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DEVELOPMENT AND SPREAD OF *PHYTOPHTHORA RAMORUM* IN THE PRESENCE OF GRAPEFRUIT EXTRACT

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Abstract: Amendment of V8 juice agar and soil leachate with grapefruit extract resulted in the inhibition of colony growth and sporulation of *Phytophthora ramorum*. Zoosporangia were more susceptible to the extract than pathogen hyphae and chlamydospores. Spraying of rhododendron inoculated with *P. ramorum* with grapefruit extract at conc. 165 $\mu g/cm^3$ inhibited 2–3 times the spread of necrosis on stems and leaves.

Key words: growth, formation, inhibition, hyphae, zoosporangium, chlamydospore, shoot, necrosis

INTRODUCTION

Phytophthora ramorum Werres, de Cock and Man in't Veld (sp. nov.) is the harmful pathogen that has been found on at least 17 plant species including 4 oaks and ericaceous plants (Orlikowski and Szkuta 2003; Werres et al. 2001). Pathogenicity trials which were done by some researchers showed different *Viburnum* and *Rhododendron* species as well as *Camellia* spp., *Chamaecyparis lawsoniana, Fagus silvatica, Fuchsia* spp., *Photinia* spp., *Picea sitchensis, Quercus ilex, Q. rubra, Sambucus* spp. and *Ulmus* spp. as potential hosts for *P. ramorum* (Brasier et al. 2002; Inman et al. 2002; Orlikowski and Szkuta 2003; Werres 2002a; b). In 2000 the pathogen was isolated from rhododendrons with leaf lesions and shoot dieback symptoms in one container Polish nursery (Orlikowski and Szkuta 2002). The formation of sympodial, semipappilate and cadocous zoosporangia of *P. ramorum* combined with production of chlamydospores is unreported within the genus *Phytophthora* (Rizzo et al. 2002).

Zoosporangia formed at temperature between 10–20°C may be splashed by rain, wind, irrigation and spread aerially with water to infect new plants. Fast spread of the pathogen from one to other plant species may be dangerous not only in ornamental nurseries, gardens and other plant arrangements but especially in natural

forest environment. High losses attributed to that pathogen have been reduced through the use of phosphonate product or coating of oak trunk with copper sulfate (Garbelotto and Rizzo 2001). Chemicals could not be used, however, in natural environment like parks, copses or forests. Studies of Orlikowski (2001) showed that grapefruit extract applied as substratum drench strongly reduced population density of *P. cryptogea* Pethybr. et Laff. by suppresion of hyphae growth and formation of zoosporangia.

The objective of this study was to evaluate grapefruit extract activity in the reduction of growth and development of *P. ramorum* and suppression of the pathogen spread on rhododendron.

MATERIALS AND METHODS

Grapefruit extract (GE). Biosept 33 SL (33% of GE) supplied by Cintamani Poland was used. The product was applied at concentration from 1.6 to 1000 $\mu g/cm^3$.

Phytophthora ramorum. The pathogen isolated from diseased stem of rhododendron was used in an *in vitro* and greenhouse trials. Stock culture was maintained on V8 juice agar at 20°C in the dark.

***In vitro* trials.** Influence of GE on colony growth of *P. ramorum* was estimated on V8 juice agar. Medium amended with GE was seeded with 5 mm diam disks (4 per plate) taken from the edge of 6-day-old colony. After 5 and 7-day-growth diam of colony was measured. Formation of zoosporangia and chlamydospores were observed in soil leachate (Orlikowski 1979) on the edge of 5 mm diam pathogen disks taken from 10-day-old culture grown on oatmeal agar and from infested rhododendron leaves. Sporangia were counted after 24, 48 and 96 hr of incubation at 20°C in the dark.

In the next trials rhododendron leaves treated with GE at conc. 165 and 330 $\mu g/cm^3$ were taken from plants 24, 48 and 72 hr after their spraying, transferred into plastic boxes on moist, sterile blotting paper and infected with 3 mm diam disks of *P. ramorum* taken from 6-day-old colony grown on V8 juice agar. Boxes were covered with plastic and incubated at 20–22°C in the dark. After 5 days diam of lesions was measured. Experimental design was completely randomised with 4 replications and 1 Petri dish in each rep. Trials were repeated 2–3 times.

