Iprodione Resistance of *Alternaria alternata* pv. *citri* from Minneola Tangelo in Israel and Florida

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ABSTRACT

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In an orchard of Minneola tangelo at Suffa, Israel, where iprodione had been used in the preceding 3 years, iprodione treatments in the current season failed to control Alternaria brown spot, caused by *Alternaria alternata* pv. *citri*. All 200 *A. a.* pv. *citri* isolates from this orchard were iprodione resistant, as evidenced by growth on potato-dextrose agar (PDA) amended with 25 mg of iprodione per liter. The average ED_{50} of 10 resistant isolates was 280 mg of iprodione per liter. In addition, in Florida and Israel, many isolates of *A. a.* pv. *citri* were recovered from brown spot lesions of Minneola tangelo in randomly selected orchards differing in iprodione history. The average ED_{50} of these iprodione-sensitive isolates ranged from 0.20 to 0.62 mg of iprodione per liter. All Florida isolates of *A. a.* pv. *citri* were iprodione sensitive, with ED_{50} values of 0.20 to 0.62 mg of iprodione per liter. When plates with mycelial plugs were incubated for 7 to 14 days on PDA amended with 6 to 200 mg/liter, most isolates from Florida and Israel developed resistant colonies as sectors. The subcultures from these sectors, designated as laboratory-selected iprodione-resistant isolates, grew well on PDA supplemented with 100 mg of iprodione per liter. Detached leaves of Minneola tangelo that had been sprayed with iprodione at 250 or 500 mg/liter were well-protected from infection by iprodione-sensitive isolates of *A. a.* pv. *citri*. However, when leaves were inoculated with an iprodione-resistant isolate from Suffa, disease was not controlled. The severity of infection of leaves inoculated with laboratory-selected resistant isolates from Florida or Israel was not affected by spraying iprodione at 250 mg/liter, but was reduced slightly at 500 mg/liter.

Additional keywords: citrus, dicarboximides, fungicide resistance

Alternaria brown spot, caused by *Alternaria alternata* (Fr.:Fr.) Keissl. pv. *citri* Solel (14), inflicts severe damage on Minneola tangelo (*Citrus reticulata* Blanco × *C. paradisi* Macfady), some other mandarins, and their hybrids, in Israel (14), the United States (Florida) (18), and other countries (4,5,12). Foliar lesions occur on young growth throughout the growing season, resulting in severe defoliation and blight of shoots. On fruit, small dark lesions develop, mainly in spring and early summer, and render the fruit unmarketable (14,18). Iprodione (Rovral, Rhone-Poulenc Ag. Co.) was found to be the most effective fungicide for control of this damaging disease (4,10,17). In Israel, some Minneola tangelo orchards have been treated with iprodione on a regular basis since 1991. The standard spray schedule was to apply fungicide at 2-week intervals, commencing soon after petal fall. Iprodione (500 mg a.i./liter) was used for the first, second, and fourth treatments, and copper hydroxide was used for the third and fifth sprays. The latter is less effective than iprodione but was alternated with it in hope of delaying the development of resistance of *A. a.* pv. *citri* to iprodione. In Florida, some orchards have been treated annually with iprodione up to three times early in the season, followed by copper fungicides later.

In summer 1994, a severe epidemic of Alternaria brown spot was observed in a Minneola orchard at Suffa (Northern Negev, Israel), where disease control was based on the standard spray schedule. Apparently, an iprodione-resistant population of *A. a.* pv. *citri* had developed and become dominant, thereby rendering iprodione ineffective. This study was undertaken to evaluate the iprodione sensitivity of the population in this orchard. In addition, field surveys were conducted in Israel and Florida in search of iprodione resistance among populations of *A. a.* pv. *citri* in citrus orchards.

