Prangolarin Is a Chemical Facilitator for the Enhanced Development of the Infection Process in the Epicarp of *Citrus limon* by *Penicillium digitatum*

Yutaka Arimoto, Fumio Sugawara,* Shigeo Yoshida, and Isamu Yamaguchi

The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan

The application of a water suspension of conidia of *Penicillium digitatum* caused complete "green mold" symptoms to develop on 65% of wounded lemon (*Citrus limon*) epicarps after 4 days. However, wounded lemon epicarps, exhaustively washed with water, exhibited only 2% green mold symptoms when inoculated with spores of *P. digitatum*. However, when a comparable amount of isolated lemon epicarp oil was topically applied to the washed wounds, 92% of the inoculated wound sites developed green mold symptoms. These observations indicated that some component of the lemon epicarp oil is essential for the development of *P. digitatum* in epicarp tissues and suggest that the fungal facilitating factor(s) could be present in the lemon epicarp oil. One of promoting factors from the oil was isolated by bioassay-guided fractionation and was identified as prangolarin (**1**) by spectrometric analyses. Prangolarin, by itself, enhanced disease development by *P. digitatum* on the wounded epicarp of lemon.

Keywords: *Penicillium digitatum Sacc.; prangolarin; oxypeucedanin; green mold of lemon; facilitator for conidial infection*

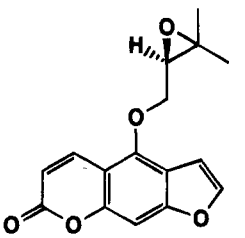
INTRODUCTION

"Green mold" commonly develops on postharvested citrus fruits after infection by *Penicillium digitatum* Sacc. Because of the wide range of hosts affected, its severity, and its distribution, green mold is the most economically important disease of citrus production in the world. Green mold alone is responsible for 90% of citrus loss due to diseases occurring during storage from summer to autumn (Pelser and Eckert, 1977).

Generally, the spores of *P. digitatum* do not germinate on the surface of the citrus fruit until the epicarp (peel) is injured (Smoot et al., 1971). However, once the epicarp of lemons is wounded, during harvesting or subsequent processing, *P. digitatum* spores germinte, begin infection, and eventually develop complete green mold symptoms on decaying fruit. If there is no wound on a fruit surface, the spores of *P. digitatum* cannot usually infect it.

Interestingly, the production of green mold in citrus can be reduced by washing the fruit that may have been wounded (Brown, 1973). Complete green mold symptoms could not be induced by the inoculation of *P. digitatum* spores on wounded epicarp tissues after washing with water, while all symptoms developed after the addition of peel oil at the time of inoculation (Kawai et al., 1984). One of the compounds from the peel oil that enhances the development of green mold was identified as limonene; however, other compounds from citrus epicarps also have an effect on the biology of *P. digitatum* (Arimoto, 1994). For instance, Eckert and Ratnayake (1994) reported that a mixture of limonene, α-pinene, sabinene, β-myrcene, acetaldehyde, ethanol, and CO_2 from wounded oranges induced germination of *P. digitatum* conidia on water agar.

Given the fact that water-washed wounds do not support full development of green mold symtoms and that peel oil extracts added to wounded epicarps can fully restore the disease development potential to *P. digitatum*, we investigated the possibility that one or more compounds in the peel oil extract can facilitate the disease-causing potential of *P. digitatum*. This potential is expressed by the complete fully developed "green symptoms" on infected fruit. The appearance of green mold is the result of the production of masses of conidiophores and conidia, yielding a greenish fungal mass. This study reports on the isolation and identification of prangolarin (Figure 1) from epicarp extracts and implicates its role in facilitating the complete development of green mold symptoms in *P. digitatum* infected lemons; in contrast; plant tissues, although infected, do not show the massive fungal development commonly recognized as green mold.



1: prangolarin

Figure 1. Structure of prangolarin.

MATERIALS AND METHODS

Inoculum. *P. digitatum* was isolated from green mold of *Citrus unshu* and was grown on *Citrus limon* fruit at 23 °C under 100% humidity. After 5–6 days, the conidia were collected in mass on a waxed paper by tapping of the diseased fruit. These spores were stored at 0 °C and periodically used in the infection assays.

