Effect of Culture Media, Temperature and pH on Growth of *Sphaeropsis Tumefaciens*, Hedges[1,2]

Sol D. Rodríguez, Rocío Rodríguez and Pedro L. Meléndez[3]

ABSTRACT

Of twenty-eight culture media evaluated for *S. tumefaciens* growth and sporulation, Czapek solution agar was the most effective medium for the induction of sporulation. Optimum mycelial growth was observed on citron fruit peel agar, V-8, and citron branch agar 3 days after inoculation at 28° C. However, most media were effective in stimulating mycelial growth after 7 days of incubation. Optimum fungal growth was obtained at 30° to 35° C and at pH 4.0.

INTRODUCTION

Since 1911 *Sphaeropsis tumefaciens* has been known to be the causal organism of knots in lime and orange. Its action as pathogen on other species of *Citrus* was reported by Naylor (3) and Prasad and Bhatangar (4). Beyond this recognition, little is known about the growth requirements of this fungus. Information on the nutritional, acidity and temperature requirements of *S. tumefaciens* may be useful for its laboratory culture as well as for clues leading to better understanding of its distribution and possible control measures.

To meet this objective we studied the behavior of the citron isolate of *S. tumefaciens* cultivated on different culture media and over a range of temperatures and pH.

MATERIALS AND METHODS

The fungus was isolated from diseased citron branches as described by Rodríguez and Meléndez (5) and kept as pure colonies in potato dextrose agar (PDA) petri plates.

EFFECT OF CULTURE MEDIUM ON RADIAL SPREAD AND MORPHOLOGY

Twenty-eight culture media were evaluated, four of which were prepared with citron stems, branches, fruit peel and leaves. The latter four were prepared by adding in a blender 62.5 g of plant tissues to 125 ml of distilled water. The plant tissues thus macerated were filtered through

[1] Manuscript submitted to Editorial Board June 14, 1984.

[2] Part of a thesis submitted to the Graduate School in partial fulfillment of the requirements for the Master of Science degree in Crop Protection. This research was sponsored by Agricultural Experiment Station Project C-486.

[3] Graduate Student, Assistant Phytopathologist; and Phytopathologist, Crop Protection Dept. College of Agricultural Sciences, University of Puerto Rico, Mayagüez, P.R. 00708.

cheesecloth and 2 g of agar was added to the filtrate. All media were autoclaved at 120° C and 15 lb/in^2 for 15 min. The centers of 85 mm diameter plastic petri plates containing approximately 15 cm^3 of the medium were inoculated with 6 mm diameter mycelial disks from 8-day-old culture of *S. tumefaciens* on PDA. Diameter of the colony was recorded after 3, 5 and 7 days of incubation at 28° C. Presence or absence of fructifications and morphology of the colony was visually assessed. Each culture medium was replicated four times in a randomized design. Significance of growth differences was determined by Duncan's Multiple Range Test.

EFFECT OF TEMPERATURE ON RADIAL GROWTH

The center of 85-mm-diameter plastic petri plates containing approximately 15 cm^3 of Czapek solution agar (CZSA) was inoculated with 6-mm-mycelial disks of 8-day old *S. tumefaciens* on CZSA. In a randomized design each of four plates was incubated at 1, 20, 25, 30 and 40° C for 8 days, at the end of which time radial spread was measured. The relation between radial growth and temperature was determined by applying regression analysis and comparing the linear and quadratic equations (6).

EFFECT OF PH ON MYCELIAL GROWTH

Growth was measured by gain of dry weight after 8 days of incubation at 30° C. Four Ehrlenmeyer flasks (125 ml) containing 50 cm^3 each of buffered Czapek dox broth were inoculated with mycelial 6-mm-disks 4-days-old *S. tumefaciens* on CZSA. The pH ranged from 2 to 10. Each desired level was obtained by adding dehydrated Czapek dox broth (Difco)[4] to a previously calibrated buffered solution. Checks were incubated with sterile 6-mm-CZSA disks.

Filter papers (Whatman #3) were previously dried for 24 hr at 70° C, after which time they were put at room temperature in a glass drier for 1-hr, and weighed. To stabilize their weight, papers were kept in the dryer for an additional hour and again weighed. The 8-day-old cultures were filtered through suction at 15 lb/in^2 and weighed as described previously. Mycelial dry weight was obtained from the difference of both measures. Relation between pH and dry weight was determined by comparing linear and quadratic equations of the regression analysis (6). Treatments were arranged in a randomized design.

