J Plant Dis Prot (2025) 132:101
https://doi.org/10.1007/s41348-025-01099-w

SHORT COMMUNICATION

Broccoli (*Brassica oleracea* var. *italica*) biomass as a resource for obtaining glucosinolate extracts to control postharvest fungal diseases

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Received: 29 July 2024 / Accepted: 3 May 2025

Abstract
Broccoli (*Brassica oleracea* var. *italica*) is a crop of great agronomic and economic importance worldwide. Because its edible parts are the inflorescences, large quantities of non-commercial biomass are produced each year in the field and in the food industry. In order to develop a circular economy around the broccoli crop, the present work develops glucosinolates (GSL) extracts with antimicrobial capacity for postharvest use in tomato, apple and table white grape against fungal diseases produced by the pathogens *Botrytis cinerea*, *Alternaria alternata* and *Penicillium expansum*. GSL extracts from organic crop management reported a higher content of GSLs than conventional management. These extracts are not effective in the control of *A. alternata* and *P. expansum*, possibly due to the absence of sinigrin. Furthermore, the extracts were ineffective in the control of *B. cinerea* on table white grapes, possibly due to the non-climacteric fruit condition and an absence in the induction of ethylene-mediated plant defenses. However, intact GSL extracts were effective in controlling *B. cinerea* on apple, while the addition of myrosinase enzyme caused effectiveness also on tomato and apple. Therefore, obtaining GSL extracts with biopesticidal capacity against *B. cinerea* in postharvest could be a circular economy strategy for broccoli agriculture and industry.

Keywords Tomato Cherry · *Botrytis cinerea* · Myrosinase enzyme · Glucosinolate hydrolysis products · Glucobrassicin

Introduction

Broccoli (*Brassica oleracea* var. *italica*) is a crop belonging to the Brassicaceae or cruciferous family, of great agronomic and economic importance worldwide (Han et al. 2021). The harvested part of broccoli is the hypertrophic reproductive organs (floral head and stalk), which contribute important nutraceutical benefits to the diet (Han et al. 2021). In 2022, 1.4 million hectares of broccoli and cauliflower was grown worldwide, from which 26 million tons of edible product was harvested (FAOSTAT 2024). Broccoli harvesting begins when the flower primordia are uniformly blue green and protrude above the leaves, being the only part of the plant used for food (Bhattacharjee and Singhal 2018). Leaves in particular represent around 50% of total plant biomass in broccoli, thus producing a significant non-marketable biomass (Liu et al. 2018). The edible part of broccoli is called a "superfood" because of its vitamins (A, C and K), minerals (Ca, K and Fe), fiber, glucosinolates (GSLs) and phenolic compounds content, with important antioxidant, anti-inflammatory, anticancer, antimicrobial, metabolic disorder regulatory, neuroprotective and renoprotective effects on human health (Li et al. 2022; Syed et al. 2023). Specifically, broccoli presents very important quantities of GSLs, up to 2% of dry matter weight (Ilahy et al. 2020).

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Published online: 27 May 2025

GSLs are sulfur-rich secondary plant metabolites present in plants within the order Brassicales (Nguyen et al. 2020; Wu et al. 2021). Of the about 200 different GSLs described so far, crops of the genus *Brassica* present between 10 and 40 different ones (Bischoff 2021). These are classified into three groups, depending on the amino acid present in their structure, being aromatic (phenylalanine or tyrosine), indole (tryptophan) or aliphatic (alanine, leucine, isoleucine, methionine or valine) (Bischoff 2021). GSLs are mainly defensive secondary metabolites due to their antimicrobial and insecticidal capacity, being potent biocides against bacteria (Liu et al. 2021), fungi (Poveda et al. 2020), oomycetes (Poveda et al. 2020), nematodes (Eugui et al. 2022) and insects (Liu et al. 2021). This biocidal capacity is mostly caused as a consequence of the action of the system called GSL–myrosinase, based on the hydrolysis of GSLs by binding to the enzyme myrosinase after plant cell rupture or pathogen recognition. This GSL–myrosinase binding results in the chemical modification of these secondary metabolites to nitriles, epithionitriles, thiocyanates, oxazolidine-2-thiones and/or isothiocyanates, called as glucosinolate hydrolysis products (GHPs), with high biocidal activity (Chhajed et al. 2020). Specifically, against fungi, GSLs and GHPs act directly, causing damage to the cell membrane and cell wall (Poveda et al. 2020), or indirectly, by inducing plant defenses (Rodríguez et al. 2023).

