Use of microbial inoculants during cultivation maintain the physiological, nutritional and technological quality of fresh-cut romaine lettuce

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Abstract

Nutrition-sensitive agriculture is a novel concept in the agri-food system, which considers the implementation of techniques able to guarantee the nutritional value of the produce, the sustainability of the production and, at the same time, to reduce the ecological impact of agricultural practices. These principles can be also introduced in the fresh-cut market with the aim of maintaining the produce quality during shelf life. In this context, the use of bio-based products is rapidly increasing for improving economic and environmental sustainability of cropping systems during cultivation and shelf life. The aim of this work was to evaluate the effects of three different bacterial-based formulations (*Paenibacillus pasadenensis, Bacillus amyloliquefaciens, Pseudomonas syringae*) applied during romaine lettuce cultivation by monitoring the changes of several quality indexes at

harvest and during storage. Results showed that the application of microbial inoculants during romaine lettuce cultivation contributed to the maintenance of nutritional, functional and perceived quality attributes of leaves during shelf life. The microbial inoculants prevented the development of postharvest fungal pathogen *B. cinerea*. Moreover, the study evidenced different modes of action of the different inoculants and, in the case of *Pseudomonas syringae* 260-02 application, a direct involvement of ascorbic acid-mediated antioxidant mechanisms was observed.

Keywords: Antioxidants, Bio-based products, Botrytis cinerea, Leafy vegetables, Nutrition-sensitive agriculture, Postharvest quality

1. Introduction

The fresh-cut market has been constantly increasing in importance and economic relevance ~~interested by a significant increment~~ in the recent years. Within this sector, salads are among the most important vegetables used and appreciated by the consumers. The growing demand for fresh and high quality minimally processed products and the awareness of consumers towards the rise of environmental issues, are pushing toward the adoption of novel agronomical and technological practices aiming to preserve both the product quality and the environment sustainability (Shabbir et al., 2019).

The list of banned pesticides is constantly being updated, including more and more molecules, ~~increasing~~ and this phenomenon is driving the research effort towards ~~pushing the to~~ finding new effective and reliable alternatives. In this context, the adoption of nutrition-sensitive agriculture (NSA) is of particular~~ly~~ importance. NSA is a novel concept in the agri-food system, which considers the implementation of techniques able to guarantee the nutritional value of the produce, the sustainability of the production and, at the same time, to reduce the environmental impact of agricultural practices (Shetty, 2018). Fresh-cut salads production pipeline is characterized by a sequence of mild operations (including washing, cutting, drying, packaging, and storage) which, on a physiological point of view, represent a stress for the leaf tissue. In fact, it is important to point out that at this stage, leaves are composed by living cells and that most of

the plant metabolic processes are still ongoing. Thus, the quality of fresh product at harvest must be the highest possible and it should be maintained, if possible, in all the production phases. The quality of leafy vegetables is the sum of various indices, including sensorial and textural properties and nutritional value (including the presence of health-related compounds, particularly appreciated by the consumers) (Tudela and Gil., 2020). The loss of quality during postharvest can be due to enzymatic phenomena and/or by the proliferation of saprophytic and pathogenic bacteria or fungi, which rapidly lead to the total loss of product marketability (Lugtenberg et al., 2017).

This is particularly true when speaking of a major post-harvest pathogen for lettuce: *Botrytis cinerea*. While it is known that this pathogen can cause crown and bottom rot in salads pre-harvest (Sowley et al., 2010, Chatzidimopoulos and Pappas, 2019; Sanogo et al., 2019) this necrotrophic pathogen is most devastating for the grey mold it causes on leaves in post-harvest conditions (Van Kan, 2005; Shim et al., 2013; Barrière et al., 2014)

The use of bio-based products in agriculture is rapidly ~~increasing~~growing in order to~~for improving~~ increase the economic and environmental sustainability of cropping systems. These novel agronomic tools can enhance plant productivity, produce quality and tolerance to abiotic stresses. Biostimulants composition can include bacteria, fungi, seaweeds, higher plant extracts, animals, and humate-containing raw materials (Yankhin et al., 2017). From a regulatory point of view, products that act against abiotic stresses can be classified as biostimulants, while if they are active against biotic stresses must be classified as biocontrol agents even if both can share the same bioactive compounds. Biostimulants and biocontrol agents are characterized by a low environmental impact, especially in comparison to synthetic fertilizers. Scientific efforts have been dedicated in the last years in the identification of the mode and mechanism of action of various bio-based products to be used in agriculture (including biostimulants and biocontrol agents). It has been demonstrated that the application of bio-based products could improve the agronomical performance of crops, and at the same time it could also enhance the nutritional value of produce, by stimulating the biosynthesis and the accumulation of bioactive molecules (such as phenolic compounds) and vitamins (such as ascorbic acid, or carotenoids) (Cocetta and Ferrante, 2020). While several studies have been conducted

evaluating the effect of these products during cultivation and at harvest, few experiments have been performed to assess the persistency of the positive effects of in-field biostimulants application during postharvest. Also, there is no clue regarding the effect on postharvest quality deriving from the field application of potential biocontrol agents.

