

## THE ASSOCIATION OF *SCLEROTINIA SCLEROTIORUM* RESISTANCE WITH GLUCOSINOLATES IN *BRASSICA NAPUS* DOUBLE-LOW DH POPULATION

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### SUMMARY

Rapeseed, one of the most important oilseeds worldwide, suffers from *Sclerotinia* stem rot. Glucosinolates are important because their hydrolysates are physiological functional to plant growth and development and are poisonous to livestock. However, previous reports only emphasized predominant glucosinolates and ignored trace glucosinolates. In our study a double haploid oilseed rape population was produced to study its interactions with two isolates of *Sclerotinia sclerotiorum*. Eleven glucosinolates were identified and evaluated for their roles in plant-parasite interactions. It was found that the 2-hydroxy-4-pentenyl glucosinolate induced susceptibility to one fungal isolate, whereas the 1-methoxy-3-indole-methyl glucosinolate induced resistance to the other. In contrast with previous reports, total glucosinolate was not correlated with *Sclerotinia* resistance. On the other hand, partial correlation was used to understand the association of the biosynthesis between different glucosinolates. The association between seed glucosinolate content and *Sclerotinia* resistance as well as the association between different biosynthesizing pathways of different glucosinolates is discussed.

**Keywords:** *Brassica napus*, *Sclerotinia* resistance, seed glucosinolates, host-pathogen relationship.

### INTRODUCTION

Oilseed rape (*Brassica napus* L.) is one of the most important oilseed crops worldwide. Most of current cultivars have “double-low” quality (zero erucic acid and low total glucosinolate), but earlier such varieties were more susceptible to stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary than those with a high glucosinolate content (Liu, 2000).

Stem rot is the most devastating disease of rapeseed in China, causing yield losses of 10 to 80% and low oil

quality (Oilcrop Research Institute, Chinese Academy of Sciences, 1975). This is believed to be due largely to their low glucosinolate content deriving from the Polish spring cultivar Bronowski (Liu, 2000; Zhao and Meng, 2003b). However, the situation has begun to improve because of the introduction of the new sources of *Sclerotinia* resistance from Chinese native cultivars. For example, the double-low restorer line of the elite cv. Zhongshuang 9 was released as *Sclerotinia* resistant (Wang *et al.*, 2004).

Glucosinolates present in crops of the family Brassicaceae have fungicidal and bactericidal properties which can give resistance against fungal pathogens (Zukalová and Vašák, 2002). However, some degradation products present in seeds can be toxic to animals. Thus, it would be desirable to reduce glucosinolate components that are toxic to livestock and increase those that can strengthen the resistance through breeding programs.

In previous studies (Sodhi *et al.*, 2002; Hill *et al.*, 2003; Zhao and Meng, 2003a, 2003b), a high and a low glucosinolate line of *B. napus* were used to investigate the genetics of total glucosinolate content and the relation between specific glucosinolate components and *Sclerotinia* resistance. In these studies, trace glucosinolate components were masked by the prevailing ones or were overlooked by the authors, although they probably played an important role in disease resistance or in plant growth and development. Furthermore, few studies have addressed the genetic relation between different glucosinolate components.

The objective of this study was to investigate the relationship between different glucosinolate components and resistance to *S. sclerotiorum* using *B. napus* lines with low total glucosinolate content.

### MATERIALS AND METHODS

**Plant material.** Two lines of *B. napus* with double-low quality in seeds and different resistance to *S. sclerotiorum* were used, i.e. the susceptible P28 line, kindly provided by Prof. Yang [Huazhong Agriculture University (HAU), China] and the highly resistant B11 line, se-

lected from cv. Zhongshuang 9 (Wang *et al.*, 2004).

In spring 2003 microspores from F1 plants of the cross P28×B11 were cultured as reported by Shi and Liu (1993) to produce a double-haploid (DH) population consisting of 77 genotypes. Each genotype was self-pollinated to produce enough seeds in the spring 2004. The 77 lines, along with the two parents and the high glucosinolate resistance control Zhongyou 821 (Li *et al.*, 2001), were planted in autumn 2004 using a randomized complete block design with two replicates in the Huazhong Agricultural University (HAU) Experiment Station. Each replicate was composed of 80 plots with 30 plants in three rows. The distance was 0.3 m between rows and 0.18 m between plants within rows.

