J.A. Torés (1990) *Phytoparasitica* 18(4):321–329

IN VITRO VARIABILITY OF A NUMBER OF CHARACTERISTICS OF *SCLEROTINIA SCLEROTIORUM*

J.A. TORÉS[1]

Studies were made of the variability of *Sclerotinia sclerotiorum* (Lib.) de Bary: (i) growth rate when cultivated on potato-dextrose agar (PDA); (ii) the *in vitro* resistance of this organism to the fungicides benomyl, vinclozolin and procymidone; and (iii) the size and number of sclerotia formed on PDA. The isolates used were collected from eggplants, French beans and tomatoes cultivated in the same locality, but although marked differences were observed among them, especially in growth rate and formation of sclerotia, it was not possible to establish groups or categories among the isolates studied, or to relate the observed variability to their origins. Temperature was a decisive factor for formation of sclerotia on PDA. No correlation was found between size and number of sclerotia, and growth rate. The ED_{50} level for both vinclozolin and procymidone *in vitro* was 2 mg l^{-1}, whereas that for benomyl was below the minimum level used, and therefore could not be established.
KEY WORDS: *Sclerotinia sclerotiorum; in vitro* variability; mycelial growth; fungicide effectiveness; number and size of sclerotia.

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is one of the most damaging plant pathogens in those areas of southern Spain in which horticultural crops are grown under plastic sheeting. The commonly used fungicides do not give effective control (7) and the problem is compounded by two circumstances: the sclerotia maintain their viability in soil for a long time (8), and many different plant genera are susceptible to this pathogen (10). The present work is a preparatory investigation for a wider epidemiological study of the organism and was designed to determine the possible differences between isolates of several origins in the affected area studied.

Received June 13, 1989; received in final form Sept. 14, 1990.
[1] Estación Experimental "La Mayora" (CSIC), Algarrobo-Costa, 29750 Málaga, Spain.

MATERIALS AND METHODS

1. *Origin of the isolates.* Sclerotia removed from plants attacked by *S. sclerotiorum* were disinfected by immersing them in a mixture of commercial bleach (20%), ethyl alcohol (10%), and distilled water (70%) for 3 min. They were then dried on sterile filter paper in a laminar-flow chamber, after which they were placed in petri dishes containing Difco potato-dextrose gar (PDA). When the mycelia started to develop, they were transferred to new petri dishes containing PDA. Three strains were randomly selected from those originally obtained from eggplants (*Solanum melongena* L.), three from French beans (*Phaseolus vulgaris* L.) and three from tomato (*Lycopersicon esculentum* Mill.).

The nine strains, all taken from polyethylene-greenhouse crops from the same geographical area (the coastal zone of Malaga province, southern Spain), were used in all the experiments. All belonged to the species *Sclerotinia sclerotiorum* (Lib.) de Bary, as confirmed by the observation that their ascospores were of uniform size with a length-width ratio greater than 2, and were binucleate (5).

2. *Growth in vitro.* In all cases, the fungus was grown in 9-cm-diam petri dishes on PDA. Inoculation was by a 5-mm-diam disk punched from the edge of an actively growing colony of the pathogen. Incubations were carried out at 18 and 25°C, and growth was determined by measuring colony diameters after 24 h and 48 h. Some of the cultures had almost reached the edge of the petri dish after 48 h (2).

3. *In vitro sensitivity to fungicides.* Stock solutions of each of the three fungicides used in the study, benomyl, vinclozolin, and procymidone, were prepared from the commercial products containing 50% of the active substance, by dissolving 1 g of the product in 250 ml of sterile deionized water. Sets of PDA culture media with five different concentrations (2, 4, 8, 10 and 20 mg l^{-1}) were prepared from each fungicide stock solution following the methodology of Stack (12) and Almeida and Yamashita (2). For each isolate two petri dishes without fungicides were prepared to serve as controls.

To determine whether the sterilization temperature would alter fungicide effectiveness, two series of incubations were conducted; in one, the media containing fungicides were autoclaved; in the other, the media were sterilized before adding the fungicides.

Inoculation and incubation followed the method described in section 1, except that mycelial growth was measured after 25 days.

The ED_{50} of each fungicide was calculated from its linear regression.

4. *Number and size of sclerotia formed on PDA.* Fungal growth was as described in section 1 and was allowed to continue until the sclerotia could be separated with ease from the media (approx. 2 to 3 weeks). Maximum length, breadth, and thickness were measured with a vernier caliper to an accuracy of 0.1 mm. Five replicates were employed

for the number of sclerotia, and all the sclerotia formed in one randomly selected petri dish were used to determine size and variability within the population.

