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Nutritional requirements of the mycoparasitic fungus Verticillium biguttatum

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Abstract

Verticillium biguttatum was able to grow axenically in a synthetic liquid medium with a compound containing ammonium or amino group as nitrogen source, glucose as carbon source and biotin as growth factor. Among various carbon and nitrogen compounds tested, highest mycelial production was achieved with mannitol and with two ammonium salts and glutamine; sporulation reached highest values with galactose and glutamine. Highest yields of mycelium and conidia were obtained at pH 4.3 and 5.1, respectively. Although neutral and alkaline conditions were growth-limiting in the synthetic medium, some growth of *V. biguttatum* occurred on solid media at pH 7.0 and on sclerotia of *Rhizoctonia solani* in natural soil at pH 7.2-7.3.

Additional keywords: biological control, *Rhizoctonia solani*, sclerotia.

Introduction

Verticillium biguttatum has recently been characterized as an ecologically obligate mycoparasite of *Rhizoctonia solani* (Van den Boogert, 1989); for its development in the field the fungus is strongly dependent on *R. solani*, whilst in culture growth and sporulation are supported by several common agar media.

Malt extract agar, supplemented with peptone (malt peptone agar; MPA) was shown to meet the nutritional requirements of the mycoparasite in culture (Van den Boogert and Jager, 1984). The chemical composition of MPA varies to some extent and the amounts of the different nutritional components are presumably higher than required for fungal growth. The non-adjusted pH of MPA (6.4 ± 0.2) seems to be suitable for growth. Recently, I found that growth of *V. biguttatum* was stimulated in soils with a relatively low pH, particularly in those with a coarse texture, e.g. sandy loam soils. To apply *V. biguttatum* as a biological control agent against *R. solani* in potatoes large quantities of conidia are needed for inoculation of the tubers (Jager and Velvis, 1985, 1986, 1988).

The present study was undertaken to find a suitable synthetic medium for growth and sporulation of *V. biguttatum* in axenic culture.

Materials and methods

Media. Malt peptone agar was prepared according to Van den Boogert and Gams (1988).

A synthetic medium (BM) was used which contained the following basic ingeredients per litre: 0.5 g NaCl, 0.35 g K_2SO_4, 0.25 g Mg-glycerophosphate, 0.25 g Ca-glycerophosphate, and 5 ml of a minor-element solution containing per litre 10 mg $FeCl_3$, 10 mg $MgCl_2.4H_2O$, 5 mg $ZnSO_4.7H_2O$, 1 mg $CuSO_4.5H_2O$, 0.5 mg MoO_3 and 0.25 mg H_3BO_3. The BM was buffered with 50 mM KH_2PO_4/Na_2HPO_4 and adjusted to pH 5.0 with 0.1 M NaOH or HCl. Unless indicated otherwise, BDH chemicals were used.

Various carbon and nitrogen sources were tested in BM with NH_4Cl or with glucose, and with biotin (Serva) at a final concentration of 0.4 μM. Unless indicated otherwise, the carbon and nitrogen sources amounted to 10 and 2 g l^{-1}, respectively.

To determine the vitamin requirements, BM supplemented with glucose and NH_4Cl was used. The glucose was freed from any vitamin contaminants by boiling it in deionized water (50 g l^{-1}) together with charcoal powder (5 g l^{-1}); the charcoal was removed from the solution by vacuum filtration.

For pH experiments, either a half strength malt peptone solution (MP) or biotin-supplemented BM with glucose and glutamine was used. The pH in both media was buffered with 0.1 M citric acid/0.2 M Na_2HPO_4 (McIlvaine's buffer) to the desired value.

Radial growth was assessed on MP media with 1.2% agar (Oxoid L 13; No. 3) or potato dextrose agar (PDA, Oxoid CM 139).

Fungal inoculum. *V. biguttatum*, isolate M73 (CBS 228.80) and *R. solani*, isolate 41 AHa, were used in all experiments. Mycelial agar discs (3 mm diam., 2 mm thick) were punched from the edge of a culture growing on MPA and used as inoculum. Conidial suspensions were collected from two week-old cultures growing on MPA by scraping the conidia from the mycelial mat into 20 ml deionized water and filtering the crude suspension through a double layer of cheese cloth. The density was determined with a hemocytometer and adjusted to 1.5×10^5 ml^{-1}. The viability of the inoculum of *V. biguttatum* was determined according to Van den Boogert and Gams (1988).

