In: Controlled Environment Agriculture
Editor: Md. Asaduzzaman
ISBN: 978-1-63484-489-5

Chapter 6

PRIMING WITH S-METHYLMETHIONINE INCREASES NON-ENZYMATIC ANTIOXIDANT CONTENT OF LETTUCE LEAVES EXPOSED TO SALT STRESS

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ABSTRACT

Tolerance of plants to various environmental stress factors that impair normal develop-ment and reduce biomass production may be enhanced by priming with exogenously supplied compounds which stimulate metabolic processes involved in hardening, and protect molecular structures that support antistress reactions. During the last years, several agents (e.g., salycilic acid, polyamines, hydrogen sulfide, hydrogen peroxide, glutathione, ascorbic acid) have been used, both under controlled laboratory conditions and in field experiments, to alleviate the deleterious effects of abiotic stress factors on metabolic and developmental processes in different crop plants. High salinity of soil water was identified as one of the major environmental stresses that limit yield of crop plants and induce metabolic adjustments that result in qualitative changes of the consumable crop. Enhancement of salt stress tolerance may be related to accumulation of health-promoting metabolites, especially of antioxidants that are not digested and enter the animal and human organism upon consumption of plants exposed to unfavorable growth conditions and stimulated in their defence against harmful environmental factors. In this context, the aim of the present study is to reveal the beneficial influence of priming with S-methylmethionine (also known as vitamin U) on non-enzymatic anti-oxidant production of lettuce leaves exposed to salt stress in hydroponic cultures. There are very few data about influence of this metabolite (a non-proteinogenic amino acid) on membrane stability, stimulation of antioxidant enzyme activities, enhancement of

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poliamine and dimethylsulfoniopropionate biosynthesis and phloem transport of sulfur compounds, especially under low temperature stress conditions, but there are no reports on its possible influence on non-enzymatic antioxidant content of the marketable yield of crop plants. This is the first report on influence of priming with S-methylmethionine on carotenoid pigment, water-soluble phenoloid, ascorbate and glutathione content of lettuce leaves developed under controlled hydroponical conditions in the presence of salt stress exerted by high concentrations of sodium chloride in the nutrient solution. One week after germination of seeds, a moderately salt-tolerant lettuce variety (Paris Island), selected in previous investigation for cultivation on saline soils, was grown hydroponically for two weeks (in 3 L tanks with continuous aeration of roots), under controlled conditions of illumination, air humidity and temperature, then for three days the plants were grown in ¼ Hoagland nutrient solution without and with supplementation of 0.05 mM S-methylmethionine. After this period, the plants were transferred to Hoagland media containing 75 mM and 150 mM sodium chloride, to induce mild and severe salt stress. After three and six days of exposure to high salinity, the third oldest leaves of control plants and of lettuces primed with S-methylmethionine were used for spectrophotometric determination of carotenoid pigment, water-soluble phenoloids, ascorbate and glutathione concentrations. The reduced to oxidized vitamin C and reduced to oxidized glutathione ratios were also determined. The results reveal that priming with S-methylmethionine enhances production of protective antioxidants (especially of glutathione and phenolic compounds) in lettuce leaves grown under high salinity of nutrient solution, thus increasing their health-promoting quality upon consumption.

Keywords: ascorbate, carotenoids, glutathione, health-promoting antioxidants, hydroponic culture, lettuce, phenoloids, priming, salt stress, S-methylmethionine

INTRODUCTION

Plant species are evolutionarily adapted to various environments characterized by very different combinations of physical, chemical and biological factors. There is no environment with optimal ranges of every factor for all kind of plants, and normal life conditions for one species may be unfavorable for others. In this context, plants have developed a wide range of adaptive strategies based on heritable anatomical and physiological traits, and on metabolic plasticity that enables quick and reversible biochemical and biophysical changes as specific reactions to signals from the immediate surrounding of the individuals. Environments characterized by extreme conditions (e.g., drought, very high or very low temperatures, high salinity, lack of oxygen) are inhabited by specialized species, highly adapted to those habitats but with low ability to live and reproduce in other types of environments. Whenever abiotic or biotic factors impair the normal metabolic and developmental processes in a plant, they induce specific changes known as stress reactions, representing an integrated network of genetic and metabolic modifications regulated upon perception and processing of external and internal signals generated by the harmful environmental factors that act as stressors. Among the various stress reactions, biosynthesis of secondary metabolites with protective properties is widespread in the plant kingdom, and the various specific phytochemicals may have similar protective effects also in the organisms which consume those plants, i.e., animals and humans (Gupta & Singh, 2014; Martin, 2013; Rejeb et al., 2014a).

Crop plants are generally not adapted to extreme conditions, but they possess the ability to acclimatize to certain environmental constraints, depending on the time course and intensity of stress factors, as well as on the physiological state of the individuals. Under controlled conditions of cultivation, crop plants may be hardened upon the induction of metabolic processes that lead to an increasing tolerance of the disturbing external factors, and biochemical changes associated with this process may improve the nutritional quality of the marketable yield, mainly by production and accumulation of health-promoting substances, with the capacity to maintain normal functions, to counteract harmful effects or to repair damages that occur in cells on the molecular level. Furthermore, the ability of crop plants to produce phytochemicals with protective properties may be stimulated by appropriate cultivation techniques and treatments in different stages of their ontogenesis, or by a more costly, but heritable genetic manipulation towards increased resistance or tolerance to stress factors. By means of breeding towards withstanding unfavorable growth conditions, both the quantity and the quality of marketable yield can be improved, and efficiency of crop production may be increased in areas which are less suitable for a performant classical agriculture (Hollington & Steele, 2007; Macedo, 2012; Trivedi et al., 2014).

Inherited Resistance and Acquired Tolerance to Environmental Stress Factors

In environments dominated by specific extreme conditions, natural selection has led to highly adapted species with inherited characteristics that enable survival and reproduction without impairment of physiological processes by external factors that would represent stressors for other plant types. The specific adaptive traits existing constantly in every individual of the given species ensure avoidance of stress conditions, because life functions are not disturbed by the extreme conditions, thus no modification is needed in genetic and biochemical processes in order to repair previously produced damages. This means that the specifically adapted species are resistant to those environmental factors which represent stressors for other species. For example, succulents are resistant to drought stress, because they accumulate and bind in their body a quantity of water high enough to prevent any harmful effect of dehydration. The so-called resurrection plants withstand very long dry periods in a dormant state with minimal metabolic processes and with ceased growth, thus avoiding the deleterious effects of pronounced water shortage (Fodorpataki et al., 1995). These resistant species do not need to develop mechanisms of stress tolerance, because they do not experience stress if they avoid any negative change caused by the extreme environmental condition in internal processes.

But in any environment there are periodical fluctuations of the main physical and chemical parameters which define the life conditions in a habitat, so fine and reversible tuning of physiological processes to short-term variations of adverse external conditions is needed for a successful survival of plants, and these changes become more pronounced when plants spread into new habitats. This is why inherited, but irreversible resistance to adverse factors has to be complemented with reversible readjustments of the metabolic processes in order to diminish further damages caused by temporary stress factors at individual level. When a specific stress signal is perceived by a plant, it initiates a signal transduction that leads to up- and down-regulation of biochemical reactions that are sensitive to stressors, in

order to establish a new steady-state level of physiological processes that reduces further dysfunctions. This is the process of hardening, which results in an increased tolerance to the stress factor, i.e., disturbances caused initially are compensated and the functions become less and less damageable by the same intensity of stressor. Tolerance is acquired through physiological acclimation to stress conditions, which is a distinctive type of adaptation to the environment. E.g., savanna grasses tolerate drought stress by effective osmoregulation (rapid accumulation of osmotically active compatible solutes) and hydroactive stomata closure, reversible processes that counteract the negative influences of water loss from metabolically active tissues, without storing significant amounts of water reserve. Usually, regulatory processes that reprogram the physiological functions in order to cope more successfully with stress factors and to compensate for damages that were produced, are temporary and are not fixed in the genetic program, so they are not inherited, and they occur on an individual level. In some cases, when tolerance is based on epigenetic modifications that take place in reproductive cells, it may be inherited, thus the progeny receives from the parent organisms a stress memory, and will be predisposed to cope more successfully with future stressors (Dyachenko et al., 2006; Peng & Zhang, 2009). Even though in contexts of plant stress physiology resistance and tolerance are quite often used improperly and the two concepts are mixed with each other, they rely on distinct mechanisms (of avoidance by prevention and of subsequent adjustment by hardening), but they do not exclude each other, as temporary functional adjustments are performed on the background of innate capacities, tolerance and resistance being two contrasting manifestations of adaptation to environmental stress conditions. In this context, it becomes obvious that stress reactions that enable plants to grow and reproduce better under adverse conditions, are closely related to mechanisms of tolerance, based on biochemical and physiological acclimation (Osakabe et al., 2014; Soni et al., 2015).

When different external stress factors cause similar effects in physiological processes (e.g., high photon flux density, UV-B radiation, low temperature, high salinity, some herbicides, heavy metals and air pollutants all enhance the production of harmful reactive oxygen species in different cell compartments), a cross-tolerance may develop, i.e., a stress reaction induced by one environmental factor may confer protection to other stress factors. Cross-tolerance relies on interactions between signal transduction pathways and effectors activated by different stressors (Huang et al., 2012), and knowledge of genetic and metabolic fundaments of the mechanisms that result in cross-tolerance will offer a powerful tool for breeders to obtain crop plants with increased tolerance to a combination of several stress factors that interact in limiting production and quality of the marketable yield (Fraire-Velazquez et al., 2011; Mahajan & Tuteja, 2005; Shukla & Mattoo, 2014).

