Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2009;17:124–135
DOI: 10.1159/000226589

Published online: June 26, 2009

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

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Key Words

Phytochemicals · Alkenyl glucosinolates · Methylsulfinyl glucosinolates · 2-Phenylethyl glucosinolate · Persistence of plant growth-promoting bacteria · Real-time PCR

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 Drypteres 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

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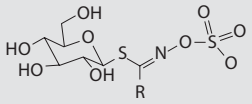
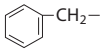
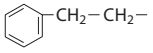
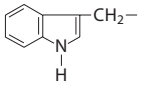
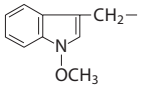
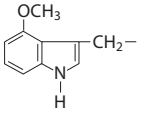
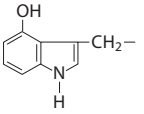
Table 1. Structural formulae of glucosinolates assessed in this study

 Basic structure of glucosinolates			
Glucosinolate group	Trivial name	Semisystematic name	Structure of R group
Aliphatic GS			
Alkyl GS	glucoiberin	3-methylsulfinylpropyl	$CH_3–SO–CH_2–CH_2–CH_2–$
	glucoraphanin	4-methylsulfinylbutyl	$CH_3–SO–CH_2–CH_2–CH_2–CH_2–$
	glucoalyssin	5-methylsulfinylpentyl	$CH_3–SO–CH_2–CH_2–CH_2–CH_2–CH_2–$
Alkenyl GS	sinigrin	2-propenyl	$CH_2=CH–CH_2–$
	gluconapin	3-butenyl	$CH_2=CH–CH_2–CH_2–$
	glucobrassicanapin	4-pentenyl	$CH_2=CH–CH_2–CH_2–CH_2$
Hydroxy-Alkenyl GS	progoitrin	2-hydroxy-3-butenyl	$CH_2=CH–CH(OH)–CH_2–$
	gluconapoleiferin	2-hydroxy-4-pentenyl	$CH_2=CH–CH_2–CH(OH)–CH_2–$
Aromatic GS	glucotropaeolin	benzyl	
	gluconasturtiin	2-phenylethyl	
Indole GS	glucobrassicin	3-indolylmethyl	
	neoglucobrassicin	N-methoxy-3-indolylmethyl	
	4-methoxy-glucobrassicin	4-methoxy-3-indolylmethyl	
	4-hydroxy-glucobrassicin	4-hydroxy-3-indolylmethyl	

GS = Glucosinolates.

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-affected 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

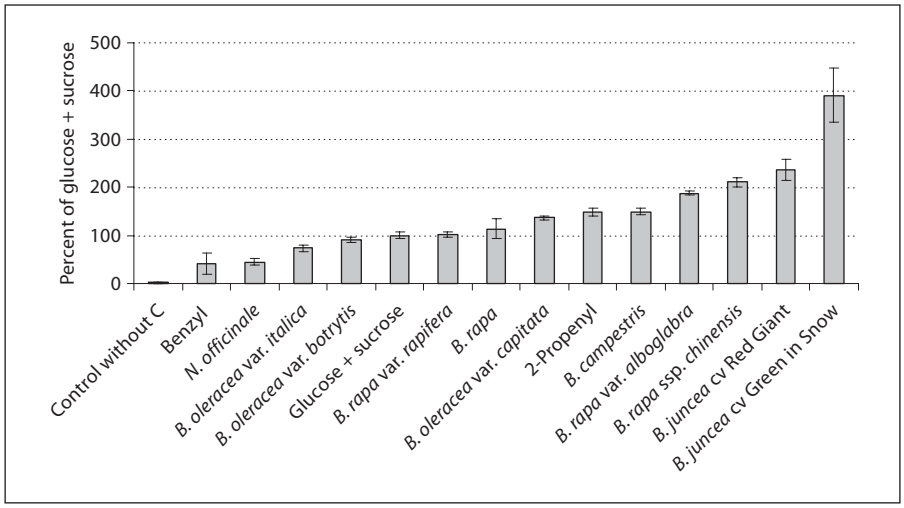


Fig. 1. Bacterial growth of *E. radicincitans* DSM16656 with glucosinolate standards (2-propenyl and benzyl), glucose + sucrose and *Brassica* plant extracts as C sources (4 μg C μl^{-1}) on minimal medium (in vitro experiment). Bacterial growth response was calculated relatively to the growth on glucose + sucrose (= 100%). Bars indicate the standard deviation of the mean values from three replicates.

