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Short Communication

FIRE BLIGHT MANAGEMENT: PHYSIOLOGICAL ASSESSMENT OF CULTURAL CONTROL BY PRUNING IN PEAR ORCHARDS

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The aim of this work was to evaluate the photosynthetic performance of Pear trees (cv. 'Rocha') infected with *Erwinia amylovora*, three months after suffering a pruning of infected branches (P-trees) compared with asymptomatic trees (C-trees) of the same orchard. Three months after pruning, P-trees looked healthy and were negative for the presence of *E. amylovora*. In September of 2018, fully expanded leaves of both P- and C- trees were sampled and analysed for photosynthetic parameters related to chlorophyll *a* fluorescence and gas exchange, alongside with pigments, total soluble sugars, starch, and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) contents. No significant differences were found in chlorophyll and carotenoids levels, but anthocyanins significantly decreased in P-trees. Also, despite the maximum quantum yield (Fv/Fm) significantly decreased in P-trees, the effective quantum yield of the PSII was maintained, paralleled with no changes in gas exchange parameters (P_N, gs, Ci, E, iWUE, P_N/gs), nor in RuBisCO relative content. Finally, the maintenance of the levels of total soluble sugars and starch also supports that the photosynthetic performance of P-trees, three months after pruning, reached values similar to those of the C-trees, contributing to the normal development and ripening of the fruit. Data support that pruning represents a reliable control measure against this quarantine pathogen. This work is the first evaluation of pruning in fire blight management regarding carbon metabolism in *P. communis* trees.

Key words: *Erwinia amylovora,* photosynthesis, pruning, RuBisCO, pear tree

Fire blight, caused by the quarantine gram-negative bacteria *Erwinia amylovora* (Burril), is a highly destructive disease of Rosaceae, namely affecting the chain of value pears (*Pyrus communis*), apples (*Malus domestica*), loquats (*Eriobotrya japonica*), and quinces (*Cydonia oblonga*) (Piqué *et al*. 2015;

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Evrenosoğlu *et al*. 2019), and some ornamental species (EPPO 2013; Vrancken *et al*. 2013; Azarabadi *et al*. 2017; Català-Senent *et al*. 2017). Despite the intensive studies on this quarantine disease, there is no efficient way to cure infected plants, so preventive ways must be applied including the choice of the orchard site, shortening the duration of flowering, prevention of off-season blooming, and proper fertilisation and irrigation (Shtienberg *et al*. 2015; Chen *et al*. 2018). When preventive measures fail, different control methods have been applied, including cultural control (e.g., phytosanitary pruning), biological control (e.g., antagonist bacteria), and chemical control (e.g., copper application and antibiotics) (Mikiciński *et al.* 2016; Buttimer *et al.* 2017; Martins *et al.* 2018). In more severe cases, the countries' legislations (e.g., Portugal, Israel) demand the destruction of the infected trees (DGAV 2013; Shtienberg *et al.* 2015). However, due to the increasing restriction to the use of antibiotics on crops (e.g., streptomycin) (EC 2004; Lamichhane *et al.* 2018), and the increase of pathogens showing acquired resistance to antibiotics/copper, the need of different control strategies is increasing (Khan *et al.* 2012; Martins *et al.* 2018).