**Assessment of resistance to *S. sclerotiorum*.** Two isolates of *S. sclerotiorum*, coded BN and SF, were used. BN was isolated from severely infected rapeseed stems in the HAU experimental field, whereas SF was from sunflower (kindly provided by Mr. W. Zhang, Department of Plant Science and Technology, HAU). Previous investigations (Li *et al.*, 2000) had shown that the two isolates belonged in different genetic groups. *S. sclero-*

*tiorum* was routinely grown on potato-dextrose-agar (PDA) medium.

Stem resistance to *S. sclerotiorum* was assayed using the tooth-pick method for inoculation (Zhao and Meng, 2003a). To distinguish BN from SF, toothpicks for BN were painted black before culturing. The plants grew in HAU experimental fields. In spring 2005, after flowering, nine plants were selected randomly and were inoculated with BN and SF isolates. Lesion length was measured at the 3<sup>rd</sup>, 5<sup>th</sup>, and 7<sup>th</sup> day after inoculation.

**Glucosinolate analysis.** Healthy non-inoculated plants were self-pollinated and seeds were harvested. Samples from two plants were thoroughly mixed before glucosinolate analysis. Total seed glucosinolate content and components were detected by high performance liquid chromatography (HPLC). The HPLC protocol conformed to the national standard of P.R.China 'ISO9167-1:1992'. Seed glucosinolate contents were expressed as  $\mu\text{mol g}^{-1}$ .

**Statistical analysis.** The mean lesion length was used as the index of resistance level. SAS 8.0 was used to

**Table 1.** Total seed glucosinolate content and average concentration of eleven components detected in the two parents (P28 and B11) and in the control (Zhongyou 821).

Glucosinolate		Content ( $\mu\text{mol g}^{-1}$ )		
Class	Side chain R-	P28	B11	Zhongyou 821
Aliphatic	2-hydroxy-3-butenyl	3.75 (28.43%)*	3.57 (28.58%)	39.43 (54.02%)
	2-hydroxy-4-pentenyl	0.19 (1.44%)	0.00	1.40 (1.92%)
	Methylsulfoxide-amyl	0.27 (2.05%)	0.72 (5.76%)	0.47 (0.64%)
	3-butenyl	2.09 (15.85%)	1.57 (12.57%)	23.62 (32.36%)
	4-pentenyl	0.03 (0.23%)	0.00	0.48 (0.66%)
Aromatic	Phenylethyl	1.62 (12.28%)	0.61 (4.88%)	0.48 (0.66%)
	Benzyl	0.33 (2.50%)	0.57 (4.56%)	0.00
Indolic	4-hydroxy-3-indole-methyl	3.36 (25.47%)	4.71 (37.71%)	2.73 (3.74%)
	3-indolyl-methyl	1.29 (9.78%)	0.58 (4.64%)	2.20 (3.01%)
	4-methoxy-3-indole-methyl	0.1 (0.83%)	0.06 (0.48%)	2.12 (2.90%)
	1-methoxy-3-indole-methyl	0.15 (1.14%)	0.10 (0.80%)	0.06 (0.08%)
Total		13.19	12.49	72.99

\* Data in brackets are the percentage of total glucosinolate content in seeds.

