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Effect of biocontrol agent *Bacillus amyloliquefaciens* and 1-methyl cyclopropene on the control of postharvest diseases and maintenance of fruit quality

Mohamed Salia Osman [a], Dharini Sivakumar [b,*], Lise Korsten [c]

[a] Department of Horticultural Science, Faculty of Agriculture and Natural Resources, University of Bakht Er-Ruda, Ed Duiem, Sudan
[b] Department of Crop Sciences Tshwane University Of Technology, Pretoria West, Pretoria 0001, South Africa
[c] Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0002, South Africa

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ABSTRACT

Efficacy of biocontrol agent *Bacillus amyloliquefaciens* PPCB004 was evaluated on the control of anthracnose and phomopsis rot in 'Solo' papaya pre-treated with 1-methyl cyclopropene (100 μl) (1-MCP) during storage. This treatment was compared to the untreated control, commercial treatment (washing in chlorinated water), stand alone 1-MCP and PPCB004 treatment. Although fruit pre-treated with 1-MCP delayed the ripening (100% yellow) after cold storage by 9–10 d, it showed higher incidence and severity of anthracnose and phomopsis rot than the fruit subjected to commercial treatment. Application of PPCB004 after 1-MCP pre-treatment (1-MCP + PPCB004) reduced the anthracnose and phomopsis incidence and severity after cold storage (10 °C, 85% RH for 14 d) and ripening at 25 °C. The 1-MCP + PPCB004 treatment helped to retain the fruit firmness, overall quality and uniform yellow skin (100%) and flesh colour after ripening. The PPCB004 was effectively recovered from stand alone PPCB004 and 1-MCP + PPCB004 treated fruit after cold storage and ripening. The PPCB004 population showed an increase by 1 log units after ripening in 1-MCP + PPCB004 treated fruit. After ripening the recovery of PPCB004 population was higher (0.7 log units) in 1-MCP + PPCB004. The total recovery of fungal population on the fruit surface after ripening was lower in 1-MCP + PPCB004 and stand alone PPCB004 treated fruit. It can be concluded that application of *B. amyloliquefaciens* PPCB004 with 1-MCP pre-treated papaya (at 25–30% skin yellow stage) can significantly reduce disease incidence associated with 1-MCP treatment. This treatment has the potential for commercial application in the 'organic' papaya industry.

1. Introduction

Papaya (*Carica papaya* L.) is a popular desert fruit cultivated in the tropical and subtropical regions of the world. Although this exotic fruit is favoured on the local markets due to its excellent taste and well known health benefits and nutritional value, it has not emerged as a major traded fresh fruit. This is mainly due to rapid flesh softening after harvest resulting in reduced shelf life, thin skin that can easily be damaged during harvesting and handling often coupled with high incidence of postharvest decay. Postharvest diseases were identified as the major cause of quality loss in the supply chain. Anthracnose, caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz., is identified as a major postharvest disease in the tropics affecting many fruit crops (Snowdon, 1990). Postharvest diseases caused by classic wound pathogens such as *Phomopsis* Petr. & Cif and *Rhizipous stolonifer* (Ehrenb.Fr.) Vuill. (=*R. nigricans* Ehrenb.) are also considered important postharvest pathogens particularly during poor field and facility sanitation and improper harvesting and handling practices.

Postharvest application of prochloraz or propiconaziole (Sepiah, 1993) or combination of fungicides and hot water treatments (Couey and Farias, 1979) were recommended to control postharvest diseases on papaya. However, hot water dip treatment was reported to affect fruit ripening (Paull, 1990) and is often difficult to manage properly in commercial settings. Due to global concern over the often indiscriminate use of pesticides and its hazardous side effects on nature and human health, more stringed product registration requirements have been developed. Due to perceived low profit margins by major agricultural chemical companies, re-registration of existing pesticides for small niche crops has not been considered a priority. The lack of strategic development of new chemical products for exotic fruits does not favor future growth in this market segment. On the other hand, due to the emergence of fungicide resistant strains, postharvest fungicide application is

* Corresponding author. Tel.: +27 12 382 5303; fax: +27 12 382 4162.
E-mail address: dharinisivakumar@yahoo.co.uk (D. Sivakumar).