Cultural measures are thus the current mandatory control strategy, being pruning (immediately after detection) of the infected branches the most effective technique (Chen *et al.* 2018). During pruning, cuts should be made with 15 to 30 cm of distance from the visible symptom, using surface-disinfected tools (Shtienberg *et al.* 2003; Johnson & Temple 2017). Studies on pruning effectiveness were only focused on how this technique should be applied, if it controls the infection rate of *E. amylovora;* which period time should be applied; and if pruning influences the epidemiological dynamics (Shtienberg *et al.* 2003; 2015). To the present, the effects of pruning on the host physiology namely if and how these plants may display reduced photosynthesis and sugars mobilization, thus compromising flowering/fruiting apical parts, is still unknown. Whilst never explored in fire-blight hosts, the photosynthetic parameters related to chlorophyll a fluorescence and gas exchange have been used with success to understand the host-pathogen impact on carbon metabolism (Tatagiba *et al.* 2015; Sterling & Melgarejo 2018; Sekulska-Nalewajko *et al.* 2019). This technique is predominantly non-invasive providing highly informative endpoints on the photosynthetic response under conditions of pathogen-induced biotic stress (Bermúdez-Cardona *et al.* 2015; Sterling & Melgarejo 2018). Besides fluorescence parameters, sugars/starch quantifications are robust and simple complementary tools to better discriminate the physiological condition of plants, as sugars are essential for plant development and production, and are involved in crucial signalling and metabolomic processes (e.g., metabolic resource and regulators) (Zhang *et al.* 2017). Leaves infected by pathogens showed inhibition of net carbon assimilation rate, reduced ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity, stomatal conductance, mesophyll conductance, and chlorophyll content (Polanco *et al.* 2014; Halldorson & Keller 2018). These photosynthetic endpoints are thus critical to assess if pruning is being effective – as a cultural control strategy – in maintaining the plant homeostasis and carbohydrates availability for the normal plant development, including fruiting/ripening. The aim of this work was to assess if the photosynthetic performance was maintained, with no changes in the final carbohydrate levels, of pruned pear trees with infection of *E. amylovora,* after three months. To demonstrate this, chlorophyl a fluorescence and gas exchange measurements, along with pigments, total soluble sugars, starch, and RuBisCO quantification were applied to evaluate the carbon metabolism of the pruned-infected trees versus noninfected ones.

Plant material

Twelve-year-old *P. communis* cv. 'Rocha' trees grafted on Sydo rootstock from an orchard (Alcobaça, Portugal) (39.548393, −8.961914) planted at 4.5 m × 1.7 m spacing, were studied. The trees were well watered with a frequency of 4 times per week, from June to September. Six trees (control: C) never showed symptoms of fire blight and before the assay trees were not subjected to cultural controls. The other six trees had evidenced symptoms of fire blight (supplementary data 1) and were subjected to cultural control by pruning (P) immediately after detection. All trees were previously subjected to biological control with Serenade Max and Aliette Flash (both from Bayer, Leverkusen, Germany). Pruning took place in early June 2018, in stems and trunks infected (Figure S1). The pruning removed around

50% of the initial canopy (Figure 1). All cuttings took place according to the recommended practices, and the cuts were applied 50 cm below the last visible symptom, which is considered an intensive pruning as described previously by Shtienberg *et al*. (2015). On 13th September 2018 (~3 months after pruning), samples were used for photosynthetic analysis, and collected for biochemical assays, from both groups. All leaf samplings took place between 12h – 15h (the period of the day corresponding to the highest solar PAR intensity, and higher transpiration pressure). The measurements were performed under a or the clear sky with a total solar radiance of 27,914 ± 2,531 Lux and relative humidity (HR) of 50.5 ± 6.5% and temperature of 27 ± 1ºC, recorded by a weather station in the middle of the orchard. Climate conditions were monitored in 2018 for the months of the experiment (May-September), which corresponds to the period of time of the plant vegetative growth stage (Figure S2).

Nutritional data from the soil were measured at the same time as the pruning (Table S1). To confirm that asymptomatic pear trees were uninfected, samples were collected for the detection of *E. amylovora* following the EPPO guidelines (EPPO, 2013). Colonies presenting *E. amylovora* like colonies were further analysed by 16S rRNA partial gene sequencing (Table S2, one Enterobacteriaceae was identified, namely, *Pantoea* spp.).

Pigment content, fluorescence, and gas exchange

Photosynthetic pigments were quantified according to Sims and Gamon (2002). Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), carotenoids (Car), and anthocyanins (Ant) were extracted from frozen leaf powders in acetone: 50 mM Tris-HCl pH 7.8 buffer (80:20, v/v). Absorbance at 470, 537, 647, and 663 nm was read (Multiskan™ Microplate Spectrophotometer, Thermo Fisher Inc., MA, USA). Results are expressed as mg/kg (FM).

