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Postharvest control of citrus green mold caused by *Penicillium digitatum* using a LAB-activated lemon-peel-derived formulation

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ABSTRACT

Green mold, caused by *Penicillium digitatum*, is the most damaging postharvest citrus fruit disease. Conventionally, it is managed with synthetic fungicides. This study evaluated a cell-free, non-viable, lactic acid bacteria (LAB)-activated lemon peel-derived formulation (LM + LAB), produced by fermenting a lemon peel-based medium with *Lactiplantibacillus plantarum* N3B2, under two application scenarios. LM + LAB was compared with imazalil (standard synthetic fungicide), the yeast *Candida oleophila* strain O, a potassium phosphite-based formulation, phosphorous acid, and an untreated control., Sweet orange fruit were treated either before inoculation or 4 h after inoculation with *P. digitatum*. Fruit were stored for 7 days at 6 °C and then at 18 °C until 14 days post-inoculation. Disease incidence was assessed at 7, 10, and 14 days, sporulation reduction at 14 days, and fruit quality throughout storage. Imazalil provided the most effective control. LM + LAB significantly reduced disease incidence under both application scenarios. At 14 days, incidence was reduced from 78% to 38% when LM + LAB was applied before inoculation and from 79% to 15% when it was applied 4 h after inoculation. Sporulation was reduced by 43% and 64%, respectively. When applied post-inoculation, LM + LAB was as effective as imazalil and *C. oleophila* in reducing disease incidence and suppressed sporulation more effectively than the yeast. Potassium phosphite and phosphorous acid were the least effective substances. None of the treatments caused weight loss or significantly affected firmness, total soluble solids, juice pH, or titratable acidity of fruit. Results show LM + LAB is a promising natural product for management of citrus green mold.

1. Introduction

Citrus are a major fruit commodity worldwide (Aloi et al., 2021; Huang et al., 2024; Khanchouch et al., 2017). Postharvest rots caused by *Penicillium* spp. are a serious constrain for the citrus fresh fruit supply chain (Cheng et al., 2020; Riolo et al., 2023b; Rovetto et al., 2024a; Tennant et al., 2009; Wang et al., 2022). Green mold, caused by *Penicillium digitatum*, is regarded as the most destructive postharvest disease of citrus fruit, while blue mold, caused by *P. italicum*, also contributes to substantial postharvest losses (La Spada et al., 2021; Palou, 2014; Yu et al., 2012). Both fungi are obligate wound pathogens that infect in the field, in packinghouses, and during storage and distribution. They spread rapidly at warm temperatures through the production of abundant, coloured, airborne conidia (Ismail and Zhang, 2004; Palou, 2014; Smilanick et al., 2019). Symptoms of green mold consist of watery soft rot around wounds, growth of white mold on the lesion and, subsequently, a dense powdery conidiation (Costa et al., 2019). Oranges infected by *Penicillium* spp. can contain mycotoxins, such as patulin and rubratoxin B, which raise food-safety concerns for juice processing (Moss, 2008; Riolo et al., 2025; Rovetto et al., 2023).

Commercial handling of fresh citrus fruit includes several operational phases: washing, disinfection, coating, and distribution under diverse time–temperature regimes, which commonly include refrigerated storage followed by retail shelf-life conditions. These steps expose fruit to wounding and warm phases that increase the risk of infections in packinghouses and during shelf-life (Cao et al., 2019; Defraeye et al., 2015). In this context, effective and residue-compliant control of Penicillium rots is essential to limit postharvest losses.

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Conventional postharvest management of Penicillium rot of citrus fruit relies on synthetic fungicides and wax coatings applied in the packing line. The fungicide imazalil is widely used by dip, drench, or incorporation into coatings. Its efficacy is influenced by pH, temperature, and exposure time (Kanetis et al., 2008; Palou et al., 2015; Smilanick et al., 2005). In the EU, maximum residue limits for imazalil on citrus fruit are defined by Regulation 2019/1582, and labelling rules require that postharvest treatment is indicated on the packaging, with additional specifications on peel edibility in some countries. Increasingly stringent residue regulations, environmental concerns, consumer expectations, and reports of fungicide-resistant *Penicillium* populations have fostered the search for integrated or alternative management strategies (Palou, 2014; Sánchez-Torres and Tuset, 2011; Smilanick et al., 2019). These strategies include biological control agents (BCAs), generally recognized as safe (GRAS) substances, physical means, innovative edible coatings, biostimulants, plant-resistance inducers and bioproducts, such as plant and animal extracts or natural matrices activated by microbial fermentation (Angilè et al., 2025; Arslan et al., 2024; Bhatta, 2022; Cavallaro et al., 2024; La Spada et al., 2020; Maurizzi et al., 2023; Palou et al., 2015; Plaza et al., 2004; Riolo et al., 2024; Stracquadanio et al., 2021; Zacarias et al., 2020).

In organic agriculture, regulations on pesticide use are particularly stringent, further restricting postharvest fungicide options for citrus fruit. Potassium phosphite and other phosphorous acid derivatives are widely used in agriculture and forestry as systemic, broad-spectrum fungicides, resistance inducers and biostimulants (Gómez-Merino and Trejo-Téllez, 2015; Manghi et al., 2021; Reis et al., 2024). Their potential to control *Penicillium* rot of citrus when used alone is still debated, but their effectiveness can be improved when applied in conjunction with other control means (Cerioni et al., 2013a, 2013b; La Spada et al., 2021; Strano et al., 2015). In some citrus-exporting countries, phosphonate treatments are combined with imazalil for fruit shipped to distant destinations, whereas in the EU phosphonates are banned from organic agriculture and some large-scale retail chains do not accept their residues irrespective of the production system (Manghi et al., 2021; Rovetto et al., 2024a, 2024b).

BCAs are a promising, eco-friendly and toxicologically safe option in postharvest citrus disease management. Among them, the yeast *Candida oleophila* has been demonstrated to be effective in controlling green mold and other *Penicillium* rots and is already used in commercial settings (Brown et al., 2000; Lahlali and Jijakli, 2009; Perez et al., 2017; Rovetto et al., 2024b; Zacarias et al., 2020).

Lactic acid bacteria (LABs) and LAB-activated matrices are another promising, eco-friendly and sustainable option to control postharvest fruit diseases (Dopazo et al., 2022; La Bella et al., 2024; La Spada et al., 2024; Leyva Salas et al., 2017; Ma et al., 2019; Riolo et al., 2023a). LABs can transform citrus by-products into fermentation broths enriched in organic acids and antimicrobial metabolites with inhibitory activity against postharvest pathogens. A cell-free supernatant from a lemon peel-based medium fermented by *Lactiplantibacillus plantarum*, alone or in a microbial consortium with the cyanobacterium *Arthrospira platensis* (syn. *Spirulina platensis*), showed *in vitro* inhibitory activity on *Penicillium* spp. and reduced the growth and sporulation of these fungi on citrus fruit (La Bella et al., 2024; Riolo et al., 2024). Among these biobased approaches, LAB-activated lemon-derived formulations are of particular interest because, according with the principles of circular economy, they combine antifungal activity with the valorisation of citrus-derived matrices. However, it remains unclear whether the efficacy of LAB-activated lemon-derived formulations is restricted to preventive applications or can also be retained when treatment is applied after pathogen infection.

