Phytopathologia Mediterranea (2018), 57, 1, 37–47
DOI: 10.14601/Phytopathol_Mediterr-22478

RESEARCH PAPERS

Genetic diversity and infection sources of *Rosellinia necatrix* in northern Israel

Mery DAFNY-YELIN[1], Orly MAIRESSE[1], Jehudith MOY[1], Shlomit DOR[2] and Dan MALKINSON[3,4]

[1] Northern Agricultural Research and Development, MIGAL Galilee Technology Center. Kiryat Shemona, 11016 Israel
[2] MIGAL Galilee Technology Center, Kiryat Shemona, 11016 Israel
[3] Shamir institute, Haifa University, Katzrin, 12900 Israel
[4] Department of Geography and Environmental Studies, University of Haifa, Haifa, 31905 Israel

Summary. Symptoms of white root rot (caused by *Rosellinia necatrix*) of fruit trees (including apple, cherry and peach) are rotting of the roots and yellowing of the leaves, followed by wilting and death. Undecomposed organic material in forest soils is favourable for growth of *R. necatrix*. Genetic tools and mycelial compatibility assays can be used to group the fungus into genetically similar groups. This study identified and located the sources of root infection, using broad surveys of infested plots in various locations, and assessed infection probability as a function of distance from potential inoculum source. Fifty-five infested plots in 14 settlements at different altitudes were surveyed. About 60% of the infested plots, at altitudes up to 540 m above sea level, were located near Mediterranean oak maquis forests, and the infections spread inward from the edges of the fruit orchards. These results indicated four possible sources of infection: (i) Mediterranean maquis forest near agricultural lands; (ii) soil transferred to low-lying sections within orchards; (iii) infection source carried by farmers from plots on the same farm; and (iv) infections via roots of adjacent trees within orchards. No correlation was found between genetic variation and virulence, but isolates that grew quickly on potato dextrose agar plates at 28ºC were more virulent than slow growing isolates.

Key words: *Dematophora necatrix,* Mediterranean oak maquis forest, genetic diversity, white root rot, virulence.

Introduction

White root rot, caused by the fungus *Rosellinia necatrix* Hartig (anamorph *Dematophora necatrix*, phylum: Ascomycotina) is destructive to many commercially important hosts, including ornamental plants and fruit trees including apple, sweet cherry, nectarine, peach and avocado (Ten Hoopen and Krauss, 2006; Pliego *et al.*, 2012). Symptoms of the disease include rotting of roots and yellowing of leaves, followed by wilting and death of the trees. Growth of *R. necatrix* mycelia is strongly dependent on soil temperature: optimal growth occurs at 23°C; 35°C is lethal (García-Jiménez *et al.*, 2004). Sztejnberg *et al*. (1987) showed that 1 h of exposure to 38°C reduced survival of the fungus.

Farmers in northern Israel have reported widespread wilting of deciduous trees, and spread of the disease to new trees and new agricultural land every year. Deciduous orchards cannot be replanted because of the presence of *R. necatrix*, and orchards are being abandoned. The teleomorph phase of the fungus has rarely been seen in fruit orchards, and never in Israel. Stromata of *R. necatrix* have been found on dead trees in nonagricultural lands such as yards and forests (Nakamura *et al.*, 2000). In Israel, Galilee and the Golan Heights are rich in Mediterranean oak maquis. Optimal conditions for *R. necatrix* include forest soils, that are rich in undecomposed organic material (Carlucci *et al.*, 2013). *Rosellinia* spp. have been observed in oak forests in Europe and Western Asia. *Rosellinia*

Corresponding author: M. Dafny-Yelin
E-mail: merydy@gmail.com

www.fupress.com/pm
Firenze University Press
ISSN (print): 0031-9465
ISSN (online): 1593-2095

quercina has been shown to damage oak trees in Romanian forests (Bercea, 2010), and various *Rosellinia* spp. have been isolated from severely damaged oak forests in Iran (Davari *et al*., 2003).

Mycelial compatibility assays are commonly applied to filamentous fungi, including ascomycetes, and they have useful tools for identifying intraspecific diversity within field populations of fungal plant pathogens (Armengol *et al*., 2010) distinct barrage lines form. In these assays, when mycelia of *R. necatrix* encounter mycelia of different genetic strains, distinct barrage lines form, indicating incompatibility. These barrages develop even between single ascospore isolates from the same perithecium resulting from sexual reproduction. They do not form between colonies that originated from the same ascospore (Ikeda *et al*., 2005).

