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A rapid method for *in vitro* evaluation of systemic fungicides against Phytophthora crown rot of fruit trees

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Abstract. A new method was developed to evaluate *in vitro* the systemic ability of fungicides against crown rot diseases caused by *Phytophthora* spp. Excised shoots of peach were inoculated with *P. cactorum* or *P. citrophthora* and inserted vertically into vermiculite, 2 cm deep, and flooded with test fungicide, in a storage jar. After incubation, shoots were removed from the vermiculite and stripped of their periderms and the length of necrosis was measured. The systemic fungicides metalaxyl and fosetyl-Al inhibited necrosis whereas copper hydroxide, captan and dimethomorph, which gave protection in bark strip and trunk inoculation assays had no effect. This confirmed that the new assay discriminated between systemic and non-systemic effects. The new method is not laborious, allows ample replications and is inexpensive.

Introduction

Phytophthora root and crown rot is a serious and widespread disease problem in fruit orchards in Greece (Elena and Tsipouridis 2000) and elsewhere. It is especially prevalent in orchards subjected to flood-irrigation as well as in plantings on susceptible rootstocks (Wilcox 1993).

Control of *Phytophthora* diseases in orchards is based mainly on the use of chemicals. Protectant fungicides kill germinating spores but have no effect on mycelium after it has penetrated the plant. The introduction of systemic fungicides that are taken up and translocated within the plant has facilitated control of *Phytophthora*.

A variety of techniques has been developed to evaluate the effectiveness of fungicides against *Phytophthora* spp. The use of poisoned agar is most common (Thomidis and Michailides 2002). Timmer (1977) used bark disks from the trunk to evaluate the fungicide activity on treated trees. Matheron and Matejka (1988) reported that the bark strip assay is a reliable and rapid technique for testing efficacy of fungicides for control of Phytophthora gummosis of citrus. Most of these laboratory-based methods evaluate only the protective functions of fungicides, but not systemic activity. Evaluating systemic activity in the field is time-consuming and expensive, especially for orchard crops.

The purpose of this study was to develop a quick and reliable laboratory method to determine the systemic activity of fungicides against diseases of fruit trees caused by *Phytophthora* spp. The results of the new assay on six fungicides were compared with those of two widely used assays that do not differentiate between protectant and systemic activity.

Materials and methods

An isolate of *P. cactorum* from almond and an isolate of *P. citrophthora* from citrus were used in this study. Both had been maintained on cornmeal agar at 22°C. Their pathogenicity to peach trees had been confirmed in previous studies (Thomidis 2001). For preparing fresh cultures, agar plugs with mycelium were transferred to 9-cm-diameter plates containing cornmeal agar and incubated at 22°C until mycelium growth covered the agar surface.

The following fungicides were used in this study: metalaxyl (Ridomil 2E; Ciba Geigy), fosetyl-Al (Aliette WP; Rhone Poulec Hellas), captan (Captan 83WP; Euthimiadis ABEE), dimethomorph (Acrobat WP, BASF), cymoxanil (Diametan WP, Bayer Hellas ABEE) and copper hydroxide (Kocide 101 WP; Euthimiadis ABEE) at the rates given in Table 1.

Excised shoot assay

Fungicide solutions were prepared by mixing appropriate concentrations of product with tap water (Table 1). The rates were similar to those used by Greek growers. Coarse-textured vermiculite (100 mL) was dispensed into jars to give a depth of 1.5 to 2 cm. The vermiculite was then flooded with 100 mL of the tested fungicide solutions.

Woody shoots without leaves were collected from GF677 and KID I peach rootstocks (Stylianides *et al.* 1988) growing in an experimental field at the Pomology Institute of Naoussa in spring 2000. Segments 15 cm long and about 2 cm in diameter were cut from the central part of shoots. They were inoculated about 10 cm above the basal end. Using a flamed sharp knife, 6 mm of bark periderm was removed to expose the cambium and agar plugs, about 4-mm-diameter, with mycelium of *P. cactorum* or *P. citrophthora* were placed directly onto the cambium. Wounds were then wrapped with adhesive tape to avoid desiccation. Ten of these shoots were inserted vertically, distal end up, into flooded vermiculite in each jar. The jars were placed in incubators at 22°C for 4 days, after which shoots were removed from the jars and stripped of their periderms with a sharp scalpel to expose any necrosis. The length of necrosis was recorded.

