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Efficacy of the cyclolipopeptides fengycin and iturin A against postharvest pome fruit pathogens

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Botrytis cinerea (gray mold) and *Penicillium expansum* (blue mold), are major pome fruit postharvest pathogens and their control relies heavily on the use of one postharvest fungicide (fludioxonil). This study aimed to evaluate the efficacy of the cyclolipopeptides (CLPs), in the form of fengycin and iturin A, in crude metabolite extracts from *Bacillus amyloliquefaciens* as an alternative biofungicide. The crude extract containing CLPs was applied with an edible coating (i.e., zein as a carrier) as a postharvest treatment against *B. cinerea* on "Packham's Triumph" pears and *P. expansum* on "Cripps Pink" apples. Treatments were applied either as dips in combination with a zein edible coating at pH 2.0 or pH 8.0, or as sprays with a zein edible coating at pH 2.0 or pH 8.0. The efficacy of CLP applications was measured in comparison to the standard registered synthetic fungicide fludioxonil at a concentration of 299.0 mg/L. Treatments were applied to the fruit either preventatively (as sprays) or curatively (as dips or sprays). The lowest mean blue mold incidence (68.6%) was achieved when the edible coating containing the crude extract of *B. amyloliquefaciens* (CLP at pH 2.0) was applied as a curative dip treatment. *B. cinerea* infection was reduced by 92.6%, resulting in a 5.7% gray mold incidence for the curative application of CLPs at pH 2.0. This result was not significantly different from the inhibitory action of the fungicide fludioxonil. These results indicate that CLPs are an effective alternative biofungicide that can be used for the control of *B. cinerea* on pome fruit, especially if their formulation and application are improved and optimized.

KEYWORDS

***Bacillus amyloliquefaciens*, alternative control, fruit decay, fungicides, postharvest treatment**

1 Introduction

A major constraint of pome fruit production worldwide is the occurrence of postharvest diseases, which can result in significant crop losses. Fruit is susceptible to damage by fungal diseases during cultivation, transportation, and storage, which results in 50% of the fruit harvested in countries with limited cold-chain capacity being lost due to decay (Qu et al., 2016). *Botrytis cinerea* Pers., causing gray mold, and *Penicillium expansum* Link, causing blue mold, are among the postharvest pathogens that extensively reduce the shelf and marketing life of apples and pears (Lutz et al., 2013; Spadaro and Droby, 2016).

Currently, postharvest fungal control is primarily conducted using synthetic fungicides, i.e., fludioxonil or pyrimethanil. However, owing to the pressure of consumer demands for reduced chemical residues in fruit because of perceived health risks, the increasing resistance of fungal pathogens to frequently used fungicides, and environmental concerns, there is a major drive toward the development of innovative non-chemical measures to control postharvest diseases (Conway et al., 2004; Zhang et al., 2007; Kefi et al., 2015; Spadaro and Droby, 2016). These growing concerns have resulted in the need to develop other methods to control postharvest decay, which can be complemented with antimicrobial agents and physical treatments (Nunes, 2012; Ali et al., 2015; Bordoh et al., 2020).

Cyclolipopeptides (CLPs) are secondary metabolites produced by microorganisms, primarily bacteria (e.g., *Bacillus amyloliquefaciens* Priest, Goodfellow, Shute & Berkeley, *B. subtilis* Cohn and *B. vallismortis* Roberts). They are a potential alternative to synthetic fungicides and can therefore both limit the overuse of postharvest synthetic fungicides and meet consumer demands for lower synthetic fungicide residue on fruit. Several studies report the use of these organisms as biocontrol agents; however, their variable efficacy when applied as living cells has hampered their application (Nunes, 2012). Instead of applying living bacteria as a fungicide, an alternative route is to produce the CLPs in fermentation, separate and purify the active component in the biocontrol agent, and apply them directly as biofungicides. Few studies have investigated methodologies to harvest significant quantities of CLPs, post fermentation, or investigated their efficacy when applied without the bacterial biocontrol agent present (Pretorius et al., 2015). Edible coatings, such as zein and carnauba waxes, have a positive influence on the quality and shelf life of fruit (Bai et al., 2003; Wibowo et al., 2021). Moreover, they can act as carriers of antimicrobials and antioxidants to protect produce from being attacked by pathogens. Zein is a protein-based coating that has been reported to be an excellent barrier that increases shelf life and prevents the weight loss of postharvest produce (Bai et al., 2003), whereas carnauba wax is a natural wax mainly used to improve fruit quality and appearance, provided it does not inhibit respiration, i.e., choose specific wax registered/used for pome fruit (Barman et al., 2011; Nascimento et al., 2016).

