In vitro activity of glucosinolates and their degradation products against *Brassica* pathogenic bacteria and fungi

Running tittle: *Brassica* glucosinolates against plant pathogens

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ABSTRACT

Glucosinolates (GSLs) are secondary metabolites found in *Brassica* vegetables, which confer them with resistance against pests and diseases. Both GSLs and glucosinolate hydrolysis products (GHP) have shown positive effects in reducing soil pathogens. Information about their *in vitro* biocide effect 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 effect of 17 GSLs and GHPs and 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 scletoriorum*) *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 effect of the different compounds studied was dependent on the species and race of pathogen. These results indicate that GSLs and their GHPs, as well as extractions of different *Brassica* species, have potential to inhibit pathogen growth, and offer new opportunities to study the use of *Brassica* crops on biofumigation for the control of multiple diseases.

INTRODUCTION

The *Brassica* genus belongs to the *Brassicaceae* family (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 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 highly affected by several diseases, which result in substantial economic losses (2). Black rot, caused by bacteria *Xanthomonas campestris* pv. *campestris* (Pammel) (*XCC*), is considered to be one of the most important pathogens affecting vegetable *Brassicas* worldwide (3). There are nine races of *XCC*: races 1 to 6 are 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 (6).

Bacterial leaf spot, caused by *Pseudomonas syringae* pv. *maculicola* (McCulloch) (*PSM*) (7), is very significant on cauliflower but also occurs on broccoli, Brussels sprouts and other *Brassicas*. *PSM* 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 (*SS*), is a widespread fungal disease in temperate areas of the world, which 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. (*AB*). 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. This 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 (8, 9), including some of the aforementioned pathogens (10). 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* (11).

GSLs are nitrogen and sulphur-containing plant secondary metabolites that occur mainly in Capparales and almost exclusively in the *Brassicaceae* family. GSLs are β-thioglucoside N-hydroxysulphates 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 (GHPs) and indole glucosinolate metabolites (in particular indol-3-carbinol) are two major groups of autolytic breakdown products of GSLs. It is believed that GSLs can confer resistance to *Brassica* crops against pests and diseases (12-16).

Giamoustaris and Mithen (17) tested the hypothesis that *B. napus* varieties with high GSL levels were more resistant to *Alternaria* spp. and *Leptosphaeria maculans* than those with low GSL. 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 (18)

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 (18-20). Brader et al. (21) 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. (11) evaluated the *in vitro* effect 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 1) to evaluate the *in vitro* biocide effect of 17 GSLs and GHPs in suppressing the *in vitro* growth of two bacterial (*Xanthomonas campestris* and *Pseudomonas syringae*) and two fungal (*Alternaria brassicae* and *Sclerotinia sclerotiorum*) pathogens of Brassica crops, and 2) to evaluate the *in vitro* biocide effect of methanolic extracts of different *Brassica* crops with different GSL profiles against the same pathogens.

MATERIALS AND METHODS

Pathogen isolates and growing conditions

Brassica pathogens used in this study were: *Xanthomonas 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 WHRI-Wellesbourne, UK and isolates CFBP 4953, CFBP 1124 and CFBP 6650, representing races 7 to 9, were provided by CFBP- INRA, Beaucouzé Cedex, France), *Pseudomonas syringae* pv. *maculicola* (two bacterial isolates, MBG-*PSM* 147.1 (*PSM*147) from Misión Biológica de Galicia (MBG-CSIC) and CFBP 1657 (*PSM*1657) from the CFBP-INRA, Beaucouzé Cedex, France), *Sclerotinia sclerotiorum* and *Alternaria brassicae*, (two fungal isolates obtained from MBG-CSIC).

Bacterial isolates of *XCC* and *PSM* were plated on Petri dishes containing potato dextrose agar (PDA) and King B medium, respectively, and incubated at 32 ºC for 24 h. A loop of bacterial growth was then subcultured in nutrient broth overnight in a shaker at 30 ºC and in the dark. Then, 200 µl were spread uniformly by using a sterile plastic inoculation loop on 9cm diameter plates containing PDA and King B media for *XCC* and *PSM*, respectively. For fungal pathogens, a 6 mm portion of PDA medium containing the fungus was placed in the centre 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 Cyclohexamide®, also obtained from Sigma–Aldrich). The lowest concentration (0.015 µM) was ommitted for testing fungal pathogens. Finally, a disc containing the negative control (15µl of solvent DMSO) was manually inserted in the centre of each plate. After incubation for 18 h in daylight at a temperature of 30±1 ºC, the inhibition of the pathogen growth was measured as the diameter (mm) of clear zones around the disc. For each compound and pathogen, five replicates were made and the antibacterial and antifungal activity was expressed as the mean of inhibition zone diameters (mm).

GLS standards, their GHPs and leaf methanolic extract

In the present study 10 GSLs and 7 GHPs (5 isothiocyanates and 2 indoles) were used. The effect of these substances was tested using the methodology described by Aires et al. (11), with some modifications.

In order to check if methanolic extracts from Brassica leaves (which contain predominantly GSLs) have an effect which is similar to the effect of GSL standards, 17 methanolic extracts of different Brassica local and commercial varieties were evaluated including four extracts of *B. rapa* (turnip top); ten methanolic extracts of *B. oleracea* (kale, cabbage, tronchuda, broccoli, cauliflower) and three extracts of *B. napus* (nabicol). All varieties were planted in multipot-trays and seedlings were transplanted into the field at the five or six leaf stage, with three replications. One bulk 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) 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 mL of the methanolic extraction (described below) was diluted by a factor of 3, 10, 100, 1000 and 10,000 (Table S1) and tested against the four above mentioned pathogens by using the disc method in a similar way to the experiment with GSLs. In the *XCC* 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 Kliebenstein et al. (22) with minor modifications. Five microlitres of the desulfo-GSL extract from leaves were used in order to identify and quantify the GSLs. Chromatographic analyses were carried out on an ultra-high-performance liquid chromatography (UHPLC Nexera LC-30AD; Shimadzu) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector. The UHPLC column was an Acquity UPLC HSS T3 (1.8µm particle size, 2.1 x100 mm i.d.) from Waters (Waters Corporation, MA, USA) protected with a Van Guard pre-column. The oven temperature was set at 30 ºC.

Compounds were detected at 229 nm and were separated by using the following method in aqueous acetonitrile (ACN), with a flow of 0.4mL min^{-1}: 1.5 minutes at 90% A; a 3.5 min gradient from 10% to 25% (v/v) B; a 4 min gradient from 25% (v/v) to 50% (v/v) B; a 4.5 minute gradient from 50% to 100% (v/v) B; a 1 minute gradient from 100% to 0% (v/v) B and a final 3 min at 90% A. Solvents used were: ultrapure water (A) and 25% of ACN (B). Data were recorded on a computer with the LabSolutions software (Shimadzu). Specific GSLs were identified by comparing retention times with 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 standard and expressed in µmol g^{-1} dry weight (DW).

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 y= 148818x (R^2 = 0.99) and y= 263822 x (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 (23). 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 (23). Simple regression was analyzed in order to study the relation among the concentration studied and the growth inhibition of the different pathogens by using PROC REG of SAS v 9.2 (23).

RESULTS

Potential role of GLS standards and their GHPs in suppressing the *in vitro* growth of bacterial and fungal *Brassica* diseases

The analysis of variance combined for compounds and pathogen 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 inhibition caused by different GSL concentrations adjusted to a linear regression with an R^2 between 0.80 and 0.99. Mean concentrations for each pathogen and each compound were compared (17 compounds × 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, results may be different if we use isolates from other parts of the world.

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

Bacterial pathogens: *Xanthomonas campestris* pv. *campestris* and *Pseudomonas syringae* pv. *maculicola*

For bacterial pathogens (*XCC* and *PSM*) results were dependent on the race or the isolate tested in each case.