This study evaluated a LAB-activated lemon-derived formulation in comparison with imazalil, *C. oleophila*, potassium phosphite and phosphorous acid for the control of citrus green mold caused by *P. digitatum* under pre-inoculation and post-inoculation application conditions. Specifically, the aims were: (i) to evaluate the efficacy of the treatments under two distinct application scenarios, namely application before pathogen inoculation and application 4 h after inoculation, considering disease incidence and sporulation reduction; and (ii) to assess whether the treatments affected key physicochemical properties of the fruit, including weight loss, firmness, total soluble solids, pH and titratable acidity.

2. Materials and methods

2.1. Microbial isolates

Penicillium digitatum isolate P1PP0 (El Boumlasy et al., 2021), sourced from the microbial collection of the Laboratory of Molecular Plant Pathology, Department of Agriculture, Food and Environment, University of Catania, was used for the artificial inoculation of sweet orange fruit. *Candida oleophila* strain O was used as the yeast biocontrol agent, whereas *Lactiplantibacillus plantarum* N3B2 was used for the preparation of the LAB-activated lemon-derived formulation. Both microorganisms were sourced from the same microbial collection and were previously characterised (Riolo et al., 2024; Rovetto et al., 2024b).

2.2. Test substances

The substances and microbial treatments used in this study were imazalil, potassium phosphite, phosphorous acid, *Candida oleophila* strain O and a LAB-activated lemon-derived formulation. Their composition, application dosage and source are reported in Table 1. Sterile distilled water (sdw) was used for untreated control fruit.

2.3. Fruit material and inoculum preparation

Sweet orange fruit (*Citrus sinensis* cv. Tarocco) harvested at commercial maturity from organic orchards located in eastern Sicily were used in all the experiments. Fruit were selected for uniform size and physiological stage and checked for the absence of mechanical injuries or visible physiological disorders. Before treatment and inoculation, fruit were surface-disinfected by immersion in 1% sodium hypochlorite for 2 min, rinsed twice in sterile distilled water and air-dried at room temperature. For inoculum preparation, *P. digitatum* isolate P1PP0 was grown on Potato Dextrose Agar (PDA) at 25 °C for 7 days. A conidial suspension was prepared in sdw supplemented with 0.05% (v/v) Tween 20 and adjusted to 10^6 conidia mL^{-1} using a haemocytometer.

2.4. Experimental design, fruit treatment, inoculation and storage conditions

Sweet orange fruit were randomly assigned to six experimental groups corresponding to the substances or microbial treatments reported in Table 1: untreated control, imazalil (POL), potassium phosphite-based formulation (RIO1), phosphorous acid (FOL), *Candida oleophila* strain O (M01) and the LAB-activated lemon-derived formulation (LM + LAB).

Two application-timing assays were performed: (i) a pre-inoculation treatment assay, in which treatments were applied before artificial inoculation with *P. digitatum*, and (ii) a post-inoculation treatment assay, in which fruit were inoculated before treatment application. For each treatment and application timing, 90 fruit were used in each experimental repetition and arranged into three replicate groups of 30 fruit each. The three replicate groups were maintained separately throughout treatment, inoculation and storage. Both assays were conducted twice as independent experiments under the same experimental conditions.

2.4.1. Pre-inoculation treatment assay

Fruit were immersed in the respective treatment solution or microbial suspension for 2 min and then air-dried at room temperature until the fruit surface was visibly dry. Untreated control fruit were immersed in sdw. After drying, fruit were inoculated with *P. digitatum*. For

Table 1
Substances and microbial treatments used for fruit application in this study.

ID	Product category	Active substance/nature	Application dosage	Notes/source
POL	Synthetic fungicide (emulsifiable concentrate)	Imazalil (IMZ-50, 50% w/v a.i.)	1000 mg a.i. L^{-1}[a]	Standard packinghouse rate; prepared in sterile distilled water.
RIO1	Potassium phosphite-based formulation	Potassium phosphite 57% (w/v)	0.25% (formulation)[b]	Used as an aqueous solution at tested concentration.
FOL	Phosphorous acid	Phosphorous acid (≥99% purity; CAS 13598-36-2)	0.5% (w/v, a.i.)[c]	Aqueous solution freshly prepared.
M01	Biocontrol agent (living yeast)	*Candida oleophila* strain O	10^8 CFU mL^{-1}	Suspension in sterile distilled water.
LM + LAB	Bioactive, non-viable formulation	Cell-free supernatant (CFS) from lemon peel medium fermented by *Lactiplantibacillus plantarum* N3B2	250 g L^{-1}	Prepared as in Riolo et al. (2024).

[a] Active ingredient (a.i.).
[b] Dosage expressed on product formulation.
[c] Dosage expressed as phosphorous acid active ingredient.

inoculation, one wound, 2 mm deep, was made in the equatorial region of each fruit using a sterile needle of 1 mm diameter, and an aliquot of 20 μL of the conidial suspension was applied to the wound.

2.4.2. Post-inoculation treatment assay

For the post-inoculation treatment assay, fruit were first wounded and inoculated with *P. digitatum* following the same procedure described above. After inoculation, fruit were incubated for 4 h at 20 °C before treatment application. Fruit were subsequently immersed for 2 min in the respective treatment solution or microbial suspension and air-dried at room temperature until the fruit surface was visibly dry. Control fruit were immersed in sdw following the same procedure.

2.4.3. Storage conditions and disease assessment

After completion of the respective inoculation–treatment sequence, fruit from both assays were stored at 6 °C for 7 days to simulate refrigerated storage and then transferred to 18 °C until 14 days post-inoculation (dpi) to simulate retail shelf-life conditions. The three replicate groups of each treatment were maintained separately during storage.

2.5. Disease incidence and sporulation reduction

Disease incidence was recorded separately for the pre-inoculation and post-inoculation assays at 7, 10 and 14 dpi and expressed as the percentage of infected fruit. For each treatment, application timing and assessment date, disease incidence was calculated independently for each replicate group of 30 fruit, providing three replicate incidence values in each experimental repetition.

At 14 dpi, sporulation was quantified separately in the two application-timing assays by recovering conidia from the surface of each infected fruit, followed by serial dilution plating. Briefly, the surface of each infected fruit was rinsed with 1 mL sdw containing 0.05% (v/v) Tween 20. The resulting suspension was collected and vortexed for 30 s to disperse the conidia. The suspension was serially diluted (10-fold series), and an aliquot of 100 μL of each dilution was plated on PDA. Colony-forming units (CFUs) were counted after 48–72 h incubation at 25 °C and expressed as CFUs per infected fruit (Riolo et al., 2024). For each replicate group, the mean CFU value was calculated from the infected fruit belonging to that replicate. For each independent experimental repetition, sporulation reduction for each treated replicate group was calculated relative to the mean CFU value obtained from the three untreated-control replicate groups of the same experimental repetition. Sporulation reduction was calculated separately for the pre-inoculation and post-inoculation assays according to the formula:

$$\text{Sporulation reduction (\%)} = \left(\frac{\overline{\text{CFUs control}} - \text{CFUs treatment}}{\overline{\text{CFUs control}}}\right) \text{x } 100$$

where $\overline{\text{CFUs control}}$ is the mean CFU s per infected fruit calculated across the three untreated-control replicate groups within the corresponding experimental repetition, and CFU treatment is the mean CFU s per infected fruit calculated for each individual treated replicate group. This procedure generated three sporulation-reduction values per treatment in each independent experiment and six values per treatment after pooling the two experiments.