[4] Trade names in this publication are used only to provide specific information. Mention of a trade name does not constitute a warranty of equipment or materials by the Agricultural Experiment Station of the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

RESULTS AND DISCUSSION

Sphaeropsis tumefaciens grew well on almost all culture media. In some media the lag period was longer, demonstrating a critical growing point during the first 3 days of incubation evidenced by the significant differences detected (table 1). CFPA, V8-A, CBA, OA, CSA, CLA, CCMA and LBA were very effective in stimulating initial radial spread. Although these were not significantly different, the first three culture media stand out for their remarkable influence on this fungus growth. After 3 days of incubation, colonies covered almost all the available plate surface. Later

TABLE 1.—*Growth, differentiation and morphology of* **S. tumefaciens** *on several culture media*

Medium	Key	Radial spread (cm) Days			Colony morphology[1]
		3	5	7	
Citron fruit peel agar	CFPA	8.24 a[2]	8.50 a	8.50 a	Abundant
V-8 juice agar	V8-A	8.18 a	8.50 a	8.50 a	Abundant
Citron branch agar	CBA	8.00 a	8.43 a	8.50 a	Scanty
Oatmeal agar	OA	7.72 ab	8.50 a	8.50 a	Abundant
Citron stem agar	CSA	7.68 ab	8.50 a	8.50 a	Abundant
Citron leaves agar	CLA	7.58 ab	8.50 a	8.50 a	Abundant
Coconut milk agar	CCMA	7.05 abc	8.50 a	8.50 a	Scanty
Lima bean agar	LBA	6.91 abcd	8.50 a	8.50 a	Abundant
Sabouraud dextrose agar	SDA	6.30 bcde	8.50 a	8.50 a	Granular
Litmus milk agar	LMA	6.17 bcde	8.50 a	8.50 a	Compact
Bean pod agar	BPA	6.15 bcde	8.50 a	8.50 a	Abundant
Prune agar	PA	6.08 bcde	8.50 a	8.50 a	Scanty
Potato dextrose agar	PDA	5.94 cde	8.50 a	8.50 a	Abundant
Trychophyton 3 agar	TA	5.81 cde	8.45 a	8.50 a	Compact
Malt extract agar	MEA	5.72 cde	8.50 a	8.50 a	Compact
Antibiotic medium 4 agar	AMA	5.71 cde	8.50 a	8.50 a	Compact
Mycologic agar	MLA	5.39 cde	7.81 abc	8.37 a	Abundant
Corn meal agar	CMA	5.32 de	8.50 a	8.50 a	Compact
Czapek solution agar	CZSA	5.30 de	6.93 abcd	8.50 a	Scanty
Rice agar	RA	5.04 e	8.15 ab	8.37 a	Scanty
Middlebrook 7H10 agar	M7H10A	3.41 f	6.35 cd	6.42 b	Scanty
Water agar[3]	WA	3.38 f	6.69 bcd	7.81 a	Scanty
Chlamidospore agar[3]	CA	3.04 f	5.65 d	5.76 b	Compact
Levine agar[3]	LA	2.97 f	6.63 bcd	7.58 a	Compact
Nutrient agar	NA	2.80 f	7.62 abc	8.50 a	Scanty
Brain heart infusion agar	BHIA	2.70 f	7.67 abc	8.50 a	Compact
Littman oxagall agar	LOA	1.10 g	1.89 e	2.49 c	Compact
Mycobiotic agar[3]	MA	0.00 g	0.00 f	0.00 d	—

[1] Observed after 8 days of incubation.

[2] Means in the same column followed by one or more letters in common do not differ significantly at the 0.01 level.

[3] No sporulation.

growth was somewhat similar on most of the culture media, with maximum possible growth (8.5 cm) at the end of 7 days of incubation. However, colonies growing in M7 H10 and LOA were comparatively smaller, and in MA, this fungus was incapable of getting established.

Colonies growing in OA, SDA, PDA, BPA, CLA, LMA, CZSA and CBA were actively sporulating after 4 days of incubation (fig. 1). However, sporulation was not uniform among replicates of the same medium. In three plates of OA, SDA, LMA and CLA, masses of conidia were observed, whereas in BPA and PDA sporulation occurred in two plates and one plate, respectively.