The use of CLPs, in the form of fengycin and iturin A, as potential biofungicidal applications has previously been reported (Ongena et al., 2005; Arrebola et al., 2010a). Although limited work has examined their application in plant pathogen control directly, CLPs have been found to be effective fungitoxic metabolites (Touré et al., 2004; Conway et al., 2004). The mode of action of fengycin and iturin A is thought to be the destruction of the fungal cell membrane, inhibition of spore germination, and inhibition of mycelial growth (Arrebola et al., 2010b; Qu et al., 2016; Calvo et al., 2017). Other studies have reported an indirect effect of CLPs as host defense inducers (Ongena et al., 2005; Waewthongrak et al., 2014). However, limited information has been published on the possibility of using CLP metabolites in the application of fruit fungicides (Dukare et al., 2018).

Furthermore, actual fungicide application to fruit requires that an application method be chosen, and each method differs in terms of its efficacy; some application methods that are widely used industrially include dipping, thermofogging, and spraying (Russouw et al., 2021). A consistent composition of CLPs can be achieved if metabolites are produced using a bioreactor (Tan et al., 2022). Further work is needed to verify the activity of each fungicidal lipopeptide component to achieve consistent results when usedas post harvest treatment.

This study aimed to evaluate the efficacy of the CLPs fengycin and iturin A from a crude extract from *B. amyloliquefaciens* fermentation broth against postharvest decay (gray or blue mold) on inoculated pome fruit either as a curative dip or spray application (preventatively or curatively).

2 Materials and methods

2.1 Bacterial strain cultivation and extraction of lipopeptides

B. amyloliquefaciens strain DSM 23117, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, was cultivated in growth media (as described in Pretorius et al., 2015) at 30°C for 48 h, at 150 rpm in an orbital shaker–incubator [Labcon model FSIM-SPO35); Labcon laboratory equipment (Pty.) Ltd., Krugersdorp, South Africa]. A 10% v/v of late-exponential-phase culture was transferred, axenically, to the liquid medium and incubated for 48 h at 30°C and 150 rpm. To remove bacterial cells, the broth containing the secondary metabolites was then centrifuged at 5,000 rpm for 35 minutes (Eppendorf 5702 R).

The cell-free culture supernatant was acidified to pH 2.0 through the drop-wise addition of 16% v/v hydrochloric acid (HCl) and the solution was stored for 16 h at 4°C so that complete precipitation could take place. The off-yellow colored acid precipitate, containing CLPs with some impurities, was concentrated, and recovered by centrifugation at 5,000 rpm for 15 minutes (Eppendorf 5702 R). The supernatant was discarded while the acid precipitate was collected from the pellet. The pellet was oven dried at 37°C for 24 h. The resulting dry acid precipitate was crushed into a powder using a pestle and mortar, weighed, and stored in a 50-mL Falcon tube at −18°C until required for further experimental use. The CLP extract was adjusted to pH 8.0 in 80%

ethanol for the dip and spray application using 1 M sodium hydroxide (NaOH), with a final concentration corresponding to 10 times the dry weight of acid precipitate to culture supernatant (Mazibuko, 2018).

Analysis of the composition of the actives in the CLP extract was conducted using high-performance liquid chromatography (HPLC) to quantify fengycin and iturin A metabolites; this was undertaken at the Central Analytical Facilities (CAF) at Stellenbosch University. The CLPs were compared with pure technical grade standards (Sigma cat. Nr. I1774 and SMB00292) and the results corresponded to iturin A. However, the retention time of fengycin varied slightly between batches, likely indicating differing congeners.

2.2 Pathogen inoculum preparation and fruit used

Virulent strains of the postharvest pathogens *B. cinerea* (STE-U 9254) and *P. expansum* (STE-U 9255), obtained from the Department of Plant Pathology, Stellenbosch University culture collection (STEU), were maintained on potato dextrose agar (PDA) and periodically transferred to fresh fruit to induce infection and spore production.