Analysis techniques for characterizing genetic diversity in several groups of fungi, including *R. necatrix*, include Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeats PCR (ISSR-PCR), and the Universal Primed PCR (UP-PCR), have yielded results that were consistent with the mycelial compatibility groups (Ikeda *et al*., 2005; López *et al*., 2008; Armengol *et al*., 2010) *Rosellinia necatrix*, is a devastating soil-borne pathogen of many plant species. Biocontrol with the hypovirulence factor is promising, but disease symptoms, signs or culture morphology of the pathogen cannot be reliably used as markers for hypovirulence in this fungus. Comparative studies of the genetic diversity between isolates, based on DNA polymorphism analysis and the incompatibility assay can reveal genetic distribution, and can elucidate the propagation of *R. necatrix* infections. Biological virulence assays can be used to compare *R. necatrix* virulence against different hosts. Kanematsu *et al*. (2004) tested *R. necatrix* virulence against apple seedlings, Ikeda *et al*. (2005) tested the fungus against *Lupinus luteus* L., and garden strawberry transformed with a *Trichoderma harzianum* gene was tested for sensitivity to *R. necatrix* infection. The objectives of the present study were: (i) to identify the source/s of root infection by reference to a comprehensive geographic survey of infection in orchard plots; (ii) to use genetic and phenotyping tools such as RAPD and mycelial compatibility assays to study the genetic diversity of the fungus; and (iii) to assess the effects of temperature on mycelial growth rate and virulence.

Materials and methods

Spatial analysis

During 2012–2015, 95 orchard plots in northern Israel were sampled for presence of *R. necatrix*, to assess the probability of occurrence of *R. necatrix* infection in the orchards related to potential inoculum sources. Root samples collected from all plots known or suspected to be infested were sent for laboratory identification, and the plots were inspected for presence of typical hyphal with pear-shaped swellings above the hyphal septum. The plots were sampled after reports from farmers, agronomists and people from the Agriculture Extension Service, who suspected that tree wilting was resulting from soil-borne diseases. In the light of the results of Nakamura *et al.* (2000) that *R. necatrix* stromata could be found on dead plants such as forest trees in non-agricultural lands, we considered that these sources might include adjacent infested orchards or *Quercus* spp. maquis. Distances from the closest potential infection sources, maquis or known infested orchards, were measured by means of the ArcMap platform (ESRI, 2013). Logistic regression was applied to assess infection probability as a function of the distance (in km) from the closest potential source of infection. Because the minimal distance, D_{min} was the explanatory variable, we examined the infection-probability/distance relationship using three models: (i) minimal distance from closest infested orchard; (ii) minimal distance from closest maquis; and (iii) minimal distance from closest infested orchard or maquis. The binary response variable was infested/uninfested orchard, and the explanatory variable was distance:

$$p(Y_{infected}) = \frac{1}{1 + e^{\beta_0 + \beta_1 Dmin}}$$

where $p(Y_{infected})$ is the probability for an orchard to be infested. In the present case β_0 represents mortality probability in the absence of potentially infecting neighbors, and β_1 the effect of distance on infection probability. This analysis essentially quantifies the degree of spatial auto-correlation among the infested sites, in the light of the hypothesis that the shorter the distances among the sampled sites, the greater is the probability of infection.

Isolate collection and plot information

Thirty-three *R. necatrix* isolates from apple roots, six from grapevines, five from cherry, two from *Pistacia pa-*

laestina, and two from *Quercus* spp. (see Table 1) were obtained during 2012–2015 from various locations in northern Israel, the Golan Heights and Galilee. All the isolates were collected from dead trees in 26 orchards, and some of these hosts were replanted by us in research plots. Infested plots were located near 14 settlements in four regions in the north of Israel (Figure 1). These were: (i) the northern Golan at 1,010–1,100 m above sea level (a.s.l), where infected trees were found in Ein Qiniyye, Mas'ade, Majdal Shams, Bokata, Merom Golan and in Alonei HaBashan (Figure 1.1); (ii) in Galilee near Mount Meron (640-830 m a.s.l) in the villages of Gish, Netoua, Tzuriel, Alma, and Sasa (Figure 1.2); (iii) in the Ramim region (692-848 m a.s.l.) in Manara and Margaliot (Figure 1.3); and (iv) in orchards in Metula, located at about 442 m a.s.l (Figure 1.4). Three isolates were collected in orchards in Netoua (Rn-D plots), nine from Metula (Rn-J plots), 11 from Mas'ade (Rn-L), and four isolates from Gish (Rn O).

Four 1 mm pieces of infected root tissue containing white mycelia, taken from under the peel of the root neck of each sample, were placed on potato dextrose agar (PDA), made from 10% potato dextrose broth (Difco) amended with agar at 15 g L^{-1} and chloramphenicol at 250 mg L^{-1}. These cultures were grown for 4 d at 25°C. Typical hyphal mycelia of all isolates were tipped, to guarantee genetic uniformity, and then transferred to PDA plates for further growth. Stock cultures were maintained on PDA slants at 4°C.

