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The effect of seedling chilling on glutathione content, catalase and peroxidase activity in *Brassica oleracea* L. var. *italica*

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Abstract

The study was designed to determine the possible relationship between *Brassica oleracea* var. *italica* seedlings stored at 2°C in the dark for seven and fourteen days, respectively, and the level of certain antioxidant parameters in particular organs. A parallel objective of the experiment was to determine if the reaction of seedlings to low temperature might be persistent in fully developed plants until harvest time. After 14 days of chilling a significant increase in the glutathione content was observed in the seedling leaves in comparison to the non-chilled plants. During vegetation in field conditions this effect was maintained in leaves up to the stage of formation of flower buds. At harvest the highest content of glutathione was demonstrated in broccoli heads, obtained from plants, which were previously chilled in the seedling phase for two weeks. Peroxidase activity in broccoli seedlings increased each year of the three-year study due to the duration of the cooling time, whereas in the case of catalase the changes were not so distinct. At harvest time the activity of both enzymes in the leaves and flower buds fluctuated according to the particular year of study.

Keywords: broccoli, *Brassica oleracea* L. var. *italica*, chilling stress, development stage

Introduction

Proper growth and development of plants depends on an efficient defense system, mainly against reactive oxygen species. Studies on how plant genotypes respond to all kind of stresses indicate a similar mechanism of adaptation to unfavorable environmental conditions. These reactions are mostly connected with neutralizing free radicals, particularly reactive oxygen species (ROS) which frequently appear as the effect of different stress factors [1–3]. Many experiments indicate significance changes in the enzymatic and non-enzymatic antioxidant system during environmental stresses in plant tissues. The most active in this respect are usually the classified enzymes: catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) as well as chemical compounds such as glutathione, ascorbic acid or different polyphenols [4,5]. It is well established that the mechanisms of tolerance to chilling, freezing, drought or high salinity include the action of common genes. For this reason chilling transplants before planting might result in a better adaptation of plants cultivated from such transplants to various stresses via a well-defined increase in the antioxidant activity system in particular plant organs [6,7]. In recently carried out investigations that were focused on the reaction of wild species or cultivated plant cultivars to abiotic stresses, only early vegetative stages of development in the studied genotypes were applied [7,8]. Moreover, these experiments were mainly carried out in strictly controlled conditions. As a rule, neither the natural environment of plant growth nor the stage of full generative development was considered.

The *Brassica oleracea* L. var. *italica* that was used in the presented experiment is a vegetable of great economical value, and in Europe it is grown on a large scale that could be compared to that of the cauliflower. Broccoli flower buds contain an elevated level of health-promoting substances, which are active during the neutralization of free radicals [9,10]. A crucial example of such is glutathione, a non-enzymatic hydrophilic antioxidant that commonly occurs in plants belonging to the Brassicaceae family. This thiol compound is an important substrate of the enzymatic defense system in the cytoplasm and in the chloroplasts of plant cells. Because of its anticarcinogenic functions it is very important in the human diet. In some cultivars of broccoli the glutathione content may exceed 200 nmol g^{-1} of fresh weight [11].

The cultivar italica of *B. oleracea* can optionally be planted from transplanted seedlings in spring, summer or autumn, but this practice sometimes requires storage of the transplant material. The storage may be necessary because of unfavorable weather conditions. For that reason offering an efficient way

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of storing seedlings is important from an economical point of view and is scientifically interesting.

According to the reasons mentioned above, a three-year study was undertaken to determine the influence of storing broccoli seedlings at 2°C in darkness, on the level of glutathione, and the activity of CAT and POD in the leaves of the seedlings. A parallel aim of the undertaken experiment was to determine if the reaction of the seedlings to low temperature might be persistent in fully developed plants. We were also interested whether the intensity of the reaction could be dependent on the age of the seedlings subjected to stress conditions of low temperature.

