Control of citrus green mold with Aspire is impacted by the type of injury

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Abstract

Aspire, a formulation of the yeast *Candida oleophila* registered for postharvest application to citrus for the control of green mold (*Penicillium digitatum*), competes with the pathogen for nutrients at injuries to prevent infection. A major factor affecting efficacy is how quickly and well the yeast colonizes injuries to the fruit surface, including minor injuries involving only oil vesicles. Colonization of puncture-related injuries that either encompassed oil glands or individually ruptured glands was achieved within 1–2 days at 21°C. Colonization of puncture injuries by *C. oleophila* was comparable after 2 days at 21 and 30°C, but no colonization occurred at 13°C. Ruptured oil glands were colonized more effectively if treated 7 h after injury rather than immediately. Peel oil was toxic to cells of *C. oleophila* but not to spores of *P. digitatum*. *Candida oleophila* colonized punctures more uniformly than individually damaged oil glands, and provided more effective control of green mold originating at punctures than at oil gland injuries. Incubating treated fruit at 30°C for 2 days before storage at 21°C enhanced the control of green mold, and control was significantly improved by the addition of Aspire in one of two trials. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Postharvest pathology; *Candida oleophila*; *Penicillium digitatum*; *Citrus sinensis*

1. Introduction

Green mold is a major postharvest disease of citrus fruit caused by the pathogen *Penicillium digitatum*. The fungus infects fruit only through injuries where moisture and nutrients are available to stimulate spore germination and infection. Infection (Bates, 1933; Nadel-Schiffmann and Littauer, 1956; Kavanagh and Wood, 1967, 1971) can occur through very minor injuries that involve damage to individual oil glands (Thomson et al., 1976) of the fruit exo- and mesocarp (flavedo), and through more extensive puncture injuries encompassing oil glands that extend deeper into the mesocarp (albedo) (Green, 1932; Nadel-Schiffmann and Littauer, 1956; Kavanagh and Wood, 1967, 1971).

Aspire (*Candida oleophila*) is a commercially registered yeast formulated for application to citrus fruit for biological control of green mold.

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The product is applied in water to washed fruit before drying and waxing. The major mode of action of Aspire is through competition with the pathogen for nutrients released by the injury (Droby et al., 1989).

Much of the published work with antagonistic yeasts (Chalutz et al., 1988; Wilson and Chalutz, 1989; Chalutz and Wilson, 1990; Droby et al., 1991) has been conducted with artificially inoculated fruit where rather large injuries have been used for evaluation of efficacy. Under natural conditions minor punctures to the rind are not easily detected during grading, and are frequent sites for infection in commercially handled fruit. These injuries may occur during harvesting from thorns or twigs within the tree canopy, or from nails or wooden splinters on the surfaces of pallet boxes and packing equipment used for handling citrus fruit. Other injuries may occur to oil glands when they rupture during impact at handling, or from sand grains that accumulate in picking bags and pallet boxes, or on surfaces of the conveyor belts of the packingline.

In pilot plant studies using commercially harvested fruit treated with Aspire the day after harvest (Brown and Chambers, 1996), the natural incidence of green mold was usually not controlled as effectively as with labeled rates of the commercially used fungicides thiabendazole and imazalil. In these trials, much of the green mold in Aspire-treated fruit occurred at minor injuries.

In some instances, minor injuries to the rind can involve the rupture of oil glands. The released oil in these injuries may affect the efficacy of Aspire. This study was initiated to determine the impact of either puncture- or oil gland-related injuries on control of green mold with Aspire. Preliminary results have been reported previously (Brown et al., 1997).

2. Materials and methods

2.1. Fruit treatments

Oranges (Citrus sinensis) cultivars ‘Hamlin’ and ‘Valencia’ were obtained from a nearby commercial packinghouse the morning after harvest or harvested from trees in experimental blocks of the Citrus Research and Education Center, Lake Alfred. Fruit were used immediately or held at 21°C, 92–94% relative humidity for no longer than 2 days before use. Fruit were washed with commercial fruit detergent, rinsed with potable water and dried at 40°C. Fruit were graded and randomized into treatment lots.

