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RESEARCH PAPERS

Ozone for post-harvest treatment of apple fruits

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Summary. Different biotic contaminations can affect apple production. Among these, infections by *Penicillium expansum*, the causal agent of blue-green post-harvest rot and patulin production, is particularly important. Fruit of the apple varieties: 'Royal Gala', 'Golden Delicious' and 'Fuji' were challenged with a patulin-producing *P. expansum* strain and stored at 1 ± 1°C in presence of gaseous ozone at 0.5 μL L^{-1} for 2 months. During the storage period, fungal populations, the biosynthesis of patulin and the activity of some Pathogenesis Related Proteins (glucanase, peroxidase and phenylalanine ammonia-lyase) were evaluated. Ozone treatment reduced fungal populations and patulin production. The activity of the assayed enzymes was not directly or clearly correlated with the inhibiting effect of ozone. These results indicate that ozone could be used to increase storage duration of apple varieties to maintain their quality.

Key words: Patulin, *Penicillium expansum*, glucanase, peroxidase, phenylalanine ammonia-lyase.

Introduction

Apple production in Europe amounts to about 12 million tonnes (Pesticide Action Network (PAN) Europe, May 2007), which represents 15% of the world production. Product losses occur all along the production chain. In particular, losses due to post-harvest diseases are costly since these include the cumulative value of growing, harvesting and storing apple fruit. As a general rule, apples can be stored for up to 10 months in cold storage combined with controlled-atmosphere (CA) environment prior to being sold. The most important diseases with economic importance for apples are caused by fungi [e.g. *Pezicula malicorticis* (H. Jacks), *Botrytis cinerea* Pers.:Fr., *Mucor* spp., *Gloeosporium perennans* Zeller & Childs, *Penicillium expansum (Link)*, *Phialophora malorum* Kidd & Beaum]. In particular, *P. expansum* is the causal agent of blue-green rot, one of the most economically important post-harvest rots of apples. The fungus is the most important patulin (PAT) producing organism in fruits, especially on pomefruits (Andersen *et al.*, 2004). PAT is a mycotoxin with genotoxic, neurotoxic, immunotoxic and immunosuppressive effects (CAST, 2003; Moake *et al.*, 2005). Toxic effects caused by PAT are hazards to public health, and its presence has therefore been regulated in different countries. Controlling *P. expansum* development and PAT contamination, to obtain cosmetically perfect produce in conventional apple growing, requires many preventive fungicide treatments. However, the development of resistance to fungicides and the health concerns due to contamination of the environment with pesticides, have resulted in restrictions in the use of these materials, which has resulted in increased research on alternative control measures, including ozone (O_3) (Suslow, 2004).

Ozone has been evaluated for post-harvest disease control and other storage uses for many years; in 2001 it was listed as a secondary direct food additive permitted in food for human consumption (FDA, 2009). The use of O_3 in the management of post-harvest quality of horticultural products has been extensively studied in the last decade. In par-

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ticular, the effects of ozone were evaluated on the growth of *Penicillium digitatum*, *P. italicum*, and *B. cinerea* on table grapes (Ozkan *et al*., 2011; Yaseen *et al*., 2014); on the sporulation of *P. digitatum* and *P. italicum* on packed oranges (Palou *et al*., 2003); on the growth of *Monilinia fructicola* on inoculated peach (Palou *et al*., 2002); on *P. digitatum* and *P. italicum* on artificially inoculated orange fruits (Di Renzo *et al*., 2005; Yaseen *et al*., 2013); on decay of carrots caused by *Sclerotinia sclerotiorum* and *B. cinerea* (Hildebrand *et al*., 2008), on tomato using low-level atmospheric ozone exposure (Tzortzakis *et al*., 2011; González-Fernández *et al*., 2014), on kiwifruit affected by stem-end rot caused by *B. cinerea* (Hur *et al*., 2005; Minas *et al*., 2010), and for inhibition of ethylene biosynthesis (Minas *et al*., 2014).

The overall aim of the study reported in the present paper was to investigate the use of O_3 to maintain the quality of apples during storage. For this purpose the widely marketed apple fruit varieties 'Royal Gala', 'Golden Delicious' and 'Fuji' were challenged with a patulin-producing *P. expansum* strain and stored in presence of gaseous O_3 at 0.5 μL L^{-1} for 2 months. This concentration of O_3 was selected based on previous small scale *in vitro* experiments. During the storage period fungal populations, biosynthesis of patulin and the activity of some Pathogenesis Related Proteins (glucanase, peroxidase and phenylalanine ammonia-lyase) were evaluated.

