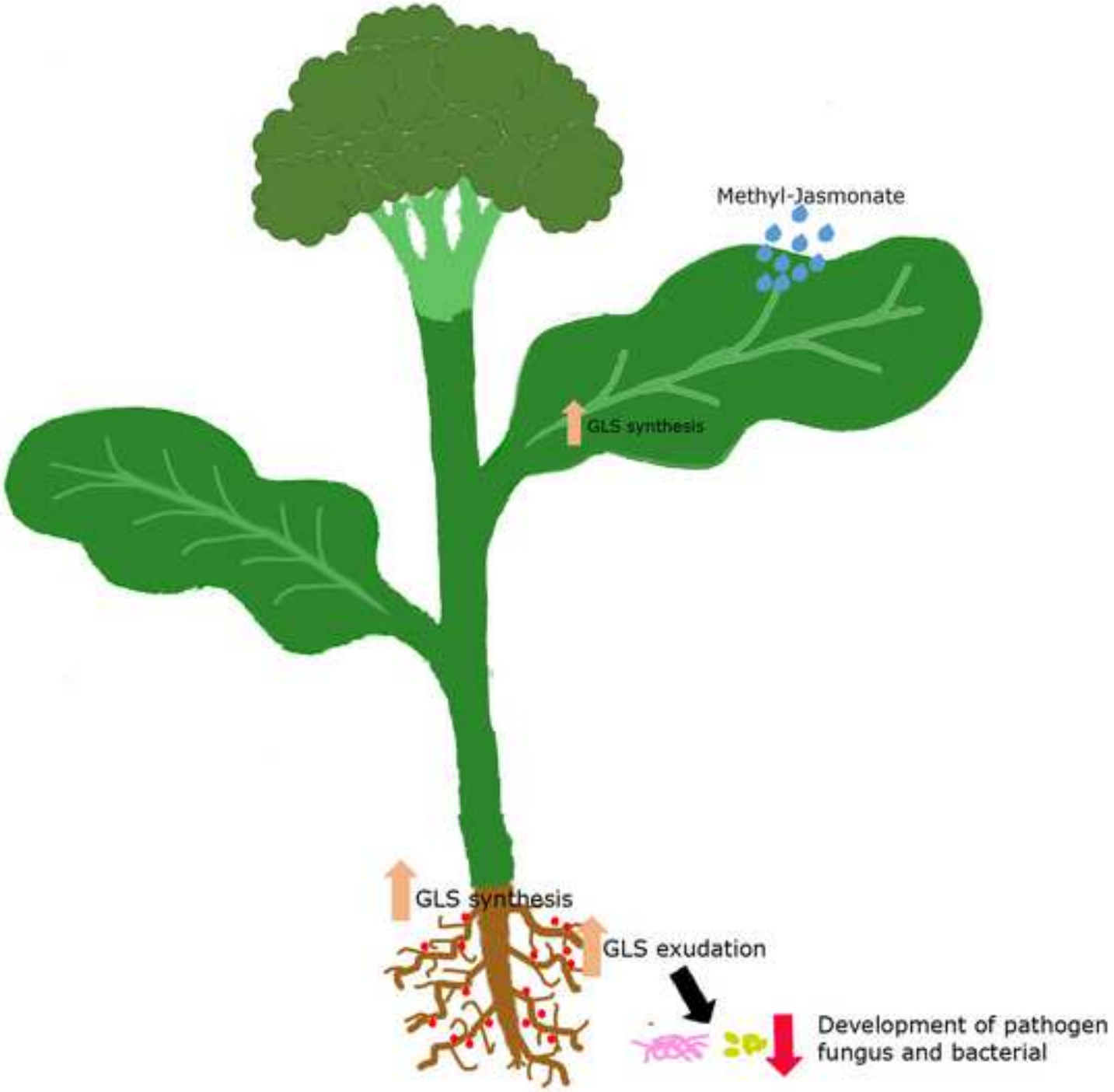
Graphical Abstract



Highlight

- Broccoli plants exudate secondary compounds.
- Foliar MeJA provoked a systemic response, enhance of exudation of these compounds.
- Exudates compounds show a strong effect on plant pathogen growth.

Title page

Influence of foliar Methyl-jasmonate biostimulation on exudation of glucosinolates and their effect on root pathogens of broccoli plants under salinity condition.

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Influence of foliar Methyl-jasmonate biostimulation on exudation of glucosinolates and their effect on roots pathogen of broccoli plants under salinity condition.

Abstract

The rhizosphere is the closest area of soil around plant roots. Root exudates are the main plant factor responsible for changes in microbial communities. As brassica plants have a defence system based on secondary metabolites such as glucosinolates (GLS) and isothiocyanates (ITC), the aim of this study was to determine the root exudation of these metabolites by broccoli plants and the influence of elicitors on this process. Also, the effect of these exudates on plant pathogens *Fusarium oxysporum*, *Pseudomonas syringae* and *Sphingomonas suberifaciens*. Broccoli plants were grown under controlled conditions. Biostimulation treatment with methyl-jasmonate (MeJA) with/without NaCl conditions were applied. The glucosinolates and isothiocyanates were measured in the leaves, roots and growth solution. Also, these compounds present in the exudates were applied to cultures of pathogens to check the effect on their growth. The results indicate that broccoli plants exude both GLS and ITC, and that foliar application of MeJA has a systematic positive effect, enhancing their biosynthesis and exudation by root cells, however, the biostimulation produced a less plant growth under salinity conditions. The intact GLS

plus the ITC detected in the growth solution showed a strong effect against the pathogens *F. oxysporum, P. syringae, S. suberifaciens*, providing the first evidence of their suppression to be implemented under agronomical conditions.

Keywords: biostimulation; exudate; glucosinolates; isothiocyanates plant pathogens; salinity.

1. Introduction

The rhizosphere is the soil immediately surrounding plant roots. Plants grown in natural environments have interactions with bacteria and fungi, and their roots are continuously in contact with the soil microorganisms present in the rhizosphere (Schmidt et al., 2016). Different rhizospheres have been described for crop plants such as lettuce, barley, maize and beet depending on the agricultural conditions, soil physiochemical properties, and plant metabolic stage (Sasse et al., 2018; Souri et al., 2019; Souri and Hatamian, 2019). Also, the effect of the roots on the microbial communities in the rhizosphere changes with the plant phenological stage (age) and environmental conditions (Chaparro et al., 2014; Shi et al., 2015).

