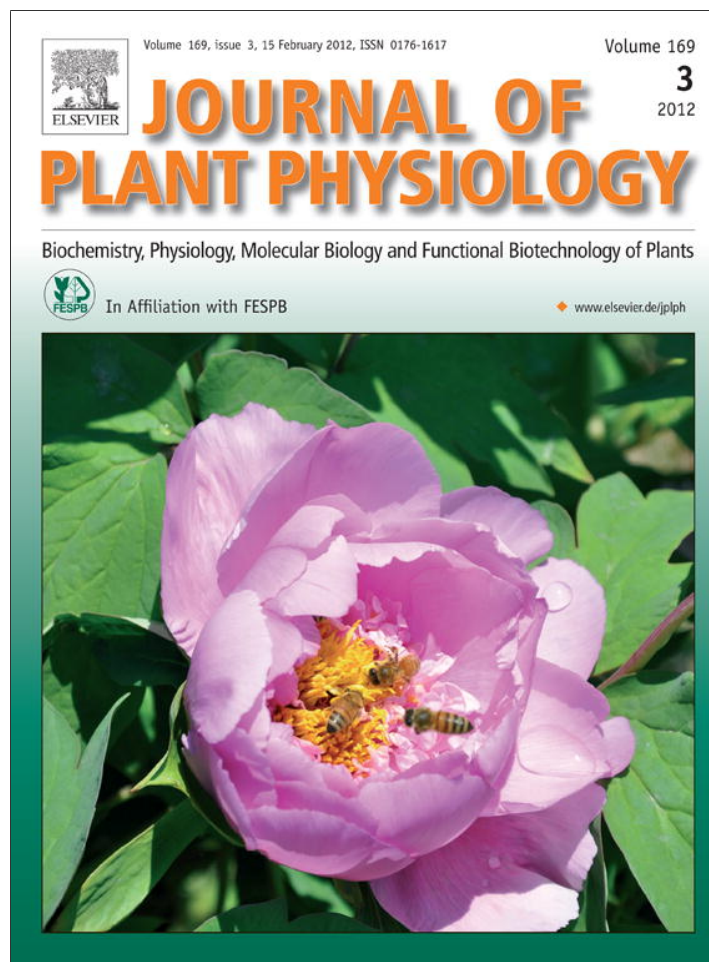


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Tipburn in salt-affected lettuce (*Lactuca sativa* L.) plants results from local oxidative stress

Luciano R. Carassay^{a,1}, Dolores A. Bustos^b, Alberto D. Golberg^a, Edith Taleisnik^{b,*}

^a Facultad de Agronomía, UNLPam, Ruta 35 Km 334, L6300 Santa Rosa, La Pampa, Argentina

^b CIAP-INTA (formerly IFFIVE), Camino a 60 Cuadras Km 5.5, X5020ICA Córdoba, Argentina

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ABSTRACT

Tipburn in lettuce is a physiological disorder expressed as a necrosis in the margins of young developing leaves and is commonly observed under saline conditions. Tipburn is usually attributed to Ca²⁺ deficiencies, and there has very limited research on other mechanisms that may contribute to tipburn development. This work examines whether symptoms are mediated by increased reactive oxygen species (ROS) production.

Two butter lettuce (*Lactuca sativa* L.) varieties, Sunstar (Su) and Pontina (Po), with contrasting tipburn susceptibility were grown in hydroponics with low Ca²⁺ (0.5 mM), and with or without 50 mM NaCl. Tipburn symptoms were observed only in Su, and only in the saline treatment. Tipburn incidence in response to topical treatments with Ca²⁺ scavengers, Ca²⁺ transport inhibitors, and antioxidants was assessed. All treatments were applied before symptom expression, and evaluated later, when symptoms were expected to occur. Superoxide presence in tissues was determined with nitro blue tetrazolium (NBT) and oxidative damage as malondialdehyde (MDA) content. Superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) activities were assayed.

Under control and saline conditions, tipburn could be induced in both varieties by topical treatments with a Ca²⁺ scavenger (EGTA) and Ca²⁺ transport inhibitors (verapamil, LaCl₃) and reduced by supplying Ca²⁺ along with an ionophore (A 23187). Tipburn symptoms were associated with locally produced ROS. O₂^{•-} and oxidative damage significantly increased in leaf margins before symptom expression, while topical antioxidant applications (Tiron, DPI) reduced symptoms in treated leaves, but not in the rest of the plant. Antioxidant enzyme activity was higher in Po, and increased more in response to EGTA treatments, and may contribute to mitigating oxidative damage and tipburn expression in this variety.

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Introduction

Tipburn in lettuce (*Lactuca sativa* L.) is a significant source of economic losses. It is a physiological disorder expressed as a necrosis in the margins of young developing leaves and it is usually attributed to Ca²⁺ deficiencies (Collier and Tibbitts, 1982; Saure, 1998). Calcium is a xylem-mobile element, and deficiency symptoms are observed in young expanding leaves in leaf vegetables such as

lettuce, in enclosed tissues in head-forming vegetables and in celery, and in developing fruits depending on phloem rather than xylem water supply (White and Broadley, 2003). Localized Ca²⁺ deficiencies in leaf blades can cause tipburn in lettuce (Barta and Tibbitts, 2000) and blossom end rot (BER) in fruits of tomato and pepper (Ho and White, 2005).

Calcium is an essential plant macronutrient with key structural and signaling roles. Calcium ions act as osmotic agents within vacuoles, as a membrane stabilizing elements, as strengthening agents in cell walls; and as secondary messengers for a multitude of signals, in the regulation of enzyme synthesis (Gilliham et al., 2011) and is required for normal cell growth. Nevertheless, and in contrast to observations that sustain a link between vigorous growth and tipburn incidence (White and Broadley, 2003), tipburn and BER (Adams and Ho, 1992) are also promoted under adverse growing conditions such as salinity or water stress (Saure, 1998). Calcium imbalance is a common consequence of substrate salinity (Lauchli, 1990). Salinity restricts water uptake and transpiration and was

Abbreviations: APX, ascorbate peroxidase; BER, blossom end rot; CAT, catalase; DPI, diphenyleneiodonium; EGTA, ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid; MDA, malondialdehyde; NBT, nitro blue tetrazolium; PI, plastochron index; Po, Pontina; ROS, reactive oxygen species; SOD, superoxide dismutase; Su, Sunstar; TCA, trichloroacetic acid.