Nine races of *XCC* were tested against GSLs and GHPs. The analysis of variance showed a significant interaction of race × compound. Therefore, the effects of compounds were race-dependent ($p \leq 0.001$), and 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 races 1 (11.75 mm) and 4 (11.19 mm), which are the widespread races of XCC 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, 3BITC) inhibited the growth of races 2, 5, 6, 8 and 9, and finally, sinigrin (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 *PSM* 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 isolate *PSM*147 ($p \leq 0.001$), GNA was significantly more effective than any other compound (12.22 mm); glucobrassicin (GBS) was the second most effective (11.91 mm), and then gluconasturtiin (GST) and sinigrin (SIN) (11.23 mm and 11.21 mm, respectively). SNB, 4PITC and glucoerucin (GER) were the least effective compounds (Fig. 1). Against isolate *PSM*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: *Sclerotinia sclerotiorum* and *Alternaria brassicae*

The analysis of variance for *SS* 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 SFN (8.84 mm). Glucoiberin (GIB) (7.20 mm) and GBN (7.65 mm) were the least effective compounds against the development of *SS* (Fig. 2a).

The analysis of variance of *AB* showed significant differences among compounds ($p \leq 0.001$). Mean comparisons showed that I3C, GNA and PRO were the compounds with the highest inhibitory effect (11.69 mm, 11.59 mm and 11.58 mm, respectively). On the other side, BITC, SIN and GER were the compounds with the weakest activity (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 *AB*) or GST (against *SS*).

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 content in 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, of three different species, was studied. These extracts contained GSLs but other compounds like phenolics may also have been present. It is therefore possible that any antibiotic effect may have been due to compounds other than GSLs.

The analysis of variance combined 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 against 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 ($p \leq 0.001$) for races 1 and 4 of *XCC*. Extracts of all the varieties studied had an inhibitory effect on *in vitro* growth of both races. For race 1, MBG-BRS0062 (kale- 12.39 mm) was the variety with the greatest inhibitory effect. 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 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 *SS* and *AB* 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 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 *AB* followed by variety MBG-BRS0259 (turnip top- 9.58 mm) (Fig.4a). In the case of *SS*, varieties MBG-BRS0066 (turnip top- 9.88 mm) and MBG-BRS0226 (tronchuda cabbage- 9.83 mm) were the most effective, followed by varieties MBG-BRS0259 (turnip top- 9.56 mm) and MBGBRS0425 (cabbage-8.85 mm) (Fig.4b).

In order to check if the inhibitory effect of these varieties could be due to GSLs present in leaves, correlations were made between 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 diameter of some pathogens. However correlation between GSL concentration and inhibition were higher than those found in the previous assays using the compounds: correlations between SIN and *SS*, *AB* and race 1 of *XCC* were highly significant and positive (0.63, 0.74 and 0.55 respectively), as were those between race 4 of *XCC* and GIB, neoglucobrassicin (NeoGBS) and total GSLs (0.76, 0.73 and 0.62 respectively) (Table3).

As GSLs with 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 content and the inhibition diameter of some pathogens, only for crops of this species. 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 SS, AB and race 1 of XCC whereas GIB and NeoGBS appears to have a biocide effect on the growth of race 4 of XCC.

DISCUSSION

The biological effects of GSLs and GHPs have been known since the early 90s when several authors investigated their effects on the growth and development of bacteria (20, 24), insects (25-27), fungus (28, 29) and nematodes (30, 31), 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 (17). Other studies have shown the impacts of GSLs-containing plants on successive plant communities growing in close proximity: for example, Vera et al. (32) showed that *Brassica* herbage reduced stand establishment of five crop species, more than double of what happened with barley (*Hordeum vulgare*). *Brassica* plants also inhibited the germination of annual grasses (33). Residues of broccoli (*B. oleracea*) amended to soil inhibited the germination and growth of lettuce (34).

However, the effect of different glucosinolates profiles in *Brassica* crops on the development of *Brassica* pathogens has scarcely been investigated, and the few studies that have been found show contradictory results (11, 35, 36). For this reason, a complete evaluation of the effects of the most important GSLs and GHPs in plant defenses is necessary.