Root exudates are the main driver of changes in rhizosphere microbial communities, since they determine the interactions among plants, microorganisms and solutes (Broeckling et al., 2008; Haichar et al., 2008). Exudates include many different compounds, varying from simple molecules to complex ones, such as sugars, organic acids, secondary metabolites and polymers (Sasse et al., 2018). In fact, different accessions of *Arabidopsis thaliana* L. showed variation with respect to secondary metabolites exudation in the same growth conditions (Monchgesang et al., 2016). Furthermore, changes along the plant life cycle have been reported; thus, sugar exudation by Arabidopsis plants decreased with increasing plant age, but the exudation of secondary compounds increased (Monchgesang et al., 2016). Biotic interactions also induce changes in the exudation of allelopathic compounds, biocidal compounds and other secondary compounds (Sasse et al., 2018; Souri and Neumann, 2009).

Thereby, some specific plant-pathogen interactions provoke systemic acquired resistance that could modify the microbial diversity in the rhizosphere (Hein et al., 2008). Members of the Brassicaceae have a defence system that involves the synthesis of secondary metabolites such as GLS. The GLS are sulphur- and nitrogen-containing glycosides and so far about 200 types have been described (Ishida et al., 2014). They can be considered chemically stable under optimal conditions (Fahey et al., 2001). However, in plants suffering biotic or abiotic stress, GLS can be released from the vacuole into the

cytoplasm, where they are easily hydrolysed by myrosinase (Petersen et al., 2002), producing ITC. The ITC are compounds with fungicidal, bactericidal, nematocidal and allelopathic properties (Sasse et al., 2018). They have also been reported as having anticancer and antioxidant properties in humans after their ingestion (Ávila et al., 2013).

The synthesis of GLS in broccoli (*Brassica oleracea* L.) leaves and florets can be induced by elicitors such as jasmonic acid, applied as MeJA or by abiotic stress conditions (Hassini et al., 2019; Yi et al., 2016). An enhanced of GLS synthesis have been observed under abiotic stress conditions as salinity in broccoli plants (Zaghdoud et al., 2016, 2012). Then changes on GLS concentration as response against salt conditions have been related to their possible role in plant defence metabolism under abiotic stress (Martínez-Ballesta et al., 2013). Although the GLS concentration and involved may vary according to the type of glucosinolates, and plant genotype (Rios et al., 2020). However, there aren´t studies that related how the elicitors could increase GLS synthesis and improve the plant tolerance under salinity conditions.

Kniskern et al., (2007) demonstrated that jasmonic and salicylic acids act as signal molecules, reducing the natural endophytic and epiphytic bacterial diversity in leaves of Arabidopsis. However, there is little research that demonstrates the systemic effect of these elicitors in all plant parts, particularly in the roots. Therefore, although in

Arabidopsis and *Brassica napus* L. roots GLS exudation has been demonstrated (Gimsing et al., 2006; JG et al., 2006), in *Brassica rapa* L. only an increase in GLS exudation when elicitors were applied directly to roots has been reported (Schreiner et al., 2011). The potential of these root exudates has not been widely studied. In this regard, there is strong interest in exploiting the root exudates of brassicas in agriculture, due to their use as break crops in rotation systems (Halkier and Gershenzon, 2006), based on the description of the antifungal and antibiotic effects of such exudates on the soil microbial community (Omirou et al., 2011). This would help to maintain rotation systems without high infection rates (Wang et al., 2012). However, few studies have focused on the power of the compounds present in root exudates against non-brassica-specific pathogens.

Fusarium oxysporum Schlectend. Fr is a fungus with more than 150 plant host species (Smolinska et al., 2003; Srinivas et al., 2019), affecting numerous agricultural crops and causing important economic losses such melon, tomato and lettuce, although it is not a common pathogenic fungus for the Brassicaceae (Garibaldi et al., 2002; Nirmaladevi et al., 2016; Sebastiani et al., 2017). In the same way, *Pseudomonas syringae* and *Sphingomonas suberifaciens* are pathogens bacteria common in crop soils, affecting tomato lettuce, in particular iceberg lettuces, pome fruits and citrus (Ivanović et al., 2017; Uppalapati et al., 2008; van Bruggen et al., 2015, 2014); and

could produce losses between 30 to 80% in field (O'Brien, 1992; Van Bruggen et al., 1993, 1990).

Recent legislation in Europe, aiming to reduce the use of chemical products in agriculture, has encouraged research into natural compounds that control soil pathogens. In this regard, as there are no studies of the effect of elicitors on GLS rhizo-secretion and the roles of GLS and ITC in the rhizosphere, the objectives of this work were (1) to determine GLS exudation by broccoli plants, (2) to elucidate the effect of MeJA on growth and on GLS exudation under different salt conditions (0 or 50 mM NaCl), and (3) to check the potential protective role of these elicited exudates against the common soil pathogens *F. oxysporum, P. syringae* and *S. suberifaciens*. For this, we have determined the plant biomass and the GLS concentrations in plant tissues and exudates. Also, fungal and bacterial growth after GLS and ITC application has been analysed.

2. Material and Methods

2.1. Plant growth conditions

Broccoli seeds (cv. Parthenon Commercial), provided by SAKATA Seed Ibérica (Valencia, Spain), were pre-hydrated for 24 hours, with aeration, before being sown in trays filled with vermiculite and incubated in a germination chamber at 28 °C. After 3 days, they were transferred to a controlled environment chamber with a 16-h light and

