Synergism of *Geotrichum candidum* and *Penicillium digitatum* in Infected Citrus Fruit

S. C. Morris

Gosford Horticultural Postharvest Laboratory, New South Wales Department of Agriculture, P.O. Box 355, Gosford, N.S.W. Australia 2250.

The advice and initial assistance of B. L. Wild and L. E. Rippon, senior research horticulturists at the Gosford Laboratory, and statistical analysis of the results by P. J. Nicholls, senior biometrician, N.S.W. Department of Agriculture, are gratefully acknowledged.

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ABSTRACT

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Consistently high levels of sour rot of citrus fruit by *Geotrichum candidum* were obtained by adding *Penicillium digitatum* spores to the inoculum and incubating the fruits at high relative humidities and at either 30 or 25 C after dipping them in benomyl. Synergism was greatest in mature fruit and occurred over a wide range of spore concentrations of *G. candidum* and *P. digitatum*. The ability of spores of *G. candidum* to induce disease was particularly increased by adding nutrients to the inoculum. The spores, rather than the sterile filtrate of a *P. digitatum* spore suspension, were found necessary for synergism to be expressed. Benomyl prevented development of symptoms caused by *P. digitatum*, but not spore germination and therefore permitted the synergistic increase of *G. candidum* to occur. Therefore, fungicides that prevent germination of *P. digitatum* spores should markedly reduce sour rot losses due to *G. candidum*.

Postharvest losses of citrus fruit due to sour rot caused by *Geotrichum candidum* (Lk. ex Pers.), although highly variable (14,17), are now more frequent (13). The control of more aggressive pathogens such as *Penicillium digitatum* Sacc., and *P. italicum* Wehmer, particularly by benzimidazole fungicides, has made sour rot more noticeable. However, results by some workers using these fungicides (18) indicate that the increased incidence of sour rot is greater than would be expected due merely to the removal of a competing pathogen.

Artificial inoculations with spore suspensions of *G. candidum* had resulted in low and variable levels of infection (4,10,19). Butler et al (4) attributed unsuccessful infection following artificial inoculation to inadequate fruit injury and found successful infection could only be achieved by deep injury (≥10 mm deeper). Gutter (8) attributed the low success rate of artificial inoculation to poor inoculum and found it necessary to smear wounded fruit with a puree of *G. candidum*-infected fruit to produce high levels of infection. Neither of those conditions normally occur commercially, at least during initial stages of infection and thus could not account for the high wastage that may occur.

The presence or absence of *P. digitatum* may influence the rate of growth of *G. candidum* on agar plates (7) and in mixed culture on citrus fruit (15). However, in neither study was the infection process observed, nor were both organisms examined individually and in combination on citrus fruit in comparable experiments.

Results of previous work (12) indicated that the growth rate of lesions caused by *G. candidum* is influenced by the presence of *P. digitatum* spores. This paper reports the results of further experiments to verify this and to identify factors responsible for the synergistic effect.

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MATERIALS AND METHODS

Fruit. Fruit of Eureka lemons and Valencia oranges grown in the Gosford district of New South Wales were washed, surface sterilized with 70% ethanol, and allowed to dry prior to inoculation.

Spore suspensions. *G. candidum* and *P. digitatum* were cultured on PDA plates. Spores were suspended by gently brushing into sterilized distilled water containing 0.05% Lissapol (ICI, Sydney, Australia) and the concentration of the suspension was determined (11). Inocula were prepared by diluting spore suspensions to the required concentration.

A sporeless filtrate was prepared from spore suspensions of *P. digitatum* by filtration through 0.45-μm Acrodisc filters (Gelman Instrument Co., Ann Arbor, MI 48106). The retained spores were rinsed from the filter and made up to the original concentration with distilled water.