Chl *a* fluorescence was measured *in situ* in fully expanded leaves and measurements were performed with a pulse amplitude modulation system (FluorPen FP 110, Photon Systems Instruments, Czech Republic) according to Dias *et al*. (2018). After 30 min of adaptation to the dark, leaves were sampled by applying a weak modulated light. For the determination of the maximum fluorescence (Fm), a saturating pulse of white light was applied. In 30 min light-adapted expanded leaves (exposed to sunlight) the corresponding Fm' and the steady-state fluorescence (F') were obtained. The potential maximum quantum yield of PSII [Fv/Fm = (Fm – F0)/Fm] and the effective quantum yield of PSII [Φ_{PSII} = (Fm' – F')/Fm'] was then calculated.

In fully expanded leaves, the net CO_2 assimilation rate [P_N, µmol (CO_2)/m^2/s], stomatal conductance [g_s, mmol (H_2O)/m^2/s], the intercellular CO_2 concentration (C_i, ppm), and transpiration rate [E, mmol (H_2O)/m^2/s] were measured *in situ* with an infrared gas analyser (LI-COR 6400 Portable Photosynthesis Systems, USA). Also, the intrinsic water-use efficiency [iWUE = P_N/gs, µmol (CO_2)/mmol (H_2O)], and the intrinsic carboxylation efficiency [P_N /C_i, µmol (CO_2)/ppm] were calculated.

Total soluble sugars and starch

Total soluble sugars (TSS) and starch contents were quantified by the anthrone method as described by Dias *et al.* (2018). Absorbance at 625 nm was read at a Multiskan™ Microplate Spectrophotometer (Thermo Fisher Sci, MA, USA). Results are expressed as mg/g (FM).

Relative RuBisCO quantification

Soluble proteins extracted from N_2-frozen leaves were quantified by the Bradford method (Sigma-Aldrich, USA). SDS-PAGE was used for ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) analysis according to Li *et al*. (2013), using 15 µg of protein, and a Protein Molecular Weight Marker (DynaMarker® Protein MultiColor Stable, BioDynamics Lab. Inc., Japan). Bands were stained with 1% Coomassie Brilliant Blue R250. Relative RuBisCO quantification of large and small subunits was done after 8h incubation in 2 mL formamide at 50ºC. Absorbance was read at 595 nm in a Multiskan™ Go Microplate Spectrophotometer (Thermo Fisher Sci. Inc., MA, USA), and the results are expressed as $ABS_{Rubisco\ content}/ABS_{Protein\ content}$.

Statistical analysis

For fluorescence and gas exchange measurements six fully expanded leaves (with similar age/size), of each tree (biological replicates) were used (n = 6). For pigments, carbohydrates, and relative RuBisCO quantification, a pool of leaves from each group, C and P [each pool consisting of 30 leaves from the six trees (5 leaves of each tree) were used

(n = 6)]. Five technical replicates of each pool were analysed. Comparisons between the C and P conditions were made using the T-test with Welch's correction using GraphPad Prism.v6 for Windows (GraphPad Software, La Jolla, USA). Results were considered statistically different when $p < 0.05$.

Three months after pruning, the six P-trees showed a healthy status and no evidence of exudates or symptoms of fire-blight. Branches looked healthy and the leaves did not have symptoms of chlorosis (Figure 1). The sequencing of the partial 16S gene to determine the presence of the genus *Erwinia* of the collected samples of the P-trees did not present results for any *Erwinia* spp., thus confirming an absence of *E. amylovora* in the P-trees studied.