* Corresponding author. Tel.: +54 351 4973636; fax: +54 351 4974330.

E-mail addresses: etaleisnik@conicet.gov.ar, etaleisnik@yahoo.com (E. Taleisnik).

¹ CONICET fellow.

shown to reduce Ca^{2+} uptake and transport to young lettuce leaves (Lazof and Bernstein, 1999), which may partially explain tipburn stimulation by salinity.

In some cases, no relation between improvement in leaf Ca^{2+} and decrease in tipburn incidence has been observed (Cresswell, 1991; Saure, 1998), yet there has been very limited research on other mechanisms contributing to tipburn development. BER developing in fruits of pepper and tomato grown under saline conditions has been recently associated with the production of reactive oxygen species (ROS) in the affected zone (Aktas et al., 2005; Ho and White, 2005). This is a novel view in tipburn etiology and it has not been investigated before whether tipburn in lettuce is also related to ROS.

ROS are highly reactive oxygen derivatives and include singlet oxygen, superoxide radical, hydrogen peroxide, and hydroxyl radical. ROS production generally increases under biotic and abiotic stress conditions, as reviewed by Miller et al. (2010). On the other hand, controlled ROS production appears to be a general characteristic of expanding plant cells and organs (Rodríguez et al., 2002). While excess ROS exert adverse effects (oxidative damage) stemming from their interaction with macromolecules: lipids, proteins, nucleic acids and carbohydrates, low ROS levels participate in signaling events (Dat et al., 2000; Mittler et al., 2011) that regulate ion channel activity and gene expression.

ROS production occurs in almost all cell compartments (Mittler et al., 2004). ROS balance is controlled by diverse antioxidant mechanisms (Dat et al., 2000), including enzymatic and non-enzymatic components (Foyer and Noctor, 2005) acting also in various cell compartments. Antioxidant activity during hypersensitive response has been characterized in lettuce (Bestwick et al., 2001). Salt stress commonly results in ROS production (Hernández et al., 2001) and activation of the antioxidant system (Mhadhbi et al., 2011). The contribution of increased antioxidant activity to salt tolerance has been repeatedly reported in the literature (Munns and Tester, 2008), but the association between antioxidant activity and tipburn susceptibility has not been evaluated.

In this work we examined whether reduced apoplastic Ca^{2+} stimulates tipburn expression in lettuce leaves, and whether symptoms are mediated by increased ROS production. To this end, tipburn incidence in response to topical treatments with Ca^{2+} scavengers, Ca^{2+} transport inhibitors and antioxidants were compared in two lettuce varieties differing in tipburn susceptibility, grown under saline conditions. Antioxidant enzyme activity was measured in leaf zones where tipburn symptoms were later expected to either develop or not.

Materials and methods

Plant material and growth conditions

This study was performed in butter lettuce (*Lactuca sativa* L.) genotypes Sunstar (Su) and Pontina (Po). In preliminary experiments (Carassay et al., unpublished) these varieties had shown contrasting responses to tipburn predisposing conditions: Su was susceptible to this physiogenic disease and Po was not.

Seeds were sown in germination trays according to ISTA recommendations (ISTA, 1996) and kept at in a growth chamber at 20 °C under a 12 h photoperiod. Thirty days later, seedlings were transplanted to 7-L black trays containing modified Hoagland solution (Fernández and Johnston, 1986) with a reduced Ca^{2+} level (0.5 mM), which, according to the preliminary experiments stimulated symptom expression. There were 8 plants per tray and the nutrient solution was changed weekly. Electrical conductivity, pH and temperature were monitored to insure constant conditions, light intensity was $350 \mu\text{mol m}^{-2} \text{s}^{-1}$ with a 16 h photoperiod.

Table 1

Topical treatments to modify Ca^{2+} concentration and oxidative stress.

Topical treatment	Effect
EGTA (50, 25 and 10 mM, pH 7.8)	Chelator of apoplastic Ca^{2+}
Verapamil (10 mM)	Ca^{2+} ion blocker
LaCl_3 (10 mM)	Ca^{2+} ion blocker
Ionophore (A 23187) (5 μM + Ca^{2+} 1 mM)	Promotes Ca^{2+} entry through plant membranes
Tiron (50 mM)	Antioxidant, ROS scavenger
DPI (50 μM)	Inhibitor of plasma membrane NADPH oxidase and peroxidases

Temperature was initially set at 22 °C, daytime temperature was raised to 25 °C at the onset of salt treatments and gradually increased till the end of the trial when temperatures were 36 °C and 27 °C by day and night, respectively. This temperature change scheme was previously observed to increase symptom expression. Seven days after transplantation, salt treatments were given to half of the plants by providing NaCl at gradually increasing concentrations (10, 25 and 50 mM) with the nutrient solution. Since salinity imposes growth restrictions, plants were compared at similar development stages by using the plastochron index (PI) developed by Erickson and Michelini (1957), and under these experimental conditions, tipburn expression under salinity occurred at PI 22–23.5, according to preliminary experiments.

Tipburn assessment

Combined tipburn incidence and severity (tipburn index, TI) was evaluated according to Frantz et al. (2004) as $\text{TI} = \{[(S \cdot 5) + (M \cdot 3) + (L \cdot 1)] / P \cdot 5\} \cdot 100$, where S = number of plants with severe tipburn; M is the number of plants with medium tipburn; L is the number of plants with light tipburn and P is the total number of plants. The proportion of leaf area affected by tipburn was estimated in digital photographs by measuring necrotic areas and total leaf area with the image processing software ImageJ (<http://rsbweb.nih.gov/ij>).

Modulation of tipburn expression

The purpose of the following treatments was to assess the association between tipburn symptoms, local Ca^{2+} concentrations and oxidative stress. Briefly, Ca^{2+} concentration was locally modified by painting the edge of leaves with a Ca^{2+} chelator (EGTA), Ca^{2+} channel blockers LaCl_3 and verapamil, and by adding 1 mM Ca^{2+} along with a Ca^{2+} ionophore (A 23187). Oxidative stress development was modified by supplying Tiron (sodium 4,5-dihydroxybenzene-1,3-disulfonate), a ROS scavenger and diphenyleneiodonium (DPI). DPI is a suicide inhibitor of the phagocytic NADPH oxidase and also an inhibitor of NADH-dependent H_2O_2 production by peroxidase (Frahry and Schopfer, 1998) and has been used to reduce ROS production in plant systems. Treatments are detailed in Table 1.

