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Botrytis cinerea response to pulsed light: Cultivability, physiological state, ultrastructure and growth ability on strawberry fruit

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ABSTRACT

Botrytis cinerea causes postharvest spoilage in important crops such as strawberry and other berries. Pulsed light (PL) treatment could be an environmentally friendly postharvest alternative to synthetic fungicides in berries. Cultivability, physiological state, ultrastructure of *Botrytis cinerea* suspended in peptone water and irradiated with PL (fluence = 1.2 to 47.8 J/cm^2) were investigated by using conventional plate count technique, flow cytometry analysis (FCM) and transmission electron microscopy. In addition, PL effect on *B. cinerea* development in artificially contaminated strawberries throughout storage at (5 ± 1) °C was evaluated.

PL reduced fungus' ability to form colonies on agarized culture media. Survival curve fitted with the Weibullian model evidenced a wide distribution of conidia sensitivity to PL. FCM showed that most of irradiated conidia entered in a viable non-culturable state, although a subpopulation without esterase activity and compromised membranes and a subpopulation with active esterase and intact membranes were also detected. PL attacked multiple targets in *B. cinerea*. Ultrastructural changes varied with the dose and within the conidia population, supporting FCM results. Damage included plasmalemma detachment from cell wall, cytoplasm collapse, and vacuolization of cytoplasm, disruption of cell wall and plasmalemma with massive loss of cytoplasm and/or disruption of organelles. In strawberries artificially contaminated with *B. cinerea*, a 2-day delay on the onset of the infection and a lower incidence in PL-treated strawberries (11.9 and 23.9 J/cm^2) compared to control (16–20%) up to 10 days of cold storage was observed. Results indicated that PL significantly reduces *B. cinerea* growth in peptone water and in inoculated strawberries. However, other preservation factor(s) in combination would be needed to increase PL action for a better control of this fungus.

1. Introduction

Microbial decay is one of the main factors that compromise the quality of fresh produce in the field and during the postharvest phase (Mari et al., 2010). *Botrytis cinerea* is a necrotrophic fungus that causes gray mold on many economically important crops (Elad et al., 2007). It has been reported to be the main problem in cold storage for table grape, strawberry, tomato and kiwifruit. *B. cinerea* uses a wide range of infection strategies and often causes quiescent infections or minor damage in unripe fruit, protected by induced or preformed antifungal substances and mechanical barriers in the peel (Adikaram et al., 2010). During fruit ripening and senescence, the fungus transforms to necrotroph causing typical decay symptoms, in concordance with a decrease in antifungal compounds and softening of epidermis structure. Control of disease is very important during cold storage of fruit because the fungus is able to grow effectively at temperatures just above freezing (−0.5 °C) (Elad et al., 2007).

Current crop protection techniques rely on synthetic conventional fungicide application and on horticultural practices. However, the wide consumption of fresh fruit in human diet increased concerns for the possible toxicity of agrochemical residues and has led to the development of new strategies for *B. cinerea* control. *B. cinerea* could also develop fungicide resistance, reducing the effectiveness of chemical protection. Alternative methods to fungicide treatments include: a)

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biological control agents, b) plant bioactive compounds, c) physicochemical methods (heat, ionizing and UV- C irradiation, etc.), and d) combinations among the cited methods (Mari et al., 2010; Romanazzi et al., 2016).

Pulsed light (PL), approved by FDA in 1996, is a promising innovative technology to decontaminate foodstuffs that reduces microbial populations in very short time intervals (tens of seconds). It involves short-duration pulses (1 μs – 0.1 s) of high power with a broad emission spectrum ranging from UV to near-infrared (200–1100 nm). It is ecologically friendly, cost effective and can be easily incorporated in processing lines (Oms-Oliu et al., 2010). Only few studies have been dealing with the efficacy of PL against mold decay in berries after harvest (Duarte-Molina et al., 2016; Luksiene et al., 2013; Marquenie et al., 2003). Moreover, data reported in the literature are inconclusive. Therefore, deeper investigations are needed for better understanding the antifungal action of PL and evaluating the potential of this technique for commercial purposes.

This study investigated *in vitro* the impact of pulsed light dose on: a) the *B. cinerea* conidia cultivability (ability to form colonies on malt extract agar), b) the physiological state (membrane integrity, esterase activity) of conidia population on a single cell level, and c) the changes in conidia ultrastructure. PL effect on *B. cinerea* development in artificially contaminated strawberries stored at refrigeration temperature was also assessed.

2. Materials and methods

2.1. Microorganism and preparation of the inoculum

Botrytis cinerea BAFC 3003 was provided by BAFC Culture Collection (Faculty of Exact and Natural Sciences, University of Buenos Aires). Fungal strain was cultivated on malt extract agar (MEA) (Britania, Argentina) for 14 days at 25 °C. Conidia were harvested by washing with sterile peptone water (0.1 g/100 mL) (Biopack, Argentina) containing Tween 80 (0.05 mL/100 mL) (Biopack, Argentina) and shaking gently on a vortex mixer. The conidia suspension was filtered through sterile gauzes to remove the large mycelium fragments. The resulting conidia suspensions (approximately 10^4–10^5 conidia/mL) were immediately used for subsequent experiments.

2.2. Inoculation of strawberries

Strawberries (*Fragaria x anannasa Duch* cv Fortuna) with 100% of red colour in their surface were harvested in an orchard from Pilar in the province of Buenos Aires and immediately transported to the laboratory. Fruit were immersed in a hypochlorite solution (200 mg/L) for 2 min and rinsed three times with sterile distilled water to decontaminate the surface. Decontaminated fruit were randomly distributed in polypropylene trays (10 strawberries each). A spot-inoculation method was used to inoculate *B. cinerea* in strawberries. Two small and superficial injures were made on the fruit skin, approximately midway between the calix and the cap, and 10 μL of inoculum suspension ($\approx 10^4$ conidia/mL) was applied in a small drop in each injury. The inoculated strawberries were kept in a biosafety hood at (20 ± 2) °C for 22 h to allow attachment of the fungus (Supplemental Fig. 1).

