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Glucosinolates in cauliflower as biochemical markers for resistance against downy mildew

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Abstract

Glucosinolate contents were quantified by HPLC in three susceptible (Billabong, Fanch and Jakavan) and two resistant (C300 and Maudez) cauliflower plants (*Brassica oleracea* var. *botrytis*) infected by *Peronospora parasitica* to determine a possible correlation between glucosinolates and resistance against downy mildew. Uninfected plants were used as controls. Three aliphatic (glucoïberin, sinigrin and glucoïberverin) and four indole glucosinolates (glucobrassicin, 4-methoxyglucobrassicin, 4-hydroxyglucobrassicin and neoglucobrassicin) were detected. Six days after inoculation with downy mildew spore suspension, the glucosinolate pattern of susceptible and resistant varieties differed. Sinigrin content was higher in resistant varieties than in susceptible ones. Glucobrassicin and methoxyglucobrassicin amounts expressed as a percentage to total indole glucosinolates were compared in infected and healthy seedlings. The susceptible seedlings exhibited a 12% decrease in glucobrassicin and a 25% increase in methoxyglucobrassicin when compared with healthy ones six days after treatment whereas no difference in glucobrassicin and a 10% increase in methoxyglucobrassicin were observed between healthy and inoculated resistant seedlings. Based on the ratio between methoxyglucobrassicin and glucobrassicin, resistant varieties were distinguished from susceptible ones. The susceptible varieties had a ratio greater than 1.5 but the resistant varieties showed a ratio of less than 1. This feature can be readily utilised as a biochemical inducible marker in screening seedlings resistant/susceptible to *P. parasitica*.

Keywords: *Brassica oleracea* var. *botrytis*; Cruciferae; Cauliflower; *Peronospora parasitica*; Downy mildew; Sinigrin; Indole glucosinolates

1. Introduction

Glucosinolates are secondary sulfur-containing metabolites found in every organ of cruciferous plants. The chemical nature of their side chain is divided into three classes: aliphatic, aromatic and indole compounds derived from methionine, phenylalanine and tryptophan. Myrosinase enzyme hydrolysis (thioglucosidase EC 3.2.3.1.) gives rise to products such as nitriles, thiocyanates and isothiocyanates. These breakdown products are known to contribute to the characteristic unpalatable flavor of Brassica crops and to cause toxic effects on animals and humans (Fenwick, Heaney & Mullin, 1983; Rosa, Heaney, Fenwick & Portas, 1997). In order to restrict these negative effects, Cruciferae crops such as oilseed rape with low glucosinolate contents were obtained by selective breeding. As greater susceptibility to diseases and pests was observed in these cultivars (Greenhalgh & Mitchell, 1976), glucosinolates and/or their breakdown products were suspected to participate in the resistance mechanisms. Indeed, their toxicity to insects, fungi or viruses has often been reported. Sinigrin and/or its breakdown products are known as powerful antifungal products. Mithen, Lewis and Fenwick (1986) have noticed their toxicity to *Leptosphaeria maculans*; Mari, Iori, Leoni and Marchi (1993) have observed that they completely inhibit the conidial germination of postharvest fruit

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Table 1
Variations of total glucosinolate contents, relative aliphatic and indole glucosinolate contents in three susceptible (S) and two resistant (R) cauliflower varieties, healthy and inoculated with *Peronospora parasitica*, at day 0 prior to inoculation (D0), day 3 (D3) and day 6 (D6) after treatment (Values represent ±sd of *n* replicates)

