Influence of Nutrition on the Formation of Basidia and Basidiospores in *Thanatephorus cucumeris*

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ABSTRACT

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Isolates of *Thanatephorus cucumeris* and related fungi were grown on agar media in two-compartment petri plates. One compartment of each plate contained an inoculated growth medium. After 2–3 days, mycelium grew into the other compartment, which contained a sporulation medium. Amounts of $NaNO_3$ and glucose were varied primarily in the growth medium and to a limited extent in the sporulation medium. The optimum levels of $NaNO_3$ and glucose for inducing the perfect state were defined for several isolates belonging to anastomosis groups (AG) AG-1 and AG-4, and one isolate of AG-5. The nutritional conditions favoring the formation of basidia by isolates in AG-1 and AG-4 were the initial level of nitrogen and glucose in the growth medium and growth from this medium onto a medium very low in nutrients. Formation of basidia on the sporulation medium was greatly enhanced by solidifying it with Difco Bacto agar rather than Difco purified agar. A water-soluble factor present in Bacto agar stimulated basidial formation when added to the sporulation medium solidified with purified agar. No isolate of AG-2, AG-3, or other *Rhizoctonia* or *Sclerotium* species sporulated on agar media.

Additional key words: Rhizoctonia solani, Ceratobasidium cornigerum.

The reliable production of basidia and basidiospores of *Thanatephorus cucumeris* (Frank) Donk (anamorph: *Rhizoctonia solani* Kühn) in culture is essential for the understanding of the genetic basis of pathogenicity, environmental adaptation, sexuality, discerning the process of heterokaryosis, the relationship to other species, and the relationships within and among anastomosis groups. In addition, it would enhance understanding of the epidemiology of foliar disease (web-blight).

Two methods have been commonly employed to induce sporulation. One method involves transferring the fungus from agar medium high in nutrients to agar medium relatively poor in nutrients (eg, 2% water agar [7,9,11,23,27], soil extract agar [3,8,14], Coon's medium [10], and plant decoctions [19,21]). The other method involves placing moist pasteurized soil on a culture growing in a petri plate (13,21,22,24,25). Both methods share in common a well-hydrated substrate and a transition from a medium high in nutrients on which mycelium grows to a low-nutrient substrate on which sporulation occurs. The success of these methods has been dependent on the particular isolates used. For nearly all other isolates the methods are unpredictable and results seldom repeatable.

This study concerns the influence of nutrition on sporulation. We first compared isolate 239 (AG-1), which fruits readily on water agar, to isolate 610 (AG-4), which is more fastidious in its requirements. The influence of nutrients on the formation of the sexual state by these isolates on agar media was determined and the

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optimum conditions for fruiting were defined. Other isolates from these groups were then tested under the optimum conditions to determine if isolates within a group responded similarly to similar nutritional conditions. We also examined the nutritional requirements for sporulation of isolates from all anastomosis groups, as well as fungi similar to *R. solani.* These latter fungi included binucleate species of *Rhizoctonia* considered to have the sexual state *Ceratobasidium cornigerum* (Bourd.) Rogers, as well as *Rhizoctonia oryzae* Ryker and Gooch, *Rhizoctonia oryzae-sativa* Sawada, *Rhizoctonia endophytica* Saksena and Vaartaja, *Sclerotium hydrophilum* Sacc., *Waitea circinata* Warcup and Talbot, and miscellaneous isolates of uncertain affinity.

MATERIALS AND METHODS

Sixty-one isolates of *R. solani* were studied; these are listed by anastomosis groups (AG) as follows:

AG-1: 43, 65, 189, 199, 239, 245, 272, 309, 311, 316, 404, 434, 465, 466, 539, and 658.

AG-2: 289, 455, 457, 460, 472, 480, and 512.

AG-3: 141, 293, 423, and 624.

AG-4: 31, 41, 93, 107–110, 113, 132, 142, 153, 195, 208, 219, 223, 233, 250, 266, 281–286, 320, 436, and 608–610.

AG-5: 440, 441, 462, 463, and 464.