2.3. Pulsed light treatments

PL treatments were performed with a RS-3000B Steripulse-XL system (Xenon Corporation, Woburn, MA, U.S.A.) as described by Gómez et al. (2012). Treatments were carried out by placing the samples (conidia suspension or inoculated strawberries) at 0.1 m distance from the quartz window of the xenon lamp and within a uniform area of the radiation field (beneath the lamp and around the central point) to minimize variations in radiation dose absorption. The PL fluences assayed were obtained by applying different number of pulses. Fluence measurements were taken by a pyroelectric head model ED500 (Gentec Electro-Optics, Québec, Canada) connected to an oscilloscope model TDS 2014 (Tektronix, Beaverton, USA), with an aperture cover of 20.3 cm^2. The terms PL "fluence" or "dose" were used interchangeably throughout the manuscript. However, it is important to clarify that the term "fluence" is more appropriate when applying PL since it refers to the "incident" energy in the sample, rather than "dose" which refers to the "absorbed" energy (Bolton and Linden, 2003).

The temperature build-up of samples during treatments was monitored using a T-type thermocouple connected to a data logger Digi-Sense model 69202-30 (Barnant Company Division, Barrington, USA).

For PL processing of conidia suspension, 3 mL ($\approx 10^5$ conidia/mL) were poured into a 50 mm diameter Petri dish and PL-treated for 1 to 40 s (fluences: 1.2 to 47.8 J/cm^2, respectively; final temperature ≤ 20 °C). Three replicates were done for each condition. For PL processing of inoculated strawberries, fruit were placed in a sterile glass tray (4 fruit per tray) and exposed to PL firstly on one side, and then were turned upside down on another sterile tray, and treated during the same period of time on the opposite side. In each side, fruit were irradiated for 10 and 20 s (fluences: 11.9 and 23.9 J/cm^2, respectively; final temperature < 30 °C). Fruit were packed in closed plastic boxes permeable to air (26 cm × 19 cm × 6 cm) (10 fruit per box) and stored at (5 ± 1) °C for 12 days. Three replicates of 10 strawberries were made for each condition. PL-treated conidia suspensions and strawberries were compared against controls (non-treated samples).

2.4. Determination of cultivability and modelling of survival curve

Immediately after PL treatments, conidia suspensions were serially diluted in peptone water (0.1 g/100 mL) and subsequently spread plated (100 μL) onto MEA in duplicate. In addition, conidia suspensions (1 mL) without dilution were directly pour-plated into Petri dishes with MEA. Plates were incubated for 5 days at (25 ± 1) °C. Survival population was counted using the standard colony count technique. Survival curve was generated from experimental data by plotting log N/N_o (N: number of CFU/mL at a given PL treatment time; N_o: initial number of CFU/mL) versus time of treatment.

Survival curve was modelled with the cumulative form of a Weibull type distribution of resistances (Peleg and Cole, 1998):

$$S\ (t) = \log(N/N_0) = -bt^n \tag{1}$$

where *S(t)* is the fraction of survivors at a given time and *b* and *n* are the scale and the shape parameters, respectively. The *b* value in the Weibull distribution function is related to the rate of inactivation of microbial cells, while *n* indicates the concavity of the survival curve ($n > 1$ indicates a downward concavity and $n < 1$, an upward concavity; a log linear shape is a special case when $n = 1$). The values of *b* and *n* were then used to generate the resistance frequency curves using the following equation:

$$\frac{d\varphi}{dt_c} = bnt_c^{n-1}\exp(-bt_c^n) \tag{2}$$

where t_c is the inactivation time (a measure of the organism's resistance or sensitivity to PL) and $\frac{d\varphi}{dt_c}$ is the Weibull distribution corresponding to t_c. Other statistical parameters which better explain the observed frequencies (distribution mode, t_{cm}; mean, $\bar{t}_c$; variance, $\sigma_{tc}{}^2$; and coefficient of "skewness", υ_1) were calculated from the equations reported by Peleg and Cole (1998) and Peleg (1999). The distribution mode, t_{cm}, represents the treatment time at which the majority of population dies or is inactivated. The mean, $\bar{t}_c$, corresponds to the inactivation time on average with its variance, $\sigma_{tc}{}^2$. The "skewness" coefficient, υ_1, represents the skew of the distribution.