198 8-h dark cycle, with air temperatures of 25 and 20 °C, day/night. The
199 relative humidity (RH) was 60–80% and the photosynthetically active
200 radiation (PAR) was 400 μmol m^{-2} s^{-1}, provided by a combination
201 of fluorescent tubes (TLD36W/83, Philips, Hamburg, Germany and
202 F36W/GRO, Sylvania, Danvers, MA, USA) and metal-halide lamps
203 (HQI, T 400 W; Osram, München, Germany). After 5 days the
204 seedlings were transferred to 15-L containers, with five per container.
205 Each container was full of Hoagland solution (pH 5.5–6.0) that was
206 continuously aerated. The composition of the Hoagland solution was:
207 6 KNO_3, 4 $Ca(NO_3)_2$, 1 KH_2PO_4 and 1 $MgSO_4$ (mM), and 25 H_3BO_3, 2
208 $MnSO_4$, 2 $ZnSO_4$, 0.5 $CuSO_4$, 0.5 $(NH_4)_6Mo_7O_{24}$ and 20 Fe-EDDHA (μM).
209 The solution was replaced every week. The plants were grown for 28
210 days. After this period four treatments were applied:
211 (i) Control: plants were grown in Hoagland solution.
212 (ii) Salinity: plants were grown with 50 mM NaCl in the growth solution.
213 (iii) Foliar elicitor: plants were treated with 100 μM MeJA (SAFC, St.
214 Louis, MO, USA). It was dissolved in 0.2% ethanol, containing 0.1%
215 non-ionic, organo-silicon surfactant, and applied as a foliar spray.
216 (iii) Foliar elicitor and salinity: 100 μM MeJA (as above) plus 50 mM
217 NaCl in the growth solution.
218 The plants were grown for 10 days under the treatments
219 conditions. The foliar treatments were applied on days 1, 3, 5, 7 and
220 9. The application were realized early in the morning on full expanded
221 leaves until they were completely wet. Previous last application, the

plants were transferred into individual 1-L containers for one day, maintaining the treatments for collecting the exudates.

2.2. Plant harvest and exudate collection

At harvest, the shoots and roots were separated, washed three times with distilled water and blotted dry with filter paper. The samples were weighed to obtain the fresh weight (FW) and then, after being frozen in liquid nitrogen, they were freeze-dried. After this, the samples were weighed, to obtain the dry weight (DW), and then ground to a fine powder for analysis of GLS.

For exudate collection, the nutrient solution of each plant was totally frozen in liquid nitrogen and freeze-dried. Then, the solutes were resuspended in 70% methanol and filtered through a 0.22-µm PVDF filter, prior to analysis of GLS.

2.3. Glucosinolates analysis

The intact GLS and phenolic compounds were analysed according to Baenas et al., (2014). Briefly, 100-mg samples of freeze-dried, ground powder of shoots or roots were extracted in 1 mL of 70% methanol. The incubation of these samples and the exudate solutions lasted 30 min at 70 °C, with vortexing every 5 min. The tissue extracts were then centrifuged for 15 min at 13000 rpm, at 4 °C. The supernatants and exudate solutions were collected and the methanol was removed using a rotary evaporator; the dried residue

was re-dissolved in ultrapure MilliQ water, to the initial volume of the supernatant, and filtered through a 0.22-µm PVDF filter. The samples were kept in ice during the whole procedure. The separation of GLS was achieved on a Luna C18 100A column (250 x 5 mm, 5 µm particle size; Phenomenex, Macclesfield, UK). Ultrapure water (MilliQ) + 0.1% trifluoroacetic acid (TFA) and acetonitrile + 0.1% TFA were used as mobile phases A and B, respectively, with a flow rate of 1 mL/min. The linear gradient started with 1% solvent B; reaching 17% solvent B at 15 min, which was maintained to 17 min; 25% at 22 min; 35% at 30 min; and 50% at 35 min, which was maintained to 45 min. The injection volume was 20 µL. Chromatograms were recorded at 227 nm. The GLS were identified according to their UV spectra, order of elution and MS/MS fragmentations, as previously described for similar acquisition conditions, and were quantified using sinigrin and glucobrassicin (GB) as external standards for aliphatic and indole GLS, respectively (Phytoplan; Diehm & Neuberger, GmbH, Heidelberg, Germany).

2.4. Metabolites analysis

Measurement of metabolites in the leaves, roots and growth solutions (GLS; ITC) was performed, following their MRM transition, by a rapid, sensitive and high-throughput UHPLC-QqQ-MS/MS (Agilent Technologies, Waldbron, Germany) method. The protocol of Dominguez-Perles et al., (2014) was modified for the optimisation of

new compounds: sulforaphane (SFN), GB and indol-3-carbinol (I3C); assigning their retention times, MS fragmentation energy parameters and preferential transitions. For identification, the glucoraphanin (GRA) standard was obtained from Phytoplan (Diehm & Neuberger GmbH, Heidelberg, Germany) and ITC and indoles were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.5. Microbiological assays

To determine the effects of GLS and ITC on different pathogens, solutions from pure chemical compounds of 1, 2 and 4 mM, due to these concentrations could be reached the area closet to root epidermis, according with exudates data observed, were made of the main compounds detected in the exudates. Standard solutions of GB, GRA, SFN and I3C were filtered through a 0.22-µm PVDF filter to sterilise them.

2.6. Assay of F. oxysporum growth

To measure *F. oxysporum* growth, Petri plates with potato dextrose agar (PDA) were prepared and the different compounds were applied: 500 µL of GB, GRA, SFN, I3C and control (without any compound) (1 mM, 2 mM or 4 mM) were added on the surface of the medium and spread using a digralsky handle. Each treatment was replicated three times.

For this purpose, a *F. oxysporum* suspension was obtained by washing a 7-day *F. oxysporum* Petri dish culture with 5 mL of sterilised water. After this, 50 µL of this suspension were added in the middle of each assay plate. The plates were incubated at 28 ºC for 5 days. Each day, the fungal growth was measured by taking pictures and the growth area was determined using the ImageJ program.

2.7. *Assay of P. syringae and S. suberifaciens growth*

To measure the effect of different compounds on the growth of phytopathogenic bacteria, two common pathogens not specific to brassicas - *P. syringae* pv. *tomato* and *S. suberifaciens* - were selected. For 3 days, both were individually grown in Tryptone soya broth (TSB), in Erlenmeyer flasks that had been previously autoclaved for 20 minutes at 28ºC. In 24-well plates, 50 µL of each culture were added into 1 mL of TSB and 30 µL of each solution of the experimental compounds (1, 2 or 4 mM GR, GB, SFN or I3C) per well. Each treatment was replicated three times for each bacterium. After incubation at 28ºC for 5 days, the growth rate was measured by recording the absorbance at λ= 595 nm in a spectrophotometer.