**Table 2.** Pearson correlation and partial correlation between the glucosinolate components.

	HyBut	HyPen	MetA	But	HyMetI	Pen	Ben	MetI	Phe	4MIM	1MIM
HyBut		0.1193	0.1918	0.5543**	-0.0684	0.0721	0.3662**	0.0939	0.3178	0.0805	-0.0685
HyPen	0.4119**		0.2832*	-0.0374	0.2184	-0.1390	-0.0104	-0.3242**	0.0998	0.4622**	-0.1360
MetA	0.7435**	0.4606**		0.4160**	-0.0362	0.0630	0.2695*	0.0106	-0.0705	-0.1481	0.1470
But	0.7862**	0.3243**	0.7463**		0.0473	0.0587	-0.2231	-0.0645	0.1229	-0.1128	-0.0674
HyMetI	0.0652	0.1856	0.0517	0.0526		0.4515**	0.0671	0.4984**	0.2652*	0.2538*	0.0381
Pen	0.0605	-0.1180	0.0425	0.0906	0.5704**		-0.1794	0.1299	-0.2619*	-0.1065	0.1247
Ben	0.5503**	0.2963**	0.5210**	0.3601**	-0.0478	-0.1433		-0.0444	-0.0786	-0.0176	-0.0261
MetI	-0.0671	-0.2663*	-0.1205	-0.0875	0.5658**	0.5226**	-0.1424		-0.1866	0.0051	-0.0018
Phe	0.1212	0.2408*	0.1351	0.1744	0.1593	-0.1353	0.0136	-0.1706		-0.2661*	0.4756**
4MIM	0.0156	0.4724**	-0.1151	-0.0993	0.4363**	0.0968	0.0036	0.1005	0.1026		0.4795**
1MIM	-0.0282	0.2081	0.0341	-0.0427	0.3825**	0.1550	-0.0660	0.1006	0.4392**	0.5313**	
Total	0.9213**	0.5011**	0.7711**	0.8555**	0.3312**	0.1986	0.5204**	0.0481	0.2990**	0.1694	0.1517

Pearson correlation and Partial correlation are shown at the lower left and upper right quarter, respectively;

\*\* Significant at P=0.01; \*significant at P=0.05;

Hybut: 2-hydroxy-3-butenyl; HyPen: 2-hydroxy-4-pentenyl; MetA: Methylsulfoxide-amyl; But: 3-butenyl; HyMetI: 4-hydroxy-3-indole-methyl; Pen: 4-pentenyl; Ben: Benzyl; MetI: 3-indolyl-methyl; Phe: Phenylethyl; 4MIM: 4-methoxy-3-indole-methyl; 1MIM: 1-methoxy-3-indole-methyl.

compute Pearson correlation between resistance level and glucosinolate content as well as the partial correlation between different glucosinolate components.

## RESULTS

**Seed glucosinolate content variation in parents and DH population.** A total of 11 components were identified by HPLC. Compared with that of line Zhongyou 821, the contents of main aliphatic glucosinolates, especially 2-hydroxy-3-butenyl and 3-butenyl glucosinolate were sharply reduced in the seeds of both parents. Indolic glucosinolates, especially 4-hydroxy-3-indole-methyl glucosinolate, were the prevailing component. The total percentage of 4-hydroxy-3-indole-methyl glucosinolate had a sharp increase (Table 1), whereas some glucosinolate components differed in the two parents. For example, 2-hydroxy-4-pentenyl and 4-pentenyl glucosinolate was detected in P28 but not in B11 seeds.

Although the total glucosinolate content in the seeds of both parents was similar, there was an obvious segregation in the DH population (data not shown) in accordance with Zhao and Meng (2003b). The Pearson correlation analysis showed that the total glucosinolate content was significantly positively correlated with all components, except for 4-pentenyl, 3-indolyl-methyl, 4-methoxy-3-indole-methyl and 1-methoxy-3-indole-methyl. Correlation coefficients were also highly significant between several components (Table 2). However, judging from partial coefficients, only 9 groups were found highly significant (P=0.01) between each other.

**Relation between disease resistance and glucosinolate components.** Following inoculation of parent lines with the two fungal isolates it was observed that the lesions developed in P28 stems were always longer than B11 and the control Zhongyou 821. No obvious difference was detected between B11 and the control Zhongyou 821 (Table 3).

**Table 3.** Average lesion length (cm) in the stems of two parents (P28 and B11) and the control Zhongyou 821 inoculated with two isolates of *S. Sclerotiorum* (SF and BN).

Day after inoculation	SF			BN		
	P28	B11	Control	P28	B11	Control
3 <sup>rd</sup> day	5.17	2.4	2.49	7.9	4.9	4.49
5 <sup>th</sup> day	10.17	8.58	8.48	13.00	8.75	8.14
7 <sup>th</sup> day	15.50	13.98	13.28	19.33	13.00	12.28

Figures in the table represent the lesion length on the stem.