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often not considered a long term solution for the industry. Therefore, the search for natural environmental friendly alternative products and processes becomes important for the exotic fruit industry.

Physiologically papaya is a climacteric fruit and during ripening it shows a climacteric rise in respiration and ethylene production. The application of ethylene inhibitor 1-methyl cyclopropene (1-MCP) has been adopted for some climacteric fruit including papaya (Hofman et al., 2001; Ergun and Huber, 2004) to prevent non-homogenous ripening due to exogenous ethylene exposure or rapid fruit softening because of poor postharvest handling practices. According to Manenoi et al. (2007), disease development was delayed and -severity reduced with 1-MCP treated fruit than in non-treated (control fruit) 'Solo' papaya. Fruit treated with 1-MCP at more than 25% ripe stage also showed a delay in fruit softening and may have commercial utility. However, according to Hofman et al. (2001), although the 1-MCP treatment delayed ripening in fruit harvested at commercial maturity, the incidence of post-harvest diseases, anthracnose, black rot (*Phoma caricae-papayae* (Tarr) Punith), black spot (*Asperisporium caricae* (Speg.) Maubl) and stem black rots (*Lasiodiplodia theobromae* Syn. *Botryodiplodia theobromae* Patouillard), were observed to increase after ripening. Therefore, to ensure even ripening, extend shelf life and reduce decay, 1-MCP could provide a commercial solution given it does not increase disease incidence. In order to assess the effect of 1-MCP treatment during prolonged cold storage (14 d at 10 °C) and after ripening at 25 °C under simulated marketing conditions, an additional protectant should be included to prevent 1-MCP disease-associated development.

The biocontrol agent, *Bacillus amyloliquefaciens* PPCB004 was selected as a potential antagonist to control *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer* on peach fruit (Arrebola et al., 2010a). The HPLC data of PPCB004 indicated the lipopeptides iturin A, fengycin and surfactin as secondary metabolites (Arrebola et al., 2010a). The GC/MS analysis of PPCB004 showed 3-hydroxy-2-butanone as dominant compound (Arrebola et al., 2010b). The objective of this study was therefore, to determine the effect of *B. amyloliquefaciens* PPCB004 application on papaya pre-treated with 1-MCP on decay control and quality retention of 'Solo' papaya after cold storage (for 14 d, 10 °C and 80% RH) and after ripening at 25 °C market simulated conditions.

2. Materials and methods

2.1. Pathogen inoculum

Phomopsis caricae-papayae and *C. gloeosporioides* were isolated from symptomatic papaya fruit. Purified cultures were maintained on Potato Dextrose Agar (PDA, Merck, Johannesburg, South Africa) slants 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% of Tween 80 (Merck) for better spore separation. Spore suspensions were filtered through sterile double-layered cheesecloth and the spore concentration was determined using a haemocytometer and diluted to obtain a final concentration of 10^5 spores ml^{-1} for each fungus according to Arrebola et al. (2010b).

2.2. Biocontrol agent

B. amyloliquefaciens PPCB004 isolated from the surface of citrus 'Valencia' was used in this study. Molecular identification of *B. amyloliquefaciens* PPCB004 was confirmed according to Arrebola et al. (2010a). The biocontrol PPCB004 formulation of PPCB004 was prepared for this trial by Stimuplant cc., Pretoria, South Africa. The formulation was mixed with water (1:6 v/v) to obtain a final concentration of 10^9 cfu ml^{-1} according to the manufacture's instruction.

