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NITRATE METABOLISM IN LETTUCE AND ROCKET

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NOME DEL DOTTORANDO
Nadia Podetta
Matr. num. R09143

NOME E COGNOME DEL TUTOR
Dr. Antonio Ferrante

NOME E COGNOME DEL COORDINATORE DEL DOTTORATO
Prof. Piero Attilio Bianco

A.A.
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1. Abstract

Intensive farming techniques with highly fertilization management lead to produce leafy vegetables with high accumulation of nitrates. The Reg. UE 1258/2011 imposes threshold on nitrate content beyond which the vegetables (lettuce, rocket and spinach) cannot be placed on the market. It is so important to adopt strategies which reduce nitrate levels in leaves. This objective can be achieved through the studies on the metabolism of nitrates in model and greatest commercial interest species. This study focuses on cultivation in floating system because was shown that this cultivation technique has the ability to reduce the intake of macronutrients in the nutrient solution, making more efficient the assimilation of nitrogen by the plants and so reducing the level of nitrates in vegetables (Rouphael et al., 2004). Two leafy vegetables were chosen as study-species, lettuce (*Lactuca sativa* L.) and rocket (*Diplotaxis tenuifolia* L.) that have different efficiency of use and organication of nitrates. Some researchers have found levels of nitrate in *Diplotaxis* higher than 9300 $mg*kg^{-1}$ FW (Santamaria et al., 1999; Cerutti et al., 1996), in fact rocket it is considerate a hyper accumulator of nitrates. This research focuses on comparative biology studies between the two species and on their nitrate metabolism to understand which are the factors that make the difference in the nitrate accumulation. Nitrate reductase, NR, (E.C.1.7.1.1-3) is the first enzyme from which start NO_3^- organication. Concentration in nitrates in the nutrient solution and other abiotic factors like light intensity and exposure, diurnal alternation of light and dark, temperature, CO_2 levels, hormones (cytokinin, ethylene) (Dordas, 2009), anoxia, availability of sugars and nitrogen metabolites such as glutamine all play regulatory roles in NR activity (Crawford, 1995). The growing experiments performed for this thesis were planned to characterize the nitrate metabolism in lettuce and rocket plants grown in nutrient solutions containing different nitrate concentrations and under different light exposure. To achieve this objective the plants were cultivated, on the one hand, in conditions very similar to reals one, in greenhouse and with nutrient solution containing 2, 10 and 20 mM NO_3^-, but also with nutrient solution with low nitrate concentrations, 0.25, 0.5, 1 and 2 mM NO_3^-, gave after 1 day of nitrogen starvation, to highlight the high sensitiveness of nitrate transporters, and consequently on nitrate reductase, at small differences of nitrate in the nutrient solution. On the other hand the cultivations were carried out in a growth chamber in order to eliminate the influence on the results of some environmental parameters, which are difficult to evaluate in an uncontrolled environment and the aforesaid low nitrate concentrations were tested.

The qualitative parameters, as content of chlorophylls, carotenoids, nitrates, nitrites, sucrose, reducing and total sugars were determinate to understand the status of the plants sampled at different environmental conditions and to evaluate how the different concentration of nitrates in the nutrient solutions affect these parameters. Then the activity of NR was measured using two different assays to understand the response of this enzyme at the different conditions used and to be correlated with the qualitative parameters, in order to deepen the mechanisms that affect the first step of reduction of the nitrates and, consequently, their organication to glutamate and amino acids.

The study was integrated with the gene expression analysis for the main enzymes involved in the nitrate metabolism of lettuce and rocket: nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamate synthase and nitrate transporter (NTR). These analysis were performed using quantitative retro-transcriptase PCR (qRT-PCR) to study the transcriptional regulation under the different nitrate concentrations availability.

2. Introduction

Leafy vegetables ready to use represent one the most growing share of the entire fruit and vegetables market; in Italy in the last 10 years, to the end of the 2012, ISMEA registered an increase in consumption of the 380% despite prices much higher than the traditional fresh.

	Volume of purchases		Expenditure		Average price	
	% Variation 2012/2011	% Variation Jan-Aug 2013/Jan-Aug 2012	% Variation 2012/2011	% Variation Jan-Aug 2013/Jan-Aug 2012	% Variation 2012/2011	% Variaton Jan-Aug 2013/Jan-Aug 2012
Ready to eat vegetables	14.8	- 3.5	11.0	- 7.5	- 3.3	- 4.2
Salads	14.4	- 4.5	10.6	- 8.4	- 3.3	- 4.0
Monovarietals salads	10.1	- 4.5	7.8	- 6.3	- 2.1	- 1.9
Mixed salads	17.7	- 4.6	13.0	- 9.7	- 4.0	- 5.4
Other vegetables	16.0	-7.0	11.2	- 11.5	- 4.1	- 4.8
Other vegetables to be cooked	15.7	2.8	13.7	0.9	- 1.7	- 1.8

Tab. 2.1: Recent dynamics of the purchases of the ready-to-eat vegetables. Data from ISMEA 2013.

The current crisis of consumption is lead to declines in sales in this area, less 3.5% of the volume purchased from the same period of 2012 and a decrease in expenditure of 7.5%, due to a concomitant reduction in average retail prices (-4.2%), however the market for this type of products remains active. (ISMEA -Istituto di servizi per il mercato agricolo alimentare - 2013).

In Italy the cultivation of leafy vegetables for the ready to eat market are in open field and in greenhouse.

Geographical areas	Cultivation area (ha)	Harvest (t)
North	972.42	30262.5
Center	1427.25	43178.1
South	1666.80	58396.8
ITALY	**4066.47**	**131837.4**

Tab. 2.2: Surface of cultivation and production of lettuce in different Italian geographical areas. Data from ISTAT, 2012.

One of the most debated topics linked to this kind of production is the nitrate question.

In fact high nitrate content in edible vegetables affect human health and quality of the products besides that pollute the environment.

Nitrates, nitrite and N-nitrosocompounds are mainly derived in mammals from external sources, generally drink and food, but derivates of this compounds are also present after endogenous formations (Santamaria, 2006), as demonstrated by Ohshima and Bartsch in 1981. In fact NO thanks to the NO synthase (NOS), is produced starting from L-arginine, which is oxidates to have L-citrulline and NO (Stuehr, 1999). In plants nitrite can be converted in nitric oxide (NO) with witch NR, using O_2, forms a toxic peroxynitrite ($ONOO^-$).

The intake of nitrates depend from the dietary habits and the method of preparation of food, for example boiling reduce nitrates content since they goes towards the boiling water during cooking (Rutkwoska et al., 1993). Nitrates derived from vegetables can reach the 85%, generally vary between 60 and 80% of the total intake

(Hmelak Gorenjak, 2012), the 15-20% of nitrates is introduced with drinking water and conserved processed meat (10-15%), (Weitzberg. and Lundberg, 2013) where nitrates and nitrites are used, often in association with antioxidants like ascorbic acid, as additives and preservatives to enhance taste and appearance of products (Skibsted, 2011). Nitrite acts fixing colors, amends flavors, inhibit microorganisms and the oxidation of lipids controlling rancidity (Sindelar and Milkowski, 2012).

Different parts of the plants show different accumulation capacity of nitrates and petioles and leaves are the parts with greater accumulation of nitrates, since many nitrates are contained in vacuoles. Then we found stems, roots, inflorescences, tubers, bulbs, fruits and seeds (Santamaria, 2006).

			Nitrate concentration (mg/kg)		
Vegetable group	Examples	Sample size	Median	Mean	Range (P5-P95)
Leafy	Spinach Lettuce Rocket Beet	25306	1140	1614	66-4556
Herb	Basil Parsley Dill Chives	492	791	1240	10-4040
Stem	Asparagus Fennel Celery Rhubarb	1379	302	698	3-2923
Root and tuber	Potato Beetroots Carrot Celeriac	7579	152	506	15-2302
Brassica	Broccoli Cabbage Kale Cauliflower	3192	241	279	7-758
Legume	Beans Peas String beans	882	56	221	1-748
Bulb	Garlic Onions	243	60	159	1-601
Fruiting	Cucumber Tomato Aubergine Pumpkin	2822	83	149	1-486
Fungi	Mushroom	12	41	59	31-100

Table 2.3: Nitrate concentrations in vegetables. From Weitzberg and Lundberg, 2013. *Eur. Food Saf. Auth.* 2008. Nitrate in vegetables: scientific opinion of the Panel on Contaminants in the Food Chain. *EFSA J.* 689: 1–79.

Different species have different accumulation ability; rocket, *Diplotaxis tenuifolia,* belong to Brassicaceae family, show the higher accumulation of nitrates reaching up to 9300 $mg*kg^{-1}$, content almost twice the statutory limit allowed.

European regulation, Reg. UE 1258/2011 (amending Reg. CE 1881/2006), impose maximum thresholds of nitrates in some leafy vegetables beyond which the vegetables cannot be commercialized, in order to overcome the differences among the limits of the different member states, facilitating trade, and to prevent diseases ascribed by nitrosation of nitrates. This thresholds change depending primarily on the species, then on seasonality of production, the cultivation and finally vary for the fresh and the preserved greens. The methods of cultivation affect the nitrate contents; hydroponic systems can reduce nitrate accumulation in vegetables (Santamaria et al., 2002). Usually the rocket plants grown in floating system have higher yield and lower nitrate compared to those cultivated in soil (Ferrante et al., 2003). Latitude also affect nitrate in leaves: generally the problem of nitrates is great in north Europe lands compared with Mediterranean states.

About 5% of nitrate of food is converted in the mouth, from the oral bacteria, to nitrite that accumulates in the saliva and, whit the hydrochloric acid secreted by the parietal cell of the stomach, they are protonated to form nitrous acid. Nitrous acid can spontaneously turn in many nitrogen oxides like nitric oxide, nitrogen dioxide and dinitrogen trioxide that will have different destinies, reacting directly as signals, or binding to other metabolites present forming many different molecules like ethyl nitrite, S-nitrosothiols, N-nitrosamines, nitroalkenes that are absorbed and have different systemic effects in the human body (Weitzberg and Lundberg, 2013).

Nitrite can reacts with haemoglobin to form methaemoglobin and nitrate. Methaemoglobin entails a reduced efficiency of oxygen transport in the blood, causing methaemoglobinaemia or “blue baby syndrome” when the ratio of methaemoglobin is 10% of the normal haemoglobin. This disease is particularly dangerous for infants up to 3 months of age, but can also affect children and adults, led to cyanosis and then to suffocation.

N-nitrocompounds, especially N-nitrosamine, are considered carcinogenic since the first studies started in 1956 by Magee and Barnes in rats. N-nitrosodiethylamine and N-nitrosodimethylamine would be associated with a higher risk of gastroesophageal cancer (Jakszyn and González, 2006). Deiana et al. (1999) show how compounds derived from NO can cause mutagenesis. However, recent studies reject a direct correlation between nitrate content in food and cancer incidence, deepening rather positive aspects of nitrogen compounds on the cardiovascular and immune system (Weitzberg and Lundberg, 2013). European Food and Safety Authority stated that the advantages of vegetables intake outweigh the disadvantages and that it is unlikely that such an assumption poses any risk to health.

Nitrosating compounds formation can be inhibit by antioxidants like vitamins E and C because they promote the transformation of nitrite in NO. This mechanism explains why vegetables, which are high in nitrates and antioxidants, are not associated with cancer (Weitzberg and Lundberg, 2013).

The content of nitrates in vegetables depends by a lot of biotic and abiotic parameters, sunlight and nitrate supply during cultivation are the most important of them (Dapoigniy et al., 2000). For this reason this report focuses on the effects of light and nitrate uptake on the nitrate metabolism in two widespread leafy species: lettuce and rocket.

Some studies show how lettuce generally has low content of nitrates at harvest, while rocket is a hyper accumulator of nitrates: this work want to compare and deepen this physiological behaviour and connect-it to aforesaid environmental parameters through physiological studies and expression gene analyses.

Nitrate metabolism

Nitrates are uptake by plants from the nutrient solution principally by roots but also by leaves. Nitrates are actively transported through the plasma membrane of the epidermal and cortical cells of the roots across the proton symporters (NO_3^-:$2H^+$) or Cl^- canal ($2NO_3^-$:H^+); this active action exploits the driving force of

transmembrane different potential, create thanks the ATP hydrolysis by H^+ATP*ase* of the plasma membrane. The transport is regulated by a large family of nitrate transporters (NTR) well characterized in *Arabidopsis*: saturable high affinity transporters (HATS) and non-saturing low affinity transporters (LATS).

HATS work when the concentration of nitrates in the nutrients solution are between 0,2 and 0,5 mM and are constitutively expressed (cHATS), or inducible (iHATS) by the presence of nitrates and shown rapid response at different changes in nitrates concentration (Forde, 2000). LATS work when the concentrations of nitrates are higher than 1 mM and are constitutively expressed.

In *Arabidopsis* was demonstrating that cHATS and cLATS are encoded by *At*NRT1.1 and *At*NRT1.2 genes, while iHATS are encoded only by *At*NRT2.1. (Forde, 2000) belong to the major facilitator superfamily (MFS).

NRT1 transport nitrate, histidine, and nitrite, belonging to the subgroup of nitrate/nitrite transporters (Pao, 1998); NRT2 transport peptides, amino acids, nitrate, chlorate, and nitrite and belongs to the subgroup of proton-dependent oligopeptide transporters (Galvan and Fernandez 2001).

*At*NRT1.1, as we have said previously, is involved in both HATS and LATS, in fact it is a dual-affinity transporter that acts depending on phosphorylation and dephosphorylation of threonine T101. It also senses NO_3^- to activate the expression of the genes involved in the nitrate metabolism. *At*NRT1.2 is a constitutive low-affinity nitrate transporter that is expressed mainly in root hairs and the epidermis of *Arabidopsis* (Huang et al., 1999). The root to shoot long-distance nitrate translocation includes mainly NRT1.5, NRT1.8 and NRT1.9, three NRT1 family members (Dechorgnat et al., 2010; Bai, 2013).

In *Arabidopsis*, until today, 7 NRT2 genes have been found. In this specie knockout mutants of NRT2.1, NRT2.2, NRT2.4 and NRT2.7 was studied and shown to be involved in nitrate transport (Bai, 2013), especially in the roots. AtNRT2.7 was more expressed in shoot than in root (Wang et al., 2003).

Recently has been clarified that NRT2 needs contribution of another family of HATS, NRT3 or NAR2 (Nitrate Assimilation Related family), to have an optimized activity in the uptake (Okamoto et al., 2006).

After his uptake NO_3^- can be stored in vacuoles to accomplish osmotic functions, go back in the soil via apoplast, translocate via xylem and transported to other tissues, or be reduced with different redox reactions in order to organicate him. These redox reactions, both catalysed by a specific enzyme, compose the nitrate metabolism; they are energy dependent and generally exploit $NAD(P)H^+$ as electron donor.

The first step of the nitrate metabolism is the reduction of nitrate (+5) to nitrite (+3) in the cytosol, catalysed by Nitrate Reductase, NR (NAD(P)H) [EC: 1.7.1.1; 1.7.1.2; 1.7.1.3; ex E.C.1.6.6.1-3). Then nitrite is transported into the chloroplast of the leaf, or in the plastid of the root, to be reduced to ammonium by ferredoxin-Nitrite Reductase, NiR [EC: 1.7.7.1]. Nitrite and ammonium ions are cytotoxic because lead to pH changes and induce reactive nitrogen species and oxidative damages, so they cannot be accumulating inside cells (Chow, 2002). For this reasons, their incorporation into organic compounds must be relatively fast (Chow, 2002). Ammonium then start a "Glutamine syhntetase/Glutamine oxoglutarate aminotransferase" cycle (GS/GOGAT). Glutamine Oxoglutarate aminotransferase is also known as Glutamate synthase GOGAT [(NADPH/NADH) EC: 1.4.1.13, 1.4.1.14; (ferredoxin) EC: 1.4.7.1]).

Ammonium is transformed to have glutamine, in the cytosol or in the chloroplasts/plastistids, by two isoenzyme: cytosolic or cloroplastic/plastidial glutamine synthetase, GS, respectively called GS1 and GS2 [EC: 6.3.1.2], (Lancien et al., 2000). In higher plants GS1 is codifying by multiple homologous genes, while GS2 is codifying by a single nuclear gene. GS catalyse a condensation of ammonium whit glutamate to have glutamine and this process require ATP (Temple et al., 1998).

At this point, if α-chetoglutarate (or oxo-glutarate) and energy are available from photosynthesis, two amide group of glutamine can be transferred, thanks to Glutamate Synthase, to α-chetoglutarate (or oxo-glutarate) (Temple et al., 1998). Finally, one of the two molecules of glutamate can accept NH_4^+ during another GS/GOGAT cycle, while the other can be organicated in amino acids by transaminases and then transformed in protein useful for the plant. (Sun et al., 2010). Probably also other three enzymes participate to the ammonium assimilation process: cytosolic asparagine synthetise (AS), plastidial Carbamoylphosphate synthase (CPS*ase*) and mitochondrial NADH-glutamate dehydrogenase (GDH) (Masclaux-Daubresse et al., 2010). AS, using ammonia as substrate, catalyses the transfer of the amide group of glutamine and a molecule of aspartate to generate glutamate and asparagine (Masclaux-Daubresse et al., 2010).

Carbamoylphosphate synthase (CPS*ase*) uses bicarbonate, ATP, ammonium or the amide group of glutamine to makes carbamoylphosphate, a precursor of citrulline and arginine (Masclaux-Daubresse et al., 2010). GDH can deamininig glutamate or, alternatively, if there are high level of ammonium as stress, incorporate ammonium into glutamate (Masclaux-Daubresse et al., 2010).

In the mesophyll of the cells there is high expression of GS2, while GS1 expression is low in leaves, being generally limited to the phloem; these two isoenzymes have an organ-specific expression pattern (Edwards and Coruzzi, 1990).

Therefore GS1 is the major form of GS in plant roots, it is very important for the primary nitrogen assimilation and his expression is metabolically regulated by availabilities of Nitrogen and Carbon (Sun et al., 2010).

GS2 plays a crucial role in reassimilation of NH_4^+ released via photorespiration in plants.

Glutamate synthase is present with two forms in plants: Fd-GOGAT, that use ferredoxin as electron donor, and NADH-GOGAT, that use NADH. The first one is generally localized in the chloroplasts, while NADH-GOGAT is in the plastids of non-photosynthetic tissues (Masclaux-Daubresse et al., 2010).

Generally, the reduction of nitrate is more efficient in leaves than in roots due to the close dependence on photosynthesis for reductants, energy and carbon skeleton (Chen et al., 2004).

Nitrate Reductase (E.C.1.7.1.1-3)

Four types of Nitrate Reductase have been found until today: eukaryotic assimilatory NR and three different bacterial enzymes (Morozkina et al., 2007). In this work we talk about eukaryotic NR.

Nitrate Reductase (E.C.1.7.1.1-3) is the first, inducible, enzyme involved in the nitrate metabolism of plants, therefore plays a central role to which the entire pathway is influenced by his activity as in a cascade mechanism. Its activity and gene expression levels also contribute to differences between high and low nitrate accumulation in leaves.

NR is a very complex water soluble enzyme that catalyse the reaction of reduction of nitrate to nitrite, NAD(P)H dependent, in plants, algae, fungi (Campbell, 1999) and yeast. Chemically is a dimeryzed, homodimer of two polypeptides of about 100 kD of molecular mass each and with tendency to a further dimerization to a homotetramer (dimer of dimer); for the activity the dimerization is essential (Campbell, 1999; Morozkina et al., 2007). Each polypeptide is composed of three codomains structurally distinct: FAD on the terminal carbon of the molecule, a little, central, heme-iron (b5 type) and molybdenum-molybdopterin (Mo-MPT) which is the nitrate reduction site posed on the N-terminal (Campbell, 1999). This subunit structure also contains cytochrome b (Cb), NADH and a dimer interface (Campbell, 1999). NR is a little redox chain immersed in the cytoplasm, where, during

catalytic action, the three codomain are reduced and oxidized, cyclically (Campbell, 1999). FAD, Fe-Heme and Mo-MPT codomains are tied together through two protease-sensitive surface-exposed loops (Lambeck, 2010) called "hinge1" and "hinge 2", able to transfer internally electrons (Campbell, 1999). Hinge2 transfer electrons from FAD via NAD(P)H to heme-iron, while hinge1 transfer two electrons from reduced Molibdenum (IV)-MPT to nitrate (Morozkina et al., 2007). Considering that 3 states of oxidation of FAD, 2 for Fe and 2-3 for Mo are possible, we can found NR.

NR presents three sequence regions different for the different forms of NR, this regions are: 1) N terminal region, composed with different amino acids, that is generally short; 2) "Hinge 1", that contain the Serine 534 residue, that can be phosphorylated, and a trypsin proteolytic site 3) "Hinge 2", which also contains a proteinase site (Campbell, 1999).

The main reaction catalysed by this enzyme is:

$NO_3^- + NADH \rightarrow NO_2^- + NAD^+ + OH^-$ ΔG= -34,2 kcal/mol

Secondly, NR catalyses the reduction of other molecules like NADH ferric citrate, chlorate, bromate and iodate (Campbell, 1999).

Photosynthetic organisms, fungi, and bacteria present a variety of mechanisms to regulate and control the expression of the enzymatic activities involved in nitrogen assimilatory pathways (Chow, 2002). These mechanisms are very complex and simultaneously coordinate the balance between nutrients availability and environmental conditions with nitrogen and carbon metabolisms (Reda, 2013).

Nitrate reductase expression is regulated by various factors, such as levels of nitrates (Wang et al., 2000), temperature, light (Lillo, 2008), circadian rhythms (Gutierrez et al., 2008), sucrose (Lejay et al., 2003), carbon skeletons, nitrogen metabolites as amino acids and glutamine, CO_2 (Crawford, 1995; Lopes et al., 2002) and gaseous environment (Garg, 2013), oxygen and hormones like auxins (Krouk et al., 2010), and still inorganic salts and ions (eg. Molibdate and Tungsten), antibiotics and metabolic inhibitor, herbicides and fungicides, seedling age, water stress, atmospheric pollutants, and external pH (Garg, 2013).

The rate of NR protein is disconnected from the quantity of mRNA for this enzyme, as shown in various studies, demonstrating that there are different regulatory mechanisms (Crawford, 1995). In fact NR can be regulated at three levels: transcriptional (expression) by changes in NR-encoding genes, post translational (catalytic activity) with modification of existing proteins (Reda, 2013) and with proteins degradation. These regulatory mechanisms often act in synergic way and the response of NR can be short or long term type (Chow, 2012).

Post-translational regulatory mechanism

NR is reversible modulated at posttranslational level and can be inactivated through a two-step process that occurs in presence of divalent cations (especially Ca^{2+} or Mg^{2+}): phosphorylation and participation of 14-3-3 regulatory proteins. After an inhibitory signal, as can be dark or high concentration of glutamine, with divalent cations and ATP in the medium, Ca-dependent kinase family (CPK) and the calcium-independent SNF1 resembling kinase family (SNRK1) can phosphorylate the conserved serine residue (534-Ser in *Arabidopsis thaliana*; 543-Ser for *Spinacia oleracea*) in hinge1 of NR transforming it in pNR, which remains active (Lambeck et al., 2010). At this point 14-3-3 regulatory proteins, that have affinity with the phosphorylated site of pNR, can bind pNR and inactivate it; also in this case divalent cations are required. It has not yet been established because it is necessary the presence of cations in this phase, but if cations aren't available after phosphorylation of NR, NR remains

active (Kaiser and Huber, 2001). Inactive NR can be degraded or the process can be slowly reverted to have pNR by protein phosphatase (PP2A), in presence of salts.

The rate of NR in the cytosol is the result of the balance between NR synthetized and NR degraded; NR is functional only for several-hours and then is degraded. Generally the degradation occurs after 20 h in the plants exposed at light (Lillo et al., 2003). The synthesis of NR depends from lots of factors, especially light, and it is not constant, so also this mechanism contributes to modulate the reduction of nitrates in the plant.

Main factors that regulate NR

Nitrate up regulate NR activity

Since NR is highly regulated and closely connected with photosynthesis, it show fine mechanisms of regulation and it represents the rate-limiting step in nitrogen assimilation (Lambeck et al., 2010). NR is inducible and depends by the light signal and concentration of his substrate (Sehnke and Ferl, 1996). The first signal that starts the transcription of NR is the concentration of nitrates in the nutrient solution (Crawford, 1995; Kaiser et al., 2002) that involve primary sensing of the plants (Scheible, 1997). Very low concentrations of nitrate, <10 μM, induce the gene expression within minutes (Crawford, 1995). In maize and *Arabidopsis* 30 minutes are necessary to increase specific NR-mRNA after external nitrate induction (Wang et al., 2000; Wang et al., 2003). The nitrate responsive genes transcripts can be regulated involving *cis*-acting regulatory sequences or nitrate response elements (NRE) (Pathak et al., 2008; Garg, 2013) and nitrate can regulate NR activity only at transcription level (Kaiser et al., 2002). With sufficient nitrate concentrations and in plants in optimal growth conditions the activity of NR is about double compared to the needs. Plants sense nitrate as a hormone; this mean that nitrates levels can drive changes in the root-to-shoot-ratio, in the development of root hairs or similar modifications, in fact nitrate can induce enzymes and can promote proteins, perhaps including DNA regulatory proteins, involved in the metabolic response to the availability of a limiting nutrient (Campbell, 1999). Nitrate acts directly on the regulation of its metabolism or indirectly, through the downstream metabolites formed during the processes of assimilation. Some studies showed that nitrate rapidly induces the transcripts of high-affinity transporters proportional at the rate of the nitrate addiction (Scheible, 1997). In Tobacco transgenic plants with constitutive expression of NR, the deregulation of NR gene expression led to a reduced nitrate rate in the tissues (Quillere et al., 1994; Garg, 2013). External nitrate supplies in tobacco (Calza et al., 1987), in maize (Gowri and Campbell, 1989), in barley (Cherel et al., 1986) and in squash plants increased the transcript of NR. Is not yet clear, however, the proportionality between the concentration of nitrate and nitrate reductase activity, in fact Hu et al., 1992, found a negative correlation between NR activity and nitrate concentration, but other authors found the contrary, therefore the greater was the proportion of nitrate in the plant and the greater the activity of NR, in virtue of the fact that the enzyme would be induced by the substrate (Chen et al., 2004; Ivashikina and Sokolov, 1997). Chen et al., 2004 found in Rape, Cabbage and Spinach, that NR activity increased proportionally with the low nitrate supplies (from 0 to about 0.3 g $N*kg^{-1}$ soil), while at higher nitrate supplies (0.3-0.6 g $N*kg^{-1}$ soil), the activity went a plateau or even decreased.

Sugars up regulates NR activity

The levels of transcription of NR are also positive correlated with the content of soluble sugars (Sivasankar et al., 1997; Klein et al., 2000; Larios et al., 2001). Sugars are signalling molecules and primary messengers that affect all

stages of grow of plants and can regulate the expression of different genes involved in the Carbon metabolism (Gupta and Kaur, 2005) and various physiological processes (Reda, 2013). Exoses repress gene expression with intracellular signalling mechanisms that can involve hexokinase (Gupta and Kaur, 2005). NR gene expression is up-regulated by sucrose and the plants can reduce nitrate only if cells contains enough carbohydrates (Cheng et al., 1992). Already in 1976 Aslam et al. showed that NR activity was induced by glucose, in presence of nitrate, in etiolated barley leaves both in light an in dark and that the reduction of nitrate was higher at dark after treatment with exogenous fructose, glucose and sucrose. Cheng et al., in 1992 reported that, at light, sucrose stimulates the increase of transcript for NR1 in dark-adapted *Arabidopsis* plants, and even that the increase of m-RNA for NR1 is dependent on the availability of sucrose, which mimics and replacing and/or replacing the induction-effect of light.

Sugars, glucose and sucrose, can also post-translational regulate NR activity with reversible phosphorylation, but also involving redox and hysteretic modifications (Kaiser and Huber, 1997; Reda et al., 2008). Expression, activity and posttranslational regulation are increased by sugars (Morcuende et al., 2011).

