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Principal component and hierarchical cluster analysis to select natural elicitors for enhancing phytochemical content and antioxidant activity of lettuce sprouts

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ABSTRACT

Sprouts have received increasing attention in recent years because of their potential dietary value. Additionally, the efficient production of sprouts with high levels of phytochemicals and antioxidants is desirable. However, no studies were performed on lettuce sprouts. The phytochemical content and antioxidant activity of lettuce sprouts were studied. Moreover, natural elicitors (chitosan and tea tree) were applied as soaking solution during different times or as exogenous daily spraying during germination in order to enhance the phytochemical content of the sprouts. Data was analyzed using multivariate analysis. Untreated lettuce sprouts presented a significant higher content of total phenolics and flavonoids, and antioxidant activity (through DPPH and TEAC assays) than those reported for mature heads of different green lettuce cultivars. Germination percentage was negatively affected by high elicitors concentrations and long contact times. Based on multivariate analysis, only sprouts daily sprayed with elicitors (tea tree 0.18, 0.27, 0.36% v/v and chitosan 0.25, 0.5, 1.0% w/v) presented a significant higher content of phytochemicals and antioxidant activity than the others. Therefore, exogenous application of chitosan and tea tree can stimulate the biosynthesis of phytochemicals, improving the nutritional value of lettuce sprouts.

1. Introduction

Lettuce (*Lactuca sativa*) is one of the most popular leafy vegetables in the world preferably consumed fresh and in salad dishes. It is of particular interest in nutrition due to its content of antioxidants and phytochemicals including caffeic acid and its derivatives, flavonols, vitamins C and E, chlorophyll and carotenoids (Llorach et al., 2008; Viacava et al., 2014). These compounds are associated to health benefits and several studies have shown the health effects of lettuce consumption in improving the lipid status and preventing tissue lipid peroxidation in rats and increasing plasma total antioxidant capacity and antioxidant levels in humans (Nicolle et al., 2004; Serafini et al., 2002).

The enrichment of phytochemicals in plant-based foods suggests the possibility of improving public health through diet. Germination is an inexpensive and simple method for improving nutritive value and edible sprouts are one of the potentially new functional foods (Zhang et al., 2007). However, despite the nutritional composition of lettuce is well known, few studies have determined the nutritional value of lettuce sprouts. Additionally, the efficient production of sprouts with high levels of phytochemicals and antioxidant activity is desirable. In plants, phytochemicals are induced in response to biotic and abiotic stresses, acting as natural phytoalexins to protect plants against these stresses. Some compounds exhibit elicitor activity and highly induce plant defense phytoalexins, suggesting that treatment of plants with elicitors could be a feasible way to trigger the biosynthesis of bioactive metabolites (Pérez-Balibrea et al., 2011b). In this study, two natural elicitors were chosen to study their ability to stimulate the phytochemicals synthesis in lettuce sprouts: the carbohydrate polymer chitosan (CHI) and the essential oil of tea tree (TT). Chitosan is a natural biopolymer obtained by deacetylation of chitin, the linear polymer of (1–4)-β-linked *N*-acetyl-D-glucosamine, a major component of the shells of crustaceans such as crab, shrimp, and crawfish. Recently, chitosan and its oligomers have attracted notable interest due to their biolog-

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ical activities, including antimicrobial, antitumor, antioxidative, and hypocholesterolemic functions (Bautista-Baños et al., 2006). In addition, recent studies have also revealed that chitosan enhances the yield and quality of soybean sprouts and exhibit elicitor activity in broccoli sprouts (No et al., 2003; Pérez-Balibrea et al., 2011b).

The essential oil of *Melaleuca alternifolia* (tea tree) exhibits broad-spectrum antimicrobial activity. Its mode of action against Gram-positive bacteria, Gram-negative bacteria, and yeasts, as well as the native microbiota of different vegetables has been reported (Alvarez et al., 2015; Goñi et al., 2013b). The chemical composition of TT oil has been well defined and consists largely of cyclic monoterpenes of which about 50% are oxygenated and about 50% are hydrocarbons. Among them, terpinen-4-ol is the main antimicrobial constituent. In recent times, TT has gained reputation as a safe, natural and effective antiseptic and it is currently incorporated as natural preservative in many pharmaceutical and cosmetic products (Alvarez et al., 2015). In this current research we investigated if TT essential oil can stimulate the antioxidant synthesis of lettuce sprouts.

The application of chemometric tools for characterization and quality control of food products has recently become a very active research area. Multivariate mathematical approaches are powerful tools which often permit a relatively simple representation of similarities between samples on the basis of more-or-less complex analytical data. Principal component analysis (PCA) is one of the most popular multivariate techniques because it reduces the dimensionality, compresses the noise and correlates measurements in a simple informational sub-space of the data set. Hierarchical cluster analysis (HCA) is another multivariate technique that uses a defined metric to form clusters sequentially; grouping first the most similar objects which are then merged due to their similarities. HCA is used to reveal the structure residing in a data set and disclose the natural groupings existing between samples characterized by the values of a set of measured variables (Patras et al., 2011).

The hypothesis of this research is that yield parameters and phytochemical content of lettuce sprouts can be increased by treating the seeds with natural elicitors, thereby improving the nutraceutical value and seedling growth. To our knowledge, however, the effect of preharvest treatment of exogenous elicitors on bioactive compounds of lettuce sprouts has not been examined. Therefore, the present study aimed to analyze and compare the effects of chitosan and TT essential oil application on antioxidant status, total phenolic and flavonoid contents and growth parameters of lettuce sprouts based on PCA and HCA analysis to improve the healthfulness of this food product with an added value for consumers.