Inoculation. Each fruit was inoculated by dipping into the inoculum suspension a cork with a nail point protruding 3 mm, then wounding the fruit on opposite sides (penetrating into the mesocarp). When the effect of spore levels per inoculation point were being tested, each fruit was similarly injured with a sterile nail and inoculum was applied to the wound with a syringe. The uniform wounds held 40 ± 5 μl of suspension when filled, thus allowing spore numbers per inoculation site to be calculated. If the fruit was dipped in benomyl this was done after inoculation and the fruit was allowed to drain. For development of symptoms the fruit were placed in perforated plastic bags to maintain a high humidity, held at 30 C for 7 days, and then examined for typical sour rot

symptoms at the inoculation points. Only *G. candidum* was isolated from the margin of lesions showing typical sour rot symptoms.

Statistical analysis. Four replications of 30 fruit each were used per treatment. Generally these were from different growers and data from them were analyzed as blocks. The data were arc sine transformed for analysis of variance. Significant differences were determined according to the Waller-Duncan k ratio LSD rule (5) at the $k = 100$ level of statistical significance.

RESULTS

Linear growth of *P. digitatum* and *G. candidum* on PDA plates was maximum for *P. digitatum* at 26 C, with no growth at 30 C and above (similar in vivo results for *P. digitatum* at 28 and 30 C are reported by Savastano [15]), while *G. candidum* had a linear growth maximum at 30 C with inhibition only above 35 C. Results when fruit was incubated at 26 and 30 C confirmed these findings.

Successful growth of *G. candidum* required high humidity (17). The effect of humidity on disease incidence was studied by storing inoculated lemons (10^6 spores of *G. candidum* per milliliter) over six different saturated salt solutions at 30 C. Disease incidence was related ($R^2 = 0.98$) to relative humidity (RH) by the equation: Disease incidence (%) = $8.59 - 0.59$ (% RH) + 0.0089 (% RH)^2 with disease incidence rapidly dropping from 39% at 98% RH to 1% at 47% RH. Thus the optimum conditions for disease incidence and growth of *G. candidum* were unfavorable for *P. digitatum*.

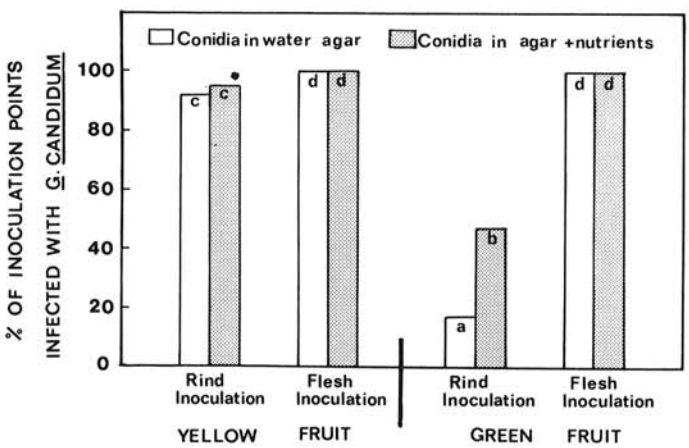

Fig. 1. Effect of inoculation depth, nutrient level, and fruit maturity on infection of Eureka lemons by *Geotrichum candidum*. Rind inoculation = wound 3 mm deep into the mesocarp and flesh inoculation = wound penetrating into endocarp. Differences between means represented by bars with different letters are statistically significant, $P = 0.05$.

Inoculation depth, exogenous nutrient, and fruit maturity effects. *G. candidum* spores (10^6 spores per milliliter) in either water agar (0.2% agar) or agar containing added nutrients (1% glucose, 1% dehydrated potato flakes, 0.2% agar) were introduced into a cut penetrating into either the rind or flesh of green and yellow lemons and held at 30 C (Fig. 1). All inoculations into the flesh were successful. Rind inoculations without added nutrients resulted in slightly lower disease incidence than did flesh inoculations in yellow fruit, but much lower levels in green fruit. The addition of nutrients caused a rise in disease incidence resulting from rind inoculation in green fruit from 17 to 48%.