Compatibility assay

Rosellinia necatrix isolates were transferred from stock cultures to PDA plates and grown at 25°C in darkness for 1 week. Mycelium incompatibility assays were performed on 2% malt extract agar as described by Armengol *et al*. (2010), in all possible combinations. Each isolate pairing was conducted at least three times. Boundary lines between incompatible reactions were recorded after 2 months.

DNA extraction, PCR, and PCR-ISSR analysis

Total DNA was extracted from pure cultures of 49 isolates that were grown on PDA plates for 1 week, with the Master Pure Yeast DNA Purification Kit (Epicentre Biotechnologies). The specific primers R5, R8 and R11 were used for conventional PCR, as described by Schena *et al*. (2002). PCR Inter-simple sequence repeats (ISSR) analysis was performed as described by Armengol *et al*. (2010) and Ikeda *et al*. (2005). Primers and melting temperatures (Tm) were: (i) ISSR1 5′-HBH$(AG)_7$-3′, Tm 50°C; (ii) ISSR2 5′-DBDA$(CA)_7$-3′, Tm 41°C; (iii) ISSR4 5′-YHY$(GT)_7$-3′, Tm 54°C; (iv) ISSR5 5′-BDB$(ACA)_5$-3′, Tm 49°C; (v) AS4 5′-TGTGGGCGCTCGACAC-3′, Tm 56°C; (vi) CA8G 5′-$(CA)_8$G-3′, Tm 45°C; and GTG 5′-$(GTG)_5$-3′, Tm 40°C. Each PCR reaction was performed in a volume of 25 μL and contained: 1 × PCR buffer, 3 mM $MgCl_2$, 1 mM dNTPs, 0.8 pmol of each primer, 0.2 units of DNA Superterm 500 Taq polymerase (GMR) and 2 μL of DNA. PCR amplifications were performed in a BIOER XP thermal cycler (Hangzhou High Tech). The amplification programme comprised an initial step of 5 min at 95°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at the primer-specific temperature for 1 min, and elongation at 72°C for 2 min. A final extension was performed at 72°C for 10 min. PCR products were separated by electrophoresis on 1.5% TAE agarose gel. All the ISSR assays were repeated at least three times, and only clear and reproducible bands were considered.

Virulence assay

Virulence of *R. necatrix* isolates was assayed on the sensitive model plant, strawberry (Mercado *et al*., 2015). Strawberries (*Fragaria* × *ananassa*) cv. 'Golan' were rooted in garden soil (Ram; Tuff Merom Golan Ltd) at 100% humidity under a shade net that blocked 50% of the sunlight. After 3 weeks, the plants were transferred to 250 mL capacity pots containing 20% perlite no. 4 and 80% unsterilized local soils from the Avni Eitan Experiment Research Center. The plants were placed in growth chambers at a controlled temperature of 25°C. Three grains of wheat, colonized with *R. necatrix* isolates, were used to inoculate the pots 7 d after planting, as described by Sztejnberg and Madar (1980). The tested isolates were designated as: Rn-A, Rn-D, Rn-E, Rn-L, Rn-N, Rn-O15, Rn-O36, Rn-O6, Rn-P, and Rn-U, with uninoculated pots used as experimental controls. The experiments were conducted three times in six replicates, each of one plant. The vitality of the plants was assessed three times per week. This was evaluated and scored into five categories: healthy green plants (score = 5); first symptoms of wilting (4); half of plant wilted (3); small amounts of visible green tissue seen (2), completely dead plant (1). Regressions were constructed between the vitality scores and day

Table 1. Genetic groups of *Rosellinia necatrix* isolates. Genetic groups were separated according to ISSR, universal primers-PCR (UP-PCR), and compatibility assays (CA). Relative virulence, as calculated from the maximum slope (plant vitality score as function of time), is presented in the virulence (V) column. More than one sample was taken in plots designated as Rn-L, Rn-J, Rn-O and Rn-D. Blank cell - not applicable.

