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Myrosinase-Glucosinolate System in Crop *Brassica* species: Variation and Association with Defensive Responses to *Pieris brassicae* Infestation

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ABSTRACT

A diverse *Brassica* germplasm collection (128) was assayed for myrosinase activity and glucosinolate content in different plant tissues/organs. Significant genotypic variation was observed. Myrosinase activity was highest in leaves, followed by developing seeds and least in the mature seeds. *B. napus* leaves had the maximum mean value (1.29 ± 0.59 nmole glucose released mg^{-1} min^{-1}), followed by *B. juncea* (0.63 ± 0.05), *B. nigra* (0.52 ± 0.09) and *B. rapa* (0.30 ± 0.03). Genotypes with very low myrosinase activity were *B. napus* ACN 40 (0.68), *B. juncea* KH 2099 (0.09), *B. rapa* VKS 11/29 (0.08) and *B. nigra* FRG 2 (0.17). Glucosinolate values were substantially higher in mature seeds than in the green tissues. Land races in general possessed higher glucosinolate content. Leaf myrosinase activity was negatively correlated with total glucosinolate content. Correlation with but-3-ethyl glucosinolate (gluconapin) was negative while a positive correlation occurred with 2-OH-but-3-ethyl glucosinolates (progoitrin). Variation was recorded for resistance to cabbage caterpillar (*Pieris brassicae*) in *B. nigra*, where genotypes namely Assam, N 17, N24, Pakistan and Mozambique showed an antibiosis reaction. Increased myrosinase activity was correlated with resistance to herbivore. This was evident from a negative correlation between larval mortality and myrosinase content. Leaf glucosinolates, on the other hand, were negatively correlated with larval mortality.

Keywords: cabbage caterpillar, defense enzymes, plant defense, rapeseed-mustard

INTRODUCTION

The myrosinase-glucosinolate system has been the subject of many chemical investigations (Heqnauer 1986). Specific β-thioglucosidases known as myrosinases are responsible for degradation of glucosinolates (β-thioglucoside-*N*-hydroxysulfatases), which are amino acid-derived thioglycosides. Both these are spatially compartmentalized within plant cells (Bones and Rossiter 1996). These are brought together when the cell is disrupted, mechanically or following herbivore invasion. Myrosinase cleaves the sugar from glucosinolates and a plethora of toxic compounds are released. These hydrolysis products are more biocidal than the intact glucosinolates, being toxic to both generalist insect herbivores as well as to crucifer specialist insects (Li *et al.* 2000). Several myrosinase isoenzymes have been reported in seeds, seedlings and vegetative tissue of oilseed rape (Lenman 1993). Most studies to date have focused on variation in glucosinolates without much emphasis on the levels of myrosinase activity in different vegetative and reproductive structures of plant growth in a range of test germplasm. Past studies regarding defensive responses of the glucosinolate-myrosinase system mostly suggest a lack of correlation with herbivory of *Brassica* specialist insects (Kliebnstein *et al.* 2002). These are in agreement with the hypothesis of a chemical co-evolution theory (Stamp 2003) which suggests that specialist insects have adapted to sequester or even utilize plant defensive chemicals which normally function as feeding deterrents for generalist herbivores. Some of these specialist herbivores can also circumvent the myrosinase activities of the plant tissue which normally hydrolyze glucosinolates after plant damage. The present study aimed to evaluate different *Brassica* accessions for their variation in myrosinase activity and total glucosinolate levels in different plant parts of four economically important oilseed *Brassica* species. Feeding experiments were also conducted to analyze the inherent genetic variation in test genotypes for resistance to cabbage caterpillar.