Priming with Exogenous Substances to Improve Stress Tolerance

Pretreatment with certain chemicals may sensitize plants in order to be better prepared for a quicker and more intense defence against subsequent stress factors. Upon the action of exogenous tolerance-enhancing agents, plants acquire the capacity to develop a more efficient defence behavior when they will be exposed to abiotic or biotic stresses. This phenomenon is called priming, and represents a physiological state that enables plants to respond to low levels of stimuli in a more rapid and more intense manner, thus being able to exhibit a stronger activation of defence reactions when subsequently challenged by adverse growth

conditions. This capacity for augmented expression of induced defence remains inactive until exposure to stressors, and fitness costs of priming are significantly lower than those of directly induced defence. Primed plants are protected against several stressors without major trade-off effects on commercially or ecologically important traits, such as biomass production and reproductive capacity (Beckers & Conrath, 2007; Ton et al., 2009). The primed state seems to result from an improved perception of defence response-inducing signals, which confers an increased alertness that helps a more efficient reaction after the installation of stress condition (Aranega-Bou et al., 2014; Jakab et al., 2005). Generally, priming is associated with enhanced transcription of defence-related genes, thus transcription factors play a basic regulatory function in the onset of this physiological state. The stress-induced signal transduction develops faster in primed cells, resulting in a quicker activation of defence-related genes, and thus in an augmented protection against the harmful effects of stress factors. Accumulation of dormant mitogen-activated protein kinases, chromatin and histone modifications, as well as alterations of primary and secondary plant metabolism are also general features of the primed state that enables a more efficient acclimation to adverse conditions, based on enhanced stress tolerance. In relation with epigenetic phenomena that occur upon priming, a so-called transgenerational priming was also observed, when the progeny of primed plants exhibited an enhanced defence response after exposure to stress factors (Luna & Ton, 2012; Migikovsky & Kovalchuk, 2013). Due to advances in the field of sensitizing crop plants with exogenous agents for a better protection against stressors by induced acclimation processes, defence priming may be considered a promising strategy for modern crop production management aiming health- and yield-increasing effects (Ahmad et al., 2010; Chinnusamy & Zhu, 2009; Conrath, 2011).

A wide variety of synthetic and naturally occurring chemicals may exert a direct influence on fundamental physiological processes of plants, interfering with signal-processing and regulatory mechanisms (Horvath et al., 1996). Some of these substances provide an enhanced defence signalling potential, contributing to the primed state of plants. Since the first report on defence priming in plant cell cultures (Kauss et al., 1992), several chemical agents, as well as biotrophic and necrotrophic pathogens, and non-pathogenic microorganisms (rhizobacteria, mycorrhizal fungi) have been proved to have specific roles in priming crops against abiotic and biotic stresses. Priming of different crop plants has been achieved with ascorbic acid (Athar et al., 2008; Shafiq et al., 2014; Shalata & Neumann, 2001), salicylic acid (Sahu, 2013; Sharma, 2014; Szepesi et al., 2009), polyamines (Gupta et al., 2014; Wen & Moriguchi, 2015), nitric oxide (Oz et al., 2015; Poor et al., 2015), hydrogen peroxide (Wahid et al., 2007), proline (Ali et al., 2007), glycine betaine (Athar et al., 2009), selenium (Hasanuzzaman & Fujita, 2011; Rios et al., 2008), silicon (Hashemi et al., 2010; Tuna et al., 2008), β-aminobutyric acid (Jakab et al., 2011), 5-aminolevulinic acid (Korkmaz, 2012), jasmonic acid, ethylene and other volatile organic compounds (Rejeb et al., 2014b), azaleic acid (Aranega-Bou et al., 2014), glutathione (Rejeb et al., 2014a), hydrogen sulfide (Fotopoulos et al., 2014), hexanoic acid (Aranega-Bou et al., 2014), thiamin or vitamin B1 (Ahn et al., 2007; Al-Hakimi & Hamada, 2001; Kaya et al., 2015), riboflavin or vitamin B2, biotine or vitamin H, pro-vitamin K (Borges et al., 2014), pipecolic acid, chitosans, oligogalacturonides (Aranega-Bou et al., 2014), quercetin, or the fungicide propiconazole (Jaleel et al., 2008). These priming agents may be applied as components of the nutrient solution in hydroponic cultures, as foliar spray or as seed soaking solutions (Ashraf & Foolad, 2005; Yasmeen et al., 2013). Most investigations concerning the priming effect of these

agents were performed for wheat, rice, maize, canola, tomato, as well as with the model plant *Arabidopsis thaliana*, in experiments concerning tolerance to chilling stress, drought, heavy metal toxicity, wounding by insects, infection with pathogens (Conrath et al., 2002; Filippou et al., 2013; Sairam et al., 2005; Saito & Matsakura, 2015; Zushi et al., 2009).

Roles of S-Methyl-L-Methionine (Vitamin U) in Plant Metabolism

S-methyl-L-methionine (SMM), also known as vitamin U (Pimenta et al., 1995) is a non-proteinogenic free amino acid, present in all flowering plants. It was first identified by McRorie and his coworkers, in 1954. It is biosynthesized from methionine mainly in leaves and translocated through the phloem vessels, its main sinks being represented by seeds, where it is reconverted into methionine (Amir, 2008; Emes, 2009; Miret & Munne-Bosch, 2014). Usually it is present in a concentration of 0.5-3.0 micromoles in 1 gram of plant dry weight, the highest amounts being measured in green leaves of dicots belonging to the *Brassicaceae* family (Bourgis et al., 1999; Racz et al., 2008; Ranocha et al., 2001). It is a donor of methyl groups for several metabolic reactions in plants, including biosynthesis of vitamin E (tocopherol) and vitamin H (biotin), histone and DNA methylation during epigenetic regulation of gene expression. It is a methylating agent also for the production of certain phenylpropanoids (essential for the biosynthesis of lignins), flavonoids, volatile methyl esters (e.g., methyljasmonate, methylbenzoate and methylsalicylate as signalling molecules), carbocyclic fatty acids, nicotianamine (an iron chelator), in biochemical reactions catalyzed by different methyltransferases (Weretilnyk et al., 2001). It is also involved in the biosynthesis of polyamines and ethylene, as universal growth regulators in cormophytes. S-methylmethionine plays a significant role in the phloem transport of reduced sulfur. Its main metabolic function is regulation of the ratio between S-adenosylmethionine (AdoMet or SAM) and methionine, being the specific constituent of the futile SMM cycle, closely linked to the methionine cycle or active methyl cycle (Khan et al., 2013; Ko et al., 2004; Kocsis et al., 2003; Ravanel et al., 2004; Zagorchev et al., 2013).

In plants S-methylmethionine is formed from L-methionine in a reaction catalyzed by S-adenosylmethionine:methionine S-methyltransferase. It can be reconverted to methionine by donating a methyl group to L-homocysteine, in a process driven by the enzyme homocysteine S-methyltransferase. The two-way conversion between methionine and S-methylmetionine, i.e., the SMM cycle, is thought to ensure a methionine pool for regulation of methionine cycle. Each turn of the SMM cycle consumes and then regenerates two methionines, while converting ATP to adenosine, pyrophosphate and ortophosphate (Mudd & Datko, 1990; Rarocha et al., 2001; Roje, 2006). In certain plant species, S-methylmethionine is the precursor of the efficient osmo- and cryoprotectant 3-dimethylsulfoniopropionate (Ogawa & Mitsuya, 2012; Trossat et al., 1998).

It was demonstrated that pretreatment of maize seedlings with micromolar concentrations of S-methylmethionine reduces cell membrane damage, stimulates the synthesis of certain polyamines (spermidine, putrescine and agmatine), reduces ethylene production, stimulates the phenylpropanoid pathway of the secondary metabolism, protects the photosynthetic apparatus of chloroplasts when plants are exposed to low-temperature stress (Kosa et al., 2011; Paldi et al., 2014; Szego et al., 2009) and to heavy metal toxicity, especially to cadmium (Fodorpataki et al., 2012). Its accumulation was also observed in aerobic-etiolated

and in anoxic rice seedlings, being involved in methyl group storage for post-stress recovery (Menegus et al., 2004). Its beneficial effect was demonstrated in maize against mosaic virus infection (Ludmerszki et al., 2011).