2. Materials and methods

2.1. Elicitors solutions preparation

Various dilutions of CHI and TT were used in order to determine the ideal concentration for maximum elicitor response. Chitosan solutions were prepared by dissolving chitosan powder (ACOFAR, Mar del Plata, Argentina; 98% deacetylation degree) in lactic acid (LA) 0.7% (v/v) and mixed overnight at 100 rpm in an orbital shaker (TS-1000, Zhejiang, China). Lactic acid was used as diluent for chitosan, because pH lower than 6 is required for its appropriate dissolution (Goñi et al., 2013a).

Tea tree (*M. alternifolia*) essential oil was provided by Nelson and Russell (London, England), which supplies food grade oils. TT was diluted in distilled water and vigorously shaken at 30 °C for 30 min to obtain reasonably stable dispersions.

2.2. Seed treatments and germination

Lettuce seeds (*L. sativa* var. Lores) were obtained from Vilmorin® (La Ménitré, France).

Two experiments were consecutively conducted. In the first experiment, 0.5 g of lettuce seeds were mixed with 30 mL of: distillated water (water control), chitosan (0.01, 0.05, 0.1, 0.5% w/v), lactic acid 0.7% v/v (acid control) and tea tree (0.18, 0.27, 0.36% v/v) in an orbital shaker at 100 rpm during 3, 6, 9, 12, 18 and 24 h. The soaked seeds (50 seeds) were then sown in plastic trays (18 × 14 cm) with two layers of Whatman filter paper #42, adequately moistened with 20 mL of distillated water. Trays were covered with plastic foil to prevent dehydration and incubated in a germination chamber (20–22 °C and 8 h photoperiod) for 7 days. The concentrations of chitosan solutions used in this experiment were selected according with previous studies of seed soaking in different vegetal species (Cho et al., 2008; Goñi et al., 2013a). In the case of TT solution, no literature was available about its application as soaking treatment; thus, concentrations were selected based on its application as preharvest sanitizers on late development stages of Butterhead lettuce (Goñi et al., 2013b).

In the second experiment, solutions of: water (water control), chitosan (0.25, 0.5, 1% w/v), lactic acid 0.7% v/v (acid control) and tea tree (0.18, 0.27, 0.36% v/v) were applied by exogenous spraying to the lettuce seeds. 50 lettuce seeds were placed in plastic trays (18 × 14 cm) with two layers of Whatman filter paper #42. 20 mL of each solution was sprayed at sowing day and then daily during 7 days. Trays were covered with plastic foil and incubated as mentioned previously. The concentrations of chitosan solutions used in the second experiment were selected according with previous studies of exogenous spraying in broccoli (Moreno et al., 2008; Pérez-Balibrea et al., 2011b). Concentrations of TT solutions were selected based on its application as preharvest sanitizers on late development stages of Butterhead lettuce, as mentioned previously (Goñi et al., 2013b).

Lettuce sprouts, containing the hypocotyls and cotyledons, were collected at day 7 after sowing and immediately used in the analysis. Percentage of seed germination was determined according to Barassi et al. (2006) by counting germinated seeds at day 7. Only those seedlings without defects were considered as germinated. In order to determine the absence of defects, each tray was thoroughly inspected with a magnifying glass while germinated seedlings were counted.

2.3. Yield parameters

After 7 days of germination, yield parameters (height and weight of sprouts) were measured. The shoot height above the soil was directly measured using a ruler and analyzing at least 10 sprouts per tray. Fresh weight of sprouts was measured after the plant was cut off from above the filter paper and results were expressed as g/50 seeds.

2.4. Extraction of phytochemicals

1 g of lettuce sprouts from each treatment was homogenized with 10 mL solution of ethanol (80% v/v). The homogenate was sonicated for 30 min and then centrifuged at 8000 rpm for 15 min at 4 °C. The supernatant was collected and the precipitate was extracted again with 10 mL of 80% ethanol, under the conditions previously described. The two supernatants were combined and filtered using Whatman filter paper #1. The final ethanolic extract was stored at −20 °C to be used in the determination of total phenolic content (TPC), total flavonoids content (TFC), and antioxidant activity by DPPH and TEAC methods.

Table 1
Germination percentage of lettuce seeds treated with the elicitors in Experiment 1. Values are lsmeans (least square means, means estimators by the method of least squares).

Time of soaking (h)	Water (water control)	CHI 0.01%	CHI 0.05%	CHI 0.1%	CHI 0.5%	Lactic acid 0.7% (acid control)	TT 0.18%	TT 0.27%	TT 0.36%
3	99.4^{aA}	100.0^{aA}	99.4^{aA}	98.9^{aA}	98.9^{aA}	94.9^{bA}	99.4^{aA}	98.3^{aA}	98.9^{aA}
6	99.8aA	98.9^{aA}	100.0^{aA}	100.0^{aA}	99.3^{aA}	57.7^{dB}	97.7^{aA}	94.3^{bAB}	69.9^{cB}
12	97.7^{aA}	99.4^{aA}	95.5^{abAB}	99.4^{aA}	100.0^{aA}	17.9^{dC}	98.2^{aA}	91.5^{bB}	77.8^{cB}
18	93.5^{bB}	98.9^{aA}	91.5^{bB}	23.3^{cB}	8.5^{dB}	0.9^{eD}	98.3^{aA}	90.9^{bB}	15.9^{cC}
24	89.6^{bB}	30.1^{cB}	5.1^{efC}	10.8^{eC}	9.7^{eB}	0.6^{fD}	97.7^{aA}	90.3^{abB}	15.9^{deC}

Mean values with different letter within the same row are significantly different ($P < 0.05$). Mean values with different letter within the same column are significantly different ($P < 0.05$).

