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Fungal infection-induced metabolites in *Brassica rapa*

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ABSTRACT

The metabolic interactions between three cultivars of *Brassica rapa* and three pathogenic fungi such as *Leptosphaeria maculans*, *Aspergillus niger*, and *Fusarium oxysporum* were investigated using ¹H nuclear magnetic resonance spectroscopy. Firstly, in order to reveal latent information on common metabolites induced by these fungi, partial least square-discriminant analysis (PLS-DA) was applied to the ¹H NMR spectra of fungi-infected and control plants of the three cultivars. It showed the presence of metabolites in infected *B. rapa* leaves such as phenylpropanoids, flavonoids and glucosinolates which were highly associated with the fungal infection. Subsequently, the difference between the metabolic responses to each type of fungal infection was evaluated. *F. oxysporum* infected plants were found to accumulate more phenylpropanoids (sinapoyl-, feruloyl- and 5-hydroxyferuloyl malate), flavonoids (kaempferol and quercetin) and fumaric acid than the infection of other two fungal species. In addition to the variation depending on fungal species, the cultivars of host plant largely affect the metabolic alteration. Nuclear magnetic resonance (NMR)-based metabolomics employed in this study reveals that fungal infection alters a manifold of secondary metabolites, though specific patterns were observed for each fungus and each cultivar.

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1. Introduction

Brassica species have become an important source of vegetable oils [1], proteins for human [2,3] and animal nutrition [2]. The large demand of *Brassica* plants due to their use not only as a vegetable but also as an industrial source for seed oils and biofuel requires a considerable increase in its production in Europe and the USA [4]. Additionally, its high level of genetic resemblance to *Arabidopsis* [5] has made it an alternative model system in the field of plant physiology. It is considered particularly interesting for the study of plant interaction with other organisms such as insects, fungi, and bacteria, because of its wide distribution in the wild.

Brassica crops are heavily challenged by various fungal pathogens and insects, whereas bacterial and viral diseases have little effect on yield. *Fusarium oxysporum* is the plant pathogen that infects *Brassica* crops causing the wilt disease leading to great loss in quantity and quality of oilseed *Brassica* [6]. *Aspergillus niger* causes black mold on certain fruits and vegetables and is a common contaminant of food. *Leptosphaeria maculans* causing blackleg or stem cankers is the most devastating fungal diseases for oilseed *Brassica*, particularly canola (*Brassica napus* and *Brassica rapa*) [7,8]. The devastating effect of *L. maculans* can lead to severe yield loss in many regions in the world.

For example the oilseed rape industry of Australia was completely wiped out due to *L. maculans* in 1970s. The annual costs of blackleg (*L. maculans*) are estimated to range between 11.3 and 30.1 million € in Australia, 36.8 and 147 million € in France, and 14 and 56 million € in the U.K. [8].

It is a well-known fact that the attack by pathogenic microorganisms triggers diverse defense mechanisms, including, among others, the production of different groups of antimicrobial or antifungal compounds. Among the induced metabolites, glucosinolates [9] and phenolics [10,11] are the well-known pathogen-induced metabolites of Brassicaceae family as well as cysteine-rich polypeptides [12]. In the case of phenolic compounds, eleven aromatic compounds have been identified in the cell wall extracts of *Arabidopsis* roots [10]. Nine of those compounds were found to be analogues of benzaldehyde, benzoic acid, and cinnamic acid. The other two were indole derivatives (indole-3-carboxylic acid and indole-3-carbaldehyde). These phenolic compounds were found to be considerably increased as result of *Pythium sylvaticum* infection. The same pattern of variation of these compounds was observed in the leaves of *Arabidopsis* infected with *Pseudomonas syringae* [10].

In the case of the infection of Chinese cabbage (*Brassica campestris* sp. *pekinensis*) with *Plasmidiophora brassicae* (an obligate biotroph which is the causal agent of club root disease in *Brassica* species) a change in levels of several glucosinolate was observed. Significant differences in the glucosinolate pattern of

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susceptible and tolerant varieties and even between the susceptible varieties themselves were detected [9]. While tolerant varieties exhibited an enhanced production of aromatic glucosinolates 14 and 30 days after infection, susceptible varieties showed an increase in aliphatic as well as indole glucosinolates. Interaction of oilseed rape and Chinese cabbage with *Alternaria brassicae* increased the level of indole glucosinolates [13,14]. Elicitation of *Arabidopsis* with *Erwinia carotovora* induced indole glucosinolate production to a high level [15]. Another example is *Peronospora parasitica* infected cauliflower, where sinigrin content was higher in resistant varieties than in susceptible ones [16].

In spite of huge diversity of existing metabolites, only a few have been found so far in fungi-infected *Brassica* species. Most of the previous studies have been focused on a specifically targeted group of compounds, such as glucosinolates [9] or phenylpropanoids as well as a few other phytoalexins [17]. Plant metabolism is a dynamic system and changes in levels of any metabolite are expected to affect that of others. Therefore, in metabolic studies it is crucial to get the whole picture of all possible connected metabolites pathways. In this context, a metabolomic approach is required in order to determine the plant metabolome after infection. One of the most interesting developments of metabolic studies nowadays is the comprehensive chemical profiling of the phenotype (i.e. metabolome) of a plant, under different conditions and the use of multivariate data analysis to find correlations in treatments and metabolic changes. This is what is known as metabolomics [18].

