Some physiological properties of citrus and noncitrus races of *Geotrichum candidum* isolated from soil in Japan

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Some physiological properties of citrus and noncitrus races of *Geotrichum candidum* isolated from soils of citrus groves and noncitrus fields in Japan were studied. Of 358 isolates of citrus race, 260 isolates required pyridoxine for growth on asparagine-glucose agar, while 98 isolates could grow in the absence of pyridoxine. On the other hand, of 11 isolates of noncitrus race, 10 isolates could grow without pyridoxine, while 1 isolate absolutely required pyridoxine for growth. The citrus race grew well in autoclaved lemon juice at pH 2.2, while the noncitrus race failed to grow. The radial growth rate of the citrus race on potato-dextrose agar was higher than that of the noncitrus race. The two races grew well within the same range of temperature and pH, the optimal temperature ranging between 25 and 30°C, and optimal pH being 6.0. Both races utilized glucose, galactose, xylose, sorbose, sorbitol and mannitol as carbon sources, but could not utilize arabinose, lactose, maltose, rhamnose and sucrose. Arginine and asparagine were the best nitrogen sources for both races, but KNO₃ could not be utilized.

Key Words——citrus race; Geotrichum candidum; noncitrus race; physiological properties.

Geotrichum candidum Link comprises a complex of asexual fungi which are saprophytic and/or pathogenic in various fruits (Butler, 1960; Butler et al., 1965; Tubaki, 1978), and which cause diseases in man and animals (Carmichael, 1957). Isolates of G. candidum from citrus fruit or from soil were pathogenic in various citrus fruits, tomato fruit, cucumber, carrot, netted melon, persimmon and pepper (Aoki, 1992; El-Tobshy and Sinclair, 1965; Kitajima, 1989; Suprapta et al., 1996). A group of isolates of G. candidum from widely separated regions of the world that exhibit pathogenicity on citrus fruit was designated as the citrus race (Butler et al., 1965) and is known as citrus sour rot pathogen. This pathogen has been reported to be an important cause of postharvest losses of citrus fruits (Brown and Eckert, 1988; El-Tobshy and Sinclair, 1965; Hershenhorn et al., 1992; Kuramoto, 1981; Kuramoto and Yamada, 1975; Smith, 1917).

Morphologically, the citrus race of *G. candidum* is indistinguishable from the noncitrus race (Butler et al., 1965) and, therefore, a pathogenicity test on citrus fruit or a physiological approach is commonly applied to distinguish the races of this fungus. Butler et al. (1965) reported that pyridoxine requirement for growth on asparagine-glucose agar (AGA) and growth in autoclaved lemon juice at pH 2.2 or 2.7 could be used to distinguish the citrus and noncitrus races of *G. candidum*. We obtained 369 isolates of *G. candidum* from soils of citrus groves and noncitrus fields of diverse plant species in Japan, of which 358 isolates were pathogenic on citrus fruit and 11 isolates were nonpathogenic on citrus fruit (Suprapta et al., 1995). All isolates were similar in morphological characteristics such as color of colony on potato-dextrose agar (PDA), formation of chains of arthrospores, shape of arthrospores and hyphal dichotomous branching at the periphery of a colony (Suprapta et al., 1995). In this study, cultural experiments were conducted on some physiological properties which could be used to characterize and distinguish the two races.

Materials and Methods

Fungus To examine pyridoxine requirement of citrus and noncitrus races of *G. candidum*, 369 isolates of this fungus from soils of citrus groves and noncitrus fields in Japan were used (Suprapta et al., 1995). Of these, 358 isolates were citrus race (pathogenic on citrus fruit) and 11 isolates were noncitrus race (non-pathogenic on citrus fruit). For other tests, 11 isolates each of citrus and noncitrus races were used (Table 1). All cultures were maintained on PDA slants. Small mycelial pieces were transferred to new PDA slants and incubated at 25° C for 5 d in the dark. Arthrospores from 5-d-old cultures were harvested and put into sterile water, and the suspension was filtered through sterile glass wool to eleminate hyphal fragments. The density of arthrospores was adjusted to 10^7 /ml before use.

Pyridoxine requirement for growth Since pyridoxine

Table 1. Origin of isolates of Geotrichum candidum used.