2.5. Total phenolic content

TPC was determined using the Folin–Ciocalteu Reagent (FCR) according to the methodology proposed by Singleton et al. (1999) with modifications. Extract samples (0.2 mL) were added to 1 mL of FCR (diluted 1/10). After 3 min of incubation at ambient temperature, 0.8 mL of Na_2CO_3 solution (7.5% w/v) was added and the reaction mixture was incubated for 2 h at the same temperature. The absorbance was measured at 765 nm and results were expressed as mg gallic acid equivalents (GAE) 100 g^{-1} of fresh weight (FW).

2.6. Total flavonoid content

TFC was determined based on the methods described by Zhishen et al. (1999), modified as required. Extract samples (0.2 mL) were mixed with 1.280 mL of deionized H_2O and 0.06 mL of $NaNO_2$ (5% w/v). After 5 min, 0.06 mL of $AlCl_3$ (10% w/v) was added and at 6 min, 0.4 mL of NaOH (1 M) was incorporated. The mixture was stirred, and absorbance was measured at 510 nm. TFC was expressed as mg of quercetin equivalents (QE) 100 g^{-1} of FW.

2.7. DPPH assay

Antioxidant activity using the DPPH radical was determined based on the methodology adjusted by Viacava et al. (2015b). 0.25 mL of sample extract or ethanol (blank) was mixed with 1 mL of an ethanolic DPPH solution (100 μM). The mixtures were shaken immediately and allowed to stand at refrigeration temperature (2 °C) in the dark. The decrease in absorbance at 517 nm was measured after 60 min. DPPH radical scavenging activity was expressed as mg of ascorbic acid equivalents (AAE) 100 g^{-1} of FW.

2.8. TEAC assay

TEAC value was determined according to Re et al. (1999). $ABTS^{\bullet+}$ cation was generated through the interaction of 19.2 mg of ABTS (2,2′-azinobis-(3-ethylbenzotriazoline-6-sulfonic acid)), dissolved in 5 mL of distilled water and 0.088 mL of potassium persulfate ($K_2S_2O_8$; 3.78% w/v). It was incubated in the dark at room temperature for 16 h; then 1 mL of ABTS activated radical was taken and 88 mL of ethanol were added. The radical was adjusted at an absorbance of 0.70 ± 0.02 at 754 nm. The reaction was initiated adding 1 mL of $ABTS^{\bullet+}$ and 50 μL of the extract and absorbance was monitored at 754 nm after 10 min. The percentage of inhibition was calculated and the results were expressed as mg of trolox equivalents (TE) 100 g^{-1} of FW.

2.9. Statistical analysis

Statistical ANOVA ($P < 0.05$) analysis was performed using R, software version 2.12 (R Development Core Team, 2011) and multivariate analysis was assessed using PC-ORD software. Statistical ANOVA was carried out to compare the germination percentage values among treated samples and differences were determined by the Tukey–Kramer multiple comparisons test ($P < 0.05$). PCA and HCA were applied to the data set after standardization using PC-ORD software. Standardization was necessary because of the different magnitudes of the variables; after standardization, each parameter contributes equally to the data set variance and carried equal weight in the analysis. The Ward's method as the amalgamation rule and the squared Euclidean distance as metric were used to establish clusters. The number of dimensions to keep for data analysis in PCA was evaluated by the respective eigenvalues (which should be greater than one) and by the total percentage of variance (that should be as higher as possible) explained by the number of principal components (PC) selected. This allows meaningful interpretations, to ensure their reliability (McCune and Mefford, 2011). Such multivariate analysis were carried out to evaluate the influence of tested parameters in the classification and differentiation of lettuce sprout samples according to the treatment applied. Each treatment was performed by duplicate and three independent runs were carried out. Values were expressed as means ± standard deviations.

3. Results and discussions

3.1. Phytochemical content of lettuce sprouts

Major phytochemicals in lettuce are phenolic compounds, including phenolic acids and flavonoids (Viacava et al., 2014). Therefore, phytochemical content of lettuce sprouts was evaluated through total phenolic content and total flavonoid content indices. Besides, two in vitro assays (DPPH and TEAC) were used as complementary methods to evaluate the antioxidant activity of the lettuce sprouts since it is known that no single assay accurately reflects the mechanism of action of all antioxidants present in the plant (Prior et al., 2005).

Untreated lettuce sprouts (grown under control conditions) presented total phenolic content of 67.9 ± 2.8 mg GAE 100 g^{-1} FW. These amounts were 2–14 times higher than those found in mature heads of different green lettuce cultivars (López et al., 2014; Oh et al., 2009; Ozgen and Sekerci, 2011; Viacava et al., 2014). Moreover, they were similar than those found in *Brassica* sprouts (i.e., broccoli and radish sprouts), which are known to be an excellent dietary source of phenolic compounds (Singh et al., 2007; Yuan et al., 2010).

Results of total flavonoid content of untreated lettuce sprouts (31.3 ± 1.8 mg QE 100 g^{-1} FW) were 2–28 times higher than those reported in methanolic extracts of different green lettuce cultivars (Crozier et al., 1997). However, they were a few lower than those informed by Pérez-Balibrea et al. (2011a) in broccoli sprouts of three different cultivars.