The hosts, geographic origins, and sources of 25 of the above isolates are given in Table 1, and similar information on the remaining isolates is in a previously published table (1). Isolates were maintained on potato-dextrose agar (PDA) prior to transfer onto synthetic media. The synthetic media utilized to investigate the influence of nutrition on fruiting contain the following: K_2HPO_4, 1.0 g; KH_2PO_4, 0.78 g; $MgSO_4 \cdot 7H_2O$, 0.75 g; $CaCl_2 \cdot 2H_2O$, 37.0 mg; $FeCl_3 \cdot 6H_2O$, 1.0 mg; $ZnSO_4 \cdot 7H_2O$, 0.9 mg; $CuSO_4 \cdot 5H_2O$, 0.8 mg; $Na_2MoO_4 \cdot H_2O$, 0.3 mg; thiamine-HCl, 0.15 mg; Difco Bacto agar, 20 g; distilled water, 1,000 ml (26), and varying amounts of $NaNO_3$ and D-glucose. When the last two compounds were present, making a complete medium, we refer to it as glucose-nitrate agar (GNA); without $NaNO_3$ and D-glucose it is referred to as salts-thiamine agar (STA).

All experiments were carried out in petri plates partitioned into two compartments. One compartment of each plate contained a medium for growth, 20 ml of GNA, which was inoculated. After 2–3 days mycelium grew into the other compartment, which contained the medium for sporulation, 10 ml of STA (Fig. 1). At this time, the lids were removed and the plates placed randomly, with respect to treatment, in 3-L closed plastic boxes (26 × 19 × 6 cm), 15 plates per box. Air, at 99% RH (2), was passed through these chambers at a rate of 1.0 L per min to prevent buildup of respiratory CO_2 (2). After 10 days, cultures were examined for basidia. If basidia were present cultures were inverted over glycerol-sorbose agar (GS) (16) and incubated for 24 hr to allow for discharge of basidiospores.

The GS medium restricted growth of colonies enabling the counting of individual colonies when few spores were discharged onto the medium. When many spores fell, the outline of the deposit was traced onto paper and the area, in square millimeters, measured with a compensating polar planimeter. The number of spores per square millimeter was estimated by counting the number of spores in 10 microscope fields, each 0.196 mm^2, with a Zeiss Epi-illuminator at ×256. The mean number of spores per square millimeter was multiplied by the total area on to which spores dropped to obtain the total number of spores per plate. This quantitative measurement of fruiting was used to compare treatment effects. The spore counts from 10 plates of a treatment were compared to those of other treatments by analysis of variance and Duncan's multiple range test.

Nutrition studies. To investigate the influence of nitrogen and carbohydrate concentration on formation of basidia, the medium for growth (GNA) was prepared with different levels of $NaNO_3$ and glucose. The following formulas of GNA (grams of $NaNO_3$/grams of glucose per liter) were tested with isolates 239 (AG-1) and 610 (AG-4): 6/20, 3/20, 1.0/20, 0.5/20, 0.25/20; 6/10, 1.0/10, 0.25/10; 6/5, 1.0/5, 0.25/5; 6/1.0, 1.0/1.0, and 0.25/1.0. The composition of STA, the medium for sporulation, was held constant. To examine group-specific responses the medium that induced the optimum sporulation of isolate 239 was subsequently inoculated with other isolates of AG-1 and likewise that of isolate 610 with other AG-4 isolates.

Isolates representative of all AGs and related fungi (Table 2) were also studied. The growth medium was varied as follows (grams of $NaNO_3$ and grams of glucose per liter): 6/10, 3/10,

TABLE 1. Isolates of *Rhizoctonia solani* used in a study of nutritional requirements for in vitro basidia and basidiospore formation by *Thanatephorus cucumeris*