Sclerotia of *R. solani* were collected after incubation for 3 weeks at 21 °C following inoculation on MPA. Fragments of the sclerotia (3 mm diam.), carefully freed from adhering agar, were then cut, air-dried and stored at 1 °C until they were used for inoculations.

Experimental design. Experiments to determine the nutrient requirement and optimal pH for growth were performed either in submerged culture in 250 ml Erlenmeyer flasks or on agar plates in 90 mm diam. Petri dishes containing 100 or 20 ml medium, respectively. After filling, the flasks were plugged with cotton wool, capped with aluminium foil and autoclaved at 121 °C for 20 min. Solutions of vitamins and minor elements were filter-sterilized separately and added to the autoclaved medium. The pH of the medium was measured after sterilization and readjusted if necessary. Each flask was inoculated with one 3-mm diam. mycelial agar disc or with 0.04 ml of conidial suspension. The submerged cultures were incubated on a reciprocal flask shaker (100 strokes min^{-1}).

As a measure of growth in submerged culture the mycelial dry weight and the conidial production were determined. The conidia were separated from the mycelial mats by passing the culture liquid through nylon gauze (0.04 mm). The numbers of conidia remaining in the culture liquid were determined with a hemocytometer. The mycelial

mats were collected from the nylon gauze, vacuum-filtered on a tared filter paper, dried at 90 °C for 24 h and weighed. The results were expressed as average numbers of conidia and average dry weights per 100 ml medium from four replications.

As a measure of growth on solid media the colony diameter was determined.

The effect of pH on mycoparasitic growth was also studied in natural soil. For this purpose pleistocene loamy sand (location Haren; pH-H_2O 5.3) and marine silt loam (location Kloosterburen; pH-H_2O 7.5) were used, both having previously been cropped with wheat. Both soils were dried, passed through a 3-mm sieve and adjusted to pH 5.0 and 7.0. The desired pH values were obtained by repeatedly spraying the soils with dilute H_2SO_4 or $CaCO_3$ solutions. After treatment the soils were stored for two weeks at 15 °C. Densities of *V. biguttatum* in the soils were determined according to Van den Boogert and Gams (1988) and then raised to 100 viable conidia per g by spraying the soils with appropriate conidial suspensions. The moisture content of the soils was adjusted to 50% of the water-holding capacity. Petri dishes (15 cm diam.) were filled with the soil and dried fragments of sclerotia of *R. solani* were buried between two layers of soil. As a measure of mycoparasitic growth the loss of viability of the sclerotia was determined. Periodically, 50 sclerotia (from two Petri dishes) were recovered from soil, and assessed for their viability according to Velvis et al. (1989). All cultures were incubated at 21 °C in darkness.

Results

Mycelial growth on solid medium. BM agar plates supplemented with glucose and NH_4Cl supported profuse radial growth of *R. solani* (6.0 mm day^{-1}), even after five successive transfers to fresh plates. Hyphal development of *V. biguttatum*, however, was observed only in close vicinity of the site of inoculation. After a second transfer the mycoparasite failed to grow out. When overgrown with mycelium of *R. solani*, however, the BM-glucose/NH_4Cl medium supported good growth of *V. biguttatum* at rates similar to those on MPA plates overgrown with *R. solani* and amounted to 1.3 mm day^{-1}.

Cultivation of *R. solani* and *V. biguttatum* in dual culture on BM-glucose/NH_4Cl plates revealed that before physical contact *R. solani* induced growth of the parasite when the inoculum discs had been placed 3 cm apart. When the colonies touched, *V. biguttatum* started to overgrow *R. solani.*

Apparently one or more unknown diffusable substances released by *R. solani* are essential for growth of *V. biguttatum* on the vitamin-free BM-glucose/NH_4Cl plates.

Effect of vitamins on growth. In submerged cultures in liquid vitamin-free BM supplemented with glucose and NH_4Cl hardly any growth of *V. biguttatum* occurred. Addition of biotin (final concentration 0.4 μM) to the medium, alone or in combination with other vitamins (final concentration 0.4 μM each), allowed the fungus to produce mycelium (Table 1). The minute yields in the biotin-free media might be due to growth factors contained in the MPA discs that were used as inoculum. The pH of the medium in which growth occurred dropped from 6.3 to 3.5 after 12 days of incubation.

