# Role of Major Glucosinolates in the Defense of Kale Against *Sclerotinia sclerotiorum* and *Xanthomonas campestris* pv. *campestris*

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## ABSTRACT

Glucosinolates (GSLs) are secondary metabolites present in *Brassicaceae* species implicated in their defense against plant pathogens. When a pathogen causes tissue damage, the enzyme myrosinase hydrolyzes GSLs into diverse products that exhibit antimicrobial activity against a wide range of bacteria and fungi *in vitro*. It was demonstrated that modulation of GSLs content *in vivo* affects plant resistance to infection by pathogens in *Arabidopsis*. However, our knowledge of the roles of specific metabolites and how they interact with pathogens is poorly understood in *Brassica* crops. We previously developed a set of populations of *B. oleracea* var. *acephala* L. (kale) differing in content of three GSLs: the aliphatics sinigrin (2-propenyl, SIN) and glucoiberin (3-methylsulphinylpropyl, GIB), and the indolic glucobrassicin (3-indolylmethyl, GBS). These populations can be used to study the effects of major GSLs in kale, with the advantage that genotypes within each selection have the same genetic background. This research aimed to explore the role of SIN, GIB and GBS in the defense of kale against the necrotrophic fungus *Sclerotinia sclerotiorum* and the bacterium *Xanthomonas campestris* pv. *campestris*. Results showed that increasing the amount of a particular GSL did not always result in disease resistance. The effects of GSLs were apparently dependent on the pathogen and the type of GSL. Thus the aliphatic SIN was inhibitory to infection by *S. sclerotiorum*, and the indolic GBS inhibitory to infection by *X. campestris* pv. *campestris*. Other factors, including the quantity and proportion of other metabolites modified during the pathogen infection process, could also modulate the degree of inhibition to the pathogen.

**Keywords**: *Brassica oleracea*, plant resistance, black rot, white mold, secondary metabolites

Plants defend themselves against pathogens by synthetizing and accumulating chemically diverse metabolites with antibiotic properties (Pastorczyk and Bednarek 2016). Constitutive synthesis and accumulation of defensive metabolites (phytoanticipins) is resource-demanding; therefore, *de novo* synthesis can be induced after plant tissue is exposed to pathogen infection (phytoalexins) (Pastorczyk and Bednarek 2016). *Brassicaceae* species accumulate up to 1% of dry weight as the sulfur-containing metabolites, glucosinolates (GSLs) (Calmes et al. 2015). GSLs can be classified into three chemical classes, depending on the side chain of their precursor amino acid. Methionine, alanine, valine, leucine and isoleucine are precursors of aliphatic GSLs. Phenylalanine and tyrosine render aromatic GSLs, and tryptophan is the precursor of indolic GSLs (Zukalova and Vasak 2002). They are constitutively synthetized and stored in plant cells, functioning mainly as phytoanticipins, although they can be induced in response to a variety of stimuli, as well as following exposure to insect damage and pathogens infection (Brader et al. 2006; Buxdorf et al. 2013). *De novo* synthesis of indolic GSLs in *Arabidopsis* can be triggered by plant pathogens, at the same time inducing synthesis of the phytoalexin camalexin through the up regulation of the genes CYP79B2 and CYP79B3, which are shared by several indolic pathways (Brader et al. 2006; Frerigmann et al. 2016; Stahl et al. 2016).

Myrosinase enzymes hydrolyze GSLs into several chemically diverse active products. Myrosinases and GSLs are stored in different plant compartments, and after tissue damage caused by injuries, pests or pathogens (Calmes et al. 2015), these

63 constituents mix, and hydrolysis of GSLs occurs. Hydrolytic products exhibit
64 antimicrobial activities against a wide range of bacterial and fungal plant pathogens *in*
65 *vitro* (Aires et al. 2009; Calmes et al. 2015; Dufour et al. 2015; Li et al. 2013; Sotelo et
66 al. 2015; Stotz et al. 2011). Antimicrobial effects depend on the dose, pathogen and the
67 specific race or isolate (Sotelo et al. 2015). Besides, the chemical structure of the
68 breakdown products determine their activity *in vitro*, with isothiocyanates (ITCs) being
69 the most toxic, even at low concentrations (Pastorczyk and Bednarek 2016). Toxicity of
70 ITCs is also related to the length of their side chain (Li et al. 2013).

71 *In planta* activation of GSLs is dependent on tissue breakdown; however, this
72 does not happen in the interaction with biotrophic pathogens (Calmes et al. 2015).
73 Production and liberation of active indole compounds to the apoplast can also be
74 mediated by the action of the genes pen2 and pen3 as described with the biotroph
75 fungus *Blumeria graminis* fsp. *hordei* (Bgh) (Johansson et al. 2014). To demonstrate the
76 role of GSLs in the immunity of *Brassicaceae* plants to various pathogens, knockout
77 mutants of genes implicated in the synthesis and hydrolysis of GSLs in *Arabidopsis*
78 have been tested against various bacteria and fungi. The double mutant CYP79B2/B3,
79 which does not accumulate indolic GSLs or camalexin, and the double mutant
80 MYB28/29, which does not accumulate aliphatic GSLs, modified the sensitivity of
81 plants against isolates of the fungus *Alternaria brassicicola* (Schwein.) Wiltshire,
82 *Botrytis cinerea* Pers. (Buxdorf et al. 2013) and *Sclerotinia sclerotiorum* (Lib.) de Bary
83 (Stotz et al. 2011). However, transgenic plants *Arabidopsis* accumulating specific GSLs
84 can increase their resistance to pathogens. Expression of the CYP79D2 gene from
85 cassava in *Arabidopsis* causes accumulation of aliphatic isopropyl and methylpropyl
86 GSLs that enhance resistance to the bacteria *Erwinia carotovora* (van Hall) Dye.
87 *Arabidopsis* expressing the sorghum CYP79A1 or over-expressing the endogenous

CYP79A2 accumulate p-hydroxybenzyl or benzyl GSLs, respectively, increasing resistance to *Pseudomonas syringae* van Hall (Brader et al. 2006). The contribution of GSLs to the defense of *Arabidopsis* is dependent on the pathogen species and even on the isolate under consideration (Brader et al. 2006; Pastorczyk and Bednarek 2016). Specialist pathogens can adapt themselves by detoxifying ITCs and/or suppressing the induction of GSLs biosynthesis (Pastorczyk and Bednarek 2016). Variability in the response among pathogens may also be due to different life styles (i.e., biotrophic versus necrotrophic) or differential host-ranges (Buxdorf et al. 2013).

*Brassica* crops are affected by several diseases that can reduce the quantity and quality of yield, resulting in economic losses worldwide (Sup et al. 2016). Research efforts are dedicated to identify resistant materials and to discover the mechanisms underlying that resistance. However, knowledge of the role of specific metabolites in defense of *Brassica* crops against pathogens is not well characterized. The content of GSLs in several varieties of *Brassica oleracea* and *B. napus* has been studied in relation to resistance to *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson, *S. sclerotiorum* and *B. cinerea* (Aires et al. 2011; Giamoustaris and Mithen 1997). But it should be noted that the host genotypes had different GSL profiles and concentrations, as well as different genetic backgrounds. Recent work found that lines of *B. napus*, which over-expressed UGT74B, an indolic GSL pathway gene, enhanced not only the production of total indolic GSLs, but also the resistance to *S. sclerotiorum* and *B. cinerea* (Zhang et al. 2015).

While these efforts show that modulating the content of GSLs may affect *Brassica* plant resistance to pathogens, our knowledge regarding the contribution of specific metabolites is poorly understood. Previously, we used mass selection to develop a set of *B. oleracea* var. *acephala* L. (kale) genotypes differing in the content of

three GSLs: the aliphatics sinigrin (2-propenyl, SIN) and glucoiberin (3-methylsulphinylpropyl, GIB), and the indolic glucobrassicin (3-indolylmethyl, GBS). Sotelo et al. (2016) evaluated these genotypes and concluded that different alleles at the GSL-ALK locus were responsible for variation in the content of SIN and GIB, whereas the CYP81F2 gene may be responsible for the variation in concentration of GBS. This material can be used to study the effects of major GSLs in kales, with the advantage that the genotypes all have the same genetic background (Sotelo et al. 2016). The aim of this research was to assess the role of SIN, GIB and GBS in the defense of kale plants against the necrotrophic pathogens, *S. sclerotiorum* (*Ss*) and *X. campestris* pv. *campestris* (*Xcc*).