In order to directly exploit the biological activity of GSLs, extracts from Brassicaceae plants with a high concentration of these metabolites are obtained (Cuellar-Núñez et al. 2020; Sheu et al. 2023). In the specific case of broccoli, it has been studied how the extraction methodology greatly conditions the activity of the extract and the profile of GSLs obtained, being mainly involved aspects such as temperature and the solvent used (Bojorquez-Rodríguez et al. 2022; Eugui et al. 2023). Glucosinolate extracts obtained from broccoli have been described as effective antifungals by inhibiting the in vitro growth of plant pathogenic fungi, such as *Alternaria alternata* and *Sclerotinia sclerotiorum*, also against *Rhizoctonia solani* if myrosinase enzyme is added to the extracts (Eugui et al. 2023).

With respect to postharvest plant pathogens, it is estimated that up to 50% of fruit and vegetables are lost, with fungi being the main causal agents (Poveda 2020). The main postharvest plant pathogenic fungi in fresh produce are *Botrytis cinerea*, *Penicillium* spp., *Colletotrichum* spp., *Alternaria* spp. and *Monilinia* spp. (Díaz-Urbano et al. 2023). Currently, the main control strategies for these pathogens in postharvest are based on physical (controlled atmosphere or UV radiation) and chemical (calcium chloride or bicarbonate) methodologies; however, biological strategies, such as antagonistic microorganisms or the use of plant extracts and/or fungicidal essential oils, are gaining prominence (Qadri et al. 2020; Díaz-Urbano et al. 2023).

The objective of this work is to characterize different GSL extracts from broccoli leaves as potential antifungal biopesticides against several postharvest pathogens in tomato, apple and table white grapes.

Materials and methods

Biological material

Cherry tomatoes (SAT Campos de Granada, Spain), Golden Delicious apple (Interlázaro, Spain) and Sugar Crisp table white grapes (Agronativa, Spain) were purchased at the local market and used as fresh postharvest products for fungal infection.

Botrytis cinerea (CECT 20973), *Alternaria alternata* (CRD 41/37/2019 JCYL 965) and *Penicillium expansum* (CECT 20906) from the Spanish Type Crop Collection (CECT) (Valencia, Spain) and from the Regional Diagnostic Center of the Junta de Castilla y León (CRD) (Salamanca, Spain) were used as postharvest pathogens.

GSL extracts obtaining and characterization

Broccoli leaves were used to obtain GSL extracts according to the methodology described in a previous work (Eugui et al. 2023). Plant tissues were obtained from broccoli fields grown under conventional conditions (fertilized with NPK 9-23-30 at 200 kg/ha before planting and then supplemented with two more fertilizations of ammonium nitrate 200 kg/ha) and grown under organic conditions (fertilized with two tons/ha of the organic fertilizer NPK 4-5-4 Fercrisa Biosuelo (Crisara S.L., Almería, Spain) before planting and another two tons/ha one month after planting). The leaves were stored at − 20 °C or − 80 °C for one month, until the extracts were obtained, using the cold methanol methodology. Starting from 100 mg of frozen plant material, samples were mixed with 1 ml of 75:25 methanol:water (v/v) in a 1.5-ml eppendorf tube and vortexed for 1 min 30 s at room temperature. The samples were incubated in the dark for 60 min at room temperature with shaking at 250 rpm and centrifuged at 2150 gs for 12 min to collect the supernatant. The methanol was evaporated at a rotary evaporator, and the samples were topped up to 1 ml with sterile deionized water and stored at − 80 °C until use.

Once the extracts were obtained, the GSLs profile was analyzed using the methodology previously 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 and identified by comparing retention times and UV spectra with the standards (Phytoplan Diehm & Neuberger GmbH, Heidelberg, Germany).

