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Photorespiration Process and Nitrogen Metabolism in Lettuce Plants (*Lactuca sativa* L.): Induced Changes in Response to Iodine Biofortification

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Abstract Iodine is vital to human health, and iodine biofortification programs help improve human intake through plant consumption. There is no research on whether iodine biofortification influences basic plant physiological processes. Because nitrogen (N) uptake, utilization, and accumulation are determining factors in crop yield, the aim of this work was to establish the effect of the application of different doses (20, 40, and 80 μM) and forms of iodine (iodate [IO_3^-] vs. Iodide [I^-]) on N metabolism and photorespiration. For this study we analyzed shoot biomass and the activities of nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamate synthase (GOGAT), aspartate aminotransferase (AAT), glutamate dehydrogenase (GDH), glycolate oxidase (GO), glutamate:glyoxylate aminotransferase (GGAT), serine:glyoxylate aminotransferase (SGAT), hydroxypyruvate reductase (HR) and catalase (CAT), nitrate (NO_3^-), ammonium (NH_4^+), organic and total N, amino acids, proteins, serine (ser), malate, and α-ketoglutaric acid in edible lettuce leaves. Application of I^- at doses of at least 40 μM reduced the foliar concentration of NO_3^- with no decrease in biomass production, which may improve the nutritional quality of lettuce plants. In contrast, the application of 80 μM of I^- is phytotoxic for lettuce plants, reducing the biomass, foliar concentration of organic N and NO_3^-, and NR and GDH activities. HR activity is significantly inhibited with all doses of I^-; the least inhibition was at 80 μM. This may involve a decrease in the incorporation of carbonated skeletons from photorespiration into the Calvin cycle, which may be partially associated with the biomass decrease. Finally, the application of IO_3^- increases biomass production, stimulates NO_3^- reduction and NH_4^+ incorporation (GS/GOGAT), and optimizes the photorespiratory process. Hence, this appears to be the most appropriate form of iodine from an agronomic standpoint.

Keywords Biofortification · Iodine · Lettuce plants · Nitrate · Nitrogen assimilation · Photorespiration

Introduction

Agriculture is the main source of all nutrients required to maintain good health. Humans need large amounts of some of these elements, such as nitrogen (N), calcium (Ca), or phosphorus (P), but only trace amounts of others, for example, iron (Fe), zinc (Zn), iodine, or selenium (Se). Deficiencies in these micronutrients are widespread and is a cause for concern in the case of some trace elements (Welch and Graham 2004; Prasad and Chetty 2008).

Humans require around 150 μg iodine/day, an essential micronutrient for mammals. Around 30% of the world's population is estimated to be iodine-deficient, and more than a million individuals are considered to be at risk of this condition (Fields and others 2005). The iodine biofortification of vegetable products has been proposed to improve human nutrition in this respect. Biofortification is a process that increases the concentration of bioavailable essential elements in the edible portions of crops by agricultural intervention or genetic selection (White and Broadley 2005; Abrahams 2006).

In China, in the region of Xingjiang, where the soil and drinking water are low in iodine, fertigation programs

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supplementing iodine have been implemented. This method has been deemed effective and economical in reducing the deficiency of this element in the population (Cao and others 1994). More recently, Mackoviak and Grossl (1999) carried out biofortification programs in rice fields with little success because iodine has little phloem mobility (Herret and others 1962) and consequently the translocation of this element to the grain was limited. Furthermore, as reflected in other studies, the accumulation of this element in the leaves depends mainly on its transport through the xylem (Herret and others 1962), implying that plants in which the edible part is the leaf constitute a more effective medium for iodine biofortification.

Different works have shown that iodine uptake by vegetables depends on both the concentration and the existing form of this element in the environment. Thus, Zhu and others (2003), while studying *Spinacea oleracea* L., demonstrated a direct and proportional relationship between the concentration of iodine in plants and the external application of this element in both its IO_3^- and I^- forms. It has also been shown that the iodine concentration in vegetables is greater when I^- is the form applied in the nutrient solution, because IO_3^-, in addition to having greater valence and molecular weight, must be reduced to I^- for uptake (Umaly and Poel 1971; Whitehead 1975). As a result, the I^- form has the greatest bioavailability for plants.

Rapid population growth over recent decades has generated an increase in the demand for vegetable products. This has created a productivity challenge for agriculture due to the shortage of additional land for farming and the pressure on water supplies in many areas (Lawlor 2002; Kmiecik and others 2004). As a result, the excessive application of fertilizers has become a common agronomic practice. This is especially true for fertilizers containing N because of the essential role of this element in biomass production (Lea and Azevedo 2006).

Different forms of N are found in soil. Nitrate (NO_3^-) is the most bioavailable form and the best assimilated by plants (von Wiren and others 1997; Lea and Azevedo 2006). The first step in NO_3^- reduction is nitrate reductase (NR) catalysis, giving rise to nitrite (NO_2^-), which is reduced to ammonium (NH_4^+) by the action of nitrite reductase (NiR) (Miller and Cramer 2004). NH_4^+ is rapidly incorporated into organic molecules by the combined action of glutamine (GS) and glutamate synthase (GOGAT) enzymes. The reaction catalyzed by GS is the main incorporation pathway for inorganic N into glutamate to form a glutamine molecule. Subsequently, GOGAT is responsible for transferring the amino group of glutamine to 2-oxoglutarate-producing glutamate molecules. One of these molecules regenerates the GS/GOGAT cycle and the other is available for the synthesis of amino acids and different nitrogen compounds. This pathway is of great value because the glutamine and glutamate produced serve as N donors for the biosynthesis of important compounds such as chlorophylls, nucleotides, polyamines, amino acids, and alkaloids (Harrison and others 2000).