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 oleracea* [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

Table 2. Individual glucosinolate concentration (mg 100 g^{-1} fm) in the leaf extract of 11 cruciferous plant species (in vitro experiment)

Plant species	Aliphatic GS								Aromatic GS	Indole GS
	2P	3B	4P	2H3B	2H4P	3MSP	4MSB	5MSP	2PE	total
Brassica rapa var. *alboglabra*	n.d.	1.42^{b}	n.d.	n.d.	n.d.	n.d.	0.58^{a}	n.d.	n.d.	0.26^{b}
Brassica rapa	n.d.	0.61^{b}	3.93^{b}	1.48^{a}	1.75^{a}	n.d.	n.d.	0.15^{b}	0.50^{b}	1.52^{ab}
Brassica rapa ssp. *rapifera*	n.d.	0.39^{b}	1.48^{b}	1.03^{b}	0.66^{b}	n.d.	n.d.	0.36^{a}	0.47^{b}	4.40^{a}
Brassica. rapa ssp. *chinensis*	n.d.	3.04^{ab}	1.27^{b}	0.21^{c}	n.d.	n.d.	n.d.	0.10^{c}	0.66^{b}	1.08^{b}
Brassica oleracea var. *capitata*	2.49^{b}	0.10^{b}	n.d.	0.16^{c}	n.d.	0.66^{a}	0.03^{c}	n.d.	0.08^{b}	1.08^{b}
Brassica oleracea var. *italica*	n.d.	n.d.	n.d.	n.d.	n.d.	0.74^{a}	0.30^{b}	n.d.	n.d.	0.44^{b}
Brassica oleracea var. *botrytis*	0.79^{b}	n.d.	n.d.	n.d.	n.d.	0.80^{a}	n.d.	n.d.	n.d.	0.64^{b}
Brassica juncea cv. Green in Snow	18.30^{ab}	0.67^{b}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.16^{b}	0.61^{b}
Brassica juncea cv. Red Giant	31.14^{a}	1.65^{b}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.36^{b}	
Brassica campestris	n.d.	8.68^{a}	1.72^{b}	0.33^{c}	n.d.	n.d.	n.d.	0.10^{c}	0.22^{b}	1.33^{b}
Nasturtium officinale	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	15.46^{a}	0.30^{b}

GS = Glucosinolate; fm = fresh matter; 2P = 2-propenyl; 3B = 3-butenyl; 4P = 4-pentenyl; 2H3B = 2-hydroxy-3-butenyl; 2H4P = 3-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 3. Total glucosinolate concentration, major aliphatic, aromatic and indole glucosinolates in 5 cruciferous plant species (plant pot experiment)

Plant species	Total GS		Major aliphatic GS						Major aromatic GS		Major indole GS	
			2P		3B		4MSB		2PE		3IM	
	mg g^{-1} dm		mg g^{-1} dm	%	mg g^{-1} dm	%	mg g^{-1} dm	%	mg g^{-1} dm	%	mg g^{-1} dm	%
Brassica rapa var. *alboglabra*	1.85^{c}	L	0.15^{c}	8	0.72^{b}	38.9	0.37^{a}	20.0	0.02^{c}	1.1	0.04^{c}	2.2
Brassica juncea	4.27^{b}	M	3.93^{a}	9	0.16^{b}	3.8	n.d.	0.0	0.12^{b}	2.8	0.03^{c}	0.7
Nasturtium officinale	7.05^{a}	H	n.d.	0	n.d.	0.0	n.d.	0.0	6.63^{a}	94.0	0.15^{a}	2.1
Brassica oleracea var. *capitata*	1.76^{c}	L	1.07^{b}	6	0.10^{b}	5.7	0.07^{b}	4.0	0.03^{c}	1.7	0.12^{ab}	6.8
Brassica campestris	5.60^{ab}	H	0.03^{c}	1	4.76^{a}	85.0	n.d.	0.0	0.70^{b}	12.5	0.07^{bc}	

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-indolylmethyl; 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.

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 4. Concentrations of phyllospheric carbon sources (mg g^{-1}dry matter) in 5 cruciferous plant species (plant pot experiment)

Plant species	Glucose	Fructose	Sucrose	C	N	C/N
Brassica rapa var. *alboglabra*	61.00^{ab}	39.15^{a}	19.94^{a}	41.38^{a}	1.78^{b}	23.78^{ab}
Brassica juncea	37.82^{b}	27.52^{ab}	11.88^{bc}	39.89^{bc}	1.72^{b}	23.65^{ab}
Nasturtium officinale	21.13^{c}	16.14^{b}	7.30^{cd}	38.78^{c}	2.95^{a}	13.32^{b}
Brassica oleracea var. *capitata*	43.86^{ab}	30.37^{ab}	5.90^{d}	40.42^{ab}	1.62^{b}	25.62^{a}
Brassica campestris	70.64^{a}	18.11^{b}	13.77^{b}	39.36^{bc}	1.55^{b}	25.74^{a}

N = Total nitrogen; C = total carbon. Each value represents the mean of 9 samples. Mean values are compared for each compound. Values followed by the same superscript are not significantly different.