"Cripps Pink" apples and "Packham's Triumph" pears were collected from Western Cape pome fruit orchards. Unblemished, asymptomatic fruits were used in the trials. Prior to each treatment, fruits were surface sterilized by dipping in 250 $mg.L^{-1}$ chlorine (HTH Chlorine; 50 $mg.L^{-1}$ calcium hypochlorite) and tap water for 1 minute and air dried in a laminar flow cabinet overnight unless stated otherwise.

2.3 The effect of CLPs on *B. cinerea* or *P. expansum* infection using dip application

Sixty fruits (15 fruits per replicate, four replicates per treatment) were dipped for 30 seconds in one of the following treatments: (a) zein only (prepared as described above); (b) fludioxonil (299 mg/L; Teacher 230 SC, ICA, SA); (c) water only (untreated); (d) the "CLP in zein" treatment at pH 2.0; and (e) "CLP in zein" treatment at pH 8.0 (for *B. cinerea* only). Fruits were treated curatively (3 hours after inoculation). The trial was repeated once.

Zein-based edible coating was prepared by adding 1 g of zein powder (Sigma-Aldrich Cat. Nr. W555025, Merck KGaA, Darmstadt, Germany) with 10 mL of ethanol (80%) and left to dissolve in a 37 °CC water bath for 20 min. CLP extracts were added to both the zein coating solutions at a pH of either 2.0 (unadjusted, directly from acid precipitation product) or 8.0 (adjusted using 1 M NaOH). The dip application CLP extract contained 2,900.5 mg/L of fengycin and 191.3 mg/L of iturin A. The commercial standard fungicide fludioxonil was applied at the recommended dose of 299 mg/L as a positive control. Untreated fruit to which only water was applied) was included as a negative control.

Two wounds (3 mm wide × 5 mm deep) were made using a wounding tool and were inoculated with a 1×10^5 spores/mL spore suspension of *B. cinerea* or *P. expansum*. All fruits were incubated at 25°C ± 2°C in plastic-covered crates with moist paper towels. Lesion sizes were measured on days 4, 6, and 8 after inoculation (DAI). All fruits were incubated at 25°C ± 2°C in plastic-covered crates with moist paper towels. Fruit decay incidence (i.e., percentage of wounds infected per fruit) and severity (i.e., lesion size in mm) were evaluated on days 4, 6, and 8 after inoculation.

2.4 The effect of cyclolipopeptides on B. cinerea and P. expansum infection using spray application

Twelve fruits were used for each treatment, and each treatment was repeated three times in each trial. The spray application was either applied preventatively (3 hours before inoculation) or curatively (3 hours after inoculation) by spraying 110 µL of the treatment on each fruit, followed by gentle brushing for uniform distribution. For the positive control treatment, fruits were dipped in fludioxonil for 30 seconds. The treatments tested were the zein treatment (alone), and the two different CLP applications, that is, the CLPs in zein treatment at a pH of 2.0 and "CLPs in zein" at a pH of 8.0. An additional control treatment combining fludioxonil (299 mg/L) with zein was included for the curative trial only (spray application as described above). The trial was repeated once. The CLP extracts contained 80.7 mg/L of fengycin and 1.1 mg/L of iturin A in the spray application. Inoculation and incubation were carried out as described above for the curative application, and the preventative application was applied in an additional trial; the fruit was wound inoculated as described above 3 hours after the preventative fungicide spray application.

2.5 Statistical analysis

The mean incidence and severity of fruit decay were calculated as a percentage for each treatment replicate. All statistical analysis was carried out in Statistica v. 13.5 (TIBCO, Software, CA, USA) at the Center of Statistical Analysis, Stellenbosch University. Data (pooled for days 4, 6, and 8) were analyzed for incidence (number of wounds infected) and severity (lesion size), using formulas (i) (D/T) × 100, where D = total number of decayed fruit wounds, and T = total number of fruit wounds for the incidence, and (ii) (lesion size from each treatment/lesion size from the inoculated-untreated fruit) × 100, respectively (Russouw et al., 2021). A two-way ANOVA was used to compare means between treatments with treatment and day as factors and Levene's test was used to determine the homogeneity of variances. A probability level of 5% was considered significant for all significance tests. In some cases, homogeneity was strongly rejected ($p < 0.01$); therefore, a Welch test was added as a *post hoc* analysis.

