



## Timing the application of *Bacillus subtilis* QST 713 in the integrated management of the postharvest decay of mango fruits

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### ABSTRACT

This work evaluated the application of a commercial formulation of *B. subtilis* QST 713 (Serenade<sup>®</sup>, Bayer AgroSciences) at different times in the development of mango fruits for the control of postharvest diseases. Initially, two experiments were conducted to define the best fruit development stage for substitute synthetic fungicides by biocontrol. The experiments were made in commercial orchards with cv. 'Kent' and 'Palmer' with five treatments: 1. Conventional – fungicides were applied from pre-bloom until the maximal growth of fruits and in the postharvest processing; 2. *B. subtilis* QST 713 substitute fungicides from the 50% flowering stage onwards and in postharvest treatment; 3. *B. subtilis* from the fruit-setting (peanut size) up to egg size and in postharvest; 4. *B. subtilis* from the fruit growth (from egg size until harvest) and in postharvest; 5. Preharvest – three weeks before harvest and in postharvest. Application of *B. subtilis* from the fruit setting and fruit growth stages onwards resulted in significantly lowest fruit rot incidence. A third experiment was conducted in an orchard with cv. Tommy Atkins and *B. subtilis* application started in three fruit development stages: 1. Conventional; 2. Fruit growth; 3. Preharvest. There was a significant reduction of rot incidence, severity, and lesion development rate through the shelf life period for the application starting fruit growth onward. Our results showed that *B. subtilis* QST 713 applied after the mid-stage of fruit development can efficiently complement the application of fungicides in the initial stages of mango fruit production. It is an important finding showing a safe substitution of conventional fungicides by a biofungicide formulation for the management of postharvest rot of mango.

### 1. Introduction

The Brazilian production of mango fruits was 1.2 million metric tons in 2015, with a gross income around US\$720 million (OECD/FAO, 2015). However, the losses caused by postharvest decay of fruits may reach up to 30% in the mango production chain. *Colletotrichum* spp. Pens., *Fusicoccum aesculi* Corda, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (1909), and *Neofusicoccum parvum* (Pennycook & Samuels) Crous are the prevalent pathogens causing mango fruit rot (Costa et al., 2010; Lima et al., 2013; Marques et al., 2013). All these pathogens are known to cause postharvest decay in most mango-producing regions in the world, and to cause quiescent fruit infections (Singh and Singh, 2012; Sivakumar et al., 2011). Quiescent infections of the mango fruits begin in the field, even in flowering (Costa et al., 2010). They are hard to be detected and treated because after infection the pathogens colonize endophytically the tissues where they are protected below the epidermis surface, and the infected fruits are asymptomatic before maturation

begins (Prusky and Lichter, 2007; Prusky et al., 2013).

*Colletotrichum* and the Botryosphaericean fungi (*F. aesculi*, *N. parvum*, and *L. theobromae*) are found in all geographic and climatic areas of the world (Phillips et al., 2013). They are the main pathogens of mango in tropical semi-arid regions where ascospores production and plant infection occurs mainly on humid conditions produced by rain and irrigation (Souza-Polito and Goes, 2017). Optimum temperature for mycelial growth of *C. gloeosporioides* was established among 20 to 25 °C (Maia et al., 2011), Botryosphaericean fungi had optimum temperature ranging from 25.4 to 31 °C (Marques et al., 2013; Pawar et al., 2012). Rain and irrigation increase the production and release of ascospores in water droplets (aerosols).

The control of postharvest decay of fruit has been based on the spraying of synthetic fungicides both before and after fruit harvest. However, fungicide residues in foods have become a major concern for consumers, pressing for the elimination of spraying in the final stages of fruit development. Consequently, postharvest application of pesticides

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in fresh fruits and vegetable production has been severely restricted. As a result, studies on biological fungicides have been gaining in influence and given rise to successful commercial products in recent years (Droby et al., 2016).