Glucosinolate type	Varieties	Disease response	D0 ($n = 6$)	Days after treatment			
				D3 ($n = 3$)		D6 ($n = 6$)	
				Healthy	Inoculated	Healthy	Inoculated
Total content (μmol g^{-1} dry wt)	Billabong	S	81 ± 8	61 ± 4	51 ± 5	47 ± 5	38 ± 6
	Fanch	S	65 ± 6	46 ± 4	46 ± 3	39 ± 4	35 ± 2
	Jakavan	S	96 ± 13	70 ± 10	69 ± 2	60 ± 7	42 ± 5
	C300	R	71 ± 6	52 ± 3	45 ± 3	44 ± 2	41 ± 1
	Maudez	R	104 ± 7	87 ± 4	87 ± 9	63 ± 4	59 ± 8
Aliphatic (%)	Billabong	S	83 ± 1	83 ± 1	82 ± 2	82 ± 1	75 ± 4
	Fanch	S	86 ± 2	85 ± 1	84 ± 1	84 ± 1	72 ± 4
	Jakavan	S	89 ± 2	88 ± 1	86 ± 1	87 ± 2	75 ± 2
	C300	R	87 ± 1	88 ± 1	86 ± 1	84 ± 1	81 ± 1
	Maudez	R	89 ± 0	89 ± 1	90 ± 1	89 ± 2	85 ± 2
Indole (%)	Billabong	S	17 ± 1	17 ± 1	18 ± 2	18 ± 1	25 ± 4
	Fanch	S	14 ± 2	15 ± 1	16 ± 1	16 ± 1	28 ± 4
	Jakavan	S	11 ± 2	12 ± 1	14 ± 1	13 ± 2	25 ± 2
	C300	R	13 ± 1	12 ± 1	14 ± 1	16 ± 1	19 ± 1
	Maudez	R	11 ± 0	11 ± 1	10 ± 1	11 ± 2	15 ± 2

pathogens. Other researchers have shown that turnip mosaic virus infectivity decreases when viral particles are suspended in a sinalbin-hydrolysed solution (Spak, Lewis & Fenwick, 1993). Several papers have dealt with the effects of indole glucosinolates or their products which are known to accumulate after wounding or pathogen attack (Bodnaryk, 1992; Doughty et al., 1991). It has been reported that the concentrations in glucobrassicin and neoglucobrassicin increased in response to infection by *Alternaria brassicae* (Doughty et al., 1991). Mechanical wounding or feeding by the flea beetle *Phyllotreta cruciferae* have also shown an increase in glucobrassicin and hydroxyglucobrassicin contents (Bodnaryk, 1992).

The present study investigated the possible correlation between the glucosinolate contents of cauliflower plants and the development of resistance to downy mildew. This disease induced by *Peronospora parasitica*, is an obligatory biotrophic organism, causing economic damages in horticultural and agricultural Brassica crops. The disease mainly affects young plants that may, in severe cases, be stunted or destroyed (Moss, Lucas & Crute, 1991; Silué, Nashaat & Tirilly, 1996). The glucosinolate contents in seedlings of three susceptible (Billabong, Fanch and Jakavan) and two resistant (C300 and Maudez) cauliflower plants (*Brassica oleracea* var. *botrytis*) were analyzed by HPLC and compared according to the susceptibility or resistance of the varieties.

2. Results

In the present study, seven main compounds common to the five cauliflower varieties were detected by HPLC. Comparing with other chromatograms previously described (Lewis, Fenwick & Gray, 1991; European Standard 9167-1, 1995; Spinks, Sones & Fenwick, 1984) and UV absorbance spectra (Quinsac, 1993), three aliphatic glucosinolates (glucoïberin, sinigrin and glucoïberverin) and four indole glucosinolates (glucobrassicin, 4-methoxyglucobrassicin, 4-hydroxyglucobrassicin and neoglucobrassicin) were identified. Another aliphatic compound identified as glucoerucin was detected in Maudez seedlings only.

The total glucosinolate concentration (Table 1) in untreated seedlings varied from 65 to 104 μmol g^{-1} dry wt without significant differences between varieties. Glucosinolate concentration regularly decreased at a 40% rate over the 6-day period. No significant differences were noticed between susceptible and resistant plants.

Percentages of indole-to-total glucosinolate were approximately 5-fold lower than aliphatic ones in the five varieties studied (Table 1). It was also observed that three days (D3) after inoculation, the percentages of indole compounds were very close to those determined in healthy plants (regardless of the susceptibility or resistance of these plants). In 6-day (D6) inoculated seedlings, the percentages of indole compounds were higher in both resistant and susceptible plants.

Table 2
Glucosinolate proportions (sinigrin, glucobrassicin, 4-hydroxyglucobrassicin and 4-methoxyglucobrassicin), in three susceptible (S) and two resistant (R) cauliflower varieties, at day 3 (D3) and day 6 (D6) after treatment (Values represent ±sd of *n* replicates)

Glucosinolates (%) in inoculated-to-healthy seedlings	Varieties	Disease response	Days after treatment	
			D3 (n = 3)	D6 (n = 6)
Sinigrin	Billabong	S	83 ± 10	44 ± 17
	Fanch	S	85 ± 2	53 ± 8
	Jakavan	S	81 ± 6	47 ± 9
	C300	R	83 ± 6	79 ± 2
	Maudez	R	98 ± 11	81 ± 11
Glucobrassicin	Billabong	S	70 ± 12	72 ± 9
	Fanch	S	116 ± 8	101 ± 13
	Jakavan	S	134 ± 26	91 ± 16
	C300	R	95 ± 14	114 ± 13
	Maudez	R	82 ± 8	144 ± 37
Hydroxyglucobrassicin	Billabong	S	64 ± 6	53 ± 9
	Fanch	S	46 ± 2	106 ± 24
	Jakavan	S	52 ± 6	71 ± 11
	C300	R	72 ± 7	67 ± 5
	Maudez	R	79 ± 5	82 ± 21
Methoxyglucobrassicin	Billabong	S	184 ± 28	262 ± 31
	Fanch	S	232 ± 10	291 ± 33
	Jakavan	S	232 ± 19	278 ± 31
	C300	R	142 ± 33	197 ± 19
	Maudez	R	155 ± 29	160 ± 26