Material and methods

Apple fruits

Apple fruits, of varieties 'Royal Gala', 'Golden Delicious' and 'Fuji', with no signs of rots, were obtained from a warehouse (Mottola, Taranto-Italy) immediately after harvest, and stored in refrigerated cells at 1 ± 1°C at 0.95% RH for 60 d in presence of O_3. The experiments were performed with non-inoculated and artificially inoculated fruits. Control apple fruits were stored under the same environmental conditions in conventional atmosphere.

Fungal isolation

Different isolates of *P. expansum* were obtained from naturally infected blue mould decayed apple fruits. All isolates were obtained using single spore purification methods on Pentachloro Rose Bengal Yeast Extract Agar (PRYES Agar), and an incubation period of 3-4 d at 25°C. The isolates obtained were assessed for patulin production using quantification by high-performance liquid chromatography (HPLC). The isolate shown to produce the greatest amount of patulin was chosen for the fruit storage experiments.

PRYES Agar contains 150 g sucrose, 20 g yeast extract, 0.1 g Pentachloronitrobenzene (PCNB), 0.025g rose Bengal, 0.05 g chloramphenicol, 0.05 g chlortetracycline, and 20 g agar per litre (Bridson *et al*., 1970).

Fungal inoculum

A suspension was prepared by washing the selected *P. expansum* culture with 4 mL of sterile distilled water containing 0.01% of Tween20, and scraping conidia by a sterile loop. Conidia were counted using a Thoma chamber and adjusted to 2.5×10^5 conidia mL^{-1} (5×10^3 conidia in 20 μL). Apples were each wounded at four points using a cork borer. Each wound (diameter 5 mm, depth 5 mm) was inoculated with 20 μl of conidium suspension of *P. expansum*.

The experimental design consisted of eight boxes of apple fruits for each variety (inoculated with *P. expansum* and non-inoculated) maintained in the cold chamber at 1 ± 1°C in the presence of O_3 for 60 d. The same arrangement of samples was used in in the conventional atmosphere. Ozone enriched atmosphere (0.5 μL O_3 L^{-1}) was obtained by an ozone generator (Air Met) supplied by MET srl. Ozone concentration was kept constant during the duration of the experiment by using a sensor. The experiments were also performed on non-inoculated apple fruits in order to evaluate the behaviour of naturally contaminated apples.

Fungal populations and patulin determination

The effect of O_3 on fungal infection was determined by the quantification of fungal colony forming units (CFUs) on fruit surfaces, by washing four apples for each variety with 500 mL distilled sterile water. After 30 min of orbital shaking at 150 rpm, 10 mL of the washing liquid was removed and 100 μl was plated onto PDA in 9 cm diam. Petri dishes. Serial dilutions (10 to 10^3) of this washing liquid were also plated onto PDA plates, and these were

incubated at 24°C for 5 d. After each sampling period *P. expansum* colonies were identified using morphological criteria (Pitt and Hocking, 1985) and counted.

Four apples from each treatment were weighed and homogenized for Patulin and protein determination. Patulin quantification in the apple puree was carried out by HPLC analysis, using the method described by Ricelli *et al.* (2007).

Protein determination

Protein concentration was determined according to Bradford (Bradford, 1976), using the Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd). Total protein concentration was determined by the following formula: [prot] = (ΔAbsorbance- 0.3138) / 0.1538 (Zor and Selinger, 1996; Carlsson *et al.*, 2011).

Four apple fruits for each sampling period for each variety were homogenized (kept in ice) and stored at -80°C until analysed to quantify β-1,3-glucanase, peroxidase, phenylalanine ammonia-lyase (PAL), using the method described by Youssef *et al.*, (2014).

All the experiments were repeated twice, carrying three replicates of each measure. The data were statistically analysed using Statistica software (ver. 7.0, StatSoft). Mean values were evaluated using ANOVA and compared using Fisher's Protected LSD Test ($P = 0.05$).

Results

During the first 15 d of incubation, the number of *P. expansum* CFUs in all considered varieties did not significantly change in any of the experimental conditions. However, within 45 d of incubation, in all samples stored in conventional atmosphere, gave significant increases in numbers of CFUs. The increasing trend was maintained until the end of the storage period. Fruits stored in conventional atmosphere showed infection percentages between 65 and 75% depending on the variety. In the ozone treated fruits, however, the infection percentages was less than 5% at the end of the experiment period. On the contrary, in samples stored in the presence of O_3, there was no significant increase in numbers of *P. expansum* CFUs, except for the non-inoculated 'Royal Gala' apples after 60 d of storage (Figure 1 a–f).

