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In Vitro Activity of Glucosinolates and Their Degradation Products against *Brassica*-Pathogenic Bacteria and Fungi

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Glucosinolates (GSLs) are secondary metabolites found in *Brassica* vegetables that confer on them resistance against pests and diseases. Both GSLs and glucosinolate hydrolysis products (GHPs) have shown positive effects in reducing soil pathogens. Information about their *in vitro* biocide effects is scarce, but previous studies have shown sinigrin GSLs and their associated allyl isothiocyanate (AITC) to be soil biocides. The objective of this work was to evaluate the biocide effects of 17 GSLs and GHPs and of leaf methanolic extracts of different GSL-enriched *Brassica* crops on suppressing *in vitro* growth of two bacterial (*Xanthomonas campestris* pv. campestris and *Pseudomonas syringae* pv. maculicola) and two fungal (*Alternaria brassicae* and *Sclerotinia sclerotiorum*) *Brassica* pathogens. GSLs, GHPs, and methanolic leaf extracts inhibited the development of the pathogens tested compared to the control, and the effect was dose dependent. Furthermore, the biocide effects of the different compounds studied were dependent on the species and race of the pathogen. These results indicate that GSLs and their GHPs, as well as extracts of different *Brassica* species, have potential to inhibit pathogen growth and offer new opportunities to study the use of *Brassica* crops in biofumigation for the control of multiple diseases.

The genus *Brassica* belongs to the family *Brassicaceae* (also known as *Cruciferae*); economically speaking, it is the most important genus within the tribe *Brassicaceae*, containing 37 different species. *Brassica* vegetables are of great economic importance throughout the world. Currently, *Brassica* crops, together with cereals, represent the basis of world food supplies. In 2007, *Brassica* vegetables were cultivated in more than 142 countries around the world, and they occupied more than 4.1 million ha (1).

The productivity and quality of important *Brassica* crops (e.g., cabbage, oilseed rape, cauliflower, Brussels sprouts, kale, and broccoli) are seriously affected by several diseases, which result in substantial economic losses (2). Black rot, caused by the bacterium *Xanthomonas campestris* pv. campestris (Pammel), is considered to be one of the most important pathogens affecting *Brassica* vegetables worldwide (3). There are nine races of *Xanthomonas campestris* pv. campestris: races 1 to 6 were described by Vicente et al. (4) and races 7 to 9 by Fargier and Manceau (5). It is recognized that races 1 and 4 are the most virulent and widespread, accounting for most of the black rot recorded around the world (4).

Bacterial leaf spot, caused by *Pseudomonas syringae* pv. maculicola (McCulloch) (6), is very significant on cauliflower but also occurs on broccoli, Brussels sprouts, and other brassicas. *P. syringae* pv. maculicola may also cause leaf blight on the oilseed species *Brassica juncea* and *Brassica rapa* (3).

Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is a widespread fungal disease in temperate areas of the world and also occurs in warmer and drier areas during the winter months or the rainy season. Since the 1950s, stem rot of oilseed brassicas has become increasingly important because of the expanding area of *Brassica napus* and *B. rapa* in Europe, Canada, India, China, and Australia (3).

Alternaria black spot is caused by the fungus *Alternaria brassicae* (Berk.) Sacc. This facultative parasite colonizes susceptible hosts, as well as dead plant material. Particularly severe epidemics in oilseed brassicas occur in India, the United Kingdom, France, Germany, Poland, and Canada. The disease produces a considerable reduction of both yield and seed quality (3).

During the past decade, a large number of compounds from different plants have been tested in order to explore their antimicrobial properties against plant-pathogenic organisms (7, 8), including some of the above-mentioned pathogens (9). Brassica crops have been shown to release toxic compounds that negatively affect bacteria, fungi, insects, nematodes, and weeds. However, few studies focused on the effects of glucosinolates (GSLs) and glucosinolate hydrolysis products (GHPs) on pathogens have been conducted *in vitro* (10).

GSLs are nitrogen and sulfur-containing plant secondary metabolites that occur mainly in Capparales and almost exclusively in the family *Brassicaceae*. GSLs are β-thioglucoside *N*-hydroxysulfates containing a side chain and a β-D-glucopyranosyl moiety. Upon cellular disruption, glucosinolates are hydrolyzed to various bioactive breakdown products by the endogenous enzyme myrosinase. Isothiocyanates (ITCs) (GHPs) and indole glucosinolate metabolites (in particular indole-3-carbinol [I3C]) are two major groups of autolytic breakdown products of GSLs. It is be-

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lieved that GSLs can confer resistance against pests and diseases on *Brassica* crops (11–15).

Giamoustaris and Mithen (16) tested the hypothesis that *B. napus* varieties with high GSL levels were more resistant to *Alternaria* spp. and *Leptosphaeria maculans* than those with low GS levels. Due to the biocide effect of GSLs, different authors have tested the effects of GHPs and GSLs on soil pathogens by incorporating *Brassica* residues into the soil or by using *in vitro* assays. Bending and Lincoln (17) demonstrated the toxic properties of crucifer tissues after their incorporation into soil, which limits the growth of weeds, fungus, and nematodes. GHPs have a positive effect in reducing soil pathogens, but their persistence varies depending on the compound (17–19). Brader et al. (20) reported that the accumulation of GSLs in *Arabidopsis thaliana* L. enhanced resistance to *Erwinia carotovora* (Jones) and *P. syringae* pv. maculicola (McCulloch). Recently, Aires et al. (10) evaluated the *in vitro* effects of GHPs on six plant-pathogenic bacteria, showing that GHPs could be an alternative tool for controlling these plant diseases.

The objectives of this work were (i) to evaluate the *in vitro* biocide effects of 17 GSLs and GHPs in suppressing the *in vitro* growth of two bacterial (*X. campestris* and *P. syringae*) and two fungal (*A. brassicae* and *S. sclerotiorum*) pathogens of *Brassica* crops and (ii) to evaluate the *in vitro* biocide effects of methanolic extracts of different *Brassica* crops with different GSL profiles against the same pathogens.