Materials and methods

Scheme of the experimental set-up and stress treatments

The three-year experiment was carried out with *Brassica oleracea* L. var. *italica* 'Monaco F1' cultivar that abounds in health-promoting substances. In every year of the study *B. oleracea italica* seeds were sown in greenhouse conditions in the second half of May and in the second half of June to obtain seedlings. Seedlings were produced in the greenhouse under natural light conditions and a temperature of 18 ±2°C. The 4- and 8-week-old seedlings that were obtained were divided into equal parts. One part was kept in a cold chamber for seven days and the other for fourteen days. The conditions in the cold chamber were as following: 2°C, a relative humidity of 85%, plants kept in darkness. Non-chilled seedlings and seedlings cooled for seven or for fourteen days were planted in the field from mid- to the end of July, respectively, with a spacing of 400 × 675 mm. The cultivated plants were fertilized according to recommendations given for broccoli under field conditions. In every year of the experiment broccoli heads obtained from 8-week-old seedlings were harvested at the end of September to the beginning of October, and from the 4-week-old ones at the end of October. The average monthly temperature recorded for the three-year study period by a weather station situated in close proximity of the fields (in Cracow-Mydlniki) is presented in Tab. 1.

Tab. 1 Maximum and minimum temperature (°C) during respective growing season of *Brassica oleracea* L. var. *italica* 'Monaco F1'.

Temperature	Month and year				
	Jun	Jul	Aug	Sep	Oct
			2008		
maximum	27.46	25.28	25.83	26.13	16.87
minimum	10.03	13.23	11.89	6.91	3.59
			2009		
maximum	21.19	27.35	27.93	23.03	17.09
minimum	8.04	12.13	11.16	6.82	2.79
			2010		
maximum	23.90	29.7	26.18	18.09	13.38
minimum	11.40	12.80	11.78	7.48	0.13

The analyses conducted on tested plant material

The sum of reduced (GSH) and oxidized (GSSG) glutathione, the activity of CAT and POD were tested in four replications for each term of every experimental treatment. The chemical analyses were conducted (1st term) just before chilling and immediately after chilling (for 7 and 14 days, respectively), and subsequently (2nd term) both in the leaves and heads obtained from the broccoli plants at harvest time. Additionally, the glutathione content in the broccoli heads was measured during the forming phase (e.g. about two weeks before harvest time when the diameter of the heads reached about 5 cm). In each experimental treatment ten plants at the seedling phase and five plants of full maturity phase were randomly chosen for the analytical studies. Intact leaves in high vigor from the middle part of the broccoli rosette were collected for analyses. In the case of seedlings, the first proper leaves were used.

The sum of reduced and oxidized forms of glutathione (GSH + GSSG) was determined spectrophotometrically according to the method of Akerboom and Sies [12]. In this method NADPH (reduced nicotinamide adenine dinucleotide phosphate) and glutathione reductase reduce GSSG to GSH, then DTNB (dithiobis nitrobenzoic acid) as an oxidizer carries the whole glutathione to the oxidizing form (GSSG). The product of the last reaction is blue in color. An increase in absorbance was monitored at 412 nm, at 60-second intervals up to 5 minutes. Catalase activity was determined following the method as described by Bartosz [13] based on a decrease of absorbance of H_2O_2 being decomposed by the enzyme (absorbance at 240 nm). Peroxidase activity was assayed according to Lück [14], with p-phenylodiamine and H_2O_2 as the enzyme substrates (an increase in absorbance at 485 nm at 60-second intervals up to 2 min was measured). In all analyses a HITACHI U-2900 UV-VIS spectrophotometer was used.

Statistical analysis of the obtained results was carried out with STATISTICA 9.0 and ANOVA software (StatSoft. Inc.). To estimate the significance of differences between the mean values, homogeneous groups were appointed using the Fisher LSD test at a significance level of $\alpha = 0.05$.

Results and discussion

Glutathione content

Glutathione is known as an important signaling and protective molecule. GSH is a key antioxidant, which participates in protecting the photosynthetic apparatus in leaves [15]. In the presented experiment a high content of glutathione was found in the leaves of broccoli in the bud-forming phase in comparison to the seedling development stage (Tab. 2). And, at harvest time (Tab. 3), a several times higher glutathione content was observed in the leaves than in the buds. These results might indicate the significant participation of glutathione in the increased of antioxidant system in the organs with highest photosynthetic activity.

