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Natural elicitors as preharvest treatments to improve postharvest quality of Butterhead lettuce

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ABSTRACT

Lettuce (*Lactuca sativa*) is one of the most popular leafy vegetables in the world, characterized by a diverse composition of phytochemical compounds but at low levels. However, their content may be increased with abiotic stresses. Accordingly, phytochemical enhancement and related microbiological and organoleptic quality and peroxidase (POD) and polyphenol oxidase (PPO) activities of Butterhead lettuce elicited during preharvest with natural compounds (chitosan 10 g L^{-1} and tea tree essential oil 2.7 mL L^{-1}) were studied. Quality indices were evaluated at harvest and during 21 days of refrigerated storage (0–2 °C). Treatments with chitosan and tea tree increased the total phenolic content of freshly lettuces by 30.5 and 21.1%, respectively, and the total flavonoids concentration by 43.3 and 36.4%, respectively, compared with control samples. The antioxidant capacity at harvest of the elicited plants, measured with DPPH and TEAC assays, was also higher. Notably, these improvements were maintained during refrigerated storage. Conversely, although a higher concentration of ascorbic acid was initially detected in treated plants with chitosan and tea tree, these differences were not observed at later storage times. No differences were detected in the organoleptic quality of elicited and control plants, meanwhile the microbiological quality and enzymatic activity were affected by the preharvest treatments. In particular, the application of chitosan exerted a fungistatic effect reducing yeast and molds population counts by 1.6 log throughout the storage, compared with control samples. Furthermore, chitosan also reduced the activities of PPO and POD, enzymes related with browning processes. Preharvest treatments with chitosan and tea tree enhanced the content of health-promoting phytochemicals in lettuce, without affecting its organoleptic quality. Moreover, chitosan treatment appears as a promising method to improve the safety and reduce the enzymatic activity of lettuce.

1. Introduction

High consumption of fruits and vegetables is associated with a reduced risk of several major health conditions, including cancer, cardiovascular diseases, and age-related functional declines (Soerjomataram et al., 2010; Wang et al., 2011). Since oxidative damage is considered as one of the main mechanisms in the onset of these diseases, it has been suggested that phytochemicals with antioxidant activity, such as some vitamins and phenolic compounds, are mainly responsible for the protective effect (Wang et al., 2011). Therefore, in the last years, research has been focused on finding means to enhance the phytochemical content of plant-based foods and, consequently, to improve public health through diet. Moreover, studies based on popular and worldwide consumed vegetables, such as lettuce, are of particular interest.

In this context, preharvest treatments of plants with elicitors could be a feasible way to induce the biosynthesis of phytochemicals (Pérez-Balibrea et al., 2011). Elicitors are exogenous chemical compounds that cause a series of defense reactions in plants, including physiological and morphological changes and phytochemical compounds accumulation through the induction of secondary metabolic pathways (Mandal 2010). In a previous work we have demonstrated that the application of chitosan (2.5, 5, 10 g L^{-1}) and tea tree essential oil (*Melaleuca alternifolia* L.) (1.8, 2.7, 3.6 mL L^{-1}) to Butterhead lettuce (*Lactuca sativa* var. Lores) seeds and sprouts during germination exhibited elicitor activity and stimulated the biosynthesis of phytochemical compounds in seven days old sprouts (Viacava and Roura, 2015). However, these treatments negatively affected lettuce sprouts biomass parameters, preventing the normal growth of the seedlings for mature lettuce

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production. An alternative to overcome this issue could be to use these elicitors in more advanced stages of lettuce growth, when plants have a more developed defense system that would render it more resistant to changes in the environment. Furthermore, the application of chitosan and tea tree essential oil in the late development stages of lettuce could also help to control the native microflora growth and enhance the microbiological quality of this produce, as demonstrated by i et al. (2013, 2014); i et al. (2013, 2014).

Appearance, color, brightness, and texture are the major marketable properties of fresh lettuce. Since these quality attributes may be influenced by abiotic and biotic factors (Złotek et al., 2014), it is important to assess the effect of preharvest treatments with elicitors on the organoleptic quality of the plant. Besides, the increased levels of phytochemicals in the elicited plant, particularly of phenolic compounds, could hasten the enzymatic browning of plant tissues, because they are substrates for the browning enzymes peroxidase (POD; EC 1.11.1.7) and polyphenol oxidase (PPO; EC 1.14.18.1). Additionally, the activity of these enzymes may rapidly change after treatment with elicitors due to their role in the phenylpropanoid metabolism and in the defense system of the plant (Mandal, 2015).

There are some studies regarding the effect of preharvest application of chemical elicitors (mainly endogenous phytohormones such as arachidonic acid, jasmonic acid, and abscisic acid) on the phytochemical content and nutritional quality of different lettuce varieties (Kim et al., 2007; Li et al., 2010a; Tierranegra-García et al., 2011; Złotek et al., 2014). However, to the best of our knowledge, the utilization of chitosan and tea tree essential oil to stimulate the biosynthesis of lettuce phytochemicals has not been examined. Besides, little has been reported about changes in the organoleptic and microbiological quality of lettuce after treatment with elicitors or in the activity of PPO and POD. Therefore, the purpose of this study was to evaluate the feasible use of chitosan and tea tree essential oil applied before harvest to increase the antioxidant phytochemicals content in Butterhead lettuce. Furthermore, the activities of PPO and POD enzymes and the organoleptic and microbiological quality of the samples were also evaluated. For the determination of the real quality of the elicited lettuces, these quality indexes were assessed at harvest and during 21 days of postharvest refrigerated storage.

