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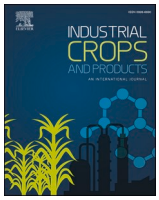

Glucosinolate-extracts from residues of conventional and organic cultivated broccoli leaves (*Brassica oleracea* var. *italica*) as potential industrially-scalable efficient biopesticides against fungi, oomycetes and plant parasitic nematodes

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ABSTRACT

This study aimed to standarize a protocol for obtaining a bioactive extract from broccoli (*Brassica oleracea* var. *italica*) crop residues, that is suitable for application on an industrial scale and effective in reducing plant disease incidence. For this purpose, the influence of several extraction factors in the glucosinolate (GSL) content was studied with leaves collected from two conventional broccoli fields and two organic broccoli fields. The analysis showed that lyophilization had no influence on the GSL content. Storage of plant material under two different temperatures (− 20ºC and − 80ºC) had no influence on the GSLs content of the extracts. Phytotoxicity of the extracts was studied with six different plant seeds, and also cytotoxicity was determined with human liver cells *in vitro*. The extracts were phytotoxic at dilutions above 10%, while cell toxicity was low. Extracts concentrations of 0.1%, 1% and 2% were tested *in vitro* against eight plant pathogenic fungi and two oomycetes in solid and in liquid media. The extracts reduced the growth of several plant pathogenic fungi at 2% dilution by up to 38.37% against *Alternaria alternata* and up to 46.55% against *Sclerotinia sclerotiorum*. When combined with myrosinase enzyme the effect of the extracts was enhanced, reaching inhibition values of 67.06% against *A. alternata* in solid medium and 68.52% against *Rhizoctonia solani* in liquid medium. In contrast, the same extracts increased the growth of the plant pathogenic oomycetes *Pythium ultimum* and *Phytophthora cactorum*. The effect of the same extracts in the free leaving larvae, J2s, of the plant-parasitic nematode (PPN) *Meloidogyne javanica* was not obvious. Minor significant differences were obtained but with no clear dose-response in nematode mortality, and no inhibition of eggs hatching was observed. These results show the industrial potential of using broccoli residues to obtain extracts with biopesticide activity against plant pathogenic fungi.

1. Introduction

Modern agriculture faces many challenges, of which biotic stresses are relevant. Plant pathogenic fungi, oomycetes, and plant-parasitic nematodes (PPNs) threaten agrosystems, compromising global food security (Fisher et al., 2020). Because of their genetically uniform populations and high densities, agrosystems foster the dissemination of pathogens, further aggravating the problem (McDonald and Stukenbrock, 2016). It is estimated that pathogens cause annual direct losses in agricultural productivity between 10% and 15%, equivalent to 220 billion US$ (Mitra, 2021). As for the type of plant pathogen, fungi are responsible for 10–35% losses in the four major human feeding crops: rice, wheat, maize and soybean (Godfray et al., 2016). The information about the economic impact of plant-pathogenic oomycetes is scarce and

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probably underestimated due to secondary infections, but the damage caused is calculated around 8–9 billion US$ (Bickel & Koehler, 2021). Estimating losses caused by PPNs is difficult as well, because of all the indirect damage and the difficulties to distinguish from unspecific aboveground symptoms. However, the annual yield loss varies from 80 to 173 billion US$ according to many estimations (Eugui et al., 2022). All this data poses the urgent need to reduce the impact of these pathogenic organisms in agriculture. It is also important to remark that the interaction between fungi, oomycetes and PPNs occupying the same ecological niche may cause even further damage to plants than the organisms alone, making more difficult to find efficient solutions (Zhang et al., 2020b). Many strategies and practices for controlling these pests are available, but those usually have associated limitations to consider, *i.e.*, chemical pesticides have been extensively used, but they were banned or their application limited, due to their potential toxic or harmful impact on human health and the environment (Singh et al., 2020; Phani et al., 2021). Some cultural practices, such as solarization, can affect soil biota, including non-target organisms (Ntalli et al., 2020); and using resistance crop varieties have fostered the emergence of new resistant strains (Fisher et al., 2020). In this context, it is imperative to develop new alternatives in controlling these plant pathogens, by obtaining new substances or practices that are both effective and safer than current options.

Brassica genus plants are cultivated worldwide and have great economic value (Poveda et al., 2020b). The primary use of these crops is as human and animal food sources, but they can also be important as bioremediation crops, green manure, soil conditioners, to obtain vegetable oils used as fuel or engine lubricants, and as sources of metabolites for different industries, as sanitary (Card et al., 2015). *Brassica* species contain health-promoting phytochemicals that have been studied in recent years, including glucosinolates (GSLs), phenolic compounds, carotenoids and vitamins (Šamec et al., 2017). In particular, broccoli (*Brassica oleracea* var. *italica*), is one the most studied *Brassica* species due to the presence of nutritional and health-promoting compounds (Huang et al., 2021). Among them, GSLs have proven to have many beneficial effects as antioxidants, anti-inflammatory, anti-carcinogenic, neuroprotectants and with anti-microbial effects (Li et al., 2021), which increased the general interest for this crop. This also enhanced considerably the value to this crop, reaching 35 million tons produced of broccoli and cauliflower worldwide, mainly in China, India, the United States, Spain, Mexico and Italy (FAO, 2020). In addition, organic farming boosted in the last decade (Rembiałkowska, 2016; Cámara-Martos et al., 2022), and broccoli is an important vegetable within that organic market due to its high demand (Renaud et al., 2014). The organic cultivation system differs from conventional cultivation as it avoids most synthetic chemical inputs, relying on biological and cultural measures and processes, such as crop rotation, biological control and overall preserving the biodiversity of the agro-system (Renaud et al., 2014).

Once harvested, broccoli heads (florets and sprouts) are selected for consumption, making non-selected plant parts by-products or residues such as discarded broccoli heads, stalks and leaves (approximately 60–75% of broccoli production) (Li et al., 2021). These plant residues are wasted during the whole transformation process and not only when harvesting, but also during processing, packing, dispatching, and retailing (Li et al., 2021). In this context, strategies to use these agronomic by-products such as green manure utilization or animal feeding should be encouraged. However, given the large amount of residues generated, new strategies are needed, and this work proposes its use for plant pathogen control which is in the spotlight of industrial interest as an environmentally safe strategy that is also compatible with integrated pest management.

The use of plant parts as botanical pesticides has been practiced for many centuries because of the suppressive effect observed in some of them, and nowadays many pesticides with botanical origin have been registered such as neem oil and pyrethrins (Acheuk et al., 2022). Plants possess a vast range of secondary metabolites suitable for controlling pests in agriculture, such as alkaloids, phenolics, pyrethrins, essential oils or fatty acids (Acheuk et al., 2022). Given the enormous amount of agricultural plant waste biomass generated every year, which may imply environmental problems if not properly managed, the potential of these residues as a source of bioactive compounds has been already remarked (Santana-Méridas et al., 2012). Despite the great effort done, there are still some challenges to face in order to consolidate botanical biopesticides in the market. The main one is posed by the regulation systems, which should be adapted to this type of pesticide; also the production, formulation and application technologies need to be further investigated and developed, in order to facilitate its successful integration (Kumar and Singh, 2015).

GSLs are thioglycosides formed by a sulfonated oxime group, a thioglucose group and an amino acid-derived side chain (Mitreiter and Gigolashvili, 2021). These compounds are hydrolyzed by myrosinase enzymes, also present in Brassicaceae tissues, and form glucosinolate hydrolysis-products (GHPs), such as isothiocyanates (ITCs), thiocyanates, nitriles, epithionitriles and/or oxazolidine-2-thiones (Wu et al., 2021). Many roles have been attributed to GSL, such as adaptation to environmental conditions (Poveda et al., 2021), signaling for flowering time (Jensen et al., 2015), stomatal closure (Hossain et al., 2013), water transport (Martínez-Ballesta et al., 2014), or auxin transport (Vik et al., 2018). However, one of the main roles of GSLs in plants is defense. Many studies have suggested that GSLs and GHPs trigger defense response mechanisms of plants against pathogens, such as the hypersensitive response or a priming effect (Evivie et al., 2019). The main mechanism however is the direct toxicity (Poveda et al., 2020a). ITCs and other GHPs decrease oxygen consumption rate, cause the intracellular accumulation of reactive oxygen species (ROS) and depolarize the mitochondrial membrane, which is effective against fungi and oomycetes (Calmes et al., 2015; Poveda et al., 2020a). GSLs and GHPs have also been described as nematicidal compounds, decreasing the population of PPNs in studies both *in vitro* and *in vivo* (Eugui et al., 2022). ITCs toxicity is the main mechanism involved, but also an increase of the competition promoting the populations of saprophytic nematodes have been related to PPN reduction (Roubtsova et al., 2007).

The most common GSL extraction method from plant material is described in ISO 9167–1 norm, which consists in 70% methanol heated at 75 °C for 10 mins (Doheny-Adams et al., 2017; Major et al., 2020), but this solvent is expensive and can be hazardous for the user (Ashurst & Nappe, 2022). A later revision of the ISO 9167 norm suggested replacing aqueous methanol by aqueous ethanol to lower toxicity (International Organization for Standardization, 2019), but each case should be validated individually (Clarke, 2010). Some authors also point out that cold methanol extraction is a safer method with similar extraction efficiencies than the boiling methanol (Major et al., 2020). Another commonly used GSL extraction is the boiling water method, with comparable extraction efficiency (Rangkadilok et al., 2002; Herzallah and Holley, 2012; Major et al., 2020) or even better efficiency than the boiling methanol method (Stoin et al., 2007).

The aim of this work is to study the influence of several factors on the GSL extraction efficiency from broccoli leaves, for an eventual industrial escalation process that may use *Brassica* crops residues as a circular economy strategy. Phytotoxicity and cytotoxicity of the broccoli extracts will also be analized as part of the health and environmental security approximation. The second part of this study consists in determining the antifungal, oomyceticide and nematicidal activities of such GSL extracts *in vitro*, in order to obtain an environmentally friendly biopesticide in an industrially scalable way.

2. Materials and methods

2.1. Plant material

Broccoli leaves were collected from different fields located in

Northern Spain (Ribaforada, Navarra). Two fields were grown under conventional conditions (CONV1 coord., 41°58'02.6"N, 1°32'28.0"W and CONV2 coord., 41°57'54.4"N, 1°32'19.9"W), fertilized with NPK 9–23–30 at 200 kg/ha before plantation, and then supplemented with two more fertilizations of ammonium nitrate 200 kg/ha. Two fields were grown under organic conditions (ORG1 coord., 41°59'45"N, 1°29'54"W and ORG2 coord., 41°59'40"N, 1°29'53"W), and fertilized with two tons/ha of the organic NPK 4–5–4 fertilizer Fercrisa Biosuelo (Crisara S. L., Almería, Spain) before plantation, and another two tons/ha one month after plantation.

Broccoli leaves located near the central inflorescence were collected in the harvest period, frozen in liquid nitrogen, crushed to powder, and stored under two different conditions. In order to evaluate the influence of the temperature storage of the material, half plant material collected from each field was stored at − 20ºC for 30 days and then stored at − 80ºC until use, and the other half was directly stored at − 80ºC until use.

2.2. Extraction of broccoli leaves

Broccoli leaf extracts were obtained through two different methods: cold methanol extraction or aqueous extraction.

The cold methanol extraction method was conducted adapting the procedures described by Velasco et al. (2021): 20 mg of lyophilized plant material or 94 mg of frozen plant material was mixed with 1 ml of 75:25 methanol:water (v/v) in a 1.5 ml eppendorf tube. The sample was vortexed for 1 min 30 s at room temperature, and then incubated in the dark for 60 min at room temperature with 250 rpm agitation. Sample was centrifuged at 3700 rpm for 12 min, and the supernatant was collected. Methanol was evaporated in a rotary evaporator and the sample was topped up to 1 ml with sterile deionized water and kept at − 80ºC until use.

For the aqueous extraction method, we followed the protocol used by Doheny-Adams et al. (2017): 5 ml of boiling water was added to 20 mg of lyophilized plant material or 94 mg of frozen plant material. Sample was heated at 100ºC for 10 min with magnetic stirring and then further heated at 70ºC for another 4 h, before centrifugation at 4000 rpm for 10 min. Supernatant was collected and topped up to 5 ml with sterile deionized water and kept at − 80ºC until use.

2.3. GSL analysis

Plant material GSL content was studied after extraction with the hot methanol extraction protocol described before (Poveda et al., 2021). As an internal standard, 20 μl of glucotropaeolin was added, and 400 μl of the extracts was injected into an ion-exchange column with Sephadex DEAE-A25 (Sigma-Aldrich, St. Louis, MO, USA). Purified sulphatase solution (Sigma-Aldrich, St. Louis, MO, USA) was added for the desulphation step, and once desulphation had occurred, 200 μl of ultrapure water and 200 μl of 70% methanol were added to each extract, and kept frozen until analysis.

Both the GSLs profiles of the plant material and the broccoli extracts obtained with the two previously described procedures and aimed for pathogen inhibition, were analyzed following the methodology described by Velasco et al. (2021). The quantification of GSLs was carried out with an Ultra High Performance Liquid Chromatograph UHPLC Nexera LC-30AD (Shimadzu Corporation, Kyoto, Japan) equipped with a Nexera SIL-30AC injector (Shimadzu, Kyoto, Japan) and a SPDM20A UV/VIS photodiode array detector (Shimadzu, Kyoto, Japan). An X Select ®HSS T3 UHPLC (2.5 μm particle size, 2.1 mm I.D., length 100 mm) column was used (Waters Corporation, Milford, MA, USA), with a VanGuard pre-column incorporated. The temperature of the oven was 35ºC, and GSLs were quantified at 229 nm, separated using the aqueous acetonitrile method described in Poveda et al. (2021) and identified by comparing retention times and UV spectra with the standards (Phytoplan Diehm & Neuberger GmbH, Heidelberg, Germany). Calibration equations for each GSL were determined with at least five points: glucoiberin (GIB) (y = 99397x; $R^2 = 0.950$), glucoraphanin (GRA) (y = 352910x, $R^2 = 0.999$), glucobrassicin (GBS) (y = 869483x; $R^2 = 0.988$), neoglucobrassicin (NEOGBS) (y = 342954x, $R^2 = 0.997$) and methoxyglucobrassicin (MEOGBS) (y = 398645x, $R^2 = 0.980$).