The radical scavenging activity measured with the DPPH assay (96.0 ± 2.6 mg AAE 100 g^{-1} FW) was considerably higher than that reported by Khanam et al. (2012) for lettuce (11.56 μg AAE g^{-1} dry weight which is approximately equivalent to 0.06 mg AAE 100 g^{-1} FW). However antioxidant capacity values measured with the TEAC technique (68.6 ± 1.5 mg TE 100 g^{-1} FW) were in the range of those reported by different authors for different cultivars of green lettuce

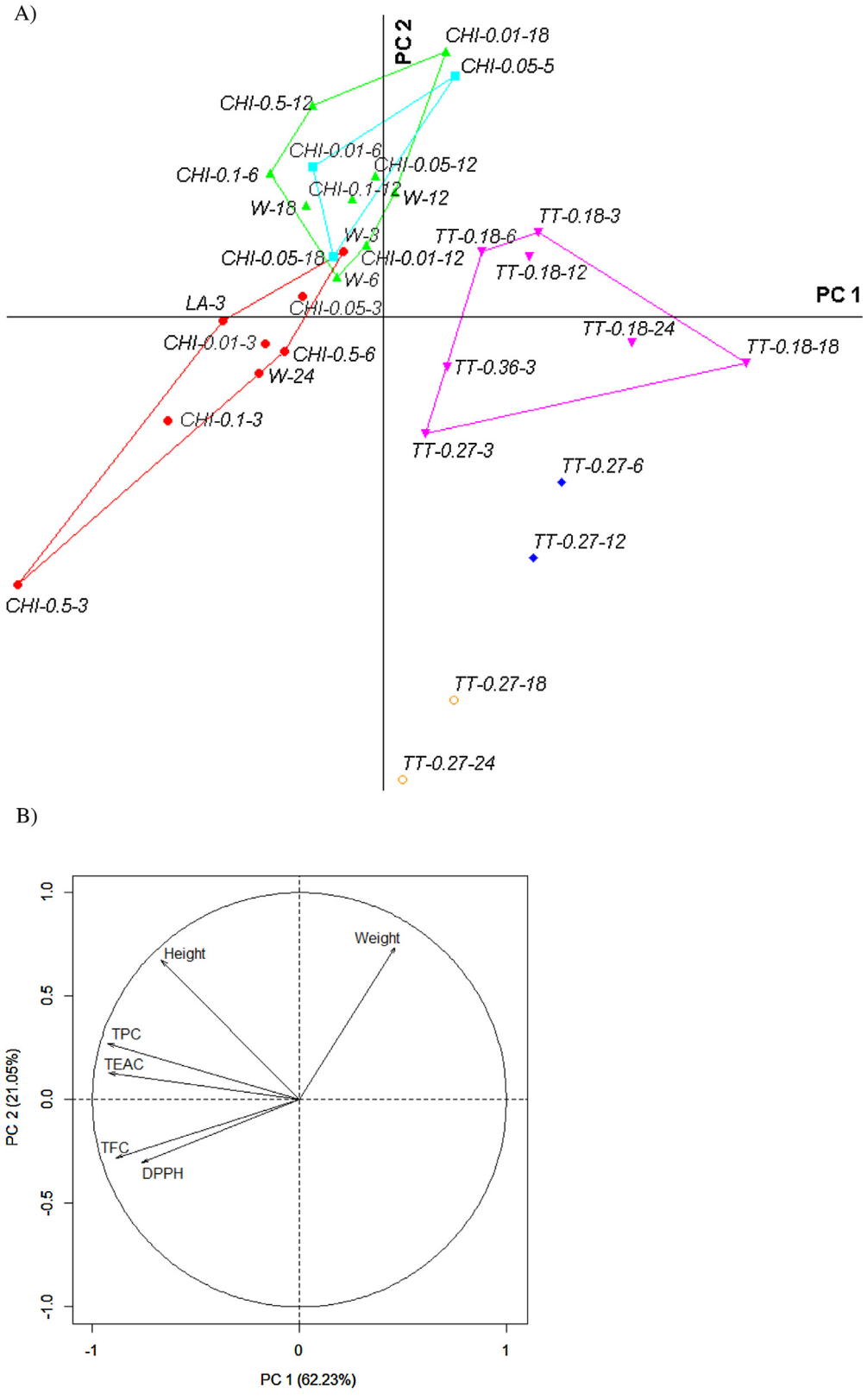

Fig. 1. PCA and HCA score plot (A) and loading plot (B) generated for the first two dimensions in Experiment 1.

(Cano and Arnao, 2005; Llorach et al., 2008; Ozgen and Sekerci, 2011).

Differences in the concentration of phytochemicals and antioxidant activity among plants depend upon genetics, development stage, environmental conditions, sample treatment, extraction process and quantification method (Pérez-Balibrea et al., 2011a; Viacava et al., 2015a). However, according to our results, lettuce sprouts are a good source of phenolics and antioxidants, being richer than mature lettuce heads in these health-promoting compounds.

Table 2
Germination percentage of lettuce seeds sprayed with elicitors (Experiment 2). Values are lsmeans (least square means, means estimators by the method of least squares).