2.3. Effect of 1-MCP treatment and B. amyloliquefaciens PPCB004 on disease development in vivo

Freshly harvested 'Solo' Papaya fruits at 25–30% yellow (maturity stage) and uniform fruit size, free from decay and defects were collected from the Rodney Copper's packhouse, Tzaneen, South Africa. Prior to inoculation trials, the fruits were disinfected with 70% ethanol spray. The fruits for inoculation trial were subjected to following treatments; 1-MCP treatment, 1-MCP + PPCB004, PPCB004 alone, and commercial treatment (200 µl l^{-1} NaOCl) for each test pathogen (*C. gloeosporioides* or *Phomopsis*) separately. Each treatment with respect to specific pathogen had 10 replicate fruits. A set of 40 fruits was placed in an air tight chamber for 100 µl 1-MCP treatment (SmartFresh™ powder, active ingredient (0.14%), Rohm and Hass, South Africa) for 24 h at 20 °C. After 1-MCP treatment, fruits were air equilibrated for 6 h at 25 °C prior to inoculation or further treatments. 1-MCP treated (40) or non-treated fruit (40) was wounded with No 2 Cork borer (1.2 cm diameter) on the fruit surface. The wound was inoculated with 100 µl of spore suspension (10^5 spores ml^{-1}) of the test pathogen separately and incubated for 24 h at 25 °C to initiate infection. Inoculated wounds in 1-MCP treated fruits (40) and non-treated fruits (40) were treated with 1 ml PPCB004 formulation (10^9 cfu ml^{-1}). After treatment fruits were held at 25 °C. A set 10 wound inoculated fruits for each pathogen without any treatments served as control. Disease incidence was recorded and disease severity was evaluated by measuring the lesion diameter (mm) after 5 d at 25 °C and the experiment was repeated twice.

2.4. Effect of 1-MCP treatment and B. amyloliquefaciens PPCB004 on control of anthracnose and phomopsis rot in naturally infected fruit

A set of fruits at 25–30% yellow was subjected to the following four postharvest treatments; 1-MCP (100 µl) treatment, 1-MCP + PPCB004 [fruits were initially subjected to 1-MCP (100 µl) treatment for 24 h at 25 °C, equilibrated in air for 8 h and thereafter, subjected to dip treatment in PPCB004 (1:7 v/v, 10^9 cfu ml^{-1})]; PPCB004 alone; commercial treatment (fruits washed in chlorinated water sodium hypochlorite, 250 µl ml^{-1}) and untreated fruits (control). At completion of these treatments, fruits were packed and stored for 14 d at 10 °C and at 80% RH. After cold storage, fruits were allowed to ripen at 25 °C. Each treatment had five replicate boxes and each box had five fruits per box. The experiment was repeated twice in order to confirm the observations.

Incidence of anthracnose or phomopsis rot was recorded as the ratio of fruits showing disease development against the total number of fruits treated. Disease severity was determined by measuring the lesion diameter in mm. For each treatment number of days to reach 100% yellow skin and for initial occurrence of disease was assessed.

2.5. Recovery of B. amyloliquefaciens PPCB004 from naturally infected fruit after cold storage and ripening

Three fruits were randomly selected from each treatment after cold storage and after ripening at 25 °C, and washed in 500 ml quarter strength Ringer's solution (Merck) in an ultrasonic bath (Ultrasonic Manufacturing Company (Pty) Ltd., Johannesburg) at 25 °C for 30 s (Govender et al., 2005). The surface washing was filtered through a 0.22 µm filter in a vacuum assembly. The filters

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were cut aseptically in quarters, of which one was transferred to 9 ml Ringer's solution and subjected to a serial dilution. Aliquots of 0.1 ml of each dilution from each postharvest treatment was transferred to Standard-1 agar (Biolab), PDA and Malt Extract gar (Biolab) plates and incubated at 32 °C, 48 h for growth of bacteria and 28 °C for 4–5 d for fungi and yeast. Observation on survival of *B. amyloliquefaciens* PPCB004 and total surface bacterial, yeast and fungal population was expressed as colony forming units per millimetre (cfu ml^{-1}) (Govender et al., 2005). The PPCB004 colonies were distinguished from background flora based on colony morphology and confirmation of identity of selected representative isolates. The background microflora was obtained from control fruits. Identity of *B. amyloliquefaciens* PPCB004 was confirmed by molecular identification according to Arrebola et al. (2010a).