2.8. Statistical Analysis

The statistical analysis of the production of GLS and ITC was carried out with 60 values (5 plants x 4 treatments x 3 analytical replicates). The analysis of biomass accumulation was carried out with 20 values

317 (5 plants × 4 treatments). The analysis of pathogen growth was
318 carried out with 15 values (3 wells x 5 treatment). All values were
319 analysed by one-way analysis of variance (ANOVA), at the 95%
320 confidence level, using the software SPSS Release 18 for Windows
321 (SPSS Inc., Chicago, IL, USA). The statistical significance was
322 considered as: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and n.s.
323 - not significant. Also, Duncan's test at $P \leq 0.05$ was chosen to
324 determine the significance of differences between treatments. The
325 values presented are the means ± SE.
326
327 **3. Results**
328
329 *3.1. Plant growth*
330
331 The results show differing effects of the elicitors on root and
332 shoot growth (Fig. 1). The application of all three treatments provoked
333 a significant decrease in root biomass with respect to control plants
334 (Fig. 1A); NaCl and the combined treatment (MeJA+NaCl) produced
335 reductions of 22% and 24%, respectively. However, in shoots, the
336 foliar application of MeJA did not produce a change in biomass, while
337 the other two treatments decreased shoot biomass relative to control
338 plants (Fig. 1B).

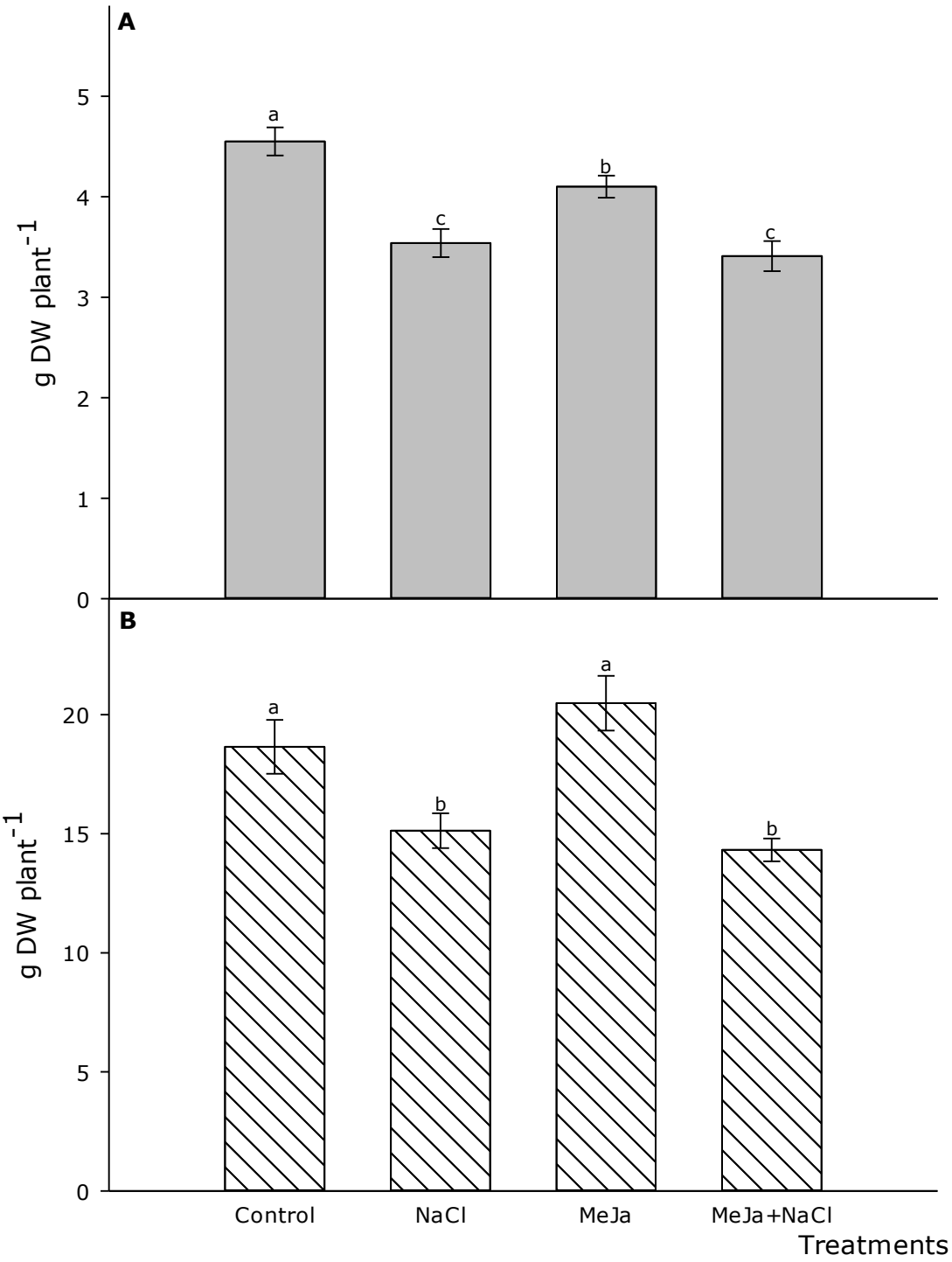

Fig. 1. Effects of different elicitors on the biomass of broccoli: (A) roots and (B) shoots. Bars with different letters show significant differences according to Duncan's test at $P<0.05$. Values are means ±SE (n=5).

344

345 *3.2. GLS concentrations in plants*

346

347 Table 1 shows the GLS concentrations in broccoli roots under the
348 different treatments. All treatments produced an increase in total GLS
349 with respect to control roots. Also, foliar application of MeJA and the
350 combined treatment provoked higher values than NaCl application
351 (Table 1). The enhancement of GLS accumulation with all the
352 treatments was observed mainly for indolics, methoxy glucobrassicin
353 (MGB) being the indole glucosinolate whose concentration increased
354 most with all treatments. On the other hand, the total aliphatic GLS
355 decreased with the combined treatment, which affected mainly the GR
356 concentration in roots. Also, the concentration of complex molecules
357 such as feruoyl-glucosinolate (F-GLS) was augmented only with the
358 MeJA treatment (Table 1).