2.6. Fruit quality analysis

To verify whether treatments may affect the shelf-life of fruit by altering their physicochemical characteristics, non-inoculated sweet orange fruit cv. Tarocco treated with POL, FOL, RIO1, M01 and LM + LAB, respectively, were stored under the same temperature conditions as those described in section 2.4.3. All fruit were from the same batch and at the same maturity stage. Physicochemical fruit characteristics monitored throughout the storage period included weight loss, firmness, and total soluble solids, pH and titratable acidity of the juice. Evaluations were performed at 7, 10, and 14 days after treatment. All measurements were performed at 20 ± 1 °C using calibrated instruments at the start of each session. The measurement order of samples was randomized to minimize instrumental drift. For each treatment and assessment time, three independent biological replicates of 10 fruit each were used. Fruit were randomly allocated to the three assessment times at the beginning of the experiment and were used only at their assigned time point, resulting in 30 fruit per treatment at each assessment time and 90 fruit per treatment overall.

2.6.1. Fruit weight loss

Weight loss was determined by weighing fruit at day 0 and at each subsequent time point. Results were expressed as percentage of the initial weight according to the following formula:

$$\text{Weight loss (\%)} = \left(\frac{W_0 - W_t}{W_0}\right) \times 100$$

where W_0 is the initial fruit weight and W_t is the weight at the time of measurement, as described by Singh and Reddy (2006).

2.6.2. Fruit firmness

Firmness was measured at two equidistant points on the equatorial region of each fruit using a digital penetrometer (Fruit Texture Analyzer, Turoni, Italy) equipped with an 8 mm probe. The two measurements obtained from each fruit were averaged to obtain a single firmness value per fruit. The same probe, instrument settings and measurement procedure were used for all treatments and sampling times. Results are expressed in newtons (N) (Zeb et al., 2020).

2.6.3. Juice total soluble solids (TSS)

TSS were measured using juice extracted from three randomly selected fruit per replicate. Measurements were performed with a digital refractometer (Atago PAL-1, Tokyo, Japan) and expressed as % TSS (Zeb et al., 2020). The refractometer was zeroed with distilled water;

measurements were taken with automatic temperature compensation (ATC) at 20 ± 1 °C.

2.6.4. Juice pH

The pH of the juice was measured using a digital pH meter (HI2211, Hanna Instruments, Woonsocket, RI, USA), calibrated with standard buffer solutions at pH 4.0 and 7.0. Measurements were taken at 20 ± 1 °C by immersing the electrode in freshly squeezed juice. The electrode was rinsed and dried between successive samples.

2.6.5. Juice titratable acidity (TA)

TA (g citric acid per 100 mL) was determined by titrating 10 mL of juice with 0.1 N NaOH until reaching an endpoint of pH 8.2, using phenolphthalein as an indicator (Talasila et al., 2012). Results were expressed as grams of citric acid equivalent per 100 mL of juice, calculated according to the formula:

$$\text{TA (g citric acid per 100 mL juice)} = \frac{(V_{NaOH} \times N_{NaOH} \times 0.064 \times 100)}{V_{sample}}$$

Where V_{NaOH} is the volume of NaOH used (mL), N_{NaOH} is the normality of NaOH, 0.064 is the equivalent weight of citric acid, and V_{sample} is the volume of juice titrated.

Each titration was performed in technical duplicate per sample; the maximum deviation between replicates was ± 0.02 mL.

2.7. Statistical analysis

Data analyses were performed in R (v4.0.3). Both the pre-inoculation and post-inoculation assays were conducted twice as independent experiments, with three replicate groups of 30 fruit per treatment in each experimental repetition. For each assay, a preliminary two-way ANOVA including treatment, experiment and their interaction detected no significant effect of experiment or treatment × experiment interaction ($\alpha = 0.05$). Therefore, data from the two experimental repetitions were pooled for subsequent analyses. For sporulation reduction, this pooling resulted in six replicate values per treatment and application-timing assay, corresponding to three replicate groups from each of the two independent experimental repetitions.

Before ANOVA, normality of model residuals was assessed using the Shapiro–Wilk test (statsshapiro.test), and homogeneity of variances was assessed using Levene's test (carleveneTest) at $\alpha = 0.05$. Percentage data were transformed using the arcsin square-root transformation. Disease-incidence data at 7 dpi were not subjected to statistical analysis because no symptomatic fruit were observed in any treatment. For both the pre-inoculation and post-inoculation assays, pooled disease-incidence data were analysed separately at 10 and 14 dpi by one-way ANOVA (statsaov). Sporulation-reduction data measured at 14 dpi were also analysed separately for the pre-inoculation and post-inoculation assays by one-way ANOVA. For fruit-quality analyses, weight loss, firmness, total soluble solids, juice pH and titratable acidity were analysed separately at each assessment time by one-way ANOVA, considering the biological replicate as the experimental unit ($n = 3$ per treatment and assessment time). All one-way ANOVAs were followed by Tukey's HSD post hoc test for multiple comparisons (statsTukeyHSD). Differences at $p \leq 0.05$ were considered significant.

To summarize treatment performance at 14 dpi, disease incidence and sporulation-reduction data were standardized to zero mean and unit variance. Heatmaps based on standardized treatment-wise mean disease incidence and sporulation reduction were generated separately for each application-timing assay using pheatmap. Hierarchical clustering based on Euclidean distance was used to order treatments and variables. Principal component analysis (PCA) was performed separately for each assay using standardized replicate-level disease incidence and sporulation-reduction data, and score plots were generated to visualize the distribution and separation of treatments.

3. Results

3.1. Incidence of Penicillium digitatum infections in orange fruit

No green mold symptoms were observed at 7 dpi in either application-timing assay, when fruit had been maintained at 6 °C. Disease symptoms became visible after transfer to 18 °C, and incidence increased between 10 and 14 dpi in most treatments (Fig. 1).

In fruit inoculated after treatment, the untreated control showed disease incidence values of approximately 56% and 78% at 10 and 14 dpi, respectively (Fig. 1a). POL provided the greatest disease control, with incidence values of approximately 9% at 10 dpi and 13% at 14 dpi. M01 and LM + LAB also significantly reduced disease incidence compared with the untreated control, reaching approximately 17% and 23% at 10 dpi and 33% and 38% at 14 dpi, respectively. At both assessment dates, M01 and LM + LAB did not differ significantly from each other. RIO1 and FOL showed lower efficacy, with disease incidence values of approximately 36% and 46% at 10 dpi and 66% and 69% at 14 dpi, respectively. At 14 dpi, neither RIO1 nor FOL differed significantly from the untreated control.

In fruit inoculated before treatment, disease incidence in the untreated control reached approximately 56% at 10 dpi and 79% at 14 dpi (Fig. 1b). POL, M01 and LM + LAB significantly reduced disease incidence at both assessment dates and did not differ significantly from each other. Their incidence values were approximately 8%, 17% and 13%, respectively, at 10 dpi, and 8%, 19% and 15%, respectively, at 14 dpi. RIO1 and FOL provided only partial control at 10 dpi, with incidence values of approximately 34% and 38%, respectively. At 14 dpi, disease incidence increased to approximately 67% for RIO1 and 70% for FOL, and neither treatment differed significantly from the untreated control.