2.6. Effect of 1-MCP treatment and B. amyloliquefaciens PPCB004 on fruit quality

Fruit surface skin and flesh colour were measured (20 fruits per treatment) using a Minolta Chromameter, expressing CIELAB Commission International de I Eclairage (CIE) colour space values (L^* *Chroma* h°). Overall quality of the fruits was assessed as; 1–2: not marketable; 3-with limited marketability; 4–5: fair, moderate defects; 6–7: good with slight defects; 8–9: excellent). Fruit firmness was assessed for 20 fruits per treatment on opposing sides of each fruit with a Chatillon penetrometer (Chatillon and Sons, New York, USA) equipped with a 6 mm diameter plunger capable of penetrating through the peel into the pulp.

2.7. Statistical analysis

Data were subjected to analysis of variance (ANOVA) using the GenStat for Windows (2004) statistical package. Fisher's protected least significant difference (LSD) at 1% level of significance was performed.

3. Results and discussion

3.1. Effect of B. amyloliquefaciens PPCB004 on disease incidence and severity in 1-MCP pre-treated papaya

The severity of anthracnose and phomopsis rot was significantly ($P < 0.001$) higher in 1-MCP treated fruits than that of the commercial treatment, PPCB004 stand alone or 1-MCP + PPCB004 combination treatment after 5 d at 25 °C (Fig. 1). The disease severity of phomopsis rot and anthracnose was significantly ($P < 0.001$) reduced in fruits treated with 1-MCP + PPCB004 treatment.

In naturally infected papaya fruits, the anthracnose and phompsis rot incidence and severity were also significantly ($P < 0.001$) higher in 1-MCP treated fruits than those of the fruits subjected to 1-MCP + PPCB004, commercial treatment, and PPCB004 alone treatment (Fig. 2A and B) Our observation supports the findings of Hofman et al. (2001) with the increase of stem-end rot in 'Kensigton Pride' Mango, and black rots and anthracnose in 'Solo' papaya after 1-MCP treatment. The 1-MCP treatment also showed increased decay incidence in non-climacteric fruits such as strawberries (Ku and Wills, 1999) and increased the incidence of *Penicillum digitatum*, *Penicillum italicum* and stem-end rots caused by *Diplodia natalensi* in Shamouthi oranges (Porat et al., 1999). One explanation may be the concentrations of antifungal compounds that are required to prevent disease development were not maintained at optimum levels, although the 1-MCP treatment delayed ripening, (Prusky and Keen, 1993). Based on Hofman et al. (2001) and our findings, the 1-MCP treatment was applied at the 25% fruit ripe stage and the ripening was further delayed by resulting in fruits close to the full ripe stage at which the fruits would have had lower concentration of antifungal compounds. In addition, the 1-MCP treatment could have inhibited the ethylene induced plant defence mechanism as shown in Japanese pear where 1-MCP reduced the expression of one of three tested plant defense-related proteins (Itai et al., 2000).