In principle, any analytical method can be applied to plant metabolomic research. However, in spite of its inherent low sensitivity, nuclear magnetic resonance (NMR) spectroscopy has been found to have several advantages, especially for the detection of secondary metabolites. In the case of the *Brassica* metabolomics studies, NMR spectroscopy coupled with multivariate data analysis has been employed as well as MS-based methods. In our recent studies, NMR and principal component analysis (PCA) were used to distinguish different cultivars and developmental stages of *B. rapa* leaves [19] as well as the effect of methyl jasmonate on the metabolome [20,21]. Other studies used NMR to study the interaction between food borne bacteria and vegetables [22]. Another demonstration of the potential of NMR and PCA for plant–environment interactions was its use to evaluate the effect of herbivores on *B. rapa* metabolites [23].

One of the greatest challenges for plant metabolomics is posed by biological variation. Even within the same genotype, many factors such as soil conditions, developmental stage or water supply might result in huge metabolic differences. For example, *Brassica* cultivars were found to have highly distinct metabolomes and even for one cultivar, metabolites were found to be largely influenced by its developmental stage [19]. Using chemometric methods that allow the selection of common features, the highly variable *B. rapa* metabolome, could be studied for metabolic alterations under different conditions.

In the present study, using the metabolomic data from our previous *Brassica* research, we applied NMR spectroscopy combined with PCA and partial least square-discriminant analysis (PLS-DA) to identify metabolites of *B. rapa* accumulated after infection with the fungi *L. maculans*, *A. niger*, or *F. oxysporum*. Additionally, differences in the metabolite profiles between the different fungal infections and different cultivars were also evaluated by NMR-based metabolomics.

2. Materials and methods

2.1. Plant material

Different cultivars from *B. rapa* were grown under uniform cultural practices. Seeds from registered cultivars including

Raapstelen (Groene Gewone), Herfstraap (Goldana) and Oleifera were germinated in soil in the cold room (4 °C) for two days. Boxes containing the pots were covered and sufficient water was provided. Pots were then transferred to the greenhouse at 25 °C, 50–60% relative humidity and a 16-h light and 8-h dark per day regime. Seven-day old seedlings were transplanted to 10 cm diameter pots with substrate and kept in the same conditions. Plants were watered daily. After six weeks the fourth leaves of the plants were inoculated with the conidia of the three mentioned fungi after wounding the leaves. Control plants were treated with sterilized water. Plants were covered with plastic sheets for two days after inoculation and the local (fourth leaves) and systemic (sixth leaves) were harvested seven days after inoculation and immediately frozen with liquid nitrogen and subsequently freeze-dried.

2.2. Fungal strains and preparation of conidia

Strains of *L. maculans*, *A. niger* and *F. oxysporum* were used for plant infection. *L. maculans* was kindly provided by Prof. Barbara Howlett. *A. niger* and *F. oxysporum* were generously donated by Department of Fungal Genetics, Leiden University, Leiden, The Netherlands. *L. maculans* and *F. oxysporum* were grown on potato dextrose agar (PDA) at 24 °C and *A. niger* was grown on complete media (CM) at 30 °C until conidia appeared. Conidia were collected from the Petri dish by immersion in sterilized distilled water and removal of the sticky spores from the agar media with sterilized tooth brushes. Suspended conidia were collected with sterilized pipettes and filtered through two layers of sterile Miracloth. The conidia concentrations were measured with haemocytometer and adjusted to 10⁶ spore/ml using sterilized distilled water.

2.3. Extraction and NMR spectra measurements

Freeze-dried plant material (60 mg) was transferred to a 2-ml microtube and vortexed at room temperature for 1 min with 1.5 ml of a mixture of KH₂PO₄ buffer (pH 6.0) in D₂O containing 0.05% trimethyl silyl propionic acid sodium salt (w/w) (TMSP) and methanol-*d*₄ (1:1). The sample was then sonicated for 20 min and centrifuged at 13,000 rpm for 10 min. An aliquot of 800 µl of the supernatant was used for NMR analysis.

¹H NMR, 2D-J-resolved spectra were recorded at 25 °C on a 500-MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany). ¹H–¹H-correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bonds coherence (HMBC) spectra were recorded on a 600-MHz Bruker DMX-600 spectrometer (Bruker). All the NMR parameters were the same to those of our previous reports [19,22].

2.4. Data analysis

¹H NMR spectra were automatically binned by AMIX software (v. 3.7, Bruker Biospin). Spectral intensities were scaled to total intensity TMSP and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ0.4–δ10.0. The region of δ4.8–δ4.9 was excluded from the analysis because of the residual signal of water as well as δ3.28–δ3.34 for residual methanol. PCA and PLS-DA were performed with the SIMCA-P software (v. 11.0, Umetrics, Umeå, Sweden) with Pareto and unit variance scaling method, respectively. The ANOVA and *t*-tests for ¹H NMR signals were performed using Multi-Experiment Viewer (v. 4.0) [24].

3. Results

A wide array of primary and secondary metabolites was identified in the ¹H NMR spectrum. All metabolites were identified

Table 1

¹H chemical shifts (δ) and coupling constants (Hz) of metabolite of *Brassica rapa* infected with *Leptosphaeria maculans* obtained from comparing with references and using 1D and 2D NMR spectra (CD₃OD–KH₂PO₄ in D₂O (pH 6.0)).