RESULTS

Table 1 shows the growth measurements for each isolate, or group of isolates, at the two time intervals and for each temperature. Table 2 gives the percentage of variation expressed by the isolate and temperature after a factorial analysis of two fixed factors. Temperature had little influence on growth at 24 h, but at 48 h it caused increased growth. On the other hand, the influence of the isolate factor was uniform at both time intervals, and greater than that of temperature.

Table 3 shows the means of fungal growth on fungicide-containing PDA media. The variation percentages for each factor were as follows: fungal isolate, 1%; fungicide, 22% (significant difference at $P = 0.01$); fungicide concentration, 16% (significant difference at $P = 0.01$); and sterilization procedure, 0%. The activities of the three fungicides in this

TABLE 1

EFFECT OF TEMPERATURE ON GROWTH OF ISOLATES (COLONY DIAMETER, IN MM) AFTER 24 H AND 48 H (Means of five replicates)

Isolates	*After 24 h*		*After 48 h*	
	18°C	*25°C*	*18°C*	*25°C*
All isolates	32 x	34 y	73 x	82 y
S. melongena isolates	33 x	33 x	76 x	82 y
P. vulgaris isolates	30 x	32 x	66 x	78 y
L. esculentum isolates	32 x	37 y	73 x	87 y
1 (*S. melongena*)	31 x a	34 x a	75 x a	85 y a
2 (*S. melongena*)	33 x a	33 x ab	80 x a	86 y a
3 (*S. melongena*)	34 x a	32 x ab	74 x a	76 x b
4 (*P. vulgaris*)	28 x a	32 x ab	65 x b	74 y b
5 (*P. vulgaris*)	31 x a	30 x b	72 x a	76 y b
6 (*P. vulgaris*)	31 x a	33 x a	75 x a	85 y b
7 (*L. esculentum*)	31 x a	36 x a	73 x a	86 y a
8 (*L. esculentum*)	33 x a	41 y c	72 x a	86 y a
9 (*L. esculentum*	34 x a	35 y a	74 x a	87 y a

Means followed by a common letter do not differ significantly ($P = 0.05$). The letters "x" and "y" indicate significant differences between temperatures according to the L.S.D. test and should be compared horizontally for each isolate and time. The letters "a", "b" and "c" indicate significant differences according to the Student-Newman-Keuls test and the isolates should be compared vertically for each time and temperature.

TABLE 2

PERCENTAGES OF VARIATION IN THE GROWTH OF *SCLEROTINIA SCLEROTIORUM IN VITRO*, AS EXPRESSED BY THE FACTORS OF TEMPERATURE AND ISOLATE

Factor	*After 24 h*	*After 48 h*
Temperature factor		
For all isolates	16**	42**
For isolates of *S. melongena*	2	28**
For isolates of *P. vulgaris*	10	13**
For isolates of *L. esculentum*	49**	89**
Isolate factor		
For all temperatures	28**	31**
For temperature of 18°C	27	52**
For temperature of 25°C	72**	82**

**Significant difference at $P = 0.01$.

TABLE 3

GROWTH OF *SCLEROTINIA SCLEROTIORUM* ISOLATES (COLONY DIAMETER, IN MM) ON MEDIA CONTAINING FUNGICIDE

(Means of 50 replicates for each isolate-fungicide combination)

Isolates	*Fungicide*			
	Benomyl	*Vinclozolin*	*Procymidone*	*Mean*
S. melongena isolates	5.0 a x	18.2 a y	32.3 a z	17.1 a
P. vulgaris isolates	7.1 b x	12.7 a x	24.5 a y	14.4 a
L. esculentum isolates	5.4 c x	16.7 a x	36.7 a y	18.3 a
Mean of all isolates	5.9 x	15.9 y	31.0 z	16.6

Means followed by the same letter do not differ according to the Student-Newman-Keuls test ($P = 0.05$). The letters "a", "b" and "c" indicate significant differences between isolates and should be compared vertically for each fungicide. The letters "x", "y" and "z" indicate significant differences between fungicides and should be compared horizontally for each isolate.

work were not reduced by sterilization and all the isolates tested were equally sensitive to each of the three fungicides. The only factors influencing mycelial growth were fungicide type and fungicide concentration. Analysis of variance revealed two groups of concentrations: one containing the two lowest concentrations (2 and 4 mg l^{-1}) and the other containing the three highest concentrations (8, 10 and 20 mg l^{-1}), the means of which differed significantly between the two groups, but not within them.