2.4. Phytotoxicity assays

The phytotoxicity of the extracts was assessed adapting the procedure described by Selim et al. (2012) with a total of six cultivated plant seeds: tomato (*Solanum lycopersicum*), lettuce (*Lactuca sativa*), maize (*Zea mays*), chard (*Beta vulgaris* subsp. *vulgaris*), radish (*Raphanus sativus*) and watercress (*Nasturtium officinale*). Seeds were surface sterilized with 1% sodium hypochlorite for two minutes with periodical agitation, and then washed two times with sterile distilled water. One ml of crude broccoli extract at 0.1%, 1%, 10% and 100% in sterile distilled water was applied to filter paper in a 9 mm diameter Petri dish, and then 5 seeds per plate (for maize and chard) or 10 seeds per plate (for tomato, lettuce, radish and watercress) were placed on the filter paper. Three Petri dishes were tested per condition, and the experiment was performed in triplicate. Petri dishes were then sealed with Parafilm and incubated in the darkness at 20ºC for 72 h in the case of maize, chard, radish and watercress, and 96 h in the case of tomato and lettuce.

The percentage of seed germination (% GER), percentage of root elongation (% ELO) and germination index (GI) were calculated using sterile distilled water as control, according to Zucconi et al. (1981) as follows:

$$\%GER = \frac{No.of\ \ seeds\ \ germinated\ \ in\ \ broccoli\ \ extract}{No.of\ \ seeds\ \ germinated\ \ in\ \ control\ \ treatment} x100$$

$$\%ELO = \frac{Mean\ \ root\ \ lenght\ \ in\ \ broccoli\ \ extract}{Mean\ \ root\ \ lenght\ \ in\ \ control\ \ treatment} x100$$

$$GI = \frac{\%GER\ \ x\ \ \%ELO}{100}$$

In addition to the seed experiments, another phytotoxicity *in planta* analysis was carried out with 3-weeks-old tomato plants in 365 ml pots with peat as substrate. Three weeks after germination, broccoli extract obtained from conventional broccoli stored at −80 °C and extracted with methanol (M4) was added at 1%, 2% and 5%, and repeated twice every 7 days. This extract was selected because it was the most toxic extract to this plant in seed experiments. Six plants per dose were selected and six more plants were left untreated as control. Fourteen days after the last treatment, the following measures were conducted: leaf number, total, shoot and root fresh weight.

2.5. Cytotoxicity

The HePG2 human cell line was obtained from the American Type Culture Collection (Manassas, VA). HePG2 cells were cultured as described previously Gomez-Torres et al. (2022) in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1% antibiotic (penicillin-streptomycin) and incubated in a humidified incubator at 37° and 5% CO_2. When cells reached about 80% confluency, they were treated with the selected broccoli extracts at 6%, 12.5%, 25% and 50% for 24 h.

The 3-(4,5-dimethyl-2-thiazolyl)− 2, 5-diphenyl-2 H-tetrazolium bromide (MTT) assay, was performed in order to evaluate cell viability. At the end of the experiments, the conditioned medium was removed and cells were washed with PBS and incubated with 0.5 mg/ml MTT dye (Sigma) in DMEM medium without phenol red, 30 min at 37ºC. Blue formazan crystals were solubilized by dimethylsulfoxide (DMSO) and colorimetric evaluation was performed using a spectrophotometer NanoDrop 2000 (BioTek) at 555 nm and 690 nm, this last one to subtract background.

2.6. Plant pathogens used

The pathogenic fungi and oomycetes used in this study were obtained from the Spanish Type Culture Collection (CECT) (Valencia, Spain) and the Regional Diagnostic Center of the Regional Government of Castilla y León (CRD) (Salamanca, Spain): *Alternaria alternata* (Card 41/37/2019 JCYL 965), *Botrytis cinerea* (CECT 20973), *Colletotrichum acutatum* (CECT 21009), *Fusarium oxysporum* f. sp. *lycopersici* (CECT 2715), *Penicillium expansum* (CECT 20906), *Pythium ultimum* (CECT 2364), *Phytophthora cactorum* (CRD Prosp. 59 JCYL 9), *Rhizoctonia solani* (CECT 2813), *Sclerotinia sclerotiorum* (CRD 345/202), and *Verticillium dahliae* (CECT 20246). Strains were routinely grown on potato-dextrose-agar (PDA, Sigma-Aldrich, Madrid, Spain).

For the reproduction of *Meloidogyne javanica* Treub (1885; identified by morphology, isozymes pattern and specific primers by PCR described in Robertson et al. (2009)), *Cucumis sativus* (L.) cv. Hoffmanns Giganta seeds (Buzzy Seeds, Catalog Number: 02186) were surface sterilized, grown *in vitro*, inoculated with larvae, J2s, and maintained as described in Díaz-Manzano et al. (2016).

2.7. GSL hydrolysis

The effect of the hydrolyzed broccoli extracts was studied as well. In this case, 5 µl of myrosinase enzyme (25 units/ml) (Sigma-Aldrich, Madrid, Spain) was added to 1 ml of broccoli extract, and they were left 2 h at room temperature for the reaction to occur (Lazzeri et al., 2004). The hydrolyzed extracts were immediately used after this time.

2.8. In vitro fungi and oomycete growth inhibition in solid medium

In vitro fungi and oomycete growth inhibition was tested by adding different volumes of the selected broccoli extracts or hydrolyzed broccoli extracts to 150 ml of sterilized PDA medium before mixing and pouring it into sterile 9 mm diameter Petri dishes. Doses tested in this experiment for broccoli extracts were 0.1%, 1% and 2%, each experiment used three Petri dishes per treatment and each experiment was conducted in triplicate.

A 6 mm diameter mycelial plug was inoculated in the center of each Petri dish and incubated in the dark at 24 ℃ until the first dish was fully colonized with the pathogen. The area colonized by the pathogen at that moment was measured with the ImageJ software (NIH, USA) and the data was normalized to untreated dishes.

2.9. In vitro fungi and oomycete growth inhibition in liquid medium

In vitro growth inhibition activity of extracts was also tested in liquid culture medium, adapting the procedures described previously by Tierens et al. (2001). Tests were performed with 2 µl of a pathogen inoculant in 78 µl of half strength Potato Dextrose Broth (PDB) (Sigma-Aldrich, Madrid, Spain), with 20 µl of the selected extracts or hydrolyzed extracts diluted at 0.1%, 1% and 2% in sterile distilled water. Control treatments were 80 µl of half strength PDB medium and 20 µl of sterile distilled water. The pathogen inoculant was a spore suspension (final concentration with PDB medium 2×10^4 spores/ml) for *A. alternata, B. cinerea, C. acutatum, F. oxysporum* f. sp. *lycopersici, P. expansum* and *V. dahliae,* and a mycelial suspension (final optical density 0.17 per ml at 520 nm) for *P. cactorum, P. ultimum, R. solani* and *S. sclerotiorum* as described in Poveda (2022). Spore suspensions were collected by adding 10 ml of sterile distilled water and scraping the surface of the mycelium with sterile pipette tips to release the spores in the water. To remove mycelium and medium fragments, the suspension was filtered through a double layer of Miracloth (VWR, Barcelona, Spain), collected in a 1.5 ml sterile eppendorf tube and adjusted at desired concentration with sterile distilled water. Mycelial suspensions were collected with the same procedure as spores, but instead of filtering two sterile glass spheres were introduced into the tube, and the tube was vortexed in order to break down the mycelium into small fragments.

Plates were incubated in an orbital shaker in the dark at 50 rpm and 24 ℃, measuring absorbance using a microplate reader Multiskan EX (Lab systems, Argentina) at 595 nm at 30 mins and 48 h times. Pathogen growth inhibition was defined as follows:

$$Growth \quad inhibition = \frac{Control - Test}{Control} x100$$

$$Control = ABS[595]Control(t48h) - ABS[595]Control(t30mins)$$

$$Test = ABS[595]Test(t48h) - ABS[595]Test(t30mins)$$

2.10. Mortality of M. javanica J2 nematodes

Mortality of *M. javanica* J2 nematodes was studied in 24-well plates. A volume of 900 µl of the corresponding treatment was added to each well, and 100 J2 were inoculated as a suspension in 100 µl of sterile water. Selected broccoli extracts were applied at 0.1%, 1% and 2%, with and without myrosinase enzyme (5 µl, final activity 25 units/ml). Control treatments were carried out with sterile distilled water, with or without myrosinase. Plates were sealed with Parafilm and incubated at 26℃ in the dark for 4 days in agitation. To distinguish dead J2 from the immobile nematodes, 1 µl of 1 N NaOH was added per well and their mobility was monitored after two minutes (Xiang and Lawrence, 2016). Three independent biological replicates per treatment were prepared with 100 nematodes each and the experiment was conducted in triplicate.

2.11. Anti-hatching effect on M. javanica nematodes

Hatching of *M. javanica* eggs was studied in 24-well plates. A volume of 900 µl of the corresponding treatment and 100 µl of sterile water containing 1 egg mass were added to each plate well. Selected broccoli extracts and hydrolyzed extracts were added to the plates at 0.1%, 1% and 2% dose, and sterile distilled water with and without myrosinase enzyme (5 µl, final activity 25 units/ml) was used for control treatment. Plates were sealed with Parafilm and incubated at 26℃ in the dark for 18 days in agitation. Hatched dead and alive J2s were assessed as described before. Unhatched eggs within the egg mass were firstly disaggregated with 500 µl of sodium hypochlorite 10%, shaking the tube followed by 4 min incubation and counted. Three independent replicates per treatment were prepared, and the experiment was conducted in triplicate.

3. Results

3.1. GSL content and GSL extractions from broccoli leaves

GSL analysis was carried out with the initial leaf plant material collected from the four different broccoli fields, two conventionally and two organically grown. The broccoli plant material collected from organic fields showed higher total GSL content (34.50 µmol/g DW), as well as aliphatic GSL content (22.90 µmol/g DW) than the conventional broccoli ($p < 0.05$; Fig. 1). Five different GSLs were detected in broccoli plant material, at different concentrations: glucoraphanin (GRA) and glucobrassicin (GBS) were the most abundant, ranging from 6.44 to 19.16 µmol/g DW; glucoiberin (GIB), methoxyglucobrassicin (MEOHGBS) and neoglucobrassicin (NEOGBS) were less abundant ranging from 1.10 to 4.05 µmol/g DW (Fig. S1). Organic broccoli extracts had higher aliphatic (GIB, GRA) and total GSL contents than conventional broccoli extracts, but indolic GSL content was similar in both groups. ($p < 0.05$; Fig. 1; Table S1).

Regarding solvent used, the extraction with methanol (M1 to M8) rendered higher total, aliphatic and indolic GSL contents than aqueous extracts (A1 to A8) (Fig. 2). Methanol extracts showed a higher content of the five GSL detected than the aqueous ones, and methanol extracts

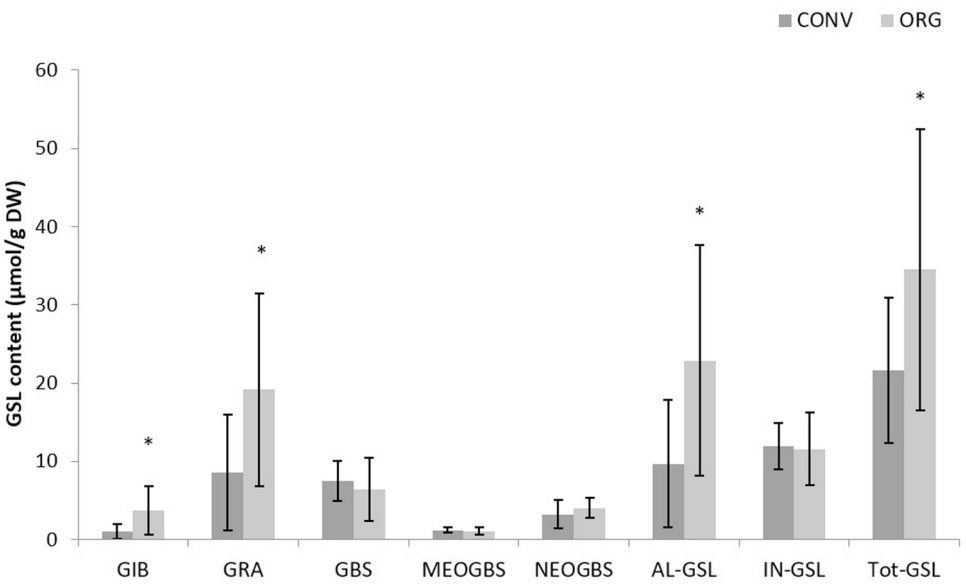


Fig. 1. : Mean of individual GSL content detected in broccoli leaves: glucoiberin (GIB), glucoraphanin (GRA), glucobrassicin (GBS), 4-methoxyglucobrassicin (MEOHGBS), neoglucobrassicin (NEOGBS), and mean of grouped aliphatic (AL-GSL), indole (IN-GSL) and total (Tot-GSL) glucosinolate content in broccoli leaves collected from conventional (CONV) and organic (ORG) fields.

obtained from organic broccoli rendered the highest GSL content of all (7.42–10.20 µmol/g DW). Aqueous extracts had a similar content in all GSL except the organic lyophilized broccoli stored at − 20 °C extracted (A7), which had a higher GRA content and similar to its respective methanol extract (Fig. 3).

Temperature storage of plant material and lyophilization had no significant influence on GSL content of the broccoli extracts (Fig. 2).

After extract analysis, four different extracts were selected for the following experiments: conventional − 80 °C non-lyophilized methanol extract (M2), conventional − 20 °C non-lyophilized methanol extract (M4), organic − 80 °C non-lyophilized methanol extract (M6) and organic − 20 °C non-lyophilized methanol extract (M8). The aqueous extracts were discarded for the following experiments because of the lower GSL extraction efficiency, and the extracts obtained from the lyophilized plant material were discarded as well according to industrial scalability criteria, after observing no significant difference with the non-lyophilized material (Fig. 2).