2. Materials and methods

2.1. Elicitors solutions preparation

Medium molecular weight Chitosan (98% deacetylation degree) was supplied by ACOFAR (Mar del Plata, Argentina). Chitosan solution (10 g L^{-1}) was prepared by dissolving chitosan powder in lactic acid 7 mL L^{-1}. The pH solution was adjusted to 5.6 with NaOH 1 mol L^{-1}. To achieve complete chitosan dispersion, the solution was stirred overnight at 100 rpm in an orbital shaker (TS-1000, Zhejiang, China) (Goñi et al., 2014).

Tea tree (*M. alternifolia*) essential oil was provided by Nelson and Russell (London, England), which supplies food grade oils. Tea tree essential oil was extracted by steam distillation from tea tree leaves of Australian origin. The main component determined by gas chromatography/mass spectrometry was terpinen-4-ol (29%). Other minor constituents detected were γ-terpinene, α-terpinene and ρ-cymene (data not shown). Tea tree was diluted in distilled water and vigorously shaken at 30 °C for 30 min to obtain a reasonably stable dispersion (2.7 mL L^{-1}) (Goñi et al., 2014).

The concentrations of chitosan and tea tree solutions used were selected based on previous studies where these compounds had been investigated as preharvest sanitizers on lettuce plants (i et al., 2013, 2014; i et al., 2013, 2014), showing promising results.

2.2. Plant material and preharvest application of elicitors

Butterhead lettuces (*L. sativa* var. Lores) were grown in Sierra de los Padres, Mar del Plata, Argentina. Lettuce plants were cultivated in a greenhouse using mulch technology (a black plastic film separating each plant from the soil). The assays were performed in the fall-early winter (April to August). At the greenhouse, plants of equal size were selected (forty lettuce plants in each treatment, $n = 160$) to be sprayed with a solution of the elicitors to be tested (7–10 mL per plant): 2.7 mL L^{-1} tea tree (TT) and 10 g L^{-1} chitosan (CHI), taking care not to spread other plants. To avoid this dissemination, a plastic cone with an upper hole was placed over each plant and the spray was applied through the cone. Control plants were sprayed with the solvents used to prepare the solutions of TT and CHI. Thus, there were two different kinds of control solutions: distillated water (W) and 7 mL L^{-1} lactic acid (LA). Elicitor and control solutions were applied in five successive applications (at 14 + 10 + 7 + 3 + 0 days previous to harvest) to each plant, following the protocol of application recommended by Goñi et al. (2013). In this period of growth most of the lettuce leaves are developed (80%) but the head is not yet fully formed, which ensures an effective contact of the elicitors with a great proportion of leaves. Previous studies (i et al., 2013, 2014; i et al., 2013, 2014) and screening experiments (data not published) demonstrated that the concentrations of elicitors used and the protocol of application did not evoke negative effects on the health and growth of plants. Therefore, all plants (controls: W and LA; and elicited: CHI and TT) presented optimal maturity at harvest, which corresponds to a complete formation of the head and a marketable size of approximately 18–22 leaves and 250 g per head.

One hundred and sixty lettuces (40 for each treatment: W, CHI, TT, and LA) were hand harvested. Once harvested, lettuce heads were immediately transported to the laboratory and eight whole plants of each treatment were analyzed within 1 h after harvest to determine the effect of preharvest treatments immediately after harvest (zero time): four of them were used to assess microbiological quality and enzymatic activity and the other four to analyze the other quality indices (organoleptic quality, reduced ascorbic acid, total phenolic content, total flavonoids content, and antioxidant capacity).

The other plants were put in polyethylene bags (with O_2 and CO_2 permeabilities of 600 and 4000 $cm^3\ m^{-2}\ d^{-1}$, respectively, and a water vapor permeability of 4 g $m^{-2}\ d^{-1}$; determined at P = 101,325 Pa, T = 25 °C), placing two plants per bag (28 × 55 cm^2, useful volume: 4 L). Bags were hermetically sealed and stored in an environmental chamber (SCT, Pharma, Argentina) at 0–2 °C and 97–99% of relative humidity. Sampling was carried out at day 3, 7, 14, and 21 of storage. At each sampling time, four bags from each treatment were used for analysis. Two bags were used to assess microbiological quality and enzymatic activity. The other two bags were used to analyze the other quality indices (organoleptic quality, reduced ascorbic acid, total phenolic content, total flavonoids content, and antioxidant capacity).

Except for organoleptic quality (which was performed on the intact plant), to assess the remaining indices, the lettuce heads were cut transversely in 2-cm portions and mixed, taking two samples from each head. Two independent experimental runs were performed.

2.3. Reduced ascorbic acid content

Reduced ascorbic acid content was determined by the titrimetric assay described by Agüero et al. (2011) with modifications. Lettuce portions (20 g) were homogenized with a tissue blender (Multiquick MR 5550 CA, Braun, Spain) with 40 mL of 2% w/v oxalic acid solution. This mixture was vacuum filtered through glass fiber. Temperature during ascorbic acid extraction was maintained at 0 °C. Two aliquots (10 mL each) of filtrates were titrated with 2,6-dichloroindophenol. Ascorbic acid content was calculated as mg per 100 g of fresh weight (FW).

2.4. Extraction of polyphenols and antioxidants

Extraction of polyphenols and antioxidants was carried out following the methodology proposed by Viacava et al. (2015). The obtained extracts were used in the determinations of total phenolic content (TPC), total flavonoids content (TFC), and antioxidant activity by DPPH and TEAC methods.

2.5. Total phenolic content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu reagent (FCR) according to the methodology proposed by Viacava and Roura (2015). Concentration of total phenolic compounds was calculated using a standard curve of gallic acid and expressed as mg gallic acid equivalents (GAE) per 100 g of FW.