Glutamine down regulate NR activity

The transcription level is negatively influenced by the nitrogen metabolites, for example by glutamine (Scheible et al., 1997; Sivasankar et al., 1997). In the last part of photoperiod the accumulation of amino acids like glutamine lead to a decrease of the NR activity and *vice versa*, in plants with good availability of nitrates and CO_2, abundant activity of NR at the end of the night is explained by a decrease in the content of amino acids (Scheible et al., 1997). Posttranslational inactivation and degradation of NR in the dark are favoured by glutamine (Morcuende et al., 1998). At the beginning of light exposure NR is maintained active if there are lower concentrations of glutamine in the plant (Morcuende et al., 2011).

Light-dark transitions affect NR

The circadian rhythm of alternation between day and night affect the NR activity as if the plants follow a mechanism regulated by an endogenous clock (Deng et al., 1990): generally the activity is high at the starting of the light exposure in the morning and tends to decrease at the end of the exposure at light, in the afternoon to the first part of the dark period of the night (Morcuende et al., 2011; Galangau et al., 1988). Light induces the synthesis and the posttranslational activation of NR and probably the most of it is due to posttranslational regulation levels (Lillo, 2008). The activation state of NR passes from 10-30% at dark to 70-90% at light (Kaiser and Huber, 2001). This behaviour however is dependent also by other factors connected with the Carbon metabolism: in fact, in Kaiser and Huber (2001) studies if CO_2 was not available, NR remained inactive despite the plants were exposed for long time at high illumination and if exogenous sugar was given to plants adapted at dark NR was activated. Often, at the end of the light period, when soluble sugars tend to rise again, NR activity decreased; this behaviour depend on the interaction among the various factors that influence NR: in fact, the reduction of activity is attributable to the degradation of NR, generally associated with a decline in the level of foliar nitrates (Kaiser et al., 2002).

Nitrate assimilation and Carbon metabolism

Generally NR activity is related with photosynthesis by regulatory mechanisms, and these two processes are maximal during the day, in fact they are activated by light, and minimal in the dark. These responses indicate that

nitrogen and carbon pathways are strictly connected. These two metabolisms interact influencing gene expression in order to optimize the balance between nutritional and metabolic conditions (Wanget al., 2000; Palenchar et al., 2004). Moreover photosynthesis, respiration and photorespiration provide carbon skeleton, ATP, NADH and ferredoxin (Fdx) essentials for the organication of nitrates in the plants. In fact, NADH or Fdx are required by NR, NiR and GOGAT; ATP is required from GS and AS; without the keto-acids and other carbon skeletons would not be possible the formation of amino acids (Masclaux-Daubresse et al., 2010). The connection of nitrogen and carbon metabolism requires an integrate regulation system between these processes, so photosynthesis and respiration are affected. Since reductants availability derived from photosynthesis are necessary for the nitrate reduction, a low photosynthetic activity negatively affect NR activity through post-translational inactivation, (Kaiser et al., 2002; Dutilleul et al., 2005). Inhibitors of electrons transport chain prevent the activation of NR (Nemie-Feyissa et al., 2013).

The photochemical efficiency of the PSII under N starvation is reduced and the absorbed excitation energy is dissipated. *Vice versa* a reduction of the conversion of photosynthetic energy influences the formation of amino acids. Due to the necessity of the carbon skeleton and ATP also the respiration is influenced by the nitrogen starvation of the plant, though the related mechanisms are not well clear presently.

3. Objectives of the thesis

The aim of this thesis encompasses the study of the nitrogen pathway in rocket, *Diplotaxis tenuifolia* L., and lettuce, *Lactuca sativa* L., carrying out two different, but parallel tracks, one more applicative and the other one more directed to basic research. The aim of this work is to evaluate the effect of the exogenous nitrate supply in two different species belong to different botanical families: *Diplotaxis tenuifolia* L., a Brassiceae, and *Lactuca sativa* L., belong to Asteraceae, at different environmental conditions in order to assess the physiological, biochemical and molecular responses. The comparison between rocket and lettuce is justified mainly because these species are very popular on the market: lettuce is the most important specie used for salads and rocket is widespread in the salad-mix to give taste and improve the content of antioxidants. From literature, moreover, it is emerged that rocket is a hyper accumulator of nitrates and can be used as a model plant, being genetically closed to *Arabidopsis*, the most studied model plant. Lettuce generally shows low levels of nitrates in the leaves and some studies show that it is able to exploits low concentration of nitrate in the nutrient solution without compromise the product quality. The question to be addressed to planning the experimental design is what are the factors that determine such big differences in the accumulation of nitrate in these two species? What are the differences in the biochemical pathway of nitrates that lead to different nitrate accumulation? There are not in literature comparative studies with the same target on these species.

Applied research activities

Since the edible parts intended for the commerce in the ready-to-use salads are leaves and nitrate is stored in vacuoles, it is important to find agronomical strategies addressed to control the content of nitrates in these organs. Furthermore the fertilizers used to produce cash crops generally exceed the culture needs, in order to avoid economic losses due to deficiencies, so it is important publicly show results that confirm with which nitrate concentrations can be possible obtain optimized quality productions without waste. In order to achieve this target the cultivation method chosen for the experiments is floating system; it is scientifically attested that this hydroponic technique optimizes the radical absorption of the macronutrients increasing the use efficiency of the minerals.

Since the content of nitrates in the leaves depend from many environmental factors, like light exposure and intensity, temperature, concentration of nitrates in the nutrient solution, the studies, addressed to deepen the main productive reality, was planned similarly to them. So the trials were performed in greenhouse with nitrates concentrations of the solutions in excess compared to the actual crop needs, similarly to how it happens in the productive real life. The concentrations of nitrates used were 2, 10 and 20 mM. In addition, crops were carried out in hot and cold season as a further comparison parameter. The content of chlorophylls and carotenoids is a good indicator of the wellness of the crops, in fact it is correlated with the nutritional status of the plants, particularly with the nitrogen levels in the leaves. These antioxidant pigments moreover, are used as parameters to define the quality of the vegetables; quality is very important in this study because allows to evaluate the applicability of the tested agronomical techniques to the market realities. Therefore chlorophyll *a*+*b* and carotenoids amount measurements were included in the experimental plan.

Basic research activities

Some studies show that little variations in the concentration of nitrate in the nutrient solution affect the NR activity. The transport of nitrates into the cortical cells depends by specific membrane transporters HATS, that act when the concentration are very low, between 0.2 to 0.5 mM and LATS that work with concentrations above of

0.5 mM. In order to highlight the response of the activity of NR and the gene expression of enzyme involved in the nitrate assimilation under this range of values covered by the nitrate transporters, 0.2, 0.5, 1 and 2 mM concentrations of nitrates were chosen for the treatments. All the experiments were conducted in greenhouse in the spring-summer and autumn-winter seasons. The environmental parameters like temperature and irradiance were measured with the purpose to give careful considerations, correctly discriminate and attribute the various contributions in changes of the activities of NR. Another part of the project has provided for the cultivation in a growth chamber in order to totally eliminate the environmental effects on the activity of NR; the gene expressions analyses were carried out on the samples obtained from plants grown under controlled environmental conditions. With the objective to see the influences of light intensity and exposure on the demeanor of NR, a very accurate timing of sampling has been planned both in greenhouse and in growth chamber, providing to analyze plants adapted at dark and after two, four and six hours of light.

4. Materials and methods

Applied experiments

Plant material and growth conditions

The cultivation in floating system of plants of *Diplotaxis tenuifolia* L. and *Lactuca sativa* L. for the applied research experiments was carried out in greenhouse starting from May 2011 to the end of 2011. Some blistered panels (51.5 cm long and 32.5 cm wide), with 228 holes, was filled with agriperlite® and wetting with water before sowing. The sowing was carried out through semi-automatic seed drill, which has placed 4-5 seeds in each hole of the polystyrene panel support allowing a density of 1150 plants*m^2; then, the panels was left to germinate in water, at dark and at room temperature for four-seven days. After germinations the panel with the seedlings were transferred into twelve tanks, with 700 liters of capacity each, (190 x 145 cm), that was filled with a standard Hoagland nutrient solution optimized for leafy vegetables in growth (nitrates changed among treatments; 2.8 P_2O_4; 8.4 K_2O; 3.5 Ca; 1.4 Mg; 2 S_2O_4; 0.04 EDTA-Fe and micronutrients) that were made bubbled through an oxygenator to a pressure of 1.2 atm in order to guarantee to the roots an amount of the 6 a 7 mg*L^{-1} of oxygen. The pH of the nutrient solution was periodically controlled and was between 5.5 and 6.5.

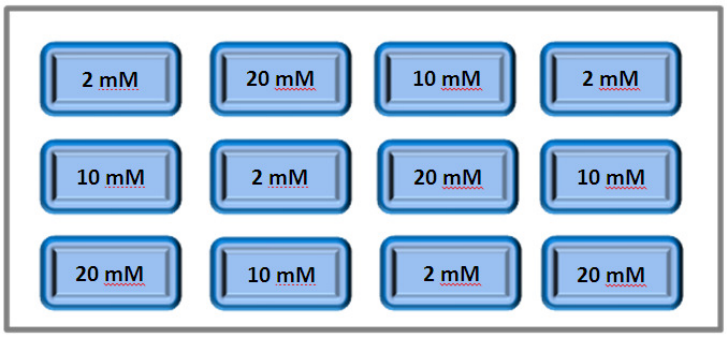

Fig. 4.1: Schematic representation of the tanks used for the cultivation in the greenhouse, with the concentration of the nutrient solutions, used for the applied experiment.

Treatments

The effects of different nutrient concentrations were studied. The standard nutrient solution is used but with three different nitrates treatments: N-NO_3^- 2, 10 and 20 mM. The measurements were carried out at baby leaf commercial stage. The plants were then collected and fresh weight was measured with an analytical scale.

Analysis

Chlorophylls and carotenoids content

Three disks of fresh leaves with a diameter of 5 mm were cutted (away from the midrib), weighted and placed in a 15 ml tube, in which 5 ml of 99.9% pure methanol were added. Three tubes for each treatment were collected. Following a brief manual shaking the tubes were placed at dark in temperature chamber at 4 °C for 24 hours, in order to obtain the maximum extraction possible from the disks. After this time, the tubes were taken to a further, brief, Vortex shaking and then the extracts were read at the spectrophotometer at different wavelengths:

665.2 nm for the chlorophyll *a*, 652.4 nm for the chlorophyll *b* and 470 nm for the total carotenoids. To obtain the concentrations of chlorophyll and carotenoids the Lichtenthaler (1987) formula was applied.

Nitrates

A colorimetric analysis for the determination of the content of nitrates in leaves was performed, as described by Cataldo et al., 1975. About 1 g of fresh or stored sample at -20 °C leaves was homogenate in a mortar with 3 or 4 ml of water, respectively for lettuce and rocket, and poured into 15 ml tubes. The tubes were then centrifugated at 4000 rpm for 20 minutes and the supernatant was collected. The colorimetric reaction was carried out by adding 20 µl of the extract and 80 µl of salicyl-solphoric acid (5% v/v) prepared new each time; then 3 ml of NaOH 1.5 N were added to stop the reaction. The spectrophotometer was set to the absorbance of 410 nm and for calibration different standards of nitrates solutions were used.

Nitrate reductase *in vivo* activity

The colorimetric determination of NR activity *in vivo* was performed as described by Aslam et al., 1984 modified. Fresh leaves, gathered in some plastic bags to avoid wetting, was immediately placing at dark in ice. Fresh leaves were quickly cut in little disks of 5 mm of diameter and putted in 15 ml tubes to reach 0.250 mg of fresh weight. After weight the tubes were closed and placed in ice in order to maintain inactive the enzyme. Three or four technical replicates were performed for each treatment; for each sample were prepared a control. The control samples were immediately boiled in water for 5 minutes in order to denature the enzyme; from these samples the content of constitutive nitrites was obtained. The incubation medium was composed of potassium phosphate 100 mM (pH 7.5); 5% v/v isopropanol and 30 mM potassium nitrate. 5 ml of the reaction buffer was added to the tubes placed in ice, and then the tubes were transferred in a water bath at 30 °C for 30 minutes to incubate. After incubation the reaction was stopped with 5 ml of 1% sulfanilamide in HCl 3.0 N, and 0.02% N naftin etilen diamide as indicator of nitrites content. The tubes were left in the dark for 15-30 minutes to wait the color development and then the spectrophotometric readings were made at 540 nm. The calibration was carried out with a standard solution of sodium nitrite.

Sucrose

The sucrose analysis was carried out modifying Kulka's method (1956). 100 µl of the plant extract, obtained as previously described, have been put in 2 ml eppendorf with 100 µl of 2N NaOH and boiled for 10 minutes. Then 750 µl of the reagent (a mixture of 17.5 mg of resorcinol, 45 mg thiourea, 12.5 ml acetic acid, 5 ml distilled water and 125 ml of hydrochloric acid 30%) was added and the tubes were put at 80 °C for 10 minutes. After cooling the samples were read at the spectrophotometer at absorbance of 500 nm. The calibration line was calculated from the absorbance obtained from different concentrated sucrose standards.

Reducing sugars

The leaves extract for this analysis was obtained as described before for nitrates assay. Miller, 1959 protocol was modified and used. 2.5 g of DNS (dinitrosalycilic acid) was dissolved in 150 ml of distilled water and mixed with a previously prepared mixture of 75 g of Rochelle salt (Potassium Sodium Tartrate) with 25 ml di NaOH 4N. This solution was stirred at 50 °C until completely dissolved, filtered and then kept at dark until the use. The reagent was not used if exceeded 2 months of storage. 100 µl of DNS reagent were added to 100 µl of leaves extract previously placed in eppendorf of 2 ml; the tubes were then incubate at 100 °C for 5 minutes. After incubation 750 µl of distilled water were added to the tubes and read at the spectrophotometer at 530 nm. For the calibration different glucose standard solutions were used.

Total sugars

The extract used for colorimetric determination was obtained as described above for the nitrates assay and diluted with water (1:10). The method described and Leyva et al., 2008, was used, modified. The anthrone reagent was each time prepared new, dissolving 0.1 g of anthrone in 50 ml of pure sulfuric acid (98%). The reagent was stirred for at least half an hour and kept in the dark until use. 1 ml of reagent was put in 2 ml eppendorf, and then 200 µl of the diluted extract was gently placed above the reagent. The eppendorf tubes were placed for 5 minutes in ice to lowering the reaction temperature. Then the tubes were agitated by inversion for 4 times and immediately placed in a water bath at 95 °C for 5 minutes. After the incubation, samples were left at room temperature for 10 minutes and then read at 610 nm of absorbance at the spectrophotometer. The calibration curve was obtained reading standards with different glucose concentrations.

Biochemistry of nitrate assimilation in rocket under natural environmental conditions

Plant material and growth conditions

Rocket plants were hydroponically grown under natural spring conditions in greenhouse equipped with environmental parameters control station. Plants were grown for four weeks in nutrition solution containing (concentrations are expressed in mM): 12 $N\text{-}NO_3$, 3.8 $N\text{-}NH_4$, 2.8 P_2O_4, 8.4 K_2O, 3.5 Ca, 1.4 Mg and Hoagland's concentration for micronutrients.

Treatments

Plants were grown under natural light conditions (natural circadian rhythm, NCR) or covered with black clothes the evening before and first sampling was performed after that the black clothes were removed (modified circadian rhythm, MCR). Three sampling times of two-three hours of interval was followed starting from 9 am. The plants under NCR had 2 h more of light exposure compared to those under MCR.

Analysis

Nitrates content and NR *in vivo* activity were measured as described above for the applied experiments.

Experiments in greenhouse

Plant material and growth conditions

The lettuce and rocket cultivation for the basic research was performed in a greenhouse. The polystyrene panels above described was filled with agriperlite® and wetting with water before manual sowing. After sowing the panels was covered with aluminum foil pending the development of seedlings, then have been discovered and placed in plastic plots of 15 liters of capacity, filled with a growth Hoagland standard nutrient solution, the same described before for the cultivation in greenhouse (10 $N\text{-}NO_3$; 3.8 $N\text{-}NH_4$; 2.8 P_2O_4; 8.4 K_2O; 3.5 Ca; 1.4 Mg; 2 S_2O_4; 0.04 EDTA-Fe and micronutrients) that were made bubbled through an oxygenator. The pH of the N.S. was periodically controlled and was about from 5.5 to 6.5.

Treatments

At the commercial stage of baby leaf the growth nutrient solution was changed with the treatments solutions, different only for nitrate concentration: 0.25; 0.5; 1 and 2 mM $N\text{-}NO_3$. The measurements were carried out at the physiological stage of baby leaf (13 cm height and 3-4 true leaves) and after 24 h of treatments. In all the trials the sampling of the plant material was take place follow an accurate timing plane: starting from plants adapted at

dark and after 2, 4 and 6 hours, to bring out the behavior of NR in plants with different light exposure and intensity. In all the sampling phases the environmental parameter was measured.

Analysis

Nitrate reductase *in vitro* activity

The Ferrario-Méry (1998) modified protocol was used for the determination of nitrate reductase activity. Leaves stored at -80 °C were homogenate with liquid nitrogen in a cool mortar to obtain a fine powder. About 1 g of the powder was dissolved in 3 ml of fresh prepared extraction buffer as follows: 50 mM MOPS-KOH buffer (pH 7.8), 5 mM EDTA, 5 mM NaF, 1 µM Na2MoO4, 2 mM β-mercaptoethanol, 1 mM PMSF, 10 µM FAD, 1 µM leupeptin, e 10 µM chymostatin or inhibitor protease cocktail. The buffer with the powder was always kept in ice to avoid of enable or denature the enzyme. The solution was centrifuged at 12,000 g at 4 °C for 15 minutes; the supernatant was the crude extract used for the assay. For each sample, replicate 3 times, four tubes was set: one for the total NR activity determination with the control, and one for the active NR determination.

Total NR activity (non phosphorylated form)

In eppendorf of 2 ml, posed in ice, 50 µl of crude extract was added to 450 µl of 50 mM MOPS-KOH (pH 7.8), 1 mM NaF, 10 mM KNO_3, 0.17 mM NADH and 5 mM EDTA fresh reaction buffer and stirred. The tube was immediately put on water bath to incubate at 30 °C for 30 minutes. After this time the reaction was stopped with 250 µl of N-naphthylethylethylenediamine dihydrochloride (0.02%, w/v). The tubes were stirred and posed at dark to promote the color development; after this time the samples was read at spectrophotometer at the absorbance of 540 nm. The control was obtained boiling the crude extract for 5 minute before adding of the reaction buffer. The calibration curve was obtained with different standards of $NaNO_2$.

Active NR activity (in presence of Magnesium)

The determination is the same described of the measure of the total NR activity, but a different reaction buffer was used: 50 mM MOPS-KOH buffer (pH 7.8), 1 mM NaF, 10 mM KNO_3, 0.17 mM NADH, e 10 mM $MgCl_2$.

Protein quantification

According with Bradford protocol (1976) the crude extracts used for the NR activity measure were analyzed with IBI SCIENTIFIC® assay kit. In eppendorf, posed in ice, 5 µl of the extract were added to 95 µl of NaCl 0.15 N; after stirring 1 ml of Bradford reagent was added at the solution and the samples was read at the spectrophotometer at 595 nm. The calibration curve was built using different concentrations standards of Bovine Serum Albumine.

Biochemical and molecular studies of nitrate assimilation in rocket and lettuce grown in controlled environment

Plant material and growth conditions

The cultivation of rocket and lettuce for basic research activities was carried out in growth chamber setting with 13 hours of photoperiod; 60% of relative humidity, 23-26 °C of temperature; 500 $W*m^{-2}$ of light intensity, with the same indications provided above for greenhouse cultivation for basic research.

Treatments

The treatments were the same described above for the greenhouse experiment aimed at basic research.

Analysis

All the qualitative analysis described above for the applied experiments were carried out.

Total RNA isolation

For the RNA isolation, 1 g of fresh leaves were collected in liquid N, stored in -80 °C and homogenized in liquid N until the use. Spectrum™ Plant Total RNA Kit by Sigma-Aldrich® was used for the extraction according with the manufacturer's instructions. The RNA was then quantified at the Nano-Drop at 260 nm and stored at -80 °C until use.

Primers design

For lettuce the primers of the genes encoding for the enzymes studied involved in the nitrate metabolism was designed based on sequences found in GenBank for this specie and with Primer3 web tool http://primer3.ut.ee/.

For rocket the sequences of the genes was obtained thanks the availability of RNA-seq libraries, built by my fellow researchers, deriving from transcriptome analysis of rocket plants subjected to nitrogen starvation (lasted 24 hours). The sequences of interest were blasted and the primers were designed using Primer3 web tool.

Retrotranscription of total RNA

First-strand cDNA was synthesized using 5 µg of total RNA that was retrotranscripted using SuperScript® III Reverse Transcriptase by Life Thecnologies™ and used according with the suggestion of the producer.

qRT-PCR

The qRT-PCR (Reverse transcriptase-Polimerase Chain Reaction) was performed with 2 µl of standardized c-DNA and with the use of SYBR® Green Real-Time PCR Master Mixes by Life Thecnologies™, as fluorescent dye for the quantification of double stranded DNA. Three technical replicates were performed for each sample. The analysis was made with ABI7300 Real Time PCR System by Applied Biosystem with the follow amplification program and for each cycle a dissociation curve (melting curve) was measured to verify the absence of primer dimers and to confirm the absence of multiple products. As internal control the EF1α gene was used and the relative expression of the genes of interest was calculated using a comparative ΔCt method.

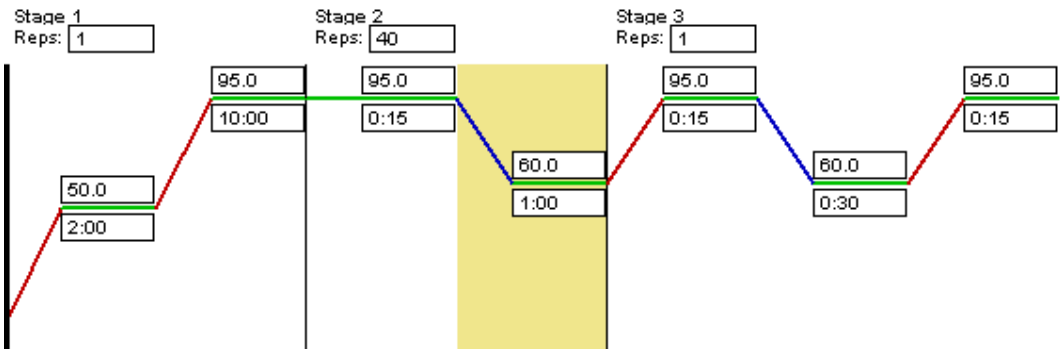

Fig. 4.2: Amplification program with the temperatures and the stages used in the qRT-PCR analysis.

Statistical analysis

The raw data obtained was statistically analyzed using GraphPad Prism software; the analysis of variance one-way and two-way-ANOVA was applied to the data with Bonferroni correction and considering the statistical significance of differences among averages ($P \leq 0.05$). The results reported are the averages ± the standard errors.

5. Results

Applied research experiments

The experiments concerning the applied research were carried out in 2011 in greenhouse. Both for lettuce and for rocket two cycles of cultivation for the spring-summer season were performed: one in May 2011 when the radiation was 310 $W*m^{-2}$ and one in June whit 278 $W*m^{-2}$; the temperatures were 15.6 and 19.1 °C respectively. In order to evaluate the seasonal effect on the antioxidants the lettuce and rocket were cultivated also In October 2011; at the harvest the temperature was 19.6 °C and the radiation 211 $W*m^{-2}$. The results showed are means with standard errors. Three concentrated in nitrates Hoagland standard were tested: 2, 10 and 20 mM. The environmental parameters have been measured and provided by an automatic weather station placed in Via Celoria 2, Milan (Dr. Parisi S.) integrated with Arpa (Agenzia Regionale per la Protezione dell'Ambiente)-Lombardia.

Date of sampling	Radiation (W/m^2)	Temperature (°C)
5 May 2011	310	15.6
9 June 2011	278	19.1
6 October 2011	211	19.6

Tab. 5.1: Environmental parameters measured referring to the dates of sampling of plants grown in the greenhouse. Data from Arpa-Lombardia.

Antioxidants content

Chlorophyll *a+b* in lettuce

In May the content of lettuce total chlorophylls between 2 and 10 mM of nitrates concentrations was significantly increased. The maximum rate of chlorophyll was 0.8 $\mu g*mg^{-1}$ of fresh weight found on the 20 mM nutrient solution (Fig. 4.1-A). No significant differences among treatments were found in the values of the content of chlorophylls of the leaves sampled in June and the values were about 0.2 $\mu g*g^{-1}$ of fresh weight, values from 2 to 4 times lower than those found for the cycle of May.

The content of chlorophylls measured from the leaves of the autumnal cycle of cultivation was constant at the three nitrate concentrated nutrient solution and amounted around 0.8 $\mu g*mg^{-1}$ of fresh weight. These values were consistent only with that found in plants collected in May at the 20 mM concentration.

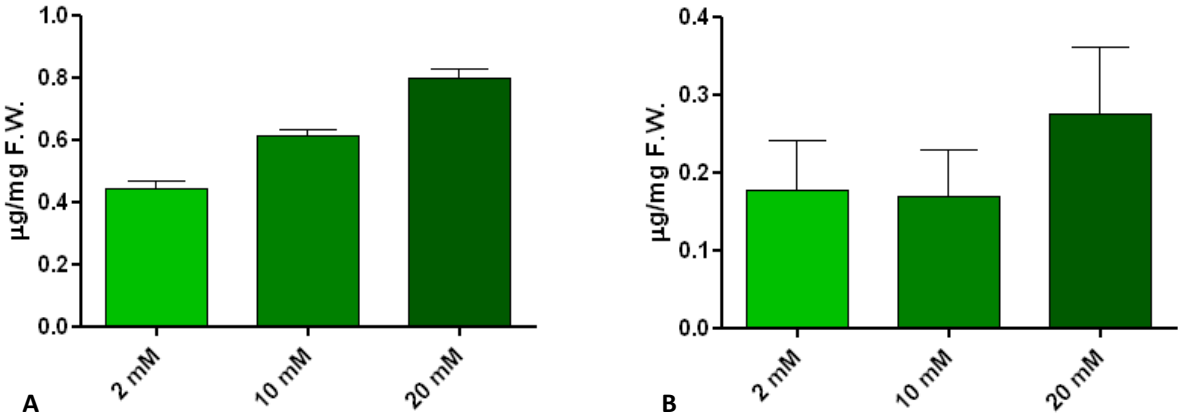

Fig. 5.1: Content of total chlorophylls in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in May (A) and in June (B). The values are means ± standard errors (n=3).

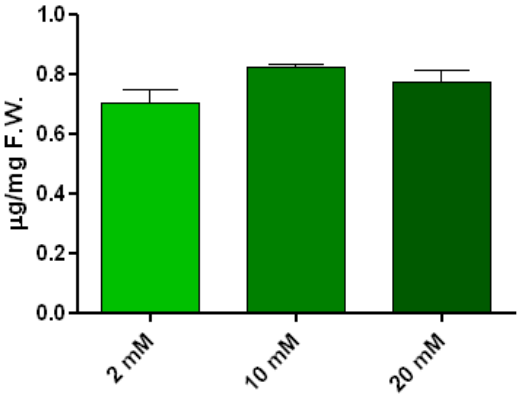

Fig. 5.2: Content of total chlorophylls in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in October. The values are means ± standard errors (n=3).

Chlorophyll *a+b* in rocket

The content of chlorophylls in rocket was the same in the three treatments and both for the two cycles of May and June. The content was about 0.9 µg*mg^{-1} of fresh weight as you can see in the Fig 4.3.

As it is showned in Fig. 4.4, the content of chlorophylls in rocket plants harvested in October was the same for the three treatments and the average, of about 1 µg*mg^{-1} of fresh weight, was in line with the contents found in May and June.