Settlement	Plot designation	Cultivar	Rootstock	Location		ISSR				UP-PCR			V
				Lat	LON	1	2	4	5	AS4	CA8G	CA	
Northern Golan Heights													
Ein Qiniyye	Rn-A	Cherry	MM2	35.740	33.229	1	1	1	1			1	-0.25
	Rn-K	Apple	Malos	33.234	35.745	2	2	2	2			2	
Majdal Shams	Rn-G	Apple	Malos	35.776	33.253	3	2	3	3			3	
	Rn-H	Apple	Malos	35.776	33.253	3	4	4	4			4	
	Rn-U	Apple	MM106	33.124	35.802	5			5			5	-0.27
Mas'ade	Rn-C	Apple	Malos	35.755	33.209	6	1	6	6			6	-0.26
	Rn-L	Apple	Malos	35.780	33.236	6	1	6	6			6	-0.22
	Rn-L1	grapevine	101/14 Mgt	35.780	33.236	6	1	6	6			6	
	Rn-L2	Apple	Malos	35.780	33.236	6	1	6	6			6	
	Rn-L2-16-20	Apple	Malos	35.780	33.236	6	1	6	6			6	
	Rn-L3	Apple	Malos	35.780	33.236	6	1	6	6			6	
	Rn-L4	Apple	Malos	35.780	33.236	6	1	6	6			6	
	Rn-L4-45	grapevine	1103 Paulsen	35.780	33.236	6	1	6	6			6	
	Rn-L4-58	Apple	Malos	35.780	33.236	6	1	6	6			6	
	Rn-L5	Apple	Malos	35.780	33.236	6	1	6	6			6	
	Rn-L6	Apple	Malos	35.780	33.236	6	1	6	6			6	
	Rn-L6-39	Apple	Malos	35.780	33.236	6	1	6	6			6	
	Rn-R	Apple	Malos	35.761	33.240	6	1	6	6			6	
	Rn-F	Apple	Malos	35.776	33.253	2	1	7	7			7	
	Rn-N	Cherry	Mahaleb	35.776	33.239	3	8	8	4			8	-0.19
	Rn-V	Apple	Malos	35.740	33.217	9	8	8	9			9	
	Rn-W	Cherry		35.754	33.209	9	8	8	10			10	
Metula													
Metula	Rn-J1	*Q. infectoria*	Hashabi	35.569	33.278	11	11	11	11	11	11	11	
	Rn-J2	grapevine	140 Ruggeri	35.569	33.278	11	11	12	12	11	12	12	
	Rn-J3	Almond × peach	GF677	35.569	33.278	13	13	13	13	11	12	13	
	Rn-J4	*Q. calliprinos*		35.569	33.278	11	11	11	11	11	11	11	
	Rn-J6	*P. palaestina*		35.569	33.278	14	13	14	14	11	14	14	
	Rn-J35	grapevine	Paulsen	35.569	33.278	11	11	12	12	11	12	12	
	Rn-J77	grapevine	3309C	35.569	33.278	11	11	12	12	11	12	12	

(Continued)

Table 1. (Continued).

Settlement	Plot designation	Cultivar	Rootstock	Location		ISSR				UP-PCR			V
				Lat	LON	1	2	4	5	AS4	CA8G	CA	
	Rn-J119	grapevine	101/14 Mgt.	35.569	33.278	2	13	15	15	15	12	15	
	Rn-J169	*P. palaestina*		35.569	33.278	16	11	16	15	16	12	16	
	Rn-E	Apple	Hashabi	35.554	33.257	3	13	17	17			17	-0.27
	Rn-I	Apple	Hashabi	35.569	33.278	18	18	18	18			18	
	Rn-M	Apple	Hashabi	35.564	33.269	13	13	19	19			19	
Miron													
Gish	Rn-O6	Apple	Hashabi	33.013	35.410	20	13	20	20	20	20	20	-0,27
	Rn-O15	Apple	Hashabi	33.013	35.410	20	13	20	20	20	20	20	-0,20
	Rn-O36	Apple	Hashabi	33.013	35.410	20	13	20	20	20	20	20	-0,19
	Rn-O41	Apple	Hashabi	33.013	35.410	20	13	20	20	20	20	20	
	Rn-P	Apple	Hashabi	35.410	33.009	21	18	21	21			21	-0.18
	Rn-X	Apple	Hashabi	33.014	35.414	9			22			22	
Netoua	Rn-D	Apple	Hashabi	35.371	33.050	21	18	23	23	23	23	23	-0.20
	Rn-D1	Apple	Hashabi	35.371	33.050	21	18	23	23	23	23	23	
	Rn-D2	Apple	Hashabi	35.371	33.050	21	18	23	23	23	23	23	
	Rn-Q	Cherry	Mahaleb	33.055	35.356	24	24	24	24			24	
Sasa	Rn-ZA	Apple	Hashabi	33.023	35.400	25			25			25	
	Rn-ZB1	Apple	Hashabi	33.023	35.401	26			26			26	
	Rn-ZC2	Apple	Hashabi	33.021	35.400	25			25			25	
Ramim													
Manara	Rn-S	Apple	Hashabi	33.179	355.463	27			27			27	
Margaliot	Rn-B	Cherry	MM2	355.436	33.207	28	8	28	28			28	

of observation. The relative virulence of *R. necatrix* isolates was calculated as the maximum slope during the linear stage of the vitality/time relationships, because a steeper slope of this relationship indicates fast development of disease.

Growth rates of mycelia at different temperatures

Forty *R. necatrix* isolates were transferred from stock cultures to PDA plates and grown at 25°C in darkness for 5–6 d. Two 4-mm-diameter disks of young hyphae from the edge of the colony of each isolate were placed on new PDA plates for further growth at 22, 25, 28, or 29°C for 1 week. The experiments at 22 and 25°C were performed twice, those at 28°C three times, and that at 29°C once. Each experiment included at least three repetitions. Colony size was measured daily to enable calculation of daily growth rates, and the maximum slope of the growth curve of each experiment was recorded. The isolates were divided into four main geographic regional groups: Metula, Ramim, Meron, and the Golan Heights. All data were pooled for Analysis of variance (ANOVA) using the JMP13 software (SAS Institute Inc.).