Extraction and Fractionation of the Facilitators. The epicarps (about 0.5 mm thickness) of mature lemons (*C. limon*) were peeled off with razor blades and mechanically blended. After passing through filter paper (No. 3), the filtrates (93 mL from 100 lemons) were centrifuged at 10000*g* for 10 min, at which time the oil moves to become the upper layer (36 mL). After removal of the limonene by HPLC equipped with a reversed phase column (C_{18} Capcell Pak) in a mixture of

0021-8561/95/1443-2283$09.00/0

Table 1. Effects of Various Additives to a Conidial Suspension of *P. digitatum* on Complete Green Mold Development in *C. limon* after 4 Days of Incubation[a]

suspension of *P. digitatum* in	green mold development (%)	suspension of *P. digitatum* in	green mold development (%)
water	2	epicarp oil without limonene and prangolarin	5
orange oil	3	limonene added to orange oil	28
epicarp oil	92	prangolarin added to orange oil	29
epicarp oil without limonene	28		

[a] One milliliter of a spore suspension of 10^6 spores of *P. digitatum* was placed on a cut epicarp surface of lemon. Each test was repeated 100 times, and the data show the number of surfaces per 100 that developed complete green mold symptoms. Note that the wounds without washing with water had 65% green mold.

acetonitrile and water (80:20 v/v; flow rate, 3 mL/min) monitored by an RI detector, the residual oil was used for the biological assay and additional facilitator isolation. The oil (1 mL), after removal of limonene, was placed on a preparative silica gel TLC plate (0.5 mm thickness, E. Merck, Darmstadt), which was developed in a mixture of ethyl acetate and acetone (95:5 v/v). Fractions were detected by UV (320 nm) and eluted with diethyl ether. After drying under a stream of nitrogen, each fraction was duly assigned for the infection assay. Ultimately, the active fraction was further purified by preparative TLC developed in chloroform on three successive passes. The above separations and biological tests were successively repeated twice. The active fraction was further purified on HPLC equipped with a reversed phase column (C_{18} Capcell Pak). The white crystals (17 mg from 100 lemons) obtained were used for spectroscopic analyses.

Biological Assay. The lemons to be tested were washed with water and then wiped with a paper towel. Twenty-five lemons were used for each sample. The epicarps of matured lemons were peeled to 0.05–0.1 mm thickness (7–10 mm^2 area) at four places with razor blades, and then the lemons were continuously washed with water for 2 h. After drying, a water suspension (2.5 μL) of *P. digitatum* conidia (10^6/mL) was inoculated onto the wounded surface. Complete disease development was determined by counting the percent by the number of wounds producing complete green mold development from the 100 wounds per test.

Samples to be tested were dissolved with commercially available orange oil (1 mL each, DoLce Orange Oil, Narizuka Corp., Tokyo) instead of water, because none of the fractions from lemon extracts were soluble in water. The orange oil used is totally ineffective at promoting green mold (see Table 1). The conidia of *P. digitatum* (10^6/mL) were suspended in the orange oil in which a test sample was dissolved.

For assay, 2.5 μL of conidial suspension involving test samples was applied to the wound. The approximate concentration of test compound was 0.6 mg/mL. The same amount of orange oil, or a water suspension of conidia, was tested as a control. In addition, unwashed wounded lemons were inoculated by conidia suspended in water. All treated fruits were incubated at 23 °C under 100% humidity. The number of infected wounds showing complete "green mold" symtoms was counted 4 days after inoculation.

Spectroscopy. NMR spectra were recorded on a JEOL JNM EX-270. Chemical shifts were expressed by δ units from TMS as internal standard. The UV spectrum was obtained from a Beckman DU 640 spectrophotometer. The mass spectrum was recorded with a Hitachi M-80A spectrometer. The melting point was measured with a Yanaco micro melting point apparatus, and the data were not corrected. The HPLC column employed was a Capcell-Pak C_{18} (10 × 250 mm, Shiseido, Tokyo). Optical rotation was determined on a Jasco DIP-370 in $CHCl_3$.

Prangolarin: mp 102–103 °C [lit. 104–105 °C (Ghoshal et al., 1963)], $[\alpha]^{23}_D$ +12.1° [*c* 0.5, $CHCl_3$, lit. +20.1° (Ghoshal et al., 1963)]; $[\lambda]^{EtOH}_{max}$ (nm) 304, 262 (sh), 247, 220; Rt 7.5 min (solvent, MeOH/water, 70:30); R_f 0.35 (TLC, ethyl acetate/acetone 95:5 v/v); LREIMS (rel int) 286 (80%, M+), 202 (100%); HREIMS, *m/z* 286.0845 (obsd), 286.0841 (calcd for $C_{16}H_{14}O_5$); ^{1}H NMR ($CDCl_3$) δ 8.13 (d, 1H, J = 9.5 Hz), 7.54 (d, 1H, J = 2.3 Hz), 7.12 (s, 1H), 6.88 (m, 1H), 6.24 (d, 1H, J = 9.9 Hz), 4.54 (dd, 1H, J = 10.9, 4.3 Hz), 4.36 (dd, 1H, J = 10.9, 6.6 Hz), 3.17 (dd, 1H, J = 6.6, 4.3 Hz), 2.11 (1H, s), 1.35 (s, 3H), 1.26 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 161.2 (s), 158.2 (s), 152.7 (s), 148.5 (s), 145.5 (d), 139.2 (d), 114.4 (s), 113.3 (d), 107.6 (s), 104.7 (d), 95.0 (d), 72.5 (t), 61.3 (d), 58.5 (s), 24.8 (q), 19.2 (q).