Table 5. Concentrations of major flavonoids and carotenoids (mg g^{-1} dry matter) in 5 cruciferous plant species (plant pot experiment)

Plant species	Quercetin	Kaempferol	Isorhamnetin	Lutein	β-Carotene
Brassica rapa var. *alboglabra*	0.43^{b}	1.94^{a}	n.d.	0.79^{b}	0.44^{b}
Brassica juncea	0.32^{b}	1.23^{b}	0.63^{b}	0.61^{c}	0.41^{b}
Nasturtium officinale	0.90^{a}	0.19^{c}	n.d.	0.92^{a}	0.39^{b}
Brassica oleracea var. *capitata*	0.54^{b}	2.02^{a}	n.d.	0.59^{c}	0.29^{c}
Brassica campestris	0.30^{b}	1.06^{b}	1.44^{a}	0.82^{ab}	0.56^{a}

Each value represents the mean of 9 samples. Mean values are compared for each compound. Values followed by the same superscript are not significantly different. n.d. = Not detectable.

trast, 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). *B. 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

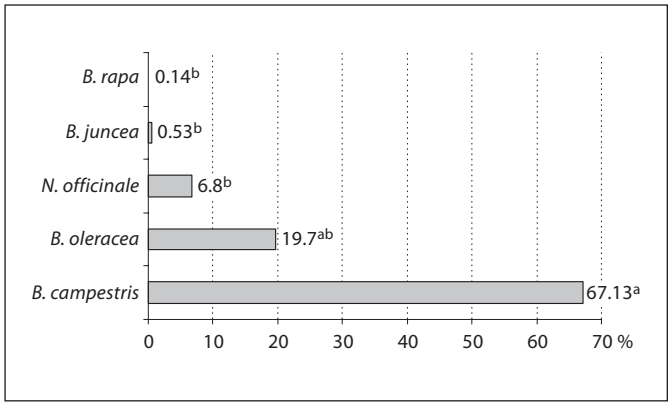


Fig. 2. Colonization ability of *E. radicincitans* bacterial cells inoculated into the phyllosphere of 5 cruciferous species (plant pot experiment). Quantification of *E. radicincitans* in the inoculated treatment sample was relative to the non-inoculated control sample and normalized to the reference TEF gene (mean values of 3 replicates, different superscripts indicate significant differences between plants).

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

Variables	Standardized regression coefficients (β)	p level[1]
2-Propenyl	0.83	0.000
3-Butenyl	0.24	0.008
4-Pentenyl	–0.03	0.718
2-Phenylethyl	–2.21	0.021

[1] Significant at $p \leq 0.05$.

by increased lutein concentrations (table 5). In addition, *B. campestris* also comprised the highest β-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 standard 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-

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., 1986].

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 (tables 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; Janzcik 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. *alboglabra,* two major aliphatic glucosinolates were found: 3-butenyl and methylsulfinylbutyl. Isothiocyanates of methylsulfinylbutyl are characterized by pronounced antimicrobial activity [Tierens et al., 2001], suggesting that the strong inhibition of *E. radicincitans* growth in *B. rapa* var. *alboglabra* could mainly be due to methylsulfinylbutyl glucosinolate. In addition, the high kaempferol concentration in *B. rapa* var. *alboglabra* 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 [Tierens 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 [Tierens 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; Tierens 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

plant's major individual glucosinolate – besides other physical and chemical factors – and its concentration as demonstrated in the phyllosphere of various cruciferous plants.

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 + sucrose and to a control without carbon in minimal medium. Additionally, the leaf extracts obtained from 11 *Brassica* species containing different concentrations and compositions of glucosinolates were investigated. These selected *Brassica* species comprised the *Brassica* species of the plant pot experiment *(B. juncea* cv Red Giant, *B. campestris* cv Mibuna Early, *B. oleracea* var. *capitata* cv Türkis, and *B. rapa* var. *alboglabra*, *N. officinale)* which were supplemented by *Brassica* species, varieties or cultivars *(B. juncea* cv Green in Snow, *B. rapa* var. *rapifera*, *B. rapa*, *B. rapa* ssp. *chinensis*, *B. oleracea* var. *italica*, *B. oleracea* var. *botrytis)* characterized by alternative glucosinolate concentrations or compositions (table 2).

