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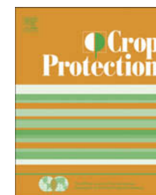

Combined application of antagonist *Bacillus amyloliquefaciens* and essential oils for the control of peach postharvest diseases

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ABSTRACT

Bacillus amyloliquefaciens PPCB004 was selected as a potential antagonist to control *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer* on peach fruit. The HPLC data of PPCB004 indicated the lipopeptides iturin A, fengycin and surfactin as secondary metabolites. The GC/MS analysis of PPCB004 showed 3-hydroxy-2-butanone as the dominant compound (97.52% of relative peak area). Thyme (TO) and lemongrass (LO) oils showed over 50% and 25% inhibition of radial mycelial growth respectively with 8 μl oil per plate for all pathogens. Combination treatment with both oils failed to increase the percentage inhibition of radial mycelial growth of the pathogens. Combined application of PPCB004 with TO or LO was tested to assess the effectiveness in the control of these pathogens during postharvest storage. The biofilm formation of PPCB004 was significantly higher in LO than TO. LO (6 μl plate^{-1}) and PPCB004 completely inhibited the mycelial growth of the pathogens. Fruit inoculation trials with PPCB004 + LO in NatureFlex™ modified atmosphere packaging (MAP), showed lower disease incidence and severity at 25 °C for 5 d than treatments with PPCB004 + MAP, PPCB004 + TO + MAP, LO + MAP, TO + MAP or stand-alone MAP. On naturally infected fruit, PPCB004 + LO + MAP and LO + MAP treatments retained the total soluble solids/titratable acidity ratio and flesh firmness but failed to stimulate the levels of total phenolic content, phenylalanine ammonia-lyase, β-1,3-glucanase and chitinase activities. Combination of PPCB004 (spray treatment) + LO (in pad delivery system) in NatureFlex™ MAP showed absence of disease and off-flavour development, retained the overall appearance and increased the overall acceptance at market shelf conditions (20 °C for 2 d) after cold storage at 4 °C for 14 d.

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1. Introduction

The major postharvest pathogens of peaches (*Prunus persica* (L.) Batsch.) include *Botrytis cinerea* Pers.:Fr. (grey mould), *Penicillium expansum* Link (blue mould) *Rhizopus stolonifer* (Ehrenb.:Fr.) (Rhizopus rot) and *Monilinia* spp. (Karabulut and Baykal, 2002; Zhang et al., 2007). There are three species: *Monilinia fructigena*, *Monilinia fructicola* and *Monilinia laxa*. The postharvest losses due to decay may increase up to 50% without fungicide treatment, although decay can be reduced to 5–10% with postharvest fungicides (Margosan et al., 1997). Because the use of synthetic fungicides is becoming more restricted due to health and environmental concerns, it is necessary to develop alternative treatments to replace these to reduce the environmental risk and satisfy consumer demands.

Application of biocontrol agents *Kloeckera apiculata* (Karabulut and Baykal, 2002), *Cryptococcus laurentii* (Zhang et al., 2007) and the use of hot water brushing and *Candida* spp. (Karabulut et al., 2002) were researched to control postharvest diseases in peaches. According to Karabulut et al. (2002), the combination of hot water brushing and *Candida* spp. gave inconsistent control of postharvest decay in peaches. *Bacillus* spp. were considered as potential biocontrol agents due to their high spore production ability, resistance and ability to survive desiccation, heat, U.V. irradiation, and organic solvents (Romero et al., 2007). The antagonist *Bacillus amyloliquefaciens* RC-2 produced antifungal compounds that inhibited the conidial germination of *Colletotrichum gloeosporioides* in mulberry leaves. Antifungal compounds isolated from strain RC-2 were identified as iturin A, a cyclic peptide with the following sequence: Asn-Tyr-Asn-Gln-Pro-Asn-Ser-3-amino-tetradecanoic acid (β-amino acid) (Yoshida et al., 2001).

The use of lemongrass oil (LO) on the control of *B. cinerea* or *R. stolonifer* was reported (Tzortzakis and Economakis, 2007). The active component of thyme oil (TO), thymol, controlled *B. cinerea*

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and retained the overall quality of table grapes in modified atmosphere packaging (MAP) (Valverde et al., 2005). Essential oils (EOs) and their components are gaining increasing interest due to their volatility, relatively safe status, wide acceptance by consumers, eco-friendly and biodegradable properties (Tzortzakis, 2007). Application of EOs is an attractive method to control postharvest diseases in postharvest systems due to their bioactivity in the vapour phase and the limitation of aqueous sanitation for many commodities, make them useful as possible fumigants.

Bacillus spp. (gram positive) form biofilms on fruit surfaces, which are multicellular matrixes of bacteria surrounded by extracellular polysaccharides called a glycocalyx. The glycocalyx acts as a physical barrier and is strongly anionic, thereby can protect the microcolony from external agents (Jeyasekaran et al., 2000). Recent studies suggested that the biofilm mode is important for the bacteria's ability to act as biocontrol agents (Bais et al., 2004). Monoterpene components of EO increased the biofilm formation in gram positive bacteria (Sandasi et al., 2008). Therefore, combined treatment of biocontrol agent *B. amyloliquefaciens* and EOs in a postharvest system may be beneficial to control the incidence of postharvest diseases during storage and transportation of peaches especially for organic growers.

The objectives of this study were to determine 1) the mode of action of the biocontrol agent *B. amyloliquefaciens* PPCB004 against the peach postharvest pathogens *B. cinerea*; *P. expansum*; and *R. stolonifer*, 2) the antifungal activity of the EOs on peach postharvest pathogens, 3) the biofilm formation of PPCB004 in presence of selected EOs, and 4) efficacy of combination treatments of PPCB004 as postharvest spray application and selected EOs in a pad delivery system in modified atmosphere packaging on disease control and disease resistance in peaches.

2. Material and methods

2.1. Pathogen inoculum

B. cinerea (Pers.:Fr.) PPRI8222, *R. stolonifer* (Ehrenb.:Fr.) Vuill PPRI9052 and *P. expansum* Link P1.1 were grown on Malt Extract Agar (MEA) (Merck, Johannesburg, South Africa) or Potato Dextrose Agar (PDA, Merck) for 7–10 d at 25 °C. Spore suspensions were prepared by removing the spores from the sporulating edges of the culture with a sterile glass rod by adding 5 ml of sterile deionised water with 0.02% Tween 80 for better spore separation. Spore suspensions were filtered through double-layered cheesecloth and the spore concentration was determined by haemocytometer (10^6 spores ml^{-1}) for each fungus.