Isolate	AG[a]	Host	Geographic origin	Source or previous reference[b]
272	1	*Oryza sativa* L.	Louisiana	Parmeter et al (15), S257
311	1	*Lespedeza stipulacea* Maxim.	N. Carolina	Parmeter
316	1	*Oryza sativa* L.	Louisiana	Parmeter
434	1	*O. sativa*	Louisiana	Parmeter
539	1	*O. sativa*	Arkansas	Butler
658	1	*Macroptilium atropurpureum* (DC) Urb.	Florida	Adams
512	2	*Beta vulgaris* L.	Colorado	Adams
423	3	*Solanum tuberosum* L.	New Zealand	Butler
624	3	*S. tuberosum*	California	Adams
93	4	*Beta vulgaris* L.	Australia	Parmeter et al (15), C93
109	4	*Pinus resinosa* Ait.	Canada	Bolkan and Butler (4), 109
153	4	(unknown)	(unknown)	ATCC 14011 (15)
195	4	*Phaseolus vulgaris* L.	England	CMI 34886 (15)
208	4	*Aster* sp.	California	Bolkan and Butler (4), 208
219	4	(unknown)	(unknown)	Parmeter et al (15), C219
223	4	*Aster* sp.	California	Parmeter
233	4	*Pinus ponderosa* Dougl.	California	Parmeter et al (15), C233
250	4	*Gossypium hirsutum* L.	California	Parmeter et al (15), C250
266	4	*Hydrangea hortensii* DC	(unknown)	Parmeter et al (15), C266
281	4	*Medicago sativa* L.	N. Carolina	Dodman et al (6), 15
282	4	Conifer	California	Parmeter et al (15), C282
320	4	*Pinus palustris* Mill.	N. Carolina	Parmeter et al (15), S276
608	4	*Beta* sp.	England	Anderson et al (3), 42
609	4	*Medicago sativa* L.	Minnesota	Anderson et al (3), 140
610	4	*M. sativa*	Minnesota	Anderson et al (3), 146

[a] Anastomosis group.
[b] Abbreviations: ATCC = American Type Culture Collection; CMI = Commonwealth Mycological Institute Collection.

0.5/10; 0.25/20, 0.25/10, 0.25/5, 0.25/2.5, and 0.25/1.25. In addition, the sporulation medium was varied (grams of $NaNO_3$/grams of glucose per liter): 0/0; 0.03/0.25; 0.06/0.5; and 0.12/1.0.

Influence of agar on sporulation. Sporulation occurred when the sporulation medium (STA) was made with Bacto agar, but not when made with Difco purified agar (Lots 674909 and 590945, respectively, Difco Laboratories, P. O. Box 1058A, Detroit, MI 48232). To determine the nature of the factor in Bacto agar that promoted formation of basidia, the agar was ashed and the ash was added to the sporulation medium. Two methods were used: either by igniting a suspension (w/w) of 1 part of agar to 2 parts of 95% ethanol (low-temperature ash) or heating the low-temperature ash in a muffle furnace for 12 hr at 550 C (high-temperature ash). Ash was added to STA made with 2% purified agar in aliquots equivalent by weight to 10, 20, 30, and 40 g Bacto agar per liter. Also, Bacto agar was leached by soaking 100 g of agar in 1 L of glass distilled water (gdw) for 7 days at 4 C. The leachate was filtered through a series of filter papers and added in place of distilled water to STA made with 2% purified agar. One portion of the leached agar was rinsed with gdw, dried, and used to solidify STA at 2%; another portion was soaked and leached again, rinsed, dried, and used to solidify STA at 2%.