Isolates	Locality	Substrate/Cultivated plant species
Citrus race		
S7ª)	Kagoshima	Soil, <i>Citrus unshiu</i> Marc.
S31	Ehime	Soil, <i>C. unshiu</i> Marc.
S152	Saga	Soil, <i>C. unshiu</i> Marc.
S161	Saga	Soil, <i>C. unshiu</i> Marc.
S175	Wakayama	Soil, <i>C. unshiu</i> Marc.
S181	Wakayama	Soil, <i>C. unshiu</i> Marc.
Gr3	Saga	Soil, <i>Vitis vinefera</i> L.
Pt3	Saga	Soil, <i>Solanum tuberosum</i> L.
R16	Saga	Soil, Oryza sativa L.
Te2	Shizuoka	Soil, <i>Thea sinensis</i> L.
Tm2	Miyazaki	Soil, Lycopersicon esculentum Mill.
Noncitrus	ace	
Cb4	Ehime	Soil, <i>Brassica oleracea</i> L. var. <i>capitata</i>
lg1	Kumamoto	Soil, Juncus effusus L. var. decipiens Buch
Mm2	Kumamoto	Soil, Prunus mume (Sieb.) Sieb. et Zucc.
R9	Saga	Soil, <i>Oryza sativa</i> L.
R10	Saga	Soil, <i>O. sativa</i> L.
R21	Hiroshima	Soil, <i>O. sativa</i> L.
R22	Hiroshima	Soil, <i>O. sativa</i> L.
S63	Kumamoto	Soil, <i>C. unshiu</i> Marc.
Tm5	Kumamoto	Soil, Lycopersicon esculentum Mill.
W4	Oita	Soil, Triticum aestivum L.
W6	Fukuoka	Soil, <i>T. aestivum</i> L.

a) This isolate is also deposited at American Type Culture Collection (ATCC) with the accession number 200598.

(vitamin B₆) requirement appeared to be useful for distinguishing between the citrus and noncitrus races of G. candidum (Butler et al., 1965), 369 isolates were tested for their requirement of pyridoxine for growth on AGA. The medium was prepared according to the formula developed by Butler et al. (1965), which contained the following (g/L): KH₂PO₄, 1; MgSO₄.7H₂O, 0.5; FeCl₃, 0.001; L-asparagine, 2; glucose, 8; and distilled water to make 1 L. Before autoclaving, 15 g/L agar powder was added to the basal medium, with or without 100 μ g/L of pyridoxine. A 10-ml portion of the medium was transferred and solidified in a Petri dish, then inoculated with arthrospores of G. candidum. Ten plates of each medium (with or without pyridoxine) were inoculated for each isolate by dipping the tip of a pin (1-mm in diam) in arthrospore suspension (107/ml), then pushing the pin into the center of the plate. The cultures were incubated at 25°C for 5 d in the dark and growth was examined by observing the development of colonies on AGA plates.

Pyridoxine requirement was also confirmed in liquid medium for 11 isolates each of citrus and noncitrus races. Fifty milliliters of asparagine glucose basal medium with or without 100 μ g/L pyridoxine was dispensed into 200-ml Erlenmeyer flasks and autoclaved at 121°C for 20 min. Each flask was inoculated with 20 μ l of arthrospore suspension (10⁷/ml) and incubated at 25°C for 5 d in the dark. The growth was examined by ob-

serving the development of a mass of white mycelia in the culture media. Six flasks were inoculated for each isolate.

Growth at various temperatures Optimal temperature for radial growth on PDA was measured for 11 isolates each of the citrus and noncitrus races of *G. candidum* at 10 different temperatures between 5 and 33° C. A 4mm diam mycelial plug from an actively growing culture was put in the center of a PDA plate (pH 6.0), which was incubated at the desired temperature in the dark for 5 d, after which the radial growth was measured. Ten plates were used for each isolate at each temperature.

Radial growth on several media In the test of growth at various temperatures, we observed that the colonies of citrus race isolates were generally larger than those of noncitrus isolates. In this test, the radial growth of 11 isolates each of the citrus and noncitrus races of *G. candidum* was examined on four media, as shown in Table 2. A 10-ml portion of each medium was solidified in a Petri dish, and a 4-mm mycelial plug from an actively growing colony was put in the center of the plate. The Petri dish was incubated at 25°C for 5 d in the dark. Ten plates were used for each isolate. Colony diameter was measured every day.

