Combining *Pantoea agglomerans* (CPA-2) and Curing Treatments To Control Established Infections of *Penicillium digitatum* on Lemons

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**ABSTRACT**

The effectiveness of the strain CPA-2 of *Pantoea agglomerans* alone or in combination with a curing treatment at 33°C for 65 h to control green mold was evaluated on lemons stored at ambient temperature and in cold storage. An application of *P. agglomerans* at 2 × 10⁸ CFU/ml effectively reduced green mold incidence on recently inoculated lemons stored at temperatures from 5 to 25°C. Moreover, a 30-s immersion of lemons in a *P. agglomerans* suspension at 2 × 10⁸ CFU/ml significantly reduced green mold incidence, even when delayed up to 15 h after inoculation with *Penicillium digitatum* at either 20°C or while in cold storage. However, it failed to control established infections of *P. digitatum* of more than 24 h. Curing *P. agglomerans*-treated lemons at 33°C for 65 h completely controlled 24-h-old infections on artificially inoculated lemons stored at 20°C for 14 days and on naturally infected lemons stored at 10°C for 3 weeks plus 7 additional days at 20°C. When applied before curing, population growth of *P. agglomerans* in wounds was similar to that within wounds of control fruits at 20°C. In contrast, when it was applied immediately after curing treatment, *P. agglomerans* populations within wounds did not increase.

Fungicides are a primary means of controlling green mold (caused by *Penicillium digitatum* Sacc.) and blue mold (caused by *Penicillium italicum* Wehmer), the main postharvest diseases on citrus fruit (11). However, with growing health and environmental concerns over pesticide disposal and residue levels on fresh commodities, the development of fungicide-resistant strains of postharvest pathogens, and the deregistration of some of the more effective fungicides, a growing interest has been generated in the development of effective alternatives that and pose no risk to human health and the environment (13).

Biological control by microbial antagonists has gained considerable attention as a promising alternative to chemicals. A number of microbial antagonists have been patented and evaluated for commercial use as a postharvest treatment. A reduction of decay in citrus fruit caused by *P. digitatum* was reported with several antagonistic yeasts, such as *Debaromyces hansenii* (5), *Pichia guilliermondii* (10), and *Candida* spp. (1, 9, 12); several bacteria, such as *Pseudomonas* spp. (14, 27); and fungal antagonists, including *Trichoderma viride* (6, 8). Currently, two yeasts, *Candida oleophila* and *Cryptococcus albidus*, and two strains of the bacterium *Pseudomonas syringae* are commercially available under the trade names ASPIRE, YieldPlus, and BIOSAVE-110, respectively (13).

Recently, studies have shown that the CPA-2 strain of *Pantoea agglomerans*, which was isolated from the surface of an apple, is an effective antagonist to the major postharvest pathogens on apples and pears (21, 32). Generally, biological control agents have a relatively narrow spectrum of activity compared with fungicides (17). Therefore, to facilitate their commercial development, it is particularly important to find antagonists with a broad spectrum of hosts in which they can be used and of diseases that they can control. Recent studies with *P. agglomerans* CPA-2 have demonstrated its efficacy to control green and blue mold on oranges (30) and mandarins (23). However, no studies have been performed on lemons.

Because infection of citrus fruit by *P. digitatum* and *P. italicum* can occur either prior to or during harvest and subsequent handling, biological products are expected to protect wounds and also eradicate previously established infections in a manner similar to synthetic fungicides (13). However currently, microbial antagonists confer only a protective effect that diminishes with ripening and provides no control of previously established infections (12, 27). Recent attempts to overcome the variable performance and augment the efficacy of existing biological approaches led to the development of a combination of complementary biological approaches for additive or synergistic effects or both (13). Enhancement of microbial biocontrol agents has been reported with CaCl₂ (4) and carbonate salts (28, 30), as well as physical methods such as curing and heat treatments (7, 14).

