DOI: 10.1007/s10535-015-0552-8 BIOLOGIA PLANTARUM 59 (4): 757-766, 2015

Nitrogen forms modulate effects of benzothiadiazole and arbutin on cucumber sugar metabolism

M. SKŁODOWSKA, M. NALIWAJSKI, M. WIELANEK, E. GAJEWSKA, and E. KUŹNIAK*

Department of Plant Physiology and Biochemistry, Faculty of Biology and Environmental Protection, University of Łódź, Banacha 12/16, PL-90237 Łódź, Poland

Abstract

The effect of benzothiadiazole (BTH) and arbutin (ARB) on sugar metabolism and plant fitness in cucumber growing hydroponically in media with different doses of NO_3^- and urea as nitrogen sources (100 % NO_3^-, 75 % NO_3^- + 25 % urea, and 50 % NO_3^- + 50 % urea) was studied on the 7th and 14th day after the treatment. The glucose, sucrose, and chlorophyll (Chl) content, acid and alkaline invertases and lactate dehydrogenase activities, as well as leaf area of the 3rd and 5th leaves were determined. Urea changed the plant sugar metabolism in a dose-, time- and leaf-age-dependent manners and influenced a cucumber response to the BTH and ARB treatments. The BTH caused a significant cessation of growth, a decrease in Chl content, a reduction of leaf area, and an enhancement of lactate dehydrogenase and alkaline invertase activities. In the older leaves of the BTH-treated plants, a high accumulation of glucose and sucrose was found. At the lower dose of urea, the metabolic changes were limited. In the ARB-treated plants, the Chl content remained unchanged in all the nitrogen variants. In these plants, decrease in glucose and sucrose content and in both invertase activities was observed mainly in younger leaves of the plants grown on the high dose of urea. The ARB improved the fitness of the cucumber plants grown in the presence of urea.

Additional key words: chlorophyll, *Cucumis sativus*, invertases, lactate dehydrogenase, urea.

Introduction

Benzothiadiazole [benzo(1,2,3)thiadiazole-7-carbotionic acid-S-methyl ester, BTH], a functional analogue of salicylic acid (SA), is well recognized as effective inducer of a systemic acquired resistance and is often used under field conditions to protect crop plants, including cucumber, from infectious diseases (Walters *et al.* 2005, Phuntumart *et al.* 2006, Sillero *et al.* 2012). However, in BTH-treated plants, reduction of growth and seed production were observed (Heil 1999). As BTH was found to cause growth cessation (Dietrich *et al.* 2004) without influencing photosynthesis (Šindelářová *et al.* 2002), this effect was suggested to result from BTH-triggered accumulation of defense-related secondary metabolites. This negative response depends on the developmental stage of treated plants as well as on the nitrogen supply (Dietrich *et al.* 2004, Walters *et al.* 2005).

Most plant species can utilize both NH_4^+ and NO_3^- as nitrogen source (Nacry *et al.* 2013). In crop production, however, urea is often used as source of nitrogen. It is considered to be degraded by soil microbes to ammonia and nitrate which are taken up by plants. It has been reported that plants possess active and passive urea transporters and utilize ammonia liberated by the action of plant ureases (Torisky and Polacco 1990, Kumar and Kayastha 2010, Witte 2011). However, plant nitrogen nutrition based only on urea leads to a reduced growth and to symptoms of nitrogen starvation (Witte 2011) resulting from altered osmotic homeostasis or lack of signals for translocation of amino acids from roots to shoots (Gerendás and Sattelmacher 1997).

Vigour and fitness of plants are correlated with the efficiency of photosynthesis. Its rate was found to decrease with increasing nitrogen deficiency (Cruz *et al.*

Submitted 27 December 2014, *last revision* 25 March 2015, *accepted* 1 April 2015.

Abbreviations: 100N - growth medium containing 100 % NO_3^-; 75N - growth medium with 75 % NO_3^- + 25 % urea; 50N - growth medium with 50 % NO_3^- + 50 % urea; AcIn - acid invertase; ADH - alcohol dehydrogenase; AlIn - alkaline invertase; ARB - arbutin; BTH - benzo-thiadiazole; Chl - chlorophyll; dpt - day post treatment; Glc - glucose; HQ - hydroquinone; LDH - lactate dehydrogenase; SA - salicylic acid; Suc - sucrose.

Acknowledgements: This work was supported by the National Science Centre Grant No. NN310302339. The authors are very grateful to Miss M. Fronczak for a linguistic correction of the manuscript.

* Corresponding author; fax: (+48) 42 6354423, e-mail: elkuz@biol.uni.lodz.pl

2003, Li *et al.* 2013). In most plants, sucrose (Suc) is the transported form of sugars, however, cucumber synthesizes stachyose in mature leaves, and this oligosaccharide is primarily transported in phloem (Miao *et al.* 2007). After long-distance transport to peduncles, stachyose is converted to Suc which is further catabolised to glucose (Glc) and fructose. Utilization of Suc as source of carbon and energy depends on its cleavage into hexoses catalyzed by sucrose synthase (EC 2.4.1.13) or invertase (EC 3.2.1.26) (Sturm and Tang 1999). Plants possess two main isoforms of invertase, acid invertase (AcIn) in vacuoles and apoplast and alkaline invertase (AlIn) in cytoplasm. The AcIn (β-fructo-furanosidase) cleaves Suc most efficiently at a pH of 4.5 - 5.0 and attacks the Suc molecule from the fructose residue. It also hydrolyzes other β-fructose-containing oligosaccharides such as raffinose and stachyose. The AlIn has an optimum activity at a pH of 7.0 - 7.8. In contrast to AcIn, AlIn is Suc specific (Sturm 1999).

Under aerobic conditions, leaves of many plant species contain a low content of a glycolytic pathway enzyme, lactate dehydrogenase (LDH, EC 1.1.1.27) (Sugiyama and Taniguchi 1997). The LDH catalyses a reversible reaction of lactate oxidation and pyruvate reduction (O'Carra and Mulcahy 1996). Its activity results in a decrease in the cell pH and creates conditions for action of alcohol dehydrogenase (ADH), the terminal enzyme of glycolysis in plants under anaerobic conditions.

Arbutin (hydroquinone-β-D-glucopyranoside, ARB) is a natural phenolic glucoside synthesised by plants (Petkou *et al.* 2002) but its function is not fully elucidated (Suau *et al.* 1991). It has been suggested that in resurrection plants in a dried state, ARB protects membranes against destruction, *e.g.*, by formation of complexes with fatty acids in their lipid bilayers (Frias *et al.* 2008), by inhibition of phospholipase A_2 activity, and by its antioxidant activity resulting in superoxide radical scavenging (Oliver *et al.* 1996). On the other hand, ARB may be metabolised to hydroquinone (HQ) and then to benzoquinone (2,5-cyclohexadiene-1,4-dione) which exhibits antimicrobial activity (Jin and Sato 2003). It has been also shown that an elevated HQ content may enhance photosynthesis (Casano *et al.* 2000, Xu *et al.* 2002).