For this reason, the aim of this work was to evaluate the effects of three different bacterial-based biostimulant formulations applied during romaine lettuce cultivation by monitoring the changes of several quality indexes at harvest and during storage. These bacteria (*Paenibacillus pasadenensis* strain R16, *Bacillus amyloliquefaciens* strain CC2, and *Pseudomonas syringae* strain 260-02) have been previously used as biocontrol agents against soilborne pathogens of lettuce *Rhizoctonia solani* and *Pythium ultimum*, showing a reduced severity of the symptoms induced by these pathogens on treated lettuce plants, while having no negative effects on the quality of the produce at time of harvest or altering the microbial diversity of bulk soil (Passera et al., 2020). The hypothesis of the work was that the three biostimulants could increase the product quality with positive effects also during the shelf life. Lettuce has been chosen as the most representative and widely used crop in the fresh-cut salads industry (Tudela and Gil, 2020). The parameters considered for the experimental evaluations included those related to the physiological responses of leaves (chlorophyll content, chlorophyll *a* fluorescence, lipid peroxidation, electrolyte leakage and leaf relative water content), the nutritional value (phenolic compounds, carotenoids, vitamin C and antioxidant capacity) and the technological quality (texture and color). Moreover, the potential biocontrol effect of inoculants was assessed by measuring the damage caused by a typical postharvest fungal pathogen (*Botrytis cinerea*).

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2. Material and methods

2.1. Microbial strains

Three bacterial strains were used as candidate biocontrol and postharvest quality-promoting agent. The applied bacterial strains were: *Paenibacillus pasadenensis* strain R16, ~~which has been already described as a potential antifungal agent in Passera et al., 2017 and had some of its mechanisms investigated by genome analysis in Passera et al., 2018;~~ *Pseudomonas syringae* strain 260-02, ~~which has already been described as~~

~~plant growth promoting bacteria and potential biocontrol agent on Solanaceae plant in Passera et al., 2019;~~ and *Bacillus amyloliquefaciens* strain CC2. Strain R16 has been isolated from grapevine leaf in 2009 (Bulgari et al., 2011), and has been characterized as an antifungal agent *in vitro* against *Botrytis cinerea* and *Phomopsis viticola* (Passera et al., 2017), and *in vivo* against *B. cinerea*, *Rhizoctonia solani*, and *Pythium ultimum* (Passera et al., 2020); based on the obtained results and the characterization of the genome (Passera et al., 2018), the main modes of action of this strain seem to be the production of chitinase, antifungal volatile organic compounds, and indirect effects that strengthen plant defenses. Strain 260-02 has been isolated from roots of apple trees in 2012, and has been characterized as an *in vitro* antifungal agent against *B. cinerea*, and *in vivo* against *B. cinerea*, *R. solani*, and *P. ultimum* (Passera et al., 2019; Passera et al., 2020); based on the previously obtained results and the characterization of the genome, the main modes of action of this strain seem to be the production of toxins, siderophore, and antifungal compounds, as well as the activation of plant defense mechanisms, as suggested by effective biocontrol against also against a viral pathogen (Passera et al., 2020). Strain CC2 has been characterized as ~~an~~a potential ~~-~~*in vivo* antifungal agent against *R. solani* and *P. ultimum* (Passera et al., 2020). All these strains were successfully applied during romaine lettuce cultivation and their effectiveness in containing the damage caused by soilborne fungal pathogens has been demonstrated (Passera et al., 2020). All strains were cultivated on LB High Salt Agar plates (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, sodium chloride 10 g L^{-1}, agar 15 g L^{-1}) at 25 °C and were stored in a 20 % glycerol solution at -80 °C for long conservation periods.

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2.2 Plant material and Inoculum with bacterial strains

Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) seedlings were grown in 13 cm pots filled with commercial potting soil. Two weeks old seedlings were inoculated with the bacterial strains, which were administered as soil drenching. The treatments were: R16, 260-02, or CC2 (10^5 CFU mL^{-1} in Ringer's solution), while sterile Ringer's solution was used as non-treated control (NT). Seven plants were used for each treatment. The plants were grown in an experimental greenhouse under monitored conditions (25 ± 3 °C, 14 h photoperiod) and were harvested after three weeks from transplant.

2.3. Botrytis cinerea inoculation

Additional lettuce plants, 10 per treatment, were prepared as detailed in paragraph 2.2. These plants were used for inoculation of the postharvest foliar pathogen *Botrytis cinerea*, strain MG53 (which will be identified as BC for the rest of the study). The inoculum of BC was composed by a conidia suspension (10^5 conidia mL^{-1}) obtained by adding sterile water and scraping the surface of well-developed BC mycelium, grown on PDA medium for ten days. The suspension was filtered on double-layer sterile gauze to remove the mycelium fragments. The concentration and purity of the conidia suspension was assessed by visual analysis in optical microscopy (20X; Easylab CX40, Olimpus), evaluating five 10 µL drops per 5 mL batch of suspension in a Kova counting grid, and then diluted to the final concentration of 10^5 conidia mL^{-1}. The inoculum of BC conidia suspension was carried out five days after the inoculum with the bacterial strains and consisted of spraying 15 mL of conidia suspension on each plant, ensuring a homogeneous distribution of the droplets on the leaves. These BC-inoculated plants were kept in a different greenhouse from the non-BC-inoculated plants, albeit with the same conditions. The non-BC-inoculated plants were used as healthy control plants to compare the development of symptoms.