2.6. Total flavonoids content

Total flavonoids content (TFC) was determined based on the methodology described by Viacava and Roura (2015). TFC was expressed as mg of quercetin equivalents (QE) per 100 g of FW using a standard curve of quercetin.

2.7. DPPH assay

Antioxidant activity using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical was determined based on the methodology described by Viacava et al. (2015). DPPH radical scavenging activity was expressed as mg of ascorbic acid equivalents (AAE) per 100 g of FW.

2.8. TEAC assay

TEAC (Trolox Equivalent Antioxidant Capacity) value was determined according to Viacava and Roura (2015). Results were expressed as mg of trolox equivalents (TE) per 100 g of FW.

2.9. Peroxidase activity

Peroxidase (POD) activity was determined spectrophotometrically at 470 nm using guaiacol as substrate and H_2O_2 as hydrogen donor, according to Ponce et al. (2008). Peroxidase activity was expressed as activity units (AU) per gram of FW, where an activity unit was defined as a 0.001 change in absorbance per minute.

2.10. Polyphenol oxidase activity

Polyphenol oxidase (PPO) activity was measured by the colorimetric method, following the rate of catechol oxidation at 400 nm for 60 s according to Ponce et al. (2008). The PPO activity was expressed as activity units (AU) per gram of FW where an activity unit was defined as a 0.001 change in absorbance per minute.

2.11. Microbiological quality

Enumeration of the microbial populations was made according to Ponce et al. (2004a). Mesophilic aerobic bacteria microorganisms was performed on plate count agar incubated at 35 °C for 24–48 h. Yeast and molds were counted in yeast-glucose-chloramphenicol medium incubated at 25 °C for 5 days. All culture mediums were from Britania, Buenos Aires, Argentina. Microbial counts were performed in duplicate and expressed as log colony forming units (CFU) per gram of FW.

2.12. Organoleptic quality: overall visual quality (OVQ)

Overall visual quality (OVQ) was evaluated following Agüero et al. (2011). A sensory panel comprised of 9 trained judges, aged 30–55 years, all members of the Food Engineering Group and with sensory evaluation experience in leafy vegetables, proceeded to the assessment. Each individual lettuce was presented one at a time in random order to the judges who made independent evaluations. Quality parameters including color (shade and uniformity), brightness, crispness, wilting, bacterial decay, and physiological disorders (mainly midrib and edge browning of lettuce heads) were analyzed and scored with a 5-point scale: 5 = excellent, 4 = good, 3 = fair (accept limit), 2 = poor, and 1 = extremely poor. The average of the indices was used as an estimation of OVQ.

2.13. Statistical analysis

A completely randomized design was used to assign treatments to the experimental units (lettuce heads). Results reported in this paper are LSMEAN values (least square mean, means estimators by the method of least squares) together with their standard deviations (Lenth 2016).

Statistical ANOVA analysis was performed using R, software version 2.12 (R Development Core Team, 2011). A statistical model was used to evaluate the effect of preharvest treatments on the quality indices of Butterhead lettuce immediately after harvest (day 0). For this purpose, a one-way ANOVA was applied with the factor TREATMENT (W, CHI, TT, and LA) as source of variation. A second statistical model was used to evaluate the effects of preharvest treatments and postharvest storage on the quality indices of Butterhead lettuce. Variation sources used as factors of the two-way ANOVA were TREATMENT (W, CHI, TT, and LA), STORAGE TIME (0, 3, 7, 14, and 21 days) and TREATMENT * STORAGE TIME interaction. For each model, differences among results obtained for different factor levels were evaluated with the multiple comparisons Tukey-Kramer test (Kuehl 2001). A P-value of < 0.05 was considered statistically significant.

Linear regression fittings were performed using SYSTAT 5.0 (SYSTAT INC., 1992).

3. Results and discussion

3.1. Reduced ascorbic acid content

The initial ascorbic acid (AA) content for control lettuce plants (W) was 15.13 ± 0.94 mg 100 g^{-1} FW. Preharvest application of elicitors introduced significant differences ($P < 0.05$) in the initial AA content. Treatments with TT and CHI led to higher initial values of AA (21.01 ± 2.09 and 17.48 ± 2.59 mg 100 g^{-1} FW, respectively, which constitute an increase of 39 and 16% in relation to the control). Conversely, plants treated with LA contained at harvest the lowest content of AA (13.10 ± 2.31 mg 100 g^{-1} FW).

It is known that biotic and abiotic stresses induce changes in primary and secondary plant metabolism, stimulating the synthesis of diverse metabolites such as polyphenols, amino acids and sugars, among others (Dixon and Paiva, 1995). Since elicitors can act like biotic and abiotic stresses, the AA increase observed in lettuces elicited with TT and CHI could be linked to the indirect activation of ascorbic acid synthesis by the production of carbohydrates such as sucrose and glucose, key factors in the biosynthetic pathway of L-ascorbate (Li et al., 2010b). Previous studies have proved that some abiotic elicitors increased the levels of ascorbic acid of different plants (Wolucka et al., 2005; Złotek et al., 2014). However, only scarce data are available on the direct effect of chitosan on the ascorbic acid content of plants. Moreover, it is the first time that the effect of tea tree essential oil on plant ascorbic acid accumulation is reported. Moreno et al. (2008) indicated that chitosan application (10 g L^{-1}) during broccoli head formation increased the AA/DHAA ratio (ascorbic acid to dehydroascorbic acid) of inflorescences, but not the total content of vitamin C. Additionally, these authors reported a very different effect on the broccoli leaves, where chitosan treatment induced the lowest content of vitamin