Study of the antifungal capacity of GSL extracts in postharvest

To study the protective antifungal effect of different GSL–broccoli extracts on tomato, apple and table white grape, the methodology described by Fernández-San Millán et al. (2022) was carried out, with some modifications. Initially, the fruits were washed with 70% (v/v) ethanol for 1 min, 1% (v/v) sodium hypochlorite for 15 min and 3 washes with sterile distilled water, finally leaving the fruits to dry in the laminar flow chamber for 15 min. Three wounds of 1 mm depth and 1 mm width were made on each fruit, around the insertion point of the peduncle. Subsequently, different treatments were applied, with 5 µl in each wound, leaving the fruit to dry for 15 min in the flow cabinet. The treatments were distilled water (as control), 1, 10 and 100% (v/v) dilutions of the four GSL extracts in distilled water, and those same dilutions adding myrosinase enzyme (methodology described at the end of this section) in order to check if the antifungal effect is carried out by the GSLs or the GHPs. Infections with the pathogens were carried out with 5 µl at 10^5 conidia/mL of each pathogen, keeping the fruits in sterile humid chambers at 25 ºC and in darkness. Each humid chamber contained 6 fruits in the case of Cherry tomato and table white grape and 2 fruits in the case of Golden Delicious apple, with 3 humid chambers per treatment. The entire experiment was repeated 3 times. After 7 days of incubation, disease incidence (DI) was evaluated as the percentage of infected wounds over the total. At 14 days, disease severity (DS) for Cherry tomato and table white grape was evaluated using a qualitative scale: 0 = fruit with no visible damage; 1 = 1 to 25% of the fruit damaged; 2 = 26 to 50% of the fruit damaged; 3 = 51 to 75% of the fruit damaged; 4 = 76 to 100% of the fruit damaged. The DS index was then calculated with the formula:

$$DS(tomato,\ grape) = \frac{\sum (No.\ infected\ fruits\ in\ each\ scale \cdot scale\ value)}{Total\ fruits \cdot highest\ scale\ value} \cdot 100$$

For Golden Delicious apple, the DS was calculated as follows: The lesion diameter for each wound was measured and the maximum diameter from each individual experiment was set as 100%, expressing the other values according to this:

$$DS(apple) = \frac{Lesion\ diameter}{Maximum\ lesion\ diameter} \cdot 100$$

A volume of 5 µL of myrosinase (E.C. 3.2.1.147 from *Sinapis alba*) (25 units/mL) (Sigma-Aldrich, Madrid, Spain) was added to 1 mL of broccoli extract and incubated 2 h at room temperature for the reaction to occur. The hydrolyzed extracts were used immediately afterward.

Statistical analysis

The statistical analysis of the data was carried out with the Statistix 8.0 software. To perform the data normality confirmation analysis, the Shapiro–Wilk test was performed. One-way ANOVA with Tukey's comparison t-tests at $p \leq 0.05$ was used in GSL analysis; significant differences are denoted using different letters. Student's t-test was used for comparison of means at $p \leq 0.05$ in fruit experiments; significant differences are denoted using one asterisk. The group means were represented in columns in the graphs, representing the standard error in the form of error bars.

Results

GSLs profile of the extracts

The analysis of GSLs reported that the extracts obtained from organic broccoli contained a significant higher content of total GSLs, aliphatic and indole GSLs. In particular, the extract obtained from organic broccoli stored at − 20 °C presented the highest content of GSLs of all 4 extracts (Fig. 1).

Protective effect of GSL extracts on postharvest fungal diseases

After applying different GSL extracts, no significant differences against the water control were reported for DI and DS in tomatoes infected with *A. alternata* and *P. expansum*, apples infected with *P. expansum* and table white grapes infected with *B. cinerea* (data not shown). Although different intact extracts used did not significantly reduce the incidence and severity of *B. cinerea* disease on tomatoes, compared to the control water treatment, the addition of myrosinase enzyme caused a significant reduction in the disease (Fig. 2). Specifically, GE1, GE2 and GE3 extracts incubated with myrosinase enzyme significantly reduced disease severity compared to the water control treatment (Fig. 2). On the other hand, the treatment of apples with different