The objective of the present work was to assess the influence of nitrogen nutrition on the effects of BTH and ARB on plant vigour and sugar metabolism. We examined cucumber leaves at different developmental stages from plants grown hydroponically in media with different concentrations of urea and NO_3^-.

Materials and methods

Cucumber (*Cucumis sativus* L. cv. Polan) seedlings were grown hydroponically in a medium consisting of [mg dm^{-3}]: N - 224; P - 39; K - 312; Ca - 160; Mg - 33; S - 44; Fe - 0.84; Mo - 0.98; Cu - 0.84; Mn - 8.4; B - 4.2; and Zn - 4.2. The plants were divided into three groups growing in the media containing the same nitrogen concentration but differing in the ratio of inorganic (NO_3^-) to organic (urea) nitrogen. The N nutrition variants were as follows: 100 % NO_3^- (100N), 75 % NO_3^- and 25 % urea (75N), 50 % NO_3^- and 50 % urea (50N). In order to limit urea degradation by microorganisms, the nutrition solutions were changed every day. The plants were cultured in a greenhouse at a temperature of 24 °C, an air humidity of 60 %, a 16-h photoperiod, and an irradiance of 174 μmol m^{-2} s^{-1}. Three-week-old plants from each group were divided into three subgroups: a non-treated control and plants sprayed once with BTH or ARB. The water solution of a commercial preparation *BION 50 WG* (*Novartis Protection AG*, Basel, Switzerland) containing 0.1 mM BTH as active ingredient or 2.0 mM water solution of ARB (Sigma-Aldrich, St. Louis, USA) were used. The non-treated plants were sprayed with distilled water. On the 7th and 14th day post treatment (dpt), the 3rd and 5th leaves (from the bottom) were taken for analyses. A leaf sample without main veins (0.5 g) was immediately homogenized in a mortar and pestle in 5 cm^3 of an ice-cold 0.05 M Na-phosphate buffer, pH 7.0, containing 1 mM Na_2EDTA and 1 % (m/v) polyvinylpyrrolidone. Immediately after centrifugation (20 000 *g*, 4 °C, 20 min) the supernatant was used for measurement of enzyme (LDH, AlIn, and AcIn) activities as well as protein content.

For chlorophyll (Chl) determination, frozen leaf tissue stored at -20 °C was extracted three times with 80 % (v/v) acetone and centrifuged at 33 000 *g* for 15 min. After measurement of absorbance of the supernatant at 663.2 nm and 646.8 nm using a spectrophotometer (*Unicam UV 300*, *Thermo Spectronic,* Cambridge, UK), Chl *a* and Chl *b* content was calculated according to the method of Wellburn (1994). The leaf area of the 3rd and 5th leaves was assessed on the 14th dpt by a computer method of image analysis (Gocławski *et al.* 2012).

The Glc and Suc content was determined in the extract obtained from a fresh leaf sample (0.5 g) after triple extraction with 80 % (v/v) ethanol. The ethanolic extract was evaporated to dryness at 50 °C and the residue was resolubilized in distilled water. The content of sugars was assayed using a commercial enzymatic test (*Roche Diagnostics*, Basel, Switzerland) and expressed in micrograms per milligram of protein determined in the extract used for enzyme activity by the Bradford (1976) method with bovine serum albumin as standard. There was no significant difference in the protein content among the experimental variants.

Invertase activity was determined according to Miller and Ranwala (1994). A reaction mixture consisted of 40 mM Suc and a 50 mM Na-acetate buffer (pH 5.0 for

AcIn and pH 7.5 for AlIn). When the extract was added, the reaction mixture was incubated at 37 °C. After 30 min, the reaction was stopped by heating at 90 °C for 5 min. The amount of Glc released was determined as described above. One unit of invertase activity was defined as the amount of enzyme that catalyzed the production of 1 μmol of Glc per min.

Lactate dehydrogenase activity was measured by decrease in absorbance at 340 nm resulting from oxidation of NADH (ε = 6.22 mM^{-1} cm^{-1}) according to Sugiyama and Taniguchi (1997). An assay mixture consisted of a 50 mM K-phosphate buffer (pH 7.25), 0.5 mM NADH, 4 mM sodium pyruvate, 2.3 mM 4-methypyranozole, and 2.3 mM NaCN. One unit of LDH activity was the amount of the enzyme that catalyzed oxidation of 1 nmol of NADH per min.

The results presented are means of 8 to 10 independent experiments. Sample variability is given as the standard deviation of the mean. The significance of differences among the mean values for the non-treated and treated groups, among the 75N or 50N nutrition variants in relation to 100N, as well as the significance of time course changes (in relation to 7 dpt) were determined by the non-parametric Mann-Whitney rank sum test (*STATISTICA* software, *v. 10*, *StatSoft*). Differences at $P < 0.05$ were considered as significant.

Results

Urea supplied to the nutritional solution changed the area of both the 3rd and 5th leaves of the non-treated plants (Table 1). The 3rd leaves were largest in the 75N plants, their area was 17 and 33 % greater than in the 100N and 50N, respectively. In the plants from both urea variants, the 5th leaf areas were smaller by 20 - 25 % than in the 100N. Among all tested variants, the smallest leaf area (3rd and 5th) was observed in the plants treated with BTH. The 3rd leaves achieved from 30 to 60 % of the area of those from the non-treated and ARB-treated plants. These differences were also visible in the 5th leaves. In the BTH-treated plants, the addition of urea to the growing medium significantly decreased the area of the 3rd leaves (Table 1).

Table 1. Areas [cm^2] of 3rd and 5th cucumber leaves 14 d after treatments with benzothiadiazole (BTH) or arbutin (ARB). Means ± SD, n = 8; ∗ indicates values significantly ($P < 0.05$) different from non-treated plants; # indicates significant differences between 75 % NO_3^- + 25 % urea (75N) or 50 % NO_3^- + 50 % urea (50N) variants compared to plants grown in 100N for a given time point.