Plants have other metabolic pathways for the generation of NH_4^+, including phenylpropanoid biosynthesis, amino acid catabolism, and, especially, photorespiration. Photorespiration can produce 20-fold more NH_4^+ than is generated by the reduction of NO_3^- and is considered the major source of this cation, especially in C_3 plants (Hirel and Lea 2001). Photorespiration commences in the chloroplast with the oxygenase action of the bifunctional enzyme ribulose-1.5-bisphosphate carboxylase/oxygenase (Rubisco), which produces one molecule of glycerate-3-phosphate (3-PGA) and one molecule of glycolate-2-phosphate (2-PG). This 2-PG is hydrolyzed by phosphoglycolate phosphatase to glycolate that is transported to the peroxisome and oxidized to glyoxylate by glycolate oxidase (GO). Glyoxylate is transaminated to glycine by the reaction catalyzed by glutamate glyoxylate aminotransferase (GGAT) and is subsequently transported to the mitochondria. Subsequently, gly is transformed into ser by the action of the enzymes glycine decarboxylase and serine hydroxymethyltransferase. Ser formed in the mitochondria is transported to the peroxisome, where it is transformed by the action of serine:glyoxylate aminotransferase (SGAT) into hydroxypyruvate, which is reduced to glycerate by hydroxypyruvate reductase (HR). Finally, glycerate passes to the chloroplast, where it is phosphorylated by glycerate kinase to give a molecule of 3-PGA that will enter the Calvin cycle (Wingler and others 2000).

In the passage from glycine to serine, there is a release of CO_2 and NH_4^+, which may be phytotoxic for plants and must be incorporated by GS/GOGAT action into organic compounds. As a result of this simultaneous release and incorporation of NH_4^+, the GS/GOGAT cycle becomes a binding nexus between N metabolism and photorespiration. Furthermore, glutamate dehydrogenase (GDH0) is a very active enzyme in the presence of large amounts of NH_4^+ and constitutes another possible withdrawal pathway for this cation given that it catalyzes the reversible reaction of 2-oxoglutarate and NH_4^+ to glutamate. This double physiological function has recently stimulated considerable debate, because GDH has been found to have a low K_m for NH_4^+, indicating that it is improbable that it would act in favor of biosynthesis. This enzyme also participates in the assimilation of N in the presence of GS when the concentration of NH_4^+ is elevated (Harrison and others 2000; Frechilla and others 2002).

Photorespiration has been considered a wasteful process because around 25% of the fixed CO_2 is released during this metabolism (Wingler and others 2000; Igarashi and

others 2006). However, suppression of photorespiration has negative effects on plants, producing a decrease in the CO_2 assimilation rate, poor vegetable growth, and alterations in chloroplast structure (Shi-Wei and others 2007). Reported benefits of photorespiratory CO_2 are that it supplies substrates for other processes, provides protection against photoinhibition, and increases protection against different types of stress (Shi-Wei and others 2007).

Lettuce is being subjected to numerous iodine biofortification programs that may affect the physiology and nutritional value of the plant (Blasco and others 2008). Research is therefore required into the effect of these trace elements on the development of crops. The objective of the present study was to analyze nitrogen metabolism and photorespiration in plants subjected to iodine biofortification.

Materials and Methods

Plant Material and Growing Conditions

Seeds of *Lactuca sativa* L. var *longifolia* were germinated and grown for 35 days in cell flats (cell size = 3 cm × 3 cm × 10 cm) filled with perlite mixture, and the flats were placed on benches in an experimental greenhouse in southern Spain (Granada, Motril, Saliplant S.L.). The 35-day-old seedlings were transferred to a growth chamber under controlled environmental conditions with a relative humidity of 60-80%, temperature of 25/15°C (day/night) and 12/12-h photoperiod at a photosynthetic photon flux density (PPFD) of 350 μmol m^{-2} s^{-1} (measured at the top of plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). The plants were grown in individual 8-L pots (25 cm upper diameter, 17 cm lower diameter, 25 cm in height) filled with vermiculite. Throughout the experiment the plants received a growth solution composed of 4 mM $Ca(NO_3)_2$, 6 mM KNO_3, 2 mM $MgSO_4{\cdot}7H_2O$, 1 mM $NaH_2PO_4{\cdot}2H_2O$, 50 μM H_3BO_3, 2 μM $MnCl_2{\cdot}4H_2O$, 1 μM $ZnSO_4{\cdot}7H_2O$, 0.1 μM $Na_2MoO_4{\cdot}2H_2O$, 0.25 μM $CuSO_4{\cdot}5H_2O$, and 10 μM Fe-EDDHA. The nutrient solution (pH 5.5–6.0) was renewed every 3 days and the vermiculite was partly rinsed with Millipore-filtered water to avoid excessive nutrient accumulation.