3.2. Phytotoxicity and cytotoxicity of broccoli leaves extracts

Determining the phytotoxicity of broccoli extracts was evaluated by the seed germination method (Selim et al., 2012), in which seed germination and root elongation of the treated seeds are combined in the GI value and compared with untreated seeds. In this index, values of 50% or below would be considered strongly toxic, while values ranging from 50% to 80% would be considered moderately toxic and above 80% would indicate no toxicity (Zucconi et al., 1981). The different seed species used to determine phytotoxicity responded differently to the selected broccoli extracts (Fig. 4). Radish and watercress were the most tolerant species to the broccoli extracts. These species were able to germinate with the pure extracts, reaching GI values of 40% while for the rest of species the GI value was practically 0%, and with 10% diluted extracts the GI value was > 70% in case of radish, and > 100% in case of watercress. Tomato and chard were also highly tolerant to the extracts. Pure extracts were toxic to tomato and chard, but the 10% diluted extracts were similar to the untreated control, with GI values above 95%. Chard obtained GI higher than 100% with extracts dilutions 10%, 1% and 0.1%, reaching values over 300% with the extracts obtained from the organic broccoli (M6 and M8). On maize, the pure extracts were toxic, the 10% dilutions were moderately toxic and the 1% and 0.1% dilutions were not toxic. Lettuce was the most sensible species among the six tested. Pure extracts inhibited germination of lettuce, while 10% and 1% dilutions were toxic or moderately toxic. Methanol extract from conventional broccoli stored at − 20 °C diluted at 0.1% was the only non-toxic treatment in lettuce seeds (Fig. 4).

Phytotoxicity was also studied in tomato plants with the conventional broccoli stored at − 80 °C and extracted with methanol (M4), because this was the most toxic extract to this plant in seed experiments (Fig. 4). No significant differences were detected due to a low number of plants per treatment, but values were found to be lower as the concentration of the extract increased (Fig. S2), and the 5% dilution caused older leaves to be light green or yellow.

Cytotoxic activity was evaluated for the four selected broccoli extracts, with MTT assay with different doses: 6%, 12.5%, 25% and 50%. Cell viability was not affected with 6% and 12.5% treatments, while the methanol extract from conventional broccoli stored at − 20 °C (M2) significantly reduced cell viability at 25% dilution (Fig. 5). Cell viability was also significantly decreased by all selected extracts at 50%, reaching values of 63–85% cell viability compared to untreated control. No differences among the four selected extracts were detected with the 50% dilution (Fig. 5).

3.3. Antifungal and oomyceticide activities of broccoli leaves extracts

In order to evaluate the antifungal activity of the selected broccoli extracts, two different assays were conducted, one with solid medium and one with liquid medium (Fig. 6 and Fig. 7); pictures of the solid medium test plates are shown in Fig. S3. The growth of *F. oxysporum* was significantly reduced in solid medium when treated with 2% of the conventional broccoli extracts (M2 and M4) by 2–19% reduction, compared to untreated control. However, in liquid medium, conventional broccoli stored at − 20 °C extract (M4) increased significantly its growth by 7%, compared to untreated control. When hydrolyzed with myrosinase, all four extracts reduced significantly pathogen growth, by around 20% in solid, and by more than 20% in liquid medium, compared to untreated control.

V. dahliae was only affected in solid medium by the organic broccoli stored at − 20 °C extract (M8), which reduced its growth by 16% compared to untreated control. However, pathogen growth was reduced significantly by hydrolyzed extracts by 54% and 47% with the

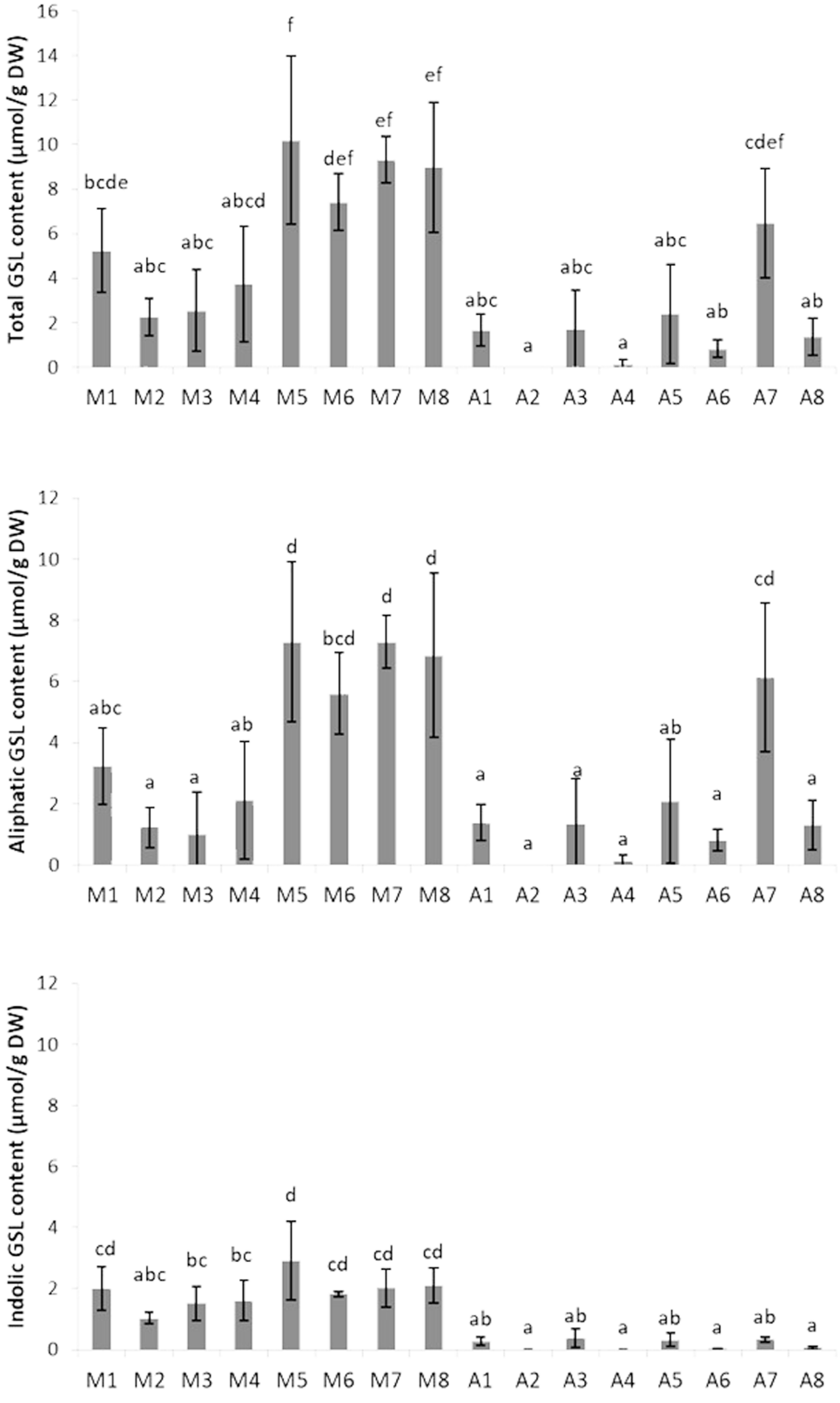
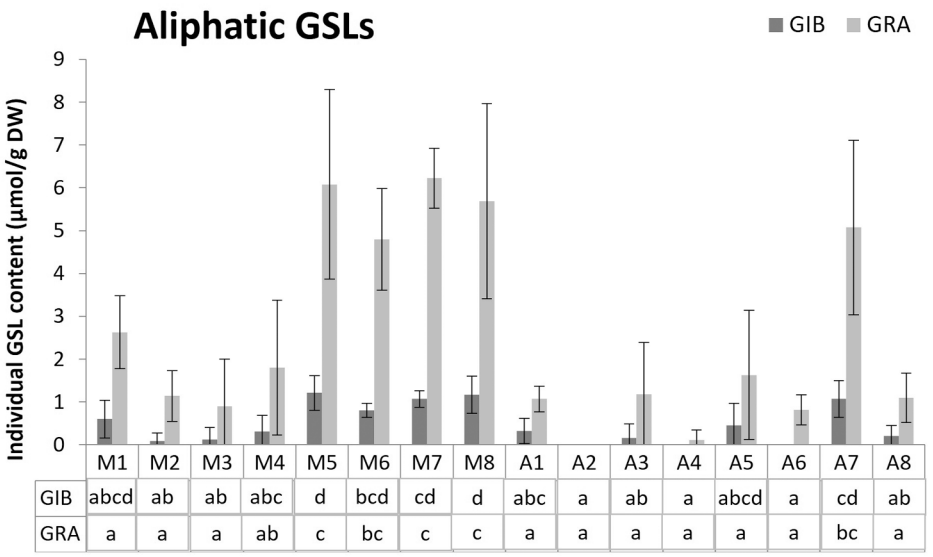
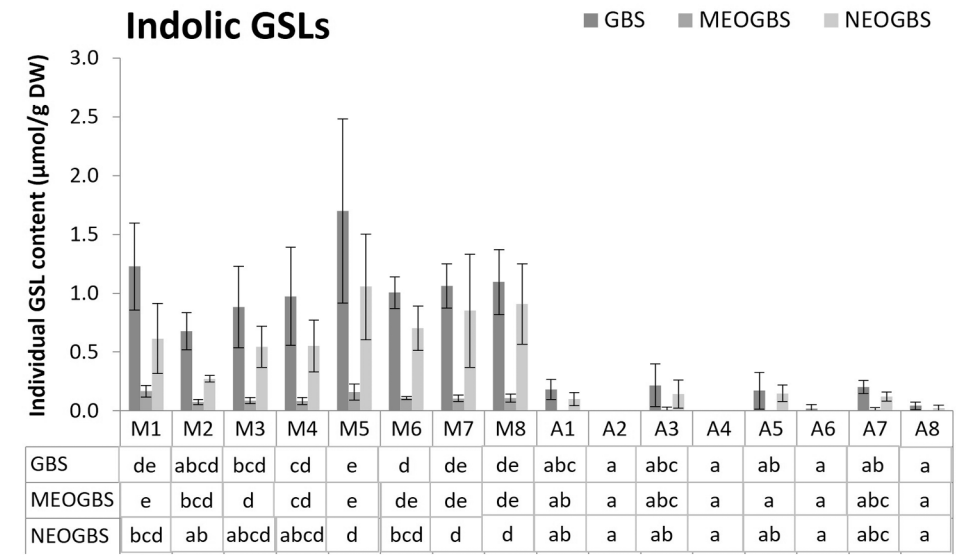


Fig. 2. : Total, aliphatic and indole GSL content of the different broccoli extracts obtained: M1 Conventional lyophilized broccoli stored at − 80 °C and extracted with methanol. M2 Conventional broccoli stored at − 80 °C and extracted with methanol. M3 Conventional lyophilized broccoli stored at − 20 °C and extracted with methanol. M4 Conventional broccoli stored at − 20 °C and extracted with methanol. M5 Organic lyophilized broccoli stored at − 80 °C and extracted with methanol. M6 Organic broccoli stored at − 80 °C and extracted with methanol. M7 Organic lyophilized broccoli stored at − 20 °C and extracted with methanol. M8 Organic broccoli stored at − 20 °C and extracted with methanol. A1 Conventional lyophilized broccoli stored at − 80 °C and extracted with water. A2 Conventional broccoli stored at − 80 °C and extracted with water. A3 Conventional lyophilized broccoli stored at − 20 °C and extracted with water. A4 Conventional broccoli stored at − 20 °C and extracted with water. A5 Organic lyophilized broccoli stored at − 80 °C and extracted with water. A6 Organic broccoli stored at − 80 °C and extracted with water. A7 Organic lyophilized broccoli stored at − 20 °C and extracted with water. A8 Organic broccoli stored at − 20 °C and extracted with water.

conventional broccoli stored at − 80 °C (M2) and with organic broccoli stored at − 20 °C (M8) extracts, respectively. In liquid medium all hydrolyzed extracts reduced the growth, up to 24% with 1% dilution and up to 44% with 2% dilution compared to untreated control (Fig. 6 and Fig. 7). The growth of the pathogen *R. solani* was significantly reduced in solid medium with the selected extracts at 2% (12–32% reduction). In liquid medium, the non-hydrolyzed extracts showed no significant effect on the pathogen. Hydrolyzed extracts reduced the growth of *R. solani* by up 55% with 0.1% dilution, 70% reduction with 1% dilution and 69% reduction with 2% dilution compared to untreated control in liquid medium, but had no influence in solid medium (Fig. 6 and Fig. 7). Extracts at 2% dilution also reduced *S. sclerotiorum* growth in solid medium



	M1	M2	M3	M4	M5	M6	M7	M8	A1	A2	A3	A4	A5	A6	A7	A8
GIB	abcd	ab	ab	abc	d	bcd	cd	d	abc	a	ab	a	abcd	a	cd	ab
GRA	a	a	a	ab	c	bc	c	c	a	a	a	a	a	a	bc	a



	M1	M2	M3	M4	M5	M6	M7	M8	A1	A2	A3	A4	A5	A6	A7	A8
GBS	de	abcd	bcd	cd	e	d	de	de	abc	a	abc	a	ab	a	ab	a
MEOGBS	e	bcd	d	cd	e	de	de	de	ab	a	abc	a	a	a	abc	a
NEOGBS	bcd	ab	abcd	abcd	d	bcd	d	d	ab	a	ab	a	ab	a	abc	a

Fig. 3. : Individual aliphatic and indole GSL content of the different broccoli extracts obtained: M1 Conventional lyophilized broccoli stored at − 80 °C and extracted with methanol. M2 Conventional broccoli stored at − 80 °C and extracted with methanol. M3 Conventional lyophilized broccoli stored at − 20 °C and extracted with methanol. M4 Conventional broccoli stored at − 20 °C and extracted with methanol. M5 Organic lyophilized broccoli stored at − 80 °C and extracted with methanol. M6 Organic broccoli stored at − 80 °C and extracted with methanol. M7 Organic lyophilized broccoli stored at − 20 °C and extracted with methanol. M8 Organic broccoli stored at − 20 °C and extracted with methanol. A1 Conventional lyophilized broccoli stored at − 80 °C and extracted with water. A2 Conventional broccoli stored at − 80 °C and extracted with water. A3 Conventional lyophilized broccoli stored at − 20 °C and extracted with water. A4 Conventional broccoli stored at − 20 °C and extracted with water. A5 Organic lyophilized broccoli stored at − 80 °C and extracted with water. A6 Organic broccoli stored at − 80 °C and extracted with water. A7 Organic lyophilized broccoli stored at − 20 °C and extracted with water. A8 Organic broccoli stored at − 20 °C and extracted with water. Statistical analysis was carried out individually for each GSL.