MATERIALS AND METHODS

Pathogen isolates and growth conditions. The *Brassica* pathogens used in this study were *X. campestris* pv. campestris (nine bacterial isolates belonging to races 1 to 9; HRI 3811, HRI 3849A, HRI 5212, HRI 1279A, HRI 3880, and HRI 6181, representing races 1 to 6, were provided by Warwick HRI [WHRI], Wellesbourne, United Kingdom, and isolates CFBP 4953, CFBP 1124, and CFBP 6650, representing races 7 to 9, were provided by CFBP-INRA, Beaucouzé Cedex, France), *P. syringae* pv. maculicola (two bacterial isolates, MBG-*P. syringae* pv. maculicola 147.1 [*P. syringae* pv. maculicola 147] from Misión Biológica de Galicia [MBG-CSIC] and CFBP 1657 [*P. syringae* pv. maculicola 1657] from the CFBP-INRA, Beaucouzé Cedex, France), *S. sclerotiorum*, and *A. brassicae* (two fungal isolates obtained from MBG-CSIC).

Bacterial isolates of *X. campestris* pv. campestris and *P. syringae* pv. maculicola were plated on petri dishes containing potato dextrose agar (PDA) and King B medium, respectively, and incubated at 32°C for 24 h. A loop of bacterial growth was then subcultured in nutrient broth overnight in a shaker at 30°C in the dark. Then, 200 μl was spread uniformly by using a sterile plastic inoculation loop on 9-cm-diameter plates containing PDA and King B medium for *X. campestris* pv. campestris and *P. syringae* pv. maculicola, respectively. For fungal pathogens, a 6-mm portion of PDA medium containing the fungus was placed in the center of each plate. Six sterile filter paper discs (6 mm in diameter) were situated on each plate by using a disc dispenser (Oxoid) and then impregnated with 15 μl of the compound being tested, applied at five different concentrations (0.015, 0.15, 0.75, 1.5, and 3.0 μM in dimethyl sulfoxide [DMSO]). The sixth disc was a positive control (for bacterial pathogens, 10 μg disc^{-1} of commercial gentamicin obtained from Sigma-Aldrich Chemie GmbH [Steinheim, Germany]; for fungal pathogens, 10 μg disc^{-1} of cycloheximide, also obtained from Sigma-Aldrich). The lowest concentration (0.015 μM) was omitted for testing fungal pathogens. Finally, a disc containing the negative control (15 μl of the solvent DMSO) was manually inserted in the center of each plate. After incubation for 18 h in daylight at a temperature of 30 ± 1°C, the inhibition of pathogen growth was measured as the diameter (in mm) of the clear zone around the disc. For each compound and pathogen, five replicates were made, and the antibacterial and antifungal activities were expressed as the mean of the inhibition zone diameters (mm).

TABLE 1 Glucosinolates and glucosinolate hydrolysis products used in this study

Compound	Supplier
GSLs	
2-Propenyl (SIN)	Phytoplan Diehm & Neuberger GmbH
3-Methylsulphinylpropyl (GIB)	Phytoplan Diehm & Neuberger GmbH
4-Methylsulphinylbutyl (GRA)	Phytoplan Diehm & Neuberger GmbH
2-Hydroxy-3-butenyl (PRO)	Phytoplan Diehm & Neuberger GmbH
3-Butenyl (GNA)	Phytoplan Diehm & Neuberger GmbH
4-Pentenyl (GBN)	Phytoplan Diehm & Neuberger GmbH
4-Methylthiobutyl (GER)	Phytoplan Diehm & Neuberger GmbH
4-Hydroxybenzyl (SNB)	Phytoplan Diehm & Neuberger GmbH
2-Phenylethyl (GST)	Phytoplan Diehm & Neuberger GmbH
Indole-3-ylmethyl (GBS)	Phytoplan Diehm & Neuberger GmbH
GHPs	
Allyl (AITC)	Sigma Aldrich Co.
Benzyl (BITC)	Sigma Aldrich Co.
3-Butenyl (3BITC)	TCI Europe N. V.
4-Pentenyl (4PITC)	TCI Europe N. V.
Phenetyl (PEITC)	Sigma Aldrich Co.
Sulforafane (SFN)	Sigma Aldrich Co.
Indole-3-carbinol (I3C)	Sigma Aldrich Co.

GLS standards, their GHPs, and leaf methanolic extract. In the present study, 10 GSLs and 7 GHPs (5 isothiocyanates and 2 indoles) were used (Table 1). The effects of these substances were tested using the methodology described by Aires et al. (10), with some modifications.

In order to check if methanolic extracts from *Brassica* leaves (which contain predominantly GSLs) have an effect that is similar to the effect of the GSL standards, 17 methanolic extracts of different *Brassica* local and commercial varieties were evaluated, including four extracts of *B. rapa* (turnip top), 10 methanolic extracts of *Brassica oleracea* (kale, cabbage, tronchuda, broccoli, and cauliflower), and three extracts of *B. napus* (nabicol). All the varieties were planted in multipot trays, and seedlings were transplanted into the field at the five- or six-leaf stage, with three replications. One mix was taken from each replication of leaves. Samples were transferred to the laboratory and conserved at −80°C until processing. All samples were lyophilized (Beta 2-8 LD plus; Christ GmbH, Osterode am Harz, Germany) for 72 h. The dried material was powdered by using an IKA-A10 (IKA-Werke GmbH & Co. KG) mill, and the fine powder was used for GSL extraction. One milliliter of the methanolic extract (described below) was diluted by factors of 3, 10, 100, 1,000, and 10,000 (see Table S1 in the supplemental material) and tested against the four above-mentioned pathogens by using the disc method in a similar way to the experiment with GSLs. In the *X. campestris* pv. campestris experiment only races 1 and 4 were tested because they are the most common races worldwide.