Nutrition	Non-treated		BTH		ARB	
	3rd leaves	5th leaves	3rd leaves	5th leaves	3rd leaves	5th leaves
100N	126.7±29.35	84.43±1.66	75.40±18.3∗	26.22±7.43∗	129.1±8.39	55.15±11.03∗
75N	148.4±45.84	68.17±7.37	44.93±4.60∗#	25.43±8.14∗	127.4±16.16	67.72±23.11
50N	99.5±16.94	64.12±17.96#	39.94±2.50∗#	16.37±5.40∗	109.1±10.94	56.04±18.22

In the non-treated plants, the content of Chl *a* or Chl *b* was similar in all N nutrition variants (Fig. 1). The treatment with BTH decreased the Chl content in both older and younger leaves. The lowest Chl *a* content was observed in the 50N, it was about 60 and 50 % of the control on the 7th and 14th dpt, respectively. In the BTH-treated plants, these values were higher by about 10 % in the 75N than in the 100N. In the ARB-treated plants, the significant differences in Chl *a* content were observed only in the 3rd leaves of the 75N variant on the 14th dpt (126 %) and in the 5th ones of the 100N on the 7th dpt (115 %) in comparison to the non-treated plants (Fig. 1*A,C*). The addition of urea to the growing medium augmented the negative effect of BTH on Chl *b* content in the 5th leaves on the 7th dpt (Fig. 1*D*).

We observed that urea modified Glc and Suc content in the leaves of the non-treated plants (Fig. 2). The highest Glc content in the 3rd leaves was observed in the plants from the 75N medium and in the 5th leaves in the plants from 50N medium (Fig. 2*A,C*). The high dose of urea (the 50N) decreased the Suc content at both time points in the 3rd non-treated leaves by more than 30 % in comparison to the 100N and 75N (Fig. 2*B*). In the 5th non-treated leaves, the Suc content was similar in all nitrogen variants except the 50N where its content on the 14th day was higher by about 37 and 32 % in comparison to the 100N and 75N, respectively (Fig. 2*D*).

Benzothiadiazole increased the Glc and Suc content in both examined types of leaves regardless of the type of N nutrition. Two weeks after the BTH application, a massive accumulation of Glc and Suc took place in the 3rd leaves (Fig. 2*A,B*). In comparison with the respective controls for 100N, 75N, and 50N, the Glc content was 83, 88, and 250 % higher, respectively (Fig. 2*A*), and the content of Suc 113, 59, and 125 % higher, respectively (Fig. 2*B*). On the 7th dpt, BTH induced the accumulation of Suc only in the older leaves of the 50N plants (192 % of the control). However, in the BTH-treated 3rd leaves, the rate of Suc accumulation between 7th and 14th dpt diminished with the increasing urea concentration in the medium. On the 14th dpt, the Suc content was by 192, 124, and 73 % higher than on the 7th dpt for the 100N, 75N, and 50N, respectively (Fig. 2*B*). A reverse trend was observed in Glc accumulation. In the 3rd leaves, the

Glc content on the 14th dpt was 79, 108, and 136 % above values observed on the 7th dpt for the 100N, 75N, and 50N, respectively (Fig. 2*A*). The influence of BTH on Glc and Suc content was observed earlier in the younger (5th) leaves than in the older ones, but this effect also depended on the type of N nutrition (Fig. 2). In the 5th leaves on the 7th dpt, the Glc content was above the control level only in the 100N (84 %) and 50N (54 %) (Fig. 2*C*). On the 14th dpt, only in the 75N leaves, the Glc content was different (lower by 32 %) than in the non-treated ones. A similar observation was made for Suc, but its accumulation on the 7th dpt was found only in the variants with urea (Fig. 2*D*).