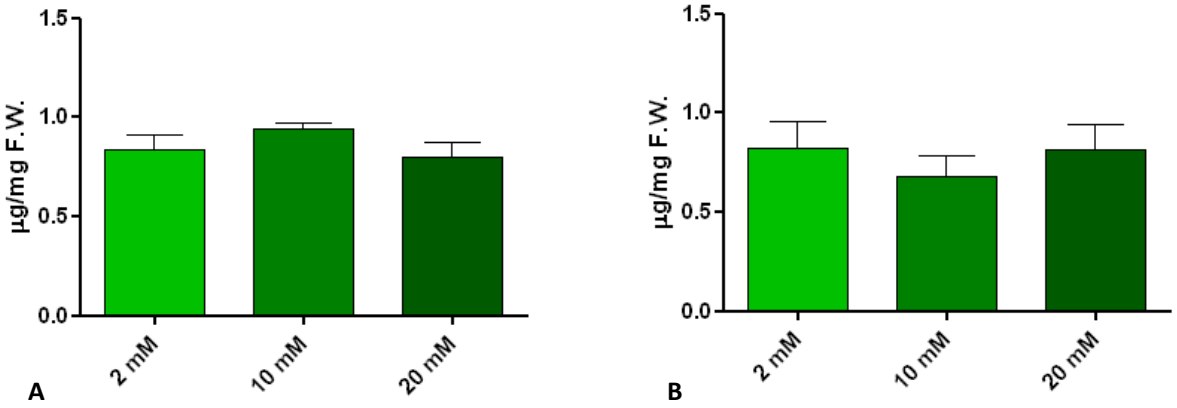

Fig. 5.3: Content of total chlorophylls in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in May (A) and in June (B). The values are means ± standard errors (n=3).

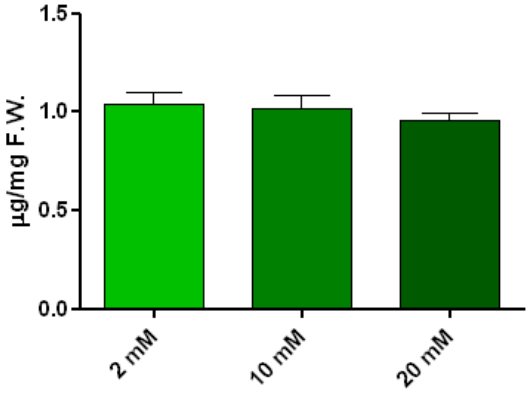

Fig. 5.4: Content of total chlorophylls in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in October. The values are means ± standard errors (n=3).

Carotenoids in lettuce

In Fig. 5.5 it was shown that in May the content of carotenoids was significantly increased passing from the lower nitrate treatment, with 0.10 µg*mg^{-1} of fresh weight to the 10 mM one, with 0.15 µg*mg^{-1} of fresh weight and, finally, to the 20 mM reaching 0.2 µg*mg^{-1} FW. In June the content of carotenoids was the same in all the concentrations of nitrate tested. The content of carotenoids in average obtained in June was higher compared to the values obtained in May.

The carotenoids in October (Fig. 5.6) showed significant differences among the 2 mM treatment, where there were 0.075 µg*mg^{-1} FW, to the other two treatments where the values were about 0.1 µg*mg^{-1} FW. No differences were observed between the carotenoids extract from leaves grown on the 10 mM nutrient solution and the 20 mM one. These contents were lower in average compared to the values achieved in spring-summer.

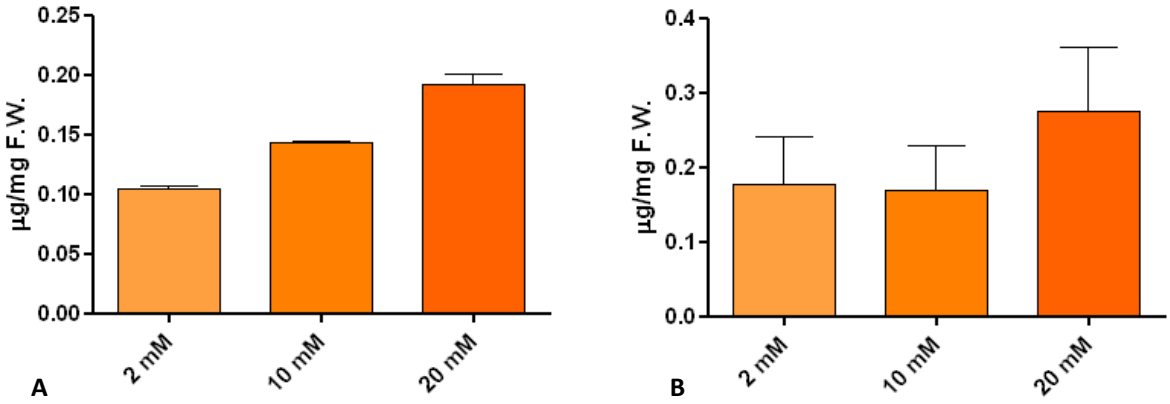

Fig. 5.5: Content of total carotenoids in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in May (A) and in June (B). The values are means ± standard errors (n=3).

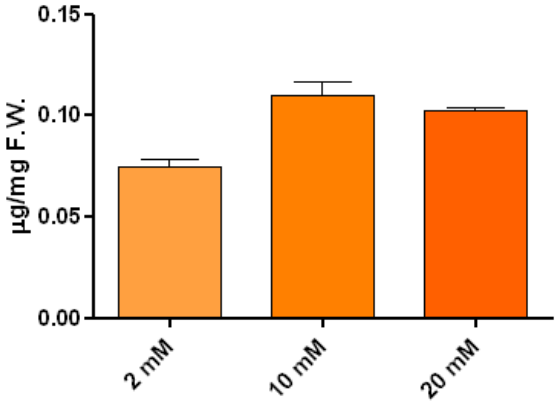

Fig. 5.6: Content of total carotenoids in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in October. The values are means ± standard errors (n=3).

Carotenoids in rocket

The content of carotenoids found in plants grown in May was significantly different between the 10 mM nutrient solution to the 20 mM one. The higher value was 0.2 µg*mg^{-1} FW measured in the 10 mM treatment. In June there were not differences in the content of carotenoids among treatments and the average of the values was about 0.8 µg*mg^{-1} FW.

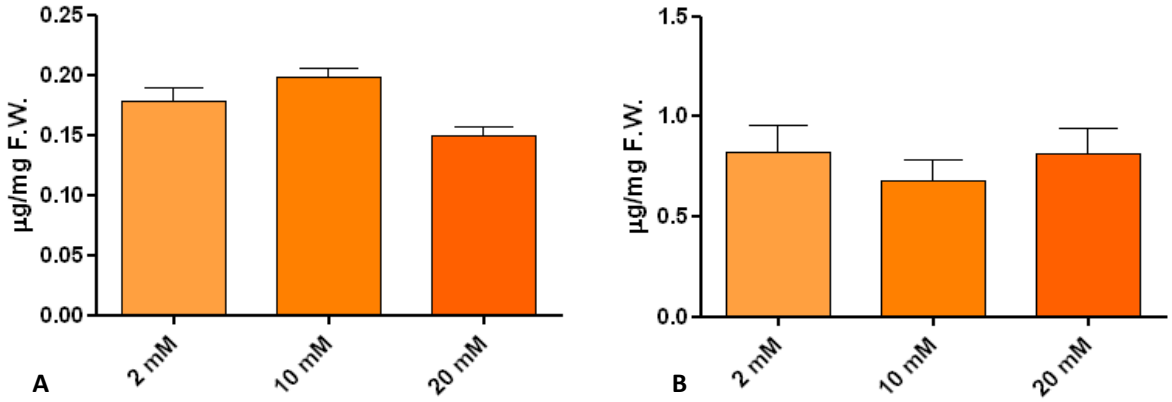

Fig. 5.7: Content of total carotenoids in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in May (A) and in June (B). The values are means ± standard errors (n=3).

In October the statistical significant difference of content of carotenoids were observed between the lower concentrated solution and the 20 mM one. The average of the content was about 0.13 µg*mg^{-1} FW.

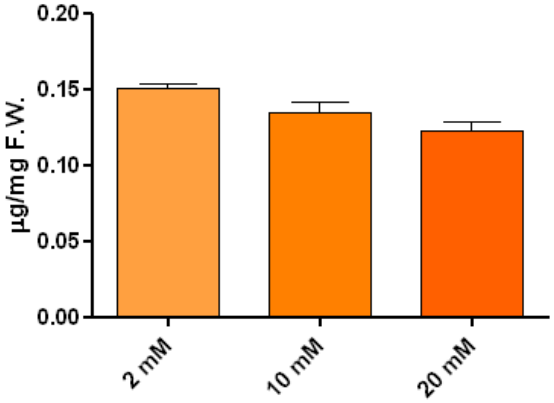

Fig. 5.8: Content of total carotenoids in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in October. The values are means ± standard errors (n=3).

Nitrates content

Nitrates content in lettuce

No significant difference among treatments was observed in nitrate content of the lettuce plant grown in May and in June. The contents were under the threshold imposed by European Regulation of 4000 mg NO_3^-*kg^{-1} FW, and were, in average, about 1830 mg NO_3^-*g^{-1} FW in May and about 2400 mg NO_3^-*kg^{-1} FW as shown in Fig. 5.9.

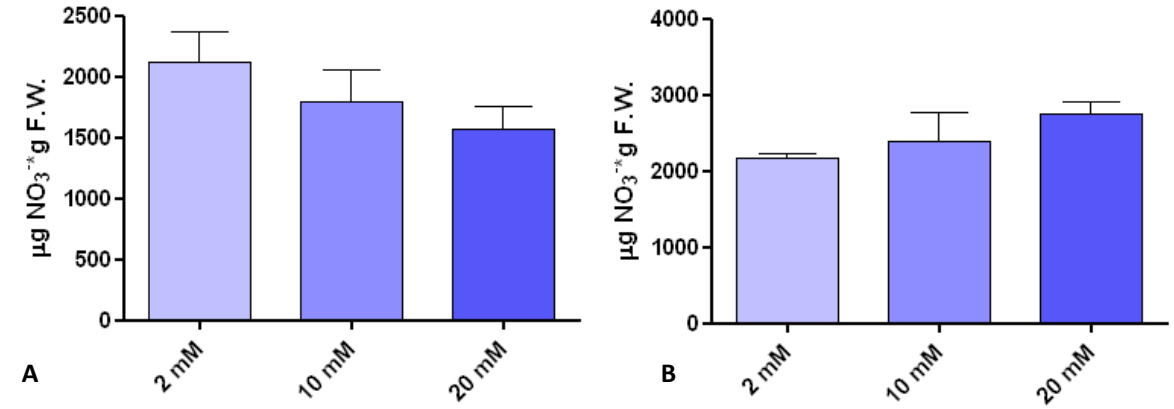

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Fig. 5.9: Content of nitrates in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in May (A) and in June (B). The values are means ± standard errors (n=4).

Also in leaves sampled in October the content of nitrates did not change in the three treatments and the average was about 1955 µg NO_3^-*g^{-1} FW, under the threshold of 5000 µg NO_3^-*g^{-1} FW imposed by European Regulation.

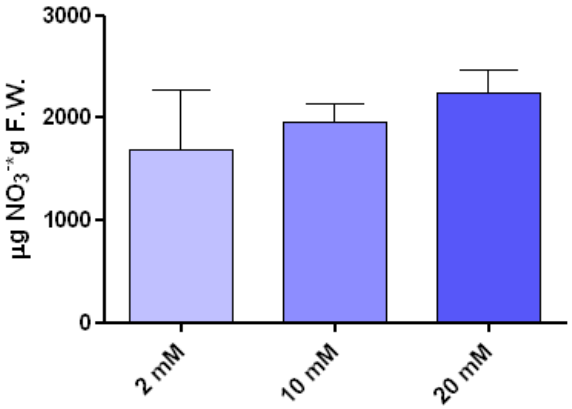

Fig. 5.10: Content of nitrates in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in October. The values are means ± standard errors (n=4).

Nitrate content in rocket

In the experiment of May and June no differences were found among nitrates treatments as shown in the Fig. 5.11. The average of the values was 5330 and 3500 µg NO_3^-*g^{-1} FW of nitrates respectively, under the 6000 µg NO_3^-*g^{-1} FW of threshold fixed by the UE Reg. 1258/2011.

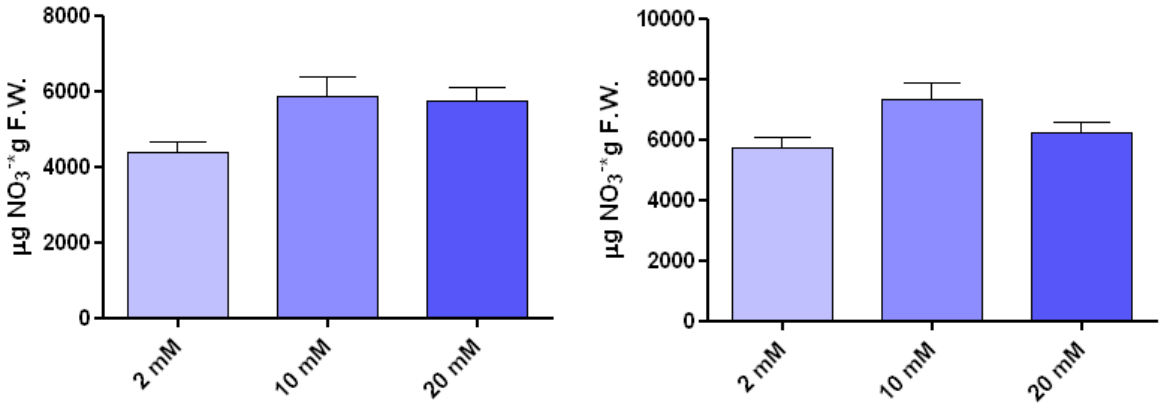

Fig. 5.11: Content of nitrates in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in May (A) and in June (B). The values are means ± standard errors (n=4).

In the autumn the nitrates did not differ among treatments and the values were within the limits imposed.

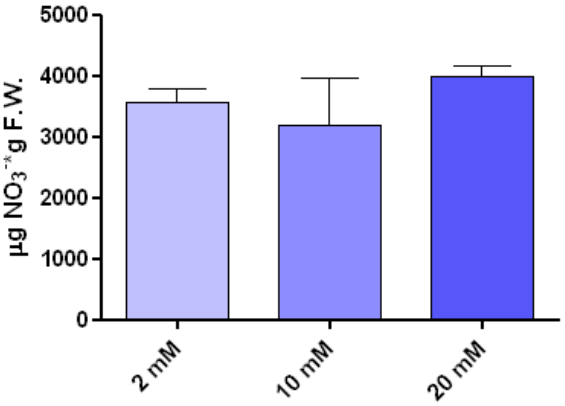

Fig. 5.12: Content of nitrates in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in October. The values are means ± standard errors (n=4).

Enzymatic activity

Nitrate reductase activity in lettuce

Nitrate reductase activity in lettuce did not change among treatments in the harvest of May as shown in Fig 5.13-A; the activity was, in average, of about 110 µg $NO_2^-*g^{-1}$ of fresh weight*$hour^{-1}$. Nitrate reductase showed in the plants cultivated in June a negative dose-response effect, in fact the activity, started high, tend to decrease at the increasing of the nitrate concentration in the nutrient solution. In average the values were 35 µg $NO_2^-*g^{-1}*h^{-1}$.

In autumn the measured activity of NR did not change among different nitrates treatments as shown in Fig. 5.14, and was, in average, about 85 µg $NO_2^-*g^{-1}*h^{-1}$.

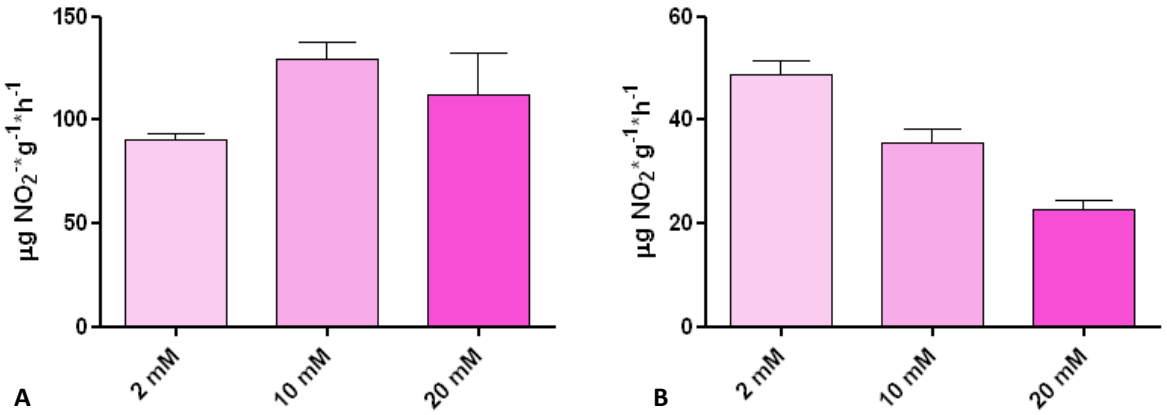

Fig. 5.13: Nitrate reductase activity in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in in May (A) and in June (B). The values are means ± standard errors (n=3)

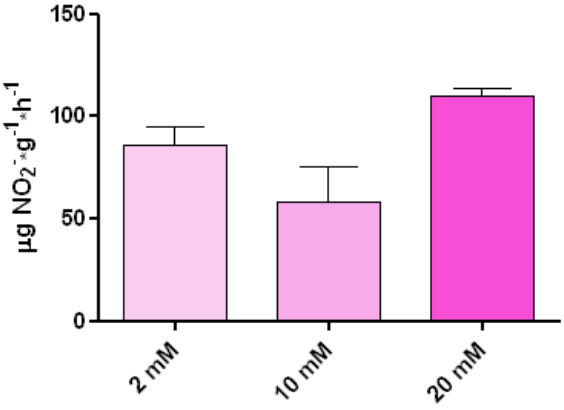

Fig. 5.14: Nitrate reductase activity in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in in October. The values are means ± standard errors (n=3).

Nitrate reductase activity in rocket

Fig. 5.15 shows results obtained in rocket leaves grown in spring-summer; significant difference of the activity was found passing from the 2 mM concentrated nutrient solution to the higher concentrated one. The activity tended to decrease at the increase of the nitrate concentration treatment. In June none significant difference among nitrates treatments was measured. The activities were, in average, lower compared with those measured in May.

The results obtained for the cultivation in October did not show significant differences passing from 2 mM nitrate concentrated nutrient solution to the more concentrated solutions. The activity was 245 µg NO_2^-*g^{-1}*h^{-1} of average, the highest measured among the three harvests.

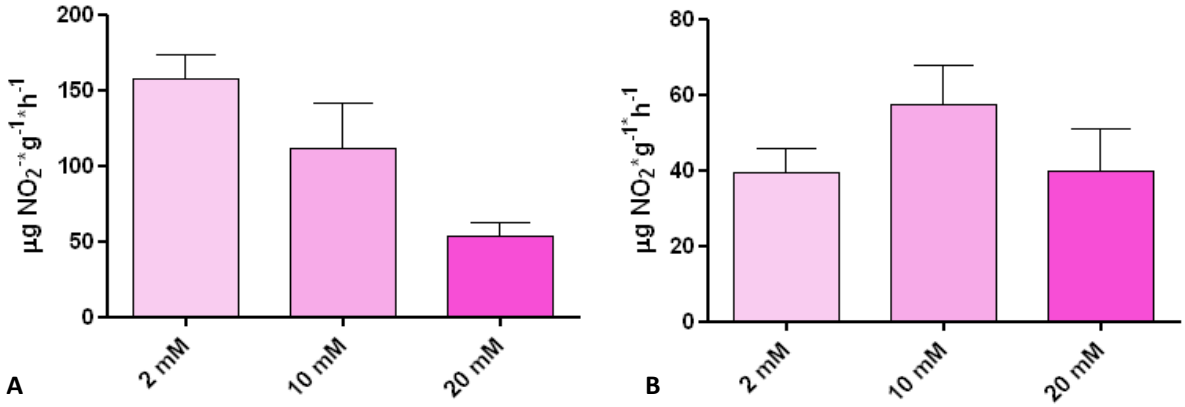

Fig. 5.15: Nitrate reductase activity in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in in May (A) and in June (B). The values are means ± standard errors (n=3).

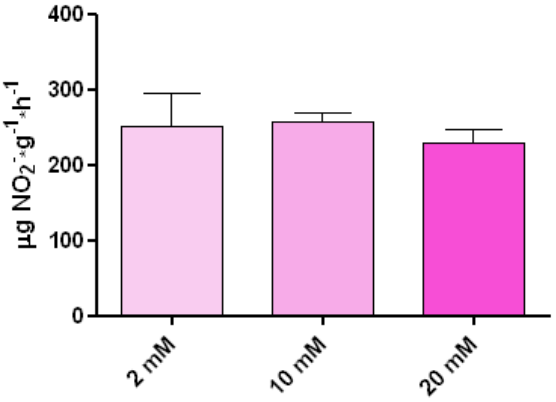

Fig. 5.16: Nitrate reductase activity in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in in October. The values are means ± standard errors (n=3).

Nitrite content

Nitrite in lettuce

In the cultivations carried on in May and June there were not differences among treatments and the average content vas about 2.2 µg $NO_2^-*g^{-1}$ of fresh weight in May and 1.7 µg $NO_2^-*g^{-1}$ of fresh weight in June.

The content of nitrites in the leaves of lettuce harvested in October was constant among treatments and correspond at an average of 2.4 µg $NO_2^-*g^{-1}$ of fresh weight.

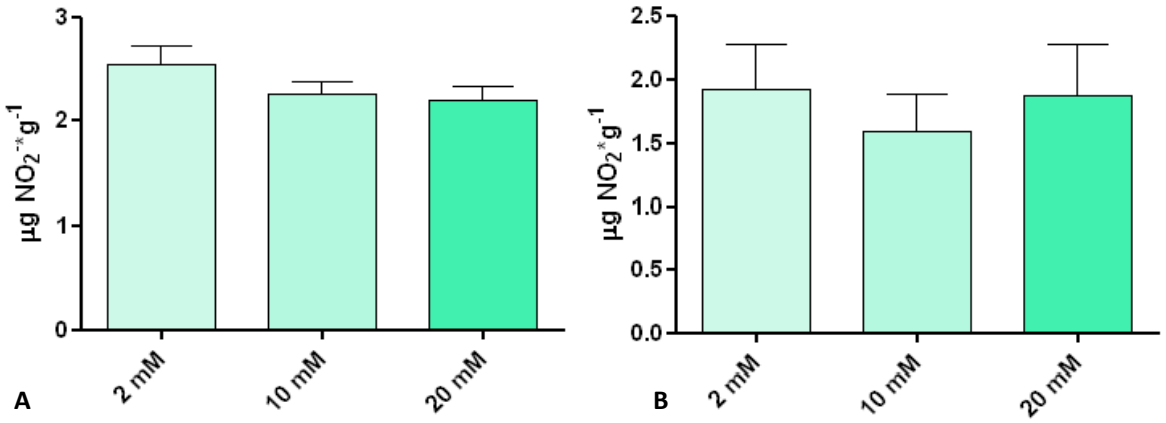

Fig. 5.17: Nitrites in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in in May (A) and in June (B). The values are means ± standard errors (n=3).

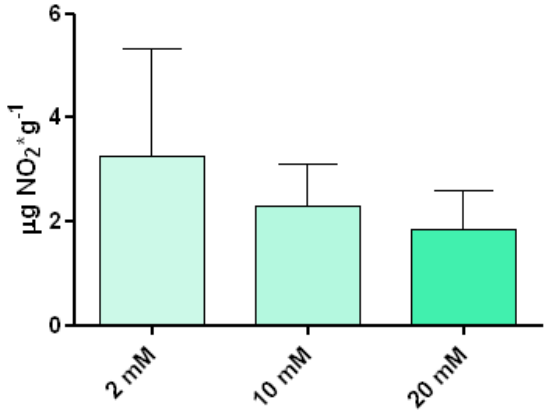

Fig. 5.18: Content of nitrites in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in October. The values are means ± standard errors (n=3).

Nitrite in rocket

The content of nitrites did not change among the different concentrations of nitrates of the nutrient solutions at the harvest of May and June. In May the rate of nitrates was about 1.8 µg $NO_2^-*g^{-1}$ while in June was attested about 1.6 µg $NO_2^-*g^{-1}$.

In October the nitrite content in the plants of rocket not varied passing from 2, 10 and 20 mM nitrate concentrated nutrient solutions, but reach the higher levels compared with all the other experiments with an average of about 4.4 µg $NO_2^-*g^{-1}$.

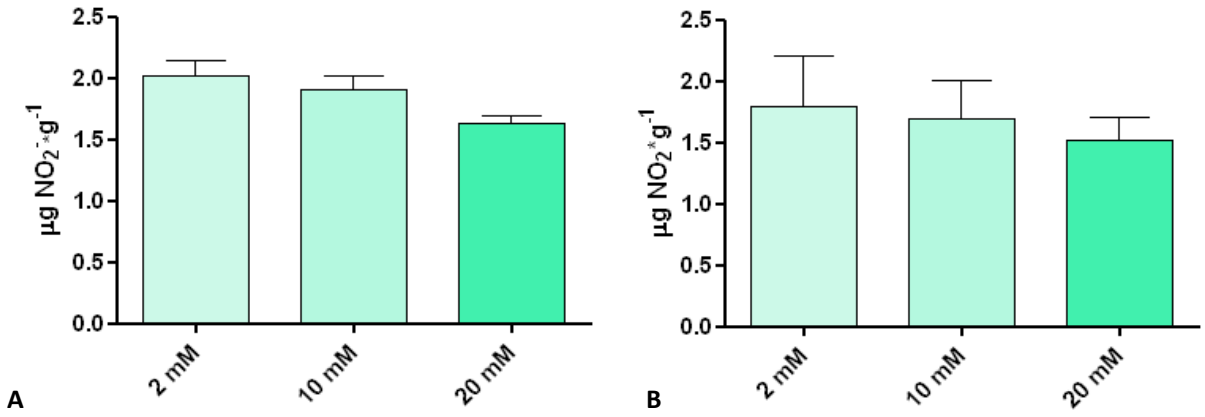

Fig. 5.19: Nitrites in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in in May (A) and in June (B). The values are means ± standard errors (n=3).

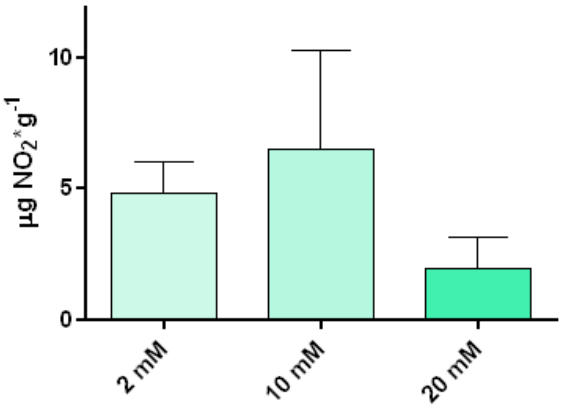

Fig. 5.20: Nitrite in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in in October. The values are means ± standard errors (n=3).

Sugars content

Sucrose in lettuce

The content of sucrose in the leaves sampled in May did not change passing from the lower concentrated nutrient solution to the higher one and the values gave an average of about 0.7 mg*g^{-1} FW.

At the October harvest none significant differences were found in sucrose content, so the leaves showed a quite constant content of sucrose among the different concentrations of nitrates in the nutrient solution with an average of about 0.8 mg*g^{-1} FW.