The ED_{50} obtained for both vinclozolin and procymidone *in vitro* was 3 mg l^{-1}, but

that for benomyl could not be calculated because it was very far below the minimum dose employed.

TABLE 4

SIZE AND NUMBER OF SCLEROTIA FORMED ON POTATO DEXTROSE AGAR AT TWO TEMPERATURES
(Means of five replicates for number; all the sclerotia in one random-sampling petri dish for size)

Isolates	*Length (in mm)*		*Number of sclerotia*	
	18°C	*25°C*	*18°C*	*25°C*
All isolates	4.3 x	2.6 y	27.2 x	40.0 y
S. melongena isolates	4.4 x	3.4 y	30.1 x	31.7 x
P. vulgaris isolates	4.1 x	2.2 y	25.2 x	46.9 y
L. esculentum isolates	4.4 x	2.5 y	26.3 x	41.3 y
1 (*S. melongena*)	3.4 x a	2.7 y a	27.7 x a	19.7 y a
2 (*S. melongena*)	5.8 x d	3.8 y b	30.7 x a	41.0 x bc
3 (*S. melongena*)	3.9 x ab	3.4 y c	32.0 x a	34.3 x b
4 (*P. vulgaris*)	3.8 x ab	2.4 y a	28.0 x a	45.7 y bc
5 (*P. vulgaris*)	4.3 x bc	2.3 y a	23.0 x a	47.7 y bc
6 (*P. vulgaris*)	4.3 x bc	2.0 y d	24.7 x a	47.3 y bc
7 (*L. esculentum*)	4.5 x bc	2.6 y a	25.7 x a	35.7 y b
8 (*L. esculentum*)	3.8 x ab	2.4 y a	30.0 x a	52.0 y c
9 (*L. esculentum*	5.0 x c	2.6 y a	23.3 x a	36.3 y b

Means followed by a common letter do not differ significantly ($P = 0.05$). The letters "x" and "y" indicate significant differences between temperatures according to the L.S.D. test and should be compared horizontally for each isolate or group of isolates. The letters "a", "b", "c" and "d" indicate significant differences according to the Student-Newman-Keuls test and the isolates should be compared vertically for each temperature.

TABLE 5

PERCENTAGES OF VARIATION IN THE SIZE AND NUMBER OF SCLEROTIA FORMED *IN VITRO*, AS EXPRESSED BY THE TEMPERATURE FACTOR FOR EACH GROUP OF ISOLATES AND PARAMETER

Parameter	*Isolate*			
	S. melongena	*P. vulgaris*	*L. esculentum*	*Mean*
Length	6^{**}	34^{**}	31^{**}	22^{**}
Width	9^{**}	35^{**}	34^{**}	26^{**}
Thickness	7^{**}	25^{**}	22^{**}	18^{**}
Number	1	79^{**}	59^{**}	39^{**}

** Significant difference at $P = 0.01$.

Table 4 shows the length and number of sclerotia formed on PDA (width and thickness were omitted for clarity, and because they were not particularly informative). The percentages of variation expressed by each of the factors (isolate and temperature) are listed in Tables 5 and 6. The variation percentages for sclerotia size were similar, but those relating to number show that formation temperature was the only determinant.

TABLE 6

PERCENTAGES OF VARIATION IN THE SIZE AND NUMBER OF SCLEROTIA FORMED *IN VITRO* AS EXPRESSED BY THE ISOLATE FACTOR FOR EACH TEMPERATURE AND EACH PARAMETER

Parameter	*Temperature*		
	18°C	*25°C*	*Both temperatures*
Length	12**	23**	21**
Width	5**	19**	25**
Thickness	5**	10**	17**
Number	36	79**	20

**Significant difference at $P = 0.01$.

The correlation coefficients between the size and number of sclerotia formed at each of the temperatures are presented in Table 7. The correlation coefficients between number of sclerotia formed and growth rate at both temperatures were non-significant.

TABLE 7

CORRELATION COEFFICIENTS BETWEEN SIZE AND NUMBER OF SCLEROTIA FORMED AT 18 AND 25°C

	Number of sclerotia	
	at 18°C	*at 25°C*
Length	−0.10	−0.37
Width	−0.72*	−0.61
Thickness	−0.74*	−0.78*

*Significant difference at $P = 0.05$.

