Suppression of Green and Blue Mold in Postharvest Mandarin Fruit by Treatment of *Pantoea agglomerans* 59-4

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In order to control postharvest spoilage of satsuma mandarin fruits, rhizobacteria were isolated from soil samples. The *Pantoea agglomerans* strain 59-4 (Pa 59-4) which suppresses the decay of mandarin fruit by green and blue mold, was tested for the control efficacy and its mode of action was investigated. Pa 59-4 inhibited infection by green and blue mold on wounded mandarins, which were artificially inoculated with a spore suspension of *Penicillium digitatum* and *P. italicum* with control efficacies of 85-90% and 75-80%, respectively. The biocontrol efficacy was increased by raising the concentration of cells to between 10^6 and 10^7 cfu/ml, and pretreatment with the antagonist prevented subsequent infection by green mold. The population of Pa 59-4 was increased more than 10 fold during the 24 hr incubation in infection by green mold. Despite poor antifungal activity, the Pa 59-4 isolate completely inhibited the germination and growth of *P. digitatum* spores at 1 x 10^4 cfu/ml. We argue that the control efficacy was mediated by nutrition competition. Overall, the effective rhizobacterium, Pa 59-4, was shown to be a promising biocontrol agent for the postharvest spoilage of mandarin fruits by green and blue mold.

**Keywords**: Competition, Mandarin, *Pantoea agglomerans*, *Penicillium digitatum*, Postharvest disease

Postharvest green and blue molds of citrus, caused by *Penicillium digitatum* and *Penicillium italicum*, respectively, are major factors limiting the storage of satsuma mandarins (mandarins) which are the major citrus crop in Jeju province, Korea, and the pathogens are responsible for severe economic losses worldwide (Spadaro and Gullino, 2004; Sharma et al., 2009).

Control of postharvest pathogens still relies mainly on the use of synthetic fungicides (Eckert and Ogawa, 1988). However, the use of fungicides is becoming restricted due to the development of fungicide-resistant pathogens and public concerns regarding health (Droby et al., 2009; Spadaro and Gullino, 2004). Biological control using microbial antagonists has received a great deal of attention as a promising alternative to synthetic fungicides for controlling postharvest diseases in citrus.

Several bacteria and yeasts have been reported as effective in laboratory and pilot tests for controlling postharvest diseases in fruits. Bio-Save110 and 1000 (Eco Science Corp., FL. USA) with *Pseudomonas syringae* as active ingredient, and Aspire (Ecogen Inc., Langhorne, PA) with *Candida oleophila* strain I-182 have been registered for the biocontrol of postharvest diseases of pome and citrus fruits (Bull et al., 1997; Droby et al., 1998). Serenade (AgraQuest Inc., CA.) containing *Bacillus subtilis* strain QWT713 is also available as a wettable powder for the control of postharvest disease of pome and stone fruits (Nakkeeran et al., 2005, Sharma et al., 2009).

Many bacteria, such as *B. subtilis* (Singh and Deverall, 1984; Demoz and Korsten, 2006), *Pantoea agglomerans* (Nunes et al., 2001; Torres et al., 2007), *Pseudomonas cepacia* (Huang et al., 1993), *Pseudomonas fluorescense* (Mikani et al., 2008) and *Serratia plymuthica* (Meziane et al., 2006) have been reported as effective biological agents against postharvest pathogens of citrus and apple fruits.

Among bacteria used as biocontrol agents, *P. agglomerans* CPA-2, originally isolated from apple surface, was effective in controlling green and blue mold on citrus fruits as well as major postharvest diseases in apples and pears (Nunes et al., 2001; Teixidó et al., 2001; Usall et al., 2008). *P. agglomerans* strain EPS125 decreased the incidence of blue mold in apple and pear, and brown rot and soft rot in stone fruits.
Materials and Methods

Isolation and culturing of bacterial strains. Bacteria were isolated from soil samples collected from different kinds of fields and stored in 15% glycerol at −70°C. The promising bacterium which was identified as Pa 59-4 was cultured for efficacy and population assays. A bacterial suspension was prepared from bacteria grown in a shaking incubator at 28°C, 200 rpm, in tryptic soy broth (TSB). Each bacterial cell was harvested at the beginning of stationary phase (24 hr) by centrifugation at 6,000 g for 10 min. The bacterial cells were resuspended in 0.05 M phosphate buffer (pH 6.5) to the desired concentration.

