

# Carbohydrate, phenolic and antioxidant level in relation to chlorophyll *a* content in oilseed winter rape (*Brassica napus* L.) inoculated with *Leptosphaeria maculans*

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**Abstract** The relationships between the level of chlorophyll *a*, and the content of soluble carbohydrates, phenolics and low molecular antioxidants in the leaves of three oilseed winter rape varieties with different resistance to *Leptosphaeria maculans* were determined. During pathogenesis, an increase in the content of chlorophyll *a* in the resistant and medium-sensitive rape varieties was observed. In these varieties, the level of chlorophyll *a* significantly correlated with the content of primary metabolites in the form of soluble sugars, as well as with the level of free and cell wall-bound phenolics, and low-molecular antioxidants. In contrast, a decrease in soluble carbohydrates, observed during the pathogenesis in the susceptible variety, was accompanied by lower level of chlorophyll *a* and with high activity of reactive oxygen species. Significant correlations were also confirmed for the cell wall-bound phenolics and water content in the leaves of the resistant and medium-sensitive variety.

**Keywords** Chlorophyll *a* · *Leptosphaeria maculans* · Leaf water content · Low-molecular antioxidants · Phenolics · Winter rape

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## Introduction

Stem canker attacking the cruciferous plants is one of the most dangerous and the most common fungal diseases of an oilseed rape (Dawidziuk et al. 2012; Liu et al. 2014). A fungus causing this disease, *Leptosphaeria maculans* [anamorph: *Phoma lingam* (Tode ex Fr.) Desm.] may attack the rape throughout the whole growing period. Visible symptoms of infestation of the aboveground plant organs involve characteristic yellowish or light gray spots with black dots called pycnidia that contain conidia. As a plant grows, the spots become larger and deeper, leading to premature maturation of plants or, if the disease attacks a stem, to conidia germination and consequently huge losses in yield (Abuamsha et al. 2011).

Substantial knowledge on the epidemiology of *L. maculans* has been gathered, however, the information on the biochemical response of the oilseed rape at early stages of infection, before the infestation symptoms become visible, is still limited (Hura et al. 2014a). *L. maculans* is a hemibiotrophic pathogen, which in the initial phase of infestation may behave as a biotrophic fungus (Šašek et al. 2012), but also a necrotrophic one (Jindřichová et al. 2011; Hayward et al. 2012). An important element of a necrotrophic infestation is the stimulation of the attacked tissues to reactive oxygen species (ROS) production, which cause cell death and facilitate the infestation of healthy plant tissues (Torres 2010). It should be mentioned that necrotrophs can control plant metabolism by secreting specific proteins that induce defense mechanisms typical for biotrophs (Oliver and Solomon 2010). A result of this ‘tricking’ of the host

plant may be a less effective response in the form of activated biochemical defense mechanisms.

At the initial stage of pathogenesis *L. maculans* is a necrotroph and it benefits from the overproduction of ROS, which are toxic to the plant cellular structures, including the photosynthetic apparatus (Mayer et al. 2001). In our previous work we described the changes in the activity of the photosynthetic apparatus and chlorophyll content in the cotyledons of winter rape growing on an agar medium containing a toxic culture of *L. maculans* / *L. biglobosa* filtrates (Hura et al. 2014b, c). Reactive oxygen species are claimed to be the main cause of chlorophyll degradation, and this is in turn one of the symptoms of progressive cell death (Mur et al. 2010). It is well known that chlorophyll level is crucial for the activity of the photosynthetic apparatus, and hence, indirectly, for the level of carbohydrates (primary metabolites) that are the basis for the synthesis of secondary metabolites (including phenolic compounds), influencing the plant-fungal pathogen interaction (Hura et al. 2014d). The phenols that form cross-bridges between the cell wall carbohydrates are an important element of a plant defense against a fungal pathogen. These compounds bind to the cell wall carbohydrates with ester and/or ether bonds. Saturation of the cell wall with phenolic compounds makes it more compact and impenetrable, and thus a more effective barrier separating a fungal pathogen from a plant cell interior (Santiago et al. 2009). Moreover, reinforced cell wall prevents water loss by healthy plant cells (Hura et al. 2012). Proper cell hydration improves the effectiveness of enzymatic and non-enzymatic antioxidants, as well as the solubility of the primary and secondary metabolites.

The aim of this study was to determine the relationships between the level of chlorophyll *a*, and the content of soluble sugars, free and cell wall-bound phenolics and low molecular antioxidants in the leaves of seedlings of three winter rape varieties with different resistance to *Leptosphaeria maculans*. In contrary to our previous works (Hura et al. 2014a, b) all biochemical analyzes were performed 7 and 14 days after inoculation with *L. maculans* spores. Our research hypothesis is based on an assumption that the level of chlorophyll *a* may be important for increasing the efficiency of defense mechanisms in the plants inoculated with the spores of *L. maculans*. Apart from the biochemical analyzes, we also investigated the interaction between different winter rape varieties and *L. maculans* based on histochemical determination of superoxide anion content and the activity of chitinase and  $\beta$ -1,3-glucanase.

Both enzymes belong to a group of PR proteins, and their function is to destroy the cell wall of a fungal pathogen (Žur et al. 2013).

## Materials and methods

### Plant materials and stress treatment

The experiments involved three varieties of winter rape (*Brassica napus* L.) with different susceptibility to *L. maculans* infection: *cv.* Bojan (sensitive), *cv.* Lisek (medium-sensitive), and *cv.* ‘Liclassic’ (resistant). Susceptibility to fungal infection was based on field trials carried out in Polish conditions, breeding observations and own experiments, whose results have been published (Hura et al. 2014a, b, c, d). The plants were grown in plastic pots (15 cm in diameter, 18 cm in height) filled with a mixture of soil, peat and sand (2:1:1 v/v/v). Until the inoculation the plants were maintained in an air-conditioned greenhouse at a temperature of 16 °C ( $\pm 2$  °C) day/night and air humidity 50 %. The plants were illuminated (12 h light/12 h dark) with PPFD (photosynthetic photon flux density) of about 150–160  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ .

The plants, at a stage of two cotyledons and one leaf, were inoculated with *Leptosphaeria maculans* spores, as described by Jędryczka et al. (1991). The isolate (DH28) of *L. maculans* fungus, was obtained from the Institute of Plant Genetics PAS in Poznań. There were two injection points per a cotyledon. Each half of a cotyledon was centrally injected with 10  $\mu\text{l}$  of the inoculum with a density of  $1.5 \times 10^7$  spores per 1 ml, and the control seedlings were injected with 10  $\mu\text{l}$  of sterile water. The seedlings were injected at a shady place, at a temperature of 18 °C and relative humidity of 80 %.

### Measurements

The measurements were performed 7 and 14 days after the inoculation. The analyses involved the first true leaves that were not directly inoculated. The whole true leaf from a single seedling was collected for a single sample. The biochemical analyzes were performed in five replicates. A replication means the whole true leaf from a single seedling (e.g., five replicates means five leaves from five seedlings). In total, about 70 seedlings of each cultivar were used in the biochemical analysis. Quantitative analyzes (carbohydrates, phenols, antioxidants) were carried

out using freeze-dried plant material. Before lyophilization, three discs were cut out from each leaf for osmotic potential measurements. Then, each leaf was weighed and freeze-dried. After freeze-drying the leaves were re-weighed and powdered using the Qiagen grinder (TissueLyser II, Germany). The powdered leaf material served as a source of three 5 mg samples for the analysis of sugars, phenolic compounds and antioxidants. Summing up, for each individual leaf we determined its water content, osmotic potential, and the level of sugars, phenolic compounds and antioxidants.

#### Detection of superoxide radical ( $O_2^{\cdot-}$ )

Detection of superoxide radical ( $O_2^{\cdot-}$ ) was done according to Doke and Ohashi (1988). The plant samples were infiltrated in darkness for 20 min under the pressure of 0.8 MPa with 0.5 % (*w/v*) nitroblue tetrazolium (NBT) containing 10 mM potassium phosphate buffer (pH 7.0) and 0.005 % (*w/v*) Triton X-100. Then, the samples were exposed to light for 15 min and after that rinsed with hot ethanol (96 %) to remove chlorophyll from the tissues.  $O_2^{\cdot-}$  accumulation, manifested by dark blue spots, was scanned.

#### Activity of $\beta$ -1,3-glucanase (EC 3.2.1.39) and chitinase (EC 3.2.1.14)

Activity of  $\beta$ -1,3-glucanase was determined according to the procedure given by Fink et al. (1988), using the Somogyi reagent (Somogyi 1952), and the Nelson reagent (Nelson 1944). The amount of glucose released from laminarin by the enzyme present in the plant extract was determined spectrophotometrically at 540 nm. The activity of  $\beta$ -1,3-glucanase was expressed in katal per gram of fresh weight. The measurements were taken in five replicates.

Chitinase activity was analyzed according to the procedure provided by Legrand et al. (1987). The amount of N-acetyl-glucosamine (Glc-Nac) released from chitin by the enzyme present in the plant extract was determined spectrophotometrically at 585 nm. Chitinase activity was expressed in nanokatal per gram of fresh weight. The measurements were taken in five replicates.

#### Leaf water content and osmotic potential

Leaf water content (LWC) was assessed by quantitative sampling of leaf fresh weight ( $L_{FW}$ ), followed by

lyophilization for 72 h with Freeze Dry System/Freezone<sup>®</sup>4.5 (Labconco, USA). The resulting leaf dry weight ( $L_{DW}$ ) was measured and the water content was calculated according to the following equation and expressed as a percentage:

$$LWC = ((L_{FW} - L_{DW}) / L_{FW}) \times 100\%$$

the measurements of LWC were taken in five replicates.

Leaf osmotic potential was measured using a dew point microvoltmeter (Wescor Inc., USA), equipped with leaf sample chambers C-52 (Wescor Inc., USA). The measurements were performed on paper discs 5 mm in diameter, soaked with a cell juice squeezed with a syringe from the collected leaf discs (5 mm in diameter). The measurements were taken in a dew point mode in five replicates.

#### Total carbohydrate content

Total content of carbohydrates was estimated by anthrone method according to Ashwell (1957). Sugar extraction from plant samples was performed using 96 % ethanol. The absorbance was measured spectrophotometrically at 490 nm. Glucose was used to prepare the calibration curve. The measurements were taken in five replicates.

#### Phenolics analysis

The total content of soluble and cell wall-bound phenolics was estimated according to Singleton and Rossi (1965), with the use of Folin-Ciocalteu reagent. Phenolics were extracted with 80 % ethanol. Cell-wall bound phenolics (ester- and ether-bond fraction) were removed from the insoluble material by alkaline hydrolysis (NaOH 3 N) according to Hura et al. 2012. The absorbance was measured at 760 nm. Chlorogenic acid was used to prepare the calibration curve. The measurements were taken in five replicates.

#### Low-molecular antioxidants

Quantitative analysis of low-molecular antioxidants was performed using 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to Pieroni et al. (2002). First, 3 ml of DPPH were added to 100  $\mu$ l of plant extract. After 30 min the sample absorbance was measured at 515 nm. Ascorbic acid was used to prepare the calibration curve. The measurements were taken in five replicates.

## Chlorophyll and carotenoids content

The content of the assimilation pigments were determined in ethanol (96 %) extracts. Absorbances at 663, 646 and 470 nm were read and the concentration of chlorophyll and carotenoids was then calculated as described by Lichtenthaler and Wellburn (1983). Analysis of chlorophyll and carotenoids were completed in five replicates.

## Average severity index (ASI)

Severity of infection was estimated 35 days after inoculation, using the visual rating system (0–5) described by Hartman et al. (1984), where: 0 - plant without symptoms; 1-trace symptoms; 2 - less than half of the leaves infected; 3 - half of the leaves with disease symptoms; 4 - more than three-quarters of the leaves infected; 5 - whole plant infected. ASI was calculated according to the formula:

$$\text{ASI} = [(n \times 0) + (n \times 1) + \dots (n \times 5)] \times N^{-1},$$

where n is the number of plants regarding to each disease rating (0–5) and N is the total observations. ASI was calculated as an average from seven pots (five plants in pot, each pot means one replicate). Increased values of ASI mean a decrease in plant resistance to *L. maculans*.

## Statistical analysis

The results were statistically evaluated with the help of Statistica software for Windows, version 9.0. Analysis of variance was used to determine the main effects of inoculation on the biochemical parameters within each studied cultivar. Duncan's multiple range test at the 0.05 probability level was performed to determine the significance of differences among treatment means within each cultivar and between cultivars in the case of ASI. Correlations were tested at a probability of  $P < 0.05$ .