Compound	Chemical shifts (ppm) and coupling constants (Hz)
Glutamic acid	2.13 (m), 2.43 (m), 3.72 (t, <i>J</i> = 6.8 Hz)
GABA	1.92 (t, <i>J</i> = 7.5 Hz), 2.35 (t, <i>J</i> = 7.5 Hz)
Alanine	1.49 (d, <i>J</i> = 7.4 Hz), 3.73 (q)
Threonine	1.33 (d, <i>J</i> = 7.0 Hz), 3.53 (d, <i>J</i> = 12.0 Hz), 4.24 (m)
Valine	1.00 (d, <i>J</i> = 7.0 Hz), 1.05 (d, <i>J</i> = 7.0 Hz), 2.3 (m)
Fumaric acid	6.52 (s)
Malic acid (free)	2.58 (dd, <i>J</i> = 16.0, 7.4 Hz), 2.76 (dd, <i>J</i> = 16.0, 4.6 Hz), 4.31 (dd, <i>J</i> = 7.2, 4.0 Hz)
Malic acid conjugated with phenylpropanoids	2.71 (dd, <i>J</i> = 16.0, 12.0 Hz), 2.85 (dd, <i>J</i> = 16.0, 3.0 Hz), 5.23 (dd, <i>J</i> = 11.0, 3.0 Hz)
Lactic acid	1.36 (d, <i>J</i> = 7.0 Hz), 4.14 (d, <i>J</i> = 7.0 Hz)
Choline	3.24 (s)
Sucrose	5.4 (d, <i>J</i> = 3.6 Hz)
β-Glucose	4.59 (d, <i>J</i> = 7.8 Hz)
α-Glucose	5.19 (d, <i>J</i> = 3.7 Hz)
Progoitrin	2.86 (dd, <i>J</i> = 16.0, 10.0 Hz), 4.63 (m), 5.21 (dt, <i>J</i> = 11.0, 2.0 Hz), 5.34 (dt, <i>J</i> = 16.0, 2.0 Hz), 5.92 (m)
Neoglucobrassicin	4.09 (s, MeO), 7.17 (t, <i>J</i> = 7.8 Hz), 7.30 (t, <i>J</i> = 7.8 Hz), 7.49 (d, <i>J</i> = 7.8 Hz), 7.48 (s), 7.73 (d, <i>J</i> = 7.8)
Indole acetic acid	3.25 (d, <i>J</i> = 16.0 Hz), 3.39 (d, <i>J</i> = 16.0 Hz), 7.12 (s), 7.13 (t, <i>J</i> = 7.8 Hz), 7.21 (t, <i>J</i> = 7.8 Hz), 7.47 (d, <i>J</i> = 7.8 Hz), 7.72 (d, <i>J</i> = 7.8 Hz)
Kaempferol analogues	6.46 (d, <i>J</i> = 2.1 Hz), 6.77 (d, <i>J</i> = 2.0 Hz), 7.00 (d, <i>J</i> = 8.8 Hz), 8.08 (d, <i>J</i> = 8.8 Hz)
Quercetin analogues	6.47 (d, <i>J</i> = 2.1 Hz), 6.77 (d, <i>J</i> = 2.0 Hz), 6.9 (d, <i>J</i> = 8.8 Hz), 7.54 (dd, <i>J</i> = 6.5, 3.0 Hz), 7.84 (d, <i>J</i> = 2.0 Hz)
Sinapoyl malate	6.49 (d, <i>J</i> = 16.0 Hz), 6.98 (s), 7.66 (d, <i>J</i> = 16.0 Hz)
Coumaroyl malate	6.46 (d, <i>J</i> = 16.0 Hz), 6.85 (bd, <i>J</i> = 8.8 Hz), 7.57 (bd, <i>J</i> = 9.2 Hz), 7.66 (d, <i>J</i> = 16.0 Hz)
Caffeoyl malate	6.4 (d, <i>J</i> = 16.0 Hz), 6.83 (d, <i>J</i> = 8.8 Hz), 7.13 (dd, <i>J</i> = 8.4, 2.0 Hz), 7.15 (d, <i>J</i> = 2.0 Hz), 7.61 (d, <i>J</i> = 16.0 Hz)
Feruloyl malate	6.48 (d, <i>J</i> = 16.0 Hz), 6.87 (d, <i>J</i> = 8.4 Hz), 7.06 (dd, <i>J</i> = 8.4, 2.3 Hz), 7.26 (d, <i>J</i> = 2.0 Hz), 7.65 (d, <i>J</i> = 16.0)
5-Hydroxyferuloyl malate	6.44 (d, <i>J</i> = 16.0 Hz), 6.82 (d, <i>J</i> = 2.0 Hz), 6.87 (d, <i>J</i> = 2.0 Hz), 7.59 (d, <i>J</i> = 16.0 Hz)