Extraction and determination of GSLs from *Brassica* species. Sample extraction and desulfation were performed according to the method of Kliebenstein et al. (21) with minor modifications. Five microliters of the desulfo-GSL extract from leaves was used in order to identify and quantify the GSLs. Chromatographic analyses were carried out on an ultra-high-performance liquid chromatograph (UHPLC) (Nexera LC-30AD; Shimadzu) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV-visible (Vis) photodiode array detector. The UHPLC column was an Acquity UPLC HSS T3 (1.8-μm particle size; 2.1 by 100 mm inside diameter [i.d.]; Waters Corporation, Massachusetts, USA) protected with a Van Guard precolumn. The oven temperature was set at 30°C.

Compounds were detected at 229 nm and were separated by using the following method in aqueous acetonitrile (ACN), with a flow rate of 0.4

TABLE 2 Inhibitory *in vitro* effects of 10 GSLs and 7 GHPs on growth of 9 races of *X. campestris* pv. campestris

Compound	Inhibition[a]								
	R1	R2	R3	R4	R5	R6	R7	R8	R9
GSLs									
GIB	11.54 GHI	8.94 K	11.75 AB	13.57 AB	10.71 DE	10.91 GH	10.67 H	10.12 CDEF	11.20 B
PRO	11.69 FGH	9.92 FGHI	10.33 FGH	12.18 DE	10.10 G	10.86 H	11.31 DEF	9.60 GH	9.56 GH
GRA	14.21 A	10.19 CDE	10.95 CDEF	11.22 FGHIJ	10.32 EFG	11.86 B	11.44 D	9.26 I	10.23 E
SIN	12.19 CDE	10.08 CDEF	12.36 A	11.03 HIJ	11.37 B	11.65 CD	11.36 DEF	10.31 BCD	10.24 E
GNA	11.89 EFG	10.85 B	9.97 H	11.19 GHIJ	11.30 BC	12.16 A	11.31 DEF	9.83 EFG	11.32 B
SNB	11.09 I	10.29 C	10.77 DEFG	11.23 FGHIJ	12.07 A	10.57 IJ	12.37 A	10.50 AB	10.94 C
GER	10.59 J	9.70 IJ	10.96 CDEF	14.20 A	11.08 BCD	10.94 GH	11.88 BC	10.13 CDEF	12.00 A
GBS	12.49 BCD	10.18 CDE	10.97 CDE	11.90 DEFG	10.99 BCD	11.00 FG	11.44 D	10.67 A	10.24 E
GBN	12.31 CDE	10.26 CD	11.19 BCD	12.52 CD	11.96 A	10.21 K	12.27 A	10.29 BCD	10.69 D
GST	11.14 I	9.60 J	10.94 CDEF	10.68 IJ	9.69 H	11.29 E	11.08 G	10.31 BCD	10.72 D
GHPs									
AITC	12.62 BC	9.95 EFGH	12.19 A	11.44 EFGHI	10.54 EF	11.08 F	10.51 I	9.51 HI	10.29 E
BITC	9.66 K	9.75 HIJ	9.82 H	10.47 J	10.16 FG	10.45 J	10.44 IJ	10.41 ABC	9.48 H
3BITC	11.40 HI	10.27 CD	10.37 EFGH	8.55 K	11.12 BC	9.67 L	11.84 C	10.65 A	9.08 I
4PITC	12.30 CDE	10.02 DEFG	10.20 GH	11.54 EFGH	10.22 FG	11.11 F	10.30 J	9.82 FGH	9.58 GH
PEITC	11.29 HI	9.80 GHIJ	10.01 H	11.97 DEF	8.17 I	11.68 C	12.00 BC	9.85 EFG	9.87 F
SFN	12.87 B	11.24 A	11.48 BC	11.23 FGHIJ	10.94 CD	10.67 I	11.27 EF	10.14 CDE	9.53 H
I3C	12.10 DEF	10.24 CD	10.17 GH	13.11 BC	11.10 BC	11.54 D	11.19 FG	10.02 DEF	9.75 FG

[a] Observed by the disc diffusion assay (dose, 3.0 μM) and measured as the diameter of the inhibition zone (mm). The values are the means of five replicates. R, race. Values followed by the same letter are not significantly different.

[b] Aliphatic glucosinolates, GIB, glucoiberin; PRO, progoitrin; GRA, glucoraphanin; Sin, sinigrin; GER, glucoerucin; SNB, sinalbin; GBN, glucobrassicanapin. Indolic glucosinolate, GBS, glucobrassicin. Aromatic glucosinolate, GST, gluconasturtiin. GHPs, AITC, allyl; BITC, benzyl; 3BITC, 3-butenyl; 4PITC, 4-pentenyl; PEITC, phenetyl; SFN, sulforaphane; I3C, indole-3-carbinol.

ml min^{-1}: 1.5 min at 90% A, a 3.5-min gradient from 10% to 25% (vol/vol) B, a 4-min gradient from 25% (vol/vol) to 50% (vol/vol) B, a 4.5-min gradient from 50% to 100% (vol/vol) B, a 1-min gradient from 100% to 0% (vol/vol) B, and a final 3 min at 90% A. The solvents used were ultrapure water (A) and 25% ACN (B). The data were recorded on a computer with LabSolutions software (Shimadzu). Specific GSLs were identified by comparing retention times with the standards and by UV absorption spectra.

GSLs were quantified by using sinigrin (SIN) (sinigrin monohydrate from Phytoplan, Diehm & Neuberger GmbH, Heidelberg, Germany) and glucobrassicin (GBS) (glucobrassicin potassium salt monohydrate from Phytoplan, Diehm & Neuberger GmbH, Heidelberg, Germany) as external standards and expressed in μmol g^{-1} (dry weight).