The development of symptoms was monitored during growth in greenhouse and in shelf life conditions, obtained by keeping the leaf material in high humidity conditions (95 %) and 24 °C of temperature for ten days. The leaf material collected at harvest was divided into two separate trials: i) excising from the leaves disks with a diameter of 2 cm, making three biological replicates of 10 disks each per treatment, stored on 1 % agar-water plates and ii) storing a whole leaf per plant per treatment in a humid chamber. For both types of samples, the symptoms were visually assessed and assigned to a symptom severity class ranging from 0 (asymptomatic material) to 7 (material showing 100 % of BC infection and sporulation) as previously reported (Vercesi et al., 2013). The symptom classes were then converted to an infection percentage index (I%I) using the formula presented by Townsend and Heuberger in 1953.

2.4. Fresh-cut management

Fully expanded lettuce leaves, both from plants that were not treated (NT) or inoculated with the bacterial strains (R16, 260-02, CC2), prepared as detailed in paragraph 2.2., were collected and immediately subjected to fresh-cut processing. Leaves were cut in three parts and randomized, rinsed for 3 min with tap water, washed with a chlorine solution (0.11 g L^{-1} active chlorine) for 3 min, rinsed with tap water for 3 min. Leaves were gently dried in a centrifuge and packed in sealed plastic (BOPP, 30 µm, O_2TR 1800 cm^3 m^{-2} d^{-1}, WVTR 6.0 g m^{-2} d^{-1}; Taghleef Industries, Italy) bags (about 80 g per each bag).

All the bags (three for each treatment per time) were stored at 8 °C for up to 8 d. All the analyses were performed at harvest as well as after 1, 3, 6 and 8 d of shelf life. Then aliquots of each bag for chemical test were stored at -80 °C till the analysis time.

2.5. Evaluation of physiological and stress-related properties

2.5.1. Chlorophyll a *fluorescence*

In vivo chlorophyll *a* fluorescence was measured at harvest as well as during shelf life on six leaves per treatment, using a hand-portable fluorometer (Handy PEA, Hansatech, Kings Lynn, UK). Chlorophyll *a* fluorescence was measured on dark adapted leaves, kept for 30 min at room temperature. Measurements were taken on the leaf surface (4 mm diameter) exposed to an excitation light intensity [ultra-bright red-light emitting devices (LEDs) with a peak at 650 nm] of 3000 µmol m^{-2} s^{-1} (600 Wm^{-2}) emitted by three diodes. Fluorescence detection was measured by fast response PIN photodiode with RG9 long pass filter (Technical manual, Hansatech, Kings Lynn, UK).

2.5.2. Total chlorophylls

Leaf discs (around 40 mg) were placed in pure methanol (99.9 %) and extracted overnight at 4 °C in the dark. Leaf pigments were assessed by measuring the absorbance readings at 665.2 and 652.4 nm and the chlorophyll concentration was calculated using Lichtenthaler's formula (Lichtenthaler, 1987).

2.5.3. Lipid peroxidation (TBARS)

Lipid peroxidation was estimated by measuring the thiobarbituric acid reactive substances (TBARS) (Heath and Packer, 1968). One gram of leaf tissue was homogenized in 5 mL of trichloroacetic acid (TCA) of 0.1 % w/v and centrifuged for 10 min at 2900 x g. The supernatant (1 mL) was mixed with 4 mL of 20 % (w/v) TCA, 25 µL of 0.5 % thiobarbituric acid (TBA), and distilled water. After mixing, the solution was heated at 95 °C for 30 min in a water bath and then cooled on ice. The absorbance at 600 nm was subtracted from the one at 532 nm (as an index of non-specific turbidity) and the concentration of TBARS were expressed as malondialdehyde (MDA) equivalents (µmol kg^{-1}) on fresh weight basis, calculated by using the Lambert-Beer law with an extinction coefficient (ε = 155 mM^{-1} cm^{-1}).

2.5.4. Electrolyte leakage

The electrolyte leakage was measured to estimate the integrity and permeability of cell membranes. Leaf disks (around 100 mg) were placed in distilled water and the electric conductivity (EC) of the solution was determined using a conductivity meter (FE30, Mettler Toledo, Shanghai, China). Then, the EC was determined on the same samples after freezing (−20 °C) and thawing. ~~electrolyte leakage.~~ Electrolyte leakage is expressed as percent total electrolytes (Kim et al., 2005).

2.5.5. Leaf water content (WC)

To measure the leaf water content (WC), a thermogravimetric analysis was carried out by using a Sanyo Gallenkamp OMT drying oven (Gallenkamp, Sanyo, UK). One gram of sample was weighed and heated at 105 °C until reaching a constant weight. Analyses were performed on 6 specimens (each consisting on 5 pooled replicates) per treatment, per storage time. The water content (H) was calculated as grams of water per 100 g of sample.