359 All elicitors gave significant increases in total GLS in broccoli
360 leaves (Table 2). There was a strong enhancement of the
361 concentrations of all GLS with MeJA application, to values almost 3-
362 times the control plant concentrations, mainly for neoglucobrassicin
363 (NGB). Also, as in roots, we observed a greater effect on the
364 abundance of indole GLS than on aliphatic GLS. Application of MeJA,
365 alone or together with NaCl in the nutrient solution, provoked an
366 increase in F-GLS in broccoli leaves, while the NaCl treatment did not
367 affect this compound.

Table 1

GLS concentrations in broccoli roots under distinct elicitor treatments. Values are means ± SE (n=5)

Treatments	GRA (µmol/gDW)	PE (µmol/gDW)	GB (µmol/gDW)	MGB (µmol/gDW)	NGB (µmol/gDW)	F-GLS (µmol/gDW)	Total Aliphatic (µmol/gDW)	Total Indolic (µmol/gDW)	Total GLS (µmol/gDW)
Control	0.20 ± 0.03b	0.45 ± 0.07a	0.88 ± 0.08b	1.21 ± 0.19a	0.64 ± 0.16a	0.41 ± 0.05a	0.65 ± 0.07b	3.14 ± 0.18a	3.79 ± 0.23a
NaCl	0.18 ± 0.02b	0.56 ± 0.06b	1.27 ± 0.11c	2.20 ± 0.11b	0.87 ± 0.13b	0.43 ± 0.03a	0.74 ± 0.05c	4.77 ± 0.07b	5.51 ± 0.08b
MeJA	0.21 ± 0.03b	0.60 ± 0.15c	0.71 ± 0.07a	3.10 ± 0.32c	1.34 ± 0.12c	0.61 ± 0.11b	0.75 ± 0.12c	5.76 ± 0.46c	6.51 ± 0.61c
NaCl+MeJA	0.12 ± 0.02a	0.43 ± 0.07a	0.81 ± 0.17b	2.35 ± 0.24b	1.38 ± 0.10c	0.62 ± 0.15b	0.41 ± 0.10a	5.16 ± 1.35bc	5.57 ± 0.81c
P-value	**	**	*	***	***	***	**	**	**

GLS concentrations are expressed as µmol g^{-1} DW. GRA: glucoraphanin, PE: gluconasturtin, GBS: glucobrassicin, MGB: methoxyglucobrassicin, NGBS: neoglucobrassicin, F-GLS: cinnamoyl (feruloyl)-indol-GLS. Levels of significance: *P < 0.05, **P < 0.01 and ***P < 0.001. For each glucosinolate, different letters show significant differences according to Duncan's test at P<0.05.

Table 2

GLS concentrations in broccoli leaves under distinct elicitor treatments. Values are means ± SE (n=5).

Treatments	GRA (µmol/gDW)	GB (µmol/gDW)	MGB (µmol/gDW)	NGB (µmol/gDW)	F-GLS (µmol/gDW)	Total Aliphatic (µmol/gDW)	Total Indolic (µmol/gDW)	Total GLS (µmol/gDW)
Control	0.83 ± 0.05a	0.61 ± 0.18ab	2.44 ± 0.57ª	3.44 ± 0.41a	0.81 ± 0.10a	0.83 ± 0.05a	7.30 ± 0.60a	8.12± 0.64a
NaCl	1.63 ± 0.22c	1.04 ± 0.10c	3.83 ± 0.39b	3.95 ± 0.23a	0.78 ± 0.04a	1.63 ± 0.22c	9.59 ± 0.54b	11.22 ± 0.44b
MeJA	1.75 ± 0.23c	0.66 ± 0.09b	3.45 ± 0.32b	16.92 ± 0.62c	1.75 ± 0.24c	1.75 ± 0.23c	22.78 ± 0.45d	24.53 ± 0.37d
NaCl+MeJA	0.98 ± 0.02b	0.53 ± 0.03a	3.51 ± 0.13b	9.51 ± 0.51b	1.10 ± 0.08b	0.98 ± 0.02b	14.64 ± 0.48c	15.74 ± 0.34c
P-value	***	***	*	**	*	**	***	***

GLS concentrations are expressed as µmol g^{-1} DW. GRA: glucoraphanin, GBS: glucobrassicin, MGB: methoxyglucobrassicin, NGBS: neoglucobrassicin, F-GLS: cinnamoyl (feruloyl)-indol-GLS. Levels of significance: *P < 0.05, **P < 0.01 and ***P < 0.001. For each glucosinolate, different letters show significant differences according to Duncan's test at P<0.05.

3.3. Metabolites in exudates

The broccoli plants exuded GLS and/or ITC. In fact, among the metabolites identified in the exudates were MGB and F-GLS as GLS and SFN and I3C as ITC (Table 3). The total concentration of GLS in the exudate showed significant increases with the treatments that increased the compound exudate with respect to control plants. Also, MeJA and NaCl produced higher exudation of MGB. Moreover, the exudation of F-GLS was promoted by the combined treatment (NaCl+MeJA) and by MeJA application. The ITC abundance, especially that of SFN, was enhanced, relative to control plants, with all the treatments. Also, exposure to NaCl or the combined treatment (NaCl+MeJA) provoked higher values than MeJA application alone.

Table 3

GLS and ITC concentrations as exudates into the nutrient solution of broccoli plants under distinct elicitor treatments. Values are means ± SE (n=5).

	GLS			ITC		
Treatments	MGB (nmol/g root DW)	F-GLS (nmol/g root DW)	Total GLS (nmol/g root DW)	SFN (nmol/g root DW)	I3C (nmol/g root DW)	Total ITC (nmol/g root DW)
Control	5.70 ± 0.78a	2.66 ± 0.27a	8.36 ± 0.54a	0.069 ± 0.007a	0.013 ± 0.001a	0.082 ± 0.010a
NaCl	10.45 ± 0.52c	3.76 ± 0.52b	14.21 ± 0.20b	0.289 ± 0.017c	0.049 ± 0.007b	0.338 ± 0.013c
MeJA	11.56 ± 0.68c	6.26 ± 0.42c	17.82 ± 0.78c	0.125 ± 0.003b	0.040 ± 0.012b	0.165 ± 0.002b
NaCl+MeJA	8.65 ± 0.74b	5.86 ± 0.14c	14.51 ± 0.30b	0.238 ± 0.020c	0.109 ± 0.008c	0.347 ± 0.029c
P-value	**	**	***	***	***	***

GLS and ITC concentrations are expressed as nmol g^{-1} DW. MGB: methoxyglucobrassicin, F-GLS: cinnamoyl (feruloyl)-indol-GLS, SFN: sulforaphane and I3C: indol-3-carbinol.

