

Inhibitory effect of the glucosinolate–myrosinase system on *Phytophthora cinnamomi* and *Pythium spiculum*

FRANCISCO T. ARROYO^{1*}, ROCÍO RODRÍGUEZ ARCOS², ANA JIMÉNEZ ARAUJO²,
RAFAEL GUILLÉN BEJARANO², MARÍA JOSÉ BASALLOTE¹, CARMEN BARRAU¹

¹IFAPA Centro Las Torres-Tomejil, Alcalá del Río (Sevilla), Spain; ²Departamento de Fitoquímica de los Alimentos, Instituto de la Grasa, CSIC, Sevilla, Spain

*Corresponding author: franciscot.arroyo@juntadeandalucia.es

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Abstract: Glucosinolate extracts from sprouts of common *Brassica nigra*, *B. juncea* cv. Scala, *B. carinata* cv. Eleven, and *Sinapis alba* cv. Ludique were analysed by reversed phase high-performance liquid chromatography-diode array detection-mass spectrometry. The effect of the glucosinolate–myrosinase system on *in vitro* mycelial growth of *Phytophthora cinnamomi* Rands and *Pythium spiculum* B. Paul was assessed. Likewise, sinigrin and sinalbin monohydrate commercial standards were also tested. The extracts from *B. carinata*, which contained 159 mmol/g plant DW equivalent (85% sinigrin, 5% gluconapin, and 3% glucotropaeolin), were the most effective against *Phytophthora* and *Pythium* isolates used in this study. However, the extract from *S. alba*, which contained 1 180 mmol/g (100% sinalbin), did not inhibit the mycelial growth of the isolates tested. The use of the glucosinolate-myrosinase system provides important additional information to advance in the implementation of field application of brassicaceous amendments for the control of soil-borne pathogens.

Keywords: isothiocyanates; plant defence; *Brassica*; *Phytophthora* spp.; *Pythium* spp.

Phytophthora and *Pythium* are two plant damaging oomycetes (water moulds) affecting a large number of hosts worldwide, including woody plants (UTKHEDE *et al.* 1991; LOWE *et al.* 2000). The species *Phytophthora cinnamomi* Rands and *Pythium spiculum* B. Paul have been pointed out as main agents of the *Quercus* decline in southern Spain and Portugal (BRASIER *et al.* 1993; TUSET *et al.* 1996; SÁNCHEZ *et al.* 2002; RODRÍGUEZ-MOLINA *et al.* 2003). Symptoms of this oak disease appear as defoliation and eventually death of the trees affecting important areas of oak forests, which represents a serious concern for owners and authorities (SERRANO *et al.* 2012; RÍOS *et al.* 2016a). The natural character of these rangeland forests, which are included as type habitats protected within the Directive on Habitats (Annex I, Council Directive 92/43/EEC) of the European Union, means that control

methods must be respectful and environmentally friendly. In this way, the biofumigation through *Brassica* residues appears to be a potential tool for the oak disease management in this ecosystem (RÍOS *et al.* 2016b). These plants are widely used as cover crops for biofumigation and this is due to a high level of glucosinolates, which could be hydrolysed by the enzyme myrosinase to yield volatile compounds, mainly isothiocyanates that possess a potent biocidal activity. In fact, the use of *Brassica* amendments into soils represents a sustainable alternative to chemical control of soil-borne pathogens (CHAN & CLOSE 1987; KIRKEGAARD & SARWAR 1998; LAZZERI & MANICI 2001; ZURERA *et al.* 2009; KRASNOV & HAUSBECK 2015).

These secondary metabolites have no biological toxicity but their hydrolysis products called isothio-

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cyanates are produced in a reaction catalysed by thioglucosidases or myrosinases. The glucosinolate–myrosinase system constitutes a part of the plant defence against pathogens and insects and it has been suggested that this system evolved from the more prevalent system of cyanogenic glucosides and corresponding *O*- β -glucosidases (RASK *et al.* 2000).