(25–47% reduction, compared to untreated control), while organic broccoli extracts reduced pathogen growth when hydrolyzed (Fig. 6). In liquid medium only the hydrolyzed extracts reduced pathogen growth (8–10% reduction, compared to untreated control) (Fig. 7).

A. alternata was not affected by the extracts in liquid medium without myrosinase enzyme, but in solid medium the 2% dilution reduced its growth (9.5–38% reduction compared to untreated control). Once myrosinase was added in solid medium, the inhibitory effect increased and pathogen inhibition reached 69%. In liquid medium, organic broccoli extracts (M6 and M8) with myrosinase reduced *A. alternata* growth by up to 30%, compared to untreated control (Fig. 6 and Fig. 7). The selected extracts had no clear effect on *B. cinerea* growth, since 1% dilutions reduced its growth in solid medium (15–20% reduction compared to untreated control), but 2% dilution did not. No significant differences were obtained in liquid medium. The addition of myrosinase enzyme had no influence on *B. cinerea* growth (Fig. 6 and Fig. 7). *P. expansum* was not affected by the broccoli extracts both in solid as in liquid medium, but when myrosinase was added, the extract from conventional broccoli stored at − 20 °C (M4) reduced its growth at 2% dilution (18% reduction, compared to untreated control). While the extract from organic broccoli stored at − 20 °C (M8) increased its growth at 1% dilution (16% increase compared to untreated control) (Fig. 6 and Fig. 7).

The growth of the pathogen *C. acutatum* was reduced in solid medium when treated with 2% of the non-hydrolyzed extracts (14–18% reduction, compared to untreated control). While in liquid medium no significant differences were detected. Hydrolyzed extracts had no influence in solid nor in liquid medium against *C. acutatum* (Fig. 6 and Fig. 7).

In addition to fungal pathogens, the extracts inhibitory capacity was also tested in solid and in liquid media against the oomycete *P. cactorum* (Fig. 6 and Fig. 7). Broccoli non-hydrolyzed extracts increased *P. cactorum* growth in solid medium at 1% and 2% dilutions (11–19%, and 38–61% increase, respectively). With hydrolyzed extracts the increase was even greater (21–48%, and 50–99% increase, respectively). However, in liquid medium only the extract obtained from organic broccoli stored at − 20 °C (M8) decreased the pathogen growth by 66% compared to untreated control, while the rest of treatments showed no significant differences (Fig. 6 and Fig. 7).

No differences in pathogen inhibitory activity were found among the selected extracts. The myrosinase enzyme alone had no influence in plant pathogen growth. The addition of myrosinase together with the extracts increased its effectiveness in reducing pathogen growth against certain pathogens, but had no influence or had opposite effect with others (Fig. 6 and Fig. 7).

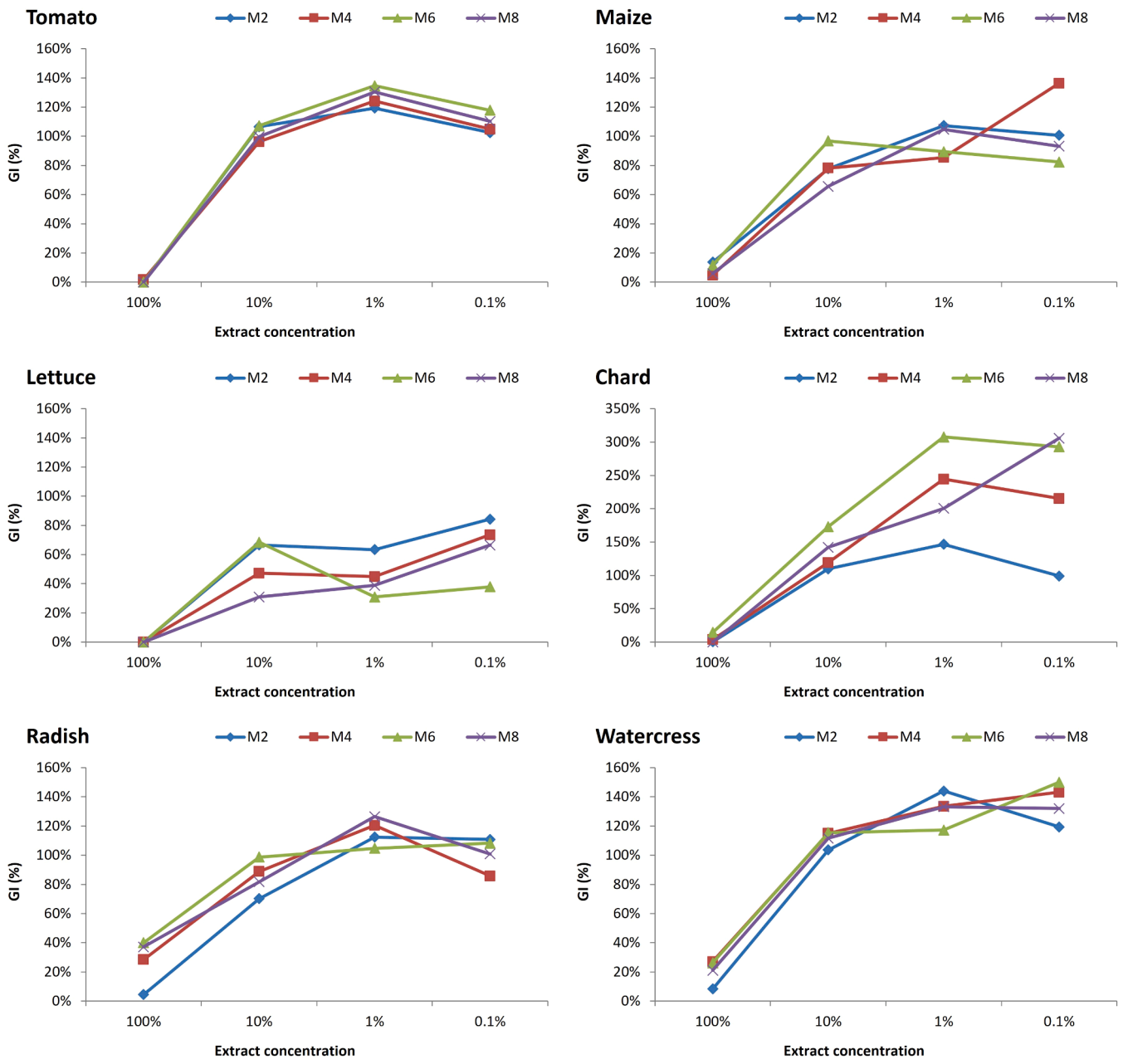


Fig. 4. : Germination index (GI) of different plant seeds grown with different concentrations 0,1%, 1%, 10% and 100% of conventional broccoli leaves stored at − 80 °C or − 20 °C and extracted with methanol (M2 and M4, respectively) or organic broccoli leaves stored at − 80 °C or − 20 °C and extracted with methanol (M6 and M8, respectively) for 72 h in the dark and compared with the water treatment.

3.4. Nematicidal activity: mortality and egg hatching

The effect of selected broccoli extracts on *M. javanica* nematode J2 mortality and egg hatching was also studied (Fig. 8). Organic broccoli extracts at 0.1% dilution reduced J2 nematode mortality, while 2% extract of organic broccoli stored at − 20 °C (M8) showed higher nematode mortality than control treatment. When hydrolyzed, extracts obtained from conventional broccoli (M2 and M4) significantly increased J2 nematode mortality with dilutions 0.1%, 1%, and 1%, 2%, 5%, respectively (Fig. 8). No significant differences were found with organic broccoli hydrolyzed extracts.

No effect on nematode egg hatching was observed with the application of broccoli extracts alone. However, when hydrolyzed, the organic broccoli extracts (M6 and M8) reduced nematode egg hatching at 5% dilution. Extract from conventional broccoli stored at − 20 °C (M4) increased nematode egg hatching with 1% and 2% dilutions (Fig. 8).

4. Discussion

4.1. Differences in GSL content in broccoli leaves from organic or conventional farming

In the present study, higher total and aliphatic GSL content, in particular GIB and GRA, were found in organic broccoli compared to conventional broccoli. Some authors have previously hypothesized that the organically-cultivated crops contain a higher level of secondary metabolites and phytochemicals that the conventional farming (Sousa et al., 2008), but the data show a less conclusive reality. To date, a wide number of studies has been conducted obtaining a higher GSL content in

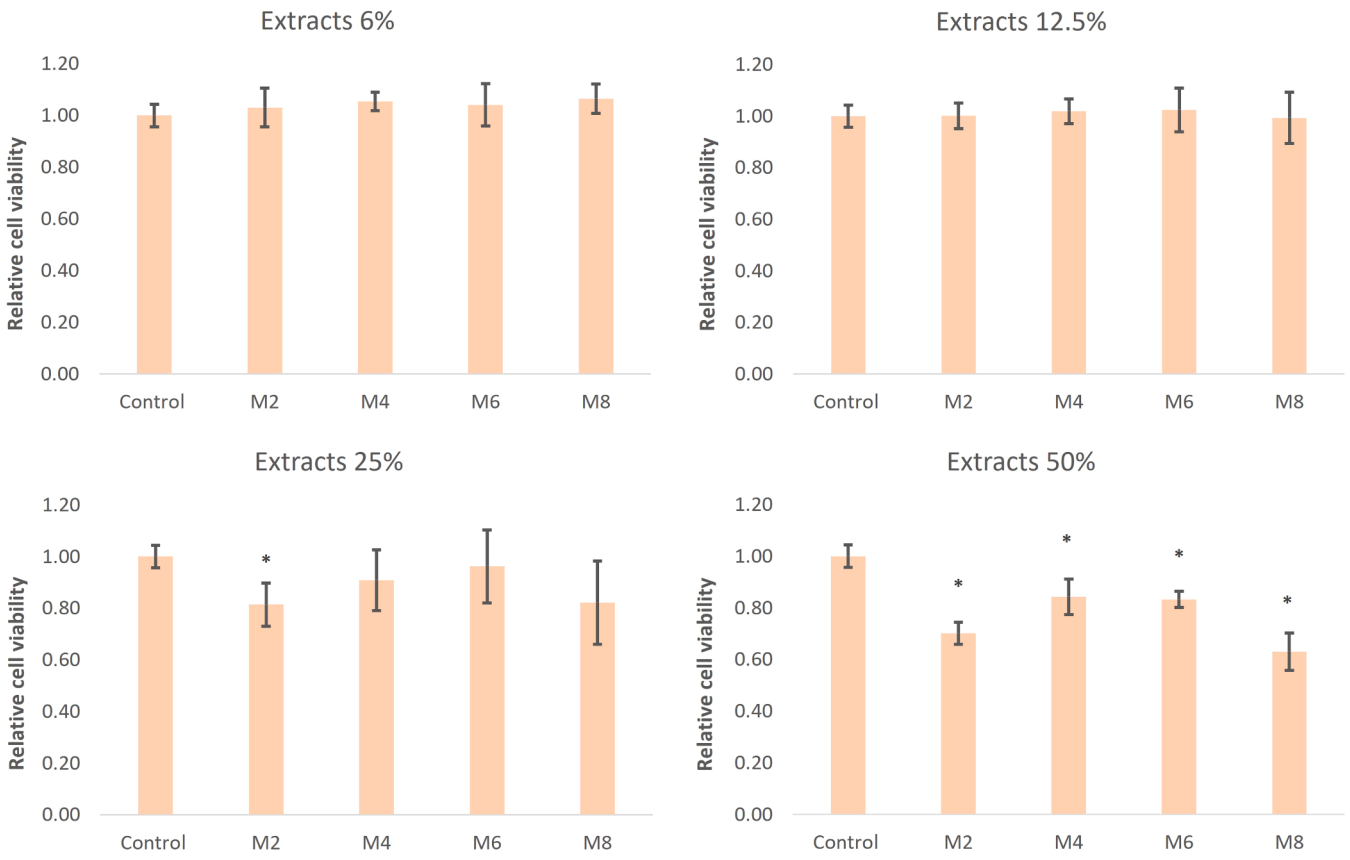


Fig. 5. : Effects of broccoli extracts at different concentrations 6%, 12,5%, 25% and 50%, on cell viability in HePG2 cells. Cells cultured with conventional broccoli leaves stored at − 80 °C or − 20 °C and extracted with methanol (M2 and M4, respectively) or with organic broccoli leaves stored at − 80 °C or − 20 °C and extracted with methanol (M6 and M8, respectively) were compared with the untreated cell cultured (Control).

organic samples than in conventional ones, in broccoli (Meyer & Adam, 2008; Miranda-Rossetto et al., 2013), cabbage (Meyer & Adam, 2008; Sousa et al., 2008), cauliflower (Picchi et al., 2012) or collar green, rocket and watercress (Miranda-Rossetto et al., 2013). However, many studies obtained opposite results and conventionally cultured *Brassica* showed higher GSL content than organic ones (Robbins et al., 2005; Cámara-Martos et al., 2022), or even no differences between both systems (Renaud et al., 2014; Conversa et al., 2016). After reviewing previous studies and taking into account the nutritional inputs of these plants, we came to the conclusion that the conventional broccoli was fertilized with a high nitrogen level and no sulfur addition, and thus a high nitrogen-sulfur rate, could have stimulated vegetative growth over secondary metabolism and GSL production.