Greenhouse trials. Rhododendron plants cv. Nova Zembla were placed on greenhouse bench and their leaves and top of stems were inoculated with 3 mm diam disks of *P. ramorum* taken from cultures grown on V8 juice agar. After 12 hr plants were sprayed once or twice (at 3-day-interval) with grapefruit extract at conc. 165 and 330 $\mu g/cm^3$ (1 dm^3 of solution/10 m^2). Control plants were sprayed with water only. Oxadixyl at conc. 160 μg /cm^3 was used as the standard chemical. After treatments plants were covered with plastic tunnel to keep air moisture on the level above 92% and incubated at temperature varying from 15° to 21°C. One and two weeks after the second treatment of plants with GE length of necrosis on stems and diam of lesions on leaf blades were measured. Experimental design was completely randomised with 4 replications and 5 plants in each rep. Trial was repeated twice at 3 week interval. Similar results were obtained so results from the first experiments are presented.

RESULTS AND DISCUSSION

Growth and development of *P. ramorum*. Inhibition of colony growth was observed at concentration of GE from 1.6 to 1000 $\mu g/cm^3$ whereas 50% inhibition of the pathogen growth was observed on medium containing GE at doses between 40 and 200 $\mu g/cm^3$ (Fig. 1).

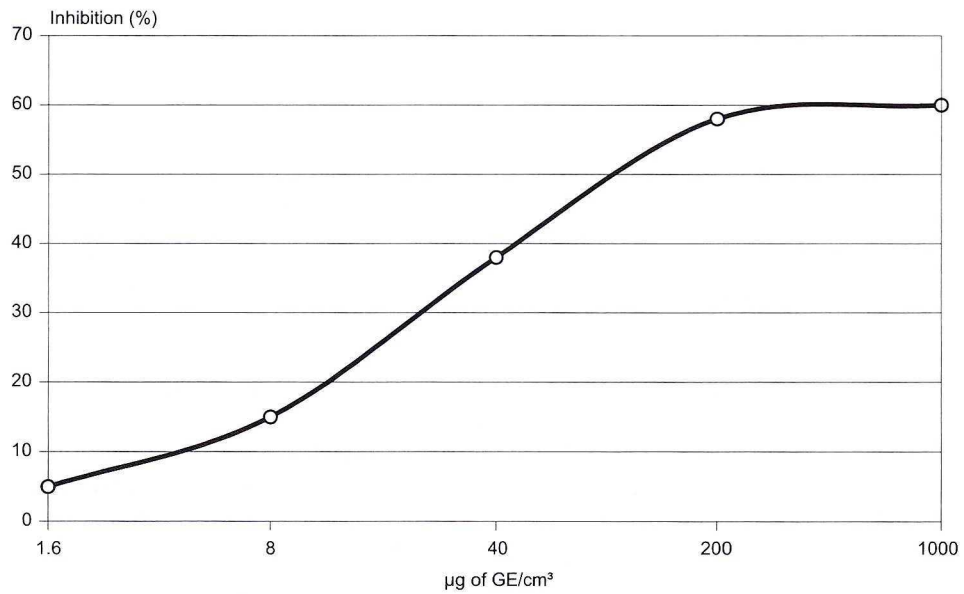

Fig. 1. Linear growth of *Phytophthora ramorum* on PDA amended with grapefruit extract (GE)

Transferring of mycelial disks into soil leachate amended with 8 μg of GE/cm^3 drastically suppressed zoosporangia formation within 24 and 48 hr incubation (Tab. 1). Sporangia were not observed in medium containing 40 or more μg of the product/cm^3 (Tab. 1). On invaded disks of rhododendron leaves sporulation of the pathogen was already inhibited in soil leachate amended with 8 μg of GE/cm^3 and zoosporangia were not observed at 200 μg of the product/cm^3 (Tab. 1).

Table 1. Inhibition of *Phytophthora ramorum* sporulation in soil leachate amended with grapefruit extract: number of zoosporangia/mm^2 of mycelium on OMA disks (A) and leaf plugs (B)

μg of grapefruit extract/cm^3	Hrs of incubation			
	24		48	
	A	B	A	B
0	31 b	16 b	171 c	51 c
8	0 a	0.7 a	0.6 a	1.4 a
40	0 a	0.6 a	0 a	0.6 a
200	0 a	0 a	0 a	0 a

Note: means followed by the same letter, do not differ with 5% of significance (Duncan's multiple range test)

Observation of agar and leaf disks under microscope showed that already at 8 μg of GE/cm^3 zoosporangia were not formed in clusters but only singly. They were longer than in nonamended media. At conc. 40 μg of GE/cm^3 disintegration of hyphae was observed.