The effectiveness of disease suppression by amendments depends on the *Brassica* species as well as on the developmental stage of plants (CHAN & CLOSE 1987; BARRAU *et al.* 2009). Natural extracts of *Brassica* spp. and pure isothiocyanates have demonstrated an *in vitro* suppressive effect on several fungal pathogens like *Fusarium* spp. (MAYTON *et al.* 1996), *Monilinia laxa* (MARI *et al.* 2008), *Botrytis cinerea* (UGOLINI *et al.* 2014), *Sclerotinia sclerotiorum* (KURT *et al.* 2011), *Rhizoctonia* spp. (MAZZOLA *et al.* 2001), *Arpergillus parasiticus*, and *Penicillium expansum* (WU *et al.* 2011; MANYES *et al.* 2015). Similarly, *Phytophthora* spp. and *Pythium* have shown the inhibition of mycelial growth and spore production by volatiles released from Brassicaceae plants (SARWAR *et al.* 1998; LAZZERI & MANICI 2001; ZURERA *et al.* 2009; MORALES-RODRÍGUEZ *et al.* 2012). However, diverse responses of different pathogens exposed to the same *Brassica* compounds have been found. In addition, the extracts of these plants show a distinct inhibitory activity depending on the glucosinolate profile.

The aim of this study was to identify and characterize the glucosinolate extracts from Brassicaceae sprouts, as well as to assess the antimicrobial activity of the glucosinolate–myrosinase system against soil pathogens responsible for *Quercus* decline, in order to search safer and environmentally friendly control strategies.

MATERIAL AND METHODS

Plant material. Four Brassicaceae species were selected and used for glucosinolate analysis and *in vitro* antifungal activity assay because of a high content of glucosinolates in these plants. Seeds of common *Brassica nigra*, *B. juncea* cv. Scala, *B. carinata* cv. Eleven, and *Sinapis alba* cv. Ludique, which were obtained commercially, were sown in plastic containers (1.362 ml) filled with vermiculite, and they were maintained in a growth chamber under light and saline stress with a 60 ppm SO_4K_2 solution and 20 h photoperiod at 20–25°C light/dark, respectively. The salt stress has been proved to significantly increase

the glucosinolate content and inhibit the myrosinase activity in radish sprouts (YUAN *et al.* 2010). After 10 days, sprouts of each species were harvested for the glucosinolate assay. Three containers were used for each species and assay.

Chemicals and reagents. Glucanapin, glucotropaeolin and progoitrin were purchased from Chromadex Chemical (Barcelona, Spain). Sinigrin, sinalbin, formic acid and acetonitrile, HPLC grade, were purchased from Sigma Chemical (St. Louis, USA). Pure deionised water was obtained from a Milli-Q 50 system (Millipore, Bedford, USA).

Glucosinolate extraction. Prior to performing glucosinolate extraction and characterisation, the influence of different parameters (solvent type, solvent/solid ratio, simple or sequential extraction, microwave power, temperature, and extraction time) on glucosinolate extraction efficiency was studied.

The optimum conditions of extraction were set as follows: 5 g of sprouts and 45 ml of water were placed in a 500-ml beaker and then extracted in a microwave oven for 2 min, at 250 W. After cooling, the mixture was homogenised in a VDI 12 homogeniser (VWR International, Barcelona, Spain) for 1 min, and then centrifuged at 2 500 g. The residue was washed with 10 ml ($\times 2$) of water and extracted in the same conditions.

The supernatants containing glucosinolates were concentrated under vacuum, and then lyophilised. The glucosinolate extracts were stored at –20°C until analysis by HPLC. Three replicates were used for glucosinolate extraction.

Analysis and quantification of glucosinolates by HPLC-DAD. Glucosinolates were analysed by reversed phase high-performance liquid chromatography–diode array detection–mass spectrometry (HPLC-DAD-MS). Analyses of glucosinolates were carried out using a Jasco-LC-Net II ADC liquid chromatograph system equipped with a diode array detector (DAD). Individual compounds were separated using a MEDITERRANEA SEA₁₈ reverse-phase analytical column (25 cm length \times 4.6 mm i.d., 5 mm particle size; Teknokroma, Barcelona, Spain). An elution gradient was used with solvents A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid): 0–7 min, 100% A; 7–12 min, linear gradient to 40% B; 12–20 min, linear gradient to 80% B and 20–30 min, linear gradient to 100% B; then maintained at 100% B for 5 min and finally returned to the initial conditions over the next 5 minutes. The flow rate was 0.8 ml/min and

the column temperature was 30°C. Spectra from all peaks were recorded in the 200–600 nm range and chromatograms were acquired at 235 nm. Calibration curves were established on seven data points that covered a concentration range of 25–500 µg/ml for each glucosinolate. Seven concentrations of the mixed standards water solution were injected in triplicate. The quantities of individual compounds were calculated according to concentration curves constructed with authentic standards.