At 45 days after germination, the different treatments were applied with the above nutrient solution. Treatments were I^- (20, 40, 80 μmol L^{-1} as KI) and IO_3^- (20, 40, 80 μmol L^{-1} as KIO_3), which were added to the growth solution and maintained for 21 days. The different application rates of iodine were selected as the most appropriate for a biofortification program with this trace element in lettuce plants (Blasco and others 2008). We also carried out a control treatment in which the complete growth solution was applied without an iodine supplement. We used a randomized complete block design with seven treatments, arranged in individual pots with six plants per treatment and three replications each. The experiment was repeated three times under the same conditions.

Plant Sampling

Edible lettuce leaves (12-17 leaves per plant) were sampled on day 66 after sowing. The plant material was rinsed three times in distilled water after disinfection with 1% nonionic detergent and then blotted on filter paper. A part of the plant material was used for the assay of fresh weight (FW), amino acids, proteins, ser, malate, and α-ketoglutaric acid and of NR, NiR, GS, GOGAT, aspartate aminotransferase (AAT), GO, GGAT, SGAT, HR, GDH, and catalase (CAT) enzymatic activities The rest of the plant material was lyophilized and used to determine dry weight (DW), NO_3^-, NH_4^+, and organic and total N.

Plant Analysis

Analysis of N Forms and Photorespiration Metabolites

NO_3^- was analyzed from an aqueous extraction of 0.2 g of DW in 10 ml of Millipore-filtered water. A 100-μl aliquot was taken for NO_3^- determination and added to 10% (w/v) salicylic acid in sulfuric acid at 96%, measuring the NO_3^- concentration by spectrophotometry as performed by Cataldo and others (1975).

NH_4^+ was analyzed from an aqueous extraction and was determined by using the colorimetric method described by Krom (1980).

For the organic-N determination, a sample of 0.1 g DW was digested with sulfuric acid and H_2O_2 (Wolf 1982). After dilution with deionized water, a 1-ml aliquot of the digest was added to the reaction medium containing buffer (5% potassium sodium tartrate, 100 μM sodium phosphate, and 5.4% w/v sodium hydroxide), 15%/0.03% (w/v) sodium silicate/sodium nitroprusside, and 5.35% (v/v) sodium hypochlorite. Samples were incubated at 37°C for 15 min, and organic N was measured by spectrophotometry according to the method of Baethgen and Alley (1989).

Total N concentration was assumed to represent the sum of organic N and NO_3^-.

Amino acids and proteins were determined by homogenization of 0.5 g FW in 50 mM cold KH_2PO_4 buffer at pH 7 and centrifugation at 12,000 *g* for 15 min. The resulting supernatant was used for the determination of total amino acids by the ninhydrin method (Yemm and Cocking 1955). Soluble proteins were measured with Bradford G-250 reagent (Bradford 1976).

Serine, malate, and α-ketoglutaric acid were analyzed at the University of California Davis Genome Center. The metabolite assay followed the method of Weckwerth and others (2004) and Fiehn (2006).

Enzyme Extractions and Assays

Leaves were ground in a mortar at 0°C in 50 mM KH_2PO_4 buffer (pH 7.5) containing 2 mM EDTA, 1.5% (w/v) soluble casein, 2 mM dithiothreitol (DTT), and 1% (w/v) insoluble polyvinylpolypyrrolidone. The homogenate was filtered and then centrifuged at 30,000 *g* for 20 min. The resulting extract (cytosol and organelle fractions) was used to measure enzyme activity of NR, NiR, GOGAT, and GDH. The extraction medium was optimized for these enzyme activities so that they could be extracted together according to the same method (Groat and Vance 1981; Kaiser and Lewis 1984; Lillo 1984; Singh and Srivastava 1986).

The NR assay followed the methodology of Kaiser and Lewis (1984).The NO_2^- formed was colorimetrically determined at 540 nm after azocoupling with sulfanilamide and naphthylethylenediamine dihydrochloride according to the method of Hageman and Hucklesby (1971).

NiR activity was defined by the disappearance of NO_2^- from the reaction medium (Lillo 1984). After incubation at 30°C for 30 min, the NO_2^- content was determined colorimetrically as above.

GOGAT activity was assayed spectrophotometrically at 30°C by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance (1981) and Singh and Srivastava (1986), always within 2 h of extraction. Two controls, without ketoglutarate and glutamine, respectively, were used to correct for endogenous NADH oxidation. The decrease in absorbance was recorded for 5 min.

GDH activity was assayed by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance (1981) and Singh and Srivastava (1986). The reaction mixture consisted of 50 mM KH_2PO_4 buffer (pH 7.5) with 200 mM NH_4^+ sulfate, 0.15 mM NADH, 2.5 mM 2-oxoglutarate, and enzyme extract. Two controls, without ketoglutarate and NH_4^+ sulfate, respectively, were used to correct for endogenous NADH oxidation. The decrease in absorbance was recorded for 3 min.