2.5. Flow cytometry analysis

PL-treated and non-treated conidia were double stained with

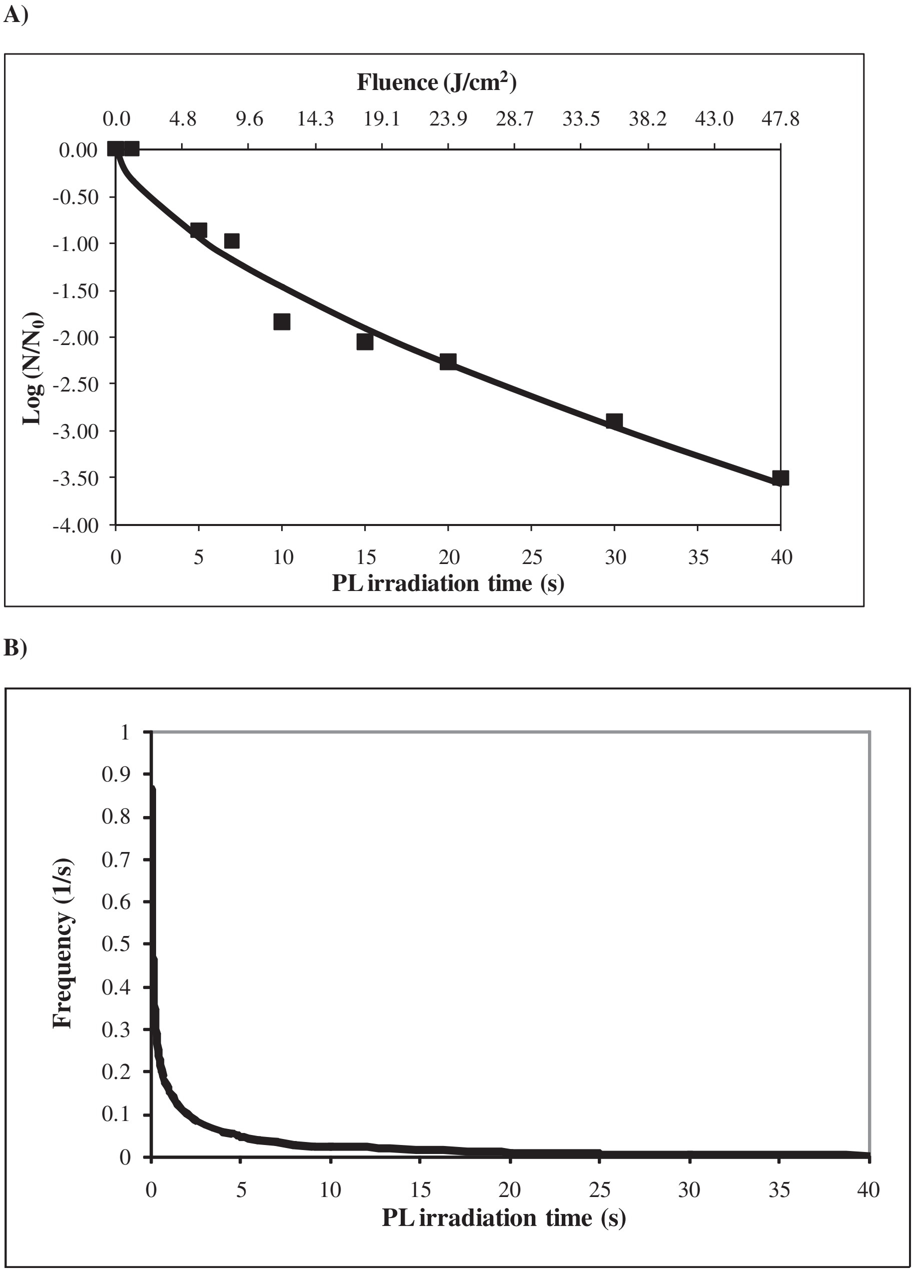

Fig. 1. A) Experimental survival curve (points) and fitted values derived from Weibull type model (solid line) for *B. cinerea* conidia treated with PL (each point represents the mean value; standard deviation $\leq$0.04). B) Frequency distributions of resistances of *B. cinerea* to PL treatments, obtained by applying Eq. (2).

fluorescein diacetate (FDA) (Calbiochem, Darmstadt, Germany) and propidium iodide (PI) (Sigma Aldrich St Louis, MO, USA) using the methodology described by Schenk et al. (2011). Briefly, two microliters of FDA (5 mg/mL acetone) were added to 1 mL of conidia suspension, and after incubation for 30 min at (37 ± 1) °C, the mixture was washed twice with PBS buffer (pH 7.0). The pellet was re-suspended in 1 mL PBS (pH 7.0) and 2 μL of PI (1 mg/mL sterile water) was added. The sample was subsequently incubated for 15 min at room temperature in the dark before analysis. Non-irradiated conidia heated at 100 °C for 60 min and stained with PI were used as PI- positive control, whereas non PL-treated conidia stained only with FDA were employed as FDA-positive control. In addition, a non-stained-non-treated control was used for determining the autofluorescence of conidia.

Samples were analyzed on a flow cytometer BD FACSAria II (Becton Dickinson, USA), equipped with an air-cooled 488 nm argon laser. The 530/30- FITC filter was used for FDA detection, whereas the 585/42-IP and 695/40-PerCP filters were employed for PI detection. Flow rate of cells was set at 200 events per second. A total of 20,000 events were registered per sample. Analyses were done in duplicate. The software WinMDI 2.8 (Scripps Research Institute, La Jolla, CA) was used to analyze flow cytometric data. Results were represented in a two-dimensional dot plot of red fluorescence (PI) versus green fluorescence (F). Side scatter light (SSC) values (associated with complexity) and forward scatter light (FSC) values (associated with particle size) were also analyzed.

2.6. *Transmission electron microscopy*

Samples of treated and non-treated conidia suspensions were centrifuged (1.5 mL, 7880 g, 5 min) (Eppendorf, model 5804 R, Hamburg, Germany) and the pellets were re-suspended in 1.5 mL of sample. This procedure was repeated twice in order to concentrate the suspension.

