INTERACTION AMMONIUM-NITRATE: RESPONSE TO OXIDATIVE STRESS IN CHICORY PLANTS

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Abstract

The aim of this work was to study, as a function of the different availability of nitrogen in the reduced form, mineral and organic, the induction of the synthesis of some ROS-scavenging molecules and the evolution of some enzymatic activities such as ascorbate peroxidase (APX) and polyphenoloxidase (PPO).

Chicory seedlings were grown in nutritive solution for 35 days in controlled conditions. On the 14^{th} day, one third of the plants was transferred into a nutritive solution containing $(NH_4)_2SO_4$ 60 mM, one third was transferred into a medium containing Urea 60 mM, and the remaining was let grow into the nutrition solution, as a control. Three samplings of leaves were performed, respectively after 21, 28 and 35 days of growth.

The urea and ammonium sulphate-treated samples showed higher ascorbic acid and polyphenol contents than the control, together with a lower anthocyanins content. APX showed the highest activity in the urea-treated samples, while the highest PPO activity was to refer to samples treated with ammonium sulphate.

The variations of the organic components showed the incidence of the nitrogen supply in the reduced form on the cell redox potential, confirming the importance of fertilization for obtaining high amounts of antioxidant molecules.

Introduction

The induction of secondary metabolism in the plants, with the consequent synthesis of antioxidant compounds, depends on biotic and abiotic inputs produced by natural processes and anthropic actions. Metabolic components produced by secondary metabolism, such as ascorbic acid, polyphenols, anthocyanins, ROS-scavenging molecules and some enzymatic activity involved in the biosynthesis of this organic components, such as ascorbate peroxidase (APX) and polyphenoloxidase (PPO), indicate the total antioxidant capacity (TAC) of a biological system [1]. TAC variations are representative of the metabolic requirement of equilibration of cellular redox potential. Polyphenols production in the plants depends on genetics and physiologicals factors, such as genotypes and maturation [2]; on other factors such as UV [3], osmotic stress [4] and nutrients availability. Particularly, nitrogen source has a potential effect on polyphenols formation [5]. Since nitrogen supply is indispensable to increase plant productions, the evaluation of the influence of nitrogen reduced form in nutritive solution on molecules and ROS scavenging activities production, results very interesting.

Soil favours the availability of nitrate for the plant, by inducing oxidative processes of ammonia nitrogen; the nitrogen supply in the reduced form in the nutritive solution will determine some competition with the nitrate absorption [5, 6], causing stress conditions for the plant.

The aim of this work was to study, as a function of the different availability of nitrogen in the reduced form, mineral and organic, the induction of the synthesis of some ROS-scavenging molecules and the evolution of some enzymatic activities concerned with the biosynthesis of these organic compounds, assessing, at the same time, the TAC of plants in the different experimental conditions.

Materials and Methods

Two-week chicory seedlings were grown in trays (15 plants in each tray) containing 4 l Hoagland nutritive solution (½ ionic strength). The experiment was carried out for 35 days in controlled conditions: photoperiod (16/8 h); PAR (150 μmol m^{-2} s^{-1}); temperature (day/night): 25/20°C; relative humidity (75-85%). The pH of the nutritive solution was 6.5.

On the 14^{th} day of the trial, one third of the plants was transferred into a nutritive solution containing $(NH_4)_2SO_4$ 60 mM, one third was transferred into a medium containing Urea 60 mM, and the remaining was let grow into the Hoagland solution, as a control. Three samplings of leaves were performed, respectively after 21 (I), 28 (II) and 35 days of growth (III). The following determinations were carried out on samples:

Total and inorganic phosphorus;

Total nitrogen and internal nitrate;
Total polyphenols
Total anthocyanins
Total, reduced and oxidized ascorbic acid
Total antioxidant activity (TAC)
Ascorbate peroxidase (APX) specific activity
Polyphenoloxidase (PPO) specific activity

Results are the means of three independent experiments each analysed twice ($n = 3$). Different letters indicate that means are significatively different at $P \leq 0.05$.

Inorganic phosphorus (Pi)

One gram of fresh vegetable material was homogenized in 10% (w/v) trichloroacetic acid (TCA) at 4°C. The homogenate was diluted with 5% (w/v) TCA, let in ice for 30 minutes, then centrifuged at 10000 rpm AT 4°C for 10 minutes. Six hundred µl supernatant were withdrawn and diluted to 5 ml with water. The mixture was added with 0.5 ml H_2SO_4 10 N, 0.8 ml ammonium molybdate, 0.4 ml 4-amino-3-hydroxy-1-naphthalenesulfonic ($C_{10}H_9NO_4S$).

