Mechanism of antifungal action of borate against *Colletotrichum gloeosporioides* related to mitochondrial degradation in spores

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**A B S T R A C T**

Anthracnose caused by *Colletotrichum gloeosporioides* Penz. is one of the most important diseases in harvested mango fruit. In this study, we investigated the effect of borate on spore germination of the fungal pathogen *in vitro* and anthracnose control in harvested mango fruit, and observed mitochondrial damage in the spores under borate exposure, in order to evaluate the mechanism of its antifungal action. Borate treatment significantly inhibited spore germination of the fungal pathogen and effectively controlled anthracnose in harvested mango fruit. Borate treatment at 20 mM furthermore seems to induce reactive oxygen species (ROS) generation in the fungal spores. Mitochondrial degradation after 6 h treatment of the spores with borate was observed with both laser scanning confocal and transmission electron microscopy. These results suggest that mitochondrial degradation is involved in the mechanism of antifungal activity of borate against *C. gloeosporioides*.

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1. Introduction

Mango (*Mangifera indica* Linn.) is an important tropical fruit because of its favorable flavour and high marketing value. However, diseases caused by various pathogens usually result in great economic losses in harvested mango fruit (Tian et al., 2010). Anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) is one of the most serious diseases in mango fruit, as the pathogen can attack various parts of mango and cause latent infection in small fruit (Pitkethley and Conde, 2007; Kefialew and Ayalew, 2008). Application of synthetic fungicides is the major approach for control of fruit diseases; for example, hot benomyl dips have been reported to effectively control anthracnose of harvested mango fruit (Kim et al., 2007). However, using fungicides at high concentrations over a long time can result in pathogen resistance (Tian, 2006). Additionally, public concern about fungicide residues is urging researchers to find new technologies for fruit disease control.

Recently, a number of reports have demonstrated that some antifungal compounds, such as oxalic acid (Zheng et al., 2007), salicylic acid (Tian et al., 2007), calcium (Zhu et al., 2010) and plant extracts (Regnier et al., 2008; Linde et al., 2010), can effectively control postharvest diseases in mango fruit. In previous studies, we found that borate could significantly limit the pathogenicity of *Penicillium expansum* (Qin et al., 2007), and effectively control postharvest diseases in fruit, such as gray mould rot caused by *Botrytis cinerea* in table grapes (Qin et al., 2010) and anthracnose caused by *C. gloeosporioides* in mango fruit (Shi et al., 2011). In addition, we showed that the antifungal action of borate is associated with antioxidant enzymes and oxidative damage of the fungal pathogen.

It is well known that mitochondria are the main source of cellular adenosine triphosphate (ATP) and play a central role in a variety of cellular processes (Chan, 2006; Mucha et al., 2009). Moreover, mitochondria are also the major endogenous sources of reactive oxygen species (ROS), whose accumulation can lead to oxidation of macromolecules, resulting in mtDNA mutations, aging and cell apoptosis (Osiewacz, 2002; Kowaltowski et al., 2009). However, little information is available about whether borate can cause mitochondrial damage of fungal spore cells, and whether the mitochondrial damage is specifically related to inhibiting fungal growth.

The main objective of this study was to comprehensively explore the antifungal mode of borate against the fungal pathogen, with particular attention to the changes of mitochondria in *C. gloeosporioides* spores treated with or without borate. Moreover, effects of borate on spore germination of *C. gloeosporioides* *in vitro* and on anthracnose disease control of harvested mango fruit were investigated.

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2. Materials and methods

2.1. Fungal isolates

The fungus C. gloeosporioides was isolated from diseased tissues of symptomatic mango fruit. The virulence of the isolates was verified by inoculating mango fruit with 5 μL of a spore suspension (1 × 10^6 spores mL^{-1}). Then, the pathogen was purified via single spore isolation on potato dextrose agar (PDA) and maintained in PDA slants at 10 °C according to the method of Cloh (1999).