Studies of relationships between GE concentration and sporulation of *P. ramorum* showed that the product at doses 8 and 40 μg/cm^3 stimulated chlamydospore formation. Increase of GE concentration to 200 μg/cm^3 resulted in complete inhibition of the pathogen sporulation (Tab. 2).

Analysis of necrosis development on rhododendron leaves treated with GE and inoculated with *P. ramorum* showed that the product significantly inhibited the spread of lesions (Tab. 3). GE was more effective when applied 48 or 72 hr prior the inoculation of leaf blades (Tab. 3).

Greenhouse trial. Analysis of infected stems of rhododendron indicates that GE at both concentrations suppressed the necrosis spread about twice (Tab. 4). Measurement of necrosis length 7 and 14 days after treatment showed that increase of GE concentration did not result in the decrease of necrosis spread (Tab. 4).

Observation of necrosis development on protected rhododendron leaves showed the strong inhibition of lesion spread by GE (Tab. 5). Spraying of plants twice with GE was more effective than one application, especially after 7-day-incubation. Higher concentration of GE had no significant influence on its efficacy (Tab. 5).

Recent study of Orlikowski (2001; 2002) indicate on activity of grapefruit extract toward *Phytophthora cinnamomi* and *P. cryptogea*. Inhibition of growth and sporulation of pathogens including disintegration of hyphae and deformation of zoosporangia resulted in drastical decrease of pathogen population in substrata (Orlikowski 2001). Decrease of pathogen propagule number and long activity in

Table 2. Influence of grapefruit extract on chlamydospore formation of *Phytophthora ramorum* in soil extract; number of spores/mm^2 of mycelium

μg of grapefruit extract/cm^3	Hrs of incubation		
	24	48	96
0	0.4 a	0.6 a	2.0 a
8	5.5 b	7.0 c	8.3 c
40	7.0 c	8.0 c	9.5 c
200	0 a	0 a	0 a

Note: see table 1

Table 3. Activity of grapefruit extract in the control of *Phytophthora ramorum*; diam of leaf spots in mm on rhododendron after 5-day-incubation

Grapefruit extract μg/cm^3	Inoculation of leaf blades after hrs of treatment with GE		
	24	48	72
0	25.6 d	25.6 d	25.6 d
165	18.1 c	8.7 a	15.3 bc
330	16.1 bc	12.6 ab	12.8 ab

Note: see table 1

Table 4. Biological activity of grapefruit extract in the control of *Phytophthora ramorum* spread on rhododendron stems; length of necrosis in mm

Treatment	μg of a.i./cm^3	Number of sprayings	Days after inoculation	
			7	14
Control	–	–	45.6 d	66.3 c
Grapefruit extract	165	1	22.3 b	27.3 b
Grapefruit extract	165	2	21.8 b	28.8 b
Grapefruit extract	330	1	30.0 c	34.0 b
Grapefruit extract	330	2	22.8 b	29.3 b
Oxadixyl	160	1	4.5 a	6.8 a

Note: see table 1

Table 5. Biological activity of grapefruit extract in the control of *Phytophthora ramorum* spread on rhododendron leaves; diam of necrosis in mm

Treatment	μg of a.i./cm^3	Number of sprayings	Days after inoculation	
			7	14
Control	–	–	29.8 e	43.3 c
Grapefruit extract	165	1	17.4 d	21.0 b
Grapefruit extract	165	2	8.4 bc	15.0 b
Grapefruit extract	330	1	11.3 c	19.6 b
Grapefruit extract	330	2	8.0 b	16.3 b
Oxadixyl	160	1	0 a	0 a

Note: see table 1

soil caused that at least 50% of Lawson cypress, yew and heather were protected by grapefruit extract applied as peat drench (Orlikowski 2002). In most cases, however, effectiveness of GE was significantly lower than phosethyl-Al or furalaxyl (Orlikowski 2002). Present trials with biological activity of grapefruit extract toward *P. ramorum* confirmed previous study (Orlikowski 2001; 2002). The pathogen, however, was more tolerant to GE than *P. cryptogea* but especially hyphae. ED 50 for *P. ramorum* was close to 200 μg of GE/cm^3 whereas for *P. cryptogea* about 40 μg. In case of zoosporangia formation similar reaction of both pathogens on the biocide was observed. The data obtained showed the tolerance of *P. ramorum* chlamydospores on the tested product. Those spores were formed at the same number at conc. 8 and 40 μg of GE/cm^3 but their production was completely inhibited at 200 μg. It is possible that on plant shoots protected with grapefruit extract at conc. 165 μg/cm^3 chlamydospore may be still formed, especially inside of invaded tissues. Higher activity of the product applied 48 or 72 hr before plant inoculation with *P. ramorum* in the suppression of stem rot and leaf lesion spread indicate that grapefruit extract may penetrate rhododendron tissues or induce defence reaction on the pathogen.