Regressions were made, with at least five data points, from 0.34 to 1.7 nmol for sinigrin and from 0.28 to 1.4 nmol for glucobrassicin. The average regression equations for SIN and GBS were as follows: $y = 148{,}818x$ ($R^2 = 0.99$) and $y = 263{,}822x$ ($R^2 = 0.99$), respectively.

Statistical analysis. For all experiments, analyses of variance and mean comparisons were made for the inhibition zone diameter. Mean values were separated by using Fisher's protected least significant difference (LSD) at the 0.05 level of probability. Statistical analyses were performed by using the SAS statistical package (22). Furthermore, simple correlation coefficients were computed between fungal or bacterial growth inhibition and the concentration of glucosinolates with PROC CORR of SAS v 9.2 (22). Simple regression was analyzed in order to study the relationship among the concentration studied and the growth inhibitions of the different pathogens by using PROC REG of SAS v 9.2 (22).

RESULTS

Potential role of GLS standards and their GHPs in suppressing the *in vitro* growth of bacterial and fungal *Brassica* diseases. The combined analysis of variance for compounds and pathogens showed a significant pathogen-compound interaction (data not shown). For this reason, analyses were performed separately for each pathogen.

All the compounds tested had an inhibitory effect on pathogens compared to the negative control, and this effect was dose dependent. The growth inhibitions caused by different GSL concentrations were adjusted to a linear regression with an R^2 between 0.80 and 0.99. The mean concentrations for each pathogen and each compound were compared (17 compounds times 13 pathogen isolates, giving 221 comparisons), and differences were found to be significant. Five replicates were used for each compound and concentration, and the differences between replicates were not significant, which demonstrates the reproducibility and confidence of this experiment. Only one isolate of each pathogen and race was tested. For this reason, the results might be different if we used isolates from other parts of the world.

Because the biocide effect was dose dependent, the highest concentration tested (3 μM) was selected in order to compare the effects of different GSLs and derivatives on each pathogen species and/or race.

Bacterial pathogens *X. campestris* pv. campestris and *P. syringae* pv. maculicola. For bacterial pathogens (*X. campestris* pv. campestris and *P. syringae* pv. maculicola), the results were dependent on the race or the isolate tested in each case.

Nine races of *X. campestris* pv. campestris were tested against GSLs and GHPs. The analysis of variance showed a significant interaction of race and compound. Hence, the effects of compounds were race dependent ($P \leq 0.001$), and the results are therefore shown per race. Mean comparisons were carried out among the 17 compounds tested (Table 2). The effectiveness of compounds varied between races and was generally greater on

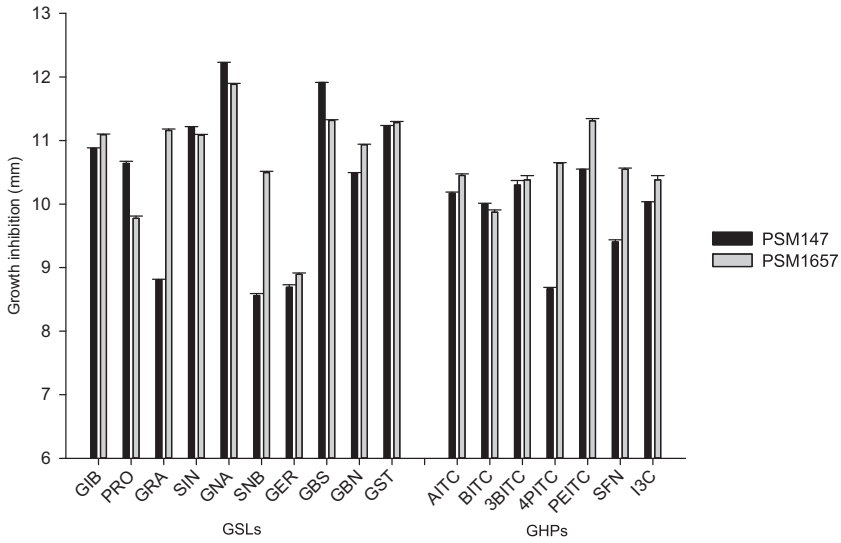

FIG 1 Inhibitory effects of 10 GSLs and 7 GHPs in suppressing the *in vitro* growth of two isolates (*P. syringae* pv. maculicola 147 and *P. syringae* pv. maculicola 1647) of *P. syringae* pv. maculicola observed by the disc diffusion assay (dose, 3.0 μM) and measured as the diameter of the inhibition zone. The values are the means of five replicates, and the error bars indicate their standard deviations.

races 1 (11.75 mm) and 4 (11.19 mm), which are the widespread races of *X. campestris* pv. campestris on *Brassica* crops around the world (Table 2). Glucobrassicanapin (GBN) was effective for races 1, 2, 3, 4, 5, and 7. Sinalbin (SNB) was among the most effective GSLs for races 2, 5, 7, 8, and 9. Gluconapin (GNA) and/or its GHP (3-butenyl ITC [3BITC]) inhibited the growth of races 2, 5, 6, 8, and 9, and finally, SIN and/or its GHP (allyl ITC [AITC]) appears to be most effective on races 1, 3, 5, 6, and 8. Conversely, benzyl ITC (BITC) was clearly the least effective compound, being among the worst five compounds for eight of the nine races studied.

The growth of two isolates of *P. syringae* pv. maculicola was tested against the 17 compounds. There was a significant isolate-compound interaction ($P \leq 0.001$), indicating that the effectiveness of compounds depends on the isolate tested. When the analysis was made for each isolate separately, significant differences were recorded between compounds. For the isolate *P. syringae* pv. maculicola 147 ($P \leq 0.001$), GNA was significantly more effective than any other compound (12.22 mm); GBS was the second most effective (11.91 mm), and then gluconasturtiin (GST) and SIN (11.23 mm and 11.21 mm, respectively). SNB, 4-pentenyl ITC (4PITC), and glucoerucin (GER) were the least effective compounds (Fig. 1). Against the isolate *P. syringae* pv. maculicola 1657, levels of inhibition again varied significantly depending on the compound ($P \leq 0.001$). Again, GNA (11.88 mm) and GBS (11.32 mm) were the most effective substances, although the levels of inhibition caused by GST (11.28 mm), phenetyl ITC (PEITC) (11.31 mm), and glucoraphanin (GRA) (11.16 mm) were not significantly different. The least effective compound was GER (8.89 mm), followed by BITC (9.87 mm) and progoitrin (PRO) (9.78 mm).