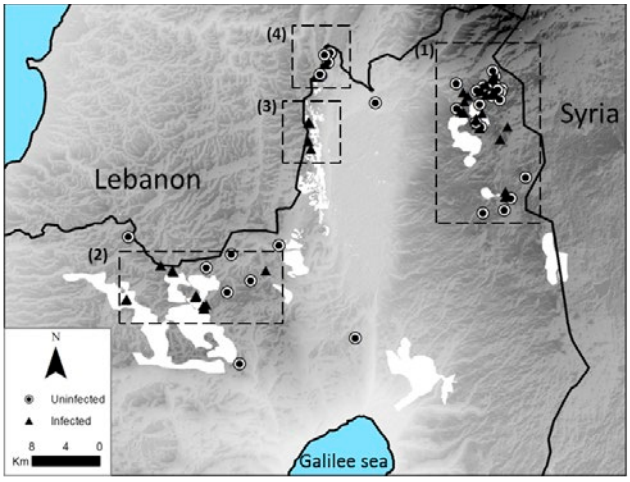

Figure 1. Map of northern Israel, where 95 orchards were sampled for presence of *Rosellinia necatrix*. Infested plots are indicated by black triangles and uninfested plots by black circles with white borders. The *Quercus* maquis are marked in white. The infested plots were distributed among four regions, as indicated within dashed rectangles: Golan Heights (1), Meron (2), Ramim (3), and Metula (4).

Results

Spatial analysis

Of the 95 surveyed orchards, 56 were found to be infested with the fungus (Figure 1). All the infested plots were near to 14 settlements, in nine of which the infested plots were located at perimeters of Mediterranean maquis oak forests. Near Mas'ade, five plots were located 250–540 m from maquis; near Bokata four plots were located 100–470 m from maquis; near Gish four plots were located 50–370 m from maquis; near Netoua three plots were located 30–200 m from maquis; near Manara and Margaliot two plots were located 440–590 and 180–200 m, respectively, from maquis; near Merom Golan and Sasa three plots were located 440–520 and 180–200 m, respectively, from maquis; and near Tzuriel one plot was located 50 m from maquis. In seven plots, located near Margaliot, Merom Golan, Netoua and Tzuriel, the first orchard trees to be affected by the fungus were located at the perimeters of Mediterranean maquis forests.

The mean distance of infested orchards from the closest maquis area was 1,271 m, whereas that of orchards unaffected by white root rot was 2,572 m. The mean distance between adjacent infested orchards was 335 m, whereas that between infested and unaffected orchards was 2,257 m. All three regression analyses yielded significant relationships between distance and infestation probability, but the overall minimal distance had the strongest effect, as expressed by the largest $ß_1$ value (Table 2). Distance to closest maquis site was the most significant model, with P=0.0007 (Table 2).

Isolate collection

In eight farms or kibbutz collective farms, between two and four widely separated plots were infested with *R. necatrix*; in two cases, neighbouring plots that did not belong to the same farmer were infested. Seventeen of the 51 isolates from 25 infested plots were identified by PCR amplification with *R. necatrix*-specific primers R5 and R8, which yielded about 400 bp, or primers R5 and R11, which yielded about 300 bp.

Genetic diversity of *Rosellinia necatrix* isolates

Genetic groups were separated according to polymorphic bands revealed by PCR analysis with ISSR primers and universal primers. PCR analysis with the ISSR1 primer yielded ten polymorphic bands, ISSR2 five bands, ISSR4 14 bands, and ISSR5 14 bands. The UP-PCR primer AS4 yielded, 12 clear, easy-to-score polymorphic bands and CA8G yielded five bands. Primer GTG was not informative because it did not yield any polymorphic bands. Three isolates from the Netu'a orchard (Rn-D, D1, D2) (see Table 1 for plot designations), four isolates from the orchard in Gish (Rn-O6, O15, O36, O41), and 11 isolates from Mas'ade (Rn-L, L1, L2, L2-16-20, L3, L4, L4-58, L5, L6, L6-39, L4-45) had the same genetic band patterns and did not form boundaries in the incompatibility assays. Nine isolates from the Metula orchard (designated Rn-J in Table 1) were in six genetic groups, most of which differed from each other, except for one pair and one group of three isolates that were genetically similar as indicated by the mycelial compatibility assays. Isolates collected from separate orchards were genetically different from each other, except for: (i) those from three neighbouring plots in Mas'ada, designated Rn-C, Rn-O and Rn-R, belonged to different farmers but were located near each other; and (ii) three neighbouring plots in Sasa that belonged to the same collective farm, where two isolates were genetically identical and one was different. These isolates

Table 2. Logistic analysis results of the distance of Roselinia necatrix infested and uninfested orchards plots from possible sources of infection.