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MATERIALS AND METHODS

Sampling and isolation of *A. a.* pv. *citri*. In Israel, samples of infected Minneola tangelo fruits and leaves were collected from an orchard at Suffa, where iprodione resistance was suspected, as well as from two adjacent Minneola orchards at Makha and Nir Yitzhak, within 10 km of the first. The orchard at Makha had been treated with iprodione for 4 years on a standard spray schedule, whereas the orchard at Nir Yitzhak had low disease incidence and had never been treated for control of Alternaria brown spot. Also, 20 samples of infected fruits and leaves were collected in four randomly selected Minneola orchards (Bet Cherut, Ramat Hakovesh, Givat Haim, and Mayan Zvi) in the center of the coastal plain, about 80 km north of Suffa, where iprodione has been applied in accordance with standard recommendations for the last 4 years.

In Florida, diseased fruits and leaves were collected from Minneola tangelo, as well as from Dancy tangerine (*C. reticulata*), Murcott tangor (*C. reticulata* × *C. sinensis* (L.) Osbeck), and Orlando tangelo in 10 orchards in Central Florida (from Polk City in the north to Arcadia in the south). Half of the orchards had histories of iprodione treatment in the last 2 years.

Isolation of *A. a.* pv. *citri* was made from segments of fruit or leaves with a single lesion. The tissue was surface-sterilized with sodium hypochlorite at 1.75%, washed once in sterile water, and placed on potato-dextrose agar (PDA, Difco). Subsequently, single-spore or hyphal-tip cultures were established, and their pathogenicity to Minneola tangelo was verified by inoculating detached young leaves (at 1/4 to 3/4 of full expansion) with a spore suspension. If cultures did not readily sporulate on PDA, spores were produced on calcium-carbonate agar (13). The suspension was applied to sterile 6-mm antibiotic assay disks, which were placed on the abaxial surface of detached young Minneola leaves in a humid chamber in the laboratory at 21 to 24°C in the dark. Disease symptoms indicating pathogenicity were monitored for up to 5 days. We examined 200 isolates from Suffa and 20 isolates from each of the six other orchards in Israel. In Florida, four to 20 isolates were obtained from each of 10 orchards.

Sensitivity of isolates of *A. a.* pv. *citri* to iprodione. Iprodione was added as an

aqueous suspension of Rovral 4SC to molten PDA just before pouring into 9-cm petri dishes. Resistance of each isolate of *A. a.* pv. *citri* to iprodione was evaluated by placing seven mycelial plugs (5 mm diameter), mycelial surface down, on PDA amended with 25 mg of iprodione per liter. After incubation for 4 days at 23°C, the extent of mycelial growth on the medium was monitored.

Iprodione-resistant isolates were placed on media with 0, 100, 200, and 400 mg of iprodione per liter; and sensitive ones were placed on media with 0, 0.31, 0.62, and 1.25 mg of iprodione per liter. Colony diameter was measured after incubation for 4 days at 23°C, and the radial growth was calculated. The EC_{50} values were calculated from linear regression equations of colony areas (11) using the above concentrations.

Laboratory-selected iprodione-resistant subcultures. Following the bioassay for iprodione resistance during the 4-day incubation of mycelial plugs on PDA amended with 25 mg of iprodione per liter, plates of sensitive isolates were incubated for an additional 3 to 10 days. If visible growth occurred, hyphal-tip subcultures were made and bioassayed for iprodione resistance. In addition, three mycelial plugs of 12 iprodione-sensitive isolates from Florida were placed on plates of a series of iprodione-amended PDA (6.25, 12.5, 25, 50, 100, and 200 mg/liter) and incubated for 10 days. If visible growth occurred, hyphal-tip subcultures were made and bioassayed for iprodione resistance.

Foliar protection assay. Two Minneola tangelo seedlings with three growing shoots were sprayed to runoff either with iprodione at 250 or 500 mg/liter, or with distilled water. After the spray on both sides of the leaves had dried, about 35 young leaves at 1/2 to 2/3 full expansion were detached, arranged abaxial side up in a moistened plastic box, and inoculated by fine droplets of a spore suspension (2 to 5 $\times 10^5$ conidia per ml) of the tested *A. a.* pv. *citri* isolate. After 4 days incubation at 24°C, disease severity was assessed as the percentage of area affected on each of the leaves. Relative infection severity was calculated as a percentage of the untreated control.