Fungal culture. *Phytophthora cinnamomi* MYC032 and MYC011 isolates were obtained from symptomatic oak trees grown in an oak forest from the province of Badajoz (Extremadura, Spain) and provided by CICITEX (Centro de Investigaciones Científicas y Tecnológicas de Extremadura, Extremadura, Spain), which were characterised by studying cultural and morphological characteristics on the PAR(PH)-V8 semi-selective medium and studied microscopically. Besides, *Phytophthora cinnamomi* PE90 isolate obtained from *Quercus ilex* in Huelva (Andalusia, Spain), GenBank accessions No. AY943301, and *Pythium spiculum* PA54 isolate obtained from *Quercus suber* in Huelva, GenBank accessions No. DQ196131, both provided by UCO (University of Córdoba, Spain) were used (DE VITA *et al.* 2013). The oomycetes were grown on potato dextrose agar (PDA) from Difco Laboratories (Detroit, MI) in darkness at 25°C for 7 days.

Glucosinolates and myrosinase enzyme. Commercial glucosinolates (GLs), sinigrin hydrate and sinalbin potassium salt were used for *in vitro* inhibition assays. Moreover, natural extracts obtained from *Brassica* plants described above were also used. Likewise, a thioglucosidase enzyme from *Sinapis alba* (white mustard) seed, ≥100 units/mg solid, which was purchased from Sigma-Aldrich was used in the present study. The samples were prepared by dissolving the GLs and the natural extracts in 0.1 M phosphate buffer at pH 7 with a myrosinase enzyme solution in 0.1 M phosphate buffer at pH 7, and additionally the myrosinase enzyme in 0.1 M phosphate buffer at pH 7 without GLs (LEONI *et al.* 1997; MANICI *et al.* 1997). Native glucosinolates were not used because they show no fungicidal activity but their hydrolysis products (SPAK *et al.* 1993; BROWN & MORRA 1996).

Antifungal activity assays/Inhibition of mycelial growth. A mycelial disk, 5 mm in diameter, was cut from cultures of *Phytophthora cinnamomi* and *Pythium spiculum* growing on PDA and transferred to a new petri plate containing PDA. For each isolate

and each substrate concentration from different plant species, four replications were prepared. A small glass dish, 2 cm in diameter, was placed inside a Petri plate together with filter paper, which was moistened with 5 ml of sterile distilled water. After this and firstly, 0.5 ml of the natural extracts from *B. nigra*, *B. juncea*, *B. carinata*, and *S. alba* at 0.5, 1.5, 3, and 6 mg/ml was added on a small glass dish. Subsequently, 0.5 ml of the enzyme myrosinase was added, adjusted to 1 mg of glucosinolate per 0.16 units of the enzyme, and finally the mixture was homogenized (LEONI *et al.* 1997). Also, pure sinigrin and sinalbin samples were prepared in the same manner as natural extracts and at the concentrations used for these natural compounds. Finally, the Petri plates were quickly sealed with plastic laboratory film (Parafilm PM-996; Bemis, USA), and incubated in darkness in a growth chamber at 25°C. Control treatment consisted of petri dish with the mycelial disk and 1 ml of 0.1 M phosphate buffer at pH 7. The radial colony growth was measured daily for 10 days. This experiment was conducted twice.

Data analysis. Analysis of variance ANOVA (Statistix 8, Analytical Software for Windows) was performed for colony diameter and to the least significant difference *LSD test*, at $P < 0.05$. Also, data are shown as percent of inhibition with respect to the control.

RESULTS

Characterization and quantification of glucosinolates from Brassicaceae species. Extraction of glucosinolates was done by a microwave-assisted extraction (MAE) method, which allowed the complete recovery of intact glucosinolates from plant tissues, while its effectiveness was comparable with that of traditional methods based on the use of organic solvents, but with the additional advantages of being easier to apply, non-polluting and inexpensive.