2.2. Antagonist identification

B. amyloliquefaciens PPCB004 was isolated from the surface of citrus cv. 'Valencia'. Molecular identification of PPCB004 was based on colony morphology, API 50 CH test system (BioMérieu, Marcy-l'Etoile, France) and analysis of the 16S rRNA region after PCR amplification with primer 41F and 1486R-P specific for gram positive bacteria (Stackebrandt and Goodfellow, 1991). Bacterial DNA was isolated from *Bacillus* strains using the Illustra™ Bacterial genomicPrep Mini Spin Kit (GE-Healthcare UK Limited, Bucks, UK). For the PCR reaction, 2 µl of 1:10 diluted DNA stock solution was used. The PCR products were cleaned up using the MSB Spin PCRapace Kit (Invitek, Berlin, Germany), and 3 µl of clean PCR product was used for the sequencing marker PCR by BigDye® Terminator Kit (AB Foster City, California, USA). The resulting PCR products were purified and used directly for sequencing. Homology studies were carried out using the NCBI program BLAST (Zhang et al., 2000).

2.3. *In vitro antagonistic effect of B. amyloliquefaciens PPCB004 against the pathogens*

The antagonistic effect of PPCB004 was determined against the postharvest pathogens by the dual culture technique (Yoshida et al., 2001). The presence of antifungal secondary metabolites in the culture filtrate of PPCB004 was determined by thin layer chromatography (TLC), using the characterised *Bacillus subtilis* strain UMAF6614 (Romero et al., 2007) as standard. Bacterial cultures were grown in a medium optimum for lipopeptide production (Ahimou et al., 2000) at 37 °C. After 5 d of incubation, cells were removed by centrifuging at 2500*g* for 10 min and the supernatants were extracted with *n*-butanol. Once the butanol layer was completely evaporated, the residue was dissolved in methanol for further chemical analysis. The methanolic fractions containing the secondary metabolites were analysed by TLC (Razafindralambo et al., 1993). TLC analysis of secondary metabolites was performed on TLC plates coated with Liesel gel 60 F254, 20 cm × 20 cm (Merck). TLC plates were developed in chloroform/methanol/H_2O (65:25:4, v/v/v) as mobile phase. Thereafter, fungal bioautography was assayed on these TLC plates by mixing 3 ml of fungal spore suspension in 50 ml of autoclaved, luke-warm (ca. 50 °C) MEA for each pathogen. The fungal spore suspension in MEA was poured on the TLC and the plates were incubated at 25 °C for 3–4 d.

2.4. *Chromatography analysis of antifungal secondary metabolites and volatile compounds produced by B. amyloliquefaciens PPCB004*

The methanolic fractions used in TLC analysis were also analysed by Reverse Phase High Performance Liquid Chromatography (RP-HPLC), using an analytical reverse phase C18 column ultrasphere, 4.6-mm diam, 150-mm long (Supelco, Bellefonte, PA 16823, USA) and solutions of 0.05% trifluoroacetic acid in acetonitrile and milliQ water, with a flow rate of 1 ml min^{-1}.

The identification of volatiles produced by PPCB004 was analysed by GC/MS using a method developed by Sivakumar et al. (2008). The samples (150 ml) were placed in a water bath at 35 °C for 3 d. The volatiles were isolated by purging the samples with 500 ml N_2 (g) (5.0, Afrox, Gauteng, South Africa) at 25 ml min^{-1}. Multi-channel open tubular silicone rubber traps (MCTs) were used to collect the volatile compounds from the bacterial strains (Ortner and Rohwer 1996). The GC column was a Zebron, ZB1 30 m × 250 µm ID × 0.25 µm film thicknesses (Phenomenex, Separations, Randburg, South Africa), the velocity of the carrier gas (helium) was 46 cm s^{-1} (1.8 ml min^{-1}) and the column head pressure was 65 kPa in the constant pressure mode. The GC oven temperature program was −20 °C (3 min) at 5 °C min^{-1} to 250 °C (5 min). The GC run time was 62 min. The GC/MS transfer line was at 280 °C, the mass scan range was 35–450 atomic mass units in full scan mode, the source (EI+) temperature 230 °C, the MS quadruple temperature 150 °C, the ionisation energy 70 eV and the electron multiplier (EM) 1753 V. Tentative identification of organic compounds was performed by a probability based match search of the Wiley spectral library. Matches of the mass spectra of the components to that of the library were ≥80%. The retention times (RT) of the components were also established. Identification of each individual compound was made by comparison of their retention times with those of pure components, matching mass spectral data with those from NIST mass spectra library software.

2.5. *Antifungal activity of EOs on mycelial growth of the pathogens*

The inhibitory effects of ten EOs (Table 2) were tested *in vitro* on mycelial growth of *B. cinerea*, *P. expansum* and *R. stolonifer*. Preliminary screening was carried out by incorporating 10 µl of

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each EO separately in 6 mm diameter Whatman filter discs and thereafter placing the discs in the centre of PDA plates spread-plated with 100 μl of either fungal spore suspension. The inhibitory effect of the EOs on each pathogen was determined by measuring the diameter of the inhibition zone. The two most effective EOs obtained from the preliminary screening, lemongrass (LO) and thyme oil (TO) were tested again as single or combination treatments *in vitro* using a disc volatilisation method, incorporating the pure EO aliquots 2, 4, 5, 6, 8 and 10 μl separately in sterile Whatman filter discs (6 mm) (Plaza et al., 2004). The EO incorporated in each disc was placed on the inner surface of the Petri dish lid and the Petri dishes were sealed with Parafilm and incubated upside down at 25 °C. The inhibition zone was measured with a Vernier calliper (Digimatic; Mitutoyo Co., Japan) in mm and the results were expressed as percentage inhibition of radial mycelial growth (IRMG). All the assays were repeated twice with six replicates.