## MATERIAL AND METHODS

*Plant and pathogen culture.* Three independent divergent selection programs were performed using mass selection at the Misión Biológica de Galicia (MBG-CSIC, Spain), so as to obtain genotypes of *B. oleracea* differing in content of three major GSLs (namely SIN, GIB and GBS) (Sotelo et al. 2016). The three divergent selection programs were all based on the landrace MBG-BRS0062. This is a heterogeneous kale variety, cultivated by farmers since ancient times in the NW of the Iberian Peninsula (Padilla et al. 2007; Tortosa et al. 2017). The race is maintained at the germplasm bank at the MBG-CSIC. MBG-BRS0062 was chosen because it had broad genetic variability in GSL content, a desirable characteristic on which to perform selection (Sotelo et al. 2016). For each one of the divergent selections (SIN, GIB and SIN), three consecutive cycles of selection were performed to obtain genotypes with high (H) or low (L) GSL content. Six genotypes were obtained: HSIN, LSIN, HGIB, LGIB, HGBS and LGBS, which were used to test the effect of SIN, GIB and GBS on infection of kale by two

pathogens (*Xcc* and *Ss*) of *B. oleracea*.

The isolate of *Xcc* belongs to race 1 (strain HRI3811, synonymous with PHW1205 collected from *B. oleracea* in the USA, Joana Vicente personal communication) and was provided by Joana Vicente (Warwick HRI, Wellesbourne, UK). Fresh bacterial colonies of *Xcc* were sub-cultured on petri dishes containing potato dextrose agar (PDA) and incubated at 32°C for 24 h in the dark. For inoculum preparation, a loop of bacteria was transferred to nutrient broth and shaken overnight at 150 rpm and 30°C in the dark. The culture was diluted in sterile tap water to a concentration of 5 $\times 10^8$ cfu·ml$^{-1}$, which corresponds to an absorbance of 0.51 at a wavelength of 600 nm, measured using a spectrophotometer (Spectra MR; Dynex Technologies, Chantilly, VA, USA). The *Ss* isolate MBG-*Ss*2 was provided by MBG-CSIC. The original isolate was collected in January 2008 from a naturally infected plant of *B. napus* in an experimental field at MBG (42°24'23"N 8°38'31"W).

Fresh colonies of *Ss* were obtained through routine transfer of mycelium-agar plugs from the margin of a colony of *Ss* growing on PDA medium. The cultures were incubated with a 14 h photoperiod, a day night mean temperature of 24/18°C. Agar plugs (4 mm in diameter) containing the advancing edge of fungal mycelia were used as the inoculum source.

*Plant-inoculation and disease severity measurements.* The reaction of six kale genotypes (LSIN, HSIN, LGBS, HGBS, LGIB, HGIB) to infection by *Xcc* and *Ss* were tested in two independent experiments in a greenhouse with a 14 h photoperiod, a day night mean temperature of 24/18°C and 70% relative humidity. Plants were grown in pots containing 2.5L of peat (Gramoflor GmbH & Co. KG Produktion, Vechta, Germany). In both experiments, thirty plants were inoculated per genotype in a

completely randomized design. Variability among the thirty individuals, which were genetically different, was considered as the error in both experiments. Six weeks after sowing, the second youngest leaf of each plant (counting from the apex) was inoculated. This was considered the first trial of the experiments (T1).

For *Xcc*, inoculum was injected into three different points of each leaf, puncturing the main veins using mouse-tooth forceps wrapped in cotton soaked in the bacterial suspension. After inoculation, plants were placed in the greenhouse with a day/night mean temperature of 24/28°C, a 14h photoperiod and a relative humidity of 90 to 100%. At 21 days post-inoculation (dpi), the inoculated leaves were collected and photographed. The area of each chlorotic lesion was measured using ImageJ software version 1.51n (LOCI, University of Wisconsin, USA). Total lesion area ($cm^2$) was calculated by summing the area of all the individual chlorotic lesions on a leaf. For the *Ss* experiment, leaves were inoculated by placing one agar plug with the fungal inoculum on the upper side of the leaf. On the $4^{th}$ dpi, inoculated leaves were collected and photographed, and the area of necrotic lesion per leaf was obtained with ImageJ software as described above. Five replicate plants per genotype were used as controls (not inoculated).

Subsequent to collecting the inoculated leaves and control leaves in both the *Xcc* and *Ss* experiments in T1, the second youngest leaf of each plant counting from the apex was inoculated as described above for T1. All experiment procedures were the same as described above for T1. This was considered the second trial (repeat) of the experiments (T2).

*GSLs identification and quantification.* After photographing inoculated leaves at T1 and T2, they were immediately frozen in solid dry ice and transferred to the laboratory and stored at -80°C. GSL extraction followed the methodology of

Kliebenstein et al. (2001) with minor modifications to perform multiple extractions in 96 plates. Identification and quantification of compounds was performed using ultra-high performance liquid chromatography (UHPLC) according to Sotelo et al. (2014). GSL concentration was measured in μmol $g^{-1}$ dry weight.

*Data analyses.* The variables lesion area and GSL concentration were subjected to analysis of variance using a generalized linear model. Analysis of variance were performed independently by experiment (plants inoculated with *Ss* or *Xcc*) and by trial (T1 and T2) within each experiment. GSL genotypes (high or low) and individual plants were considered sources of variation. Genotypes were considered fixed effects, whereas replications (plants within each GSL selection) were considered random factors. For each experiment and trial, three separate analyses of variance were performed. Firstly, genotypes for each divergent selection (SIN, GIB and GBS) and controls were analysed. Secondly, only genotypes for each divergent selection were included. Thirdly, an analysis comparing genotypes among divergent selections was performed. All analyses were completed using R Core Team (2018) and the *Rcmdr* package (Fox and Bouchet-Valat, 2018). Means comparisons were performed for all traits using Fisher's protected least significant difference (LSD) at $\alpha = 0.05$.

## RESULTS

***Disease severity and its relationship to the target and other GSLs within each selection.*** Disease severity in the three divergent selections was measured as the area of the lesion caused by *Ss* or *Xcc* in two independent experiments. GSL concentration was measured in the same leaves as disease severity. In addition to the three major GSLs

under selection, a further three GSLs were detected across the six genotypes in the control plants in *Ss* experiment: the indolics hidroxyglucobrassicin (4-hydroxy-3-indolylmethyl, OHGBS), neoglucobrassicin (1-methoxy-3-indolylmethyl, NEOGBS) and methoxyglucobrassicin (4-methoxy-3-indolylmethyl, MEGBS) (Fig. 1). The same GSLs were identified in the six genotypes inoculated with *Ss* or *Xcc* in both T1 and T2 (Fig. 2–4). We present the results in three sections related to the divergent selections to describe the relationship between disease severity and content of each GSL: SIN, GBS and GIB, and the additional GSLs.