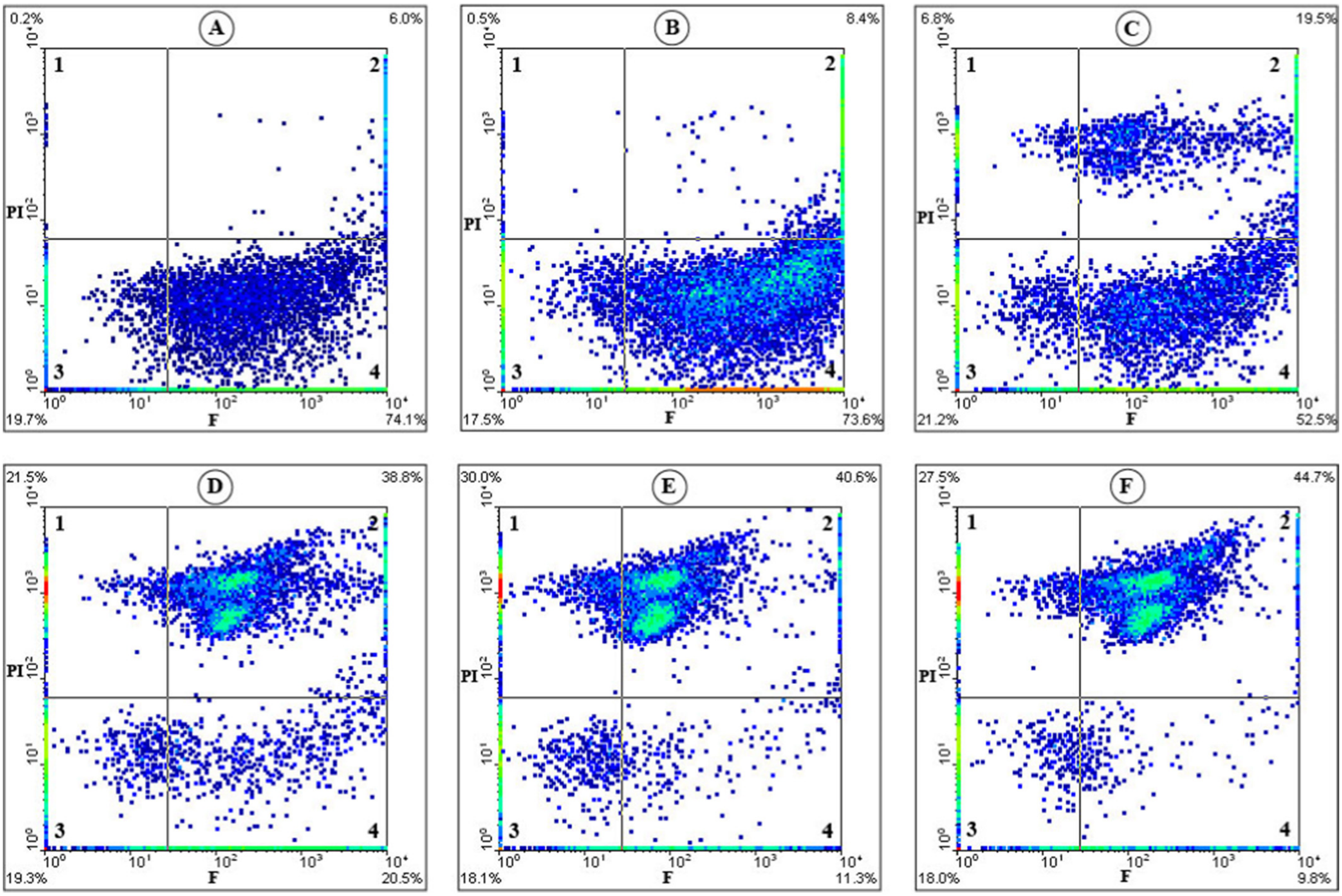

Fig. 2. Fluorescence density plots of *B. cinerea* BAFC 3003 in response to staining with PI and FDA after PL exposure during different times: A) 0 s, B) 5 s, C) 10 s, D) 20 s, E) 30 s and F) 40 s. Each dot represents one single cell, which is plotted as a co-ordinate of its green and red fluorescence value. The percentage of conidia subpopulations which falls in each quadrant is indicated in the four edges of each plot.
(Quadrant 1: F^- PI^+, Esterase activity not detectable, compromised membrane; Quadrant 2: F^+ PI^+, Active esterase, minimally damaged membrane; Quadrant 3: F^- PI^-, Esterase activity not detectable, intact membrane; Quadrant 4: F^+ PI^-, Active esterase, intact membrane).

Conidia were re-suspended in 1 mL glutaraldehyde (2.5 g/100 mL) in PBS (phosphate buffered saline), pH 7.0, and fixed for 6 h at 5 °C. Then, the pellets were washed in PBS at pH 7.0 and post-fixed with $KMnO_4$ (2 g/100 mL) in PBS (pH 7.0) for 17 h at 4 °C. Thereupon, conidia were washed twice with distilled water, dehydrated in a graded ethanol series and embedded in Epon 812 (Shell Chemical Company, USA) for 48 h. Ultrathin sections were cut and stained with uranyl acetate and lead citrate, and examined with a microscope JEOL microscope model JEM 1200 EX 259 II (JEOL, Japan) at 80 kV.

2.7. Evaluation of *B. cinerea* growth on strawberries

B. cinerea development in PL-treated and non-treated strawberries was evaluated daily up to 12 days of storage. Strawberries with visible fungal development were considered decayed. Results were expressed as percentage of infected fruit [IF (%)] (Duarte-Molina et al., 2016).

2.8. Statistical analysis

Statistical analyses were carried out using Infostat v2015 software (Córdoba, Argentina) and Statgraphics Plus for Windows 3.0-Package (Statistical Graphics, Washington, USA). Weibull model fit and performance were evaluated using the adjusted determination coefficient (R^2_{adj}) and the root-mean-square error (RMSE), respectively (Alzamora et al., 2005). Fungal decay results of inoculated strawberries were compared by analysis of variance (two-way ANOVA, testing treatment and storage time) and means comparison was performed by Duncan's test. Before conducting the analysis, the assumption of normality and homoscedasticity of residuals were checked by the Shapiro-Wilk and Levene tests, respectively. The significance level was set up at $p < 0.05$.