In our study, both cold methanol and boiling water extractions were tested, in order to obtain an effective GSL extraction method suitable for industrial adaptation. Our results showed that the cold methanol extraction was more effective than the aqueous extraction. Although some previous works report that boiling water extraction has similar efficiency than boiling or cold methanol extraction (Rangkadilok et al., 2002; Herzallah and Holley, 2012; Major et al., 2020), GSL are heat sensitive and may be degraded at temperatures above 75 °C and heating times above 10 mins (Oerlemans et al., 2006). Indole-GSL in particular, such as GBS or MEOHGBS are quickly degraded at 100 °C (Oerlemans et al., 2006; Doheny-Adams et al., 2017). In our case, it is likely that GSLs have been thermally degraded with the aqueous extraction method, and thus little GSLs content has been collected with this protocol in comparison with the methanol extraction. On the other hand, high temperatures inactivate myrosinase, but may cause thermal degradation and GSL loss (Ares et al., 2014). However, Doheny-Adams et al. (2017) showed that cold methanol 80% may also inactivate myrosinase without heating (Doheny-Adams et al., 2017). Our results suggest that myrosinase was not active during the cold methanol extraction, given no GSL degradation was detected.

Lyophilization and plant material storage at − 80 °C or − 20 °C had no influence on extracted GSL, both with methanol and aqueous extractions. One of the main objectives in this study was to determine whether some of those steps, most commonly used in GSL extraction protocols (Doheny-Adams et al., 2017), could be avoided without losing much extraction efficiency. Lyophilization is an expensive and limiting step in escalation processes due to its high energy demand, long processing times and low sample process per time (Major et al., 2020). To our knowledge, only Doheny-Adams et al. (2017) studied the effect of lyophilization step on GSL content of *Brassica* extracts, and they concluded that a cold methanol extraction was effective in inactivating myrosinase and extract GSL, with no difference between freeze-drying and direct extraction (Doheny-Adams et a, 2017). Temperature storage of *Brassica* plant material may also cause a significant GSL loss. Temperatures above 0 °C have been proven to cause GSL loss in previous works (Hansen et al., 1995; Vallejo et al., 2003; Meyer & Adam, 2008), while storage at − 20 °C storage has no clear effect (Cieślik et al., 2007; Miao et al., 2019; Wu et al., 2021). In this respect, the present work showed that storage at − 20 °C for 30 days caused no GSL loss in broccoli plant material.

4.2. *Phytotoxicity and cytotoxicity*

Phytotoxicity values obtained in the present study are higher than those obtained by Mason-Sedun et al. (1986), who compared phytotoxicity of aqueous extracts from 6 different *Brassica* species in wheat seeds, and obtained significant differences, with wheat growth

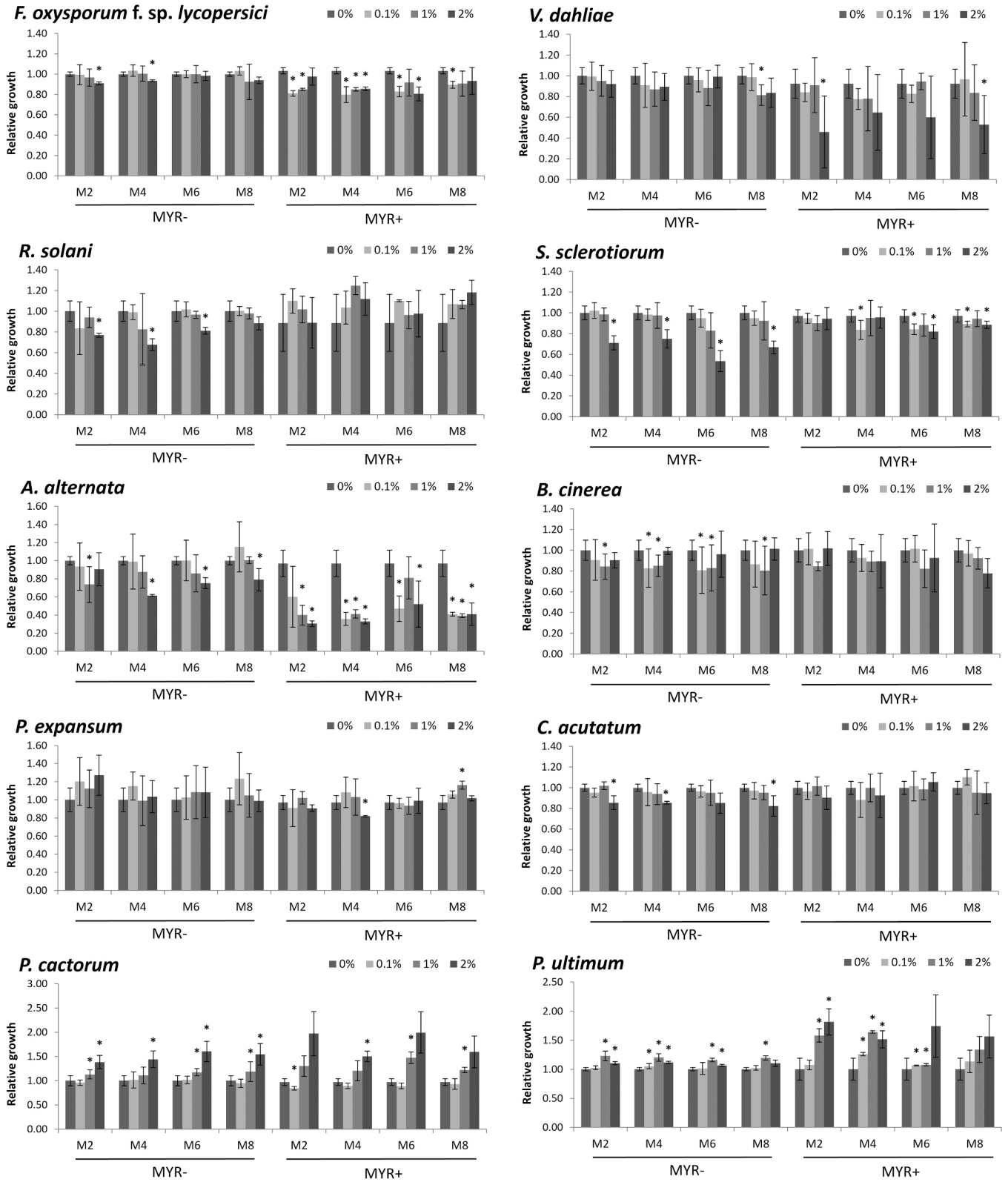


Fig. 6. : Plant pathogen relative growth with the selected broccoli extracts, conventional broccoli leaves stored at − 80 °C or − 20 °C and extracted with methanol (M2 and M4, respectively) or organic broccoli leaves stored at − 80 °C or − 20 °C and extracted with methanol (M6 and M8, respectively), at different concentrations (0,1%, 1% and 2%) with and without myrosinase enzyme (MYR), in solid culture medium (PDA).

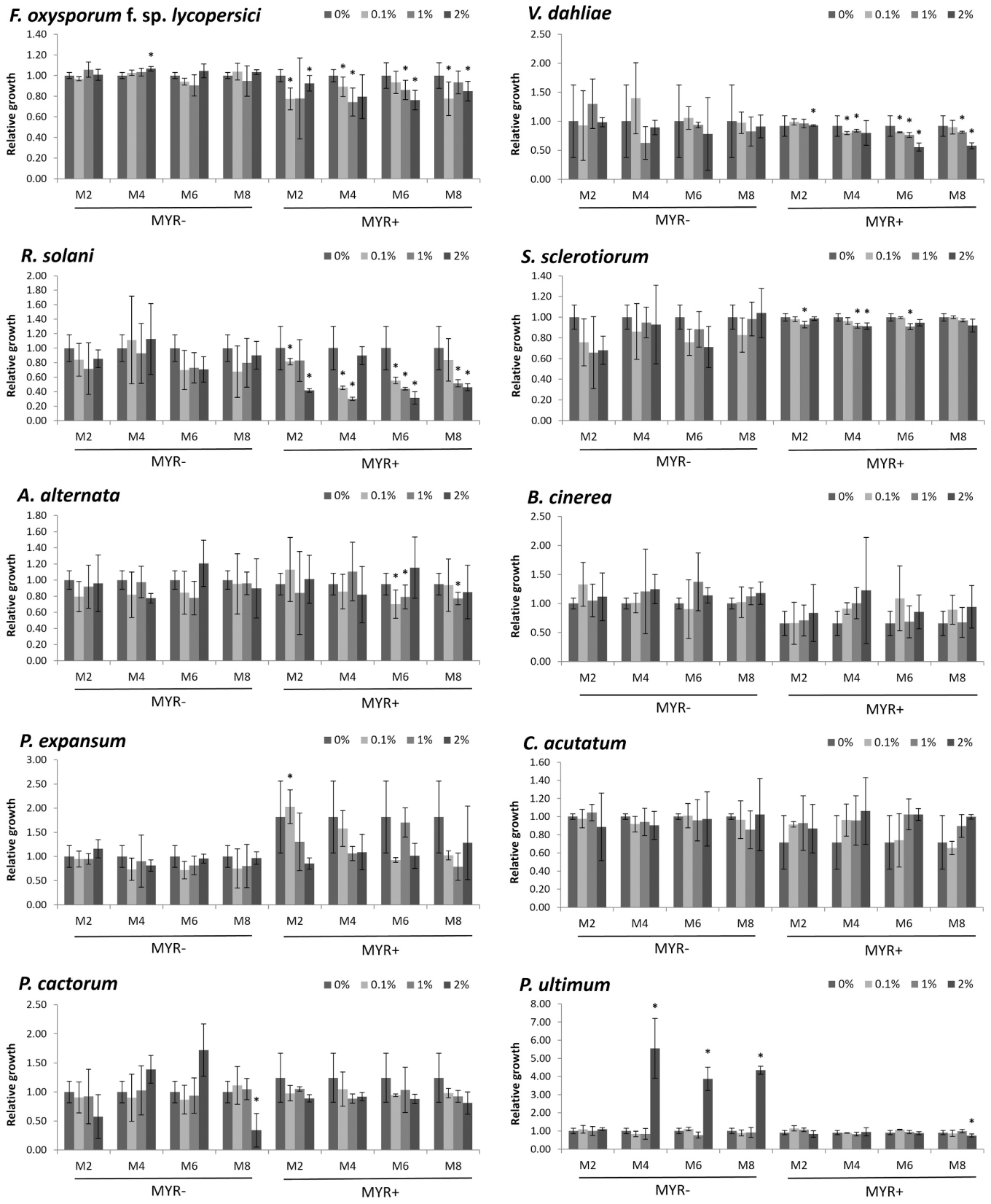


Fig. 7. : Plant pathogen relative growth with the selected broccoli extracts, conventional broccoli leaves stored at − 80 °C or − 20 °C and extracted with methanol (M2 and M4, respectively) or organic broccoli leaves stored at − 80 °C or − 20 °C and extracted with methanol (M6 and M8, respectively), at different concentrations (0,1%, 1% and 2%) with and without myrosinase enzyme (MYR), in liquid culture medium (half strength PDB).

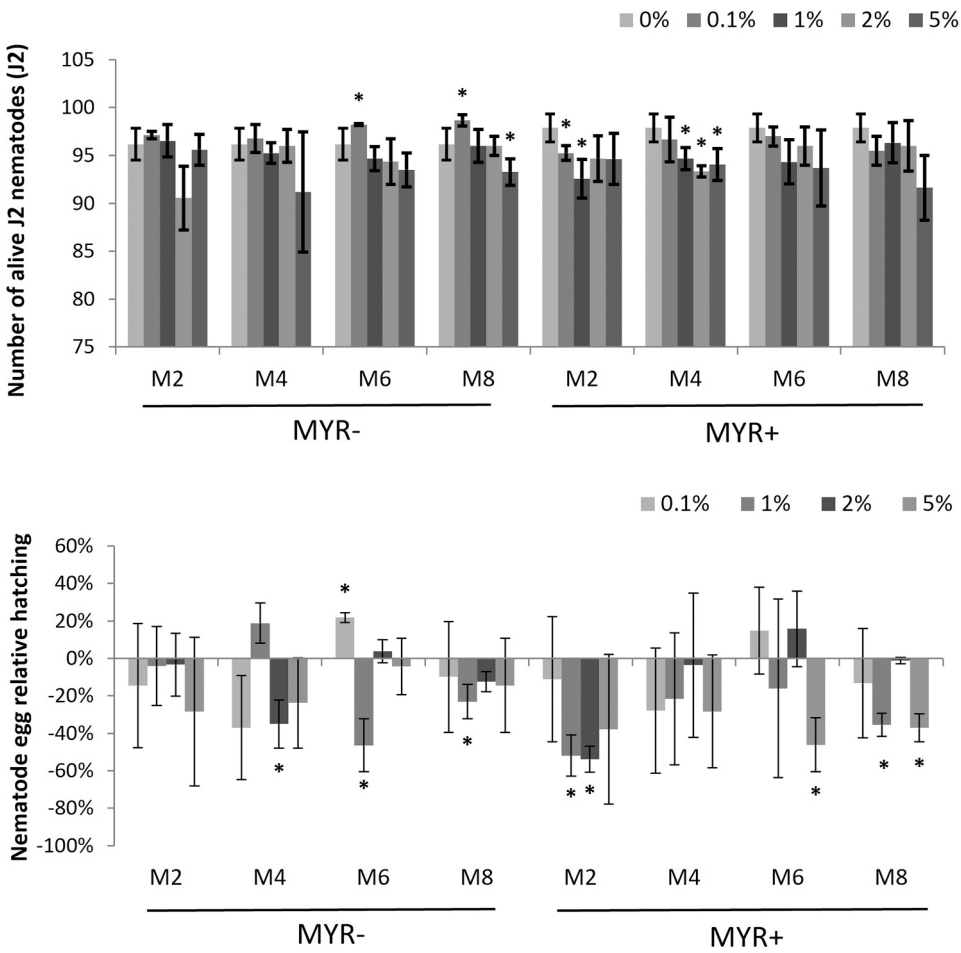


Fig. 8. : *Meloidogyne javanica* J2 mortality and relative egg hatching after treated with the selected extracts, conventional broccoli leaves stored at − 80 °C or − 20 °C and extracted with methanol (M2 and M4, respectively) or organic broccoli leaves stored at − 80 °C or − 20 °C and extracted with methanol (M6 and M8, respectively), and incubated for 4 and 18 days, respectively.

inhibition ranging from 59.1% to 97.8% (Mason-Sedun et al., 1986). Radish and watercress were the most tolerant species to broccoli extracts, probably because both are from the Brassicaceae family containing GSL themselves. Tomato was also tolerant to the diluted broccoli extracts, while lettuce, on the other hand, was the most sensible species to the broccoli extracts, that is in agreement with the results obtained by Miranda-Arámbula et al. (2021), which showed that tomato plants were in general more tolerant to GSL-containing extracts than lettuce. Interestingly, chard increased its growth when treated with broccoli extracts diluted at 10% or below compared to control, which could be explained by some mechanism capable not only of detoxifying GSLs to a certain degree, but also of using the sulfur molecules contained within them. GSL have been described as allelochemical compounds, with inhibitory effects on weeds (Abd El-Ghany et al., 2022), but these results showed that the dilutions 1% and below are not phytotoxic, and 10% and below are only moderately phytotoxic in some cases. Other biomolecules, such as flavonoids, phenolic compounds or minerals may be present in the extracts and may have acted promoting its growth.