The fundamental principle of applying antagonistic microorganisms to control quiescent pathogens that cause postharvest diseases of fresh fruits is the introduction of a biocontrol agent that, colonizing the fruit surface near or within the pathogen entry sites, impair the early stages of the infection process (Bonaterra et al., 2012). Applying mechanisms such as competition for nutrients and space, antibiosis, and parasitism, the antagonists can modify the fruit surface environment or destroy pathogen propagules, stopping the infection's development (Parafati et al., 2016; Spadaro and Droby, 2016). Besides, they can induce resistance eliciting metabolic pathways that can confine lesion development (Romanazzi et al., 2016).

Postharvest treatment is the most common strategy for applying biocontrol agents (BCA) to control fruit rot. Usually, fruits are immersed in cell suspensions in the final steps of postharvest processing, dried, and stored in the cold (Sharma et al., 2009). However, this approach has not been efficient with quiescent pathogens of mango once infection has occurred in the field. The better strategy to control such pathogens is the application of BCA as a preharvest treatment, part of the integrated management of fruit disease.

*Bacillus* strains have been pointed out as efficient biocontrol agents of plant pathogens, primarily to control soilborne plant diseases (Singh et al., 2018). However, some promising results were also obtained using *Bacillus* spp. to control postharvest rot of fruits both in pre or postharvest application (Wisniewski et al., 2016). *B. amyloliquefaciens* BUZ-14, e. g., applied after postharvest processing significantly reduced the incidence of fruit rot in orange, apple, grape, and stone fruit stored in cold temperatures when typically applied in the postharvest (Calvo et al., 2017). A similar result was obtained by Punja et al. (2016) by combining the pre and postharvest application of *B. subtilis* QST 713 and cold storage for controlling storage rot of tomatoes.

*Bacillus subtilis* QST 713 is a commercial formulation of biofungicide (Serenade<sup>®</sup>, Bayer AgroSciences), usually recommended for the control of foliar pathogens and soilborne plant disease. In this work, we evaluated the substitution of conventional fungicides by *B. subtilis* QST 713 in mango orchards in different stages of fruit development, defining a proper strategy to include the biofungicide in the integrated management of postharvest pathogens of mango.

## 2. Materials and methods

Three experiments were designed to evaluate the efficiency of *Bacillus subtilis* QST 713 (Serenade<sup>®</sup>, Bayer AgroSciences) in the control of pathogens that cause postharvest rot of mango fruits. The experiments were conducted in two commercial orchards and one semi-commercial orchard, aiming to evaluate the control of natural infections of the prevalent pathogens in the Valley of the São Francisco river region, the largest exporter of mango fruits in Brazil. The climate in the region is semi-arid, with warm and dry summer, corresponding to the “Bsh” class according to the Koppen classification (Alvares et al., 2013). All experiments were conducted in the summer.

### 2.1. Orchard pest management

Pest management in the mango orchard was limited to the application of imidacloprid against *Erosomyia mangifera* and thiamethoxam against cochineal species (*Aulacaspis tubercularis* and *Pseudococcus* sp.) when detected in the pest monitoring. The Mediterranean fruit fly *Ceratitis capitata* Wiedemann was controlled using toxic baits containing spinosad and a food attractant. Powdery mildew was controlled by additional spraying of micronized sulfur each fortnight during inflorescence and initial fruit setting.

### 2.2. Timing the preharvest application of *B. subtilis*

Two experiments were conducted in commercial orchards to evaluate the best time to substitute synthetic fungicide by the biofungicide. The first was conducted from July to November 2015 in an orchard of mango cv. ‘Kent’ with 7 years, planted in a spacing 4 × 6 m. The second was conducted from August to December 2015, in a mango orchard cv. ‘Palmer’ with 8–9 years, also planted in a spacing 4 × 6 m. Both areas were irrigated under the canopy, using micro-sprinklers. Weather conditions was monitored using an automatic meteorological station (Davis Instruments, Hayward, CA – USA). Weather data are presented in the Supplementary Fig. 1A (Fig. Sup. 1A). Briefly, the average temperature along the period was 27.7 °C, maximum average 34.7 °C (peak at 39.2 °C) and minimum average 21.5 °C (lowest 15.6 °C); average relative humidity was 49.3, with minimum average RH 25.7% (lowest 11.4%) and maximum average RH 75.3 (peak 95.1%). Accumulated rainfall in the period was 74.2 mm.