In the samples stored in conventional atmosphere, apples inoculated with PAT-producing *P. expansum* and non-inoculated fruits, both showed significant increases in PAT production after 45 or 60 d depending on the variety. For apples stored in presence of O_3, no significant increase in PAT production was detected although some increase was detected after 45 d storage in 'Golden Delicious' fruit, and after or 60 d in 'Royal Gala' (Figure 2 a–f).

Glucanase activity increased significantly in the samples stored in conventional atmosphere, both for *P. expansum* inoculated and non-inoculated 'Royal Gala' and 'Fuji' fruit, after 45 d. In 'Golden Delicious' apples, glucanase increased after 45 d of incubation in inoculated samples and after 60 d in the non-inoculated samples. On the other hand, the samples treated with O_3 showed significant increases in glucanase activity only in inoculated 'Golden Delicious' apples after 60 d of storage (Figure 3 a–f).

Peroxidase activity in the samples stored in conventional atmosphere did not increase in 'Golden Delicious' apples, while in both inoculated and non-inoculated 'Fuji' apples in both fruits this activity increased significantly after 60 d. In 'Royal Gala', the non-inoculated samples showed significant increases in peroxidase activity after 15 d of storage while in *P. expansum*-inoculated samples the increase in activity was detected after 45 d of storage. In the samples treated with O_3, the activity of peroxidase increased only in 'Royal Gala' apples after 30 d and in 'Fuji' apples after 15 d when analysed in inoculated samples (Figure 4 a–f).

The activity of phenylalanine ammonia-lyase (PAL; Figure 5) in the apples stored in conventional atmosphere decreased in 'Royal Gala' apples both in inoculated and in non-inoculated fruits, starting from 45 d of storage. In 'Golden Delicious', a decline in PAL activity was evident after 45 d in non-inoculated apples while in the inoculated fruits a succession of decreases and increases was detected. In 'Fuji', PAL activity increased significantly after 60 d of incubation in non-inoculated samples, while the inoculated samples showed a succession of reductions and increases. In the presence of O_3 a reduction of PAL activity followed by an increase occurred in all 'Royal Gala' samples, in 'Golden Delicious' apples and inoculated 'Fuji' samples alternating increases and reductions were detected, while in the non-inoculated samples no changes in enzymatic activity were detected.

