FEMS Microbiology Letters 28 (1985) 145–150
Published by Elsevier

FEM02119

Morphogenesis and free amino acid composition of the ascomycete *Sphaerostilbe repens* as influenced by nitrogen and calcium

(Organ differentiation; coremium; rhizomorph; nitrogen metabolism)

Bernard Botton and Abbes Es-Sgaouri

Université de Nancy I, Laboratoire de Physiologie Végétale, B.P. 239, F. 54506 Vandoeuvre les Nancy Cédex, France

Received 13 February 1985
Revision received 17 March 1985
Accepted 23 March 1985

1. SUMMARY

Sphaerostilbe repens utilizes nitrate and ammonium as nitrogen sources. Differentiation of mycelium into rhizomorphs and coremia was reduced in the presence of nitrate and completely inhibited in the absence of calcium. The most abundant free amino acids were, in decreasing order: alanine, glutamine, glutatomic acid, serine, aspartic acid, γ-aminobutyric acid, arginine and threonine. These compounds represented 90% of the total amino acid pool.

The free amino acid composition did not vary with cultural conditions although concentrations of individual amino acids differed. In ammonium-grown cells, γ-aminobutyric acid increased in concentration and glutamate, aspartate and alanine decreased. Calcium-deficient media reduced amino acid concentrations, especially of arginine and ornithine. Amino acid contents increased during the growth period and were higher in rhizomorphs than in vegetative mycelia.

2. INTRODUCTION

From vegetative mycelium, *Sphaerostilbe repens* gives rise to coremia and rhizomorphs which result from the coalescence of hyphae growing in a preferential direction. These two types of organs are jointly referred to hereafter as an 'aggregated unit' [1].

Morphogenetic phases of the differentiation of these organs in *Sphaerostilbe repens* have been extensively studied [1–3]. However, metabolic pathways related to the morphogenesis of this organism remain so far largely unknown. Our main target is to elucidate the biochemical steps leading to the formation of the aggregated organs, especially studying nitrogen metabolism as this element is known to play a crucial role in regulating coremium and rhizomorph formation [4]. In addition, complete differentiation of the thallus is a calcium-dependent phenomenon. The presence of this cation in culture media is a prerequisite for the formation and elongation of the aggregated structures [5].

The metabolic route for incorporating nitrogen from nitrate into amino acids has already been investigated in *Sphaerostilbe repens.* Nitrate reductase and glutamate dehydrogenase have previously been studied [6,7]. This investigation deals with the effect of mineral nitrogen compounds and calcium on the accumulation of free amino acids in the fungus in relation to its capability for differentiation.

3. MATERIALS AND METHODS

3.1. Culture of the organism

Sphaerostilbe repens (Strain 275-60) obtained from the Centraal Bureau voor Schimmelcultures of Baarn (The Netherlands) was grown in the dark at 28°C on a medium with the following composition (g/l): sucrose, 45; tartaric acid, 2.64; