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Glucosinolate profiling of *Brassica rapa* cultivars after infection by *Leptosphaeria maculans* and *Fusarium oxysporum*

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ABSTRACT

The glucosinolate contents of two different cultivars of *Brassica rapa* (Herfstraap and Oleifera) infected with *Leptosphaeria maculans* and *Fusarium oxysporum* were determined. Infection triggered the accumulation of aliphatic glucosinolates (gluconapin, progoitrin, glucobrassicanapin and gluconapoleiferin) and indole glucosinolate (4-hydroxy-glucobrassicin) in Herfstraap and of two indole glucosinolates (glucobrassicin and 4-hydroxy-glucobrassicin) in Oleifera. While total and aliphatic glucosinolates decreased significantly in Oleifera, a large increase was observed in Herfstraap after fungal infection. The indole glucosinolate glucobrassicin accumulated in Oleifera at a higher rate than Herfstraap especially after infection with *F. oxysporum*. Apparently the interaction between fungus and *B. rapa* is cultivar and fungal species specific.

1. Introduction

Knowledge of the chemical composition of Brassicaceae is not only important because of the health related properties of various vegetables and oils obtained from plants belonging to this family, but also for a better understanding of the possible changes resulting from the activation of resistance mechanisms (Abdel-Farid et al., 2006). Metabolic characterization of different cultivars of *Brassica rapa* was previously done using NMR-based metabolomics and some phenylpropanoids, flavonoids and glucosinolates were identified (Abdel-Farid et al., 2007). Among the identified compounds in Brassicaceae, the group of glucosinolates has attracted a great deal of attention as they seem to be involved as phytoanticipins in the chemical defense of the plants (Abdel-Farid et al., 2007, 2009).

Glucosinolates are classified into three different groups depending on the precursor amino acid, from which they are derived: aliphatic glucosinolates derive from methionine, aromatic glucosinolates from phenylalanine and tyrosine, and indole glucosinolates from tryptophan (Halkier and Du, 1997; Kiddle et al., 2001). Glucosinolates and their breakdown products are thought to play a role in disease resistance to fungal pathogens (Mithen, 1992; Mithen et al., 1987). Thus, the accumulation of glucosinolates can be induced after wounding (Bodnaryk, 1992), pathogen attack (Doughty et al., 1991; Ludwig-Müller et al., 1997), herbivore attack (Widarto et al., 2006), as well as after treatment with salicylic acid (SA)

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(Kiddle et al., 1994; Ludwig-Müller et al., 1997; van Dam et al., 2003), jasmonic acid (JA) (Ludwig-Müller et al., 1997) and methyl jasmonate (MJ) (Bodnaryk, 1994; Doughty et al., 1995; Liang et al., 2006; Hendrawati et al., 2006).

Some pathogenic fungi have the ability to infect Brassicaceae species and cause detrimental effects on the quality and quantity of the crop. Of these, *Leptosphaeria maculans*, responsible for crucifer blackleg occurs worldwide and can be particularly devastating for the oilseed crops of *Brassica napus* and *B. rapa* (Howlett et al., 2001). *F. oxysporum* is a common soil-borne fungus that causes damping-off and wilting of many plants including *Brassica* species, causing severe yield reductions (Gaetán, 2005). *Brassica*-fungi interactions have been extensively studied in many species of the genus, but unfortunately *B. rapa* has not received much attention yet. Most studies have focused on the interaction between *B. napus* and fungi. Among these, research carried out by Doughty and co-workers in 1991 (Doughty et al., 1991), considered to be one of the most important in this field, consisted in the monitoring of the glucosinolate content of two cultivars of *B. napus* after inoculation with *Alternaria brassicae*. Significant variations in the aliphatic glucosinolate composition were observed after inoculation of *B. napus* with *A. brassicae* following different patterns in susceptible and resistant cultivars. To our knowledge only Ludwig-Müller et al. (1997) studied the interaction between the clubroot fungus, *Plasmodiophora brassicae*, and two susceptible and two resistant varieties of *B. rapa,* reporting a significant variation of glucosinolates in the roots after infection with this fungus. In another study, the glucosinolate content of three susceptible and two resistant varieties of *Brassica oleracea* var. *botrytis* infected with *Peronospora parasitica* was evaluated, finding the sinigrin content to be higher in resistant varieties than in susceptible ones. Additionally, resistant varieties were able to be distinguished from susceptible ones according to the ratio of glucobrassicin and 4-methoxy-glucobrassicin (Ménard et al., 1999).