May et al. [16] and Soltesz et al. [17] have reported that an increasing glutathione content, especially its reduced form (GSH), plays a key role in plant responses to adverse environmental factors, including low temperature. In every year of the presented study, in comparison to the non-chilled plants, a significant stimulating effect of the two-week detention of seedlings at 2°C in the dark on the level of glutathione in the seedling leaves was shown (Tab. 2). Similarly, O'Kane et al. [18]

Tab. 2 Glutathione content (nmol g^{-1} fresh matter) in *Brassica oleracea* L. var. *italica* 'Monaco F1' plants before or after chilling, given in two developmental stages – in seedling phase, and in heads forming phase.

Age of the seedlings when stress was used (weeks)	Period of seedling chilling (weeks)					
	0	1	2	0	1	2
	Leaves of seedlings			Leaves of head forming plants		
Vegetation period 2008						
4	74.96 bc*	64.11 ab	94.77 d	99.88 ab	113.0 bc	181.31 d
8	81.71 cd	55.50 a	112.80 e	97.98 ab	78.45 a	137.27 c
Vegetation period 2009						
4	32.24 b	15.19 a	48.12 c	106.98 a	122.19 b	151.47 c
8	22.24 a	42.44 c	94.14 d	99.77 a	127.83 b	147.50 c
Vegetation period 2010						
4	5.23 a	27.75 c	71.01 e	23.76 a	39.30 b	59.24 c
8	9.15 a	20.46 b	48.41 d	45.35 b	27.90 a	57.79 c
Mean value from vegetation periods 2008–2010						
4	37.48 A	35.68 A	71.30 B	76.88 A	91.50 B	130.68 D
8	37.70 A	39.47 A	85.45 C	81.03AB	78.06 A	113.52 C

* Values presented by small characters refer to separate year and growing phase, whereas by big characters refer to interaction between chilling and age of seedlings.

Tab. 3 Glutathione content (nmol g^{-1} fresh matter) in leaves or heads of *Brassica oleracea* L. var. *italica* 'Monaco F1' plants, determined at harvest time.

Age of the seedlings when stress was used (weeks)	Period of seedling chilling (weeks)					
	0	1	2	0	1	2
	Broccoli leaves			Broccoli heads		
Vegetation period 2008						
4	85.81 a*	167.83 c	118.29 b	11.32 a	65.61 c	75.04 d
8	123.57 b	188.61 d	116.63 b	83.84 de	52.64 b	84.34 e
Vegetation period 2009						
4	37.99 a	84.84 b	135.66 e	13.68 a	18.95 a	110.35 d
8	121.39 cd	115.66 c	129.07 de	58.99 b	83.02 c	125.62 e
	2010					
4	34.77 a	48.32 b	47.01 b	33.20 bc	42.21 c	101.08 d
8	47.90 b	45.54 b	71.44 c	18.61 a	17.44 a	30.35 b
Mean value from vegetation periods 2008–2010						
4	52.85 A	100.33 BC	100.32 BC	19.40 A	42.26 B	95.49 E
8	97.63 B	116.60 D	105.71 C	53.81 C	51.03 C	80.10 D

* Values presented by small characters refer to separate year and growing phase, whereas by big characters refer to interaction between chilling and age of seedlings.

showed that in *Arabidopsis* callus at 4°C there was an increase in the total glutathione content (GSH + GSSG) in comparison with a temperature of 23°C. After seven days of cooling the broccoli seedlings (Tab. 2), changes in the glutathione content were not distinct, what indicates that a two-week period of low temperature treatment is more effective. It is worth noticing that a significant increase in the total glutathione content in broccoli leaves was found in the bud-forming phase of the two-week cooling period. This dependence occurred regardless of the age of the seedlings when stress was used and regardless of the experimental year. How long was this effect maintained?