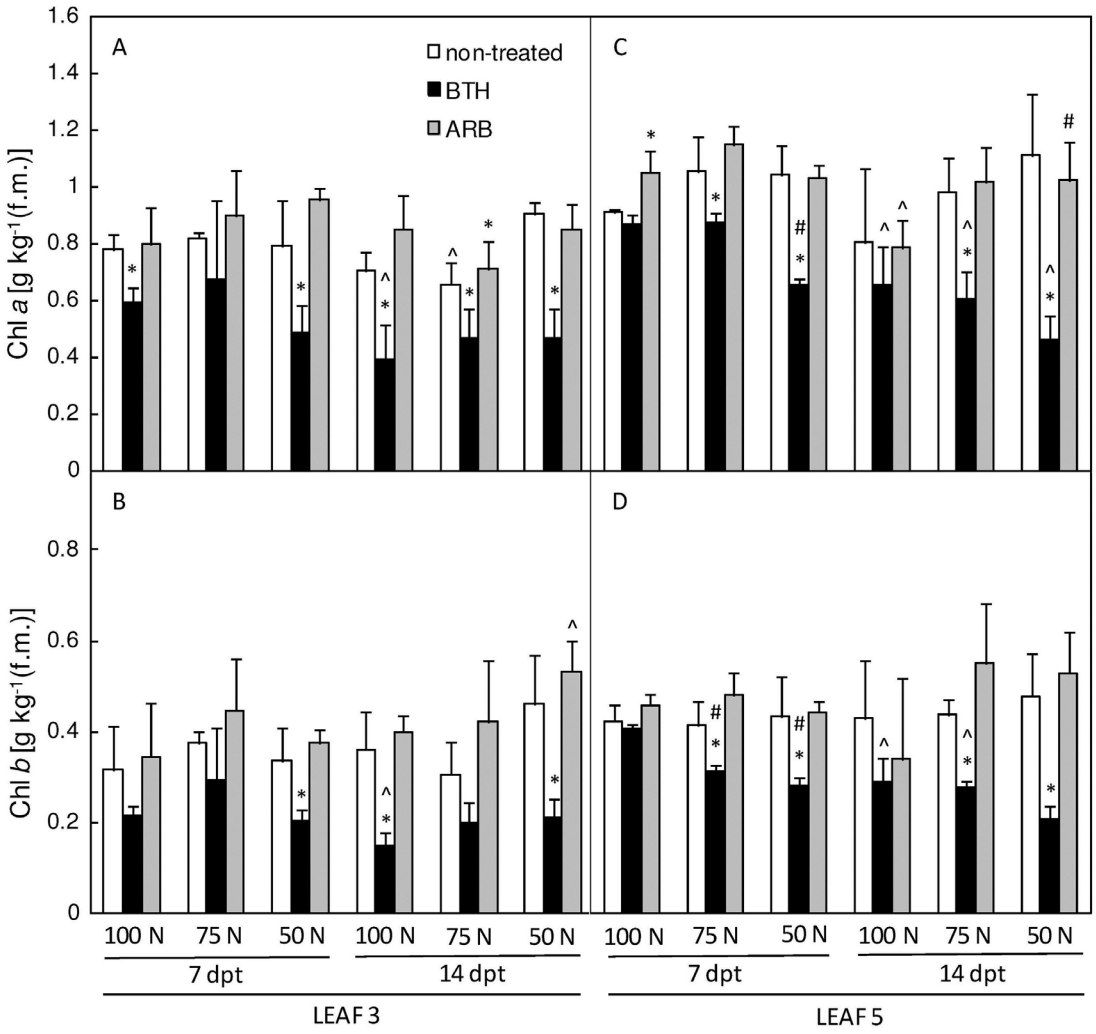

Fig. 1. The content of chlorophyll (Chl) *a* (*A*,*C*) and Chl *b* (*B*,*D*) in the 3rd (*A*,*B*) and 5th (*C*,*D*) leaves of cucumber plants grown in 100 % NO_3^- (100N), 75 % NO_3^- + 25 % urea (75N), and 50 % NO_3^- + 50 % urea (50N) nutrition media and treated or not with benzothiadiazole (BTH) or arbutin (ARB). Mesurements were done on days 7 and 14 after treatment (dpt). Means ± SD, n = 10. * indicates values significantly different ($P < 0.05$) from non-treated plants for a given time point; # indicates significant differences between the 75N or 50N nutrition variants compared to the 100N; ^ indicates values on the 14th dpt significantly different from those on the 7th dpt for a given nutritional variant.

In the ARB-treated leaves, the Glc and Suc content changed mainly in the plants growing on the urea-containing media. In the older leaves, no changes in Glc content were observed when compared with the control (Fig. 2*A*). In the ARB-treated 5th leaves, the Glc content was similar to the control except the 50N where it decreased on the 14th dpt (Fig. 2*C*). A significant increase in Suc content in the ARB-treated 3rd leaves was observed only in the 50N (the 7th and 14th dpt by 60 and 40 %) and 100N (the 14th dpt by 25 %) (Fig. 2*B*). In the 3rd leaves of the ARB-treated plants, the Suc content increased between the 7th and 14th dpt by 33 - 40 % for all examined variants. A similar tendency was found in the 5th leaves (Fig. 2*D*).

The AlIn activity significantly increased in the 3rd and 5th leaves of the BTH-treated plants (Fig. 3*A,D*). Urea added to the medium stabilized the AlIn activity (no change between the 7th and the 14th dpt) whereas in the

100N on the 14th dpt, the AlIn activity was higher by 70 % than on the 7th dpt. In the 5th leaves, changes of the AlIn activities were similar to those in the 3rd ones, *i.e.*, the AlIn activity was higher on the 14th dpt than on the 7th dpt, especially in the 100N plants (Fig. 3*D*).

The urea application did not significantly influence the AlIn activity in the non-treated plants except the younger leaves from the 50N in which on the 7th dpt it decreased by 40 and 23 % and on the 14th dpt it increased by about 40 and 80% as compared to the non-treated 100N and 75N plants, respectively (Fig. 3*A,D*).

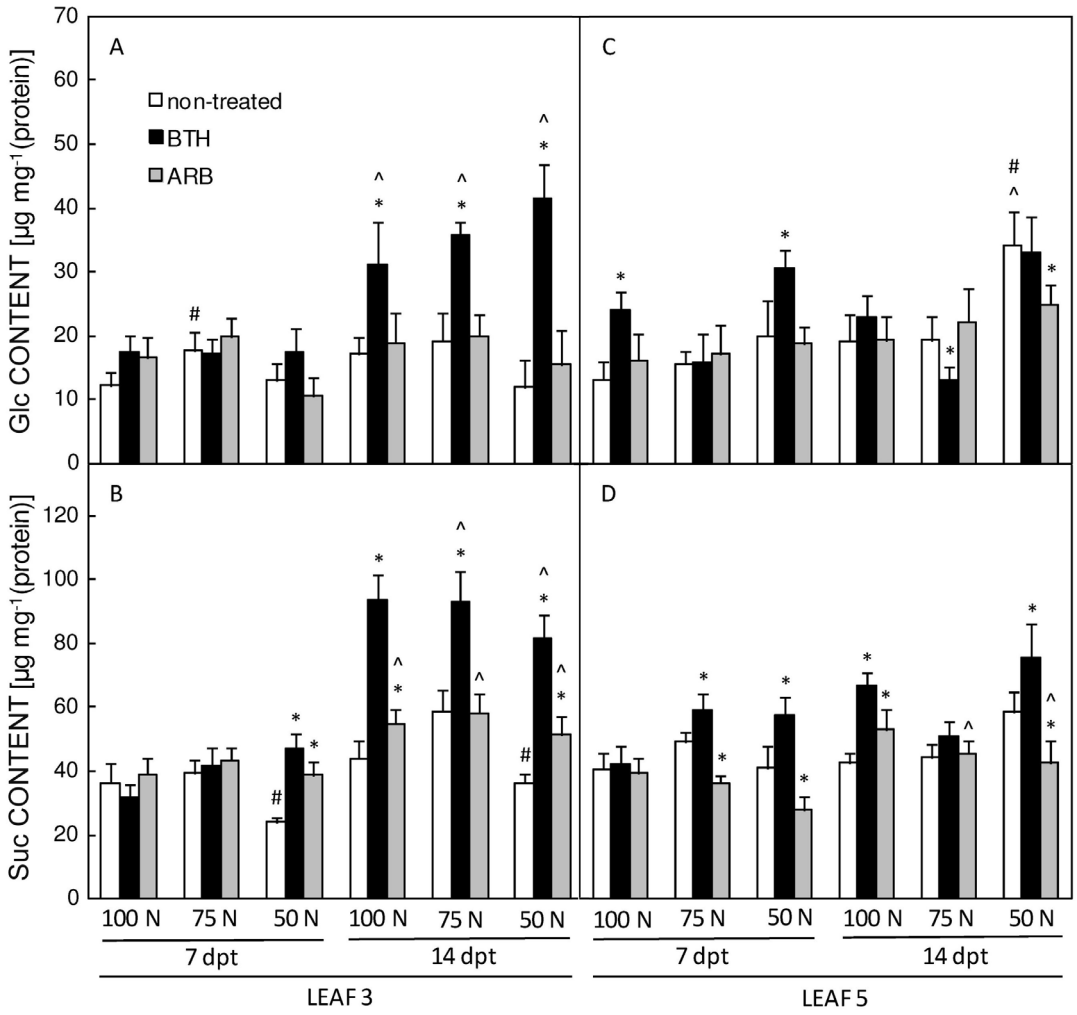

Fig. 2. The content of glucose (*A*,*C*) and sucrose (*B*,*D*) in the 3rd (*A*,*B*) and 5th (*C*,*D*) leaves of cucumber plants grown in 100 % NO_3^- (100N), 75 % NO_3^- + 25 % urea (75N), and 50 % NO_3^- + 50 % urea (50N) media and treated or not with benzothiadiazol (BTH) or arbutin (ARB) for 7 and 14 d. Means ± SD, n = 10. For other details see Fig. 1.