The aim of this study was to investigate the glucosinolate profile after infection with two strains of fungi. For this, the leaves of plants of two cultivars of *B. rapa* were inoculated with two different fungi: one responsible for blackleg disease or canker, while the other was a fungus that infects *Brassica* roots causing wilting disease. Subsequently, their glucosinolate content was investigated in order to determine its pattern of variation.

2. Materials and methods

2.1. Plant materials

The seeds of *B. rapa* cultivars, Herfstraap (Goldana) and Oleifera were germinated in soil and placed in a cold room (4 °C) for two days in the dark and in a closed container. The seedlings were then transferred to the greenhouse and grown under controlled conditions, at 25 °C, 50–60% humidity and 16 h light/8 h dark cycles. Each seven-day old seedling was transferred to a 10 cm diameter pot containing substrate and grown in the same controlled room. Plants were watered daily.

2.2. Fungal strains, preparation of conidia and inoculation

The fungus *L. maculans* kindly provided by Prof. Barbara Howlett (University of Melbourne, Australia) and *F. oxysporum* was obtained from the Department of Fungal Genetics, Leiden University (Leiden, The Netherlands). Fungi were grown on potato dextrose agar (PDA) in a 24 °C incubator until formation of conidia.

The conidia were collected as follows: the Petri dish with fungus culture was immersed in sterile distilled water. After removing the sticky pores of fungi using sterilized tooth brushes, conidia were collected by aspiration with sterilized pipettes and filtered through two layers of sterile Miracloth. The concentration of conidia was adjusted to 10^6 spore/ml with a haemocytometer. The fourth leaves (local leaves) of six week-old *Brassica* plants were inoculated with *L. maculans* or *F. oxysporum* conidia, while sterilized distilled water was applied to leaves of the control. The plants were covered by plastic sheets for the following two days. Seven days after infection, the infected (local leaves) and the leaves above the infected leaves (sixth leaves or systemic leaves) were harvested from each plant and immediately transferred to a chamber containing liquid nitrogen. Leaves were then ground under liquid nitrogen and freeze-dried.

2.3. Glucosinolate analysis

Extraction was performed using the method described by Font et al. (2005) and Padilla et al. (2007) with slight modifications. Dried leaves (100 mg) were transferred to Eppendorf tubes and 2 ml of 70% methanol/water were added. The samples were vortexed, heated for 6 min at 90 °C, sonicated for 15 min and centrifuged for 15 min at 3500 rpm. After removal of the supernatant, the extraction procedure was repeated once more. The combined supernatants were pipetted onto an ion-exchange column containing 0.5 ml of Sephadex DEAE-A25. Desulphation was carried out by the addition of 20 μl of purified sulphatase solution (E.C. 3.1.6.1, type H-1 from *Helix pomatia*) (Sigma). Desulphated glucosinolates were eluted with 2 ml (2 × 1 ml) of Milli-Q (Millipore) water and freeze-dried for at least two days. The residue was redissolved in 1 ml of Milli-Q water and subjected to HPLC analysis. The HPLC system used consisted of a Waters system equipped with a 626 pump and 600 S pump controller, a 717 plus autosampler, and a 2996 photodiode array detector set at 229 nm. Desulphated sinigrin was used as a standard and the amount of each individual glucosinolate present in the sample was calculated using sinigrin as a reference and expressed as nmole/g of dry wt. The total glucosinolate content was calculated as the sum of all individual glucosinolates present in the sample. Three replicates were analyzed for each sample.

2.4. Statistical analysis

The significance of the differences between individual and total glucosinolates in infected plants compared to controls was assessed by one-way analysis of variance (ANOVA) using Minitab version 12.21. The same method was also used to evaluate the differences between *L. maculans* and *F. oxysporum* interactions and the cultivars with regard to glucosinolate content in each cultivar of *B. rapa*.