3.2. Sporulation reduction

At 14 dpi, all treatments reduced *P. digitatum* sporulation compared with the untreated control, although their efficacy differed substantially (Fig. 2).

In fruit inoculated after treatment, POL showed the greatest sporulation reduction, reaching approximately 86% (Fig. 2a). LM + LAB was the second most effective treatment, reducing sporulation by approximately 43%, and differed significantly from all other treatments. M01, RIO1 and FOL showed lower and statistically comparable reductions of approximately 29%, 30% and 23%, respectively.

In fruit inoculated before treatment, POL again provided the highest sporulation reduction, reaching approximately 95% (Fig. 2b). LM + LAB reduced sporulation by approximately 64% and was significantly more effective than M01, RIO1 and FOL. These last three treatments showed statistically comparable reductions of approximately 43%, 43% and 36%, respectively.

3.3. Multivariate analysis of treatment performance

Heatmap and principal component analyses were used to summarize treatment performance at 14 dpi based on disease incidence and sporulation reduction.

For fruit inoculated before treatment, hierarchical clustering separated POL, LM + LAB and M01 from the untreated control, FOL and RIO1 (Fig. 3a). Within the first group, LM + LAB and M01 showed the closest response profiles, while POL was distinguished by the lowest disease incidence and the greatest sporulation reduction. Within the second group, FOL and RIO1 showed closely related profiles, whereas the untreated control was characterized by the highest disease incidence and the absence of sporulation reduction.

The PCA showed a distribution consistent with the patterns observed in the heatmap (Fig. 3b). PC1 and PC2 accounted for 78% and 22% of the total variance, respectively. FOL and RIO1 occupied closely overlapping regions of the scores plot, while LM + LAB and M01 were positioned in

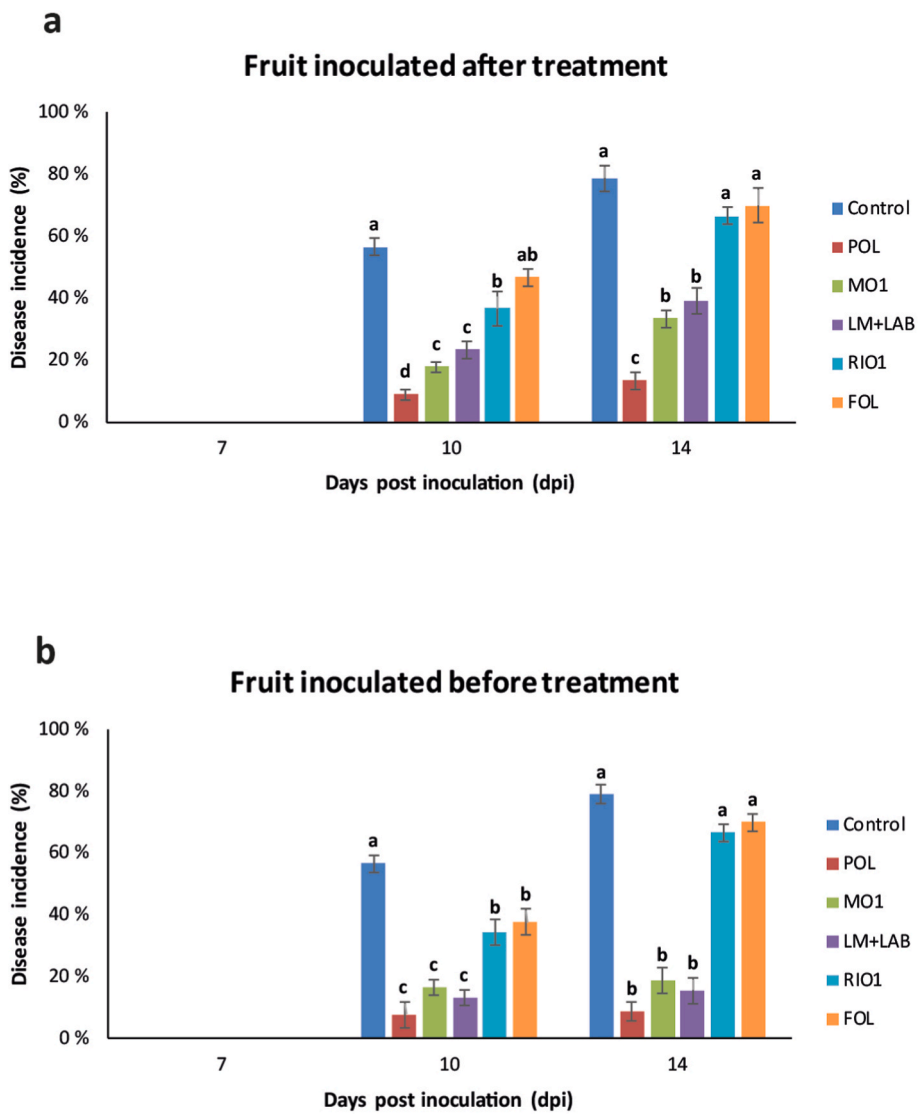

Fig. 1. Disease incidence of green mold caused by *Penicillium digitatum* in sweet orange fruit subjected to different application timings. Fruit were either inoculated after treatment (a) or inoculated 4 h before treatment (b). Disease incidence was assessed at 7, 10 and 14 days post-inoculation (dpi) and expressed as the percentage of infected fruit. Values are means ± SD of six replicate groups, obtained from three groups of 30 fruit in each of two independent experiments. Different letters above bars indicate significant differences among treatments within the same assessment date and application-timing assay according to Tukey's HSD test at $p \leq 0.05$. Treatments included POL (imazalil, 1000 mg a.i. L^{-1}), M01 (*Candida oleophila* strain O, 10^8 CFU mL^{-1}), LM + LAB (LAB-activated lemon-derived formulation, 250 g L^{-1}), RIO1 (potassium phosphite-based formulation, 0.25%) and FOL (phosphorous acid, 0.5% a.i.); fruit immersed in sterile distilled water served as untreated controls. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

adjacent regions. POL was located at positive PC1 scores, whereas the untreated control occupied a distinct region at negative PC1 scores.

For fruit inoculated after treatment, hierarchical clustering identified two closely related treatment pairs: LM + LAB and M01, and FOL and RIO1 (Fig. 4a). POL and the untreated control occupied separate branches and showed contrasting response profiles. POL was characterized by the lowest disease incidence and the greatest sporulation reduction, whereas the untreated control showed the highest disease incidence and no sporulation reduction.

The PCA supported the treatment distribution observed in the heatmap (Fig. 4b). PC1 and PC2 explained 78.7% and 21.3% of the total variance, respectively. POL and the untreated control occupied clearly separated regions at opposite sides of the scores plot. LM + LAB and M01 were positioned in adjacent regions, whereas FOL and RIO1 showed closely overlapping distributions.

3.4. Effect of treatments on physicochemical parameters of fruit during storage and shelf-life

The impact of the different treatments on the quality of sweet orange fruit was evaluated using non-inoculated fruit stored under the same conditions adopted for the disease-control assay, namely 7 days at 6 °C followed by storage at 18 °C until day 14. Physicochemical parameters assessed at 7, 10 and 14 days after treatment included fruit weight loss, firmness, juice total soluble solids (TSS), pH and titratable acidity (TA). Results are summarized in Table 2.