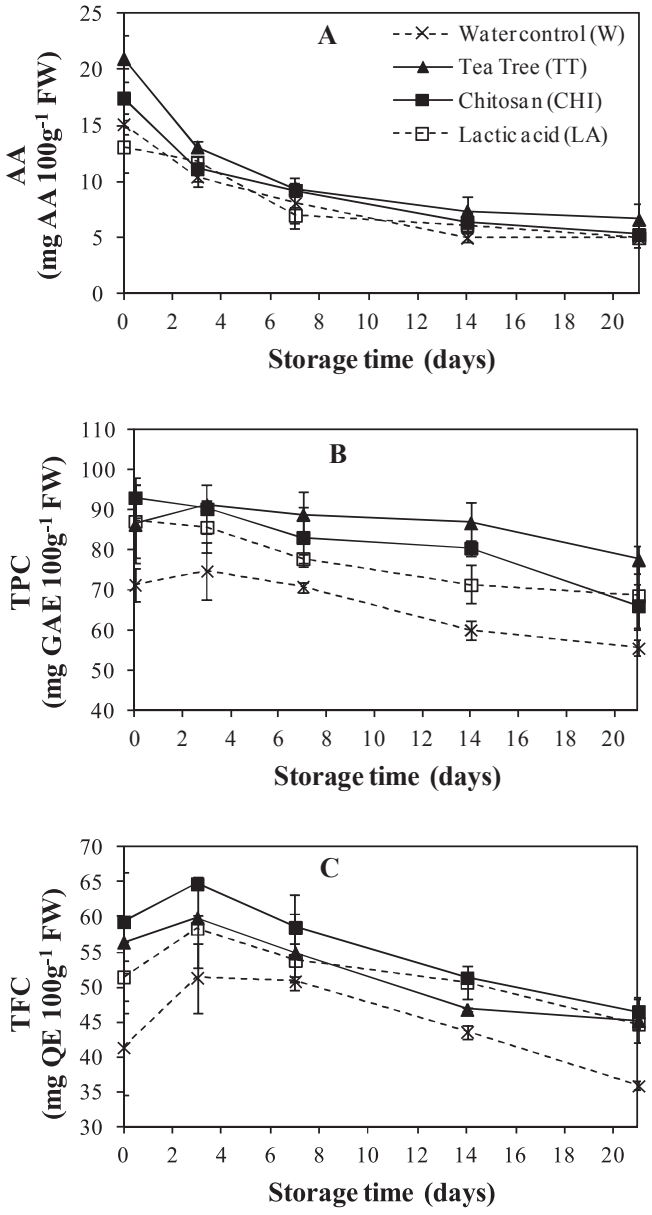

Fig. 1. Effect of elicitation on reduced ascorbic acid (A), total phenolics content (B), and total flavonoids content (C) of lettuces stored during 21 days at 0–2 °C. Data presented correspond to the LSMEAN (least square mean, estimators of means by the method of least square, $n = 4$) and the error bars represent the standard deviation associated to each LSMEAN.

C. Conversely, in edible sprouts, it has been reported that treatment with chitosan increased the vitamin C content of soybean (No et al., 2003) and broccoli (Pérez-Balibrea et al., 2011) sprouts. These discrepancies might be due to the different forms of applications and chitosan concentrations used in each study as well as the different plant species and tissues analyzed.

Fig. 1 (A) shows the change of AA content during refrigerated storage of control (W and LA) and elicited (CHI and TT) lettuce plants. ANOVA applied to AA data showed a significant interaction between factors considered in the analysis (TREATMENT and STORAGE TIME), indicating that the behavior of each sample was different during storage. In fact, although AA content decreased along storage in all lettuce plants, losses registered in TT and CHI treated plants were higher during the first 3 days of storage. This behavior caused that no differences were detected in AA values among lettuce samples from day 3 of storage, which indicate a low residual effect of elicitation for this phytochemical quality parameter. Final AA values were in the range of 4.9–6.7 mg 100 g^{-1} FW, which represent losses along storage of 67–70% for all treatments. Losses of AA during storage at optimal temperature (0–2 °C) could be associated to stress induced by harvest, tissue structural changes due to biological deterioration factors (senescence), and the role of ascorbic acid as scavenging agent. Agüero et al. (2011), analyzing changes in ascorbic acid content in Butter lettuce heads, also reported losses of about 55% during 20 days of storage at 0–2 °C.

3.2. Total phenolic and flavonoids content

In freshly harvested plants, the total phenolic content (TPC) of samples treated with CHI, TT, and LA were 93.17 ± 5.09, 86.50 ± 9.73, and 87.27 ± 9.06 mg GAE 100 g^{-1} FW, respectively, which represents a 31, 21, and 22% increase with regard to water control sample (71.40 ± 4.32 mg GAE 100 g^{-1} FW). On the other hand, the initial contents of total flavonoids (TFC) in the lettuces treated with CHI and TT were 59.34 ± 7.22 and 56.45 ± 2.71 mg QE 100 g^{-1} FW, respectively, and were significantly higher (by 43 and 36%, respectively) than that found in water control plants (41.40 ± 6.68 mg QE 100 g^{-1} FW). LA treated lettuces presented an intermediate content of total flavonoids (51.51 ± 5.06 mg QE 100 g^{-1} FW).

The biosynthesis of phenolic compounds is mediated by the action of the enzymes phenylalanine ammonium lyase (PAL) and tyrosine ammonium lyase (TAL), which participate in the shikimic acid pathway (Vogt 2010). Genes that codify for PAL are induced by the attack of pathogens and/or abiotic factors. As a consequence, the increase in TPC and TFC detected in our work in treated lettuce plants with TT and CHI could be attributed to the induction of PAL. The stimulant action of chitosan in the synthesis of polyphenols is a scientifically established fact (Khan et al., 2003; No et al., 2003; Pérez-Balibrea et al., 2011). For instance, Khan et al. (2003) demonstrated that 100 mmol L^{-1} chitosan produced a significant increase of total phenolic content in soybean leaves and this increase was associated with the induction of PAL and TAL activities. On the other hand, the recognition of tea tree essential oil as elicitor of secondary metabolism and synthesis of polyphenols is a novel result.