2.6. Antifungal activity of EOs on spore germination of the pathogens

Two 100 μl drops of 50% PDA were placed on sterile microscopy slides. A fungal spore suspension prepared in Ringer's solution with 0.02% Tween 80 was sprayed over the microscope slides. The slides were kept inside a sterile Petri dish at 100% RH, and a sterile Whatman filter disc (6 mm) incorporated with aliquots of 2, 4, 5, 6, 8 or 10 μl of the two selected pure EOs was placed separately in the centre of each slide. Thereafter, the Petri dish was sealed with Parafilm and incubated at 25 °C for 12 h. The germination was stopped and stained with lacto-phenol cotton blue, and the stained slides were dehydrated for 30 min at 50 °C. The percentage spore germination was calculated by counting the number of germinated spores out of 200 spores via microscope (optical microscope Zeiss, Oberkochen, Germany) (40× magnification). The assay was repeated twice with six replicates.

2.7. Effect of EO on biofilm formation of B. amyloliquefaciens PPCB004

A liquid culture of PPCB004 (10^8 cfu ml^{-1}) was incubated at 37 °C for 12 h to assay the biofilm formation. Biofilm formation and quantification were carried out according to Peeters et al. (2008). One hundred microlitres of PPCB004 was pipetted into the 24 wells of a microtitre plate and incubated at 37 °C for 24 h to allow cell attachment and biofilm formation. For biofilm fixation, 100 μl of ethanol (99%) was added into each well of a 96-well microtitre plate. After 15 min the ethanol was removed and the plate was air-dried. The biofilm quantification was evaluated separately in the presence of the EOs; LO or TO selected from 2.5. To assay the effect of each EO on antagonist biofilm formation, the wells of the microtitre plate fixed with PPCB004 were exposed to sterile pieces of Whatman filter paper (80 × 120 mm) incorporated with aliquots of 2, 4, 5, 6, 8 or 10 μl of EO diluted in 1 ml ethanol separately for 2 h and quantification of biofilms was made using a crystal violet assay. The negative controls contained biofilm and ethanol or biofilm and water. Following the incubation period, plates were washed with 100 μl of sterile distilled water to remove any loosely associated or planktonic bacteria. The plates were air-dried and the wells were stained with 100 μl of 0.5% crystal violet and incubated at 25 °C for 15 min after which the plates were washed under running tap water. Finally, bound crystal violet was released by adding 150 μl of 33% of acetic acid (Sigma). One hundred microlitres from each well were measured at 595 nm using a micro-plate reader. The mean of the samples and the standard deviations were determined and plotted against EO concentrations. The efficacy of biofilm formation was measured by comparing the readings of the biofilms at different concentrations of EOs to that of the positive and negative controls. The experiment was repeated twice with 24 replicates with each EO.

2.8. Effect of combined treatment on the mycelial growth of pathogens

The two EOs with the best performance (LO and TO, selected from 2.5) were used to investigate the combined effect with PPCB004 according to the volatilisation method in 2.5 with modification. A liquid culture of PPCB004, 5% (v/v), shake-incubated at 37 °C for 12 h, was added to the PDA before pouring into the Petri dishes. The inhibitory effect was measured after 5 d according to a 1–5 visual scale (1 = 0%; 2 = 25% growth, 3 = 50%, 4 = 75%, 5 = 100% growth).

2.9. Effect of B. amyloliquefaciens PPCB004 and EO on in vivo disease development

Peach fruit, cv. Transvaal, were collected from the Morvel Boerdery packhouse, Gauteng Province, South Africa. The fruit (300) were disinfected by ethanol (70%) spray, air-dried and thereafter wounded (1 × 1 mm) with a needle. From the disinfected, wounded fruit, 150 fruits were sprayed with PPCB004 (10^8 cfu ml^{-1}) 24 h prior to pathogen inoculation. Thereafter, pathogen spore suspensions (10^6 spores ml^{-1}) were sprayed on the fruit and left for 24 h to initiate infection. The PPCB004 and the pathogen were sprayed on the fruit surface via a pressured air sprayer for 10–15 min. A set of ten fruit subjected to stand-alone treatments PPCB004 or LO or TO and combination treatments PPCB004 + LO or PPCB004 + TO were packed in a biodegradable modified atmosphere packaging (MAP, NatureFlex™ Biodegradable Film (E944), Innovia Films Ltd, Cumbria, UK). EO was introduced into the packaging containing the fruit by incorporating 75 μl in a Whatman filter paper strip (2 × 5 cm) and the packaging was heat sealed. The fruit in MAP were incubated for 3–5 d at 25 °C. Fruit packed in stand-alone MAP without any treatment served as control. Disease incidence was recorded and disease severity was obtained by measuring the lesion diameter (mm). Each had five replicates and each replicate contained ten fruit. The experiment was repeated twice.

2.10. Analysis of enzyme activity and total phenolic content

Phenylalanine ammonia-lyase (PAL) activity was determined according to Jiang et al. (2001) with some modifications. Peach pulp (10 g) obtained from five fruit per treatment (cumulative sample) was homogenised by Ultra-Turrax homogeniser (Ultra-Turrax, IKA-Werk, Germany) in 15 ml of 0.1 M borate buffer (pH 8.8) containing 5 mM β-mercaptoethanol and 2 mM EDTA. The homogenate was centrifuged for 20 min at 5500*g* and the supernatant was collected and filtered using Whatman No 1 filter paper (55 mm diameter) to determine the PAL activity and total phenolic content. The PAL activity was measured by incubating 0.5 ml supernatant with 2 ml of 0.1 M borate buffer (pH 8.0) containing 3 mM L-phenylalanine for 30 min at 37 °C. Increased absorbance at 290 nm, due to the formation of *trans*-cinnamic acid, was measured spectrophotometrically. The PAL activity was expressed as change in Abs_{290} h^{-1} mg^{-1} protein (Jiang et al., 2001). Proteins were assayed by the dye-binding method of Bradford (1976) with bovine serum albumin as the standard.

1,3-β-Glucanase (β-Glu) activity was assayed by measuring the rate of reducing sugar production with laminarin (Sigma, USA) as the substrate (Rivera et al., 2002). Peach pulp (10 g sample) was extracted in 0.5 M sodium acetate (pH 5.2) with 15 mM of β-mercaptoethanol.