3. Results and discussion

3.1. Total glucosinolate profiles

A preliminary experiment was designed to study the resistance and susceptibility of two cultivars of *Brassica* (Herfstraap and Oleifera). From the number of necrotic parts after infection Herfstraap was classified as resistant and Oleifera as susceptible (data not shown). The analysis of glucosinolates of these two cultivars before infection showed higher level of glucosinolates in Herfstraap as compared with that of Oleifera (Abdel-Farid, 2009) further increasing the probability that Herfstraap might be a resistant and Oleifera is a susceptible cultivar. The glucosinolate content of the two *B. rapa* cultivars (Herfstraap and Oleifera) was analyzed after infection with *L. maculans* or *F. oxysporum*. A total of eight glucosinolates which were found to be present in all analyzed plants were identified and quantified by HPLC (Fig. 1). The glucosinolate content increased at a higher rate in the systemic leaves than in the infected local leaves of Herfstraap. Also the control of Oleifera local leaves was lost, so in the discussion part we will focus more on the changes of glucosinolates in the systemic leaves after infection with *L. maculans* and *F. oxysporum*. The local leaves of *L. maculans* infected Herfstraap cultivar plant significantly differed from control in terms of total and aliphatic glucosinolates, as well as progoitrin and gluconapin, whereas local leaves of *F. oxysporum* infected plants showed significant differences from control with regards to total, aliphatic and indole glucosinolates, as well as all detected individual glucosinolates (Figs. 2A and 3A).

The cultivars showed contrasting glucosinolate responses. In the resistant cultivar Herfstraap, aliphatic and indole glucosinolates increased significantly after infection with both *L. maculans* and *F. oxysporum* (Fig. 2A). The amounts of indole glucosinolates, however, were still lower than those of aliphatic glucosinolates (Fig. 2A). In the susceptible cultivar Oleifera the total and aliphatic glucosinolates decreased significantly after infection with *L. maculans* and *F. oxysporum*, whereas indole glucosinolates increased significantly after infection with *F. oxysporum* (Fig. 2B). Doughty et al. (1991) reported similar results with diverse cultivars of *B. napus* infected with *A. brassicae* observing a higher content of aliphatic than indole glucosinolates in resistant cultivars after infection. Our results showed an increased level of indole glucosinolates in both a susceptible cultivar (Oleifera) infected with *F. oxysporum* and the resistant cultivar (Herfstraap) (Fig. 2A and B). This is in agreement with reports of an increase in the total indole glucosinolates levels in both resistant and susceptible cultivars and varieties of different species of *Brassica* (*B. napus* and *B. oleracea*) after infection (Doughty et al., 1991; Ménard et al., 1999). Considering the changes in the levels of the different classes of glucosinolates one may hypothesize that the flux towards the precursor, tryptophan is strongly regulated. It would also be interesting to study the flux towards other secondary metabolites derived

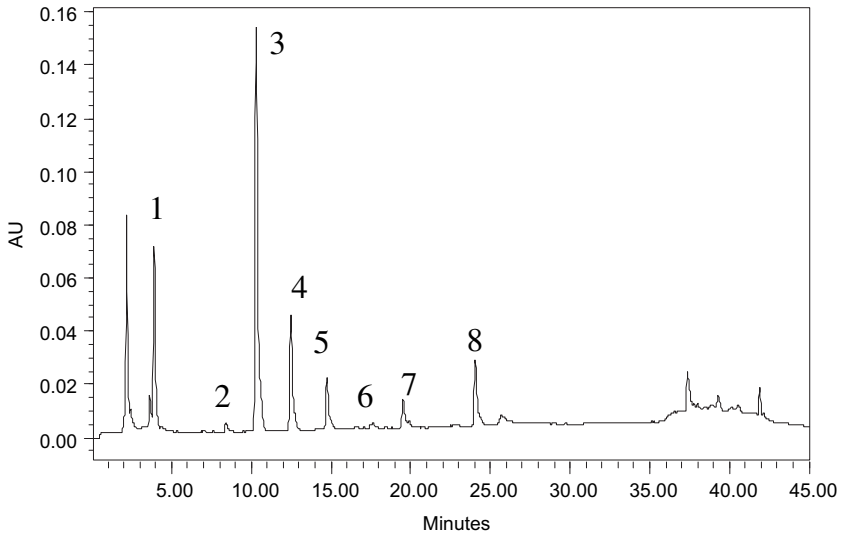

Fig. 1. HPLC chromatogram of control Herfstraap 1: progoitrin, 2: gluconapoleiferin, 3: gluconapin, 4: 4-hydroxy-glucobrassicin, 5: glucobrassicanapin, 6: glucobrassicin, 7: 4-methoxy-glucobrassicin and 8: neoglucobrassicin.