The levels of Chl *a* in C-trees are 352.5 ± 11.3 mg/kg (FW) being, as expected, the double of the Chl *b* values [145.6 ± 7.7 mg/kg (FW)]. On P-trees the values were similar [355.7 ± 16.9 mg/kg (FW) for Chl *a,* and 143.3 ± 5.1 mg/kg (FW) for Chl *b*], and thus no significant differences were detected for Chl *a*, *b*, and Chl *a*/*b* ratio. Also, no changes were found in carotenoids levels [122.7 ± 5.9 mg/kg (FW) for C-trees and 115.1 ± 7.7 mg/kg (FW)] between the two groups of trees. Interestingly, a significant decrease of anthocyanins was observed in P-treated trees [34.3 ± 0.9 mg/kg (FW) compared to 48.9 ± 4.8 mg/kg (FW) in C-Plants, $p \leq 0.001$, Figure 2]. The maximum quantum yield of photosynthesis given by the ratio F_v/F_m (Figure 3a) showed significantly lower values ($p \leq 0.05$) in P-trees (0.76 ± 0.01) compared to the C-trees (0.79 ± 0.03). Considering the effective quantum yield of PSII, it ranged between 0.69 ± 0.04 in C-trees and 0.66 ± 0.03 in P-trees, showing no significant variation ($p > 0.05$; Figure 3b). Regarding gas exchange, both C- and P-trees showed a similar behaviour regarding P_N [20.6 ± 0.7 µmol $(CO_2)/m^2/s$ for C and 19.8 ± 0.9 µmol $(CO_2)/m^2/s$ for P], gs [268.9 ± 29.1 mmol $(H_2O)/m^2/s$ for C and 298.5 ± 40.6 mmol $(H_2O)/m^2/s$ for P], E [6.1 ± 0.7 mmol $(H_2O)/m^2/s$ for C and 6.9 ± 0.9 mmol $(H_2O)/m^2/s$ for P], Ci [130.9 ± 19.8 ppm for C and 114.2 ± 8.3 ppm for P], iWUE [0.077 ± 0.009 µmol (CO_2)/mmol (H_2O) for C and 0.077 ± 0.019 µmol (CO_2)/mmol (H_2O) for P], and intrinsic carboxylation efficiency [0.16 ± 0.02 µmol (CO_2)/ppm for C and 0.18 ± 0.03 µmol (CO_2)/ppm for P] (Figure 4), with no significant differences in any parameter