Growth in autoclaved lemon and orange juices Previous reports stated that the citrus races of *G. candidum* grew well in autoclaved lemon juice at pH 2.2 or 2.7, whereas the noncitrus race failed to grow (Butler et al., 1965). In the present study, 11 isolates each of the citrus and noncitrus races were tested for their ability to grow in autoclaved lemon and orange juices of pH 2.2, 2.4, 2.7, and 3.0.

Lemon or orange juice was collected using a juicer and adjusted to the desired pH with 2 N HCl or 2 N NaOH before autoclaving at 121°C for 5 min. The juice was distributed into Petri dishes (15 ml/dish), then inoculated with 20 μ l of arthrospore suspension (10⁷/ml) and incubated at 25°C for 5 d in the dark. The growth was examined by observing the appearance of slimy and flaky mycelia on the surface of the juice. Five dishes were inoculated for each isolate and each treatment.

Growth at varying pH Isolates of citrus and noncitrus

 Table 2. Radial growth of citrus and noncitrus races of *Geotrichum candidum* on four media^{a)}.

	Mean diameter of colony (mm) ^{b)}											
Race -	PDA	PCA	Sabouraud	MEA								
Citrus race (C)	80.92	58.74	72.20	71.02								
Noncitrus race (NC)	48.02	50.72	52.02	49.74								
C-NC	32.90**	8.02**	* 20.18**	21.28**								

a) Data are means of 110 colonies from 11 isolates.

b) Diameter of colony was measured after 5 d of incubation at 25° C in the dark.

PDA, potato-dextrose agar; PCA, potato-carrot agar; MEA, malt extract agar.

**: Significantly different according to the Paired *t*-Test (P < 0.01).

races of *G. candidum* were inoculated into potato-dextrose broth (PDB) at 8 different pH levels. Broth (50 ml/flask) of a desired pH was distributed into 200-ml Erlenmeyer flasks and autoclaved at 121°C for 20 min. A 20- μ l portion of arthrospore suspension (10⁷/ml) was put into each flask, and the flasks were incubated at 25°C for 5 d in the dark. The mycelia were collected by filtration using pre-weighed Whatman glass microfibre filter paper GF/C and dried at 80°C to constant weight. Five flasks were inoculated for each isolate and each pH value.

Utilization of varying carbon and nitrogen sources Isolates of citrus and noncitrus races of Geotrichum candidum were tested for their ability to utilize various carbon and nitrogen sources, using eleven carbon sources and seven nitrogen sources. The basal medium contained the following (g/L): KH_2PO_4 , 1; $MgSO_4 \cdot 7H_2O$, 0.5; glucose, 8; NH₄NO₃, 1; FeCl₃, 0.001; pyridoxine, 0.0001, and distilled water to make 1 L. A 50-ml portion of medium was dispensed into a 200-ml Erlenmeyer flask. For the carbon utilization test, glucose in the basal medium was replaced with carbon (5 g/L) from a designated carbon source and for the nitrogen utilization test, NH₄NO₃ was replaced with nitrogen (0.5 g/L) from a designated nitrogen source. A 20-µl portion of of arthrospore suspension was transferred into the flask and which was incubated in the dark at 25°C for 5d in the dark. The growth of fungus was determined by measuring the mycelial dry weight.

Results and Discussion

Pyridoxine requirement for growth Of 358 isolates of the citrus race of *G. candidum*, 260 isolates absolutely required pyridoxine for growth on AGA medium, whereas 98 isolates could grow in the absence of pyridoxine. On the other hand, all of the noncitrus race could grow without pyridoxine, except 1 isolate (Tm5, isolated from

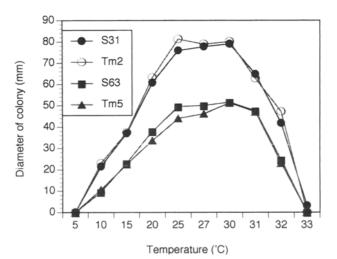


Fig. 1. Growth of *Geotrichum candidum* citrus race (S31 and Tm2) and noncitrus race (S63 and Tm2) at various temperatures on PDA after 5 d in the dark.

the soil of a tomato field). Van-Uden and Carmo-Saosa (1959) reported that two isolates of *G. candidum*, isolated from the intestinal tract of a rabbit and a horse respec-