3. Results and discussion

3.1. PL effect on *B. cinerea* cultivability

PL application to *B. cinerea* conidia population suspended in peptone water was effective for reducing fungus' ability to form colonies on MEA (Fig. 1A). Exposure to PL for 10 s (11.9 J/cm^2) decreased colony counts by 2.0 log-cycles. After 40 s treatment (47.8 J/cm^2), the number of colonies was reduced by 3.5 log-cycles. Survival pattern of *B. cinerea* conidia showed a clear deviation from log-linear death when exposed to PL (Fig. 1A). At of > 11.9 J/cm^2, counts decreased considerably slower and the survival curve exhibited an upward concavity.

The log N/N_0 vs. PL exposure time curve was described in terms of the cumulative form of the Weibull distribution. This distribution function takes biological variation into account and may be helpful to pinpoint relevant physiological effects caused by PL. This approach postulates that inactivation mechanism at the molecular level may vary from cell to cell and that the death of a single cell could be due to more than one single lethal event (Peleg, 1999). Fitting of inactivation curve resulted in high adjusted coefficient of determination (R^2_{adj}: 0.969), evidencing that 96.9% of the variation in the experimental data could be explained by the model. Calculated scale and shape parameters were (0.34 ± 0.07) s^{-n} and 0.64 ± 0.06, respectively. Estimated values of b and n were then employed to generate the frequency distribution of conidia resistances to PL (Fig. 1B) and to calculate related statistics (mode, mean, variance and coefficient of skewness). Resulted frequency distribution lacked of mode and was strongly skewed to the right (coefficient of skewness: 3.8), indicating that the majority of conidia population was inactivated at low PL exposure times. Although mean inactivation time was 7.6 s, the high variance value (σ^2: 154 s^2) pointed out that conidia sensitivity to PL was widely distributed, that is, there was a spectrum of resistances of conidia population to PL.

3.2. Physiological state of PL treated *B. cinerea* conidia

The effect of PL radiation at different doses on esterase activity and membrane integrity was evaluated by the ability of *B. cinerea* conidia to accumulate and retain fluorescein (F) as an indicator of enzyme activity, and the uptake of propidium iodide (PI) to assess membrane damage. PI is positively charged and almost membrane impermeable. It can enter cells via damaged membranes of injured or dead cells and intercalate into DNA and RNA generating red fluorescence. The nonpolar FDA molecule can pass cell membranes and inside the cell is hydrolysed by non-specific intracellular esterases into polar fluorescein molecule (F), a membrane impermeable fluorescent dye which gives green fluorescence and is retained by intact cells (Ueckert et al., 1995).

Density plots of red fluorescence (y-axis) versus green fluorescence (x-axis) for different PL doses are shown in Fig. 2. Esterase activity was detected in 74% of untreated conidia population (quadrant 4), whereas < 20% of the population apparently showed very little or even nonexistent metabolic activity (quadrant 3) (Fig. 2A). The absence of metabolic activity in this last subpopulation could be attributed to the lack of "stainability" with fluorescent dyes due to the thick and resistant cell walls that prevent conidia from successful staining (Prigione et al., 2004). After 5 s of PL irradiation, fluorescent pattern of treated conidia was similar to that of untreated ones (Fig. 2B). PL applied during 10 s affected membrane integrity, since a shift of conidia from quadrant 4 (active esterase, intact membranes) to quadrant 2 (active esterase, minimally damaged membranes) and quadrant 1 (esterase activity not detectable, compromised membranes) was detected. Only 6.8% of conidia were solely labelled by PI throughout the population, whereas 19.5% of conidia were double stained with PI and F, revealing that membrane integrity and esterase activity were not completely decreased (Fig. 2C). As PL exposure increased to 20 s and 30 s (Fig. 2D, E), a growing percentage of conidia were encountered at quadrants 1 and 2 at expenses of population at quadrant 4. No considerable further change in staining pattern was observed between 30 s and 40 s PL treated conida (Fig. 2E, F). Despite the high PL dose applied (47.8 J/cm^2), some conidia (quadrant 4, 9.8% of the population) still showed metabolic activity and non-compromised membranes. Forward-scattered light (FSC) and side-scattered light (SSC) values were not impacted in a similar manner after PL exposure (data not shown). Conidia treated during 5 s exhibited similar size and complexity than control ones. After 10 s and 20 s PL exposure, there was an increase in conidia size in ≈ 7% and 6% of the population respectively, with smaller changes in particles complexity (only 2% of the conidia displayed greater SSC values). A further increase in PL exposure did not modify these parameters. Dot plots of forward scatter (FSC) values vs PI fluorescence of conidia population (graphs not shown) revealed that the global 6–7% increase in population size was due to the enlargement of ≈ 23–25% of the subpopulation that incorporated PI. These enlarged conidia could result from the growth/swelling of yet metabolic active conidia that would be unable to germinate and initiate hyphal growth because of injured membranes.

It is worth noting that 5 s of PL exposure caused a ≈ 1 log-cycle reduction in conventional viable conidia count as revealed by Fig. 1. In contrast, this low PL dose had no effect on PI or F fluorescent values (Fig. 2B), indicating that conidia remained metabolic active and with integer membranes although 90% of the conidia lost their ability to reproduce on agar. These findings are in partial agreement with those reported by Kramer and Muranyi (2013), who found that a considerable proportion of *Listeria innocua* and *Escherichia coli* cells entered the "viable but non-culturable" (VBNC) state after PL treatment. Moreover, they did not detect an immediate shutdown of vitality functions even when the number of colony-forming units significantly decreased, attributing the loss of cultivability to the oxidative stress with damage to the DNA caused by PL. Flow cytometry studies on the PL- induced damage on *Saccharomyces cerevisiae* using similar as in this work were performed by Ferrario et al. (2014). At low doses, there was an increase in the yeast population in quadrant 2 (VBNC), but at moderate and high doses most of cells were in quadrant 1 (PI^+- F^-), revealing a greater sensitivity of the yeast to PL as compared to *B. cinerea* conidia. However, in spite of the intrinsic sensitivity, these authors found that the loss of cultivability at low doses was much higher than the correspondent increase in the subpopulation with permeabilized cells.