XCC is considered one of the most important pathogens affecting vegetable *Brassicas* worldwide. Different authors have studied the role of glucosinolates in the defense against *XCC*. Aires et al. (11) evaluated the effect of different GHPs against

several phytopathogenic bacteria, including *XCC*. They found a strong effect of GHPs, meaning that the growth of *XCC* could be limited by the addition of GHPs, especially AITC, BITC, sulforaphane (SFN) and indol-3-carbinol (I3C). Furthermore, Velasco et al. (37) evaluated the effect of different secondary metabolites against *XCC* and found that GNA and its GHP 3BITC had an antibacterial effect on the growth of this pathogen and that the effect of the GSL was strongly dependent on the concentration applied.

Our results confirm that all GSLs and their GHPs tested inhibit the growth of *XCC*, with GBN, SIN, SNB GNA and 3BITC showing the strongest inhibitory effects for most *XCC* races. It is notable that 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 pathogen, Bacterial leaf spot, caused by *Pseudomonas syringae* pv. *Maculicola* (*PSM*), has a high incidence in the oilseed species (3). In our *PSM* study, the effect 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 *PSM*. 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 with the response of *PSM* to GSLs or GHPs and therefore further research is needed to confirm these results.

Fungal pathogens such as *SS* and *AB* are present in several countries around the world and their study is important due to the considerable reduction of both yield and seed quality caused by them. In the case of *SS*, previous studies detected that different isolates of this pathogen vary in their impacts (15, 38). Fan et al. (15) studied the effects of GSL content in *B. napus* on the resistance to two different *SS* isolates, and

highlighted a complex relation between *SS* isolates and the GSLs content. In our study GST showed the strongest activity but GNA was found to be one of the most effective compounds for inhibiting *SS*. For the other fungal pathogen *AB,* 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 *Brassicas* 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 *XCC*. Furthermore, correlations found in these assays were positive but low and this could be in concordance with Njoroge et al. (39) 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 results are not shown.

The results obtained in this experiment could be in concordance with the allelochemical effects of GSLs on fungus and bacteria found in previous works. The negative impact of *Brassica* tissues on soil-borne pathogens has been reviewed by Brown and Morra (40). 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 anti-fungal activity. This idea is in concordance with the results obtained by Velasco et al. (37) relating to growth inhibition in *XCC*.

It is worth noting that GSLs accumulate in leaves, flower buds and seeds of the *Brassicaceae* family. 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 more practical use of GSLs and GHPs is, of course, merely tentative and dependent on more field studies on the use of weed control as a selective herbicide. Plants of *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 (40). This is an agronomic technique that is an alternative to chemical fumigants in order to manage soil-borne 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 effect of the standard GSL, 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 *XCC* races we have to also highlight other GSLs with potent inhibition as GBN, SIN and SNB. For *SS* isolates, GBS should be highlight due to their potential as inhibitor.

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

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Table 1: Glucosinolates and glucosinolate hydrolysis products used in this study.

Compound	Supplier
Glucosinolates	
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
Indol-3-ylmethyl (GBS)	Phytoplan Diehm & Neuberger GmbH
Glucosinolate hydrolysis products (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.
Sulforaphane (SFN)	Sigma Aldrich Co.
Indol-3-Carbinol (I3C)	Sigma Aldrich Co.

Table 2: Inhibitory in vitro effect of 10 Glucosinolates (GSLs) and 7 glucosinolate hydrolysis products (GHPs) on nine races of *Xanthomonas campestris* pv. *campestris* growth observed by the disc diffusion assay (dose 3.0 uM) and measured as diameter of inhibition zone (in mm). Values are the mean of five replicates.

	R1	R2	R3	R4	R5	R6	R7	R8	R9
Glucosinolates									
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

Aliphatic glucosinolates: GIB, Glucoiberin; PRO, Progoitrin; GRA, Glucoraphanin; Sin, Sinigrin; GER, Glucoerucin; SNB, Sinalbin; GBN, Glucobrassicanapin; Indolic glucosinolate: GBS, Glucobrassicin; Aromatic glucosinolate:GST, Gluconasturtiin; Glucosinolates hydrolysis products: AITC, Allyl; BITC, Benzyl; 3BITC, 3-Butenyl;PITC, 4-Pentenyl; PEITC, Phenetyl; SFN, Sulforafane; I3C, Indol-3-Carbinol.