Effect of nitrogen sources on growth and sporulation. In submerged cultures in BM supplemented with glucose and biotin, ammonium nitrogen compounds supported good

Table 1. Effect of various vitamins on growth of *V. biguttatum* in liquid BM-glucose/NH_4Cl after 12 days of incubation at 21 °C following inoculation with one mycelial agar disc per flask.

Vitamin[1]	Mycelial dry weight (mg per flask)
None	15 ± 4 a[3]
Thiamine[2]	16 ± 3 a
Pyridoxine[2]	18 ± 1 a
Biotin	268 ± 7 b
Biotin + thiamine	247 ± 22 b
Biotin + pyridoxine	252 ± 15 b

[1] Final concentration of each vitamin: 0.4 μM.
[2] Supplied to the medium as thiamic hydrochloride and pyridoxine hydrochloride.
[3] Values followed by the same letter do not differ significantly (P = 0.05) according to Duncan's multiple range test.

Table 2. Effect of various nitrogen sources on growth and sporulation of *V. biguttatum* in liquid MB-glucose with biotin after 18 days of incubation at 21 °C following inoculation with 0.6×10^4 condia per flask.

Nitrogen source	Concentration (mM)	Mycelial dry weight (mg per flask)	Conidia ($\times 10^9$ per flask)
None	0	4 ± 4 a	0.8 ± 0.5 a
KNO_3	19.8	10 ± 7 a	0.3 ± 0.1 a
NH_4Cl	37.4	355 ± 23 b	31.1 ± 10.0 b
Glutamine	13.7	325 ± 75 b	349.0 ± 45.7 c
Glutamine in medium without glucose	13.7	8 ± 3 a	0.5 ± 0.2 a
$(NH_4)_3$-citrate	8.9	306 ± 37 b	250.0 ± 52.1 d
$(NH_4)_3$-citrate in medium without glucose	8.9	185 ± 8 c	234.7 ± 37.8 d

Values within a single column followed by the same letter do not differ significantly (P = 0.05) according to Duncan's multiple range test.

growth and sporulation whereas nitrate nitrogen was not utilized by *V. biguttatum* (Table 2). Glutamine served only as a nitrogen source and not as a carbon source. In comparison with both ammonium salts a substantially higher yield of conidia was obtained with glutamine. The pH in the BM-glucose medium with glutamine remained unchanged, whereas use of ammonium was accompanied by a pH decrease from 6.1 to 3.5 after 18 days of incubation.

The following organic nitrogen sources (5 g l^{-1}) were also tested in solid BM-glucose and BM-glucose supplemented with biotin: peptone (Oxoid L 72), tryptone (Oxoid L 42), yeast extract (Oxoid L 21), albumin (Brocades). All these nitrogen sources supported radial growth on both solid media at rates similar to that on MPA.

Effect of carbon sources on growth and sporulation. In submerged cultures in BM

Table 3. Effect of various carbon sources on growth and sporulation of *V. biguttatum* in liquid BM-NH_4Cl with biotin after 18 days of incubation at 21 °C following inoculation with 0.6×10^4 conidia per flask.

Carbon source	Concentration (mM)	Mycelial dry weight (mg per flask)	Conidia $\times 10^5$ per flask	Conidia $\times 10^5$ per g mycelial dry weight
None	0	4 ± 3 a	< 0.1	–
Sodium pyruvate	90.1	2 ± 2 ab	< 0.1	–
Sodium succinate	61.7	2 ± 1 b	< 0.1	–
D-ribose	66.7	3 ± 1 ab	< 0.1	–
D-arabinose	66.7	43 ± 9 d	1.4 ± 0.1 a	33
D-glucose	55.6	330 ± 51 e	61.5 ± 36.5 be	186
D-fructose	55.6	376 ± 9 e	7.0 ± 5.1 c	19
D-galactose	55.6	36 ± 3 d	76.0 ± 4.0 e	2111
Sorbitol	54.9	60 ± 16 d	54.7 ± 4.6 b	911
Mannitol	54.9	483 ± 33 f	0.6 ± 4.3 a	1
Inositol	55.6	1 ± 1 b	< 0.1	–
Sucrose	29.2	7 ± 5 c	5.2 ± 2.3 c	743
Cellobiose	29.2	348 ± 17 e	13.5 ± 4.4 d	40
Maltose	29.2	1 ± 1 b	< 0.1	–

Values within a single column followed by the same letter do not differ significantly (P = 0.05) according to Duncan's multiple range test.

supplemented with NH_4Cl and biotin, the carbohydrates glucose, fructose, mannitol and cellobiose supported good growth. Other substrates (galactose, arabinose and sorbitol) yielded relatively small amounts of mycelium and the remaining carbon sources listed in Table 3 did not appear to be suitable substrates at all. The pH of all media in which significant growth occurred dropped from 6.2 to 4.0-3.5. The conidial production on galactose was superior, both per unit liquid medium and per unit mycelial dry weight.

The following polymers were tested on solid BM-NH_4Cl/biotin at 5 g l^{-1}: carboxymethyl cellulose, chitin (finely ground powder; Calbiochem 270461) and soluble starch. None of these carbon sources supported any radial growth of *V. biguttatum*.

Effect of pH on growth. Experiments on the effect of pH on mycelial growth were conducted either in liquid culture with half-strength MP medium or biotin-supplemented BM with glucose and glutamine or on solid MPA and PDA. Substantial growth occurred in the synthetic medium at low pH values (highest production at pH 4.3); sporulation was maximum at pH 5.1 (Fig. 1). When Fe-EDTA (1 ml filter-sterilized solution containing 4.0 g Na_2EDTA and 3.0 g $FeSO_4 . 7H_2O$ per litre) was used instead of $FeCl_3$, the same results were obtained, indicating that growth reduction was not due to decreased availability of Fe in the medium at pH > 4.3. In liquid MP, however, some growth of *V. biguttatum* was observed at pH 7.0 (Table 4). Growth of *R. solani* was initially poor at neutral pH in liquid MP, but after prolonged incubation times, yields were similar

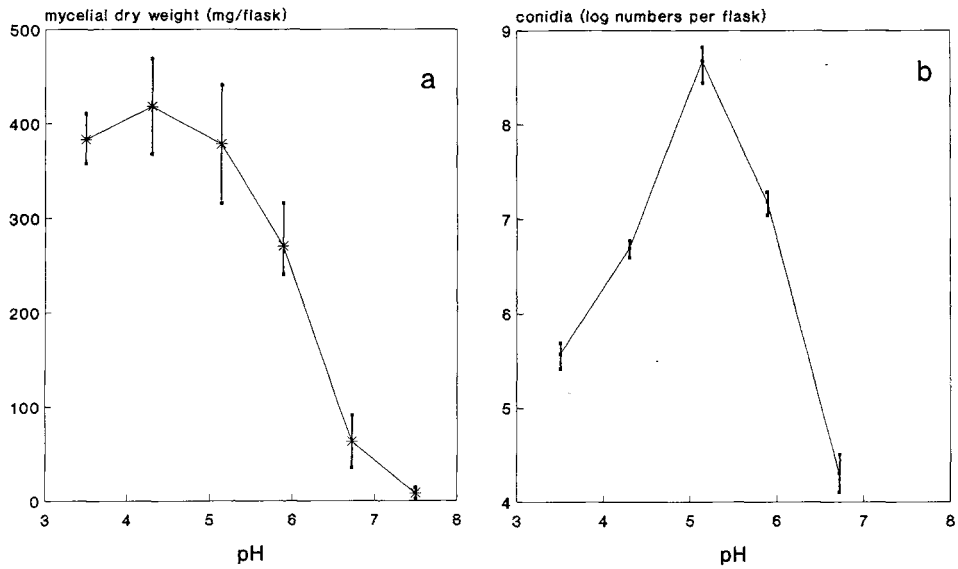

Fig. 1. Effect of pH on growth (a) and sporulation (b) of *V. biguttatum* in submerged culture with BM-glucose/glutamine/biotin after 12 days incubation at 21 °C following inoculation with one mycelial agar disc per flask.

Table 4. Effect of pH on growth of *V. biguttatum* and *R. solani* in liquid and on solid medium, expressed as mycelial dry weight per flask (mg) and colony diameter (mm), respectively, after 6 and 12 days incubation at 21 °C following inoculation with one mycelial agar disc.