Antioxidants Involved in Protection against Extreme Living Conditions

Several environmental stress factors exhibit a convergent damaging effect in living organisms by inducing overaccumulation of reactive oxygen species (singlet oxygen, superoxide radical anion, hydrogen peroxide, hydroxyl radical, alkyl peroxides and organic hydroperoxyl radicals), which damage the structure and functions of enzymes and other proteins, of membrane lipids with unsaturated fatty acids, of nucleic acids, of chlorophylls and of other molecules indispensable for normal metabolism of plants (Apel & Hirt, 2004). To cope with this very frequent oxidative stress that accompanies the more specific consequences of a plethora of abiotic stresses, plants have evolved a highly integrated protective system consisting of several antioxidants. The antioxidative protection system consists of enzymatic and non-enzymatic components. Antioxidative enzymes are localized in different compartments of plant cells, and their catalytic function is regulated by stress signals. Superoxide dismutases are the most stable constituents of this protective enzyme system, with isozymes localized in chloroplasts, mitochondria, peroxysomes, the cytosol and the apoplast (cell wall space), where they efficiently transform superoxide radicals, with production of hydrogen peroxide, which is also a harmful oxygen derivative. This is why their activity is highly correlated with that of hydrogen peroxide-scavenging enzymes. Ascorbate peroxidase, also with several isozymes in different cell compartments, has a high affinity to its inorganic substrate, it already detoxifies micromolar amounts of hydrogen peroxide, using the reducing property of vitamin C. Its activity depends on the concentration of reduced ascorbic acid, and is inhibited by high amounts of hydrogen peroxide. Its cytosolic isozyme is one of the most sensitive biochemical markers of the degree of oxidative stress. Catalase is present in peroxisomes, it is active in the millimolar range of hydrogen peroxide concentrations, and it scavenges hydrogen peroxide originated both from photorespiration and oxidative stress. It does not require an organic cosubstrate, its activity depends directly on the amount of generated hydrogen peroxide, and because of its pronounced susceptibility to photoinactivation, it is characterized by a very high turnover rate. Peroxiredoxins detoxify, beside hydrogen peroxide, organic hydroperoxides produced during membrane lipid peroxidation, and they function in combination with thioredoxins, glutaredoxins, sulfiredoxins, methionine sulfoxide reductases and other protective proteins. These enzymes that directly scavenge reactive oxygen species with reducing agents as cofactors, usually act together with reductases that enable regeneration of the reduced form of the organic cofactor, in order to ensure a continuous defence capacity (Rouhier et al., 2008). The most important reductases involved in antioxidative mechanisms are glutathione reductase, dehydroascorbate reductase, monodehydroascorbate reductase. Generally, when plants become tolerant to abiotic stress factors that induce oxidative damage, the activity of antioxidative enzymes increases, while in case of pronounced sensitivity or upon prolonged exposure to stress, their catalytic activity registers a steep depletion (Gill & Tuteja, 2010). In contrast to abiotic stresses, biotic stress caused mainly by infection with pathogenic microorganisms down-regulates catalase activity,

in order to increase the amount of hydrogen peroxide which acts as an antiseptic agent during the hypersensitive reaction to pathogen attack (Pogany et al., 2006).

The non-enzymatic constituents of the antioxidative defence system are represented mainly by glutathione and related thiol compounds, ascorbic acid (vitamin C), tocopherol (vitamin E), carotene (pro-vitamin A) and related carotenoids (mainly xanthophylls), phenolic compounds such as flavones, flavonols, anthocyanins and tannins. Glutathione has multiple roles in regulation of the redox homeostasis necessary for undisturbed metabolic processes (Kumar et al., 2010). It alleviates the harmful effect of many environmental stress factors, being involved in antioxidative protection, in post-translational regulation of protein functions under stress conditions, in immobilization of excessive amounts of heavy metals, as well as in detoxification of organic xenobiotics (e.g., pesticides), and other protective mechanisms. Ascorbic acid is the most abundant antioxidant in plants. It has an important role in scavenging toxic hydrogen peroxide, in regeneration of vitamin E consumed during the antioxidative defence, in redox homeostasis, in formation of photoprotective xanthophylls (antheraxanthin and zeaxanthin), in regulation of the reduced ferro ion pool of cell compartments (Smirnoff, 2000). Vitamin E or tocopherol is a hydrophobic molecule occurring in membranes, especially in the thylakoid system of chloroplasts. It has a main role in detoxifying the very harmful hydroxyl and alkil peroxyl radicals, thus preventing peroxidation of membrane lipids, which would adversely modify the selective permeability and the fluidity of cell membranes. Carotenoids (carotenes and xanthophylls) are photosynthetic pigments localized in the thylakoid membranes and plastoglobuli of chloroplasts, as well as in chromoplasts. They have a photoprotective function by preventing formation of singlet oxygen or by neutralizing the already generated singlet oxygen and the hydroxyl radical (Smirnoff, 2005). From among the secondary metabolites with pronounced protective functions in counteracting stress effects, water-soluble phenoloids are the most effective ones. Among them, certain flavonoids exhibit the highest antioxidative capacity, manifested in inactivation of reactive oxygen radicals and hydrogen peroxide. The flavonoids with the most powerful antioxidant capacity belong to the group of dihydroxy B-ring-substituted flavones and flavonols, especially those classified as quercetin and luteolin glycosides. They substitute ascorbic acid as cosubstrate for scavenging of hydrogen peroxide by vacuolar peroxidases. They may exhibit not only intracellular, but also epicuticular localization, being secreted on the surface of plant organs. Beside their antioxidative capacity, they possess other benefic properties in protection against UV-B radiation, in regulation of stress-induced morphogenetic processes through influence on auxin receptors and on mitogen-activated protein kinases during post-translational regulation of stress reactions (Ramakrishna & Ravishankar, 2014; Zhang & Kirkham, 1996). It is worth mentioning that the non-enzymatic antioxidants are relatively small molecules that may be produced in high concentrations, and upon ingestion of the plant material that contains them, they are not digested and enter the animal and human organism which consumed the plant. Because oxidative stress may exist and has similar harmful effects in any living organism, plant metabolites with antioxidative properties exert a health-promoting effect in animals and humans, by enforcing the immune system, by lowering the possibility of malign tumor formation, by slowing down the aging process of cells, by contributing to redox homeostasis necessary for maintenance of normal cell functions. This is how stimulation of antioxidant biosynthesis in crop plants provides health benefits to humans (Mittler, 2002; Neill et al., 2009).

Impact of High Salinity on Plant Growth and Metabolism

Beside drought and extreme temperatures, high salinity of the soil solution is a major stress factor that limits crop plant production in many cultivated areas. It is estimated that approximately 20% of the world's cultivated area and almost half of the irrigated lands are affected by salinity (Sairam & Tyagi, 2004). A soil is considered saline if the electrical conductivity of the saturated soil paste extract is higher than 4 dS m^{-1}, which roughly corresponds to 40 mM NaCl (Chimmusamy et al., 2005). Soils with high salinity may be saline soils, where the main soluble salts are sodium chloride and sodium sulfate (and the pH is usually moderately alkaline, lower than 8.5), and sodic soils with sodium carbonate and sodium bicarbonate as the main constituents, and with pH values higher than 8.5 (Hasanuzzaman et al., 2013; Rasool et al., 2013). Considering that most of the cultivated plant species are sensitive to salt stress, and that increasing the yield of crop plants in less productive lands is an absolute requirement for feeding the growing human population, the development of plants that can tolerate high soil salinity is a practical demand for solving the problem of quantitatively and qualitatively corresponding food supply (Yamaguchi & Blumwald, 2005). Exploitation of natural variations through direct selection using physiological and biochemical markers, as well as improvement of salt tolerance of the existing cultivars are two feasible, cost-effective approaches to develop crops with high productivity on saline soils, which are extending in relation to global climate changes that include extension of dry and warm regions. By selecting salt tolerant cultivars and by improving the quality of their marketable yield with adequate treatment, it is possible to increase plant production in stressful environments, even without creation of transgenic plants with introduction of novel genes or with altered expression levels of the existing genes in order to decrease salt sensitivity (Ashraf & Harris, 2004; Caliskan, 2011; Sekmen et al., 2014).

In terrestrial habitats characterized by constantly high salinity, halophytes are evolutionarily adapted plant species which do not experience stress, and consequently do not exhibit reactions of stress tolerance, because they avoid the negative effects of high concentrations of sodium and chloride ions. Most of the halophytes, like tree species constituting the mangroves and vascular plants inhabiting salt marshes, are resistant to high salinity, because they are able to avoid salt accumulation in the above-ground organs by sequestration in roots, by controlled uptake, or by salt excretion. Many halophytes develop succulency to dilute the salt solution of the cells and to prevent the dehydrating effect of high salinity. Crop plants are not resistant to salt stress, but different cultivars and local varieties may exhibit different degrees of salt tolerance or sensitivity, and salinity tolerance may be enhanced by gradual hardening (Joshi et al., 2015; Karan & Subudhi, 2012).

High salinity impairs growth and metabolism of salt-sensitive plants by inducing an osmotic stress because of more pronounced osmotic potential of the concentrated soil solution which imposes difficulties in water supply and dehydrates cells, it also exerts an ion toxicity because of excessive amounts of sodium ions which inhibit metabolic reactions and cause an imbalance in the cellular ion homeostasis, and a side-effect of high salinity is induction of oxidative stress by generation of higher amounts of reactive oxygen species. Due to its osmotic and oxidative component, salt stress exhibits several common features with drought stress, as well as with cold and heat stress. This is the reason why an obvious cross-tolerance exists for high salinity, low water availability and extreme temperatures of the environment, and improving salt tolerance will lead to a better water economy and decreased sensitivity

towards adversely low and high temperatures (Mahajan & Tuteja, 2005; Mahmoudi et al., 2010; Munns, 2002; Rajibi et al., 2014; Zhu, 2002).