All treatments were applied to leaves 17 or 18, the first ones to show symptoms in previous assays. Leaves were treated at PI 20, before symptom expression, and tipburn was evaluated at expected occurrence PI.

Oxidative stress determination and superoxide localization in leaf tissues

Malondialdehyde (MDA) content is taken as a parameter for oxidative damage in plant tissues. MDA was determined in leaf 17, at PI 21.5, according to the method described by Heath and Parker (1968). Briefly, 200 mg of tissue from the leaf margins were

extracted with 1 ml of 3% (v/v) trichloroacetic acid (TCA), liquid N was added and samples were ground with mortar and pestle to a fine powder. The extract was then centrifuged at 14,000 rpm for 10 min. The reaction mixture included 150 μ l supernatant and 150 μ l of a mixture of 20% TCA and 0.5% (v/v) thiobarbituric acid. This mixture was placed in a water bath at 95 °C for 25 min, allowed to cool down and centrifuged at 10,000 \times g for 5 min. The absorbance of the supernatant was read at 532 and 600 nm. Nonspecific absorbance at 600 nm was subtracted from that at 532 nm (Hodges et al., 1999), and MDA content was calculated using this adjusted absorbance and the extinction coefficient of 155 mM⁻¹ cm⁻¹ (Heath and Parker, 1968). Results are expressed on a fresh weight basis.

Superoxide in tissues was determined with nitro blue tetrazolium (NBT), which reacts with O₂^{•-}, producing a blue formazan precipitate. Intact detached leaves were gently infiltrated (4 min) with a 0.01% NBT solution in water, and incubated in the dark in the same solution, for 25 min at room temperature. Leaves were then placed in 80% ethanol and heated to 90 °C to remove chlorophyll, subsequently rinsed and kept in water until scanned. The resulting colour images were first inverted to obtain a negative, transformed to black and white 8-bit images, and formazan colour intensity (in the negative corresponding to the lighter tones of grey), determined by ImageJ 1.29.

Antioxidant enzyme activities

Antioxidant enzyme activities were assessed separately in samples obtained from the base and tip of leaves. Frozen leaf samples (100 mg fresh weight) were ground to a fine powder in liquid nitrogen, and homogenized in 50 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA and 1% polyvinylpolypyrrolidone (PVPP), and 5 mM ascorbate in samples for ascorbate peroxidase (APX) activity. Homogenates were centrifuged at 16,000 \times g for 25 min at 4 °C and the supernatant was used to determine protein concentration (Bradford, 1976) and enzyme activity.

Total superoxide dismutase (SOD) activity was assayed at 560 nm by measuring the inhibition of the photochemical reduction of NBT (Beauchamp and Fridovich, 1971). One unit of SOD activity was defined as the amount of enzyme which causes a 50% inhibition of the photochemical reduction of NBT, and SOD specific activity was expressed as units per mg protein. Catalase (CAT) activity was determined at room temperature by measuring the decrease in A₂₄₀ after adding 5 mM H₂O₂ to samples (Gallego et al., 1996). Total APX activity was measured according to Nakano and Asada (1981), by measuring the H₂O₂-dependent oxidation of ascorbate at 290 nm. The reaction mixture contained appropriate dilutions of the samples in 50 mM phosphate buffer pH 7.4, 0.5 mM ascorbic acid and 0.1 mM H₂O₂.

All results were analyzed using InfoStat (InfoStat/Profesional ver. 2008p, Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina).

Results

Tipburn expression in relation to local alterations in leaf Ca²⁺ concentration

Tipburn symptoms were not observed in lettuce plants grown in the absence of salinity. Under salt stress (50 mM NaCl), tipburn was expressed only in Su, with an 84% incidence. Symptoms first appeared in leaves 17–19, as small necrotic areas that gradually expanded (Fig. 1). When symptoms appeared in leaves that were less than 3 cm long, necrosis affected the distal margins of the leaf (referred to as “tip” from here on), however, if they appeared in

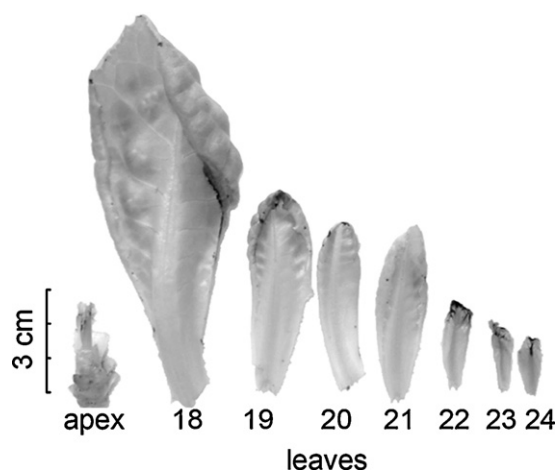


Fig. 1. Tipburn symptoms at PI 23.5 from emergence in successive leaves of salt-treated (50 mM NaCl) lettuce variety Sunstar.