Recent studies carried out by Plaza et al. (24) dem-
onstrated that a curing treatment at 33°C for 65 h effectively controlled green and blue mold on oranges and lemons stored under ambient conditions.

The objectives of this work were (i) to evaluate _P. agglomerans_ CPA-2 efficacy to control green mold on lemons stored at different temperatures, (ii) to determine the after-inoculation efficacy of _P. agglomerans_ CPA-2 to control established infections of _P. digitatum_ in wound-inoculated lemon fruit, and (iii) to determine whether the combination of curing and _P. agglomerans_ CPA-2 treatments enhances control of decay.

**MATERIALS AND METHODS**

**Fruit.** Eureka lemons (Citrus limon (L.) N. L. Burm) were obtained directly from packinghouses before any commercial postharvest treatment was applied and randomized before use in these tests. Fruits were grown in a grove in the San Joaquin Valley (California) and in the Baix Ebre-Montsià areas in Tarragona (Catalonia, Spain).

**Pathogen culture and inoculation methodology.** The isolate used in the tests conducted in California was _P. digitatum_ M6R (obtained from J. W. Eckert, University of California, Riverside); the isolate used in tests conducted in Catalonia was _P. digitatum_ PDM-1 (obtained from decayed citrus fruit in the Pathology Unit, UdL-IRTA Center, Catalonia). Fungi were maintained on potato dextrose agar with periodic transfer through citrus fruit. A conidial suspension was harvested by adding 9 ml of sterile, deionized water with 0.01% Tween 80 or Triton-X over the surface of 1- to 2-week-old cultures and rubbing the surface with a sterile glass rod. The concentration of the conidial suspensions was determined with a hemacytometer.

Fruits were inoculated by wounding the flavedo with a steel rod (1-mm-wide and 2-mm-long tip), previously immersed into the conidial suspension, on the circumference of each fruit.

**Antagonist.** _P. agglomerans_ strain CPA-2 was obtained from the UdL-IRTA Center, Catalonia. It was originally isolated from an apple surface (cv. Golden Delicious). Bacterial suspensions for efficacy and population assays were prepared by growing cultures in Erlenmeyer flasks half filled with a medium containing yeast extract (5 g/liter) and sucrose (10 g/liter) for 24 h at 30°C and stirred at 150 rpm. The medium was centrifuged at 10,000 g for 11 min at 10°C (RCSC Sovall Instruments Dupont, Newton, Conn.), and the cell paste was resuspended in 0.05 M phosphate buffer to the desired concentration.

**P. agglomerans** CPA-2 efficacy at different temperatures. Lemons from California were wounded and inoculated as previously described with a _P. digitatum_ conidial suspension adjusted to 10^6 CFU/ml by air drying. Fruits were treated with 15 μl of water (control fruits) or with 15 μl of _P. agglomerans_ suspension adjusted at 2 × 10^6 CFU/ml and stored under different storage temperatures. Twenty-five fruits constituted a single replicate, and each treatment was repeated four times. Green mold incidence was recorded after 5, 6, 10, 15, and 21 days at 25, 20, 15, 10, and 5°C, respectively.

**P. agglomerans** CPA-2 efficacy in wound-inoculated lemons with _P. digitatum_. Lemons from California were wounded and inoculated as previously described with a _P. digitatum_ conidial suspension adjusted to 10^6 CFU/ml. After 40, 24, 15, and 2 h at 20°C to allow infection establishment, fruits were treated by dipping for 30 s in an aqueous suspension containing 2 × 10^8 CFU/ml of _P. agglomerans_. Control fruits were immersed for 30 s in water. In order to test the protective effect of _P. agglomerans_, another set of fruits were first wounded with a steel rod with a 1-mm-wide and 2-mm-long tip, then treated with _P. agglomerans_ at 2 × 10^6 CFU/ml or water; after 6 h at 20°C, they were inoculated with 15 μl of _P. digitatum_ at 10^6 conidia per ml. Twenty fruits constituted a single replicate, and each treatment was repeated four times. Treated lemons were stored at 20°C for 7 days and at 10°C for 3 weeks plus 7 additional days at 20°C to simulate commercial conditions. After each storage period, data were recorded as the percentage of decayed fruits.