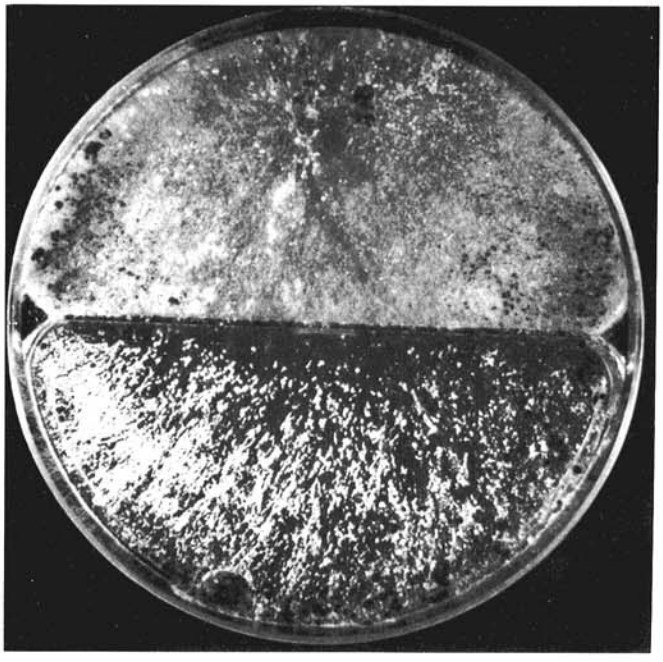

Fig. 1. Fruiting of *Thanatephorus cucumeris* in the lower compartment of a partitioned petri plate. The growth medium in the upper compartment was inoculated with isolate 239. The lower compartment, onto which the mycelium grew, contained a sporulation medium.

It was hypothesized that organic impurities would probably act as trace sources of carbohydrate or nitrogen in Bacto agar. Therefore, 0.03, 0.06, and 0.12 g of $NaNO_3$ and 0.03, 0.06, 0.12, and 0.24 g glucose per liter were added in separate tests to STA made with 2% purified agar. The number of basidiospores produced on sporulation medium of the foregoing treatments was compared to sporulation on STA made with 2% Bacto agar and STA made with 2% purified agar. The growth medium contained 0.25 g of $NaNO_3$ and 20 g of glucose per liter, solidified with 2% Bacto agar, and was inoculated with isolate 610. Ten plates were inoculated for each test.

RESULTS

Nutrition studies. *T. cucumeris* isolate 610 (AG-4) formed basidia and basidiospores on STA after growth on GNA containing less than 0.5 g of $NaNO_3$ per liter. Sporulation was

TABLE 2. Isolates of miscellaneous basidiomycetous fungi similar to *Rhizoctonia solani* used in a study of nutritional requirements for in vitro basidia and basidiospore formation by *Thanatephorus cucumeris*

Nuclear status	Isolate identity	Host	Geographic origin	Source or previous reference[a]
Binucleate				
147	*Rhizoctonia endophytica* var. *filicata* Sak and Vaar.	Conifer	Canada	ATCC 14014
155	*R. endophytica* var. *filicata*	Conifer	Canada	CBS 261.60
519	*Rhizoctonia oryzae-sativa* Sawada	*Oryza sativa L.*	California	Adams
524	*R. oryzae-sativa*	*O. sativa*	California	Adams
587	*Rhizoctonia* sp.	(unknown)	(unknown)	Richter and Sneider (17), E4
601	Anastomosis group 1	Festuca	Pennsylvania	Burpee et al (5), Bn-20
602	Anastomosis group 2	(unknown)	(unknown)	ATCC 34969 (5)
603	Anastomosis group 3	*Juniperus conferte* Perle	N. Carolina	Burpee et al (5), Bn-69
604	Anastomosis group 4	*Glycine* sp.	Georgia	Burpee et al (5), Bn-38
605	Anastomosis group 5	*Taxus* sp.	Rhode Island	Burpee et al (5), Bn-49
606	Anastomosis group 6	*Erigeron* sp.	(unknown)	ATCC 13247 (5)
607	Anastomosis group 7	*Pittosporum* sp.	Florida	FTCC 585 (5)
672	*Rhizoctonia* sp.	*Potamageton nodosus* Poir	California	Adams
595	*Sclerotium hydrophilum* Sacc.	(unknown)	Georgia	Butler
Trinucleate				
232	*Rhizoctonia* sp.	(unknown)	California	Parmeter
256	*Rhizoctonia* sp.	*Phaseolus* sp.	Mexico	Parmeter
445	*Rhizoctonia* sp.	*Medicago sativa* L.	Oman	Butler
Multinucleate isolates				
60	*Rhizoctonia* sp.	(unknown)	(unknown)	Butler
143	*Sclerotium* sp.	Soil	United States	ATCC 14008
538	*Rhizoctonia oryzae* Ryker and Gooch	*Oryza sativa* L.	Arkansas	Butler
543	*R. oryzae*	*O. sativa*	Arkansas	Butler
241	*Waitea circinata* Warcup and Talbot	Soil	Australia	CBS 447.63