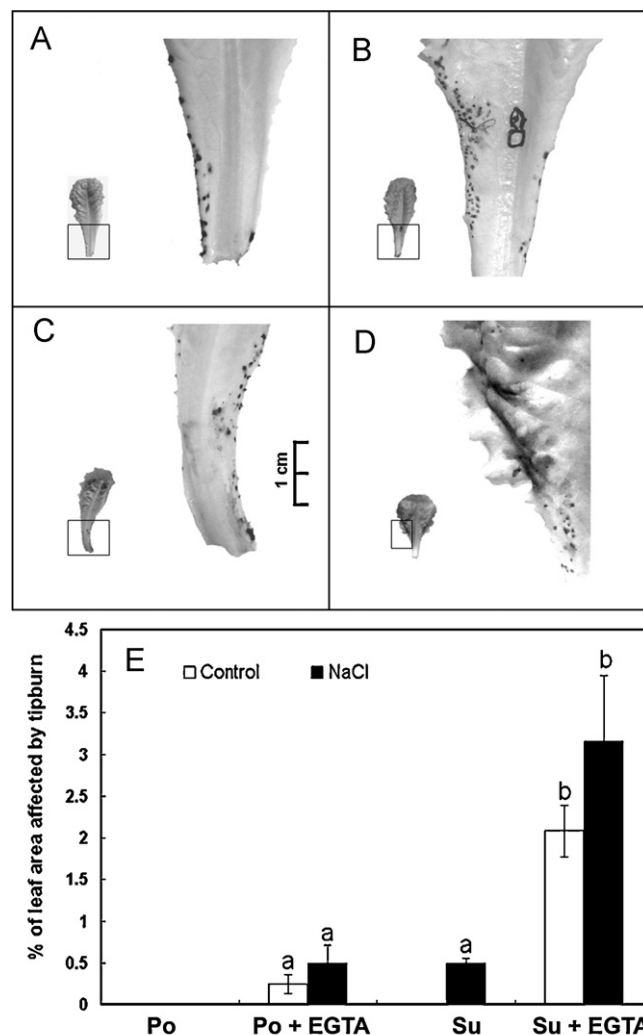


Fig. 2. Tipburn symptoms after topical applications of EGTA to the margins of leaf 17. EGTA (50 mM) was applied at PI 20 and symptoms were evaluated at PI 23.5. (A–D) Tipburn symptoms. (A) Pontina control; (B) Sunstar control; (C) Pontina salinized (50 mM NaCl); (D) Sunstar salinized (50 mM NaCl); (E) percentage of leaf area affected by tipburn. Bars are means \pm SE of 6 replicates. Different letters indicate significant differences at $p \leq 0.05$ (ANOVA).

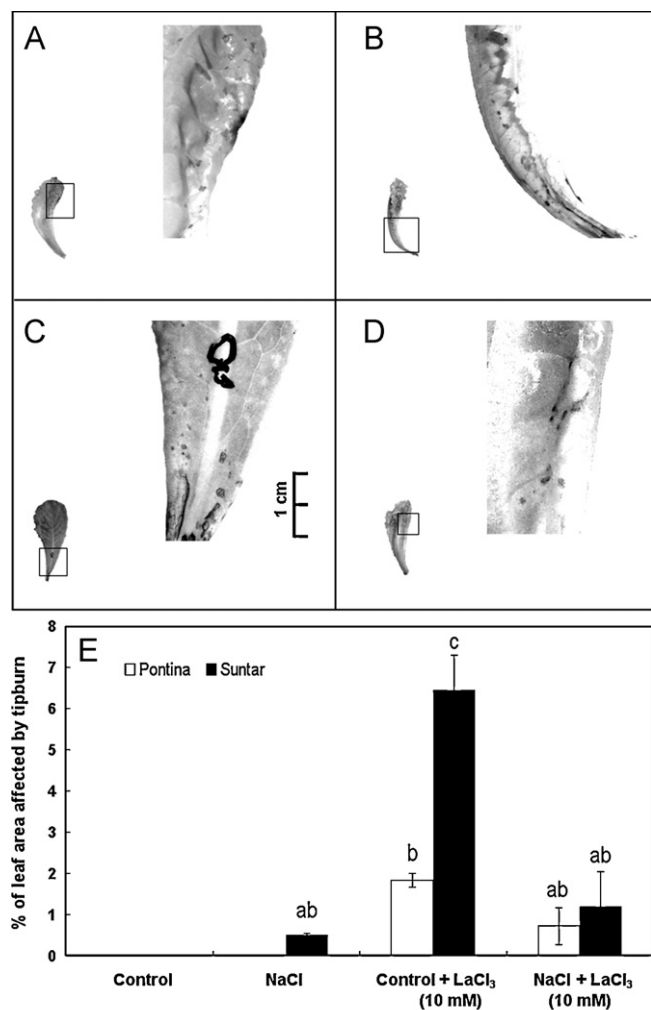


Fig. 3. Localized necrosis after topical applications of LaCl₃ to the margins of leaf 17. LaCl₃ (10 mM) was applied at PI 20 and evaluations were 24 h later. (A–D) Leaf necrosis. (A) Pontina control; (B) Sunstar control; (C) Pontina salinized (50 mM NaCl); (D) Sunstar salinized (50 mM NaCl); (E) Percentage of leaf area affected by necrosis. Bars are means ± SE of 6 replicates. Different letters indicate significant differences at $p \leq 0.05$ (ANOVA).

longer leaves, tipburn symptoms were observed in the margins of the leaf base.

Since tipburn is associated to locally decreased Ca²⁺ concentrations (Barta and Tibbitts, 1991a), it was assumed that its alteration would lead to differential tipburn expression. Calcium was locally sequestered by topical applications of EGTA to the margins of leaf 17 at PI 20, which, as mentioned above, is earlier than any symptom expression. At PI 21.5 the treatment induced tipburn in the treated area of this leaf in plants of both varieties, grown under saline as well as under non-saline conditions (Fig. 2A–D). Po plants, which otherwise were tipburn-free (Fig. 2C), also showed them, and they dramatically increased (up to five times) in salinized Su (Fig. 2E). Incidence in treatments with EGTA was 60% and 36%, for Su and Po, respectively, in non-salinized plants, and 92% and 44% in salt treated plants. Since EGTA solutions are pH 7.8, it was also tested whether dilute NaOH solutions of the same pH would induce tipburn symptoms, which they did not.

Topical application of Ca²⁺ channel blockers also modified tipburn expression. When 10 mM LaCl₃ was applied to the leaf margins, tissue necrosis increased very significantly in control