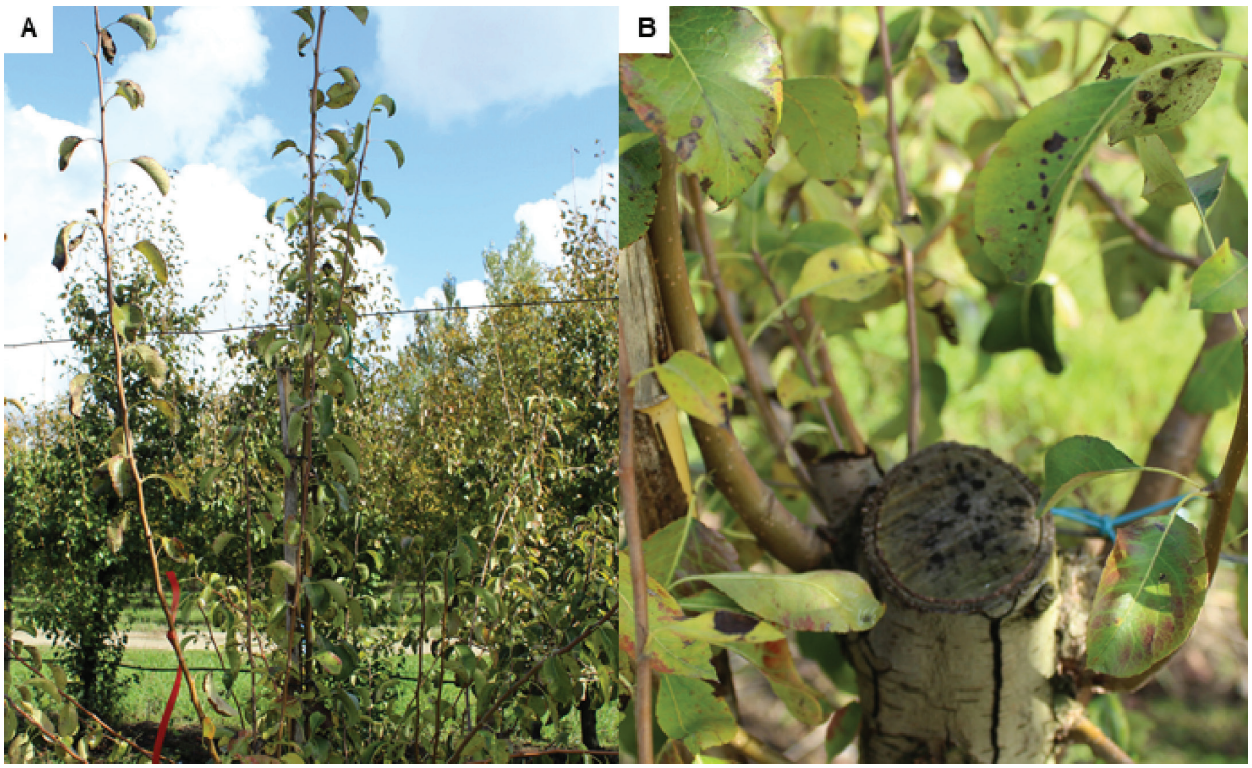

Figure 1. A) Pruned Pear tree cv. 'Rocha', not showing traces of infection by *E. amylovora,* and B) pruned trunk of the same Pear tree cv. 'Rocha'

(Figure 4, $p > 0.05$).

In the same way, the amounts of TSS [58.2 ± 2.3 mg/g (FM) for C and 54.7 ± 2.7 mg/g (FM) for P], starch [102.9 ± 5.9 mg/g (FM) for C and 109.3 ± 9.8 mg/g (FM) for P], and the relative content of RuBisCO (0.43 ± 0.02 ABS_{RC}/ABS_{TPC} for C and 0.40 ± 0.02 ABS_{RC}/ABS_{TPC} for P) remained unaffected between the two groups (Figure 5).

The Chlorophyll levels measured in Pear 'Rocha' trees are close to those of other pear cultivars like 'Abbé Fétel' and 'Passe Crassane' (Rotondi & Predieri 2002). Our data also show that three months after pruning, the levels of Chl *a*, Chl *b*, Chl *a*/Chl *b* ratio, and carotenoids in P-trees are similar to those of uninfected-unpruned control plants, indicating that the photosynthetic pigments of both light-harvesting complex II/I photosystems are not affected. A casual pruning, in consequence of fire blight or not, by reducing the canopy, this may increase the photon accessibility on pear trees. Canopy size of pear trees and compactness, resulting from different plant densities, pruning techniques, or canopy management, influence the plant´s performance, namely light interception and distribution (Rotondi & Prediari 2002; Sousa & Abreu 2015).

Anthocyanins are an end-product of one of the several pathways of the phenylpropanoid (Phe) metabolism, and their synthesis is controlled by external environmental factors including pathogen infection, and extreme temperature or light (Wu *et al*. 2019).