A main consequence of salt stress in plants is inhibition of shoot growth, manifested especially in reduced biomass production (directly related to yield of crop plants) and in limited leaf surface expansion, which finally reduces photoassimilation area. Short-term salt stress causes an osmotic imbalance by impairing water supply. This effect may be compensated by osmoregulation, through accumulation of osmotically active compatible solutes (e.g., proline, sugar alcohols, glycine betaine in certain species adapted to salty habitats), and through reduction of transpirational water loss by hydroactive stomata closure. These antistress processes are regulated by plant hormones, especially by abscisic acid (Tilbrook & Roy, 2014; Upadhyaya et al., 2013). The most specific manifestation of salt stress develops upon long-term exposure to high salinity, when sodium ions accumulate in plant organs in an amount which causes toxicity, mainly through imbalance in potassium and calcium homeostasis. This ion toxicity may cause the premature senescence of older leaves and a delayed and decreased fruit formation. This toxic effect may be prevented or diminished by sequestering the sodium ions in cell compartments where they cannot exert their inhibitory influence (Parida & Das, 2005). This is why one of the most important components of salt stress tolerance is an enhanced activity of the sodium ion - hydrogen ion antiporter in the plasma membrane and in the tonoplast of plant cells (associated with an increased activity of proton pumps), ensuring excretion on the cell surface (in the cell wall space) and sequestration into the vacuolar sap of the excess sodium, were there are no metabolic processes to be damaged, e.g., through antagonism with potassium ions in regulating enzyme activities (Lauchli & Grattan, 2007). For developing salt tolerance, some crop plant species translocate sodium ions through the xylem sap and store them in the vacuoles of leaf cells, thus using the accumulated sodium ions in osmoregulation, to increase the internal water-absorbing power (the osmotic potential) of cells (Bartha et al., 2015), while other species protect themselves against salt toxicity by retention of sodium and chloride ions in the root tissues, with no translocation to other organs (Munns & Tester, 2008). Finally, because high salinity induces oxidative stress in plants, a less specific component of salt stress tolerance is related to enhanced antioxidative activity, in order to protect cell components from the harmful effect of reactive oxygen species that are generated in an increased quantity (Andriolo et al., 2005; Ruiz & Blumwald, 2002; Younis et al., 2009; Zushi et al., 2009).

For a correct and efficient selection of salt tolerant crop plant varieties and for evaluation of the results of pretreatment with priming agents in the attempt to obtain a marketable yield which provides human health benefits, controlled environmental conditions are needed. For this purpose, hydroponic cultures are the best suited technical implementations, because they exclude the unknown and uncontrollable parameters of different soils, because all the inorganic nutrients are directly available for uptake by roots, because the exact concentration of substances applied for different treatments can be set and monitored in time, and because constant growth conditions can be created to ensure repeatability and to reduce the influence of factors other than the ones selected for investigation (Al-Maskri et al., 2010; Resh, 2015; Shavrukov et al., 2012).

The aim of the present chapter is to present the benefic influence of priming with the naturally occurring S-methylmethionine on increased accumulation of non-enzymatic anti-oxidants in fresh leaves of lettuce plants exposed to high salinity in hydroponic cultures, thus

obtaining a specialty crop with improved health-promoting quality. An updated scientific background of priming for environmental stress tolerance and its prospective application in obtaining crop plants with human health benefits through antioxidative protection capacity is presented synthetically.

METHODS

Plant Material and Experimental Conditions

Sixteen lettuce (*Lactuca sativa* L.) cultivars, largely used in nutrition as fresh vegetables (mainly in salads), were evaluated in previous experiments for their salt stress tolerance (Bartha et al., 2010). The Paris Island variety was found to be moderately tolerant to high salinity and was selected for experiments concerning priming with S-methylmethionine to enhance antioxidative defense during stress acclimation. Seeds were purchased from the seed bank of B &T World Seeds (Paguignan, France), and after one day of prehydration with distilled water were germinated for one week in Linhardt germinators, being watered with ¼ diluted Hoagland nutrient solution. The most similar thirty plantlets were transferred for two weeks in hydroponic cultures, on 3 L vessels filled with ¼ strength Hoagland solution, with continuous aeration of roots (Hoagland & Arnon, 1950; Trejo-Tellez & Gomez-Merino, 2012). The nutrient solution was refreshed every three days. The hydroponic cultures were placed under controlled environmental conditions in a growth chamber, where the temperature was kept at 20ºC and the relative air humidity at 65%. The daily photoperiod was set to 14 hours of light and 10 hours of darkness, the photon flux density, provided by a combination of fluorescent tubes and metal halide lamps, was 330 μM photons m^{-2} s^{-1} photosynthetically active radiation (Fodorpataki & Bartha, 2008). Afterwards, half of the three weeks old plants (15 out of 30) were placed for three days of pretreatment in 3 L vessels containing ¼ strength Hoagland solution supplemented with 0.05 mM S-methylmethionine (vitamin U), while the other 15 plants were grown, as controls, in ¼ Hoagland solution, under the same external conditions. pH of every nutrient solution was kept at 5.8. Salinization treatment was initiated in parallel with control plants grown in normal Hoagland solution and with individuals primed with 0.05 mM S-methylmethionine. Both primed and not primed plants were grown for six days at two salt concentrations: 75 mM sodium chloride (p.a.), which creates conditions of moderate salt stress for this lettuce cultivar, and 150 mM sodium chloride, which induces severe salt stress. Five plants were kept for the same six days in Hoagland solution without any supplementation (control), while five other lettuces continued to grow in the nutrient solution supplemented only with 0.05 mM S-methylmethionine. This means that for the last six days of the experiments five different plants were grown in ¼ Hoagland solution, five of them were in nutrient solution supplemented only S-methylmethionine, other five individuals were treated with 75 mM sodium chloride without being pretreated with S-methylmethionine, five lettuces were subjected to the influence of 75 mM sodium chloride after being primed for three days with 0.05 mM S-methylmethionine, another five not primed plants were treated with 150 mM sodium chloride, and the same treatment was applied for five lettuces primed with S-methylmethionine. Growth conditions were similar during the whole period. On the third day and on the sixth day of salt treatment, the

third and the fourth oldest leaves were used for determining the content of non-enzymatic antioxidant (ascorbic acid, glutathione, carotenoids and water-soluble phenoloids). This means that one set of analyzes was perform after a shorter time period of exposure to high salinity, and another similar set was conducted upon long-term exposure to salt stress. Every measurement was repeated three times (there were five biological repetitions and three technical repetitions for each determination).

Determination of Ascorbic Acid Content and of the Reduced to Oxidized Vitamin C Ratio

Ascorbic acid content was determined by homogenizing 0.5 g lettuce leaf with 4 mL of 6% trichloroacetic acid in a prechilled mortar. The mixture was centrifuged for 15 min at 15600 g and 4ºC, then 0.2 mL of supernatant was transferred to sodium phosphate buffer (pH 7.4) containing trichloroacetic acid, dithiothreitol, ortophosphoric acid, ethanolic solution of 2,2'-dipyridyl, N-ethylmaleimide and ferric chloride. After one hour of incubation of this extract mixture at 42ºC with permanent mixing, absorbance of the solution was measured at 525 nm with a spectrophotometer. The ferro ions resulting from the reaction of ferric chloride with the reduced ascorbic acid forms a coloured product with 2,2'-dipyridyl. The oxidized form of vitamin C present in leaf tissues (dehydroascorbate) is reduced to ascorbate by dithiothreitol. Then the total ascorbic acid content is determined photometrically by the 2,2'-dipyridyl method, and concentration of dehydroascorbate is calculated as the difference between total and reduced ascorbate. Standard curve was obtained with known concentrations of pure ascorbic acid dissolved in 6% trichloroacetic acid, in the range of 25-100 nM (Kampfenkel et al., 1995).

Measurement of Glutathione Content and Evaluation of the Reduced to Oxidized Glutathione Ratio

Glutathione concentration was determined using 0.5 g lettuce leaf homogenized in 3 mL of 5% sulfosalicylic acid and centrifuged for 10 min at 14000 g and 4ºC. 2 µL of 3-ethanolamine and 2 µL of 2-vinylpyridine were added to 0.1 mL of supernatant, and the mixture was incubated for one hour at room temperature. This serves for determining the oxidized glutathione. Glutathione concentration in the samples was determined as increase of absorbance at 412 nm upon reduction of 5,5-dithio-bis (2-nitrobenzoic acid), in a reaction mixture with potassium phosphate buffer (pH 7.5), ethylenediamine-tetraacetic acid and NADPH. This mixture was incubated for 10 min at 30ºC, then 10 µL of glutathione reductase solution (50 units in one mL) were added to 50 µL of extract sample (Fodorpataki et al., 2015; Zushi et al., 2009).

Quantification of Carotenoid Content of Lettuce Leaves

Carotenoid pigments were extracted using 0.25 g of lettuce leaves finely homogenized in a final volume of 5 mL pure methanol heated until complete extraction. The pigment extract was centrifuged for 10 min at 4000 g, and the carotenoid content of the supernatant was determined spectrophotometrically, by measuring the absorbance of the solution at the wavelengths 470 nm, 653 nm and 666 nm (Lichtenthaler & Wellburn, 1983).

Determination of Water-Soluble Phenoloid Content

For determination of phenolic content 1 g fresh leaf was homogenized in a mortar with 6 mL of 80% acetone (v/v), then the samples were incubated for 8 hours at 4ºC and centrifuged for 3 min at 3000 g. 50 µL of supernatant was transferred to a mixture containing 135 µL distilled water, 750 µL 1/10 diluted Folin-Ciocalteu reagent and 600 µL of 7.5% (w/v) sodium carbonate. After a thorough mixing for 30 seconds, the mixture was incubated at 45ºC for 15 min, then it was allowed to cool down to the room temperature and its absorbance was measured at 765 nm. Instead of 50 µL leaf extract, 50 µL of 80% (v/v) acetone was used as blank. The standard curve was obtained with 1 mg mL^{-1} gallic acid solution in 80% (v/v), freshly prepared from stock solution, and phenoloid content was expressed in gallic acid equivalents in unit of leaf fresh weight (Oh et al., 2009a).