From a practical point of view, the occurrence of unculturable yet viable conidia would prove valuable in developing appropriate decontamination processes. Under suitable conditions, the cells in the VBNC state might be repaired, regain cultivability and the renewed ability to cause spoilage. Thus, conventional plate counts would underestimate the number of survivors after PL treatments.

3.3. Ultrastructure features of PL treated *B. cinerea* conidia

The effects of PL on the ultrastructure of the conidia of *B. cinerea* examined by TEM are illustrated in Figs. 3 and 4. Untreated conidia, oval in shape, presented an ultrastructure similar to that described in previous studies (Hawker and Hendy, 1963; Buckley et al., 1966; Pârvu et al., 2008; Pârvu et al., 2010; Adrian and Jeandet, 2012) (Fig. 3). Cell walls were composed by a thin electron dense outer layer and a thicker electron transparent inner one. Many short protuberances, randomly distributed, were observed in the conidia's surface. A well-defined plasmalemma was closely adhered to the cell wall (Fig. 3A, C). In the highly dense cytoplasm, numerous pleomorph mitochondria, abundant ribosomes, dark-appearing storage bodies, nuclei, stellate vacuoles and lipid bodies were visualized (Fig. 3A, B, C, D). Myelinic membranes and wormlike structures were also noted within these storage inclusions (Fig. 3D, E). Endoplasmic reticulum mainly appeared as double strands marginally located near the plasmalemma (Fig. 3A).

The ultrastructure of *B. cinerea* conidia was differently modified by PL exposure (Fig. 4). Observations of 20 s-PL treated conidia demonstrated that PL affected different targets in the cells. Moreover, ultrastructure changes induced by PL in the population were highly heterogeneous (Fig. 4A). In general, detachment of the plasma membrane from the cell wall was noted (Fig. 4A–E). In many conidia, walls looked with lower electron density, with loss of integrity and rigidity, and cells appeared more elongated or deformed (Fig. 4A, C–E). Surface protuberances were altered. Damage to the cell wall and rupture of cytoplasmic membrane led to a massive loss of cytoplasmic content and many conidia were observed emptied with only small inclusions and membrane debris (Fig. 4A, C–E). In other conidia, walls apparently maintained their integrity but great vacuoles in the cytoplasm, damage in organelles and plasmalemma breakage occurred (Fig. 4B, C). Conidia exposed to PL during 40 s showed more severe alterations in the cytoplasm. Some conidia showed only disrupted membranes reorganized into vesicular structures (Fig. 4F, G). In others, disruption and degeneration of fungal organelles were notorious. No recognizable cellular organelles could be visualized except mitochondria, which appeared very clear but with a complete disorganization of the cristae (Fig. 4H–J). Cytoplasmic contents without attached walls and plasma membrane were also detected (Fig. 4K).