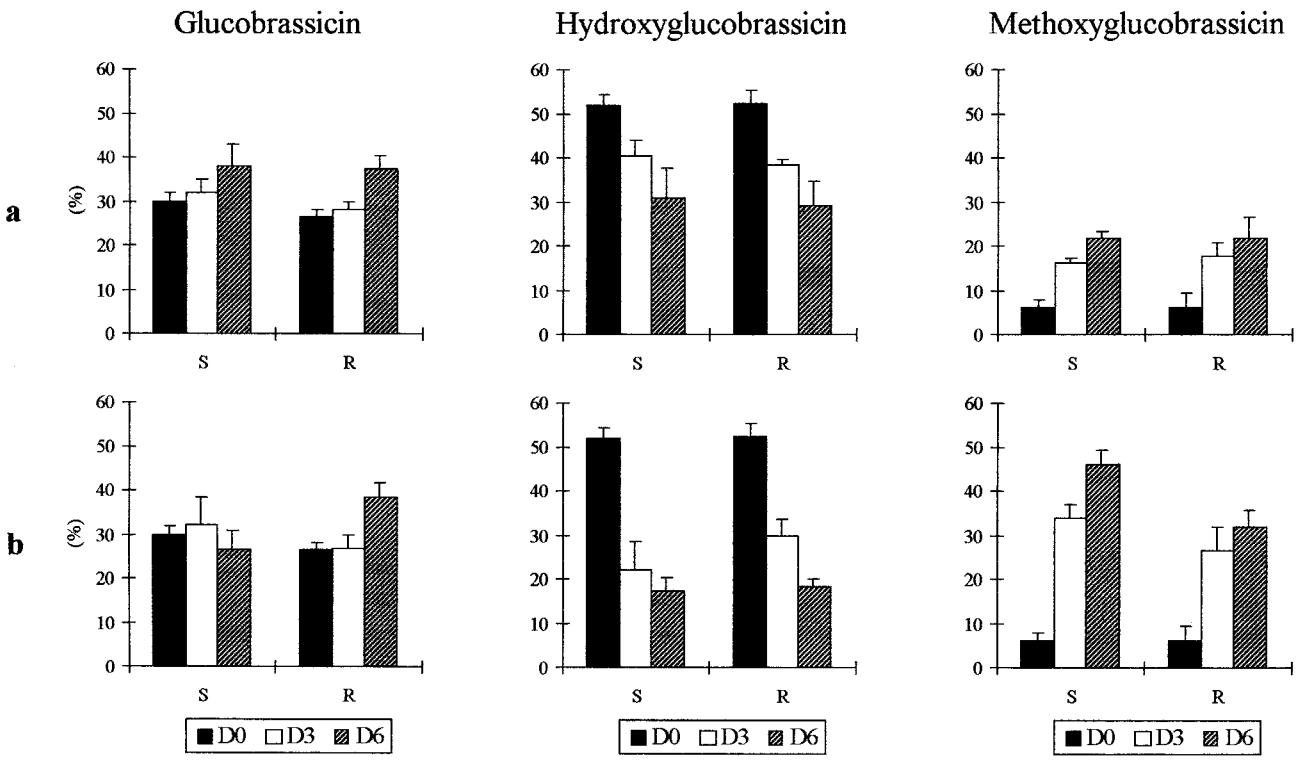

Fig. 1. Glucobrassicin, hydroxyglucobrassicin and methoxyglucobrassicin expressed in percentage of their content-to-total indole glucosinolates in three susceptible (S) and two resistant (R) varieties, healthy (a) and inoculated (b) by *Peronospora parasitica*.

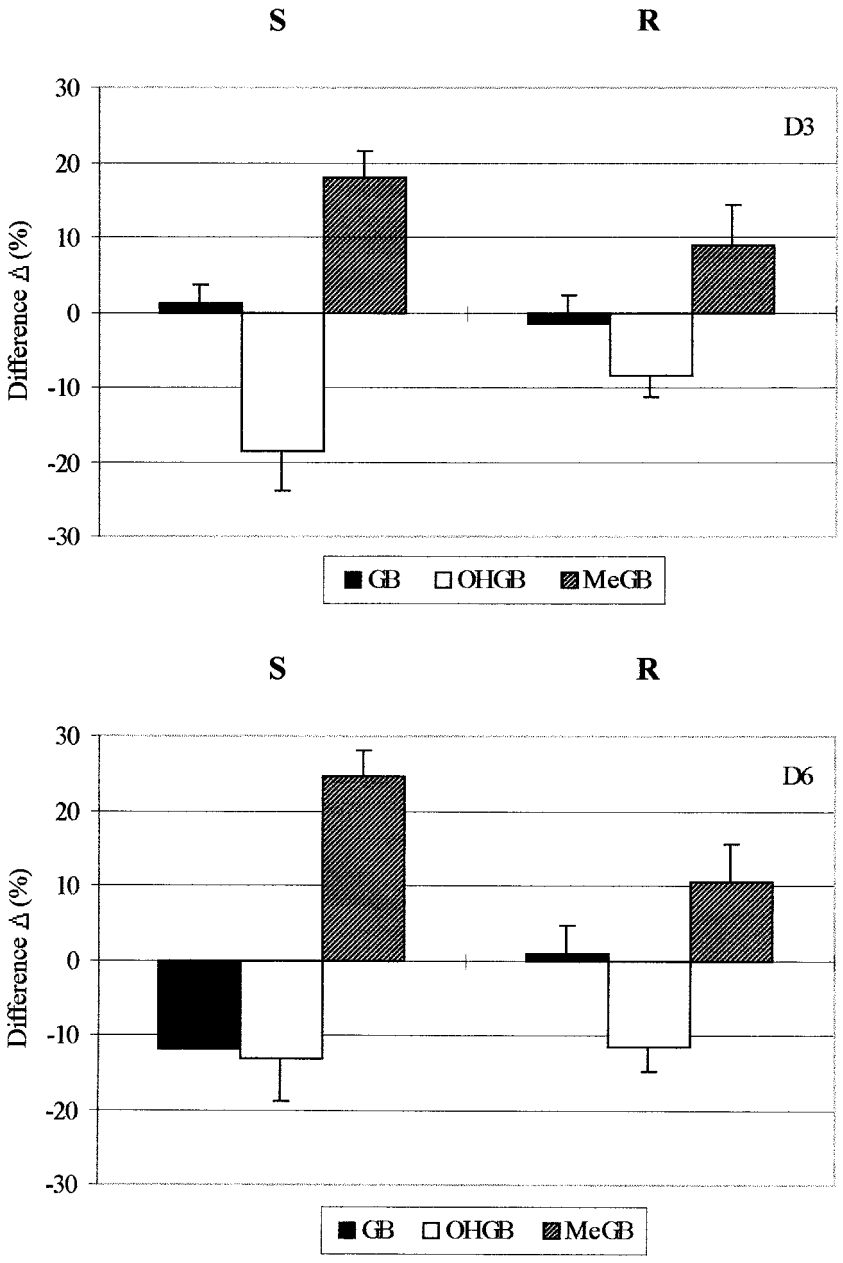

Fig. 2. Difference (Δ) between the percentages determined in healthy and inoculated seedlings (as shown in Fig. 1) for glucobrassicin (GB), hydroxyglucobrassicin (OHGB) and methoxyglucobrassicin (MeGB) at day 3 (D3) and day 6 (D6) after treatment. Values represent means of the three susceptible (S) and the two resistant (R) varieties.