Fig. 1 (B) and (C) shows the change of TPC and TFC, respectively, during refrigerated storage of control (W and LA) and elicited (CHI and TT) lettuce plants. ANOVA applied to both TPC and TFC data showed that interaction between TREATMENT and STORAGE TIME was not significant while both factors resulted highly significant, indicating that all lettuce samples evolved similarly during storage. Regardless of preharvest treatment, all lettuce samples revealed a TPC decrease during storage, with an average reduction of 20% respect to initial values. With regard to TREATMENT factor, the TPC of lettuces treated with CHI, TT and LA were the highest during the entire sampling period and were not statistically different among themselves, with a mean value of 82.59 ± 11.01 mg GAE 100 g^{-1} FW throughout storage. These results might suggest a long-term effect of elicitation in secondary metabolism induction. Similar results were obtained for TFC whose values were higher in plants treated with CHI, TT, and LA (by 26, 18, and 16%, respectively) than in control plants (Fig. 1 (C)). Additionally, a reduction of 16% in TFC was found for all lettuces when compared with values obtained at harvest.

Although it has been reported that phenolic compounds are generally stable during cold storage (< 4 °C) of most leafy vegetables (Martínez-Sánchez et al., 2012), some authors have also reported losses of polyphenols during the storage of lettuce (DuPont et al., 2000; Gil et al., 1998). According to these studies, flavonoid losses were associated to demalonation of quercetin glycosides. Another possible cause of polyphenol losses during storage may be related to the enzymatic oxidation of phenolic compounds by PPO and POD (Zhan et al., 2012). Some authors have recently studied the implication of these enzymes in

minimally processed lettuce (Mai and Glomb, 2013; Zhan et al., 2012) and although in the present study lettuces were not subjected to cutting procedures before storage, an enzymatic oxidation of phenolic compounds could also take place.

3.3. Antioxidant activity

Two complementary *in vitro* assays (DPPH and TEAC) were used to evaluate the antioxidant activity of lettuce. At harvest, antioxidant activity of control plants was 74.62 ± 3.11 mg AAE 100 g^{-1} FW for DPPH and 68.25 ± 1.39 mg TE 100 g^{-1} FW for TEAC. No significant differences were found in the initial antioxidant activity of treated lettuces with CHI, TT and LA (with mean values of 95.13 ± 4.16 mg AAE 100 g^{-1} FW (DPPH) and 90.43 ± 4.68 mg TE 100 g^{-1} FW (TEAC)), although they were significantly higher (between 27 and 33% depending on the technique used) than that found for control plants.

Antioxidant activity of lettuce is mainly associated with its content of phenolic compounds, vitamins C and E, chlorophyll and carotenoids (Nicolle et al., 2004). Consequently, the higher antioxidant activity observed in freshly lettuces treated with CHI and TT could be related to the higher phenolic compounds and ascorbic acid obtained in these plants. Additionally, some studies have reported that both chitosan and tea tree essential oil present antioxidant activity themselves. Kim et al. (2004) showed that the antioxidant activity of TT was attributed to its content of α-terpinene, α-terpinolene, and γ-terpinene. On the other hand, previous works have indicated that chitosan is a good chelator of metal ions and free radicals scavenger due to its content of amine groups (Xie et al., 2001). Therefore, results obtained in this study for antioxidant activity indices could be attributed to the potential elicitor activity of TT and CHI as well as to their own antioxidant activity.

During storage, antioxidant activity of lettuces determined with both DPPH and TEAC techniques presented a similar behavior (Fig. 2 (A) and (B)). Results of statistical analysis applied to DPPH and TEAC data did not show a significant interaction between factors under consideration, but both TREATMENT and STORAGE TIME resulted significant. For all samples, antioxidant activity decreased with increasing storage time, with losses of 13% (DPPH) and 20% (TEAC) by day 21 compared to initial values. With regard to preharvest treatments, differences detected at harvest were maintained during storage, i.e. the antioxidant capacity of lettuces treated with CHI, TT and LA remained 19% (DPPH) and 23% (TEAC) (average throughout storage) higher than that of control plants.

3.4. Enzymatic activity

Polyphenol oxidase (PPO) and peroxidase (POD) are enzymes of phenylpropanoid metabolism of lettuce, responsible of tissue browning. These enzymes catalyze the oxidation of phenolics with subsequent formation of dark compounds, which greatly reduces the visual quality of plants (Tomás-Barberán et al., 1997). As demonstrated in Section 3.2, elicitation affects the phenol metabolism of lettuce and induces the synthesis and accumulation of phenolics, which might trigger enzymatic browning reactions. Therefore, in this study PPO and POD activities were monitored in order to determine how phenol oxidizing enzymes were affected by the preharvest treatments with CHI and TT.

Immediately after harvest, no significant differences were detected in PPO activity among W, CHI and LA lettuces (Table 1). Instead, lettuces treated with TT exhibited a significant higher PPO activity (by 23%) when compared with control plants (Table 1). On the other hand, the initial POD activity of CHI and TT treated lettuces was 52 and 24% lower, respectively, in comparison with control plants (W) (Table 1). Instead, it was observed a slight but significant increase of 5% in initial POD activity in LA treated lettuces (Table 1).