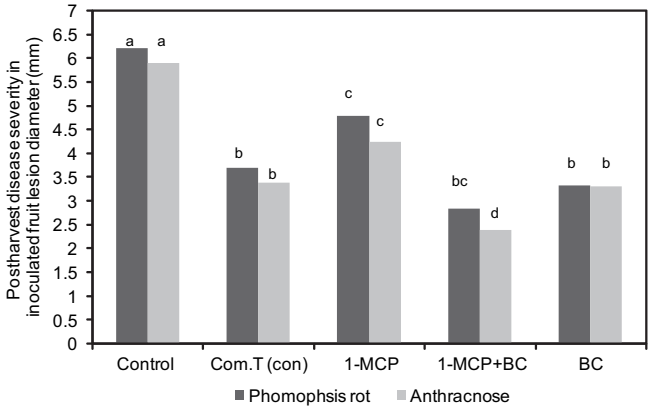

Fig. 1. Effect of *Bacillus amyloliquefaciens* PPCB004 on anthracnose and phomopsis severity in inoculated papaya pre-treated with 1-MCP. Means in each bar followed by the same letter are not significantly different at $P < 0.001$ by Fisher's protected least significant test. Con = Control (untreated fruit); Com.T = Commercial treatment (NaOCl 250 µl l^{-1}); 1-MCP + BC = 1-methyl cyclopropene + *Bacillus amyloliquefaciens* PPCB004; BC = *Bacillus amyloliquefaciens* PPCB004.

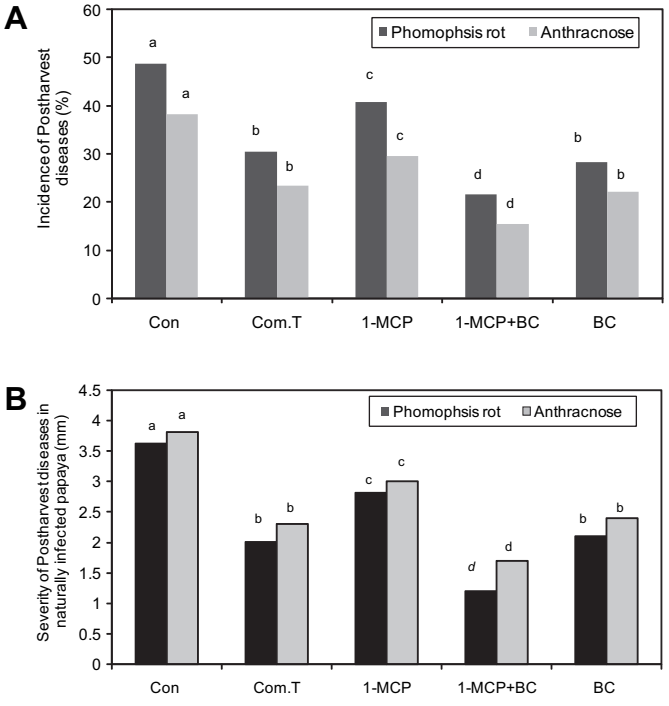

Fig. 2. Effect of *Bacillus amyloliquefaciens* PPCB004 on anthracnose and phomopsis. (A) incidence and (B) severity in naturally infected papaya pre-treated with 1-MCP. Means in each bar followed by the same letter are not significantly different at $P < 0.001$ by Fisher's protected least significant test. Con = Control (untreated fruit); Com.T = Commercial treatment (NaOCl 250 vµl l^{-1}); 1-MCP + BC = 1-methyl cyclopropene + *Bacillus amyloliquefaciens* PPCB004; BC = *Bacillus amyloliquefaciens* PPCB004.

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The 1-MCP + PPCB004 application effectively reduced the anthracnose and phomopsis rot incidence in naturally infected fruits during storage and after ripening than the commercial treatment (chlorinated water). However, the effectiveness of PPCB004 stand alone treatment on phomopsis rot and anthracnose disease control was similar to the currently adopted commercial treatment (Fig. 2A).

The disease severity of anthracnose and phomopsis rot in naturally infected fruits was shown in Fig. 2B. The postharvest disease severity was significantly ($P < 0.001$) higher in 1-MCP treated fruit than in fruits subjected to PPCB004 stand alone or commercial treatment. The application of PPCB004 reduced the disease severity of anthracnose and phomopsis rot as commercial control. However, among all treatments tested in this study the 1-MCP + PPCB004 treatment significantly ($P < 0.001$) reduced the anthracnose and phomopsis rot severity in naturally infected fruits after ripening.

