Plant Pathol. J. 16(4) : 222-226 (2000)

The Plant Pathology Journal

In Vitro Sensitivity to Metalaxyl of *Phytophthora parasitica* var. *nicotianae* Isolates from Burley Tobacco in Korea

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(Received on June 15, 2000)

In disease surveys from 1986 to 1998, disease incidence of tobacco black shank was gradually increased in burley tobacco from 1996. To study the causes of the disease occurrence, one hundred and fourteen isolates of *Phytophthora parasitica* var. *nicotianae* (Ppn) were collected from burley tobacco-growing areas in the southern part of Korea during 1996-1997, and tested *in vitro* for metalaxyl sensitivity which was determined by measuring the mycelial growth on corn meal agar (CMA) amended with metalaxyl. Of the tested isolates, 78.1% showed sensitive to metalaxyl, having ED_{50} values less than 1.0 μg/ml, while 1.7% was resistant with ED_{50} greater than 100 μg/ml. Ppn isolates from three provinces, Chungnam, Chonbuk and Chonnam showed similar distributions of metalaxyl sensitivity. Metalaxyl-resistant isolates were not significantly different from metalaxyl-sensitive ones in mycelial growth rate, chlamydospore formation capacity and size of the spore, and pathogenicity on tobacco plant (cv. Burley 21). These results suggest that the metalaxyl-resistant Ppn in burley tobacco may be one of the major factors to cause the higher occurrence of the tobacco black shank in the burley tobacco-growing area.

Keywords : fungicide resistance, metalaxyl, *Phytophthora parasitica* var. *nicotianae*, tobacco black shank.

Tobacco black shank caused by *Phytophthora parasitica* var. *nicotianae* (Breda de Hann) Tucker, occurs worldwidely and causes a destructive root and stem rot disease in all types of cultivated tobacco (Lucas, 1975; Shew and Lucas, 1991). In Korea, tobacco black shank, one of the soil-borne diseases, was reported first in 1918 (Nakata and Takimoto, 1928). The occurrence of the disease surveyed was 0.8% in flue-cured tobacco growing area and 1.2% in burley tobacco growing areas, respectively (Ro et al., 1971; Heu, et al., 1975). After polyethylene film-mulching system was introduced in tobacco cultivation in the early 1970s, black shank had drastically increased in all over the tobacco-growing areas. Soil drenching of metalaxyl solution (125 μg a.i./ml) which was recommended to control black shank in 1981 provided a satisfactory control at most field conditions during first five years. However, black shank is still one of the most potentially devastating soil-borne diseases in burley tobacco growing areas (Kim et al., 1995). Furthermore, failure of the disease control occasionally increased the disease incidences in burley tobacco fields, and in severe cases resulted in almost total destruction of tobacco plantation. The failure of the chemical control could be derived from the development of chemical-resistant strains, which is now a serious problem for the control of plant diseases in many crops (Ferrin and Kabashima, 1991; Staub, 1991).

Therefore, in this study, the occurrence of metalaxyl-resistant isolates of *P. parasitica* var. *nicotianae* and distribution of the populations in burley tobacco-growing areas were investigated to understand the cause of increased disease occurrence and to obtain more information for the development of effective control measures. The differences in several fitness components between the metalaxyl-sensitive and -resistant isolates were measured.

Materials and Methods

Disease survey. Seventy to ninety burley tobacco fields in the southwestern parts of Korea were surveyed, and disease incidences of black shank were examined yearly (late July, 1986-1998) with 200 plants at each field. Disease incidence (DI) was calculated by following formula:

$$DI = \Sigma A_i D_i / \Sigma A_i$$

Where, DI = Disease incidence (%)
A = Total areas of surveyed field (are)
D = Percent of diseased plants in a sampling field.

Isolation of *Phytophthora parasitica* var. *nicotianae* isolates. Two or three tobacco samples exhibiting symptoms of black shank in the burley tobacco fields were collected in late July from 1996 to 1997 at Chungnam, Chonbuk, and Chonnam provinces, Korea. *P. parasitica* var. *nicotianae* was isolated from infected tissue by placing infected tobacco pith tissue on corn meal agar (CMA), which was amended with 400 mg of pimaricin (2.5% a.i.,

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Sigma), 200 mg of vancomycin hydrochloride (Sigma), 100 mg of pentachloronitrobenzene (PCNB 70% a.i., Sigma), and 50 μg of hymexazol (99.4% a.i., Sungbo Chemicals Co, Ltd.) per liter (Mitchell et al., 1986). One hundred and fourteen isolates were collected from tobacco fields; 32 from 10 localities in Chungnam, 42 from 10 localities in Chonbuk, and 40 from 9 localities in Chonnam. The isolates were maintained on CMA for the test at room temperature.