2.6. Evaluation of nutritional properties

2.6.1. Ascorbic and dehydroascorbic acid

For each treatment/time, three replicates of 2 g of leaf tissue were ground in pre-chilled mortar with liquid nitrogen and the powder was immediately added to 5 mL of 3 % metaphosphoric acid. The homogenate was then centrifuged at 25,000 x g for 15 min at 4 °C, and the supernatant immediately analyzed. L-ascorbic acid (AsA) was quantified by HPLC as previously described (Picchi et al., 2012). The oxidized form (dehydroascorbic acid, DHA) was determined by the "subtractive" method after measurement of the total ascorbate (AsA + DHA) content following reduction with 100 mM Tris-carboxyethyl phosphine (TCEP) in 0.1 M HCl. The reduction was carried out according to Wechtersbach and Cigić (2007). Reduced extracts were then diluted with 0.02 M orthophosphoric acid and immediately analyzed by HPLC. The analytical column was a 250 x 6 mm i.d., Inertsil ODS-3, maintained at 40 °C. The isocratic elution was performed using 0.02 M mobile phase orthophosphoric acid at a flow rate of 0.7 mL min^{-1}. Samples of 20 µl were injected and monitored at 254 nm. The identity of the AsA peak was confirmed by coelution with authentic standards and the concentration of AsA was calculated from the experimental peak area by analytical interpolation in a standard calibration curve (range 0.0025-0.02 g L^{-1} AsA).

2.6.2. Phenolic index and total anthocyanins

Phenolic compounds were extracted from leaves disks (around 50 mg) that were placed in 5 mL of acidified methanol (1 % HCl v/v) and maintained at 4 °C for 24 hours in the dark. The total phenolics content was expressed as phenolic index, calculated as the absorbance measured at 320 nm (Ke and Saltveit, 1989). Total anthocyanins were assayed from the same extracts by spectrophotometric readings at 535 nm and the concentration expressed as cyanidin-3-glucoside equivalents (g kg-1) was calculated using the extinction coefficient (ε) of 29,600 mM^{-1} cm^{-1} (Klein and Hagen, 1961).

2.6.3. Total carotenoids

Total carotenoids were extracted following the same procedure described for chlorophylls. For total carotenoids, spectrophotometrical readings were performed at and 470 nm and carotenoid concentrations were calculated using Lichtenthaler's formula (Lichtenthaler, 1987).

2.6.4. Antioxidant capacity (DPPH)

The antioxidant capacity was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical quenching method. For each treatment/time, three replicates of 2 g of leaf tissue were ground in pre-chilled mortar with liquid nitrogen and the powder was immediately added to 5 mL of 1:1 v/v mixture of ethanol and 0.06 N HCl. The homogenate was then centrifuged at 25,000 x g for 15 min at 4 °C, and the supernatant was used as extract. The DPPH quenching capacity was measured using electronic paramagnetic resonance (EPR) with a MiniScope MS200 Magnettech (Berlin, Germany) following the protocol detailed in Picchi et al. (2012). Data are presented as ascorbic acid equivalents (g kg^{-1}) on fresh weight basis.

2.7. Evaluation of instrumental sensory properties of leaves

2.7.1. Mechanical analysis

In order to evaluate the mechanical properties of leaves both from untreated and treated romaine lettuce plants along the shelf life measurement, and to obtain instrumental texture parameters, a mechanical bending test was performed with the TA.TX2 Stable Micro Systems Texture Analyzer (Stable Micro Systems, Godalming, UK) as reported in Roversi et al. (2016). A single leaf was fixed on an annulus-bounding fixture plate with a central testing area of 7 mm diameter. A round-ended stainless-steel plunger of 4 mm diameter was moved to the film surface at 10 mm s^{-1} constant speed until the probe passed through the specimen. During the test, the imposed mechanical loading develops a state of flexural stress deforming the leaf up to failure. A uniform one-dimensional stress distribution within the film thickness was assumed. Results of the mechanical test are expressed in force/distance coordinates. The mechanical-parameters taken from the curves, i.e. bending force applied to the leaf-film sample up to failure (g), the slope (kg m^{-1}) of the linear force/probe distance dependence , that provides a quantitative information on the consistency of the leaf material (consistency index); the work at break (m*kg) that is quantified through the area underlying the force/probe distance curve up to the sample breakage. The mechanical properties of salad leaves were evaluated at room temperature at harvest time. For each treatment combination and each storage time, 30

specimens were analyzed. From the recorded curves, mechanical discrete parameters were extracted by means of Texture Exponent Exceed TEE32 (Stable Micro Systems, Godalming, UK) software.

2.7.2. Color

For color measurement, 30 replicate samples were analyzed for each treatment and storage time. The CIELAB color rectangular coordinates L*, a*, b* (CIE, 1986; Pace et al., 2014) were determined by a reflectance spectrophotometer Minolta Chroma Meter II™ (Konica-Minolta, Tokio, Japan). Standard illuminant C was used as reference. The CIELAB colorimetric parameters were interpreted as follows: L* values indicate lightness read from 0 (black) to 100 (white). Positive a* value indicates the red color while the negative a* value represents the green color. Similarly, positive and negative b* values indicate the yellow and the blue colors, respectively.

2.8. Statistical analysis

All data were subjected to the analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. Statistics were performed using GraphPad Prism version 6 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

3. Results

3.1. Evaluation of eeffectiveness of microbial strains in controlling development of Botrytis cinerea

No visual symptoms caused by *Botrytis cinerea* (BC) were detected during crop cultivation. During the post-harvest shelf life monitoring, in both leaf disk and whole leaf condition, no symptoms were detected in non-BC-inoculated leaves. In contrast, leaves from plants that were inoculated with BC showed varying grade of rotting and molding, compatible with infection from BC. The results of this evaluation are reported in Figure 1.