RESULTS AND DISCUSSION

Complete green mold symptoms formed on over 65% of the wounds in the epicarps of mature lemons after inoculation with a water suspension of *P. digitatum* conidia. The incidence was reduced to 2% if the lemon epicarp was washed with water immediately after wounding. Suspension of fungal conidia in commercially prepared orange oil (DoLce) induced less than 3% of green mold production on the wounded lemons. Since this result was nearly identical to that obtained with water, we used it as a solvent for compounds isolated from lemon epicarp oil. When the wounds were washed with water followed by application of the suspension of *P. digitatum* conidia in lemon epicarp oil, 92% of the wounds developed complete green mold symptoms (Table 1).

The residual epicarp oil, lemon oil with the limonene removed, promoted the production of complete green mold symptoms on 28% of the wounds. These indirect observations suggested that the limonene was a facilitator of green mold formation on wounded lemons (Arimoto, 1994). However, the presence of another facilitator was also suggested by these results.

The residual oil was separateed by preparative TLC into three fractions which were estimated by the biological assay. The orange oil solution containing the R_f 0.35 fraction promoted complete green mold development on 28% of epicarp wounds. This fraction was separated further into three fractions on a preparative TLC in chloroform and the fraction of R_f 0.32 promoted green mold formation. Fractionations and biological tests were repeated at least two times to yield an active fraction. The active fraction was further purified by HPLC, and a white crystalline compound was obtained. This compound promoted complete green mold formation on 26% of the wounds when applied at a concentration approximately that of its normal abundance in the epicarp.

The molecular formula was suggested as $C_{16}H_{14}O_5$ by HRMS. The Proton NMR spectrum showed the presence of two olefins at δ 8.13 and 6.24 with the coupling constant J = 9.9 Hz and at δ 7.54 and 6.88 with J = 2.3 Hz. An epoxidic proton at δ 3.17 coupled with a low field shifted methylene at δ 4.54 and 4.36 with the coupling constants J = 6.60 and 4.29 Hz associated with a geminal coupling of J = 10.9 Hz was also observed.

In the ^{13}C NMR spectrum, 16 carbon signals were observed. Eleven carbons were assigned for a furocoumarin ring at δ 161.2 (–OC=O, C-2), 113.2 (=CH, C-3), 139.2 (=CH, C-4), 107.6 (C, C-4a), 148.5 (C, C-5), 114.4 (C, C-6), 158.5 (C, C-7), 95.0 (=CH, C-8), 152.2 (C, C-8a), 145.5 (=CH, C-2′), and 104.7 (=CH, C-3′). The rest of the other carbon signals suggested the presence of an epoxidic prenyl group at δ 72.5 (OCH_2–, C-1‴), 61.3

(OCH−, C-2‴), 58.5 (OC−, C-3‴), 24.8 (CH_3, C-4‴), and 19.2 (CH_3, C-5‴). At this point, a computer search of carbon signals was performed with CSEARCH (Japan Bio-Rad Laboratories Ltd., Tokyo) on IRIS Indigo (Silicon Graphics Inc., CA). All carbon signals fit those of oxypeucedanin, which was isolated from *Ligusticum seguieri* (Lemmich et al., 1971), *Ammi majus* (Ivie, 1978), *Angelica officinals* (Harker et al., 1984), and lemon oil (Nagamura et al., 1985). Thus, the additional promoter, besides limonene, present in the epicarp oil of lemon was identified as oxypeucedanin. However, it is known that oxypeucedanin has an optical isomer called prangolarin (Ghoshal et al., 1963; Hata et al., 1981). In some cases, the name oxypeucedanin has been used for a racemic mixture showing a mp of 140−143 °C (Lemmich et al., 1971; Harkar et al., 1984). The melting point of (*S*)-(−)-oxypeucedanin was reported to be 103−104 °C ($[\alpha]_D$ −14.4°) (Lemmich et al., 1971), while that of the (*R*)-(+)-epimer was 104−105 °C ($[\alpha]_D$ +20.1°) (Ghoshal et al., 1963) or 103−104 °C ($[\alpha]_D$ +13.2°) (Hata et al., 1981); this epimer was called prangolarin.