A typical chromatogram of glucosinolates from *Brassica* species aqueous extracts is presented in Figure 1. The analytical method allowed the separation of up to five distinct aliphatic glucosinolates, sinigrin being the major compound, in the three *Brassica* samples analysed.

This was the only glucosinolate detected in *B. nigra* (Figure 1A). In *B. juncea*, sinigrin was the most abundant compound, representing about 90–95% of the total glucosinolate complement, but it was

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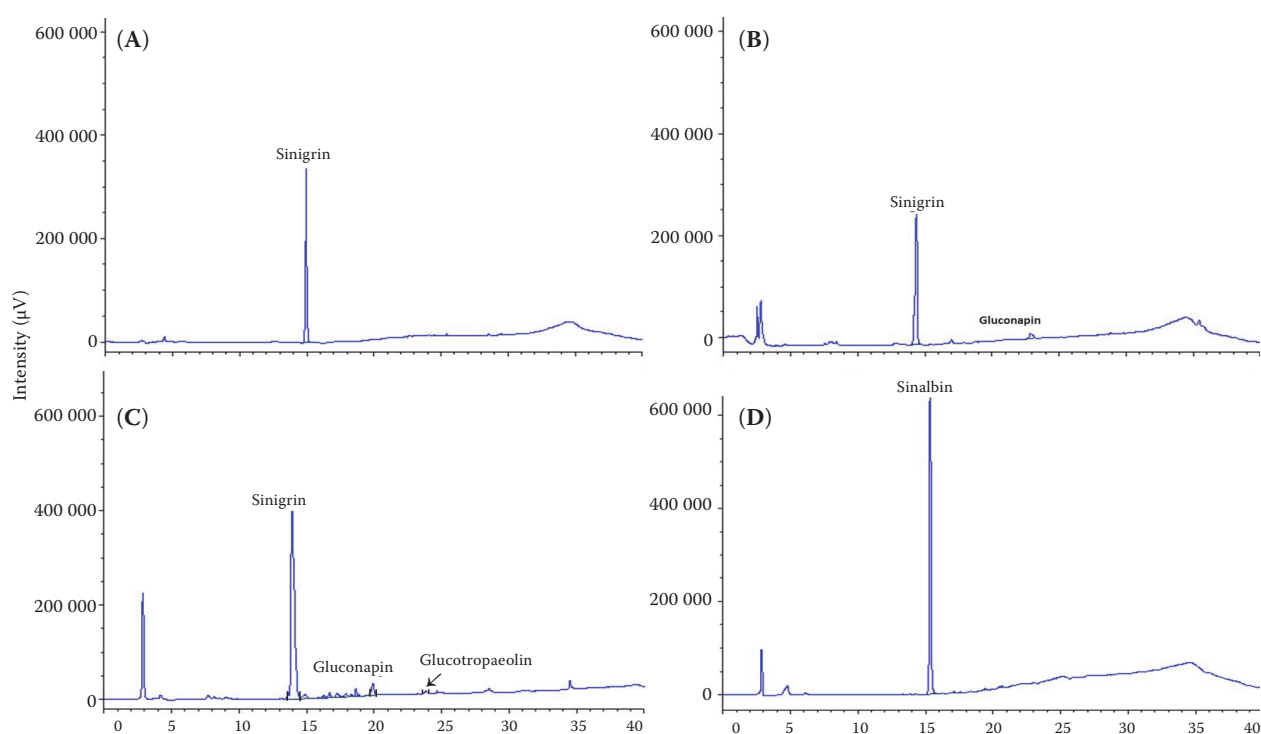


Figure 1. HPLC profile of glucosinolates from common *Brassica nigra* (A), *B. juncea* cv. Scala (B), *B. carinata* cv. Eleven (C), and *Sinapis alba* cv. Ludique (D) sprouts

Glucosinolates were isolated by microwave-assisted extraction method. Individual compounds were measured at 229 nm and eluted with a gradient that combined water and acetonitrile

accompanied by minor quantities of gluconapin (Figure 1B). Finally, *B. carinata* presented a lower content of total glucosinolates, but a greater variety of compounds, since, in addition to sinigrin (over 80%), it was shown to contain significant amounts of gluconapin and glucotropaeolin (Figure 1C). Our data, for the four investigated species in the present work, are in consonance with those reported by KUMAR and ANDY (2012), who reviewed the bioactive compound profiles from *Brassica*. The glucosinolate profiles of *B. nigra* and *B. juncea* reported by SMALLEGANGE *et al.* (2007) and MALABED *et al.* (2014), respectively, were also similar to those described in the present manuscript.