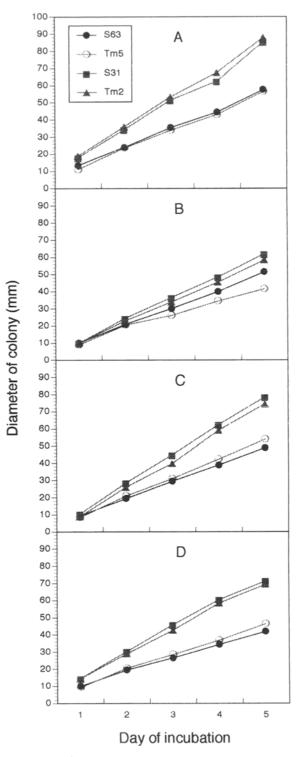


Fig. 2. Radial growth of *Geotrichum candidum* citrus race (S31 and Tm2) and noncitrus race (S63 and Tm5) on PDA (A), PCA (B), Sabouraud's agar (C) and MEA (D), at 25°C in the dark.

tively, required pyridoxine for growth, but their virulence on citrus fruits was not tested. Butler et al. (1965) found that all isolates of the citrus race of *G. candidum* showed an absolute requirement for pyridoxine for growth on AGA, while the noncitrus race could grow in the absence of pyridoxine. In general, our results are in accordance with the findings of Butler et al. (1965), in that most of the citrus race isolates of *G. candidum* required pyridoxine for growth, and the noncitrus races could grow without pyridoxine. Some of the citrus isolates, however, did not require pyridoxine and one isolate of the noncitrus race absolutely required pyridoxine for growth. This result suggests that the use of pyridoxine requirement alone is not appropriate for distinguishing between the citrus and noncitrus races of the fungus.

Growth at varying temperature Both citrus and noncitrus races of *G. candidum* could grow on PDA over a relatively wide range of temperatures. Optimal temperature ranged between 25 and 30°C (Fig. 1). All 22 isolates tested could grow between 10 and 32°C, but no visible growth was observed at 5°C. Five out of 11 isolates of the citrus race showed a trace growth at 33°C, with a colony diameter of 2.8 mm in 5 d, while all 11 isolates of the noncitrus race failed to grow at 33°C.

Van-Uden and Carmo-Saosa (1959) used cultures of *G. candidum* from several substrates, including the intestinal tract of human, cattle, horse, rabbit and sheep, swine embryo, Camembert cheese, Roquefort cheese, kefir grains and yogurt. They found that in liquid medium composed of 2% glucose, 1% peptone and 0.5% yeast extract, *G. candidum* could grow, at a maximum temperature between 35 and 38°C. El-Tobshy and Sinclair (1965) tested one plant isolate and one human isolate of *G. candidum* and found that the optimal temperature for growth was 27°C for the former and 30–31°C for the latter. The plant isolate grew over a wider range

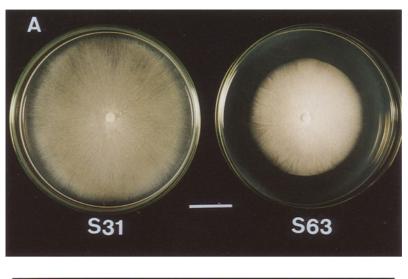




Fig. 3. A: Colonies of *Geotrichum candidum* citrus race (S31) and noncitrus race (S63) on PDA incubated for 5 d at 25°C in the dark. Bar represents 2.5 cm.

B: Growth of *Geotrichum candidum* citrus race (S31) and noncitrus race (S63) in autoclaved lemon juice pH. 2.2, incubated for 5 d at 25°C in the dark.

of temperature than did animal isolate. The maximal temperature for growth of both isolates was 37°C. Although the maximal temperature was slightly different from previous reports (El-Tobshy and Sinclair, 1965; Van-Uden and Carmo-Saosa, 1959), the range of temperatures in which *G. candidum* could grow well were similar to the result of El-Tobshy and Sinclair (1965).

Growth on several media The radial growth rates of citrus race isolates of *G. candidum* were faster than those of noncitrus race isolates on all four solid media tested (Fig. 2). The mean diam of colonies of the citrus race on PDA, PCA, Sabouraud's agar and MEA were significantly higher (P<0.01) than those of the noncitrus race (Table 2). The citrus race isolates generally produced white, flat and relatively sparse colonies, which grew rapidly, while the noncitrus isolates grew slowly, producing white and dense colonies of powdery appearance (Fig. 3A). The results suggested that the radial growth on PDA can be used as a criterion for distinguishing between the citrus and noncitrus races of *G. candidum*.