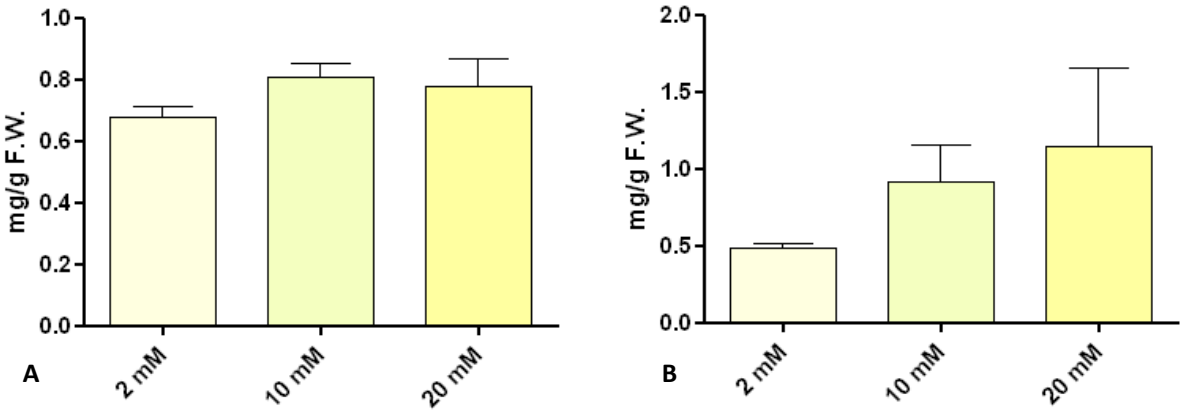

Fig.5.21: Sucrose in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in in May (A) and in October (B). The values are means ± standard errors (n=3).

Sucrose in rocket

The sucrose content in the rocket plants sampled in May was constant among nitrates treatments with values slightly under 1 mg*g^{-1} FW.

In October the content of sucrose in the leaves was lower compared to the contents measured in May and attested about 0.3 mg*g^{-1} FW. No significant changes were detected among treatments.

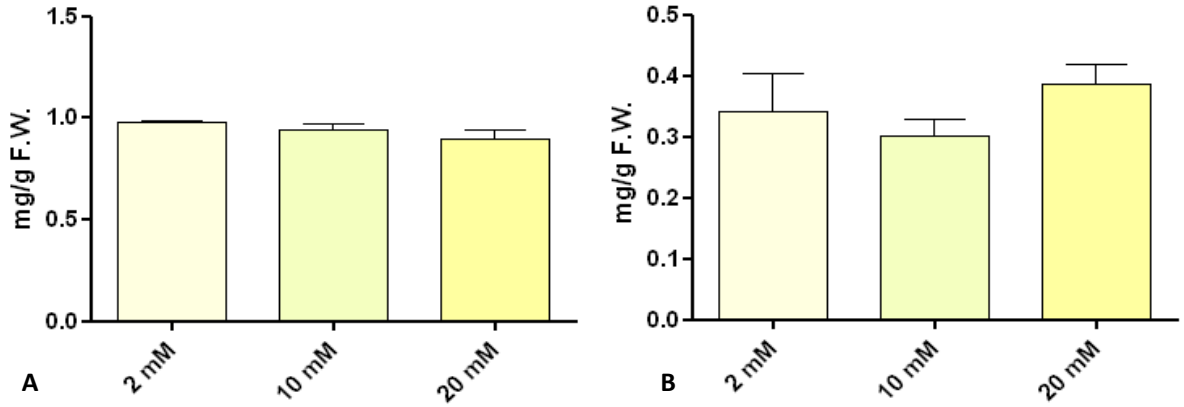

Fig. 5.22: Sucrose in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in in May (A) and in October (B). The values are means ± standard errors (n=3).

Reducing sugars in lettuce

The content of reducing sugars in lettuce tended to increase at the increasing of the concentration of nitrate treatment. Leaves sampled in May showed significant differences between the 10 and the 20 mM concentrated nutrient solution, passing from 1.5 mg*g^{-1} FW to 3.9 mg*g^{-1} FW, and between the lower concentrated solution to the 20 mM, passing from 0.3 to 3.9 mg*g^{-1} FW. In October the trend was confirmed, tended to increase at the

increasing of the nitrates content in the nutrient solutions. Significant difference was found between 2 mM *vs* 10 mM treatments and between 2 mM and 20 mM of nitrates in the nutrient solution.

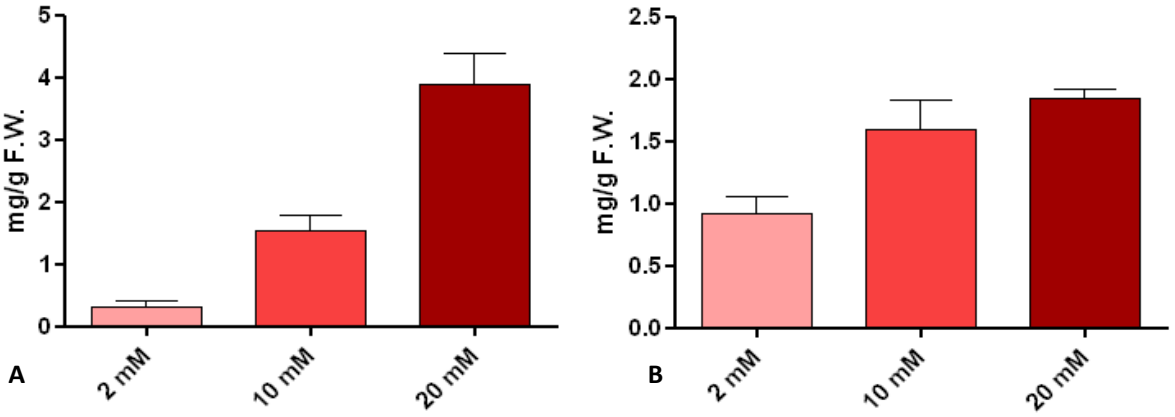

Fig. 5.23: Reducing sugars in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in in May (A) and in October (B). The values are means ± standard errors (n=3).

Reducing sugars in rocket

In the harvest of May the sampled leaves had not different content of reducing sugars for the different concentration of nitrate in the nutrient solution; the average was about 1.5 mg*g^{-1} FW. Also in the sample of October none differences on the reducing sugar content among treatments were observed and the content was around 0.8 mg*g^{-1} FW.

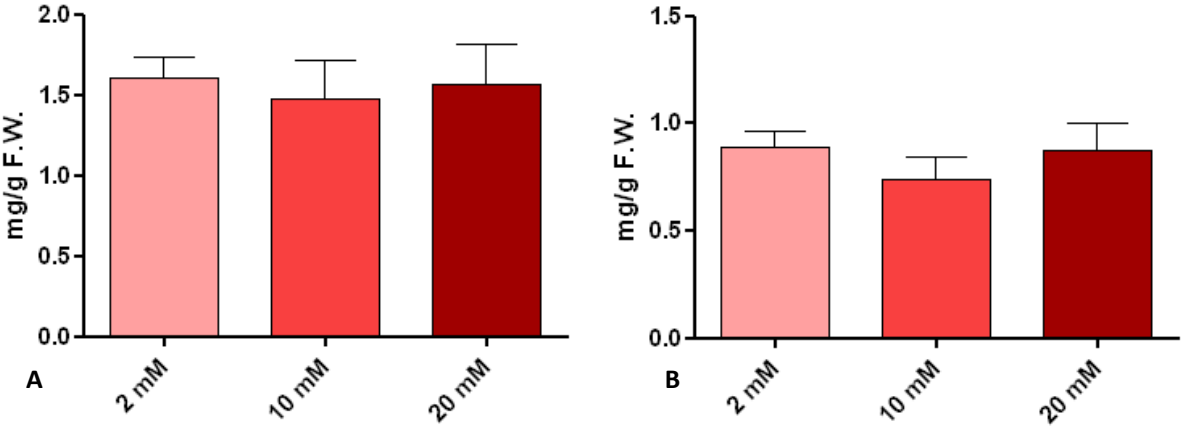

Fig. 5.24: Reducing sugars in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in in May (A) and in October (B). The values are means ± standard errors (n=3).

Total sugars in lettuce

In May no significant differences were found among the different nitrate treatments and the average of 4.3 mg*g^{-1} FW was measured. In October the content of total sugars was constant and the average of the contents was of about 7.6 mg*g^{-1} FW.

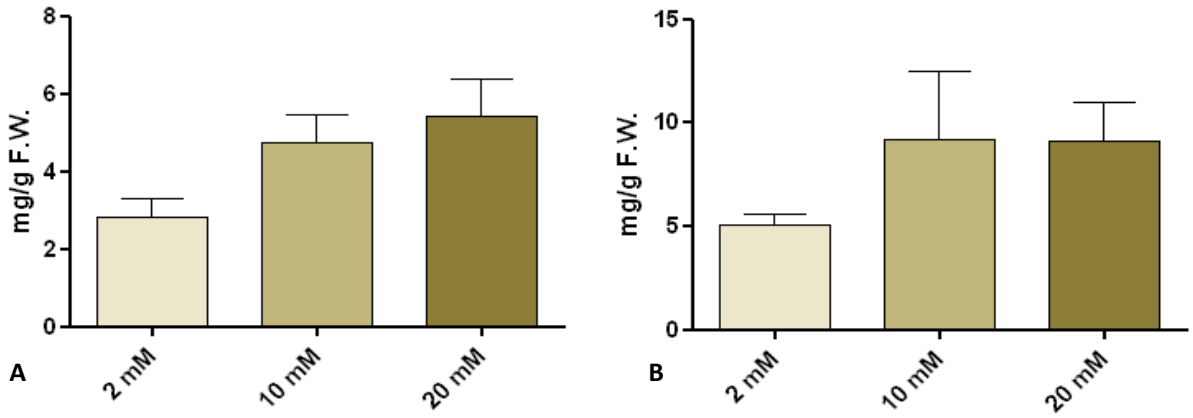

Fig. 5.25: Total sugars in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in in May (A) and in October (B). The values are means ± standard errors (n=3).

Total sugars in rocket

There were no difference among the content of total sugars in rocket grown in May at different concentrations of nitrate in the nutrient solution and the average of the content was about 3.5 mg*g^{-1} FW.

Also for the harvest of October the results showed content of total sugars constant among the treatments and attested about 4.4 mg*g^{-1} FW.

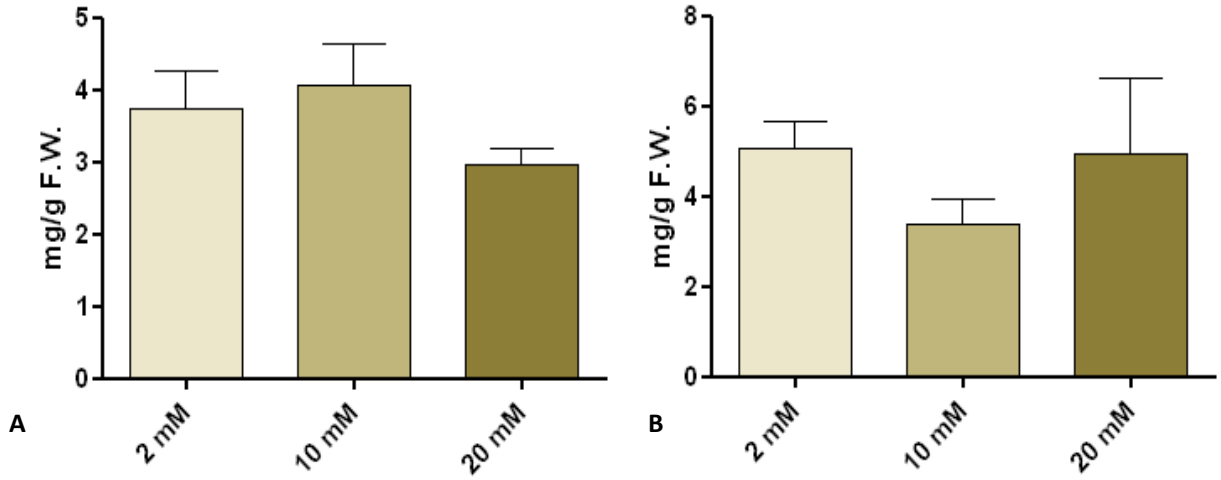

Fig. 5.26: Total sugars in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 2, 10 and 20 mM in in May (A) and in October (B). The values are means ± standard errors (n=3).

Biochemistry of nitrate assimilation in rocket under natural environmental conditions

Nitrates contents

The nitrate contents in rocket leaves sampled under natural circadian rhythm, NCR, reached, at the beginning of the sampling, the higher value of 85 µmol*g^{-1} FW, after 2 hours the content was drastically decreased to 55 µmol*g^{-1} FW and then slightly increased again. Instead, in plants that were adapted to the dark, the content of nitrates at 9 am was around 45 µmol*g^{-1} FW; this content has been kept fairly constant even in leaves taken in the other sampling times as you can see in Fig. 5.27-A.

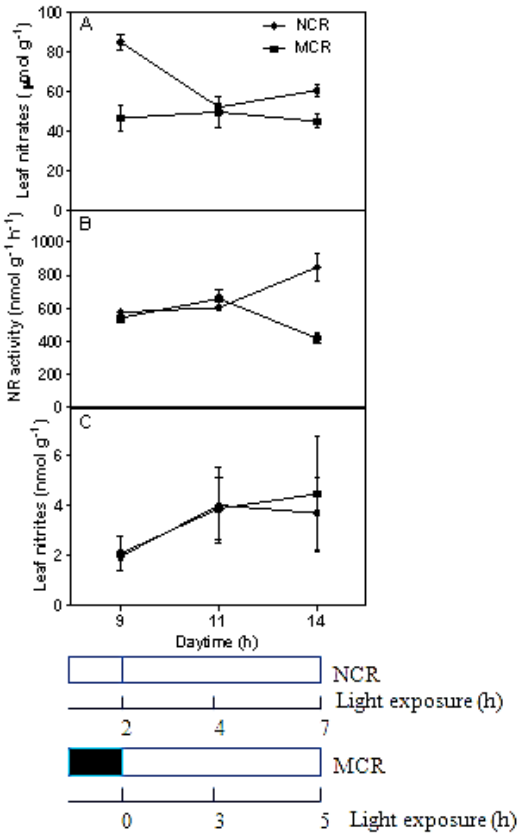

Figure 5.27: Nitrate content (A), NR *in vivo* activity (B) and nitrite content (C) in rocket leaves grown under natural circadian rhythm (NCR) or modified circadian rhythm (MCR). Values are means with standard errors (n=4).

NR activity

The activity of NR at the beginning of the sampling times was slightly less than 600 nmol*g^{-1}*h^{-1} both for the plants under NCR and for the plants under modified circadian rhythm, MCR. This behavior was maintained also after 2 hours of exposure at light, when the measured activity among treatments differed slightly and was fainly higher in plants with MCR. Passing from 4 to 7 hours of light the activity of the enzyme was opposite for the two treatments: in plants under NCR the activity increased passing from 600 to about 800 nmol*g^{-1}*h^{-1}, and for plants placed under MCR the activity decreased to about 400 nmol*g^{-1}*h^{-1}.

Nitrite content

The nitrites at the first time of sampling were low for each treated plants, with 2 nmol*g^{-1} of content. After 2 hours of exposure at light the contents increased in the same way for the two treatments, reaching the 4 nmol*g^{-1} of content. Passing from the 11 am to the 14 the plants under NCR slightly decreased in content of nitrites while the plants under MCR slightly increased their content of nitrites.

Basic research experiments

The second and third year of the PhD project was dedicated to the experiments concerning the basic research activity. The experimental tests were carried out both in greenhouse and in grown chamber, for lettuce and for rocket. For these experiments four different concentrations of nitrates in the nutrient solution were tested: 0.25; 0.5; 1 and 2 mM. The environmental parameters have been measured integrating data provided by Arpa-Lombardia and data obtained from a portable weather sensor posed in greenhouse. 2 cycles of cultivation were performed both for lettuce and rocket in greenhouse and in grown chamber. In order to test the seasonal effects on the measured parameters the cycles were conducted in spring-summer and in autumn-winter for each species of study.

Biochemical analysis of nitrate assimilation in different seasons

The lettuce plants during spring seasons were exposed to from 98 to 185 W*m^{-2} in the first 4 h and lowered to from 79 to 6 h of light, with a mean temperature ranging from 13.5 to 15.2 °C. In autumn-winter the lettuce plants were grown under 29 to 150 W*m^{-2} with a temperature ranging from 12.1 to 16.5 °C. Rocket plants in summer (June) were grown under 217 to 766 W*m^{-2} from 07:00 to 13:00, with a temperature ranging from 20 to 26.1 °C (Tab. 5.2). In winter time (January) plants were harvested with a light intensity ranging 6 to 267 W*m^{-2} and a temperature ranging from 3.1 to 6.9 °C.

Species	Sampling times	Hours of exposure at light	SEASONS					
			spring-summer			**autumn-winter**		
			Month of sampling	Radiation (W/m^2)	Temperature (°C)	Month of sampling	Radiation (W/m^2)	Temperature (°C)
LETTUCE	First	0	April	98	13.5	October	29	12.1
	Second	2		106	14.4		147	11.3
	Third	4		185	15.2		150	14.8
	Fourth	6		79	14.1		150	16.5
ROCKET	First	0	June	217	20	January	6	3.1
	Second	2		537	21.1		152	4.6
	Third	4		791	23.5		256	5.9
	Fourth	6		766	26.1		267	6.9

Tab. 5.2: Environmental parameters measured referring to the times of sampling of plants grown in the greenhouse in two different seasons. Integrated data from Arpa-Lombardia and Dott. Parisi S., University of Milan.

Nitrate, NR activity, nitrite and sugars content.

Lettuce in spring

The effects of nitrate concentrations were evaluated in different seasons in both species in order to understand the physiological behavior of plants in the nitrate organication. The first sampling point was performed at

darkness in order to have the plants without nitrate assimilation. The differences among the treatments were not statistically different, but an increase of the nitrate content was observed in the plants grown with 0.25, 0.5 and 2 mM (Fig. 5.28), while a slight reduction was observed in the 1 mM treatment. The values of nitrates were comprised from 1800 to 2700 mg*kg^{-1} FW.

The activity of nitrate reductase was significantly different only between the 0.25 and the 1 mM at dark, with values, respectively of 28 µg NO_2^-*g^{-1}*h^{-1} FW and 5.4 µg NO_2^-*g^{-1}*h^{-1} FW.. The activity was low in all the treated plants and constant during the time course.

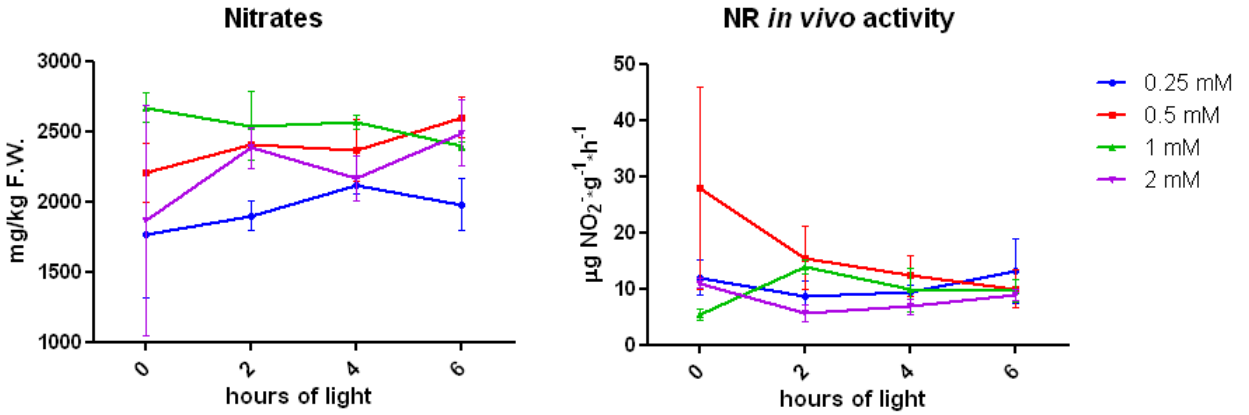

Fig. 5.28: Content of nitrates and NR *in vivo* activity in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in spring. The values are means ± standard errors (n=3).

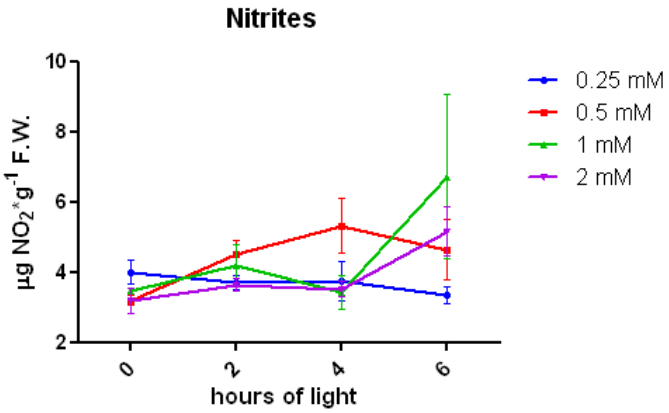

Fig. 5.29: Content of nitrites in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in spring. The values are means ± standard errors (n=3).

The nitrite contents were low and ranged from 3 to 6.7 mg*kg^{-1} FW. In the 0.25 mM the amount did not change during the time course, while in the treatments with 2 and 1 mM increased after 6 hours of light. On the contrary, the treatments with 0.5 mM decreased until 4 h of light exposure (Fig. 5.29).

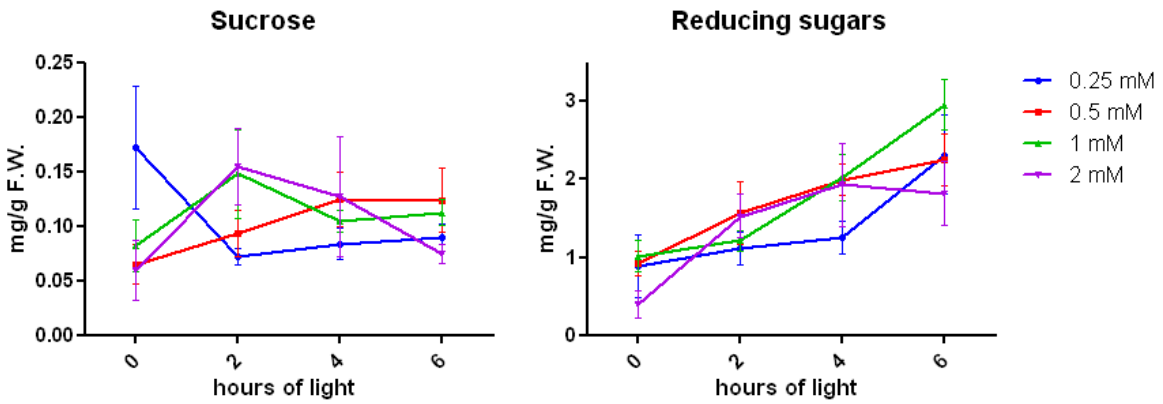

Fig. 5.30: Content of sucrose and reducing sugars in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in spring. The values are means ± standard errors (n=3).

The sucrose content in darkness at first time sampling was higher in the 0.25 mM and lower in the other treatments. The sucrose ranged in the different treatments and in the different time points from 0.06 to 0.2 mg*g^{-1} FW (fig. 5.30). The sucrose in leaves treated with 0.25 mM of nitrate immediately declined after 2 h and remained constant. In the treatments with 1 or 2 mM the sucrose content in leaves increased after 2 h of illumination and then slightly decreased. The plants treated with 0.5 mM of nitrate showed an increase of sucrose until 4 h and then remained constant.

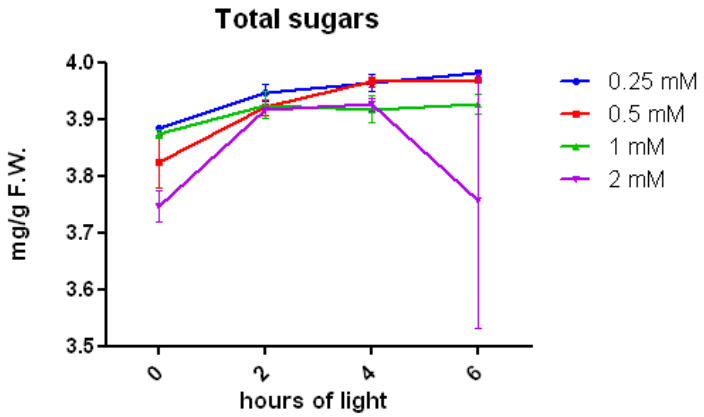

Fig. 5.31: Content of total sugars in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in spring. The values are means ± standard errors (n=3).

The reducing sugars increased progressively in all treatments with the increase of light exposure. The lowest value was observed in 2 mM at dark (0.39 mg*g^{-1} FW) and the highest (2.9 mg*g^{-1} FW) was found after 6 h at 1 mM treatment (Fig. 5.30). The total sugars did not show any significant changes during the whole experimental period and ranged from 3.7 to 4.0 mg*g^{-1} FW (Fig. 5.31).

Rocket in spring

The rocket plants are hyper accumulator of nitrate content in leaves and different nitrate concentrations and light exposure affected the nitrate assimilation in plants.

The nitrate concentrations in leaves ranged from 4000 mg*kg^{-1} FW to 7500 mg*kg^{-1} FW. (Fig. 5.32). In the darkness nitrate content was lower in the 0.25 and 0.5 mM nitrate treatments with an average of 5500 mg*kg^{-1} FW, while in the 1 and 2 mM treatments was higher with 6500 mg*kg^{-1} FW. The nitrate content was higher in

plants exposed to higher concentration of nitrates in the nutrient solutions. The differences were significant after 2 and 4 h of light exposure. The nitrate content increased during the light exposure in the 2 mM treatments until 4 h, then declined with concentrations lower than the first sampling (0 hours of light).

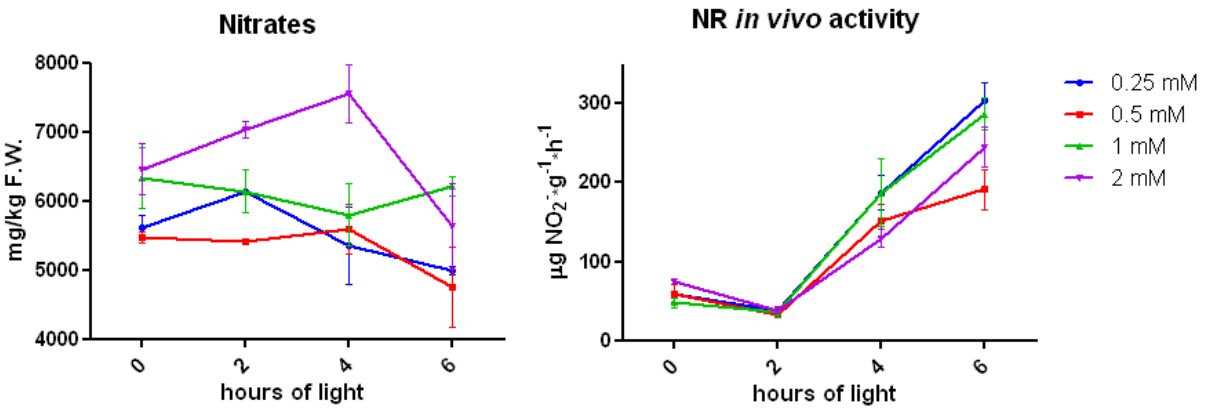

Fig. 5.32: Content of nitrates and NR *in vivo* activity in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in spring. The values are means ± standard errors (n=3).

The NR *in vivo* activities were very low below 100 µg NO_2^-*g^{-1}*h^{-1} FW until 2 h of light exposure. After 2 h the NR *in vivo* activities increased in all treatments until 6 h. The highest values were found at 0.25 mM and 1 mM, while in 0.5 and 2 mM were lower (Fig. 5.32).