2.2. Effect of borate on spore germination of C. gloeosporioides

The effect of borate on spore germination of C. gloeosporioides was assayed in potato dextrose broth (PDB) medium following the method described by Qin et al. (2003). Spores were collected by adding sterile distilled water to the surface of the culture and gently scrubbing with a sterile spatula. Then, spores were incubated in PDB medium with different concentrations (10, 20 and 30 mM) of potassium tetraborate with or without pH adjustment. The PDB medium without borate served as the control. The inhibition efficacy of borate at different concentrations was evaluated on spore germination after 8 h of incubation at 25 °C. At least 100 spores in each treatment were investigated. Each treatment contained three replicates and the entire experiment was repeated twice.

2.3. Effect of borate on reactive oxygen species generation

The oxidant-sensitive probe 2', 7'-dichlorodihydrofluorescein diacetate (DCHF-DA) was used to assess the intracellular ROS levels in C. gloeosporioides according to the methods of Chen and Dickman (2005). Spores of C. gloeosporioides were cultured in PDB medium supplemented with 0 or 20 mM potassium tetraborate (without adjusting the pH) and incubated for 2, 4, 6 and 8 h. Then, the spores were washed with 10 mM potassium phosphate buffer (pH 7.0), and incubated for 5 min in the same buffer containing 10 μM DCHF-DA (dissolved in dimethyl sulfoxide). After being washed twice with potassium phosphate buffer, spores were examined under a Zeiss Axioskop microscope (Carl Zeiss, Oberkochen, Germany) and the percentage of spores stained by DCHF-DA in each treatment calculated. DCHF-DA is commonly used to determine intracellular ROS, particularly H_2O_2 (Chen and Dickman, 2004).

2.4. Effect of borate on spore mitochondria by laser scanning confocal microscopy and transmission electron microscopy (TEM)

The spores were incubated in liquid PDB medium containing 20 mM potassium tetraborate as described above or suspended in PDB medium without borate as a control for 2, 4, 6 and 8 h at 25 °C. MitoTracker® Orange CMTMRos probes (Invitrogen) were added to the fungal inoculation at final concentrations of 500 nM for 5 min. Fluorescence of MitoTracker® Orange CMTMRos stained spores was detected using a Zeiss LSM 510 META laser scanning confocal microscope (LSCM). At least 20 spores were examined for each treatment with three replications. MitoTracker® Orange CMTMRos is a mitochondrion-selective stain, and has been widely used for studying mitochondrial distribution and functionality (Chida et al., 2004; Czarna et al., 2010). Accumulation of the stain in cells is dependent upon membrane potential which is one of the most important parameters indicating mitochondrial functionality.

For TEM analysis, the spores treated with 0 and 20 mM potassium tetraborate for 2, 4, 6 and 8 h as described above were fixed overnight in 2.5% formaldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (SCB) at a pH 7.2, and centrifuged (16,000 × g for 5 min at 4 °C). Gels of 1–2 mm^3 were prepared by adding 3% low gelling temperature agarose in SCB to the pellet. After thorough rinsing with 0.1 M SCB, the gels were post-fixed with 1% osmium tetroxide in 0.1 M SCB for 4 h at room temperature, and dehydrated with 15 min stages in an ascending acetone series. The samples were embedded in spurr’s resin. Ultrathin sections were obtained using a diamond knife and stained by soaking in 2% uranyl acetate for 15 min, post-stained in lead citrate for 1 min. Mitochondrial alteration in the spores was analysed using a JEOI 1230 transmission electron microscope at 80 kV.

2.5. Effect of borate on anthracnose disease on mango fruit

Fresh, almost ripe mango fruit (Mangifera indica L. cv. Tai Nong 1) were harvested from commercial orchards in the southern part of Hainan province, Lingshui county. The surface of the mango was sterilized by immersion in 70% ethanol for 1 min and prepared for inoculation by infecting a single 1-mm deep wound in the middle of each fruit with a sterile needle. Each wound was then inoculated with the pathogen C. gloeosporioides by placing 5 μL of spore suspension (1 × 10^6 spores mL^{-1}) on the wound. The inoculated fruit were incubated overnight in a sterile box at 28 °C before dipping it in the potassium tetraborate at concentration of 20 mM for 30 min. Fruit dipped in sterile distilled water served as the control. In addition, the effect of borate treatments was also compared with 100 μg a.i. mL^{-1} benomyl. The treated fruit were incubated in a moist plastic box at 28 °C for 6 d and disease development was assessed by measuring the diameter of the anthracnose lesion on mango fruit. Each treatment consisted of 50 fruit with 3 replications and the experiment was repeated twice.