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The extracts were centrifuged at 5500g for 30 min at 4 °C and the supernatants were used in β-Glu activity assay. The absorbance was measured spectrophotometrically at 660 nm.

Chitinase activity: peach pulp (10 g) obtained from five fruit per treatment (cumulative sample) was extracted in 0.1 M phosphate buffer (pH 7.2) with 15 mM of β-mercaptoethanol. The extracts were centrifuged at 5500g for 30 min at 4 °C and the supernatants were used in a chitinase assay according to Liu et al. (2003) using chitin azure (Sigma–Aldrich, Missouri USA) as substrate. The absorbance was measured spectrophotometrically at 550 nm. For the above mentioned enzyme assays three replicate samples were taken from each treatment

Total phenolic content: determined using the Folin–Ciocalteu procedure (Jiang et al., 2001), and measured at 690 nm and expressed as mg of equivalent gallic acid g^{-1} fresh weight. Three replicates per treatments were used to determine the enzyme activity and the total phenolic content, and the experiment was repeated twice.

2.11. *Effect of postharvest spray application of* B. amyloliquefaciens *PPCB004 in combination with MAP natural decay control and overall fruit quality*

In the PPCB004 + MAP and PPCB004 + LO + MAP treatments, the antagonist PPCB004 (10^8 cfu ml^{-1}) was initially sprayed on the fruit and allowed to air dry for 1 h. Thereafter, fruit subjected to all three treatments were packed in biodegradable NatureFlex™ packaging. For treatments that included LO, pads to release the LO within MAP during storage were made with heat sealable grade 126/3 tea bag paper (Schnabel and Mercier, 2006) cut into squares 4 cm × 5 cm and sealed on each side with a heat sealer. A Whatman filter paper strip (3 cm × 3 cm) impregnated with 75 μl LO was inserted into each tea bag and heat sealed together with the MAP containing the fruit. Fruit subjected to all four treatments were stored at 4 °C and 90% RH for 14 d. Each postharvest treatment had five replicates and each replicate consisted of ten fruit.

After cold storage, the fruit were held at 20 °C for 48 h to simulate market shelf conditions and observations were made on incidence of decay, severity, fruit quality [flesh firmness, total soluble solids (TSS) and titratable acidity (TA)] and sensory parameters. Head-space gases CO_2 and O_2 were measured from five replicates per treatment using a PBI Dansensor CO_2/O_2 gas analyser (Checkmate 9900, Ringsted, Denmark) at the end of the 4 °C storage (14 d) and during market shelf conditions. Incidence of decay was expressed as %decay severity measured according to surface percentage of decay score rating (score 1: 0%, score 2: 25%, score 3: 50%, score 4: 75% and score 5: 100%). Flesh firmness was evaluated from 10 fruit per treatment and measured on opposite sides of each fruit using a Chatillon digital penetrometer (John Chatillon & Sons, New Cork, USA) fitted with an 8-mm fruit tester probe and expressed in Newton (N). The TSS were determined with a digital refractometer (PR-100 Atago, Tokyo, Japan) and expressed in percentages. Percentage TA was determined by titration of juice with 0.01 M NaOH and calculated as malic acid equivalent. For sensory evaluation, ten fruit per treatment were given to a panel of ten members who are familiar with peach fruit sensory characters. The samples, coded with three-digit numericals, were presented at random one at a time. Sensory profiles were assessed using a nine-category scale. The intensity of the descriptors increased from 1(=none or poor) to 9 (=extreme or excellent) for aroma, flavour, taste, overall appearance (decay and skin colour) and acceptance. Overall appearance was based on decay control and skin colour retention (absence of skin browning).

2.12. *Statistical analysis*

A complete randomised design was adopted in this study. The data obtained were subjected to analysis of variance (ANOVA) using SPSS 8.0 software for Windows (SPSS Inc., Chicago, IL, USA). The mean values were compared using Tukey's HSD test at $P < 0.01\%$. The effect of LO, TO stand-alone and combination against pathogens was also analysed by a regression statistical test. For the sensory evaluation based on score rating, the means were separated using least significant difference (LSD) at $P < 0.01\%$. Limpel's formula, as described by Richer (1987), was used to determine synergistic interactions between antagonist and LO or TO. Limpel's formula is $E_e = X + Y - (XY/100)$, in which E_e is the expected effect from additive response of two treatments and X and Y are the percentages of disease reduction relative to each agent used alone.

Table 1
High performance liquid chromatography (HPLC) of secondary metabolites and gas chromatography/mass spectrometry (GC/MS) analysis of volatile compounds of the antagonist *Bacillus amyloliquefaciens* PPCB004.

HPLC analysis				GC analysis		
RT[a] (min)	RPA[b] (%)	Height (AU)[c]	Product	RT (min)	RPA (%)	Product
17.089	5.39	0.831	Iturin A	13.92	0.18	3-Methyl 1-butanol
21.742	2.26	0.366	Iturin A	11.88	97.52	3-Hydroxy-2-butanone (Acetoin)
22.527	2.34	0.402	Iturin A	19.41	0.36	2-Heptanone
24.122	7.95	1.215		26.56	0.42	2-Nonanone
				32.86	0.60	2-Undecanone
40.919	7.57	0.888	Fengycin	34.72	0.12	2-Dodecanone
41.519	22.79	0.399	Fengycin	38.43	0.48	2-Tridecanone
43.848	5.21	0.550	Fengycin	20.88	0.30	*n*-(Diphenylmethylene) aminoacetonitrile
46.671	1.54	1.777	Fengycin			
47.437	5.42	0.343	Fengycin			
49.359	4.77	0.278	Fengycin			
78.825	2.30	0.372	Surfactin			
88.251	12.55	0.579	Surfactin			
95.075	15.43	0.458				
95.923	4.46	0.390				

[a] RT: retention time.
[b] RPA: relative peak area.
[c] AU: absorbance units.

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Therefore, if the combination of the two agents produces any value of disease reduction greater than E_e, then synergism exists.