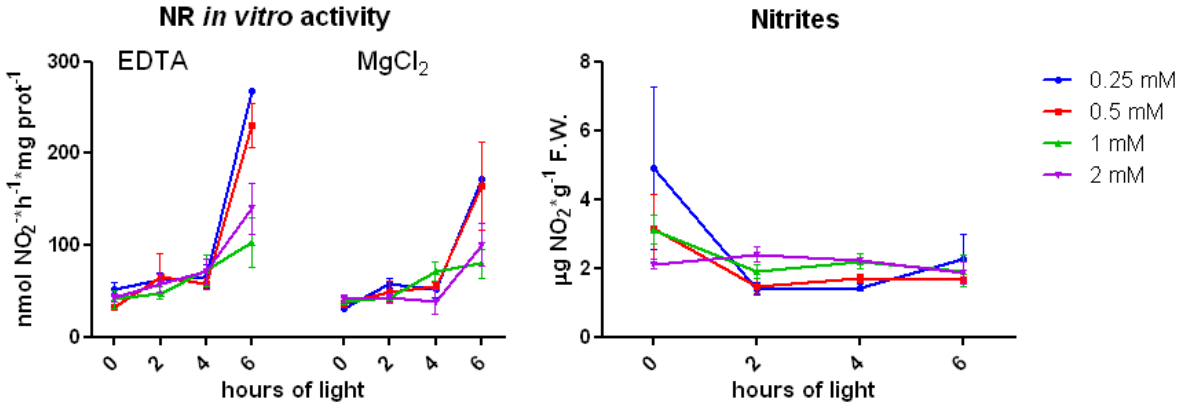

Fig. 5.33: Content of NR *in vitro* activity and nitrites in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in spring. The values are means ± standard errors (n=3).

The NR *in vitro* activities, the total and active forms in rocket leaves of all treatments were similar, with a sharp increase after 4 h especially in 0.25 and 0.5 mM treatments. The values of total NR activities in dark conditions were in average 32-51 nmol NO_2^-*h^{-1}*mg $protein^{-1}$ (Fig. 5.33) analogous results were found in the active form but with lower values.

The nitrite contents were higher in plants grown with lower nitrate concentrations and declined during the time course. In plants exposed to 2 mM of nitrate concentration did not change during the time course (Fig. 5.33).

The sucrose content in leaves of plants placed in 0.25 mM of nitrate remained almost constant, with increase in 1 mM treatment. In leaves of plants treated with 2 mM the sucrose increased after 2 h and declined maintaining the values of the first sampling point.

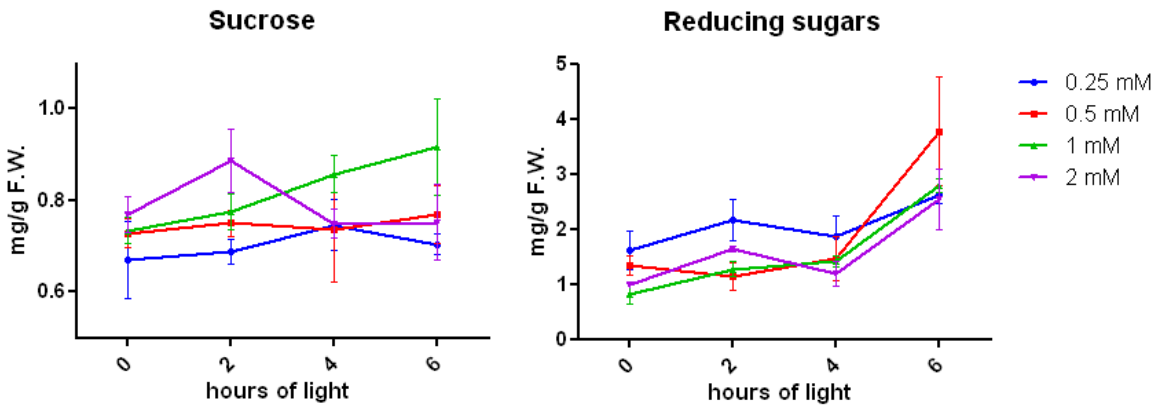

Fig. 5.34: Content of sucrose and reducing sugars in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in spring. The values are means ± standard errors (n=3).

Reducing sugars increased after 4 h and no significant changes were observed among treatments. The values ranged from 1 to 4 mg*g^{-1} FW (Fig. 5.34).

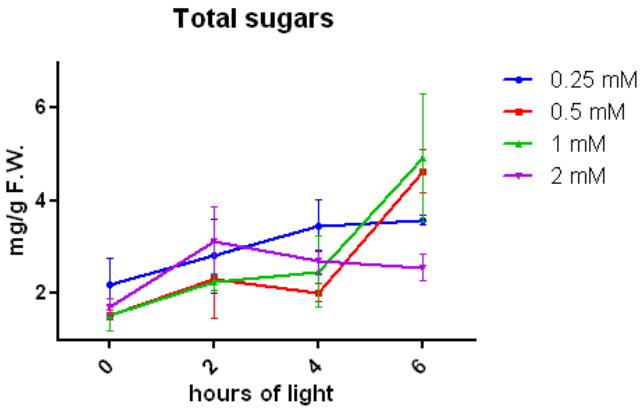

Fig. 5.35: Content of total sugars in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in spring. The values are means ± standard errors (n=3).

Total sugars increased in all treatments, the highest values were found after 6 h of light exposure in 0.5 and 1 mM of nitrate concentration in the nutrient solution (Fig. 5.35). The total sugars ranged from 1.8 to 5 mg*g^{-1} FW.

Lettuce in autumn

The lettuce grown in the autumn and placed on nutrient solutions with different nitrate concentrations did not show significant differences among treatments. At the dark sampling the nitrate content in leaves ranged from 2000 to 3000 mg*kg^{-1} FW (Fig. 5.36). The plants treated with 0.5 and 1 mM of nitrate increased the nitrate content after 2 to 4 h, while declined after 6 h. The 0.25 mM and 2 mM treatments remained constant during the whole experimental period. The NR *in vivo* activities were low during the first two sampling times and increased after 4 h and at 6 h the values reached 800 µg NO_2^-*g^{-1}*h^{-1} in the treatments with 1 and 2 mM, while 500 and 600 µg NO_2^-*g^{-1}*h^{-1} were found in 0.25 and 0.5 mM, respectively (Fig. 5.36).

The lowest values of total NR *in vitro* activity at 0 h light exposure were observed in 0.25 and 1 mM of nitrate concentration treatments. In all treatments the NR activities declined after 4 h of illumination and increased after 6 with the exception of the 2 mM treatment. In this treatment the NR activity showed the highest value after 2 h

(Fig. 5.37). The active form of NR showed lower oscillations in the 2 mM and the nitrate concentration remained constant, while in 0.25, 0.5 and 1 mM the NR activity had the same trend of the total NR activity (Fig. 5.37).
The content of nitrites in the leaves (Fig. 5.37) did not showed significantly differences among nitrate treatments and, after a slightly decrease found during 2 h of exposure at light they maintained constant their values.

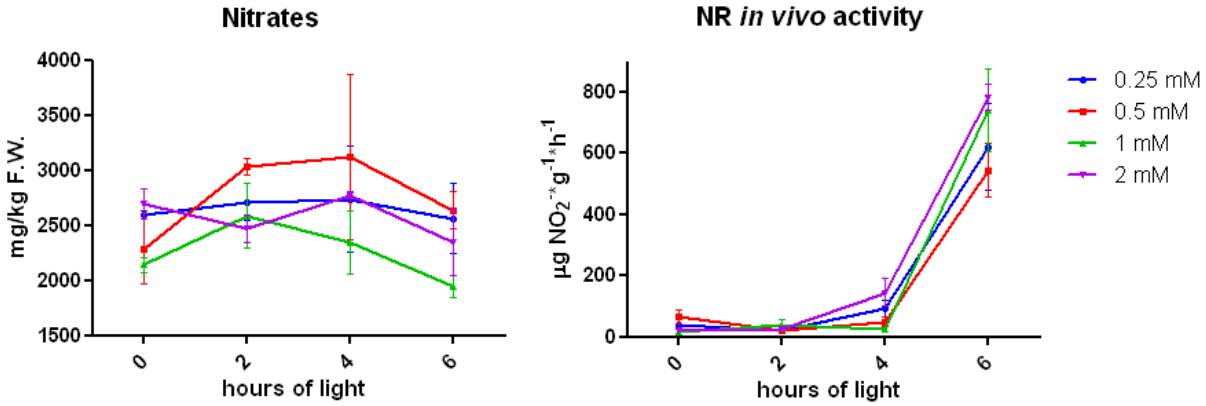

Fig. 5.36: Content of nitrates and NR *in vivo* activity in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in autumn. The values are means ± standard errors (n=3).

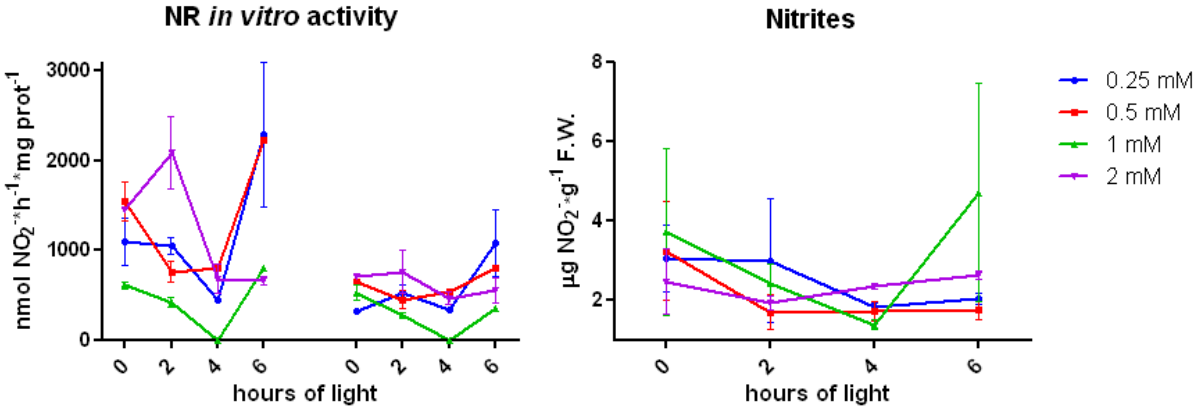

Fig. 5.37: Content of NR *in vitro* activity and nitrites in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in autumn. The values are means ± standard errors (n=3).

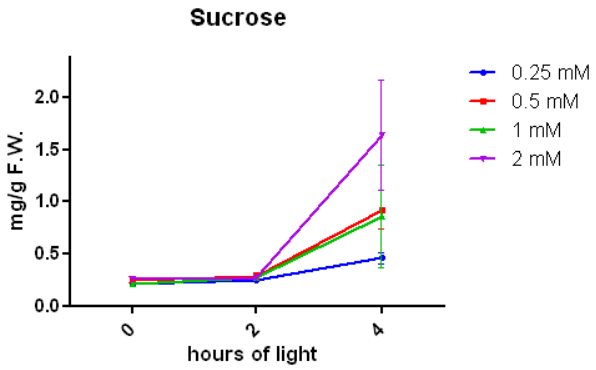

Fig. 5.38: Content of sucrose in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in autumn. The values are means ± standard errors (n=3).

Sucrose content was not affected by treatments during the first two h then increased and the highest value, 1.5 mg*g^{-1} FW was found in the 2 mM treatment (Fig. 5.38). The reducing sugars content progressively increased with light exposure up to 4-5 folds (Fig. 5.39).

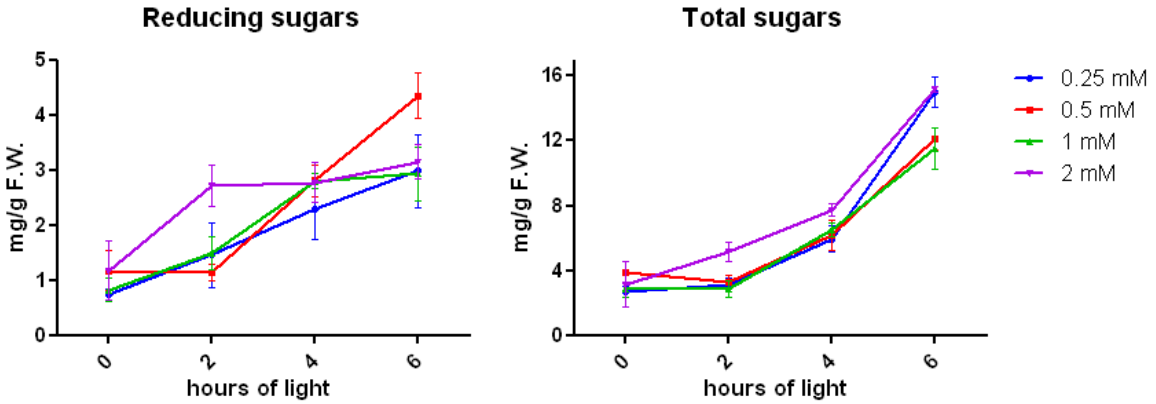

Fig. 5.39: Content of reducing sugars and total sugars in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in autumn. The values are means ± standard errors (n=3).

Total sugars showed the same trend of reducing sugars. The total sugars immediately increased with illumination in leaves of plants treated with 2 mM, while in all other treatments increased after 2 h of light exposure. After 6 h the highest values in average 15 mg*g^{-1} FW were observed in 0.25 and 0.5 mM. Treatments with intermediate nitrate concentrations (0.5 and 1 mM) had in average 12 mg*g^{-1} FW (Fig. 5.39).

Rocket in winter

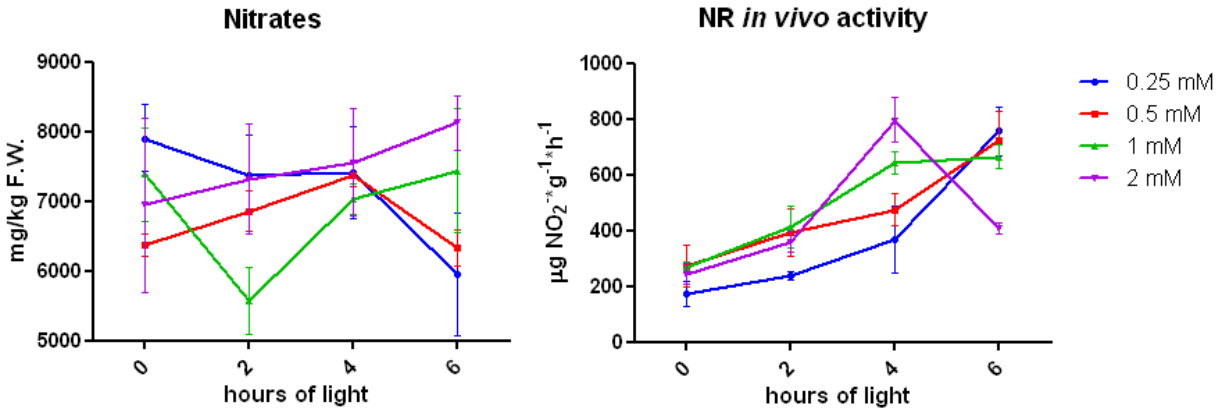

Fig. 5.40: Content of nitrates and NR *in vivo* activity in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in winter. The values are means ± standard errors (n=3).

The rocket grown in winter showed different effect on the nitrates accumulation in plants treated with the nutrient solutions at various concentrations of nitrates at dark. The highest value, 8000 mg*kg^{-1} FW was on the 0.25 mM, the lower was in the 0.5 mM with 6300 mg*kg^{-1} FW; the other treatment, 1 and 2 mM, had intermediate values of ranging 7000-7500 mg*kg^{-1} FW. The plants treated with the 1 mM of nitrates reduced the content to 5700 mg*kg^{-1} FW at the second sampling time, but then increased to the initial values. The 0.25 mM

treated plants reduced the content of nitrates throughout the period of sampling to 6.3 while the nitrates slightly increased in the leaves subjected to the highest level of nitrates (2 mM) in the nutrient solution passing from 6900 mg*kg^{-1} FW found in the dark to 8000 mg*kg^{-1} FW at 6 h of light (Fig. 5.40).

The NR *in vivo* activity (Fig. 5.40) was very low at dark, with lowest value at 0.25 mM with 172 µg NO_2^-*g^{-1}*h^{-1} and highest value at 0.5 mM with 272 µg*NO_2^-*g^{-1}*h^{-1}. During the time course all the treatment increased; 0.25, 0.5 and 1 mM nitrate concentration treatment affect the NR *in vivo* activity found in these leaves by tripling. The most concentrated treatment, 2 mM, increased to 795 µg NO_2^-*g^{-1}*h^{-1} until 4 h of light exposure, and then heavily decreased to 408 µg NO_2^-*g^{-1}*h^{-1}.

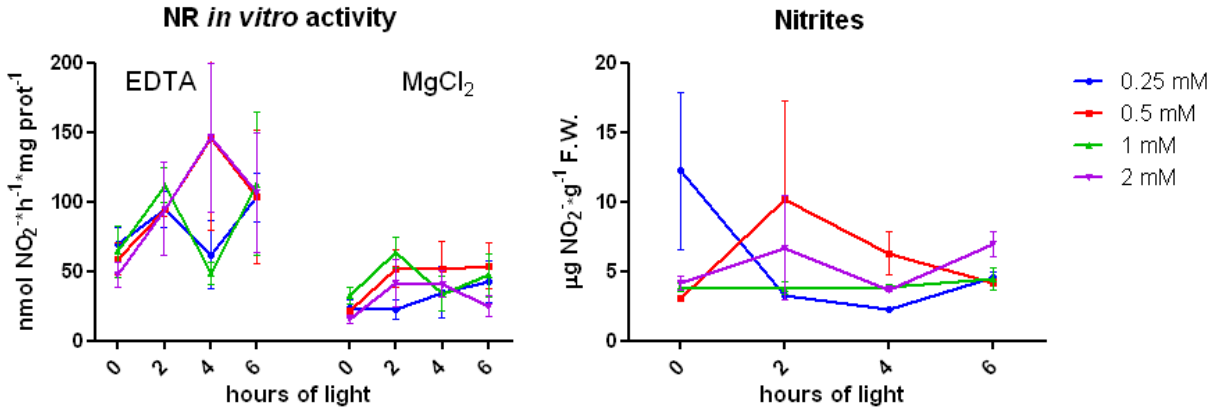

Fig. 5.41: Content of NR *in vitro* activity and nitrites in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in autumn. The values are means ± standard errors (n=3).

The total NR activity was low at dark for the most concentrated treatment (2 mM) while the others had similar activity of 64 nmol NO_2^-*h^{-1}*mg protein in average. During the sampling times the 2 mM treatment increased the NR activity and reached 146 nmol NO_2^-*h^{-1}*mg^{-1} protein at 4 h, then decreased to 106 nmol NO_2^-*h^{-1}*mg^{-1} protein. The other treatments showed oscillatory trend during the sampling times, with similar activity values; at the end of the sampling time all the treatments doubled their activity compared to the initial values. The active form of NR at dark had the lowest activity in plant with 2 mM nitrate concentration, as happened for the total NR activity. The treatment with the lowest concentration of nitrate in the nutrient solution slightly increased the active NR activity in the leaves after 2 h of light exposure. The plants treated with 0.5 and 1 mM nitrate showed similar trends during the time course, with an initial increase followed by assets rather constant. The plants under 1 mM treatment showed an oscillatory pattern of the NR activity during the sampling, reached the highest NR activity, of 63 nmol NO_2^-*h^{-1}*mg^{-1} protein after 2 h of exposure at light. At the last sampling time the most concentrated treatment showed the lowest NR activity, of 25 nmol NO_2^-*h^{-1}*mg^{-1} protein (Fig. 5.41).

Nitrites was high at dark, 12 µg NO_2^-*g^{-1} FW, in plants treated with the lowest concentration of nitrates and was low for the other treatments with 3.6 µg*NO_2^-*g^{-1} FW in average (Fig. 5.41). The content of nitrites decreased in 0.25 mM treated plants to 4.6 µg NO_2^-*g^{-1} FW, while, for the other treatments, the content was slightly increased after 6 h of exposure at light. The nitrites in the leaves treated with the most concentrated nutrient solution showed an oscillatory trend during the time course, 1 mM showed a quite constant trend and in the 0.5 mM concentration nitrites strongly increased at the second sampling time, but then decreased to 4.1 µg NO_2^-*g^{-1} FW, the lowest content found at 6 h of light exposure.

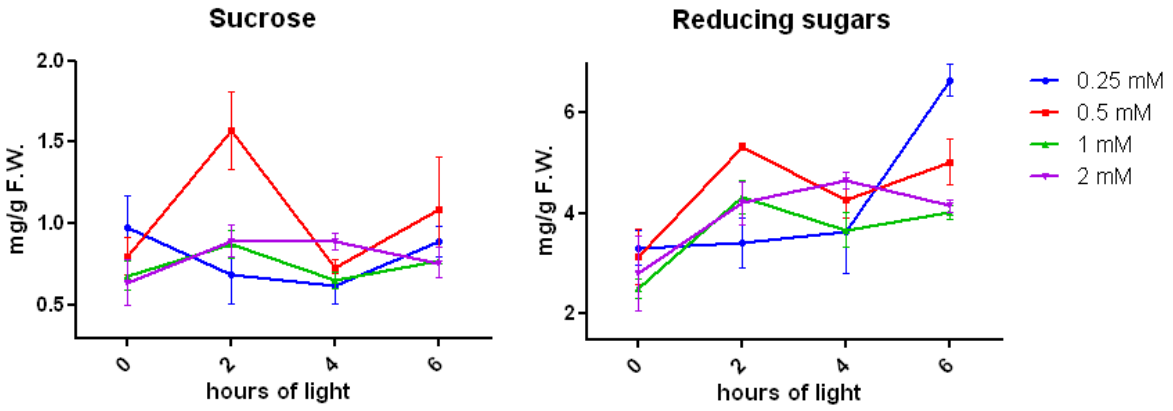

Fig. 5.42: Content of sucrose and reducing sugars in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in winter. The values are means ± standard errors (n=3).

The content of sucrose found at dark and also at the 6 h exposure at light was inversely proportional to the concentration of nitrates in the nutrient solutions, having the highest value for the 0.25 mM of about 1 mg*g^{-1} FW. During the time course the 0.25 and 2 mM treatment showed opposite trends while 0.5 and 1 mM treatment showed similar oscillatory trends. The highest content of sucrose was in the plants treated with 0.5 mM nitrates, with highest value after 2 h of light exposure, of 1.6 mg*g^{-1} FW (Fig 5.42).

Reducing sugars at dark was higher in the lower concentrated treatments, with values of about 3 mg*g^{-1} FW, the lowest content of reducing sugars was 2.5 mg*g^{-1} FW, found in plants at 1 mM nitrate concentration. All the treatment increased their initial content of reducing sugars, showing varying trends along the sampling times. The lowest concentrated treatment markedly increased passing in the last phase of exposure to light, reaching 6.6 mg*g^{-1} FW of reducing sugars content(Fig 5.42).

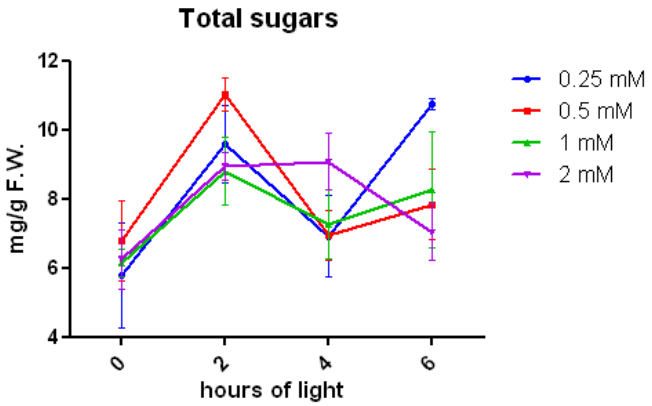

Fig. 5.43: Content of total sugars in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in winter. The values are means ± standard errors (n=3).

Total sugars in rocket (Fig. 5.43) showed similar oscillatory trend for the 0.25, 0.5 and 1 mM nitrate treatments. The plants put in the highest concentrated solution increased the reducing sugar from dark to 2 h of light, as shown also in the other treatments, but the sugars content strongly decreased with the highest illumination exposure to 7 mg*g^{-1} FW, contrary to what happens for the other treatments. The 0.25 mM treated plants

doubled their content of sugars at 6 h of illumination with 10.7 mg*g^{-1} FW. The highest content of sugars was found in the 0.5 mM nitrate treatment of 11 mg*g^{-1} FW.

Biochemical and molecular studies of nitrate assimilation in rocket and lettuce grown in controlled environment

Nitrate, NR activity, nitrite and sugars content.

Lettuce (first biological replicate)

Since the biochemical parameters vary widely and are affected by changes in the environmental conditions the cultivation of lettuce and rocket were carried out in the growth chamber in order to discriminate the effect of the nitrates concentration in the nutrient solutions by the environmental effects.

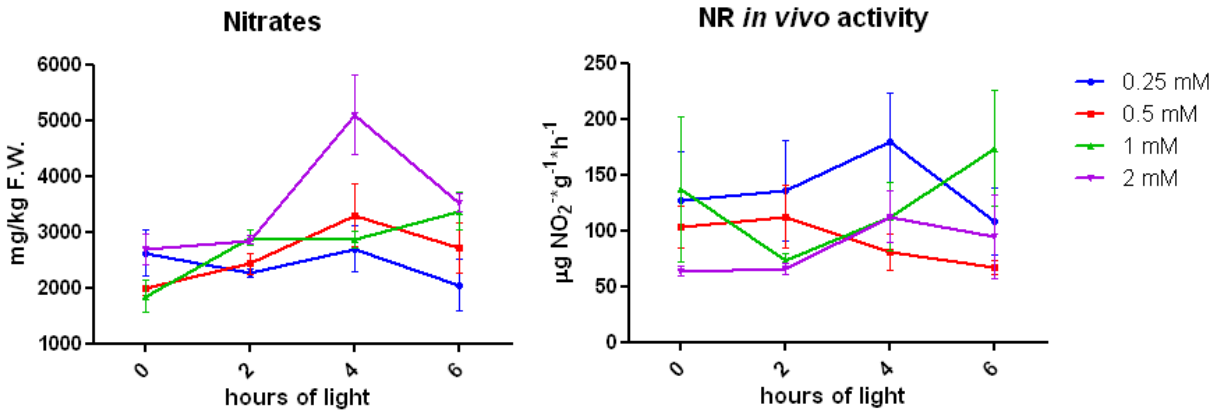

Fig. 5.44: Content of nitrates and NR *in vivo* activity in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

At the dark the higher content of nitrate in lettuce was about 2600 mg*kg^{-1} FW, found in plants treated with 0.25 and 2 mM nitrates in the nutrient solution. Significantly differences among treatments were evident at 4 hours of light among the 2 mM treatment and the others. All the plants treated increased their content of nitrates after 6 h of exposure at light (2400 mg*kg^{-1} FW in average), with the exception for the lowest concentrated, where the nitrates slightly decreased passing from an initial value of 2600 mg*kg^{-1} FW to 2050 mg*kg^{-1} FW and was significantly different from the content found in the 2 mM at 4 and 6 h of light. Higher nitrates content were found in the plants under the most concentrated treatment, where a peak of 5089 mg*kg^{-1} FW was measured after 4 h of light; this value exceeds the maximum permissible threshold of nitrate in lettuce imposed by UE Regulation n. 1258/2011 of 5000 mg*kg^{-1} FW and was statistically different from the contents found in the other treatments (Fig.5.44).