The solution, after agitation, was diluted to 10 ml with water. Spectrophotometer readings at 660 nm were performed after incubation for 10 minutes at 37°C, and the values were reported to concentration by means of a calibration curve obtained with suitable dilutions of KH_2PO_4 (80 µg/ml), respectively: 8 µg, 16 µg, 24 µg, 32 µg, 40 µg [7].

Organic components

The organic compounds, total polyphenols, anthocyanins and ascorbic acid total and reduced, were determined as previously described [8, 9, 10].

Determination of the total antioxidant capacity (TAC)

Samples of leaves were extracted with methanol and sonicated for 60 s. The homogenate was centrifuged at 10000 rpm for 10 min at 4°C. The total antioxidant capacity was assayed on the supernatant according to [11]. Spectrophotometric readings at 730 nm were performed on the reaction mixture made of: 100 µl extract; 2 mM ABTS; 0.1 mM H_2O_2, 0.25 nM HRP in 50 mM phosphate buffer at pH 7.5. TAC was determined by means of a calibration curve obtained with suitable dilutions of a solution of pure ascorbic acid standard.

Polyphenoloxidase (PPO) assay

PPO extraction was carried out according to [12], using a 50 mM potassium-phosphate buffer (pH 6.5). The homogenate was filtered and centrifuged at 20000 g for 20 minutes at 4°C. The activity, following the method of [13], was essayed on a reaction mixture made up with 0.2 ml caffeic acid, 0.1 mM ethanol and 0.5 ml of the extract. Absorbance was measured at 480 nm for 5 minutes. The PPO activity was expressed as μmoles *o*-quinone min^{-1} mg^{-1} enzymatic protein.

Ascorbate peroxidase (APX) assay

APX extraction was performed with a 50 mM potassium-phosphate buffer (pH 7.0) in presence of 1 mM ascorbic acid to avoid the enzyme inactivation during extraction. The activity was essayed following [14]. APX activity was evaluated on a reaction mixture made up of 0.5 ml enzyme extract in 50 mM potassium-phosphate buffer (pH 6.6), AsA 1 mM, H_2O_2 4 mM, Na_2EDTA 0.4 mM, following the extinction rate of AsA due to its oxidation by H_2O_2. The reaction was started with the addition of H_2O_2 and the AsA degradation was followed monitoring the decrease of absorbance at 290 nm at 25°C.

Enzymatic proteins determination

The enzymatic proteins content was determined according to the method of [15], using Coomassie Brillant Blue G250 which shows, in the free form, a maximum absorbance peak at 465 nm. The reagent, which binds mainly to the residues of arginine and, to a less extent, to lysine, histidine, tyrosine, tryptophan and phenylalanine of the enzyme, shows a maximum absorbance peak at 595 nm. One ml Coomassie was added to variable aliquotes of enzyme. After 15 minutes the absorbance at 595 nm was read and the protein amounts were calculated using a calibration curve obtained with bovine serum albumine (BSA) at concentrations ranging from 2 to 10 μg.

Results and Discussion

The observed increase of nitrates (Tab. 1), which progressed during the tests development compared to the control, might be due to the nitrate availability splitted through time in these samples due to the antagonistic action exerted by the ammonium ion towards the nitrate assimilation at the root level [6, 16]. The lack of nitrate absorbance might be due to a decrease in the transcription rate of the high-affinity inducible protein, in charge of the transport of NO_3^-, which is not detected when the ion is absent, as highlighted in barley roots grown on a nutritive solution where ammonium and nitrates were contemporarily present [17]. It is known that the presence, in the nutritive solution, of the ammonium ion after the addition of the fertilizer, determines an initial

acidification of the medium, which is a favourable condition for the absorbance of nitrogen in the reduced form. The absorbance of ammonia nitrogen allowed to increase the development of plants, as can be inferred from the organic nitrogen contents comparable with those of the control samples. The following nitrate assimilation is testified by the increments of this component in the treated samples, which can be attributed to the decrease of photosynthetic organic matrix. The phosphate contents decrease and, on the other hand, the increase in the nitrate content, observed in the three tests (Tab. 1), might be due to a response determined by the need for an equilibrium of cellular homeostasis [18, 19]. The variations of the reduced and oxidized forms of ascorbic acid (Tab. 2) is in agreement with the higher involvement registered for the APX activity in comparison with the PPO activity (Tab. 3). The rise in the APX activity registered in the treated samples implies an oxidative process of AsA, substrate of the enzyme, and this event is confirmed by the decrease of this latter component and by the increase of its oxidized form. APX, glycoprotein which contains an *eme* group, seems to be codified by a wide multigenic family and to be involved in various physiological processes [20]. This enzyme plays a fundamental role in oxidative processes, such as lignification, cross-linking of the cell walls protein structures and defence against pests attacks [21]. The variations in the AsA/AsA tot ratio, show an increase of the oxidized form in the ammonium sulphate treated samples in comparison with the other tests. The highest activation of APX is in agreement with the variation of the AsA/AsA tot ratio, while DHA represents 76.5% of the total for this test. The oxidative stress registered from the increment of the oxidized form is confirmed by the higher PPO activity (39 and 44% higher if compared with the urea-treated and control samples, respectively). The variations of AsA tot and AsA contents are related with the anthocyanins content (R=0.5566, R=0.8043), which, in turn, is also correlated with the PPO (R= 0.5556), thus pointing out a sequential induction of the biosynthesis of these organic components with a response aimed at re-equilibrating the cell redox potential.