[a] Abbreviations: ATCC = American Type Culture Collection; FTCC = Florida Type Culture Collection; and CBS = Centraalbureau voor Schimmelcultures.

sparse or absent with 0.5 g, abundant with 0.25–0.15 g, and sparse or absent with <0.10 g of $NaNO_3$ per liter (Fig. 2). This was in marked contrast to isolate 239 (AG-1), which produced the greatest numbers of basidiospores after growth on GNA containing 6 g of $NaNO_3$ per liter, the highest level tested (Fig. 2). If GNA contained less than 1.0 g of $NaNO_3$ per liter, isolate 239 usually did not fruit. However, if the sporulation medium shrank from evaporative loss of water, abundant sporulation occurred when GNA contained less than 1.0 g of $NaNO_3$ per liter (2). Data representing the influence of $NaNO_3$ on sporulation of isolate 239 are presented in a bar graph (Fig. 2) although a linear response ($y = 4.64 + 11.58x$, $r = 0.73$) to increased $NaNO_3$ would be evident if data of markedly dried plates had been excluded. The linear relationship was complicated by nonuniform drying (2). Levels of $NaNO_3$ greater than 6 g/L were not tested because isolate 239 overgrew the plates at 6 g/L and sporulated on the walls of the chambers with high RH.

Within the range of glucose levels tested, sporulation of isolate 610 after growth on GNA with 0.25 g of $NaNO_3$ per liter was not markedly affected by the glucose level in GNA. However, sporulation was optimum when GNA contained 20 g of glucose per liter. Isolate 239 fruited best when GNA contained high levels of $NaNO_3$ regardless of the different glucose levels, but 10–20 g of glucose per liter was optimal. Isolate 239 sporulated on the growth medium when the growth medium contained glucose levels <1 g of glucose per liter.

Six of 16 isolates of AG-1 fruited and all six fruited in 7–10 days. Five fruited optimally on STA after growth on GNA containing 6 g $NaNO_3$ and 10–20 g of glucose per liter (numbers 43, 65, 189, 239, and 272). These five isolates form small discrete almost spherical sclerotia on PDA and match Sherwood's (18) description of *R. solani* AG-1 type 3. A sixth isolate (309) sporulated sparsely on STA after growth on GNA with 3 g of $NaNO_3$ and 10 g of glucose per liter. Also, it sporulated on growth medium that contained 0.25 g of $NaNO_3$ and 1.25 g of glucose per liter rather than on the adjacent sporulation medium. This isolate is darkly pigmented and unlike many other AG-1 isolates, forms sclerotia under the agar surface of PDA. The remaining 10 isolates (isolates 199, 245, 311, 316, 404, 434, 465, 466, 539, and 658) did not sporulate. Isolates 404, 434, 539, and 658 characteristically form large sclerotia and cause rice sheath blight (ie, AG-1 "sasakiitype" [13]).

Fifteen of 29 isolates of AG-4 fruited in 7–10 days when GNA contained 0.25 g of $NaNO_3$ and 20 g of glucose per liter. These isolates were 41, 93, 107, 108, 109, 110, 113, 142, 195, 233, 281, 282, 608, 609, and 610. Most of the 14 other isolates that did not fruit on this medium either were darkly pigmented or formed sclerotia on STA (Fig. 3). Decreasing $NaNO_3$ in GNA to 0.125 g / L decreased the pigmentation, but did not induce sporulation. However, several of the darkly pigmented AG-4 isolates fruited on other media. Isolates 266 and 285 sporulated on STA when grown on GNA containing 3 g of $NaNO_3$ and 10 g of glucose per liter, a nitrogen level that was prohibitively high for fruiting of the majority of AG-4 isolates. Isolate 285 fruited on growth medium containing 0.25 g of $NaNO_3$ and 5 g of glucose per liter, rather than on the sporulation medium, but the process required 40 days. Isolate 283 fruited after growth on 0.25 g of $NaNO_3$ and 5 g of glucose per liter. Isolates 436 and 107 grown on GNA with nitrogen and glucose levels of 3 g of $NaNO_3$ and 10 g of glucose per liter, and 1.0 g of $NaNO_3$ and 10 g of glucose per liter, respectively, fruited only on sporulation medium containing 0.03 g of $NaNO_3$ and 0.25 g of glucose per liter.