The non-treated plants inoculated with BC showed high levels of infection in both assays, having an average value of infection percentage index (I%I) of 44 % and 90 % in leaf disk and whole leaf assay, respectively.

Plants that were treated with the bacterial inoculants all showed statistically significantly lower I%I values in both assays: CC2 had average I%I of 6 % and 42 %, 260-02 had average I%I of 12 % and 33 %, and R16 had average I%I of 11 % and 9 %, for leaf disk and whole leaf assay respectively.

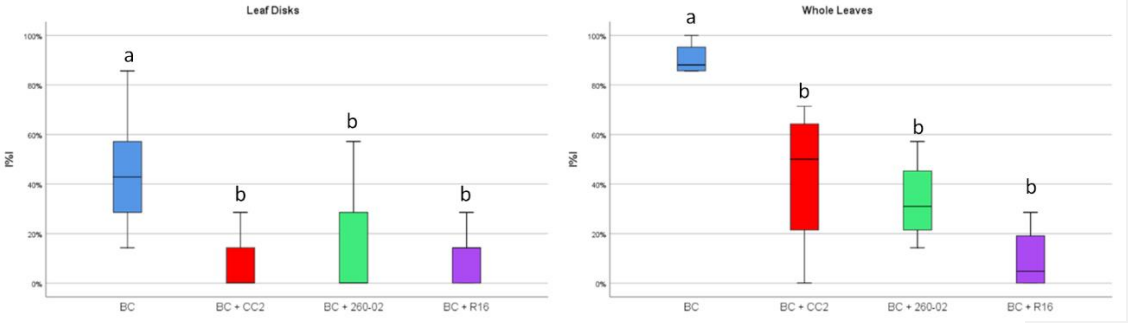

Figure 1. Results of *B. cinerea* inoculation trials. The graphs report on the X-axis the different treatments (plant inoculated with BC conidia and either non-treated[NT + BC], or treated with strains CC2 [CC2 + BC], 260-02 [260-02 + BC], and R16 [R16 + BC]), while the Y-axis reports the infection % index (I%I). Data are means ± SE. Different letters (a, b) indicate statistically significant differences in the results, according to a One-Way ANOVA followed by Bonferroni post-hoc test (P < 0.05).

3.2. Evaluation of physiological and stress-related parameters of fresh-cut lettuce

3.2.1. Chlorophyll a fluorescence and total chlorophylls

Two chlorophyll *a* fluorescence-related parameters were considered: the maximum quantum efficiency of the photosystem II (Fv/Fm) (Fig. 2 A) and the performance index (PI) (Fig. 2 B), which give a general indication of the physiological status of the leaf. In both cases there were no significant changes due to treatments with the only exceptions of 260-02 at d 1 that determined a slight decrement in Fv/Fm, and R16 which significantly increased the PI value at the same time point.

No significant changes were observed at any time point regarding the concentration of total chlorophylls (Fig. 2 C).

3.2.2. Lipid peroxidation (TBARS) and electrolyte leakage

307 In general, the leaves harvested from plants treated with 260-02 showed higher levels of TBARS. This
308 increment in the lipid peroxidation was already significant at harvest as well as after 8 d of storage (Fig. 2
309 D). Also, the same treatment determined a higher stress at membrane level after 1 d of storage, with a
310 significantly higher electrolyte leakage value. However, at the end of the storage period, all treatments
311 allowed a lower incidence of stress compared to untreated control plants (Fig. 2 E).
312
313 *3.2.3. Leaf water content (WC)*
314 No significant difference was recorded in treated and non-treated samples, which maintained a relative
315 humidity (RH) percentage between 93.80 % and 95.34 % throughout all the shelf life (Fig. 2 F). Constant
316 hydration of leaf tissues allows a correct evaluation of the modification of texture parameters of the
317 materials over the shelf-life time, since the lubrication of the structural components will not interfere with
318 the instrumental assessment of mechanical parameters.
319