**Control of 24-h-old infections of _P. digitatum_ with _P. agglomerans_ CPA-2.** Lemons from California were wounded and inoculated as previously described with _P. digitatum_ suspensions adjusted to 10^6, 10^7, or 10^8 conidia per ml. Twenty-four hours after pathogen inoculation at 20°C, wounds were inoculated with 15 μl of _P. agglomerans_ at 2 × 10^5, 2 × 10^6, or 2 × 10^7 CFU/ml. Control fruits were inoculated with 15 μl of water. Five lemons constituted a single replicate, and each treatment was repeated four times. Treated lemons were stored at 20°C and 90% relative humidity. Data were recorded as the percentage of decayed fruit after 7 days of inoculation.

**The combination of _P. agglomerans_ CPA-2 and a curing treatment to control 24-h-old infections of _P. digitatum_.** Fruits from California were wounded and inoculated as previously described with _P. digitatum_ suspension at 10^6 conidia per ml and kept at 20°C. After 24 h, four treatments were applied as follows: (i) treated with 15 μl of _P. agglomerans_ at 2 × 10^6 CFU/ml; (ii) cured at 33°C for 65 h; (iii) treated with 15 μl of _P. agglomerans_ at 2 × 10^6 CFU/ml immediately after the curing treatment at 33°C for 65 h; and (iv) treated with 15 μl of _P. agglomerans_ at 2 × 10^6 CFU/ml before the curing treatment at 33°C for 65 h.

Control fruits were kept at 20°C for 7 days. Twenty fruits constituted a single replicate, and each treatment was repeated four times. Treated lemons were stored at 20°C and 90% relative humidity. Data were recorded as the percentage of decayed fruits after 14 days of inoculation time. The experiments were repeated twice.

**Semicommercial trials combining _P. agglomerans_ CPA-2 and a curing treatment under cold storage conditions.** To evaluate the efficacy of combining _P. agglomerans_ with the curing treatment at 33°C for 65 h on a semicommercial scale, naturally infected lemons (grown in Catalonia) were used.

Fruits were treated by dipping for 30 s in an aqueous suspension of _P. agglomerans_ at 2 × 10^6 CFU/ml (_P. agglomerans_ treatment), by curing at 33°C for 65 h (curing treatment), and by curing at 33°C for 65 h after a 30-s immersion in _P. agglomerans_ suspension (_P. agglomerans_ + curing treatment). Treated lemons were stored under cold storage at 10°C for 3 weeks. Control fruits were kept at 10°C. After the storage period, fruits were kept 7 additional days at 20°C, simulating commercial shelf life. Data were recorded as the percentage of decayed fruits. Fifty lemons constituted a single replicate, and each treatment was repeated four times. The experiment was repeated twice.

**Population dynamics of _P. agglomerans_ CPA-2 in fresh or treated wounds.** Fruits were wounded with a steel rod with a 1-mm-wide and 2-mm-long tip, inoculated with 15 μl of _P. agglomerans_ at 2 × 10^6 CFU/ml, and then divided into three groups. The first group was kept at 20°C, the second was placed in the curing room at 33°C for 65 h and then stored at 20°C, and the third was inoculated with 15 μl of _P. agglomerans_ at 2 × 10^6 CFU/ml into wounds immediately after the curing treatment at 33°C for 65 h and then stored at 20°C.
TABLE 1. Incidence of green mold on Eureka lemons inoculated with P. digitatum at 10^6 conidia per ml, treated with 15 µl of P. agglomerans at 2 × 10^6 CFU/ml (●) or 15 µl of water (■). Fruits were stored at 25, 20, 15, 10, and 5°C, and the number of infected fruits was recorded after 5, 6, 10, 15, and 21 days, respectively. Asterisks indicate that treatments for each temperature are significantly different (P < 0.05) according to t tests.