Two types of injuries were made for inoculation. A single puncture, 1 mm in diameter by 2 mm in depth, was made at the fruit equator with a blunt dissecting needle. Other injuries were made at the fruit equator into three adjacent oil glands with the point of a sewing needle 0.25 mm in diameter by pricking the glands to a depth of 1 mm.

Dry spores of P. digitatum, harvested from sporulating lesions of infected oranges, were suspended in 0.04% TRITON X-100, and adjusted to a concentration of 10^6 ml with a spectrophotometer (Morris and Nicholls, 1978).

Commercial formulations of Aspire (Ecogen, Langhorne, PA 19047) were stored at 4°C and prepared at the highest labeled rate of 3 gms/l (6 x 10^7 colony forming units (cfu)/ml). Suspensions were stirred at least 2 h before use and during the application. Viability of the formulation was evaluated before application (Droby et al., 1991) and, in some trials, the amount of formulated product was increased to compensate for some loss in viability of C. oleophila to maintain the desired concentration.

Fruit were treated while rotating on brushes saturated with Aspire by dripping the material through two rows of closely spaced irrigation drip emitters (Brown and Chambers, 1996) in a manner comparable to commercial applications. Fruit were covered to excess and run-off. Aspire was applied simultaneously with spores of P. digitatum in a similar manner to evaluate the control of green mold. Fruit were air dried or dried by heat (54°C) and non-waxed or waxed (Sta-fresh 590HS, FMC, Lakeland, FL 33802), and placed in plastic crates and stored at 13, 21, or 30°C and 92–94% relative humidity for evaluation of growth of C. oleophila in injuries and control of green mold.
2.2. Colonization of injuries by Aspire

For injury colonization studies, three puncture or oil gland injuries were made at three locations on a fruit at the equator. Tissue (10 mm diameter plug of rind) from the three areas was removed with a cork borer and composited for each of three fruit (replications) at each sampling time. The three plugs of rind tissue (nine injuries) from each fruit were extracted aseptically in 100 ml sterile deionized water at high speed for 2 min with a stomacher. Various dilutions of the extract were plated on nutrient yeast dextrose agar (NYDA) with 100 μg chloroamphenicol/ml of media (Droby et al., 1991) at 25°C for 48 h before counting cfus.

2.3. Toxicity of orange peel oil

Commercially prepared Valencia orange peel oil used for the study was stored under nitrogen at 4°C. Droplets (10 μl) of C. oleophila or P. digitatum containing approximately 25 propagules were placed on NYDA at four equidistant areas of a petri dish (100 × 15 mm) and air dried. The dish was inverted upon four comparably spaced glass microdishes (25 × 10 mm) placed within the lid, each containing oil (80 μl), to evaluate the effect of volatiles on cell viability. An enclosed environment containing volatiles of the peel oil was formed by contact of the microdish with the media. Microdishes (four replications/sample time) were removed from the culture plates during incubation at 25°C, and the plates were incubated as described to measure cfu and lethality.

Propagules of P. digitatum or C. oleophila were suspended in water and continually agitated with a magnetic stirrer during the addition of peel oil and subsequent sampling to evaluate the toxicity of the peel oil. A sample was removed, diluted immediately, and 10 aliquots of 10 μl each, containing approximately 30 propagules/aliquot, were placed on Difco potato dextrose or NYDA media to assay viability of P. digitatum and C. oleophila, respectively.

2.4. Histology

Injured tissue treated with yeast and/or inoculated with P. digitatum was removed from mature fruit and fixed in 3% glutaraldehyde in 0.1 M potassium phosphate buffer (K₂HPO₄), pH 7.2, for 3–4 h at room temperature. The tissue was then washed three times with phosphate buffer, post-fixed for 4 h in phosphate buffered 2% OsO₄ at room temperature, and was washed again three times with 0.1 M phosphate buffer. Tissue was dehydrated in a graded acetone series, and embedded in Spurrs resin (Spurr, 1969). Longitudinal sections approximately 1 μm in thickness were prepared with a Richert Ultra-cut E ultra-microtome, stained with 0.1% toluidine blue (O’Brien et al., 1964) and viewed with light microscopy. Colonization of injuries by the two organisms was viewed with scanning electron microscopy using similarly fixed material. After dehydration the tissue was critical point dried, sputter-coated with gold, and viewed with a Hitachi S530 scanning electron microscope at 20 kV.

