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Research article

Elevated CO_2 and salinity are responsible for phenolics-enrichment in two differently pigmented lettuces

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A B S T R A C T

Both salt stress and high CO_2 level, besides influencing secondary metabolism, can affect oxidative status of plants mainly acting in an opposite way with salinity provoking oxidative stress and elevated CO_2 alleviating it. The aim of the present work was to study the changes in the composition of phenolic acids and flavonoids as well as in the antioxidant activity in two differently pigmented lettuce cvs (green or red leaf) when submitted to salinity (200 mM NaCl) or elevated CO_2 (700 ppm) or to their combination in order to evaluate how a future global change can affect lettuce quality. Following treatments, the red cv. always maintained higher levels of antioxidant secondary metabolites as well as antioxidant activity, proving to be more responsive to altered environmental conditions than the green one. Overall, these results suggest that the application of moderate salinity or elevated CO_2, alone or in combination, can induce the production of some phenolics that increase the health benefits of lettuce. In particular, moderate salinity was able to induce the synthesis of the flavonoids quercetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide and quercitrin. Phenolics-enrichment as well as a higher antioxidant capacity were also observed under high CO_2 with the red lettuce accumulating cyanidin, free chlorogenic acid, conjugated caffeic and ferulic acid as well as quercetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, luteolin-7-*O*-glucoside, rutin, quercitrin and kaempferol. When salinity was present in combination with elevated CO_2, reduction in yield was prevented and a higher presence of phenolic compounds, in particular luteolin, was observed compared to salinity alone.

1. Introduction

Globally averaged concentration of CO_2 in the atmosphere reached the symbolic and significant milestone of 400 ppm for the first time in 2015 (WMO Greenhouse Gas Bulletin, 2016) and its increase is predicted in conjunction with environmental stress factors including drought and salinity, especially in arid and semiarid regions (Sgherri et al., 2000b; Geissler et al., 2015). In particular, soil salinity will contribute to a more and more serious threat to agriculture, already affecting more than 6% of the global total land area and more than 30% of the global irrigated farmland (Geissler et al., 2015). Moreover, the controlled use of alternative water resources, such as diluted seawater, could be a valid tool to face drought in some regions (Sgherri et al., 2007). Thus, high CO_2 and salinity are among the major environmental constraints plants have to face in the next future.

Environmental stresses generally induce reactive oxygen species (ROS) production; consequently, an oxidative stress comes into play determining yield losses (Sgherri et al., 2007). Activation of antioxidative defense mechanisms could be observed depending on stress intensity (Sgherri and Navari-Izzo, 1995), cultivar tolerance to stress (Sgherri et al., 2000a), stage of development, tissue and stress duration (Ranjit et al., 2016), and moderate salinity has been seen to induce the synthesis of antioxidants (Sgherri et al., 2007). This is because there must be a balance between the production of ROS and their removal by antioxidant systems, which play a crucial role during oxidative stress. When the produced ROS

Abbreviations: ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid); DAS, days after sowing; EDTA, ethylenediaminetetraacetic acid; GAE, gallic acid equivalent; PB, Blonde of Paris Batavia; OL, Oak Leaf; ROS, reactive oxygen species; TEAC, trolox equivalent antioxidant capacity.

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are not counterbalanced by antioxidant pool damage to tissues occurs.

The main source of ROS production in photosynthetic tissues is represented by the leakage of electrons from the photosynthetic electron transport system to oxygen (Sgherri and Pérez-López, 2013). In particular, during environmental stresses, plants close their stomata and a CO_2-limited carbon fixation occurs, decreasing the availability of $NADP^+$ as an electron acceptor of photosystem I. Under these conditions, oxygen can compete with $NADP^+$ as a Hill reductant and superoxide may be produced (Sgherri et al., 1993). In this way, although the so called "Mehler reaction" provides a pathway for the removal of excess electrochemical energy an oxidative stress comes into play. Under elevated CO_2 conditions a higher concentration gradient exists for CO_2 both inside and outside the leaf. As a consequence, elevated CO_2 usually allows plants to cope better with those situations where stomatal conductance is decreased (Tyree and Alexander, 1993), reducing the risk of oxidative stress (Sgherri et al., 2000b; Idso and Idso, 2001; Pérez-López et al., 2009). Moreover, the increased CO_2/O_2 ratio at the photoreduction sites usually increases the photosynthetic rate and eventually growth and yield (Champigny and Mosseau, 1999).

Lettuce (*Lactuca sativa* L.) is the most important salad vegetable consumed worldwide, known as an important source of phytochemicals such as phenolic compounds. The Food and Agriculture Organization of the United Nations (FAO, 2015) reported that world production of lettuce and chicory reached about 24 million tons in 2013. The increase in CO_2 can stimulate plant growth but, it can also change the antioxidant activity and plant phytochemical composition. Thus, it is more and more interesting to know if the ongoing rise in the air's CO_2 content will cause an increase in lettuce production as well as in its health-promoting constituents in concomitance with environmental factors such as salinity which can affect yield.