RESULTS

All 200 isolates of *A. a.* pv. *citri* from Suffa (Israel) grew well on PDA amended with 25 mg of iprodione per liter. The average ED_{50} of 10 arbitrarily selected isolates, assessed on a concentration series of 0 to 400 mg of iprodione per liter, was 280 mg/liter (range 264 to 292 mg/liter). All 10 isolates grew on PDA at a rate of about 4 mm/day, sporulated abundantly, and readily infected Minneola tangelo leaves. All the isolates from Makha orchard, in the vicinity of Suffa, were iprodione sensitive and failed to grow on PDA amended with 25 mg of iprodione per liter. Their average ED_{50} assessed on an iprodione concentration series of 0 to 1.25 mg/liter was 0.3 mg/liter (range 0.25 to 0.34 mg/liter). Of the 20 isolates from the nearby orchard of Nir Yitzhak, only one isolate of *A. a.* pv. *citri* grew on PDA amended with 25 mg of iprodione per liter.

None of the cultures of *A. a.* pv. *citri* from the four Minneola orchards in central Israel or from the 10 Florida orchards grew within 4 days on PDA containing 25 mg of iprodione per liter. The ED_{50} values of the Florida isolates, assessed on an iprodione concentration series of 0 to 1.25 mg/liter, ranged from 0.20 to 0.62 mg/liter.

When plates with mycelial plugs of iprodione-sensitive cultures were incubated for 3 to 10 days beyond the standard 4 days of the sensitivity test at 25 mg of iprodione per liter, most isolates developed colonies from at least one of the seven mycelial plugs. Usually these colonies developed as outgrowths from sectors of the original mycelial disk. When mycelial disks of 12 Florida iprodione-sensitive cultures of *A. a.* pv. *citri* were placed on plates amended with from 6.25 to 200 mg of iprodione per liter, resistant outgrowths became apparent after about 1 week. More outgrowths developed at the higher concentrations (50, 100, and 200 mg/liter) than at the 25 mg/liter or lower. Mycelial tips from sectors of both Israeli and Florida isolates were transferred to PDA, and the cultures were designated as laboratory-selected iprodione-resistant subcultures. All mycelial plugs of these cultures grew well on PDA amended with 100 mg of iprodione per liter. The morphology and the grayish green color of the colonies were similar to those of the wild-type colonies. The average growth rate of 12 Florida laboratory-selected iprodione-resistant isolates on PDA with 100 mg of iprodione per liter was 85% of that on nonamended medium.

The foliar protection assay revealed that iprodione spray treatment of leaves did not protect them from infection by iprodione-resistant *A. a.* pv. *citri* from Suffa, even at the higher rate (500 mg/liter) (Table 1). Laboratory-selected iprodione-resistant isolates, from either Israel or Florida, caused severe disease of iprodione-sprayed leaves (Table 1). While the lower rate of iprodione (250 mg/liter) did not affect disease severity compared to the control, the higher concentration (500 mg/liter) gave a slight reduction in disease severity (Table 1). Iprodione spray markedly reduced disease development by iprodione-sensitive isolates, especially at the field-recommended concentration of 500 mg/liter (Table 1).

DISCUSSION

Resistance of pathogens to fungicides has been well documented and reviewed (2,16). Field resistance of a pathogen to a fungicide occurs when resistant mutants, which maintain their fitness, become prevalent in the affected crop as a result of selection under repeated treatment with a particular fungicide. The severity of the problem increases with a progressive increase in the proportion of resistant isolates. Iprodione was effective in controlling various diseases caused by species of *Alternaria*, *Monilinia,* and *Botrytis* until development of resistance rendered it ineffective (3,6,7).