After 6 days of incubation, colonies growing in CSA, CFPA, PA, CCMA, LBA and V8-A were sporulating. At the end of the trial all colonies developed fructifications, except for those growing in CA. However, weak sporulation was observed in colonies cultivated on NA, LOA and H10 A, and none was detected in colonies on LA and WA.

Table 1 summarizes observations on colony morphology. Broad differences among the colonies were detected. *S. tumefaciens* grew in some culture media producing vigorous and abundant mycelium, whereas in other media growth was compact with mycelia adhering to the surface of the medium. In other media, growth was scanty and thin and only when cultivated in SDA the colony showed a granular appearance. However, these differences are not directly related to the fungus capacity to grow or to sporulate, since actively growing and sporulating cultures are represented in all morphological categories. A positive relationship between radial growth and differentiation was detected. In colonies whose entire surfaces were covered after 7 days of incubation abundant fructifications and active sporulation were observed. Conversely, in colonies showing radical growth, differentiation was weak or absent.

EFFECT OF TEMPERATURE ON RADIAL GROWTH

Optimum temperature for radial growth ranged from 30° to 35° C with colonies totally covering the surface of the plate after 4 days of incubation. A significant relationship between the temperature ranges tested and growth was detected, demonstrating that temperature fluctuations induce changes in the mycelial growth of *S. tumefaciens*. Although visually assessed, changes in the differentiation of this fungus were observed. No sporulation was detected in colonies growing at 15° and 40° C, whereas in the intermediate ranges some differences were observed. Colonies incubated at 30° C were found in active sporulation after the fourth day. However, when incubated at 25° and 35° C they required 6 days to reach the same stage of differentiation, and in those growing at 20° C sporulation was limited to the disk area.

The capacity of this fungus to sporulate is directly related to its capacity to grow. At 30° C the fungus reached its maximum radial spread and was in active sporulation after 4 days of incubation, whereas at 15° and 40° C growth was scanty and no sporulation was observed.

EFFECT OF PH ON DRY WEIGHT

Maximum growth was attained at pH 9; optimum at pH 4. A significant difference between pH levels and mycelial growth was observed. Ingold (2) reported that for most fungi, optimum pH is in the range of pH 5 to 6.5. Decidely the citron isolate of *S. tumefaciens* preferred the acid side. After completion of the trial, pH value of the filtered medium was assessed. The pH values of *S. tumefaciens* medium appeared to vary little before and after fungus incubation as shown below.

At 0 days	*After 8 days*
2	2.3
3	3.2
4	4.3
5	5.1
6	6.2
7	6.8
8	7.6
9	8.5
10	9.3
7[5]	7.0

Variations in the behavior of *S. tumefaciens* induced by changes in its growing environment are evident. These variations involve the type and capability to grow and differentiate. Most of the culture media evaluated are recommended for the isolation of pathogenic fungi, but as was expected, growth and differentiation of this fungus is stimulated by media prepared from host tissues. However, a significant increment in growth was observed in colonies growing in V8-A. It is well known that vitamins are a growth requirement for all living organisms, and fungi are no exception. Apparently, the high vitamin content of the vegetable juice may account for the initial ability of this fungus to grow in this medium.

Hedges and Tenny (1) studied some of the cultural charcteristics of *S. tumefaciens* lime isolate. With slight differences the citron isolate behaved similarly as that from lime. They reported that optimum growth of the lime isolate was detected at 30° C and that minimum and maximum temperatures are 35° and 44°C, respectively. Although we did not deter-

[5] Check.

mine minimum and maximum temperatures for the citron isolate, when comparing our results with those reported by these authors we found that there are no evident physiologic differences between the two isolates.

RESUMEN

Se evaluaron 28 medios de cultivo y se determinó que el agar de la solución Czapek es el más eficaz para inducir la esporulación del hongo *Sphaeropsis tumefaciens*. A los 3 días de iniciada la prueba, el crecimiento micelial óptimo se observó en los medios agar de cáscara de la cidra, agar de jugo V8 y agar de ramas del cidro. El crecimiento óptimo de *S. tumefaciens* se obtuvo entre 30 y 35° C y a un pH de 4.00.

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