2.5. Analysis of data

Data were analyzed using analysis of variance. Decay data were transformed with arc-sine transformation before analysis, but are reported as percentage values. Treatment mean comparisons were made with Duncan’s multiple range test or by orthogonal contrasts.

3. Results

3.1. Development of C. oleophila in injuries

Populations of C. oleophila after application were approximately 2 log units higher in the larger puncture injuries (Fig. 1A). However, an increase in growth of yeast cells of approximately 1 log unit occurred in both types of injury after only 1 day, and the respective populations remained relatively constant during the 7 days of the time-course study.

Initial deposits of C. oleophila in fresh or 7 h puncture injuries (Fig. 1B) were similar (P =
0.091), and increased 1 log unit after a day of growth. Populations were less \((P = 0.04)\) in the 7 h injury after 3 days of growth, but after 7 days they were similar to populations in fresh injuries. In injuries to oil glands (Fig. 1C), fewer cells survived from the initial deposit to fresh injuries, and this difference persisted for at least 2 days \((P = 0.03)\), but the populations were similar by the end of the experiment at 7 days.

Initial survival of \(C.\) oleophila in puncture injuries was not impacted by waxing (Fig. 1D). Non-treated fruit contained populations of log 5.5 cfu, and populations in waxed fruit were near log 5.3 \((P = 0.61)\). Growth rates after 1 day at 21 or 30°C increased approximately 1 log and were similar whether fruit were waxed or non-waxed. Growth was prevented by storage at 13°C where propagules of the initial population remained inactive during the following 2 days.

### 3.2. Histology

At 24 h after treatment of fresh puncture injuries, \(C.\) oleophila had colonized injured tissue near the fruit surface (Fig. 2A), and particles of damaged tissue (Fig. 2B) scattered along the surface of subepidermal parenchyma within the injury. Growth of the yeast was often quite intense.

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**Fig. 1.** Population kinetics of \(C.\) oleophila in injuries to oranges. (A) Growth in puncture and oil gland injuries during 7 days at 21°C. (B) Growth in fresh and 7-h-old puncture injuries during 7 days at 21°C. (C) Growth in fresh and 7-h-old oil gland injuries during 7 days at 21°C. (D) Growth after drying without waxing (---) or with waxing (-----) in puncture injuries during 2 days at 13, 21, or 30°C.
through physically injured and oil damaged epidermal and subepidermal parenchyma cells of the exocarp (Fig. 4A) at the top of the oil gland. Occasionally, infection occurred directly through the cells of the oil gland envelope at the side or bottom of the gland (Fig. 4B). *P. digitatum* caused obvious swelling and dissolution of host cell walls in advance of hyphal penetration (Fig. 4A and B).

3.3. Toxicity of cold pressed Valencia orange peel oil

Exposure of cells to a concentration of 0.01% peel oil for as long as 80 min did not affect viability (Fig. 5A), but a concentration of 0.1% reduced the viability by 70% within 10 min, and by 90% after an additional 10 min. Cells of *C. oleophila* were sensitive to volatiles of orange peel

on these particles where nutrients were readily available.

Under comparable conditions after 24 h at freshly damaged oil glands (Fig. 3A), growth of *C. oleophila* near the fruit surface (Fig. 3B) was less extensive than at similar puncture injuries (Fig. 2A). The yeast colonized injury sites moderately at the gland wall (Fig. 3C), but colonization upon pieces of damaged tissue that fell into the gland was similar to that observed on puncture injuries shown in Fig. 2B.

Hyphae of *P. digitatum* rapidly invaded puncture injuries within 24 h, and by 48 h hyphae had extensively penetrated healthy tissue surrounding the injury (not shown). Penetration of injured oil glands by *P. digitatum* occurred by two avenues. Invasion after 24 h was observed frequently

Fig. 2. Growth of *C. oleophila* in a puncture at the (A) injured epidermis and (B) upon debris scattered along the surface of subepidermal parenchyma within the injury.