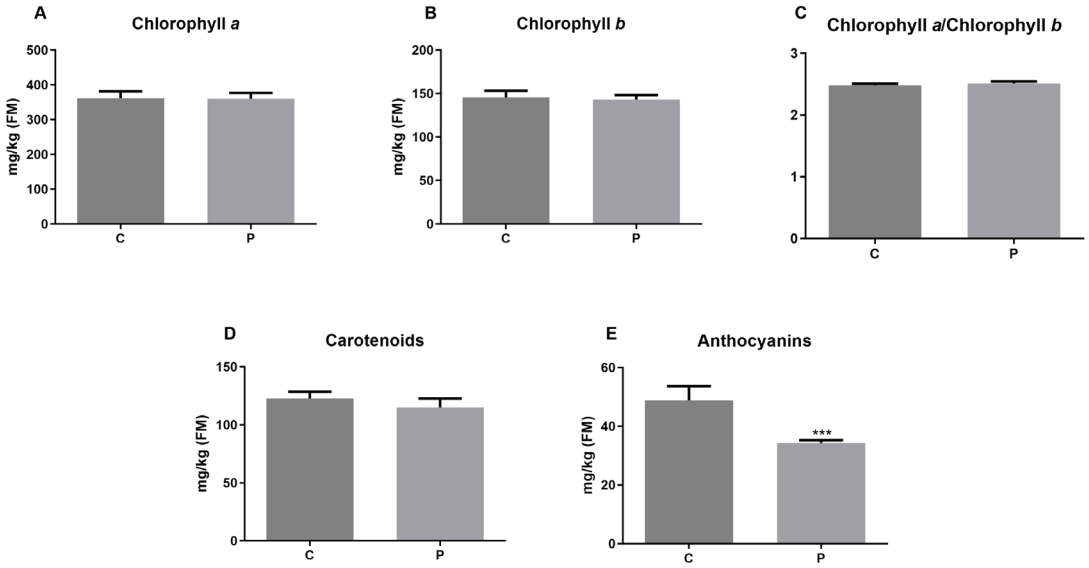

Figure 2. A) Levels of chlorophyll *a,* B) chlorophyll *b,* C) Chl *a*/Chl *b,* D) carotenoids, and E) anthocyanins, in control trees (C) and in infected pruned trees after 3 months (P). Vertical bars: mean value with standard deviation (n = 6), $p < 0.001$.

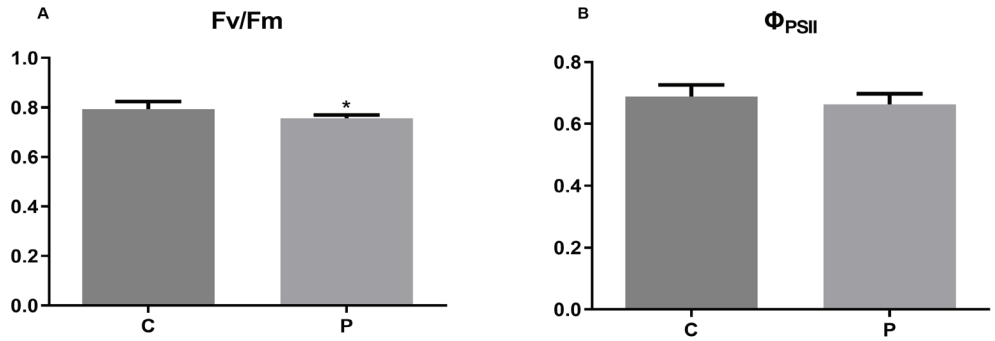

Figure 3. A) Maximum quantum yield (Fv/Fm), and B) effective quantum yield of PSII (Φ_{PSII}), in control trees (C) and in infected pruned trees after 3 months (P). Vertical bars: mean value with standard deviation (n = 6), $p < 0.05$.

Despite anthocyanin synthesis and degradation occurring simultaneously, anthocyanin degradation is often associated with plant-specific developmental stages or changes in those environmental factors (Wu *et al*. 2019). Stimulation of Phe biosynthesis under pathogen infection (Wallis & Chen 2012) may lead to anthocyanins accumulation. Also, pruning was demonstrated to increase anthocyanin levels in several species such as *Malus domestica* (Matsuoka 2019) and *Hibiscus sabdariffa* (Susanto *et al*. 2015). A possible explanation for this decrease in P-plants may involve specific shifts in secondary metabolism as a result of *E. amylovora* infection and/or pruning, with a lower investment in the chalcone pathway, decreasing the synthesis of anthocyanins. Shifts in secondary metabolism have been suggested in other *Erwinia* spp., for example, it was reported that *Erwinia mallotivora* infected tissues contained a higher concentration of total phenolic compounds than control plants (Shahida *et al*. 2016).