Treatment	Germination (%)
Water (water control)	93.6^{A}
CHI 0.25%	90.0^{AB}
CHI 0.5%	88.8^{AB}
CHI 1%	86.9^{B}
Lactic acid 0.7% (acid control)	0.0^{C}
TT 0.18%	92.5^{AB}
TT 0.27%	90.0^{AB}
TT 0.36%	84.6^{B}

Mean values with different letter within the same column are significantly different ($P < 0.05$).

3.2. Experiment 1

Table 1 shows the germination percentage when lettuce seeds were soaked in the elicitor solutions during different times (Experiment 1). Some treatments evidenced a clear inhibitory effect compared to control samples. In general, it was observed a negative correlation between the germination percentage and the concentration of elicitors or the contact time. Higher chitosan and tea tree concentrations reduced the germination percentage. In the same way and for almost all treatments, the germination percentage was significantly reduced as contact time increased, probably due to higher amount of damage on the cell coat of the seeds. These results were in concordance with those of Goñi et al. (2013a) who also found that increasing chitosan concentration to 2.0% w/v decreased the germination percentage (10–20%) of lettuce seeds imbibed for 5, 10, 15, and 20 min. The authors also informed that longer contact times, significantly reduced germination. The lactic acid solvent (acid control) presented a higher adverse effect on lettuce seeds germination when compared with chitosan-dissolved solutions. This may be a result of the acidity of the lactic acid solvent. Germination is a complex process, tightly regulated by hormones and biochemical processes (Matilla and Matilla-Vazquez, 2008), and dependent on the active transport of substances through the seed coat. Given that many biochemical processes are regulated by enzymes and it is well known that enzymatic activity is strongly affected by pH, germination inhibition might be expected in the presence of acid solutions (Goñi et al., 2013a). When chitosan is dissolved in the acid, an acid-base reaction may take place, which neutralizes the negative effect of the acid. An effective seed elicitor treatment must enhance phytochemical content, while preserve seed viability, germination, and vigor. In the present study, the high reduction in the germination percentage obtained with some treated seeds, makes the proposed treatments highly uneconomical. Therefore, selection of the most suitable treatments was focused on finding those with a germination percentage above 80%. Then, the next treatments were consider unsuitable for continuing with the experiment and were discarded: CHI-0.01% and 0.05% soaked for 24 h; CHI-0.1% and 0.5% soaked for 18 and 24 h; LA soaked for 6, 12, 18 and 24 h; TT-0.36% soaked for 6, 12, 18, and 24 h.

Antioxidant activity, TPC, TFC, and yield parameters of lettuce sprouts obtained from Experiment 1 and adequately germinated were compared based on PCA and HCA analysis. These statistical techniques were used to gain an overview on the interrelationships among TPC, TFC, antioxidant activities, and yield parameters of lettuce sprouts, and to understand the similarities and differences between treated samples. The PCA score plot generated for the first two dimensions is shown in Fig. 1A, and the loading plot (Fig. 1B) illustrated the relationship between phytochemical content, antioxidant activity, and yield parameters. Axis 1 (PC1) represented the bioactive content of lettuce sprouts, and explained 62% of the total variance in the data set and axis 2 (PC2) expressed changes in growth parameters and explained 21%. From these results, the soaking of the lettuce seeds in the different solutions had more impact on the phytochemical content of lettuce sprouts with respect to yield parameters. The left half location of the lettuce sprouts treated with chitosan solutions, except CHI-0.01-18 and CHI-0.05-6, may be explained by their high content of phenolic compounds and antioxidant activity measured with the DPPH assay, variables that are co-located in this region of the PC space. Instead, lettuce sprouts treated with TT solutions were situated on the right side of the PC1.

Through HCA analysis, samples were divided into six groups (Fig. 1A): Group 1 – contained samples TT-0.27-6 and TT-0.27-12 with a low content of total phenolics (mean values of 52.01 mg GAE 100 g^{-1} FW) and antioxidant activity measured by DPPH method (84.90 mg AAE 100 g^{-1} FW), Group 2 – represented for lettuce sprouts treated with TT-0.27-18 and TT-0.27-24 was located at one end of the axis 2, whose bioactive content not differ much from water controls but whose height was significantly lower (33% less in average); Group 3 – conformed by sprouts treated with CHI-0.01-6, CHI-0.05-6, and CHI-0.05-18 with only lower values of DPPH radical scavenging activity (88.15 mg AAE 100 g^{-1} FW in average); Group 4 – represented for samples treated with CHI-0.01-3, CHI-0.05-3, CHI-0.1-3, CHI-0.5-3, CHI-0.5-6, LA-3, W-3, and W-24; CHI-0.1-3 and CHI-0.5-3 were located to the bottom left of this group due to their higher flavonoid content (37.57 mg QE 100 g^{-1} FW) and lower weight values (9% less in average); Group 5 – represented for sprouts treated with CHI-0.01-12, CHI-0.01-18, CHI-0.05-12, CHI-0.1-6, CHI-0.1-12, and CHI-0.5-12, that not significantly differed to water controls soaked for 6, 12 and 18 h, i.e., treatments not affected the samples; and finally Group 6 conformed by samples TT-0.18-3, TT-0.18-6, TT-0.18-12, TT-0.18-18, TT-0.18-24, TT-0.27-3 and TT-0.36-3, with similar behavior than samples of Group 1 but higher weight values (1.36 g/50 seeds).

Despite no reports about the effect of TT application to seeds from different plants are available, the effect of chitosan on the phytochemical content and germination growth of several plant sprouts (broccoli, soybean, sunflower) has been previously studied. However, results from these studies are controversy and highly dependent on chitosan concentration and type of seed plant assayed. For instance, Barrientos Carvacho et al. (2014) found that soaking broccoli seeds in chitosan 90 μmol L^{-1} for 4 h reduced the antioxidant capacity of the sprouts, but Cho et al. (2008) reported that sunflower seeds soaked in chitosan 0.5% for 18 h slightly improved the DPPH radical scavenging activity of sprouts. Chitosan has also been previously reported as stimulating the growth and yield of soybean and sunflower sprouts (Cho et al., 2008; No et al., 2003), although the height of the 11-day old broccoli sprouts was not influenced after application of chitosan 10 and 90 μmol L^{-1} (Barrientos Carvacho et al., 2014).

Based on the premise that the treatments selected must not affect lettuce growth parameters as well as maximize phytochemical content, the following treatments were selected as suitable to be applied to lettuce seeds: CHI-0.01-3, CHI-0.05-3, CHI-0.1-3, CHI-0.1-6, CHI-0.5-3, CHI-0.5-6, CHI-0.5-12, LA-3.

From these results, it is possible to observe that TT essential oil did not improve the phytochemical content and antioxidant activity of the sprouts, regardless of the concentration and time of soaking used. Therefore, TT did not act as elicitor under conditions of Experiment 1. With respect to soaking time, in general, lower soaking times accomplished the objective of enhancing the phytochemical content of lettuce sprouts. Longer soaking times may increase the damage in the cell coat of the seeds and thus produced an increase leaching of bioactive compounds to the soaking solution. Therefore, any improvement in the phytochemical content

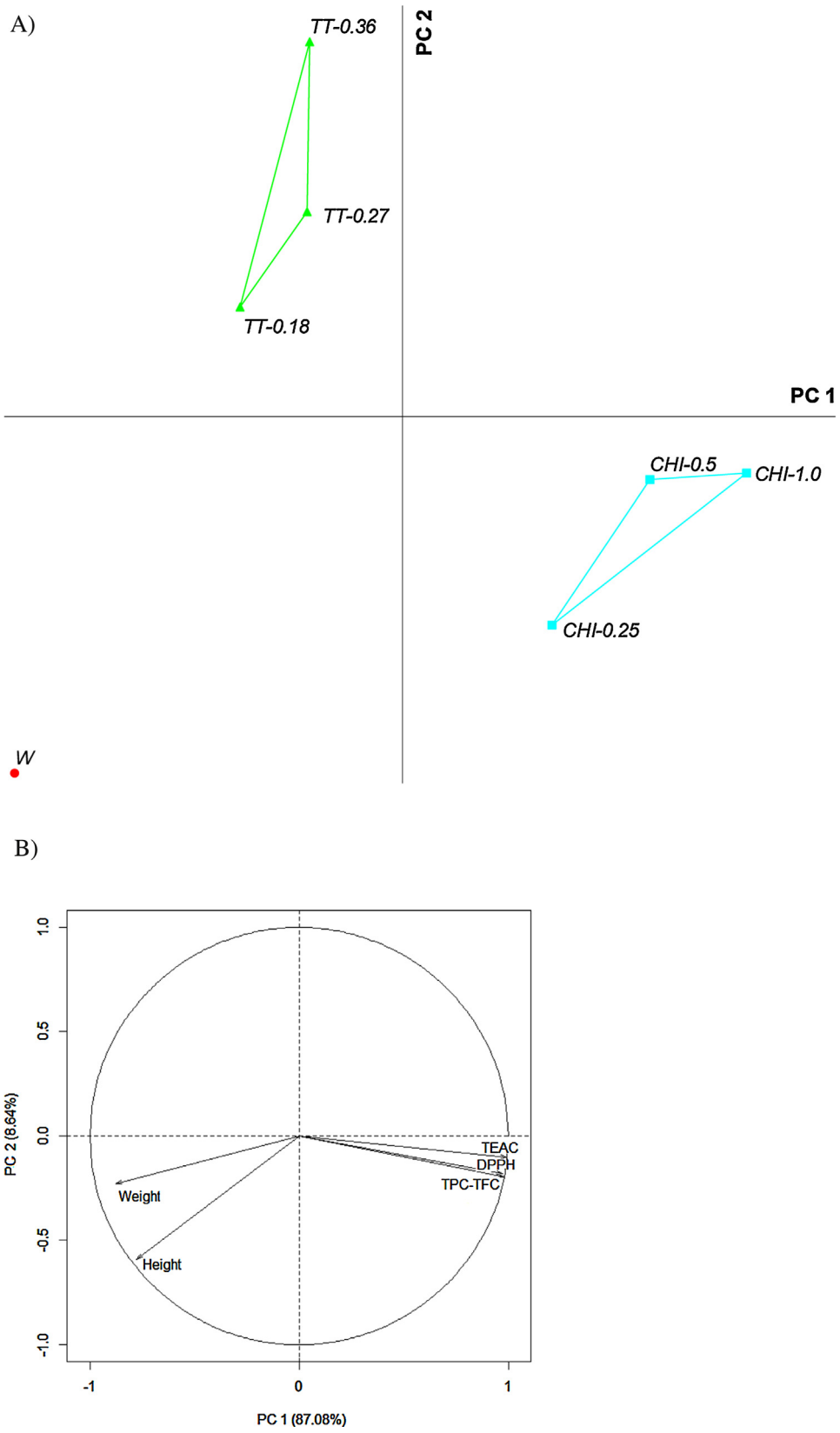

Fig. 2. PCA and HCA score plot (A) and loading plot (B) generated for the first two dimensions in Experiment 2.