No significant difference was found in the NR *in vivo* activity among the treatments. The NR *in vivo* activity at dark was very low for the highest concentrated nutrient solution, of 64 µg NO_2^-*g^{-1}*h^{-1}; the highest activity, of 136 µg NO_2^-*g^{-1}*h^{-1}, was in the plants treated with the 1 mM solution. The 0.25 and 2 mM treatments showed similar oscillatory trends in the NR *in vivo* activity, with a decrease in the last part of the sampling. The activity slightly decreased in the 0.5 mM nitrate treatment during the time course, oppositely at the increased observed in the leaves under 1 mM nitrate treatment (Fig.5.44). The total NR *in vitro* activity was very high of 522 and 313 nmol NO_2^-*h^{-1}*mg^{-1} protein in the plants treated with 0.25 and 0.5 mM nitrate solutions and these values were

statistically different; the activity for the most concentrated solutions was about 90 nmol NO_2^-*h^{-1}*mg^{-1} protein in average. The differences found among treatments at low and high concentration of nitrates were statistically significant. The 0.5 mM nitrate treatment showed opposite trends than the others and the difference was significant at 2 h of light exposure. At the end of the sampling times the total NR activity decreased for the 0.25 and 0.5 mM treatments (281 and 163 nmol NO_2^-*h^{-1}*mg^{-1} protein respectively), increased for the 1 mM one (137 nmol NO_2^-*h^{-1}*mg^{-1} protein) and remained constant in the 2 mM treated plants.

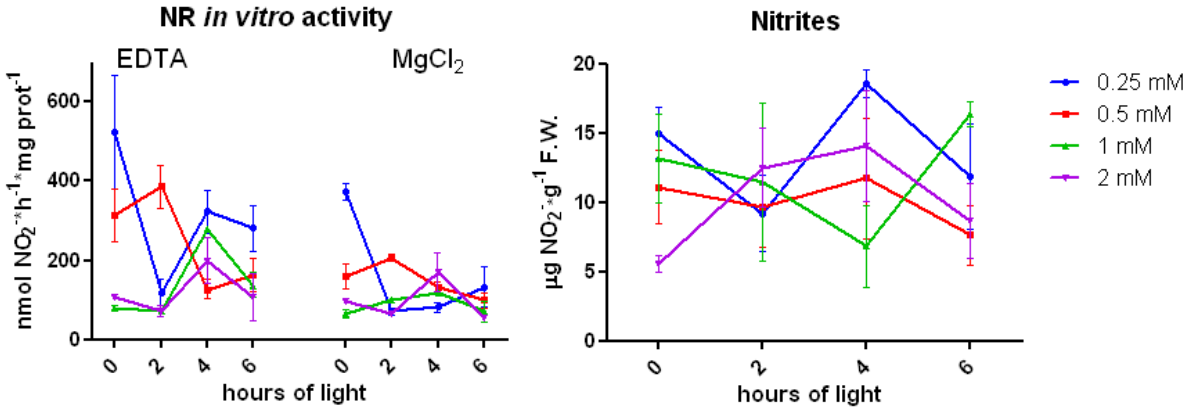

Fig. 5.45: Content of NR *in vitro* activity and nitrites in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

The active NR activity found in leaves treated with 0.25 mM was significantly different from the activity found in the others treatments at dark and was very high, 372 nmol NO_2^-*h^{-1}*mg^{-1} protein (Fig. 5.45). The intermediate value of activity was found in 0.5 mM treated plants, 158 nmol NO_2^-*h^{-1}*mg^{-1} protein and was significantly different from the value found in the 1 mM one, of 62 nmol NO_2^-*h^{-1}*mg^{-1} protein. The activities found in the plants under low concentration of nitrates in the nutrient solutions were opposite, but both decreased their activity at the end of the test, as happened in leaves treated with the most concentrated nutrient solution. The 1 mM nitrate concentration maintained the value of active activity quite constant during the time course.

The highest nitrite content, 15 µg NO_2^-*g^{-1} FW, was found in the plants under the lowest nitrate concentration in the nutrient solution and the lowest content of 5.6 µg NO_2^-*g^{-1} FW, was in the plants treated with the 2 mM nutrient solution (Fig. 5.45). The content of nitrite is variable and followed different oscillatory trends depending by the treatment, the higher value, 18.5 µg NO_2^-*g^{-1}*FW, was in the 0.25 mM treatment after 4h of exposure at light, fell by the content found in the 1 mM treatment at the end of the sampling, of 16,3 µg NO_2^-*g^{-1} FW. The 1 mM presented a pattern opposite to the 2 mM one during the time course. At 6 h of exposure at light the samples from the low nitrate treatments decreased their content of nitrites of 3-4 µg NO_2^-*g^{-1} FW, while the others increased the content with the same rate.

Sucrose content was comprised in a range of values of 0.18 and 0.27 mg*g^{-1} FW at dark and during the time sampling gradually decreased in the plants treated with the lowest nitrate concentrations of the nutrient solutions, until 4 h of light. There was a difference between the 1 and 2 mM treatment in the content of sucrose, after 2 h light. All the treatments increased the content of sucrose passing from 4 to 6 h of light but only the 2 mM treatment increased its initial level of sucrose, the others gave, in average, 0.1 mg*g^{-1} FW less than the initial values. Significant differences were among the 1 and 2 mM treatment and the 0.25 mM after 6 h of exposure at light (Fig. 5.46).

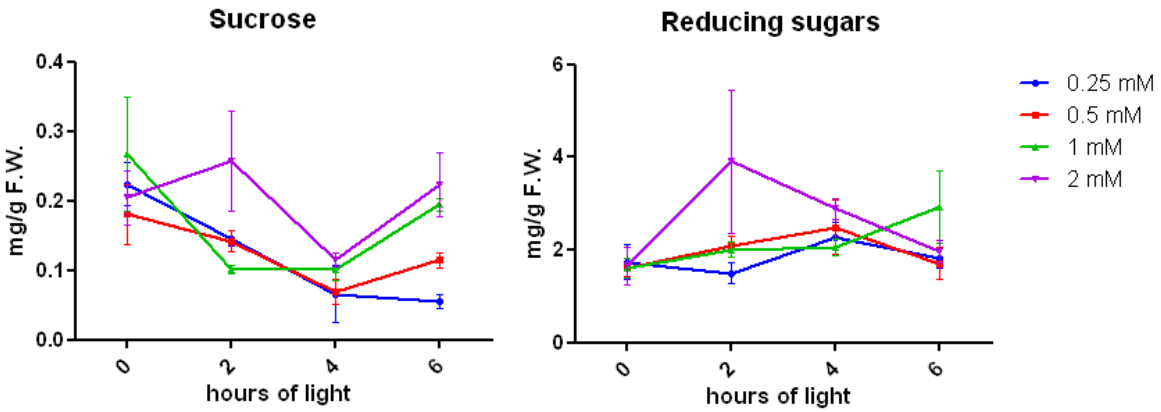

Fig. 5.46: Content of sucrose and reducing sugars in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

Reducing sugars content was the same for all the treatments tested at dark and it was quite constant during the time course for the 0.25, 0.5 and 1 mM nitrate concentrations tested (Fig. 5.46). The highest content, 3.9 mg*g^{-1} FW, was found in plants 2 mM treated after 2 h of exposure at light, significantly different from the value found in the 0.25 mM, then decreased to about 2 mg*g^{-1} FW, in line with the content of the plants treated with 0.25 and 0.5 mM nitrate. The leaves from 1 mM nitrate concentration gave, after 6 h of exposure, the higher level of reducing sugars, of 2.9 mg*g^{-1} FW.

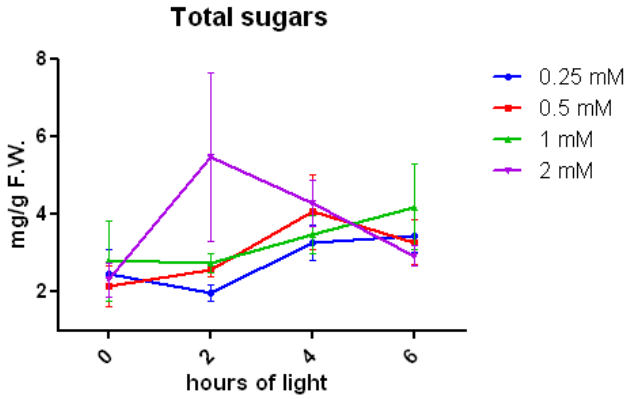

Fig. 5.47: Content of total sugars in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

The total sugars in lettuce were, in average, 2.5 mg*g^{-1} FW in all the nitrate concentrations tested and slightly increased during the time course, especially in the 1 mM treatment, that incremented passing from 2.7 to 4.2 mg*g^{-1} FW while the others of about 1 mg*g^{-1} FW (Fig. 5.47). The 2 mM treated plants showed a rapid increase at 2 h of light exposure and achieved a pick of 5.5 mg*g^{-1} FW, highest value found and significantly different from the lowest content of sugars, measured at the same time in leaves treated with the 0.25 mM nitrate concentration.

Lettuce (second biological replicate)

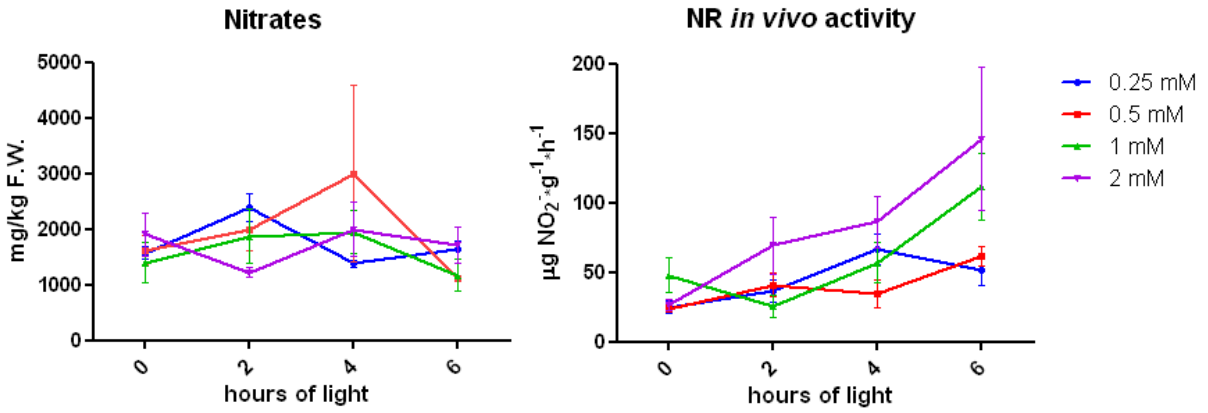

Fig. 5.48: Content of nitrates and NR *in vivo* activity in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

The content of nitrates found in the second biological replicate of lettuce was, in average, 1630 mg*kg^{-1} FW, lower compared with the values found in the first cultivation. No significant differences among the values of the content of nitrates were found and after 6 h of exposure at light the content was the same for the lowest nitrate treatment and slightly decreased in the plants treated with the others nutrient solutions (Fig. 5.48).

The NR *in vivo* activity was high, 48 µg NO_2^-*g^{-1}*h^{-1} in the plants under 1 mM nitrate treatment at dark compared with the others that had, in average, 25 µg NO_2^-*g^{-1}*h^{-1}. The activity increased in all the treatments during the time of exposure at light but more for the most concentrated solutions (1 and 2 mM), compared to 0.25 and 0.5 mM, in fact significant differences were found among the activities found in the 2 mM treatment, of 26.5 µg NO_2^-*g^{-1}*h^{-1}, and the concentrations of 0.25 and 0.5 mM with an average of 24 µg NO_2^-*g^{-1}*h^{-1} (Fig. 5.48).

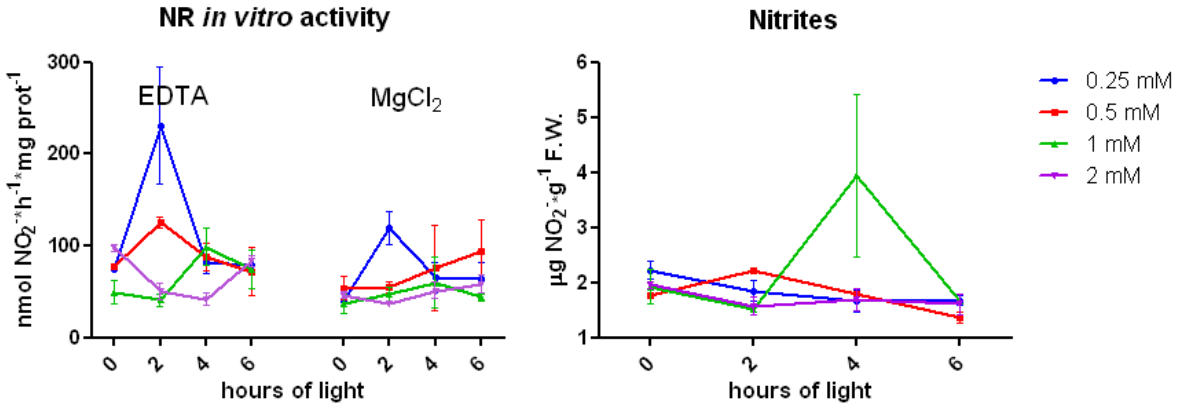

Fig. 5.49: Content of NR *in vitro* activity and nitrites in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

The total activity of NR found at dark was not significantly different among the different nitrate treatments and was, in average, 74.5 nmol NO_2^-*h^{-1}*mg^{-1} protein. After 2 h of exposure at light however, the activity found in the plants at 0.25 mM and 0.5 mM were the highest, with, respectively, 230 nmol NO_2^-*h^{-1}*mg^{-1} protein and 126

nmol NO_2^-*h^{-1}*mg^{-1} protein. The NR activity showed oscillatory patterns during the sampling times. The total NR activity increased at the end of the samplig in the 0.25 and 1 mM treatments, while the 0.5 and 2 mM decreased.

The active NR activity, measured in presence of $MgCl_2$ in the reaction buffer, was high in the plants treated with the 0.5 mM of 54 nmol NO_2^-*h^{-1}*mg^{-1} protein. The activity is statistically different among the 0.25 mM nitrate concentration in the nutrient solution and the 1 and 2 mM treatments, at 2 h of light. In the plants under 0.5 and 2 mM nitrate concentration the activity slightly increased during the time course; in all the treatments increased the NR activity after 6 h of illumination and the higher value was at 0.5 mM with 94 nmol NO_2^-*h^{-1}*mg^{-1} protein, fell by the 0.25 mM (Fig. 5.49).

The differences in the nitrites content among treatments were not significantly different but the higher value at 0 h of light was at 0.25 mM nitrate concentration in the nutrient solution. Along the sampling times the 0.25 mM treatment showed a little decrease (Fig. 5.49). The 0.5 and 2 mM treatments had opposite trends but non significant differencese were found. The higer content of nitrites in the leaves was found in the 1 mM nitrates concentration, of 3.9 µg NO_2^-*g^{-1} FW and it was significantly different from the 0.25 and 2 mM treatments. The content of nitrites ranged from 1.5 to 3.9, with an average of 1.9 µg NO_2^-*g^{-1} FW.

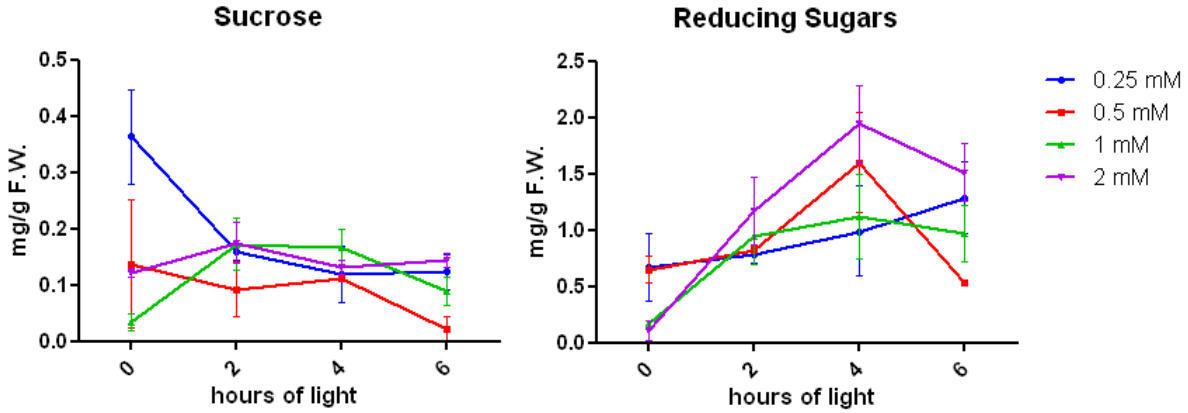

Fig. 5.50: Content of sucrose and reducing sugars in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

The highest content of sucrose was found in the leaves under 0.25 mM nitrate concentration in the nutrient solution of 0.36 mg*g^{-1} FW and this content was significantly different from the values found at the same sampling time in all the other treatments. The content of sucrose increased a bit in the two most concentrated nutrient solutions while decreased in the others. The sucrose content was included in a range of 0.03 and 0.36 mg*g^{-1} FW with an average of about 0.135 mg*g^{-1} FW (Fig. 5.50).

The reducing sugars vary in a range of 0.1 to 1.9 mg*g^{-1} of FW and both the higest and the lowest values were found in in the 2 mM treatment. The most concentrated solutions showed similar trends; there were not significant differences among the treatments and all the nitrate concentration tended to increase during the first times of sampling (Fig. 5.50).

The content of total sugars was not statistically different among the treatments with an exception between the 2 mM and the 0.5 mM after 6 h of illumination. The total sugars showed the same trends described for the reducing sugars but they were higher. The lowest value in the content of total sugars was 0.35 found in the 1 mM treatment at dark and the higher was 2.4 mg*g^{-1} FW reached in the 0.5 mM nitrate concentration (Fig. 5.51).

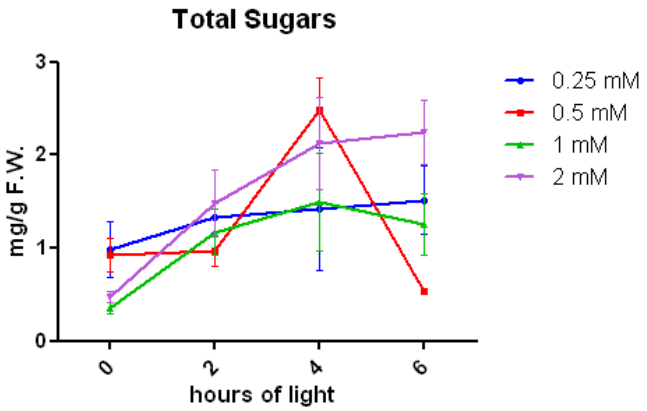

Fig. 5.51: Content of total sugars in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

Rocket (first biological replicate)

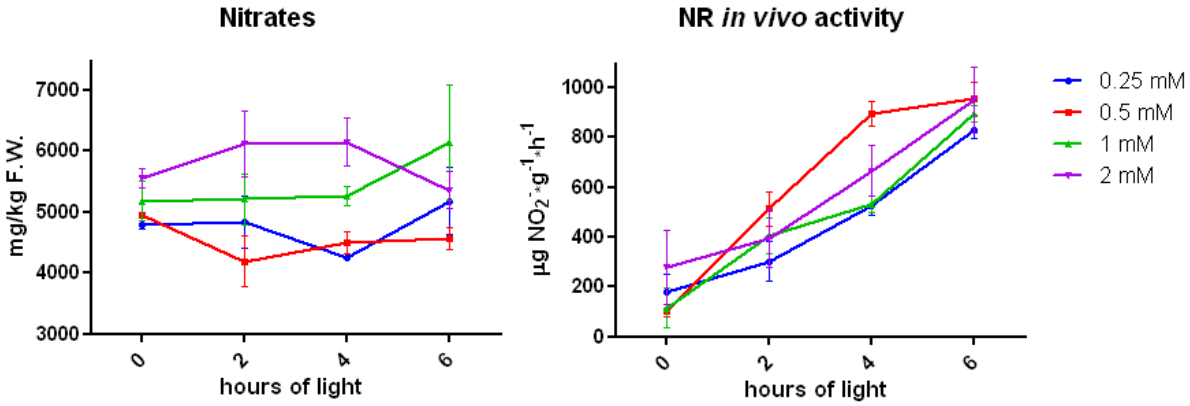

Fig. 5.52: Content of nitrates and NR *in vivo* activity in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

The content of nitrates in rocket was quite constant in the rocket plant for all the treatment tested with an average of about 5140 mg*kg^{-1} (Fig. 5.52). Some significant differences were found between the 0.5 mM and 1 mM treatment at 6 h of light and between 0.5 and 2 mM treatments at 2 and 4 h of light. The content of nitrates was low in the lowest cocncentrated nutrient solutions and high for 1 and 2 mM nutrient solutions.

The NR *in vivo* activity showed differences between 2 mM (146 µg NO_2^-*g^{-1}*h^{-1}) and 0.25 mM (51.5 µg NO_2^-*g^{-1}*h^{-1}) treatments at 6 h of exposure at light and between 0.5 mM and 1 mM after 4 h of illumination (Fig. 5.52). Plants exposed to lowest nitrate concentration showed the lowest activity. The NR *in vivo* activity increased in all the treatments during the time course.

The total activity of NR was high in the lowest concentrated treatment with an average of 275 nmol NO_2^-*h^{-1}*mg^{-1} protein and the values found at 4 and 6 h of exposure at light were statistically different among the values found in the 0.5 mM (Fig. 5.53). The 0.5 mM treatment increased strongly at 6 h of light and was statistically different also among the 1 and 2 mM nitrate concentrated solutions. At dark the significative difference was between the

0.25 mM treatment, where the activity was 286 nmol NO_2^-*h^{-1}*mg^{-1} protein, and the 1 mM one with 81.2 nmol NO_2^-*h^{-1}*mg^{-1} protein. The different treatments showed oscillatory trends.

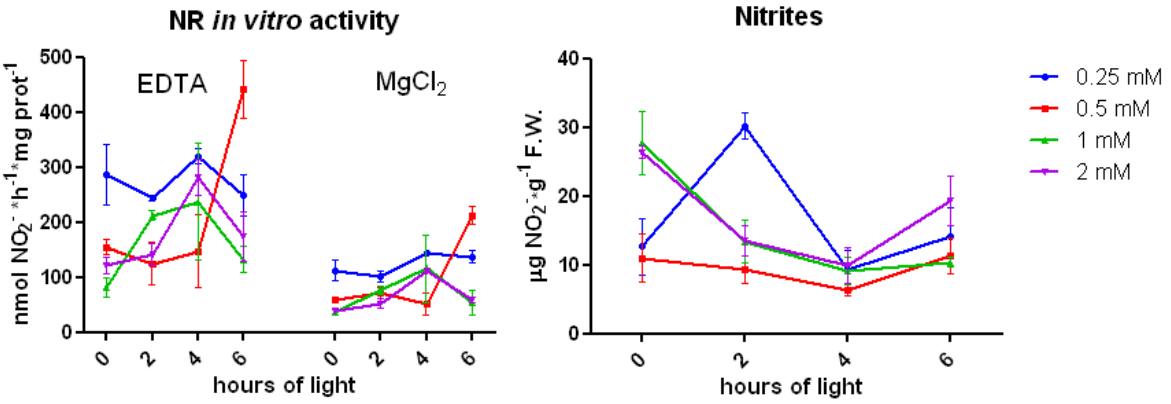

Fig. 5.53: Content of NR *in vitro* activity and nitrites in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

The active NR activity followed the pattern showed for the total NR activity with lower values. The statistical analysis highlights differences between 0.25 mM and 0.5 mM treatment after 4 and 6 h of light exposure and among 0.25, 1 and 2 mM treatments at 6 h (Fig. 5.53).

The lowest treatment showed statistical differences in the content of nitrites between the most concentrated nutrient solutions in the first two sampling times. At 0 h a difference between 0.5 mM and 2 mM was measured and at 2 h of illumination there was a difference between the 0.25 and the 0.5 mM of nitrates in the nutrient solution. None differences was observed between the two most concentrated treatments (Fig. 5.53).

The content of sucrose is not affected by the nitrate treatments and it was quite constant for the 0.25, 0.5 and 1 mM treatments (Fig. 5.54). The most concentrated treatment increased the content of sucrose in the leaves during the time course. The sucrose was, in average, 0.88 mg*g^{-1} FW and it was included in a range of 0.73-1.1 mg*g^{-1} FW, found both in the 2 mM nitrate treatment.

The reducing sugars did not present significative differences among the treatments, except at 2 h of light exposure between 0.25 and 2 mM nitrate concentration in the nutrient solution, where the highest value of reducing sugars, 2.4 mg*g^{-1} FW, was measured. In average the reducing sugars were 1.9 mg*g^{-1} FW. They showed some oscillations during the light exposure (Fig. 5.54).

The total sugars showed the same trends saw in the reducing and the same statistical differences were detected between 0.25 mM and 2 mM treatments. The content was 3.6 mg*g^{-1} FW in average and the highest value was 5 mg*g^{-1} FW in the 2 mM nitrate concentration (Fig. 5.55).

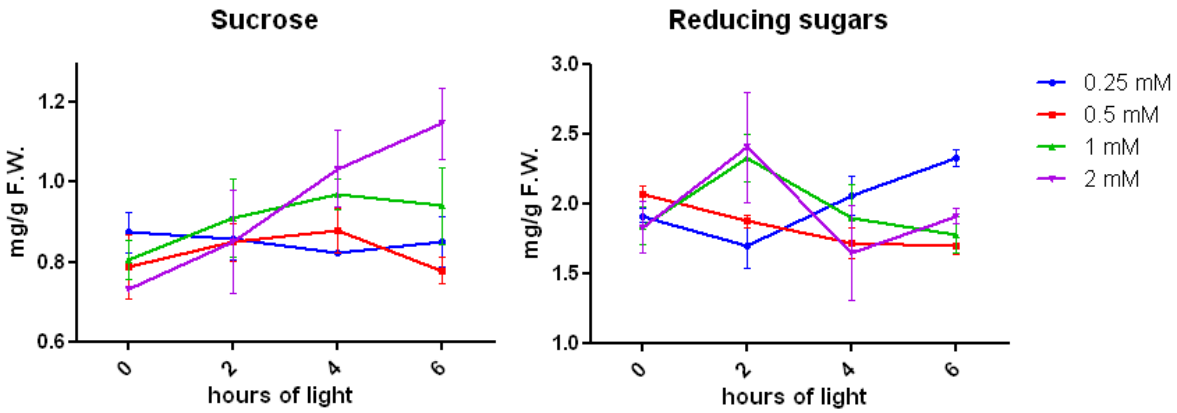

Fig. 5.54: Content of sucrose and reducing sugars in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

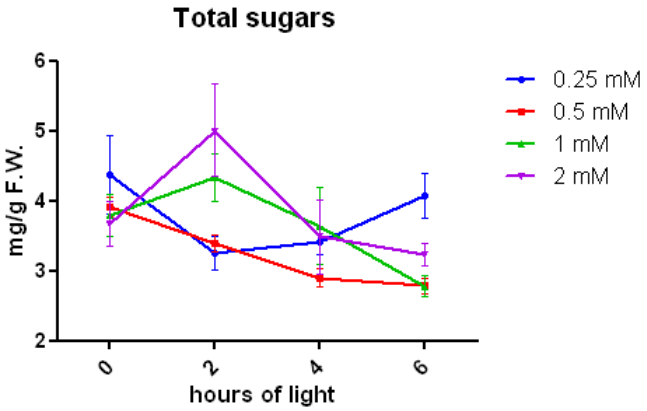

Fig. 5.55: Content of total sugars in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

Rocket (second biological replicate)

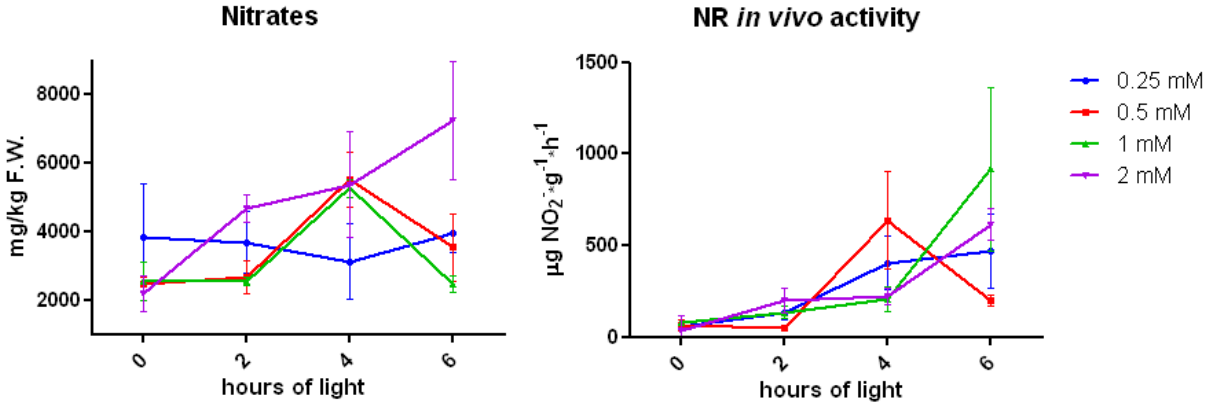

Fig. 5.56: Content of nitrates and NR *in vivo* activity in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

The nitrates content in rocket did not show significant differences due to the nitrate concentration of the nutrient solutions with an exception at 6 h of illumination between the 0.5 and 2 mM. The 0.5 and 1 mM treatments showed the same patterns, while 0.25 manteined constant the nitrates and 2 mM increased during the light exposure (Fig. 5.56). The content was, in average about 3809 mg*kg^{-1} FW.