GS was determined by an adaptation of the hydroxamate synthetase assay published by Kaiser and Lewis (1984). Leaves were ground in a mortar at 0°C in 50 ml maleic acid-KOH buffer (pH 6.8) containing 100 mM sucrose, 2% (v/v) β-mercaptoethanol, and 20% (v/v) ethylene glycol. The homogenate was centrifuged at 30,000 *g* for 20 min. The resulting extract was used to measure enzyme activity of GS. The reaction mixture used in the GS assay was composed of 100 mM KH_2PO_4 buffer (pH 7.5) with 4 mM EDTA, 1000 mM L-sodium glutamate, 450 mM $MgSO_4 \cdot 7H_2O$, 300 mM hydroxylamine, 100 mM ATP, and enzyme extract. Two controls were prepared, one without glutamine and the other without hydroxylamine. After incubation at 28°C for 30 min, the formation of glutamylhydroxamate was colorimetrically determined at 540 nm after complexing with acidified ferric chloride (Wallsgrove and others 1979).

AAT activity was assayed spectrophotometrically at 340 nm using the method published by Gonzalez and others (1995). AAT enzyme was extracted in identical conditions to GS. The reaction mixture consisted of 50 mM Tris–HCl buffer (pH 8), 4 mM $MgCl_2$, 10 mM aspartic acid, and enzyme extract. The decrease in absorbance was recorded for 3 min.

For the GO determination, fresh leaf tissue (0.25 g) was ground in a chilled mortar with PVPP and 1 ml of 50 mM Tris–HCl buffer (pH 7.8) with 0.01% Triton X-100 and 5 mm dithiotreithol (DTT). The homogenate was centrifuged at 30,000 *g* for 20 min. The supernatant was decanted and immediately used for the enzyme assay. GO was assayed as described by Feierabend and Beevers (1972) with modifications. A volume of assay mixture containing 50 mM Tris–HCl buffer (pH 7.8), 0.009% Triton X-100, 3.3 mM phenylhydrazine HCl (pH 6.8), 50 µl plant extract, and 5 mM glycolic acid (neutralized to pH 7 with KOH) was used to start the reaction. GO activity was determined by following the formation of glyoxylate phenylhydrazone at 324 nm for 2 min after an initial lag phase of 1 min.

For determination of GGAT, SGAT, and HR, leaves were ground in a chilled mortar in 100 mM Tris–HCl buffer (pH 7.3) containing 0.1% (v/v) Triton X-100 and 10 mM DTT. The homogenate was centrifuged at 20,000 *g* for 10 min. The resulting extract was used to measure enzyme activity. The extraction medium was optimized for the enzyme activities such that they could be extracted together using the same method (Hoder and Rej 1983).

GGAT activity was measured by coupling the reduction of 2-oxoglutarate by NADH in a reaction catalyzed by GDH. The reaction was assayed in a mixture containing 100 mM Tris–HCl (pH 7.3), 20 mM glutamate, 1 mM glyoxylate, 0.18 mM NADH, 0.11 mM pyridoxal-5-phosphate, 83 mM NH_4Cl, and 0.3 U GDH in a final volume of 0.6 ml (Igarashi and others 2006).

SGAT activity was measured by determining the rate of glycine formation from glyoxylate. The reaction was assayed in a mixture containing 100 mM Tris–HCl (pH 7.3), 5 mM serine, 1 mM glyoxylate, 0.11 mM pyridoxal-5-phosphate, 83 mM NH_4Cl, and 0.3 U GDH in a final volume of 0.6 ml. After 20 min of incubation, the reaction was terminated by adding four volumes of absolute ethanol and heating at 80°C. The rate of glycine was determined by the method of Yemm and Cocking (1955).

HR assay was performed with 100 mM Tris–HCl (pH 7.3), 5 mM hydroxypyruvate, and 0.18 mM NADH. Activity was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm (Hoder and Rej 1983).

CAT activity was determined by following the consumption of H_2O_2 at 240 nm for 5 min (Rao and others 1997). The reaction mixture (3 ml total volume) contained 25 mM Tris–acetate buffer (pH 7.0), 0.8 mM EDTA-Na, and 20 mM H_2O_2, and the enzyme assay was performed at 25°C.

Statistical Analysis

Data were subjected to a simple ANOVA at 95% confidence, using the Statgraphics 6.1 program. A two-tailed ANOVA was applied to ascertain whether the iodine application rate and forms applied significantly affected the results, and the means were compared by Fisher's least-significant differences (LSD). The significance levels for both analyses were expressed as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, or NS (not significant).