Interestingly, while in C-trees Fv/Fm values are ≈0.8, typical of healthy plants, in P-trees these values were lower (≈0.75). A reduction in the Fv/Fm is often linked to higher senescence (Kumagai *et al*. 2009), which in the present case indicates that 3 months after pruning, infected trees still have some indicators of stress (possibly some inactivation/damage of PSII) resulting in photoinhibition or in increased quenching (Murchie & Lawson 2013). However, this decrease is not paralleled by a reduction of the effective quantum yield of PSII, suggesting that in both C- and P- conditions there is relative stability of the proportion of incident photons that are used to drive photochemistry. It can be assumed

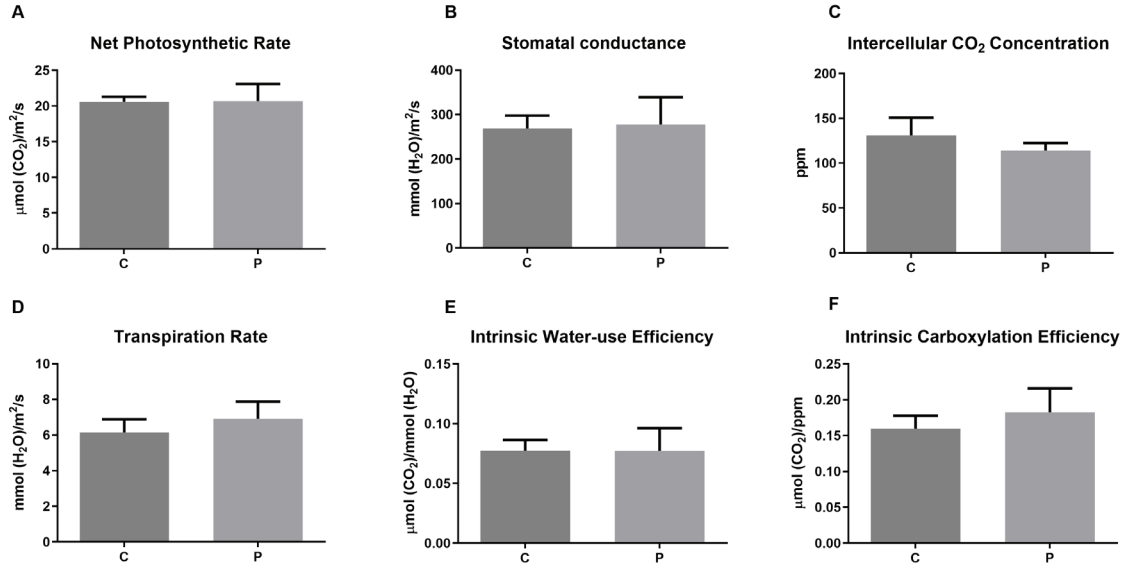

Figure 4. A) Net photosynthetic rate (PN), B) stomatal conductance (gs), C) intercellular CO2 concentration (Ci), D) transpiration rate (D) intrinsic water-use efficiency (iWUE), and F) instrinsic carboxylation efficiency(PN/gs), in control trees (C) and in infected pruned trees after 3 months (P). Vertical bars: mean value with standard deviation (n = 6).

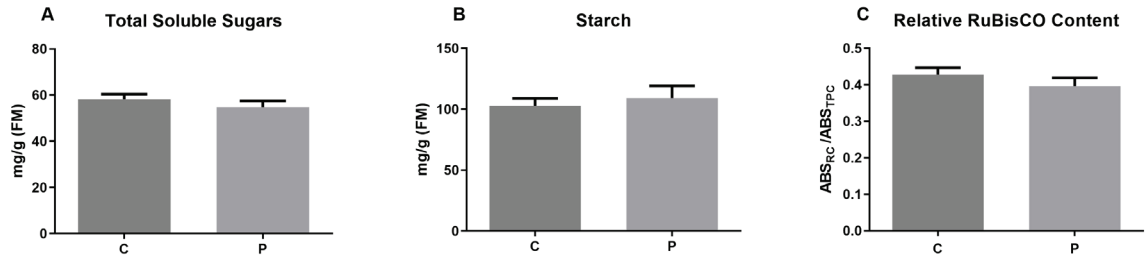

Figure 5. A) Levels of total soluble sugars, B) starch, and C) relative RuBisCO content, in control (C) and in infected pruned trees after 3 months (P). Vertical bars: mean value with standard deviation (n = 6).

that Φ_{PSII} [i.e., (Fm'–F')/Fm'] is intimately linked to the closure/opening of PSII during primary photosynthetic reactions, and the fluorescence emission of light-harvesting pigment molecules (Ye *et al*. 2017).