The compound isolated from lemon epicarp oil in this experiment showed a positive optical rotation of $[\alpha]_D$ +12.1° ($CHCl_3$) and mp 102−103 °C, which strongly suggested its identity as prangolarin rather than oxypeucedanin. Nagamura et al. (1985) did not report the optical rotation of oxypeucedanin.

We have shown that both limonene and prangolarin promote and facilitate the development of complete green mold symptoms associated with conidial infection of wounded lemon fruit. Therefore, these compounds have a role in facilitating, by a still undetermined mode of action, the infection of *P. digitatum* of wounded lemons. The orange oil (DoLce) used as a solvent does not contain prangolarin but does contain as little as 2% of limonene as demonstrated by HPLC analyses. This result further supports that complete green mold symptoms are not facilitated by the orange oil (DoLce) but by lemon oil since the latter contains some limonen.

Because isomers of prangolarin exist, we are currently examing the absolute stereochemistry of the structure necessary for the bioactivity of this compound. More interesting, however, is the quest to understand how prangolarin can influence the development of a pathogenic fungus so late in the infection process.

ACKNOWLEDGMENT

We acknowledge Dr. J. Uzawa and Mrs. T. Chijimatsu for collecting NMR data. We thank Mr. Y. Esumi for measuring MS data. Special acknowledgment is given to Professor G. Strobel of Montana State University for his fruitful comments.

LITERATURE CITED

Arimoto, Y. Limonene, an infection promoter of *Penicillium digitatum* on lemon fruit. *J. Nat. Prod.* **1994**, submitted for publication.

Brown, G. E. Development of green mold in degreened oranges. *Phytopathology* **1973**, *63*, 1104−1107.

Eckert, J. W.; Ratnayake, M. Role of volatile compounds from wounded oranges in induction of germination of *Penicillium digitatum* conidia. *Phytopathology* **1994**, *84*, 743−750.

Ghoshal, C. R.; Sen, S.; Gupta, S. S.; Chatterjee, A. Prangolarin, the optical isomer of oxypeucedanin, isolated from the roots of *Prangos pabularia* Lindl. (Umbellifarae). *Chem. Ind.* **1963**, 1430−1431.

Harker, S.; Razdan, T. K.; Qaight, E. S. Steroids, chromone and coumarins from *Angelica officinalis*. *Phytochemistry* **1984**, *23*, 419−426.

Hata, H.; Nishino, T.; Hirai, Y.; Wada, Y.; Kozawa, M. On coumarins from the fruits of *Angelica pubescens* Maxim. *Yakugaku Zasshi* **1981**, *101*, 67−71.

Ivie, G. W. Linear furocoumarins (psoralens) from the seed of Texas Ammi majus L. (bishop's weed). *J. Agric. Food Chem.* **1978**, *26*, 1394−1403.

Kawai, M.; Homma, Y.; Arimoto, Y.; Misato, T. Studies on the infection mechanism of *Penicillium digitatum* on citrus fruits (1). *Sci. Papers Inst. Phys. Chem. Res.* **1984**, *78*, 19−25.

Lemmich, J.; Pedersen, P. A.; Nielsen, B. E. Coumarins and terpenoids of the fruit of *Ligusticum seguieri*. *Phytochemistry* **1971**, *10*, 3334−3335.

Nagamura, M.; Hirose, S.; Nakayama, Y.; Nakajima, K.; Someya, T. A study of the phototoxicity of lemon oil. *Arch. Dermatol. Res.* **1985**, *278*, 31−36.

Pelser, P. du T.; Eckert, W. Constituents of orange juice that stimulate the germination of conidia of *Penicillium digitatum*. *Phytopathology* **1977**, *67*, 747−754.

Smoot, J. J.; Houck, L. G.; Johnson, H. B. Merket diseases of citrus and other subtropical fruits. In *Agricultural Handbook 398*; USDA, ARS: Washington, DC, 1971.

Received for review December 29, 1994. Revised manuscript received May 26, 1995. Accepted June 7, 1995.[⊗]

JF940738Y

[⊗] Abstract published in *Advance ACS Abstracts,* July 15, 1995.