Fungal pathogens *S. sclerotiorum* and *A. brassicae*. The analysis of variance for *S. sclerotiorum* showed significant differences among compounds ($P \leq 0.001$). GST showed the strongest activity (9.81 mm) and was significantly different from the other compounds. PEITC was the second most effective compound (9.59 mm) and differed from a third group composed of AITC (8.90 mm), GNA (8.85 mm), and sulforaphane (SFN) (8.84 mm). Glucoiberin (GIB) (7.20 mm) and GBN (7.65 mm) were the least effective compounds against the development of *S. sclerotiorum* (Fig. 2A).

The analysis of variance of *A. brassicae* showed significant differences among compounds ($P \leq 0.001$). Mean comparisons showed that I3C, GNA, and PRO were the compounds with the greatest inhibitory effects (11.69 mm, 11.59 mm, and 11.58 mm, respectively). On the other hand, BITC, SIN, and GER were the compounds with the weakest activities (8.48 mm, 8.89 mm, and 9.02 mm, respectively) (Fig. 2B).

GNA, SFN, and PEITC, therefore, all had important inhibiting effects on both fungal pathogens, and it follows that these compounds could play an important role as general fungicides, in addition to the more specific effects of other compounds, such as I3C (against *A. brassicae*) or GST (against *S. sclerotiorum*).

When considering the results for bacterial and fungal pathogens together, it is possible to highlight GNA as a general bactericide and fungicide. In order to corroborate these results, another experiment was done with methanolic extracts from different species and cultivars of *Brassica* with high contents of these GSLs.

Potential role of leaf methanolic extracts in suppressing the *in vitro* growth of bacterial and fungal *Brassica* diseases. The antibiotic effect of methanolic extracts from the leaves of several *Brassica* crops (three different species) was studied. These extracts contained GSLs, but other compounds, such as phenolics, may also have been present. It is therefore possible that any antibiotic effect may have been due to compounds other than GSLs.

The combined analysis of variance for compounds and pathogens showed a significant pathogen-compound interaction (data not shown). For this reason, analyses were made separately for each pathogen. All the extracts studied had an inhibitory effect on the development of the pathogens tested compared to the negative control, and this effect was dose dependent. The analysis of variance showed significant differences between varieties ($P \leq 0.001$)

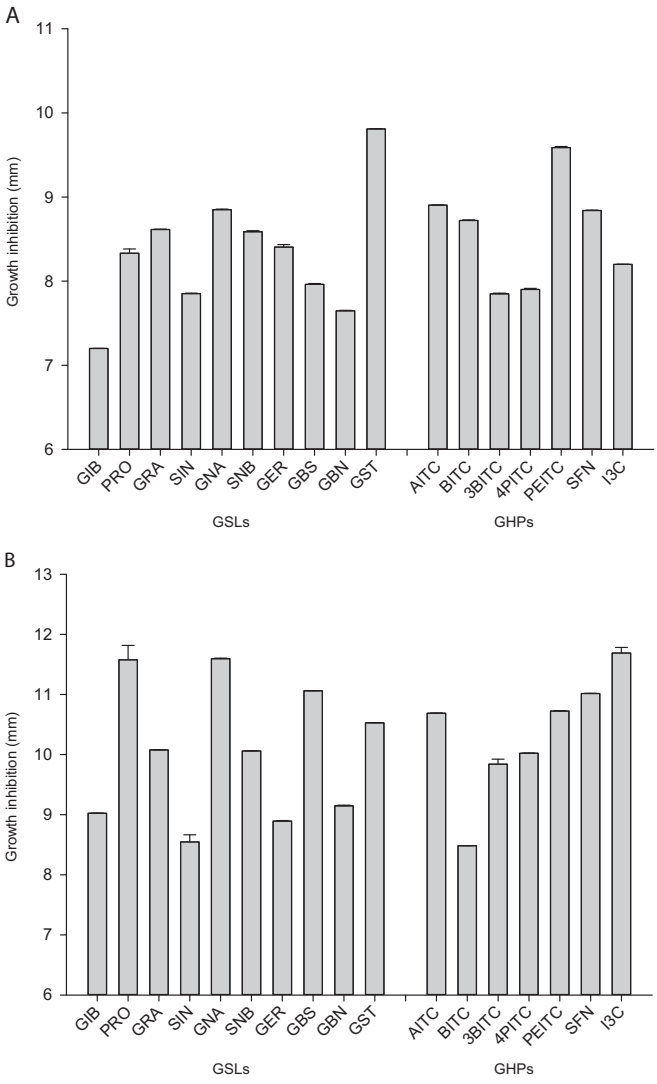

FIG 2 Inhibitory effects of 10 GSLs and 7 GHPs in suppressing the *in vitro* growth of *S. sclerotiorum* (A) and *A. brassicae* (B) as observed by the disc diffusion assay and measured as the diameter of the inhibition zone. The values are the means of five replicates. The error bars indicate standard deviations.

for races 1 and 4 of *X. campestris* pv. campestris. Extracts of all the varieties studied had an inhibitory effect on the *in vitro* growth of both races. For race 1, MBG-BRS0062 (kale; 12.39 mm) was the variety with the greatest inhibitory effect. The varieties MBG-BRS0259 (turnip top; 11.99 mm), MBG-BRS0452 (cabbage; 11.85 mm), and MBG-BRS0155 (turnip top; 11.76 mm) also showed important inhibitory effects. In contrast, the commercial hybrid of broccoli (Brocoletto; 10.19 mm), along with the local varieties MBG-BRS0072 (cabbage; 10.55 mm) and MBG-BRS0121 (tronchuda cabbage; 10.78 mm), showed weak inhibitory activity (Fig. 3).

