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Inhibitory effect of exogenous sodium bicarbonate on development and pathogenicity of postharvest disease *Penicillium expansum*

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ABSTRACT

Penicillium expansum, a widespread filamentous fungus, is a major causative agent of fruit decay and may lead to the production of patulin which causes harmful effects on human health. In this study, *P. expansum* isolated from naturally infected pears (*Pyrus bretschneideri* Rehd.) was identified by morphological observation and rDNA-internal transcribed spacer analysis. Then, the effects of exogenous sodium bicarbonate ($NaHCO_3$) on development and pathogenicity of *P. expansum* were evaluated in vitro and in vivo. Results indicated that $NaHCO_3$ at 0.6% (w/v) significantly ($P < 0.05$) reduced the germination of *P. expansum* conidia by up to 80% compared to the control after 10 h incubation. $NaHCO_3$ also showed good ability to inhibit germ tube elongation, mycelia expansion and hypha production of *P. expansum*. The lesion diameter and disease incidence of blue molds were markedly reduced by 0.6% $NaHCO_3$ treatment. Loss of membrane integrity was examined and quantified under 0.6% $NaHCO_3$ condition by the method of propidium iodide fluorescent staining. Furthermore, we found that $NaHCO_3$ obviously affected the relative expressions of four genes involved in patulin biosynthesis and effectively prevented patulin accumulation. These data will provide theoretical foundations for improving the application potential of $NaHCO_3$ on postharvest horticulture pathogens control.

1. Introduction

Penicillium expansum is one of major pathogens of pome fruits. This pathogen not only induces blue mold rot that causes considerable economical losses and shortens the shelf-life of harvested fruits, but also produces patulin in infected fruits, a secondary metabolite that is toxic to humans and animals (Sanzani et al., 2012). Low-temperature preservation has limited effects because *P. expansum* is a psychrophilic mold that grows well at 0 °C (Morales et al., 2010). The efficiency of cold storage in controlling patulin accumulation is also controversial (Morales et al., 2008). So far synthetic fungicides are the primary means to control this fungal disease, but increasing resistance of fungal pathogens to fungicides and the growing public concern over the human health and environment impact have enhanced developing alternative methods to control postharvest blue mold rot.

During the last two decades, a huge information and advances concerning novel antifungal agents (such as sodium bicarbonate, phosphite, essential oil, borate) with the lowest possible toxicological risk have been achieved (Qin et al., 2010; Xing et al., 2010; Amiri and Bompeix, 2011; De Costa and Gunawardhana, 2012). Among these, sodium bicarbonate ($NaHCO_3$) has been reported to play an important role in inhibition of postharvest diseases in several fruits (Dore et al., 2010; De Costa et al., 2012; Letscher-Bru et al., 2013; Youssef et al., 2014). As a common food additive widely used for leavening, pH control, taste and texture modification, it is listed as generally recognized as safe (GRAS) compound by the US Food and Drug Administration and exempted from residue tolerance on all agricultural commodities by the US Environmental Protection Agency. $NaHCO_3$ is a very attractive alternative also because it is easily available, inexpensive and the control measures can be implemented without much professional expertise.

When applied alone or in combination with other treatments (such as microbial biocontrol agents), $NaHCO_3$ has beneficial effects on reducing the incidence of postharvest diseases. In a previous study, $NaHCO_3$ at 3% (w/v) proved to effectively reduce up to 100% disease incidence of *Penicillium digitatum* on clementines and oranges (Youssef et al., 2014). Postharvest dip treatment in 300 mM $NaHCO_3$ for 10 min not only reduced the lesion area of anthracnose on artificially inoculated banana fruits, but also significantly reduced natural infections of anthracnose, crown rot and blossom end rot in banana fruits (De Costa et al., 2012). In addition, $NaHCO_3$ has been found very successful for controlling postharvest diseases when coordinated with *Rhodosporidium*

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paludigenum (Zhu et al., 2013), *Bacillus amyloliquefaciens* HF-01 (Hong et al., 2014), *Metschnikowia pulcherrima*, *Cryptococcus laurentii* (Janisiewicz et al., 2008) or *Trichoderma harzianum* DGA01 (Alvindia, 2013). It was also able to improve the efficacies of fludioxonil and thiabendazole in controlling green mold decay in postharvest citrus fruits (D'Aquino et al., 2013). Furthermore, Dore et al. (2010) and Youssef et al. (2014) found $NaHCO_3$ could induce crystalline wax generation, activate host-resistance and increase imazalil level in the rind wound of oranges.

The directly antifungal effects of $NaHCO_3$ were reported as well in recent years. Letscher-Bru et al. (2013) has investigated the antifungal activity of $NaHCO_3$ on 70 fungal strains of three fungal groups (yeasts, dermatophytes and molds) responsible for human skin and nail infections. De Costa et al. (2012) found $NaHCO_3$ could inhibit spore production and germination, mycelia growth and appressoria production of *Colletorichum musae* in vitro. But so far there have been no researches about the effect of $NaHCO_3$ alone on the control of blue mould rot and patulin producing in postharvest fruits.

The research goal of our work was to determine the effects of $NaHCO_3$ on pathogenicity of *P. expansum*, the causal organism of penicilliosis and patulin producing. Firstly, *P. expasum* was isolated from naturally infected pear fruits, and its genetic background was determined by rDNA-ITS (rDNA-internal transcribed spacer) analysis. Then, physiological and biochemical assays were carried out in order to determine the effects of $NaHCO_3$ on development and pathogenicity of *P. expansum*. Further, the expression of four genes involved in patulin biosynthesis was evaluated by quantitative real-time PCR (qRT-PCR) analysis. The results will provide novel insights into exploring the potential of $NaHCO_3$ to control postharvest horticulture pathogens of fruit and vegetable.