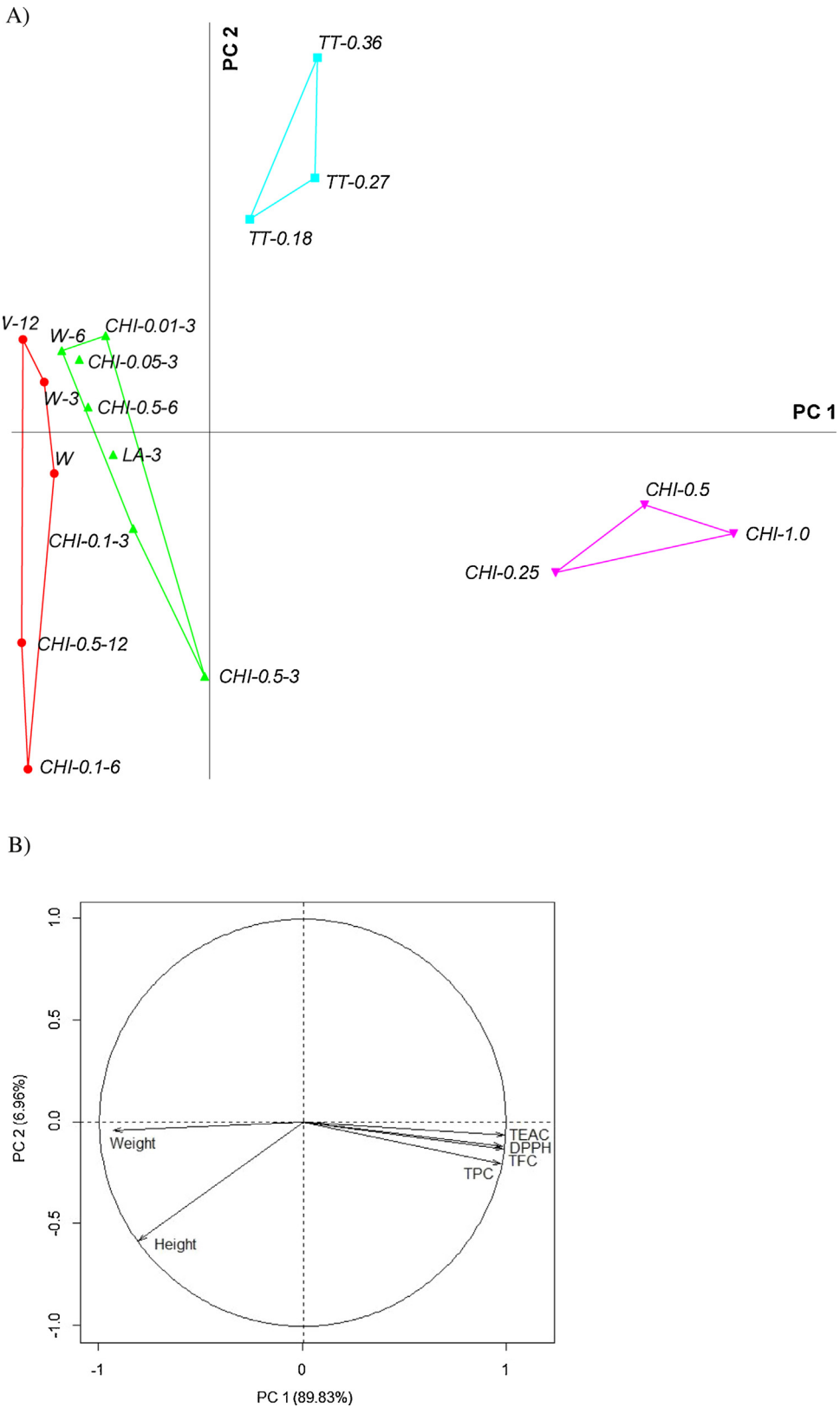

Fig. 3. PCA and HCA score plot (A) and loading plot (B) generated for the first two dimensions for best treatments of Experiments 1 and 2.

due to the elicitors could get diluted when using longer soaking times.

3.3. Experiment 2

Table 2 shows the germination percentage when lettuce seeds were sprayed with the natural elicitors (Experiment 2). The dairy application of lactic acid completely inhibited the germination of the lettuce sprouts. This result is similar to that obtained in Experiment 1 with 18 or 24 h of soaking with the acid. Additionally, similar to Experiment 1, the germination percentage was negative correlated with CHI and TT concentrations. Nevertheless, both elicitors and at all the concentrations assayed allowed lettuce seeds germination. Therefore, the application of LA as sprayed solution was the only treatment consider as unsuitable for continuing with the experiment and was discarded. The PCA score and loading plots generated using the data of the second experiment for the first two dimensions are shown in Fig. 2A and B. Axis 1 (PC1), which corresponded mainly to bioactive content variations, explained 87% of the total variance in the data set and axis 2 (PC2), which corresponded to variations in lettuce sprouts height, explained only 9%. It is noted that antioxidant properties and phenolic and flavonoid contents were highly correlated with each other. Previous studies have also shown that TPC generally correlates with antioxidant techniques such as TEAC and DPPH (Tabart et al., 2007; Viacava et al., 2014). As we observed in the previous experiment, lettuce sprouts treated with CHI and TT solutions were located on opposite sides of PC1. This may be a result of the higher values of bioactive compounds and antioxidant activity found again for chitosan-treated samples. From HCA analysis, the samples were divided into three groups: Group 1 – represented for samples treated with TT 0.18, 0.27, and 0.36%, with higher bioactive content (13% more TPC and 19% more TFC) and antioxidant activity (15% and 20% measured with DPPH and TEAC assays, respectively) than water control but lower values of height (16% less); Group 2 – represented for seeds treated with CHI 0.25, 0.5 and 1%, with the highest content of bioactive compounds (99.61 mg GAE 100 g^{-1} FW and 56.14 mg QE 100 g^{-1} FW) and antioxidant activity (136.81 mg AAE 100 g^{-1} FW and 103.30 mg TE 100 g^{-1} FW measured with DPPH and TEAC assays, respectively) but also the lowest values of weight and height (0.87 g/50 seeds and 29.85 mm, respectively); and Group 3 – represented for control sample (W). In this experiment, the difference between the solutions applied was clear since almost all lettuce sprouts treated with the same elicitor solution formed a group which was different from another.