Moreover, in susceptible varieties the percentages varied from 25% to 28%, and were thus significantly higher than in resistant ones, $P < 0.001$, which ranged from 15% to 19% only. Over the same period the percentages of aliphatic compounds decreased more in susceptible varieties than in resistant ones.

At D6 after inoculation the percentage decrease in aliphatic compounds was related to the reduction of sinigrin concentrations, major fraction in all the five varieties accounting for 20–57% of total glucosinolates. Sinigrin proportion expressed in percentage of healthy seedlings (Table 2) did not significantly vary between resistant and susceptible varieties at D3 whereas the difference ($P < 0.001$) was marked at D6. The three susceptible varieties contained about 50% sinigrin in infected seedlings when compared to healthy seedlings whereas the two resistant ones contained at least 80%.

Among indole glucosinolates glucobrassicin and hydroxyglucobrassicin proportions did not show any significant differences between susceptible and resistant varieties at D3 or D6 stages (Table 2). At later stages of inoculation, methoxyglucobrassicin proportion (Table 2) increased in all varieties. At D3 and D6

Table 3
Variations of methoxyglucobrassicin-to-glucobrassicin ratio six days after treatment (D6) in healthy and inoculated seedlings in three susceptible (S) and two resistant (R) varieties (Values represent ±sd of n replicates)

Varieties	Disease response	Six days after treatment (D6) ($n = 6$)	
		Healthy	Inoculated
Billabong	S	0.6 ± 0.1	2.3 ± 0.4
Fanch	S	0.6 ± 0.0	1.6 ± 0.2
Jakavan	S	0.5 ± 0.0	1.5 ± 0.1
C300	R	0.5 ± 0.1	0.9 ± 0.1
Maudez	R	0.7 ± 0.2	0.8 ± 0.2

methoxyglucobrassicin proportion was higher in susceptible varieties than in resistant ones: at D6, methoxyglucobrassicin proportion reached almost 300% in susceptible varieties whereas it did not exceed 200% in resistant ones.

The structural similarities between indole glucosinolates are such that possible conversions among these compounds may be possible. Variations in each indole glucosinolate-to-total indole pool in susceptible and resistant varieties are shown in Fig. 1.

The healthy susceptible and resistant seedlings contained a 38% glucobrassicin at D6. Glucobrassicin proportion did not change in resistant seedlings after inoculation (38%) whereas susceptible seedlings exhibited a lower glucobrassicin proportion (26%).

At D3 the decrease in hydroxyglucobrassicin percentage was more marked in infected seedlings than in healthy ones and was more pronounced in susceptible than in resistant varieties. However, at D6 there was no difference between susceptible and resistant varieties.

The increase in methoxyglucobrassicin percentage at D3 and at D6 was more marked after inoculation. The healthy susceptible and resistant seedlings contained a 21% methoxyglucobrassicin at D6. Susceptible seedlings showed a 46% proportion after inoculation whereas resistant ones showed a lower methoxyglucobrassicin proportion (32%).

For each of the parameter studied in Fig. 1, i.e. glucobrassicin-, hydroxyglucobrassicin- and methoxyglucobrassicin-to-total indole glucosinolates percentages, the difference (Δ) between the percentage in healthy seedlings and that observed in infected ones was determined and represented on Fig. 2.

It was observed that at D3 and D6, regardless of the varieties (susceptible or resistant), $\Delta_{\text{hydroxyglucobrassicin}}$ (ΔOHGB) decreased while $\Delta_{\text{methoxyglucobrassicin}}$ (ΔMeGB) increased. It was observed that ΔMeGB at D3 and D6 in susceptible seedlings was twice that determined in resistant ones. At D3, ΔOHGB decrease in susceptible varieties was twice that observed in resistant ones whereas at D6, ΔOHGB decreased similarly in all the varieties studied. On the other hand, at D3, $\Delta_{\text{glucobrassicin}}$ (ΔGB) is close to 0 in both susceptible and resistant varieties whereas at D6, ΔGB in susceptible seedlings was equal to (−12%) but remained close to 0 in resistant ones.