Commercial cauliflower (Bola de Nieve; 12.43 mm), MBG-BRS0452 (cabbage; 12.00 mm), MBG-BRS0026 (turnip top; 11.84 mm), and MBG-BRS0113 (leaf rape; 11.84 mm) were the most effective varieties against the growth of race 4. The only other varieties to show a significant difference from the least effective variety were MBG-BRS0062 and MBG-BRS0066 (Fig. 3).

Fungal growth of *S. sclerotiorum* and *A. brassicae* was significantly affected by the presence of leaf extracts from two varieties of turnip top (MBG-BRS0066 and MBG-BRS00259), which showed around 80% of the total concentration of GNA, and one tronchuda kale variety (MBG-BRS0226).

Two local varieties, MBG-BRS0226 (tronchuda cabbage; 9.85 mm) and MBG-BRS0066 (turnip top; 9.88 mm), were the most effective against the development of *A. brassicae*, followed by variety MBG-BRS0259 (turnip top; 9.58 mm) (Fig. 4A). In the case of *S. sclerotiorum*, varieties MBG-BRS0066 (turnip top; 9.88 mm) and MBG-BRS0226 (tronchuda cabbage; 9.83 mm) were the most effective, followed by the varieties MBG-BRS0259 (turnip top; 9.56 mm) and MBGBRS0425 (cabbage; 8.85 mm) (Fig. 4B).

In order to check if the inhibitory effects of these varieties could be due to GSLs present in leaves, correlations were made between the leaf GSL concentration and growth inhibition of all pathogens (Table 3). In general, correlations were low and not significant, but there were some positive and significant correlations between aliphatic GSLs and the inhibition diameters of some pathogens. However, correlations between the GSL concentrations and inhibition were higher than those found in the previous assays using the compounds: correlations between SIN and *S. sclerotiorum*, *A. brassicae*, and race 1 of *X. campestris* pv. campestris were highly significant and positive (0.63, 0.74, and 0.55, respectively), as were those between race 4 of *X. campestris* pv. campestris and GIB, neoglucobrassicin (NeoGBS), and total GSLs (0.76, 0.73, and 0.62, respectively) (Table 3).

As the GSLs with the highest correlation coefficients were typical of *B. oleracea* crops but were not present in *B. rapa* or *B. napus*, a second correlation analysis was made between the GSL contents and the inhibition diameters of some pathogens only for crops of *B. oleracea*. These correlations were higher than those found in the first correlation analysis. SIN appears to have a significant effect in suppressing the *in vitro* growth of *S. sclerotiorum*, *A. brassicae*, and race 1 of *X. campestris* pv. campestris, whereas GIB and NeoGBS appear to have a biocide effect on the growth of race 4 of *X. campestris* pv. campestris.

DISCUSSION

The biological effects of GSLs and GHPs have been known since the early 1990s, when several authors investigated their effects on the growth and development of bacteria (19, 23), insects (24–26), fungi (27, 28), and nematodes (29, 30), and our knowledge about the deterrent or attractant effects of the main glucosinolates on different pests (generalists and specialists) and parasitoids is well documented. Other authors have tested the effects of GHPs and GSLs on soil pathogens by incorporating *Brassica* residues into soil or by testing their effect by using *in vitro* assays. GHPs have been shown to have a positive effect in reducing soil pathogens, but with varying degrees of persistence depending on the compound (16). Other studies have shown the impacts of GSL-containing plants on successive plant communities growing in close proximity: for example, Vera et al. (31) showed that *Brassica* herbage reduced the stand establishment of five crop species, more than double what happened with barley (*Hordeum vulgare*). *Brassica* plants also inhibited the germination of annual grasses (32). Residues of broccoli (*B. oleracea*) amended to soil inhibited the germination and growth of lettuce (33).

However, the effects of different glucosinolate profiles in *Brassica* crops on the development of *Brassica* pathogens has scarcely

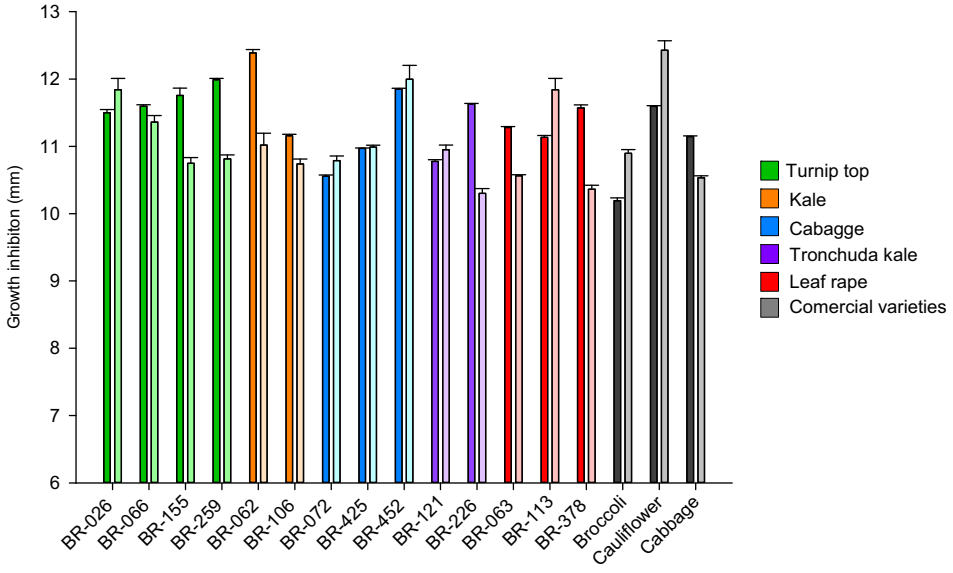

FIG 3 Inhibitory effects of the leaf methanolic extracts from 17 varieties belonging to three *Brassica* species in suppressing the *in vitro* growth of races 1 and 4 of *X. campestris* pv. campestris. The error bars indicate standard deviations.

been investigated, and the few studies that were found show contradictory results (10, 34, 35). For this reason, a complete evaluation of the effects of the most important GSLs and GHPs on plant defenses is necessary.