To determine whether there was induction of GSLs after inoculation with *Ss*, the concentration of GSLs of inoculated plants was compared to that of controls in the three divergent selections. No difference was found in the profile of GSLs; however, significant differences among GSL concentrations (SIN selection T1: SIN: $p<0.001$; MEGBS: $p = 0.001$; OHGBS: $p = 0.02$; SIN selection T2: SIN: $p<0.001$; MEGBS: $p = 0.03$; OHGBS: $p = 0.02$; GIB selection T1: GIB: $p<0.001$; MEGBS: $p<0.001$; NEOGBS: $p = 0.005$; OHGBS: $p<0.001$; GIB selection T2: SIN: $p = 0.02$; GIB: $<.001$; MEGBS: $p<0.001$; OHGBS: $p<0.001$; GBS selection T1: GBS: $p = 0.009$; MEGBS: $p = 0.001$; GBS selection T2: SIN: $p<0.001$; GBS: $p = 0.002$; NEGBS: $p = 0.02$; NEOGBS: $p<0.001$; OHGBS: $p = 0.04$) were detected (Table 1). Generally speaking the indolics MEGBS and NEOGBS were induced in inoculated plants compared to controls (Fig. 1 A–C), although this is more apparent in the GIB selected genotype compared with the SIN or GBS genotypes. The aliphatics SIN and GIB were actually repressed in T2 in the inoculated plants of the GIB selection (Fig. 1B).

***SIN selected plants.*** Subsequent to inoculation with *Ss*, there were significant differences between HSIN and LSIN for lesion area in both trials ($p = 0.05$, $p<0.001$ in T1 and T2, respectively) (Table 2). Plants with HSIN had significantly smaller lesion

areas in both T1 and T2 compared to plants with LSIN (Fig. 2A). In contrast, those HSIN plants inoculated with *Xcc* had larger lesions compared to the LSIN plants in T1, but a similar response in T2. SIN concentration was significantly higher in HSIN plants compared to LSIN plants in T2, but the difference was not significant in T1 (Fig. 2B, C).

The concentration of the indolics MEGBS and NEOGBS was significantly higher in *Ss*-inoculated HSIN plants compared to *Ss*-inoculated LSIN plants in T1 and T2, respectively. OHGBS was significantly higher in LSIN selected plants compared to HSIN selected plants in T2 (Fig. 2B). When SIN selected plants were inoculated with *Xcc*, OHGBS concentration was higher in HSIN plants, whereas MEGBS and NEOGBS were higher in LSIN plants in T1 (Fig. 2C).

***GIB selected plants***. When GIB selected plants were inoculated with *Ss*, there were significant differences ($p<0.001$) between genotypes for lesion area in T1 in the analysis of variance (Table 2). HGIB plants had a smaller lesion area in T1 when compared to lesion size on the LGIB plants (Fig. 3A). GIB content was significantly higher in the HGIB plants compared to the LGIB plants in both T1 and T2 (Fig. 3B). Furthermore, SIN content was higher in LGIB plants in T1 and OHGBS was higher in HGIB plants in both T1 and T2. There were no significant differences between genotypes for lesion area when inoculated with *Xcc* (Table 2). However, the content of GIB was significantly higher in HGIB plants compared with that in LGIB plants in both T1 and T2. The concentration of OHGBS was significantly higher in HGIB plants compared to LGIB plants in T1, whereas SIN was lower in HGIB plants when compared to that in LGIB plants in T2 (Fig. 3C).

259 ***GBS selected plants.*** No significant differences among genotypes were found
260 when plants of the GBS selection were inoculated with *Ss* (Table 2). When genotypes
261 were inoculated with *Xcc*, the HGBS plants exhibited a resistant response with
262 noticeably smaller lesion areas (Fig. 4A) compared to the LGBS plants in both T1 and
263 T2. HGBS plants had a significantly higher concentration of GBS when compared with
264 LGBS plants in both experiments in both T1 and T2. However, the total concentration
265 of GBS and the difference in GBS concentration between the HGBS plants and the
266 LGBS plants was higher when plants were inoculated with *Ss* than when inoculated
267 with *Xcc* (Fig. 4B, C). In GBS selected plants, the HGBS plants that were inoculated
268 with *Ss* had significantly higher concentrations of GIB in T1 when compared to the
269 LGBS plants. The HGBS plants had a higher concentration of SIN and NEOGBS in T2,
270 whereas MEGBS was higher in the LGBS plants. In both T1 and T2, the concentration
271 of NEOGBS was significantly higher in HGBS plants inoculated with *Xcc* compared to
272 the LGBS plants inoculated with *Xcc*.

273 ***Disease severity and its relationship to SIN, GBS and GIB, and the additional***
274 ***GSLs.*** There were significant differences for lesion area in the analysis of variance
275 among the three selections when plants were inoculated with *Ss* ($p<0.001$ in T1 and T2)
276 or *Xcc* ($p = 0.006$ in T1 and $p = 0.04$ in T2) (Table 3). In plants inoculated with *Ss*, the
277 LGIB plants had significantly larger lesions compared to the other genotypes in T1 (Fig.
278 5A). The HSIN plants exhibited a significantly smaller lesion area compared to the
279 other genotypes, with the exception of lesions on the LGBS plants in T2. The HSIN
280 plants had significantly more SIN compared with the other genotypes in T2.
281 Furthermore, the NEOGBS content of HSIN plants was numerically highest, although it
282 was not significantly different to that in the other genotypes. However, the

283 concentration of GSLs cannot explain the relatively small lesion size observed in LGBS
284 plants, which have a low SIN and NEOGBS content (Fig. 5A).

285 In plants inoculated with *Xcc*, the HGBS plants had smaller lesions compared to
286 the other genotypes, although the lesion size did not differ significantly from those on
287 the LSIN, HGIB and LGIB plants in T1, nor from the lesions on the HSIN, LSIN and
288 HGIB plants in T2 (Fig. 5B). Thus the resistance exhibited by HGBS plants to *Xcc*
289 compared to the other genotypes cannot be explained GBS content, since the HGBS
290 plants had a low GBS content in both T1 and T2.

291

292 **DISCUSSION**

293 We demonstrated that genotypes selected for higher GSL content invariably had
294 a greater concentration of the target GSL compared with the corresponding genotypes
295 selected for low GSL content. It is established that changes in a single GSL gene may
296 lead to pleiotropic effects on the concentrations of other GSLs. When divergent
297 selection modifies the content of SIN, effects on the content of GIB, GBS or other GSLs
298 may also occur (Sotelo et al. 2016). Therefore, we included minor GSLs presents in kale
299 leaves for each selection in studies of the relationship with disease severity. We
300 observed differential performance of the divergent selections after inoculation with *Ss*
301 or *Xcc*. Genotypes selected for high SIN content were more resistant to *Ss* and
302 genotypes selected for high GBS content were more resistant to *Xcc*. However, the
303 effect of GIB content was not clear, since the relationship with lesion area was time
304 dependent in the *Ss* experiment, and there was no relationship with lesion area when
305 genotypes were inoculated with *Xcc*.

306 Our results showed that the aliphatic GSL, SIN inhibited the growth of *Ss*, i.e.,
307 HSIN genotypes had smaller lesions (less severe disease). These results are congruent

with those of a previous report showing a role of SIN in reducing severity of disease (Stotz et al. 2011). The authors reported that content of aliphatic GSLs in *Arabidopsis* were correlated with changes in susceptibility to *Ss*. In addition, when genotypes of the SIN selection were inoculated with *Ss*, the concentrations of two indolic GSLs (NEOGBS and MEGBS) were significantly higher in HSIN than in LSIN. Therefore, the resistance observed with the SIN selection against *Ss* may be attributed to a combined effect of the aliphatic SIN and the indolic GSLs that together enhanced the plant defense response.

The effect of GIB selection was not clear, since the response of the HGIB and LGIB genotypes was dependent upon time when plants were inoculated with *Ss*. Previous reports indicate that different alleles at the GSL-ALK locus are selected for in the divergent selections for SIN and GIB (Sotelo et al. 2016). GSL-ALK alkenized GIB to render SIN. Therefore, when the content of one increases, the content of the other decreases. SIN inhibited the growth of *Ss* as demonstrated in SIN selection. HGIB had lower concentration of SIN than LGIB. This could explain why the selection to modify GIB content did not have a clear effect on lesion area as a result of infection by *Ss*.