As the susceptible and resistant varieties differ in glucobrassicin and methoxyglucobrassicin proportions, a characteristic feature in differentiating susceptible from resistant could be determined by calculating the methoxyglucobrassicin-to-glucobrassicin ratio (Table 3). The ratio was nearly the same in susceptible and resistant healthy seedlings and never exceeded 0.7. It differed significantly ($P < 0.001$) at D6 after inoculation between susceptible and resistant varieties. It was greater than 1.5 in susceptible varieties and less than 1.0 in resistant ones. Therefore, susceptible infected seedlings had 3-times the ratio observed in susceptible healthy ones whereas the ratio remained unchanged between resistant inoculated and resistant healthy seedlings.

3. Discussion

The lower decrease in sinigrin observed in resistant varieties when compared with susceptible ones at D6 after inoculation may suggest first that the former group of varieties synthesizes more of this compound than the latter. Secondly it is possible that sinigrin is involved in the process of resistance to *P. parasitica*. Greenhalgh and Mitchell (1976) have already shown in in vitro studies, that sinigrin isothiocyanate was highly toxic to this fungus. If sinigrin-derived compounds are known as antifungal products, the role of indole glucosinolates in resistance mechanisms is not so well documented. Among the four indole glucosinolates detected throughout the study, only the toxicity of glucobrassicin and neoglucobrassicin and/or their breakdown products to *L. maculans* (Mithen et al., 1986) and to turnip mosaic virus (Spak et al., 1993) has been evidenced in vitro.

Ludwig-Müller, Schubert, Pieper, Ihmig and Hilgenberg (1997) have reported that after inoculation with *Plasmodiophora brassicae* indole glucosinolates increased in roots of two susceptible varieties of Chinese cabbage whereas no difference was observed between inoculated and healthy roots in two resistant varieties. The study reported here exhibited similar results. The percentages of indole glucosinolates were lower at D6 in inoculated resistant seedlings by comparison with inoculated susceptible ones where these recorded high levels resulted mainly from high contents of methoxyglucobrassicin. It can be hypothesised that indole glucosinolates degradation increased in resistant varieties which could produce other biochemical

compounds involved in downy mildew resistance. Indole glucosinolates can be included in a complex metabolic process (Rosa, Heaney, Rego & Fenwick, 1994) where they are not only considered as just metabolic end-products but also as precursors of molecules, such as phytoalexins (Hanley, Parsley, Lewis & Fenwick, 1990; Monde, Takasugi & Ohnishi, 1994) or auxins (Helminger, Rausch & Hilgenberg, 1983; Rausch, Butcher & Hilgenberg, 1983), known for their involvement in microorganism resistance.

Differences between resistant and susceptible varieties were mainly noticed at D6 after inoculation when indole glucosinolate variations were expressed in percentages. The comparison of glucosinolate proportions in healthy seedlings with those in infected ones (Figs. 1 and 2) showed lower variations in resistant varieties than in susceptible ones. Resistant varieties were characterised by only a slight increase in methoxyglucobrassicin proportion or no change in glucobrassicin proportion. Resistant varieties may control glucosinolate regulation better than susceptible ones by either reducing the synthesis of glucosinolates or increasing their degradation.