Table 3: Simple correlations between the inhibition diameter of the pathogens tested and the glucosinolate concentration found on leaf extracts of all species (A) and the glucosinolate concentration found on leaf extracts of *B. oleracea* species (B). SS: *Sclerotinia sclerotiorum*; AB: *Alternaria brassicicola*; *XCC*: *Xanthomonas campestris* pv. *campestris*.

A)

	GIB	PRO	GRA	SIN	ALY	GNA
SS	-0.056	-0.180	-0.156	0.482*	-0.196	0.050
AB	-0.168	-0.033	-0.204	0.461*	0.005	0.159
XCC Race 1	0.082	-0.006	-0.466*	0.217	0.008	0.360
XCC Race 4	0.443*	-0.144	-0.146	-0.220	-0.250	0.069
	GIV	GBN	GBS	GST	NeoGBS	TOTAL
SS	0.035	-0.122	0.252	-0.150	0.103	0.207
AB	0.191	-0.036	0.168	-0.139	0.045	0.224
XCC Race 1	0.297	0.045	-0.258	-0.154	0.121	0.245
XCC Race 4	0.181	0.078	-0.194	0.046	0.527**	0.448*

B)

	GIB	PRO	GRA	SIN	ALY	GBS	GST	NeoGBS	TOTAL

SS	-0.227	-0.011	-0.206	0.632**	-0.078	0.420	0.030	0.133	0.271
AB	-0.234	-0.025	-0.327	0.742**	-0.118	0.445	-0.144	0.062	0.210
XCC Race 1	0.433	-0.174	-0.401	0.549*	-0.043	-0.089	0.003	0.239	0.239
XCC Race 4	0.761**	-0.254	-0.120	-0.376	-0.286	-0.292	0.371	0.728**	0.616**

Aliphatic glucosinolates: GIB, Glucoiberin; PRO, Progoitrin; GRA, Glucoraphanin; Sin, Sinigrin GBN, Glucobrassicanapin; Indolic glucosinolates: GBS, Glucobrassicin; NeoGBS, Neoglucobrassicin: Aromatic glucosinolate:GST, Gluconasturtiin

*, ** Significant at P≤0.05 and 0.001, respectively.

Table S1: Glucosinolate content (umol g^{-1} of dry weight) on leaves of the local and commercial varieties used in this study.

			Aliphatics								Indolics		Aromatic	Total
Species	**Crop**	**Varieties**	**GIB**	**PRO**	**GRA**	**SIN**	**ALY**	**GNA**	**GIV**	**GBN**	**GBS**	**NeoGBS**	**GST**	
B. rapa	**Turnip top**	**MBG-BRS0026**	1.01	1.19	0.00	0.00	0.00	42.18	3.77	1.07	4.98	0.85	0.09	55.14
B. rapa	**Turnip top**	**MBG-BRS0066**	0.00	1.00	0.00	0.00	0.00	13.44	1.04	0.26	0.50	0.17	0.21	16.61
B. rapa	**Turnip top**	**MBG-BRS0155**	0.93	2.14	0.00	0.08	0.00	36.11	1.81	1.81	6.42	1.82	0.24	51.35
B. rapa	**Turnip top**	**MBG-BRS0259**	1.01	1.12	0.00	0.10	0.00	39.23	2.23	0.94	1.60	1.04	0.09	47.37
***B. oleracea acephala* group**	**Kale**	**MBG-BRS0062**	5.90	0.00	0.29	4.22	0.00	0.00	0.00	0.00	11.97	10.12	0.14	32.66
***B. oleracea acephala* group**	**Kale**	**MBG-BRS0106**	4.42	0.53	0.39	3.86	0.00	0.00	0.00	0.00	26.58	1.49	0.15	37.43
***B. oleracea capitata* group**	**Cabbage**	**MBG-BRS0072**	3.69	0.97	2.97	1.80	0.00	0.04	0.00	0.00	38.10	9.94	0.36	57.86