## Results

### Superoxide anion ( $\text{O}_2^{\cdot -}$ )

Due to the limited amount of plant material, a histochemical detection of superoxide anion in the

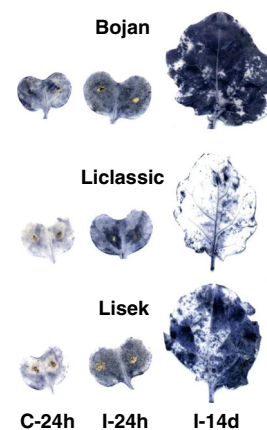
cotyledons was performed 24 h after the inoculation and 14 days later in the true leaves.

Twenty four hours after the inoculation an increase in superoxide anion content was detected in the cotyledons of the investigated winter rape cultivars, as compared with healthy seedlings (Fig. 1). The presence of superoxide anion was also detected in the control seedlings, with the highest level in 'Bojan' cultivar, and this was probably caused by the injury related stress during leaf injection. After 14 days from the inoculation, the greatest accumulation of  $\text{O}_2^{\cdot -}$  was observed in the leaves of 'Bojan' cultivar, with slightly lower values for 'Lisek' cultivar. The lowest level of superoxide anion was detected in *L. maculans* inoculated leaves of 'Liclassic' cultivar.

### Activity of $\beta$ -1,3-glucanase and chitinase

Glucanase activity in 'Bojan' cultivar was similar to the healthy plants 7 days after the inoculation and significantly lower 14 days after the inoculation (Table 1). In 'Liclassic' cultivar an increased  $\beta$ -1,3-glucanase activity was noticed both 7 and 14 days after the inoculation, and in 'Lisek' plants only 7 days following the inoculation.

Chitinase activity in 'Bojan' cultivar was higher 7 days after the inoculation. In the other two cultivars chitinase activity was significantly higher than that observed for the control plants 7 and 14 days after the inoculation. It should be emphasized that the activity of chitinase in the control 'Liclassic' plants was about three



**Fig. 1** Histochemical identification of superoxide radicals ( $\text{O}_2^{\cdot -}$ ) in the cotyledons 24 h after the inoculation and in the true leaves 14 days after the inoculation with *L. maculans* spores. C refers to control plants; I to inoculated plants

**Table 1** Changes in the activity of  $\beta$ -1,3-glucanase (G; [Kat g<sup>-1</sup> (FW)]) and chitinase (Ch; [nKat g<sup>-1</sup> (FW)]) observed 7 and 14 days after the inoculation (I) with *L. maculans* spores in the leaves of studied cultivars

		'Bojan'		'Liclassic'		'Lisek'	
		C	I	C	I	C	I
G	7	3.16±0.17a	2.87±0.26a	2.03±0.21a	3.83±0.36b	1.78±0.07a	2.56±0.18b
	14	2.99±0.18a	1.40±0.10b	2.08±0.19a	4.01±0.25b	1.55±0.13a	1.43±0.16a
Ch	7	55.3±5.4a	72.4±6.1b	155.6±7.6a	245.8±8.3b	65.7±5.9a	102.1±3.0b
	14	54.0±3.5a	49.2±4.7a	146.1±8.7a	290.6±10.1c	56.9±6.7a	136.2±6.4c

C represents control plants. Data are means±SE. Means indicated with the same letters within cultivar are not significantly different ( $P=0.05$ )

times higher than that calculated for control 'Bojan' and 'Lisek' plants.

Leaf water content (LWC), leaf osmotic potential ( $\Psi_O$ ) and soluble carbohydrate content (SC)

Leaf water content (LWC) in 'Bojan' seedlings 7 and 14 days after the inoculation did not change significantly compared to the healthy plants (Table 2). A significant increase in water content was observed in the leaves of 'Liclassic' cultivar both 7 and 14 days after the inoculation, and 14 days following the inoculation in 'Lisek' cultivar.

No significant changes in osmotic potential ( $\Psi_O$ ) were recorded for 'Bojan' leaves (Table 2). Statistically significant and considerable increase in the osmotic potential was observed 7 and 14 days after the inoculation in 'Bojan' plants. In 'Lisek' cultivar the increase was also significant, but less spectacular.

Figure 2 shows the resulting relationships between LWC and  $\Psi_O$  for winter rape cultivars 7 and 14 days after the inoculation. The correlations were statistically significant for 'Liclassic' (Fig. 2b) and 'Lisek' plants (Fig. 2c), and in the first cultivar enhanced LWC was accompanied by higher osmotic potential. On the contrary, in 'Lisek' cultivar increased LWC was accompanied by a decrease in the osmotic potential.

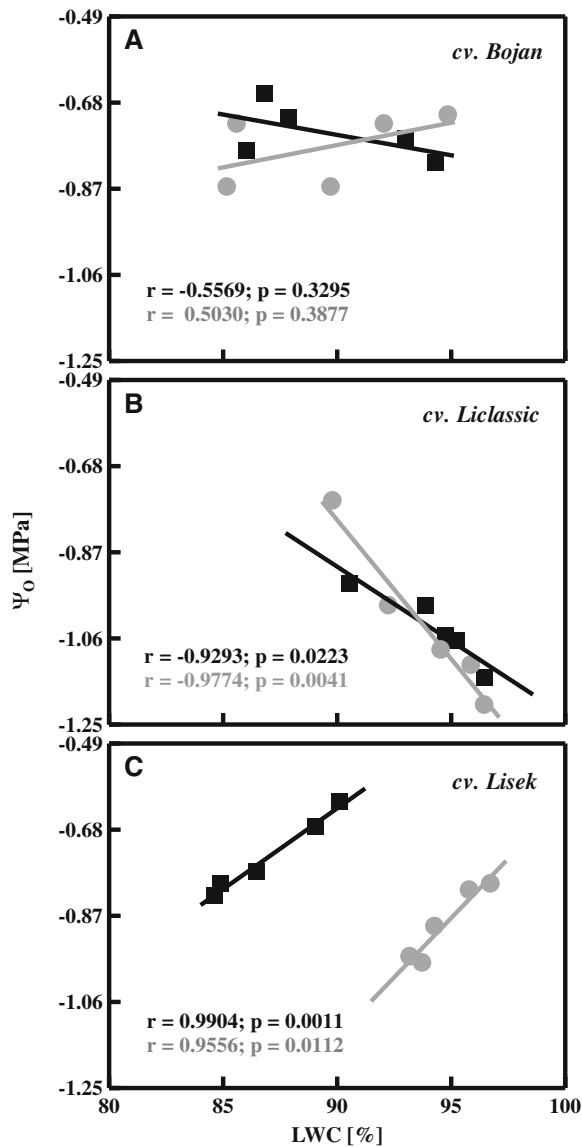
The changes in LWC and  $\Psi_O$  were associated with changes in the content of soluble carbohydrates (SC) (Table 2). In 'Bojan' cultivar, SC content was significantly lower than that observed in the control plants 14 days after the inoculation. An opposite tendency was noticed in 'Liclassic' and 'Lisek' plants. In the first cultivar, an increase was noticed both 7 and 14 days after the inoculation and in 'Lisek' plants 14 days following the inoculation.