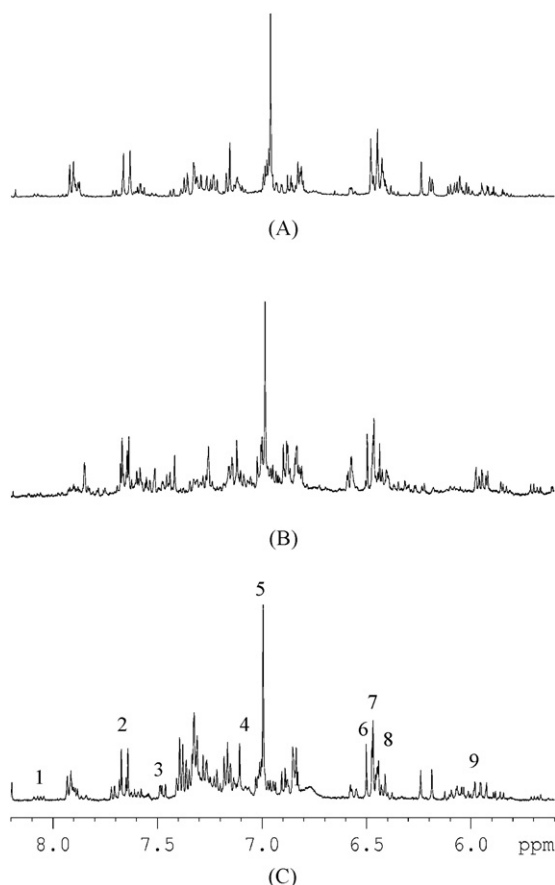


Fig. 1. ¹H NMR spectra of three cultivars of *Brassica rapa* in the range of δ5.6–δ8.2. (A) Herfstraap, (B) Oleifera, (C) Raapstelen. 1: H-2' and H-6' of kaempferol, 2: H-7' of phenylpropanoids, 3: H-7'' of indole glucosinolates, 4: H-3' and H-5' of kaempferol, 5: H-2' and H-6' of sinapoyl malate, 6: fumaric acid, 7: H-8' of phenylpropanoids, 8: H-6 and H-8 of flavonoids, 9: H-4 of aliphatic glucosinolates.

by comparison with the chemical shifts of reference compounds or with the help of two-dimensional (2D) NMR spectra including J-resolved, COSY, HSQC, and HMBC spectra (Table 1) [19–22]. The ¹H NMR of the three *B. rapa* cultivar leaves showed considerably different metabolic profiling. As an example, the phenolic region of the metabolic profile of each cultivar was found to be unique in terms of the amount of phenylpropanoids, glucosinolates, and flavonoids (Fig. 1). Following the visual inspection of the ¹H NMR spectra, multivariate data analysis was applied in order to select the ¹H NMR resonances which were altered in fungi-infected samples. For the alignment, all ¹H NMR signals were binned with a size of 0.04 ppm resulting in 256 bins.

Firstly, PCA was applied to the grouping of plants by reducing the binned ¹H NMR signals. As shown in the comparison of their ¹H NMR spectra, the large biological variation of the *B. rapa* leaves evaluated in this study hampered to obtain a reasonable grouping in PCA results (Fig. 2A). Although PCA is undoubtedly a reliable grouping method in metabolomics, if variation within the sample of the same group is bigger than between groups, a clear separation of each group cannot be expected because the separation of PCA is achieved from unbiased maximum variation within the samples tested. Therefore, a supervised multivariate data analysis using covariance was required to associate metabolites with fungal infection in this study.

Partial least square-discriminant analysis is such a supervised method which was used to identify common metabolic changes after infection with fungi, regardless of cultivars of *B. rapa*, developmental stage, and fungal species. The Y-matrix consists of two discrete classes, control and fungus-infected plants. Compared to PCA results (Fig. 2A), the score plot of PLS-DA evidently improved the separation between control and infected plants (Fig. 2B). PLS-component 1 is the main factor to distinguish the two classes. The loading plot of PLS-component 1 vs. 2 revealed that all the fungi-infected plants were characterized by high levels of fumaric acid, aliphatic glucosinolates (progoitrin), flavonoids (kaempferol analogue) and phenylpropanoids (sinapoyl and feruloyl malate) (Fig. 2C). On the other hand, the control plants were characterized by high levels of γ-aminobutyric acid (GABA), choline and sucrose (Fig. 2C). For the confirmation of statistical