Bacteria grown in standard I nutrient solution (Merck KGaA, Darmstadt, Germany) from exponential growth phase were washed twice by centrifugation at 8,000 *g* for 20 min in sterile 0.05 M NaCl solution to remove all other nutrients and carbon sources. 10 μl of the bacterial solution (optical density $A_{620} = 0.7$) was inoculated into 190 μl minimal medium [Gerhardt et al., 1994] supplemented with 0.4 μg N μl^{-1} as NH_4NO_3 using microtiter plates. The glucosinolates 2-propenyl and benzyl and a mixture of glucose + sucrose were filter sterilized (0.2 μm Millipore, Billerica, Mass., USA) and applied at a final concentration of 4 μg C μl^{-1}. Plant extracts were sterilized by microwave treatment [Song and Thornalley, 2007]. Sterile controls containing nitrogen and carbon sources were prepared to check the sterility in the absence of bacterial inoculates and were used as blanks. Plant extracts of 11 *Brassica* plants (10 μl per well) were tested likewise. All treatments were replicated 6 times. Microtiter plates were covered by clear transparent lids and incubated at 30°C in a microplate reader (anthos htIII, Eugendorf, Austria) for 28 h. Bacterial kinetic growth was monitored photometrically at 620 nm every 10 min during the incubation time. Bacterial growth responses to phytochemicals and plant extracts were calculated versus growth on glucose + sucrose.

Plant Pot Experiment

Five plant species of the Brassicaceae family *(B. juncea* cv Red Giant, *B. campestris* cv Mibuna Early, *B. oleracea* var. *capitata* cv Türkis, and *B. rapa* var. *alboglabra*, *N. officinale)* characterized by a particular major aliphatic or aromatic glucosinolate and by different concentrations of indole glucosinolates (table 3) were inoculated with the PGPB strain *E. radicincitans*. The experiment was carried out in block design with 3 replicates in growth cabinets (type VB1014, 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. *alboglabra* 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 10^8 cells per plant by spraying the bacterial suspension onto the plant 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 A_{260}/A_{280} ratio calculation to be between 1.9 and 2.1 and the A_{320} measurement was nearly 0. The colonization of inoculated *E. radicincitans* cells was calculated using the $\Delta\Delta C_t$ 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 A-25 ion exchanger (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. Desulfoglucosinolate 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 $ml \cdot min^{-1}$ and a wavelength of 229 nm. Desulfoglucosinolates were identified by HPLC-APCI–MS^2 using Agilent 1100 series in the positive ionization mode [Krumbein et al., 2005; Zimmermann et al., 2007]. Glucosinolate concentration was calculated using 2-propenyl glucosinolate as internal and external standard and the response factor of each compound relative to 2-propenyl glucosinolate. Glucosinolates were assessed in duplicate.

Flavonoid Analysis

Flavonoids were determined as their aglycones after acid hydrolysis as described in Krumbein et al. [2007]. 40 μl of 62.5% aqueous methanol was added to 0.5 g of the freeze-dried sample. 10 ml of 8 M HCl was added to this extract. Thus, the extraction solution consisted of 1.6 M HCl in 50% aqueous methanol (v/v). After refluxing at 90°C for 2 h, the extract was allowed to cool, was adjusted to 100 ml with 50% methanol, and sonicated for 5 min. The extract was then filtered through a 0.45-μm filter for HPLC analyses.

The flavonol composition (quercetin, kaempferol, isorhamnetin) and concentration were determined using a series 1100 HPLC (Agilent, Waldbronn, Germany) equipped with a diode array detection system. A Prodigy column ODS(3) (250 × 4.6 mm, 5 μm, 100 Å) (Phenomenex, Aschaffenburg, Germany) was used with a security guard C18 (4 × 3.0 mm) at a temperature of 25°C. Solvent A was water + 0.1% trifluoroacetic acid + 2% tetrahydrofuran; solvent B was acetonitrile. The following gradient was used: 30–35% B (5 min), 35–39% B (12 min), 39–90% B (5 min), 90% B isocratic (2 min), 90–30% B (5 min), 30% B isocratic (5 min). The chromatograms were monitored at 270 nm with a flow rate of 1 $ml \cdot min^{-1}$. Contents were quantitatively determined by calibration curves of the related pure standards. Flavonols were identified by HPLC-ESI–MS^2, using Agilent 1100 series in the negative ionization mode [Krumbein et al., 2007].

Carotenoid Analysis

Carotenoids (lutein, β-carotene) were also determined by HPLC [Krumbein et al., 2005]. To 15 g of cut plant material, 1 g calcium carbonate, 30 g sodium sulfate and 30 ml acetone were added, and the samples were homogenized for 2 min. The extract was then filtered under suction, and the solid materials were extracted repeatedly 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 Lichosphere 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 $ml \cdot min^{-1}$. 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 (Elemtar Analysensysteme GmbH, Hanau, Germany) using Dumas' burning method [VDLUFA, 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 $p \leq 0.05$. Using the REG procedure, linear multiple regression analysis based on the F test was conducted at a significance level of $p \leq 0.05$. 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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