The occurrence of resistance of *A. alternata* to iprodione was reported in a pathotype causing postharvest mold of cherries (8). About one of 120,000 spores formed a colony that grew on PDA amended with 100 mg of iprodione per liter. Resistance of *A. a.* pv. *citri* to iprodione was observed in a Murcott orchard in Australia after eight applications of iprodione per season for 4 years (4). Therefore, occurrence of resistance of *A. a.* pv. *citri* to iprodione in Israel in 1994 was not completely unexpected, although the orchard at Suffa had not been treated with

Table 1. Severity of infection of leaves sprayed with two concentrations of iprodione and inoculated with different isolates of *Alternaria alternata* pv. *citri*

		Iprodione 250 mg/liter				Iprodione 500 mg/liter		
Type of isolate	Isolate no.	Treated[a]	Control	Relative infection[b]	Isolate no.	Treated	Control	Relative infection
Isolates from Suffa, Israel	S-17	100.0	90.0	111.1	S-17	83.5	74.6	111.9
Laboratory-selected iprodione resistant, Israel	RH-502	90.0	90.0	100.0	RH-502	65.2	76.5	85.2
Laboratory-selected iprodione resistant, Florida	10-D-R	90.0	90.0	100.0	10-H-R	36.0	45.0	80.0
Iprodione sensitive, Israel	BC-93	15.8	88.0	18.0	GC-7	8.0	75.5	10.6
Iprodione sensitive, Florida	Mixed	2.5	24.5	10.2	19-F	5.1	55.0	9.3

[a] Disease severity was assessed as percentage of affected leaf area. Each value is the mean of 10 to 35 replicates; SE ranged from 3.70 to 7.41.
[b] Relative infection expresses the severity of infection of the treated leaves as a percentage of the untreated control.

iprodione as intensively as the one in Australia. Cultures of *A. a.* pv. *citri* from the Minneola orchard in Suffa tolerated 1,000 times more iprodione than sensitive isolates. Laboratory inoculations of leaves did not indicate any loss of fitness. The mycelial growth of 12 Australian iprodione-resistant isolates was inhibited by 20% on media amended with 10 mg of iprodione per liter (4). This slight growth inhibition is similar to that observed with the Florida laboratory-selected iprodione-resistant isolates when grown on 100 mg of iprodione per liter. Growth of isolates from Suffa was not inhibited at 100 mg/liter (results not shown).

Whenever cultures of *A. a.* pv. *citri* were exposed to a substantial selection pressure of iprodione, resistant sectors developed rapidly. The selection was enhanced by increasing the concentration of iprodione in the medium. The magnitude of iprodione resistance of these laboratory-selected subcultures was similar to that of the naturally occurring iprodione-resistant mutant in Suffa grove. A similar situation has been described by Biggs (1) for *A. alternata*, cause of storage rot of apples, where iprodione-resistant sectors developed within 12 days when mycelial plugs were placed on PDA plates amended with 25 or 250 mg of iprodione per liter. The EC_{50} values for the laboratory-selected resistant isolates of apple were about 80 mg of iprodione per liter, compared to about 3 mg/liter for the original iprodione-sensitive ones. The selected isolates remained pathogenic and caused lesions on apples treated with iprodione.

There is no evidence that iprodione is a mutagen. Therefore, we assume that the genome of the multinucleate and apparently heterokaryotic mycelium of *A. a.* pv. *citri* has the genetic tendency for iprodione resistance when placed under selection pressure. A heterokaryotic nature was assumed for several *Alternaria* species (9,15). Similarly, multinucleation and heterokaryosis accounted for the genetic variability of *Botrytis*, for which numerous observations of resistance to dicarboximide fungicides have been reported (7).

Iprodione resistance was identified as a problem in only one orchard in Israel, where all 200 isolates of *A. a.* pv. *citri* proved to be resistant. Only one iprodione-resistant culture was isolated from a nearby orchard. However, the iprodione-resistant isolate potentially could disperse rapidly to Minneola orchards throughout the region. The potential of rapid dispersal of *A. a.* pv. *citri* has been observed in Florida (18) and Israel (Z. Solel, *unpublished*). The ease with which iprodione-resistant mutants have been developed under laboratory conditions suggests that occurrence of resistance could be a threat to the citrus growers. Alternation of iprodione treatments with fungicides having a different mode of action, such as copper, may reduce the risk of resistance developing to some extent; however, this strategy may result in an unacceptable level of control. Mixing iprodione with a different class of fungicide, even with one that is not as effective, might be a safer strategy, but it will increase significantly the cost of control treatment, since each fungicide might need to be used at or near the full recommended rate.

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