Fig. 3. Growth of *C. oleophila* in an (A) injured oil gland at the (B) epidermis and (C) within the oil vesicle.
Two trials were conducted to evaluate the effect of temperature following treatment on efficacy of Aspire (Table 2). In both trials green mold developed the least in fruit stored at 30°C for 2 days after treatment. In trial 1, the effect was significantly improved by treating with Aspire, but in trial 2 Aspire did not enhance control of green mold at any temperature.

4. Discussion

The type of injury and its location on the rind of a citrus fruit has a major impact on the level of infection by *P. digitatum* (Green, 1932; Bates, 1933; Nadel-Schiffmann and Littauer, 1956; Kavanagh and Wood, 1967, 1971). Injuries that protrude into the mesocarp, usually 2 mm or greater, consistently lead to higher infection rates. More shallow injuries, 1 mm or less, result in less infec-

3.4. Control of green mold with Aspire

Aspire significantly reduced green mold at puncture injuries in two of four tests (Table 1) when it was applied simultaneously with *P. digitatum* to Hamlin or Valencia oranges. No control was achieved with similar applications of Aspire to inoculated injuries at oil glands in the four tests.

Exposure of spores of *P. digitatum* to similar volatiles and concentrations of peel oil had no effect on germination or growth (data not shown). In additional trials (data not shown), volatiles absorbed by the media did not affect viability of the yeast or fungus.

oil (Fig. 5B). A 50% reduction in viability occurred after 15 min of exposure, 80% after 1 h, and little survival after 2 h.

Exposure of spores of *P. digitatum* to similar volatiles and concentrations of peel oil had no effect on germination or growth (data not shown). In additional trials (data not shown), volatiles absorbed by the media did not affect viability of the yeast or fungus.
Table 1
Control of green mold with Aspire applied to oranges immediately following injury by puncture or rupture of oil glands

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Injurya</th>
<th>Green mold trials (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1. Control</td>
<td>Puncture</td>
<td>93.9</td>
</tr>
<tr>
<td>2. Aspire</td>
<td>Puncture</td>
<td>61.2</td>
</tr>
<tr>
<td>3. Control</td>
<td>Oil glands</td>
<td>34</td>
</tr>
<tr>
<td>4. Aspire</td>
<td>Oil glands</td>
<td>37.8</td>
</tr>
</tbody>
</table>

Orthogonal contrasts $P>F$

1 vs. 2 $<0.01$ 0.16 $<0.01$ 0.34
3 vs. 4 0.56 0.44 1 0.53

a Injuries were formed by puncture (1 × 2 mm) or rupture of three adjacent oil glands to a depth of 1 mm.
b Hamlin or Valencia oranges were treated in trials 1 and 2 or 3 and 4, respectively. Treatments in trials 1, 2, and 3 were applied to 55, 25, and 25 fruit, respectively, in each of three replications. Treatments in trial 4 were applied to four replications, each containing 25 fruit.

Colonization of injured tissue by *C. oleophila* was essentially complete at 1 to 2 days after the application of Aspire. In puncture injuries, distribution of the yeast cells was fairly uniform on damaged tissue except in areas within the injury that involved damaged oil glands. Here, and in injuries to single oil vesicles, development of the yeast was not as extensive. Most growth appeared to occur at cracks or pockets of injured tissue of the vesicle wall, presumably because of the availability of nutrients. Growth of *C. oleophila* was not significantly impacted by commercial waxes normally applied to improve fruit shine and reduce moisture loss.

Table 2
Effect of temperature of storage on control of green mold with Aspire applied to oranges immediately following puncture injury and inoculation with *P. digitatum*

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Green mold trials (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1. Control, 30°C</td>
<td>22.2</td>
</tr>
<tr>
<td>2. Control, 13°C</td>
<td>25.1</td>
</tr>
<tr>
<td>3. Control, 21°C</td>
<td>21.5</td>
</tr>
<tr>
<td>4. Aspire, 30°C</td>
<td>11.3</td>
</tr>
<tr>
<td>5. Aspire, 13°C</td>
<td>15.3</td>
</tr>
<tr>
<td>6. Aspire, 21°C</td>
<td>18.9</td>
</tr>
</tbody>
</table>