Across the 14-day storage, weight loss increased gradually in all treatments, from 0.9 to 1.1% at day 7 to 2.9–3.1% at day 14, with the untreated control showing the highest numerical values at each time point. Firmness declined moderately over time (32.5 N at day 7 to 28.8–29.2 N at day 14), with no treatment-related differences. TSS remained stable (10.6–11.3%), pH varied narrowly around 3.74–3.80, and titratable acidity decreased slightly (0.79 to 0.73–0.75 g citric acid

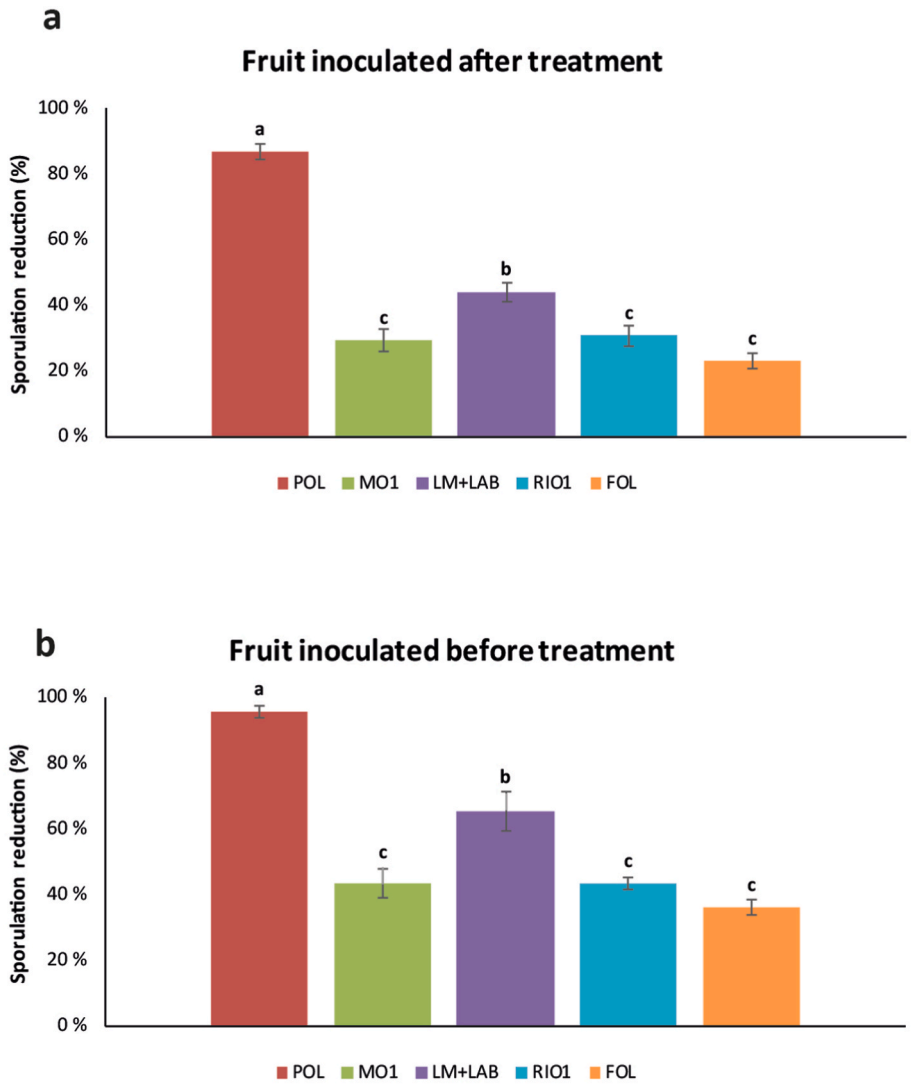

Fig. 2. Reduction of *Penicillium digitatum* sporulation on artificially inoculated sweet orange fruit following different application timings. Fruit were either inoculated after treatment (a) or inoculated 4 h before treatment (b). Sporulation was assessed at 14 days post-inoculation and expressed as percentage reduction relative to the corresponding untreated control within each application-timing assay. Values are means ± SD of six replicate sporulation-reduction values per treatment, obtained from three replicate groups in each of two independent experiments. Different letters above bars indicate significant differences among treatments within the same application-timing assay according to Tukey's HSD test at $p \leq 0.05$. The untreated control corresponded to 0% sporulation reduction and is not shown. Treatments included POL (imazalil, 1000 mg a.i. L^{-1}), M01 (*Candida oleophila* strain O, 10^8 CFU mL^{-1}), LM + LAB (LAB-activated lemon-derived formulation, 250 g L^{-1}), RIO1 (potassium phosphite-based formulation, 0.25%) and FOL (phosphorous acid, 0.5% a.i.). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

per 100 mL). No significant differences were detected among treatments for any of the physicochemical parameters assessed at each time point (Tukey's HSD test, $\alpha = 0.05$). Visual inspection also did not reveal rind injury or visible treatment deposits on treated fruit during the storage period.

4. Discussion

This study advances previous research on LAB-activated lemon-derived matrices by moving beyond the demonstration of preventive activity and evaluating the formulation against established chemical and biological benchmarks under two distinct application scenarios simulating operational conditions. Direct comparison with imazalil, an established postharvest synthetic fungicide against citrus green mold (Palou, 2014; Smilanick et al., 2019), and with *Candida oleophila* strain O, a well-characterized biological control agent for citrus fruit (Brown et al., 2000; Rovetto et al., 2024b), allows LM + LAB treatment to be applied and scheduled more rationally and effectively than in previous studies. Imazalil provided the highest overall level of control in both assays, confirming its role as the established chemical reference. Nevertheless, LM + LAB was consistently among the most effective non-synthetic treatments. When applied before pathogen inoculation, LM + LAB significantly reduced disease incidence and sporulation (Figs. 1a and 2a). Under these conditions, its effect on disease incidence was comparable to that of *C. oleophila*, while its suppression of sporulation was greater. More importantly, LM + LAB retained substantial efficacy when applied 4 h after inoculation. In this assay, LM + LAB significantly reduced both disease incidence and sporulation (Figs. 1b and 2b). Disease incidence did not differ significantly from that observed with imazalil or *C. oleophila*, whereas sporulation reduction was second only to that achieved with imazalil and was significantly greater than that provided by *C. oleophila*. By contrast, potassium phosphite and phosphorous acid produced only partial and less persistent control, particularly at 14 dpi. A previous study demonstrated the antifungal activity and preventive potential of the