A commercial formulation of *B. subtilis* QST 713 (Bayer SA, São Paulo, Brazil) was used to prepare tank mixing containing 1.0% suspension, added with 0.5% of Agro Oil (Samaritá Agrociência, Artur Nogueira, SP - Brazil), a commercial formulation of emulsifiable vegetal oil (partially esterified soybean oil). Spraying was performed in the morning, using a backpack sprayer equipped with a standard solid cone nozzle directed to the fruits.

The treatments are presented in Supplementary Table 1 (Table S1) and were applied as follow: 1. Conventional – Tebuconazole (triazole) and copper hydroxide were sprayed alternately weekly since the vegetative flush, flowering, and fruit set; azoxystrobin (strobirulin) was applied from the final of fruit set until the maximal growth of fruits (95 days after anthesis - DAA). *B. subtilis* was applied in the postharvest processing; 2. Flowering – Conventional fungicides were applied during vegetative development, while *B. subtilis* was applied weekly from 50% of anthesis until preharvest and in the postharvest processing; 3. Fruit setting – conventional fungicides were applied until flowering; one spray of azoxystrobin (strobirulin) was applied on fruit set (peanut size), and QS713 was applied weekly until harvest and postharvest; 4. Fruit growth – conventional treatment was applied until 83 days after anthesis when fruits achieved egg size (DAA), and QS713 was applied weekly until harvest and in the postharvest; 5. Preharvest – conventional treatment was applied until 83 DAA, no spray was applied along fruit growth until maturation stage 1, and *B. subtilis* was applied three weeks before harvest and postharvest.

### 2.3. Validation experiment in a mango orchard cv. Tommy Atkins

A third experiment was conducted from August to December 2016 applying *B. subtilis* to the biological control of mango rot in a semi-commercial orchard with 12-year old mango cv. “Tommy Atkins” located on the experimental farm of Embrapa Semiárido (Petrolina – PE, Brazil) to confirm the results from the first experiments. The orchard was planted in 4 × 6 m spacing and irrigated using micro-sprinklers. Weather conditions was monitored using an automatic meteorological station (Campbell Scientifics, Logan UT – USA). Climatic variation during the experiment is showed in Fig. S1B. Briefly, the average temperature was 27.8 °C, maximum average was 35.6 °C (peak 39.0) and minimum average 21.6 (lowest 16.3); average RH was 53.1%, maximum average 78.1 (peak 99.1) and minimum average 30.8% (lowest 13.2%); accumulated rainfall in the period was 250.5 mm, concentrated in the final of the fruit growth and maturation.

The treatments applied were: 1. Conventional – conventional fungicides were applied similarly to the previous experiments (Table S1) until the maximal growth of fruits. *B. subtilis* was applied in postharvest; 2. Fruit growth – conventional treatment was applied until 64 DAA, and *B. subtilis* was applied weekly until harvest and in the postharvest processing; 3. Preharvest – conventional treatment was applied until the maximal growth of fruits (72 DAA), and no spray was applied until

maturation stage 1. *B. subtilis* was applied for two weeks before harvest (125 DAA) and in the postharvest processing.

#### 2.4. Mango fruits harvest and processing

Mango harvest started when fruits reached maturation stage 2–3 (OECD/FAO, 2011). One hundred-twenty mango fruits were harvested from each plot and initially selected for no apparent damage. They were conditioned in plastic containers, previously lined with bubble wrap and transported to postharvest processing. Briefly, the fruits were washed with detergent under tap water, and their peduncles standardized at 20 mm. The fruits were selected for the absence of mechanical lesions and uniformity (size and maturation) and immersed in a suspension containing 2 liters of the commercial formulation of *B. subtilis* per 100 l of water for 5 min, removed and dried using forced air provided by an industrial blower.