FIGURE 1. Incidence of green mold on Eureka lemons inoculated with P. digitatum at 10^6 conidia per ml, treated with 15 µl of P. agglomerans at 2 × 10^6 CFU/ml (●) or 15 µl of water (■). Fruits were stored at 25, 20, 15, 10, and 5°C, and the number of infected fruits was recorded after 5, 6, 10, 15, and 21 days, respectively. Asterisks indicate that treatments for each temperature are significantly different (P < 0.05) according to t tests.

FIGURE 2. Influence of the interval between inoculation with P. digitatum at 10^6 conidia per ml and a 30-s immersion in water (●) or in a P. agglomerans suspension at 2 × 10^6 CFU/ml (■) on Eureka lemons stored at 20°C for 7 days (A) or at 10°C for 3 weeks plus 7 days at 20°C (B). Asterisks indicate that treatments for each interval are significantly different (P < 0.05) according to t tests.

FIGURE 3. Influence of P. digitatum concentration on P. agglomerans biocontrol of 24-h-old infections of P. digitatum on Eureka lemons stored at 20°C for 7 days. Columns with the same letter are not statistically different (P < 0.05) according to the least significant difference test.
INCIDENCE OF GREEN MOLD AMONG EUREKA LEMONS INOCULATED WITH P. DIGITATUM AT 10^6 CONIDIA PER ML 24 H BEFORE TREATMENT WITH 15 µL OF P. AGGLOMERANS AT 2 X 10^8 CFU/ml BEFORE OR AFTER CURING AT 33°C FOR 65 H. DATA WERE RECORDED AFTER 14 DAYS OF INOCULATION. COLUMNS WITH THE SAME LETTER ARE NOT STATISTICALLY DIFFERENT (P < 0.05) ACCORDING TO THE LEAST SIGNIFICANT DIFFERENCE TEST.

FIGURE 5. INCIDENCE OF DECAYED FRUITS BY P. DIGITATUM, P. ITALICUM, AND OTHER PATHOGENS ON NATURALLY INFECTED EUREKA LEMONS TREATED WITH A 30-S DIP IN A P. AGGLOMERANS SUSPENSION AT 2 X 10^8 CFU/ml BEFORE A CURING TREATMENT AT 33°C FOR 65 H. DATA WERE RECORDED AFTER 3 WEEKS AT 10°C PLUS 7 DAYS AT 20°C. COLUMNS WITH THE SAME LETTER ARE NOT STATISTICALLY DIFFERENT (P < 0.05) ACCORDING TO THE LEAST SIGNIFICANT DIFFERENCE TEST.

DISCUSSION

The results of this study demonstrated that P. agglomerans CPA-2 is an effective biocontrol agent against P. digitatum. Control was achieved on recent infections at a wide range of storage temperatures as well as when applied 15 h after pathogen inoculation, protecting lemons from green mold decay under ambient storage and cold storage conditions.

Control efficacy of P. agglomerans CPA-2 has been...
reported by Teixido et al. (30) on oranges to control *P. digitatum* and *P. italicum*. In their work, the antagonist was effective against both green and blue mold on artificially inoculated oranges when applied immediately after infection. The efficacy of *P. agglomerans* CPA-2 to control green mold under a wide range of storage temperatures would be an advantageous feature compared with other biocontrol agents. Teixido et al. (30) also demonstrated that *P. agglomerans* CPA-2 was adapted to cold temperatures, achieving similar population sizes in wounds on fruits stored at 20 and 3°C.