Reduced POD activity in TT treated lettuces could be related to the antioxidant properties of tea tree constituents, as mentioned earlier. In fact, Ponce et al. (2004b) demonstrated the effectiveness of several

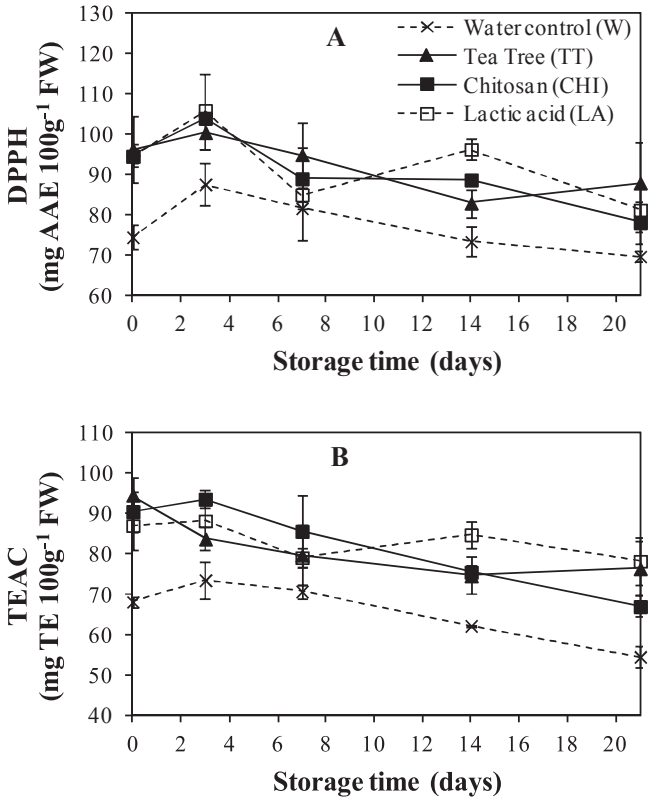

Fig. 2. Influence of preharvest treatments with natural elicitors on antioxidant activity determined by DPPH (A) and TEAC (B) methods of lettuces stored during 21 days at 0–2 °C. Data presented correspond to the LSMEAN (least square mean, estimators of means by the method of least square, $n = 4$) and the error bars represent the standard deviation associated to each LSMEAN.

essential oils including tea tree in reducing POD activity of leafy vegetables by *in vitro* assays. On the other hand, several authors have studied the influence of postharvest chitosan treatments on the enzymatic activity of diverse fruits and vegetables. Different effects were reported. For example, El Hassni et al. (2004) informed an increase in POD and PPO activities of date palm roots treated with chitosan (0.1, 0.5, and 1.0 g L^{-1}). Instead, in agreement with our findings, several authors have reported a reduced PPO and POD activities in different fruits treated with chitosan (Pasquariello et al., 2015; Ruoyi et al., 2005; Wang and Gao, 2013). In general, these authors indicated that chitosan application forms an edible coating on fruits that provides a semi-permeable barrier against oxygen, thereby reducing enzymatic oxidation reaction rates. In the present study, preharvest applications of chitosan on lettuce leaves might have formed a biopolymer film that acts as a gas barrier and reduces the tissue oxygen intake, which is necessary for enzymatic reactions.

Results of statistical analysis applied to PPO and POD data during refrigerated storage indicated a significant TREATMENT * STORAGE TIME interaction for both enzymes (Table 1). However, PPO and POD activities of elicited (CHI and TT) and control (W and LA) lettuces changed little during storage. Although lettuce is highly sensitive to enzymatic browning (Zhan et al., 2012), the storage of the uninjured vegetable under optimal refrigerated temperature could be responsible for the delayed increase in PPO and POD activities. Differences in PPO activity found at harvest with regard to preharvest treatment were not observed during storage and CHI treated plants presented, in general, a lower PPO activity throughout storage (Table 1). As regard POD, similar results than those presented at harvest were in general observed during storage. Samples treated with CHI and TT presented a lower POD activity when compared to control plants (W and LA) during the entire sampling period (Table 1).

Table 1
Polyphenol oxidase (PPO) and peroxidase (POD) activities ((AU g^{-1} FW) × 1000) of control and elicited samples during refrigerated storage. Values are mean ± standard deviation of 4 replications.

Storage time (day)	Treatments			
	Water control	Tea Tree	Chitosan	Lactic acid
PPO				
0	4.22^{bAB} ± 0.34	5.07^{aA} ± 0.38	4.16^{bA} ± 0.22	4.05^{bB} ± 0.22
3	3.80^{aBC} ± 0.25	3.73^{aB} ± 0.32	3.46^{aBC} ± 0.40	3.31^{aC} ± 0.22
7	4.66^{abA} ± 0.76	4.88^{aA} ± 0.16	4.00^{bAB} ± 0.04	4.80^{aA} ± 0.09
14	3.15^{aC} ± 0.23	2.85^{abC} ± 0.36	2.65^{bC} ± 0.32	3.23^{aC} ± 0.32
21	4.23^{aAB} ± 0.31	3.21^{bBC} ± 0.16	3.00^{bC} ± 0.09	3.41^{bBC} ± 0.35
POD				
0	3.40^{bA} ± 0.07	2.59^{cA} ± 0.12	1.65^{dC} ± 0.05	3.58^{aAB} ± 0.04
3	3.49^{aA} ± 0.28	2.83^{bA} ± 0.10	2.24^{cAB} ± 0.27	3.48^{aB} ± 0.08
7	2.75^{bC} ± 0.11	2.26^{cB} ± 0.03	2.07^{dB} ± 0.12	3.06^{aB} ± 0.04
14	3.27^{bAB} ± 0.02	2.82^{cA} ± 0.14	2.63^{cA} ± 0.32	4.13^{aA} ± 0.03
21	3.03^{abBC} ± 0.13	2.81^{bA} ± 0.16	2.57^{bA} ± 0.07	3.30^{aB} ± 0.08

^{ab}Values in the same row with different low case letters were significantly different at $P < 0.05$ according to the Tukey-Kramer's multiple comparisons test.
^{ABC}Values in the same column with different capital letters were significantly different at $P < 0.05$ according to the Tukey-Kramer's multiple comparisons test.