Media	Incubation time (days)	Initial pH	Growth of	
			V. biguttatum	*R. solani*
Liquid				
BM-glucose/glutamine + biotin	12	5.0	277 ± 37[1]	341 ± 32
		7.0	12 ± 6*	367 ± 63
MP	6	5.0	284 ± 33	197 ± 18
		7.0	18 ± 9*	69 ± 8*
	12	5.0	317 ± 22	357 ± 26
		7.0	69 ± 20*	344 ± 11
Solid				
MPA	12	5.0	31 ± 2	33 ± 2[2]
		7.0	37 ± 2*	42 ± 2[2]*
PDA	12	5.0	31 ± 2	22 ± 2[2]
		7.0	41 ± 2*	22 ± 1[2]

[1] Pairs of values followed by * differ significantly (P = 0.01), according to Student's t-test.
[2] Values obtained after 3 days of incubation.

Table 5. Effect of pH (artificially adjusted) in two natural soils on the viability of sclerotia of *R. solani*, expressed as an index (0 = minimum and 100 = maximum viability), after different incubation periods at 18 °C.

Soil type (location)	pH-H_2O	Viability index (0-100) after an incubation time (days)		
		14	20	27
Loamy sand	5.1	94.5	56.5	18.5
(Haren)	7.2	99.0	77.7	34.5
Silt loam	5.2	96.0	58.9	31.5
(Kloosterburen)	7.3	100	80.5	53.1

to those at pH 5.0. On solid MPA medium, however, relatively small differences in colony diameter were observed at pH 5.0 and 7.0 for both *R. solani* and *V. biguttatum* (Table 4). The pH of MPA, PDA and MP had not changed at the end of the incubation period.

In an experiment on the effect of pH on mycoparasitic growth on sclerotia of *R. solani* in natural soil almost all sclerotia (90-100%) were found to be colonized by *V. biguttatum* 14 days after they had been placed in the soils. On both soil types tested, the viability of the sclerotia was more severely affected at pH 5.1-5.2 than at pH 7.2-7.3 (Table 5).

Discussion

Only a few mono- and disaccharides, in combination with NH_4Cl as an inorganic nitrogen source, were utilized by *V. biguttatum* at the concentrations tested. The addition of biotin was essential for fungal development.

To require one or more vitamins is not unusual for filamentous fungi (Fries, 1964) and vitamin requirements have been reported for biotrophic (Barnett, 1970) and destructive mycoparasites (e.g. *Sporidesmium sclerotivorum*; Barnett and Ayers, 1981). Some other mycoparasites grow readily on synthetic nutrient agar without vitamins, e.g. *Trichoderma* spp. (Elad et al., 1981).

R. solani does not require any particular growth factor, which was also found by Sherwood (1970). In dual culture both fungi were found to grow on solid vitamin-free synthetic medium. Apparently the host can satisfy the vitamin requirements of *V. biguttatum*.

Glutamine can serve only as a nitrogen source. Unlike growth on glutamine, growth on ammonium salts led to a distinct decrease in pH of the synthetic medium, apparently without affecting the mycelial yield. This is in agreement with the acidophilic nature of the fungus, which has been shown to reach highest mycelial yields in the synthetic medium at pH 3.5-5.1. Some growth occurred under alkaline conditions on solid media after a longer incubation time.

Mycoparasitism of *V. biguttatum* on sclerotia of *R. solani* in the soil was most effective at relatively low pH values, although under slightly alkaline conditions some activity was found.

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Samenvatting

Voedingseisen van de mycoparisitaire schimmel Verticillium biguttatum

Een synthetisch vloeibaar medium met glucose als koolstofbron, een ammonium- of aminogroep bevattende verbinding als stikstofbron en biotine als groeifactor voldeed aan de voedingseisen van *Verticillium biguttatum*. Van de verschillende koolstof- en stikstofbronnen leverden mannitol en twee ammoniumzouten en glutamine de hoogste opbrengsten aan mycelium; de opbrengst aan conidiën was het hoogst met galactose en glutamine. De hoogste opbrengsten aan mycelium en conidiën werden bereikt bij respectievelijk pH 4,3 en 5,1. Ofschoon neutrale en alkalische omstandigheden de groei van *V. biguttatum* in het synthetische medium beperkten, werd enige groei van *V. biguttatum* waargenomen op vaste voedingsbodems bij pH 7,0 en op sclerotia van *R. solani* in natuurlijke grond bij pH 7,2-7,3.

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