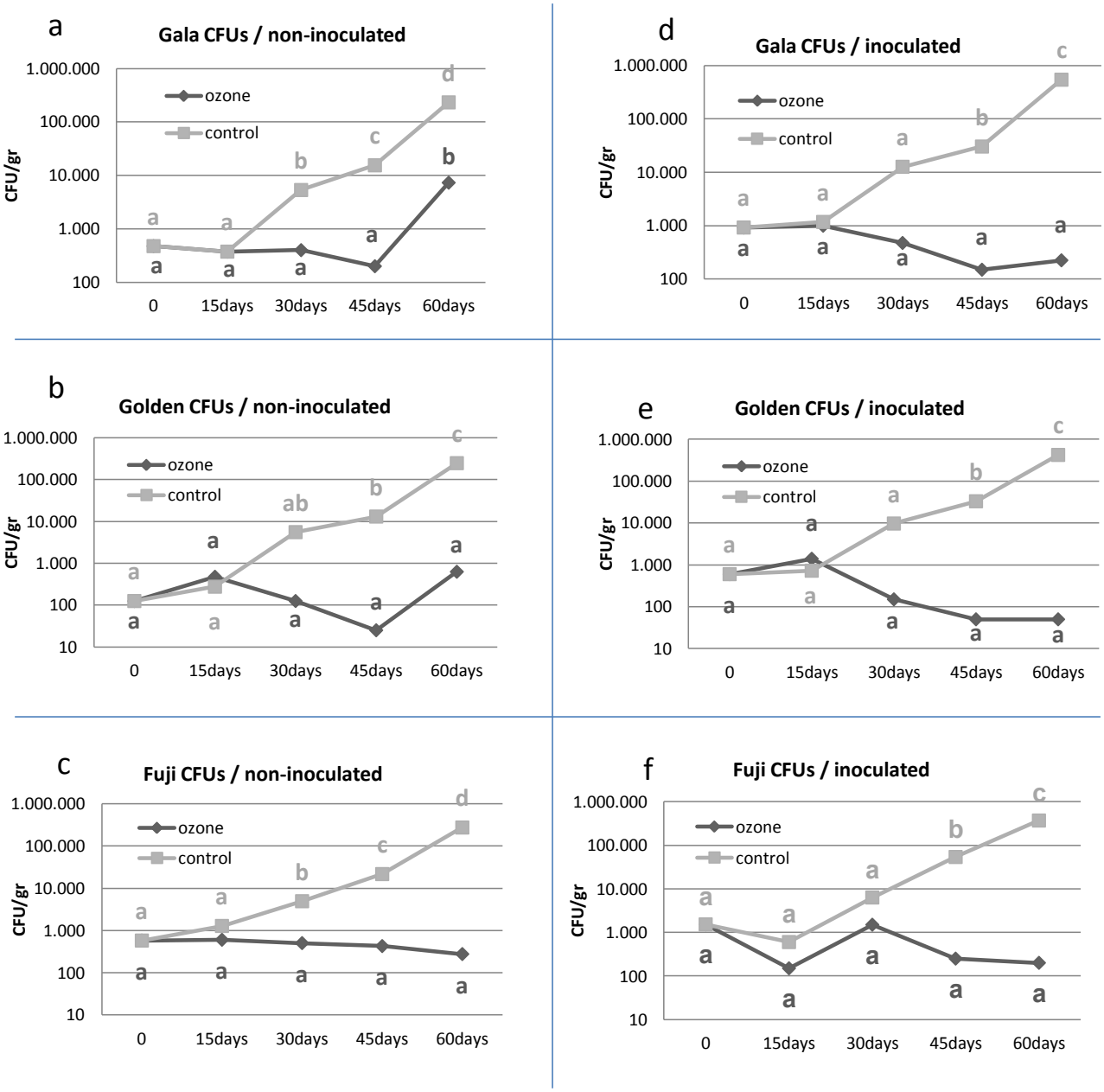

Figure 1. *Penicillium expansum* colony forming units (CFUs) from the surface of non-inoculated (a, b, c) and *P. expansum*-inoculated (d, e, f) apple fruit ('Royal Gala', 'Golden Delicious' and 'Fuji'), either non-treated (control) or treated with ozone (0.5 μL L^{-1}) and stored at 1 ± 1°C in the dark. Means followed by the same letter are not significantly different (Fisher's Least Significance Difference test (LSD, $P = 0.05$)).

Discussion

Little is known about effects of O_3 treatment of apples during post-harvest storage (Smilanick *et al.*, 1999; Skog and Chu, 2001).

The data obtained during the present study showed that the use of O_3 during post-harvest storage reduced the populations of *P. expansum*, as well as patulin biosynthesis associated with *P. expansum*