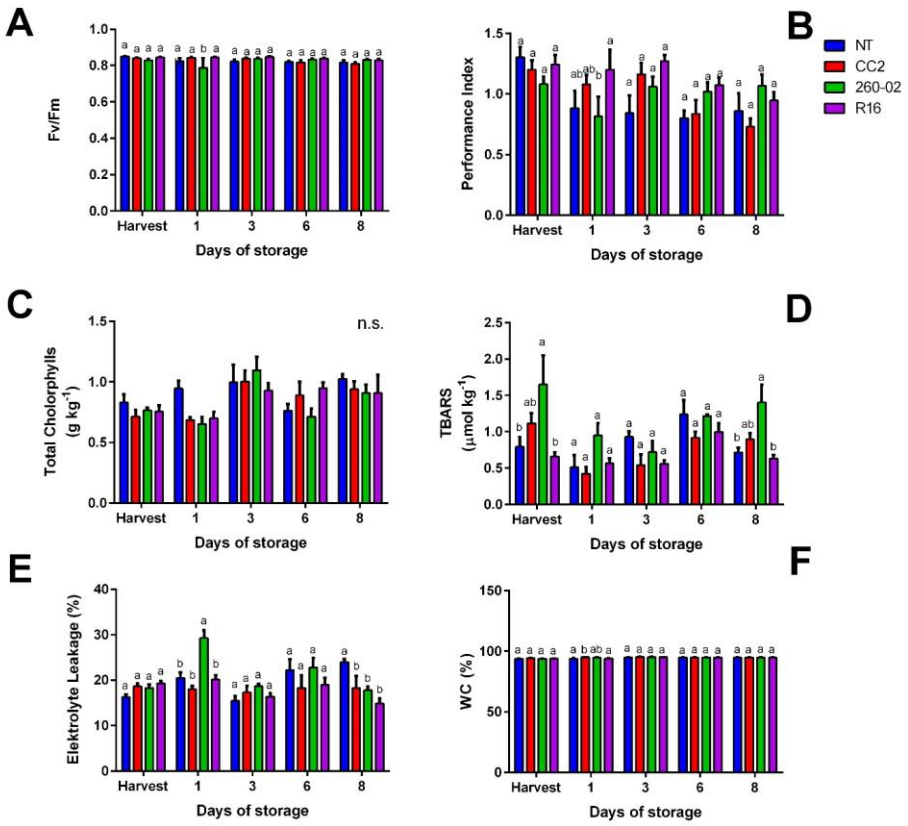

Figure 2. Physiological status of romaine lettuce leaves at harvest and during storage, as affected by different treatments. A: Quantum efficiency of the PSII (Fv/Fm); B: Performance index (PI); C: Total chlorophyll; D: Lipid peroxidation; E: Electrolyte leakage; F: Water content. Data are means ± SE. At each time point, different letters indicate statistically significant differences among treatments, according to a One-Way ANOVA followed by Bonferroni post-hoc test ($P < 0.05$).

3.3. Evaluation of nutritional properties of fresh-cut lettuce

Ascorbic acid (AsA) (Fig. 3 A) and total vitamin C (AsA + DHA) (Fig. 3 B) showed a significant decline during storage (statistics not shown) and in general, treatments allowed maintaining higher levels of both AsA and DHA. The positive effect of treatments was significant in case of AsA at harvest and after one day of storage. The maximum AsA content (0.081 g kg^{-1}) was registered in leaves from 260-02 treated

plants at harvest. The highest amount of AsA+DHA was recorded in leaves from R16-treated plants at the end of shelf-life.

No changes were found in the phenolic index, total anthocyanins, and total carotenoids (Fig. 3 C, D, E).

A marked increment in the antioxidant capacity was recorded at harvest, as a response to the application of 260-02 (Fig. 3 F), and after 1 d of shelf-life, 260-02-treated lettuce maintained significantly higher DPPH quenching capacity.

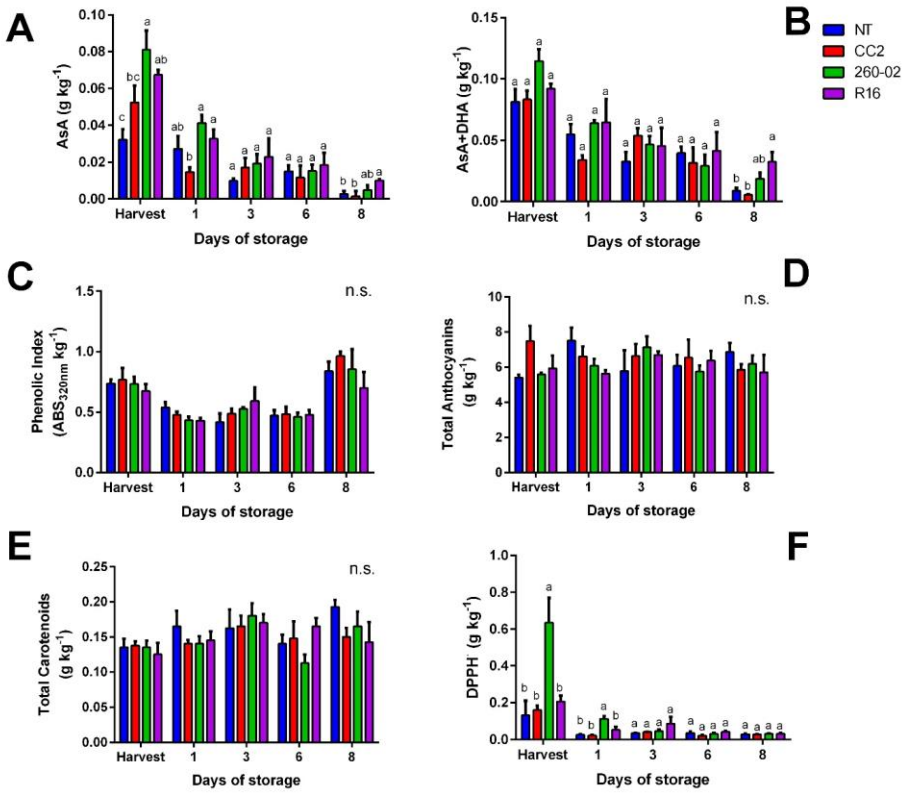

Figure 3. Nutritional properties of romaine lettuce leaves at harvest and during storage, as affected by different treatments. A: Ascorbic acid; B: Total vitamin C; C: Phenolic index; D: Total anthocyanins; E: Total carotenoids; F: Antioxidant capacity. Data are means ± SE. At each time

point, different letters indicate statistically significant differences among treatments, according to a One-Way ANOVA followed by Bonferroni post-hoc test (P < 0.05).