The chromatogram from the aqueous extract of *S. alba* was very similar to that obtained from *B. nigra*, but in this case the aromatic glucosinolate sinalbin was the only compound detected, as can be observed in Figure 1D.

Regarding the content and composition of glucosinolates (Table 1), the results of our analysis revealed that the natural extracts from *B. nigra*, *B. juncea*, and *B. carinata* contained 344 $\mu\text{mol/g}$ plant DW equivalent of glucosinolates (100% sinigrin), 288 $\mu\text{mol/g}$ plant DW equivalent (92% sinigrin and 8% gluconapin), and 159 $\mu\text{mol/g}$ plant DW equivalent (85% sinigrin, 5% gluconapin, 3% glucotropaeolin, and 7% other compounds that possess absorption spectra

Table 1. Total and individual glucosinolate content ($\mu\text{mol/g}$ dry weight) in sprouts of common *Brassica nigra*, *B. juncea* cv. Scala, *B. carinata* cv. Eleven, and *Sinapis alba* cv. Ludique. Sinigrin (SIN), gluconapin (GN), glucotropaeolin (GTR), sinalbin (SIB)

	Total GLs	SIN	GNA	GTR	Others	SIB
<i>B. nigra</i>	344.16 \pm 1.68	344.16 \pm 1.68	–	–	–	–
<i>B. juncea</i>	287.49 \pm 2.92	264.97 \pm 2.17	23.04 \pm 0.32	–	–	–
<i>B. carinata</i>	159.08 \pm 2.82	135.15 \pm 2.39	7.95 \pm 0.20	4.77 \pm 0.39	11.13 \pm 1.06	–
<i>S. alba</i>	1180.55 \pm 53.32	–	–	–	–	1180.55 \pm 53.32

Table 2. Maximum radial growth (mm) of three isolates of *Phytophthora cinnamomi* (Pc MYC 032, Pc MYC011, and Pc PE90) and *Pythium spiculum* (Py PA54) at different concentrations of pure sinigrin (SIN) and sinalbin (SIB) after 10 days

	Dose of glucosinolate (mg/ml)						
	0	1.5		3		6	
	(Control)	SIN	SIB	SIN	SIB	–	SIB
Pc MYC032	80 ± 0	80 ± 0	80 ± 0	0	80 ± 0	–	80 ± 0
PC MYC011	80 ± 0	80 ± 0	80 ± 0	0	80 ± 0	–	80 ± 0
Pc PE90	80 ± 0	80 ± 0	80 ± 0	0	80 ± 0	–	80 ± 0
Py PA54	80 ± 0	80 ± 0	80 ± 0	0	80 ± 0	–	80 ± 0

Each value is the mean of four replicates; each replicate is the mean of two samples from one colony; glucosinolate concentration and myrosinase activity were adjusted to 1 mg of glucosinolate per 0.16 units of the enzyme

characteristics of glucosinolates), while the natural extracts from *S. alba*, contained 1180 µmol/g plant DW equivalent of glucosinolates (100% sinalbin). However, the former extracts were very effective against the oomycetes, showing up to 100% inhibition of mycelial growth, whereas the extracts from *S. alba* did not show any inhibitory activity, as it is explained in the next paragraph.

Antifungal activity of the glucosinolate–myrosinase system. Colony diameters of the isolates of *Phytophthora cinnamomi* and *Pythium spiculum* growing on PDA and exposed to different doses of glucosinolates and myrosinase enzyme from commercial products and Brassicaceae species natural extracts were measured daily and at day ten they were compared to determine the growth activity inhibition. Pure sinigrin and sinalbin showed differences in their effectiveness to prevent the *in vitro* growth of selected oomycetes when sinigrin was more effective than sinalbin, as it totally inhibited the radial growth of all isolates used at 3 mg/ml (Table 2). However, pure sinalbin did not even inhibit the growth at a higher dose of 6 mg/ml.