3. Results and discussion

3.1. Antagonistic effect of B. amyloliquefaciens PPCB004 on fungal pathogens

It is evident from the dual culture, that strain PPCB004 is an efficient antagonist to inhibit the mycelial growth of *B. cinerea* (inhibition zone 31 ± 0.28 mm), *P. expansum* (24 ± 0.27 mm) and *R. stolonifer* (11 ± 0.1 mm). However, the non-antibiotic producer antagonist used as control failed to show any inhibitory zones. The HPLC data on the secondary metabolites of PPCB004 showed the presence of lipopeptides which were identified as iturin A, fengycin and surfactin (Table 1). The spot on TLC-bioautographies from the PPCB004 extract that showed sufficient antifungal activity to inhibit the growth of the three pathogens was identified as iturin A (Fig. 1). The antifungal effect of iturins produced by *B. amyloliquefaciens* has been previously shown by Yu et al. (2002). The GC/MS analysis of the volatiles produced by PPCB004 showed 3-hydroxy-2-butanone (acetoin) as the dominant compound with 97.52% of relative peak area (RPA) (Table 1). According to Choudhary et al. (2008), acetoin showed antifungal activity in a low oxygen atmosphere environment, like MAP used in this study.

3.2. Effect of essential oils on the growth and germination of the pathogens

The preliminary screening of the EOs mentioned in Table 2 showed different inhibitory effects on the target pathogen. *B. cinerea* was the most resistant pathogen. The inhibitory potency of different EOs on the three pathogens was as follows: peppermint and tea tree oils < basil oils < LO and TO (data not presented). Grape fruit oil showed an inhibitory effect similar to basil oil, with a 3 cm inhibition zone. Eucalyptus, and lemon failed to show any inhibitory effect on any of the fungal species. The LO and TO have proved their effectiveness against several postharvest pathogens (Feng and Zheng, 2007; Tzortzakis and Economakis, 2007). Therefore, LO and TO were selected for further investigation. The mode of action of the EOs is attributed to their hydrophobicity, which enables them to partition in the lipids of the cell membrane, thus disturbing its integrity and the inorganic ion equilibrium (Lambert et al., 2001).

In order to determine the most effective doses of TO and LO on the control of postharvest diseases in peaches, and to investigate their possible synergistic effect, increasing volumes of TO and LO were used in experiments. Table 3 illustrates the observed percentage of IRMG for each pathogen with the two EO treatments showing the best results, i.e. LO and TO. Significant differences ($P < 0.01$) in percentage IRMG were observed for the three tested pathogens between the stand-alone LO and TO treatments. Stand-alone treatment with TO showed over 50% IRMG with 8 µl oil per plate for all three pathogens but, stand-alone treatment with LO did not. The combination treatment (TO + LO) showed lower percentage IRMG ($P < 0.01$) especially for *B. cinerea*, than the stand-alone TO treatment, clearly showing the absence of a synergistic effect in combination treatment (LO and TO). Although all treatments completely inhibited the spore germination of *B. cinerea*, *P. expansum* and *R. stolonifer* with oil volumes of 2 µl per plate, the data from the percentage IRMG confirmed that TO showed strongest antifungal activity against the three pathogens tested in this study. The antifungal activity of EOs has been demonstrated usually using a direct contact assay such as diffusion or dilution methods (Dafarera et al., 2000; Tzortzakis and Economakis, 2007). In diffusion assays, the EO components are partitioned through the agar according to their affinity for water, and in dilution methods low water solubility has to be overcome by addition of emulsifiers or solvents such as Tween 80 or ethanol, which may alter the activity

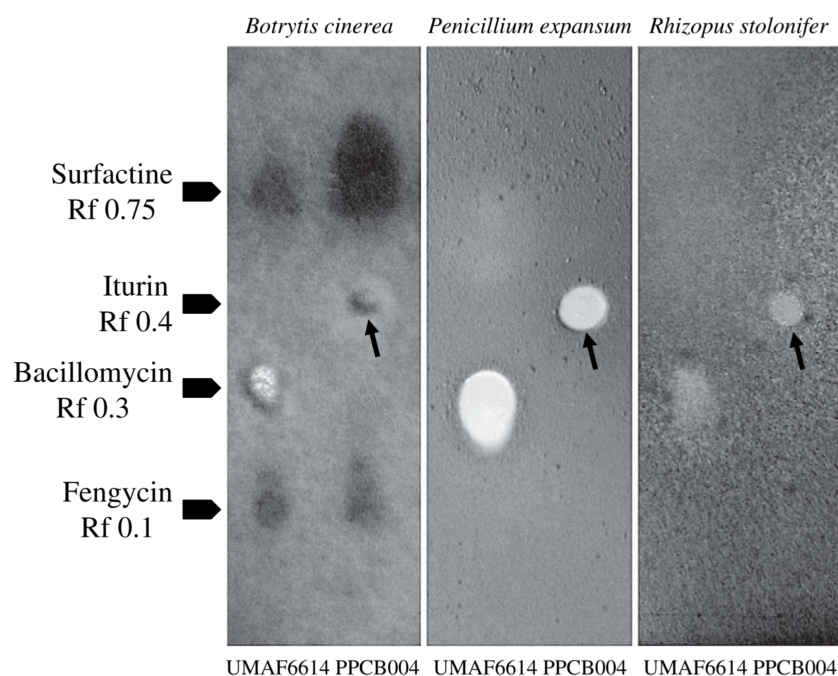

Fig. 1. Thin Layer chromatography bioautography showing the absence of mycelial growth of *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer*. The spot marked with arrow showed Rf = 0.3 corresponded to bacillomycin produced by *Bacillus subtilis* UMAF6614 (standard), and the spot marked with arrow showed Rf = 0.4 corresponded to iturin A produced by *Bacillus amyloliquefaciens* PPCB004.

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Table 2
List of essential oils tested in this study against *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer*.

Common name	Botanical name	Active compound	Source[a]
Basil	*Ocimum basilicum* L.	Estragole, linalool	Burgess & Finch
Eucalyptus	*Eucalyptus melliodora* L.	Grandinol	Dis-Chem
Grapefruit	*Citrus paradisi* Macf.	Triclosan	Burgess & Finch
Lemon	*Citrus limonum* Risso	D-Limonene	Burgess & Finch
Lemongrass	*Cymbopogon citratus* Stapf.	Citral	Burgess & Finch
Peppermint	*Mentha piperita* L.	Allyl isothiocyanate	Burgess & Finch
Tea tree	*Melaleuca alternifolia* L.	α-Terpinene	Dis-Chem
Thyme	*Thymus vulgaris* L.	Thymol, carvacrol	Burgess & Finch

[a] Burgess & Finch (Vital Health Foods S.A. Distributor, Kuils River, South Africa); Dis-Chem (Pty) Ltd. Randburg, South Africa.

of the EOs. Therefore, a disc volatilisation method was adopted in this study to identify the antifungal effect of selected EOs in a vapour phase using small volumes of EOs.