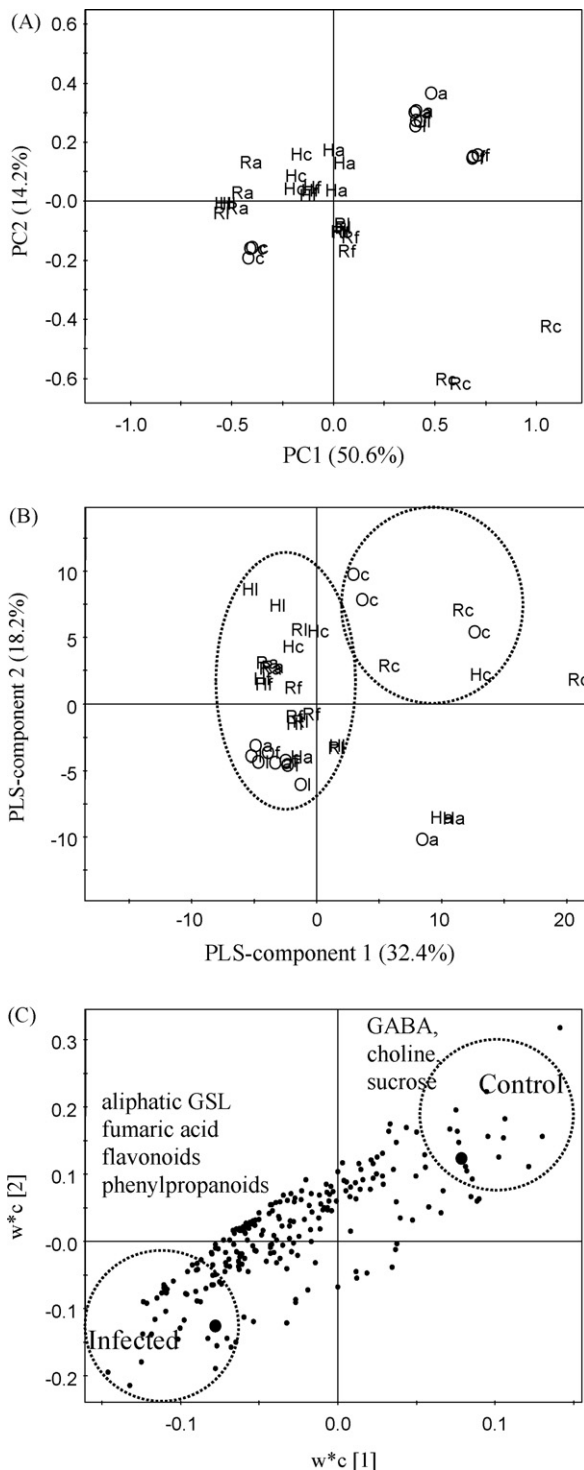


Fig. 2. Score plot of PCA (A) and PLS-DA (B) of control and infected plants, and loading plot of PLS-DA (C). Rc: Raapstelen control, Ra: Raapstelen infected with *Aspergillus niger*, Rl: Raapstelen infected with *Leptosphaeria maculans*, Rf: Raapstelen infected with *Fusarium oxysporum*, Hc: Herfstraap control, Ha: Herfstraap infected with *Aspergillus niger*, Hl: Herfstraap infected with *Leptosphaeria maculans*, Hf: Herfstraap infected with *Fusarium oxysporum*, Oc: Oleifera control, Oa: Oleifera infected with *Aspergillus niger*, Ol: Oleifera infected with *Leptosphaeria maculans*, Of: Oleifera infected with *Fusarium oxysporum*.

significance, *t*-test was applied for the signals responsible for the separation in PLS-DA ($p < 0.05$).

The next question was to determine whether or not there was a significant difference in the metabolic response of *B. rapa* to each fungus species. For this, PLS-DA was used to evaluate the effect of

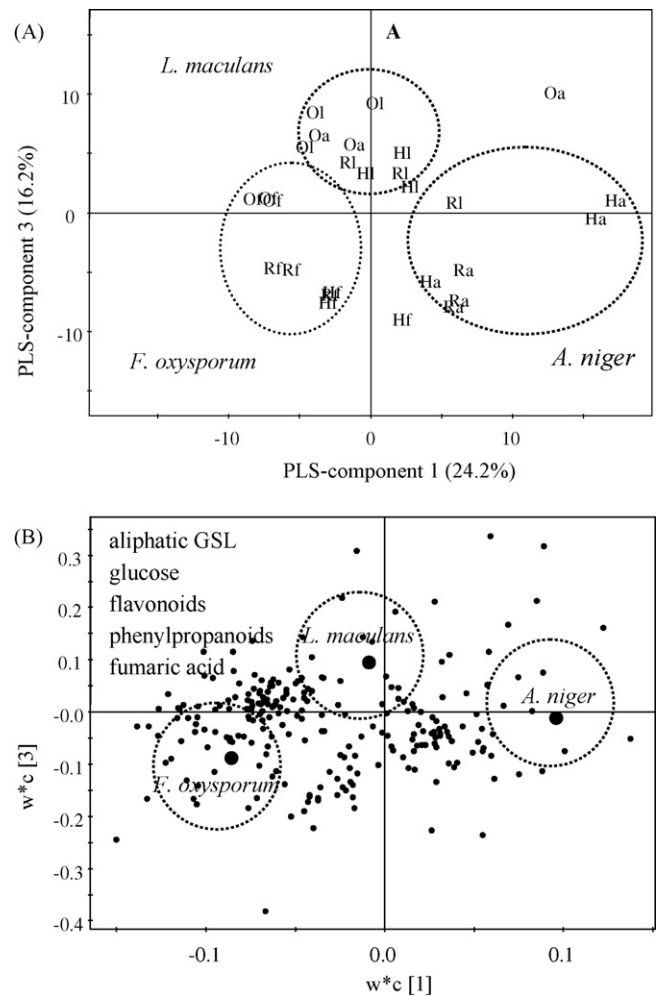


Fig. 3. Score (A) and loading plot (B) of PLS-DA of three groups of fungus-infected *Brassica rapa*. Labelling of each group are the same as Fig. 2.

each fungus on the expression of metabolites in the infected plants, using three classes derived from the three species excluding control plants. The score plot of PLS-component 1 vs. 3 showed the best separation of infected plants according to the type of fungus (Fig. 3A). The loading plot of PLS-component 1 vs. 3 followed by *t*-test indicated that the plants infected with *F. oxysporum* produced higher concentration of glucose, fumaric acid, aliphatic glucosinolate (progoitrin), flavonoids (kaempferol and quercetin analogs).

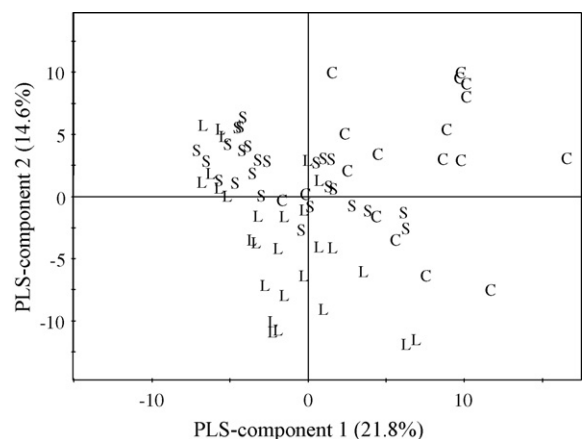


Fig. 4. Score plot of PLS-DA of local and systemic leaves of *Brassica rapa* infected with fungi. C: control leaves, L: local leaves, S: systemic leaves.

gues) and phenylpropanoids (sinapoyl, feruloyl and 5-hydroxyferuloyl malate) (Fig. 3B).