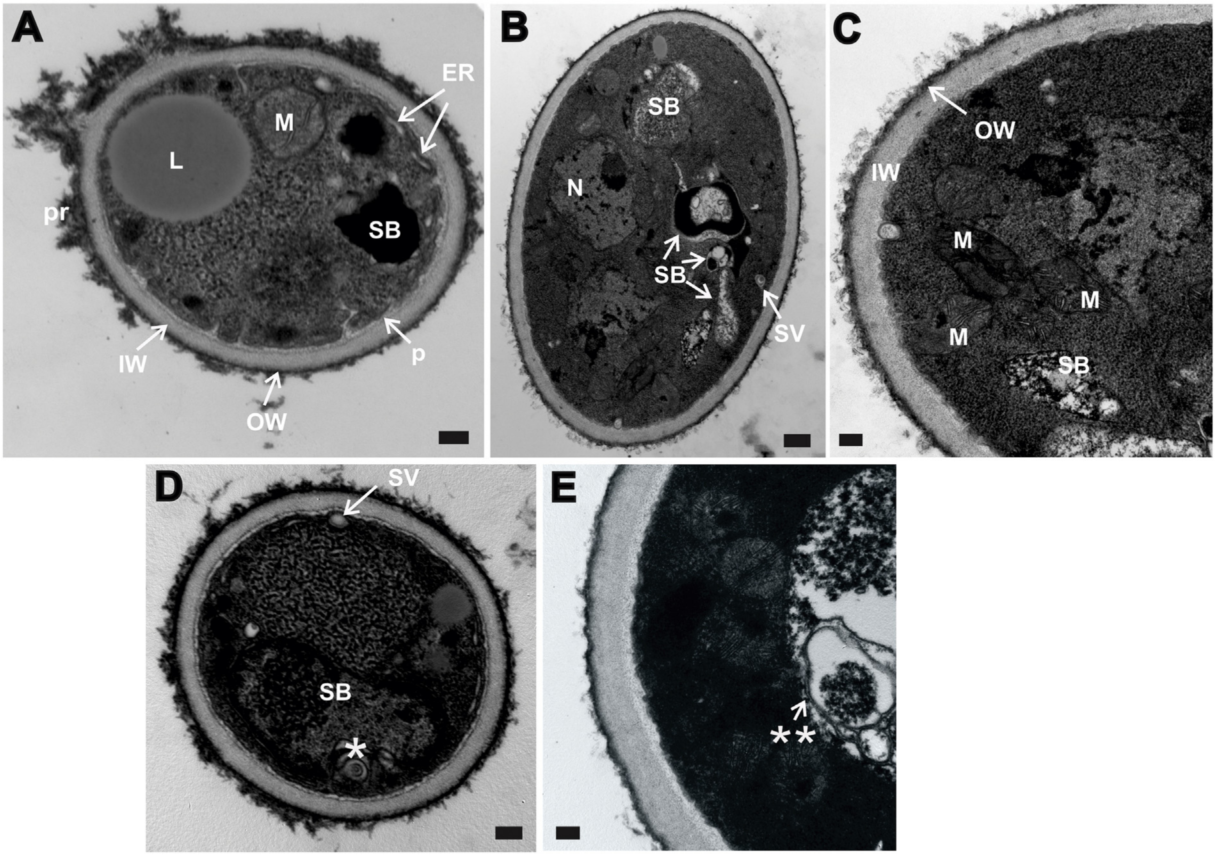

Fig. 3. TEM images of untreated conidia of *B. cinerea.* Scale: A, C, D, E, 2 µm; B, 5 µm. *IW*: inner wall; *OW*: outer wall; *pr*: surface protuberance; *p*: plasmalemma; *M*: mitochondria; *N*: nuclei; L: lipid body; *ER*: endoplasmic reticulum; *SB*: storage body; *SV*: stellate vacuole;*, wormlike structure; **, myelinic membrane.

3.4. Growth dynamics of PL treated B. cinerea conidia inoculated in strawberries

Accumulated percentages of *B. cinerea* incidence [FI (%)] along 12 days of storage at (5 ± 1) °C for untreated and 10 s and 20 s PL exposed strawberries are shown in Fig. 5. Non-significant interaction between "treatment" and "storage time" was found ($F_{6,24} = 0.97$; $p = 0.465$), although the main effect of the factors was significant ($p < 0.002$). Treated and untreated fruit showed a significant increase in the FI (%) during storage. However, there was a 2-day delay on the onset of the infection on strawberries exposed to PL for 10 s or 20 s compared to the control. In general, *B. cinerea* incidence was ~ 16–20% lesser than control up to 10 days of storage, but FI (%) values were similar in treated and untreated fruit at day 12. It is important to note that these PL fluences did not have a detrimental effect on the surface colour and the mechanical properties of strawberries throughout cold storage (Contigiani, 2019; Duarte-Molina, 2015). Moreover, depending on the strawberry cultivar these PL fluences could induce a delay of softening along 8 day storage at 5 °C (Duarte-Molina et al., 2016).

These results are partially in agreement with literature findings. Duarte-Molina et al. (2016) investigated the effect of different PL doses (2.4–47.8 J/cm^2) on fungi spoilage of strawberries stored for up to 8 days at 6 °C. They found that incidence of postharvest molds was reduced by over 16–42% with PL application. Luksiene et al. (2013) observed that yeasts and fungi distributed on the surface of strawberries were inactivated by 1 log when applying a PL dose of 3.9 J/cm^2. They concluded that shelf-life of strawberries was significantly prolonged without negative impact on their nutritional quality and overall appearance. On the other hand, Marquenie et al. (2003) used PL (40–250 s, doses not reported), alone or combined with heat or UV-C, for surface decontamination of strawberries inoculated with *B. cinerea* conidia. In contrast with present results, they found that PL alone, even at the longest treatment assayed, had no positive or negative effect on fungus development, nor did it induce resistance against fungal infections in treated fruit.

When comparing the effect of PL on *B. cinerea* conidia in *in vitro* (Fig. 1) and *in vivo* studies (Fig. 5), different responses between planktonic and attached microorganisms were found. Cells associated to food surfaces are much more heterogeneous and resistant to preservation factors than planktonic, freely suspended cells (Chmielewski and Frank, 2003).

In addition, the lower efficacy of PL treatment in fruit could be associated to the absorption, reflection, and scattering of light by the fruit matrix, and to the "shade" effect or irregular surfaces that protect microorganisms from light irradiation (Lagunas-Solar et al., 2006; Gómez-López et al., 2008). In spite of these differences, and in line with staining results, this in vivo study would indicate a recuperation of the treated fungus along storage for both PL doses assayed, or/and growth of refractory/irradiation protected conidia.

4. Conclusions

Physiological and ultrastructural characteristics of PL exposed *B. cinerea* conidia as well as cultivability loss test revealed the heterogeneous response of conidia population to PL. Structure study showed a heterogeneous, multitarget and dose-dependent effect of PL. Survival curve of *B. cinerea* conidia exhibited an upward concavity and was adequately fitted by a Weibull distribution model, with a mean inactivation time equal to 7.6 s and a very high variance value, pointing out a widely distributed sensitivity of conidia to PL. Flow cytometry analysis of metabolic activity and integrity of membranes detected conidia subpopulations in all quadrants for energy doses ≥11.9 J/cm^2,