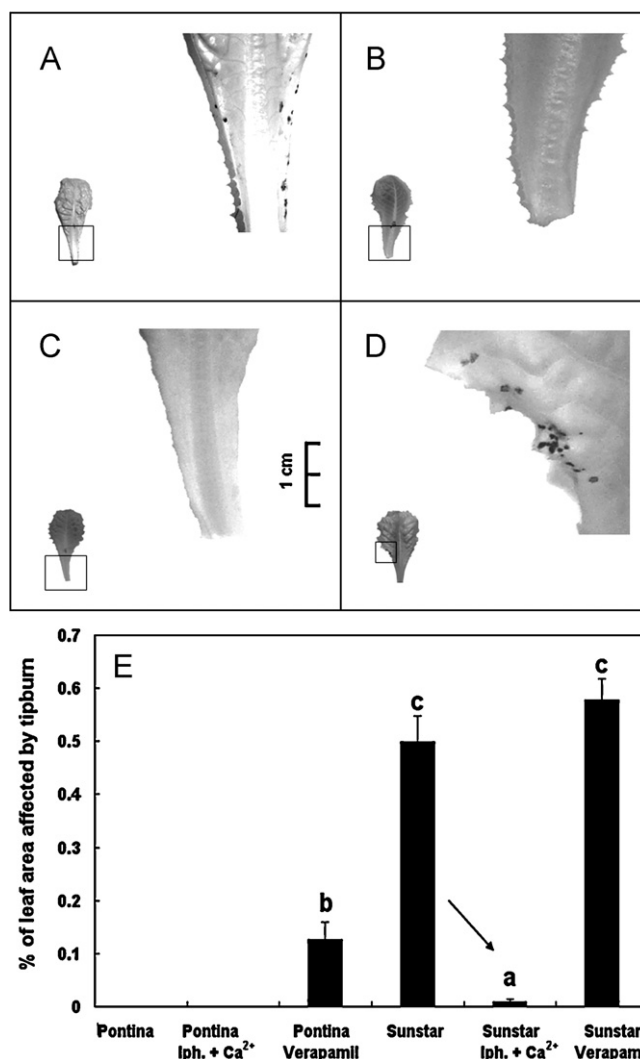


Fig. 4. Tipburn symptoms after topical applications of verapamil (10 mM) and a Ca²⁺ ionophore (A 23187, 5 μM) + Ca²⁺ (1 mM) to the margins of leaf 17 from control and salt-treated (NaCl 50 mM) lettuce plants. Treatments were applied at PI 20 and symptoms were evaluated at PI 23.5. (A–D) Tipburn symptoms. (A) Pontina NaCl + verapamil; (B) Sunstar NaCl + ionophore + Ca²⁺; (C) Sunstar control + verapamil; (D) Sunstar NaCl + verapamil. (E) Percentage of leaf 17 area affected by tipburn in salt-treated plants, lph: ionophore. Bars are means ± SE of 6 replicates. Different letters indicate significant differences at $p \leq 0.05$ (ANOVA).

treatments, more than in salinized ones (Fig. 3A–D). Po always showed less symptoms than Su. The necrotic symptoms induced by LaCl₃ were not exactly like tipburn symptoms: necrotic spots appeared throughout the leaf lamina, not only in the treated areas. Though salt treated plants initially showed less symptoms than controls in response to the LaCl₃ treatment (data in Fig. 3 was collected within 24 h of the application), later, at 36 h, those leaves were completely necrosed. Another channel blocker, verapamil, induced necrosis in salt treated plants but not in controls (Fig. 4A–D).

In order to locally increase Ca²⁺ concentration, 1 mM Ca²⁺ was applied to the leaf margin along with a ionophore. Results are also shown in Fig. 4B and E to illustrate how increased local Ca²⁺ supply reversed symptom expression.

Taken together, these results show that local changes in Ca²⁺ concentration can alter tipburn symptoms, even in Po, a variety that did not otherwise express them.

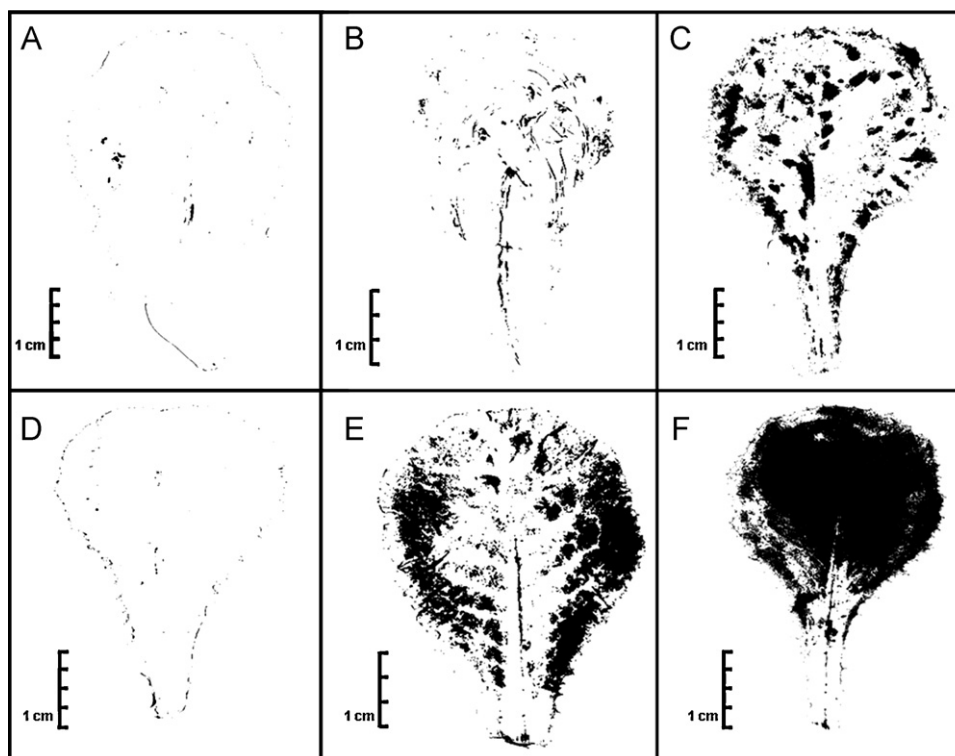


Fig. 5. NBT staining for $O_2^{\bullet-}$ in leaf 17 of lettuce varieties Sunstar and Pontina at PI 21.5, before tipburn symptom development. (A–C) Pontina; (D–F) Sunstar. (A and D) Control plants; (B and E) Salt-treated plants 50 mM NaCl; (C and F) NaCl 50 mM + EGTA 50 mM. Grayscale images of stained leaves were transformed into black and white.

Tipburn symptoms, ROS production and oxidative stress

Necrosis in leaf margins suggested a possible link with ROS, which tend to increase under stress conditions and can lead to cell death. Superoxide was assessed with NBT in leaves at PI 21.5, before symptom development. Staining was imperceptible in leaves from non-salinized plants (Fig. 5A and D), and it significantly increased in salinized plants (Fig. 5B and E), especially in Su (Fig. 5E). In this variety, staining was observed in the leaf borders, and more in the base of the leaves, where tipburn symptoms later developed. EGTA treatments to the leaf margins induced $O_2^{\bullet-}$ across the leaf surface, and intensity was much higher in Su (Fig. 5F) than in Po (Fig. 5C).