Results and Discussion

NH_4^+ Production: Reduction of NO_3^- and Photorespiration

N is the most abundant element in vegetable tissues, representing 2% of the dry weight of the plant. It can pass to the trophic chain largely in the form of NO_3^- and NH_4^+ (Miller and Cramer 2004), factors with a major effect on the quality, growth, and development of lettuce plants (Abu-Rayyan and others 2004; Niu and others 2007). One of the main constraints to the assimilation of NO_3^- is NR activity. After performing its catalytic function and producing NO_2^-, which is highly toxic for plants, the action of NiR gives rise to the formation of NH_4^+. Table 1 shows how crops respond in a different manner to the application of iodine according to the form used. Treatments with I^- at doses of at least 20 μM produce a decrease in NO_3^- concentration and NR activity (Table 1). Furthermore, the application of IO_3^- had no effect on the foliar accumulation of NO_3^-; however, IO_3^- treatments increased NR activity, especially at 20 μM (Table 1). The iodine applications had no effect on NiR activity (Table 1). According to our results, the application of I^- may reduce the absorption of NO_3^- because it decreases both the concentration of this anion and NR activity, more markedly in treatments with 80 μM (Table 1). Treatments with IO_3^- induced a larger reduction of NO_3^- by an increase of NR (Table 1), although this was not reflected in the foliar concentration of this anion, which remained constant. This may be because IO_3^- also would induce a larger absorption of NO_3^-, which stimulates NR activity and therefore NO_3^- utilization. This may explain why the levels of NO_3^- in the different IO_3^- treatments are similar to those of control plants (Table 1). Likewise, the enzyme NiR was not affected by any of the treatments, possibly because it is not a constitutive enzyme that modifies its degree of expression according to the levels of NO_2^- in the medium (Heldt 2005).

In general, plants do not tend to accumulate large amounts of NH_4^+, which may cause toxicity symptoms such as chlorosis of mature leaves, leaf roll, and the appearance of necrotic areas. These symptoms are possibly due to problems with pH balance, imbalance of the cation:anion ratio, and/or energy exhaustion due to the efflux of the ion (Lea and Azevedo 2006). In the present experiment, iodine application had no significant effect on the foliar concentration of NH_4^+ in lettuce plants (Table 1).

NH_4^+ not only originates from the reduction of NO_3^- but also is generated when there are high photorespiration rates due to the oxidation of glycine (Hirel and Lea 2001). GO acts first by forming glyoxylate. In this step, electrons are transferred to O_2, giving rise to the formation of H_2O_2 that is subsequently detoxified by CAT. In our experiment, GO activity was affected only in plants treated with 20 μM of IO_3^-, observing a significant increase with respect to control plants (Table 2). However, CAT activity significantly increased after the application of either form of

Table 1 Response of NO_3^- reduction and NH_4^+ concentration in lettuce leaves submitted to different application rates and forms of iodine

Treatments	NO_3^-		NR		NiR		NH_4^+	
	I^-	IO_3^-	I^-	IO_3^-	I^-	IO_3^-	I^-	IO_3^-
Doses								
0	89.2	89.2	16.3	16.3	1.62	1.62	2.31	2.31
20	58.0	81.6	12.9	29.5	1.45	3.71	2.07	2.05
40	56.7	88.2	9.71	24.7	1.06	3.34	2.23	2.19
80	52.0	93.1	9.31	24.3	1.44	3.43	2.26	2.29
P value	***	NS	*	**	NS	NS	NS	NS
LSD	4.89	10.9	6.46	3.52	1.95	2.68	0.29	0.28
Analysis of variance								
Doses (D)	***		*		NS		NS	
Form (F)	***		***		*		NS	
D × F	***		**		*		NS	
LSD	5.53		3.38		1.52		0.19	

NO_3^- and NH_4^+ are expressed as mg g^{-1} DW. Nitrate reductase (NR) is expressed as mM NO_2^- mg $prot^{-1}$ min^{-1}. Nitrite reductase (NiR) is expressed as mM NO_2^- mg $prot^{-1}$ min^{-1}

The levels of significance are represented by $P > 0.05$: NS (not significant); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 2 Response of some photorespiration enzymes in lettuce leaves submitted to different application rates and forms of iodine

Treatments	GO		CAT		GGAT		SGAT		HR	
	I^-	IO_3^-	I^-	IO_3^-	I^-	IO_3^-	I^-	IO_3^-	I^-	IO_3^-
Doses										
0	14.0	14.0	0.015	0.015	0.17	0.17	69.5	69.5	53.7	53.7
20	10.9	18.9	0.037	0.027	0.20	0.22	65.6	72.5	28.7	52.5
40	12.0	12.1	0.026	0.027	0.59	0.44	65.7	65.5	38.4	53.2
80	13.1	14.1	0.027	0.051	0.42	0.34	64.9	70.6	45.1	61.4
P value	NS	***	*	**	***	*	NS	NS	***	**
LSD	3.12	1.93	0.010	0.009	0.14	0.15	10.2	6.24	8.23	3.55
Analysis of variance										
Doses (D)	***		***		***		NS		***	
Form (F)	***		NS		NS		NS		***	
D × F	***		**		*		NS		***	
LSD	1.06		0.007		0.09		5.50		4.12	

Glycolate oxidase (GO), catalase (CAT), glutamate:glyoxylate aminotransferase (GGAT), and hydroxypyruvate reductase (HR) activities are expressed as Δ OD mg $prot^{-1}$ min^{-1}. Serine:glyoxylate aminotransferase (SGAT) activity is expressed as μM glycine mg $prot^{-1}$ seg^{-1}

The levels of significance are represented by $P > 0.05$: NS (not significant); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

iodine in all treatments and was slightly higher when applying 80 μM of IO_3^- (Table 2). The increase in CAT activity without an increase in GO activity after applying I^- may be due to the continuous generation of H_2O_2 from various sources during normal metabolism in plant cells. Thus, this compound is formed in the electron transport process during photosynthesis and respiration and in the conversion of fatty acids to succinate, and it is even spontaneously formed by the reactive oxygen species $O_2^{\cdot-}$ and $HO_2^{\cdot}$ (Laloi and others 2004; Li and others 2009).