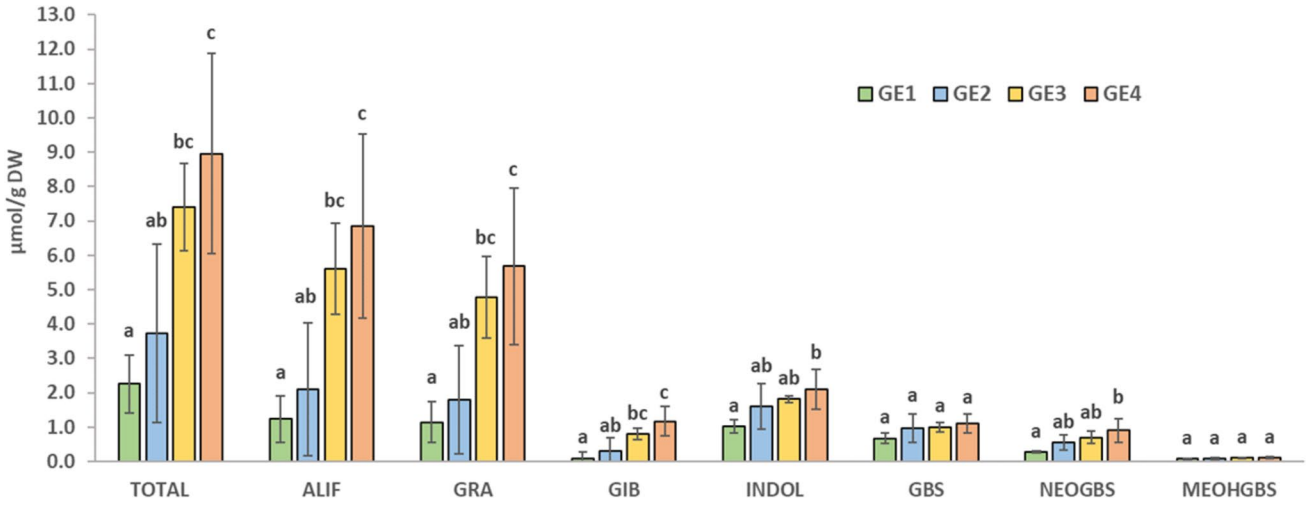

Fig. 1 Total, aliphatic and indole GSL content of different broccoli leaf extracts obtained. GE1: from conventional broccoli stored at −80 °C; GE2: from conventional broccoli stored at −20 °C; GE3: from organic broccoli stored at −80 °C; GE4: from organic broccoli stored at −20 °C. The amounts of total GLS (TOTAL), aliphatic (ALIF), indole (INDOL), glucoraphanin (GRA), glucoiberin (GIB), glucobrassicin (GBS), neoglucobrassicin (NEOGBS) and 4-methoxyglucobrassicin (MEOHGBS) were quantified. One-way ANOVA with Tukey's comparison t-tests ($p \leq 0.05$) was used in GSL analysis; significant differences are denoted using different letters. Data are represented as the means ± SE (n = 5)

GSL extracts (with and without myrosinase) also did not significantly reduce the DI caused by *B. cinerea* (Fig. 3). With respect to DS, the intact GSLs extracts GE1, GE3 and GE4 significantly reduced the disease affection of apples, as well as GE1 and GE4 after incubation with the myrosinase enzyme (Fig. 3).

Discussion

Broccoli GSLs extracts presented different biochemical profiles according to their agronomic management, conventional or organic. Specifically, a higher amount of GSLs was obtained from leaves under organic management. These results could be due to a higher N fertilization in conventional management, causing a higher vegetative growth and a lower accumulation of secondary metabolites in leaves, such as GSLs. However, the results reported in other works so far are very diverse. Higher contents of GSLs have been found in crops under conventional versus organic management (Robbins et al. 2005; Cámara-Martos et al. 2022), similar under both management (Renaud et al. 2014; Conversa et al. 2016) and even higher in organic management compared to conventional (Meyer & Adam 2008; Miranda-Rossetto et al. 2013). Therefore, more research is still needed to understand what causes and mechanisms are involved in these differences and each case needs to be studied individually.