Isolate 441 anastomosis group 5 was induced to sporulate on STA after growth on GNA with 0.25 g of $NaNO_3$ and 10–20 g of glucose per liter, similar to levels required by most AG-4 isolates.

An atypical isolate, 232 (Table 2), with trinucleate and occasionally binucleate cells fruited on STA after growth on media with nutrient levels similar to those required by most AG-4 isolates. Heterokaryons formed when basidiospores of 232 were paired with those of isolate 610 (Ag-4), proving that isolate 232 belongs to AG-4. No *R. solani* isolates from AG-3, AG-2 (AG-2 type 1 [13], and AG-2 type 2 [13]), or related fungi (Table 2) fruited on any of the media.

Influence of agar on sporulation. Sporulation of isolate 610 (AG-4) always was abundant on Bacto agar, but often no fruiting occurred on Difco purified or Noble agars. Fortunately, different lots of Bacto agar did not vary significantly. Ashing Bacto agar and amending purified agar with the residue did not stimulate fruiting comparable to Bacto agar, whatever the type or quantity of ash added. Apparently, both ashing procedures destroyed the stimulating factor. Soaking and rinsing Bacto agar once before use

TABLE 3. Nutritional effects of kind of agar used in a salts-thiamine sporulation medium on basidiospore formation in 24 hr by *Thanatephorus cucumeris* isolate 610 (AG-4), in response to the type of agar used in the sporulation medium (salts-thiamine medium)

Amendments[a]	Average total spores $\times 10^4$ discharged per plate[b]
2% Difco Bacto agar	49.0 A
2% Difco Purified agar plus leachate from Bacto agar	17.3 AB
2% Difco Bacto agar leached with water once	5.8 BC
2% Difco Bacto agar leached with water twice	0 C
2% Difco purified agar	0 C

[a] Agars were added to the salts-thiamine medium at 2%.
[b] Means of 10 replications. Means followed by different letters in the same column are significantly different, $P = 0.05$, according to Duncan's multiple range test. $F = 13.5^{**}$.

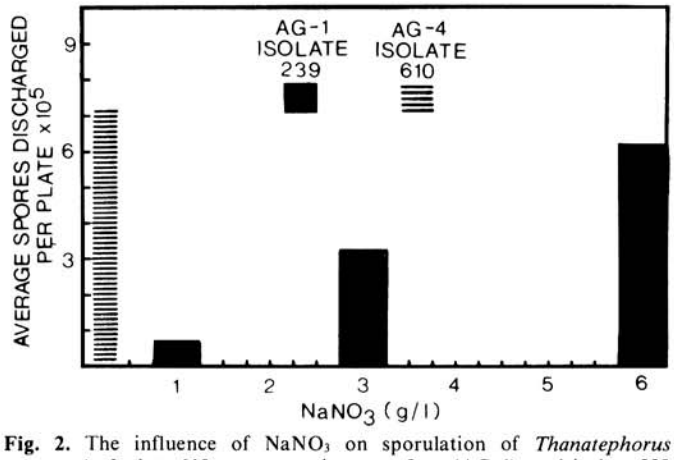

Fig. 2. The influence of $NaNO_3$ on sporulation of *Thanatephorus cucumeris*. Isolate 610, anastomosis group four (AG-4), and isolate 239 (AG-1) were grown on media containing 20 g of glucose per liter and different amounts of $NaNO_3$. Isolates grew from these media onto a sporulation medium where sporulation occurred if nutrient conditions had been favorable. The mean numbers of spores discharged in 24 hr were estimated for 10 cultures per isolate for each treatment.