Statistically significant correlations were found between the content of soluble sugars and osmotic potential in the inoculated leaves of 'Liclassic' (Fig. 3b) and

**Table 2** Changes in the leaf water content (LWC; [%]), leaf osmotic potential ( $\Psi_O$ ; [MPa]) and soluble carbohydrates content (SC) observed 7 and 14 days after the inoculation (I) with *L. maculans* spores in the leaves of studied cultivars

		'Bojan'		'Liclassic'		'Lisek'	
		C	I	C	I	C	I
LWC	7	88.3±1.77a	89.6±1.69a	86.1±1.36a	94.2±1.00b	91.2±1.47a	90.7±1.10a
	14	89.0±1.79a	89.5±1.86a	86.9±1.32a	93.8±1.23b	91.9±1.20a	96.4±0.78b
$\Psi_O$	7	-0.74±0.039a	-0.76±0.028a	-0.58±0.030a	-1.04±0.035b	-0.72±0.031a	-0.74±0.039a
	14	-0.75±0.027a	-0.78±0.036a	-0.56±0.038a	-1.03±0.077b	-0.73±0.032a	-0.89±0.036b
SC	7	34.6±1.29a	36.9±0.77a	33.7±1.05a	43.8±1.00b	33.7±1.29a	35.8±1.40a
	14	35.7±0.94a	28.9±1.04b	32.1±2.08a	47.0±1.51b	33.5±1.33a	42.7±1.33b

C represents control plants. Data are means±SE. Means indicated with the same letters within cultivar are not significantly different ( $P=0.05$ )

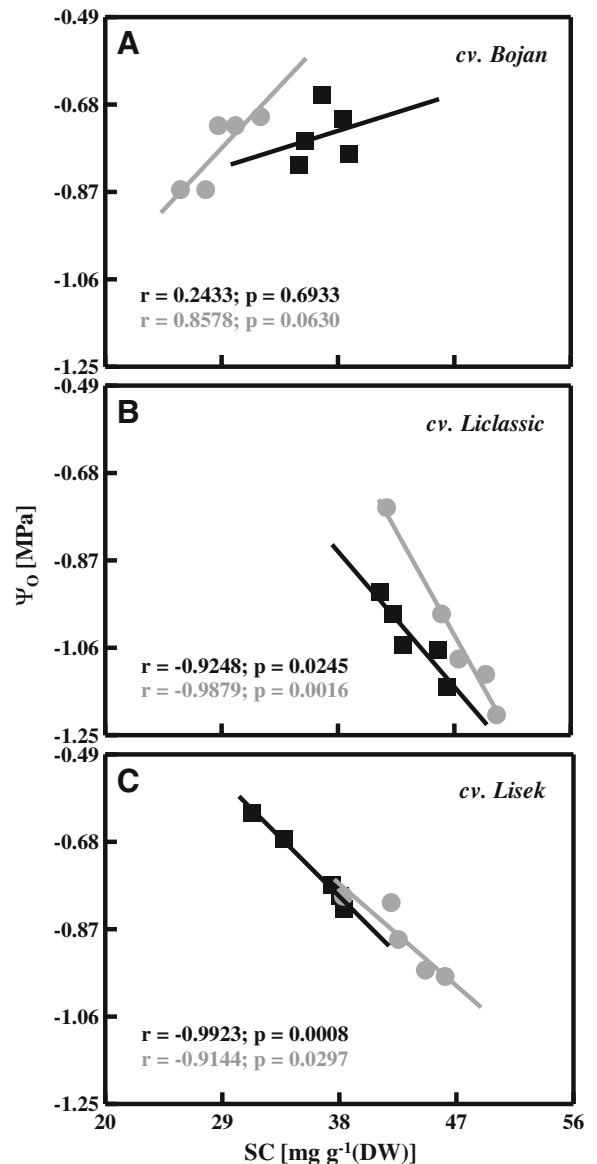


**Fig. 2** Correlations between the leaf osmotic potential ( $\Psi_O$ ) and leaf water content (LWC) in the leaves of *cv. Bojan* (a), *cv. Liclassic* (b) and *cv. Lisek* (c). *Black squares* – 7 days after the inoculation; *gray circles* – 14 days after the inoculation. The lines represent linear adjustment at a probability level  $P < 0.05$

‘Lisek’ cultivars (Fig. 3c). In both cases, higher osmotic potential corresponded to an increase in the content of soluble sugars.

#### Changes in the content of phenolic compounds and low-molecular antioxidants

In the inoculated ‘Bojan’ seedlings the level of soluble phenolic compounds (SPh) after 7 days was close to that



**Fig. 3** Correlations between the leaf osmotic potential ( $\Psi_O$ ) and soluble carbohydrates content (SC) in the leaves of *cv. Bojan* (a), *cv. Liclassic* (b) and *cv. Lisek* (c). *Black squares* – 7 days after the inoculation; *gray circles* – 14 days after the inoculation. The lines represent linear adjustment at a probability level  $P < 0.05$

recorded for the control seedlings and was significantly lower after 14 days (Table 3). In ‘Liclassic’ cultivar SPh content was significantly higher 7 and 14 days after the inoculation. The same increase in SPh was recorded after 14 days in ‘Lisek’ plants.