MATERIALS AND METHODS

A germplasm collection comprising 128 commercial varieties, breeding lines and land races belonging to four crop *Brassica* species namely, *B. juncea*, *B. napus*, *B. rapa* and *B. nigra* were evaluated for myrosinase activity and glucosinolate content in different plant tissues/organs over two years..This germplasm collection is being maintained at Punjab Agricultural University. Young leaves, pod wall, developing and mature seeds were collected at defined plant growth stages (pre-anthesis, termination of flowering on main shoot and at maturity) from field-grown plants. Standard agronomic practices were followed throughout the growing season to raise the crop. Myrosinase activity was measured as described by Li *et al.* (2002). Total glucosinolate content in mature seeds was estimated by the method of Kumar *et al.* (2004). Individual glucosinolates were analyzed using high performance liquid chromatography (Spinks *et. al.* 1984). Insect herbivory assays involving feeding experiments were conducted in a randomized complete block design. There were three replications with 10 larvae per replication. For insect bioassays, freshly moulted 2nd instar larvae of *P. brassicae* from laboratory culture were released in glass jars containing the leaves of different genotypes. The top of the glass jar was covered with muslin cloth fastened with elastic bands. These jars were kept in a BOD incubator at 22±1°C. Fresh leaves of different test genotypes were provided every day. Daily observations were recorded of larvae dying or reaching the pupal stage. Similar observations were also made for pupae until adult emergence. The experiment was repeated three times from 2004-05 to 2006-07. The data were analyzed following randomized complete block design to find out least significant difference (LSD)/critical difference (CD) for means of glucosinolate content,

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Table 1 Variation of mean myrosinase activity (nmol mg^{-1} min^{-1}) in different developmental tissues of crop *Brassica* species.

Species	Samples analyzed	Myrosinase activity (nmol mg^{-1} min^{-1}) (Mean ± SE)					CD (P≤0.05)
		Leaves	Developing seeds	Mature seeds	Developing pod wall	Mature pod wall	
Brassica napus	45	1.29 ± 0.59 (0.68-2.07)	1.12 ± 0.05 (0.53-2.15)	0.67 ± 0.03 (0.32-1.17)	0.56 ± 0.02 (0.25-1.11)	0.27 ± 0.01 (0.10-0.47)	0.093
Brassica juncea	49	0.63 ± 0.05 (0.08-1.50)	0.90 ± 0.09 (0.34-2.47)	0.25 ± 0.01 (0.10-0.63)	0.44 ± 0.03 (0.18-1.15)	0.11 ± 0.01 (0.08-0.20)	0.069
Brassica rapa	20	0.30 ± 0.03 (0.08-0.53)	0.94 ± 0.06 (0.45-1.55)	0.29 ± 0.02 (0.17-0.44)	0.59 ± 0.04 (0.21-0.78)	0.15 ± 0.01 (0.09-0.20)	0.021
Brassica nigra	14	0.52 ± 0.09 (0.13-1.37)	1.02 ± 0.06 (0.61-1.37)	0.21 ± 0.01 (0.16-0.28)	0.44 ± 0.02 (0.31-0.58)	0.09 ± 0.01 (0.07-0.11)	0.079

Averaged over two years
Figures in parentheses indicate range

Table 2 Correlation for myrosinase activity (nmol mg^{-1} min^{-1}) at various developmental stages in *Brassica* species.

Developmental stage	Species	Developing seed	Developing pod wall	Mature seed	Mature pod wall
Leaves	*B. napus*	0.435**	0.344**	0.093	-0.106
	B. juncea	-0.179	-0.173	-0.290	-0.022
	B. rapa	0.745**	0.894**	0.818**	0.730**
	B. nigra	-0.380	-0.288	-0.355	-0.648**
Developing seed	*B. napus*		0.836**	-0.036	-0.044
	B. juncea		0.949**	0.866**	0.807**
	B. rapa		0.997**	0.615**	0.585**
	B. nigra		0.824**	0.707**	0.446**
Developing pod wall	*B. napus*			-0.088	-0.017
	B. juncea			0.908**	0.835**
	B. rapa			0.667**	0.817**
	B. nigra			0.929**	0.508
Mature seed	*B. napus*				0.218
	B. juncea				0.861**
	B. rapa				0.927**
	B. nigra				0.557*

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

larval, pupal, total mortality and adult emergence at the 95% probability level. All the statistical analysis including analysis of variance and correlation coefficients were carried out using the software OPSTAT.