The synergistic effect of *P. digitatum* on *G. candidum* infection. Two series of inocula were used, one containing only *G. candidum* (over the range 2.5×10^3 to 2.5×10^7 spores per milliliter) and the other with the same concentrations of *G. candidum*, but with *P. digitatum* added at 2.5×10^7 spores per milliliter (Fig. 2). When *P. digitatum* was added to *G. candidum* spores, disease incidence increased. *P. digitatum* induced high levels of disease in combination with low concentrations of *G. candidum*, even when the same concentration of *G. candidum* spores alone induced virtually no disease.

In a further experiment a suspension of *P. digitatum* spores cultured on oranges was added to *G. candidum* (2.5×10^6 spores per milliliter). The same large synergistic increase in *G. candidum* disease incidence occurred. This increase was not affected by varying the final concentration of added *P. digitatum* spores between 2.5×10^5 and 2.5×10^7 spores per milliliter.

Efficacy of *P. digitatum* spore suspension in causing synergism. The *P. digitatum* spore suspension obtained from PDA plates (2.5×10^5 spores per milliliter) was separated by filtration into spores and filtrate, then each of these were mixed with *G. candidum* spores (2.5×10^5 spores per milliliter) and introduced into lemons held at 30 C (allowing infection only of *G. candidum*). No significant infection resulted from inoculation with the *G. candidum* spore suspension alone (0.7% infection), or with the addition of the sterile *P. digitatum* filtrate to the inoculum (0.0%). A strong synergistic

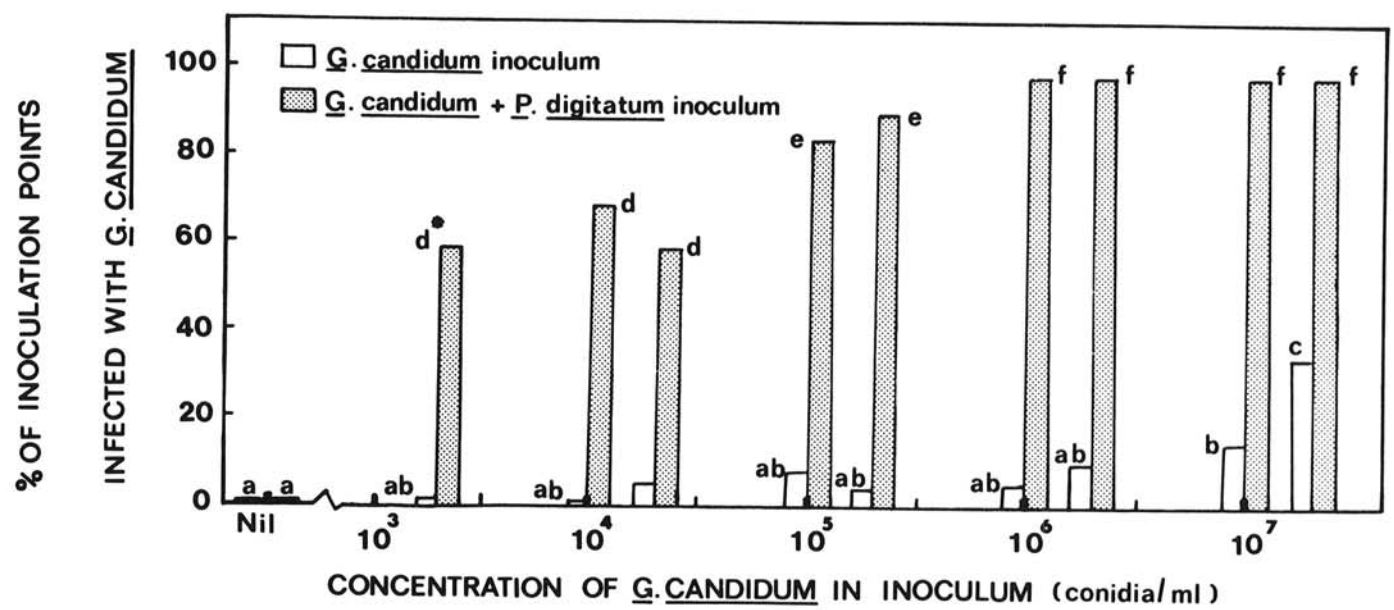

Fig. 2. Synergistic increase in infection of Valencia oranges by *Geotrichum candidum* at 30 C due to the presence of *Penicillium digitatum* in the inoculum. All *P. digitatum* concentrations were 2.5×10^7 spores per milliliter. Differences between means represented by bars with different letters are statistically significant, $P = 0.05$.