The conversion of a given indole compound into another one is possible because of close similarities in their chemical structures: glucobrassicin is known as the precursor of methoxyglucobrassicin, hydroxyglucobrassicin and neoglucobrassicin (Iqbal, Röbbelen & Möllers, 1995; McDanell, McLean, Hanley, Heaney & Fenwick, 1988). (It should be noted that the levels of the latter compound (data not given) were too low, i.e. <2.5 μmol g^{-1} dry wt, to detect significant differences). From the results in Fig. 2, it is likely that a hydroxylation of glucobrassicin into hydroxyglucobrassicin takes place which is further methylated into methoxyglucobrassicin. In resistant varieties, inoculation only modified the balance between hydroxyglucobrassicin and methoxyglucobrassicin proportions indicating a direct conversion between these two compounds while glucobrassicin proportion did not vary between healthy and inoculated seedlings (ΔGB close to 0). At D6 in susceptible varieties, inoculation modified not only hydroxyglucobrassicin and methoxyglucobrassicin proportions but also glucobrassicin one. Indeed, the variation of ΔMeGB ($+25\%$) was compensated by a variation of ΔGB (-12%) and of ΔOHGB (-13%) indicating that methoxyglucobrassicin could derive from both glucobrassicin (possibly via hydroxyglucobrassicin) and hydroxyglucobrassicin.

The metabolic differences in susceptible and resistant varieties, highlighted by glucosinolate or especially by methoxyglucobrassicin variations, result from their direct or indirect involvement in resistance mechanisms. Thus, methoxyglucobrassicin-to-glucobrassicin ratio is very fruitful to evidence resistance as illustrated by the present study which showed a ratio lower than 1.0 in resistant seedlings whereas it was greater than 1.5 in susceptible varieties. Extending this approach to other resistant and susceptible accessions will help us to conclude on the possible use of this ratio in cauliflower as a biochemical marker of resistance against downy mildew.

4. Experimental

4.1. Preparation of plant materials and fungal isolate

Seeds of Fanch, Jakavan, C300 and Maudez (*B. oleracea* var. *botrytis*) were provided by OBS (Organisation Bretonne de Sélection, Plougoulm, France) and seeds of Billabong by Limagrain (Paris, France).

Peronospora parasitica isolate (2389) originated from cauliflower crops in Yonne (France) was provided by Limagrain.

4.2. Inoculation process

Seeds sown in propagators and grown in a greenhouse were maintained at $25 \pm 2°C$ (day) and $18 \pm 2°C$ (night) with a 12 h/12 h light-dark regime. Seedlings were watered through the holes at the bottom of propagator tray. Seven day-old cauliflower seedlings were inoculated with two 20-μl droplets of spore suspension (20000 spores ml^{-1}) on each cotyledon (i.e. four per seedling). Experiment using distilled water was repeated in healthy plants which served as controls. Propagators were sealed and placed first in a dark growth room for 16 h followed by a 12 h/12 h photoperiod.

4.3. Sampling for glucosinolates analysis

For each variety and treatment, three successive samples were taken, the first one prior to inoculation and the other two on the third and sixth day after inoculation. For each sample, approximately 20 seedlings were cut and immediately frozen in liquid nitrogen. They were then freeze-dried for glucosinolate extraction. All the values presented in this study are the means of six experiments except the samples taken for three days after infection (only three replicates). LSD test was used for the statistical evaluation of data.

4.4. Glucosinolate extraction and analysis

One-hundred milligrams of each freeze-dried sample was crushed in liquid nitrogen with mortar and pestle. Glucosinolates were then extracted, desulfated and purified according to the EEC method (European Standard 9167-1, 1995). To increase recovery, desulfo-

glucosinolates were then eluted with distilled water (4×0.5 ml). HPLC analyses were performed using a 127P pump (Beckman, Fullerton, CA, USA) and a 166 UV-detector (Beckman) or a 168 diode array detector module (Beckman). Seventy-five μl of desulfoglucosinolate extract were injected onto 250×4 mm Lichrosorb RP 18 (5 μm) column at room temperature. The mixture of solvents used consisted of H_2O (A) and acetonitrile (B) with 0.7 ml min^{-1} flow rate. The gradient programme was: 7 min A–B (95:5), 3 min gradient to A–B (80:20), 13 min gradient to A–B (60:40), 3 min gradient to A–B (0:100), 5 min A–B (0:100) and 5 min gradient to A–B (95:5). Desulfoglucosinolates were detected at 229 nm. Glucotropaeolin isolated and purified from natural source (Hanley, Heaney & Fenwick, 1983) was used as internal standard in all the analyses. As glucoerucin and glucotropaeolin had the same retention time in HPLC, additional samples of Maudez seedlings were extracted, purified and desulfated without addition of internal standard to correctly estimate the concentration of glucotropaeolin.

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