RESULTS

Variation of myrosinase activity

Analysis of variance revealed significant differences for mean square values of myrosinase activity among the genotypes of different *Brassica* species at almost all the stages of crop growth excepting for the developing pod wall of *B. napus*. In leaves, grand mean values for myrosinase activity were generally lower (**Table 1**) in diploids (*B. rapa* and *B. nigra*) than the digenomics amphiploid species (*B. napus* and *B. juncea*). These ranged from 0.68 (ACN-40) to 2.07 (ACN-50), 0.08 (KH-2099) to 1.50 (ALM-129), 0.08 (VKS-11/6) to 0.53 (Sunshine) and 0.13 (FRG-2) to 1.37 (N-17) in *B. napus*, *B. juncea*, *B. rapa* and *B. nigra*, respectively. During seed development, myrosinase activity was mostly higher than that observed in the leaves (**Table 1**). Mature seeds generally reflected the trend observed in developing seeds. Myrosinase activity was lower in seeds, except for *B. rapa*, where it was almost similar to that recorded in leaves. Interestingly, in the developing pod walls, myrosinase activity in *B. rapa* was higher than in *B. napus*. In all other stages evaluated, *B. napus* revealed maximum average myrosinase activity.

In *B. napus*, leaf myrosinase activity was positively correlated with that in green tissues (developing seeds and developing pod wall), but no such correlation was recorded with other tissues/organs. In *B. juncea* and *B. nigra* myrosinase activity of developing seeds and the developing pod wall was strongly correlated with that of the developing pod wall and mature seeds (**Table 2**). *B. rapa* showed a strong positive correlation between myrosinase activity and all developmental stages.

Variation for glucosinolate content

Extensive genotypic variation was observed for the total glucosinolate content (**Table 3**) in the test species and plant tissues/organs assayed. The leaf glucosinolate content varied from 10.14 (ACN 54) – 22.31(ACN 21), 3.82 (ALM129)-21.19 (KH 2096), 8.32 (Sunbean)-32.32 (VKS 11/29), 10.66 (N17)-18.99 (FRG 1) in *B. napus*, *B. juncea*, *B. rapa* and *B. nigra*, respectively. Overall mean values were generally similar in *B napus*, *B. rapa* and *B. nigra*, but were significantly lower for *B. juncea.* In the developing seeds the glucosinolate values were mostly higher than those observed in the leaves. These were also lower in *B napus* and *B. juncea* as compared to those recorded for *B. rapa and B nigra*. In the developing pod walls, excepting *B. nigra* which showed very high value, other three species had lower but similar values. In the mature seeds the glucosinolate content was 4-5 times higher than the one recorded in the leaves and pod tissues. Land races in general had more glucosinolate content. Significant correlation between leaf glucosinolate content and the corresponding values in developing seeds and pod walls in *B. napus* was indicated. Almost similar trend was apparent in *B. juncea* and *B. nigra.* In *B. rapa*, however, positive correlations were observed among glucosinolate values in all the plant tissues sampled (**Table 4**).

Association between myrosinase activity and glucosinolates

A negative correlation between myrosinase activity and glucosinolates content was recorded. It was especially true for green tissues (**Table 5**). Only exception was *B. nigra*, which showed a positive correlation between myrosinase activity in leaves and glucosinolate content in the developing pod walls. Myrosinase activity in developing seeds revealed a positive correlation with glucosinolate content in developing seeds of *B. napus.* In *B. rapa*, it was also positively associated with glucosinolate levels in developing seeds,

Table 3 Variation of mean glucosinolate content in different developmental tissues in crop *Brassica* species.