The level of cell wall-bound phenolics (CWPh) in ‘Bojan’ cultivar did not change either 7 or 14 days after the inoculation with the fungal spores (Table 3). In ‘Liclassic’ plants CWPh content was significantly

**Table 3** Changes in the content of soluble phenolics (SPh; [mg g<sup>-1</sup> (DW)]), cell wall-bound phenolics (CWPh; [mg g<sup>-1</sup> (DW)]) and low-molecular antioxidants (LWC; [mg g<sup>-1</sup> (DW)])observed 7 and 14 days after the inoculation (I) with *L. maculans* spores in the leaves of studied cultivars

		‘Bojan’		‘Liclassic’		‘Lisek’	
		C	I	C	I	C	I
SPh	7	26.5±0.69ab	30.3±0.90a	22.0±1.10a	35.1±1.73b	19.4±0.91a	19.4±1.31a
	14	27.5±2.59a	22.8±0.28b	21.3±0.89a	36.8±1.95b	20.5±0.58a	27.9±0.69b
CWPh	7	4.51±0.27a	4.23±0.29a	4.10±0.31a	6.40±0.22b	3.07±0.22a	3.63±0.26a
	14	4.20±0.33a	4.22±0.35a	3.96±0.28a	9.79±0.30c	2.96±0.30a	5.24±0.37b
LMA	7	63.8±4.81a	47.6±2.88b	127.6±6.8a	277.7±9.3b	149.7±8.5a	135.3±9.7a
	14	62.7±4.60a	50.1±3.29b	136.7±9.9a	277.2±22.7b	158.1±11.4a	359.1±40.9b

C represents control plants. Data are means±SE. Means indicated with the same letters within cultivar are not significantly different ( $P=0.05$ )

higher 7 and 14 days after the inoculation, and in ‘Lisek’ plants raised CWPh was observed 14 days after the inoculation with *L. maculans* spores.

Figure 4 shows the correlations between LWC and CWPh. Statistically significant correlation was found after 14 days from the inoculation of ‘Liclassic’ cultivar (Fig. 4b) with the fungal spores, and after 7 and 14 days for ‘Lisek’ cultivar (Fig. 4c). In both of these cultivars an increase in the leaf water content was correlated with enhanced CWPh content.

Statistically significant correlations between SC and SPh and CWPh were reported only in ‘Liclassic’ plants (Fig. 5b and e) 7 and 14 days after the inoculation. Higher level of both types of phenolic compounds found in this cultivar correlated with an increase in the content of soluble sugars. The relationships calculated for ‘Bojan’ cultivar (Fig. 5a and d) were statistically insignificant. In the case of ‘Lisek’ cultivar significant correlations were obtained only after 14 days from the inoculation and an increase in SC content corresponded to an increase in SPh (Fig. 5c) and a decline in CWPh (Fig. 5f).

LMA content in ‘Bojan’ cultivar was significantly lower than that observed for the healthy plants 7 and 14 days after the inoculation with *L. maculans* spores (Table 3). LMA content in ‘Liclassic’ plants was significantly higher 7 and 14 days after the inoculation. In ‘Lisek’ cultivar increased content of low-molecular antioxidants was recorded 14 days after the inoculation.

#### Content of assimilation pigments

The content of the assimilation pigments in ‘Bojan’ cultivar was significantly lower than that recorded for

the control plants 7 and 14 days after the inoculation with *L. maculans* spores (Table 4). At the same time, the healthy ‘Bojan’ plants were characterized by a higher content of chlorophyll and carotenoids than ‘Liclassic’ and ‘Lisek’ cultivars. A significant increase in the assimilation pigments was observed in ‘Liclassic’ cultivar after 7 and 14 days, and in ‘Lisek’ plants 14 days after the inoculation.

Significant relationships between the content of chlorophyll *a* and SC, SPh, CWPh, and the LMA both after 7 and 14 days from the inoculation were observed only in ‘Liclassic’ plants (Fig. 6). Increased content of chlorophyll *a* in ‘Liclassic’ cultivar corresponded to an increase in sugars (Fig. 6b), phenols (Fig. 6e and h), and LMA level (Fig. 6k). The relationships calculated for ‘Bojan’ cultivar were statistically insignificant. In ‘Lisek’ cultivar significant correlations were observed mainly 14 days after the inoculation with *L. maculans* spores. Increased content of chlorophyll *a* in ‘Lisek’ cultivar was accompanied by higher level of carbohydrates (Fig. 6c), SPh (Fig. 6f), LMA (Fig. 6l) and a decrease in CWPh (Fig. 6i). Only the relationship between chlorophyll *a* and SC content 7 days after the inoculation was statistically significant (Fig. 6c) in ‘Lisek’ cultivar.

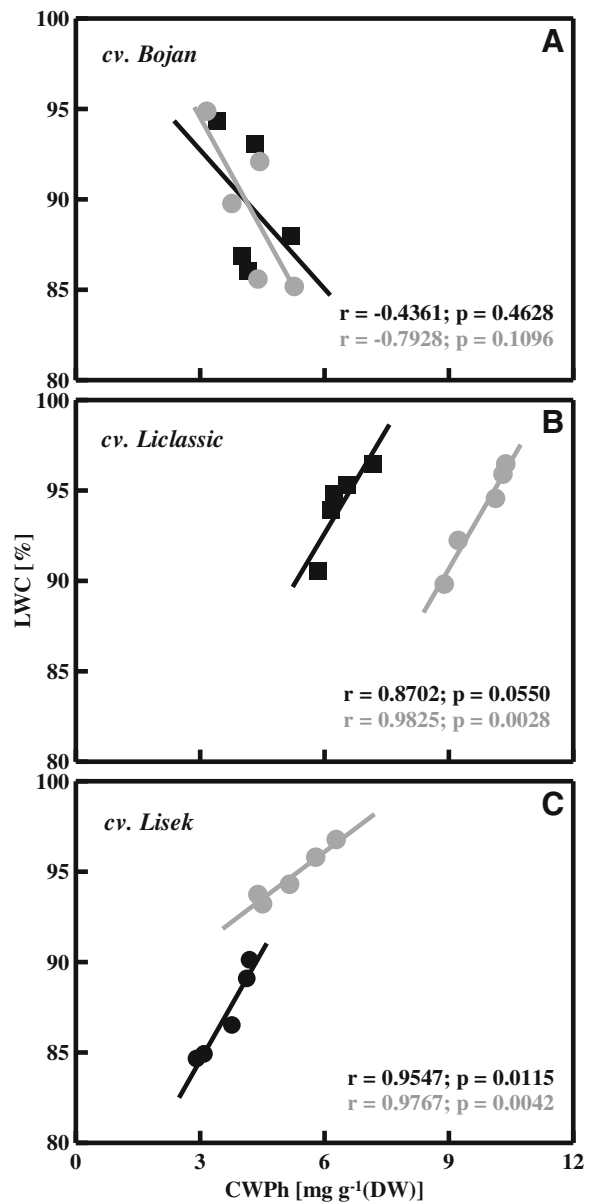
#### *L. maculans* susceptibility

Average severity index (ASI) calculated 35 days after inoculations with *L. maculans* spores is shown in Table 5. Fungal pathogen susceptibility was in ‘Bojan’ cultivar (ASI=3.64) significantly higher than that recorded for ‘Liclassic’ (ASI=1.15) and ‘Lisek’ (ASI=2.11) plants (they demonstrated a lower degree of infection).