Assessment of metalaxyl sensitivity. Mycelial growth of isolates was measured on CMA amended with different concentrations of metalaxyl previously dissolved in methanol. Final concentration of methanol did not exceed 0.5%. Mycelial plugs (7 mm diameter) were cut from the margins of 7-day-old cultures of the isolates and transferred to CMA plates containing metalaxyl at concentration of 0, 0.1, 1, 10, and 100 μg/ml, respectively. After the plates were incubated for 7 days at 28°C, colony diameters were measured. Metalaxyl sensitivity was determined by comparing colony diameter of metalaxyl-amended cultures with controls and expressed in effective dose for 50% growth inhibition (ED_{50}) for each isolate. ED_{50} was calculated by plotting growth inhibition rates (%) vs. metalaxyl concentrations and applied by a linear regression to produce respective values of 50% inhibition, which was estimated on the basis of regression equation using logarithmic values of metalaxyl concentrations (Finney, 1971). For the isolates which grew well on the plates with 100 μg/ml metalaxyl like on the control plates, ED_{50} values were designated over 100 μg/ml. For the isolates which did not grow on the plates with 0.1 μg/ml metalaxyl, ED_{50} values were designated below 0.1 μg/ml.

Comparison of fitness-related characteristics between metalaxyl-sensitive and resistant isolates. Through *in vitro* experiment, two strains of metalaxyl-sensitive (<0.1 μg/ml of ED_{50}: P96-2 and P97-1) and metalaxyl-resistant (>100 μg/ml of ED_{50}: P97-22 and P97-61) in *P. parasitica* var. *nicotianae* were used to compare the fitness-related characteristics such as mycelial growth on the dry weight basis, chlamydospore production, and virulence. Three of 7 mm plugs in diameter were inoculated in the Erlenmeyer flasks (250 ml) which contained 100 ml of V-8 juice broth (20% Campbell's V-8 juice plus 4.5 g/ml $CaCO_3$ and cleared by centrifuge for 20 min at 4000 rpm), adjusted with different concentrations (0, 0.01, 0.1, 1, 10, 100, and 1000 μg/ml) of metalaxyl. After 9-day incubation at 28°C, grown mycelia were filtered with a coarse porosity filter paper (Toyo No. 2) and dried in an oven dryer for 24 hrs at 80°C before weigh. All measurements were made on each of five replicate flasks per isolate per concentration. A linear growth rate was determined on CMA. The center of each plate was inoculated with an inoculum plug as described above. Plates were incubated in a dark room at 28°C for 3, 6, 9, and 12 days, and the diameters of colonies were measured. Chlamydospore production was followed by Tsao (1971). Inoculum plugs of 7 mm in diameter were taken from the edge of 7-day-old culture grown on the CMA and placed in the Erlenmeyer flasks (250 ml) which contained 25 ml of V-8 juice broth. After incubation in a dark room for 24 hrs at 25°C, each flask was shaken vigorously by hand to obtain the fragments of hyphae which were grown from the original plugs. The flasks were then horizontally incubated at 25°C for 7 days as stationary cultures. The sterile deionized water (100 ml) was added to each flask and the flasks were re-incubated at 18°C for 3 weeks. Occasionally, gentle shaking of the flasks for several times was needed to facilitate sinking a mycelial mat. After 4 weeks of incubation, the cultures were filtered and washed on a Buchner funnel with sterile distilled water and comminuted twice for 30 seconds in a microblender (Sorvall Omni-Mixer) with sterile distilled deionized water (25 ml) per culture. The resulting suspension was subjected to 60% maximum sonication with an ultrasonic homogenizer (Cole-Parmer Instrument Co.) for 60 seconds to disrupt mycelial fragments. After sonication, the number of chlamydospores in the suspension was immediately counted at six fields (10 μl) for each of 4 samples using a standard haemacytometer. The sizes of ten chlamydospores per each sample were measured four times by a micrometer.