Levels of significance: *P < 0.05, **P < 0.01 and ***P < 0.001. For each compound, different letters show significant differences according to Duncan's test at P<0.05.

Table 4.

Fusarium oxysporum growth under GLS or ITC treatments. Values are means ± SE (n=3).

Treatments (1 mM)	**Day 1** (cm^2)	**Day 2** (cm^2)	**Day 3** (cm^2)	**Day 4** (cm^2)	**Day 5** (cm^2)
Control	0.40±0.04a	1.80±0.20a	5.83±0.30a	14.59±0.46a	30.71±0.10a
GRA	0.35±0.03a	1.76±0.04a	5.67±0.25a	14.71±0.25a	30.17±0.27a
GBA	0.35±0.01a	1.83±0.03a	6.07±0.07a	14.83±0.16a	30.22±0.57a
SFN	0.00±0.00c	0.21±0.04c	0.532±0.08c	1.31±0.19c	5.42±0.92c
I3C	0.28±0.02b	1.60±0.04b	3.90±0.14b	8.78±0.05b	21.08±1.41b
P-value	***	***	***	**	**

Treatments (2 mM)	**Day 1** (cm^2)	**Day 2** (cm^2)	**Day 3** (cm^2)	**Day 4** (cm^2)	**Day 5** (cm^2)
Control	0.40±0.04a	1.80±0.20a	5.83±0.30a	14.59±0.46a	30.71±0.95a
GRA	0.39±0.03a	1.81±0.23a	3.95±0.60b	10.84±0.39ab	27.75±1.28a
GBA	0.49±0.12a	1.85±0.08a	4.45±0.24b	9.70±0.87b	29.20±1.09a
SFN	0.00±0.00c	0.00±0.00c	0.00±0.00d	0.13±0.07d	0.30±0.09c
I3C	0.21±0.01b	1.23±0.06b	3.30±0.13c	7.28±0.72c	12.78±1.71b
P-value	***	**	***	***	***

Treatments (4 mM)	**Day 1** (cm^2)	**Day 2** (cm^2)	**Day 3** (cm^2)	**Day 4** (cm^2)	**Day 5** (cm^2)
Control	0.40±0.04a	1.80±0.20a	5.83±0.30a	14.59±0.46a	30.71±0.95a
GRA	0.26±0.01b	1.13±0.05b	4.45±0.19b	8.52±0.72b	24.03±2.12b
GBA	0.29±0.01b	1.90±0.13a	5.75±0.51a	13.25±0.56a	18.69±0.21c
SFN	0.00±0.00d	0.00±0.00d	0.00±0.00d	0.00±0.00d	0.00±0.00c
I3C	0.15±0.03c	0.94±0.04c	2.74±0.28c	7.27±0.20c	14.87±0.47d
P-value	***	**	***	**	**

Fungal growth is expressed as cm^2 of mycelium on the Petri plate day^{-1}. Levels of significance: *P < 0.05, **P < 0.01 and ***P < 0.001. For each day, different letters show significant differences according to Duncan's test at P<0.05.

443 *3.4. Effects of metabolites on F. oxysporum growth*

444

445 The effects of GLS and ITC on *F. oxysporum* development are
446 shown in Table 4. The results indicate that at the concentration of 1
447 mM only SFN and I3C had inhibited fungal growth after 5 days of
448 incubation: SFN provoked a strong inhibition, approximately 82%, and
449 I3C, almost 30% inhibition (Table 4). The other compounds (GRA,
450 GBA) had no effect at 1 mM.

451 When the compounds were applied at 2 mM, the results showed
452 the same pattern after 5 days of incubation, although the inhibition of
453 growth was greater than at 1 mM. When compared with the control
454 plates, I3C inhibited fungal growth by 41.6%, while SFN inhibited it
455 almost completely, with a decrease of 99.02%.

456 In addition, at the highest concentration added (4 mM) a decrease in
457 fungal development was observed for all compounds. The results
458 indicate that GLS (GRA and GBA) were less effective than ITC (SFR and
459 I3C). Therefore, whereas GRA and GBA diminished fungal growth by
460 21.8 and 41.3%, respectively, I3C provoked a decrease of 55% and
461 SFN inhibited it completely during the 5 days of the experiment.

462

463 *3.5. Effects of metabolites on bacterial growth*

464

465 Both GLS and ITC had a statistically significant ($P<0.001$) and
466 negative effect on bacterial growth only at a concentration of 4 mM

467 (Fig. 2). In contrast, lower concentrations of these compounds had
468 little or no effect on bacterial growth (P>0.05: n.s.; data not shown).
469 In general, ITC showed higher efficiency than GLS. *S. suberifaciens*
470 was more sensitive than *P. syringae*, with lower growth rates under all
471 treatments after 5 days of incubation. In particular, GRA and GB
472 reduced growth of *S. suberifaciens* by almost 52% and growth of *P.*
473 *syringae* by 16.6% and 22.2%, respectively, with respect to the
474 untreated bacterial cultures. Also, bacterial growth was drastically
475 inhibited when SFN or I3C was added. *S. suberifaciens* growth was
476 decreased by approximately 89% for both ITC treatments, while
477 growth of *P. syringae* was reduced by 16% in the SFN treatment and
478 by 78% in the I3C treatment, with respect to the control cultures.
479