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Table 1. Effect of six fungicides on the length of necrosis on excised shoots of two peach rootstocks placed in vermiculite soaked in fungicide and inoculated with *Phytophthora cactorum* or *P. citrophthora*

Fungicide (active ingredient)	Rate (g/L)	Vertical distance of necrosis[A] (cm) *P. cactorum*	*P. citrophthora*	Rate (g/L)	Vertical distance of necrosis (cm) *P. cactorum*	*P. citrophthora*
		Rootstock GF677				
Nil	—	3.82 a[B]	2.95 a	—	3.20 a	2.98 a
Captan[C]	5	3.78 a	2.90 a	10	2.88 a	3.16 a
Copper hydroxide	5	3.72 a	3.07 a	10	3.05 a	3.09 a
Dimethomorph	2.5	3.65 a	2.88 a	10	2.95 a	2.92 a
Cymoxanyl	2.5	3.63 a	3.00 a	10	3.10 a	3.13 a
Fosetyl-Al	2.5	0.28 b	0.25 b	5	0 b	0 b
Metalaxyl	2.5	0.24 b	0.20 b	5	0 b	0 b
		Rootstock KID I				
Nil	—	4.58 a[B]	3.86 a	—	4.25 a	3.55 a
Captan	5	4.52 a	3.59 a	10	4.32 a	3.68 a
Copper hydroxide	5	4.44 a	3.62 a	10	4.05 a	3.59 a
Cymoxanil	2.5	4.38 a	3.72 a	10	3.99 a	3.60 a
Dimethomorph	2.5	4.35 a	3.78 a	10	4.11 a	3.67 a
Fosetyl-Al	2.5	0 b	0.20 b	5	0 b	0 b
Metalaxyl	2.5	0 b	0 b	5	0 b	0 b

[A]Each value is the mean of three experiments each with 20 replicates for each fungicide.
[B]Values in the same column followed by different letters are significantly different ($P = 0.05$) according to Duncan's multiple range test.
[C]Fungicide solutions were prepared by mixing appropriate concentration of fungicide with tap water.

Ten shoots were placed in each jar, and two jars of each rootstock were used for each *Phytophthora* species at each concentration of fungicide. Fungicide-free jars contained vermiculite flooded with tap water. This experiment was conducted three times.

Sections from the margin of lesions were placed in 0.5% sodium hypochlorite for 1–3 min and washed three times with sterile distilled water. Tissue sections were blotted with sterile paper towel and placed on P_5ARP medium (Jeffers and Martin 1986). Petri dishes were then sealed with parafilm and incubated at 23 and 25°C for recovery of *P. cactorum* and *P. citrophthora*, respectively.

Bark strip assay

These experiments were conducted to confirm the protectant activity of fungicides against *P. cactorum* and *P. citrophthora.* Vertical bark strips, about 10 cm in length and 1.5–2 cm wide, were collected from the trunks of 3-year-old GF677 and KID I peach rootstocks in spring 2000. These bark strips were soaked in each of the fungicide solutions (Table 2) and dried on sterile paper towel. Strips were inoculated by placing a 6-mm-diameter agar plug with mycelium of the pathogen in the centre of the inside surface. The inoculation point was wrapped with adhesive tape to avoid desiccation. Inoculated strips were incubated for 4 days at 22°C in a moist chamber after which the lengths of lesions were recorded.

Twenty bark strips of each rootstock were inoculated with each pathogen at each fungicide concentration. Controls consisted of 40 non-treated bark strips for each peach rootstock (20 bark strips for each *Phytophthora* species). This experiment was conducted three times. Pathogens were isolated as described above.

Trunk inoculation assay

This experiment was conducted in the experimental field of the Pomology Institute, Naoussa, in May and again in September 2000. GF677 and KID I peach rootstocks were bought from a commercial tissue culture station and planted in a high-density plantation (1 × 1 m).

Two-year-old plants were wounded, about 10 cm above the soil surface, by removing a 6 mm portion of bark periderm to expose the cambium and painted with one of the tested fungicide solutions (Table 3). Inoculations were then performed by placing an agar plug with mycelium of *P. cactorum* or *P. citrophthora* directly onto the cambium. Wet cotton wool was placed on the wound which was then wrapped with adhesive tape to avoid desiccation. Fifteen days later, the periderm was scraped and the length of necrosis was recorded.