Growth in autoclaved lemon and orange juice All of the 11 isolates of the citrus race of *G. candidum* grew well in autoclaved lemon and orange juice at pH 2.2, 2.4, 2.7

Table 3. Growth of citrus and noncitrus races of *Geotrichum* candidum in autoclaved lemon and orange juices at various pHs.

Isolates	Len	non ju	lice at	рH	Orange juice at pH					
isolates	2.2	2.4	2.7	3.0	2.2	2.4	2.7	3.0		
Citrus race	_									
S7	+	+	+	+	+	+	+	+		
S31	+	+	. +	+	+	+	+	+		
S152	+	+	+	+	+	+	+	+		
S161	+	+	+	+	+	+	+	+		
S175	+	+	+	+	+	+	-+-	+		
S181	+	+	+	+	+	+	+	+		
Gr3	+	+	+	+	+	+	+	+		
Pt3	+	+	+	+	+	+	+	+		
R16	+	+	+	+	+	+	+	+		
Te2	+	+	+	+	+	+	+	+		
Tm2	+	+	+	+	+	+	+	+		
Noncitrus race										
Cb4	-	+	+	+	+	+	+	+		
lg1	-	-		+	-	+	+	+		
Mm2	_		_	+	+	+	+	+		
R9	_	-	-	+	+	+	+	+		
R10		_	+	+	+	+	+	+		
R21	_	_	+	+	+	+	+	+		
R22	—	_	-	+	-	+	+	+		
S63	_	+	+	+	+	+	+	+		
Tm5	-	—		+	_	_	+	+		
W4	_	-	_	+	-	_	+	+		
W6	_	—	_	+	_	_	+	+		

-: No growth was observed; +: Grew with flaky and slimy mycelia.

and 3.0 (Table 3). The mycelium of the citrus race was slimy with a flaky appearance as shown in Fig. 2B. On the other hand, all of the noncitrus isolates failed to grow in lemon juice at pH 2.2, but grew well in lemon juice at pH 3.0. This result agreed with previous report (Butler et al., 1965). Two isolates of the noncitrus race (S63 and Cb4) could grow in lemon juice at pH 2.4, and four isolates (S63, Cb4, R10 and R21) at pH 2.7. The result suggested that the growth of *G. candidum* in autoclaved lemon juice at pH 2.4, 2.7 and 3.0 to some extents could not be used as a distinguishing criterion.

All isolates of the citrus race tested grew well in autoclaved orange juice at any pH tested (Table 3). Some of the noncitrus race isolates failed to grow in orange juice at pH 2.2 or 2.4. The failure of the noncitrus race to grow in lemon and orange juices at pH 2.2 and 2.4 is probably due to the effect of the low pH on certain constituents of citrus juice, which act as inhibitors to the fungal growth.

Growth in PDB at various pH Geotrichum candidum could grow over a relatively wide range of pH (between pH 2.0 and 9.0) in PDB. All isolates tested grew well at pH 4.0 though pH 7.0, with maximal growth at pH 6.0 (Fig. 4). At pH 2.0, however, all the isolates showed only a trace of growth. Butler et al. (1965) found that all isolates of G. candidum grew well in PDB at pH 2.7 in 72 h, while most isolates of either citrus or noncitrus race, did not grow or showed only a trace of growth at pH 2.2. The ability of G. candidum to grow over such a wide range of pH probably allows it to survive in diverse habitats, as suggested by previous reports (Butler, 1960; Carmichael, 1957; Suprapta et al., 1995; Tubaki, 1978). Carbon and nitrogen sources utilization The citrus and the noncitrus races of G. candidum were similar in respect to the utilization of carbon and nitrogen sources. All isolates could utilize glucose, galactose, xylose, sorbose, sorbitol and mannitol (Table 4), the first three being utilized better than the others, but could not utilize

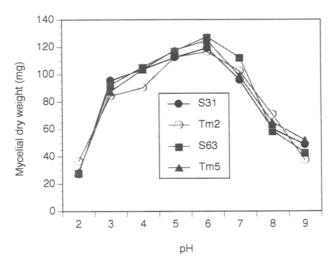


Fig. 4. Growth of *Geotrichum candidum* citrus race (S31 and Tm2) and noncitrus race (S63 and Tm5) in PDB at various pHs after 5 d at 25°C in the dark.