GGAT is responsible for the formation of glycine, an amino acid that is subsequently oxidized in the internal membrane of the mitochondria, releasing CO_2, large amounts of NH_4^+, and ser. GGAT activity was reported to show an even closer direct proportional relationship with the amount of ser than with glycine, its direct final product (Igarashi and others 2006). As shown in Table 2, the application of both forms of iodine at doses of at least 40 μM increased GGAT activity and produced a three- to fourfold increase in ser with respect to control plants (Fig. 1, $P < 0.001$). SGAT is found "in excess" under normal growth conditions, implying that this enzyme may exert an appreciable control over photorespiration under stress conditions (Wingler and others 2000). The hydroxypyruvate formed after its action is transformed by HR into glyceric acid, which forms part of the Calvin cycle as 3-phosphoglyceric acid, hence completing the photorespiratory oxidation cycle of carbon. The present data showed that iodine application had no significant effect on SGAT with respect to control plants (Table 2). In contrast, treatments with I^- decreased HR activity (Table 2), whereas the application of IO_3^- only increased the HR activity at 80 μM with the consequent formation of glyceric acid (Table 2).

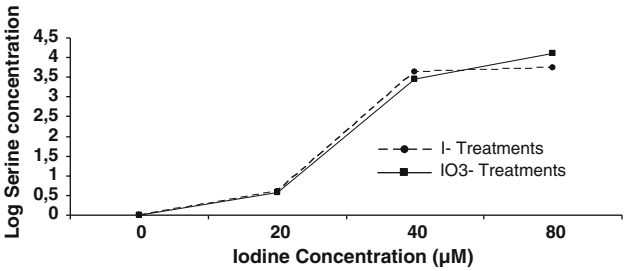

Fig. 1 Effect of different application rates of I^- and IO_3^- on the foliar log serine concentration

Incorporation of NH_4^+ and Assimilation Products

GS/GOGAT constitutes the main assimilation and reassimilation pathway of NH_4^+. In this experiment, treatments with I^- and IO_3^- produced a significant increase in GS activity (Table 3), whereas iodine application had no significant effect on GOGAT activity (Table 3). After IO_3^- treatment, the induction of GS may explain the absence of an increase in the concentration of NH_4^+ despite high NO_3^- reduction pathway activity and photorespiration (Tables 1, 2), because NH_4 is withdrawn and incorporated into glutamate via GS/GOGAT. It was previously reported that plants with high GS activity are more tolerant to NH_4^+ (Cruz and others 2006). The availability of carbonated skeletons in the form of α-ketoglutarate is essential for NH_4^+ assimilation and the correct functioning of GOGAT. This α-ketoglutarate originates largely in the respiration of sugars and the transamination reactions of amino acids

Table 3 Response of enzymes responsible for NH_4^+ assimilation in lettuce leaves submitted to different application rates and forms of iodine

Treatments	GS		GOGAT		AAT	
	I^-	IO_3^-	I^-	IO_3^-	I^-	IO_3^-
Doses						
0	16.3	16.3	4.66	4.66	1.35	1.35
20	28.2	18.3	3.42	3.62	2.95	3.36
40	27.9	19.1	5.49	3.63	4.56	2.28
80	24.5	20.0	6.50	5.89	7.58	1.78
P value	*	**	NS	NS	***	**
LSD	5.90	1.10	3.51	2.56	0.57	0.77
Analysis of variance						
Doses (D)	***		NS		***	
Form (F)	***		NS		***	
D × F	**		NS		***	
LSD	2.88		1.99		0.44	

Glutamine synthetase (GS) is expressed as μM glutamylhydroxamate mg $prot^{-1}$ min^{-1}. Glutamate synthase (GOGAT) and aspartate aminotransferase (AAT) are expressed as Δ OD mg $prot^{-1}$ min^{-1}

The levels of significance are represented by $P > 0.05$: NS (not significant); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

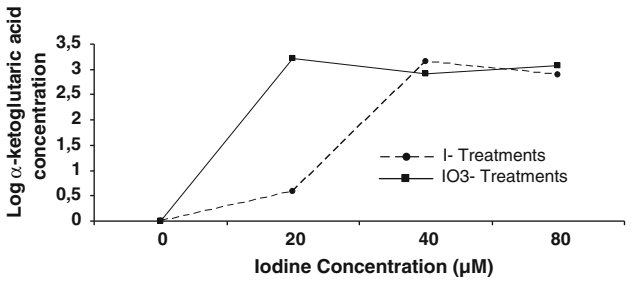

Fig. 2 Effect of different application rates of I^- and IO_3^- on the foliar log α-ketoglutaric acid concentration

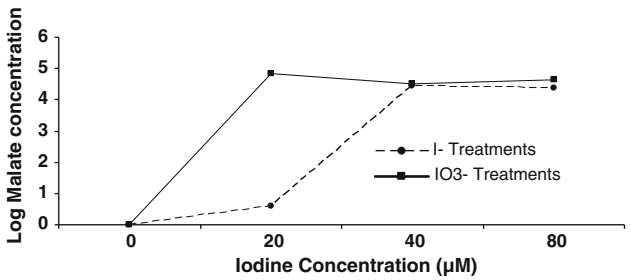

Fig. 3 Effect of different application rates of I^- and IO_3^- on the foliar log malate concentration

(Hodges 2002). In our experiments, the concentration of α-ketoglutarate was two- to threefold higher in plants treated with 40 or 80 μM of I^- (Fig. 2, $P < 0.001$) and two- to threefold higher in plants treated with any dose of IO_3^- (Fig. 2, $P < 0.001$) than in control plants.