effect was only demonstrated with the addition of the complete *P. digitatum* suspension (28.6%) and the "spores only" suspension (37.8%) to the inoculum. The higher disease incidence occurring with the "spores only" suspension, compared to the complete spore suspension, is possible due to a soluble inhibitor present in the latter.

Temperature, fungicide, and maturity effects on synergism. To ascertain the effects of synergism at other temperatures and to simulate commercial conditions more closely, an experiment was designed to study the effects of fruit maturity, spore levels of *G. candidum* and *P. digitatum* (inoculated either separately or together), dipping in benomyl, and incubation at 25 or 30 C.

The results (Fig. 3) showed that the synergistic effect of *P. digitatum* was apparent at 30 and 25 C after benomyl dipping. The synergistic increase was greatest at 30 C and was also significantly greater on mature fruit.

When fruit was not dipped in benomyl and was incubated at 25 C, virtually all points inoculated with *G. candidum* and *P. digitatum* developed green mold. Unlike the effects of maturity on sour rot levels there was no significant effect of fruit maturity on green mold.

Effect of water and benomyl dipping on *G. candidum* infection without *P. digitatum* spores present. Lemons were inoculated with *G. candidum* (10^5 spores per milliliter), incubated for 4 hr, then either not dipped or else dipped in water or benomyl and allowed to drain dry. The fruit was then placed in open boxes or perforated plastic bags, and incubated for 5 days at 60–70% RH and 25 C.

Sour rot in fruit stored in open boxes averaged 0.5% while 18% of those held in perforated plastic bags developed symptoms. When held in plastic bags disease incidence was control, 7%; water-dipped, 20%; and benomyl-dipped, 27%. These differences were significant at $P = 0.01$, except between water-dipped and benomyl-dipped).

In situ germination of *G. candidum* and *P. digitatum*. Aqueous suspensions containing 2.5×10^7 spores of *G. candidum* and *P. digitatum* per milliliter were inoculated into oranges that were incubated at 30 C, or at 25 C with or without benomyl dipping. When spores were inoculated together, the high levels of germinating *P. digitatum* spores made assessment of *G. candidum* germination impossible. The results given, therefore, are for separate inoculations of the two fungi. Germination was assessed by taking transverse sections through the wound site after 24 hr and examining microscopically.

Conidia of *G. candidum* germinated poorly (Table 1), with growth of germinated spores favored by high temperatures and humidity due to dipping in benomyl. However, *P. digitatum* spores germinated even under conditions that inhibited further development of infection. These conditions induced considerable branching and distortion of the germ tubes.