2.6. Statistical analysis

All statistical analyses were performed with SPSS version 11.5 (SPSS Inc., Chicago, IL, USA) and analysed by one-way analysis of variance (ANOVA). Mean separations were performed by Duncan’s multiple range tests. Differences at P = 0.05 were considered significant.

3. Results

3.1. Effect of borate on spore germination of C. gloeosporioides

As shown in Table 1, borate was effective in inhibiting spore germination and germ tube elongation of C. gloeosporioides following a concentration-dependent trend (P < 0.05). After incubation for 8 h at 25 °C, germination of C. gloeosporioides spores was strongly inhibited by borate at the concentration of 20 mM. The inhibitory efficacy of borate solutions with normal pH was better than that of solutions with an adjusted pH of 7.0.

3.2. Measurement of reactive oxygen species (ROS)

ROS generation was monitored when C. gloeosporioides was exposed to 20 mM borate for 2, 4, 6 and 8 h at 25 °C (Fig. 1). After 2 h of incubation, only 5.4% spores in the control treatment were stained by DCHF-DA, implying poor ROS production at the time. However, in the borate treatment about 58.2% of spores were stained. With an increased incubation period, even higher levels of ROS were detected in borate-treated C. gloeosporioides spores compared with the control. The highest percentage of stained spores was observed following 6 h of incubation with 20 mM borate.

3.3. Effect of borate on mitochondria distribution by laser scanning confocal microscopy

As shown in Fig. 2A, mitochondria in untreated spores were present throughout the intracellular space during incubation. In
Table 1
Effects of borate on spore germination of *C. gloeosporioides* after 8 h incubation at 25 °C.

<table>
<thead>
<tr>
<th></th>
<th>CK (PDB)</th>
<th>Borate (mM, pH 7.0)</th>
<th>Borate (mM, pH not adjusted)</th>
</tr>
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<tr>
<td>Spore germination (%)</td>
<td>97.5 ± 7.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.5 ± 6.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.5 ± 5.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Germ tube length (μm)</td>
<td>144 ± 24.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.5 ± 8.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.5 ± 4.2&lt;sup&gt;c&lt;/sup&gt;</td>
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Mean values followed by the different letters differ significantly within a row according to Duncan’s multiple range tests (P<0.05), respectively.

However, after 4 h borate incubation, the number of mitochondria in the spore was markedly fewer and many small vacuoles were found (Fig. 3F). After 6 h of incubation in borate, distortion of mitochondrial cristae appeared. After 8 h, mitochondria were almost completely degraded (Fig. 3G and H).

3.5. Effect of borate on anthracnose disease on mango fruit

Borate was effective in inhibiting the lesion diameter of anthracnose (P<0.05). The application of borate reduced the mean lesion diameter from 2.6 cm in the untreated control fruit to 1.4 cm in borate-treated fruit after 6 d storage at 28 °C. This was as effective as the regular fungicide used for anthracnose control in mango, Benomyl (at a concentration of 100 μg a.i. mL<sup>−1</sup>) (Fig. 4A and B).