α-Ketoglutarate is produced in numerous cell compartments and is transported into the chloroplast for incorporation into the GS/GOGAT cycle by antiport with malate (Hodges 2002). The malate concentration was fourfold higher in plants treated with at least 40 μM of I^- (Fig. 3, $P < 0.001$) and fourfold higher in plants treated with any dose of IO_3^- (Fig. 3, $P < 0.001$) than in control plants. In short, we observed a parallel increase of malate and α-ketoglutarate in all iodine-treated plants.

AAT catalyzes the transformation of glutamate and oxalacetate into aspartate and α-ketoglutarate. Therefore, besides generating transport amino acids to remobilize N, it is also a source of carbonated skeletons for the GS/GOGAT cycle. A significant increase in AAT activity was observed after all I^- treatments, which was much more marked at a dose of 80 μM, and after treatment with 20 μM IO_3^- (Table 3). GDH catalyzes the reversible reaction in which α-ketoglutarate and NH_4^+ can produce glutamate or glutamate can be recycled to its two constituting molecules. GDH has been reported to confer resistance against different types of stress, and it is particularly active in the presence of large amounts of NH_4^+ in the cell (Cruz and others 2006). GDH activity depends on the form of iodine applied, increasing after treatment with I^-, with the highest value at a dose of 80 μM (Fig. 4, $P < 0.001$), but decreasing after treatment with IO_3^- at doses of at least 40 μM (Fig. 4, $P < 0.01$). I^- is the most bioavailable and phytotoxic form of iodine for plants, and the increase in GDH activity found with its use supports its protective role against stress.

The result of the incorporation of NH_4^+ can be quantified by analyzing organic N, which is generally a product of NO_3^- assimilation and is formed mainly by amino acids and structural, functional, and storage proteins. Total N, the result of adding NO_3^- to organic N, is considered a critical parameter for determining the nutritional status of N of plants (Ruiz and Romero 1999). A significant reduction in organic N was observed only after treatment with I^-, when the dose was at least 40 μM (Fig. 5, $P < 0.05$), whereas a

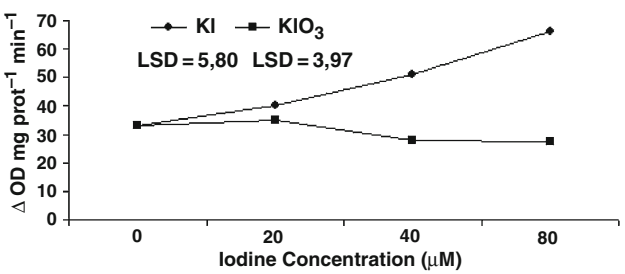

Fig. 4 Effect of different application rates of I^- and IO_3^- on the foliar activity of GDH

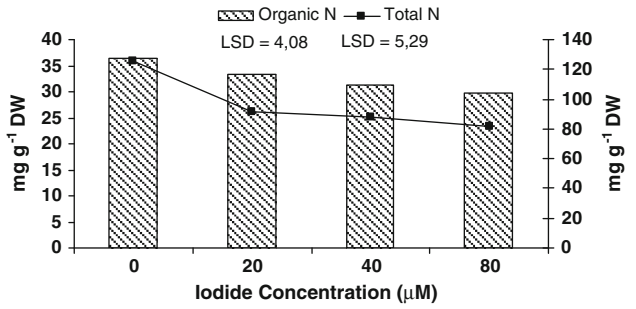

Fig. 5 Effect of different application rates of I^- on the foliar concentration of organic and total N

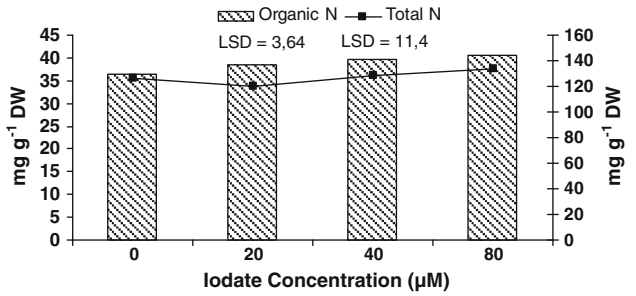

Fig. 6 Effect of different application rates of IO_3^- on the foliar concentration of organic and total N

significant reduction in total N was observed after all I^- treatments, with the lowest value at 80 μM (Fig. 5, $P < 0.001$). In contrast, no IO_3^- treatment had a significant effect on organic N or total N (Fig. 6, $P > 0.05$). The decrease in total N with all I^- is an expected result because the NO_3^- concentration decreased in parallel with total N, and total N results from the addition of organic and inorganic forms of N.