Changes of the AlIn activity caused by ARB, similarly as those by BTH, depended on the urea content. In the 3rd leaves of the ARB-treated 100N plants, the AlIn activity was significantly enhanced in comparison with the control (238 and 149 % on the 7th and 14th dpt, respectively) (Fig. 3*A*). On the 7th dpt, a marked increase in AlIn activity (244 % of the control) was found in the 3rd leaves of the ARB-treated 50N plants whereas on the 14th dpt, the activity of this enzyme was significantly enhanced in the 75N plants (168 % of the control). In the 3rd leaves of the ARB-treated plants, the AlIn activity in the 75N variant increased between the 7th and 14th dpt by about 98 % whereas in the 50N, it decreased by about 55 %. In the 5th leaves of the ARB-treated plants, urea changed the AlIn activity only on the 14th dpt as shown by its increase in the 75N plants (193 % of the control) and its decrease in the 50N (59 % of the control). In the 100N plants, the AlIn activity decreased to 47% of the control value on the 7th dpt (Fig. 3*D*).

Urea did not significantly influence the AcIn activity in the non-treated plants (Fig. 3*B,E*). However, urea affected AcIn activity changes resulting from the BTH and ARB treatments (Fig. 3*B,E*). In the 3rd leaves of the BTH-treated plants, the AcIn activity increased on the 7th dpt in all the N nutrition variants. On the 14th dpt, the AcIn activity in the 75N and 50N plants returned to the

control level whereas in the 100N, it exceeded the control value by 74 % (Fig. 3*B*). In the younger leaves of the BTH-treated plants, no changes in AcIn activity were found (Fig. 3*E*).

In the ARB-treated 100N plants in comparison to the control, a diminution in AcIn activity was found in the 3rd leaves on the 14th dpt (to 68 %) and in the 5th leaves on the 7th dpt (to 49 %). The high dose of urea (in the 50N) in combination with ARB enhanced the AcIn activity in the 3rd leaves on the 7th dpt (259 % of the control) and decreased it (51 % of the control) in the 5th leaves.

No influence of urea on the LDH activity was observed in the non-treated plants except for a 40 % decrease found on the 14th dpt in the 50N nutrition variant

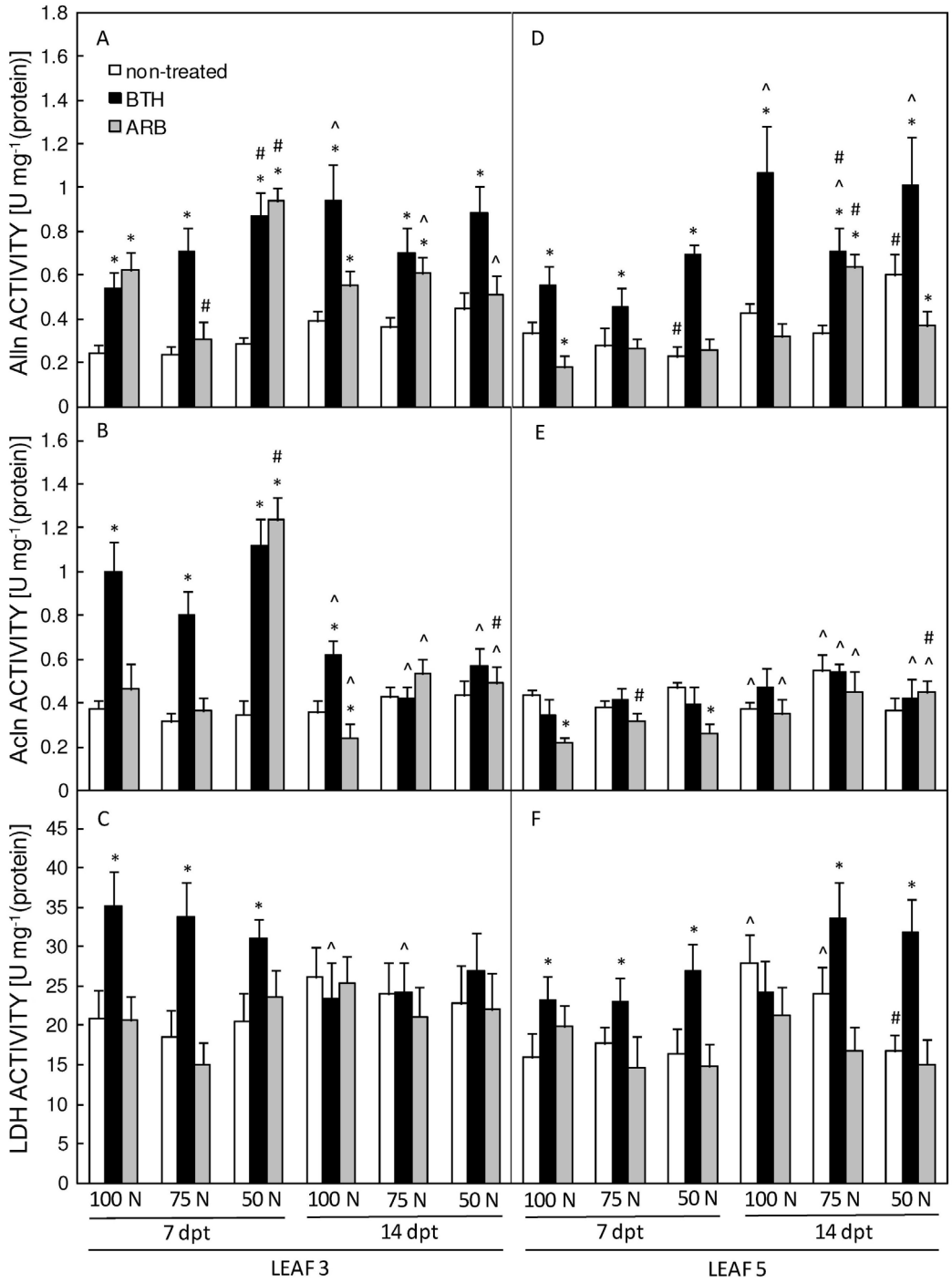

Fig. 3. The activity of alkaline invertase (*A*,*D*), acid invertase (*B*,*E*), and lactate dehydrogenase (*C*,*F*) in the 3rd (*A*,*B*,*C*) and 5th (*D*,*E*,*F*) leaves of cucumber plants grown in 100 % NO_3^- (100N), 75 % NO_3^- + 25 % urea (75N), and 50 % NO_3^- + 50 % urea (50N) media and treated or not with benzothiadiazol (BTH) or arbutin (ARB) for 7 and 14 d. Means ± SD, n = 10. For other details see Fig. 1.