Several reports indicate that indolic GSLs are inhibitory against *Ss in vivo* (Li et al. 1999; Zhang et al. 2015). However, we did not find a clear effect of the HGBS genotypes reducing lesion area caused by *Ss*. Lack of an effect may be due to variations in the GBS content in the divergent selection used in our study. Such variations are reported to occur in expression of the CYP81F2 gene (Sotelo et al. 2016). The CYP81F2 gene converts GBS into OHGBS, and should not affect other metabolic pathways. However, research that relates indolic GSLs with resistance to *Ss* in *Arabidopsis* through mutations in the UGT74B1 or CYP79B genes indicate they affect not only the content of indolic GSLs, but also other plant immune system metabolites

(Zhang et al. 2015). Therefore, it is not clear if the effect can be attributed to an increase in total indolic GSLs or to the induction of other plant immune system metabolites (i.e., camalexin or auxins). Thus, defense responses against *Ss* are probably regulated by a complex network of hormonal and signaling pathways (i.e., jasmonic acid, ethylene, abscisic acid) promoting the production of GSLs and other metabolites, working together to enhance plant resistance.

Little is known about the mechanisms of resistance to *Xcc* in *Brassica* crops. Aires et al. (2011) concluded that both aliphatic and indolic GSLs may play a complex role in the defense, depending on the species of *Brassica*. Velasco et al. (2013) reported that the aliphatic gluconapin (3-butenyl) can protect *B. rapa* against *Xcc.* We found that HGBS plants were more resistant to *Xcc,* whereas the aliphatics SIN and GIB did not have an effect in reducing lesion area. Overall, these findings suggest that an increment of individual GSLs does not always lead to enhanced disease resistance.

GSLs are present in all tissues of the plants. They are produced constitutively, but are also inducible in response to biotic stress (Brader et al. 2001; Buxdorf et al. 2013). To explore the relationship between the endogenous concentration of GSLs and the induced component in GSLs as a result of infection by *Ss*, the content of GSLs in the high and low selections of each genotypes were compared to that of the controls. Aires et al. (2011) related the suppression of *de novo* synthesis of defense compounds and the inhibition of myrosinase activity with the mode of infection by a necrotrophic pathogen. Whereas, hypersensitive cell death can be an effective defense strategy against biotrophs, it may not be efficient against necrotrophic pathogens. However, a reduction in lesion size caused by *Ss* in oilseed rape (*B. napus*) was related to induction of indolic and aromatic GSLs (Li et al. 1999). Induced synthesis of GSLs may be related to the plant genotype and to its interaction with specific pathogens. Similar to

the observations of Li et al. (1999), we found that indolic GSLs were induced after inoculation with *Ss* in all three selections, but it was most pronounced with the GIB genotype. However, aliphatic GSLs were repressed in the GIB genotype. These apparently opposing responses among GSLs could contribute to the poor performance of GIB against *Ss*.

The process of GSL hydrolysis may be related to the differential performance of the divergent selections after infection with *Ss* and *Xcc*. Myrosinases and GSLs are localized in two different specialized cells. When there is tissue destruction, for example if a necrotroph infects, the GSLs are exposed to the myrosinases which hydrolyze the β-D-thioglucosidic bond of the GSL to release β-D-glucose and an unstable aglucone (Calmes et al. 2015; Shirakawa and Hara-Nishimura 2018). The aglucone spontaneously forms an ITC by rearrangement or decomposes to a nitrile. Formation of ITCs is favored at pH values of 5–6, while nitriles are preferentially formed at a pH <4 or at increased $Fe^{2+}$ concentrations (Hanschen et al. 2018). In addition, epithiospecific proteins may promote the formation of nitriles and epithionitriles by modulating myrosinase activity. As a consequence, tissue disruption typically leads to the release of complex mixtures of GSL breakdown products (Burow et al. 2009; Hanschen et al. 2018). We measured the quantity of intact GSLs and not their degradation products after inoculation. We would expect a reduction in the concentration of GSLs when comparing inoculated plants with controls as a part of the process of degradation. However, this does not always happen, as several GSLs were induced upon *Ss* infection. Therefore, the concentrations of GSLs in inoculated plants are the product of their degradation and of their induced synthesis upon infection.

When comparing the three divergent selections, the HSIN and HGBS plants had greater resistance to *Ss* and *Xcc*, respectively. However, the resistance is not only related

to the content of GSLs: in the SIN divergent selection group, the content of SIN was related to resistance. But the relationship with resistance was not observed in the GBS selection, where the HGBS plants had a higher content of SIN compared to the LGBS plants. Other processes in the plant could modify the plant response as the three divergent selections were performed independently, resulting in different genetic backgrounds for each GSL selection. Modifying the content of GSLs could affect other processes in the plant.

## Conclusions

The toxic effect of GSL-derived products has been demonstrated with a broad range of fungi and bacteria by *in vitro* experiments. However, the role of GSLs in plant defense is more complex. Increasing the content of individual GSLs does not always result in reduced disease severity. The effect of GSL is clearly dependent on the pathogen and the chemical class of the GSL. We observed the aliphatic SIN was inhibitory to disease caused by *Ss*, but the indolic GBS was inhibitory to disease caused by *Xcc*. Additional factors, including the proportions of other metabolites in the host during the pathogen infection, could modulate the response of the plant. Differential performance of genotypes across divergent selections cannot be attributed solely to variation in GSL content. We conclude that other processes in the plant that impact disease response were probably modified when the divergent selections were developed.

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**Table 1.** Results of an analysis of variance performed to compare the content of glucosinolates in kale plants either inoculated with *Sclerotinia sclerotiorum* or noninoculated as control plants in two trials (T1 and T2). The results presented are for each of the three divergent selections for different glucosinolate types (SIN, GIB, GBS; content of MEGBS, NEOGBS and OHGBS were also measured in the three divergent selections).

| | | Glucosinolate[1] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Selection | | T1 | | | | | | T2 | | | | | |
| | | SIN | GIB | GBS | MEGBS | NEOGBS | OHGBS | SIN | GIB | GBS | MEGBS | NEOGBS | OHGBS |
| **SIN** | | | | | | | | | | | | | |
| | F-value[2] | 13.1 | 2.4 | 2.1 | 6.8 | 1.2 | 3.5 | 12.9 | 0.5 | 1.2 | 3.2 | 2.4 | 3.6 |
| | p-value | <0.001 | 0.08 | 0.1 | 0.001 | 0.3 | 0.02 | <0.001 | 0.7 | 0.3 | 0.03 | 0.08 | 0.02 |
| **GIB** | | | | | | | | | | | | | |
| | F-value | 2.6 | 7.7 | 2.1 | 7.00 | 4.8 | 7.6 | 3.7 | 29.4 | 0.7 | 10.7 | 2.5 | 14.4 |
| | p-value | 0.06 | <0.001 | 0.1 | <0.001 | 0.005 | <0.001 | 0.02 | <0.001 | 0.6 | <0.001 | 0.07 | <0.001 |
| **GBS** | | | | | | | | | | | | | |
| | F-value | 0.05 | 2.6 | 4.3 | 7.0 | 2.2 | 1.5 | 7.2 | 1.5 | 5.7 | 3.6 | 8.1 | 3.0 |
| | p-value | 1.0 | 0.06 | 0.009 | 0.001 | 0.1 | 0.2 | <0.001 | 0.2 | 0.002 | 0.02 | <0.001 | 0.04 |

[1] SIN (sinigrin, 2-propenyl), GIB (glucoiberin, 3-methylsulphinylpropyl), GBS (3-indolylmethyl, glucobrassicin), OHGBS (hidroxyglucobrassicin, 4-hydroxy-3-indolylmethyl), NEOGBS (neoglucobrassicin, 1-methoxy-3-indolylmethyl), MEGBS (methoxyglucobrassicin, 4-methoxy-3-indolylmethyl).

[2]F value of the F distibution and p-value indicating the probability of the effect.

