7:3, 70°C). From this, 5 ml of the extract was applied to a 250-μl DEA-Sephadex (acetic acid-activated, Sigma-Aldrich Chemie) and rinsed with 10 ml bidistilled water. Next, 250 μl of a purified solution of aryl sulfatase (Boehringer-Mannheim GmbH, Mannheim, Germany) was applied and left for 12 h before flushing the desulfocompounds with 5 ml bidistilled water. Desulfo-glucosinolate analysis was conducted by HPLC (Merck Hitachi, Darmstadt, Germany) using a Spherisorb ODS2 column (5 μm, 250 × 4 mm). A gradient of 0–20% acetonitrile in water was selected (2–34 min), followed by 20% acetonitrile in water (up to 40 min), and then 100% acetonitrile (10–50 min). Determination was conducted at a flow of 1.3 ml·min⁻¹ and a wavelength of 229 nm. Determination was repeated with acetone until the resulting filtrate was colorless. The extract was then filtered through a 0.45-μm filter for HPLC analysis. Carotenoid concentrations were determined by HPLC using a C-18 reversed-phase column Lichrosphere 100 (5 μm, 250 × 4 mm; Merck) with an isocratic eluent of 75% acetonitrile, 15% methanol and 10% methylene chloride. The analysis was carried out at a flow rate of 1 ml·min⁻¹. Wavelengths of 448 and 455 nm were used to determine lutein and β-carotene, respectively. Concentrations were determined quantitatively by calibration curves of the related pure standards. Chemical analyses were performed in duplicate.

Sugar Analysis
The concentrations of various free sugars (glucose, fructose, sucrose) were analyzed enzymatically [Boehringer Mannheim, 1989].

Nitrogen and Carbon Analysis
For the nitrogen and carbon analyses, finely ground samples were burned in the element analyzer Vario EL (Elementar Analyse- systeme GmbH, Hanau, Germany) using Dumas’ burning method [VDLUF, 1991]. Total nitrogen and carbon concentrations were also assessed in duplicate.

Statistical Analysis
Comparison of mean values of 3 or 4 replicates was performed using ANOVA and Tukey’s HSD test at a p ≤ 0.05. Using the REG procedure, linear multiple regression analysis based on the F test was conducted at a significance level of p ≤ 0.05. All calculations were carried out using the software package Statistica for Windows (version 6.1, Statsoft Inc. 2001).

Acknowledgements
This research was supported by the German Federation and the Federal States Brandenburg and Thuringia. We would like to thank A. Jankowsky for her phytochemical analyses, B. Wernitz for her microbial work and E. Büsch, U. Zentner, and A. Maikath for their technical assistance.
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