3.2. Recovery of *B. amyloliquefaciens PPCB004*

The recovery of the biocontrol agent PPCB004 was observed in the 1-MCP + PPCB004 and PPCB004 stand alone treatments after cold storage. However, no significant difference ($P < 0.001$) was found in the PPCB004 populations between the two treatments (Fig. 3A). After ripening at 25 °C, an increase in the PPCB004 population was also observed in both treatments. The 1-MCP treated fruits showed an increase of ~0.7 log units compared to the PPCB004 stand alone treatment (Fig. 3B). A similar increase in antagonist *Metschnikowia Pulcherrima* strain ST1-D9 on 1-MCP treated apples was reported in comparison with the 1-MCP untreated fruits (Leverentz et al., 2003). The PPCB004 *B. amyloliquefaciens* showed multiple modes of action by producing iturin A, fengycin and surfactin (Arrebola et al., 2010a) and 3-hydroxy-2-butanone (Acetoin) as dominant volatile compound (Arrebola et al., 2010b). Therefore, PPCB004 can be applied as a protectant for 1-MCP treated fruit. According to our findings the PPCB004 population showed an increase after ripening at 25 °C. In addition, a higher fungal population was observed on the fructoplane of 1-MCP treated fruits and it increased by log 1.8 units in naturally infected fruits after ripening. The integrated treatment with 1-MCP + PPCB004 and PPCB004 stand alone treatment reduced the fungal population by maintaining a higher bacterial population in the fructoplane. Although the commercially adopted treatment, washing the fruit in chlorinated water significantly reduced the fructoplane microbial population, the total fructoplane population increased significantly ($P < 0.001$) after ripening.

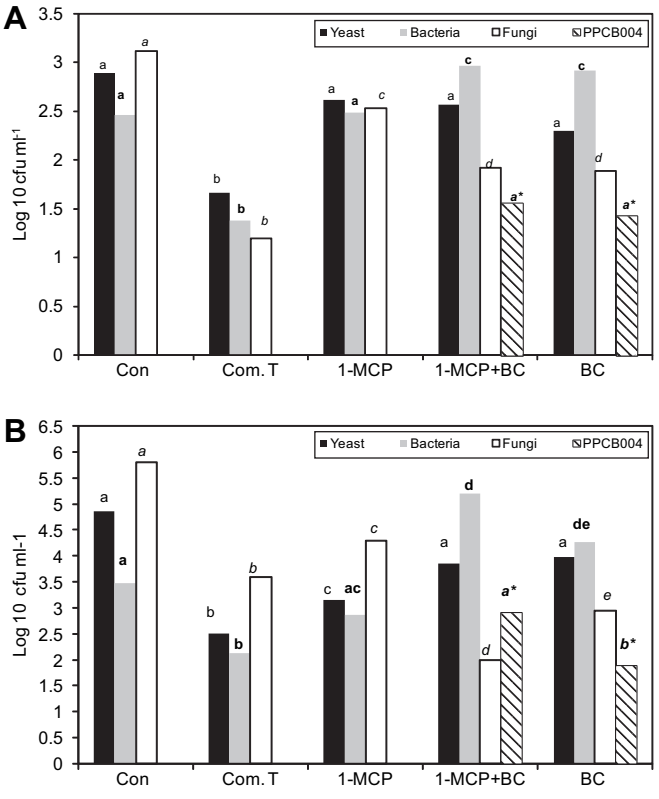

Fig. 3. Effect of different postharvest treatments on the survival of *Bacillus amyloliquefaciens* PPCB004 and the total microbial population (A) after cold storage (B) after ripening. Means in each bar followed by the same letter are not significantly different at $P < 0.001$ by Fisher's protected least significant test. Con = Control (untreated fruit); Com.T = Commercial treatment (NaOCl 250 µl l^{-1}); 1-MCP + BC = 1-methyl cyclopropene + *Bacillus amyloliquefaciens* PPCB004; BC = *Bacillus amyloliquefaciens* PPCB004.