Regarding the application of GSL extracts in the management of postharvest fungal diseases, neither the intact extracts nor their hydrolysis with myrosinase enzyme were effective in the control of *A. alternata* and *P. expansum* in tomato and apple. Many studies conducted with *Penicillium* and *Botrytis* have linked the fungicidal activity of GSL-based materials to the presence of AITC, a compound derived from sinigrin hydrolysis (Mari et al. 2002; Wu et al. 2011; Ugolini et al. 2014). In a previous work, a reduction in postharvest disease caused by both pathogens was achieved by the application of GHP allyl isothiocyanate (Brader et al. 2006). This GHP is the result of the hydrolysis of sinigrin, a GSL not present in our GSL extracts. The limited effectiveness of our extracts against *Botrytis* could have multiple explanations. The absence of sinigrin in our extracts could limit their effectiveness against this pathogen, given its derivative AITC has the most fungicidal potential against this pathogen, but other GSLs or compounds could be involved, and their concentration could be insufficient as well.

The intact GSL extracts used were effective only against *B. cinerea* on apple and after incubation with myrosinase on tomato and apple. Therefore, the release of GHPs from GSLs present in the extracts enhances their antifungal capacity. These results are contrary to those reported also in tomato with intact GSL extracts, effectively inhibiting *B. cinerea* (Damas-Job et al. 2023). However, they are consistent with the higher sensitivity of *B. cinerea* to GHPs than other pathogens reported in other postharvest work (Mari et al. 1993; Wu et al. 2011). Mechanisms of fungal inhibition by GSLs have been described in previous studies and include cell membrane damage, alteration of cell wall (Wang et al. 2020; Zhang et al. 2020), alteration of pathogen metabolism