		Cargon sources										Nitrogen sources						
Isolate	Arabinose	Galactose	Glucose	Lactose	Maltose	Mannitol	Rhamnose	Sorbitol	Sorbose	Sucrose	Xylose	KNO ₃	NH₄NO ₃	(NH4) ₂ SO ₄	NH4H2PO4	(NH ₄) ₂ -tartrate	Arginine	Asparagine
Citrus race																		
S7		++	+++	_	_	+	_	+	+++	_	+++	_	++	++	++	++	+++	+++
S31	-	+++	+++	_		+	_	++	++	_	+++		++	++	++	++	+++	+++
S152		+++	+++	_	_	+	_	+	++		+++	_	++	++	++	┿╋╊	+++	+++
S161	—	+++	+++		_	+	_	+	++	_	+++		++	+	++	++	+++	+++
S175	—	╋╋	+++	—		+	_	+	+	_	+++	_	++	++	++	++	+++	+++
S181	—	+++	+++	—		++	_	++	++	_	+++	-	+	++	++	++	+++	+++
Gr3		╋╋╋	+++		—	++	_	+	++	-	+++	—	++	++	+	+++	+++	+++
Pt3	-	+++	┼┼┾	_	_	+		+	+	_	╆┾┿	—	++	++	++	++	+++	+++
R16	_	+++	+++		—	+	—	+	+++	_	+++	_	++	+	++	++	+++	+++
Te2	-	+++	+++		_	++	_	+	++	_	+++	<u></u>	++	++	++	++	+++	+++
Tm2	-	+++	+++	-	-	+	_	++	+++	_	+++		++	++	++	++	+++	+++
Non-citrus ra	ice																	
Cb4		┾┾┾	+++	—	-	+	—	+	++	—	┾┾┼	—	++	++	++	+++	+++	+++
lg1	-	++	} } ∤	_	-	+	-	+	++	_	+++	—	++	++	++	++	+++	┾┾┾
Mm2	_	+++	+++	_	-	+	_	+	++	_	+++	_	++	+	++	++	+++	+++
R9	_	+++	++++		-	+	_	+	+	_	++	-	++	++	+	++	+++	+++
R10	_	++	++++	_		+	_	+	+	-	++	-	++	++	++	++	+++	+++
R21	_	┾┾┾	+++	_	_	+	—	+	+	_	┾┿┿	-	+	++	++	++	+++	+++
R22	-	+++	↓ ↓↓	_	_	+		+	+	_	+++	_	÷	++	++	++	+++	+++
S63	-	+++	+++	_	_	+	-	++	┿┾┼	_	+++	_	++	++	+	++	+++	+++
Tm5	-	+++	+++		_	+	_	+	++	_	+++		++	++	++	++	+++	+++
W4	_	++	+++	_	_	+	-	+	+	_	++	-	++	++	++	↓ ₩	+++	+++
W6	_	++	+++	_		+	—	+	+		++	_	++	++	++	++	+++	+++

Table 4. Ability of Geotrichum candidum citrus and noncitrus races to utilize carbon and nitrogen from varying sources.

-: No growth; +: mycelial dry weight: 1-50 mg; ++: 51-100 mg; +++: more than 100 mg.

arabinose, lactose, maltose, rhamnose and sucrose. Carmo-Sousa (1959) reported that all isolates of *G. candidum* tested utilized glucose, galactose, L-sorbose, D-xylose, D-mannitol and D-sorbitol, but did not utilize sucrose, maltose, lactose, L-arabinose, L-rhamnose and dulcitol.

All isolates utilized asparagine, arginine, $(NH_4)_2$ -tartrate, $NH_4H_2PO_4$, $(NH_4)_2SO_4$, and NH_4NO_3 for growth, but did not utilize KNO_3. Asparagine and arginine were utilized better than other nitrogen sources (Table 4). No report is available on the utilization of nitrogen sources by *G. candidum*. This information probably can be used as a physiological criterion to characterize *G. candidum*.

Results of the present study suggested that the citrus race of *G. candidum* is similar to the noncitrus race in respect to some physiological properties, such as temperature for growth, pH for growth and utilization of carbon and nitrogen sources. The two races, however, showed a different ability to grow in autoclaved lemon juice at pH 2.2 and different growth rates and colony appearance on PDA, which could be employed as criteria for differentiating the races of *G. candidum*.

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