3.3. *Combined effect of* B. amyloliquefaciens *PPCB004 and EOs on* in vitro *mycelial growth and biofilm formation of PPCB004*

Torres et al. (2007) showed that the combination of two or more different non-fungicidal postharvest treatments showed synergistic effects and increased their efficacy in reducing the decay development in citrus. On this basis, the synergistic effect of LO and PPCB004 or TO and PPCB004 combined treatments on radial mycelial growth of the three pathogens was tested *in vitro*. In the presence of PPCB004; 40% culture plates inoculated with *B. cinerea* and 80% with *P. expansum* and 100% with *R. stolonifer* showed inhibition of mycelial growth according visual score 1 *in vitro* (data not presented). When LO was introduced *in vitro* the volume needed to show complete inhibition of mycelial growth was decreased to 6 μl per plate for *B. cinerea*, 5 μl per plate for *P. expansum* and 2 μl per plate for *R. stolonifer*. More or less similar observations were recorded with TO; the volume of oil required to result in complete inhibition of mycelial growth was decreased to 6 μl per plate for *B. cinerea* and 2 μl per plate for *P. expansum* and *R. stolonifer*.

Due to the antimicrobial activity of EOs (Sandasi et al., 2008) it is necessary to test the effect of EO on biofilm formation of PPCB004 exposed to LO or TO. The effect of TO and LO treatments on biofilm formation is shown in Fig. 2. TO treatment significantly ($P < 0.01$) affected the biofilm formation of PPCB004, with increasing volumes of oil. However, LO treatment showed less inhibition on the biofilm formation of PPCB004 and 5 μl per plate was selected as optimal volume for *in vivo* fruit trials. For *in vivo* and naturally infected fruit trials, 75 μl of LO was used in an MAP postharvest system (75 μl per MAP) which was calculated according to 5 μl of LO per Petri dish.

Table 3
Effect of lemongrass or thyme oils as stand-alone or combined treatments on the percentage inhibition of radial mycelial growth of peach postharvest pathogens.

Treatments	*Botrytis cinerea*	*Penicillium expansum*	*Rhizopus stolonifer*
Untreated (control)	0.00a[c]	0.00a	0.00a
Lemongrass oil			
R^2	0.82	0.80	0.84
2 μl plate^{-1}	9.4ab	30.4b	0.00a
4 μl plate^{-1}	10.6ab	37.5bc	29.6bc
5 μl plate^{-1}	13.5abc	43.0cd	26.3b
6 μl plate^{-1}	15.4abc	45.3cd	30.3bc
8 μl plate^{-1}	22.8bcd	49.8def	34.6bc
10 μl plate^{-1}	27.8bcd	45.1cd	43.6c
Thyme oil			
R^2	0.57	0.52	0.24
2 μl plate^{-1}	22.9bd	37.1bc	64.8d
4 μl plate^{-1}	33.5de	53.8ef	83.5ef
5 μl plate^{-1}	40.3ef	57.8fg	77.8def
6 μl plate^{-1}	49.8gf	61.9g	70.9de
8 μl plate^{-1}	53.5gf	62.5g	72.9def
10 μl plate^{-1}	56.1g	67.1fg	78.6def
Lemongrass + Thyme oils			
R^2	0.71	0.57	0.31
LO[a] (2 μl plate^{-1}) + TO[b] (2 μl plate^{-1})	18.4abd	39.6bc	65.0de
LO (4 μl plate^{-1}) + TO (4 μl plate^{-1})	20.0abd	45.9cd	80.9ef
LO (5 μl plate^{-1}) + TO (5 μl plate^{-1})	30.6de	37.5bc	76.0def
LO (6 μl plate^{-1}) + TO (6 μl plate^{-1})	28.8cde	51.6de	66.6de
LO (8 μl plate^{-1}) + TO (8 μl plate^{-1})	29.1cde	60.8g	65.4de
LO (10 μl plate^{-1}) + TO (10 μl plate^{-1})	50.0fg	57.3fg	69.7de

R^2 coefficients.
[a] LO: Lemongrass oil.
[b] TO: Thyme oil.
[c] Means followed by the same letter within columns for each pathogen are not significantly different at $P < 0.01$ according to Tukey's statistic test.

3.4. *Effect of EO and* B. amyloliquefaciens *PPCB004 combined treatment on disease incidence, enzyme activity and total phenolic content in artificially inoculated peach fruit*

The observations from *in vivo* fruit trials showed differences ($P < 0.01$) in disease inhibition and severity between the control and treated fruits (Table 4). Combined treatment PPCB004 + LO showed higher percentage of disease inhibition ($P < 0.01$) and severity reduction than PPCB004 + TO treatment on fruit subjected to artificial inoculation with *B. cinerea*, *P. expansum* and *R. stolonifer*. The expected additive response (E_e), determined according to Limpel's formula for *B. cinérea*, *P. expansum* and *R. stolonifer* disease inhibition was 44%, 26% and 46.2% respectively in combined treatment PPCB004 + LO. The observed values of disease reduction were 50% for *B. cinerea*, 42% for *P. expansum* and 82% for *R. stolonifer*, which indicated a synergistic effect between PPCB004 and LO in inhibiting the disease incidence.