Phenolic acids and flavonoids belong to a large family of secondary plant metabolites, comprising anthocyanins, also present in lettuce and responsible for the major part of the antioxidant activity of the hydrophilic fraction (Pérez-López et al., 2014). Differently colored lettuce cultivars present differences in phenolic composition, with the red ones showing a higher antioxidant capacity (Llorach et al., 2008; Pérez-López et al., 2014). Due to their role as antioxidants, phenolic compounds have been recognized as phytonutrients able to lower the incidence of some types of cancer and cardiovascular diseases (Hooper and Cassidy, 2006), and thus as indicators of lettuce quality. Following the increasing consumer interest in health-promoting foods, attention has been shifted from concerns over quantity alone to concerns over food compounds that can optimize health (Idso and Idso, 2001). Considering the above, accumulation of antioxidants in plant tissues can be reached essentially in two ways: inducing the synthesis of antioxidants under a moderate abiotic stress (Sgherri and Navari-Izzo, 1995) or reducing their consumption because the oxidative stress is lower.

In addition to humans, many functions have been ascribed to phenols in plants. In particular, phenolic acids have been recognized to play a role in the plant vacuoles in the removal of hydrogen peroxide taking part in a cycle where it is involved a peroxidase (Zancani and Nagy, 2000). The phenoxyl radicals resulting from the reaction can be reduced back by ascorbic acid, whose enhancement under some altered environmental conditions can be responsible of the increase in phenolic acids amounts (Sgherri et al., 2004). The relationship between the main antioxidative mechanisms and phenol metabolism could thus increase the plant resistance to adverse conditions. Beckman (2000) pointed out that phenolic acids can also be synthesized by plants in response to physical injury, infection or other stresses and that they are often stored primarily in the apoplast or in the vacuole, strategically playing either a signaling or a direct role in defense. In fact, secondary metabolites help plants defend themselves against both vertebrate and invertebrate herbivory as well as the attacks of plant pathogens (Langenheim, 1994). This role is particularly worthy of being investigated under CO_2 enrichment which has the potential to change carbon-based secondary compounds altering plant palatability and, consequently, plant's degree of protection from the ravages of foraging insect pests (Idso and Idso, 2001).

To our knowledge, no report has investigated till now the effects of the interactions of a mild salt stress with elevated CO_2 on phenolic compound enrichment and composition in lettuce. The aim of this work was to study the changes of phenolic acids and flavonoids composition as well as of the antioxidant activity in two differently pigmented lettuce cvs (Blonde of Paris Batavia, green leaf and Oak Leaf, red leaf) when submitted to salinity (200 mM NaCl) or elevated CO_2 (700 ppm) or their combination in order to evaluate whether the next climate change may affect the production of secondary phenolic metabolites improving lettuce quality.

2. Materials and methods

2.1. Chemicals

All reagents were of the highest purity and were purchased from Sigma-Aldrich (Milan, Italy). Water was of Milli Q grade. All solvents and water were accurately degassed before use in the analyses. The standards gallic, protocatechuic, *p*-hydroxybenzoic, chlorogenic, chicoric, vanillic, caffeic, syringic, *p*-coumaric and ferulic acids as well as 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma (Milan, Italy). The standards luteolin-7-*O*-glucoside, rutin, myricetin, quercitrin, quercetin-3-*O*-glucuronide, quercetin-3-*O*-glucoside, quercetin, luteolin, kaempferol, cyanidin and cyanidin-3-*O*-glucoside were purchased from Extrasynthèse (Genay, France).

2.2. Plant material and growth conditions

Two differently pigmented cvs of *Lactuca sativa* L., Blonde of Paris Batavia (PB, green leaf) and Oak Leaf (OL, red leaf), were grown in an environmental controlled growth chamber. Growth conditions were a day/night temperature of 25/18 °C, a relative humidity of 60/80% day/night and a daily light regimen of 14 h of light and 10 h of darkness with a photosynthetic photon flux of 400 μmol photons m^{-2} s^{-1} during the light period. Every week plants were randomly repositioned inside the chamber to minimize intra-chamber effects. Plants were continuously subjected to ambient (400 ± 20 μmol/mol) or elevated (700 ± 20 μmol/mol) CO_2 from sowing. The atmospheric CO_2 concentration was continuously recorded by a CO_2 sensor, the signal being received by a computer which activated CO_2 injection into the chamber to maintain a constant level. Six seeds were sown per pot containing a mixture of perlite/vermiculite (3:1). Seven days after sowing (DAS), most uniformly sized plants were selected, leaving one plant per pot. Lettuce was watered with Hoagland's solution (Arnon and Hoagland, 1940) every two days till 35 DAS. From this date to the end of experiment, some plants, grown either at ambient or elevated CO_2, were subjected to salt treatment supplying each day Hoagland's solution supplemented with 200 mM NaCl considered as moderate salinity stress (Adnan et al., 2016). At harvest (39 DAS), the external fully matured leaves were randomly selected to obtain more homogenized results since all the leaves have received light at the same extent. Fresh weight was recorded, and samples were taken for dry weight measurements. For the biochemical analyses, leaves were first kept in a tray for two minutes with deionized water and after soaked for 15 s in ultrapure MilliQ water,