At harvest time (Tab. 3), changes in the glutathione content in the broccoli leaves were not as evident as discussed above. However, in comparison to the non-chilled plants, the mean values from all the vegetation periods (2008–2010) confirmed the stimulating effect of seedling cooling at 2°C on an increase in the concentration of glutathione in leaves during harvest time. On the other hand, in broccoli heads in every group of plants, and every year of testing the positive effect of the 2-week chilling of broccoli seedlings on glutathione content was clearly shown (except the year 2008 in combination with 8-week age of seedlings). These results suggest that the processes, which stimulate synthesis of this valuable compound initiated in the early stages of plant growth, could be established provided that there is adequate time for the stimulus effect. The influence of weather conditions cannot be excluded, although according to data presented in Tab. 1 the increase of glutathione in the broccoli heads did not seem to be induced by the lower temperatures that were present in that harvest month.

Catalase and peroxidase activities

The activity of antioxidant enzymes, catalase and peroxidase, that was determined during the experiment was variable with regard to the period of chilling, developmental stage and plant organ (Tab. 4, Fig. 1, Fig. 2). The increase in catalase activity after plant exposure to various abiotic and biotic stresses was recorded previously [3,5]. These reactions may depend on factors affecting gene expression of this enzyme. In the Prasad et al. study [19], CAT activity in maize seedlings increased after treatment for seven days with a temperature of 4°C. This dependence was confirmed by some results of the presented study (Tab. 4). In 2008 and 2009 significantly higher catalase activity in the broccoli seedling leaves after a two-week period of cooling compared with non-chilled plants was observed. However, this effect did not occur in the third year of the study. Other authors have observed a decrease in catalase activity in cucumber leaves under low temperature treatment accompanied with an increase in other antioxidant enzyme activities [20].

High CAT activity in Brassicaceae plants, especially in their green parts, was noted by Singh et al. [21]. Lower values were observed in the generative organs of plants from this family. Starzyńska et al. [8] reported that in the broccoli heads of 'Lord F1' the activity of this enzyme was, on average, 75 μmol H_2O_2 g^{-1} min^{-1}, and in the experiment of Leja et al. [22] it reached a level of up to 150 μmol H_2O_2 g^{-1} min^{-1}. In the presented study on the 'Monaco F1' cultivar (Fig. 1), CAT activity in the heads ranged between 146.4 and 168.2 μmol H_2O_2 g^{-1} min^{-1} and was several times lower than in the leaves. This dependence could be explained by the large number of chloroplasts occurring in the leaves, which are especially sensitive to reactive forms of oxygen. For this reason well-functioning antioxidant systems, including enzymatic antioxidants, act in the leaves. Two-week period of seedling chilling increased the activity of catalase in seedlings compared to non-chilled plants. This dependence was not observed in leaves and heads of broccoli during harvest time. So the effect of seedling chilling was not reflected in mature plants. Peroxidase is a successive enzymatic component of defense against ROS, which plays a key role in the response of plants to stressful conditions. Its higher activity was recorded during exposure to various stresses, including low temperature [5,23]. In every year of the presented study (Tab. 4), with the prolongation of the chilling period (to a 2-week period) of the broccoli seedlings, POD activity gradually and significantly increased regardless of the age of the seedlings. In the case of this enzyme, the broccoli seedlings' reaction to low temperature was more explicit than catalase. The question posed was if this

Tab. 4 Activity of CAT (μmol H_2O_2 min^{-1} g^{-1}), and POD (U min^{-1} 100 g^{-1}) in fresh matter of *Brassica oleracea* L. var. *italica* 'Monaco F1' seedlings before chilling or after chilling.