Increased $O_2^{\bullet-}$ detection suggested consequent oxidative damage, which was assessed as MDA, a product of lipid peroxidation. Salt treatments increased MDA concentration, and EGTA even more (Fig. 6). Differences among varieties were surprisingly low, though MDA was slightly higher in Su than in Po.

Since tipburn expression seemed to be associated with ROS production, it was proposed that ROS scavenging or the inhibition of ROS sources would mitigate symptom expression. This was tested only in variety Su, the one that expressed symptoms under salinity. Topical applications of Tiron, a ROS scavenger, reduced the area affected by tipburn symptoms (Fig. 7B and C). DPI completely inhibited symptom expression in the treated leaf, however, other, untreated leaves in the same plant did express tipburn symptoms (Fig. 7A). These results confirmed that tipburn symptoms were associated with locally produced ROS.

Antioxidant activity in lettuce varieties differing in tipburn susceptibility

It was then enquired whether tipburn expression was associated to the modulation of oxidative stress. Antioxidant enzyme activities were assessed at the base and tip of leaves at PI 21.5, before

symptom expression. Tipburn was expected to appear a few days later in the margins of the basal third of the leaf, but not at the tip.

SOD activity increased under salinity, more markedly in the leaf base of Po (Fig. 8). EGTA treatments, which had lead to increased symptom expression (Fig. 2), increased superoxide presence (Fig. 5) and oxidative damage (Fig. 6), also increased SOD activity, and, again, more in Po than in Su. A similar pattern was observed in APX activity, EGTA-related increases were higher in Po (Fig. 9A and B). However, a higher level of CAT activity was induced in Su (Fig. 9C and D), which may have been induced by the increased H_2O_2 resulting from SOD activity. Thus, conditions that lead to differential symptom expression in both varieties, were accompanied by higher SOD and APX activity in the less susceptible genotype.

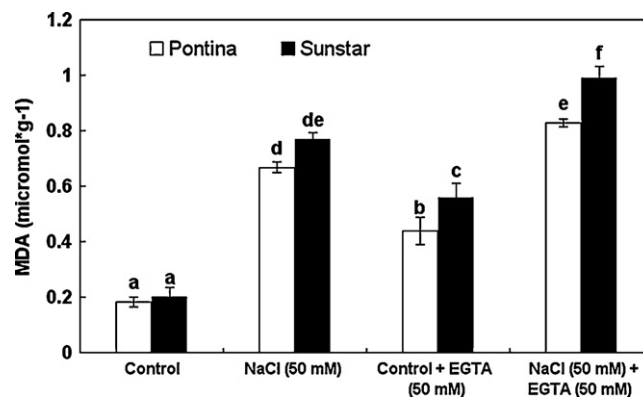


Fig. 6. Effect of salt treatment and EGTA topical application to leaf margins on membrane lipid peroxidation. Data are from leaf 17 at PI 21.5. Results are means and SE of $n = 5$ replicates. Different letters indicate significant differences ($p \leq 0.05$). DMS test.

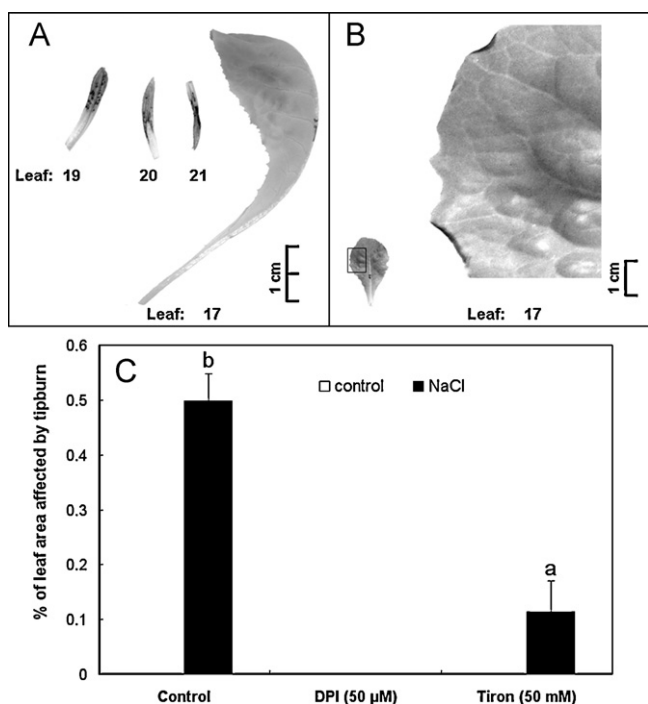


Fig. 7. Effect of topical applications of antioxidants on tipburn expression in salt-treated Sunstar lettuce. Symptoms were assessed in leaf 17, 24 h after application of antioxidants to the leaf margin. (A) 50 µM DPI treatment. Leaves 19–21 from the same plant were not treated, notice tipburn symptoms. (B) 50 mM Tiron treatment. (C) Percentage of tipburn-affected leaf areas in the above treatments and their controls. Results are means ± SE of *n* = 6 leaves, different letters indicate significant differences (*p* ≤ 0.05). DMS test.

Discussion

Tipburn expression modulated by altering Ca²⁺ concentration and supply to leaf cells

Two lettuce varieties differing in tipburn susceptibility when subject to salt stress were studied. Su develops tipburn symptoms while Po does not. Tipburn is a physiogenic disorder related to Ca²⁺ deficiencies in actively growing leaves (Collier and Tibbitts, 1982). Decreased Ca²⁺ availability in the substrate has been reported to increase tipburn symptoms, in both intact plants (Barta and Tibbitts, 1991a) and detached leaves (Aloni et al., 1986). In agreement, preliminary trials indicated that low (0.5 mM) Ca²⁺ concentration in the growth medium simulated tipburn symptoms in salt-treated plants and this study was carried out in this condition.