3.4. Evaluation of technological instrumental sensory properties of fresh-cut lettuce

3.4.1. Mechanical analysis

Leaves obtained from plants treated with the selected strains experienced a significant decrease of values for the three parameters at d 1. Leaves from lettuce plants treated with strains CC2 and 260-02 showed a slight but statistically significant decrement in mechanical properties which are expressed in terms of consistency index (71.01±2.01 kg m^{-1} and 65.74±1.71 kg m^{-1}, respectively) (Fig. 4 A) but not force at break, at d 1, compared to the other treatments, and that were recovered during the shelf life . Indeed, plants treated with R16 and CC2 strains showed higher consistency index (m*kg) (Fig. 4 B) values at d 6, in comparison to the other tested strains. The same behavior was observed for force at break which was significantly higher for plants treated with the two bacterial inoculants (R16, force at break = 116.65 ± 4.27 g; CC2, force at break = 122.57 ± 4.05 g) compared to the plants treated with 260-02, but similar to the values registered for non-treated samples (115.66 ± 6.33 g, Fig 4 C). It is indeed interesting to notice that there were no statistical differences among treated and non-treated samples at both harvest time and at d 8 in all the three tested parameters.

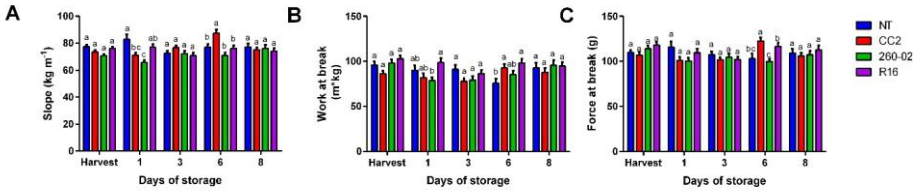

Figure 4. Mechanical properties of romaine lettuce leaves at harvest and during storage, as affected by different treatments. A: Slope (b); B: Work at break; C: Force at break (g) needed to bend the leaf sample up to failure. Data are means ± SE. At each time point, different letters indicate statistically significant differences among treatments, according to a One-Way ANOVA followed by Bonferroni post-hoc test (P < 0.05).

3.4.2. Color

Considering lightness (L*) (Fig. 5 A) and color coordinates (Fig. 5 B and C), there were few differences among treated and non-treated plants during the storage days and some significant differences among them were recorded at harvest. Significant changes were observed at day for both color parameters and at harvest for CC2 (a* and b*) and R16 (a*). A statistically significant higher value of green (a*, negative values) and yellow (b*, positive values) colors for all treatments along the storage period provide some results of relevance in a view of maintenance of appearance characteristics of salads.

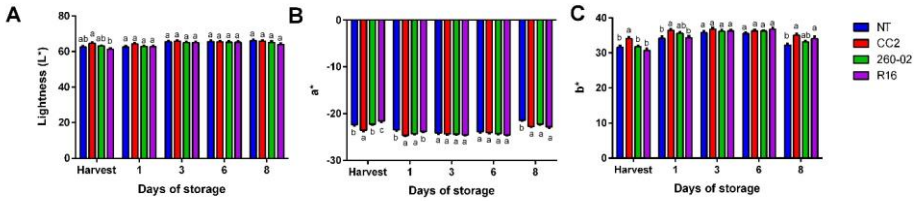

Figure 5. Color parameters of romaine lettuce leaves at harvest and during storage, as affected by different treatments. A: Lightness (L*); B: a* values; C: b* values. Data are means ± SE. At each time point, different letters indicate statistically significant differences among treatments, according to a One-Way ANOVA followed by Bonferroni post-hoc test (P < 0.05).

Discussion

The damage caused by *B. cinerea* on salads is so relevant that solutions for this problem has been sought for a very long time (Wood, 1951). The most direct and obvious solution to the problem is the use of fungicides, but this strategy has several limitations: the most evident is the economic impact of these treatments, that can often cost more than the damage caused by the pathogen (Fortunati et al., 2017); the risk of selecting resistant strains of the pathogen by repeated applications of the same fungicide is also a concern (Spotts et al., 1986); moreover, the environmental impact of the treatment with synthetic fungicides (Komarek et al., 2010), is an aspect that must be considered, especially under the guidelines of NSA that advocate a more sustainable production. For these reasons, several alternatives to the use of synthetic

fungicides have been researched, including both postharvest treatments with molecules with a lower environmental impact, such as chitosan (Fortunati et al., 2017), and preharvest treatments of different nature, including organic compounds (Zlotek and Wojcik, 2014), UV light treatments (Vasquez et al., 2017), and biocontrol agents (De Meyer et al., 1998). The pre-harvest approaches in particular offer several benefits as they do not rely on the application of active substances on the edible part of the plant, but rather reinforce the plant defense systems which, in addition to providing resistance to the pathogen, can improve the quantity of desirable molecules (such as antioxidants) in the produce increasing its nutritional value, as expected for the nutrition sensitive agriculture (NSA) proposition. All three bacterial strains tested in this study provided a noticeable amount of protection against *B. cinerea* to the treated lettuce plants through an indirect biocontrol mechanism, as evidenced by the fact that reduced symptoms were observed without a direct contact between the beneficial microorganisms (inoculated in the soil) and the pathogen (inoculated directly on the leaves). The indirect nature of this biocontrol effect is further reinforced when taking into consideration the results previously obtained with these same strains in the control of soilborne pathogens: microbiota analysis of rhizosphere, roots, and bulk soil revealed that the inoculated strains were no longer detectable in any of these compartments three weeks after inoculation, but operated a restructuring of the plant-associated microbiota (rhizosphere and root) while leaving the bulk soil largely unaltered (Passera et al., 2020). Considering these previous results that suggest a very little environmental impact of the inoculation with these strains, and the previously obtained results on soilborne pathogens, ~~also the effect seen in the biocontrol of soilborne fungal pathogens and the little environmental impact of these strains, which were not found colonizing the treated plants or growing in the soil at the time of harvest, (Passera et al., 2020)~~ the selected bacteria show very promising results for a future application, in particular *P. pasadenensis* strain R16.