**Table 2.** Results of an analysis of variance performed to compare the content of glucosinolates and lesion area in kale plants either inoculated with *Sclerotinia sclerotiorum* or *Xanthomonas campestris pv. campestris* or noninoculated as control plants in two trials (T1 and T2). The results presented are for each of the three divergent selections for different glucosinolate types (SIN, GIB, GBS; content of MEGBS, NEOGBS and OHGBS were also measured in the three divergent selections)..

| Selection | Glucosinolate[1] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T1 | | | | | | | T2 | | | | | | |
| | SIN | GIB | GBS | MEGBS | NEOGBS | OHGBS | LESION AREA | SIN | GIB | GBS | MEGBS | NEOGBS | OHGBS | LESION AREA |
| *Sclerotinia sclerotiorum* | | | | | | | | | | | | | | |
| **SIN** | | | | | | | | | | | | | | |
| F-value[2] | 33.7 | 2.1 | 3.9 | 5.9 | 2.5 | 2.0 | 2.4 | 29.8 | 0.2 | 3.7 | 0.00 | 5.3 | 4.7 | 20.4 |
| p-value | <0.001 | 0.16 | 0.05 | 0.02 | 0.1 | 0.2 | 0.05 | <0.001 | 0.7 | 0.06 | 1.0 | 0.03 | 0.04 | <0.001 |
| **GIB** | | | | | | | | | | | | | | |
| F-value | 8.0 | 12.5 | 1.0 | 0.03 | 0.9 | 16.8 | 23.9 | 0.8 | 14.4 | 1.1 | 0.01 | 1.3 | 8.7 | 0.1 |
| p-value | 0.007 | 0.001 | 0.334 | 0.9 | 0.4 | <0.001 | <0.001 | 0.4 | 0.001 | 0.3 | 0.9 | 0.3 | 0.006 | 0.7 |
| **GBS** | | | | | | | | | | | | | | |
| F-value | 0.00 | 6.2 | 5.9 | 0.8 | 2.2 | 0.2 | 0.04 | 12.8 | 1. 9 | 16.3 | 4.7 | 20.6 | 4.0 | 3.6 |
| p-value | 1.0 | 0.02 | 0.02 | 0.4 | 0.1 | 0.7 | 0.9 | 0.001 | 0.2 | <0.001 | 0.03 | <0.001 | 0.05 | 0.07 |
| *Xanthomonas campestris pv. campestris* | | | | | | | | | | | | | | |
| **SIN** | | | | | | | | | | | | | | |
| F-value | 1.4 | 0.03 | 1.3 | 6.6 | 4.4 | 4.9 | 11.0 | 31.5 | 2.0 | 2.8 | 1.3 | 0.01 | 0.8 | 0.05 |
| p-value | 0.2 | 0.9 | 0.3 | 0.01 | 0.04 | 0.03 | 0.002 | <0.001 | 0.2 | 0.1 | 0.3 | 0.9 | 0.4 | 0.8 |
| **GIB** | | | | | | | | | | | | | | |
| F-value | 2.0 | 10.0 | 2.3 | 1.3 | 3.2 | 13.3 | 0.6 | 9.7 | 28.4 | 2.1 | 0.3 | 0.7 | 0.00 | 0.3 |
| p-value | 0.2 | 0.003 | 0.1 | 0.3 | 0.08 | 0.001 | 0.4 | 0.003 | <0.001 | 0.2 | 0.6 | 0.4 | 0.9 | 0.6 |
| **GBS** | | | | | | | | | | | | | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F-value | 7.0 | 1.4 | 7.9 | 1.0 | 10.8 | 1.3 | 7.4 | 0.1 | 1.4 | 7.4 | 0.8 | 16.1 | 1.3 | 7.6 |
| p-value | 0.01 | 0.2 | 0.007 | 0.3 | 0.002 | 0.3 | 0.009 | 0.7 | 0.2 | 0.009 | 0.4 | <0.001 | 0.3 | 0.008 |

[1] SIN (sinigrin, 2-propenyl), GIB (glucoiberin, 3-methylsulphinylpropyl), GBS (3-indolylmethyl, glucobrassicin), OHGBS (hidroxyglucobrassicin, 4-hydroxy-3-indolylmethyl), NEOGBS (neoglucobrassicin, 1-methoxy-3-indolylmethyl), MEGBS (methoxyglucobrassicin, 4-methoxy-3-indolylmethyl).

[2]F value of the F distibution and p-value indicating the probability of the effect.

**Table 3.** Results of an analysis of variance performed to compare the content of glucosinolates and lesion area in kale plants either inoculated with *Sclerotinia sclerotiorum* or *Xanthomonas campestris pv. campestris* or noninoculated as control plants in two trials (T1 and T2). The results presented are combined through the three divergent selections for different glucosinolate types (SIN, GIB, GBS; content of MEGBS, NEOGBS and OHGBS were also measured in the three divergent selections)..

| Species | T1 | | | | | | | T2 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glucosinolate[1] | | | | | | Lesion area | Glucosinolate[1] | | | | | | Lesion area |
| | SIN | GIB | GBS | MEGBS | NEOGBS | OHGBS | | SIN | GIB | GBS | MEGBS | NEOGBS | OHGBS | |
| *Sclerotinia sclerotiorum* | | | | | | | | | | | | | | |
| F-value[2] | 6.9 | 4.9 | 6.7 | 3.3 | 3.9 | 9.2 | 7.8 | 12.5 | 3.4 | 4.5 | 2.9 | 4.0 | 10.5 | 8.2 |
| p-value | <0.001 | <0.001 | <0.001 | 0.008 | 0.002 | <0.001 | <0.001 | <0.001 | 0.006 | 0.001 | 0.02 | 0.002 | <0.001 | <0.001 |
| *Xanthomonas campestris pv. campestris* | | | | | | | | | | | | | | |
| F-value | 5.7 | 3.37 | 5.1 | 3.4 | 5.1 | 3.4 | 3.4 | 14.1 | 13.0 | 7.2 | 2.7 | 3.9 | 5.2 | 3.2 |
| p-value | <0.001 | 0.006 | <0.001 | 0.006 | <0.001 | 0.006 | 0.006 | <0.001 | <0.001 | <0.001 | 0.02 | 0.002 | <0.001 | 0.04 |

[1] SIN (sinigrin, 2-propenyl), GIB (glucoiberin, 3-methylsulphinylpropyl), GBS (3-indolylmethyl, glucobrassicin), OHGBS (hidroxyglucobrassicin, 4-hydroxy-3-indolylmethyl), NEOGBS (neoglucobrassicin, 1-methoxy-3-indolylmethyl), MEGBS (methoxyglucobrassicin, 4-methoxy-3-indolylmethyl).

[2]F value of the F distibution and p-value indicating the probability of the effect.

**Figure legends**

**Figure 1.** Comparison of the content of glucosinolates in kale plants inoculated with *Sclerotinia sclerotiorum* and control plants in two trials (T1 and T2) in A) a SIN divergent selection, B) a GIB divergent selection and C) a GBS divergent selection. Values are the average of 30 replicates ± standard error. Means with different letters are significantly different at $\alpha \leq 0.05$ according to Fisher's protected LSD test. SIN (sinigrin, 2-propenyl), GIB (glucoiberin, 3-methylsulphinylpropyl), GBS (3-indolylmethyl, glucobrassicin), OHGBS (hidroxyglucobrassicin, 4-hydroxy-3-indolylmethyl), NEOGBS (neoglucobrassicin, 1-methoxy-3-indolylmethyl), MEGBS (methoxyglucobrassicin, 4-methoxy-3-indolylmethyl).