Orthogonal contrasts $P>F$

1 vs. 4 $<0.01$ 0.69
2 vs. 5 $<0.01$ 0.01
3 vs. 6 0.69 0.13
4 vs. 5 0.27 $<0.01$
4 vs. 6 0.02 $<0.01$
5 vs. 6 0.15 0.49
1 and 4 vs. 2 and 5 0.20 $<0.01$
1 and 4 vs. 3 and 6 0.11 $<0.01$
2 and 5 vs. 3 and 6 0.75 0.99

a Valencia oranges in trials 1 and 2 were injured by puncture (1 × 2 mm) and stored after treatment at 30, 13, or 21°C for 2 days before continuous storage at 21°C for 19 and 23 days, respectively. Treatments were applied to five replications, each containing 55 fruit.
Oil released from oil glands involved in puncture injuries may be more rapidly absorbed by the larger amount of interglandular damaged tissue than oil released in smaller injuries that essentially remains in the oil vesicle. By delaying the application of Aspire to oil gland injuries we achieved better survival of yeast cells from the application apparently because less oil remained than in fresh injuries. The impact of peel oil on efficacy of Aspire is probably greatest in fresh injuries, those that occur during handling in the packinghouse immediately before the application of Aspire.

Efficacy of Aspire could possibly be enhanced by storing fruit after treatment at temperatures that favor growth of the yeast more than the pathogen. Growth of *P. digitatum* is reduced at 30°C (Fawcett and Barger, 1927), but *C. oleophila* colonized injuries as well at this temperature as it did at 21°C. Temperatures of 30°C also favor wound healing (Hopkins and Loucks, 1948), and increased resistance of minor injuries to infection by *P. digitatum*. The combined effect of wound healing and favorable activity of *C. oleophila* at 30°C could lead to more consistent disease control than either treatment applied alone.

Essential oils of citrus (Shaw, 1977) exhibit antimicrobial activity (Maruzella and Liguori, 1958; Maruzella et al., 1959; Murdock and Allen, 1960; Suba et al., 1967; Dabbah et al., 1970) and have been suggested as possible preservatives in food products (Dabbah et al., 1970). Antimicrobial activity of orange oil is selectively more active against *C. oleophila* than *P. digitatum*. Oil and its volatiles exhibited no activity against *P. digitatum* under the conditions of our tests, but were quite active against Aspire. Oil volatiles have actually shown a stimulatory effect on germination of *P. digitatum* in situations where nutrients necessary for germination were not available (Kavanagh and Wood, 1967; Eckert and Ratnayake, 1994). Oil is not a preferred substrate for *P. digitatum*, however, because as the fungus invades the rind it usually surrounds the oil vesicles rather than penetrate them (Klotz, 1930).

In much of the previous work, control with biological yeasts has been with artificially inoculated fruit where large injuries were made and the biological agent was frequently added to the injury before the pathogen (Wilson and Chalutz, 1989; Chalutz et al., 1988; Chalutz and Wilson, 1990; Droby et al., 1991). It may be inaccurate to extrapolate results of these studies to results expected under commercial conditions where fruit with larger injuries may be eliminated during grading, and infection by the pathogen may occur at less severe injuries and may frequently precede postharvest treatments (Brown and Chambers, 1996).

Poor eradicant activity (Chalutz and Wilson, 1990) and less effective control of infection at minor injuries associated with oil glands than at larger injuries may be two factors that contribute to efficacy and inconsistency of control observed with naturally infected fruit (Brown and Chambers, 1996). Prevalence of minor oil gland injuries to citrus may vary with geographical regions, cultivars, and harvesting and handling practices and relate to the efficacy of biological control. Constituents of citrus oils differ among citrus types (Shaw, 1977) and concentrations can change with maturity (Scora et al., 1969). These oils vary in antimicrobial activity (Maruzella and Liguori, 1958; Suba et al., 1967; Dabbah et al., 1970), and if similar variability in activity occurs to biological antagonists, some differences in decay control efficacy may be observed among citrus types at various stages of maturity.

Other factors, such as fruit susceptibility to decay, inoculum level of *P. digitatum*, and treatment methods, also may impact the efficacy of Aspire.

References