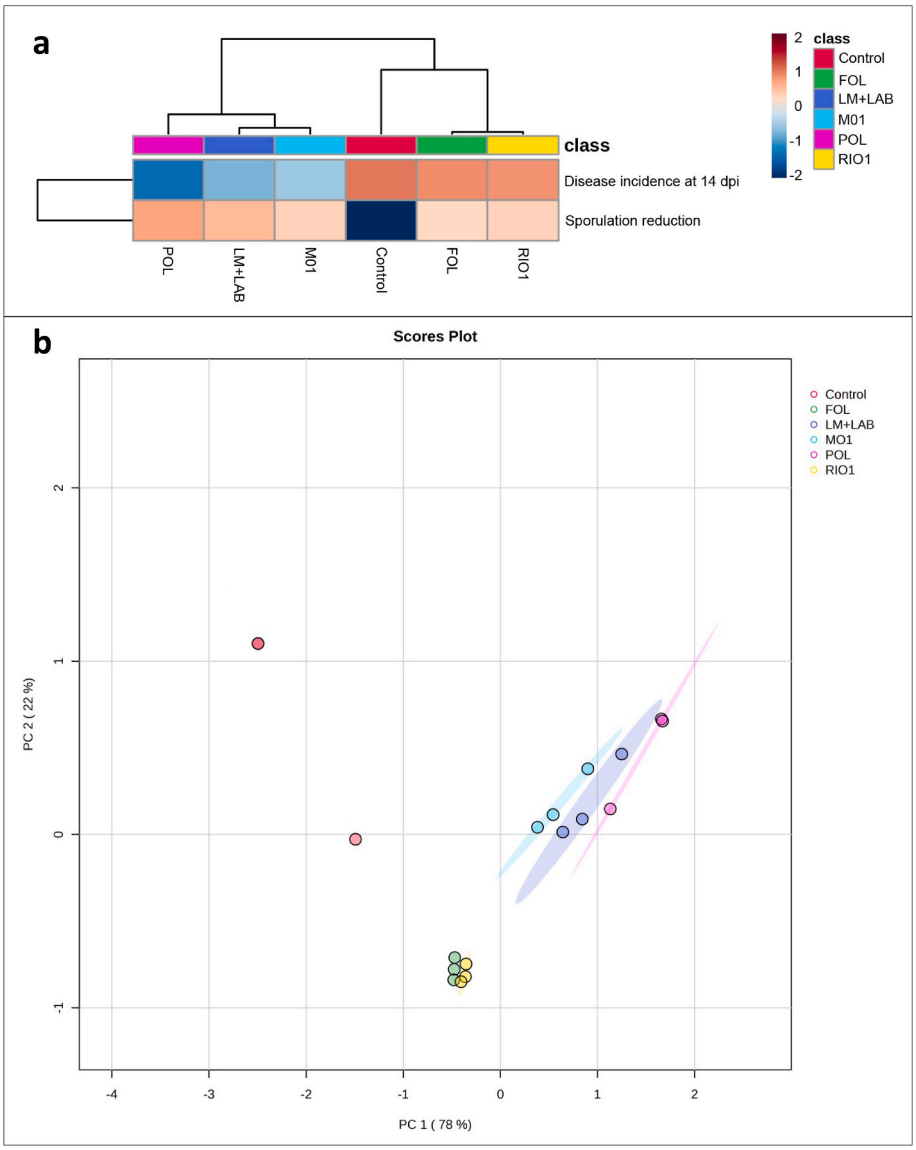

Fig. 3. Multivariate assessment of treatment performance at 14 days post-inoculation (dpi) in sweet orange fruit inoculated 4 h before treatment application. (a) Heatmap of standardized treatment-wise mean disease incidence and sporulation reduction. Dendrograms show hierarchical clustering of treatments and variables based on Euclidean distance; blue and red indicate lower and higher standardized values, respectively. (b) Principal component analysis (PCA) score plot generated from standardized replicate-level disease-incidence and sporulation-reduction data. Each point represents a replicate-level observation, and colors identify treatments. PC1 and PC2 explained 78% and 22% of the total variance, respectively. Treatments were POL (imazalil), M01 (*Candida oleophila* strain O), LM + LAB (LAB-activated lemon-derived formulation), RIO1 (potassium phosphite-based formulation), FOL (phosphorous acid), and the untreated control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

LAB-activated lemon-derived matrix (Riolo et al., 2024); the present results extend that evidence by demonstrating the activity of this product, which is preventive, certainly, but is retained when it is applied shortly after the infection by the pathogen, as shown by the notable reduction of both disease incidence and sporulation observed in the post-inoculation assay.

The inclusion of *C. oleophila* strain O, a BCA already developed as a commercial product for post-harvest treatments of citrus fruit, provided a well-established biological reference for evaluating LM + LAB. The efficacy of this yeast against postharvest *Penicillium* rots of citrus fruit is well established and has been associated with several complementary modes of action. Competition for nutrients and colonization sites within fruit wounds is considered a major component of its antagonistic activity (Droby et al., 2002; Liu et al., 2019; Rovetto et al., 2024b), while induction of host defence responses may further contribute to disease suppression (Droby et al., 2002). Biofilm formation and adaptation to the physicochemical conditions of the fruit surface and wound environment have also been linked to the effectiveness and persistence of antagonistic yeasts under postharvest conditions (Hammami et al., 2022; Lahlali and Jijakli, 2009). These characteristics explain why the performance of *C. oleophila* may depend on wound type, application timing, environmental conditions and the interval available for the antagonist to establish before pathogen development (Brown et al., 2000; Lahlali and Jijakli, 2009; Rovetto et al., 2024b). In the present study, *C. oleophila* significantly reduced disease incidence in both application-timing assays, confirming its robustness as a biological control agent even when applied after pathogen inoculation. However, its effect on sporulation was more limited than that of LM + LAB, particularly when treatment was performed 4 h after inoculation. This distinction is important because disease incidence and pathogen sporulation describe different components of treatment performance: the former reflects the ability to prevent visible decay, whereas the latter reflects the capacity to limit pathogen reproduction and secondary inoculum