After processing, the fruits were placed in a standard paper box containing 6 kg of fruits (10–12 fruits) with corrugated paper at the bottom and covered with a waxed paper sheet (hygroscopic internal surface and hydrophobic external surface). Fruit boxes were stored under controlled temperature ( $10 \pm 2^\circ\text{C}$  and 90% RH) during 20 days, followed by a shelf life evaluation under natural conditions ( $25^\circ\text{C}$  and 70% RH) during 10 days in the first experiment and 12 days in the second. Mangoes rot incidence, meaning the occurrence of one lesion in the fruit, and severity, the increase of the lesions along the time, were evaluated daily during storage and shelf life periods in each experiment.

#### 2.5. Data processing and statistical analysis

A randomized block experimental design was applied in the three experiments, with four replicates, and the experimental plot was composed of 5 plants per plot. After harvesting and processing, 5 fruit boxes became the experimental subunits for each field plot.

In this work, disease incidence was recorded when fruits showed first discernible rot lesion in each box, while severity was presented by the disease severity index estimated from the percentage of lesioned tissue on the fruit surface. Disease indexes for rot severity were obtained using the equation  $DI(\%) = Dxn/N \times 100$ , adapted from the McKinney index (Madden et al., 2007), where D is the dimension of the lesions (mm), n the number of fruits injured in each box, and N the number of fruit boxes. Data obtained as a percentage were transformed using the equation  $X'_{ij} = \arcsene X_{ij}/100$ , in which  $X_{ij}$  are the observed values, and  $X'_{ij}$  are the transformed values. Data homoscedasticity and homogeneity of variance were evaluated using the Lilliefors and the Levene tests, respectively and submitted to the analysis of variance. Treatment means were compared using the Tukey test ( $p < 0.05$ ) using Statistica for Windows v. 12 (StatSoft Inc.). Results were presented with untransformed values (mean  $\pm$  standard error).

### 3. Results and discussion

#### 3.1. Timing the preharvest application of *B. subtilis* QST 713

Two experiments were designed to evaluate the effect of different times of application of *Bacillus subtilis* in commercial orchards on the control of mango fruit rot. The biofungicide treatments were applied in different stadia of the fruit development, and the reference treatment (conventional) did not receive either fungicides or biocontrol spraying after maximal fruit growth but received *B. subtilis* in preharvest. All treatments received pre-bloom sprays with conventional fungicides that were prolonged to the different time when the *B. subtilis* application started. There was insignificant rainfall during the two experiments that were conducted from august to December 2015 and average RH was commonly lower than 60% (Fig. S1B) resulting in low disease incidence and severity.

There were no rot symptoms along the refrigerated storage in both experiments. First rot lesions were detected in the shelf life period, 2 days after removal from the cold chamber (22 days from harvest). Despite the low disease incidence in the first experiments, significant control of mango postharvest decay was obtained with the application of the biocontrol formulation in both experiments with cv. 'Kent' and 'Palmer' (Fig. 1). The conventional treatment and the application of *B. subtilis* exclusively in preharvest (three weeks before harvest) were statistically similar to each other in both experiments by the Tukey test ( $p > 0.05$ ). Rot incidence with the application of *B. subtilis* starting since flowering was also similar to the conventional preharvest treatments in the 'Kent' orchard for all the evaluation period, but there was a significant difference in the 'Palmer' orchard experiment. The application of *B. subtilis* after fruit setting and fruit growth showed the lowest incidence of fruit rot in both experiments.

The application of QST 713 in initial stages of fruit development (fruit setting and growth) also reduced the development of rot lesions in fruits as shown by the severity index (DI) in Fig. 2A and B. Rot severity in the cv. 'Kent' was significantly higher than the others in the preharvest treatment at the first evaluation by the Tukey test ( $p < 0.05$ ) (Fig. 2A), rapidly evolving until 29 days, when it was also statistically similar to the treatments applied in flowering, and remained stable at the end of the shelf life evaluation. Applying *B. subtilis* after fruit setting and during fruit growth stadia showed the lowest values for rot severity through the storage and shelf life period.