Statistical Analysis

Experimental data were statistically processed in R environment (version 2.14.1), using one-way ANOVA and the post-hoc Tukey HSD test for the significance of differences between treatments. The results were expressed as mean ± standard error, and $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Influence of Priming with S-Methylmethionine on Vitamin C Content and on Reduced Ascorbic Acid to Oxidized Dehydroascorbate Ratio of Lettuce Leaves Exposed to Salt Stress

S-methylmethionine by itself does not exert any significant influence on the vitamin C content of lettuce leaves, when it is added in concentration of 0.05 mM to the nutrient solution of hydroponic cultures of the Paris Island lettuce cultivar. Moderate salt stress exerted by 75 mM sodium chloride, with no priming, also does not cause changes in ascorbic acid concentration of leaves, neither after three days of exposure, nor after six days. On the contrary, high salinity of the nutrient medium, given by 150 mM sodium chloride, increases vitamin C content in the same degree both after shorter (three days) and longer (six days) exposure time, without priming. When pretreatment with 0.05 mM S-methylmethionine was

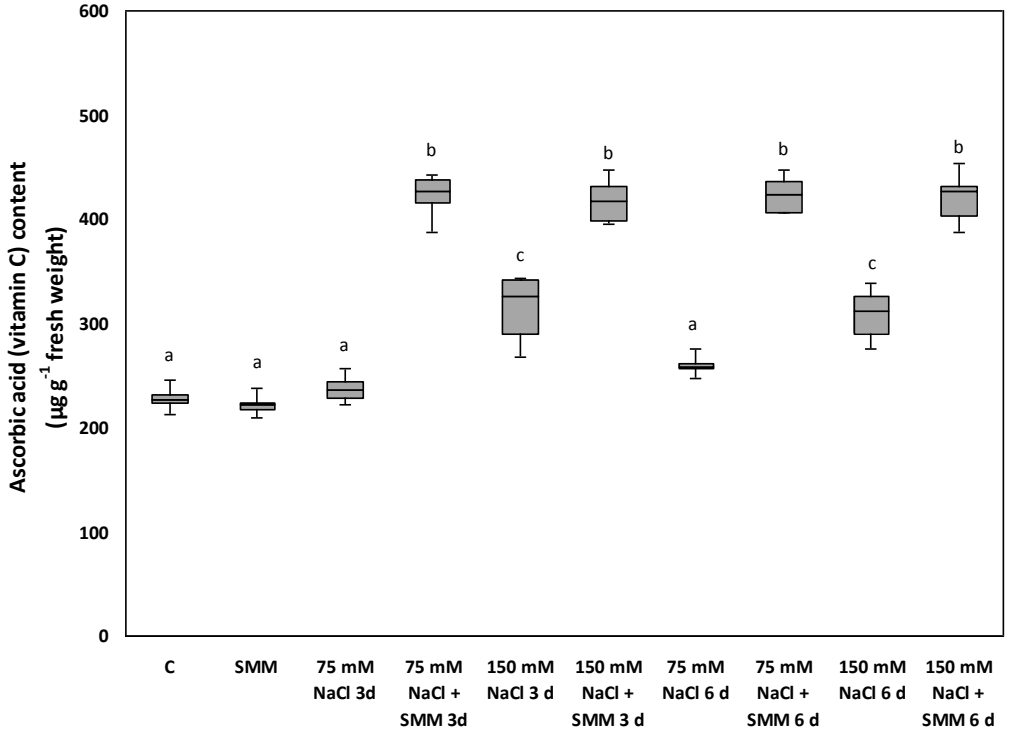
applied in hydroponic cultures for three days before subjecting the plants to salt stress, a higher vitamin C content of the lettuce leaves was recorded in every experimental variant, irrespective of the salt concentration and of the exposure period (Figure 1).

Vitamin C cannot be produced by human organism, and upon ingestion it is regularly eliminated through excretion, so a systematic supply with this vitamin through alimentation is required for normal cell functions and for avoidance of severe symptoms of avitaminosis. Plants synthesize vitamin C in the highest amounts, especially under environmental conditions which cause oxidative stress. Under these circumstances, vitamin C may accumulate in photosynthetic leaf cell in concentrations as high as 1-5 mM, and chloroplasts may contain up to 25 mM ascorbic acid (Wheeler et al., 1998). Its oxidized form is unstable, being transformed into other metabolites in a process stimulated by heat. In this context, fresh lettuce leaves may constitute an important source of vitamin C for humans, and a higher ascorbate content confers higher nutritional value to the vegetable. Its crucial role in detoxifying excessively accumulated hydrogen peroxide in cell compartment which do not possess catalase (i.e., except for peroxisomes and related microbodies) is ubiquitous for all living organisms. Its content in plant tissues may increase under several stress conditions, when the enhanced activity of ascorbate peroxidase and ascorbate oxidase up-regulates the biosynthesis of ascorbic acid from simple sugars (mannose and galactose). This increment was observed mainly under severe or prolonged stress conditions (Tuna et al., 2013). The fact that pretreatment with S-methylmethionine (vitamin U) significantly increases vitamin C content of plants exposed to salt stress which also implies enhanced production of reactive oxygen species, reflects that under the influence of the priming agent plant cells become prepared to receive stress-induced signals and to respond more intensely to the oxidative component of salt stress by an enhanced accumulation of the non-enzymatic antioxidant represented by vitamin C. The experimental results also indicate that there is no dose-effect and time-effect relationship between salinity and increment of vitamin C content induced by priming with vitamin U, because similar ascorbate concentrations were detected in all lettuce leaves, irrespective of sodium chloride concentration and of exposure period.

Upon exposure to oxidative stress, in several crop plants there is no significant change in the absolute vitamin C content, but the ratio between its reduced and oxidized form is being lowered. This is why this ratio was also monitored during development of salt stress in lettuce plants primed with S-methylmethionine. It was found that in a shorter period of three days of salinity stress only the higher salt concentration caused a slight decrease of the ratio between the reduced ascorbic acid and the oxidized dehydroascorbate (from around 88% in control to around 75%), but after six days of exposure both salt concentrations induced a more pronounced decrease of this ratio (to averages of around 60% and 50%, respectively). Priming with S-methylmethionine resulted in the maintenance of this ratio at the increased level registered in control plants, except for the lettuces being exposed for a longer period to high salt concentration, in which case priming also maintained a higher ratio, but not as high as in control (Figure 2).

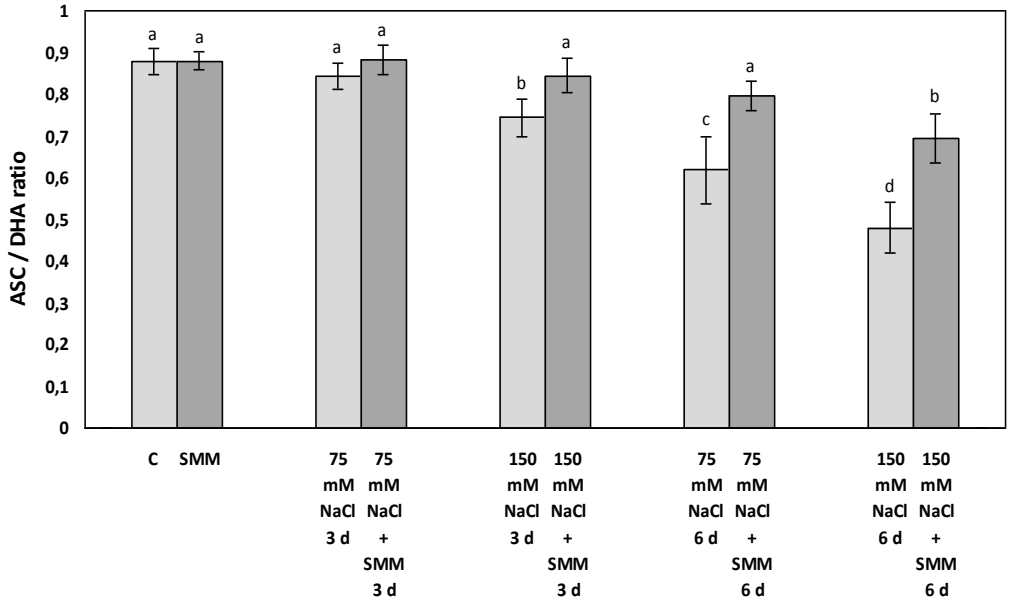
Reduction of the ratio between the reduced and the oxidized form of vitamin C in plants grown under adverse conditions associated with oxidative stress may be related to the fact that increased activity of different isozymes of ascorbate peroxidase and of ascorbate oxidase, as part of the antioxidative defence mechanism in which reduced vitamin C is used as cosubstrate for reduction of hydrogen peroxide to water molecules, is not followed by a similar increment of the catalytic activity of enzymes which regenerate the reduced vitamin C

from its oxidized form (i.e., dehydroascorbate reductase and monodehydroascorbate reductase). Higher oxidative stress requires higher consumption of reduced ascorbate, and this leads to lowering of the above mentioned ratio. Priming with S-methylmethionine increases this ratio, regeneration of the reduced form of vitamin C being stimulated in the primed state of lettuce plants. This is also a benefic effect, because only the reduced form of vitamin C is able to decompose the harmful hydrogen peroxide, while its oxidized form is less stable and inactive in redox regulation (Lee & Kader, 2000). Considering the time course of vitamin C accumulation in leaves of lettuce plants primed with vitamin U and then subjected to salt stress, one can observe that the increment of ascorbate concentration occurs during the first few days of exposure to salt stress, both in primed plants and in those subjected to severe stress without priming. Between the third and the sixth day of exposure to salt stress the lettuces pretreated with 0.05 mM S-methyl-methionine maintained a constantly higher ascorbate level in their leaves (Figure 3). This reflects that induction of enhanced vitamin C accumulation occurs during the first days of stress reaction, and S-methylmethionine stimulates this process, in relation with a quicker preparedness to counteract the upcoming oxidative challenge.