Species	Samples analyzed	Glucosinolates (µmol g^{-1}dry tissue) (Mean ± SE)				
		Leaves	Developing seeds	Developing pod wall	Mature seeds	Mature pod wall
Brassica napus	45	15.50 ± 0.37	23.67 ± 0.63	40.74 ± 0.95	12.03 ± 0.34	10.23 ± 0.32
		(10.14-22.31)	(18.17-33.06)	(28.08-54.51)	(7.90-17.81)	(7.04-17.87)
B. juncea	49	10.25 ± 0.66	24.41 ± 0.95	82.74 ± 2.80	12.35 ± 0.52	9.26 ± 0.41
		(3.82-21.19)	(10.54-38.94)	(51.08-138.48)	(6.18-21.13)	(4.76-17.55)
B. rapa	20	15.86 ± 1.73	33.55 ± 3.31	85.76 ± 7.34	14.97 ± 1.14	13.12 ± 1.00
		(8.32-32.32)	(16.97-53.97)	(41.69-139.98)	(8.11-22.64)	(7.18-20.29)
B. nigra	14	14.63 ± 0.71	37.09 ± 1.88	108.78 ± 8.22	24.62 ± 2.15	16.72 ± 1.47
		(10.66-18.99)	(26.41-48.91)	(58.63-163.48.)	(11.88-35.10)	(8.96-28.64)

Averaged over three years; Figures in parentheses indicate range

Table 4 Correlation for glucosinolate content (µ mol g^{-1}dsm) among various developmental stages in *Brassica* species.

Developmental stage	Species	Developing seed	Developing pod wall	Mature seed	Mature pod wall
Leaves	*B. napus*	0.781**	0.427**	0.155	-0.217
	B. juncea	0.843**	0.861**	0.246	0.828**
	B. rapa	0.893**	0.810**	0.761**	0.810**
	B. nigra	0.985**	0.957**	0.442	0.860**
Developing seed	*B. napus*		0.462**	0.279	0.250
	B. juncea		0.937**	0.483**	0.246**
	B. rapa		0.942**	0.905**	0.934**
	B. nigra		0.979**	0.510**	0.848**
Developing pod wall	*B. napus*			0.042	0.404**
	B. juncea			0.435**	0.958**
	B. rapa			0.976**	0.989**
	B. nigra			0.557**	0.876**
Mature seed	*B. napus*				0.078
	B. juncea				0.501**
	B. rapa				0.983**
	B. nigra				0.450

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

Table 5 Correlation between myrosinase activity and Glucosinolates content at various developmental stages in *Brassica* species.

Myrosinase activity in green tissues		Total glucosinolates content				
		Leaves	Developing seed	Developing pod wall	Mature seed	Mature pod wall
B. napus	Leaves	-0.921**	-0.762**	-0.386**	-0.121	-0.229
B. juncea		-0.291*	-0.811**	-0.774**	-0.329*	-0.747**
B. rapa		-0.937**	-0.956**	-0.880**	-0.834**	-0.876**
B. nigra		-0.898**	-0.904**	0.922**	0.350	-0.810**
B. napus	Developing	-0.834**	0.388**	0.093	0.022	0.048
B. juncea	seed	-0.046	0.041	-0.128	-0.296*	-0.051
B. rapa		-0.834**	0.737**	0.669**	0.607**	-0.658**
B. nigra		0.307	0.367	0.421	0.343	0.129

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

Table 6 Correlation of myrosinase activity and individual glucosinolates profile in *Brassica* species.

Myrosinase activity	3-methyl sulphinyl propyl (Glucoiberin)	2-hydroxy but-3-enyl (progoitrin)	2-propenyl (allyl sinigrin)	But-3-enyl (Gluconapin)	Pent-4-enyl (Glucobrassicanapin)
B. napus	-0.504	0.956*	0.768	-0.995*	0.674
B. juncea	0.045	0.164	0.198	-0.534	-0.532
B. rapa	0.300	-0.080	-0.446	-0.915*	0.512
B. nigra	0.528	-0.794*	-0.797*	-0.814*	0.638
Overall	-0.164	0.515*	-0.148	-0.510*	-0.283

* Significant at P= 0.05

developing pod wall as well as mature seeds. The correlation with glucosinolates in mature pod wall was, however, negative.