3 Results

3.1 The effect of CLPs on *B. cinerea* or *P. expansum* infection while using the dip application

Curative fludioxonil treatment showed the lowest blue mold and gray mold incidence in all trials, with complete inhibition being observed for *B. cinerea* when fludioxonil was administered as a dip. The CLPs in the zein treatment at pH 2.0 was also highly effective, resulting in 5.7% gray mold incidence and 3.8% severity (not significantly different from fludioxonil control; Table 1, Figure 1), whereas CLPs in the zein treatment at pH 8.0 was less effective, resulting in 34.4% gray mold incidence and 32.9% severity. The application of zein only slightly reduced gray mold incidence (85.4%) and significantly lowered gray mold severity (36.9%), whereas untreated and inoculated fruits had high gray mold incidence (98.3%) and severity (98.2%). The CLPs in the zein treatment at pH 2.0 reduced blue mold incidence to 68.6%, which was a significant reduction compared with the incidence for the untreated control (95.0%). The CLPs in the zein treatment at pH 2.0 also reduced the severity of *P. expansum* to 37.5% (compared with

TABLE 1 Mean incidence of blue mold and gray mold infection after dip and spray trials with CLPs at pH 2.0 or pH 8.0.

Pathogen	Application	Treatment[a]	Final fengycin con. [mg/L] (µL per fruit)	Final iturin A con. [mg/L] (µL per fruit)	Mean incidence (%)[b] (SE)
B. cinerea	Curative dip	untreated	0	0	100.00 a (0.0)
		zein	0	0	85.41 ab (3.32)
		CLP pH 2.0 + Z	2900.5	191.3	5.69 de (3.32)
		CLP pH 8.0 + Z	2900.5	191.3	34.44 c (3.32)
		Fludioxonil	0	0	0.00 e (0.00)
	Curative spray	untreated	0	0	100.00 d (0.00)
		zein	0	0	100.00 ab (0.00)
		CLP pH 2.0 + Z	80.7 (110 µL)	1.1 (110 µL)	100.00 a–d (0.00)
		CLP pH 8.0 + Z	80.7 (110 µL)	1.1 (110 µL)	100.00 cd (0.00)
		Fludioxonil + Z	0	0	57.18 f (1.48)
		Fludioxonil	0	0	13.23 g (1.48)
	Preventative spray	untreated	0	0	100.00 a (0.00)
		zein	0	0	100.00 a (0.00)
		CLP pH 2.0 + Z	80.7 (110 µL)	1.1 (110 µL)	100.00 a (0.00)
		CLP pH 8.0 + Z	80.7 (110 µL)	1.1 (110 µL)	100.00 a (0.00)
		Fludioxonil + Z	0	0	77.31 d (2.00)
		Fludioxonil	0	0	79.51 d (2.34)
P. expansum	Curative dip	untreated	0	0	100.00 a (0.00)
		zein	0	0	89.86 a (3.32)
		CLP pH 2.0 + Z	2,900.5	191.3	68.61 b (3.32)
		CLP pH 8.0 + Z	n.t.	n.t.	n.t.
		Fludioxonil	0	0	21.36 cd (3.32)
	Curative spray	untreated	0	0	89.81 b (4.45)
		zein	0	0	94.91 ab (4.45)
		CLP pH 2.0 + Z	80.7 (110 µL)	1.1 (110 µL)	100.00 a (0.00)
		CLP pH 8.0 + Z	80.7 (110 µL)	1.1 (110 µL)	88.43 ab (7.71)
		Fludioxonil + Z	0	0	49.69 c (4.45)
		Fludioxonil	0	0	1.85 d (5.45)

(Continued)

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TABLE 1 Continued

Pathogen	Application	Treatment[a]	Final fengycin con. [mg/L] (µL per fruit)	Final iturin A con. [mg/L] (µL per fruit)	Mean incidence (%)[b] (SE)
	Preventative spray	untreated	0	0	100.00 a–c (0.00)
		zein	0	0	100.0 ab (0.00)
		CLP pH 2.0 + Z	80.7 (110 µL)	1.1 (110 µL)	100.00 a (0.0)
		CLP pH 8.0 + Z	80.7 (110 µL)	1.1 (110 µL)	87.50 cd (6.68)
		Fludioxonil + Z	0	0	78.12 d (4.09)
		Fludioxonil	0	0	49.31 e (4.72)

[a]Z zein as carrier.
[b]Different letters indicate significant differences for least significant means at 95% confidence interval using the Welsh test.
n.t., not tested.