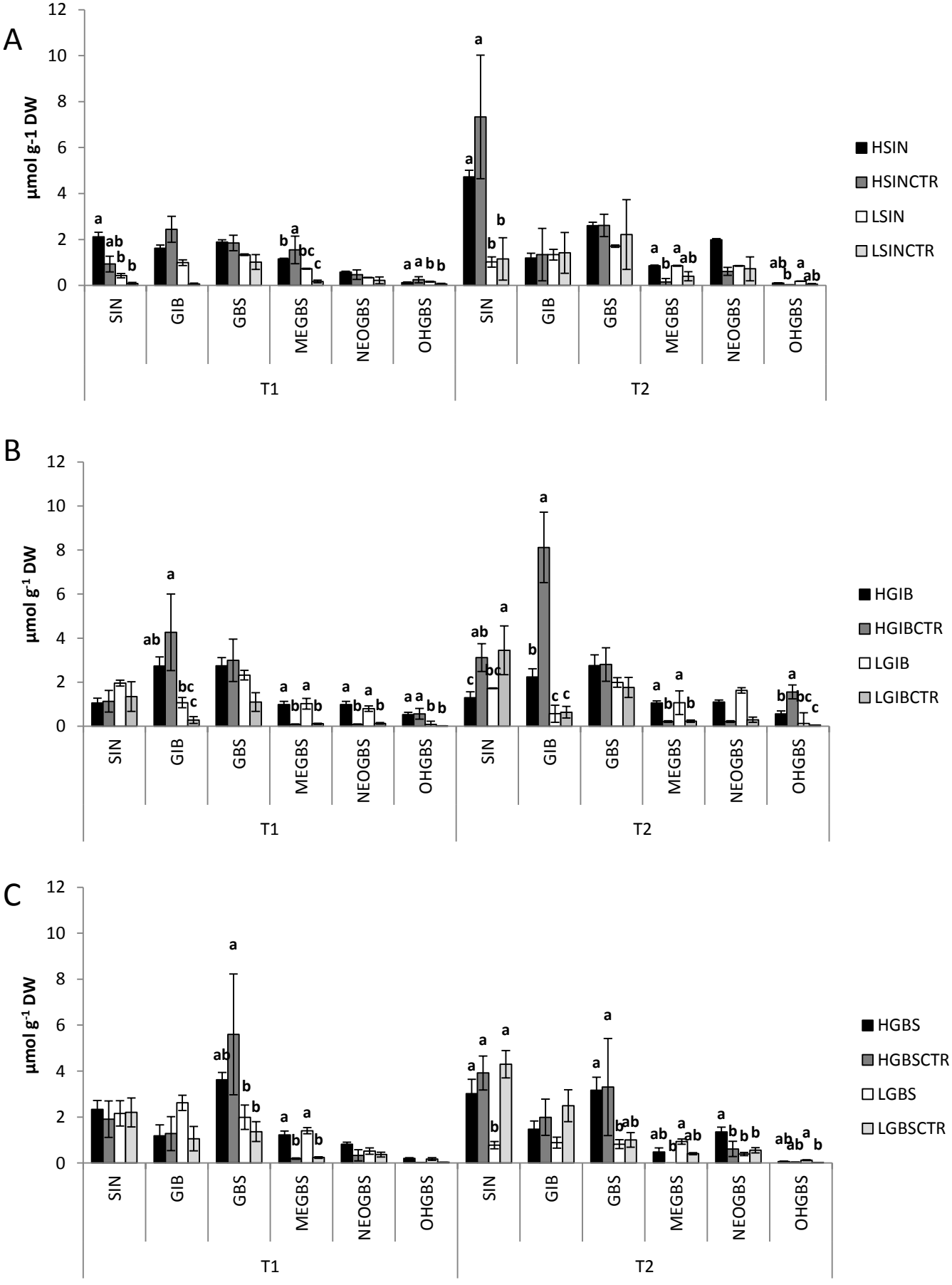
**Figure 2.** The effect of high (HSIN) and low (LSIN) SIN content in a selection of kale genotypes on (A) lesion area after inoculation with *Sclerotinia sclerotiorum* (*Ss*) or *Xanthomonas campestris pv. campestris* (*Xcc*) in two trials (T1 and T2), (B) content of SIN, GIB, GBS and minor GSLs in the HSIN and LSIN genotypes inoculated with *Ss*, and (C) content of SIN, GIB, GBS and minor GSLs in HSIN and LSIN genotypes inoculated with *Xcc*. Values are the average of 30 replicates ± standard error. Means with different letters are significantly different at $\alpha \leq 0.05$ according to Fisher's protected LSD test. SIN (sinigrin, 2-propenyl), GIB (glucoiberin, 3-methylsulphinylpropyl), GBS (3-indolylmethyl, glucobrassicin), OHGBS (hidroxyglucobrassicin, 4-hydroxy-3-indolylmethyl), NEOGBS (neoglucobrassicin, 1-methoxy-3-indolylmethyl), MEGBS (methoxyglucobrassicin, 4-methoxy-3-indolylmethyl).

**Figure 3.** The effect of high (HGIB) and low (LGIB) GIB content in a selection of kale genotypes on (A) lesion area after inoculation with *Sclerotinia sclerotiorum* (*Ss*) or *Xanthomonas campestris pv. campestris* (*Xcc*), in two trials (T1 and T2), (B) content of SIN, GIB, GBS and minor GSLs in the HGIB and LGIB genotypes inoculated with *Ss*, and (C) content of SIN, GIB, GBS and minor GSLs in HGIB and LGIB genotypes inoculated with *Xcc*. Values are the average of 30 replicates ± standard error. Means with different letters are significantly different at $\alpha \leq 0.05$ according to Fisher's protected LSD test. SIN (sinigrin, 2-propenyl), GIB (glucoiberin, 3-methylsulphinylpropyl), GBS (3-indolylmethyl, glucobrassicin), OHGBS (hidroxyglucobrassicin, 4-hydroxy-3-indolylmethyl), NEOGBS (neoglucobrassicin, 1-methoxy-3-indolylmethyl), MEGBS (methoxyglucobrassicin, 4-methoxy-3-indolylmethyl).

**Figure 4.** The effect of high (HGBS) and low (LGBS) GBS content in a selection of kale genotypes on (A) lesion area after inoculation with *Sclerotinia sclerotiorum* (*Ss*) or *Xanthomonas campestris pv. campestris* (*Xcc*), in two trials (T1 and T2), (B) content of SIN, GIB, GBS and minor GSLs in the HGBS and LGBS genotypes inoculated with *Ss*, and (C) content of SIN, GIB, GBS and minor GSLs in HGBS and LGBS genotypes inoculated with *Xcc*. Values are the average of 30 replicates ± standard error. Means with different letters are significantly different at $\alpha \leq 0.05$ according to Fisher's protected LSD test. SIN (sinigrin, 2-propenyl), GIB (glucoiberin, 3-methylsulphinylpropyl), GBS (3-indolylmethyl, glucobrassicin), OHGBS (hidroxyglucobrassicin, 4-hydroxy-3-indolylmethyl), NEOGBS (neoglucobrassicin, 1-

methoxy-3-indolylmethyl), MEGBS (methoxyglucobrassicin, 4-methoxy-3-indolylmethyl).