Myrosinase activity and glucosinolate profile

B. napus genotypes (ACN 40, ACN 50) with maximum myrosinase activity, had a higher concentration of but-3-enyl than 2-0H-but-3-enyl followed by other aliphatic compounds. In contrast, the genotypes having minimum myrosinase activity (ACN 30, ACN 36), had the highest proportion of 2-0H-but-3-enyl followed by but-3 enyl, penty-4-enyl and 3-methylsulpinyl propyl. In *B. juncea*, the genotype with maximum myrosinase activity (ALM 22, Jatahi sarson, KH 2000, etc.) showed 4-6 times higher concentration of but-3-enyl than 2-propanyl, whereas in the genotypes having minimum myrosinase activity (ALM 22-2, ALM 25-3 and ALM 129), the concentration of but-3-enyl was only 2-3 times more than 2-propanyl followed by 2-0H-but-3-enyl, pent-4-enyl and 3methyl sulphinyl propyl. *B. rapa* and *B. nigra* genotypes had greater proportion of 2 propanyl and but-3-enyl respectively, irrespective of myrosinase activity. Across all the species, a negative and significant correlation between myrosinase activity and but-3-enyl (gluconapin) was indicated. In contrast, a positive correlation was recorded with 2-OH-but-3 enyl (progotrin) (**Table 6**).

Association of leaf myrosinase activity and glucosinolate content with tolerance to cabbage butterfly

All the test genotypes, included in the present investigations were also evaluated for their resistance to cabbage butterfly,

Table 7 Association of leaf myrosinase activity/glucosinolate content with tolerance to *P. brassicae*.

Genotype	Myrosinase	Glucosinolates	Larval mortality	Pupal mortality	Total mortality	Adult emergence
Assam	0.54	13.44	63.33	8.33	71.7	44.2
Canada local-1	0.13	18.82	40.00	13.33	53.3	46.7
Canada local-2	0.57	13.22	38.33	6.67	45.0	55.8
Delhi	0.21	18.04	42.50	8.33	50.8	49.2
FRG-2	0.12	18.99	41.67	25.83	67.5	36.7
Hindia	0.37	14.76	45.00	9.17	54.2	45.8
India	0.74	12.63	50.00	7.50	57.5	42.5
India Punjab	0.24	17.28	42.50	5.00	47.5	52.5
Mozambique	0.55	13.69	59.17	12.50	71.7	28.3
N-17	1.38	10.66	57.50	20.00	77.5	22.5
N-24	1.00	11.83	56.67	3.33	60.0	40.0
Pak Punjab	0.36	14.43	40.00	11.67	51.7	48.3
Pakistan	0.78	12.34	56.67	17.50	74.2	25.8
UP	0.35	14.76	50.83	12.50	63.3	36.7
C.D. (P=0.05)			**NS**	**NS**	**NS**	**NS**

Averaged over three years

a specialist crucifer herbivore under laboratory conditions over three years. No variation in defensive responses of *B. rapa*, *B. napus* and *B. juncea* was recorded. All of them were susceptible to infestation by the insect. Only *B.nigra* genotypes differed in their response for larval mortality, pupal mortality as well as adult emergence. On the basis of three years average, Assam, N 17, N24, Pakistan and Mozambique showed antibiosis against cabbage caterpillar (**Table 7**). We attempted to correlate this variation for insect tolerance in *B. nigra* genotypes with variation for leaf myrosinase activity as well as leaf glucosinolate content. Myrosinase activity was found to be correlated positively with larval mortality (0.64) and adult emergence (0.58). Pupal mortality as well as total mortality was not influenced by variation in the myrosinase activity. A negative correlation (-0.66) existed between leaf glucosinolate content and larval mortality.