X. campestris pv. campestris is considered one of the most important pathogens affecting vegetable brassicas worldwide. Different authors have studied the role of glucosinolates in the defense against *X. campestris* pv. campestris. Aires et al. (10) evaluated the effects of different GHPs against several phytopathogenic bacteria, including *X. campestris* pv. campestris. They found a strong effect of GHPs, meaning that the growth of *X. campestris* pv. campestris could be limited by the addition of GHPs, especially AITC, BITC, SFN, and I3C. Furthermore, Velasco et al. (36) evaluated the effects of different secondary metabolites against *X. campestris* pv. campestris and found that GNA and its GHP 3BITC had an antibacterial effect on the growth of the pathogen and that the effect of the GSL was strongly dependent on the concentration applied.

Our results confirm that all the GSLs and their GHPs tested inhibit the growth of *X. campestris* pv. campestris, with GBN, SIN, SNB, GNA, and 3BITC showing the strongest inhibitory effects for most *X. campestris* pv. campestris races. It is notable that the compounds were most effective on races 1 and 4, the most widespread races globally; this suggests that plants have evolved to cope with these two races. It should also be noted, however, that only one isolate per race was used for this study, and more isolates are needed to confirm these conclusions.

Another common disease, bacterial leaf spot, caused by *P. syringae* pv. maculicola, has a high incidence in the oilseed species (3). In our *P. syringae* pv. maculicola study, the effects of compounds on the growth of isolates varied depending on the dose and on the isolate studied. From our results, we can highlight GNA and GBS as the most effective compounds against the different isolates of *P. syringae* pv. maculicola. Again, GNA and GBS are two of the most important glucosinolates in oilseed species such as *B. rapa* and *B. napus*. However, there are no other *in vitro* studies related to the response of *P. syringae* pv. maculicola to GSLs or GHPs, and therefore, further research is needed to confirm these results.

Fungal pathogens, such as *S. sclerotiorum* and *A. brassicae*, are present in several countries around the world, and their study is important due to the considerable reduction in both yield and seed quality caused by them. In the case of *S. sclerotiorum*, previous studies found that different isolates of the pathogen vary in their impacts (14, 37). Fan et al. (14) studied the effects of GSL content in *B. napus* on resistance to two different *S. sclerotiorum* isolates and highlighted a complex relationship between *S. sclerotiorum* isolates and the GSL content. In our study, GST showed the strongest activity, but GNA was found to be one of the most effective compounds in inhibiting *S. sclerotiorum*. For the other fungal pathogen, *A. brassicae*, GNA was again found to be the compound with the greatest inhibitory effect. In the second part of our study, we evaluated the potential role of leaf methanolic extracts from different cultivars and species of *Brassica* in suppressing the *in vitro* growth of different pathogens. Methanolic extracts contain GSLs, phenolics, and other compounds. Differences in the bacterial pathogen tests were dependent on the race or the isolate tested; however, these differences were less than the differences observed in the fungal pathogens, suggesting that, besides GSLs, other metabolites may influence the resistance to *X. campestris* pv. campestris. Furthermore, correlations found in these assays were positive but low, and this could be in accordance with the findings of Njoroge et al. (38), who found that induced resistance was mediated by compounds other than GSLs, such as phenolics and lignin, in the resistance to *Verticillium dahli*. In our case, other compounds besides GSLs may have had an influence on the inhibition of these pathogens. Phenolic compounds of these extracts (flavonoids, mainly kaempherol, and hidroxycinnamic acids) were quantified, but no relationships were found with the levels of resistance, and the results are not shown.

The results obtained in this experiment could be in accordance with the allelochemical effects of GSLs on fungi and bacteria found in previous works. The negative impact of *Brassica* tissues