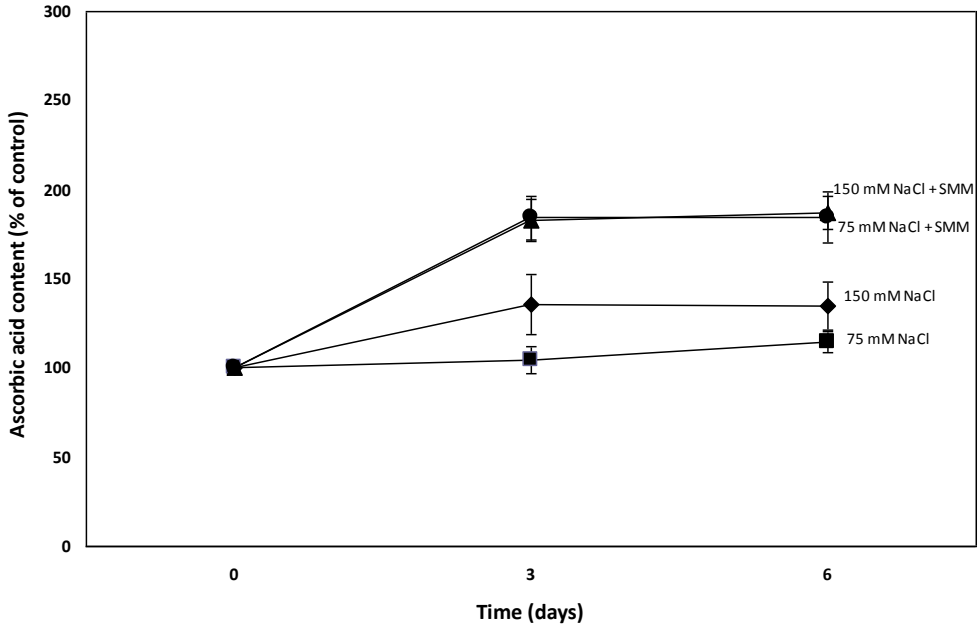
C – control grown in Hoagland's nutrient solution with no SMM and no NaCl. Vertical bars represent ± SE from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Figure 1. Influence of priming with S-methylmethionine (SMM) on ascorbic acid concentration in leaves of lettuce plants exposed to two levels of salinity stress (75 mM NaCl and 150 mM NaCl) aftre three days (3 d) and six days (6 d) of exposure.



C – control grown in Hoagland's nutrient solution with no SMM and no NaCl. Vertical bars represent ± SE from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Figure 2. Reduced ascorbic acid (ASC) to oxidized dehydroascorbate (DHA) ratios in leaves of lettuce plants subjected, without and with pretreatment with S-methylmethionine (SMM), to two salinity levels (75 mM NaCl and 150 mM NaCl), after three days (3 d) and six days (6 d) of exposure in hydroponic cultures.



Bars represent ± SE from means (n = 5).

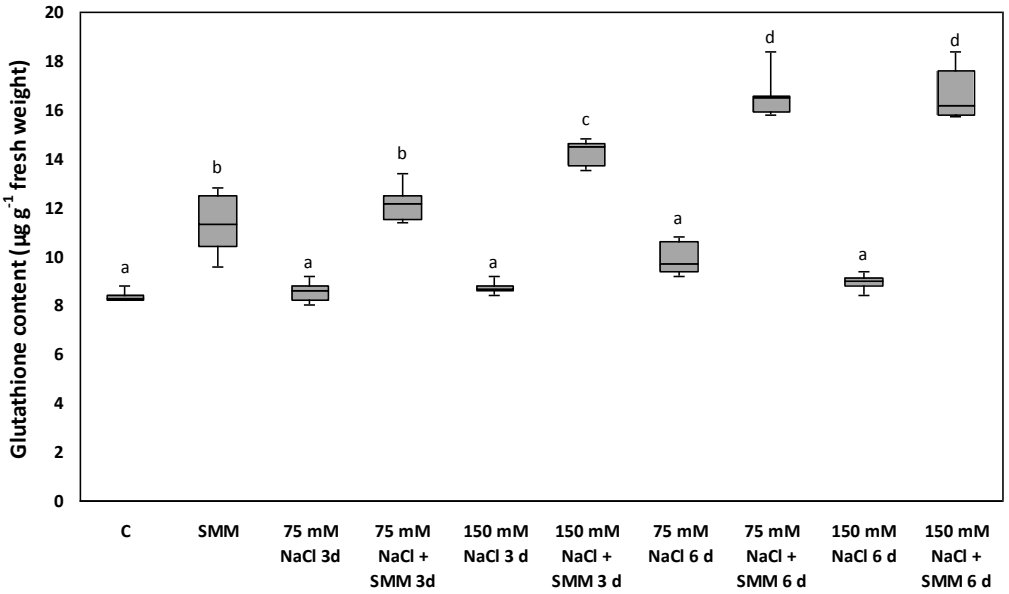
Figure 3. Time-dependent changes in ascorbic acid (vitamin C) content of leaves of lettuce plants exposed to 75 mM and 150 mM NaCl with or without priming with S-methyl-methionine (SMM).

Changes in Glutathione Content and in Reduced to Oxidized Glutathione Ratio of Salt-Stressed Lettuce Leaves Pretreated with S-Methylmethionine

Glutathione content of lettuce leaves was not significantly modified by none of the applied salt concentrations, but it was moderately increased by the presence for three days of 0.05 mM S-methylmethionine in the nutrient solution surrounding the root system in the hydroponic cultures. When primed plants were subjected to salt stress, a further increment of glutathione concentration was registered. While in SMM-primed plants subjected to 75 mM sodium chloride, after three days the glutathione content was similar to the one measured in plants treated with S-methylmethionine with no subsequent salt exposure, this amount further increased slightly as salt stress became prolonged to six days.

Upon severe salt stress exerted by 150 mM sodium chloride, pretreatment with S-methylmethionine resulted in an even higher glutathione content (approximately twice as much as in control), both after three and six days of stress exposure (Figure 4).

Glutathione is, beside ascorbic acid, the other major non-enzymatic component of the antioxidative defence system, with several roles in redox regulation during normal metabolism and in mechanisms of tolerance to various environmental stress factors, related to oxidative stress (Chattopadhyay, 2014). Being a tripeptide with a sulfur-containing amino acid, it is in some extent metabolically related to S-methylmethionine as derivative of the sulfur-containing methionine. As an organic storage compound of reduced sulfur, which can be easily translocated through xylem and phloem vessels, it may serve as source for synthesis of new amounts of glutathione, but at present this possibility is not adequately documented.

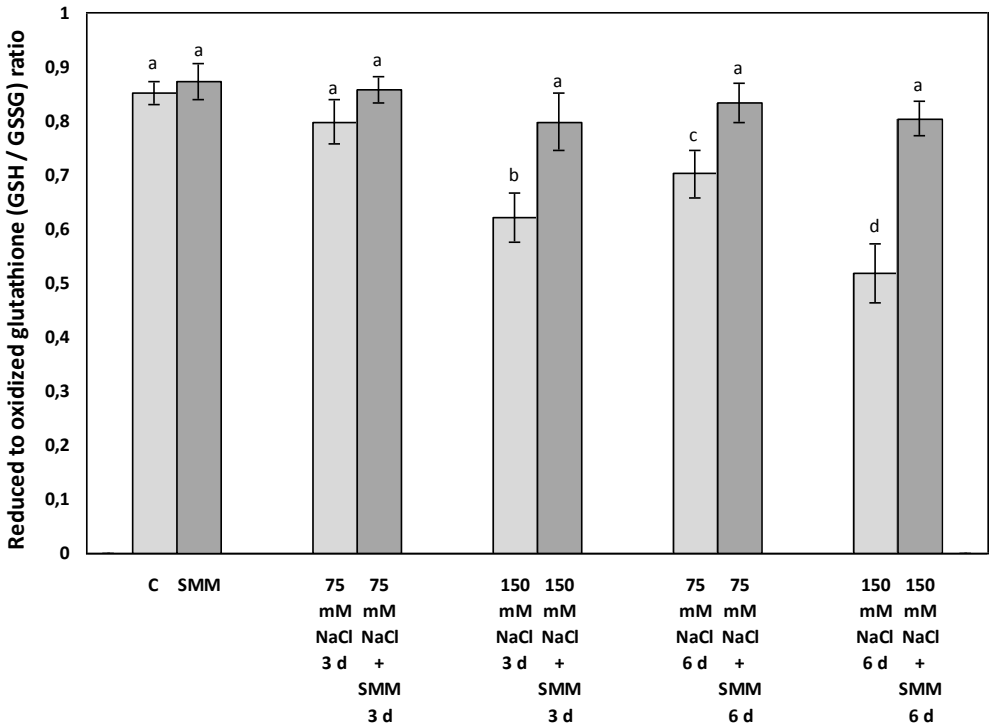


C – control grown in Hoagland's nutrient solution with no SMM and no NaCl. Vertical bars represent ± SE from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Figure 4. Influence of priming with S-methylmethionine (SMM) on glutathione concentration in leaves of lettuce plants exposed to two levels of salinity stress (75 mM NaCl and 150 mM NaCl) after three days (3 d) and six days (6 d) of exposure.