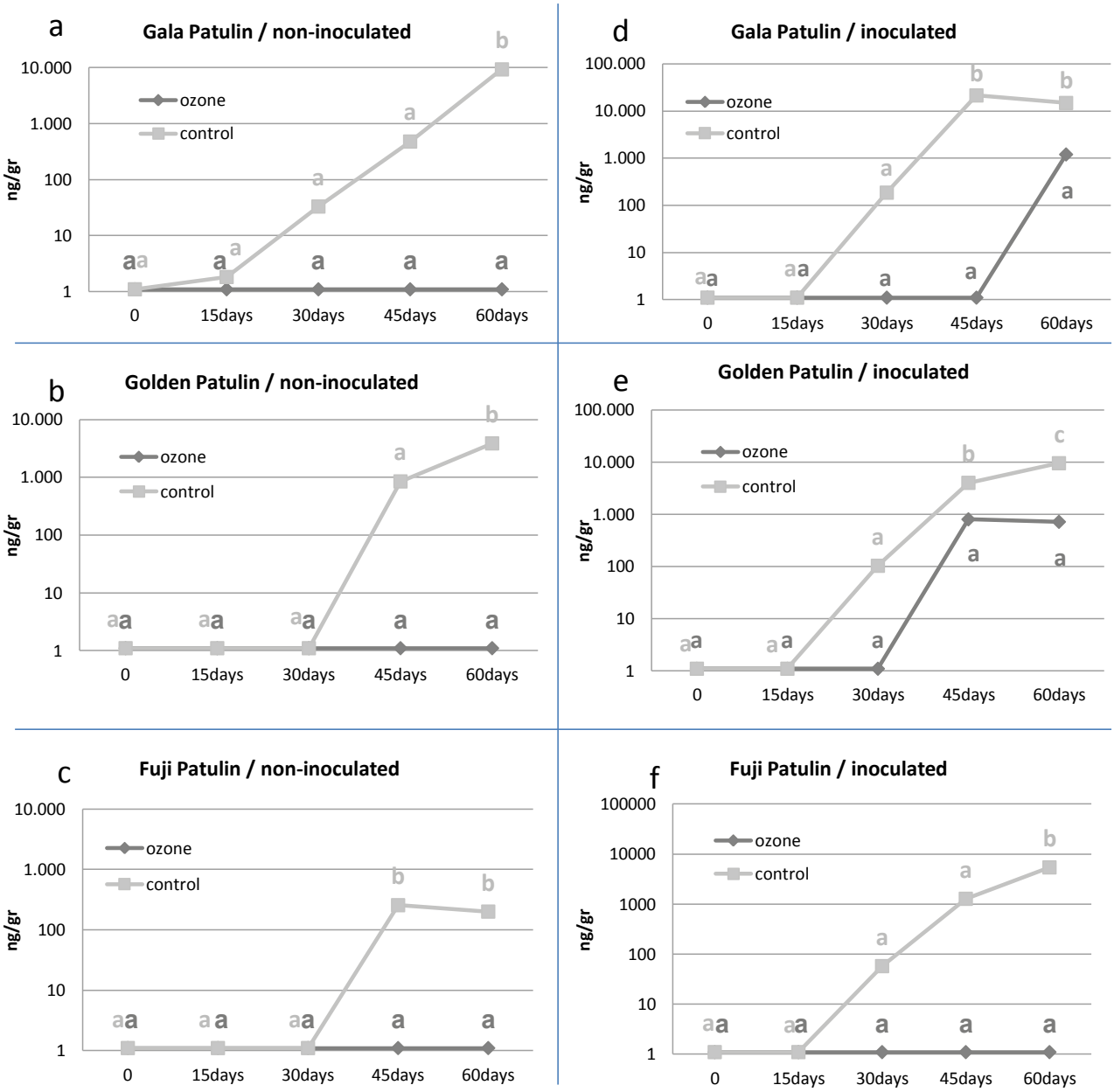

Figure 2. Mean patulin content in non-inoculated and *Penicillium expansum* inoculated apple fruits ('Royal Gala', 'Golden Delicious' and 'Fuji-), either non-treated (a, b, c) or treated by ozone at 0.5μL L^{-1} (d, e, f) and stored at 1 ± 1°C in the dark. Means followed by the same letter are not significantly different (Fisher's Least Significance Difference test (LSD, $P = 0.05$)).

development, in three varieties of apples. The inhibition obtained in non-inoculated apples reproduces a common situation in which the contamination by *P. expansum* is probable but not homogeneous. On the other hand, the inhibition obtained in apples challenged with *P. expansum* further confirms the results obtained, since the presence of *P. expansum* was much greater and homogeneous on the inoculated apples. The inhibition of fungal growth resulting from O_3 treatment also led to the inhibition of the production