However after ripening, a significant ($P < 0.001$) increase in fungal population was noted in the fructoplane of fruit washed in chlorinated water (commercial treatment) than the 1-MCP + PPCB004 and PPCB004 stand alone treatments (Fig. 3B). Postharvest disinfectant treatments had shown negative effects on the litchi and mango microflora (Govender et al., 2005). It is also evident from this study that the addition of PPCB004, the biocontrol agent in the integrated treatment with 1-MCP helped in maintaining the natural microbial ecological balance (Gerhardson, 2002; Ippolito and Nigro, 2000). Furthermore, the integrated treatment with 1-MCP + PPCB004 reduced the incidence and severity of postharvest diseases by reducing the total fungal population and maintaining higher bacterial and yeast populations on the fruit surface. The antagonist, *B. amyloliquefaciens* PPCB004 used in this study was isolated from the surface of citrus cv. Valencia. *B. amyloliquefaciens* and other members of the *Bacillus subtilis* group are considered as safe and have "Generally Recognized As Safe" status, GRAS (Food and Drug Administration., 1999).

According to the reports of European Food Safety Authority. (2008), some stains of *B. amyloliquefaciens* do not 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. However, a critical assessment of the safety of *B. amyloliquefasciens* strain used in this study on fresh fruit surface needs to be investigated prior to commercialization.

3.3. Fruit ripeness and 1-MCP + Bacillus amyloliquefaciens PPCB004 treatment

The colour development associated with ripening (100% edible ripe stage) was noted in untreated fruit (control), in fruit subjected to commercial treatment (washed in chlorinated water) and PPCB004 treated fruit after 3–4 d at 25 °C. Whereas the 1-MCP and 1-MCP + PPCB004 treated fruits took 9–10 d to ripen after cold storage at 25 °C before showing uniform yellow colouring (100% edible ripe stage). However, in order to consider the marketing potential the skin and flesh colour h° was also determined. The flesh became darker during ripening and showed non-significant difference between all the treatments (data not shown). The increase in flesh colour due to the increase in carotenoid content could be linked to the observed decrease in flesh h° value. A similar trend in flesh colour retention was reported by Paulo Fabi et al., (2007). The 1-MCP or 1-MCP + PPCB004 treated fruit further retained the uniform yellow skin (Fig. 4A). The fruit subjected to the other three treatments showed slightly lower skin and flesh h° value (orangish-yellow colour) towards the end of the 5th d. This observation coincides with the findings of Manenoi et al. (2007),