Every circumstance in which the cell homeostasis is altered, can lead to an oxidative stress which depends on an imbalance between the molecules and enzymes having antioxidant activity (AOX) and the ROS, with a consequent increase of the production of oxygen reactive species [22]. The ammonium ion absorption determines a momentary increase of pH, with consequences on the cell redox potential, inducing an oxidative response. The correlation between the nitrate and dehydroascorbate contents (R=0.9596) is a consequence of the ammonium ion supply. The oxidative stress determined by the presence of ammonium ion in the nutritive solution seems to influence, especially, the ascorbic acid content. This ROS-scavenger is able to remove directly the superoxide anion, hydroxyl radicals, singlet oxygen and hydrogen peroxide, through a reaction catalyzed by the ascorbate peroxidase [23]. In agreement with our results, a significative increase of

AsA was observed in mulberry-tree leaves in conditions of nitrogen and phosphorus deficiency [24]. The supply of nitrogen in the reduced form to the nutritive solution, influencing the redox cell equilibrium, determined increments of the polyphenol component (Tab. 4), with an induction of the secondary methabolism, as already highlighted by [5]. In correspondence with the increments of the polyphenol component, the ammonium supply caused increments in leaves of the PPO and peroxydase activities (Tab. 3). The natural ability of PPO is expressed by its capacity of oxidizing phenols and polyphenols, establishing a defence mechanism towards proteins [25]. PPO is, indeed, an enzyme which catalyzes the oxidations O_2-dependent of mono-phenols or di-phenols to o-dichinone [26]. Many plants codify the gene in charge for the production of the enzymatic protein and the correspondent peptide carrier of the PPO, which allow the transport of the enzyme from the chloroplasts to the inside of the thylacoids. Although it is not possible yet a thorough understanding of the functions of PPO in plants (Mayer, 2006), various functions have been attributed to this enzyme, such as: tissue browning [27]; the regulation of the electron cycle and/or of O_2, in the Mehler reaction [28]; the protection function in plants towards parasites and pathogens [29]. The increment of the polyphenol component, on the other hand, was realized at the expense of the anthocyanins production (Tab. 4), as highlighted by the decrease of the anthocyanins/polyphenols ratios [30]. Indeed, together with an increase of polyphenols, the content of the reduced form of ascorbic acid and of anthocyanins is less expressed in the samples fertilized with nitrogen in the reduced form, with slight variations of the AsA tot and AsA contents, which could be correlated with the same anthocyanins contents (R=0.8043, R=0.5566). The correlation observed between the anthocyanins content and the PPO activation (R=0.5556) link the induction of the biosynthesis of anthocyanins to a condition of oxidative stress. The variations of the organic and enzymatic components induced by a ROS-scavenging action were validated with the TAC measurement (date not shown), which was more expressed in the samples treated with simultaneous ammonium and nitrates supply. TAC, which is a measure of the total antioxidant capacity, resulting from a balance between the enzymatic activities and the ROS-scavenging molecules [31, 32], is especially dependent on the differentiated forms of ascorbic acid and on polyphenols. In particular, the increase of TAC in the samples treated with nitrogen in the reduced form has to be attributed to the polyphenol and total AsA components registered in these samples. [31] observed a direct correlation in cereals between these two parameters. Even if the polyphenol component did not show any correlation with TAC in wheat plants [33], such correlability was highlighted in different bean cultivars [9].

The response of the organic species investigated, therefore, indicate that the biosynthesis of ROS-scavenging molecules is influenced by the supply of nitrogen in the reduced form (Nguyen et al., 2008).

Conclusions

The variations of the organic components show the incidence of the nitrogen supply in the reduced form on the cell redox potential.