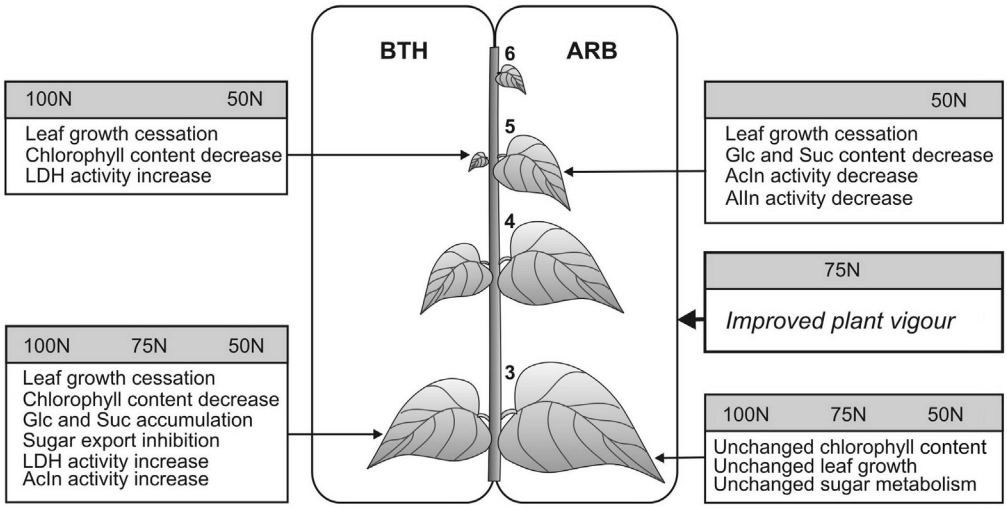

Fig. 4. The growth and metabolic effects of benzothiadiazol (BTH) or arbutin (ARB) on cucumber grown in 100 % NO_3^- (100N), 75 % NO_3^- + 25 % urea (75N), and 50 % NO_3^- + 50 % urea (50N) media. Urea in the nutrient medium modified the response of cucumber to BTH and ARB with respect to plant fitness and sugar metabolism. The improved vigour of the ARB-treated plants grown in the75N medium could be attributed to a benefit of the low dose of urea to those plants. For simplicity of the figure, a non-treated plant is not shown.

(Fig. 3*C,F*). The changes in LDH activity appeared in the BTH-treated plants. In the 3rd leaves, significant increases in LDH activity (by about 68, 82, and 51 % in the 100N, 75N, and 50N, respectively, in comparison with the control) were determined on the 7th dpt, but a week later, these values returned to the control levels (Fig. 3*C*). Similar changes were observed on the 7th dpt in all the variants in the 5th leaves, but on the 14th dpt, the LDH activity was still higher than in the control (by 39 % for the 75N and 90 % for the 50N) (Fig. 3*F*). The ARB application did not influence the LDH activity (Fig. 3*C,F*).

Discussion

Our study shows a dose-dependent effect of urea added as a partial nitrogen source on the non-treated cucumber plant growth. The highest urea concentration (50N) was more harmful resulting in a slower plant growth and a smaller leaf area as compared to the other two N nutrition variants. Moreover, among all studied non-treated variants, only the 50N plants exhibited disturbance of sugar metabolism, particularly in the younger leaves, with a tendency to accumulate Glc between the 7th and 14th dpt. This accumulation was accompanied by an increase in AlIn activity, which can indicate acceleration of processes supplying carbon skeletons for NH_4^+ assimilation or energy for adaptation processes which protect growing tissues and organs against an increased content of toxic NH_4^+ ions.

It has been postulated that increased sugar content induces leaf senescence (Yoshida 2003). Glucose accumulation in cells induces expression of genes characteristic for nitrogen remobilization which is typically enhanced during senescence (Masclaux *et al.* 2000). The 3rd non-treated leaves of cucumber from the 50N nutrition medium exhibited symptoms, such as growth depression and accelerated yellowing, similar to those observed by Merigout *et al.* (2008) for *Arabidopsis* fed only with urea as nitrogen source. These observations may suggest that a high dose of urea accelerates maturation of younger leaves and senescence of older ones, but in contrast to the findings of these authors, we did not notice a decline in invertase activities, thus the observed changes might result from a high content of NH_4^+ ions. Earlier, Jang and Sheen (1997) showed that the cellular Glc content does not determine senescence, and Loreti *et al.* (2001) postulated that some sugar-response pathways may be regulated by the rate of fluxes rather than the absolute content of sugars and sugar metabolites. The non-treated plants from the 100N and 75N variants did not differ significantly in the examined biochemical parameters. However, in the 75N plants, a better growth and vigour as well as a larger area of the 3rd leaves were noted. The lower dose of urea (75N) prolonged viability of the 3rd leaves because they tended to keep a relatively higher content of sugars and Chl *a* and *b* and exhibited a larger area in comparison with the plants growing in the 100N and 50N at the same time.

In the cucumber plants, the BTH treatment led to a decrease in chlorophyll content and to a reduction of leaf area, which could be due to the fact that BTH acted similarly as SA at higher concentrations, which reduced the activity of carbonic anhydrase and chlorophyll content (Fariduddin *et al.* 2003). In the cucumber plants,

BTH caused cessation of growth. This is in agreement with the observation of Lawton *et al.* (1996) that BTH causes biomass loss. In this study, the BTH-mediated limitation of plant growth might result from utilization of Glc and Suc for synthesis and glucosylation of secondary metabolites in older leaves because in these leaves, the high content of Glc and Suc and the high AlIn activitiy were found. These data together with the decrease in Chl *a* and *b* content seem to suggest that older BTH-treated leaves were not efficient exporters of assimilates to other organs among them to younger leaves.

The urea addition to the medium changed the response of the cucumber plants to the BTH application but in different manner in younger and older leaves. In contrast to the 100N, in the 75N and 50N plants, the application of BTH did not lead to increase in AlIn activity between the 7th and the 14th day of experiment in the 3rd leaves. This suggests that the response to the BTH treatment was delayed in the plants grown on the urea-containing media. Moreover, the 100N plants exhibited the enhanced AcIn activity whereas those from both urea containing media did not.