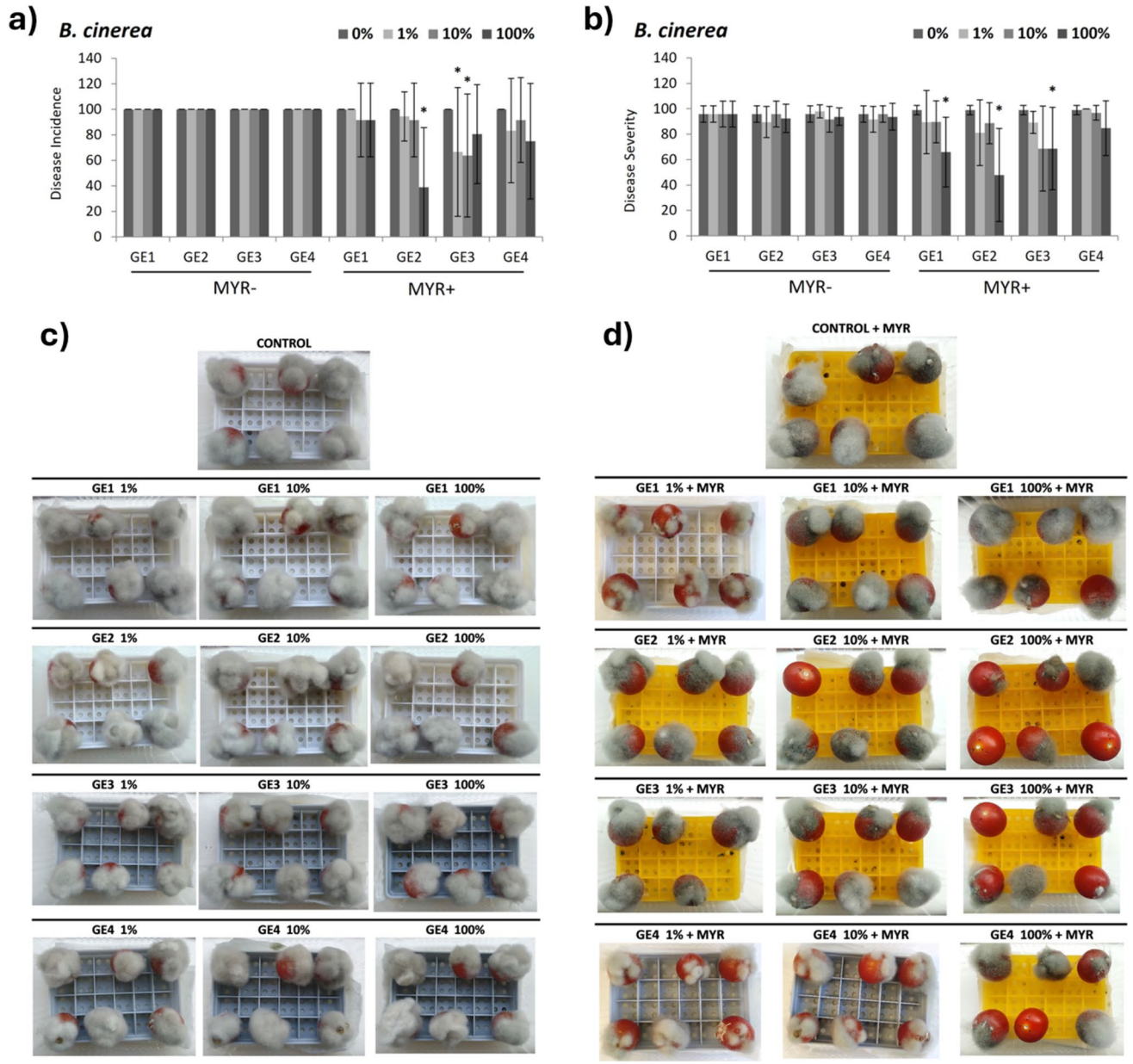

Fig. 2 DI (**a**) and DS (**b**) in tomatoes infected with *B. cinerea* and treated with different broccoli GSL extracts, without the addition of myrosinase enzyme (MYR-) or with the addition of myrosinase (MYR +). Photographs of infected tomatoes treated with GSL extracts without myrosinase (**c**) and with myrosinase (**d**). GE1: from conventional broccoli stored at − 80 °C; GE2: from conventional broccoli stored at − 20 °C; GE3: from organic broccoli stored at − 80 °C; GE4: from organic broccoli stored at − 20 °C. Student's t-test was used for comparison of means ($p \leq 0.05$) in fruit experiments; significant differences are denoted using one asterisk. Data are represented as the means ± SE (n = 18)

(Borges et al. 2015) and oxidative stress induction (Jakubikova et al. 2005). In any case, although in our study we have not been able to avoid the disease, the symptoms of the disease decrease and delay its appearance, which could be an interesting option to increase the shelf life of fruits and vegetables in a safer way for the consumer.

B. cinerea was more sensitive to GSL extracts on apples and tomatoes, but not on grapes. One of the mechanisms involved in the effectiveness of GSLs and GHPs in disease control in non-cruciferous plants has recently been described to be the activation of systemic defenses (Rodríguez et al. 2023). In the case of postharvest fruits, this defensive pathway could be activated by the ethylene route and its absence in a non-climacteric fruit such as grapes would reduce the biocontrol capacity of GSL extracts. Differences in sugar content in fruits could affect the virulence of *B. cinerea* and