***B. oleracea capitata* group**	**Cabbage**	**MBG-BRS0425**	5.24	0.60	0.82	3.70	0.00	0.00	0.00	0.00	27.16	2.16	0.35	40.03
***B. oleracea capitata* group**	**Cabbage**	**MBG-BRS0452**	5.62	0.67	2.64	2.06	0.00	0.09	0.00	0.00	16.88	7.02	0.27	35.26
***B. oleracea costata* group**	**Tronchuda cabbage**	**MBG-BRS0121**	7.20	0.00	1.28	0.12	0.00	0.00	0.00	0.00	14.84	4.63	0.45	28.51
***B. oleracea costata* group**	**Tronchuda cabbage**	**MBG-BRS0226**	3.57	0.00	0.00	4.78	0.00	0.00	0.00	0.00	30.88	11.49	0.47	51.18
***B. oleracea italica* group**	**Broccoli[1]**	**Brocoletto**	1.39	0.09	2.89	0.00	0.00	0.00	0.00	0.00	5.96	6.48	0.18	16.99
***B. oleracea botrytis* group**	**Cauliflower[1]**	**Bola de nieve**	11.09	0.00	0.61	0.54	0.00	0.00	0.00	0.00	16.96	91.38	0.59	121.17
***B. oleracea capitata* group**	**Cabbage[1]**	**Corazón de Buey**	2.66	2.10	5.36	1.35	0.04	0.36	0.00	0.00	14.71	0.95	0.22	27.75
***B. napus pabularia* group**	**Nabicol**	**MBG-BRS0063**	0.00	11.35	2.54	0.00	8.66	5.52	0.00	3.55	5.90	16.51	0.79	54.82

***B. napus* *pabularia* group**	**Nabicol**	**MBG-BRS0113**	0.00	9.13	0.04	0.00	2.76	3.96	0.00	5.98	3.88	3.52	0.55	29.83
***B. napus* *pabularia* group**	**Nabicol**	**MBG-BRS0378**	0.00	8.42	0.00	0.00	5.70	4.79	0.00	2.63	10.72	5.81	0.46	38.54

Aliphatic glucosinolates: GIB, Glucoiberin; PRO, Progoitrin; GRA, Glucoraphanin; SIN, Sinigrin; ALY, Glucoalyssin; GNA, Gluconapin; GIV, Glucoiberverin; GBN, Glucobrassicanapin. Indolic glucosinolates: GBS, Glucobrassicin; NeoGBS, Neoglucobrassicin. Aromatic glucosinolate: GST, Gluconasturtiin. [1] Commercial origin: Rainbow hortícolas.

Figure 1: Inhibitory effect of 10 Glucosinolates (GSLs) and 7 Glucosinolate Hydrolysis Products (GHPs) in suppressing the *in vitro* growth of two isolates (*PSM*147 and *PSM*1647) of *Pseudomonas syringae* pv. *maculicola* observed by the disc diffusion assay (dose 3.0 m) and measured as diameter of inhibition zone (in mm). Values are the mean of five replicates.

Figure 2: Inhibitory effect of 10 Glucosinolates (GSLs) and 7 Glucosinolate Hydrolysis Products (GHPs) in suppressing the *in vitro* growth of *Sclerotinia sclerotiorum* (A) and *Alternaria brassicae* (B) observed by the disc diffusion assay and measured as diameter of inhibition zone (in mm). Values are the mean of five replicates.

Figure 3: Inhibitory effect 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 *Xanthomonas campestris* pv. *campestris*. Growth inhibition areas are expressed in mm.

Figure 4: Inhibitory effect of the leaf methanolic extract from 17 varieties belonging to three *Brassica* species in suppressing the *in vitro* growth of *Alternaria brasssicae* (A) and *Sclerotinia sclerotiorum* (B). Growth inhibition areas are expressed in mm.