The phytotoxicity experiment with tomato plants, ranging 1–5% concentration of the broccoli extract had no significant differences, but biomass values decreased as the extract concentration increased. Visual symptoms were detected in older leaves with the 5% extract concentration. Considering this data, the maximum concentration for subsequent *in vitro* experiments was established at 2%.

Broccoli extracts were tested for their cytotoxic activity against HePG2 human cell line and the results obtained can be considered of low cytotoxic activity to the human cell line tested *in vitro*. This indicates that it could potentially be a safe biopesticide which will increase the sustainability of the agrosystems.

4.3. Broccoli extracts show high antifungal and oomicetycide activities

Plant pathogen inhibition of broccoli extracts was tested against a wide collection of different fungi and oomycetes in two different *in vitro* systems, in solid medium and in liquid medium. Many authors tested GSLs activity against plant pathogens, but most directly using plant tissues or commercial GSLs and GHPs, and little studies have been done with *Brassica* plant extracts (Poveda et al., 2020a; Eugui et al., 2022). An exhaustive list of previous works of *Brassica* plants GSL-containing matrices as antifungal compounds was compiled by Plaszkó et al. (2021).

In the present study, we have demonstrated that broccoli extracts combined with myrosinase reduce *F. oxysporum* f. sp. *lycopersici* growth *in vitro*, indicating that this pathogen is more sensitive to GHPs than to GSLs. Similarly, previous authors have used GSLs and GHPs to reduce *F. oxysporum* growth, by using powdered *Brassica* plant residues (Villalta et al., 2016) and *Brassica* seed meals (Ma et al., 2015), with success. In 1999, Smolinska & Horbowicz correlated the capacity of nine cruciferous plants to inhibit chlamydospore germination from the pathogen *F. oxysporum* f. sp. *lycopersici* to the AITC activity, despite its variable content (Smolinska and Horbowicz, 1999). Regarding the soil pathogen *V. dahliae*, non-hydrolyzed broccoli extracts did not reduce its growth, both in solid or in liquid medium, but hydrolyzed extracts significantly reduced *V. dahliae* growth. These results could indicate that *V. dahliae* is

not affected by GSLs, but after hydrolysis, the GHPs produced could affect its growth, which agrees with previous works that state that GHPs such as 2-propenyl ITC inhibit *V. dahliae* in soil (Neubauer et al., 2014). In soil experiments, green manures and seed meals from *Brassica* plants such as Indian mustard (Neubauer et al., 2014, 2015) or broccoli (Ochiai et al., 2007), have been used to reduce *V. dahliae* microsclerotia or pathogen population in soil, probably due to the release of ITCs after hydrolysis. Non-hydrolyzed broccoli extracts reduced *R. solani* and *S. sclerotiorum* growth *in vitro* at 2% dilution. Hydrolyzed broccoli extracts proved to be more effective against them in liquid medium, suggesting that these pathogens are more sensitive to GHPs, but not in solid medium. The efficacy of the extracts against *R. solani* was higher than the results obtained by Charron and Sams (1999) with volatiles from broccoli macerates (26,2% inhibition) and fairly similar to those obtained with macerates from other cruciferous plants (20,4% - 72,6% inhibition) by the same authors. More recent works had similar results against *S. sclerotiorum* to those obtained in the present study. In 2016, Warmington & Clarkson reduced *S. sclerotiorum* sclerotia germination using dried brassica plant material both *in vitro* (57.6–75% inhibition) and in microcosm experiments (37.1–92.1% inhibition) (Warmington & Clarkson, 2016). It is known that some GSLs and GHPs reduce *R. solani* growth *in vitro*, such as ITC (Tang et al., 2018), AITC (Handiseni et al., 2016) and 2-propenyl GSL (Villalta et al., 2016), and also SIN, GBS, GRA and glucoiberverin (GIV) are involved in *S. sclerotiorum* resistance (Augustine and Bisht, 2015; Abuyusuf et al., 2018; Madloo et al., 2019). According to these previous works, the specific GSLs profile of the extracts and the GHPs they produced could explain the inhibition activity against these plant pathogens.

In our experiments, *A. alternata* was one of the most sensitive pathogens to the broccoli extracts in solid medium. GSLs and GHPs toxicity to *Alternaria* has been previously observed (Kaur et al., 2011; Flores-Córdova et al., 2013), and some GHPs, such as ITCs, have been described to cause cell membrane disruption in this species (Wang et al., 2020; Zhang et al., 2020a). *B. cinerea* was not affected by broccoli extracts in liquid medium,while the experiments in solid medium showed high variability. In earlier studies, GHPs have been used to reduce *B. cinerea* growth (Ugolini et al., 2014), but previous authors have postulated that different *B. cinerea* strains have different susceptibility to GSLs, due to the virulence factor *mfsG* (Vela-Corcía et al., 2019). According to these researchers, *B. cinerea* strains with *mfsG* would be more tolerant to GHPs. Our results indicate that the strain tested in this study was not susceptible to broccoli extracts or its GHPs at the dilutions tested, which could be explained by the presence of the *mfsG* virulence factor. *P. expansum* growth was highly variable both in solid and in liquid media, and the effect of the broccoli extracts was not significant. Accordingly, opposite effects were encountered with different application methods, such as ITCs applied *in vitro* with the disc diffusion technique that showed antagonistic against the postharvest pathogen *P. expansum* (Mari et al., 1993; Manyes et al., 2015), but were ineffective in pear artificial infection assays (Mari et al., 1996).

In our study, non-hydrolyzed broccoli extracts significantly reduced *C. acutatum* growth in solid medium with 2% dilution compared to control treatment. GSL and GHP inhibition in *Colletotrichum* species has been previously studied: indol GSLs may limit the entry of C. *gloeosporioides* in *Arabidopsis thaliana* by blocking its appressorium entry mode (Hiruma et al., 2010), and glucoraphanin inhibited *C. gloeosporioides* conidia germination *in vitro* (Lara-Viveros et al., 2014), but to our knowledge no studies have been done assessing antifungal activity of brassicaceous extracts with these fungi.

Our results report also an increase in the oomycetes *P. ultimum* and *P. cactorum* growth when exposed to broccoli extracts, more prominent when hydrolyzed with myrosinase. Similarly other authors previously reported an increase in oomycete growth with *Brassica* seed meals in soil, although the mechanisms involved are still unclear (Mazzola et al., 2001; Cohen and Mazzola, 2006; Mazzola et al., 2007). However, other reports indicate the potential of GSLs, GHPs and *Brassica* amendments in oomycete control (Ren et al., 2018), as for example in *P. cinnamomi* (Ríos et al., 2016, 2017), and in *P. cinnamomi* and *Pythium spiculum* (Arroyo-Cordero et al., 2019). For example, in volatile experiments AITC has previously proven to be effective inhibiting *P. ultimum* (Mayton et al., 1996; Charron and Sams, 1999).

4.4. Nematidical activity

The nematicidal effect of the broccoli extracts was determined with a J2 mortality assay and an egg hatching assay with the PPN *M. javanica*. The potential of GSLs and GHPs as nematicides was studied previously with different biological systems, but only a few of them reported the effect of *Brassica* aqueous extracts (Eugui et al., 2022). In our study, *M. javanica* J2 mortality increased with the 2% dilution of broccoli extracts, and in some cases with the 5% dilution, but no difference was detected when hydrolyzed with myrosinase. No clear positive dose-effect response has been observed, which may be explained by the thick cuticle of the nematode as stated previously (Kruger et al., 2015). Nematode egg hatching was only affected by the hydrolyzed extracts, which indicates more sensibility to GHPs than GSLs, possibly because GHPs have the ability to be absorbed through lipophylic barriers (Zhang, 2010). Egg hatching was reduced by the hydrolyzed organic broccoli extracts at 5% dilution, which agree with previous works stating that GSLs and GHPs such as epiprogoitrin, reduce egg hatching (Tarini et al., 2020). Several works have showed antinematicidal potential of GSL-containing extracts, with broccoli aqueous macerates were applied in tomato plants reducing the *M. incognita* gall index (Silva et al., 2018), or with aqueous extracts of *Ochradenus baccatus* causing 100% immobilization of *M. javanica in vitro*, and reducing in soil the number of recovered nematodes by 95–100% (Oka et al., 2014). Nematode egg hatching may also be affected by these compounds, as demonstrated by several authors with *Brassica* seed meals in *Meloidogyne* species (Yu et al., 2007; Mocali et al., 2015; Handiseni et al., 2017).

5. Conclusions

The analysis of four broccoli fields have shown that organic broccoli had greater GSL content than conventional broccoli. We also assayed a simple and effective GSL cold methanol extraction method from broccoli leaves, which does not need to lyophilize plant material and does not require − 80 °C storage. We confirmed that those leaf extracts obtained have inhibitory effect *in vitro* against plant pathogenic fungi, such as *F. oxysporum*, *A. alternata*, *S. sclerotiorum*, *C. acutatum* or *R. solani*, effect which increased when hydrolyzed with myrosinase, confirming the potential of this strategy in controlling plant diseases and pests. However, broccoli extracts showed little nematode *M. javanica* J2 mortality and the effect on egg hatching *in vitro* was not clear.

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CRediT authorship contribution statement

D.E. performed the experiments and analyzed the data. D.E. and J.P. designed the research conducted and wrote the first version of the manuscript. J.P. supervised D.E.'s doctoral thesis. P.V. contributed to the GSL profile analysis. P.A.-U. and C.E. contributed to the design and conduct of the nematode assays. O.G.-T. contributed to the cytotoxicity study. P.V., P.A.-U., C.E., O.G.-T. and S.C. contributed to the manuscript correction and critical reading. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

We declare that the research was funded by a private corporation, Delso Fertilizantes Family S.L., which is dedicated to agricultural R+D+i, and by public financing (DIN2018–009852); however, we ensure the research is free of bias.

Data Availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2023.116841.

References

Abd El-Ghany, S.E.D., Ahmed, M.A.E.W., Abd El Rahman, S., Mohamed, N.K.M., 2022. Impact of phenolic compounds and glucosinolates of two Brassicaceae seeds powder as natural herbicides. Egypt. J. Chem. 65, 707–714. https://doi.org/10.21608/EJCHEM.2022.116122.5283.

Abuyusuf, M., Robin, A.H.K., Lee, J.H., Jung, H.J., Kim, H.T., Park, J.I., Nou, I.S., 2018. Glucosinolate profiling and expression analysis of glucosinolate biosynthesis genes differentiate white mold resistant and susceptible cabbage lines. Int. J. Mol. Sci. 19, 4037. https://doi.org/10.3390/ijms19124037.

Acheuk, F., Basiouni, S., Shehata, A.A., Dick, K., Hajri, H., Lasram, S., Ntougias, S., 2022. Status and prospects of botanical biopesticides in Europe and Mediterranean countries. Biomolecules 12, 311. https://doi.org/10.3390/biom12020311.

Ares, A.M., Nozal, M.J., Bernal, J.L., Bernal, J., 2014. Optimized extraction, separation and quantification of twelve intact glucosinolates in broccoli leaves. Food Chem. 152, 66–74. https://doi.org/10.1016/j.foodchem.2013.11.125.

Arroyo-Cordero, F.T., Rodríguez-Arcos, R., Jiménez-Araujo, A., Guillén-Bejarano, R., Basallote, M.J., Barrau, C., 2019. Inhibitory effect of the glucosinolate–myrosinase system on *Phytophthora cinnamomi* and *Pythium spiculum*. Plant Prot. Sci. 55, 93–101. https://doi.org/10.17221/98/2018-PPS.

Ashurst J.V., Nappe T.M. Methanol Toxicity. In: StatPearls. StatPearls Publishing, Treasure Island (FL); 2022. PMID: 29489213.

Augustine, R., Bisht, N.C., 2015. Biofortification of oilseed *Brassica juncea* with the anti-cancer compound glucoraphanin by suppressing GSL-ALK gene family. Sci. Rep. 5, 18005.

Bickel, J.T., Koehler, A.M., 2021. Review of *Pythium* species causing damping-off in corn. Plant. Health Prog. 22, 219–225. https://doi.org/10.1094/PHP-02-21-0046-FI.

Calmes, B., N'Guyen, G., Dumur, J., Brisach, C.A., Campion, C., Iacomi, B., Pigné, S., Dias, E., Macherel, D., Guillemette, T., Simoneau, P., 2015. Glucosinolate-derived isothiocyanates impact mitochondrial function in fungal cells and elicit an oxidative stress response necessary for growth recovery. Front. Plant Sci. 6, 414. https://doi.org/10.3389/fpls.2015.00414.

Cámara-Martos, F., Obregón-Cano, S., de Haro-Bailón, A., 2022. Glucosinolates, Ca, Se contents, and bioaccessibility in *Brassica rapa* vegetables obtained by organic and conventional cropping systems. Foods 11, 350. https://doi.org/10.3390/foods11030350.

Card, S.D., Hume, D.E., Roodi, D., McGill, C.R., Millner, J.P., Johnson, R.D., 2015. Beneficial endophytic microorganisms of *Brassica*–A review. Biol. Control. 90, 102–112. https://doi.org/10.1016/j.biocontrol.2015.06.001.

Charron, C.S., Sams, C.E., 1999. Inhibition of *Pythium ultimum* and *Rhizoctonia solani* by shredded leaves of *Brassica* species. J. Am. Soc. Hort. Sci. 124, 462–467.