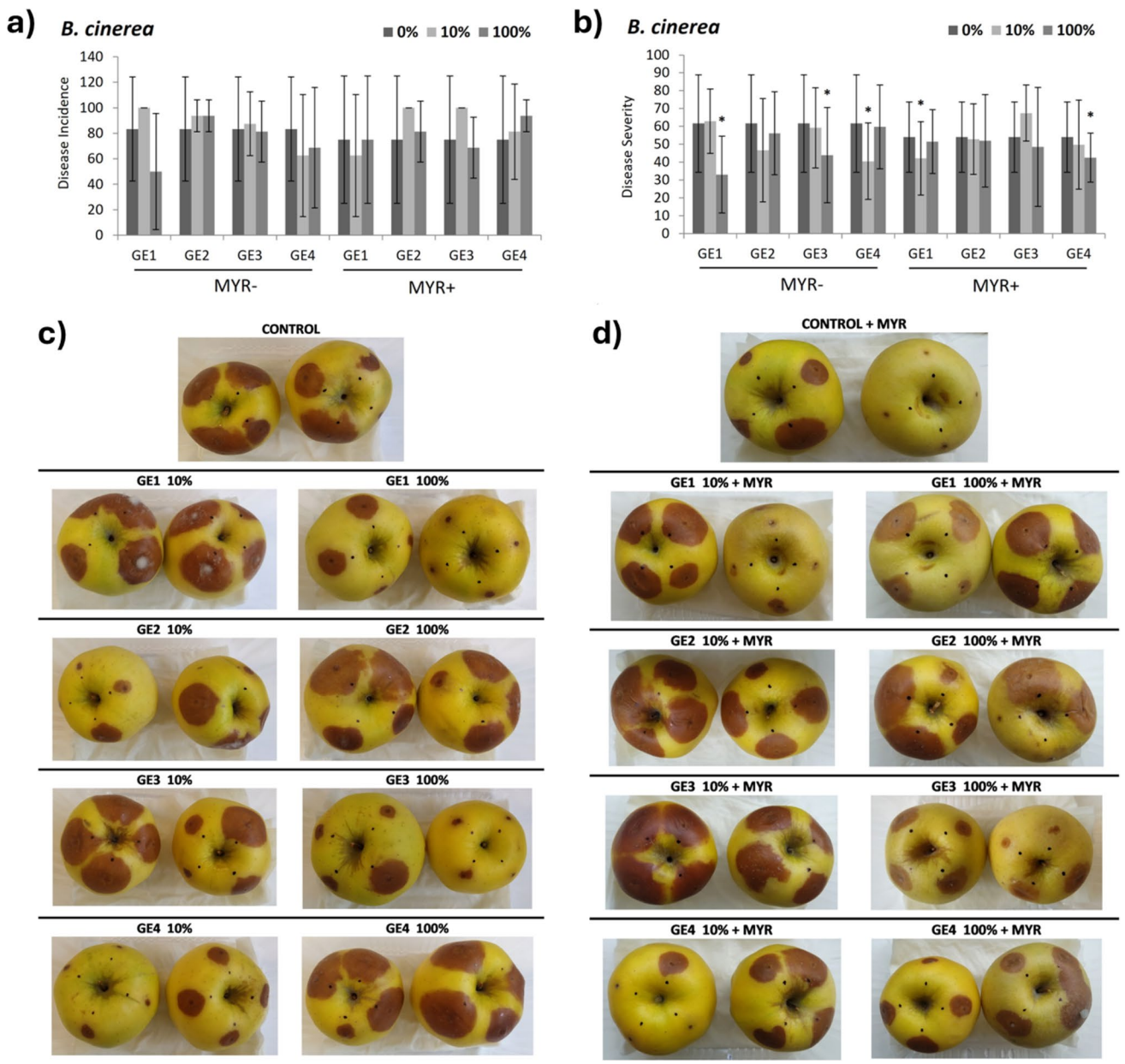

Fig. 3 DI (**a**) and DS (**b**) in apples infected with *B. cinerea* and treated with different broccoli GSL extracts, without the addition of myrosinase enzyme (MYR-) or with the addition of myrosinase (MYR+). Photographs of infected apples treated with GSL extracts without myrosinase (**c**) and with myrosinase (**d**). GE1: from conventional broccoli stored at −80 °C; GE2: from conventional broccoli stored at −20 °C; GE3: from organic broccoli stored at −80 °C; GE4: from organic broccoli stored at −20 °C. Student's t-test was used for comparison of means ($p \leq 0.05$) in fruit experiments; significant differences are denoted using one asterisk. Data are represented as the means ± SE (n = 6)

its ability to overcome GSLs toxicity as well, when using sugar as carbon sources in fruits could influence its activity (Vercesi et al. 1997).

In conclusion, organic management of broccoli crop could be involved in a higher content of GSLs in the extracts obtained. GSL extracts from broccoli are not effective against *A. alternata* and *P. expansum*, possibly due to the absence of sinigrin. However, intact GSL extracts, after hydrolisis, reduced the disease caused by *B. cienerea* on apple and on tomato.

Authors contribution D.E. performed the experiments and analyzed the data. J.P. and D.E. designed the research conducted. J.P. drafted the first version of the manuscript and supervised D.E.'s doctoral thesis. P.V. and V.M.R. contributed to the analysis of the GSL profile. A.F.S.M. and J.V. assisted in the postharvest studies. All authors contributed to

the proofreading and critical reading of the manuscript. All authors have read and accepted the published version of the manuscript.

Funding Open access funding provided by FEDER European Funds and the Junta de Castilla y León under the Research and Innovation Strategy for Smart Specialization (RIS3) of Castilla y León 2021-2027. D.E. is beneficiary of an Industrial Doctorate (DIN2018-009852) by the State Research Agency of Spain.

Declarations

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.

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