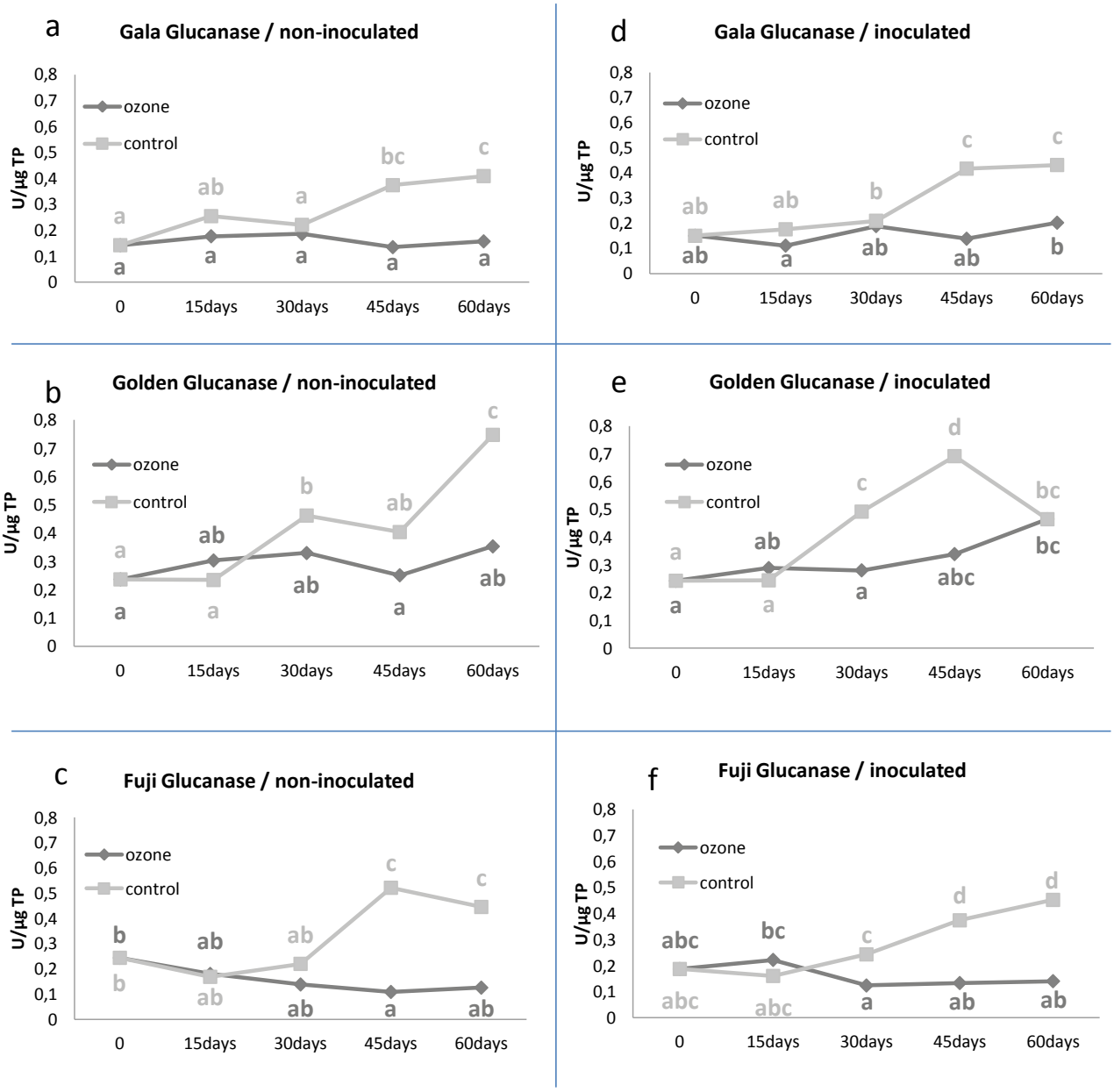

Figure 3. Mean glucanase specific activity in non-inoculated and in *Penicillium expansum* inoculated apple fruits ('Royal Gala', 'Golden Delicious', 'Fuji'), either non-treated (a, b, c) or treated by ozone at 0,5 µL L^{-1} (d, e, f), and stored at 1°±1C in the dark. Means followed by the same letter are not significantly different (Fisher's Least Significance Difference test (LSD, $P = 0.05$)).

of patulin. Patulin is a secondary metabolite; so its biosynthesis is not necessarily correlated with fungal growth. Some treatments able to inhibit the growth of *P. expansum* can cause increase in mycotoxin production (Schmidt-Heydt *et al.*, 2013).

Glucanase is a well-known biochemical marker of Systemic Acquired Response (SAR), peroxidase helps to protect plants against oxidative damage reducing free radical production from H_2O_2. Increase in these compounds is usually observed following fungal at-