## Discussion

The literature describing *Brassica napus* - *Leptosphaeria maculans* pathosystem contains contradictory data on the parasitic strategy of *L. maculans* at the initial phase of infection. Hammond and Lewis (1987) showed that *L. maculans*, being a hemibiotroph, can act as a biotroph at the beginning of the infection and switch to a necrotrophic mode at the later stages of pathogenesis. However, recent works indicate that at an early stage of plant infection *L. maculans* can be a necrotroph, then it enters a long biotrophic stage, and finally it switches back to the necrotrophic stage (Rouxel and Balesdent 2005; Jindřichová et al. 2011; Hayward et al. 2012). Furthermore, it was shown that some fungi at their necrotrophic stage can manipulate the host plant by secreting specific effector proteins that may affect the host metabolism in a manner typical for biotrophs (Oliver and Solomon 2010). The described plasticity of *L. maculans* life cycle at early stages of infection may result in an incorrect selection of resistant cultivars, if the selection is based only on the observed symptoms of the disease. Therefore, an identification of reliable biochemical or physiological indicators of rape resistance to *L. maculans* would provide the farmers with an additional tool that, in addition to molecular biology methods, would help them to evaluate the cultivars for their sensitivity to this pathogen.

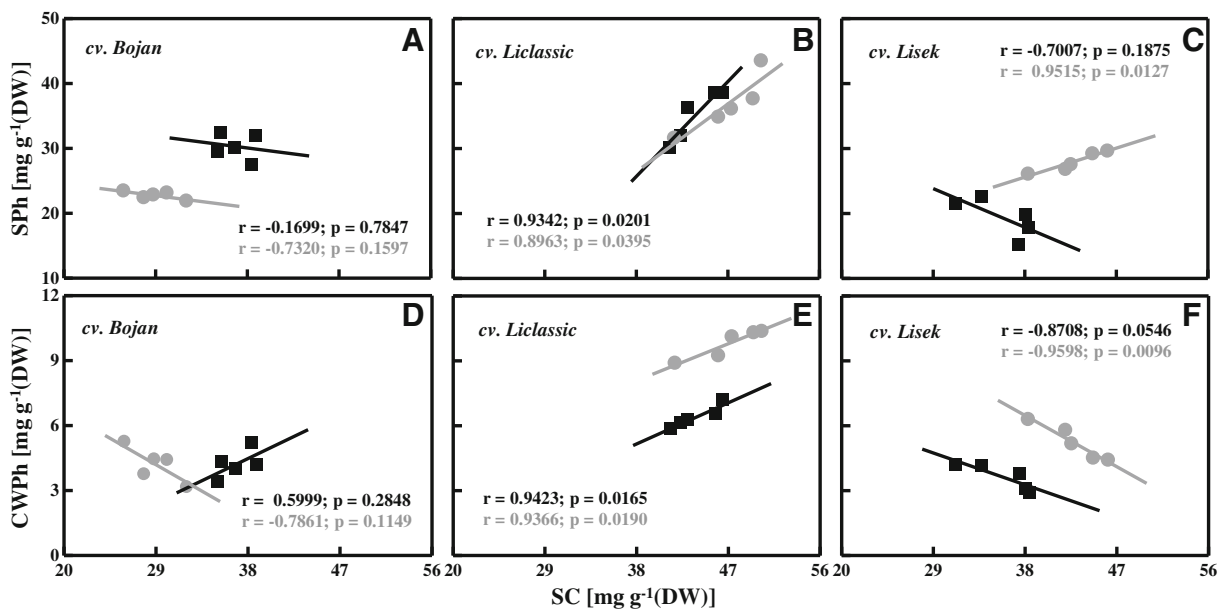
In our earlier experiments we found that the toxins excreted to a medium by *L. maculans* can change the activity of the photosynthetic apparatus, including the level of assimilation pigments in the cotyledons of winter rape (Hura et al. 2014b, c). The results presented in this paper indicate increased content of chlorophyll *a* during pathogenesis in the resistant ‘Liclassic’ and mildly resistant ‘Lisek’ cultivars after the inoculation with the fungal spores (Table 4). An increase of chlorophyll *a* content in non-directly infected the first true leaves after inoculation could evidence rather to a systemic defence response. However, it should be underlined that pathogenic fungi can stimulate host metabolic activity (Scholes and Farrar 1986; Roberts and Walters 1988) including e.g., increase in the level of photosynthetic pigments. In ‘Liclassic’ and ‘Lisek’ plants, the level of chlorophyll *a* correlated with the level of primary metabolites in the form of soluble carbohydrates (SC), as well as with the level of phenols and low-molecular antioxidants (Fig. 6). In the sensitive ‘Bojan’ cultivar a decline in carbohydrate content 14 days



**Fig. 4** Correlations between the leaf water content (LWC) and the content of cell wall-bound phenolics (CWPh) in the leaves of cv. Bojan (a), cv. Liclassic (b) and cv. Lisek (c). Black squares – 7 days after the inoculation; gray circles – 14 days after the inoculation. The lines represent linear adjustment at a probability level  $P < 0.05$

after the inoculation (Table 2) was accompanied by a decrease in chlorophyll content, which may be regarded as one of the signs of a destructive action of reactive oxygen species, including superoxide anion (Fig. 1). The other possible causes for the reduction in the level of carbohydrates can be due to a decline in the content/activity of Rubisco, or to end-product inhibition of





**Fig. 5** Correlations between the content of soluble phenolics (SPh; A, B, C), the cell wall-bound phenolics (CWPh; D, E, F) and the content of soluble carbohydrates (SC) in the leaves of

studied cultivars. *Black squares* – 7 days after the inoculation; *gray circles* – 14 days after the inoculation. The lines represent linear adjustment at a probability level  $P < 0.05$