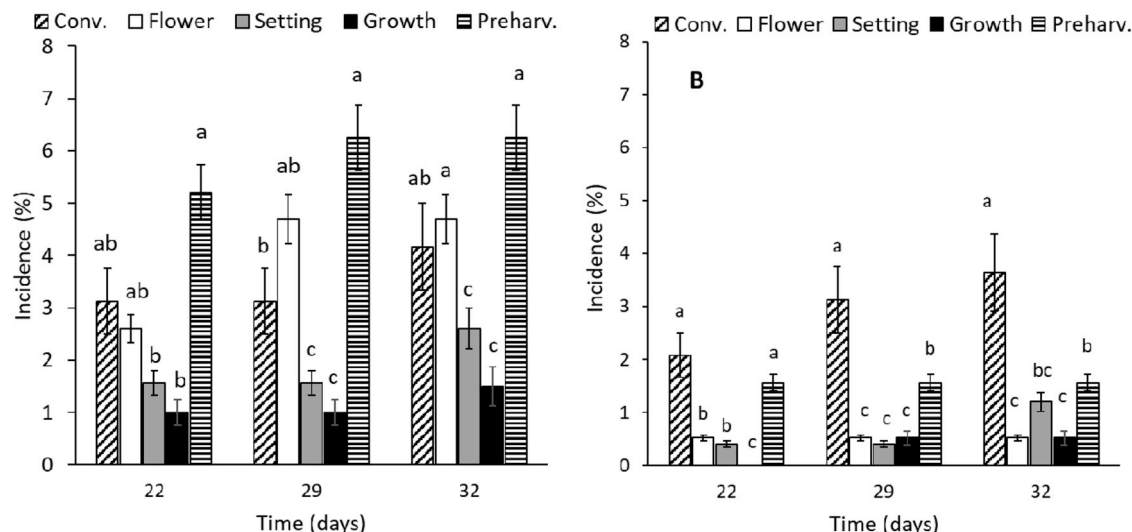
Mango rot severity was generally lower in the experiment with cv. 'Palmer' than in experiment in the orchard with cv. 'Kent'. The results for severity in Fig. 2B show that the application of *B. subtilis* since flowering, fruit setting and fruit growth resulted in statistically similar rot severity (Tukey test,  $p > 0.5$ ) that was significantly lower than the conventional treatment throughout the evaluation period. Severity increased slightly at the end of the experiment for the treatment which received *B. subtilis* spraying only in preharvest.

The experiments simulated the current management of pests and diseases by mango growers in the São Francisco Valley, including postharvest fruit-rot management practices. Conventional treatment simulated growers' conditions, where fungicide application cannot be risked at the end of the production cycle and during postharvest to meet the exigencies of different importers. Usually, fungicide sprays are scheduled from pre-bloom until maximum fruit growth when they are completely interrupted. In such management, fruits are kept for 50–60 days in the field without spraying to avoid having fungicide residues. This is a long period when fruits are exposed to quiescent infections, mainly by Botryosphaeriacean fungi (Costa et al., 2010).