For following approach the difference between directly infected leaves (local) and systemic ones (leaves upper of local ones) were analyzed by PLS-DA using three groups: control plants (upper and lower leaves), local infected leaves, and systemic infected leaves were used. Interestingly, the systemic leaves were also separated from control leaves although to a lesser extent than local leaves (Fig. 4). Local infected leaves accumulated higher levels of lactic acid and aliphatic glucosinolates which significantly differed from systemic leaves.

The responses of each cultivar of *B. rapa* to the infection with three fungi was investigated. The relative level for the metabolites in infected cultivars comparing to 100% of the control was calculated. Among the cultivars evaluated, Raapstelen showed significant increase in primary metabolites including amino acids (alanine, valine, threonine and glutamic acid) and secondary metabolites including phenylpropanoids (sinapoyl-, coumaroyl-, caffeoyl-, feruloyl- and 5-hydroxyferuloyl malate), flavonoids (kaempferol) and indole acetic acid (IAA). Among phenylpropanoids, sinapoyl- and 5-hydroxyferuloyl malate showed higher level when Raapstelen interacting with *F. oxysporum*. Raapstelen

interacting with fungi showed significant decrease in progoitrin, neoglucobrassicin and GABA (Fig. 5). Herfstraap infected with fungi showed significant increase in progoitrin and neoglucobrassicin. Among phenylpropanoids, caffeoyl- and 5-hydroxyferuloyl malate together with IAA significantly decreased after fungal infection of Herfstraap. Oleifera showed significant decrease in amino acids levels with the three fungi and showed significant increase in phenylpropanoids (caffeoyl-, feruloyl- and 5-hydroxyferuloyl malate) and flavonoids (quercetin) with higher levels of these compounds when Oleifera interacting with *F. oxysporum* (Fig. 5). Aliphatic and indole glucosinolates showed significant increase in Oleifera interacting with fungi and IAA showed significant decrease.

4. Discussion

Plants challenged by pathogens may exhibit several biochemical defense responses such as enzyme synthesis, cell wall deposition of lignin and suberin and accumulation of specific metabolites [25]. Of these diverse plant defense mechanisms, the metabolite-involved defense mechanism is mainly associated with plant secondary metabolites.

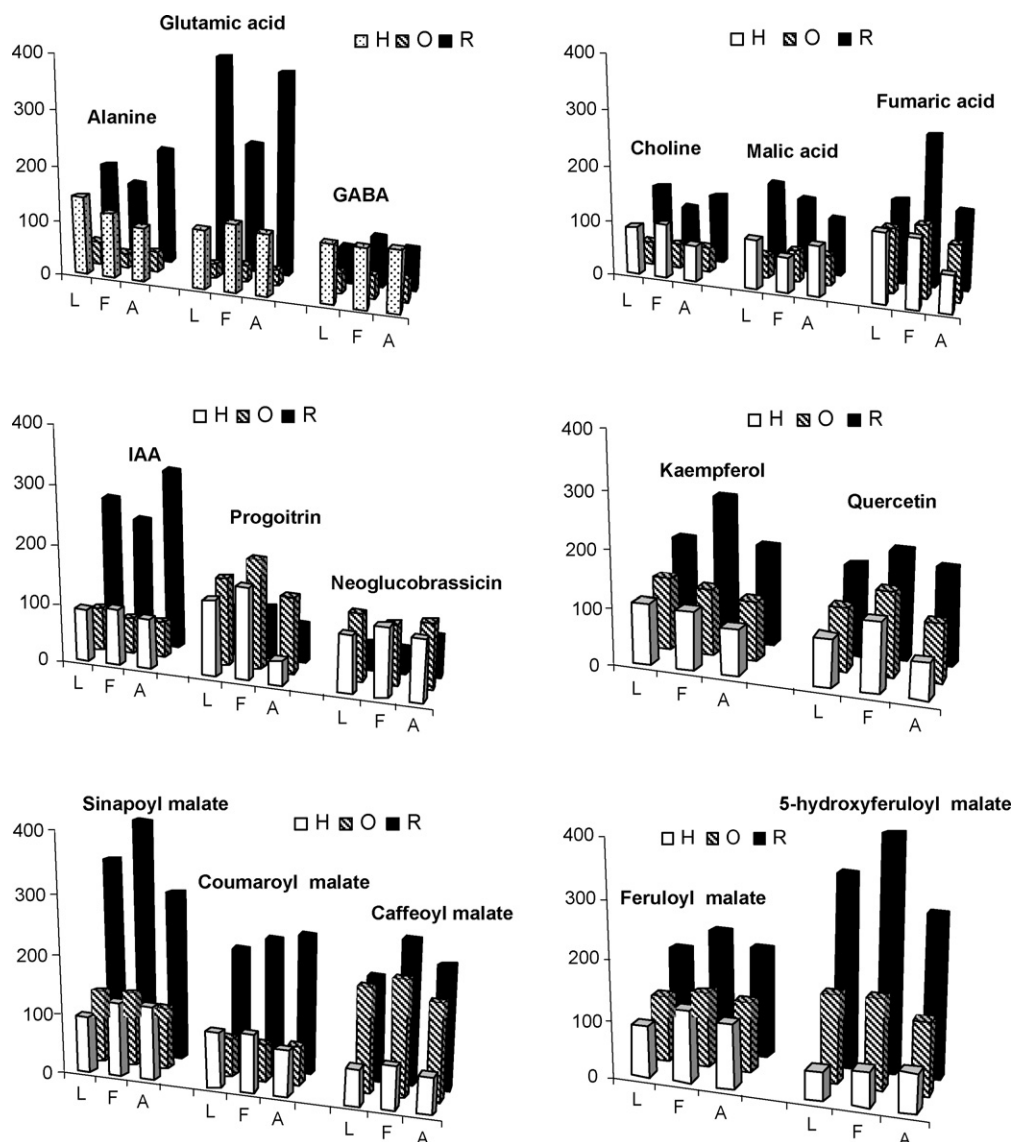


Fig. 5. The relative levels of metabolites comparing to 100% ^1H NMR intensities of control after infection of three cultivars of *Brassica rapa* with three fungi.