Regarding the natural extracts from Brassicaceae species, they also showed differences in their in-

hibitory activity of *in vitro* growth of the isolates. The natural extracts from *Brassica carinata*, which contained sinigrin (85%), gluconapin (5%), gluco-tropaeolin (3%), and other compounds that possess absorption spectra characteristics of glucosinolates (7%), were the most effective, completely inhibiting the *in vitro* growth of *Phytophthora* isolates at 0.5 mg/ml and of *Pythium spiculum* at 3 mg/ml. The natural extracts from *B. juncea* and *B. nigra*, which contained sinigrin (100%), and sinigrin (92%) and gluconapin (8%), respectively, inhibited 100% of the *in vitro* growth of both MYC 032 and MYC011 *Phytophthora* isolates, and 96.72 and 93.1% of PE90 *Phytophthora* isolate and Py PA54 *Pythium* isolate, respectively, at a dose of 3 mg/ml (Table 3). However, the natural extracts from *Sinapis alba*, which contained only sinalbin as glucosinolates, did not inhibit the *in vitro* growth of any of the isolates at the highest dose of 6 mg/ml used in this trial. Results also revealed a different response of the isolates to the glucosinolate–myrosinase system, especially to the natural extracts, the Pc MYC032, Pc MYC011, and Pc PE90 *Phytophthora* isolates being the most susceptible to *B. carinata* extracts at the lowest con-

Table 3. Maximum radial growth (mm) of three isolates of *Phytophthora cinnamomi* (Pc MYC 032, Pc MYC011 and Pc PE90) and *Pythium spiculum* (Py PA54) at different concentrations of natural extracts from *Brassica nigra* + *B. juncea* (Bj+Bn), *B. carinata* (Bc) and *Sinapis alba* (Sa) after 10 days

	Dose of natural extracts (mg/ml)												
	0	0.5			1.5			3			6		
	(Control)	Bj+Bn	Bc	Sa	Bj+Bn	Bc	Sa	Bj+Bn	Bc	Sa	Bj+Bn	Bc	Sa
Pc MYC032	80 ± 0	80 ± 0	0	80 ± 0	80 ± 0	0	80 ± 0	0	0	80 ± 0	0	–	80 ± 0
PcMYC011	80 ± 0	80 ± 0	0	80 ± 0	80 ± 0	0	80 ± 0	0	0	80 ± 0	0	–	80 ± 0
Pc PE90	80 ± 0	80 ± 0	0	80 ± 0	80 ± 0	0	80 ± 0	7.6 ± 0.97	0	80 ± 0	0	–	80 ± 0

Each value is the mean of four replicates; each replicate is the mean of two samples from one colony; glucosinolate concentration and myrosinase activity were adjusted to 1 mg of glucosinolate per 0.16 units of the enzyme

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centration used, 0.5 mg/ml of these natural extracts. The Py PA54 *Pythium* isolate was also more susceptible to *B. carinata* extracts than the other natural extracts but it was completely inhibited at 3 mg/ml. Furthermore, some differences were observed when they were exposed to *B. juncea* + *B. nigra* extracts, when the Pc PE90 *Phytophthora* and Py PA54 *Pythium* isolates were not inhibited completely at 3 mg/ml but they needed 6 mg/ml for full inhibition, whereas the MYC 032 and MYC011 *Phytophthora* isolates were inhibited completely (Table 3).

Results revealed that isothiocyanates released from the glucosinolate–myrosinase system from both commercial sources and natural extracts had a fungitoxic effect. After 10 days of exposure, the discs that had not grown were removed and replaced in a new Petri dish containing only PDA but they did not show any growth after 7 days of incubation.

DISCUSSION

Cruciferous plants are among the most important cultivated vegetables and they have several potential uses, including plant food and biofumigation. Among several phytochemicals they contain, GSLs are of special relevance since they are mainly responsible for the strong antimicrobial activity present in plant tissues from *Brassica* plants (KIRKEGAARD & SARWAR 1998).