Our study shows that in all the variants, the ARB-treated plants exhibited a better vigour and fitness than the non-treated and BTH-treated ones. The higher Chl content in the ARB-treated plants than in the non-treated and BTH-treated ones and a similar content of Glc and Suc in the ARB-treated and non-treated plants suggest that the ARB-treated plants were most efficient at utilizing assimilates for their growth. Arbutin is a well-known substrate for β-glucosidase, and the produced HQ could be involved in diverse processes. Hydroquinone can positively influence H_2O_2- and HQ-specific mitochondrial peroxidase, thus increase the efficiency of antioxidative protection in mitochondria (Hadži-Taśković Šukalović *et al.* 2007). Peroxidase with a high affinity to HQ was also found in thylakoid membranes (Casano *et al.* 2000). It was demonstrated that HQ acts as urease inhibitor (Pandey *et al.* 2005), thus HQ molecules resulting from ARB catabolism (Jin and Sato 2003) may limit urea hydrolysis and diminish the content of released ammonia ions. On the other hand, HQ may enhance the productivity of photosynthesis (Casano *et al.* 2000, Xu *et al.* 2002).

Assimilation of NH_4^+ is an energy-consuming process. The enhanced LDH activity in the BTH-treated plants observed 7 dpt might result from disturbance of oxygen metabolism and a decreased ATP production. It has been shown that SA inhibits both cytochrome oxidase and alternative oxidase pathways leading to the inhibition of ATP synthesis (Xie and Chen 1999). Assuming that BTH acted in a similar way, we suggest that an insufficient ATP supply in the BTH-treated plants might result in transient cell growth arrest, especially in young leaves. Heil (1999) indicated that after some time, plants compensate the BTH-mediated growth inhibition. This process was also observed in our study (data not shown). Although the LDH activity was similar to the control in the BTH-treated 3rd leaves, it remained elevated on the 14th dpt in the 5th leaves of the BTH-treated plants from the urea nutrition variants. It seems to indicate that in younger leaves, urea prolonged the BTH-mediated changes leading to an insufficient ATP content.

It is widely accepted that the fermentative pathway in plant cells starts when oxygen supply is drastically limited (Kato-Noguchi 2006, Mustroph and Albrecht 2007). However, plant tissues have a propensity to drive themselves into anoxia. Plant tissues that have a high metabolic activity can become hypoxic even in a well oxygenated environment (Geigenberger 2003). A lowered oxygen content in a cell leads to coordinated inhibition of respiration and ATP biosynthesis (Klok *et al.* 2002) which subsequently results in a decreased activity of invertase whose genes are strongly repressed by low oxygen concentrations (Zeng *et al.* 1999). These and other processes connected with them might influence the primary metabolism. The increase in LDH activity in the BTH-treated leaves may indicate that supply of the oxidized form of NADH for the tricarboxylic acid cycle is inadequate and may limit carbon skeleton supply and mitochondrial respiration (Hodges 2002). It should be noted that LDH can also catalyze a reverse reaction depending on a pH value in cells whereas ADH cannot do it. Taking together, besides supply of the oxidised form of NADH and participation in ATP production, other functions of LDH, such as carbon skeleton preservation and regulation of pH value in a cell, should be also taken into consideration in the BTH-treated plants.

In conclusion, we found that the urea supply into the nutrition solutions changed cucumber sugar metabolism in a dose- and time-dependent manners and influenced its response to the BTH and ARB treatments (Fig. 4). In cucumber, BTH contributed to a significant reorganization in sugar metabolism. In the BTH-treated plants, the massive accumulation of Glu and Suc in older leaves correlated with the high AlIn activity and the lower Chl content, indicating their decreased capacity to export assimilates to other organs. This might be responsible for the growth arrest found mainly in younger leaves. This negative effect was not found in the ARB-treated plants. Moreover, the improved fitness of the ARB-treated 75N plants indicates that the low dose of urea in the medium was to their advantage. In our opinion, the use of ARB as natural compound which may improve growth and vigour of cucumber plants should be taken into consideration in a future study.

References

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Casano, L.M., Zapata, J.M., Martín, M., Sabater B.:

Chlororespiration and poising of cyclic electron transport. Plastoquinone as electron transporter between thylakoid NADH dehydrogenase and peroxidase. - J. biol. Chem. **275**: 942-948, 2000.

Cruz, J.L., Mosquim, P.R., Pelcani, C.R., Araùjo, W.L., DaMatta, F.M.: Carbon partitioning and assimilation as affected by nitrogen deficiency in cassava. - Photosynthetica **41**: 201-207, 2003.

Dietrich, R., Ploss, K., Heil, M.: Constitutive and induced resistance to pathogens in *Arabidopsis thaliana* depends on nitrogen supply. - Plant Cell Environ. **27**: 896-906, 2004.

Fariduddin, Q., Hayat, S., Ahmad, A.: Salicylic acid influences net photosynthetic rate, carboxylation efficiency, nitrate reductase activity, and seed yield in *Brassica juncea*. - Photosynthetica **41**: 281-284, 2003.

Frias, M.A., Winik, B., Franzoni, M.B., Levstein, P.R., Nicastro, A., Gennaro, A.M., Disalvo, E.A.: Lysophosphatidylcholine-arbutin complexes form bilayer-like structures. - Biochim. biophys. Acta **1778**: 1259-1266, 2008.

Geigenberger, P.: Response of plant metabolism to too little oxygen. - Curr. Opin. Plant Biol. **6**: 247-256, 2003.

Gerendás, J., Sattelmacher, B.: Significance of N source (urea *vs*. NH_4NO_3) and Ni supply for growth, urease activity and nitrogen metabolism of zucchini (*Cucurbita pepo* convar. Giromontiina). - Plant Soil **196**: 217-222, 1997.

Gocławski, J., Sekulska-Nalewajko, J., Kuźniak, E.: Neural network segmentation of images from stained cucurbits leaves with colour symptoms of biotic and abiotic stresses. - Int. J. appl. Math. Comp. Sci. **22**: 669-684, 2012.

Hadži-Taśković Šukalović, V., Kukavica, B., Vuletić, M.: Hydroquinone peroxidase activity of maize root mitochondria. - Protoplasma **231**: 137-144, 2007.

Heil, M.: Systemic acquired resistance: available information and open ecological questions. - J. Ecol. **87**: 341-346, 1999.

Hodges, M.: Enzyme redundancy and the importance of 2-oxoglutarate in plant ammonium assimilation. - J. exp. Bot. **53**: 905-916, 2002.

Jang, J.-C., Sheen, J.: Sugar sensing in higher plants. - Trends Plant Sci. **2**: 208-214, 1997.