**Table 4.** Pearson correlation between glucosinolate and lesion length.

Glucosinolate	7 <sup>th</sup> day		5 <sup>th</sup> day		3 <sup>rd</sup> day	
	SF	BN	SF	BN	SF	BN
HyBut	0.0576	0.2089	0.0707	0.1618	0.0784	-0.0395
HyPen	0.0512	0.2396*	0.0065	0.1893	0.0163	0.0115
MetA	0.0008	0.1279	0.0144	0.1243	-0.0354	0.0445
But	0.1019	0.2115	0.1529	0.1454	0.0628	-0.0663
HyMetI	0.1023	-0.0063	-0.0806	-0.1046	0.0186	-0.0957
Pen	0.0187	0.0649	-0.1354	-0.0719	-0.0094	-0.1141
Ben	0.0169	0.1313	0.0901	0.2109	0.0346	-0.0570
MetI	0.0656	-0.1078	0.0265	-0.0994	0.1106	0.0401
Phe	-0.1547	0.0817	-0.1009	0.0207	-0.0374	-0.1453
4MIM	0.0658	0.0650	-0.0657	0.1055	-0.0110	0.0394
1MIM	-0.2322*	0.0034	-0.2817*	0.0505	-0.2284*	0.1063
Total	0.0457	0.2243	0.0372	0.1579	0.0439	-0.0724

\* Significant at  $p=0.05$

Hybut: 2-hydroxy-3-butenyl; HyPen: 2-hydroxy-4-pentenyl; MetA: Methylsulfoxide-amyl; But: 3-butenyl; HyMetI: 4-hydroxy-3-indole-methyl; Pen: 4-pentenyl; Ben: Benzyl; MetI: 3-indolyl-methyl; Phe: Phenylethyl; 4MIM: 4-methoxy-3-indole-methyl; 1MIM: 1-methoxy-3-indole-methyl.

The DH population could be infected by both isolates of *S. sclerotiorum*, but the mean lesion length induced by BN and SF at the 3<sup>rd</sup>, 5<sup>th</sup> and 7<sup>th</sup> day was significant different (T-test was used at  $P=0.0001$ , data not shown), suggesting the two isolates may have different pathogenic ability.

When Pearson correlation was used to determine whether total seed glucosinolate and its components had any correlation with lesion length or not, no significant correlation was found between total glucosinolate and *Sclerotinia* resistance. However, 2-hydroxy-4-pentenyl content was positively correlated with lesion length at the 7<sup>th</sup> day but not at the 5<sup>th</sup> or 3<sup>rd</sup> day for BN. As far as SF, 1-methoxy-3-indole-methyl content was negatively correlated with lesion length at the 3<sup>rd</sup>, 5<sup>th</sup>, and 7<sup>th</sup> day

(Table 4). These findings are indicative of the complexity of the relation between each isolate of *S. sclerotiorum* and glucosinolate components.

**Selecting for low glucosinolate lines with disease resistance.** Compared with the lesion length at the 7<sup>th</sup> day in the control Zhongyou 821, 10 lines with less than 12 cm mean lesion length were selected from the DH population. Among them, 8 lines (marked with \* in Table 5) had low total glucosinolate. Nevertheless, compared with the control Zhongyou 821 whose indolic class was 9.74% of the total glucosinolate (see Table 1), all the 8 lines had obvious higher percentage of total indolic glucosinolate (Table 5).

**Table 5.** Glucosinolate content and average lesion length in 10 selected lines.

Selected line	Lesion length (cm)		Glucosinolate Content ( $\mu\text{mol g}^{-1}$ )			
	SF	BN	Aliphatic	Bentyl	Indolic	Total
DH43*	3.03	9.25	11.77 (61.78%)	2.40 (12.60%)	4.88 (25.62%)	19.05 (100.00%)
DH60*	5.40	9.00	3.47 (52.90%)	0.95 (14.48%)	2.14 (32.62%)	6.56 (100.00%)
DH125	9.30	10.00	28.73 (78.41%)	3.18 (8.68%)	4.73 (12.91%)	36.64 (100.00%)
DH134*	3.00	7.00	3.08 (27.92%)	3.43 (31.10%)	4.52 (40.98%)	11.03 (100.00%)
DH84	3.50	6.75	41.91 (86.39%)	3.28 (6.76%)	3.32 (6.84%)	48.51 (100%)
DH156*	0.00	8.83	1.47 (16.93%)	2.26 (26.04%)	4.95 (57.03%)	8.68 (100%)
DH186*	10.50	11.67	11.00 (64.52%)	1.93 (11.32%)	4.12 (24.16%)	17.05 (100.00%)
DH24*	0.00	11.00	8.52 (63.44%)	0.95 (7.07%)	3.96 (29.49%)	13.43 (100.00%)
DH28*	3.50	6.17	1.15 (18.731%)	0.56 (9.12%)	4.43 (72.15%)	6.14 (100.00%)
DH39*	5.50	9.83	11.45 (69.48%)	1.00 (6.07%)	4.03 (24.45%)	16.48 (100.00%)

\* Selected resistant lines with low total glucosinolate.