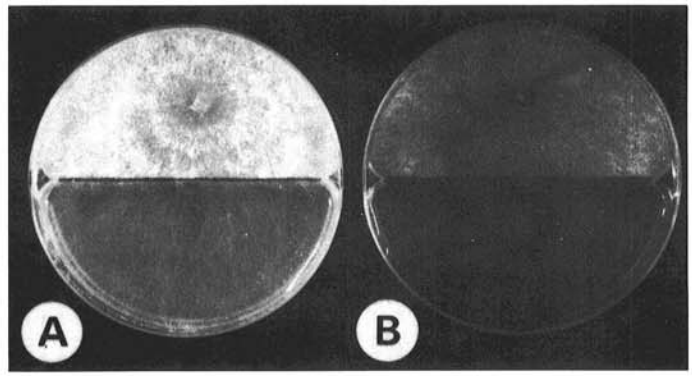

Fig. 3. *Thanatephorus cucumeris*, AG-4, was cultured in partitioned plates on a growth medium containing 0.25 g of $NaNO_3$ and 20 g of glucose per liter in the upper compartment. Isolates grew from this medium onto a sporulation medium in the lower compartment. **A,** Isolates that sporulated were lightly pigmented. **B,** Nonsporulating isolates characteristically were darkly pigmented.

partially removed the sporulation stimulating factor; soaking and rinsing Bacto agar twice caused it to become as ineffective in inducing formation of basidia as purified agar (Table 3). However, the leachate contained some of the stimulatory factor. The stimulatory factor was water soluble, but recovery by leaching was poor.

No fruiting occurred either on STA made with 2% purified agar or on STA made with purified agar and amended with trace quantities of glucose or $NaNO_3$. The amendments reduced sporulation when added to STA solidified with 2% Bacto agar.

The use of STA as a sporulation medium seemed to induce more sporulation than the use of water agar when both media had been made with 2% Bacto agar; however, test results were inconsistent STA made with 2% purified agar was not better than water agar made with 2% purified agar.

DISCUSSION

The methods developed in this study have defined the nutritional requirements for inducing sporulation of many isolates of *T. cucumeris*. The use of a synthetic medium and the partitioned petri plate helped considerably in showing the importance of nitrogen nutrition. Hymenium formation in AG-1 and AG-4 isolates was more sensitive to the level of nitrogen in the medium than carbohydrate. Another important factor was the nutrient step-down from a complete medium supporting vigorous growth to a medium devoid of carbon or nitrogen sources, a transition facilitated by the partitioned plate. Such a step-down in nutrition triggers sexual differentiation in many fungi. As early as 1928, Leonian (12) stated that "a transfer from a solution of high food concentration to a greatly dilute solution induced a maximum reproduction of perithecia and suppressed the development of pycnidia" in *Valsa leucostoma*.

J. B. Sinclair (20) wrote that techniques for inducing sporulation at will may have to be developed for particular isolates of *R. solani*. In this regard, we have discovered group-specific differences in requirements for sporulation between AG-1 and AG-4 in CO_2 tolerance and substrate drying (2). Most AG-4 isolates also exhibited a distinct nitrogen level that controlled subsequent fruiting, in contrast to AG-1 isolates. Also, AG-1 isolates differed from AG-4 in response to low levels of carbohydrate. However, intragroup differences were common. Cultures of AG-4 isolates 266, 283, 285, and 436 and AG-1 isolate 309 each had different nutritional requirements for fruiting. Hopefully, most nutritional and environmental requirements for fructification will be similar for groups and subgroups of *R. solani* rather than different for each individual isolate.

Previous investigations have succeeded in inducing sporulation of AG-1 and AG-4, but not AG-2 or AG-3 isolates on agar media (10,27). However, this is the first report of the sporulation of an isolate of AG-5 on agar. Isolates of AG-4 and AG-5 had similar nutritional requirements for fruiting, and perhaps other similarities between AG-4 and AG-5 may become evident on further investigation. Also, differences in sporulation requirements within an anastomosis group may reflect genetic divergence. We have noted some apparent barriers to interfertility in AG-1. Single basidiospore isolates derived from parent cultures 239 and 309 do not appear to form heterokaryons when paired with each other. However, heterkaryons are formed between the progeny of AG-4 isolates despite the disparate nutrient requirements of the parents. Investigation of progeny from such pairings could contribute significantly to knowledge about the inheritance of factors that regulate fruiting.

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