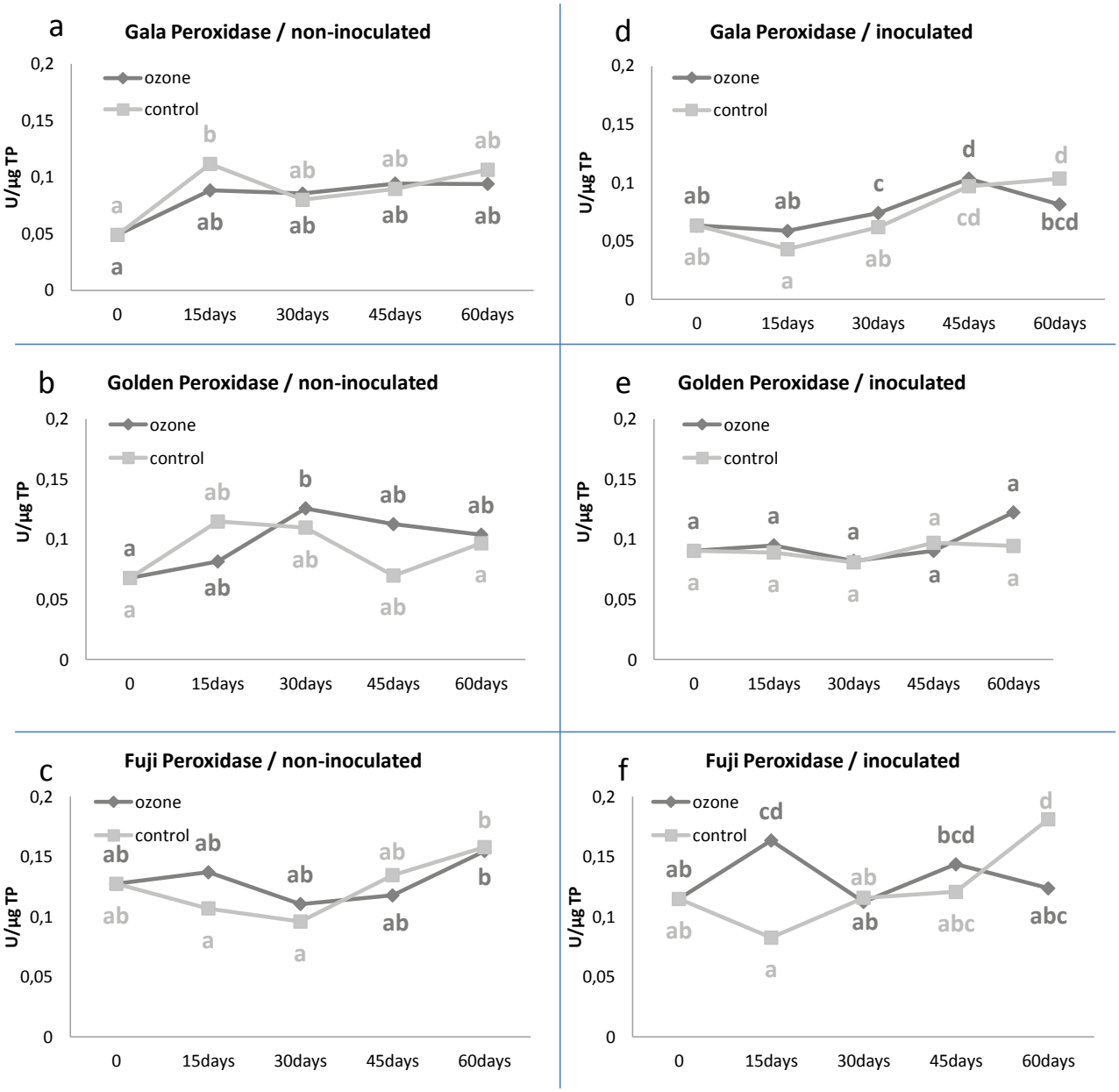

Figure 4. Mean peroxidase specific activities in non-inoculated and *Penicillium expansum* inoculated apple fruits ('Royal Gala, 'Golden Delicious, 'Fuji'), either non-treated (a, b, c) or treated by ozone at 0,5µL L^{-1} (d, e, f), stored at 1°±1C in the dark. Means followed by the same letter are not significantly different (Fisher's Least Significance Difference test (LSD, $P = 0.05$)).

tack, and the onset of the resistance has often been correlated with the accumulation of β-1,3-glucanases (Wang *et al.*, 2013). PAL catalyzes the first step of the phenylpropanoid pathway which regulates the biosynthesis of different bioactive small molecule aromatic compounds and lignin. Increased PAL activity was observed following *P. expansum* attack of apple fruits (Schovánková and Opatová, 2011).

The activity of glucanase is generally inhibited by treatment with O_3, while for the other investigated en-