#### 3.2. Applying *B. subtilis* QST 713 in the integrated management of mango rot

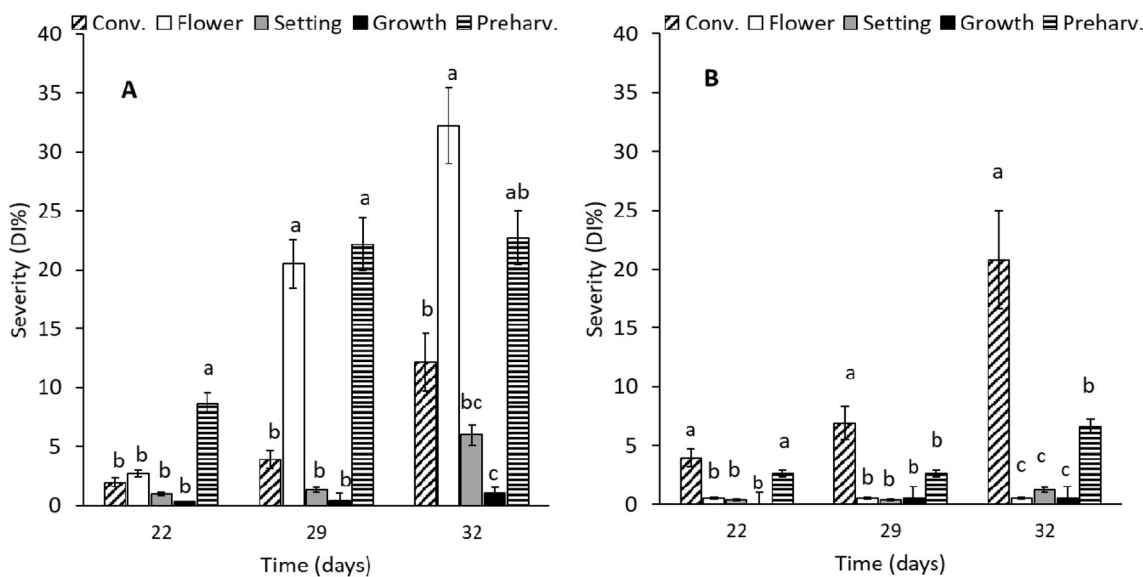
A third experiment was conducted in an orchard of cv. Tommy Atkins to validate the results from the previous experiments. The fruit growth stage (64 DAA) was selected for the beginning of the application of *B. subtilis*. From flowering to the final fruit setting, the orchard was sprayed with conventional fungicides as described in Table S1. There was no significant difference in rot incidence among the treatments in the first evaluation (Fig. 3A). However, the severity of the lesions was significantly lower at the fruit growth treatment by the Tukey test (Fig. 3B). Incidence and severity increased in subsequent evaluations, but the rot incidence on fruits treated with *B. subtilis* starting from fruit growth remained significantly lower than the others during the shelf life.

The treatments with *B. subtilis* QST 713 controlled efficiently postharvest mango rot when applied in the field and combined with a postharvest application of the product. Lesion severity developed significantly slower in the treatment with *B. subtilis* sprays starting in the fruit growth period, resulting in a significantly lowest rate of lesion development as shown by the area under the disease progress curve (AUDPC) in Fig. 4. The conventional treatment showed a fast increase of



Treatments with different letters on the same dates were significantly different from each other in the Tukey test ( $p < 0.05$ ).

**Fig. 1.** Natural incidence of mango rot in fruits from commercial orchards of cv. ‘Kent’ (A) and ‘Palmer’ (B) with the application of *B. subtilis* QST 713 after different times since flowering. The fruits were stored in a cold chamber for 21 days. In the figure Conv. is the conventional treatment; Flowering – QST 713 spraying starting after 50% of anthesis; Setting – QST 713 spraying after fruit set (peanut size); Growth – QST 713 starting after egg size; Preharvest – QST 713 starting in maturation stage 1. Details of the treatment are presented in Table 1. Treatments with different letters on the same dates were significantly different from each other in the Tukey test ( $p < 0.05$ ).