Ten plants of each rootstock were inoculated with each pathogen at each concentration of fungicide. Ten untreated plants of each rootstock were inoculated with each pathogen as controls. This experiment was conducted twice. Both pathogens were recovered as in experiment 1.

Statistical analysis

The experiments were established using a randomised design. Data were analysed by one-way analyses of variance and treatment means were separated by Duncan's Multiple Range Test ($P = 0.05$). Results of repeated experiments were similar according to Bartlett's test of homogeneity of variance, so data were combined.

Results

Excised shoot assay

For both *Phytophthora* species, the length of necrosis differed according to the fungicide used. Necrosis extended both upward and downward from the point of inoculation.

Non-treated shoots and shoots treated with captan, cymoxanil, dimethomorph or copper hydroxide developed extended necrosis. Necrotic areas were observed externally and shoots showed the symptom of gummosis. After removing bark, lesions showed typical orange-brown coloration with distinct borders. No significant difference in length of necrosis was observed between captan, copper

Table 2. Effect of six fungicides on the length of necrosis on bark strips of two peach rootstocks inoculated with *Phytophthora cactorum* and *P. citrophthora*

Fungicide (active ingredient)	Rate (g/L)	Vertical distance of necrosis[A] (cm) *P. cactorum*	*P. citrophthora*	Rate (g/L)	Vertical distance of necrosis (cm) *P. cactorum*	*P. citrophthora*
			Rootstock GF 677			
Nil	—	3.50 a[B]	3.15 a	—	3.54 a	2.98 a
Captan[C]	5	3.45 a	2.95 a	10	0 b	0 b
Copper hydroxide	5	3.38 a	2.99 a	10	0 b	0 b
Cymoxanil	2.5	3.36 a	3.20 a	10	3.41 a	3.17 a
Dimethomorph	2.5	0 b	0 b	2.5	0 b	0 b
Fosetyl-Al	2.5	0 b	0 b	2.5	0 b	0 b
Metalaxyl	2.5	0 b	0 b	2.5	0 b	0 b
			Rootstock KID I			
Nil	—	4.53 a[B]	4.25 a	—	3.95 a	3.72 a
Captan	5	4.50 a	4.35 a	10	0 b	0 b
Copper hydroxide	5	4.42 a	4.41 a	10	0 b	0 b
Cymoxanil	2.5	4.29 a	4.21 a	10	4.22 a	3.60 a
Dimethomorph	2.5	0 b	0 b	2.5	0 b	0 b
Fosetyl-Al	2.5	0 b	0 b	2.5	0 b	0 b
Metalaxyl	2.5	0 b	0 b	2.5	0 b	0 b

[A]Each value is the mean of three experiments each with 20 replicates for each fungicide.
[B]Values in the same column followed by different letters are significantly different ($P = 0.05$) according to Duncan's multiple range test.
[C]Fungicide solutions were prepared by mixing appropriate concentration of fungicide with tap water.

hydroxide, cymoxanil, dimethomorph and non-treated shoots.

Shoots treated with metalaxyl or fosetyl-Al showed little or no necrosis. There was no significant difference in length of necrosis between metalaxyl- and fosetyl-Al-treated shoots (Table 1). Both *P. cactorum* and *P. citrophthora* were recovered from at least one shoot treated with each of copper hydroxide, captan, cymoxanil, dimethomorph or from a non-treated shoot.

Bark strip assay

Metalaxyl, dimethomorph and fosetyl-Al applied at rates of 2.5 g/L inhibited the growth of both *P. cactorum* and *P. citrophthora* on GF677 and KID I bark strips (Table 2). The development of *P. cactorum* and *P. citrophthora* was inhibited on bark strips of both peach rootstocks treated with captan or copper hydroxide at 10 g/L. These fungicides did not affect the growth of either *P. cactorum* or *P. citrophthora* at 5 g/L (Table 2). Cymoxanil was ineffective even at rates of 10 g/L. Both *P. cactorum* and *P. citrophthora* were recovered from at least one shoot treated with cymoxanil and from a non-treated shoot.