Age of the seedlings when stress was used (weeks)	Period of seedling chilling (weeks)					
	0	1	2	0	1	2
	CAT			POD		
			2008			
4	605.5 b*	371.8 a	679.3 c	636.0 a	839.1 b	1133.3 d
8	405.5 a	576.3 b	733.4 c	562.0 a	984.7 c	1264.0 e
			2009			
4	413.7 a	385.0 a	664.3 b	664.3 a	1492.0 b	1835.2 c
8	781.0 c	1097.3 e	905.7 d	1368.7 b	3040.0 d	3739.2 e
			2010			
4	1081.0 b	889.6 ab	1044.8 b	405.0 a	494.4 b	574.8 c
8	938.8 ab	781.0 a	770.7 a	753.0 d	866.4 e	976.2 f

* Values presented by the characters refer to separate year.

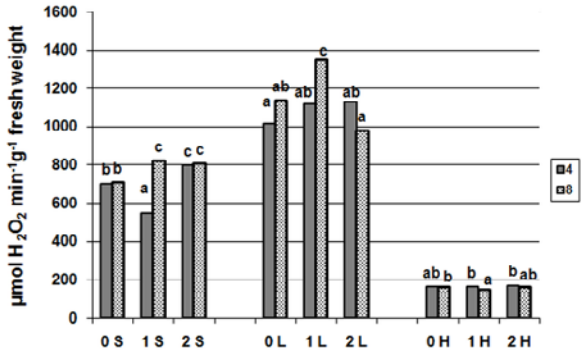

Fig. 1 Catalase (CAT) activity in *Brassica oleracea* L. var. *italica* 'Monaco F1' seedlings (S) and during harvest time in leaves (L) and heads (H) respectively. Mean values from 12 replications, obtained in vegetation periods 2008–2010. Statistical analyses were conducted separately for broccoli seedlings, leaves and heads. 0, 1, 2 – seedlings chilling in weeks; 4, 8 – age of seedlings in weeks.

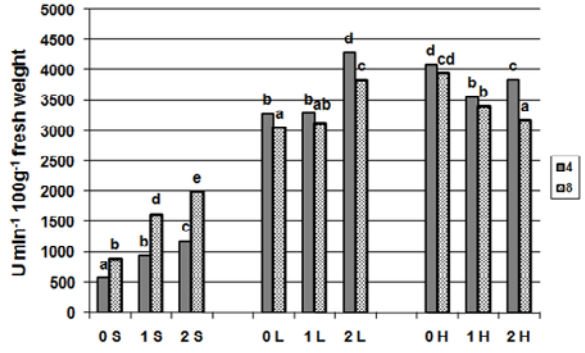

Fig. 2 Peroxidase (POD) activity in *Brassica oleracea* L. var. *italica* 'Monaco F1' seedlings (S) and during harvest time in leaves (L) and heads (H) respectively. Mean values from 12 replications, obtained in vegetation periods 2008–2010. Statistical analyses were conducted separately for broccoli seedlings, leaves and heads. 0, 1, 2 – seedlings chilling in weeks; 4, 8 – age of seedlings in weeks.

clear dependence could also be observed at harvest time (Fig. 2). At this stage the highest level of peroxidase activity occurred in the leaves of plants whose seedlings were subjected to chilling for fourteen days: first – at an age of 4 weeks, second – at an age of 8 weeks. It seems that only in this case was the effect of seedling cooling maintained. In broccoli heads the opposite trend was observed. Moreover, significantly higher activity of POD was noticed both in the leaves and heads at harvest time and for younger instead of older broccoli plants subjected to a 2-week chilling period.

It can be concluded that in every year of the experiment after two weeks of seedling chilling a significant increase in the glutathione content of the leaves was observed in comparison that of the non-chilled plants. During vegetation in the field this effect was maintained in the leaves of head-forming plants and in flower buds at harvest time. The highest glutathione content was determined in the flower buds of broccoli obtained from 4-week-old seedlings chilled for fourteen days. Peroxidase activity in the broccoli seedlings significantly increased due to the cooling time. This effect was maintained at harvest time in the leaves of plants that had been previously chilled for two weeks.

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Authors' contributions

The following declarations about authors' contributions to the research have been made: responsible for experimental design, data analysis, paper writing, and contribution to all the experimental processes: RW; performed research: EHF, AK, IK, AG; paper writing and manuscript preparation: EHF; designed research: EK.

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