The observed effect of combined treatment PPCB004 + TO on disease inhibition and severity reduction could be due to the higher antibacterial effect of TO on the biofilm formation of PPCB004 on the fruit surface as shown in Fig. 2. The presence of phenolic rings and hydroxyl groups on the phenol rings of thymol (44.7% of the TO fraction) and carvacrol (2.4% of the TO fraction), the active volatile components of TO, could have enhanced its antimicrobial activity

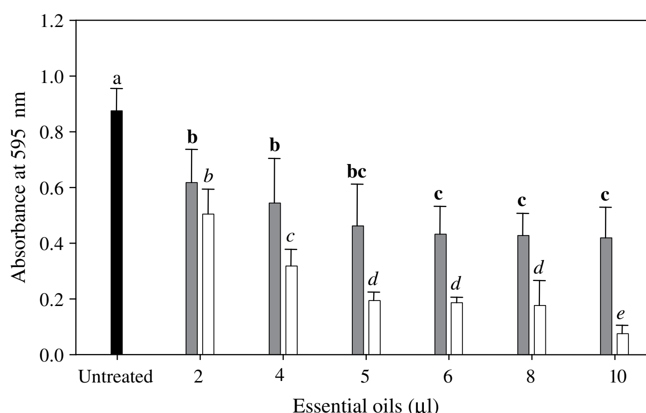

Fig. 2. Biofilm formation of *Bacillus amyloliquefaciens* PPCB004 in presence of lemongrass and thyme oils at different volumes *in vitro*. Bars show lemongrass (■) and thyme (□) compared with untreated (control) (■) biofilm. Means in each bar followed by the same letter are not significantly different at $P < 0.01$ Tukey's HSD test.

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Table 4
Percentage of disease inhibition and severity reduction in artificially infected fruit with postharvest pathogens.

	Botrytis cinerea		*Penicillium expansum*		*Rhizopus stolonifer*	
	Incidence reduction (%)	Severity reduction (%)	Incidence reduction (%)	Severity reduction (%)	Incidence reduction (%)	Severity reduction (%)
Untreated (control)	0a[d]	0.0a	2a	0.0a	8a	0.0a
PPCB004[a]	30c	47.6c	14b	51.0c	74cd	28.0c
LO[b]	20b	37.6b	14b	16.6b	16b	0.0a
TO[c]	30c	33.5b	8a	20.8b	23b	0.0a
PPCB004 + LO	50e	44.3c	42d	58.9d	82d	40.0d
PPCB004 + TO	24b	27.5ab	24c	51.4c	74cd	14.5b

Peach fruit cv. Transvaal were inoculated with *Botrytis cinerea*, *Penicillium expansum* or *Rhizopus stolonifer* separately and after 24 h treated with the antagonist. The combined treatments of PPCB004 + LO and PPCB004 + TO were also evaluated against the pathogens.
[a] *Bacillus amyloliquefaciens* PPCB004.
[b] Lemongrass (LO).
[c] Thyme (TO) oils separately.
[d] Means followed by the same letter within columns for each pathogen for disease incidence or severity reduction are not significantly different at $P < 0.01$ according to Tukey's statistic test.

(Bagamboula et al., 2004). However, in LO the active volatile component, citral (65–80% of the LO fraction), does not have the phenolic groups in its chemical structure. Furthermore, more severe skin browning was observed in inoculated fruit treated with TO treatments. This clearly stated the phytotoxic effect of TO on the fruit, and making TO an unsuitable agent to use in combination with PPCB004. There was no significant increase in PAL activity in inoculated fruit subjected to different treatments (data not shown). However, a slight non-significant increase in PAL activity was observed in inoculated fruit subjected to TO treatments. The reason for the observed difference in PAL activity with respect to TO (stand-alone and in combination treatments) and other treatments could be due to the observed skin browning in TO treated fruit, which had resulted in stress-induced PAL activity and is not due to defence mechanisms (Cisneros-Zevallos, 2003).

No increase in total phenolic content was observed with respect to different treatments in inoculated fruit (data not presented) and β-Glu and chitinase activities were not detected in inoculated fruit subjected to these treatments (data not presented). Neither induced resistance nor production of defence enzymes seem to be involved with respect to all treatments adopted in this study. Poppe et al. (2003) reported the absence of induced resistance in citrus fruit treated with *Pantoea agglomerans* CPA-2 (biocontrol agent) against *Penicillium digitatum* and *Penicillium italicum*. Although LO or TO failed to show induced resistance in this study, natural volatile compounds such as methyl jasmonate have been demonstrated to enhance disease resistance responses in peaches (Jin et al., 2009). This investigation also clearly demonstrated that the antagonistic effect of PPCB004 on the three pathogens was due to the production of bacterial secondary metabolites mentioned in Table 1 the iturin A, fengycin and surfactin and also due to the volatile compounds, mainly 3-Hydroxy-2-butanone (acetoin), and biofilm formation.

3.5. *Effect of EO and* B. amyloliquefaciens *PPCB004 combination treatments on decay incidence, fruit quality and sensory parameters of peach fruit*

It is evident from this investigation that the inhibitory potency of different treatments on the postharvest decay incidence of peach fruit was as follows: PPCB004 + LO + MAP > LO + MAP > PPCB004 + MAP > stand-alone MAP. The severity rating for naturally infected fruit subjected to the different treatments was; score ∼5 for stand-alone MAP, score ∼2 for PPCB004 + MAP, score ∼1 for LO + MAP, score 0 for PPCB004 + LO + MAP. Flesh firmness was higher ($P < 0.01$) in fruit subjected to PPCB004 + LO + MAP and LO + MAP (Fig. 3A) treatments and at harvest the flesh firmness was ∼30 N. The observed postharvest decay at market shelf conditions was the primary reason for the loss of firmness in PPCB004 + MAP and stand-alone MAP treatments. Flesh firmness was considered as the best indicator of ripening and one predictor of the potential shelf life. None of the treatments tended to increase the total phenolic content in peach fruit during storage. Tzortzakis (2007) reported different observations in tomatoes (main crop and cherry) and strawberry with respect to EO treatments and total phenolic content. In strawberry the total phenolic content was observed to decrease with EO treatments, whereas in tomatoes a non-significant increase of total phenolic content was reported. The TSS/TA at harvest was ∼28. Differences ($P < 0.01$) in TSS/TA ratio were observed between the fruit subjected to PPCB004 + LO + MAP or