However, in commercial citrus culture, wounds are inflicted and become inoculated during harvest and subsequent handling. Therefore, an application of the antagonistic microorganism to fruit on packing lines that both eradicates infections and protects wounds from further infection is of value (27). Most of the antagonists tested as biocontrol agents reduce decay initiated at wounds if applied before or with pathogen spores (8, 10, 15, 33). As seen in this study, *P. agglomerans* CPA-2 was able to protect wounds from further infections and also prevent the development of *P. digitatum*, even if the treatment was delayed 15 h from inoculation of the pathogen. Similar results were obtained by Smilanick and Denis-Arrue (27) with *Pseudomonas cepacia* and *P. corrugata* on lemons inoculated with *P. digitatum* at different intervals. They found that *P. cepacia* effectively controlled green mold decay on fruits inoculated 12 h before treatment.

Because microbial antagonists typically fail to control established infections, studies that combine antagonist applications with other treatments, such as heat treatments, has been done. Karabolut et al. (18) reported that the combination of hot water brushing at 60°C followed by *Candidatea* spp. applications effectively controlled blue mold decay on peaches and nectarines previously inoculated 24 h earlier with *P. expansum*. On grapefruits inoculated with *P. digitatum*, D’Hallewin et al. (7) significantly improved the control of green mold decay by combining a yeast (strain 22D) with a curing treatment at 37°C for 72 h in comparison with the curing treatment alone on citrus fruits inoculated 1 or 36 h earlier.

Our study shows that immersion in a *P. agglomerans* suspension for 30 s immediately before a curing treatment at 33°C for 65 h successfully controlled established infections of *P. digitatum* on artificially and naturally infected lemons, either under ambient or cold storage temperatures. It is known that curing treatments reduce decay by direct inhibition of the pathogen and by stimulating certain host defense responses, such as the production of lignin-like polymers, synthesis of phytoalexins, and biogenesis of several pathogenesis-related proteins and heat shock proteins (26). The efficiency of curing in reducing decay by various wound pathogens has been described for several citrus fruits (2, 20, 31), although such studies have focused on controlling recent *P. digitatum* infections. Our study shows that a curing treatment at 33°C for 65 h also significantly reduced green mold decay on established infections of *P. digitatum* inoculated 24 h earlier. However, the improvement obtained by combining *P. agglomerans* and the curing treatment compared with each treatment alone indicates a synergistic effect. Curing lemons that were previously treated with *P. agglomerans* could inhibit or delay germination and growth of *P. digitatum*; meanwhile, antifungal materials are being synthesized during the curing process and allowing *P. agglomerans* to grow in the wound at a similar rate as in fresh wounds kept at 20°C. Thus, *P. agglomerans* would be able to colonize wound sites and prevent fungal development.

*P. agglomerans* CPA-2 has previously been reported to be an effective antagonist to *P. expansum*, *Botrytis cinerea*, and *Rhizopus stolonifer* on apples and pears (22) and to *P. digitatum* and *P. italicum* on oranges in recent infections (30). The mode of action of *P. agglomerans* CPA-2 has not been determined conclusively. However, Poppe et al. (25) stated that competition of nutrients appears to be an important mechanism in the antagonistic activity of *P. agglomerans* CPA-2 and that no evidence was found for a role of antibiosis or induced resistance. This mode of action could be an advantage over other strains of *P. agglomerans* that produce antibiotics in vitro (16, 19), and might facilitate its regulatory approval as a postharvest biological control product. Our results on population dynamics reinforce their conclusions. During the curing treatment, lignin-like polymers are synthesized in wounds that constitute a passive barrier to the pathogen (3). This process would reduce nutrients both to the pathogen, which would reduce the number of infections, and to the antagonist, which would reduce its capacity to grow within cured wounds. Because
growth of the antagonist was limited in cured wounds, we suspect this is why we saw no increase in curing efficacy when P. agglomerans was applied after curing.

The application of P. agglomerans CPA-2 followed by a curing treatment at 33°C for 65 h could be used effectively to control established infections of P. digitatum on lemons stored either at room temperature or in long-term cold storage. We recommend large-scale commercial evaluation of these treatments to confirm their value to the citrus industry. In particular, rigorous evaluation of the effect of the treatments on the quality of the fruit is recommended, although these treatments caused no harm to the fruit that we observed.

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