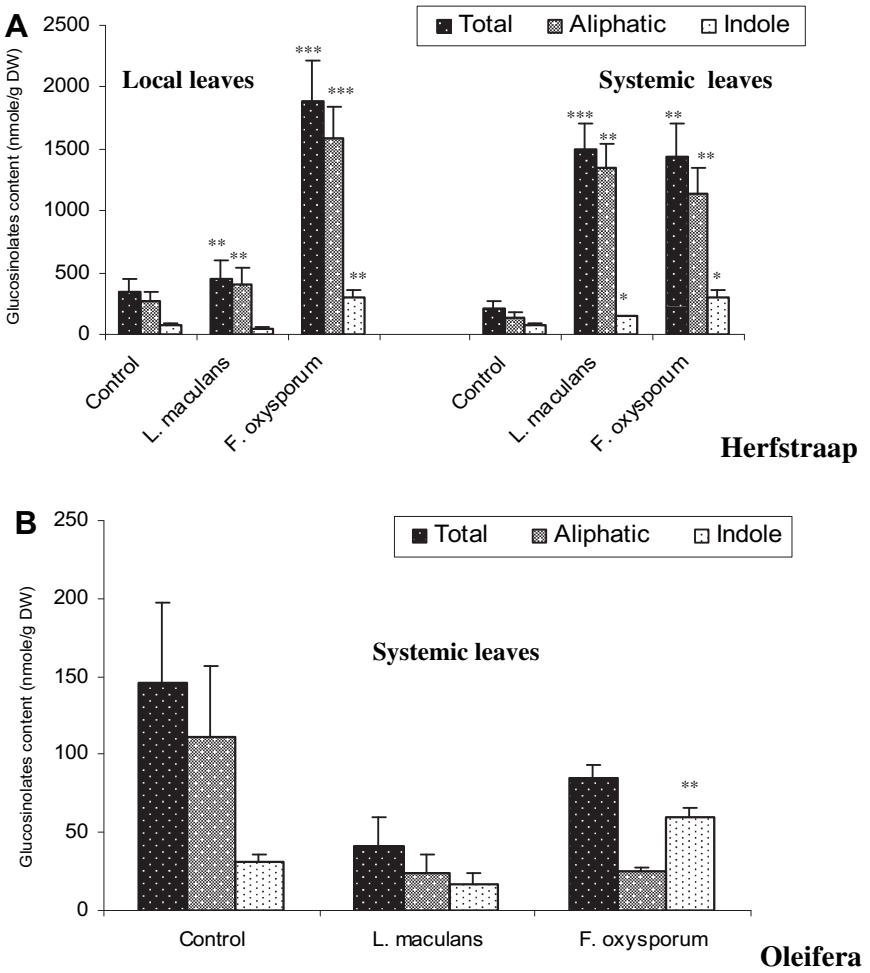

Fig. 2. Distribution of total, aliphatic and indole glucosinolates (nmole/g DW) in local leaves and systemic leaves of Herfstraap cultivar of *B. rapa* infected with *Leptosphaeria maculans* and *Fusarium oxysporum* (A), and systemic leaves of Oleifera cultivar infected with *L. maculans* and *F. oxysporum* (B).

from this precursor. Competition between the pathways for aromatic and aliphatic amino acid derived glucosinolates must occur at the level of pyruvate which is a precursor for methionine and is also incorporated in chorismate (Fig. 4).