Involvement of glutathione in tolerance of salt stress was observed in different crop plants, and was correlated with the oxidative side-effect of high salinity in plant cells. The fact that SMM increases glutathione pool even if salinity by itself does not lead to the same result, reflects that priming with S-methylmethionine sensitizes plants for a more efficient defence against a later oxidative danger, by inducing early accumulation of glutathione reserves. Its general role in regulating redox homeostasis needed for normal metabolic processes in different cell compartments of all organisms makes plant food with higher glutathione content more valuable as health-promoting nutrient (Noctor & Foyer, 1998). In this context, increased concentration of glutathione in leaves of SMM-primed lettuce plants subjected for several days to salt stress confers a higher quality with respect to human health benefits.

As in the case of vitamin C, only the reduced form of glutathione acts directly in antioxidative defence processes, and the capacity to regenerate it from the oxidized form is a prerequisite for sustained protection against reactive oxygen species. In leaves of salt-stressed lettuce plants priming with S-methylmethionine prevents decrease of the reduced to oxidized glutathione ratio, which can be observed in not primed plants exposed for a longer period to moderate salt stress, or for both shorter or longer period to severe salt stress. In non-stressed plants S-methylmethionine does not influence this ratio (Figure 5).



C – control grown in Hoagland's nutrient solution with no SMM and no NaCl. Vertical bars represent ± SE from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Figure 5. Reduced to oxidized glutathione (GSH/GSSG) ratios in leaves of lettuce plants subjected, without and with pretreatment with S-methylmethionine (SMM), to two salinity levels (75 mM NaCl and 150 mM NaCl), after three days (3 d) and six days (6 d) of exposure in hydroponic cultures.

As in case of ascorbic acid, maintenance of a high ratio between the reduced and the oxidized form of glutathione reflects that enzymes that use reduced glutathione as substrate for reduction of hydrogen peroxide through regeneration of ascorbic acid (dehydroascorbate reductase, monodehydroascorbate reductase) and those which regenerate the reduced glutathione from its oxidized form (isozymes of glutathione reductase) act in a coordinated manner, and no depletion of reduced glutathione pool is caused by enhanced antioxidative defence upon accumulation of reactive oxygen species.

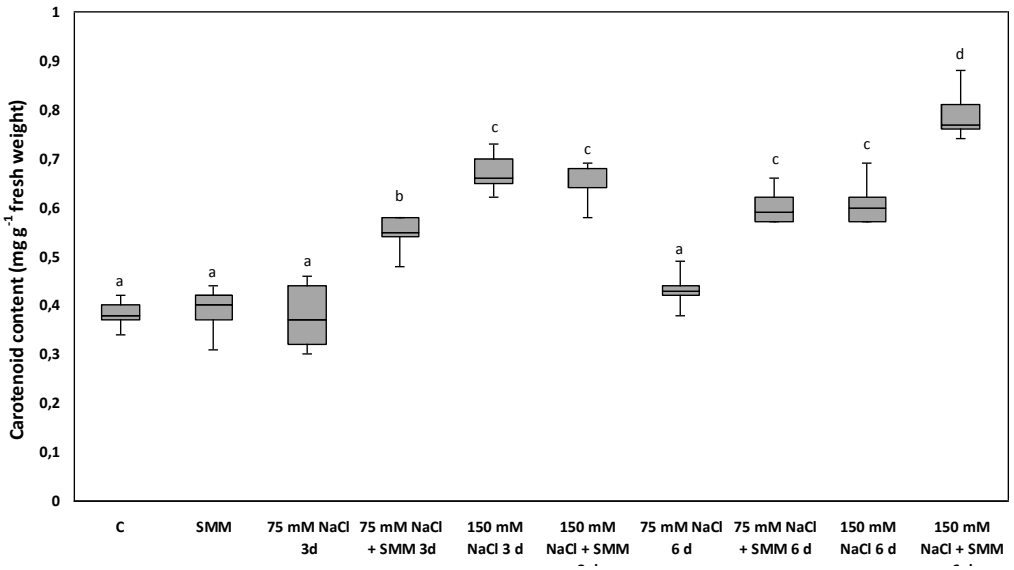
This is why maintenance of a highly reduced state of the glutathione pool in salt-stressed plants is benefic for an efficient and sustained antioxidative defence needed for enhanced stress tolerance (Kumar et al., 2010).

Carotenoid Content of Leaves of Hydroponically Grown Lettuce Plants Primed with S-Methylmethionine and Subjected to High Salinity of the Nutrient Medium

Carotenoid content of lettuce leaves is not influenced by the presence of S-methyl-methionine under stressless growth conditions and by moderate salt stress in not primed plants.

But if 75 mM sodium chloride is supplied for lettuces pretreated with SMM, carotenoid content slightly, but significantly increases. More severe salinity stress generated by 150 mM sodium chloride increases total carotenoid pigment concentration with or without priming with SMM, and the highest carotenoid level is reached in leaves of plants pretreated with 0.05 mM S-methylmethionine and then subjected to severe salt stress for six days (Figure 6).

Carotenoids are photosynthetic pigments produced specifically by plants, localized in thylakoid membranes and plastoglobuli of chloroplasts, as well as in chromoplasts. Beside absorbing blue light photons and transferring a part of their energy content to chlorophylls to sustain photochemical reactions, they have a pronounced protective role against photooxidative damages caused to proteins, membrane lipids, nucleic acids and chlorophylls by singlet oxygen, hydroxyl radical and alkyl peroxyl radicals generated under excessively high light intensities (Hernandez et al., 2000). Carotenoids (e.g., antheraxanthin and zeaxanthyn of the xanthophyll cycle, activated by excess light) may harmlessly dissipate excess light energy by converting it to heat. Because of their photoprotective role, carotenoids accumulate in photosynthetic cells mainly under light intensities that exceed the amount of energy that can be used for carbon assimilation under the given environmental conditions. Based on their capacity to neutralize singlet oxygen, hydroxyl and organic peroxyl radicals that represent harmful oxygen derivatives especially for membrane structures, carotenoids have a general protective role in any organism against oxidative damage (Smirnoff, 2005). This is why their increased quantity in leaves of salt-stressed lettuce plants primed with 0.05 mM S-methylmethionine for three days confers a health-promoting quality upon consumption of these lettuce leaves. We note that under the controlled hydroponic growth conditions created during the experiments referred to in this chapter, light conditions were far from being excessive and were constant during the illumination periods, so irradiance could not significantly influence carotenoid biosynthesis and breakdown, and the registered changes were related to the applied chemical treatments.



C – control grown in Hoagland's nutrient solution with no SMM and no NaCl. Vertical bars represent ± SE from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Figure 6. Influence of priming with S-methylmethionine (SMM) on carotenoid pigment concentration in leaves of lettuce plants exposed to two levels of salinity stress (75 mM NaCl and 150 mM NaCl) aftre three days (3 d) and six days (6 d) of exposure.

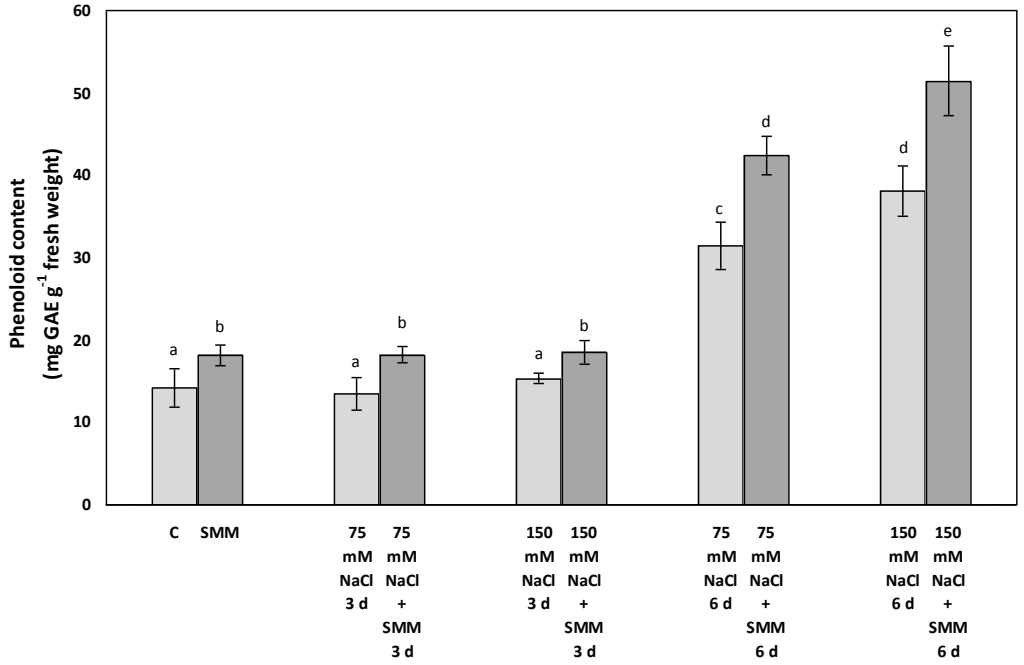
Water-Soluble Phenoloid Content of Fresh Intact Lettuce Leaves Exposed to Salt Stress Following Pretreatment with Exogenous S-Methylmethionine

The total amount of water-soluble phenolic compounds was moderately but significantly increased by supplementation of the nutrient medium of non-stressed lettuce plants with 0.05 mM S-methylmethionine, as well as by short-term exposure to 75 mM and 150 mM sodium chloride of SMM-pretreated plants. Short-term salt stress in not primed plants did not result in increased phenoloid content of leaves. Phenoloid content increased in a higher extent upon long-term exposure to salt stress, and in this case priming with S-methylmethionine further intensified the accumulation of these secondary plant metabolites with protective properties. Thus, the highest phenoloid concentration was registered in leaves of lettuce plants treated for six days with 150 mM sodium chloride, previously exposed to the priming agent (Figure 7).