Pathogenicity test on tobacco plants. The four isolates were used in mycelial growth and chlamydospore production tests were selected and tested for their pathogenicity to tobacco cv. Burley 21 using a method of Csinos and Bertrand (1994). Briefly, wood-tooth-picks cut by half were sterilized twice in an autoclave at 121°C. They were placed on petri plates filled with V-8 juice agar, and the center of each plate was inoculated with 6-mm cork borer plugs of mycelium from the growing edge of the tested isolates and incubated for 3 weeks at 28°C in a dark to allow the fungus to infest the toothpicks. Tobacco seedlings (Burley 21), which were to 8-9 leaf stages on the plastic pots (17 × 20 cm) for 5 weeks after transplanting in a greenhouse, were inoculated with the fungus by pricking the infested toothpicks into the stems. Then, 50 ml of metalaxyl solution (100 μg/ml) or water (as control) was poured into a rhizosphere of each plant, and eighteen plants were used for each treatment. Diseased plants were counted at two weeks after inoculation.

Results

Survey of tobacco black shank occurrence in burley tobacco growing areas. Occurrence of black shank on burley tobacco was surveyed at the entire burley tobacco-growing areas in the southwestern parts of Korea, including Chungnam, Chonbuk, and Chonnam provinces, from 1986 to 1998. The disease incidence was investigated from mid to late July every year in 70 to 90 tobacco fields selected randomly from 9 to 10 localities in each province. The incidence of diseased plants was ranged from 1 to 5% during the years and fluctuated year to year, but gradual increase was observed from 1986 (Fig. 1).

Sensitivity of *P. parasitica* var. *nicotianae* isolates to metalaxyl. One hundred and fourteen isolates were collected from diseased burley tobacco plants in three provinces, Chungnam, Chonbuk, and Chonnam, and tested for *in vitro* sensitivity to metalaxyl. Most of tested isolates (78.1%) had ED_{50} values of less than 1.0 μg/ml and twenty-two isolates (19.3%) had ED_{50} values ranging from 1.1 to 10.0 μg/ml. Two isolates (1.7%) were the most resistant to metalaxyl

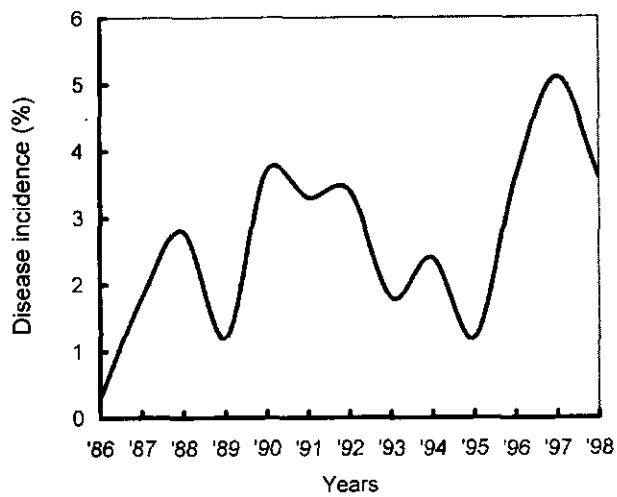

Fig. 1. The occurrence of tobacco black shank in burley tobacco growing areas during 1986-1998.

Table 1. Frequency distribution of *in vitro* sensitivity to metalaxyl of *Phytophthora parasitica* var. *nicotianae* isolates from burley tobacco

Province collected	No. of isolates tested	% isolates with ED_{50} (μg/ml)[a] of				
		<0.1	0.1-1.0	1.1-10.0	10.1-100	100<
Chungnam	32	3.1	80.3	12.5	3.1	0
Chonbuk	42	7.1	76.2	14.3	0	2.4
Chonnam	40	15.0	60.0	22.5	0	2.5
Total	114	8.8	69.3	19.3	0.9	1.7

[a]Determined by measuring radial growth on corn meal agar (CMA) amended with metalaxyl.

with ED_{50} values more than 100 μg/ml, which could grow readily on the medium with metalaxyl up to 10.0 μg/ml compared to the control medium (Table 1). The isolates from the three provinces showed similar patterns and distributions in the sensitivity to metalaxyl (Table 1).