3.2. Individual glucosinolate profiles

For the individual glucosinolates the picture is much more complex than for the biosynthetic classes. Progoitrin, gluconapoleiferin, gluconapin and glucobrassicanapin increased significantly in Herfstraap after infection. Conversely, these glucosinolates decreased in infected Oleifera (Fig. 3A and B). Coincidentally, progoitrin content in *B. napus* infected with *A. brassicae* was reported to be higher in a resistant cultivar and lower than control in a susceptible one after 5d from the infection (Doughty et al., 1991). 4-hydroxy-glucobrassicin content increased significantly in Herfstraap infected with both interactions (*L. maculans* and *F. oxysporum*). *B. napus* ssp. *rapifer*a inoculated with turnip mosaic virus showed higher concentration of 4-hydroxy-glucobrassicin (Stobbs et al., 1991). Increase of 4-hydroxy-glucobrassicin and decrease of glucobrassicin in resistant cultivar (Herfstraap) may be attributed to hydroxylation of glucobrassicin to form 4-hydroxy-glucobrassicin after infection with fungi (Fig. 3A), a simple conversion considering the close similarity in their chemical structures (Ménard et al., 1999).

In Oleifera the content of glucobrassicin increased after infection and showed significant variation with both fungal infections (*Leptosphaeria macuans* and *F. oxysporum*) (4 and 13-fold), respectively (Fig. 3B). On the other hand, the 4-hydroxy-glucobrassicin content increased significantly after infection with *F. oxysporum* (3-fold) (Fig. 3B). 4-Methoxy-glucobrassicin and neoglucobrassicin decreased in the susceptible cultivar (Oleifera) with both fungi (Fig. 3B). Doughty et al. (1991) reported that glucobrassicin and neoglucobrassicin increased in both susceptible and resistant cultivars after infection. Similarly, a three to five-fold increase in the indole glucosinolate content of the root tissue of *Brassica campestris* ssp. *pekinensis*

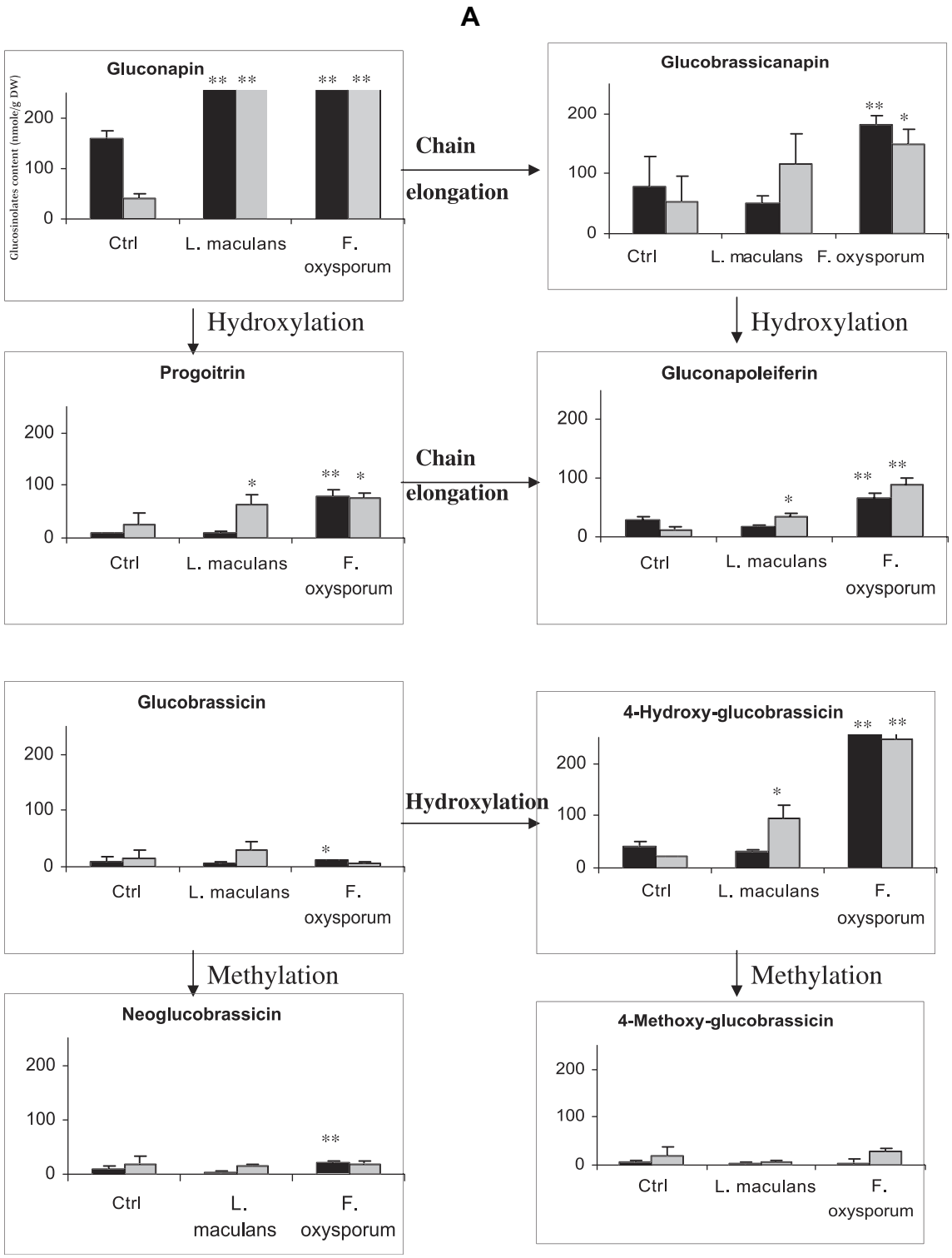

Fig. 3. Distribution of individual glucosinolates (nmole/g DW) in local (black column) and systemic leaves (gray column) of Herfstraap cultivar of *Brassica rapa* infected with *Leptosphaeria maculans* and *Fusarium oxysporum* (A), and systemic leaves of Oleifera infected with *L. maculans* and *F. oxysporum* (B). * = significant ($p < 0.05$), ** = significant ($p < 0.01$), *** = significant ($p < 0.001$).