Model	Mean distance of infested plot (km)	Mean distance of uninfested plot (km)	β_0 (*p* value)	β_1 (*p* value)
Closest infested orchard	0.335	2.257	-1.604 (0.0002)	0.985 (0.0029)
Closest maquis	1.271	2.572	-1.277 (0.0003)	0.496 (0.0007)
Closest maquis OR orchard	0.289	1.134	-0.891 (0.0014)	1.061 (0.0133)

shared the same band pattern in ISSR1-4 and did not form boundaries in the incompatibility assays.

Growth rates of isolates in PDA plates, and virulence estimation

The mean growth rate of each isolate at each of the tested temperatures is presented in Figure 2A. The growth rates on PDA plates at 22°C ranged from 1.16 to 1.85 cm d^{-1}, and no significant differences between the isolates were observed (F = 1.3194, *P*=0.2255). The growth rate range at 25°C was 0.83–1.7 cm d^{-1}, at 28°C 0.21–0.92 cm d^{-1}, and at 29°C 0.01–0.15 cm d^{-1}. Statistically significant differences were detected between isolates: at 25°C (F = 6.3410, *P*<0.0001); at 28°C (F = 3.6399, *P*<0.0001; and at 29°C (F = 12.3307, *P*<0.0001). Estimates of all parameters revealed significant differences among the four study regions (*P*=0.0026) and among the four tested temperatures (F = 4.8101, *P*=0.0026). Significant interactions between temperature and region were also detected (F = 4.7887, *P*<0.0001). Temperature was the parameter most closely correlated with the *R. necatrix* colony growth rate: significant differences were found among all the tested temperatures, with the greatest highest growth rates at 22°C and the least at 29°C (F = 441.1358, ANOVA, Tukey's Honestly Significance Difference (HSD) test, *P*<0.0001). No significant differences were observed for isolate growth rates among the four regions at 22°C (F = 0.8220, *P*=0.4859). However, at 25°C, isolates from the northern, Golan region grew more slowly than those from the other regions (F = 10.5053, *P*<0.0001). At 28°C, growth rates of isolates from Metula, the Golan Heights and Meron differed significantly from each other; and at 29°C the Golan Heights and Ramim isolates differed from each other (F = 17.2104, *P*<0.0001) (Figure 2B). Isolates were virulent to strawberry plants, and there was correlation (F = 3.9038, *P*=0.082) between isolate group virulence (maximum slope) and isolate growth rate on PDA plates at 28°C. Discarding isolate Rn-A, which exhibited very slow growth relative to virulence, there was significant correlation between virulence and growth rates at 28°C (F = 9.6580, *P*=0.0209).

Discussion

About 100 root samples were collected from fruit tree plots suspected to be infested with *R. necatrix*, from 27 settlements in northern Israel. In seven of 14 settlements with *R. necatrix*-infested plots, the first orchard trees affected by the fungus were located at the edges of Mediterranean maquis forests. In some of the plots, sprouting oak trees could be seen near the irrigated fruit trees. The other infested plots were not close to forest. The fungus was probably transmitted to these plots as inoculum adhering to farmers' agrotechnical tools. Circumstantial evidence supporting this hypothesis is that most of the individual farmers or kibbutz collectives had more than one infested plot. However, contrary evidence includes: one case in Kibbutz Sasa, where isolates from two of three sampled plots, but not the third, belonged to the same genetic group; and a case in Ein Qiniyye, where isolates from two plots belonging to one farmer did not belong to the same genetic group.

In Sasa, two *R. necatrix* isolates were genetically identicle (Rn-ZA and Rn-ZC2). However, the plants arrived from different nurseries. In addition, two isolates (Rn-ZA and Rn-ZB1) from trees from one nursery belonged to different genetic groups. These results are similar to those of Pasini *et al.* (2016), who showed that white root rot disease did not originate from nurseries. The importance of the location of the infested plots relative to maquis forest is supported by results from other studies. *Rosellinia necatrix* was pre-