Cieślik, E., Leszczyńska, T., Filipiak-Florkiewicz, A., Sikora, E., Pisulewski, P.M., 2007. Effects of some technological processes on glucosinolate contents in cruciferous vegetables. Food Chem. 105, 976–981. https://doi.org/10.1016/j.foodchem.2007.04.047.

Clarke, D.B., 2010. Glucosinolates, structures and analysis in food. Anal. Methods 2, 310–325. https://doi.org/10.1039/B9AY00280D.

Cohen, M.F., Mazzola, M., 2006. Resident bacteria, nitric oxide emission and particle size modulate the effect of *Brassica napus* seed meal on disease incited by *Rhizoctonia solani* and *Pythium* spp. Plant Soil 286, 75–86. https://doi.org/10.1007/s11104-006-9027-1.

Conversa, G., Bonasia, A., Lazzizera, C., Elia, A., 2016. Bio-physical, physiological, and nutritional aspects of ready-to-use cima di rapa (*Brassica rapa* L. subsp. *sylvestris* L. Janch. var. *esculenta* Hort.) as affected by conventional and organic growing systems and storage time. Sci. Hortic. 213, 76–86. https://doi.org/10.1016/j.scienta.2016.10.021.

Díaz-Manzano, F.E., Olmo, R., Cabrera, J., Barcala, M., Escobar, C., Fenoll, C., 2016. Long-term *in vitro* system for maintenance and amplification of root-knot nematodes in *Cucumis sativus* roots. Front. Plant Sci. 7, 124. https://doi.org/10.3389/fpls.2016.00124.

Doheny-Adams, T., Redeker, K., Kittipol, V., Bancroft, I., Hartley, S.E., 2017. Development of an efficient glucosinolate extraction method. Plant Methods 13, 1–14. https://doi.org/10.1186/s13007-017-0164-8.

Eugui, D., Escobar, C., Velasco, P., Poveda, J., 2022. Glucosinolates as an effective tool in plant-parasitic nematodes control: Exploiting natural plant defenses. Appl. Soil Ecol. 176, 104497 https://doi.org/10.1016/j.apsoil.2022.104497.

Evivie, E.R., Ogwu, M.C., Cang, W., Xu, R., Li, J., 2019. Progress and prospects of glucosinolate pathogen resistance in some brassica plants. J. Appl. Nat. Sci. 11, 556–567. https://doi.org/10.31018/jans.v11i2.2117.

FAO, 2020. Food and agriculture organization of the United Nations. food and agriculture data. http://www.fao.org/faostat/en/ (accessed 27 July 2022).

Fisher, M.C., Gurr, S.J., Cuomo, C.A., Blehert, D.S., Jin, H., Stukenbrock, E.H., Stajich, J. E., Kahmann, R., Boone, C., Denning, D.W., Gow, N.A.R., Klein, B.S., Kronstad, J.W., Sheppard, D.C., Taylor, J.W., Wright, G.D., Heitman, J., Casadevall, A., Cowen, L.E., 2020. Threats posed by the fungal kingdom to humans, wildlife, and agriculture. MBio 11, e00449–20. https://doi.org/10.1128/mBio.00449-20.

Flores-Córdova, M.A., Martínez Damián, M.T., Ángel, D.N., Rodríguez Pérez, J.E., Colinas León, M.T., Solís, J.M., 2013. Reduction in the *in vitro* germination of conidia of *Alternaria alternata* isolated from *Eruca sativa* with juice of broccoli. Rev. Mex. Fitopatol. 31, 180–190.

Godfray, H.C.J., Mason-D'Croz, D., Robinson, S., 2016. Food system consequences of a fungal disease epidemic in a major crop. Philos. Trans. R. Soc. 371, 20150467. https://doi.org/10.1098/rstb.2015.0467.

Gomez-Torres, O., Amatya, S., Kamberov, L., Dhaibar, H.A., Khanna, P., Rom, O., Yurdagul, A., Orr, A.W., Nunez, K., Thevenot, P., Cohen, A., Samant, H., Alexander, J.S., Burgos-Ramos, E., Chapa-Rodriguez, A., Cruz-Topete, D., 2022. SLAMF1 is expressed and secreted by hepatocytes and the liver in nonalcoholic fatty liver disease. Am. J. Physiol. Gastrointest. Liver Physiol. 323, G177–G187. https://doi.org/10.1152/ajpgi.00289.2021.

Handiseni, M., Jo, Y.K., Lee, K.M., Zhou, X.G., 2016. Screening brassicaceous plants as biofumigants for management of *Rhizoctonia solani* AG1-IA. Plant Dis. 100, 758–763. https://doi.org/10.1094/PDIS-06-15-0667-RE.

Handiseni, M., Cromwell, W., Zidek, M., Zhou, X.G., Jo, Y.K., 2017. Use of Brassicaceous seed meal extracts for managing root-knot nematode in bermudagrass. Nematropica 47, 55–62.

Hansen, M., Møller, P., Sørensen, H., de Trejo, M.C., 1995. Glucosinolates in broccoli stored under controlled atmosphere. J. Am. Soc. Hortic. Sci. 120, 1069–1074. https://doi.org/10.21273/JASHS.120.6.1069.

Herzallah, S., Holley, R., 2012. Determination of sinigrin, sinalbin, allyl-and benzyl isothiocyanates by RP-HPLC in mustard powder extracts. LWT 47, 293–299. https://doi.org/10.1016/j.lwt.2012.01.022.

Hiruma, K., Onozawa-Komori, M., Takahashi, F., Asakura, M., Bednarek, P., Okuno, T., Takano, Y., 2010. Entry mode–dependent function of an indole glucosinolate pathway in *Arabidopsis* for nonhost resistance against anthracnose pathogens. Plant Cell 22, 2429–2443. https://doi.org/10.1105/tpc.110.074344.

Hossain, M.S., Ye, W., Hossain, M.A., Okuma, E., Uraji, M., 2013. Glucosinolate degradation products, isothiocyanates, nitriles, and thiocyanates induce stomatal closure accompanied by peroxidase-mediated reactive oxygen species production in *Arabidopsis thaliana*. Biosci. Biotechnol. Biochem. 77, 977–983. https://doi.org/10.1271/bbb.120928.

Huang, J., Liu, Y., Han, F., Fang, Z., Yang, L., Zhuang, M., Zhang, Y., Lv, H., Wang, Y., Li, J., Li, Z., 2021. Genetic diversity and population structure analysis of 161 broccoli cultivars based on SNP markers. Hortic. Plant J. 7, 423–433. https://doi.org/10.1016/j.hpj.2021.05.002.

ISO-ISO 9167:2019-Rapeseed and rapeseed meals—determination of glucosinolates content—method using high-performance liquid chromatography. Available online: https://www.iso.org/standard/72207.html (accessed on 17 November 2020).

Jensen, L.M., Jepsen, H.K.S., Halkier, B.A., Kliebenstein, D.J., Burow, M., 2015. Natural variation in cross-talk between glucosinolates and onset of flowering in *Arabidopsis*. Front. Plant Sci. 6, 697. https://doi.org/10.3389/fpls.2015.00697.

Kaur, R., Geetanjali, R., Adarsh, P.V., 2011. Evaluation of antifungal and antioxidative potential of hydrolytic products of glucosinolates from some members of Brassicaceae family. J. Plant Breed. Crop Sci. 3, 218–228.

Kruger, D.H.M., Fourie, J.C., Malan, A.P., 2015. Control potential of Brassicaceae cover crops as green manure and their host status for *Meloidogyne javanica* and *Criconemoides xenoplax*. South Afr. J. Enol. Vitic. 36, 165–174.

Kumar, S., Singh, A., 2015. Biopesticides: present status and the future prospects. J. Fertil. Pest. 6, 100–129. https://doi.org/10.4172/jbfbp.1000e129.

Lara-Viveros, F.M., Nieto-Ángel, D., Nava-Díaz, C., Gutiérrez-Alonso, G., Ayala-Garay, Ó. J., Aguilar-Pérez, L.A., Martínez-Damián, T., 2014. Efecto del glucorafano aislado de floretes de brócoli sobre la germinación de esporas de *Colletotrichum gloeosporioides*. Rev. Fitotec. Mex. 37, 141–147.

Lazzeri, L., Curto, G., Leoni, O., Dallavalle, E., 2004. Effects of glucosinolates and their enzymatic hydrolysis products via myrosinase on the root-knot nematode Meloidogyne incognita (Kofoid et White) Chitw. J. Agric. Food Chem. 52, 6703–6707. https://doi.org/10.1021/jf030776u.

Li, H., Xia, Y., Liu, H.Y., Guo, H., He, X.Q., Liu, Y., Wu, D., Mai, Y., Li, H., Zou, L., Gan, R. Y., 2021. Nutritional values, beneficial effects, and food applications of broccoli (*Brassica oleracea* var. *italica* Plenck). Trends Food Sci. Technol. 119, 288–308. https://doi.org/10.1016/j.tifs.2021.12.015.

Ma, Y., Gentry, T., Hu, P., Pierson, E., Gu, M., Yin, S., 2015. Impact of brassicaceous seed meals on the composition of the soil fungal community and the incidence of Fusarium wilt on chili pepper. Appl. Soil Ecol. 90, 41–48. https://doi.org/10.1016/j.apsoil.2015.01.016.

Madloo, P., Lema, M., Francisco, M., Soengas, P., 2019. Role of major glucosinolates in the defense of kale against *Sclerotinia sclerotiorum* and *Xanthomonas campestris* pv. campestris. Phytopathol 109, 1246–1256. https://doi.org/10.1094/PHYTO-09-18-0340-R.

Major, N., Prekalj, B., Perković, J., Ban, D., Užila, Z., Ban, S.G., 2020. The effect of different extraction protocols on *Brassica oleracea* var. *acephala* antioxidant activity, bioactive compounds, and sugar profile. Plants 9, 1792. https://doi.org/10.3390/plants9121792.

Manyes, L., Luciano, F.B., Mañes, J., Meca, G., 2015. *In vitro* antifungal activity of allyl isothiocyanate (AITC) against *Aspergillus parasiticus* and *Penicillium expansum* and evaluation of the AITC estimated daily intake. Food Chem. Toxicol. 83, 293–299. https://doi.org/10.1016/j.fct.2015.06.011.

Mari, M., Iori, R., Leoni, O., Marchi, A., 1993. *In vitro* activity of glucosinolate-derived isothiocyanates against postharvest fruit pathogens. Ann. Appl. Biol. 123, 155–164. https://doi.org/10.1111/j.1744-7348.1993.tb04082.x.

Mari, M., Iori, R., Leoni, O., Marchi, A., 1996. Bioassays of glucosinolate-derived isothiocyanates against postharvest pear pathogens. Plant Pathol. 45, 753–760. https://doi.org/10.1046/j.1365-3059.1996.d01-5.x.

Martínez-Ballesta, M.D.C., Muries, B., Moreno, D.Á., Dominguez-Perles, R., García-Viguera, C., Carvajal, M., 2014. Involvement of a glucosinolate (sinigrin) in the regulation of water transport in *Brassica oleracea* grown under salt stress. Physiol. Plant. 150, 145–160. https://doi.org/10.1111/ppl.12082.

Mason-Sedun, W., Jessop, R.S., Lovett, J.V., 1986. Differential phytotoxicity among species and cultivars of the genus *Brassica* to wheat. Plant Soil. 93, 3–16. https://doi.org/10.1007/BF02377141.

Mayton, H.S., Olivier, C., Vaughn, S.F., Loria, R., 1996. Correlation of fungicidal activity of *Brassica* species with allyl isothiocyanate production in macerated leaf tissue. Phytopathol 86, 267–271.

Mazzola, M., Granatstein, D.M., Elfving, D.C., Mullinix, K., 2001. Suppression of specific apple root pathogens by *Brassica napus* seed meal amendment regardless of glucosinolate content. Phytopathol 91, 673–679. https://doi.org/10.1094/PHYTO.2001.91.7.673.

Mazzola, M., Brown, J., Izzo, A.D., Cohen, M.F., 2007. Mechanism of action and efficacy of seed meal-induced pathogen suppression differ in a Brassicaceae species and time-dependent manner. Phytopathol 97, 454–460. https://doi.org/10.1094/PHYTO-97-4-0454.

McDonald, B.A., Stukenbrock, E.H., 2016. Rapid emergence of pathogens in agro-ecosystems: global threats to agricultural sustainability and food security. Philos. Trans. R. Soc. 371, 20160026. https://doi.org/10.1098/rstb.2016.0026.

Meyer, M., Adam, S.T., 2008. Comparison of glucosinolate levels in commercial broccoli and red cabbage from conventional and ecological farming. Eur. Food Res. Technol. 226, 1429–1437. https://doi.org/10.1007/s00217-007-0674-0.

Miao, H., Lin, J., Zeng, W., Wang, M., Yao, L., Wang, Q., 2019. Main health-promoting compounds response to long-term freezer storage and different thawing methods in frozen broccoli florets. Foods 8, 375. https://doi.org/10.3390/foods8090375.

Miranda-Arámbula, M., Reyes-Chilpa, R., Anaya, L, A.L., 2021. Phytotoxic activity of aqueous extracts of ruderal plants and its potential application to tomato crop. Bot. Sci. 99, 487–498. https://doi.org/10.17129/botsci.2727.

Miranda-Rossetto, M.R., Shiga, T.M., Vianello, F., Lima, G.P.P., 2013. Analysis of total glucosinolates and chromatographically purified benzylglucosinolate in organic and conventional vegetables. LWT - Food Sci. Technol. 50, 247–252. https://doi.org/10.1016/j.lwt.2012.05.022.

Mitra, D., 2021. Emerging plant diseases: research status and challenges. In: Singh, K.P., Jahagirdar, S., Sarma, B.K. (Eds.), Emerging Trends in Plant Pathology. Springer, Singapore. https://doi.org/10.1007/978-981-15-6275-4_1.

Mitreiter, S., Gigolashvili, T., 2021. Regulation of glucosinolate biosynthesis. J. Exp. Bot. 72, 70–91. https://doi.org/10.1093/jxb/eraa479.