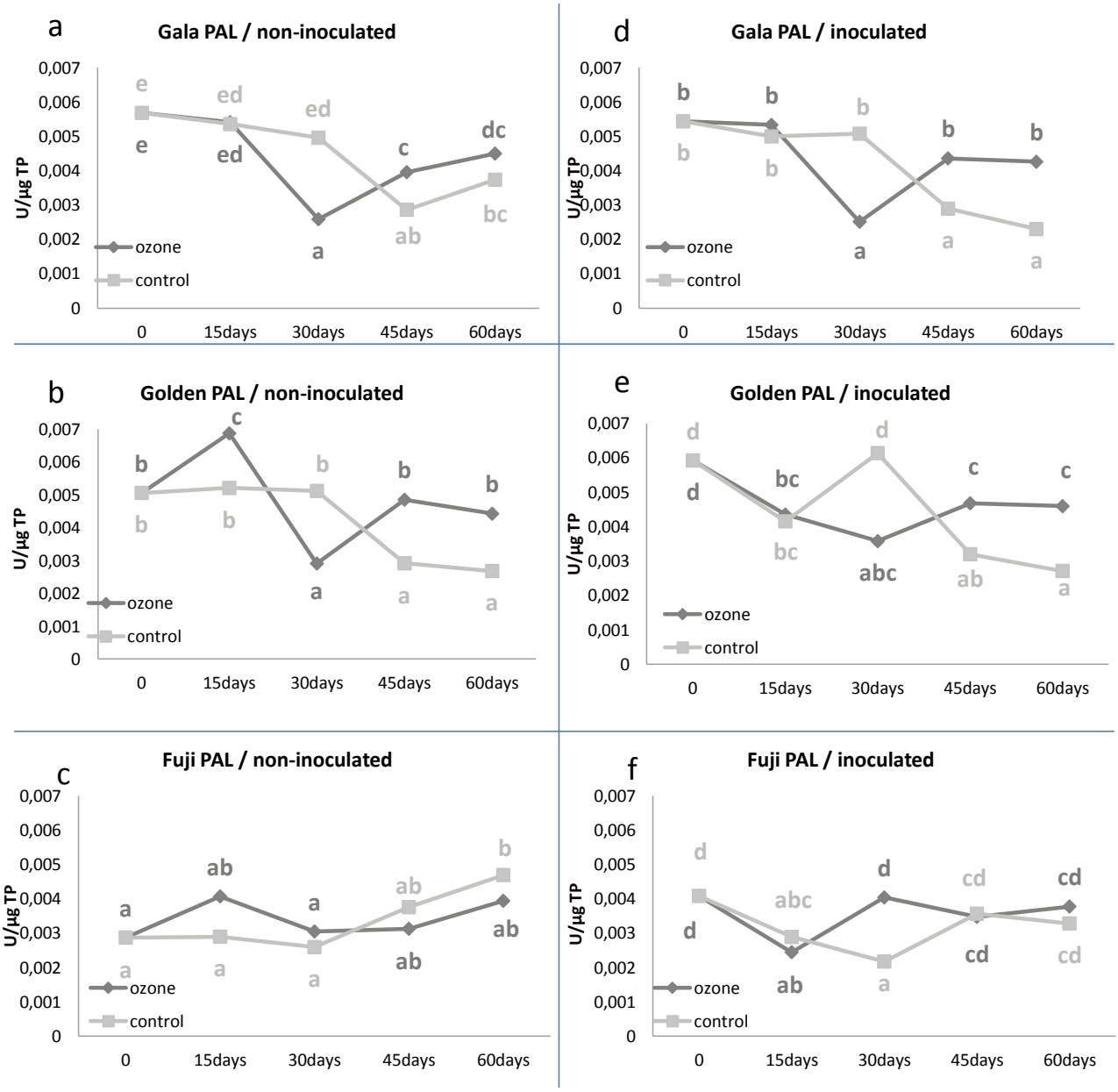

Figure 5. Mean phenylalanine ammonia-lyase specific activity in non-inoculated and *Penicillium expansum* inoculated apple fruits ('Royal Gala', 'Golden Delicious', 'Fuji'), either non-treated (a, b, c) (control) or treated by ozone at 0.5 µL L^{-1} (c, d, e), stored at 1°C±1 in the dark. Means followed by the same letter are not significantly different (Fisher's Least Significance Difference test (LSD, $P = 0.05$)).

zymes clear differences between the samples stored in conventional atmosphere and those stored in the presence of O_3 were not detected. The lack of increase of the activities of the monitored enzymes in the contaminated samples stored in presence of O_3 may indicate a reduction in stress defence mechanisms.

In general, the activities of these enzymes are probably not directly correlated with O_3 treatment,

or with the effect of O_3 on the inhibition of fungal contamination and patulin production.

The results obtained in this study confirm that O_3 treatment at 0.5 μL L^{-1} could be a suitable control system to reduce apple losses caused by *P. expansum* during post-harvest storage. The reduction of *P. expansum* infection as well as patulin biosynthesis for the tested storage period was significant and long lasting.

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