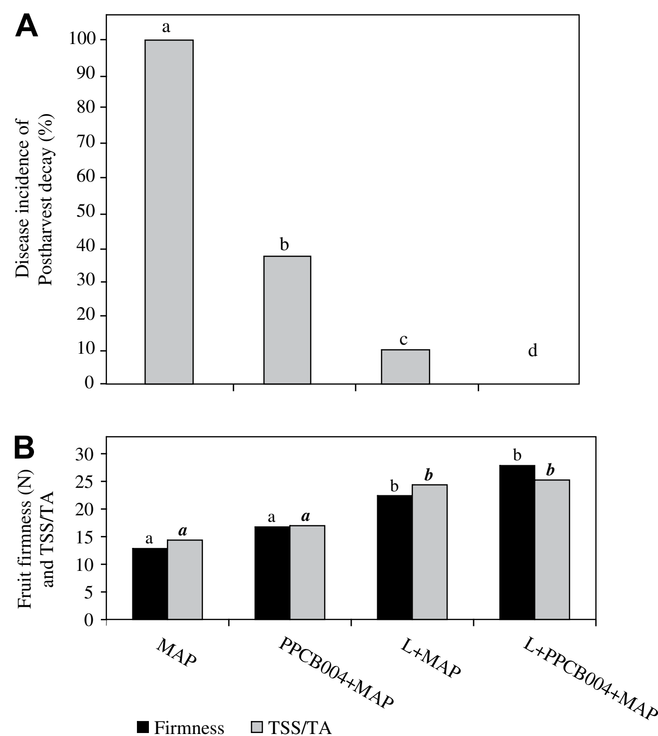

Fig. 3. Effect of postharvest treatments on (A) decay incidence of postharvest diseases (B) fruit firmness, and total soluble solid concentration/titratable acidity ratio in naturally infected peach cv Transvaal. MAP – modified atmosphere packaging (control), PPCB004 – *Bacillus amyloliquefaciens* LO – Lemmon grass oil. Fruit subjected to all four treatments were stored at 4 °C and 90% RH for 14 d. Means in each bar followed by the same letter are not significantly different at $P < 0.01$ Tukey's HSD test.

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Fig. 4. Effect of postharvest treatments on overall appearance (colour and decay) of naturally infected peach cv Transvaal stored at 4 °C for 14 d and at 20 °C for 2 d. MAP – modified atmosphere packaging (control), PPCB004 – *Bacillus amyloliquefaciens* LO – lemongrass oil, TO – thyme oil.

LO + MAP and other treatments (Fig. 3B). However, stand-alone MAP and PPCB004 + MAP showed a decrease in ($P < 0.01$) TSS and increasing TA, and thereby reduced the TSS/TA ratio mainly due to the decay developed during market shelf conditions.

Sensory evaluation was not carried out for control fruit and fruit from PPCB004 + MAP due to higher decay incidence. Although the sensory panellist data revealed a similar score (~7) for fruit treated with PPCB004 + LO + MAP and LO + MAP for taste, PPCB004 + LO + MAP treated fruit showed better overall appearance based on decay control and skin colour retention (absence of skin browning) (Fig. 4) and acceptance (~8 score). A slight odour of LO was detected immediately after opening both packages (PPCB004 + LO + MAP and LO + MAP), but it disappeared rapidly with time due to evaporation at room temperature (25 °C). However, after treatment there were no adverse effects observed in aroma (~7 score) or flavour (~7 score) in either treatment as in agreement with what was observed in banana by Anthony et al. (2003).

Usage of a pad delivery system to release the LO within the MAP enabled the avoidance of tainting on the fruit in LO + MAP and PPCB004 + LO + MAP, since peaches are thin skinned and more prone to tainting as observed before with direct contact with the EO impregnated paper in the MAP in 3.4. The use of MAP has been found to be effective in retaining the overall quality in many fruit, but the CO_2 concentration inside the packaging could not be increased to produce antimicrobial effects. Also, the increased CO_2 concentration can result in off-flavour development in fruit. In this investigation the gas composition within the MAP was ~3% O_2 and ~5.0% CO_2 after cold storage and at 20 °C ~1–1.5% increase in CO_2 was observed in the MAP. The observed changes in CO_2, and O_2 within the packaging at 2 °C and 14 °C were due to the effect of temperature and RH in the storage environment on the film permeability. There tended to be slight variations in gas compositions in different postharvest treatments adopted in this study.

This investigation demonstrated that, by combining LO with the antagonist PPCB004, the fruit can be protected from the incidence of postharvest diseases and the overall fruit quality can be retained within the MAP without increasing the CO_2 composition (higher than 10%) within the packaging. EOs and their constituents are categorised as flavourings by the European Decision (2002/113/EC). Citral which is the major component of the EO fraction of LO is reported not to cause cancer in male or female rats receiving 4000 ppm (~3.56 mg ml^{-1}) citral in the feed for 2 years (National Toxicology Program, 2003). The Acceptable Daily Intake is 5 mg citral kg^{-1} body weight and it was given Generally Recognized As Safe (GRAS) status in the United States (National Toxicology Program, 2003).

The bacterial antagonist, *Pseudomonas syringae* Van Hall, controlled green mould of citrus and grey mould of apple, by producing an antibiotic syringomycin (Bull et al., 1998). However, the production of this antibiotic was never detected on the fruit and vegetables despite extensive efforts. The antibiotics are either produced in levels below the sensitivity for the detection methods or raise the doubt on that antibiosis is the only mechanism responsible for *P. syringae* to control postharvest disease. However, in this research the antagonist *B. amyloliquefaciens* was isolated from the surface of citrus cv. Valencia and it showed multiple modes of action in decay control. It is also evident that some stains of *B. amyloliquefaciens* do not to possess the genes encoding *Bacillus* enterotoxins or the key gene implicated in the synthesis of emetic toxins, or does not demonstrate phenotypic characteristic of toxin production (European Food Safety Authority, 2008). *B. amyloliquefaciens* and other members of the *B. subtilis* group are considered as safe and have "Generally Recognized As Safe" status, GRAS (Food and Drug Administration 1999).

4. Conclusion

The present study demonstrated the potential of using *B. amyloliquefaciens* PPCB004 in combination with LO in a pad delivery system within the biodegradable MAP. The amount of LO used in the pad delivery system was reduced by combining it with the antagonist PPCB004 in order to reduce an unpleasant fruit odour and taste resulting from the use of LO. To our knowledge this is the first report on a combination with an antagonist and EO to improve the beneficial effect of MAP in retaining the overall fruit quality in peaches during storage. This treatment is also beneficial for organic peach growers and suitable for marketing chains of 14–16 d.

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