The structure of glucosinolates consists of a β -D-glucose moiety linked to a sulphated thiohydroximate. The moiety linked to thiohydroximate or side chain varies, resulting in about 130 different types of glucosinolates that can be classified into aliphatic, aromatic or indolyl ones (FABRE *et al.* 2007). Results from this study have revealed that glucosinolate profiles from the four investigated plants are distinct and seem to be phylogenetically determined, as proposed by ANGUS *et al.* (1994). Hence, the three analysed *Brassica* species contain the aliphatic GSL called sinigrin (allyl glucosinolate) as the only or major GSL, accompanied by minor quantities of other glucosinolic acid derivatives. However, sinalbin (*p*-hydroxybenzyl glucosinolate) was the only glucosinolate detected in *S. alba*.

It is well established that GSLs are not the biocidal active forms, but the volatile compounds released by the action of the myrosinase enzyme on intact glucosinolates (MANICI *et al.* 1997). Among the hydrolysis products of the glucosinolates the allyl

isothiocyanate (AITC) has proven to be the most effective against a varied range of pathogens including fungi, bacteria, and nematodes (MAYTON *et al.* 1996; SARWAR *et al.* 1998; LAZZERI *et al.* 2004); and it has even been suggested to breed *Brassica* spp. genotypes with very high allyl isothiocyanate concentrations in order to optimise biological control (MAYTON *et al.* 1996). Our results agree with those studies and show that the derivative of the hydrolysis of sinigrin, allyl isothiocyanate behaved as the most effective to inhibit the mycelial growth of the *Phytophthora cinnamomi* and *Pythium spiculum* oomycetes. On the other hand, the derivative of the hydrolysis of sinalbin, benzyl isothiocyanate, which was detected as the only glucosinolate in *S. alba*, did not show any antifungal activity at the highest dose tested.

The inhibition of mycelial growth as a result of exposure to macerated tissues of *Brassica* species or to pure chemical isothiocyanates has been observed in different species of fungi (MAYTON *et al.* 1996; SMOLINSKA *et al.* 2003; MORALES-RODRÍGUEZ *et al.* 2012). In this work, the inhibitory activity of the glucosinolate–myrosinase system is emphasised by using pure commercial products and natural extracts obtained from different *Brassica* species, and it might contribute to obtain more details about the inhibitory ability of each of the different types of volatiles released from the glucosinolate–myrosinase system. Similar *in vitro* assays of the antifungal activity of glucosinolates and their myrosinase-derived products have been performed by MARTIN and HIGUERA (2016), which were extracted from mashua, an American native tuber crop belonging to the order *Brassicales*. These authors confirmed the influence of glucosinolate content on the effectiveness of inhibitory activity. The correlation between the allyl or propenyl ITC and the inhibition of mycelial growth and spore germination have been reported for several pathogens (OLIVIER *et al.* 1999; SMOLINSKA *et al.* 2003; WANG *et al.* 2010; UGOLINI *et al.* 2014; MANYES *et al.* 2015). However, pathogens exhibit a different susceptibility to the various isothiocyanates produced by Brassicaceae plants depending mainly on the taxonomic class of fungi and it is related to the composition and structure of the membrane (SARWAR *et al.* 1998). *Alternaria alternata*, an important fungus causing post-harvest diseases in tomato fruits, has also shown a high susceptibility to the benzyl isothiocyanate, yielded from sinalbin (TRONCOSO-ROJAS *et al.* 2005). Other authors have reported a powerful inhibitory effect of the ethyl ITC

on *Penicillium expansum* (WU *et al.* 2011), of the butenyl ITC on *Monilinia laxa* (MARI *et al.* 2008). Also, MORALES-RODRÍGUEZ *et al.* (2012) reported different susceptibility of *Phytophthora nicotinae* to compounds released from Brassicaceae species. Furthermore, WILSON *et al.* (2013) confirmed that the structure of ITC is a clue to its activity against food-borne and spoilage bacteria. The structural differences in the isothiocyanates cause different antimicrobial effects, so their biological activity is a function not only of the concentration of glucosinolates contained in a determined Brassicaceae species or cultivar but also of the chemical properties (LAZZERI *et al.* 1993; SARWAR *et al.* 1998). Variability is a general characteristic of the glucosinolate–myrosinase system and it occurs at a different level including biosynthesis, regulation and breakdown playing a resistance role in an ecological and evolutionary context (KLIEBENSTEIN *et al.* 2005).