## DISCUSSION

There was an interaction between glucosinolates, DH lines and *S. sclerotiorum* isolates. Previous studies (Zhao and Meng, 2003b) showed the relation between total glucosinolate and resistance and between predominant glucosinolate components and resistance. In these studies, trace components were not paid attention. It is possible that these trace components were masked by the predominant glucosinolates although most of them may play an important role in plants. Using a DH population we have established that these minor components are important and play important role in disease resistance. Both fungal isolates could infect DH population, which indicates that there was no differentiation of pathological races. However, since significant difference in lesion length were detected, it is plausible to conclude that different isolates of *S. sclerotiorum* have a different pathogenic ability, as reported by Pratt and Rowe (1995) and Liu (1996).

There was a significant correlation between two trace glucosinolate components and resistance. The content of 1-methoxy-3-indole-methyl glucosinolate, a member of the indolic glucosinolate family, was higher in P28 than in B11 seeds. But judging from phenotype, P28 was susceptible to *Sclerotinia*. Supposing that some resistance loci were correlated with the glucosinolates, this could explain that the susceptible parent (P28) could contain the resistant locus which was also found by Zhao and Meng (2003b).

Li *et al.* (2001) found that Zhongyou 821 produced more indolic glucosinolates in the leaves after inoculation. Bones and Rossiter (1996), Kelly *et al.* (1998) and Mithen (2001) reported that indolic glucosinolate could be hydrolyzed to IAA which, in turn, could serve to regulate growth and development of the host and to delay the extension of mycelium. For BN, the negative correlation between 2-hydroxy-4-pentenyl glucosinolate and disease resistance was found only at the 7<sup>th</sup> day. Renwick and Lopez (1999) found that larvae of *Pieris rapae* were stimulated to feed by the glucosinolate sinigrin. This suggested us that some *S. sclerotiorum* isolates may be favored by specific glucosinolate components. For instance, isolate BN was recovered from field-grown rapeseeds. It is then possible that during the co-existence with the host this isolate had adapted to 2-hydroxy-4-pentenyl glucosinolate.

Plant breeding, based on the knowledge of glucosinolate biosynthesis, could play an important role, particularly in the production of improved double-low oilseed rape cultivars. Unfortunately, glucosinolates biosynthesis is so complex that we are not yet in the position to understand how every glucosinolate is synthesized in plants, with special reference to indolic and aromatic glucosinolates. In the three glucosinolate families, only the biosynthesis of aliphatic glucosinolates is rela-

tively clear. In a recent report, Andersen *et al.* (2000) found that cytochromes P-450 from cassava (*Manihot esculenta* Crantz) catalyze the conversion of L-valine and L-isoleucine into the corresponding oximes, which is the first step in the biosynthesis in cassava of the cyanogenic glucosides linamarin and lotaustralin. Based on this, we can hypothesize that the same gene may regulate the biosynthesis of different glucosinolates. Mikkelsen *et al.* (2002) and Bennett *et al.* (1995) had reported that 2-hydroxy-3-butenyl and 3-butenyl shared the same precursor. Biosynthetic relations between the two components were also detected in our research. Based on partial correlation, different components could share the same precursors in the biosynthetic pathway, thus it may be possible that the correlation between different glucosinolates found in this paper could reflect the internal relation of their biosynthesis.

Since aliphatic and indolic glucosinolates can be hydrolyzed to produce compounds toxic to livestock and IAA useful to plant growth and development, within the DH population we selected the lines with lower content of total glucosinolate and of total aliphatic glucosinolates. In fact, lines DH28, DH60, DH134 and DH156 contained low and aliphatic glucosinolate, and were resistant to both fungal isolates. Such materials will be useful for later disease-resistance breeding programs.

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