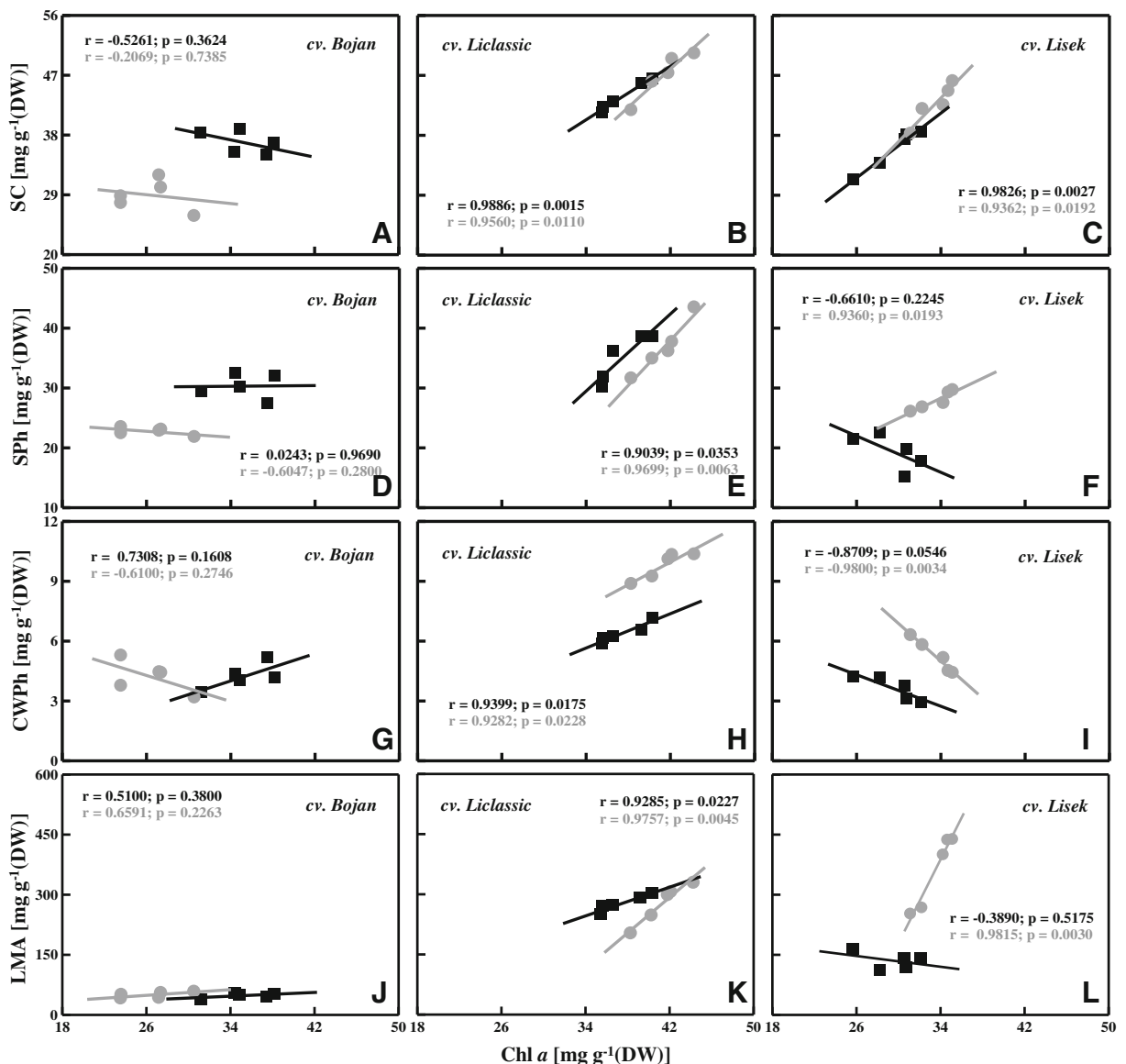
photosynthesis (Chou et al. 2000). Mur et al. (2010) showed that reactive oxygen species caused chlorophyll degradation in the course of *Pseudomonas syringae* infection and that light-excited products of chlorophyll degradation, e.g., pheophorbide *a* (Pheide) may be an additional source of ROS. It was suggested in several other studies that pheophorbide *a* can act as a photosensitizer that generates ROS in response to light (Tanaka et al. 2003; Hörtensteiner 2004; Pruzinska et al. 2005; Tanaka and Tanaka 2006). Pheophorbide *a* content was not

analyzed in this study. A decline in chlorophyll *a* in the sensitive ‘Bojan’ cultivar, accompanied by high levels of  $O_2^{\cdot -}$  14 days after the inoculation, may be the initial symptoms of cellular death. Chou et al. (2000) showed a similar decrease in chlorophyll content during infection of *Arabidopsis thaliana* leaves with *Albugo candida* (white blister rust). The decrease in chlorophyll content evoked by pathogenic fungi was also observed by other authors (Rasmussen and Sheffer 1988; Moriondo et al. 2005; Mandal et al. 2009a; Zhao et al. 2011).

**Table 4** Changes in the content ( $[mg\ g^{-1}\ (DW)]$ ) of chlorophyll *a* (Chl*a*), chlorophyll *b* (Chl*b*), total chlorophyll (Chl*a+b*) and carotenoids (Crts) observed 7 and 14 days after the inoculation (I) with *L. maculans* spores in the leaves of studied cultivars

		‘Bojan’		‘Liclassic’		‘Lisek’	
		C	I	C	I	C	I
Chl <i>a</i>	7	40.0±0.55a	35.2±1.24b	30.6±1.64a	37.5±0.98b	28.7±0.48a	29.5±1.14a
	14	39.9±1.28a	26.5±1.32c	32.8±1.11a	41.4±1.00c	28.1±1.13a	33.5±0.77b
Chl <i>b</i>	7	11.9±0.28a	11.2±0.45a	8.6±0.36a	10.9±0.43c	8.4±0.38a	8.2±0.37a
	14	11.5±0.46a	9.0±0.68b	9.8±0.38b	12.1±0.14d	8.8±0.34a	11.0±0.71b
Chl <i>a+b</i>	7	52.0±0.40a	46.4±1.24b	39.3±1.95a	48.4±1.35b	37.1±0.77a	37.7±1.49a
	14	51.4±1.68a	35.5±1.91c	42.6±1.46a	52.5±1.28b	36.8±1.31a	44.4±1.45b
Crts	7	11.6±0.16a	10.2±0.17b	8.2±0.60a	10.2±0.23bc	8.3±0.15a	8.6±0.33a
	14	11.3±0.32ab	7.3±0.59c	9.1±0.53 ac	11.3±0.44b	7.3±0.45b	8.7±0.30 ac