Several authors have studied the effect of tea tree and chitosan postharvest treatments on the PPO and POD activities of different fruits and vegetables during storage, reporting similar results than those presented in the present study. For instance, Alvarez et al. (2015) informed that the application of tea tree essential oil (15 mL L^{-1}) combined with optimal refrigeration temperature (5 °C) was able to reduce POD activity in minimally processed butternut squash and leek. Ponce et al. (2004a) also reported a reduction of 35% in POD activity of Swiss chard stored during 14 days at 5 °C and treated with TT (0.9 mL L^{-1}) previous to storage. With regard to chitosan, Zhang and Quantick (1997) indicated that the application of chitosan coating (10 and 20 g L^{-1}) on litchi fruit delayed the increase of POD and PPO activities during postharvest storage at 4 °C. In another study, chitosan coating (5 g L^{-1}) inhibited PPO and POD activities of sweet cherry stored at 2 °C for 14 days (Pasquariello et al., 2015). As far as we know, this is the first report about the application of chitosan and tea tree essential oil during preharvest and its effects on the postharvest POD and PPO activities of intact vegetables.

3.5. Microbiological quality

Recently, chitosan and tea tree essential oil have become promising alternatives to chemical decontamination because of its natural character, antimicrobial activity and elicitation of defense responses in plant tissue (Goñi et al., 2014). In the present study, the effectiveness of preharvest application of CHI and TT on Butterhead lettuce in controlling the native microflora of the vegetable was also assessed.

Changes in mesophilic population loads on lettuce samples throughout refrigerated storage are shown in Fig. 3 (A). ANOVA applied to these data indicated that only STORAGE TIME factor was significant. Therefore, none of the proposed treatments revealed to be effective in reducing mesophilic counts. Taking into account the STORAGE TIME factor, a significant increase in this population was observed for all samples during the first 3 days of storage, reaching values 1.3 log higher when compared to those obtained at harvest. After that, mesophilic counts remained steady until the end of storage. Low temperature storage might have limited mesophilic growth in the lettuce samples.

Fig. 3 (B) shows the changes in yeast and molds counts over refrigerated storage for control (W and LA) and elicited (CHI and TT) lettuce plants. ANOVA applied to these data showed that interaction between TREATMENT and STORAGE TIME was not significant while both factors resulted highly significant. Regarding preharvest treatments, no significant differences were observed in yeast and molds counts between TT treated lettuces and control plants (W) during the entire sampling period. Instead, preharvest application of CHI resulted in a significant reduction of 1.6 log throughout storage. This effect could be attributed to the known antifungal activity of chitosan (Bautista-Baños et al., 2006), but also to the acidic medium in which it is dissolved since LA treated lettuces also presented lower yeast and molds counts than control plants, with reductions of 0.8 log (average along storage). These results indicate that chitosan dissolved in lactic acid was more effective in reducing yeast and molds counts than lactic acid alone. The STORAGE TIME factor analysis showed a significant increase in yeast and molds population during storage for all samples, from 5.40 ± 0.77 log at harvest to 6.47 ± 0.55 log at the end of storage. Similar results were reported by Goñi et al. (2014).

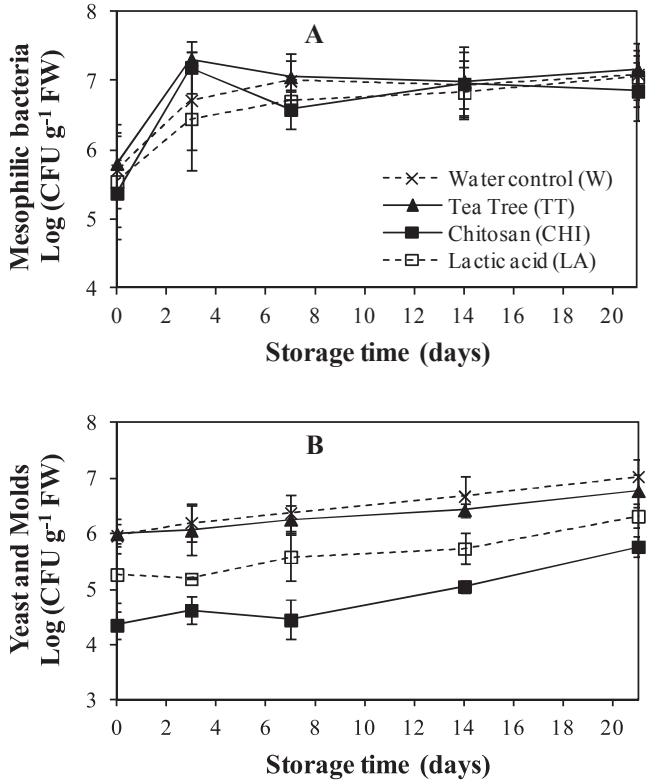

Fig. 3. Effect of elicitation on mesophilic bacteria (A) and yeast and molds (B) counts of lettuces stored during 21 days at 0–2 °C. Data presented correspond to the LSMEAN (least square mean, estimators of means by the method of least square, $n = 4$) and the error bars represent the standard deviation associated to each LSMEAN.

Table 2
Impact of storage time on sensory quality attributes of lettuce samples (values correspond to means ± standard deviation of all treatments, as TREATMENT and TREATMENT * STORAGE TIME factors were not significant).