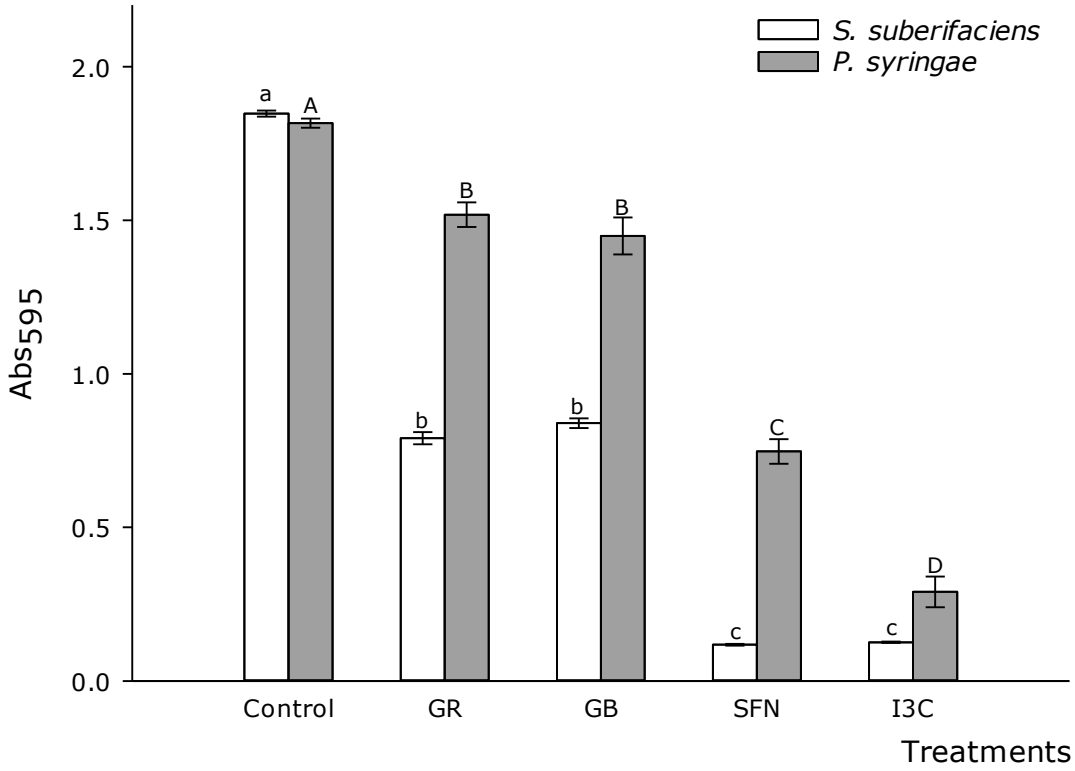

Fig. 2. Bacterial growth after 5 days of incubation, with different GLS or ITC added to the growth medium; expressed as turbidity at λ=595 nm. Bars with different letters show significant differences according to Duncan's test at $P<0.05$. Values are means ±SE (n=3).

4. Discussion

This study shows that elicitors can increase the biosynthesis of secondary compounds and have an impact on their exudation into the rhizosphere in a Brassica species, namely broccoli.

492 Elicitation in Brassica species has been studied in recent years (Chen
493 et al., 2017; Hassini et al., 2019). In our study, the effect on root or
494 shoot biomass differed according to the salinity treatment. Although
495 exogenous MeJA produced a slight reduction in root growth, no change
496 in shoot growth was observed. This could be due to the signalling role
497 of MeJA, as shown in other studies with *Brassica nigra*, *B. rapa* or
498 Arabidopsis (Chen et al., 2017; Schreiner et al., 2011; Van Dam et al.,
499 2004). Salts like NaCl can be used as elicitors (Schreiner et al., 2011)
500 but usually produce a negative effect on growth due to provoked salt
501 stress (Ahmadi and Souri, 2018; Hassini et al., 2019). The combination
502 of both MeJA and NaCl had a negative effect similar to that of NaCl
503 alone, pointing to a predominant effect of the NaCl stress. The
504 application of MeJA enhanced the biosynthesis of GLS (mainly indolic
505 ones) in our broccoli plants, yielding almost 3-times more GLS. It has
506 been reported that, in broccoli and turnip plants, content mainly *MYB34*
507 or *MYB51* promoters, which could be the target of MeJA, and enhanced
508 the transcripts abundance producing metabolism changes and a
509 strongly increased the indole GLS concentration (Hassini et al., 2019;
510 Yi et al., 2016).

511 The effect of MeJA on roots has been poorly studied. Only studies
512 in *B. rapa* and Arabidopsis showed an increase in GLS when MeJA was
513 applied to roots (Chen et al., 2017; Schreiner et al., 2011; Smetanska
514 et al., 2007). Therefore, the effect of the application of exogenous
515 MeJA in other parts of the plant distant from the treated area has not

been presented. Our results indicate that this effect is systemic, since the application of MeJA to leaves enhanced the GLS levels by 3-fold in leaves and by 2-fold in roots.

Several studies suggested that salt exposure raises the GLS concentration in Brassica species (Chen et al., 2017; Hassini et al., 2019; López-Berenguer et al., 2009; Rouphael et al., 2018). This is related to the protective roles of GLS against stress, such as NaCl, and it has been described as an adaptive response to salinity (Martínez-Ballesta et al., 2013). In our experiment, exposure of roots to NaCl produced an increase in GLS; the effects were systemic, affecting the entire plant. Although a biostimulation under salt condition increased the GLS concentration, finally the effect on growth plant weren't observed, maybe because the stress symptoms could be stronger than GLS roles or the effect could need more time of experimentation.

Due to the involvement of GLS in protection against biotic stress, the possible existence of GLS in root exudates of Brassica crops was suggested by Choesin and Boerner, (1991), since they found ITC in root exudates of *B. napus*. Later studies with the model plant Arabidopsis could not identify intact GLS in root exudates (Badri et al., 2013; Strehmel et al., 2014). However, Schreiner et al., (2011) detected intact GLS (aliphatic, indolic and aromatic) in root exudates of turnip plants. Also, these authors described increased GLS exudation after MeJA addition to the nutrient solution. However, the effects of the foliar application of elicitors on root exudation have not been described

until now; we have shown positive effects on GLS production and exudation.