Jin, S., Sato, N.: Benzoquinone, the substance essential for antibacterial activity in aqueous extracts from succulent young shoots of the pear *Pyrus* spp. - Phytochemistry **62**: 101-107, 2003.

Kato-Noguchi, H.: Pyruvate metabolism in rice coleoptiles under anaerobiosis. - Plant Growth Regul. **50**: 41-46, 2006.

Klok, E.J., Wilson, I.W., Wilson, D., Chapman, S.C., Ewing, R.M., Somerville, S.C., Peacock, W.J., Dolferus, R., Dennis, E.S.: Expression profile analysis of low-oxygen responses in *Arabidopsis* root cultures. - Plant Cell **14**: 2481-2494, 2002.

Kumar, S., Kayastha, A.M.: Soybean (*Glycine max*) urease: significance of sulfhydryl groups in urea catalysis. - Plant Physiol. Biochem. **48**: 746-750, 2010.

Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T., Ryals, J.: Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. - Plant J. **10**: 71-82, 1996.

Li, D., Tian, M., Cai, J., Jang, D., Cao, W., Dai, T.: Effects of low nitrogen supply on relationships between photo-synthesis and nitrogen status at different leaf position in wheat seedlings. - Plant Growth Regul. **70**: 257-263, 2013.

Loreti, E., De Bellis, L., Alpi, A., Perata, P.: Why and how do plant cells sense sugars? - Ann. Bot. **88**: 803-812, 2001.

Masclaux, C., Valadier, M.H., Brugiére, N., Morot-Gaudry, J.F., Hirel, B.: Characterization of the sink/source transition in tobacco (*Nicotiana tabacum* L.) shoots in relation to nitrogen management and leaf senescence. - Planta **211**: 510-518, 2000.

Merigout, P., Lelandais, M., Bitton, F., Renou, J.P., Briand, X., Meyer, C., Daniele-Vedele, F.: Physiological and transcriptomic aspects of urea uptake and assimilation in *Arabidopsis* plants. - Plant Physiol. **147**: 1225-1238, 2008.

Miao, M., Xu, X., Chen, X., Xue, L., Cao, B.: Cucumber carbohydrate metabolism and translocation under chilling night temperature. - J. Plant Physiol. **164**: 621-628, 2007.

Miller, W.B., Ranwala, A.P.: Characterization and localization of three soluble forms of invertase from *Lilium longiflorum* flower buds. - Physiol. Plant. **92**: 247-253, 1994.

Mustroph, A., Albrecht, G.: Fermentation metabolism in roots of wheat seedlings after hypoxia pre-treatment in differed anoxic incubation systems. - J. Plant Physiol. **164**: 394-407, 2007.

Nacry, P., Bouguyon, E., Gojon, A.: Nitrogen acquisition by roots: physiological and developmental mechanisms ensuring plant adaptation to a fluctuating resource. - Plant Soil **370**: 1-29, 2013.

O'Carra, P., Mulcahy, P.: Lactate dehydrogenase in plant: distribution and function. - Phytochemistry **42**: 581-587, 1996.

Oliver, A.E., Crowe, L.M., De Araujo, P.S., Fisk E., Crowe, J.H.: Arbutin inhibits PLA2 in partially hydrated model systems. - Biochim. biophys. Acta **1302**: 69-78, 1996.

Pandey, D.K., Mishra, N., Singh, P.: Relative phytotoxicity of hydroquinone on rice (*Oryza sativa* L.) and associated aquatic weed green musk chara (*Chara zeylanica* Willd.). - Pestic. Biochem. Physiol. **83**: 82-96, 2005.

Petkou, D., Diamantidis, G., Vasilakakis, M.: Arbutin oxidation by pear (*Pyrus communis* L.) peroxidases. - Plant Sci. **162**: 115-119, 2002.

Phuntumart, V., Marro, P., Metraux, J.-P., Sticher, L.: A novel cucumber gene associated with systemic acquired resistance. - Plant Sci. **171**: 555-564, 2006.

Sillero, J.C., Rojas-Molina, M.M., Avila, C.M., Rubiales, D.: Induction of systemic resistance against rust, ascochyta blight and broomrape in faba bean by exogenous application of salicylic acid and benzothiadiazole. - Crop Protect. **34**: 65-69, 2012.

Šindelářová, M., Šindelář, L., Burketová, L.: Glucose-6-phosphate dehydrogenase, ribonucleases and esterases upon tobacco mosaic virus infection and benzothiodiazole treatment in tobacco. - Biol. Plant. **45**: 423-432, 2002.

Sturm, A.: Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning. - Plant Physiol. **121**: 1-7, 1999.

Sturm, A., Tang, G.-Q.: The sucrose-cleaving enzymes of plant are crucial for development, growth and carbon partitioning. - Trends Plant Sci. **4**: 401-407, 1999.

Suau, R., Cuevas, A., Valpuesta, V., Reid, M.S.: Arbutin and sucrose in the leaves of the resurrection plant *Myrothamnus flabellifolia*. - Phytochemistry **30**: 2555-2556, 1991.

Sugiyama, N., Taniguchi, N.: Evaluation of the role of lactate dehydrogenase in oxalate synthesis. - Phytochemistry **44**: 571-574, 1997.

Torisky, R.S., Polacco, J.C.: Soybean roots retain the seed urease isoenzyme synthesized during embryo development. - Plant Physiol. **94**: 681-689, 1990.

Walters, D., Walsh, D., Newton, A., Lyon, G.: Induced resistance for plant disease control: maximizing the efficacy of resistance elicitors. - Phytopathology **95**: 1368-1373, 2005.

Wellburn, A.R.: The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with

spectrophotometers of different resolution. - J. Plant Physiol. **144**: 307-313, 1994.

Witte, C.-P.: Urea metabolism in plants. - Plant Sci. **180**: 431-438, 2011.

Xie, Z.X., Chen, Z.: Salicylic acid induces rapid inhibition of mitochondrial electron transport and oxidative phosphorylation in tobacco cells. - Plant Physiol. **120**: 217-225, 1999.

Xu, X., Boeckx, P., Wang, Y., Huang, Y., Zheng, X., Hu, F., Van Cleemput, O.: Nitrous oxide and methane emissions during rice growth and through rice plants: effect of dicyanidiamide and hydoquinone. - Biol. Fert. Soils **36**: 53-58, 2002.

Yoshida, S.: Molecular regulation of leaf senescence. - Curr. Opin. Plant Biol. **8**: 79-84, 2003.

Zeng, Y., Wu, Y., Avigne, W.T., Koch, K.E.: Rapid repression of maize invertases by low oxygen. Invertase/sucrose synthase balance, sugar signaling potential, and seedling survival. - Plant Physiol. **121**: 599-608, 1999.