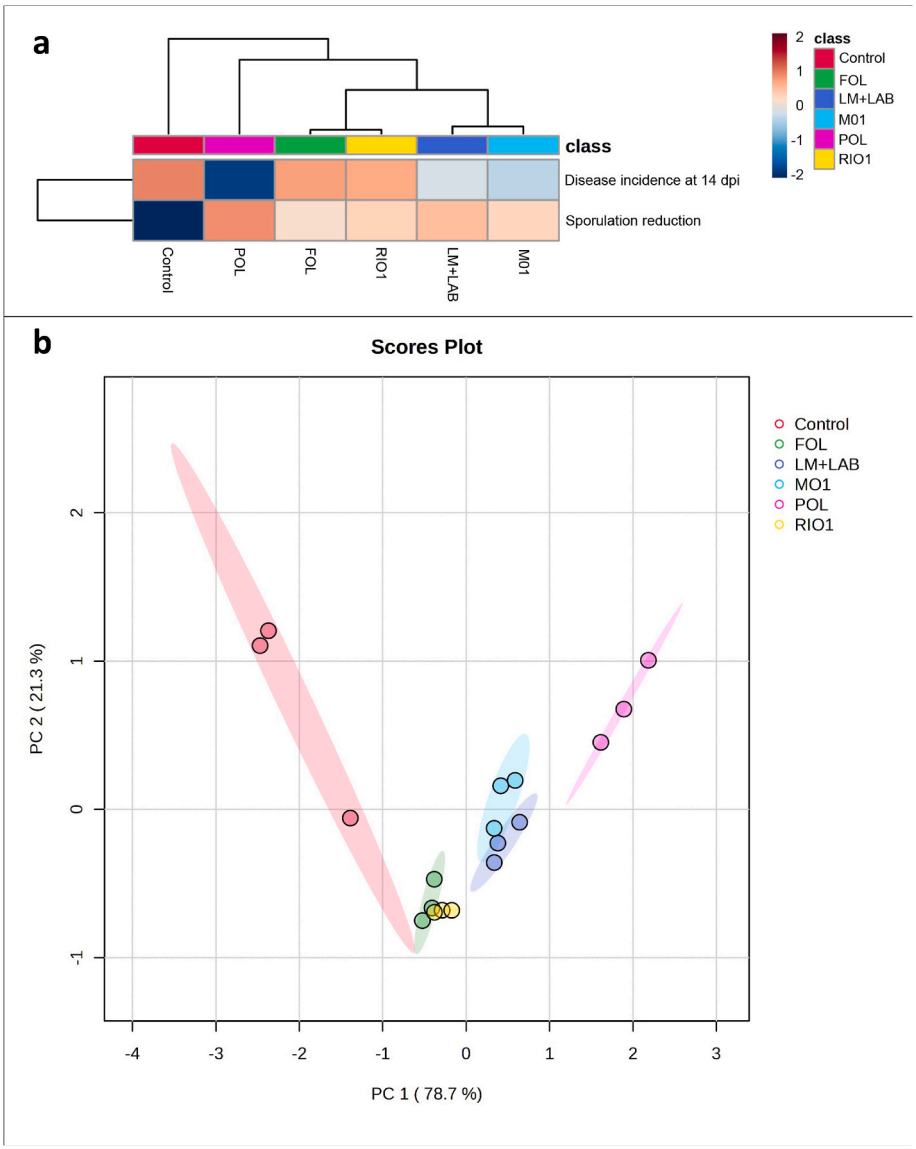

Fig. 4. Multivariate assessment of treatment performance at 14 days post-inoculation (dpi) in sweet orange fruit inoculated after treatment application. (a) Heatmap of standardized treatment-wise mean disease incidence and sporulation reduction. Dendrograms show hierarchical clustering of treatments and variables based on Euclidean distance; blue and red indicate lower and higher standardized values, respectively. (b) Principal component analysis (PCA) score plot generated from standardized replicate-level disease-incidence and sporulation-reduction data. Each point represents a replicate-level observation, and colors identify treatments. PC1 and PC2 explained 78.7% and 21.3% of the total variance, respectively. Treatments were POL (imazalil), M01 (*Candida oleophila* strain O), LM + LAB (LAB-activated lemon-derived formulation), RIO1 (potassium phosphite-based formulation), FOL (phosphorous acid), and the untreated control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

production. Therefore, the results confirm the value of *C. oleophila* as a reliable biological reference while also showing that LM + LAB can provide a stronger suppression of *P. digitatum* sporulation under the tested conditions. The commercial development of *C. oleophila*-based products, including Aspire®, and the extensive experimental evidence available for this yeast further support its use as a well-established reference for the assessment of emerging biobased formulations (Droby et al., 2002; Perez et al., 2017; Rovetto et al., 2024b).

The performance of LM + LAB is particularly relevant because the formulation differs fundamentally from a living antagonist such as *C. oleophila*. LM + LAB is a cell-free, non-viable formulation containing metabolites already produced during fermentation of the lemon-derived matrix by *Lactiplantibacillus plantarum*. Therefore, its antifungal activity is immediate, i.e. it acts soon after application, and less variable as it does not depend on microbial carposphere competence, multiplication or competitive colonization of the peel wounds. Previous chemical characterization of the fermented matrix identified several acidic components, including lactic, acetic, DL-3-phenyllactic, 3,4-dihydroxyhydrocinnamic, salicylic and vanillic acids, which were hypothesized to be responsible for its antifungal activity (Riolo et al., 2024). This interpretation is consistent with other studies showing that the inhibitory activity of LAB and LAB-fermented matrices may result from the combined action of organic acids, phenolic acids and other fermentation-derived metabolites rather than from a single active compound (Li et al., 2012, 2023; Volentini et al., 2023). Particularly notable was the strong efficacy of LM + LAB when applied 4 h after inoculation. Under these conditions, LM + LAB markedly suppressed both disease development and pathogen sporulation despite the high disease pressure observed in untreated fruit. Among these metabolites, lactic, acetic and phenyllactic acids have been associated with direct inhibition of *P. digitatum*, and phenyllactic acid has been shown to reduce spore viability and mycelial growth and to impair fungal membrane integrity (Gerez et al., 2010; Guo et al., 2022). These effects provide a

Table 2
Physicochemical parameters of orange fruit (*Citrus sinensis* cv. Tarocco) treated with different formulations and stored under simulated shelf-life conditions (6 °C for 7 days, then at 18 °C until days 10 and 14). Values determined at 7, 10, and 14 days after treatment are means ± SD (n = 3). No significant differences among treatments were detected at each time point (Tukey's HSD, α = 0.05).

Treatment	Time (days)	Weight loss (%)	Firmness (N)	TSS (%)	pH	TA (g citric acid per 100 mL)
Control	7	1.10 ± 0.07	32.5 ± 1.4	11.1 ± 0.2	3.79 ± 0.03	0.79 ± 0.01
	10	2.05 ± 0.10	30.3 ± 1.2	10.9 ± 0.3	3.78 ± 0.04	0.77 ± 0.02
	14	3.10 ± 0.15	28.8 ± 1.0	10.6 ± 0.3	3.75 ± 0.04	0.75 ± 0.02
POL	7	0.95 ± 0.05	32.8 ± 1.5	11.2 ± 0.2	3.80 ± 0.02	0.78 ± 0.01
	10	1.90 ± 0.09	30.6 ± 1.1	10.9 ± 0.2	3.78 ± 0.03	0.76 ± 0.02
	14	2.95 ± 0.14	29.1 ± 1.2	10.6 ± 0.3	3.75 ± 0.03	0.74 ± 0.02
RIO1	7	0.92 ± 0.06	33.0 ± 1.3	11.3 ± 0.3	3.80 ± 0.02	0.78 ± 0.01
	10	1.87 ± 0.08	30.7 ± 1.2	11.0 ± 0.2	3.78 ± 0.03	0.76 ± 0.01
	14	2.90 ± 0.13	29.0 ± 1.1	10.7 ± 0.3	3.75 ± 0.04	0.74 ± 0.02
FOL	7	0.90 ± 0.05	33.1 ± 1.4	11.2 ± 0.2	3.79 ± 0.02	0.77 ± 0.01
	10	1.85 ± 0.09	30.8 ± 1.3	10.9 ± 0.2	3.76 ± 0.03	0.75 ± 0.02
	14	2.85 ± 0.12	29.2 ± 1.1	10.7 ± 0.2	3.74 ± 0.03	0.73 ± 0.02
M01	7	0.93 ± 0.06	32.7 ± 1.3	11.1 ± 0.3	3.78 ± 0.02	0.78 ± 0.01
	10	1.89 ± 0.10	30.5 ± 1.2	10.9 ± 0.3	3.76 ± 0.03	0.75 ± 0.02
	14	2.97 ± 0.15	28.9 ± 1.1	10.6 ± 0.2	3.73 ± 0.04	0.73 ± 0.02
LM + LAB	7	0.91 ± 0.05	33.2 ± 1.4	11.3 ± 0.2	3.80 ± 0.02	0.77 ± 0.01
	10	1.88 ± 0.09	30.9 ± 1.3	10.9 ± 0.2	3.77 ± 0.03	0.75 ± 0.02
	14	2.93 ± 0.14	29.1 ± 1.2	10.7 ± 0.3	3.75 ± 0.03	0.73 ± 0.02