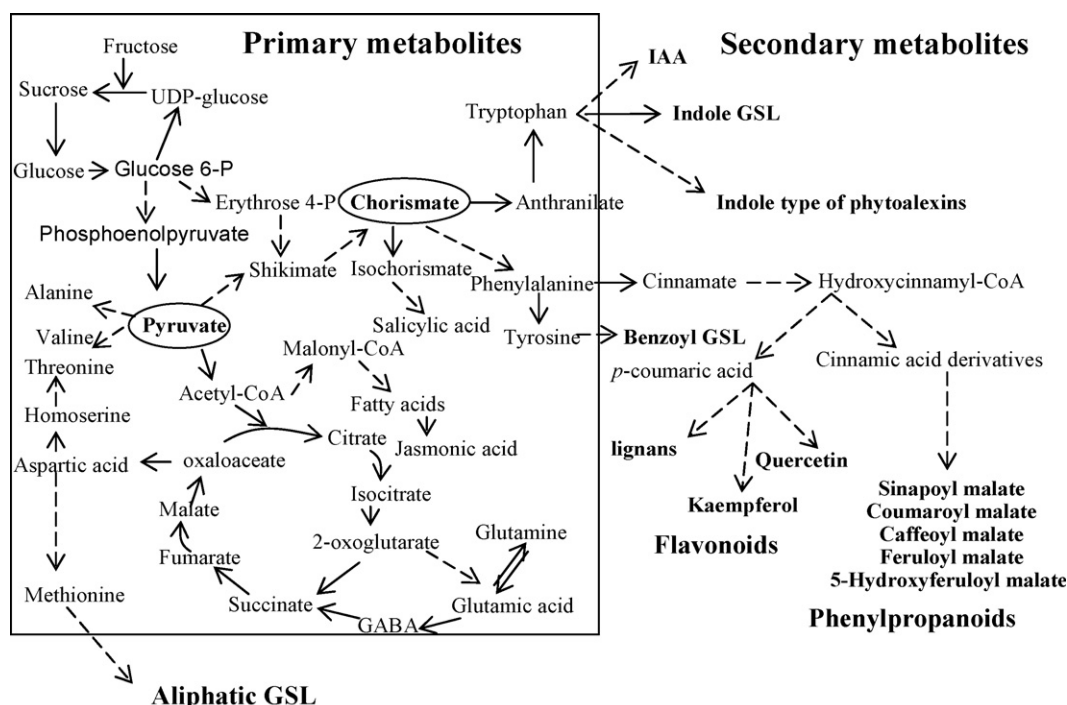


Fig. 6. General biosynthetic pathway of secondary metabolites in *Brassica rapa*. Dashed lines: multi-steps reactions, solid lines: one-step reactions.

In most cases of fungal infection, phenylpropanoids are often detected as biomarkers of fungal infected plants. The fungal infected *B. rapa* plants evaluated in this study showed a higher level of phenylpropanoids such as sinapoyl- and feruloyl malate. Although the infection significantly increased phenylpropanoids in different cultivars of *B. rapa*–fungi interactions, the infection with *F. oxysporum* showed higher levels of sinapoyl-, feruloyl- and 5-hydroxyferuloyl malate. In previous studies carried out by our group, treatment of *B. rapa* with the signal molecule methyl jasmonate (MJ) involved in plant defense was also found to enhance the accumulation of phenylpropanoids in *B. rapa* [20]. Similarly, MJ treated *Arabidopsis*, showed increased levels of sinapoyl malate as compared to control plants [26]. Another study showed that *Arabidopsis* phenylpropanoids increased in concentration upon infection of roots with *P. sylvaticum*, as well as after infection of leaves with *P. syringae* [10].

Hydroxycinnamic acid esters play a role in resistance of some plants against different fungi such as in cucumber against the powdery mildew fungi [25] and in Chick pea (*Cicer arietinum*) against *Sclerotium rolfsii* [27].

In addition to the change in phenylpropanoids level, the present study showed a significant increase of flavonoids (kaempferol and quercetin analogues) in infected plants with higher levels in *F. oxysporum* infected plants. This is in agreement with other cases such as the increased level of anthocyanins observed in Chinese cabbage leaves as a response to the fungal pathogen *A. brassicae* [14]. Similarly, methyl jasmonate treated *Arabidopsis* showed higher levels of kaempferol and quercetin analogues [26]. Actually, induction of the synthesis of flavonoids in plants following fungal pathogen attack is a well-known phenomenon [28]. For example, epidermal anthocyanin production in cotton leaves is an indicator of bacterial blight *Xanthomonas campestris* resistance [29]. The increase of cinnamic acid derived products in several plants species has been linked with the activity of the enzyme phenylalanine ammonia lyase (PAL) which is the first and key regulatory enzyme of the phenylpropanoids pathway. It links the primary metabolism to the secondary metabolism by catalyzing the deamination of the primary metabolite L-phenylalanine to produce *trans*-cinnamic

acid, leading, thus, to the formation of a wide array of secondary metabolites with the phenylpropane skeleton including coumarins, lignans, and flavonoids [30] (Fig. 6). In *Arabidopsis*, PAL responded strongly to abiotic stress. Kaempferol, quercetin and anthocyanins showed significantly increased levels in *Arabidopsis* as a result of nitrogen depletion [30].