The possible systemic effect of elicitors were observed by Chen et al., (2015) in Arabidopsis plants, where the authors described an increase of defence metabolism proteins and GLS on leaves close to those treated with MeJA. This phenomenon has been observed for the first time in our experiment with broccoli plants, suggesting that these elicitors could have a long systemic effect, not only in the nearby leaves but it reaches other tissues as the roots, or quite possibly the entire plants. Also, the elicitor effect would be produce on both GLS biosynthesis in plant cells and GLS-specific transport proteins, described recently in Arabidopsis by Xu et al., (2017). In the same way, only a recent previous study in Arabidopsis without elicitation (Xu et al., 2017), detected intact GLS and ITC detected in exudates at the same time. Our results show that proximate and more genetically evolved plants as broccoli maintain the capacity of exudes intact GLS and, also, complex compounds such as F-GLS that probably are more resistant to soil conditions. It is likely that GLS are exuded as intact chemical forms, by specific transporters in root exodermal cells, into the rhizosphere, or they could be released to rhizosphere in a passive form through cell debris and detached external cells (Souri and Neumann, 2009). Aliphatic GLS were not found intact; this could be because they were easily degraded to ITC by the effect of the pH of the growth solution or microorganisms within it (Grubb and Abel,

2006). In this sense, we obtained ITC in the form of SFN and I3C in the exudates. Therefore, there was strong enhancement of the presence of ITC (mainly SFN) due to the elicitors, suggesting conversion of this aliphatic glucosinolate to SFN in the rhizosphere, and that the effect of elicitors on both GLS and ITC could be determined by the soil conditions and microorganism population.

The importance of our results is due not only to the confirmation of the presence of GLS in root exudates of broccoli plants, but also to the impact of these compounds on the root microbiota, especially on common plant pathogens (two species of bacteria and one fungus). Compounds (GLS and ITC) exuded by broccoli roots had a strong, negative effect on three plant pathogens: *F. oxysporum, P. syringae* and *S. suberifaciens*.

Previous studies have determined the effects of GLS on these plant pathogens (Abdel-Farid et al., 2010; Prasad and Kumar, 2017; Smolinska et al., 2003). Smolinska et al., (2003) checked the effect of ITC alkylation on fungal growth and showed that propenyl and ethyl-ITC were more efficient against *F. oxysporum*. These authors found these compounds in shoots of three mustard species - *B. carinata*, *B. nigra* and *B. juncea* - although their profile and content in roots were not studied. Abdel-Farid et al., 2010 reported that an increase in GLS accumulation in tissues of *B. rapa* decreased fungal pathogenicity and infection. And Kurt et al., (2011), show antifungical effect of ITC from brassicas in vitro and in vivo against other fungus, such *Sclerotinia*

588 *sclerotiorum*, indicated the possible roles of these compounds as
589 biocontrol against pathogen fungus. These results confirm those of
590 previous studies (Abdel-Farid et al., 2010; Prasad and Kumar, 2017;
591 Smolinska et al., 2003), but we have focused on exuded compounds
592 to verify their fungus-biocontrol effect in the rhizosphere; their strong
593 effect at low concentrations could be achieved close to the roots.
594 However, none of these studies took into account the GLS or ITC profile
595 in the species studied, or if these compounds were exuded. Also,
596 anyone used GSL that could be more resistant and broke-down slowly
597 in soil, by soil proprieties effect, producing a longer time effect

598 Few studies have shown the bactericidal effect of ITC on
599 pathogenic bacteria that affect brassicas. In our study, although it was
600 performed in vitro conditions, an inhibitory effect of GLS and ITC was
601 demonstrated on common bacteria and non-bassicas specific bacteria.
602 It as shown significantly higher for ITC than for GLS, and it could be
603 extended to field conditions. In general, the ITCs are chemically very
604 reactive (Aires et al., 2009). They can react with the -SH group in
605 glutathione (thus affecting the redox status of cells) and in proteins
606 (e.g. potential enzyme and signal transduction pathway interactions),
607 forming dithiocarbamates. They can also react with -NH_2 groups of
608 proteins, forming thioureas, again potentially leading to the inhibition
609 of enzymes or effects on signal transduction pathways (Holst and
610 Williamson, 2004; Juge et al., 2007). Furthermore, a negative effect
611 of GLS on bacterial growth *in vitro* (in liquid TSB medium) has also

been demonstrated for the first time. The doses required to inhibit plant pathogen growth were higher for *F. oxysporum* than for both phytopathogenic bacteria; of the latter, *S. suberifaciens* was affected more than *P. syringae*. This can be attributed to the fact that the type of bioassay used for the fungus differed from that of the bacteria; this could have affected the way in which the compounds interacted with the microorganisms (Barth and Jander, 2006; GIAMOUSTARIS and MITHEN, 1995; Rask et al., 2000). Also, the effects would depend on the different characteristics of bacteria and fungi (GIAMOUSTARIS and MITHEN, 1995), and on the particular nature of each species. Although this current study does not show the mechanism underlying the resistance of the microorganisms, the sensitivity to the tested GLS and ITC may be related to the metabolic resistance to enzymatic inactivation, as was observed by Aires et al., (2009) in a similar study with different pathogens. Therefore, the potential of these compounds for plant pathogen biocontrol has been demonstrated under *in vitro* conditions; as mentioned above, they could be effective in the rhizosphere following exudation, but this would depend on their stability and hence on the soil conditions.

5. Conclusion

In conclusion, we have firstly evidenced that broccoli roots exude GLS as intact chemical forms. The fact that ITC also appeared could

636 indicate chemical degradation, direct root exudation or both. The
637 mechanism of exudation need to be elucidated but it is likely that they
638 reach the rizhosphere by specific transporters in root exodermal cells.
639 Also, elicitors – MeJA without other abiotic stress as sanility- provoked
640 an enhancement of their exudation due to the increase in metabolic
641 synthesis leaded by the rhizosphere dynamic flux. However, the
642 influence of GLS and ITC (MGB, SFN and I3C) reported in our work
643 could be a promising approach using elicited broccoli for its potential
644 phytosanitary effects.
645
646 **Author Contributions**
647 JJR, JAP, MC: Conception and design; JJR: Analysis and
648 interpretation of data and Drafting of the article; MC: Critical revision
649 of the article for important intellectual content; JJR: Final approval of
650 the article: JJR, ALM, MG: Provision of study materials or patients; ALM
651 and MG: Statistical expertise; MC: Obtaining of funding; MC and JAP:
652 Administrative, technical, or logistic Support; JJR: Collection and
653 assembly of data.
654
655
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JJR, JAP, MC: Conception and design; JJR: Analysis and interpretation of data and Drafting of the article; MC: Critical revision of the article for important intellectual content; JJR: Final approval of the article: JJR, ALM, MG: Provision of study materials or patients; ALM and MG: Statistical expertise; MC: Obtaining of funding; MC and JAP: Administrative, technical, or logistic Support; JJR: Collection and assembly of data.