The NR *in vivo* activity was the same in all the treatments at dark, with an average of 61 µg NO_2^-*g^{-1}*h^{-1} and they increased during the time course but only one significant difference was found at 6 h between the 0.5 and 1 mM treatment (Fig. 5.56). The activity was higher in the plants under 1 mM nitrates exposed at 6 h of illumination and reached 918 µg NO_2^-*g^{-1}*h^{-1}. The NR activity, in average, was 277 µg NO_2^-*g^{-1}*h^{-1}.

The total NR activity was very high and was not affected by the nitrate concentration of the nutrient solutions, but from the h of illumination. The most concentrated solution showed the highest values of the activity, with a pick of 2980 nmol NO_2^-*h^{-1}*mg^{-1} protein but also the lowest activity, at dark, of 231 nmol NO_2^-*h^{-1}*mg^{-1} protein. The 0.25, 1 and 2 mM treatments had oscillatory trends while the 0.5 mM gradually increased with the exposure at light. The active NR activity showed the same behaviours found in the total NR activity, but the highest activity was in the 0.5 mM treatment, of 1395 nmol NO_2^-*h^{-1}*mg^{-1} protein (Fig. 5.57).

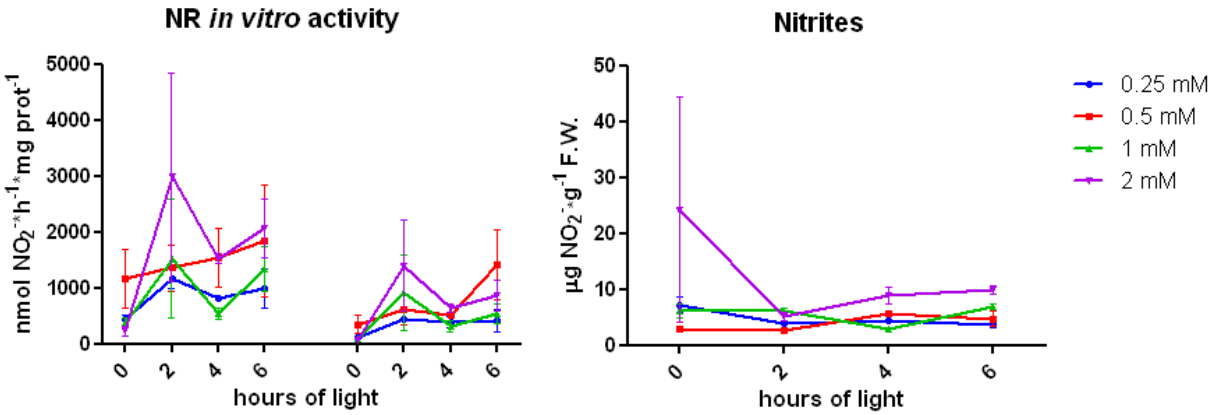

Fig. 5.57: Content of NR *in vitro* activity and nitrites in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

Nitrites content was different at dark between 0.5 mM and 2 mM of concentration of nitrates in the nutrient solution (Fig. 5.57). The content was quite constant during the sampling times and was not affected by the concentration of the nutrient solution. Nitrites measured were about 6.6 µg NO_2^-*g^{-1}.

At 4 h of light exposure a difference in the content of sucrose, due to nitrate concentration in the nutrient solution was found between the 0.5 mM and 2 mM treatments (Fig. 5.58). The others presented constant values of sucrose that did not change neither with the light exposure, and they were, in average, 1.2 mg*g^{-1} FW.

Reducing sugars were not affected by the different nitrate content in the nutrient solutions. The contents were ranged 0.6-4.4 mg*g^{-1} FW (Fig. 5.58).

The total sugars were very similar to the reducing and they did not highlight differences among treatments. In average the total sugars in rocket were 3 mg*g^{-1} FW (Fig. 5.59).

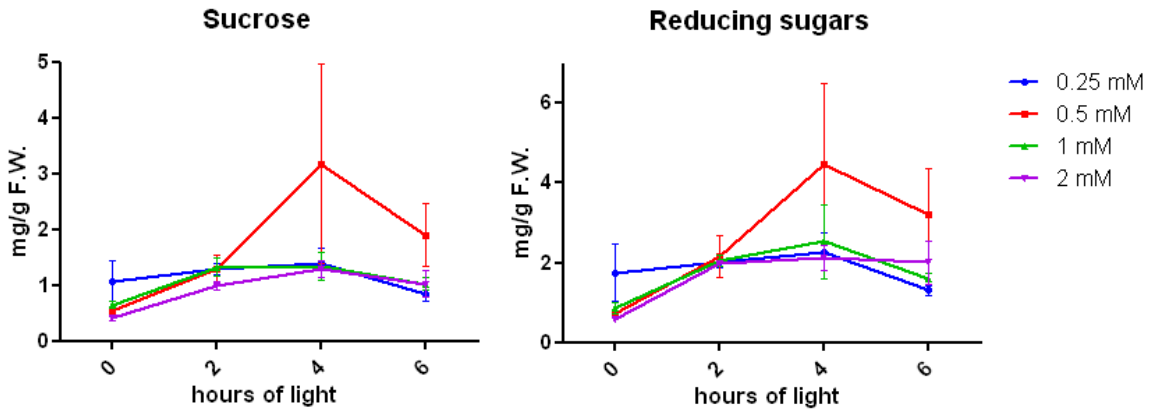

Fig. 5.58: Content of sucrose and reducing sugars in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

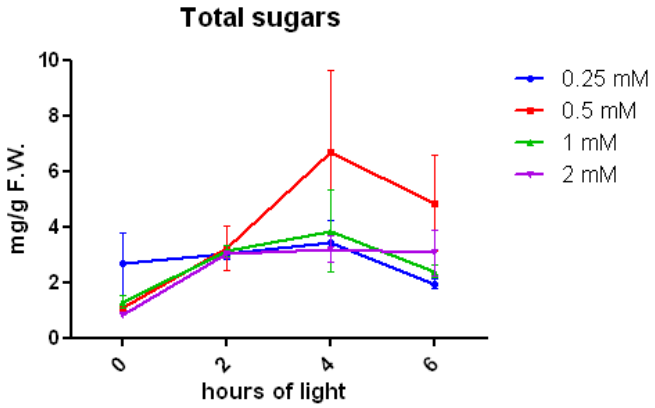

Fig. 5.59: Content of total sugars in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

Gene expression analysis

Lettuce

The expression of the genes that encodes for the enzymes involved in the nitrates metabolism was carried out on the samples obtained from cultivation in controlled environmental conditions. The results are the average of the ΔΔCt calculated for the different treatments and sampling times.

The transcripts of *LsNR*, the first and key enzyme of the assimilation pathway, were higher in dark at the first sampling time in leaves exposed for 24 h to the lower concentrations of nitrates (0.25 and 0.5 mM) in the nutrient solutions. The higher value of ΔΔCt was 1.5 and was found in lettuce leaves treated with 0.5 mM of nitrate. Leaves treated whit 0.25 mM nitrate decreased their expression during the time course, when the ΔΔCt were 1, to 4 hours of light when the value is 0.1 (Fig. 5.60). Leaves of plants treated with 0.5 mM NO_3^- concentration at dark had with the highest and decreased after 2 h of light exposure 0.04; then increased to 0.28 and at 6 hours of exposure the NR transcripts were halved. In leaves harvested from treatment with 1 mM concentration the NR gene expression was stable at time of sampling 0 and 2, with values of about 0.35, then decreased their

expression at 4 hours of exposure and maintained these values, of 0.15, at 6 hours of light. The plants treated with 2 mM showed a *LsNR* gene expression with an oscillatory trend: the level of transcript was 0.44 at dark, after 2 hours was 0.56, at the fourth time of sampling the expression was the lower found for this nitrate treatment, 0.08, and with the maximum exposure at light the transcript increased to 0.61.

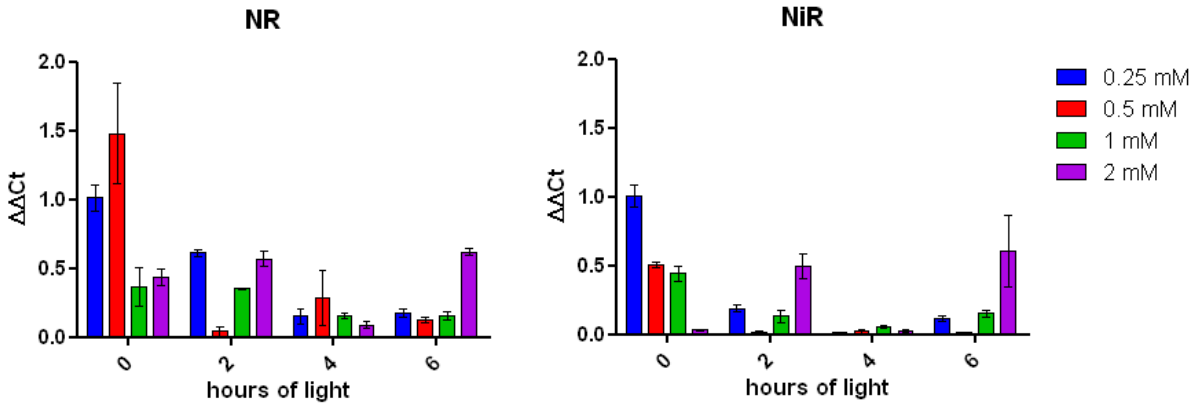

Fig. 5.60: Relative quantification of the expression of NR and NiR in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

In dark adapted plants the behavior of the expression of *LsNiR* gene decreased with the increase of nitrate concentrations in the N.S.; the highest level of expression was achieved in the leaves treated with 0.25 mM nitrate concentration in dark, with ΔΔCt of 1. After 2 hours of light the transcripts decreased to 0.1 and it continued to decrease at up to the third sampling time, when the value was 0.01 (Fig. 5.60). After 6 h the transcription levels slightly increased to 0.11. At 4 hours of light all the treatments tested showed low expression of *LsNiR*, of ranging 0.02-0.05. Under the 0.5 mM treatment the expression tended to decrease by increasing the hours of light exposure, passing from 0.5 at dark, to 0.1 in the other times. Leaves of plants treated with 1 mM nitrates treatment showed expression levels that decreased by increasing after 4 hours of light exposure, passing from a value of ΔΔCt of 0.4 to 0.05 found at 4 hours; after 6 hours of light the levels return to 0.1. The *LsNiR* expression found in the 2 mM treated leaves were very low at dark and at after 4 hours of light with values of ΔΔCt of 0.03 and 0.02 respectively. At 2 and 6 hours of light higher expression of NiR in 2 mM nitrate concentration was observed with values of 0.4 and 0.6.

Cytosolic Glutamine Synthetase (GS1) showed higher expression levels at dark in all the treatments in lettuce. The values of expressions were 1, 0.8, 0.4 and 0.1 starting from the lower nitrate concentration in the nutrient solution to the 2 mM. The *Ls*GS1 expression decreased with the increasing of hours of light in all the different nitrate concentrations. The GS1 expression in leaves exposed to 0.25 mM nitrate was 0.1, while in the 0.5 mM treatments increased up to 0.24, in the 1 mM was 0.08 and at the 2 mM treated plants the expression was 0.04 (Fig. 5.61).

The expression of chloroplastic Glutamine Synthetase (GS2) at dark was high in all treatments even if the transcripts declined by increasing the nitrate concentrations, while at 6 hours this trend was inverted (Fig. 5.61). The leaves treated with 1 and 2 mM nitrate presented a similar oscillatory pattern of transcription levels; also leaves sampled from plants treated with 0.25 mM nitrate concentration showed an oscillatory pattern, but opposite compared to the other, in fact when one of the two decreased, the others increased.

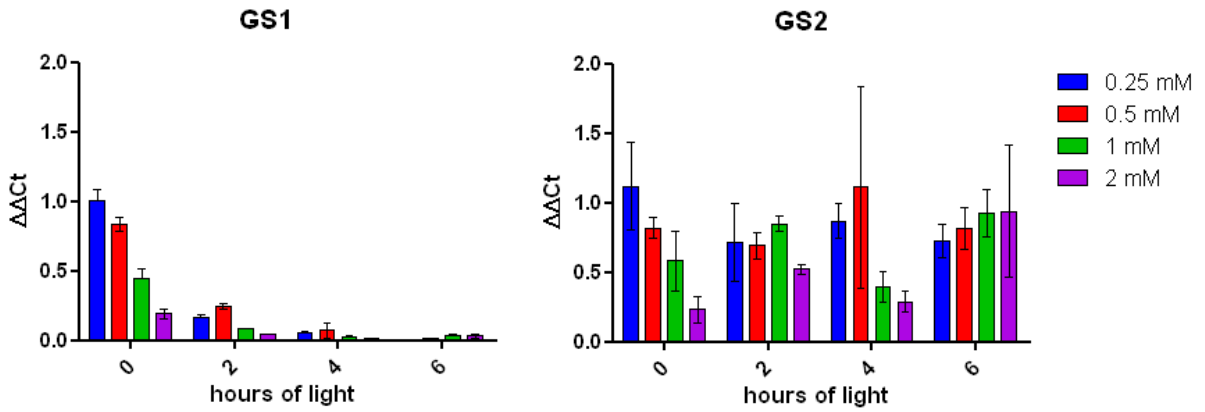

Fig. 5.61: Relative quantification of the expression of GS1 and GS2 in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

The transcript level of Glutamate Synthase (GLU) in lettuce at 0.25 mM of nitrate concentration tended to decrease passing from 0 when the expression was 1.09 to 4 hours of light when the value of ΔΔCt was 0.28, while increased at 6 hours of exposure at light achieved little more that the same levels obtained at dark, of 1.19. At 6 hours of light higher transcription levels were found in 0.25 mM treatment, with 1.19; 1 mM of nitrate with ΔΔCt of 1 and 2 mM treatments when the value was about 2.2 (Fig. 5.62).

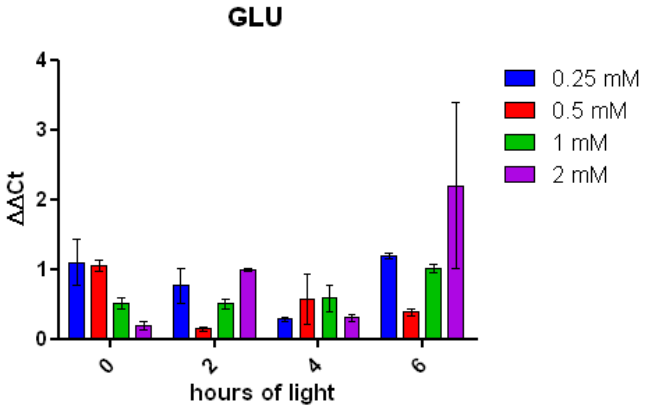

Fig. 5.62: Relative quantification of the expression of GLU in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

Lettuce

The transcription levels of *LsNR* in lettuce were high at dark, in plants treated with the 1 and 2 mM of nitrates in the nutrient solution, with values of ΔΔCt of 2.2 and 3 respectively (Fig. 5.63). The behavior of the transcripts for the lower concentrated treatments was oscillatory trend passing from the first to the last time of sampling. The *LsNR* transcripts in the plants treated with 0.5 mM of nitrates were quite constant from dark to 2 hours of light exposure, then the ΔΔCt decreased reaching 0.15 at 4 hours of light. At 6 hours of light there was a little increase of expression. The high value of ΔΔCt obtained at dark in the 1 mM nitrate concentration, of 2.2, drastically

decreased passing to 2 hours of light exposure, with 0.5 mM nitrate. Then the transcripts slightly increased by increasing the hours of exposure at light. The levels of transcripts in plants treated with 2 mM nitrate concentration decreased at the increasing of the exposure at light until 4 hours. The values of ΔΔCt passed from 3, found in the dark to 0.4, found after 4 hours of exposure at light. After 6 h exposure the *LsNR* transcripts were 0.6.

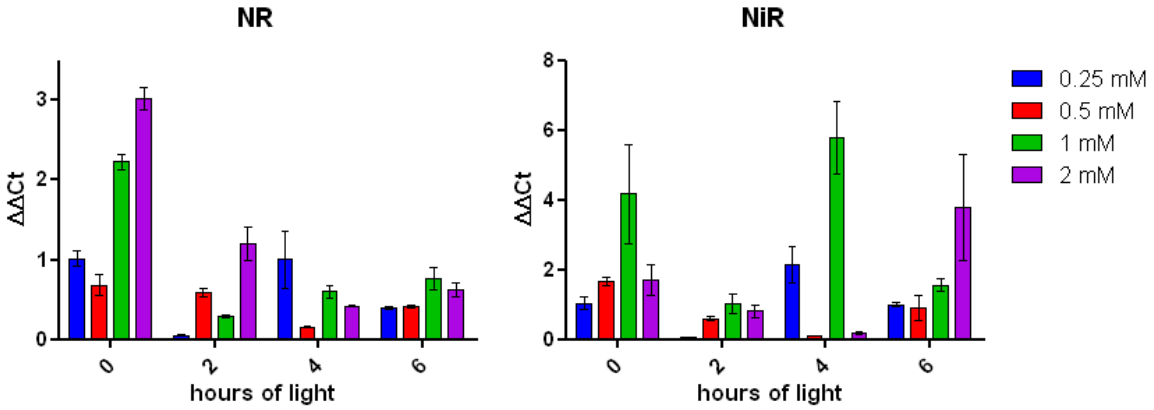

Fig. 5.63: Relative quantification of the expression of NR and NiR in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

The expression of Nitrite Reductase in lettuce showed the higher expression in the plants treated with the 1 mM nitrate concentration in the nutrient solution, which was different among the values found in the plants treated with the other concentrations at dark, and after 4 h of exposure at light, when the expression reached ΔΔCt of 5.8 (Fig. 5.63). The higher expression, of 3.8, was showed also in the plants with the 2 mM nutrient solution and after 6 h of exposure at light, where the expression increased at the increase of the concentration of the nutrient solution and was significantly different among the expressions found at the same time in the lowest concentrated treatments. The lowest concentrated treatment gave the lowest expressions; the 0.25 mM showed an oscillatory trend during the time course, as the 1 mM one, while the 0.5 and the 2 mM treatments decreased from dark to 4 h of exposure at light and then increased again.

The transcription levels of cytosolic Glutamine Synthetase showed oscillatory patterns in all the treatments tested during different light exposure. In the plants treated with the lower concentrations of nitrates (0.25 and 0.5 mM) the transcripts increased passing from dark to 2 hours of light, then decreased passing from 2 to 4 hours of light and then increased again (Fig. 5.64). The plants with 1 mM of nitrate concentration showed a slight decrease of transcription levels passing from dark to 2 hours of light and then the transcript tended to increase at the increasing of the exposure at light. For the highest concentration of nitrates the transcripts were high at dark, with value about 3 and then it strongly decreased after 2 hours of exposure; passing from 2 to 6 hours of exposure at light the levels increased again reaching ΔΔCt value of 3.8.

The chloroplastic Glutamine synthetase (GS2) in leaves showed the higher levels of transcripts at 6 hours of light in the lowest nitrate concentrations, with a value of ΔΔCt of 4.24 (Fig. 5.64). The *LsGS2* transcripts in this treatment in the other different light conditions showed an oscillatory trend, then, at the second time of sampling, decreased to 0.5 and after 4 hours of light exposure returned to 1 again. The 0.5 mM nitrate concentration treatment induced transcription levels of 0.8 in plants sampled at dark and after 2 hours of exposure at light, then decreased to about 0.2. A high value of the transcripts was observed for the plants treated

with 2 mM concentrations of nitrates at dark, with 2.2, but after 2 hours of light exposure the value drastically decreased to 0.2. At 4 and 6 hours of light exposure the transcripts were quite constant with values of 0.5.

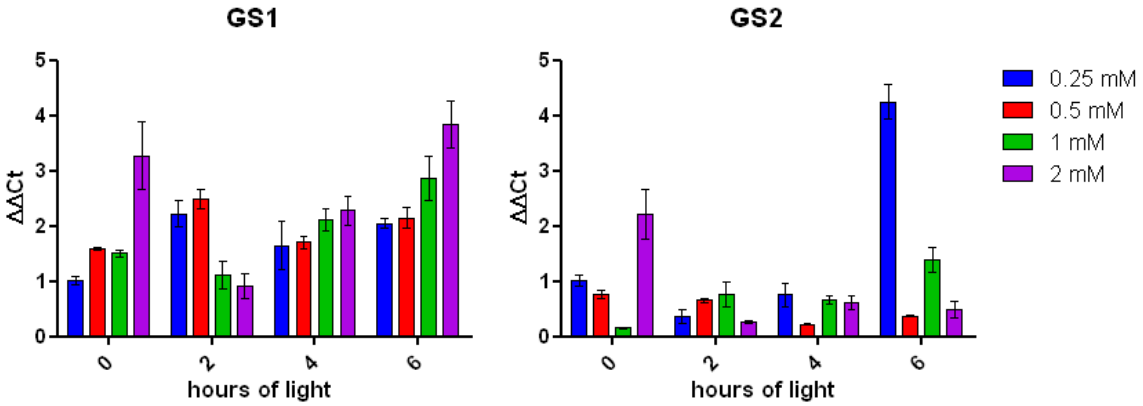

Fig. 5.64: Relative quantification of the expression of GS1 and GS2 in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

The gene encoding for Glutamate Synthetase (GLU) in lettuce showed in dark leaves low expression, with an exception for the 2 mM treatment, where the value of ΔΔCt was 4 (Fig. 5.65). The expression of this gene in 0.5 and 1 mM nitrate treatments plants had oscillatory pattern of the transcripts for *LsGLU*. The gene expression in the intermediate nitrate concentrations increased after 2 h, while the lowest did not change and the 2 mM declined. In the 0.25 treatment the increase of *LsGLU* transcripts was observed after 4 h. After 6 hours of light only the plants with 1 mM and 2 mM showed the higher gene expression. The highest value was 5.7 in the 1 mM treatment, while the 0.5 mM one remained constant with 0.8. The plants treated with higher concentration of nitrates in the nutrient solution had higher values of the transcripts at dark and with the maximum exposure at light, but at the 2 and 4 sampling times the values were lower and constant and reached values of 0.9-1.2.

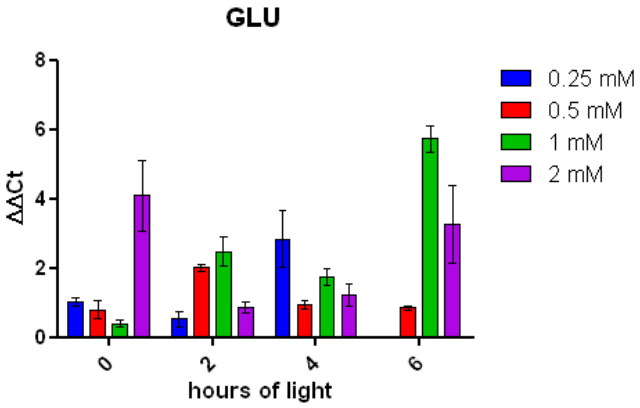

Fig. 5.65: Relative quantification of the expression of GLU in lettuce leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

Rocket

The transcripts of the *DtNR* in rocket leaves were high in the 1 mM, 7-8 fold higher than the lowest nitrate concentration at dark, while the 0.5 mM treatment had lowest expression and the 2 mM treatment 2 fold higher than the lowest nitrate treatment (Fig. 5.66). After 2 hours the transcripts were similar in all treatments. After 4 h the gene expression lowered with differences that followed the gradient of nitrate concentrations. After 6 h the higher values of *DtNR* expression were found in 0.5 and 1 mM treatments. The treatment with 0.5 mM of nitrates showed an oscillatory pattern of the transcription levels: at dark they were 0.35, after 2 hours increased more than double to about 1.9, at 4 hours decreased to 0.6 and at 6 hours of light achieved ΔΔCt of 3.45. The *DtNR* transcripts for the plants treated with the 1 mM concentration of nitrates in the nutrient solution started at 7.6 and, at the increase of the hours of light, the values decreased to 1; passing from 4 to 6 hours of exposure at light the transcription levels reached the value of about 2. In the 2 mM treatment the transcripts decreased with the increase of the light exposure, and passed from 0.6 to 1.8.

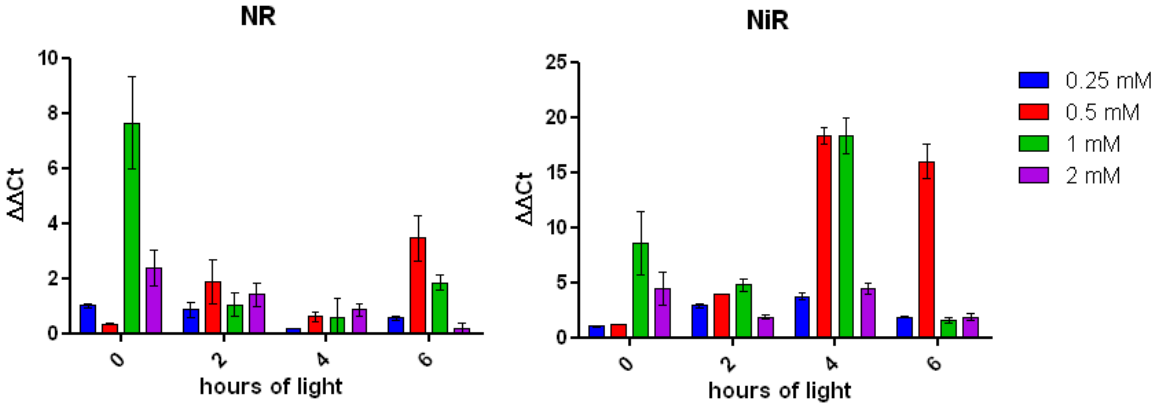

Fig. 5.66: Relative quantification of the expression of NR and NiR in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

The expression of *DtNiR* in the different treatments was similar to *DtNR* in leaves sampled in dark; higher expressions were found in the 1 and 2 mM treatments, while the 0.25 and 0.5 mM concentration of nitrates were lower and almost the same (Fig. 5.66). After 2 h of light exposure, the differences of expression were attenuated and lower values were found in the 2 mM. After 4 h the higher *DtNiR* expression was observed in the 0.5 and 1 mM treatments. The expression of these genes in the 1 mM treatment was 18 and at 6 hours of exposure at light the expression declined to 1.9. After 6 h the highest expression was found in the 0.5 mM treatments while in all other treatments the expression was similar.

Glutamine synthetase (*DtGS1*) transcription levels at dark sampling were very low for the plants treated with the lower concentrated nutrient solutions: 0.25, 0.5 and 2 mM. After 2 h the gene expression was not influenced by treatments even if higher values were observed in the intermediate nitrate concentrations. After 4 h the highest values were observed again in 0.5 and 1 mM treatments while the others remained constant. After 6 h the only increase was found in 0.5 mM (Fig. 5.67).