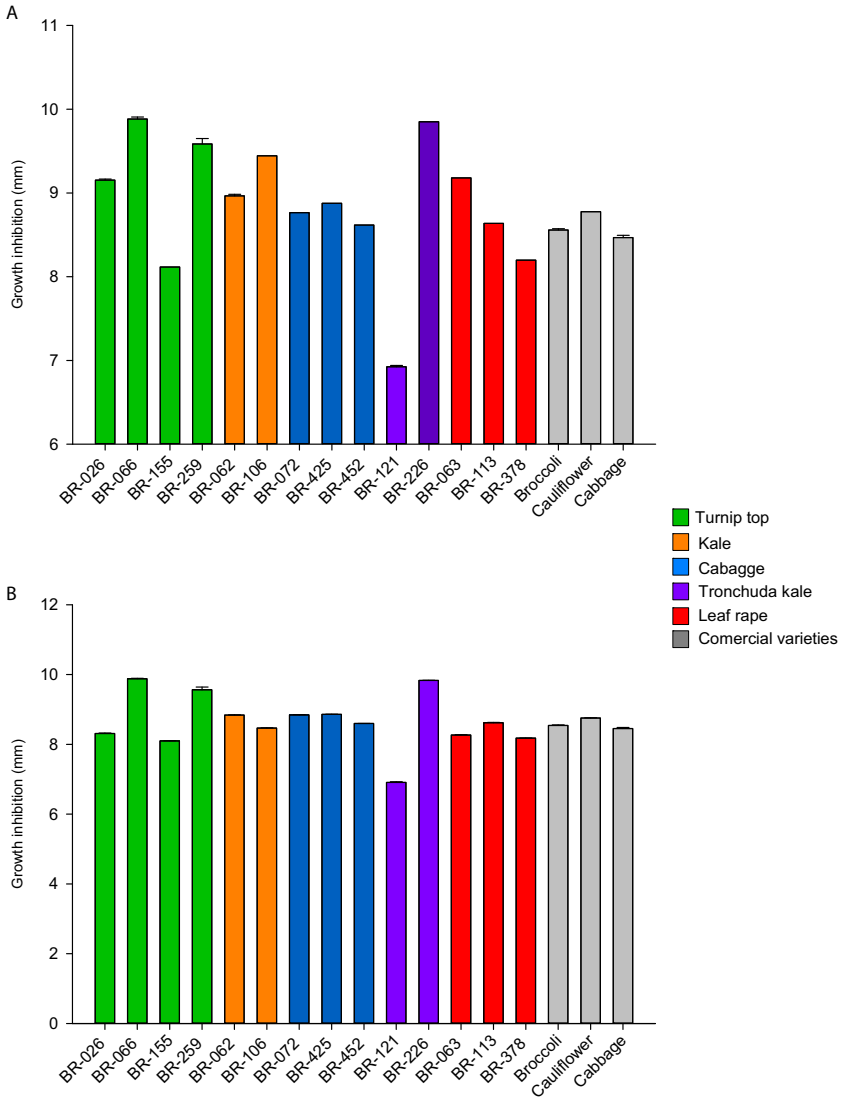

FIG 4 Inhibitory effects of the leaf methanolic extracts from 17 varieties belonging to three *Brassica* species in suppressing the *in vitro* growth of *A. brasssicae* (A) and *S. sclerotiorum* (B). The error bars indicate standard deviations.

on soilborne pathogens has been reviewed by Brown and Morra (39). They reported that GSLs and GHPs may greatly influence fungal and bacterial populations, with GHPs being the most potent products, suspected to be the major inhibitors of microbial activity.

In our study, it was notable that leaf tissue prepared from two varieties of turnip top was the most effective for inhibiting fungal growth. As GNA is the major GSL in this crop, we can therefore support the idea that this GSL is the major agent of antifungal activity. This idea is in concordance with the results obtained by Velasco et al. (36) relating to growth inhibition in *X. campestris* pv. campestris.

It is worth noting that GSLs accumulate in leaves, flower buds, and seeds of members of the family *Brassicaceae*. Mulch composed of plant waste derived from *Brassica* crops could therefore potentially be applied directly to soil without any need to isolate or synthesize GSLs. Any such conclusion regarding the practical use of GSLs and GHPs is, of course, merely tentative and dependent on more field studies on the use of weed control as an herbicide. Plants of the *Brassicaceae* have been recognized as having a potential use in biofumigation practices, based on the production of active volatiles released after enzyme hydrolysis as GHPs (39). This is an agronomic technique that is an alternative to chemical fumigants in order to manage soilborne pests and diseases in an integrated way. Previous evidence strongly supports the idea that GSLs or GHPs are biologically active, and they have considerable potential for use in pest control strategies and biofumigation.

Conclusions. Our results demonstrate that pure GSLs and GHPs, as well as leaf extracts, had an antibiotic effect on the development of the four *Brassica* pathogens studied.

The biocide effects of the standard GSLs, GHPs, and 17 different leaf extracts were dependent on the pathogen under study and the concentration applied, but in general, GNA showed a potent increase effect for fungal and bacterial pathogens. In *X. campestris* pv. campestris races, we also have to highlight other GSLs with potential to increase the inhibitory effect, such as GBN, SIN, and

TABLE 3 Simple correlations between the inhibition zone diameters of the pathogens tested and the glucosinolate concentrations found on leaf extracts of all species and of *B. oleracea* species

Leaf extracts	Pathogen	Correlation[a]											
		GIB	PRO	GRA	SIN	ALY	GNA	GIV	GBN	GBS	GST	NeoGBS	Total
All species	*S. sclerotiorum*	−0.056	−0.180	−0.156	0.482[b]	−0.196	0.050	0.035	−0.122	0.252	−0.150	0.103	0.207
	A. brassicicola	−0.168	−0.033	−0.204	0.461[b]	0.005	0.159	0.191	−0.036	0.168	−0.139	0.045	0.224
	X. campestris pv. campestris race 1	0.082	−0.006	−0.466[b]	0.217	0.008	0.360	0.297	0.045	−0.258	−0.154	0.121	0.245
	X. campestris pv. campestris race 4	0.443[b]	−0.144	−0.146	−0.220	−0.250	0.069	0.181	0.078	−0.194	0.046	0.527[c]	0.448[b]
B. oleracea	*S. sclerotiorum*	−0.227	−0.011	−0.206	0.632[c]	−0.078				0.420	0.030	0.133	0.271
	A. brassicicola	−0.234	−0.025	−0.327	0.742[c]	−0.118				0.445	−0.144	0.062	0.210
	X. campestris pv. campestris race 1	0.433	−0.174	−0.401	0.549[b]	−0.043				−0.089	0.003	0.239	0.239
	X. campestris pv. campestris race 4	0.761[c]	−0.254	−0.120	−0.376	−0.286				−0.292	0.371	0.728[c]	0.616[c]

[a] Aliphatic glucosinolates: GIB, glucoiberin; PRO, progoitrin; GRA, glucoraphanin; Sin, sinigrin; GBN, glucobrassicanapin. Indolic glucosinolates: GBS, glucobrassicin; NeoGBS, neoglucobrassicin. Aromatic glucosinolate: GST, gluconasturtiin.
[b] Significant at a *P* value of ≤0.05.
[c] Significant at a *P* value of ≤0.001.

SNB. For *S. sclerotiorum* isolates, GBS should be highlighted due to its potential as an inhibitor.

More research is needed to further determine the optimal concentrations of these compounds in order for them to be used *in vitro* against different pathogens. In order to further assess the biofumigation potentials of these compounds for crop protection, their effectiveness should be investigated under field conditions.

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