plausible basis for restricting further fungal development when treatment is applied after inoculation. Consistent with this interpretation, phenyllactic acid has been shown to control citrus green mold in fruit assays (Guo et al., 2022), while a LAB-derived cell-free supernatant whose antifungal activity depended on acidic metabolites reduced Penicillium rot incidence on lemons by approximately 80% (Volentini et al., 2023). Moreover, acetic acid fumigation significantly reduced decay in *P. digitatum*-inoculated mandarins even when applied without curing (Venditti et al., 2009). Taken together, these findings support the hypothesis that the acidic metabolites present in LM + LAB may contribute directly to the therapeutic effect observed under post-inoculation conditions. By demonstrating that LM + LAB remains highly effective after pathogen inoculation, and by comp aring this activity with established chemical and biological reference treatments, the present study considerably broadens the functional and practical potential applications of this formulation. LM + LAB emerges as a biobased postharvest treatment capable of suppressing green mold across different application windows, including situations in which pathogen contamination has already occurred. This broader efficacy spectrum strengthens its potential integration into citrus postharvest management and adds practical value to a circular-economy strategy based on the conversion of citrus-derived materials into antifungal products. The findings therefore complement and extend other biobased approaches developed for citrus preservation, including LAB fermentates and natural, edible or biodegradable coatings (Arslan et al., 2024; La Bella et al., 2024; Piancatelli et al., 2026; Riolo et al., 2026).

The potassium phosphite-based formulation and phosphorous acid occupied a clearly different efficacy range from imazalil, *C. oleophila* and LM + LAB. Previous laboratory and semi-commercial studies have shown that phosphite salts can inhibit *Penicillium* conidial germination and reduce citrus postharvest decay, although effective concentrations and treatment performance vary considerably according to formulation and application conditions (Cerioni et al., 2013a, 2013b; Reis et al., 2024). In the present study, both the potassium phosphite-based formulation and phosphorous acid provided partial disease control at 10 dpi when applied either before or after inoculation. However, this effect was not maintained through 14 dpi, when disease incidence in fruit treated with either product no longer differed significantly from that of the untreated control (Fig. 1). Their limited ability to prevent the final development of visible decay indicates that, at the concentrations tested, neither treatment was sufficiently effective as a stand-alone intervention against green mold. Nevertheless, both treatments retained measurable activity against pathogen reproduction (Fig. 2). This partial dissociation between disease incidence and sporulation suggests that these treatments may interfere with fungal growth or reproductive development even when their activity is insufficient to prevent infection from progressing to visible decay. Limiting sporulation remains relevant in postharvest disease management because conidial production contributes directly to inoculum accumulation and secondary contamination within packinghouses and storage environments (Erasmus et al., 2015). However, the magnitude of the reductions observed with the potassium phosphite-based formulation and phosphorous acid was consistently lower than that achieved with imazalil and LM + LAB. These findings are consistent with previous evidence that the postharvest efficacy of potassium phosphite is frequently enhanced when integrated with other control measures, including heat, oxidizing compounds or conventional fungicides, rather than used alone (Adaskaveg et al., 2015; Cerioni et al., 2013b; Palou, 2013). Accordingly, the present results position the potassium phosphite-based formulation and phosphorous acid primarily as potential components of integrated treatment strategies aimed at contributing to inoculum reduction, rather than as substitutes for treatments capable of providing sustained suppression of citrus green mold.

The absence of treatment-related changes in fruit physicochemical properties is an important complementary outcome because the practical value of a postharvest treatment depends not only on disease suppression but also on its compatibility with fruit quality during storage and marketing. Over the 14-day experimental period, none of the treatments significantly affected weight loss, firmness, juice total soluble solids, pH or titratable acidity. The progressive increase in weight loss and the moderate decline in firmness observed across all treatments were therefore attributable mainly to the storage period rather than to treatment application. This result is particularly relevant for LM + LAB because the formulation contains fermentation-derived acidic metabolites that could potentially alter peel tissues or juice characteristics if applied at unsuitable concentrations. However, LM + LAB did not modify juice pH or titratable acidity and did not produce visible rind injury or deposits, indicating that its antifungal efficacy was achieved without detectable adverse effects on the measured quality attributes under the tested conditions. A similar compatibility with citrus fruit quality has been reported for *C. oleophila* and other biological or low-impact postharvest treatments that do not form persistent physical barriers on the fruit surface (Bhatta, 2022; Hammami et al., 2022; Rovetto et al., 2024b; Strano et al., 2022). Imazalil likewise did not alter the evaluated physicochemical parameters, consistently with evidence that postharvest residues of this fungicide remain predominantly associated with the peel and have limited effects on juice composition (Brown and Dezman, 1990). Taken together, the maintenance of firmness, weight-loss patterns and juice composition strengthens the technological relevance of LM + LAB by showing that its disease-control activity, including when applied after pathogen inoculation, was not accompanied

by measurable deterioration of the principal physicochemical attributes assessed during simulated refrigerated storage and subsequent shelf life.

Overall, the comparative evaluation revealed distinct efficacy profiles among the tested treatments under the two application timings. Imazalil consistently provided the highest level of disease and sporulation control, confirming its suitability as the chemical reference treatment, while *C. oleophila* maintained a robust effect on disease incidence and confirmed its value as an established biological reference. The most relevant outcome concerned LM + LAB, which was effective under both application timings and showed particularly strong activity when applied 4 h after pathogen inoculation. Under these conditions, its control of disease incidence was statistically comparable to that achieved with imazalil and *C. oleophila*, whereas its reduction of sporulation exceeded that achieved with *C. oleophila* and was second only to imazalil. This response indicates that LM + LAB is not limited to a protective effect established before pathogen arrival, but can also act effectively when contamination and inoculation have already occurred. The formulation therefore combines a broad application window with direct antifungal potential, substantial suppression of secondary inoculum production and compatibility with the physicochemical characteristics of the fruit. By contrast, the potassium phosphite-based formulation and phosphorous acid provided only partial control and appear more suitable as complementary components of integrated strategies than as stand-alone treatments. Taken together, these findings substantially expand the potential use of LAB-activated lemon-derived formulations in postharvest citrus disease management and provide a stronger experimental basis for their development as practical biobased treatments. It is noteworthy that in this study all products were tested under high inoculum pressure and on 'Tarocco' sweet orange, a blood orange cultivar very susceptible to Penicillium rot (Rovetto et al., 2023). Further evaluation under semi-commercial packinghouse conditions, different inoculum pressures and a wider range of citrus cultivars will be important to define optimal dosage, persistence and operational integration, while targeted studies on pathogen viability and infection development could clarify the fungicidal mechanisms underlying the marked post-inoculation activity observed here.

CRediT authorship contribution statement

Federico La Spada: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Mario Riolo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alessandra Benigno:** Investigation, Formal analysis. **Salvatore Moricca:** Writing – review & editing, Validation, Supervision, Project administration, Conceptualization. **Antonella Pane:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Santa Olga Cacciola:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Conceptualization.

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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.

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Data availability

Data will be made available on request.

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