Regarding the physiological evaluation of romaine lettuce leaves in response to treatments during storage, a general maintenance of leaf functionality and integrity has been observed and the considered parameters indicated no marked stress responses or strong damages. Only few changes were observed in the WC, indicating that the water status of the leaves was not affected by excessive transpiration during storage. It

is important to point out the pivotal role of low storage temperature in reducing the metabolic activities of the plant tissue during the whole post-harvest phase (Spinardi and Ferrante, 2012).

However, the analyses conducted, allowed to point out few detrimental effects induced by 260-02 application. In fact, leaves from plants treated with this formulation, showed in few cases a slight loss of PSII functionality and a marked increment in those indices related to cellular oxidative stress (TBARS and Electrolyte leakage). For the evaluation of the maximum quantum efficiency of the PSII (Fv/Fm), 0.83 is generally recognized as the threshold between stressed and non-stressed tissue (Maxwell and Johnson, 2000). In most of the cases the values were higher than 0.83 and were never below 0.81, but in plants treated with 260-02, after 1 day of storage, Fv/Fm dropped to 0.78.

These observations have been corroborated by the evaluation of the nutritional status of the leaves. In fact, lettuce treated with 260-02, showed a significant increment in AsA content and improved *in vitro* antioxidant capacity, in correspondence to the abovementioned increment in stress responses. Considering that this *Pseudomonas syringae* strain has been already proven to be an effective biocontrol agent (Passera et al. 2019; Passera et al., 2020), it is possible to hypothesize that it would have stimulated the plant defense mechanisms involving the production of reactive oxygen species (ROS). In this case we observed a prompt response of lettuce leaves, which immediately activated a series of defense mechanisms, which would had probably involved the AsA metabolism instead of other antioxidants. The predominant role of AsA is further supported by the lack of changes in the phenolic compounds, anthocyanins and carotenoids content. Among antioxidant molecules, AsA has been reported to be one of the most sensitives to stressful conditions and it is often reported as a good marker of oxidative responses in fresh-cut leafy vegetables (Ferrante et al., 2009). Moreover, a recent study suggests an important role for AsA in controlling H_2O_2 accumulation during the application of a plant growth-promoting bacteria in rice (Kumar et al., 2019), so it is possible that AsA could play a similar role in case of romaine lettuce. Concerning the other treatments, it is interesting to notice, that at the end of storage, the lower oxidative stress incidence and the maximum vitamin C content was recorded in leaves from R16-treated plants. This is particularly interesting considering the promising results obtained by this treatment in the control of *B. cinerea* and suggest the

possibility of efficiently control the pathogen development by stimulating the plant own defense mechanisms, with no negative drawback on nutritional properties.

The perceived lettuce macroscopic properties are here evaluated as objective texture and color attributes. A tight relation exists between the physiological and nutritional parameters of interest and the incidence of postharvest fungal infection. In fact, data showed that treatments did not alter the lettuce sensory performance during shelf life. Significant differences were more evident at harvest and at the end of shelf life (day 8), which match with two different stressful situations for lettuce leaves. Lightness, green (a*) and yellow (b*) colors which did not highlight significant difference between NT and inoculated samples and among treated samples as well (except some differences on day 1 for color parameters). It can be concluded that the main factor affecting lettuce performance is shelf-life duration. Also, the lack of changes in color-related parameters, is consistent with the absence of variation in the chlorophyll content of the leaves.

On the other hand, mechanical analysis suggests a mild but interesting effect of CC2 and R16 strains in ameliorating textural properties of salad in middle days of storage, while the observed partial loss of mechanical properties observed in response to 260-02 application, further supports the hypothesis of a major stress condition induced by this treatment. A significant correlation ($R^2 = 0.7517$, $p<0.05$) was found in case of leaves from 260-02 treated plants, between electrolyte leakage and consistency index, indicating that the loss of membrane stability was in fact accompanied by a parallel alteration of the mechanical properties of the tissue that are finally perceived by consumers as a modified sensory acceptance of the salad.

Conclusion

Based on the results obtained it is possible to conclude that the application of microbial inoculants during romaine lettuce cultivation could contribute to the maintenance of nutritional, functional and perceived quality attributes of leaves during shelf life. At the same time, the microbial inoculants were proved to be useful in preventing the development of postharvest fungal pathogen *B. cinerea.* Moreover, the study helped in individuating potentially different modes of action of the different inoculants and, in the case of

Pseudomonas syringae 260-02, it can be hypothesized a direct involvement of AsA-mediated antioxidant mechanisms.

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