C represents control plants. Data are means±SE. Means indicated with the same letters within cultivar are not significantly different ( $P=0.05$ )



**Fig. 6** Correlations between the content of soluble carbohydrates (SC; **a**, **b**, **c**), soluble phenolics (SPh; **d**, **e**, **f**), the cell wall-bound phenolics (CWPh; **g**, **h**, **i**), the low-molecular antioxidants (LMA; **j**, **k**, **l**), and chlorophyll *a* in the leaves of studied cultivars. Black

squares – 7 days after the inoculation; gray circles – 14 days after the inoculation. The lines represent linear adjustment at a probability level  $P < 0.05$

Apart from ROS, other significant predictors of host-pathogen interaction are pathogenesis related (PR) proteins (Scherer et al. 2005). Enhanced activity of chitinase and  $\beta$ -1,3-glucanase (Table 1) observed in this experiment 7 and 14 days after the inoculation with *L. maculans* spores is probably a manifestation of the systemic plant response to the infection, and not a sign of the fungal pathogen growth in the plant cells. During the experiment and the course of the pathogenesis no

visible disease symptoms were observed on the true, not directly inoculated leaves. Increased activity of chitinase and  $\beta$ -1,3-glucanase was also reported in other studies concerning the effects of pathogenic fungi on plants (Mauch et al. 1988; Hwang et al. 1997; Krishnaveni et al. 1999; Gupta et al. 2013; Hura et al. 2014c).

The content of soluble carbohydrates considerably affects the secondary metabolism activity, involving, for example, a synthesis of phenolic compounds

**Table 5** Average severity index (ASI) calculated according to the visual scale (0–5) of fungal infection

	ASI	Result description
‘Bojan’	3.64±0.25 a	half or more than three-quarters of the leaves with disease symptoms
‘Liclassic’	1.15±0.18 b	trace symptoms of infection
‘Lisek’	2.11±0.10 c	less than half of the leaves infected

Disease symptoms were estimated 35 days after the inoculation with *L. maculans* spores. Data are means±SE. Means indicated with the same letters are not significantly different ( $P=0.05$ ).

(Arnold et al. 2004) playing a key role in defense mechanisms during pathogenesis (Rusjan et al. 2012; Hura et al. 2014a, b). The results of some recent studies provided evidence that the high level of phenolic compounds can be linked with low susceptibility of plants to fungal infection (Mikulic-Petkovsek et al. 2014a, b). This was confirmed by the results of the analyses of phenolic compounds and low molecular antioxidants, including also SPh (Table 3), and significant correlations between the content of soluble carbohydrates and SPh, CWPh and LMA for the resistant ‘Liclassic’ and medium-resistant ‘Lisek’ cultivars (Fig. 5).

Increased content of cell wall-bound phenolics is one of the defense mechanisms utilized by plants undergoing biotic (El Modafar and El Boustani 2001; De Ascensao and Dubery 2003; Mandal et al. 2009b) and abiotic stresses (Wakabayashi et al. 1997; Hura et al. 2012, 2013). Phenolic compounds present in the cell wall form cross-bridges between the cell wall carbohydrates, binding them with ester and/or ether bonds. The more such phenolic cross-bridges in the cell wall, the more compact it becomes and the better antifungal protection it provides (Fry 1982, 1987; Kamisaka et al. 1990). It is worth adding that incorporation of the phenolic compounds in the apoplast may limit the destructive action of ROS during pathogenesis, as the process requires the presence of hydrogen peroxide (Fry 1982, 1987; Schopfer 1996; Mandal et al. 2009b).

Increased leaf water content was noticed in the resistant ‘Liclassic’ and medium-sensitive ‘Lisek’ winter rape cultivars (Table 2). Elevated content of the cell wall-bound phenolics 7 and 14 days after the inoculation in the resistant and medium-sensitive cultivars (Table 3) could affect water relationships in the indirectly inoculated leaves of oilseed winter rape. In both cultivars, an increase in leaf water content corresponded to an increase in CWPh content (Fig. 4). An increase in

phenolics in the cell walls influences its mechanical properties. The cell wall becomes less stretchy, more compact and tight with the increase in the content of cell wall-bound phenolics (Wakabayashi et al. 1997). It is possible to expect that accumulation of phenolic compounds in the apoplast enhances a hydrophobic nature of the cell wall, because it increases the number of hydrophobic benzene rings in its structure, thus making it less permeable to water. The hydrophobic environment of the apoplast might significantly inhibit the water transport from the symplast to the apoplast and limit the capillary transport of water within the apoplast (Hura et al. 2012, 2013). Therefore, it should be taken into account that the hydrophobic environment of the apoplast can induce effective water management, which involves the retention of water in the symplast being the metabolically active cell structure at the cost of dead apoplast structure. Increased LWC negatively correlated with the content of soluble carbohydrates in ‘Liclassic’ cultivar, and positively in ‘Lisek’ plants (Fig. 2). In ‘Liclassic’ cultivar higher content of SC, as osmotically active substances, correlated with an increase in the osmotic potential (Fig. 3) and could be, in addition to cell wall reinforcement by phenolic compounds, another process enabling water retention in the cell. In our opinion, higher cellular water content could be an important factor improving the effectiveness of enzymatic and non-enzymatic antioxidants and the solubility of the primary and secondary metabolites.

Summing up, the study indicates a relationship between the content of chlorophyll *a* and the level of primary and secondary metabolites in oilseed winter rape inoculated with *L. maculans* spores. In the resistant cultivar of winter rape increased content of chlorophyll *a* during the pathogenesis can be considered to be an important factor directly influencing carbohydrate level, and indirectly affecting the performance of defense mechanisms related to the level of phenolic compounds and low-molecular antioxidants. The level of cell-wall bound phenolics that seal the cell wall and prevent water loss through metabolically active symplast seems to be an important indicator of plant resistance in *Brassica napus* - *L. maculans* pathosystem.

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