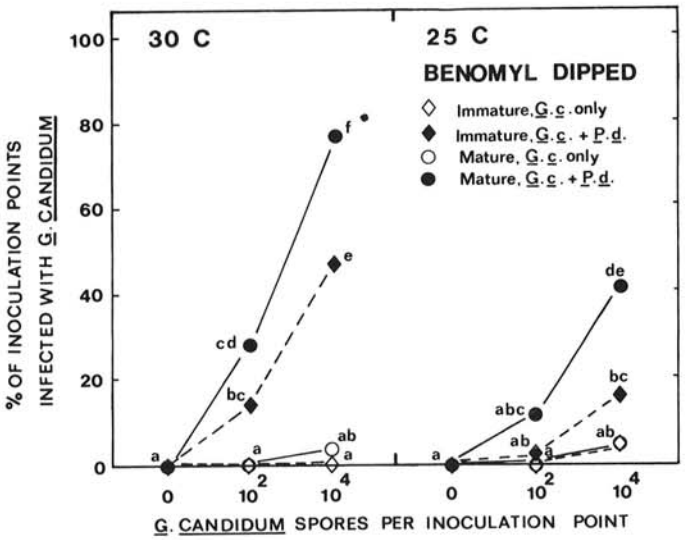

Fig. 3. Effect of temperature, fungicide and maturity on the synergistic interaction of *Penicillium digitatum* (P.d.) and *Geotrichum candidum* (G.c.) in sour rot of Valencia oranges. Differences between means represented by bars with different letters are statistically significant, $P = 0.05$. Immature oranges were >50% green.

TABLE 1. Effects of temperature and benomyl on spore germination of *Geotrichum candidum* and *Penicillium digitatum* inoculated into Valencia oranges

Organism[y]	Germination characteristics	Spore germination following incubation at: 30 C (Not dipped)	25 C (Not dipped)	25 C after benomyl dip
G. candidum	Germination	Low[z]	Very low	Low
	Germ tube length (avg)	120 μm	10 μm	150 μm
P. digitatum	Germination	High	Very high	High
	Germ tube length (avg)	150 μm	>>250 μm	50–150 μm
	Branching	Moderate	Little	Extensive

[y] Twenty fruit inoculated per treatment.

[z] Very low = 0–1% germination; low = 1–5%; medium = 5–30%; high = 30–60%; and very high = 60–90%.

DISCUSSION

Successful development of high levels of disease caused by *G. candidum* on citrus was achieved by adding *P. digitatum* spores to the inoculum and incubating the inoculated fruit at 30 C in perforated plastic bags to maintain high humidity. These conditions maximize *G. candidum* incidence and growth rates, prevent development of *P. digitatum* symptoms, and were simpler to achieve and closer to commercial practice than those previously described (4,8).

The synergistic increase of fruit disease caused by *G. candidum* in conjunction with *P. digitatum* was observed both in lemons and oranges. This increase occurred whether the *P. digitatum* spores were produced on fruit or PDA plates. The effect was most apparent at low concentrations of *G. candidum* conidia, which without *P. digitatum* present, caused practically no disease.

The major barrier to *G. candidum* infection was the rind, particularly in less mature fruit. The addition of nutrients to the *G. candidum* inoculum partially overcame the barrier presented by the mesocarp, but still did not cause the high disease incidence observed commercially. The inability of *G. candidum* (particularly on green fruit) to invade the mesocarp was possibly due to the absence of sufficient nutrients to allow penetration into the endocarp. A further contributing factor was the low germination of *G. candidum* spores introduced into the mesocarp in contrast to the abundant germination of *P. digitatum* spores even under conditions that severely inhibit further development of infection.

Dipping fruit in benomyl and storage below 30 C simulated conditions that duplicate commercial practices by citrus growers. These favored a synergistic increase in infection. When humidity was high (eg, after fruit were dipped in water or benomyl) *G. candidum* disease incidence more than trebled, even without *P. digitatum* present. Therefore, dipping in a fungicide ineffective against *G. candidum* may greatly increase losses due to sour rot.

These results demonstrate that *P. digitatum* spores are necessary to induce the synergistic increase in sour rot reported and that this increase only occurs under conditions that permit the germination of *P. digitatum*, but inhibit green mold development (30 C and benomyl dipping). Germinating *P. digitatum* spores produce high levels of several macerating enzymes (2,3,6) but these are produced only at low levels by *G. candidum* (1). Enzyme production and consequent infection seem to be a response to pectic substances at the wound site (9,16). This enzyme action should release nutrients to *G. candidum*, thus allowing invasion through the mesocarp and into the endocarp.

Increased sour rot due to synergism of *P. digitatum* with *G. candidum* was dependent upon spore germination. Thus, significant reduction of sour rot would be expected if fungicides used for control of *P. digitatum* also prevented germination.

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