Mocali, S., Landi, S., Curto, G., Dallavalle, E., Infantino, A., Colzi, C., Lazzeri, L., 2015. Resilience of soil microbial and nematode communities after biofumigant treatment with defatted seed meals. Ind. Crop. Prod. 75, 79–90. https://doi.org/10.1016/j.indcrop.2015.04.031.

Neubauer, C., Heitmann, B., Müller, C., 2014. Biofumigation potential of Brassicaceae cultivars to *Verticillium dahliae*. Eur. J. Plant Pathol. 140, 341–352. https://doi.org/10.1007/s10658-014-0467-9.

Neubauer, C., Hüntemann, K., Heitmann, B., Müller, C., 2015. Suppression of *Verticillium dahliae* by glucosinolate-containing seed meal amendments. Eur. J. Plant Pathol. 142, 239–249. https://doi.org/10.1007/s10658-015-0607-x.

Ntalli, N., Adamski, Z., Doula, M., Monokrousos, N., 2020. Nematicidal amendments and soil remediation. Plants 9, 429. https://doi.org/10.3390/plants9040429.

Ochiai, N., Powelson, M.L., Dick, R.P., Crowe, F.J., 2007. Effects of green manure type and amendment rate on Verticillium wilt severity and yield of Russet Burbank potato. Plant Dis. 91, 400–406. https://doi.org/10.1094/PDIS-91-4-0400.

Oerlemans, K., Barrett, D.M., Suades, C.B., Verkerk, R., Dekker, M., 2006. Thermal degradation of glucosinolates in red cabbage. Food Chem. 95, 19–29. https://doi.org/10.1016/j.foodchem.2004.12.013.

Oka, Y., Shuker, S., Tkachi, N., Trabelcy, B., Gerchman, Y., 2014. Nematicidal activity of *Ochradenus baccatus* against the root-knot nematode *Meloidogyne javanica*. Plant Pathol. 63, 221–231. https://doi.org/10.1111/ppa.12079.

Phani, V., Khan, M.R., Dutta, T.K., 2021. Plant-parasitic nematodes as a potential threat to protected agriculture: Current status and management options. Crop Prot. 144, 105573 https://doi.org/10.1016/j.cropro.2021.105573.

Picchi, V., Migliori, C., Scalzo, R.L., Campanelli, G., Ferrari, V., Di Cesare, L.F., 2012. Phytochemical content in organic and conventionally grown Italian cauliflower. Food Chem. 130, 501–509. https://doi.org/10.1016/j.foodchem.2011.07.036.

Plaszkó, T., Szűcs, Z., Vasas, G., Gonda, S., 2021. Effects of glucosinolate-derived isothiocyanates on fungi: a comprehensive review on direct effects, mechanisms, structure-activity relationship data and possible agricultural applications. J. Fungi 7, 539. https://doi.org/10.3390/jof7070539.

Poveda, J., 2022. AtCube: performing pathogen-root infection tests on *Arabidopsis thaliana* in a completely controlled way. Physiol. Mol. Plant Pathol. 117, 101780 https://doi.org/10.1016/j.pmpp.2021.101780.

Poveda, J., Eugui, D., Velasco, P., 2020a. Natural control of plant pathogens through glucosinolates: An effective strategy against fungi and oomycetes. Phytochem. Rev. 19, 1045–1059. https://doi.org/10.1007/s11101-020-09699-0.

Poveda, J., Francisco, M., Cartea, M.E., Velasco, P., 2020b. Development of transgenic *Brassica* crops against biotic stresses caused by pathogens and arthropod pests. Plants 9, 1664. https://doi.org/10.3390/plants9121664.

Poveda, J., Velasco, P., de Haro, A., Johansen, T.J., McAlvay, A.C., Möllers, C., Rodríguez, V.M., 2021. Agronomic and metabolomic side-effects of a divergent selection for indol-3-ylmethylglucosinolate content in kale (*Brassica oleracea* var. *acephala*). Metabolites 11, 384. https://doi.org/10.3390/metabo11060384.

Rangkadilok, N., Nicolas, M.E., Bennett, R.N., Premier, R.R., Eagling, D.R., Taylor, P.W., 2002. Determination of sinigrin and glucoraphanin in *Brassica* species using a simple extraction method combined with ion-pair HPLC analysis. Sci. Hortic. 96, 27–41. https://doi.org/10.1016/S0304-4238(02)00119-X.

Rembiałkowska, E., 2016. In: Caballero, B., Finglas, P., Toldrá, F. (Eds.), Organic food: Effect on nutrient composition, in Encyclopedia of Food and Health, first ed. Academic Press–Elsevier, Oxford, UK, pp. 178–180.

Ren, Z., Li, Y., Fang, W., Yan, D., Huang, B., Zhu, J., Cao, A., 2018. Evaluation of allyl isothiocyanate as a soil fumigant against soil-borne diseases in commercial tomato (*Lycopersicon esculentum* Mill.) production in China. Pest Manag. Sci. 74, 2146–2155. https://doi.org/10.1002/ps.4911.

Renaud, E.N., Lammerts van Bueren, E.T., Paulo, M.J., van Eeuwijk, F.A., Juvik, J.A., Hutton, M.G., Myers, J.R., 2014. Broccoli cultivar performance under organic and conventional management systems and implications for crop improvement. Crop Sci. 54, 1539–1554. https://doi.org/10.2135/cropsci2013.09.0596.

Ríos, P., Obregón, S., de Haro, A., Fernández-Rebollo, P., Serrano, M.S., Sánchez, M.E., 2016. Effect of *Brassica* biofumigant amendments on different stages of the life cycle of *Phytophthora cinnamomi*. J. Phytopathol. 164, 582–594. https://doi.org/10.1111/jph.12482.

Ríos, P., González, M., Obregón, S., Carbonero, M.D., Leal, J.R., Fernandez, P., de Haro, A., Sánchez, M.E., 2017. *Brassica*-based seedmeal biofumigation to control *Phytophthora cinnamomi* in the Spanish "dehesa" oak trees. Phytopathol. Mediterr. 56, 392–399. https://doi.org/10.14601/Phytopathol_Mediterr-20771.

Robbins, R.J., Keck, A.S., Banuelos, G., Finley, J.W., 2005. Cultivation conditions and selenium fertilization alter the phenolic profile, glucosinolate, and sulforaphane content of broccoli. J. Med. Food 8, 204–214. https://doi.org/10.1089/jmf.2005.8.204.

Roubtsova, T., López-Pérez, J.A., Edwards, S., Ploeg, A., 2007. Effect of broccoli (*Brassica oleracea*) tissue, incorporated at different depths in a soil column, on *Meloidogyne incognita*. J. Nematol. 39, 111.

Šamec, D., Pavlović, I., Salopek-Sondi, B., 2017. White cabbage (*Brassica oleracea* var. *capitata* f. *alba*): botanical, phytochemical and pharmacological overview. Phytochem. Rev. 16, 117–135. https://doi.org/10.1007/s11101-016-9454-4.

Santana-Méridas, O., González-Coloma, A., Sánchez-Vioque, R., 2012. Agricultural residues as a source of bioactive natural products. Phytochem. Rev. 11, 447–466. https://doi.org/10.1007/s11101-012-9266-0.

Selim, S.M., Zayed, M.S., Atta, H.M., 2012. Evaluation of phytotoxicity of compost during composting process. Nat. Sci. 10, 69–77.

Silva, J.C.P., Campos, V.P., Barros, A.F., Pedroso, M.P., Terra, W.C., López, L.E., de Souza, J.T., 2018. Plant volatiles reduce the viability of the root-knot nematode *Meloidogyne incognita* either directly or when retained in water. Plant Dis. 102, 2170–2179. https://doi.org/10.1094/PDIS-01-18-0143-RE.

Singh, A., Dhiman, N., Kar, A.K., Singh, D., Purohit, M.P., Ghosh, D., Patnaik, S., 2020. Advances in controlled release pesticide formulations: prospects to safer integrated pest management and sustainable agriculture. J. Hazard. Mater. 385, 121525 https://doi.org/10.1016/j.jhazmat.2019.121525.

Smolinska, U., Horbowicz, M., 1999. Fungicidal activity of volatiles from selected cruciferous plants against resting propagules of soil-borne fungal pathogens. J. Phytopathol. 147, 119–124. https://doi.org/10.1046/j.1439-0434.1999.147002119.x.

Sousa, C., Pereira, D.M., Pereira, J.A., Bento, A., Rodrigues, M.A., Dopico-García, S., Valentão, P., Lopes, G., Ferreres, F., Seabra, R.M., Andrade, P.B., 2008. Multivariate analysis of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) phenolics: influence of fertilizers. J. Agric. Food Chem. 2008 (56), 2231–2239. https://doi.org/10.1021/jf073041o.

Stoin, D., Radu, F., Dogaru, D., 2007. Researches regarding the isolation, purification and analysis of sinigrin glucosinolate from *Brassica nigra* and *Armoracia rusticana*. Bull. USAMV Agric. 63, 77–82.

Tang, J., Niu, J., Wang, W., Huo, H., Li, J., Luo, L., Cao, Y., 2018. p-Aromatic isothiocyanates: synthesis and anti plant pathogen activity. Russ. J. Gen. Chem. 88, 1252–1257. https://doi.org/10.1134/S1070363218060348.

Tarini, G., Melo, A.S., Fontana, L.F., da Silva Júnior, E., Bolanho, B.C., Moreno, B.P., Dias-Arieira, C.R., 2020. Aqueous extracts of *Crambe abyssinica* seed cake: chemical composition and potential for nematode control. Ind. Crop. Prod. 156, 112860 https://doi.org/10.1016/j.indcrop.2020.112860.

Tierens, K.F.J., Thomma, B.P., Brouwer, M., Schmidt, J., Kistner, K., Porzel, A., Broekaert, W.F., 2001. Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of *Arabidopsis* to microbial pathogens. Plant Physiol. 125, 1688–1699. https://doi.org/10.1104/pp.125.4.1688.

Ugolini, L., Martini, C., Lazzeri, L., D'Avino, L., Mari, M., 2014. Control of postharvest grey mould (*Botrytis cinerea* Per.: Fr.) on strawberries by glucosinolate-derived allyl-isothiocyanate treatments. Postharvest Biol. Technol. 90, 34–39. https://doi.org/10.1016/j.postharvbio.2013.12.002.

Vallejo, F., Tomás-Barberán, F., García-Viguera, C., 2003. Health-promoting compounds in broccoli as influenced by refrigerated transport and retail sale period. J. Agric. Food Chem. 51, 3029–3034. https://doi.org/10.1021/jf021065j.

Vela-Corcía, D., Aditya Srivastava, D., Dafa-Berger, A., Rotem, N., Barda, O., Levy, M., 2019. MFS transporter from *Botrytis cinerea* provides tolerance to glucosinolate-breakdown products and is required for pathogenicity. Nat. Commun. 10, 1–11. https://doi.org/10.1038/s41467-019-10860-3.

Velasco, P., Rodríguez, V.M., Soengas, P., Poveda, J., 2021. *Trichoderma hamatum* increases productivity, glucosinolate content and antioxidant potential of different Leafy *Brassica* vegetables. Plants 10, 2449. https://doi.org/10.3390/plants10112449.

Vik, D., Mitarai, N., Wulff, N., Halkier, B.A., Burow, M., 2018. Dynamic modeling of indole glucosinolate hydrolysis and its impact on auxin signaling. Front. Plant Pathol. 9, 550. https://doi.org/10.3389/fpls.2018.00550.

Villalta, O., Wite, D., Riches, D.A., Guiano, J., Chandolu, V., Scoble, C., Donald, C., Porter, I.J., Mattner, S.W., 2016. The concentration of 2-propenyl glucosinolate in biofumigant crops influences their anti-fungal activity (in-vitro) against soil-borne pathogens. J. Agric. Chem. Environ. 5, 38–45. https://doi.org/10.4236/jacen.2016.51B006.

Wang, T., Li, Y., Bi, Y., Zhang, M., Zhang, T., Zheng, X., Huang, Y., 2020. Benzyl isothiocyanate fumigation inhibits growth, membrane integrity and mycotoxin production in *Alternaria alternata*. RSC Adv. 10, 1829–1837. https://doi.org/10.1039/C9RA09225K.

Warmington, R., Clarkson, J.P., 2016. Volatiles from biofumigant plants have a direct effect on carpogenic germination of sclerotia and mycelial growth of *Sclerotinia sclerotiorum*. Plant Soil 401, 213–229. https://doi.org/10.1007/s11104-015-2742-8.

Wu, X., Huang, H., Childs, H., Wu, Y., Yu, L., Pehrsson, P.R., 2021. Glucosinolates in *Brassica* vegetables: characterization and factors that influence distribution, content, and intake. Annu. Rev. Food Sci. Technol. 12, 485–511. https://doi.org/10.1146/annurev-food-070620-025744.

Xiang, N., S. Lawrence, K.S., 2016. Optimization of in vitro techniques for distinguishing between live and dead second stage juveniles of *Heterodera glycines* and *Meloidogyne incognita*. PLoS One 11, e0154818. https://doi.org/10.1371/journal.pone.0154818.

Yu, Q., Tsao, R., Chiba, M., Potter, J., 2007. Elucidation of the nematicidal activity of bran and seed meal of oriental mustard (*Brassica juncea*) under controlled conditions. J. Food Agric. Environ. 5, 374.

Zhang, M., Li, Y., Bi, Y., Wang, T., Dong, Y., Yang, Q., Zhang, T., 2020a. 2-Phenylethyl isothiocyanate exerts antifungal activity against *Alternaria alternata* by affecting membrane integrity and mycotoxin production. Toxins 12, 124. https://doi.org/10.3390/toxins12020124.

Zhang, Y., 2010. Allyl isothiocyanate as a cancer chemopreventive phytochemical. Mol. Nutr. Food Res. 54, 127–135. https://doi.org/10.1002/mnfr.200900323.

Zhang, Y., Li, S., Li, H., Wang, R., Zhang, K.Q., Xu, J., 2020b. Fungi–nematode interactions: Diversity, ecology, and biocontrol prospects in agriculture. J. Fungi 6, 206. https://doi.org/10.3390/jof6040206.

Zucconi, F., Pera, A., Forte, M., de Bertoldi, M., 1981. Evaluating toxicity of immature compost. BioCycle 22, 54–57.