2. Materials and methods

2.1. Fungal isolation and identification

P. expansum was initially isolated from the naturally infected pear fruits (*Pyrus bretschneideri* Rehd.) as described by Qin et al. (2007) and maintained on potato dextrose agar (PDA) plates. Total DNA of pathogen was isolated using a DNeasy Plant Mini kit (Qiagen, Germany), following the manufacturer's instructions. The complete rDNA-ITS of the isolated was amplified using universal primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGGTAAAAGTCGTAACAAG) by routine PCR approach. The sequencing results of amplified products were analyzed in http://blast.ncbi.nlm.nih.gov/Blast.cgi.

2.2. Antimicrobial effect assays

A suitable aliquot of fresh spore suspensions of *P. expansum* was added to a 250 mL conical flask containing 100 mL potato dextrose broth (PDB) to obtain a final concentration of 1.0×10^6 spores/mL with the aid of a hemocytometer. $NaHCO_3$ was, respectively, added to the medium with final concentrations 0, 0.15%, 0.3%, 0.45%, 0.6% (m/v). After shaking culture at 25 °C, at least 100 spores per replicate were observed microscopically to determine germination rate during 6 to 10 h and germ tube length during 11 to 14 h.

The mycelial expansion rate of *P. expansum* was measured on PDA plate as follows. A 5 mm diameter plug of mycelial agar was placed on the center of a 9 cm diameter petri dish containing 25 mL PDA supplemented with 0 or 0.6% $NaHCO_3$. The petri dishes were incubated at 25 °C and the colony diameter was measured by decussation method daily. To evaluate the effect of $NaHCO_3$ on mycelial biomass accumulation, fresh spore suspensions were added into 100 mL PDB medium with 0 or 0.6% $NaHCO_3$ to obtain a final concentration of 1.0×10^6 spores/mL. After incubation of 12, 24, 36 and 48 h, the dry weight of hypha was measured after repeated washing of mycelial pellets with distilled water and during at 70 °C in hot-air oven to a constant weight. Each treatment was replicated three times and the experiment was repeated.

Pear (*P. bretschneideri* Rehd.) fruits at commercial maturity were bought from local market in Xiasha district, Hang Zhou, China. After disinfection by 2% sodium hypochlorite, the fruits were wounded at the equator using the tip of a sterile dissecting needle and inoculated with 10 μL *P. expansum* spore suspension at 1.0×10^4 spores/mL. Before inoculation, spores were cultured shakily in PDB containing 0 or 0.6% $NaHCO_3$ for 6 h at 25 °C. The decay incidence and lesion diameter of all fruits were recorded after storage for 3 to 8 days. There were 15 fruits in each treatment with three replicates and the experiment was repeated.

2.3. Transmission electron microscope analysis

Transmission electron microscope (TEM) analysis was carried out as described by Lai et al. (2014). In briefly, the spores were fixed with 2.5% formaldehyde and 2% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer (SCB, pH 7.2) overnight. Gels were prepared by adding 3% low gelling temperature agarose in SCB to the pellet. After thorough rinsing with 0.1 mol/L SCB, the gels were post-fixed with 1% osmium tetroxide in 0.1 mol/L SCB for 4 h at room temperature, dehydrated with 15 min stages in a graded acetone series. The samples were embedded in spurr resin. Ultrathin sections were obtained using a diamond knife and stained by soaking in 2% uranyl acetate for 15 min, and post-stained in lead citrate for 1 min. The sections were analyzed using a JEOL 1230 transmission electron microscope (JEOL, Japan) at 80 kV.

2.4. Prodium iodide and 4′,6-diamidino-2-phenylindole staining

P. expansum spores with or without $NaHCO_3$ treatment were collected after 2 to 10 h incubation. Then, the spores were stained by 20 μg/mL prodium iodide (PI) or 50 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) for 30 min at 30 °C in dark. After centrifugation and repeated washing with phosphate buffer solution (pH 7.4) to remove residual dye, the spores were observed on a Nikon Eclipse Ni-U microscope (Nikon, Japan) equipped with individual fluorescein rhodamine or DAPI filter set. Images were collected using a Nikon DS-Fi1c high-definition cooled color camera (Nikon, Japan).

2.5. Determination of patulin production

Patulin content was detected as described by Morales et al. (2013). In briefly, a spore suspension at 1.0×10^6 spores/mL (100 μL) was dispersed evenly with a sterile spatula on PDA medium supplemented with or without 0.6% $NaHCO_3$. After 2, 4 and 6 days of incubation at 25 °C, ten plugs (5 mm diameter) of mycelial agar (about 10 g) were obtained randomly and immersed into 10 mL ethyl acetate for 1 h. After centrifugation, a volume of 1.5 mL of supernatant was dried under N_2, and then dissolved in 1.5 mL of acidified distilled water (pH 4.0). The solution was centrifuged, filtered through a 0.22 μm syringe filter (Albet, Spain) and transferred to an autosampler vial of high performance liquid chromatography (HPLC). Patulin content was determined by reversed-phase HPLC with UV detection (Waters, USA). A Waters XTerra® RP18 column (Waters, USA) was used with mobile phases (95% water and 5% acetonitrile). The operational parameters were as follows, Flow rate: 1 mL/min, column oven temperature: 18 °C, detector wave length: 276 nm. Result was expressed as μg of patulin per 1 g PDA.