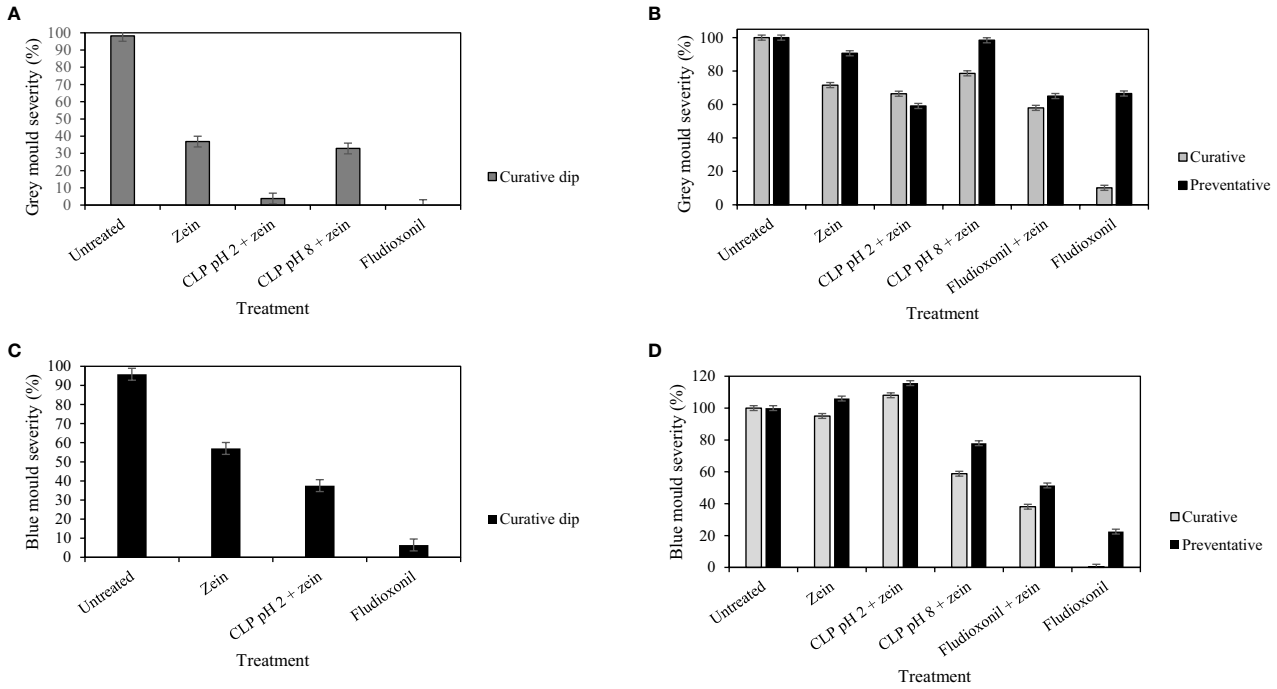

FIGURE 1
Comparison of decay severity for all treatments in the curative dip **(A, C)** or decay severity after treatment application either curatively or preventatively showing means across all 3 days **(B, D)** to control *B. cinerea* on pears **(A, B)** or *P. expansum* on apples **(C, D)**. Error bars show standard error (SE) at a 95% confidence interval.

95.8% in the untreated inoculated control). Zein dip application alone also reduced the incidence (89.9%) and severity (57.0%) of blue mold.

3.2 The effect of CLPs on *B. cinerea* or *P. expansum* infection while using the spray application

The most effective blue mold control was achieved with fludioxonil as a curative application (1.85% incidence; Table 1). Only the CLPs in the zein treatment at pH 8.0 applied preventatively marginally reduced blue mold incidence (87.5%), but not gray mold incidence. Nevertheless, the severity of blue mold was reduced in the CLPs in zein treatment at pH 8.0 (78.0%), compared with the untreated control (94.0%). Fludioxonil alone applied preventatively did not effectively protect the fruit from blue mold or gray mold infection with the very aggressive inoculation method used in this study, possibly owing to the wound that was created in the inoculation process that broke the film of the protective layer of edible coating.