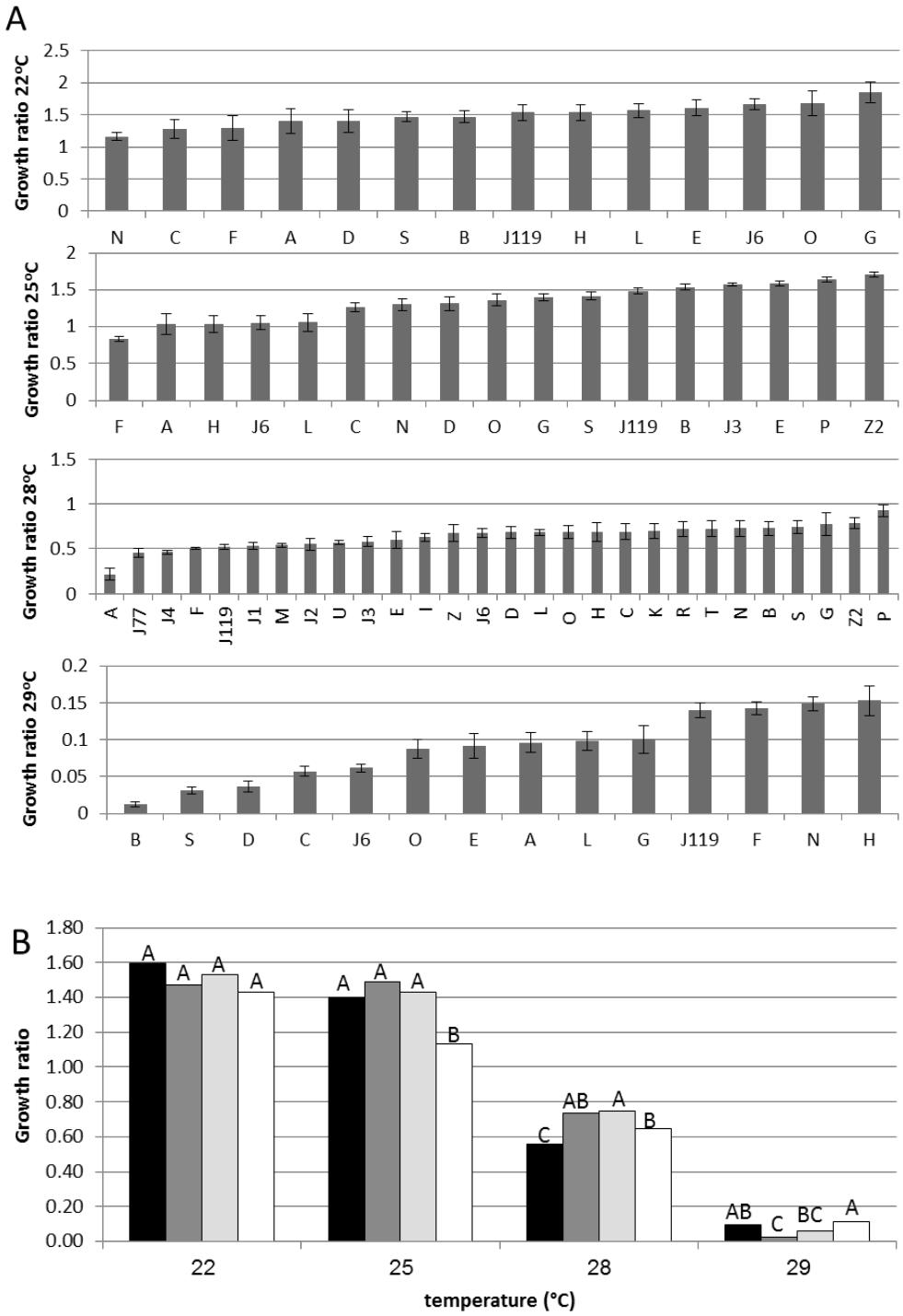

Figure 2. A. Growth rates of the isolates in PDA plates were assessed at 22, 25, 28 or 29°C. For each temperature and for each isolate, average slope ± SE of the mean cm d^{-1} growth curve (growth ratio) is presented. Data for genetically identical isolates were pooled. B. The isolates were grouped according to four geographic regions: Metula (■), Ramim (■), Meron (■) and the Golan Heights (□). All data were pooled for ANOVA. Different letters indicate statistically significant differences ($P < 0.05$) between geographic regions.

viously recorded in the Japanese forests of Hyogo and Kyoto (Ieki *et al*., 1969). Nakamura *et al*. (2000) found stromata of the fungus on dead plants in the forests, and Gonzalez and Rogers (1995) found stromata in a tropical rain forest in Mexico. Stromata of *Rosellinia* spp. have been found on dead trees in non-agricultural lands, such as plantation margins and forests, in Israel and elsewhere (Nakamura *et al*., 2000; Dafny Yelin *et al*., unpublished). Moreover, in northern Italy, orchards that replaced forests were sometimes found to be infested with *R. necatrix* (Pasini et al., 2016). This is consistent with the results of Takemoto *et al*. (2014), who demonstrated that wild plant species can act as potential inoculum sources. Comparison between the mean distances of the infested and uninfested orchards from the closest potential sources of the pathogenic fungi suggests that proximity is a significant factor in pathogen dissemination and disease spread. This interpretation is further supported by regression analysis carried out in the present study, for which we selected the minimum distance from the nearest oak stand or infested orchard. Furthermore, we cannot determine whether the source of infection was from natural areas or agricultural orchards, but distance is strongly indicated as a critical factor in determining infection probability. Carlucci *et al*. (2013) recommended keeping distance between new fruit orchards and forests in which *R. necatrix* resides, in the light of the occurrence of fungus-favourable conditions in forest soil.