Nevertheless, interesting data provided here showed that gas exchange, relative RuBisCO content, and Chl levels were not compromised after disease and pruning, namely in P-trees (Figure 2a-c and 4). As proposed elsewhere (Vicente *et al*. 2011) the ratio between RuBisCO and Chl provides a simple indication of the changes in the light-harvesting capacity to electron transport and CO_2 assimilation activity. Whereas Chl and the relative amount of RuBisCO did not change in P compared to C trees, as well as TSS and starch contents, some studies demonstrate some negative effects of infection on photosynthetic related parameters (Lindenthal *et al*. 2005). Also, the maintenance of the iWUE means that after three months, P-trees were able to show a similar rate of photosynthesis obtained for the observed stomatal conductance. The P_N/Ci is a limiting factor for CO_2 fixation, depending on the plant's carboxylation capacity, i.e., on the RuBisCO activity, thus being leaf photosynthesis conditioned by RuBisCO capacity under atmospheric CO_2 concentration (Haritha *et al*. 2017). Our data evidences that P-plants were equally efficient at carboxylation, and in the RuBisCO enzyme as C-plants.

Overall, the negative data for *E. amylovora* presence three months after pruning, suggest that pruning might have limited the fast progression of this bacteria, in those trees. Also, data show that despite pruning techniques reduce canopy size and compactness, influencing the plant´s performance, namely light interception and distribution (Rotondi & Prediari 2002; Sousa & Abreu 2015), our data show that P-trees were able to achieve a similar photosynthetic performance as C-trees.

CONCLUSIONS

The application of pruning as a cultural control after fire blight episode is demonstrated here to be an efficient method. Three months after pruning trees canopy, anthocyanins levels and the Fv/Fm significantly decreased, nevertheless the remaining pigments quantified (chl *a*, chl *b*, and caratonoids), along with TSS, starch, RuBisCO, gas exchange and Φ_{PSII} maintained their levels close to those of control trees. The pruning treatments consisted of the removal of vigorous upright shoots, as well as dead or diseased branches, immediately after the onset of the first symptoms, to control *E. amylovora* propagation and increase of the disease. This quick cultural response may prevent totally or partially infected orchards' death and is a suitable substitute for what is described in the country legislation regarding fire blight (total removal of infected and nearby trees). On the other hand, this method allows a better light distribution in the canopy, contributing to normal photosynthetic performance. This work is the first evaluation of pruning in fire blight management regarding carbon metabolism in *P. communis* trees.

Authors' contribution and Funding

R.J. Mendes, F. Tavares, and C. Santos planned the experiments and wrote the manuscript. R.J. Mendes performed all experiments, assays, and statistical analyses. N. Mariz-Ponte, C.V. Correia supported R.J. Mendes in biochemical assays. M.C. Dias and M. L. de Sousa supported in chlorophyll *a* and gas exchange measurements. All authors contributed to the manuscript revision, and state that they have no conflict of interests. FCT supported R.J. Mendes and N. Mariz-Ponte (SFRH/BD/133519/2017 and SFRH/BD/138187/2018, respectively) and the contract research of M.C. Dias (SFRH/BPD/100865/2014). Thanks are also due to FEDER/COMPET/POC (UID/QUI/50006/2019). The authors would like to thank Engineer Rui Sousa of INIAV for granting access to the pear cv. 'Rocha' orchard, and its maintenance. The authors declare no conflict of interest.

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