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# Impact of solar radiation exposure on phyllosphere bacterial community of red-pigmented baby leaf lettuce

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ABSTRACT

Solar radiation has been identified as a stress factor affecting phyllosphere associated bacteria colonization and survival during primary production. In the present study, the impact of different solar radiation doses on the phyllosphere microbiota of red-pigmented baby leaf lettuce cultivated in open field under commercial conditions was evaluated. Four weeks before harvest, the growing field was divided into four plots; each one was consecutively covered with one-week-interval with a light-excluding plastic to reduce the sunlight exposure. Four different solar radiation treatments were generated and cumulative photosynthetically active radiation (PAR) was used to differentiate treatments as follows: 4889 ± 428 μmol/m$^2$/s (uncovered), 4265 ± 356 μmol/m$^2$/s (covered for 1 week), 3602 ± 225 μmol/m$^2$/s (covered for 2 weeks) and 3115 ± 313 μmol/m$^2$/s (covered for 3 weeks). The size and composition of the phyllosphere bacterial community were determined by cultivation-depended (plate count) and independent (qPCR) techniques. Exposure to decreased levels of cumulative PAR did not produce significant differences in total bacterial community size, regardless of the chosen quantification techniques. However, total bacteria size quantified by qPCR was around 3.5 orders of magnitude higher than those obtained by plate count. The observed differences between cultivation-depended and independent techniques could be attributed to the presence of non-viable or viable but non-culturable (VBNC) bacteria. The bacterial community structure was analyzed using temperature gradient gel electrophoresis (TGGE), and significant differences were detected when the four solar treatment were compared. A qPCR approach was applied to the quantification of specific bacterial phyla and classes, previously identified in the phyllosphere of plants available literature, confirming that *Proteobacteria*, *Bacteroidetes*, *Actinobacterias* and *Firmicutes* were the most abundantly represented phyla in lettuce. Treatment comparison revealed higher proportions of *Gammaproteobacteria* as opposed to the *Betaproteobacteria* on the lettuce exposed to the lowest cumulative PAR dose (3115 ± 313 μmol/m$^2$/s). The obtained results demonstrated that the solar radiation is a relevant environmental factor influencing the relative abundance of specific-groups of phyllosphere-associated bacteria in pigmented baby leaf lettuce.



## 1. Introduction

The phyllosphere, which refers to the environment encompassing the surface and interior of the aerial parts of vascular plants, is mainly colonized by bacteria (10$^5$ to 10$^7$ cells/g) affected by different biotic and abiotic stress factors (Morris and Kinkel, 2002; Newton et al., 2010). Phyllosphere associated bacteria constitute a dynamic population in constant change during cultivation where climatic conditions and agricultural practices play an important role (Vorholt, 2012). The phyllosphere is considered to be a hostile localization for bacterial colonization due to frequent changes in water availability, incident radiation, and low nutrient availability (Lindow and Brandl, 2003). *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* are the predominant phyla detected in the phyllosphere (Bulgarelli et al., 2013; Leff and Fierer, 2013). However, the proportions of these phyla tend to vary during the growing cycle due to intrinsic and extrinsic factors including plant species (Redford and Fierer, 2009; Hunter et al., 2010; Izhaki et al., 2013), environmental conditions (Jackson and

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Denney, 2011; Asakura et al., 2016), agricultural practices (Jensen et al., 2013; Wei et al., 2016) and geographical location (Redford et al., 2010; Rastogi et al., 2012).

Solar radiation has been highlighted as a very important stress bacterial colonization and survival in the phyllosphere (Sundin and Jacobs, 1999; Sundin, 2002; Jacobs et al., 2005; Balint-Kurti et al., 2010). Jacobs and Sundin (2001) evaluated the culturable bacterial population in peanuts demonstrating that solar UV-B radiation is an important environmental stress especially for non-pigmented bacteria. Similar studies, focusing on culturable bacteria reported comparable results in leafy greens (Wood et al., 2010). Studies performed in commercial growing fields have also demonstrated that solar radiation is an important abiotic factor affecting microbial decay in leafy greens during primary production (Whitman et al., 2004; Castro-Ibañez et al., 2015). The survival of *E. coli* and *Listeria innocua* on spinach and parsley, respectively was higher in shadegrown plants than on fully-exposed ones (Dreux et al., 2007; Wood et al., 2010).

Traditionally, culture-based techniques have been used to examine the bacterial communities on fresh produce. It has become increasingly evident, however, that these methods only detect between 1 and 10% of the species in the bacterial community (Pace, 1977; Rastogi et al., 2010). Molecular biologybased methods have recently been used to overcome this deficiency and have provided more detailed description of community structure and dynamics of bacterial communities in the phyllosphere during the growing cycle (Hunter et al., 2010; Williams and Marco, 2014). Several recent studies that focused on the seasonal dynamics of bacterial communities examined using molecular approaches have suggested that environmental factors such as ambient temperature, solar radiation and frequency of the water supply, provoke significant changes in the phyllosphere associated bacteria (Jackson and Denney, 2011; Williams et al., 2013; Dees et al., 2015; Rastogi et al., 2012; Asakura et al., 2016). However, the influence of stresses such as solar radiation exposure on the kinetics of bacterial communities in crops grown under commercial conditions remains unclear. In the present study, the impact of different solar radiation doses on bacterial community phyllosphere of commercially grown pigmented baby lettuce was evaluated using a combination of cultivation-dependent and molecular approaches.