Some studies reported the effects of exogenous spraying of chitosan on the phytochemical content of broccoli sprouts. Similar with our findings, Moreno et al. (2008) found significant increases in the flavonoid content of broccoli inflorescences by 52% after the application of 0.1% chitosan. In contrast, Pérez-Balibrea et al. (2011b) reported that the TPC of broccoli sprouts was not influenced by foliar fertilization with chitosan 0.01%; although in that experiment higher concentrations of chitosan could not be evaluated since they avoided the germination of the broccoli seeds.

From the results obtained in the second experiment, treatments from Groups 1 and 2 were selected for better performance in terms of enhancing the phytochemical content with respect to control samples. However, some of these treatments also reduced the weight of the sprouts and the effects were greater when higher concentrations were applied.

3.4. Comparison of both experiments

In order to analyze the efficiency of all the applied treatments, the selected best treatments (8 in Experiment 1 and 6 in Experiment 2) were analyzed together (PCA and HCA analysis) in conjunction with their corresponding controls to determine the best ones. Fig. 3 shows the PCA and HCA score plot (A) and the loading plot (B) generated for the first two dimensions. Samples were mainly located along axis 1 (Fig. 3A), which explained 90% of the variation, while axis 2, strongly affected by height values (Fig. 3B), explained 7%. Not surprisingly, TPC, TFC and antioxidant activity (DPPH and TEAC assays) were closely loaded on PC1 (Fig. 3B), indicating again a strong correlation among them. Weight of sprouts was found in opposition to them. Pérez-Balibrea et al. (2011b) also found that treatment of broccoli seeds with elicitors (including chitosan) decreases the fresh weight of sprouts even though it stimulate the biosynthesis of vitamin C and secondary metabolites. Through HCA, 4 groups were conformed. Group 1 was composed by TT-0.18, TT-0.27 and TT-0.36. Group 2, formed by CHI-0.25, CHI-0.5, and CHI-1.0, was located on the right side of the axis 2 and presented the highest content of bioactive compounds and antioxidant activity. The other two groups were located on the left side of the axis 2. Group 3 was formed by CHI-0.01-3, CHI-0.05-3, CHI-0.1-3, CHI-0.5-3, CHI-0.5-6, LA-3 and water control W-6. Group 4, composed by CHI-0.1-6, CHI-0.5-12 and water controls imbibed for 3 and 12 h and applied as spray, presented the lowest values of phenolics, flavonoids and antioxidant activity but also the greatest values of height and weight.

When comparing all the best treatments, only sprouts from Experiment 2 presented a significant higher content of phytochemicals and antioxidant activity than the others. Moreover, this result was more evident with chitosan-sprayed lettuce sprouts, which presented increments of TPC, TFC, DPPH and TEAC antioxidant activity in the ranges of 32–48%, 44–79%, 24–43% and 26–50%, respectively, with respect to the other groups. Therefore, treatments corresponding to Groups 1 and 2 (TT-0.18, TT-0.27, TT-0.36, CHI-0.25, CHI-0.5, CHI-1.0) were chosen as those with the greatest potential to trigger the biosynthesis of bioactive phytochemicals of lettuce sprouts.

The results presented here do not provide evidences on the mechanisms involved in phytochemicals biosynthesis due to elicitors treatments. However, based on literature, several physiological changes could explain its beneficial effects. 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. Khan et al. (2003) demonstrated that 100 mmol L^{-1} chitosan produced a significant increase of the PAL and TAL activities in soybean plants. This increase was strongly correlated with an increase of total polyphenols content. As a consequence, the increase in TPC, TFC as well as antioxidant activity detected in our work when tea tree and mainly chitosan were applied can probably be attributed to the induction of PAL.

4. Conclusion

This investigation demonstrated that lettuce sprouts are a good source of phenolics and antioxidant compounds. Application of principal component and hierarchical cluster analysis to select natural elicitors for enhancing phytochemical content and antioxidant activity of lettuce sprouts was also reported. Considerable variations were observed between samples in terms of antioxidant activity, total phenolics and flavonoids content, and yield parameters. Unsupervised pattern recognition techniques enabled visualization of this complex data set and underlaying relationships responsible for clustering observed.

Exogenous application of selected elicitors can stimulate the biosynthesis of secondary metabolites, leading to accumulation of phenolics and flavonoids with antioxidant activity and improv-

ing the phytochemical and nutritional value of lettuce sprouts. However, more investigations are needed, using different application protocols and different germination conditions, in order to maximize simultaneously germination percentage, and yield and growth of sprouts.

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