The oxidative stress was confirmed by the induction of APX and PPO enzymatic activities. The equilibrium restoration was performed mainly at the expense of the variation of the ascorbic acid forms. The phenolic compounds increment was performed mainly at the expense of the anthocyanins production, as highlighted by the decrease of the anthocyanins/polyphenols ratios.

The responses observed in plants with the variations of the organic components and of the investigated enzymatic activities, supported by the total antioxidant activities, point out that the biosynthesis of ROS-scavengers is reduced by the nitrogen supply in the reduced form, therefore confirming the importance of fertilization for obtaining satisfactory productive yields and, at the same time, high amounts of antioxidant molecules.

Tab. 1 Total nitrogen (N) and phosphorus (P) contents (% DW), inorganic phosphorus and nitrate (mg g^{-1} FW) in leaves of chicory cv. Zaira in the different treatments.

Parameters	Stages	Control	Urea	$(NH_4)_2SO_4$	Means
N	I	1.67	2.72	2.78	**2.39 c**
	II	3.29	5.83	3.40	**4.17 b**
	III	6.74	6.17	6.81	**6.57 a**
	Means	**3.90 c**	**4.91 a**	**4.33 b**	
P	I	0.52	0.56	0.73	**0.60c**
	II	1.56	2.53	1.96	**2.02 b**
	III	2.13	3.17	3.25	**2.45 a**
	Means	**1.40 b**	**2.09 a**	**1.98 a**	
Pi	I	0.72	1.21	1.75	**1.23 a**
	II	0.26	0.27	0.26	**0.26 c**
	III	2.27	0.027	0.031	**0.78 b**
	Means	**1.08 a**	**0.50 c**	**0.68 b**	
NO_3^-	I	120.44	161.75	181.29	**154.49 a**
	II	183.19	193.85	128.69	**168.58 a**
	III	150.77	179.55	177.78	**169.34 a**
	Means	**151.45 b**	**178.34 a**	**162.59 ab**	

Means values followed by different letters are significantly different at $P \leq 0.05$.

Tab. 2 Total (Asa tot), reduced (AsA) and oxidate (DHA) ascorbic acid contents (µmol g^{-1} FW) in leaves of chicory cv. Zaira in the different treatments.

Parameters	Stages	Control	Urea	$(NH_4)_2SO_4$	Means
AsA tot	**I**	1.45	2.59	1.50	**1.85 c**
	II	1.50	2.11	2.60	**2.07 b**
	III	1.95	2.11	4.24	**2.77 a**
	Means	**1.63 c**	**2.27 b**	**2.78 a**	
AsA	**I**	0.22	0.38	0.36	**0.32 b**
	II	1.16	1.22	0.83	**1.07a**
	III	1.69	1.11	0.62	**1.14 a**
	Means	**1.02 a**	**0.90 b**	**0.60 c**	
DHA	**I**	1.23	2.21	1.14	**1.53 a**
	II	0.24	0.89	1.77	**1.00 c**
	III	0.26	1.00	3.62	**1.29 b**
	Means	**1.42 b**	**1.37 b**	**2.18 a**	

The significance of the letters is as in Table 1.

Tab. 3 Ascorbate peroxidase and polyphenoloxidase specific activity (U mg^{-1} protein) in leaves of chicory cv. Zaira in the different treatments.

Parameters	Stages	Control	Urea	$(NH_4)_2SO_4$	Means
APX	I	nd	nd	nd	**n.d.**
	II	0.125	0.109	0.118	**0.117 a**
	III	0.077	0.152	0.070	**0.100 b**
	Means	**0.101 b**	**0.1305 a**	**0.094 c**	
PPO	I	0.006	0.010	0.024	**0.014 a**
	II	0.006	0.006	0.005	**0,006 b**
	III	0.004	0.002	0.001	**0,003 c**
	Means	**0,006 b**	**0.006 b**	**0.010 a**	

The significance of the letters is as in Table 1.

Tab. 4 Polyphenol and anthocyanin contents (mg g^{-1} FW) in leaves of chicory cv. Zaira in the different treatments.

Parameters	Stages	Control	Urea	$(NH_4)_2SO_4$	Means
Polyphenols	**I**	45.21	76.45	72.09	**64.58 c**
	II	74.40	79.74	71.78	**75.31 b**
	III	70.56	91.29	101.48	**87.78 a**
	Means	**63.39 b**	**82.49 a**	**81.78 a**	
Anthocyanins	**I**	18.92	7.44	11.94	**12.77 b**
	II	34.04	26.08	29.06	**29.73 a**
	III	40.66	20.32	25.38	**28.79 a**
	Means	**31.20 a**	**17.95 c**	**22.13 b**	

The significance of the letters is as in Table 1.

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