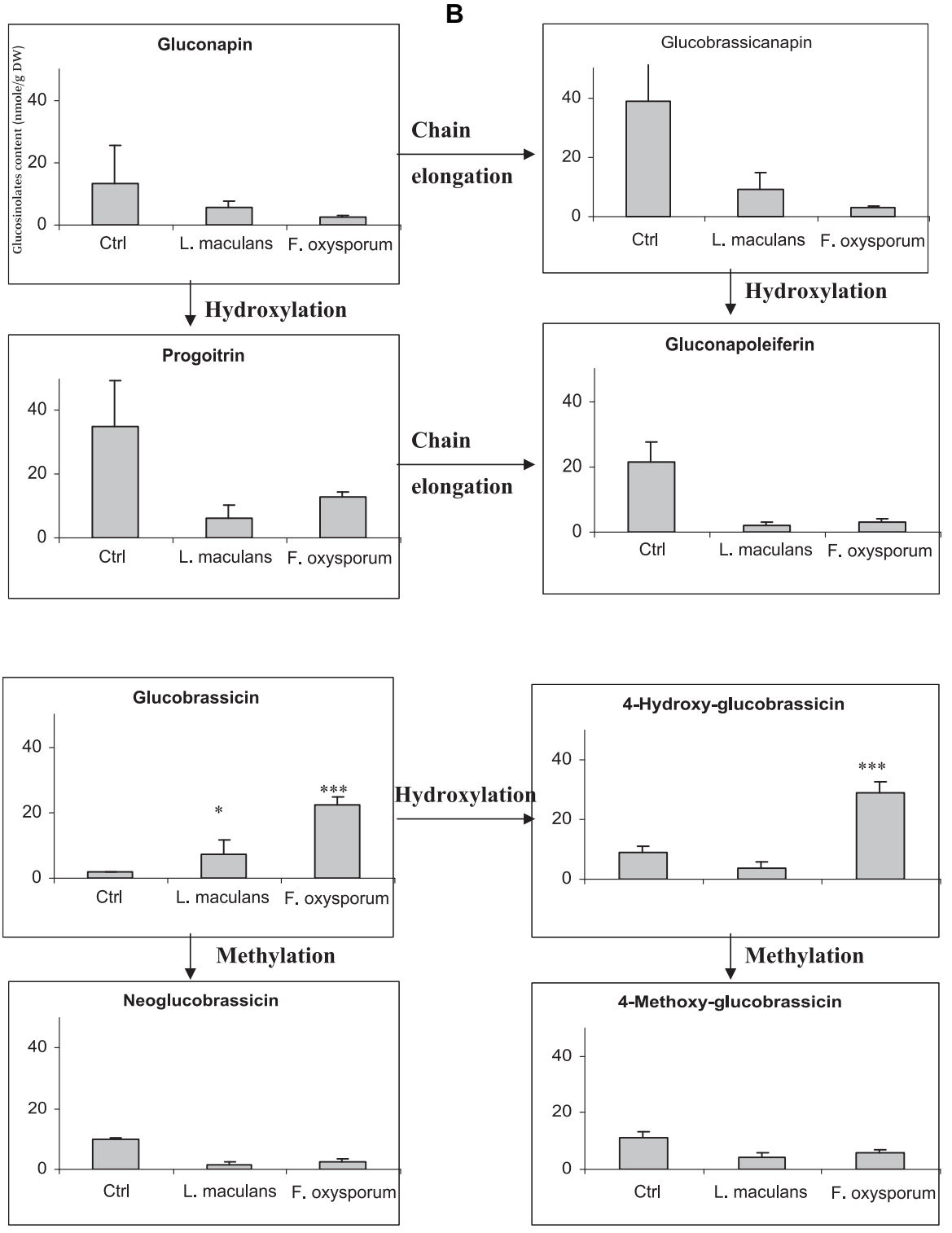

Fig. 3. (*continued*).