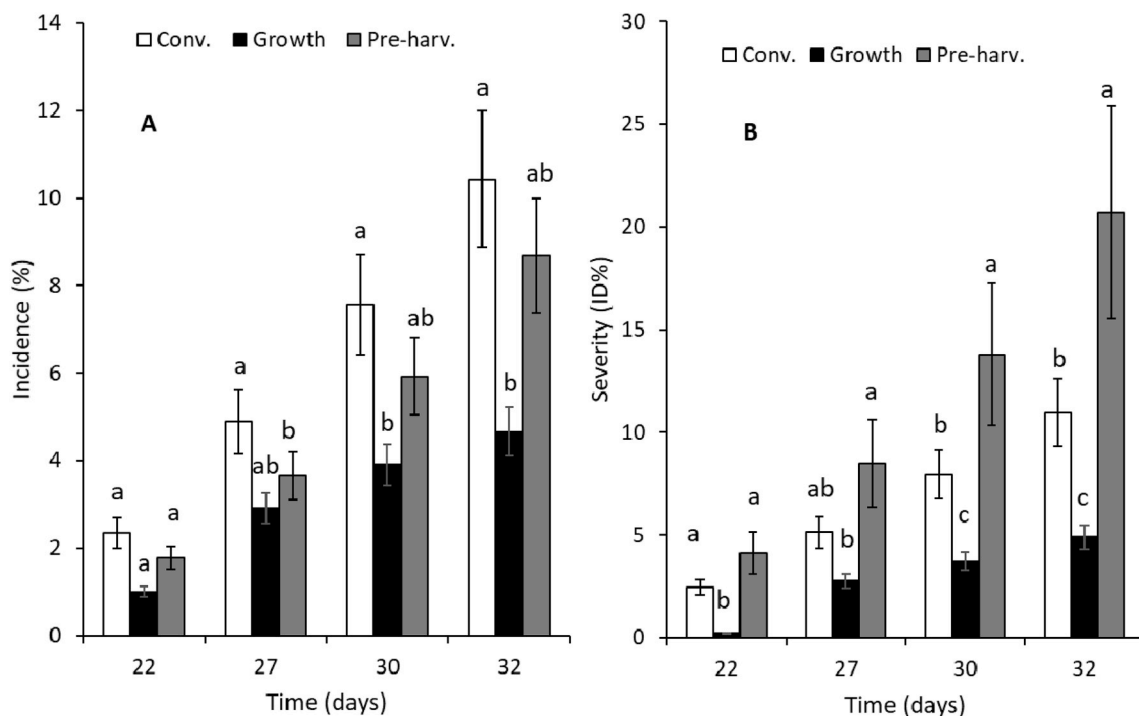
Treatments with different letters on the same dates were significantly different from each other in the Tukey test ( $p < 0.05$ ).

**Fig. 2.** Severity (McKinney disease index) of mango rot in fruits from commercial orchards of cv. ‘Kent’ (A) and ‘Palmer’ (B) with the application of *B. subtilis* QST 713 after different times since flowering. The fruits were stored in a cold chamber for 21 days. Conventional treatment, flowering, fruit set, fruit growth, and preharvest were the stadia of fruit development described in Table 1. Figure legend as in Fig. 1. Treatments with different letters on the same dates were significantly different from each other in the Tukey test ( $p < 0.05$ ).

rot incidence during the shelf-life period, while the application of *B. subtilis* exclusively in preharvest showed the largest disease severity, meaning a fast development of wounds during shelf life.

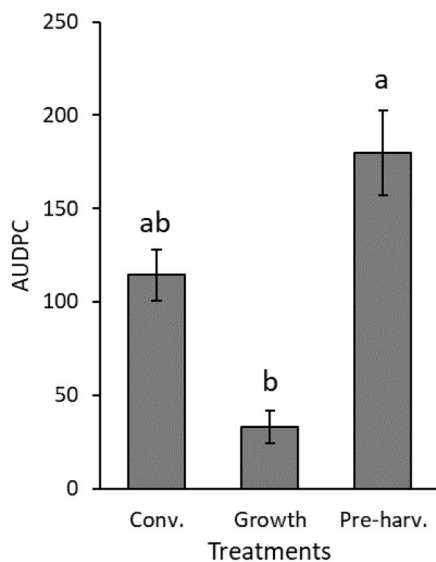
There was a strong variation in fruit rot incidence and severity among experiments that can be explained by meteorological variables, but it also is linked to the differences in cultivar susceptibility. Rainfall occurred in mid to late stages for all experiments, matching the removal

of conventional fungicides spraying. A large incidence and severity of mango rot occurred in the experiment with cv. ‘Kent’, although the experiment with cv. ‘Palmer’ was reached by rainfall in the pre-harvest stadium, strongly indicating that varietal resistance had determinant importance. The occurrence of consecutive periods of rainfall in late growth and pre-harvest in the validation experiment with cv. ‘Tommy Atkins’ explains the large incidence and severity of fruit rot in this



Treatments with different letters on the same dates were significantly different from each other in the Tukey test ( $p < 0.05$ ).