**Figure 5**. The glucosinolate content of three divergent selections of kale, each of which were selected for high (HSIN, HGIB and HGBS) or low (LSIN, LGIB and LGBS) glucosinolate content, and the lesion area on each selection after inoculation with *Sclerotinia sclerotiorum* (A), or *Xanthomonas campestris pv. campestris*, (B) in two trials (T1 and T2). Values are the average of 30 replicates ± standard error. Lesion means with different letters are significantly different at $\alpha \leq 0.05$ according to Fisher's protected LSD test. SIN (sinigrin, 2-propenyl), GIB (glucoiberin, 3-methylsulphinylpropyl), GBS (3-indolylmethyl, glucobrassicin), OHGBS (hidroxyglucobrassicin, 4-hydroxy-3-indolylmethyl), NEOGBS (neoglucobrassicin, 1-methoxy-3-indolylmethyl), MEGBS (methoxyglucobrassicin, 4-methoxy-3-indolylmethyl).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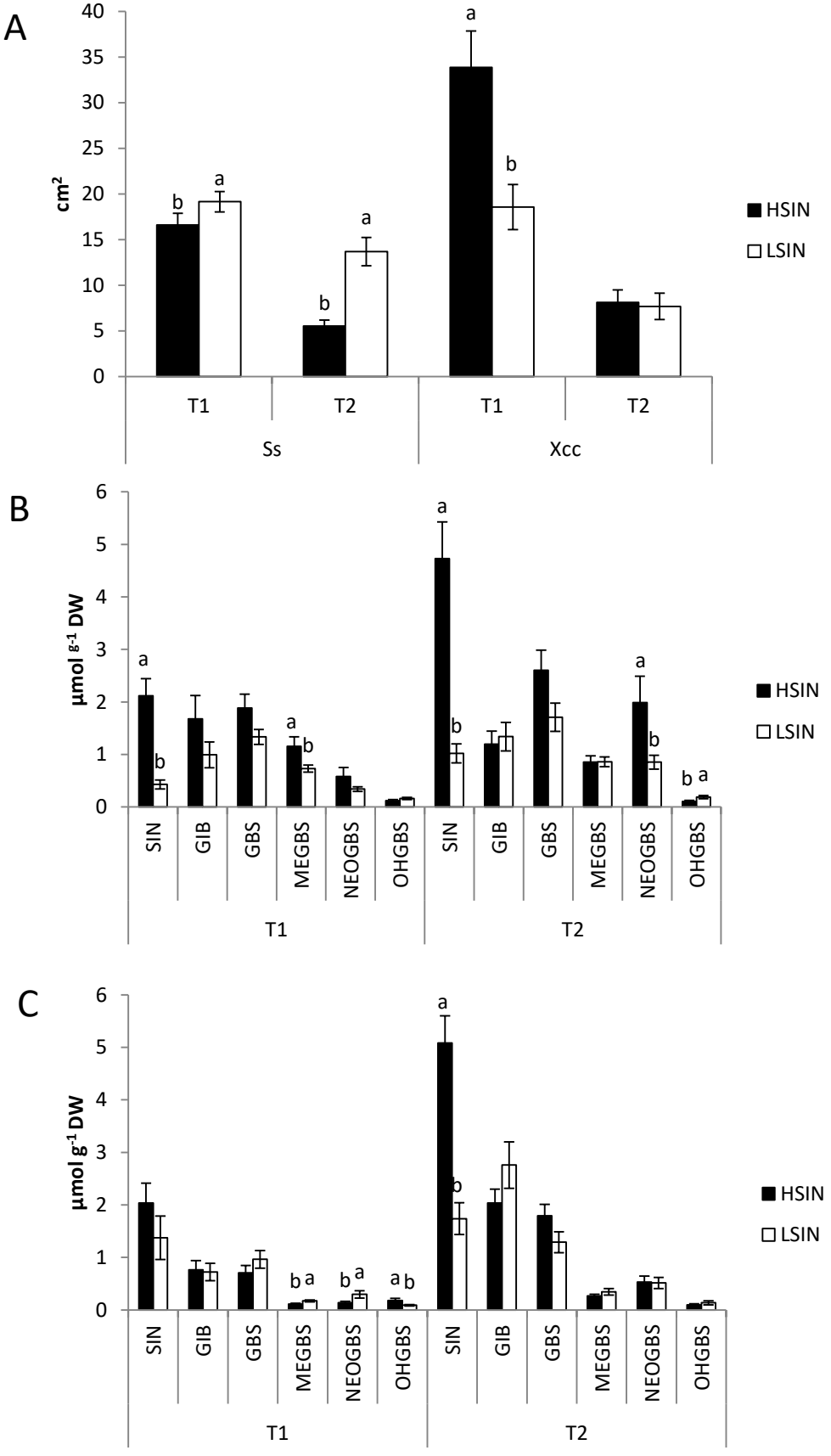
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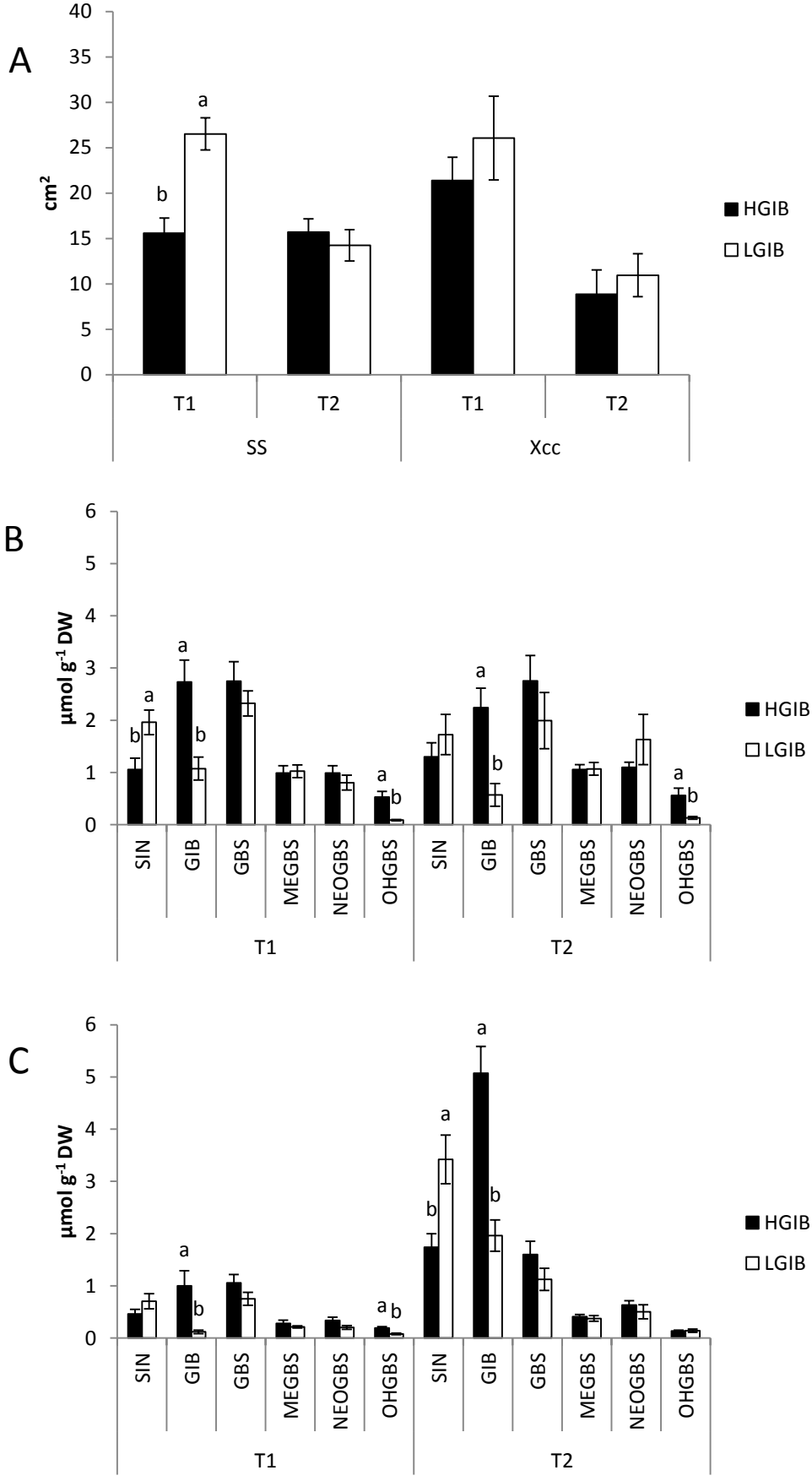
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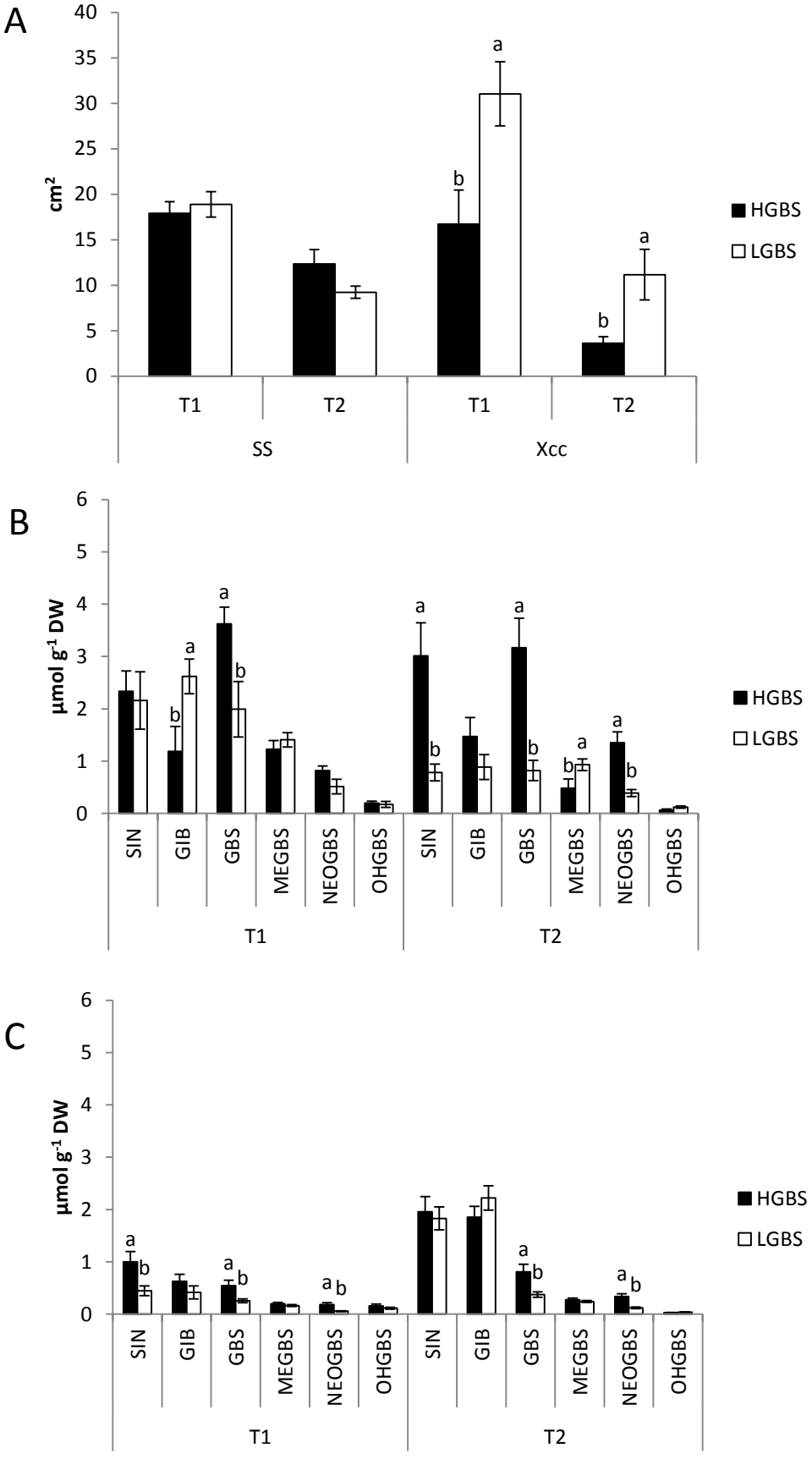
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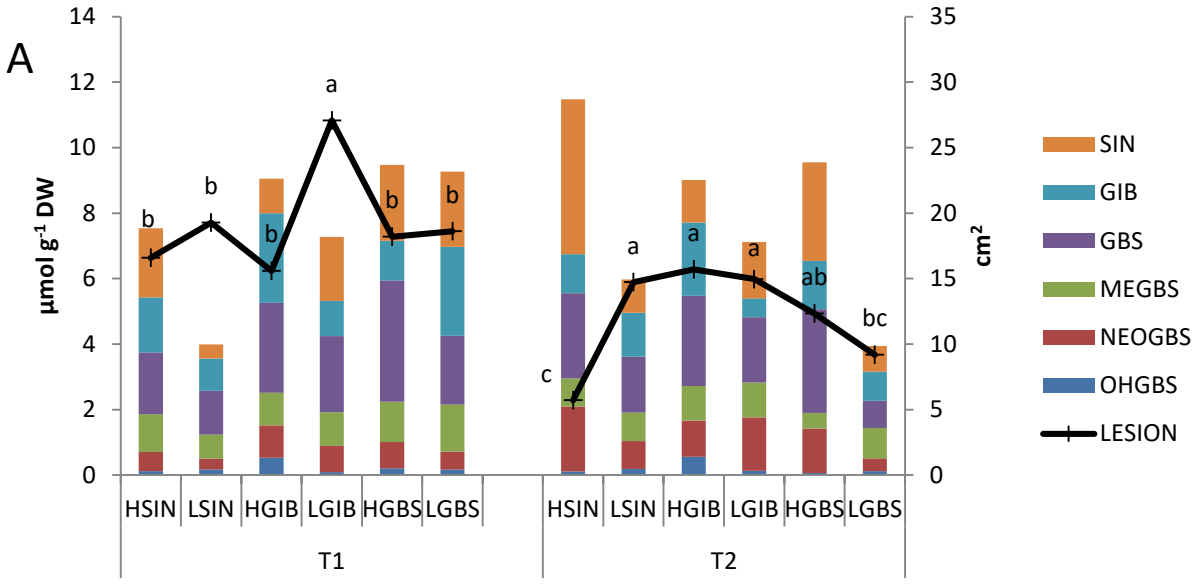
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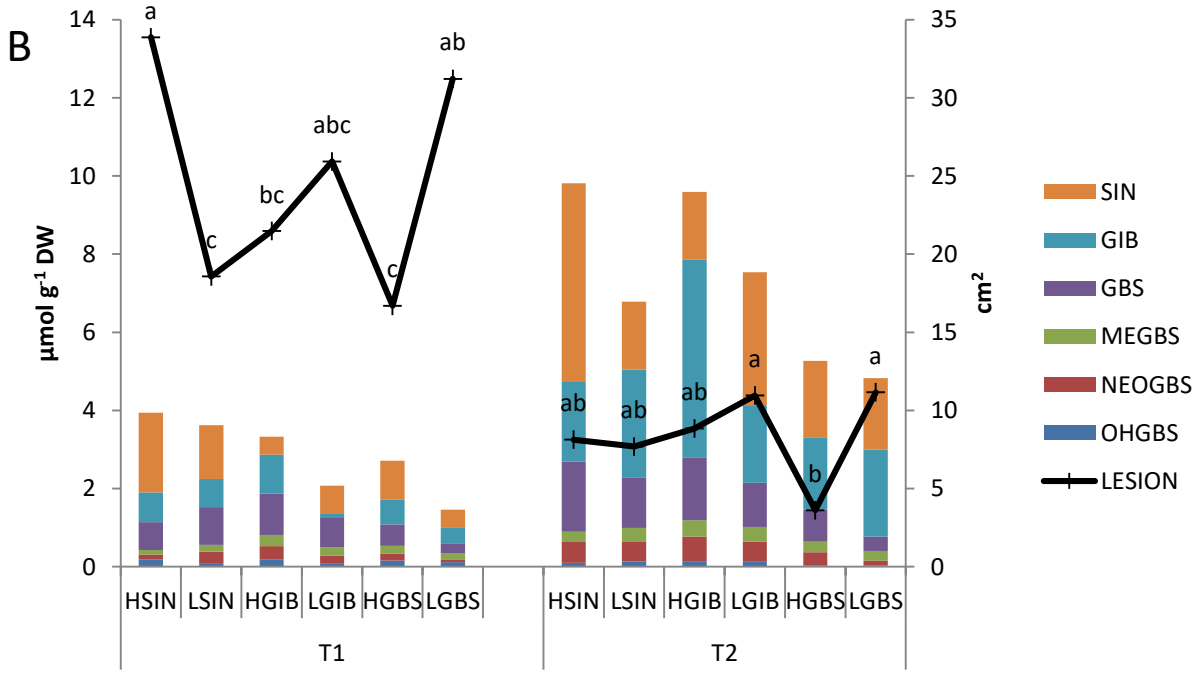
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