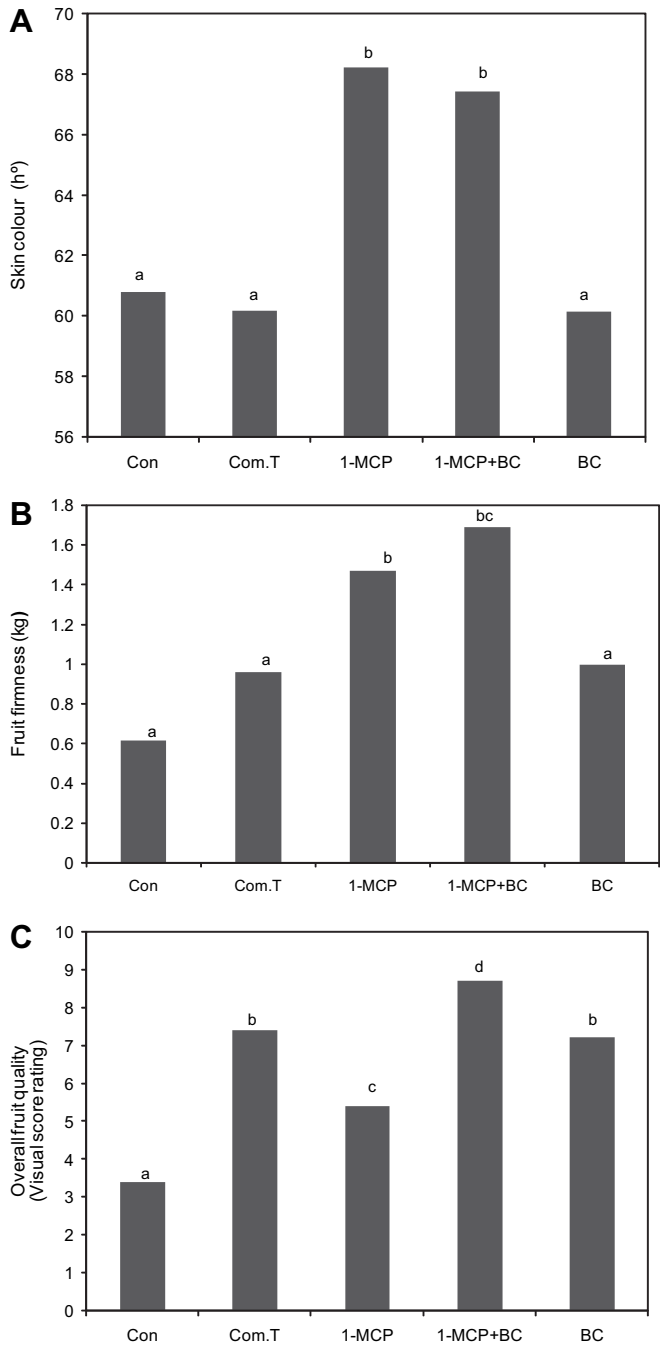

Fig. 4. Effect of *Bacillus amyloliquefaciens* PPCB004 on (A) skin colour, (B) fruit firmness and (C) overall fruit quality. Means in each bar followed by the same letter are not significantly different at $P < 0.001$ by Fisher's protected least significant test. Con = Control (untreated fruit); Com.T = Commercial treatment (NaOCl 250 μl l^{-1}); 1-MCP + BC = 1-methyl cyclopropene + *Bacillus amyloliquefaciens* PPCB004; BC = *Bacillus amyloliquefaciens* PPCB004.

where the application of 1-MCP (75 μl) to fruits at greater than 25% fruit yellow colour stage showed 96% yellow colour (skin) development after 10 d at 22 °C.

Although 1-MCP stand alone treatment showed retention of fruit firmness, the 1-MCP + PPCB004 treatment significantly ($P < 0.001$) retained the fruit firmness (moderate) compared to the other treatments adopted in this investigation (Fig. 4B). The higher incidence and severity of postharvest diseases (phomopsis and anthracnose) could have contributed to the observed loss of firmness in the untreated control fruits. Furthermore, the 1-MCP + PPCB004 treated fruits showed higher overall fruit quality with the highest number of marketable fruits (Fig. 4C) with good eating quality (data not shown) and absence of rubbery texture. According to Manenoi et al. (2007), 1-MCP treatment for fruit at 25–30% colour stage does not affect the normal softening of the fruit mesocarp and it does not show rubbery texture.

Fruits for fresh markets are generally harvested before the 25% yellow colour stage. Since fruits become more susceptible to damage such as mechanical injury or wounding during postharvest handing at the advanced colour stages (greater than 40% skin yellow), it is commercially harvested earlier. However, the impact damage can be reduced during the postharvest handling stage by using liners within the cardboard cartons and protecting the individual fruit with Styrofoam nettings. Especially in developing countries postharvest treatments that extend storage life are important where cold chain infrastructure is not well established. In those circumstances application of 1-MCP may provide a suitable alternative to extend the postharvest life of papaya ambient temperature (25 °C) especially when analysing the amount of postharvest losses during supply chain due to rapid ripening and fruit softening. Therefore, a combination of 1-MCP (100 μl) pre-treatment and *B. amyloliquefaciens* PPCB004 dip treatment for papaya fruit at 25–30% skin yellow stage provides a practical solution to ensure ultimate fruit quality for the domestic market. The extended shelf life and improved quality could also provide an opportunity to consider the export market. Commercial packhouse trials should in future studies test the feasibility of this combination treatment.

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