Trunk inoculation assay

In this experiment, metalaxyl, dimethomorph and fosetyl-Al inhibited the growth of *P. cactorum* and *P. citrophthora* on GF677 and KID I plants at 10 g/L (Table 3). Captan could also inhibit canker development when applied at 100 g/L. Copper hydroxide reduced the length of necrosis on both peach rootstocks caused by *P. cactorum* and *P. citrophthora* at 100 g/L and prevented necrosis at 150 g/L (Table 3). Cymoxanil was ineffective even at 50 g/L. Both *P. cactorum* and *P. citrophthora* were recovered from at least one shoot treated with cymoxanil and one non-treated shoot.

Discussion

The assay of excised stems described here is reliable and effective in determining the systemic fungicidal activity *in vitro* of different fungicides. While all fungicides tested, except cymoxanyl, gave control of infection in the bark strip and trunk inoculation assays, only the systemic fungicides metalaxyl and fosetyl-Al prevented lesion formation in the excised stem assay. The ineffectiveness of copper hydroxide, captan and dimethomorph in the excised shoot assay was presumably because of their lack of systemic activity, rather than lack of toxicity to *P. cactorum* and *P. citrophthora*. This confirms that the excised shoot assay works well in the evaluation of systemic activity of tested fungicides because only metalaxyl and fosetyl-Al could act systemically.

It is possible this new assay may also be applied for preliminary evaluation of the systemic activity of chemicals against diseases caused by pathogens other than *Phytophthora*. The excised shoot assay is not laborious, allows ample replication, is inexpensive and does not require applications in the field. Furthermore, fungicides can be evaluated quickly under a controlled laboratory environment. Shoots need identical wounding because

Table 3. Effect of six fungicides on the length of necrosis on two peach rootstocks inoculated with *Phytophthora cactorum* and *P. citrophthora*

Fungicide (active ingredient)	Rate (g/L)	Vertical distance of necrosis[A] (cm) *P. cactorum*	*P. citrophthora*	Rate (g/L)	Vertical distance of necrosis (cm) *P. cactorum*	*P. citrophthora*
		Rootstock GF677				
Nil	—	8.50 a[B]	6.85 a	—	7.65 a	6.40 a
Captan[C]	100	0 c	0 c	100	0 b	0 b
Copper hydroxide	100	2.10 b	1.56 b	150	0 b	0 b
Cymoxanil	10	8.65 a	6.60 a	50	7.50 a	6.25 a
Dimethomorph	10	0 c	0 c	10	0 b	0 b
Fosetyl-Al	10	0 c	0 c	10	0 b	0 b
Metalaxyl	10	0 c	0 c	10	0 b	0 b
		Rootstock KID I				
Nil	—	10.75 a[B]	9.60 a	—	11.65 a	8.60 a
Copper hydroxide	100	3.20 b	2.60 b	150	0 b	0 b
Captan	100	0 c	0 c	100	0 b	0 b
Cymoxanil	10	10.05 a	10.20 a	50	11.90 a	9.00 a
Dimethomorph	10	0 c	0 c	10	0 b	0 b
Fosetyl-Al	10	0 c	0 c	10	0 b	0 b
Metalaxyl	10	0 c	0 c	10	0 b	0 b

[A]Each value is the mean of two experiments each with ten replicates for each fungicide.
[B]Values in the same column followed by different letters are significantly different ($P = 0.05$) according to Duncan's multiple range test.
[C]Fungicide solutions were prepared by mixing appropriate concentration of fungicide with tap water.

lesions developed both upward and downward. Caution must also be taken when shoots are inserted in the flooded vermiculite to avoid splashing.

The results confirmed earlier reports that metalaxyl and fosetyl-Al have the ability to be absorbed and translocated in plant tissues (Matheron and Matejka 1991; Thomidis and Tsipouridis 2001). El-Hamalawi (1995) reported that fosetyl-Al is unique among fungicides in that it is translocated in both xylem and phloem. Thomidis and Michailidis (2002) reported that fosetyl-Al had a very limited ability to reduce the growth of *P. cactorum* and *P. citrophthora* in poisoned agar. One explanation is the mode of action of fosetyl-Al, which activates the defence mechanisms of plants against *Phytophthora* (Smillie *et al*. 1989).