Phenolic compounds represent a wide range of plant secondary metabolites with various protective and other functions related to survival, reproduction and acclimation to adverse environmental conditions. Some polycondensed phenolic compounds (mainly those constituting lignins and some tannins) cannot be hydrolised, while most of them (simple phenoloids, flavonoids and certain tannins) are water-soluble. Because of the hydroxyl groups linked to the aromatic ring, they possess an antioxidative capacity, being able to neutralize harmful reactive radicals and to reduce hydrogen peroxide. The highest reducing capacity is possessed by flavonols with several hydroxide groups. Their biosynthesis is stimulated by light, and they are localized in many cell compartments, mainly in vacuoles, in cell wall spaces, in plastids and in the nucleus. Some of them confer protection against UV-B radiations, while others play specific roles in growth regulation and in recognition of certain

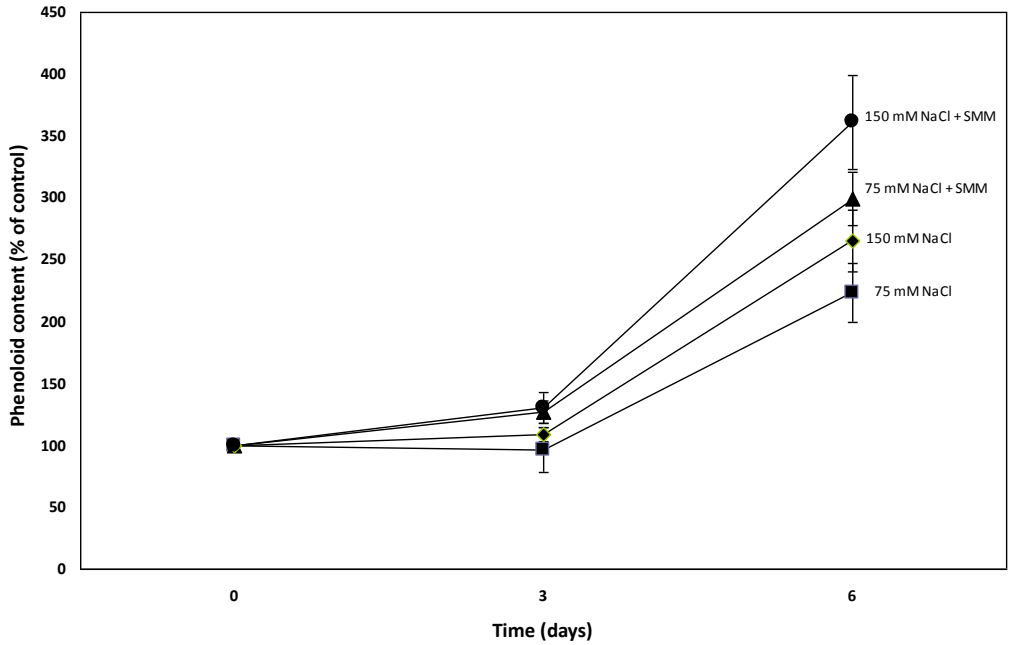
microorganisms that come into contact with plant cells. The antioxidative efficiency of different phenolic compound produced by plants vary in a wide range, the most effective ones being considered the quercetin-O-glucosides and luteolin-O-glucosides (Altunkaya & Gokmen, 2008; Bartwal et al., 2013; Ferdinando et al., 2012). Their synthesis involves methylation reactions accomplished by O-methyl transferases that use thiol-containing amino acids, which include S-methylmethionine, as well as S-adenosylmethionine (Menegus et al., 2004; Roje, 2006).

Total phenolic content was observed to increase in different crop plants following stress treatments with different physical, chemical and biological agents. For S-methylmethionine it was demonstrated that it stimulates the activity of phenylalanine ammonia lyase, the key enzyme of the phenylpropanoid biosynthetic pathway, and priming of cold stressed crop plants with SMM enhances accumulation of phenolic compounds with antioxidative properties (Kosa et al., 2011; Oh et al., 2009b; Paldi et al., 2014; Roura et al., 2008; Teklemariam & Blake, 2004). In green lettuce varieties the main phenolic compound with antioxidative properties are flavones and flavonols, anthocyanins being produced only by red lettuce cultivars (Altunkaya et al., 2009; Llorach et al., 2008; Mulabagal et al., 2010).



C – control grown in Hoagland's nutrient solution with no SMM and no NaCl; GAE – gallic acid equivalent. Vertical bars represent ± SE from means (n = 5), different letters indicate significant differences at $P < 0.05$.

Figure 7. Influence of priming with S-methylmethionine (SMM) on water-soluble phenoloid concentration in leaves of lettuce plants exposed to two levels of salinity stress (75 mM NaCl and 150 mM NaCl) after three days (3 d) and six days (6 d) of exposure.



Bars represent ±SE from means (n = 5).

Figure 8. Time-dependent changes in water-soluble phenoloid content of leaves of lettuce plants exposed to 75 mM and 150 mM NaCl with or without priming with S-methilmethionine (SMM).

Experiments presented in this chapter demonstrate that accumulation of phenolic compound in salt-stressed and S-metlylmethionine-primed lettuce is time-dependent. Only slight enhancement of their production is observed during the first few days of salt stress, while their concentration increases in a higher extent upon extended exposure (Figure 8).

The fact that phenolic compounds accumulate upon stress conditions later than other metabolites with antioxidative properties (e.g., vitamin C, see Figure 3) suggests that they have a complementary function in protection, being effective in repair of oxidative stress damages and in the re-establishment of redox homeostasis when because of long-term stress the activity of enzymes implied in antioxidative defence weakens (Hichem et al., 2009).

The exact antioxidative function of different phenolic compounds has to be established by further investigations, but it is demonstrated that their presence in human diet contributes to its health-promoting quality.

CONCLUSION

Health-promoting antioxidant content of lettuce leaves can be significantly increased if hydroponically cultivated plants exposed to high salinity stress for several days are pre-treated for three days with 0.05 mM of vitamin U (S-methylmethionine), a natural non-proteinogenic amino acid. This priming agent, absorbed through the root system from the nutrient solution, maintains a high reduced to oxidized ascorbic acid (vitamin C) and glutathione ratio, thus contributing to the redox homeostasis of cells under adverse conditions that result in increment of reactive oxygen species. Priming with S-methylmethionine enables

lettuce plants to increase the concentration of vitamin C in their leaves after a few days of exposure to moderate and high salt stress exerted by the presence of 75 mM and 150 mM sodium chloride in the nutrient solution. The content of another non-enzymatic antioxidant agent: glutathione was also increased by S-methylmethionine, in a smaller extent with no salt stress and in a more pronounced way if pretreatment of plants with this priming agent was followed by long-term exposure to high salinity.

Carotenoids, produced specifically by plants as photosynthetic pigments with a protective role under photooxidative stress conditions, accumulate in lettuce leaves mainly when plants are exposed to severe salt stress after being pretreated with vitamin U (which by itself does not influence carotenoid pigment content of leaves on a fresh weight basis).

Plant secondary metabolites belonging to the large group of water-soluble phenolic compounds (simple phenoloids, flavonoids and certain tannins), with many representatives known to possess a protective role as antioxidants in certain cell compartments, are present in higher concentrations in the leaves of lettuce plants provided with exogenous vitamin U, and their amount increases especially upon long-term exposure to salt stress, preceded by treatment with the priming agent.

Based on experimental results presented in this chapter, one can conclude that when lettuce is grown in environments characterized by high salinity of the substrate, priming with S-methylmethionine under controlled hydroponic conditions results in enhanced non-enzymatic antioxidant (vitamin C, glutathione, carotenoid and phenoloid) content of leaves, which confers higher marketable value to this crop plant because of richness in health-promoting metabolites. In this context, it is worth investigating if priming with S-methyl-methionine results in enhanced antioxidative properties or other nutritive qualities of different other crop plant species when exposed to certain abiotic or biotic stress factors or to various combinations of environmental constraints during cultivation under controlled conditions.

ACKNOWLEDGMENTS

The authors thank to Mr. Szabolcs Barna, former M.Sc. student of the "Babes-Bolyai" University, for the technical support during experimental data acquisition and processing.

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