In only two settlements, Metula and Majdal Shams, in the northern part of the Golan Heights, were the infested plots far from a Mediterranean maquis forest. In Metula and in the Druze villages in the northern Golan Heights the soil is very stoney and are shallow. In these areas, soils were imported from nearby quarries to improve the local soil profiles. One of the infested plots in the Druze villages was in an abandoned quarry that had been used in the past as a source for imported agricultural soil.

Genetic diversity in *R. necatrix* has traditionally been studied through characterization of mycelial compatibility among isolates (Armengol *et al*., 2010), in combination with molecular techniques (Ikeda *et al*., 2005; Armengol *et al*., 2010). In the present study, each infested plot yielded genetically unique isolates of *R. necatrix*, except in one case, in which three neighboring orchards belonging to different farmers yielded the same isolate genetic pattern as well as mycelial compatibility. The central plot of the three was severely infested, and the other nearby plots were probably infested later.

In four plots, several isolates were collected for analysis of genetic and mycelial compatibility. In the Metula orchard, six of nine isolates were genetically different; whereases one pair and one group of three isolates were genetically identical with one another. In the other three orchards, all the isolates (respectively four, three and 11 from each orchard) were identical to each other, even when they came from plants of different species that were replanted in the same plot. The infestation propagated from tree to tree, probably in the same way as from plot to plot, i.e., via root-to-root contact between host plants or by mycelial strand growth of the pathogen through the soil (Nowell, 1916; Ten Hoopen and Krauss, 2006). The infection process of *R. necatrix* in avocado trees was studied in detail by Pliego *et al.* (2009). They demonstrated how mycelia networks of the fungus covered root surfaces and penetrated root tissues via natural openings such as lenticels, but could also invade through epidermal, cortical and vascular cells. Armangol *et al.* (2010) showed that isolates collected from different fruit crops were genetically different, according to ISSR banding patterns and mycelial compatibility reactions, probably because of long geographical distances between crops, or different sources of infection. Their results are consistent with those of Pérez Jiménez *et al.* (2002), who showed that *R. necatrix* isolates from different avocado orchards in southern Spain all showed incompatibility, whereas isolates from nearby trees in the same orchard were somatically compatible in some cases. One explanation for the genetic variation among the Rn-J isolates from Metula could be the possible presence of the teleomorph stage of *R. necatrix*, and the existence of perithecia in the orchards, with consequent occurrence of genetic variation demonstrated by formation of incompatibility boundaries in compatibility assays (Aimi *et al*., 2002). Isolates from different orchards were genetically diverse. Variance between isolates can be expressed as differences in virulence that could be attributed to genomic variation, or to the presence or absence of specific double-strand RNA retroviruses found to be correlated with fungus virulence (Matsumoto, 1998; Kanematsu *et al*., 2004). In the present study no correlation was found between genetic types and fungal virulence, which was similar to the results of López *et al*. (2008) and Namai *et al*. (1998). Pasini *et al*. (2016) did not find any relationship between *in vitro* growth

rate and aggressiveness of an *R. necatrix* isolate to apple plantlets. However, to the best of our knowledge, the present study is the first to demonstrate correlation between hyphal growth rate of isolates in agar culture and virulence to host plants. This correlation could be seen only at 28ºC, at which there were significant differences between groups of isolates from different geographic regions.

Significant differences were observed among the growth rates of isolates from different regions at 25, 28 and 29°C, which could imply differing genetic sources. However, García-Jiménez *et al*. (2004) observed no significant growth rate differences at the optimum growth temperature of 22°C, probably because of the optimum growth conditions, whereas variation could result from less favourable conditions.

The present study suggests four main sources of *R. necatrix* inoculum in the various plots. These are: (i) Mediterranean oak forests located near agricultural land; (ii) soil transferred to augment inadequate depth of orchard soil; (iii) inoculum inadvertently carried by farmers from infested plots on the same farm; and (iv) root-to-root contact between host trees. No correlation was found between genetic type of *R. necatrix* and virulence. However, isolates that grew rapidly on PDA plates at 28°C were more virulent than slower growing isolates.

Acknowledgments

This research was carried out in the Golan Research Institute and in Northern Agricultural Research and Development, MIGAL Galilee Technology Center. The study was financed by the Israeli Ministry of Science and Technology and the Plants Production and Marketing Board of the Israeli Ministry of Agriculture and Rural Development. We thank Dr Stanley Freeman for help and advice; Dr Moshe Meron and his research group for providing help with mapping plots, Shady Nasralla for collecting root samples.

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Accepted for publication: January 24, 2018
Published online: May 15, 2018