Comparison of fitness-related characteristics between metalaxyl-sensitive and resistant isolates. The mycelial growth of metalaxyl-sensitive and -resistant isolates of *P. parasitica* var. *nicotianae* grown in V-8 juice broth for 9 days at 28°C was measured on the dry weight basis. Metalaxyl sensitive isolates were only grown at the concentration of below 0.1 μg/ml, but the growth of metalaxyl-resistant one decreased as the metalaxyl concentration increased from 0.1 to 100 μg/ml (Fig. 2). All of the tested isolates were similar in growth pattern and not significantly different in mycelial growth rate (Fig. 3). The mean numbers and sizes of chlamydospores produced in submerged culture method were examined under a microscope with four isolates. All isolates were not significantly different in numbers and sizes of chlamydospores (Table 2).

Disease development by metalaxyl-sensitive and -resistant strains in plants treated with metalaxyl. Metalaxyl treatment greatly reduced the development of black shank in tobacco plants inoculated with metalaxyl-sensitive strains (Table 3). However, in the plants inoculated with metalaxyl-resistant strains, 88.8% and 83.3% of the tested plants were diseased, respectively. However, there was no difference in virulence between the metalaxyl-sensitive and -resistant strains.

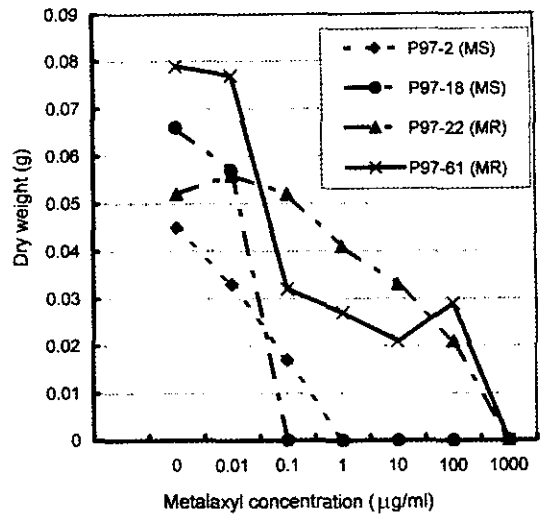

Fig. 2. The mass of mycelial growth of metalaxyl-sensitive (MS) and -resistant (MR) strains of *Phytophthora parasitica* var. *nicotianae* grown in metalaxyl contained V-8 juice broth at 28°C for 9 days.

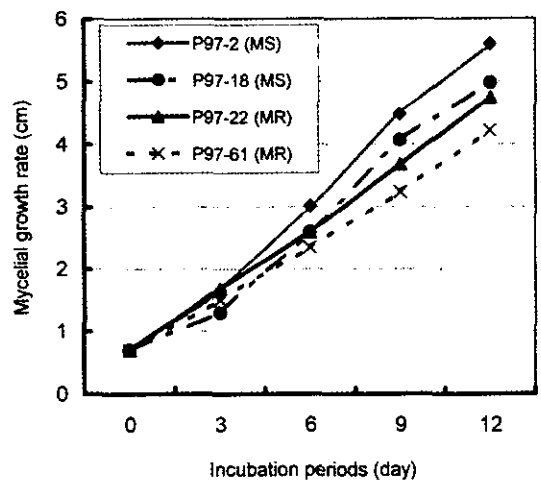

Fig. 3. Mycelial growth rate of metalaxyl-sensitive (MS) and -resistant (MR) strains of *Phytophthora parasitica* var. *nicotianae* on corn meal agar at 28°C.

Discussion

Metalaxyl has been used for the control of tobacco black shank nation-wide from 1982 in Korea. Probably because

Table 2. Formation and size of chlamydospores in metalaxyl-sensitive and -resistant strains of *Phytophthora parasitica* var. *nicotianae*[a]

Strains	Metalaxyl sensitivity (ED_{50} μg/ml)	Number of chlamydospore (spore/10 μl)[b]	Size of chlamydospores (μm)[c]	
			Range	Average
P97-2	0.40	57.6 ± 8.3	20-38	28.5 ± 6.9
P97-18	0.09	60.2 ± 11.2	20-46	31.3 ± 9.4
P97-22	213.5	55.2 ± 11.0	18-34	28.6 ± 4.8
P97-61	156.7	61.8 ± 11.5	18-44	28.7 ± 7.1

[a] Chlamydospore production was followed by Tsao (1971).
[b] The number of chlamydospores was counted six fields for each of four samples in a standard haemacytometer.
[c] Average of 40 chlamydosphores per each sample.