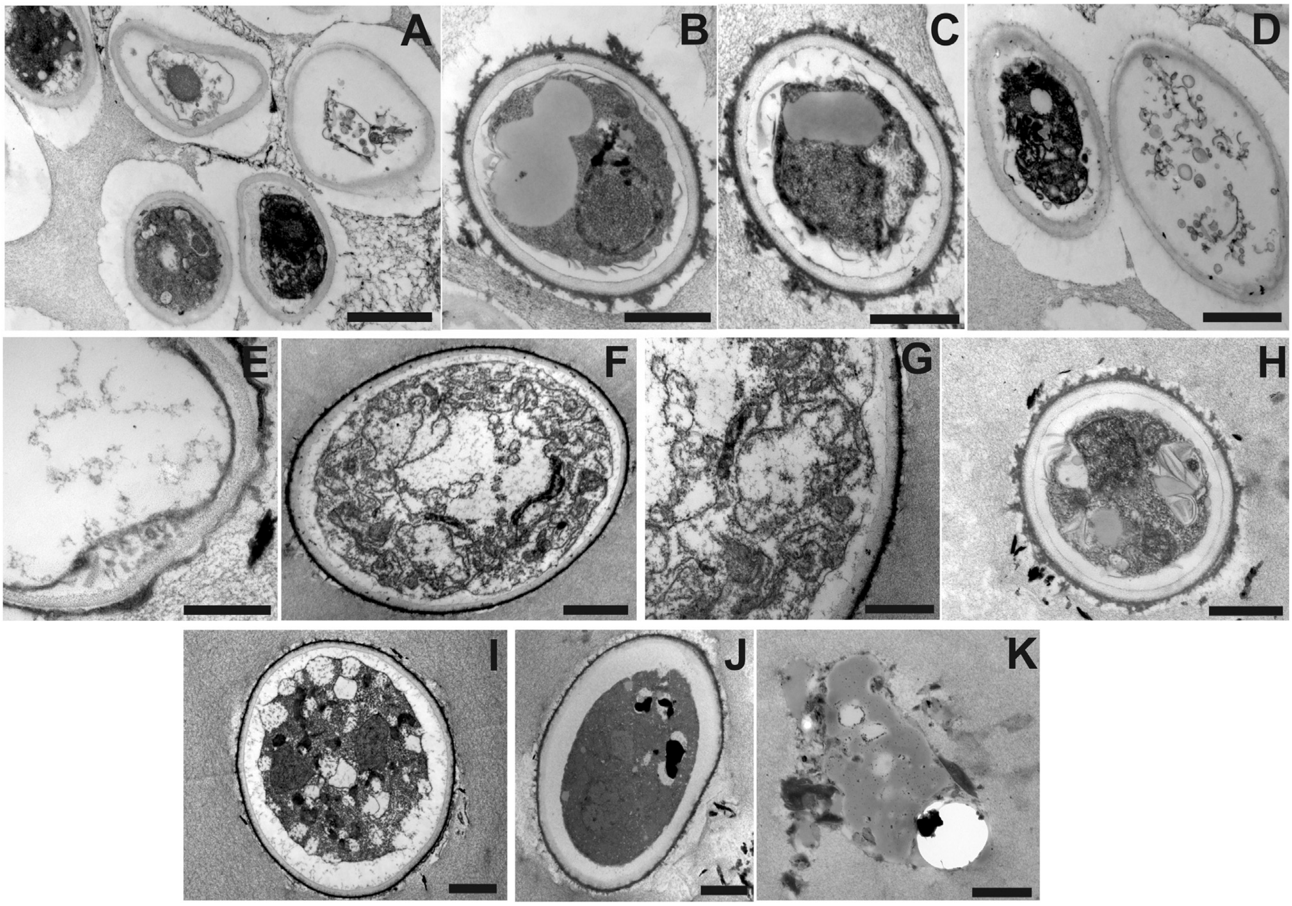

Fig. 4. TEM images of PL-treated conidia of *B. cinerea*. A-E, 20 s-PL-treated cells. F-K, 40 s-PL-treated cells. Scale: A, D, F, I, J, 2 μm; B, C, G, H, K, 1 μm; E, 500 nm.

but most of the fungal cells were inducted into VBNC state. *In vivo* studies demonstrated that PL treatment could reduce *B. cinerea* growth along cold storage in artificially inoculated strawberries. However, other stress factor(s) would be needed to increase PL action, allowing achieving higher level of *B. cinerea* inactivation while retaining the fresh keeping quality of the fruit. Studies on the ability of conidia to resuscitate from the VBNC state and return to an actively metabolizing and culturable form are in course to obtain a deeper understanding of the PL effect.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108311.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

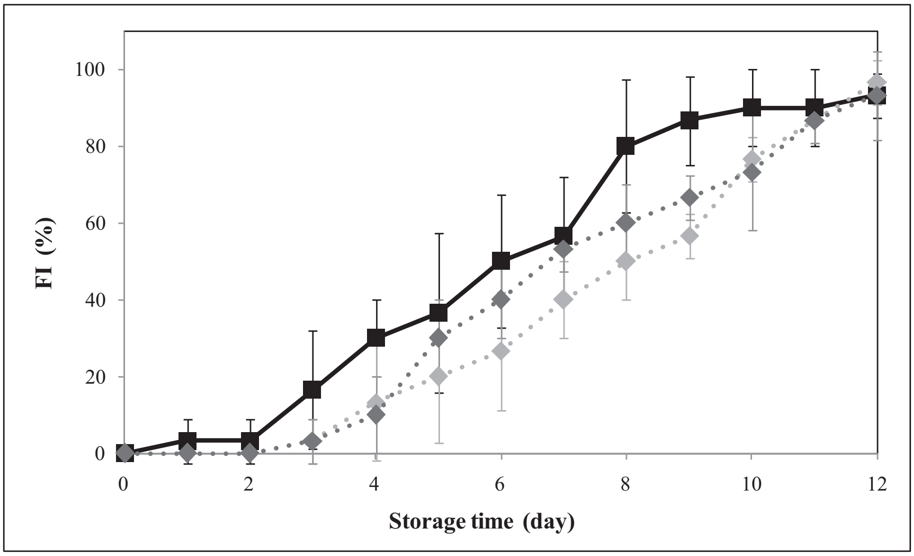

Fig. 5. *B. cinerea* decay incidence in untreated and PL exposed strawberries during storage at 5 ± 1 °C. (■) control (untreated fruit); (◆) 10 s PL; (◆) 20 s PL. Values are mean ± standard deviation.

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