DISCUSSION

Although the number of isolates in the present work was small, all showed great variability in each of the parameters measured. However, there was no indication of the

existence of groups or categories with common characteristics and the observed variability was not related to isolate origins.

Price and Colhoun (9) reported that the optimum temperature for *in vitro* growth on malt-agar was 20°C, while Khare and Bompeix (4) found that the optimum temperature for sunflower isolates on PDA was 25°C. In the present work, mycelial growth was faster at 25 than at 18°C, with the exception of an isolate obtained from eggplants. For short incubation periods (24 hours) the fungal isolate was the decisive factor, while for longer periods (48 hours) temperature had a greater effect on variability of growth rates.

No differences in sensitivity to the three fungicides were observed between the isolates. All the fungicides were very effective in comparison with the controls, in which mycelia reached the edge of the petri dishes within 48 h. The diameters given in Table 3 were measured after 25 days of growth, as described in Materials and Methods.

The poor control by fungicides for field applications could be the result of additional factors such as penetration efficiency and distribution through the different plant tissues, the timing and conditions of the application, and the type of solution or liquid vehicle employed. Steadman (13) observed that the differences noted between treatments could be attributed to the different application methods rather than to the products.

According to Almeida and Yamashita (2), the LD_{50} of benomyl against *S. sclerotiorum* is 1^{-10} mg l^{-1}, but in the present work it was less than 5 mg l^{-1}. For procymidone, Hisada *et al.* (3) reported an ED_{50} of 1.2 µm. We obtained an ED_{50} of 3 mg l^{-1} for both procymidone and vinclozolin; this is understandable, because both fungicides are dicarboximides and their action mechanisms are similar (11). Other fungicides, such as methyl thiophanate, carboxin and thiabendazole, inhibit mycelial growth. According to Abdou *et al.* (1), thiabendazole is effective *in vitro* at a concentration of 1 mg l^{-1}.

Price and Colhoun (9) found differences in the sizes of sclerotia formed on PDA by different isolates, but they could not relate the variability to the isolate origins. They obtained mean values which ranged between 5.4 [10.6–1.8] × 4.3 [6.4–1.7] mm and 2.9 [4.9–1.6] × 2.6 [4.1–1.5] mm. In the present work, the extreme values ranged from 2.6 × 2.0 × 1.6 [10.3 × 4.4 × 1.5 to 1.0 × 1.0 × 1.0] mm at 25°C, to 4.3 × 3.2 × 2.1 [13.3 × 7.0 × 3.1 to 1.0 × 0.8 × 0.7] mm at 18°C. These ranges are the extreme values of those isolates which produced significantly larger or smaller sclerotia, and not absolute maxima or minima.

Khare and Bompeix (4) found that at higher temperatures sclerotia are smaller and more numerous. In the present work, the sclerotia formed at 25°C also differed significantly in number and size from those formed at 18°C, and moreover, there was a significant negative correlation between the number of sclerotia and their thickness at all temperatures; hence the dictum that smaller numbers mean greater volume. Marukawa *et al.* (6) reported that the optimum temperature for formation of sclerotia on Czapek-Dox agar lies between 22 and 25°C, and on potato extract media — between 15 and 27°C.

There appeared to be no correlation between the growth displayed at both

temperatures at the two time intervals, on the one hand, and sclerotia size and number on the other. The correlation coefficients determined between these parameters were as positive as they were negative, they were always low, and in no case were they significant.

In conclusion, we are in agreement with Price and Colhoun (9), Purdy (10), and other authors that *S. sclerotiorum* is a highly variable species, even though we employed a smaller number of isolates than usual (nine). No categories could be established among the isolates collected in the present *in vitro* study.

The large variability observed in *S. sclerotiorum* suggests that under the usual polyethylene greenhouse conditions of southern Spain there will be a mixture of strains, each with very different characteristics such as growth rate, sclerotia formation, resistance to fungicides, apothecial formation, and spore discharge factors (14). In spite of the wide range of conditions in the greenhouse environment (temperature, relative humidity, illumination, soil characteristics, etc.), it is always possible to find at least one strain perfectly adapted to the conditions of any given moment.

This observed variability also suggests that the results of studies of any strain isolated from any greenhouse environment will provide a good model for *S. sclerotiorum* under these particular conditions, but one must not draw general conclusions from specific data.

ACKNOWLEDGMENTS

The author wishes to thank Mr. R. Moreno for his help with the statistical analyses and for his valuable comments, and Mr. David W. Schofield for his helpful suggestions in editing and translating the manuscript.

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