An adverse effect increasing blue mold infection incidence in the protective and curative application of zein was found (Table 1); zein application resulted in higher incidence when fludioxonil

control treatment was applied combined with zein, in particular when it was applied curatively as a spray (49.69% incidence vs. 21.36% when applied as a dip treatment). Blue mold severity also increased by 6% with preventative zein application alone, or by 15.7% if combined with CLPs in zein at pH 2.0 (Figure 1). On the other hand, the severity of *P. expansum infection* was significantly reduced when CLPs in zein at pH 8.0 were applied curatively, with a mean lesion diameter of 11.0 mm compared with the untreated control at 18.2 mm. This is a 40% reduction in severity compared with the untreated control (Figure 1).

4 Discussion

CLPs are known for their strong antifungal activity against postharvest disease, and among them, iturin A and fengycin have been recognized as inhibitors of most pathogens (Ambrico and Trupo, 2017). This study set out to determine the efficacy of *B. amyloliquefaciens* CLPs as *in vivo* postharvest treatments on pome fruit. The CLPs were applied in a carrier of edible coatings, that is, zein for better efficacy and to improve the ease of their application. The CLPs have not been combined with zein in previous studies; however, zein has been found to maintain the overall health of fruit well compared with other commercial coatings (Bai et al., 2003). This study aimed to find better ways to apply the CLPs to the fruit to achieve greater efficacy in controlling postharvest pathogens. The CLPs in the zein treatment at pH 2.0 controlled blue mold when applied preventatively, whereas the CLPs in the zein treatment at pH 8.0 were most effective in controlling blue mold curatively. Conversely, previous studies have not found biofungicides to have eradicative activity and claimed that they would have a reduced spectrum of activity compared with synthetic fungicides (Conway et al., 2004). Calvo et al. (2017) demonstrated that *B. amyloliquefaciens* had a limited curative effect against *B. cinerea*, *P. expansum*, *P. italicum*, and *P. digitatum*, and higher disease inhibition was achieved with a preventative treatment. As the two fungi used in the current study are latent pathogens and the treatments used in packhouses are normally applied post harvest, a curative application was tested.

In the dip application, the fruit treated with CLP with zein as a carrier at pH 2.0 was most effective in reducing *B. cinerea* lesion size compared with the other application methods, which could also be due to the higher concentration of CLPs applied in the dip trial. Similarly, Calvo et al. (2017) reported that *B. amyloliquefaciens* was more effective at a lower pH (i.e., pH 5.0) compared with pH 7.5, which they explained was beneficial for the survival of the biocontrol agent in a more acidic fruit environment. In a parallel study, the thermofogging application of CLPs at pH 8.0 did not effectively reduce *B. cinerea* and *P. expansum* incidence, although it reduced the severity of the *P. expansum* infection. Additional studies to evaluate the thermofogging application using a higher treatment volume and CLP concentration are needed, in addition to a more in-depth evaluation of the effect of pH on the infection capabilities of *B. cinerea* and *P. expansum*.

Quantification of lipopeptides from crude extracts showed that the non-fungitoxic CLP surfactin produced higher concentrations than the CLPs of interest, that is, iturin A and fengycin (Ongena and Jacques, 2008; Pérez-García et al., 2011). Furthermore, the activity of other components of the CLP extract, such as mycosubtilin alone or in combination with surfactin, and their efficacy in controlling decay, will have to be tested (Kourmentza et al., 2021).

The adverse effects seen for zein as a spray application and with lower concentrations of fengycin and iturin A in the spray, and also for zein combined with fludioxonil in the same trial, could have been a result of micro-abrasions caused by brushing of the fruit to distribute the edible coating. Other (gentler) methods, such as atomizer application and the use of rollers instead of brushes to distribute the coating will have to be investigated. Further trials should also be done to optimize the formulation of zein in combination with propylene glycol as suggested by Bai et al. (2003). In addition, a preventative application should be carried out after wounding, that is, by pipetting the spore suspension into the treated wound.

B. amyloliquefaciens CLPs have the potential to control postharvest pathogens when used in a zein edible coating as a carrier. Delivery methods to achieve consistent amounts of active compounds present, and at a larger scale, are needed to improve the ease of CLP application on fruit. New commercial products containing antagonist metabolites or with *B. amyloliquefaciens* as an active ingredient are needed to control postharvest pathogens, as available products are mostly used for preharvest applications and are not formulated to effectively control the pathogens at the postharvest stage.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

SM conducted the experiments and drafted the manusript. JM-H, RP and CL acquired funding and edited the document. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The author JM-H declared that she was an editorial board member of Frontiers, at the time of submission. This had no impact on the peer-review process and the final decision.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fhort.2023.1175251/full#supplementary-material

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