Captan, dimethomorph, cymoxanil and copper hydroxide did not show any systemic activity. However, captan, dimethomorph and copper hydroxide did act against *P. cactorum* and *P. citrophthora* as protectants. Agrios (1988) reported that captan and copper hydroxide are not absorbed or translocated by plants, but they have the ability to protect plants against *Phytophthora* (Erwin and Ribeiro 1996). Dimethomorph is a relatively new systemic fungicide that seems promising for the control of oomycete disease in the field especially in areas where phenylalanine-resistant fungal populations prevail (Cohen *et al.* 1995). However, dimethomorph showed no systemic activity in the excised shoot assay, confirming the report of Thomidis and Elena (2001) that dimethomorph did not act systemically against *P. cactorum* of peach trees when it was applied as a soil drench and trunk paint. Erwin and Ribeiro (1996) reported that dimethomorph has a limited systemic movement in the plant.

Cymoxanil has been used in fungicide mixtures to prevent the buildup of resistance to phenylamide fungicides in *Phytophthora* (Samoucha and Gisi 1987). However, it had no fungicidal activity against *P. cactorum* or *P. citrophthora* in any of the assays reported here.

References

Agrios G (1988) 'Plant pathology (3rd edn).' (Academic Press: San Diego)

Cohen Y, Baider A, Cohen B (1995) Dimethomorph activity against oomycete fungal plant pathogens. *Phytopathology* **85**, 1500–1506.

Elena K, Tsipouridis K (2000) Evaluation of resistance of stone fruit rootstocks to *Phytophthora* crown rot. *Journal of Phytopathology* **6**, 365–371.

El-Hamalawi ZA, Menge JA, Adams JC (1995) Methods of fosetyl-Al application and phosporous level in avocado tissue needed to control stem canker caused by *Phytophthora citricola*. *Plant Disease* **79**, 770–778.

Erwin D, Ribeiro O (1996) '*Phytophthora* diseases worldwide.' (APS: St Paul)

Jeffers SN, Martin SB (1986) Comparison of two media selective for *Phytophthora* and *Pythium* species. *Plant Disease* **70**, 1038–1043.

Matheron ME, Matejka JC (1988) *In vitro* activity of sodium tetrathiocarbonate on sporulation and growth of six *Phytophthora* species. *Phytopathology* **78**, 264–268.

Matheron ME, Matejka JC (1991) Effect of sodium tetrathiocarbonate, metalaxyl, and fosetyl-Al on development and control of *Phytophthora* root rot of citrus. *Plant Disease* **75**, 264–268.

Samoucha Y, Gisi U (1987) Use of two- and three-way mixtures to prevent buildup of resistance to phenylamide fungicides in *Phytophthora* and *Plasmopara*. *Phytopathology* **77**, 1405–1409.

Smillie R, Grant BR, Guest D (1989) The mode of action of phosphite: Evidence for both direct and indirect modes of action on three *Phytophthora* spp. in plants. *Phytopathology* **79**, 921–926.

Stylianides DC, Syrgianidis GD, Almaliotis DD (1988) The peach rootstocks: a review of bibliography with relative observations in Greece. *Agriculture Technology* **12**, 34–69.

Thomidis T (2001) Testing variability in pathogenicity of *Phytophthora cactorum, P. citrophthora* and *P. syringae* to apple, pear, peach, cherry and plum rootstocks. *Phytoparasitica* **29**, 47–49.

Thomidis T, Elena K (2001) Effects of metalaxyl, fosetyl-Al, dimethomorph and cymoxanil on *Phytophthora cactorum* of peach trees. *Journal of Phytopathology* **149**, 97–101

Thomidis T, Michailidis Z (2002) Preliminary evaluation of nine fungicides for control of *Phytophthora cactorum* and *P. citrophthora* with crown rot in peach trees. *Phytoparasitica* **30**, 52–60.

Thomidis T, Tsipouridis K (2001) Efficacy of metalaxyl, fosetyl-Al, dimethomorph and cymoxanyl applied as soil drench or stem paint on scraped stem cankers for controlling *Phytophthora cactorum* and *P. citrophthora* of peach tree. *Phytopathologia Mediterranea* **40**, 253–259

Timmer LW (1977) Preventive and curative trunk treatments for control of *Phytophthora* foot rot of citrus. *Phytopathology* **67**, 1149–1154.

Wilcox WF (1993) Incidence and severity of crown and root rots on four apple rootstocks following exposure to *Phytophthora* species and waterlogging. *Journal of the American Society for Horticulture Science* **118**, 63–67.

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