**Fig. 3.** Incidence (A) and severity (McKinney disease index) (B) of mango rot in fruits from a semi-commercial orchard of cv. Tommy Atkins with the application of *B. subtilis* QST 713 after different times since flowering. The fruits were stored in a cold chamber for 21 days. In the figure Conv. is the conventional treatment; Growth - QST 713 starting after egg size; Preharvest - QST 713 starting in maturation stage 1. Treatments with different letters on the same dates were significantly different from each other in the Tukey test ( $p < 0.05$ ).



Treatments with different letters were significantly different from each other in the Tukey test ( $p < 0.05$ ).

**Fig. 4.** Area under disease progress curve (AUDPC) of mango rot in fruits from a semi-commercial orchard of cv. Tommy Atkins with the application of *B. subtilis* QST 713 at different times since flowering. Figure legend as in Fig. 3. Treatments with different letters were significantly different from each other in the Tukey test ( $p < 0.05$ ).

experiment. However, starting the biocontrol treatment soon after interrupt fungicide spraying, as in the fruit growth treatment, significantly reduced disease incidence at least in 50% in the worst scenario.

Few studies evaluated the resistance of mango to Botryosphaeriacean

the fungi. However, they have shown significant differences among commercial cultivars. Batista et al. (2012), e. g., showed that cv ‘Tommy Atkins’ was moderately resistant to *F. aesculi* and *N. parvum*, cv ‘Kent’ was susceptible both to *F. aesculi* and *N. parvum*, while cv ‘Palmer’ was



moderately tolerant only to *N. parvum*. These differences were associated with the concentration of antifungal metabolites such as resorcinol and gallotannins in the fruit at the ripe stage (Karunanayake et al., 2014). The lower resistance of cv. 'Kent' help explains the highest disease incidence and severity in the first experiment. Although more resistant to the pathogens, cv. 'Tommy Atkins' showed the highest rot incidence and severity. However, it is explained by the occurrence of rainy periods between the end of the growth stage and the initial maturation stage – mid-November to mid-December - (Fig. S1B), establishing a period highly conducive for infection.

The usage of *B. subtilis* since the fruit growth stage, or 64 days after anthesis, reduced rot incidence to 48.3% and 55.3% in the shelf life period, respectively, while the reduction of rot severity was 75.8% and 80.0%. Similar results were obtained by Poleatewich et al. (2012). The authors showed that preharvest application of *Bacillus megaterium* A3-6, *B. mycoides* A1-1, and *B. cereus* FLS-5 significantly reduced fruit and foliar apple scab severity. In fact, results from several studies have shown that the inclusion of antagonist microorganisms in the integrated management strategy gave more consistent results in the control of postharvest disease (Ibrahim et al., 2016; Wisniewski et al., 2016; Nunes, 2012).

This work proposes that such an approach could include the preharvest application of conventional fungicides in the initial stages of mango fruit development, followed by an antagonistic microorganism. The results showed that *B. subtilis* QST 713 could efficiently substitute the application of contact and low residual fungicides from the middle of mango fruit development until the final stages. This strategy reduced the losses caused by mango rot and could be inserted into the integrated management of postharvest disease of mango. However, the switch from conventional fungicides to *B. subtilis* in the early development stages increased the risks of infection by quiescent pathogens, resulting in a higher incidence of lesions in storage and in the commercialization (shelf life) period. This is a significant finding since there are limited options for low residual products available for the final stages of mango fruit production.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cropro.2019.03.013>.

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