DISCUSSION

Defining the spectrum of variation and stage specificity of glucosinolates and enzyme myrosinase in the germplasm is important to understand its role in influencing the defensive responses of glucosinolate–myrosinase system following insect invasion. Aside the role of epithiospecifer (ESP) protein, varied activity and forms of myrosinase play equally important role in regulating the release of elemental sulphar, nitriles, isothiocynates and cyanoepithioalkanes for impacting the defensive responses as well as the bioavailability of proteins for the cattle fed on defatted meal. Our studies carried out over two years with a wide array of *Brassica* germplasm (including land races) have helped uncovering excellent variation for the trait. A very interesting observation was the occurrence of maximum mean values for the trait in *B. napus* canola at all the stages of crop growth. In contrast, high glucosinolate *B. juncea* showed significantly lower myrosinase values. Monogenomic *B. nigra* revealed a higher myrosinase activity as compared to another monogenomic *B. rapa* for the leaf tissues and developing seeds. Reverse was, however, true for myrosinase activity in mature seeds, developing pod walls and pod wall at maturity. Higher leaf myrosinase activity of *Brassica juncea* and *Brassica napus* than that observed in diploid species is in consonance with an earlier report (Xue *et al.* 1993). Glucosinolate contents, in general, were higher in mature seeds than in green tissues.

There appears no published evidence for correlation of myrosinase activity with total or individual glucosinolates in different plant tissues of *Brassica* species as is being reported in present communication. However, in the leaves of horse radish significant correlations between myrosinase activity and total glucosinolates (0.78 at P = 0.01) and between myrosinase activity and sinigrin have been reported (Li and Kushad 2004). Total glucosinolate content was highest in the mature seeds. Excepting for *B. rapa* there was no correlation between glucosinolate content in leaves and mature seeds. Higher glucosinolate content in the seeds might play an important role in successful germination. It may also act to prevent pathogen infection or herbivory at this crucial phase. Decrease in glucosinolate content accompanying increasing seed glucosinolates may represent turn over translocation. However, as the decrease in glucosinolate content in the pod tissue was much lower than the increase in seeds, glucosinolates or its precursor must also be remobilized from plant parts other than the pod tissues. The spatial distribution of glucosinolates within the plant or even within a leaf may be critical for plants defensive system (Shroff *et al* 2008).

Reducing the level of specific glucosinolate i.e. progoitrin to improve the commercial value of animal feed on *Brassica* is the prime goal in *Brassica* breeding. This may be possible by altering the level of myrosinase present in various plant species by genetic manipulation, since low levels of myrosinase enzyme were found to be associated with decrease in progoitrin. Increased myrosinase activity across the genotypes was correlated with the resistance to herbivore. This was evident from negative correlation between larval mortality and myrosinase content. This confirmed a previous report which suggested that resistance to specialist *Plutella xylostella* in *B. juncea* was more dependent on myrosinase activity than on glucosinolate profile (Li *et al*. 2000). Leaf glucosinolates on the other hand were negatively correlated with larval mortality. This was consonant with studies of Giamoustaris and Mithen (1995), who recorded a greater incidence of *Pieris rapae* larvae with increase in level of glucosinolates. Damage by generalist insects has, however, been reported to be reduced as glucosinolate level increased. Glucosinolates have also been suggested to be feeding stimulants, attractants or even oviposition cues for various herbivores. Among the breakdown products, allylisothiocynates are considered as the major attractants for crucifer insects from a distance. However, ovipositing insects can differentiate between plant species with different profile. Glucosinolates may also stimulate oviposition in cabbage butterflies. Effect of varying levels of glucosinolates and myrosinase on the performance of glucosinolates sequestering turnip sawfly, *Athalia rosea* has been reported (Műller and Sieling 2006). Such correlations may be misleading when the genetic backgrounds of susceptible and resistant genotypes differ in more ways than just the resistant gene(s). Use of isogenic lines, differing only for resistant gene or the gene product is always a better proposition.

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