Culturing of fungal pathogens. P. digitatum KACC 42258 and P. italicum KACC 40827, which were isolated from citrus fruit, were obtained from the Korean agricultural culture collection. The fungal pathogens were maintained on PDA with periodic transfers through mandarin fruit to maintain pathogenicity. For inoculation of fruit, a spore suspension was prepared by adding 10 ml of sterile water with 0.01% of tween 20 over the surface of 7-10 day-old cultures grown on PDA and then rubbing the surface with a sterile glass rod. The cells were counted in a haemocytometer and diluted to the optimal concentration as needed.

Biological control assay. Before each experiment, mandarin fruits were surface disinfected with 70% ethanol. The surface-sterilized fruits were wounded carefully not to penetrate juice sacs at four places with a toothpick by making injuries twice 1 mm deep above the equator of each fruit. Ten microliters of a 2 × 10⁶ spores/ml suspension of P. digitatum or P. italicum was applied to each wound, after 1 hr, followed by inoculation with the appropriate concentration of a Pa 59-4 suspension by spotting (20 µl) the challenge inoculated mandarin fruits. The bacterial concentrations were adjusted to 2 × 10⁷ cfu/ml using a spectrophotometer at 600 nm. Thirty mandarin fruits with 4 wounds constituted a single replicate, and each treatment was repeated three times. Treated fruits were incubated at 20°C and 95% RH in closed plastic containers. Data were recorded as number of infected wounds 7 days after inoculation.

Dose-response experiments of P. agglomerans. The effect of different concentrations of biocontrol agent on the incidence of green mold was assessed at several concentrations of cells of Pa 59-4 (10⁶, 10⁷, 10⁸, 10⁹ cfu/ml). The wounded mandarin fruit was inoculated with spores of P. digitatum and treated with Pa 59-4 as described above.

Preventive control effect of the antagonists. The wounded mandarin fruits as described above were immediately inoculated with 20 µl of Pa 59-4 (2 × 10⁶ cfu/ml). After 1, 24 or 48 hr, the same fruits were inoculated at the same wound with 10 µl of P. digitatum (2 × 10⁶ spores/ml), after which the treated fruits were incubated at 20°C and 95% RH. Data were recorded as the percentage of the number of infected wounds 7 days after inoculation. Twenty fruits with four wounds per fruit were used at each treatment. There were three replicates per treatment.

Population dynamics of Pa 59-4 on mandarin fruit surface. Population dynamics of Pa 59-4 were evaluated on wounded and unwounded mandarin fruits. The fruits were treated with 2 × 10⁶ cfu/ml of Pa 59-4 as described above. The treated fruits were incubated at 20°C or 4°C and 95% RH in plastic containers, and the bacterial populations were monitored 0, 1, 3, 7, 14 and 21 days after treatment. Five fruits constituted each replicate and four pieces of peel surface of 3.14 cm² from each fruit were removed using a cork borer from the inoculated points which were marked at the time of inoculation. The removed surface segments were placed in 10 ml of 0.05 M phosphate buffer, shaken on a rotary shaker for 20 min at 150 rpm and then homogenized. Serial 10-fold dilutions of washings were made and plated on TSA with 100 µg/ml of vancomycin. After incubation at 25°C in the dark for 24 hr, the colonies were counted and their number was calculated for each sample. There were three replicates per treatment.

Competition for nutrients. The effect of nutrient depletion by Pa 59-4 on the germination and growth of P. digitatum spore was tested following Janisiewicz et al. (2000) with small modifications. Briefly, 24-well tissue culture plates containing cylinder inserts with a hydrophilic polytetrafluoroethylene (PTFE) membrane (pore size 0.45 µm) attached at the bottom were used. Potato dextrose broth (PDB) diluted in sterilized D.W. (20%) was dispensed in the wells of the culture plates (0.6 ml per well), with