Likewise, the natural extract of *Brassica carinata* cv. Eleven, which contained sinigrin (85%), gluconapin (5%), glucotropaeolin (3%) and other compounds that possess absorption spectra characteristics of glucosinolates (7%), was the most effective in inhibiting *Phytophthora cinnamomi* isolates and *Pythium spiculum* isolate. However, the natural extracts of *B. nigra* and *B. juncea*, which possessed a high content of sinigrin, more than 95% of the GLs, were less effective than the natural extracts of *B. carinata*. Despite the fact that a relationship between the inhibition of *Phytophthora cinnamomi* pathogens and sinigrin content in biofumigant plants has been established (MORALES-RODRÍGUEZ *et al.* 2012; RÍOS *et al.* 2016b), other volatile compounds must also exert an inhibitory effect on fungal growth. In this work, although the extracts from *B. carinata* had a high sinigrin content but lower than *B. nigra* and *B. juncea*, they were the most effective against all isolates used. The higher effectiveness of *B. carinata* extracts may be due to their specific glucosinolate profile which comprises a greater variety of these bioactive compounds than the other Brassicaceae plants investigated. Synergistic activity among the distinct glucosinolates could be related to the higher antifungal activity found in *B. carinata* extracts. Similar findings were reported by RÍOS *et al.* (2016a) with amendments of *B. carinata*, which was demonstrated to possess the most effective inhibitory activity against the mycelial growth and sporangia production of *Phytophthora cinnamomi*. According to SMOLINSKA *et al.* (2003) and ŠPAK *et al.* (1993) the

variety of volatile inhibitory compounds may express additive or synergistic effects on the organisms. This effect was also observed by ANGUS *et al.* (1994) in isothiocyanates released from canola and Indian mustard roots on *Gaeumannomyces graminis*. Nevertheless, a great majority of studies on the antimicrobial activity of secondary metabolites from Brassicaceae species have been done by using separately pure isothiocyanates or extracts of glucosinolates and myrosinase enzyme and have proved that the allyl isothiocyanate is a potent inhibitor; this paper shows the possible synergistic action of other isothiocyanates, such as benzyl isothiocyanate. In fact, MANICI *et al.* (1997) demonstrated that the benzyl ITC, yielded from glucotropaeolin, was one of the most active compounds inhibiting various plant pathogenic fungi. Also, MARTIN and HIGUERA (2016) found that this aliphatic isothiocyanate was the most potent inhibitor of the mycelial growth of *Phytophthora infestans*. In this work the glucotropaeolin was not found in *B. nigra* + *B. juncea* extracts but it was detected in *B. carinata* extracts, which also contained other compounds with chemical properties close to those of glucosinolates that could possess some inhibitory action, making these extracts the most effective against the pathogens tested. However, the butenyl ITC, yielded from gluconapin or butenyl GL, which was present in both extracts of *B. nigra* + *B. juncea* and *B. carinata*, appeared not to have influenced the best inhibitory activity of *B. carinata* against *P. cinnamomi*. Meanwhile, the natural extract from *Sinapis alba* cv. Ludique, whose main and only glucosinolate identified was sinalbin (100% of the content), did not inhibit the *in vitro* growth of any of the isolates investigated in the present work.

Secondary metabolites of *Brassica* spp. have extensively demonstrated their inhibitory activity against a wide range of pathogens, from viruses through fungi and bacteria to nematodes. On the other hand, it is well established that the bioactive properties of glucosinolate compounds are dependent upon their structure and that minor differences in the substituents linked to the glucosinolate skeleton may lead to great differences in the bioactive properties of individual compounds. We have currently developed deeper studies on the isolation and structural characterisation of glucosinolates and derived isothiocyanates that will contribute to the knowledge of their specific action mechanism and different effectiveness against distinct plant pathogens. The breeding efforts should concentrate on the production

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of Brassicaceae species and cultivars with improved glucosinolate contents and compositions that allow selective controls of each specific pathogen. It is also interesting to design mixtures of different isothiocyanates whose synergistic effects lead to greater effectiveness in the biological control of plant diseases caused by pathogenic microorganisms.

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