In general, nitrogen compounds with high and low molecular weight (for example, amino acids and proteins) are the main product of NO_3^- assimilation (Barneix and Causin 1996). More precisely, they result from the assimilation of NH_4^+, regardless of the metabolic process from which the nitrogen is derived. The amino acid concentration was not affected by iodine application, and the protein concentration significantly increased only with IO_3^- treatments reaching the highest value at a dose of 80 μM (Table 4). Finally, the availability and utilization of N play an essential role in crop production, especially in lettuce plants, whose agricultural value depends on the growth of the aerial part. Biomass of the aerial part was lower when 80 μM of I^- was applied but significantly higher versus control plants when IO_3^- was used; the highest biomass was at a dose of 20 μM (Table 4). These data are consistent with the finding by Mackoviak and Grossl (1999) of lower shoot biomass in rice at I^- concentrations of at least 10 μM. Likewise, Zhu and others (2003) observed a direct relationship in spinach plants between higher I^- doses and

Table 4 Concentrations of amino acids and proteins and biomass production in lettuce leaves submitted to different application rates and forms of iodine

	Amino acids		Proteins		Biomass	
Treatments	I^-	IO_3^-	I^-	IO_3^-	I^-	IO_3^-
Doses						
0	0.25	0.25	9.35	9.35	3.02	3.02
20	0.28	0.25	9.50	10.8	2.85	3.75
40	0.23	0.22	9.11	10.6	2.98	3.55
80	0.25	0.28	9.76	13.8	2.34	3.37
P value	NS	NS	NS	***	***	*
LSD	0.03	0.03	0.54	1.24	0.21	0.35
Analysis of variance						
Doses (D)	*		***		***	
Form (F)	NS		***		***	
D × F	NS		***		***	
LSD	0.02		0.44		0.26	

Amino acids and proteins are expressed as mg g^{-1} FW. Biomass is expressed as g DW edible leaves

The levels of significance are represented by $P > 0.05$: NS (not significant); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

lower foliar biomass values, and Dai and others (2004) found that the IO_3^- concentration had a significant effect on the biomass of edible parts of bok-choi and spinach.

The effect of iodine appears to depend largely on the nature of the crop (Ehling 1928). I^- may exert a phytotoxic effect on the growth of lettuce plants due to an excessive accumulation of this trace element in the plant tissues (Blasco and others 2008) or due to intracellular oxidation to I_2 after uptake, which can inhibit the photosynthetic process (Mynet and Wain 1973). In this stress situation, which would be most intense after the treatments with 80 μM I^-, the lettuce plant may respond with a remobilization of amino acids toward young parts, as indicated by the increase in AAT activity (Table 3). These transport amino acids are subsequently loaded into the phloem, which would explain the absence of an increased amino acid concentration in the mature leaves in our experiment (Table 4). Likewise, treatments with I^-, especially at the 80-μM dose, produced a reduction in the concentration of organic N (Fig. 5) with no decrease in the protein (Table 4). One explanation may be that the N source for synthesis of these amino acids is not protein degradation and may be photorespiration via an increase in GGAT (Table 2), or even GDH activity (Fig. 4). NH_4^+ generated by these metabolic pathways would be rapidly assimilated due to the increased GS activity in these plants (Table 3). In addition, the decrease in HR activity impedes the integration of carbonated skeletons from photorespiration into the Calvin cycle, which may, alongside inhibition of NR activity, be a mechanism by which I^- exerts its phytotoxic effect on the plants.

IO_3^- increases the biomass, partly because this chemical form does not affect the nitrogen status of the crop (NO_3^-, organic N, and total N) (Table 1; Fig. 6). At the same time, there is an increase in the NO_3^- reduction pathway (Table 1) and a slight increase in GGAT activity (Table 2) with a decrease in GDH (Fig. 3). Consequently, a large amount of NH_4^+ is generated and rapidly incorporated into a glutamate molecule by GS, thereby avoiding an excessive accumulation of this cation (Table 1). This high ammonium assimilation may be due to the synthesis of transport amino acids to young parts, as indicated by the increase in AAT activity in 20 and 40 μM IO_3^- (Table 3) as well as to the synthesis of proteins in mature leaves, especially at the 80-μM dose of IO_3^- (Table 4). Treatments with IO_3^- also increased HR activity (Table 2), allowing a correct vegetable physical state. These findings suggest that HR may be a possible sensitive point of iodine application.

In conclusion, crop behavior differed according to the iodine form applied. Application of I^- at doses of at least 40 μM inhibited NO_3^- absorption without affecting biomass production. The application of 80 μM I^- produced stress in the plants which reduced their biomass and nitrogen status. This dose has a severe phytotoxic effect on lettuce plants, mainly due to a decrease in NR and HR activity. In contrast, the application of IO_3^- increased the growth of the edible part by stimulating NR activity, mostly at 20 μM, and the GS/GOGAT cycle, mainly at doses of at least 40 μM. In addition, the application of IO_3^- increases and optimizes the photorespiratory process. This is may be of major agronomic interest because it would allow a reduction in the use of nitrogen fertilizers and the costs and other effects of their excessive application.

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