## 2. Materials and methods

### 2.1. Experimental design

Red pigmented eazy leaf lettuce (Ezra, Enza Zaden, Spain) was seeded on February 2015 at El Jimenado, (Murcia, Spain). Sowing was performed directly on beds using a plant density of 800 plants $m^{-2}$. Cultural practices for drip irrigation and fertilization were similar to commercial conditions except that pest management was not applied. Four weeks before commercial size was reached (approx. 12–15 cm long from petiole), the field was divided into four plots (1.5 $m^2$ each). In a one-week-interval, each plot was consecutively covered with a light-excluding plastic to reduce sunlight exposure (Fig. 1). Four solar radiation treatments consisting in a non-shadow plot (Control) and the application of three different shadow intervals of 1, 2 and 3 weeks were created. The frame ends in the plastic tunnel film were left open to allow airflow and avoid condensation. The plastic tunnel film used to cover the crop was film natural of 25 μm and transmitted 95% of solar radiation (SOLPLAST S.A.).

The solar radiation with and without the use of the plastic tunnel film was measured using a linear array of photosynthetically active radiation (PAR) sensors (Ceptometer, AccuPAR LP-80, Decagon Devices, Inc). Weekly, PAR was measured above and below the plastic tunnel film (n = 20). The average PAR for open field grown lettuce was 1629 ± 209 μmol/m$^2$/s. In the case of lettuce grown under the plastic tunnel film, the average PAR values were 1038 ± 131 μmol/m$^2$/s. Therefore, the cumulative PAR during the last 4 weeks of cultivation differentiated the four treatments as follows: 1) 4889 ± 428 μmol/m$^2$/s (Control), which corresponds to the cultivation of uncovered plants 2) 4265 ± 356 μmol/m$^2$/s (1 W) which corresponds to the cultivation under the plastic tunnel film for 1 week, 3) 3602 ± 225 μmol/m$^2$/s (2 W) which corresponds to the cultivation under the plastic film for 2 weeks and 4) 3115 ± 313 μmol/m$^2$/s (3 W) which corresponds to the cultivation under the plastic film for 3 weeks. Additionally, ambient solar radiation and temperature - during the growing cycle were obtained from the nearby Torre Pacheco weather station (37° 44′ 51.81″ N, 0° 59′ 12.02″ W), using the local climatological database (SIAM, 2015). The average daily solar radiation was 929.5 ± 69.9 W/m$^2$ and the maximum and minimum temperatures were 19.9 ± 3.2 °C and 10.3 ± 1.6 °C, respectively.

### 2.2. Sampling

Pigmented baby leaf lettuces were sampled at the commercial stage (12–15 cm long measured from the petiole) from each plot. Three sampling points, randomly distributed per plot were selected. Samples of 100 g each were hand harvested using scissors from the base of petioles and stored in sterile plastic bags. Scissors were wiped with ethanol (70%) between treatments. Samples were transported under refrigerated conditions in polystyrene boxes (approximately 35 km) to the CEBAS-CSIC laboratory (Murcia, Spain) and stored at 4 °C. All the samples were processed within 4 h. Several quality parameters such as leaf weight, height and width were measured to characterize differences in plant growth between the treatments. No significant differences were observed among treatments (Table 1).

### 2.3. Microbial enumeration

#### 2.3.1. Culture-based analyses

Baby leaves were sampled for microbiological analysis as previously described (Lopez-Velasco et al., 2011). Briefly, 60 g baby leaves were sonicated with 240 ml of 2% sterile buffered peptone water (BPW; Scharlau Chemie, Barcelona, Spain) supplemented with 1% of Tween-80 (Sigma Aldrich, St Louis, MO, USA). Serial dilutions were applied to the surface of plate count agar (PCA; Scharlau Chemie, Barcelona, Spain) to estimate total bacterial populations. The plates were incubated at 30 °C for 24 h. The remaining contents of each bag were used for DNA extraction.

#### 2.3.2. Culture independent analyses

Sonicated lettuce in BPW supplemented with 0.1% of Tween-80 was centrifuged at 3000 g for 10 min and the resulting pellet was stored at −20 °C for genomic DNA extraction. Genomic DNA was extracted - using the FastDNA® SPIN Kit for soil and the FastPrep® 24-Instrument (MPBiomedicals, Germany), according to the manufacturer's indications. The quality and concentration of DNA extracts were determined by spectrophotometric measurement at 260/280 nm and 260/230 nm using a NanoDrop®ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

DNA fragments comprising, the V3-V5-V5 hypervariable region of the 16S ribosomal RNA bacterial gene (16S rRNA) were amplified by PCR using primers set 341 GC-907R (Lopez-Velasco et al., 2011). PCR amplifications were done in a T100™ Thermal Cycler (Biorad, Hercules, CA, USA) and conditions for each PCR reaction were kept as previously described (Lopez-Velasco et al., 2011). PCR products