Low Ca²⁺ levels have been detected in the zones of lettuce leaves exhibiting tipburn (Barta and Tibbitts, 1991b) and in this work, topical applications of the Ca²⁺ chelator EGTA, to the margins of growing leaves induced tipburn symptom in plants of both varieties, grown under saline as well as under non-saline conditions. Stimulated Ca²⁺ entry in treatments with a Ca²⁺ ionophore prevented symptom development in Su. These results provide experimental confirmation to the idea that tipburn is associated to decreased apoplastic Ca²⁺ supply.

In contrast to other macronutrients, a high proportion of total Ca²⁺ is found in the cell walls, where part of it is bound to the cell wall, while another portion is exchangeable at the plasma membrane. Ca²⁺ can be found in high concentrations within the vacuole but the free Ca²⁺ activity in the cytosol remains around 0.1–0.2 µM. Almost imperceptible fluctuations in cytosolic concentrations Ca²⁺ may be translated into plant growth and adaptation signals (Hirschi, 2004). Therefore, intracellular signaling requires

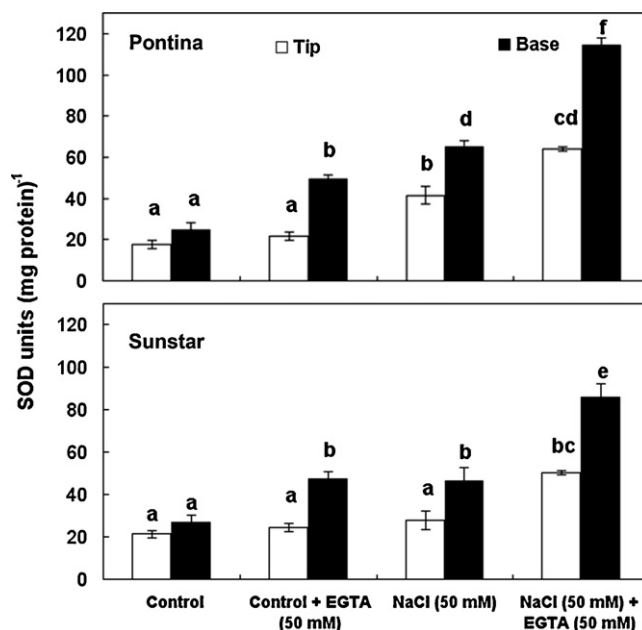


Fig. 8. SOD activity in the base and tip of lettuce leaves from control and salinized plants (50 mM NaCl), with or without topical applications of 50 mM EGTA. Leaf 17 was treated at PI 20, enzyme activity was measured 24 h later. Results are means and SE of *n* = 7 replicates, different letters indicate significant differences at *p* ≤ 0.05, after ANOVA.

cytosolic Ca²⁺ to be maintained at submicromolar levels in the resting cell and to increase rapidly in response to developmental cues or environmental challenges and prolonged high intracellular Ca²⁺ levels can lead to cell death (Hirschi, 2004). Salt stress, along with other stresses such as heat, anoxia and hyper osmotic stress can lead to transient and eventually prolonged increases in cytosolic Ca²⁺ (White and Broadley, 2003). Excess Ca²⁺ is removed from the cytosol by active transport catalized by Ca²⁺-ATPases and H⁺/Ca²⁺-antiporters (Hirschi, 2001). Transgenic tobacco overexpressing genes for H⁺/Ca²⁺-antiporters (AtCAX1), exhibited calcium deficiency disorders, such as tipburn, that could be reversed by increasing Ca²⁺ supply (Hirschi, 2001). Such phenotypes resulted from depleted cytosolic Ca²⁺.

The fluxes required for cytosolic Ca²⁺ signaling are minute compared with those required for adequate nutrition and both may be compromised by Ca²⁺ disorders (White, 2001). Transport of Ca²⁺ across the plasma membrane is mediated by several channels, which respond to a plethora of stimuli (Parekh and Putney, 2005) such as voltage (voltage operated channels, VOCs), second messengers such as inositol trisphosphate (IP₃), cyclic nucleotides and lipid derivatives (second messenger-operated channels, SMOCs), Ca²⁺ gradient channels, activated by decreasing cytosolic Ca²⁺ concentrations (store-operated channels, SOC), and those activated by receptors such as hormones (receptor-operated channels, ROC). Verapamil is an inhibitor of voltage-gated L-type Ca²⁺ channels (Davenport and Tester, 2000). Our results show that the application of this reagent lead to typical tipburn symptoms, which may suggest the blocking of this type of channel is involved in tipburn development. On the other hand, LaCl₃ can block all types of Ca²⁺ channels mentioned above, thus, it can exert significantly negative effects on cell metabolism, leading to cell death. This may explain why LaCl₃ effects were more apparent in non-salinized plants, which had a higher rate of growth, and also why symptoms were not typical of tipburn.