4. Discussion

Boron is an essential microelement and has important physiological functions for plant growth (O’Neill et al., 2001; Xuan et al., 2001; Simoglou and Dordas, 2006) and quality improvement in fruit (Strong et al., 2001; Plich and Wójcik, 2002; Hafez and Haggag, 2007). In recent years, borate has been used as an antifungal compound to control postharvest diseases in various fruit (Rolshausen and Gubler, 2005; Qin et al., 2010; Thomidis and Exadaktylou, 1010; Shi et al., 2011). In previous studies, we found that germination and germ tube elongation of *C. gloeosporioides* were significantly inhibited by the addition of 15 mM borate to the PDB. Mango inflorescences sprayed with borate significantly increased the number of fruit that set per branch compared to the water-sprayed control, and lower disease incidence of anthracnose during postharvest periods was observed (Shi et al., 2011). In addition, we found that borate at 1% concentration could inhibit blue mould rot caused by *P. expansum* in harvested apple fruit, and were able to demonstrate that the mechanisms involved changing expression of antioxidant proteins and hydrolytic enzymes, based on comparative analysis.
Fig. 3. Effect of borate on the mitochondria in spores of *C. gloeosporioides* by TEM microscopy. Untreated spores served as control were incubated in PDB medium at 25 °C for 2 h (A), 4 h (B), 6 h (C) and 8 h (D); spores treated with 20 mM potassium tetraborate were incubated in the same culturing condition for 2 h (E), 4 h (F), 6 h (G) and 8 h (H). Black arrows indicate mitochondria in spores. L: lipid bodies, N: nucleus, S: septum, V: vacuoles. Bars represent 0.5 μm.

of cellular and extracellular proteome (Qin et al., 2007). Thomidis and Exadaktylou (2010) reported that borate treatment was effective in controlling brown mould rot by *Monilinia laxa* in peach. Qin et al. (2010) proved that borate at 1% concentration could inhibit gray mould rot by *Botrytis cinerea* in table grapes stored at room temperature or at 0 °C. Furthermore, it provided evidence that boron-reduced gray mould decay of table grapes was directly related to the disruptive effect of boron on the cell envelope of the
In the current study, we found that borate significantly inhibited spore germination of *C. gloeosporioides* (Table 1) and reduced the lesion diameter of anthracnose disease in harvested mango fruit after artificially inoculating pathogen spores (Fig. 4). These results indicate that borate has a broadly inhibiting effect on postharvest fungal pathogens. Moreover, borate solutions with adjusted pH (pH 7.0) also exhibited a lower inhibitory effect, suggesting that pH only partially influenced the antifungal action of boron against *C. gloeosporioides*. The same result has been reported in *R. cinerea* causing grey mould rot of table grapes (Qin et al., 2010).

ROS may cause oxidative damage to cellular compounds and lead to cellular dysfunction or cell death (Angelova et al., 2005; Circu and Aw, 2010). In this study, we used an oxidant-sensitive probe DCHF-DA to investigate the ROS accumulation in *C. gloeosporioides* spores, and observed that borate-treated spores showed a higher ratio of stained cells than that in control (Fig. 1), indicating that borate induced ROS accumulation in *C. gloeosporioides* spores. ROS accumulation can cause oxidative damage of the fungal spore and result in lower spore germination. It is well known that the mitochondrial respiratory chain is the major endogenous source of ROS, particularly when the mitochondria are damaged (Osiewacz, 2002; Hipkiss, 2006; Shi et al., 2011). Based on confocal microscopy and TEM, we found abnormal distribution and serious degradation of mitochondria in borate-treated spores (Figs. 2 and 3), indicating that borate treatment can lead to mitochondrial damage. Avis et al. (2009) reported that abnormal mitochondria appeared in conidia of *Fusarium sambucinum* after incubation for 10 min with 100 mM AlCl3, which was also considered as a potential antifungal agent. Therefore, it suggests that mitochondrial degradation of fungal spores may be one of the important modes by which antifungal compounds inhibit fungal growth.

In summary, we conclude that borate treatment can stimulate ROS accumulation in fungal spores, resulting in mitochondrial damage, which may act as the antifungal mechanism of borate inhibiting spore germination of *C. gloeosporioides* and controlling anthracnose in mango fruit. Additionally, application of borate is promising as an alternative to synthetic fungicides for postharvest disease control in mango fruit.

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References


Fig. 4. Effect of borate treatment on disease severity in mango fruit (cv. Tai Nong 1) after inoculated with 1 × 10⁶ ml⁻¹ spores of *C. gloeosporioides*. (A) The mean disease lesion diameter was measured after the fruit were inoculated and stored in 28 °C for 6 d. Mean values present in each bar followed by different letters are significantly different according to Duncan’s multiple range tests (P<0.05). Vertical bars indicate standard deviations. for the pathogen, resulting in the breakdown of the cell membrane and loss of cytoplasmic materials from the hyphae (Qin et al., 2010).