REFERENCES

Brasier C., Rose J., Kirk A.S., Webber J.F. 2002. Pathogenicity of *Phytophthora ramorum* isolates from North America and Europe to bark of European *Fagaceae*, American *Quercus rubra* and other forest trees. Sudden Oak death Sci Symposium, Monterey, USA, 2002.12.15–18, p.34.

Garbelotto M., Rizzo D.M. 2001. Preliminary studies on chemical and cultural control of *Phytophthora* associated with sudden oak death. Phytopathology 91, p.30.

Inman A.J., Beales P.A., Lane C.R., Brasier C. 2002. Comparative pathogenicity of European and American isolates of *Phytophthora ramorum* to leaves of ornamental, hedgerow and woodland under-story plants in UK. Sudden Oak Death Sci. Symposium, Monterey, USA, 2002.12.15–18, p.24.

Orlikowski L.B. 1979. Sporulation of *Phytophthora cryptogea* from diseased gerbera in water and soil extracts. Bull. Pol. Acad. Sci., Biol. Sci., 27: 761–767.

Orlikowski L.B. 2001. Effect of grapefruit extract on development of *Phytophthora cryptogea* and control of foot rot of gerbera. J. Plant Protection Res., 41 (3): 288–294.

Orlikowski L.B. 2002. Wykorzystanie wyciągu z grejpfruta w ochronie cisa, cyprysika Lawsona i wrzosów przed *Phytophthora cinnamomi*. Sylwan CXLVI, 3: 91–97.

Orlikowski L.B., Szkuta G. 2002. First record of *Phytophthora ramorum* in Poland. Phytopathol. Pol., 25: 69–79.

Orlikowski L.B., Szkuta G. 2003. Occurrence of *Phytophthora ramorum* in Poland and colonisation of different plants by the species. Acta Mycol., 1, in print.

Rizzo D.M., Garbelotto M., Davidson J.M., Slaughter G.W., Koike S.T. 2002. *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. and *Lithocarpus densiflorus* in California. Plant Disease 86: 205–214.

Werres S. 2002a. Triebsterben an Rhododendron durch die neue *Phytophthora*-Art *P. ramorum*. *Rhododendron* und immergrüne Laubgeholze Jahrbuch 2002: 60–67.

Werres S. 2002b. *Phytophthora ramorum*. Erste Ergebnisse zum Wirtspflanzenkreis in Deutschland. Deutsche Baumschule 7, p.46.

Werres S., Marwotz R., Man in't veld W.A., De Cock A.W.A.M., Bonants P.J.M., De Weerdt M., Themann K., Iljeva E., Baayen R.P. 2001. *Phytophthora ramorum* sp. nov, a new pathogen on *Rhododendron* and *Viburnum*. Mycol. Res., 105, 10: 1155–1165.

POLISH SUMMARY

WPŁYW WYCIĄGU Z GREJPFRUTA NA ROZWÓJ I ROZPRZESTRZENIANIE SIĘ *PHYTOPHTHORA RAMORUM*

W warunkach *in vitro* dodatek wyciągu z grejpfruta do pożywki wieloważywnej V8 oraz do wyciągu glebowego powodowało zahamowanie wzrostu plechy oraz ograniczenie lub całkowite zahamowanie zarodnikowania *Phytophthora ramorum*. Okazało się, że zoosporangia patogena są wrażliwsze na działanie środka aniżeli chlamydospory. Ich formowanie było całkowicie hamowane, gdy wyciąg zastosowano w stężeniu 200 $\mu g/cm^3$ pożywki. Zastosowanie wyciągu w stężeniu 165 $\mu g/cm^3$, do opryskania różaneczników zakażonych przez *P. ramorum*, istotnie ograniczało rozwój nekrotycznych plam na blaszkach liściowych oraz rozprzestrzenianie się patogena w tkankach pędów wierzchołkowych.