High level of aliphatic and indole glucosinolates accumulated in *B. rapa* (Herfstraap and Oleifera) infected with *F. oxysporum*. An increase in levels of aliphatic glucosinolates was observed in *A. brassicae* infected *B. napus* [13]. Similarly, Chinese cabbage infected with *A. brassicae* or *P. brassicae* showed a significant increase in aliphatic and indole glucosinolates [9,14]. When treated with jasmonic and salicylic acid, this plant also exhibited higher levels of aliphatic, indole and benzoyl glucosinolates [9]. *Brassica oleracea* infected with *P. parasitica* also showed an enhancement of total aliphatic and indole glucosinolates [16].

Many primary metabolites were induced by fungal infection together with secondary metabolite. Sucrose was observed to decrease after fungal infection. Coincidentally, the sucrose level in *A. brassicae* infected leaves of Chinese cabbage decreased [14] and also in MJ treated *B. rapa* [20]. This typical pattern is an indication of the alteration of the carbohydrate metabolism following infection. A portion of the available carbon stored in sucrose is possibly diverted towards secondary metabolism, as revealed by the increased production of phenylpropanoids, kaempferol, quercetin and glucosinolates after infection. Another part of the carbohydrates provides the necessary energy to support increased secondary metabolites biosynthesis [26]. The decrease of sucrose after pathogen infection may also be associated with a mechanism for reallocation of nutrients to other non-infected plant parts for compensatory growth [14].

Regarding to the response of different cultivars and varieties of *Brassica* species to biotic stress, varieties of *B. campestris* infected with *P. brassicae* [9], cultivars of *B. napus* infected with *A. brassicae* [13] and varieties of *B. oleracea* infected with *P. parasitica* [16] showed different glucosinolate profiles according to the cultivars and varieties. These previous studies have been focused only on glucosinolate content after infection with only one fungal species.

Using the non-targeted analysis by NMR spectroscopy, we studied the response of different cultivars of *B. rapa* after infection with three different species of fungi. The cultivar Raapstelen showed different response after infection with fungi comparing to the other two cultivars (Herfstraap and Oleifera). The decrease in glucosinolate levels and increase in IAA, flavonoids and phenylpropanoids levels after infection of Raapstelen with fungi points to a different channeling in the chorismate and phenylpropanoids pathways [31] and one may hypothesize that the flux towards the precursors, tryptophan and phenylalanine or tyrosine is strongly regulated. Some of the phenylpropanoids (caffeoyl- and 5-hydroxyferuloyl malate) together with IAA decreased significantly after infection of Herfstraap, whereas aliphatic and indole glucosinolates showed significant increase. Via increasing the indole glucosinolates, the flux towards the tryptophan is strongly regulated in Herfstraap after fungal infection. In Oleifera, where phenylpropanoids, flavonoids and glucosinolates levels increased and in the same time the IAA decreased, the flux towards the precursors, tryptophan and phenylalanine or tyrosine is strongly regulated.

Although Raapstelen and Oleifera showed significant increase in phenylpropanoids and flavonoids after fungal infection, the level of these metabolites are higher in the interaction with *F. oxysporum* comparing to other two interactions (*L. maculans* and *A. niger*). The specialization of *L. maculans* in the Brassicaceae family members may have lead to the development of resistance of the fungus to the plant defense mechanisms, such as the secretion of high concentration of detoxifying enzymes that interfere with biosynthetic pathways or phytotoxins that can degrade plant metabolites, thus resulting in different metabolic profiles. The interaction between *B. rapa* and *F. oxysporum* or *A. niger* showed non-host resistance which is the most common and durable form of plant resistance in nature [32]. This type of resistance may be accompanied with high levels of antifungal secondary metabolites including phenolic and glucosinolates which are the most important compounds required for plant defense. The increase in phenylpropanoids and flavonoid in the transgenic flax plants has been the main reason that improved resistance of flax to *Fusarium*, the main pathogen of flax [33]. The susceptibility of transgenic tobacco to diseases has been increased with suppression the level of phenolic compounds [34]. Among phenylpropanoids, caffeoyl-, sinapoyl- and 5-hydroxyferuloyl malate showed higher level when Raapstelen, and Oleifera interacting with *F. oxysporum* which may be attributed to the hydroxylation and methylation of coumaroyl- and feruloyl malate at the C3 and C5 positions of the phenolic ring under the condition of the infection to form caffeoyl-, sinapoyl- and 5-hydroxyferuloyl malate.

In conclusion, this study revealed that there is a qualitative difference in plant metabolites enhanced after infection, depending on the attacking fungus. Also there are different responses to infection depending on the host cultivar. The secondary metabolites accumulated are known as antifungal secondary metabolites (phenylpropanoids, flavonoids and glucosinolates) which are related to different types of biotic stresses. The increased levels of these compounds in plant–fungal interaction support their role in plant resistance.

By means of NMR-based metabolomics, we were able to show metabolic changes in several major branches of secondary metabolism in *B. rapa* after fungal infection. A further more detailed targeted analysis of these phenylpropanoids, flavonoids and glucosinolates is of interest to possibly determine the role of individual compounds on resistance. Moreover, as it seems that chorismate plays an important role for several of the pathways involved in the defense response. To study the regulation of the fluxes through the pathways branching off the intermediate chorismate seems of interest to learn more about their role in the plant defense.

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