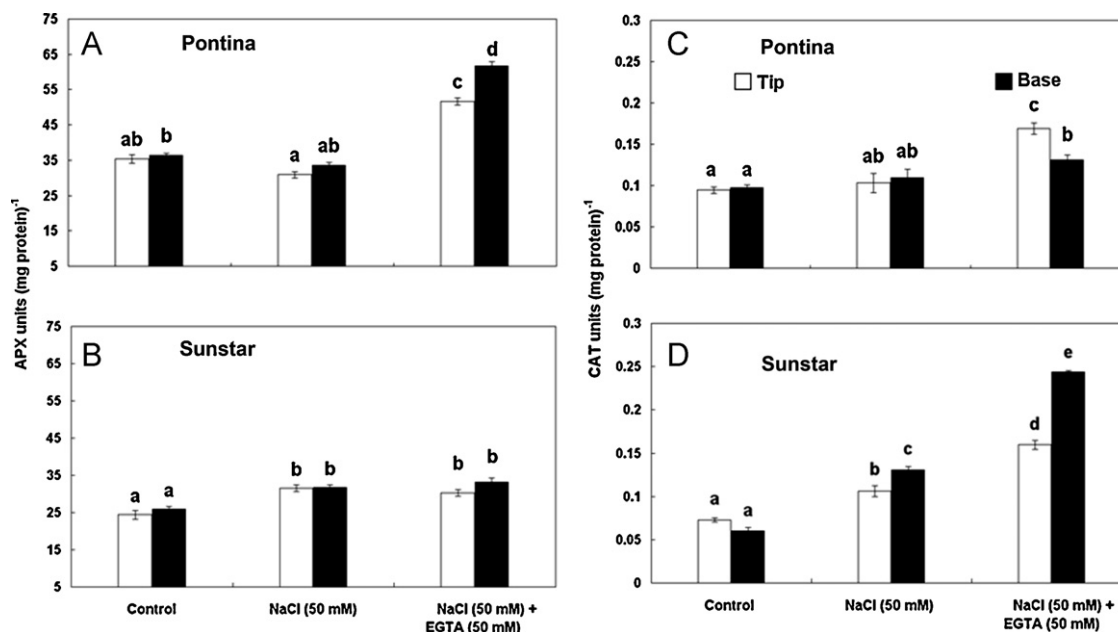


Fig. 9. APX (A, B) and CAT (C, D) activities in the base and tip of lettuce leaves from control and salinized plants (50 mM NaCl), with or without topical applications of 50 mM EGTA. Leaf 17 was treated at PI 20, assays were performed 24 h later. Results are means and SE of $n = 7$ replicates, different letters indicate significant differences at $p \leq 0.05$, after ANOVA.

ROS and tipburn expression

Increased ROS production is a common response to salinity and other stresses (Mittler et al., 2004) and its contribution to tipburn expression in lettuce had not been previously explored. Oxidative damage resulting from excess ROS may lead, among other effects, to membrane lipid peroxidation. Increased MDA accumulation, a product of lipid peroxidation (Hodges et al., 1999) is a common feature of salt-induced oxidative damage in plant tissues (Bernstein et al., 2010) and it has also been observed in lettuce under salt stress (Eraslan et al., 2007). In the present study increased superoxide levels and MDA were observed in salinized plants, and they were higher in Su than in Po, in coincidence with the differences in tipburn expression, suggesting a link between oxidative stress and symptom development. Topical applications of EGTA generated extremely high superoxide production in the whole leaf and consequently increased MDA concentrations, showing that low Ca^{2+} availability is related to the development of oxidative stress leading to tipburn symptoms. While the coincidence between Ca^{2+} deficiency and oxidative stress had been reported earlier in tomato leaves (Schmitz-Eiberger et al., 2002), there were no previous experimental tests of the association between them.

Application of a ROS scavenger (Tiron) to the leaf margins significantly (70%) reduced symptom expression, providing further evidence for the link between tipburn and ROS. One of the main sources of superoxide in plant cells is membrane NADPH oxidase (Bolwell and Wojtaszek, 1997). DPI, a suicide inhibitor of the mammal NADPH enzyme, also inhibits the plant enzyme (Levine et al., 1994) as well as apoplastic peroxidases, and its application inhibited tipburn symptoms in the treated leaf, but not in untreated leaves of the same plant. These results suggest that ROS generation is involved in the development of oxidative stress and consequent tipburn symptom expression.

On the other hand, ROS participate in signaling events that activate defence mechanisms against biotic and abiotic stress conditions (Suzuki et al., 2011). ROS signals can induce large transcriptional changes which contribute to such activation (Miller et al., 2010). In *Arabidopsis*, ROS derived from NADPH oxidase D

(RbohD) activity are required for salt acclimation responses (Xie et al., 2011). Antioxidant activities play a major role in controlling ROS levels and potential oxidative stress, not only under abiotic and biotic stress conditions but also during normal cell metabolism (Foyer and Noctor, 2005; Gill and Tuteja, 2010) and associations between antioxidant activity and tolerance to abiotic stresses are well documented in the literature. Nutrient deficiencies can stimulate antioxidant activity. In maize, oxidative stress and activities of SOD and APX were stimulated by the deficiency of Ca, N and S (Kumar Tewari et al., 2004). In our experiments, increases in ROS and antioxidant activity were observed in both varieties under salinity and EGTA, before symptom expression. SOD activity was particularly stimulated in Po, the variety exhibiting less tipburn. Within a cell, the SODs constitute the first line of defence against ROS (Scandalios, 1993) and the most important when coupled with the necessary downstream events for full detoxification of ROS (Alscher et al., 2002). Therefore, it is suggested that the higher antioxidant activity of this variety may contribute to mitigate ROS damage and decrease its tipburn susceptibility. In this context, BER in pepper was related to lowered sequestration of apoplastic ROS by non enzymatic antioxidants (Aktas et al., 2005). Red lettuce varieties are generally less susceptible to tipburn and Lorach et al. (2008) found higher antioxidant activity in these than in continental varieties. Oh et al. (2009) reported that the activation of secondary metabolism as well as antioxidative metabolism are involved in lettuce adaptation to stress conditions, thus, it is likely that other antioxidants, enzymatic as well as non-enzymatic are also involved in ROS mitigation under tipburn predisposing conditions.

While, as shown above, Ca^{2+} deficiency can generate ROS, in turn ROS can participate in cellular Ca^{2+} balance by regulating Ca^{2+} transport (Mori and Schroeder, 2004). Increases in apoplastic ROS mediated by salicylic acid resulted in increased cytosolic Ca^{2+} concentration (Kawano and Muto, 2000). ROS may activate voltage dependent Ca^{2+} channels (VOCs) (Foreman et al., 2003; Parekh and Putney, 2005), SOCs and other Ca^{2+} channels, leading to increased Ca^{2+} influx to the cytosol that changes membrane polarization originating Ca^{2+} signals (Sanders et al., 2002). On the other hand, the

cell wall Ca²⁺ reservoir may be partially released by apoplastic ROS (Kawano and Muto, 2000) contributing to transient increases in cytosolic Ca²⁺ which are inimical to normal cell metabolism.

Among other functions, Ca²⁺ is also involved in mechanisms controlling cellular Na⁺ compartmentation. Na⁺ entry into the cytosol is mediated by non-selective ion channels (Tester and Davenport, 2003) which are closed under physiological Ca²⁺ concentrations (Rus et al., 2001). While overall tissue Na⁺ concentration was not affected by EGTA topical applications (not shown), Na⁺ tissue distribution may have been affected by the treatment resulting in increased intracellular Na⁺, also leading to the stimulated ROS production (Munns and Tester, 2008) as observed in the EGTA treatments, and this may provide another link between salinity, ROS and tipburn.

In short, this paper established for the first time experimental links between oxidative stress development, antioxidant activity and tipburn tolerance in lettuce. The findings may derive in practices leading to the protection of plants against this physiogenic disease under field or greenhouse culture conditions.

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