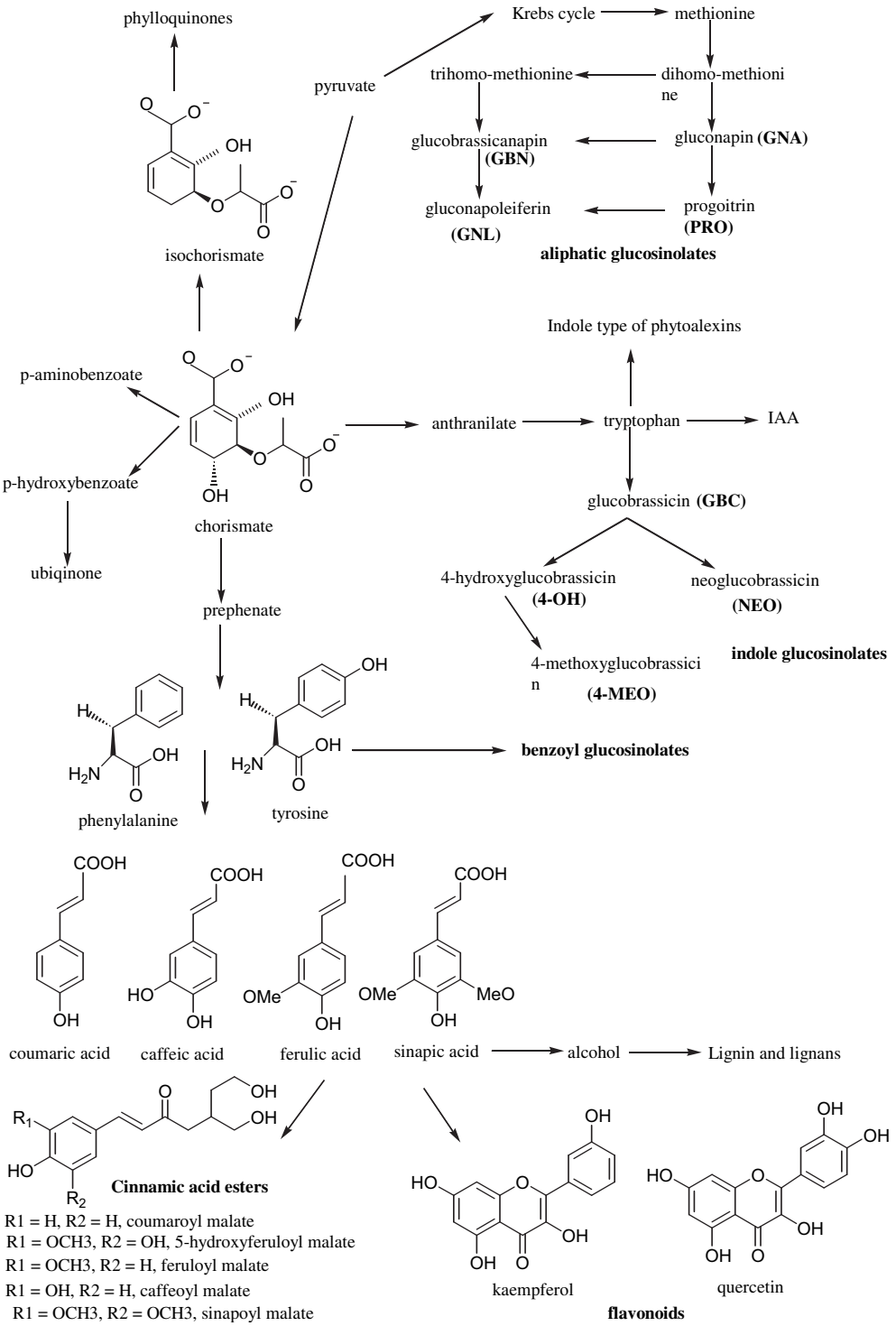

Fig. 4. Biosynthesis of glucosinolates and phenolics from their precursors.

following infection with *P. brassicae* was reported (Ludwig-Müller et al., 1997) but only glucobrassicin was affected. In Herfstraap the total indole glucosinolates increases in the systemic leaves, with a spectrum that shifted more to the 4-hydroxy-glucobrassicin and 4-methoxy-glucobrassicin derivatives in case of *F. oxysporum* infection. However, the *L. maculans* infection did not affect the indole glucosinolate levels in the local leaves, whereas in the systemic leaves a pattern rather similar to the *F. oxysporum* infection was observed though at a much lower total level (Figs. 2A and 3A).

One-way analysis of variance showed significant differences at $p < 0.05$ between *L. maculans* and *F. oxysporum* in Herfstraap cultivar regarding the indole glucosinolates level and some other individual glucosinolates such as gluconapoleferin, gluconapin, glucobrassicanapin, 4-hydroxy-glucobrassicin and 4-methoxy-glucobrassicin. Oleifera infection with *L. maculans* and *F. oxysporum* produced a significant variation of total indole glucosinolates, progoitrin, glucobrassicin and 4-hydroxy-glucobrassicin at $p < 0.05$. Thus, the variation of the total and individual glucosinolate content produced as a response to both fungi (*L. maculans* and *F. oxysporum*) is apparently dependent on both the cultivar and the infecting organism.

3.3. Concluding discussion

In conclusion, leaves of infected *B. rapa* were found to produce significant amounts of glucosinolates. The quantity seemed to be positively correlated to the resistance of cultivars studied in this investigation. The response of the studied cultivars to the infection depends on both the cultivars and the infected organism. Both fungi increased the accumulation of higher aliphatic glucosinolates in infected Herfstraap, whereas in susceptible Oleifera the levels of these compounds decreased. The indole glucosinolates showed a much more complex pattern, since the total increased in the case of the resistant cultivar, whereas in the case of the susceptible Oleifera only one of the fungi triggered an overall increase of indole glucosinolates. Within this indole glucosinolates biosynthetic network different changes occur according to the cultivars. Based on the quantitative and qualitative characterization of the two cultivars of *B. rapa,* Herfstraap and Oleifera proved to be suitable to study the effects of glucosinolates on resistance against fungi in more detail. The precise role of glucosinolates in the resistance mechanisms requires further study, but it is most likely to be involve a combination of factors of which the glucosinolates are just one (Abdel-Farid et al., 2009).

In order to better understand the regulation of the glucosinolate network after infection, a study directed to measure fluxes through the pathways in these conditions should be undertaken. For this, *L. maculans* infected *B. rapa* should be studied at different time points by both NMR and HPLC is desirable. The presence of cultivars of *B. rapa* with contrasting glucosinolate profiles, different resistance and a variable total, aliphatic and indole glucosinolate content after infection with fungi could contribute to understand the role of glucosinolates in antifungal mechanisms of the plant.

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