Storage time (day)	Leaf texture	Leaf color and brightness	Leaf browning	Browning at the cut base	OVQ
0	4.9^{A} ± 0.2	4.6^{A} ± 0.3	5.0^{A} ± 0.1	4.9^{A} ± 0.2	4.8^{A} ± 0.1
3	4.8^{A} ± 0.4	4.6^{AB} ± 0.3	4.9^{AB} ± 0.2	4.0^{B} ± 0.3	4.6^{A} ± 0.2
7	4.7^{A} ± 0.3	4.1^{B} ± 0.4	4.5^{B} ± 0.4	3.1^{C} ± 0.4	4.1^{B} ± 0.2
14	3.8^{B} ± 0.4	3.5^{C} ± 0.3	3.9^{BC} ± 0.3	2.3^{D} ± 0.4	3.4^{C} ± 0.2
21	3.8^{B} ± 0.3	3.2^{C} ± 0.2	3.5^{C} ± 0.4	1.9^{D} ± 0.3	3.1^{C} ± 0.3

^{ABCD}Values in the same column with different capital letters were significantly different at $P < 0.05$ according to the Tukey-Kramer's multiple comparisons test.

Several studies have demonstrated the antimicrobial activity of tea tree essential oil, which is principally attributed to its content of terpene hydrocarbons, mainly terpinen-4-ol (Alvarez et al., 2015; Shao et al., 2013). Most of these studies were conducted under *in vitro* conditions or with postharvest applications of the essential oil but little has been reported with regard to preharvest treatments. Recently, Goñi et al. (2014), in concordance with our results, found a low antimicrobial effectiveness of tea tree (2.7 mL L^{-1}) when applied during preharvest on Butterhead lettuce and suggested that the volatility of its active constituents might be responsible for the loss of antimicrobial activity.

The antimicrobial activity of chitosan, mainly related to its polycationic nature, has been exploited by many authors who have studied the influence of postharvest chitosan treatments on the microbiological quality of different fruits and vegetables (Alvarez et al., 2013; Badawy and Rabea, 2009). However, only few studies were conducted with preharvest applications (Bhaskara Reddy et al., 2000; Goñi et al., 2014; Romanazzi et al., 2002). For instance, Goñi et al. (2014) indicated that preharvest application of CHI (10 g L^{-1}) on Butterhead lettuce significantly reduced the mesophilic bacteria and yeast and molds initial counts, with reductions of 2.0 and 1.8 log, respectively, compared with control samples. In the present study, differences in the susceptibility of microbial populations evaluated to CHI were observed, varying from no sensible at all for mesophilic bacteria up to highly sensitive for yeast and molds, with reductions over 97% for this population compare to control plants.

3.6. Organoleptic quality

Regarding fresh lettuce, the members of the sensory panel did not find significant differences in the sensorial attributes among control (W and LA) and elicited (CHI and TT) plants and rated all samples with the maximum score (OVQ = 5). This means that preharvest treatment with elicitors (TT and CHI) or LA did not cause undesirable sensorial effects in freshly lettuces.

During storage, lettuce heads had significant decreases in OVQ values, without differences due to preharvest treatment (only STORAGE TIME factor was significant). A linear decrease of 0.09 points per day was found in all samples (Table 2). The first change observed by panelists was the presence of browning in the cut area (stem butt). At day 7, a decrease in leaf brightness was also detected with some discoloration and browning in leaf edges, principally in external leaves, but acceptable texture. At day 14, outer leaves presented moderate ruptures in leaf tissues, suggesting an increase in their mechanical fragility. Toward the end of storage, external lettuce leaves presented an extending browning in midribs, a moderate loss of texture, and severe browning in the cut base (rusty brown).

Previous works have reported that essential oils applied in fresh-cut vegetables are responsible for the increase apparition of browning (Ponce et al., 2004a). In the present study, preharvest application of tea tree essential oil did not cause undesirable sensorial effects in lettuce heads, neither at harvest nor during refrigerated storage. Accordingly, Goñi et al. (2013) indicated that preharvest application of essential oils may help to reduce the impact they have in the quality attributes when are applied during postharvest.

On the other hand, several authors have reported that fruits and vegetables treated with chitosan generally present higher scores in all its sensory attributes in comparison with untreated samples since chitosan forms a semipermeable film that regulates the gas exchange and reduces respiration rate and water loss (Bautista-Baños et al., 2006; Bhaskara Reddy et al., 2000; Moreira et al., 2011). In the present work, preharvest treatment of lettuce with CHI did not have a beneficial impact on the organoleptic quality of the produce, but neither its sensory quality attributes were negatively influenced. These results are of major importance since preharvest treatments with elicitors should not introduce deleterious effects on the sensory attributes of the produce that could negatively impact the consumer acceptability.

4. Conclusions

Based on the results presented here, preharvest treatment of lettuce with chitosan 10 g L^{-1} and tea tree essential oil 2.7 mL L^{-1} enhanced the total phenolics and flavonoids content of the vegetable, intensifying its antioxidant capacity without affecting its appearance and other sensory quality parameters. Moreover, chitosan application was the most effective in preserving the quality of the stored produce since it presented initial reductions on yeast and molds counts and exerted a fungistatic effect during refrigerated storage. Additionally, this treatment reduced the activities of PPO and POD, enzymes related to enzymatic browning.

Therefore, preharvest application of chitosan and tea tree essential oil is presented as a potential technology for stimulating the biosynthesis of health-promoting phytochemicals in lettuce and thus improving the nutritional value of the plant. Besides, it could easily be implemented by the producers, without consequences on the environment as tea tree and chitosan are generally recognized as safe substances. In particular, treatment with chitosan could also be a promising method for enhancing the phytochemical quality of minimally processed lettuce since this elicitor reduced the activities of the enzymes associated with enzymatic browning, one of the most common postharvest disorders of this kind of product. Further studies could be conducted in this regard.

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