Glutamate synthetase (*DtGLU*) expression in dark conditions was lower in the 0.25 and 0.5 mM treatments while in the others was higher, especially in the 1 mM one, where the ΔΔCt was 9. After 2 h the *DtGLU* expression was lower in 0.25 and 2 mM and higher in the intermediate treatments. After 4 h the expression of this gene increased in all treatments except the 2 mM. During the times of sampling, passing from dark to 2 and 4 hours of

exposure, the values of transcripts for the plants treated with 0.25 and 0.5 gradually increased to reach values of 13.3 and 20 for, respectively, the firs treatment and the second. The 1 mM treatments achieve his maximum at 4 hours of light, with 21.8 of ΔΔCt. The leaves treated with 2 mM nutrient solution maintained quite constant the transcripts, with 4.5, during the firsts 4 hours of sampling. At 6 hours of light exposure leaves treated with 0.5 mM nitrate concentration remained high, having ΔΔCt of 17, the others decreased and the expression levels were all about included in a range between 2.4-3.9 (Fig. 5.67).

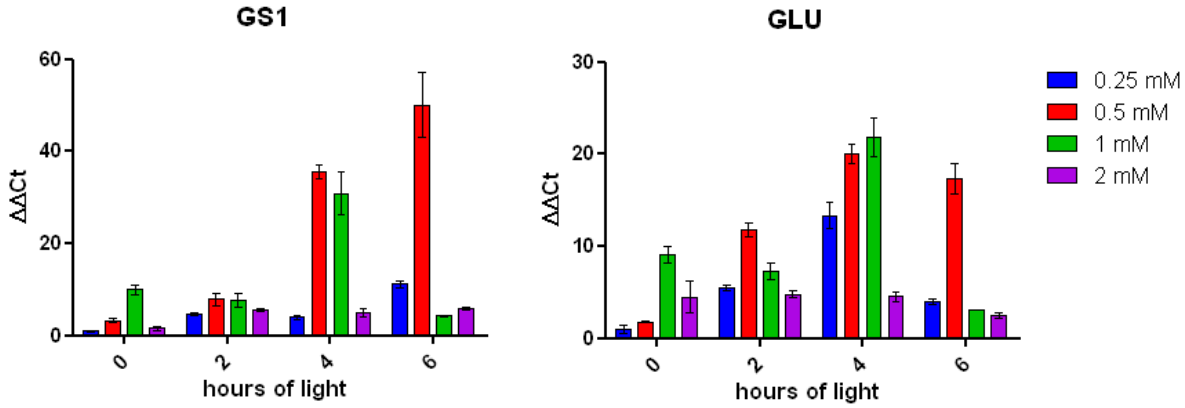

Fig. 5.67: Relative quantification of the expression of GS1 and GS2 in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

The expression of the nitrate transporter, *DtNTR*, showed an increasing trend of the transcripts in the 0.5 mM treated plants; they gradually passed from 1.8 at dark to 49.9 found at 6 hours of light exposure. At lower concentration of nitrates showed the lower expression at dark and slightly increased until the fourth sampling time, to 7.2 and then decreased to 2.9. In the leaves sampled from the 1 mM treatment the expression maintained value of about 5 in the first two sampling, then has been a surge to 44; at the last sampling time the value collapsed to 6. The behavior of the transporter for the 2 mM treatment was similar to that found for 0.25 mM: low expression at dark, with value 2, slight increased until 4 hours of light and return to low expression at 6 hours of light exposure (ΔΔCt of 3.8).

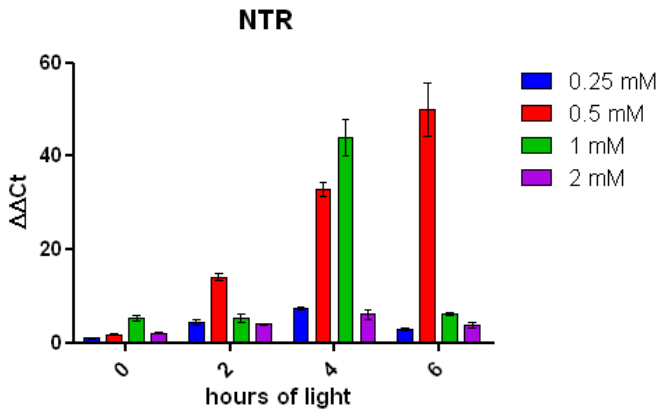

Fig. 5.68: Relative quantification of the expression of NTR in rocket leaves grown in floating system at different concentration of nitrates in the nutrient solution: 0.25, 0.5, 1 and 2 mM in growth chamber. The values are means ± standard errors (n=3).

6. Discussion

Applied research experiments

In general the content of the chlorophylls was constant for all the three different nitrate concentrations of the solution, with an exception in May. The concentration used for cultivation was chosen because are the nutrient solutions used at commercial level, so in excess of the requirements of nitrate. These considerations may suggest that already at 2 mM the plants of lettuce are in a good status of availability of nitrate and leaf color has not been affected. The amount of chlorophyll pigments was higher in the autumn compared with the data obtained in Spring-Summer. It is known that the leaves with low light intensity exposure show some modifications, like the thinning of the leaf and, specially, the increase of the content of chlorophyll on the photosynthetic reaction centers and modifications on the ratio chl *b*/chl *a* and PSII/PSI that increases. The intensity of the light in October was about 100 $W*m^{-2}$, lower compared with the intensity found in spring summer, but the temperatures was almost similar, so the differences found in the results may be attributable to this kind of modification. The nitrogen is an element that is involved in the chlorophyll biosynthesis and represents an essential part of the chlorophyll molecule. The lack of nitrogen is directly observable with chlorophyll reduction. Many old papers reported the relationship between nitrogen and chlorophyll (Tam and Magistad, 1935). On the basis of this correlation nowadays it is possible to drive the nitrogen fertilization in field using a portable chlorophyll measurement instrument such as SPAD (Bullock and Anderson, 1998). The content of chlorophyll in the cells depends on the species and it is influenced by age, growth rate, light and nutrients. The fluorescence of chlorophyll *a* is a parameter measured to evaluate of the state of stress of a plant, so chlorophylls are a very important qualitative indicator that can help to make considerations in the studies on the metabolism of nitrates.

Results show that the content of the chlorophylls in rocket are not affected by any different parameter tested, the concentration of nitrates in the nutrient solutions and the season. The content of chlorophylls in rocket was slightly higher compared with the lettuce one; this macroscopic difference was immediately evident at first sight and it is correlated with the content of nitrate in the leaves (Takebe and Yoneyama, 1989). The difference however was very little, could be because these leafy species, in average, have adjusted in the same way their production of chlorophyll in response to the treatments.

The increase of the carotenoids obtained in May passing from the nutrient solutions lower concentrated in nitrate to the others, more concentrated, followed the trend of the total chlorophylls found in the same conditions in May, in fact carotenoids are the photo-protectors of the chlorophylls. The leaves exposed with higher intensity of light tend to have high carotenoids in the tissues. The reduction of the content of carotenoids obtained in October can be explained because the intensity of light is reduced compared to the spring-summer period.

In the spring-summer cultivations the difference of about 4 times in average of the content of the carotenoid might be possibly due to very little differences in the stage of harvest. The baby leaf stage is considered when the plants achieved about 13 cm of height and 3-4 true leaves; from the physiological point of view this stage plants are in a very active phase of growth, being very young plants, so even a single day of advance or retard in the harvest may determine significant differences in the whole plant metabolism.

The content of the nitrates in the plants studied was not affected by the concentration of the nitrates of the nutrient solution. It is explained because the concentrations of nitrates used in these experiments, were in excess compared with the growth necessity of the baby leaf vegetables, which are not generally high compared to other horticultural species. In all the experiment the content of nitrates was below the safety thresholds imposed by law, confirming that the agricultural technique of the floating system is a good strategy to avoid problems of nitrates at harvest in lettuce and rocket. The data showed that lettuce did not change the content of nitrates in the different season and was, in average, always comprised in a range between 1500 and 3000 mg $NO_3^-*kg^{-1}$ FW;

this behavior suggest that lettuce has a great ability to regulate nitrates and maintain their internal levels of nitrates quite constant, despite the different environmental conditions of cultivation that, in general, are crucial regard to the content of nitrate in the plants.

The different nitrates treatment did not affected the content of nitrates in rocket. The cultivation period is the variable that influence the effect of mineral nutrition (Tuncay et al., 2011). There was a little seasonal effect on the accumulation of nitrates in rocket, in fact in October the content of nitrates in the leaves was, in average, 3600 mg NO_3^-*kg^{-1} FW, while in the other months the average was above 4000 mg NO_3^-*kg^{-1} FW. These are atypical results because generally the critical period in which there is a greater accumulation of nitrates is the cold season (Blom-Zandstra, 1989), but Shahlaei et al., 2007 reported a similar behavior for cabbage, lettuce, spinach and others vegetables with higher values of nitrates during the Winter, compared with the content in Summer. Despite the temperature at the sampling of the plants in October was highest compared with the others, the solar radiation was the lowest, so it is unlikely that the increased of nitrate obtained in October was caused by the modest increase of the temperature found. The differences in the results could be imputable at differences, although small, in the stage of the plants at the harvest time, in fact nitrate content varies with the physiological stage of the plants (Maynard et al., 1976).

The NR activity in lettuce did not showed differences among treatments in May and in October, but in May, in the sampling with the highest solar radiation, the highest activity were found with 110 µg NO_2^-*g^{-1} of fresh weight*$hour^{-1}$. The light exposure plays an essential role in the activity of NR in the plants stimulating the activity. In June the NR activity of lettuce was inversely proportional with the content of nitrate in the nutrient solutions. This behavior is not explainable and it is in disaccord with the literature. Similar result was found in rocket cultivated in May, while in the other cultivations the nitrate treatments did not affected the NR activity.
In rocket the NR activity in May was affected by the concentration of nitrates in the nutrient solutions, as described for lettuce in June. In the others samplings the activity was not affected by the nitrate concentration, but the highest activities were found in plants sampled in October, indicating an effect of the temperature on the enzyme or, more likely, a difference in the physiological stage at the harvest.

The nitrites in lettuce and rocket were constant among treatments, but the content was higher in the plants harvested in October; this may be due to the reduction of the light intensity in this month, which negatively affect the photosynthesis rate and, consequently, slows the organication of the nitrates in amino acids with an increase of the intermediate metabolites. However the nitrite is highly toxic for the plants, it is immediately transported to chloroplasts or plastids (Sechley et al., 1992) after the nitrates reduction and, in leaves, the concentration of nitrites is usually below 15 nmol g^{-1} FW (Lang and Kaiser, 1994).

The sucrose content was not affected by the concentration of the nitrates in the nutrient solutions in both the species studied. Rocket showed a seasonal effect on the sucrose, which was half lower in October compared with the content measured in May. During the photosynthesis the sucrose is synthesized, from two molecules of fructose 6-phosphate, in the cytosol of the leaves cells, and represents a readily available energy for the plant thanks to its high solubility. In October the low solar radiation limited the photosynthesis, limiting, as a consequence, the synthesis of sucrose. None differences were highlight in the content of sucrose in lettuce and in rocket.

The reducing sugars in lettuce showed an increase at the increasing of the concentration of the nitrates in the nutrient solutions, especially in the harvest of May while rocket maintained constant their content of reducing sugars among treatments. The values of reducing sugars in rocket were, in average, lower compared to the values observed in lettuce. The behavior showed could be due to the fact that the lettuce plants grown under highest levels of nitrates adapted their photosynthesis rate, if the environmental conditions permit it, to their level of

nitrates absorbed, in order to provide an amount of carbon skeletons sufficient to organicate them in amino acids.

Total sugars contents were constant in both the species among nitrate treatments. A slight increase of sugars was measure in the plants harvested in October compared with the others, may due to a temporary accumulation of the primary starch, which is included in the determination of total sugars with anthrone method (McCready et al., 1950). When plants exceed in the production of sugars that are not readily used in theirs metabolism, the glucose is quickly accumulated as little primary starch grains in the chloroplasts to be easily available later.

Biochemistry of nitrate assimilation in rocket under natural environmental conditions

Diurnal pattern of NR under a light/dark cycle of 12 hours shows strong oscillations in plants. In dark conditions the NR activity is strongly repressed and increases under light conditions up to a maximum then decreases. However, the NR activity follows the circadian rhythm but it is also regulated by substrates in a feedback inhibition way. If the natural circadian rhythm is disturbed such as dark extension with the black clothes, as observed in our work, the NR oscillations tended to flat.

Basic research experiments

Biochemical analysis of nitrate assimilation in different seasons

The content of nitrates in lettuce was not affected by the concentrations in nitrates of the nutrient solutions. The concentration used was very low in nitrates and lettuce exploited in the same way the nitrates presents in the solutions, with a low accumulate of nitrates, ranged from 1800 to 3000 $mg*kg^{-1}$ FW. The values found were always under the fixed limits imposed for the commercialization of 5000 $mg*kg^{-1}$ FW for the lettuce grown in greenhouse from the 1st of October to the 31th March and 4000 $mg*kg^{-1}$ FW for lettuce cultivated in greenhouse from the 1st April to the 30th September. None difference was found among the experiments in spring and in autumn, despite the different environmental conditions.
The activity of NR in lettuce plants grown in April did not show wide variations among treatments and was very low and neighter the exposure at light affect it. This result was unexpected in view of other data in the literature, where generally the activity of NR increase with the nitrogen supply, but also Laitfa et al., 2009 in a study on the effect of nitrate and shading in kimpul (*Xanthosoma sagittifolium*) found in an experiment that the nitrate reductase activity did not increased increasing the nitrate supply in good light conditions; they found an increase only at the highest dose of nitrate provied, despite the earlier amounts were not limiting.
The behaviour of the NR in lettuce in October was not affected by the content of nitrates in the nutrient solutions; it was slightly higher compared with the activity recorded in April, but showed a very high increase at the end of the period of exposure at light. Nicholas et al. (1976), Choo et al. (1998) and others showed that the activity of NR was proportional at the light intensity and increase at the increasing of light. The activity of NR in spring was congruent with the content of nitrates in the leaves measured, constant and without differences among treatments. This is true also for the activity found in October but the increase at 6 h of illumination is difficult to explain. Analising the environmental parameters, however, the values of the solar radiation and of the temperature in October were higher than the ones measured in April. In Autumn, after the adaptation at dark, both the parameters were lowest compared with the parameters in April, but, during the sampling times, the thermal and lighting excursion was greater and can explain the big increment found in NR activity.
Only the plants harvested in October gave positive data of the total and active NR activity, in lettuce leaves harvested in April the assay did not works.

In October the oscillatory trends found, where there was an alternation of high and low activity may be due to the negative feedback from the intermediate downstream metabolites, probably glutamine, which it can be accumulate if it cannot be deaminated in the process of organication of the nitrogen. The increase of the activity found at 6 h of light was presumably due to the high illumination and thermic increased previously described.
The nitrites in lettuce harvested at April were low, and showed little differences among treatments, at 6 h of light exposure; the differences were between the most concentrated and the lowest concentrated nutrient solutions, but these were not associated neither with a higher content of nitrates nor to an increased activity of nitrate reductase. No seasonal effect was evidenced.
The different trends showed in the content of sucrose of lettuce in April were due to the effect of the nitrate treatments combined with the high variability of the environmental conditions; the most concentrated nutrient solutions affect the photosynthesis in a way opposite at the plants treated with the lowest nitrate concentration. Druege et al. (2000) and Muchow et al. (1996) showed interactions between the nitrogen supply and the content of sucrose in the plants; generally the nitrogen supply increases the sucrose content. The lettuce harvested in October showed patterns comparable to those found for the NR *in vivo* activity at the same season. The content of sucrose was not affected by the nitrate treatments at 2 h of light exposure and the contents of sucrose at this time were, in average, similar to the contents found in the leaves harvested in April but, after, there was a rapidly increase, different for each treatment. The environmental conditions stimulated the production of sucrose from the plants.
Little effects of the nitrate treatments were observed on the content of reducing sugars in lettuce harvested in spring and in autumn. In both the experiments carried out there were not seasonal effect and the increasing of the trends during the time course indicating a strong influence of the illumination on the lettuce leaves, that probably increased the rate of photosynthesis proportionally with the light exposure, without big interferences of the treatments on theirs metabolisms.
The total sugars found in the lettuce cultivated in spring were slightly affected by the nitrate treatments; the contents were quite constant during the times of sampling, instead the total sugars of the lettuce cultivated in autumn reflected the behavior.
In rocket harvested in June the accumulation of nitrates were highest in the treatments with high content of nitrates, 1 and 2 mM, while were lowest in the treatment with low nitrates in the nutrient solutions, 0.25 and 0.5 mM. This confirm that the concentration of nitrates in the nutrient solution affect the content of nitrates in the leaves; rocket accumulate great quantity of nitrates in their leaves vacuoles, in fact rocket is considerate a hyperaccumulator of nitrates. European Regulation fixed thresholds of 7000 $mg*kg^{-1}$ FW for the plants cultivated from the 1^{st} of October to the 31^{th} March and 6000 $mg*kg^{-1}$ FW for rocket grown from the 1^{st} April to the 30^{th} September. The content of nitrates in rocket grown in greenhouse in June was higher of these thresholds. During the times of sampling the nitrates in the leaves decreased showing an effect of the environmental conditions on the nitrates metabolism of the plants, which generally increase the organication with the increasing of the light exposure. Rocket cultivated in January showed contents of nitrates above the thresholds fixed by the regulation of the 2011. The accumulation of nitrates was different in the 4 treatments tested and followed different trends with high alternation of the values during the time course.
The NR *in vivo* activity was similar for the different treatments tested in rocket; the activity increased under the effect of the environmental parameters. The activity measured in leaves harvested in January showed, in average, activities double compared to the activity found in June. The environmental parameters can suggest that in June the high temperature, associated with the high irradiance registered, and just next the night stressed the plants reducing the efficiency of the NR, while in January the good intensity of the light stimulated the activity of NR. The 0.25 mM treatment showed the lower activity but seems directly correlated with the remobilization of the nitrates from the vacuoles, in fact nitrates decreased in plants treated with this nutrient solution.
The total and active activity of NR in the rocket plants harvested in June showed the same patterns. An effect of the concentration of nitrate in the nutrient solution was showed; in fact the activity was highest in the plants

treated with lowest concentrated solutions and, oppositely was lower in the other treatments. The total NR activity found in January showed very high oscillation during the times of sampling and the activity was not affected by the differences in the nitrate concentration of the solutions. The values were in line with those measured in June. The active NR showed fewer oscillations in its activity. The behavior observed in the NR indicate high regulation of the activity, in fact the environmental parameters and the accumulation of ammonium or glutamine can temporarily induce the inactivation of NR with post translational mechanisms.
In rocket the nitrites were low in the leaves sampled in June. The plants treated with the lowest nitrate concentrations showed less nitrites during the exposure at light. This result may be due to the high NR activity saw above and to the good efficiency of the nitrate and carbon metabolisms of these plants, which maintained the nitrite concentration in their cells low to avoid potential damages from nitrites, organicating them in large quantities. The content of nitrites in January was high at dark in the 0.25 mM treatment; this result was found also in June. Probably the plants with low concentration of nitrates and adapted at the dark necessity of more time for the activation of the nitrogen/carbon metabolism, leading to a little accumulation of nitrites just after the dark period.
The content of sucrose was not affected by the treatments in rocket and it was maintained quite constant during the time course. The environmental conditions of the June sampling guaranteed a constant photosynthetic rate in all the treatment tested. In January the content of sucrose was slightly higher compared with the contents of June. Only the 0.5 mM treatment showed differences in the content of sucrose compared with the others, and followed an oscillatory trend, suggesting that the plants under this treatment sensed the variations in the environmental parameters more than the others.
None significant changes in the content of reducing sugars in the rocket leaves were observed among treatments; the results showed variability of the parameter due to post translational regulations of the plants carbon metabolism. Similar variability of the reducing sugars was found in January, but while the 0.5, 1 and 2 mM treatments showed a slight increase due to the light exposure, the 0.25 mM strongly increased their content, particularly passing from 4 to 6 hours of light and suggesting a slow response of the plants with lowest nitrates treatment to the exposure at solar radiation.
Total sugars were not affected by the treatments in rocket but only by the environmental conditions in both the experiments; the sugars in January were more sensitive to the environmental conditions, in fact the variability of the trends was highest compared with the oscillations saw in June. Also the amount was highest in average, but this may be due to little differences in the physiological stage of the plants at harvest.

Biochemical and molecular studies of nitrate assimilation in rocket and lettuce grown in controlled environment

In general the content of nitrates in the lettuce leaves is proportional with the increase of the nitrate in the nutrient solution, because the plants have a luxury consumptions in abundance of nitrates and used them for osmotic functions, while, when the concentrations of nitrates are low, these functions are supplied by the soluble sugars produced by the photosynthesis. The oscillation of the nitrates indicated high post translational regulatory mechanisms, which probably affect the nitrate metabolism already starting from the nitrates transporters. 0.25 and 0.5 mM concentrations of the solutions stimulate the cHATS and iHATS nitrate transporters, and the 1 and 2 mM the LATS, constitutively expressed, so some differences may be due to the different efficiency of the transporters (Orsel et al., 2002; Masclaux-Daubress et al., 2010).

The results confirm then the tendency of the enzyme to be stimulated by an increasing of the nitrate concentration, but also that the behavior of this highly regulated enzyme is not univocal and is extremely sensitive to small physiological variations, due to particular metabolic responses difficult to investigate instant by instant in the plants. The oscillations indicate moreover that the posttranslational adjustments are faster and cause rapid responses in the activity of the Nitrate Reductase. Galangau et al., (1988) and Lillo and Meyer (2001)

reported that the activity of NR and the mRNA strongly changes during the day and show an initial acme in the morning. Lea et al. (2006) found, in deregulate *Nicotiana plumbaginifolia* plants for NR, at the transcriptional level, that the oscillations of the activity during the day were due both to posttranslational regulation but also to the amount of total NR.

Strong oscillations of the total and active NR levels, measured in the lettuce plants, confirm the considerations on the high influence of the posttranslational regulatory mechanisms of the enzyme. In these experiments high activity were found often for the lowest concentrated in nitrate nutrient solutions. This may suggest that lettuce at low nitrate was more efficient in the reduction of nitrates than the plants treated with more nitrates.

The content of sucrose showed variabilty in the trends obtained and decreased during the time course in the lowest concentrated solutions, while were constant for the others treatments. Reducing and total sugars increased in plants treated with the 2 mM concentration of nitrates. The concentration of nitrates in the nutrient solution affect the sugars but it is difficult to discriminate or explain what effects were due to the nitrogen supply and what effects followed the C/N metabolisms.

The low variations in the content of nitrates in the rocket leaves were correlated with the NR *in vivo* activity, where only a light exposure effect was registered in the plants, which increased the NR activity at the increase of the illumination period. Some oscillations in the content could due to the oscillation in the NR *in vivo* activity that stimulates the nitrates transporters, affecting the translocation in the leaves of nitrates.

The nitrites in rocket were showed little variations due to the nitrates treatments. The levels found in rocket were higher compared with the nitrates found in lettuce.

The content of sucrose in rocket was slightly affected by the 2 mM nitrates treatment with increasing sucrose, while was quite constant in the others treatments. Rocket showed less variations in the parameters measured compared with the results obtained for lettuce

The expression of NR was high at dark in the nutrient solutions, according with how found in literature that the levels of transcripts of NR are highest at the end of the night, but rapidly decreased along the morning and during the day, to increase again in the night (Galangau et al., 1988; Matt et al., 2001). During the time course the expression was irregular with a tendency to decrease, indicating that others (post translational) mechanism of regulation of the NR activity attended. The levels of transcripts are induced by nitrate after a nitrogen starvation period, as shown by the results and affirmed in literature (Galangau et al., 1988). The lowest nitrate concentrations showed high transcripts just after nitrogen starvation, and some studies shows that also little concentration of nitrates in the nutrient solution can induce the NR expression (Wang et al., 2000). Wang et al., (2000) found in *Arabidopsis* that the two genes encode for NR were heavily and rapidly affected to low concentrations of nitrate of the treatments; the response is evident already after 20 minutes after the treatments. The results found also showed that the expression induced by nutrient solutions with low nitrate levels is temporary, and already after 2 h of exposure at light the mRNA sloped down, confirming how found in literature (Wang et al., 2000).

NiR expression followed the expression found for NR, but with lower values of $\Delta\Delta Ct$, with an only exception found in rocket at 4 h of exposure in 0.5 and 1 mM nitrates. These data confirm the co-regulation existing between NR and NiR described in literature (Vincentz et al., 1992). The gene that encode for NiR is affected by the nitrate very quickly, as well known (Lahners et al., 1988), and also in lettuce and rocket this is confirmed.

The expression of GS differs with the specie in higher plants and it is affected by lots of parameters such as physiological stage, light, and others exogen parameters (Sun et al., 2009); light can directly induce changes in the transcripts of GS or can act indirectly, with its effect on the photosynthesis (Vincentz et al., 1993; Lam et al.,

2008). An effect of nitrate was observed in maize leaves for expression of GS and GLU, but lower compared with the rensponse of NR and NiR and in long time response (Redinbaugh and Campbell, 1993).

Cytosolic GS1 mRNA showed wave patterns, probably following the availability of the carbon metabolites in the different treatments, in fact it is well known that in *Arabidopsis* the carbon metabolites, particularry 2-oxoglutarate, firsty induced the transcription of the GS1 (Oliveira and Coruzzi, 1999).

The expression of GS2 (chloroplastic) showed oscillatory trends with high variability, maybe reflecting the variability of the sucrose and glucose, or phytochrome, due to changes in the photosyntetic activity in the treatments during the sampling times. The effects of these photosyntetic metabolites were described in literature for *Arabidopsis* and *Pisum sativum* (Oliveira and Coruzzi, 1999; Edwards and Coruzzi, 1989).

Glutamate transcripts were affected by the nitrate treatments and oscillatory patterns were showed during the time course. The variability and the oscillations of the expression could be due by metabolic responses of the leaves to light and nitrate supply after a period at dark and nitrogen starvation, as saw by Pajuelo at al. (1997).

In general higher expressions were observed for rocket compared with lettuce.

7. Conclusions

The nitrate content in the leafy vegetables is considered dangerous for the human health, since they can be reduced in nitrite in the mouth and in the stomach can be transformed in nitrosamine. Recently few studies reported that nitrate can have also a positive effect on the human health. However, independently of the role of nitrate in the humans, at commercial levels the nitrate cannot overcome the limits imposed by the EU for free commercialization among the European countries.

The results of this thesis showed that the nitrate concentration can be lowered up to 2 mM especially for lettuce, without affect the internal and external quality. The 2 mM concentration can be satisfactory in any growing season. The nitrate uptake is still in the regime of low affinity transport and especially lettuce modulated the plant growth in order to achieve the commercial stage without showing physiological disorder.

The biochemical and physiological studies performed on the two species under natural conditions or in growth chamber revealed that the assimilation pathway is highly regulated by internal and external factors. All the enzymes involved in the nitrate reduction and organication undergo continuously transcription, post-transcription regulation and with clear feed-back inhibition from the metabolites along the assimilation pathway. The nitrate assimilation seems a “start and stop” pathway; the punctual investigations among different treatments may lead to wrong conclusions, therefore several time points must be including during the day

Therefore all studies regarding the nitrate assimilation in plants must be carried out under strict control of environmental parameters (especially light intensity and duration), exact nitrate availability and the plants developmental stage. Leaves have different nitrate assimilation during development since the main nitrate reduction takes place in the chloroplast.

The comparison of the two species revealed that the transcription and enzyme activity are higher in the rocket that is a hyper-accumulator than lettuce. It means that the “neck of bottle” is represented by metabolites or enzymes activity of the final steps of the nitrate assimilation pathway. In fact, the nitrite contents were higher in the rocket than lettuce. However a further metabolics study should be carried out in order to better elucidate the different behavior between the two species.

8. References

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