Table 3. Pathogenicity of metalaxyl-sensitive and -resistant *Phytophthora parasitica* var. *nicotianae*[a]

	% diseased plants[b]			
	Metalaxyl-sensitive		Metalaxyl-resistant	
	P97-2	P97-18	P97-22	P97-61
Metalaxyl treatment[c]	16.6	11.1	88.8	83.3
Control	88.8	83.3	88.8	94.4

[a] Eighteen burley tobacco (Burley 21) plants with 8-9 leaves per isolate were inoculated by pushing test fungus infested toothpicks into stem.
[b] Counted two weeks after inoculation.
[c] Soil drenched 50 metalaxyl solution (100 μg/ml) into a plastic pot (17 cm × 20 cm).

of using the fungicide, the black shank disease had rarely occurred in the fungicide-treated tobacco fields until 1985. However, as indicated in our study, the disease was frequently found in burley tobacco fields from 1986 to 1995 (1-3% of disease incidence in average) and moreover 4-5% in recent three years. But low incidences of tobacco black shank were recorded in 1989 and 1995 when it was wholly or partially drought in the surveyed areas during the May of those years (rainfall was a half of an average year). The black shank became again one of the most destructive diseases in burley tobacco-growing areas even though metalaxyl was widely used. In our study, the ratio of totally sensitive isolates was only 8.8%, while other isolates were more or less resistant to metalaxyl. A few isolates of *P. parasitica* var. *nicotianae* were very resistant to the fungicide that they could grow on CMA included with over 100 μg/ml of metalaxyl. In some cases, they could grow on the plates with lower concentration (10 μg/ml) than they did without metalaxyl. In contrast, sensitive isolates did not grow on the plates with 1.0 μg/ml of metalaxyl. Although there were no significant differences in the morphology of chlamydosphores and sporangiospores between resistant and sensitive isolates. The resistant isolates readily caused the black shank in the chemical treatment. These results suggest that recent increases of the diseases could be due to the appearance of metalaxyl-resistant isolates in tobacco fields. However, more studies on the disease epidemics are needed to obtain a confirmative relationship between the chemical resistance and disease development in natural conditions.

Metalaxyl is a phenylamide fungicide with a site-specific mode of action (inhibition of RNA synthesis). In general, fungicide with this mode of action increases the risk of buildup of pathogen populations that are fungicidal resistance. A metalaxyl is effective to reduce the damage by black shank, especially when used in combination with other management strategies such as use of resistant cultivars, crop rotation, nematode control in some fields, and use of soil fumigants (Melton, 1997; Schemaker et al., 1996). For many years, a burley 21, susceptible variety to tobacco black shank, was only grown in Korea. And according to our survey, most tobacco growers were greatly relied on the use of metalaxyl for the control of black shank. It may cause the build-up of resistance to the fungicide more rapidly in Korea than in the USA in which metalaxyl-resistant isolates of *P. parasitica* var. *nicotianae* are relatively less frequent even though it has been used longer (Shew, 1984; Csino, 1994).

Distribution of metalaxyl-sensitive and -resistant isolates was similar among the three provinces regardless of geographical and environmental differences. However, the facts that the metalaxyl-resistant isolates grew well *in vitro* and they were mostly similar in morphology and readily induced black shank disease indicated that metalaxyl-resistant isolates have a good fitness potential almost equal to metalaxyl-sensitive isolates in fields. Metalaxyl appears to be effective for the black shank control as long as the proportion of resistant isolates is low, but its use should be discontinued in the fields if the chemical-resistant strains are established. Many workers suggested that the use of fungicides in alternation or in mixture could delay the development of chemical resistance (Dekker, 1976; Delp, 1980; Edgington et al., 1980; Samoucha and Gisi, 1987; Skylakakis, 1983; Staub, 1991). Therefore, the development of alternation and mixture with other chemicals which have different modes of action from metalaxyl in chemical control system should be considered as an efficient control method.

Acknowledgement

This work was supported by the tobacco research fund of Korea Tobacco & Ginseng Corporation from 1996 to 1998.

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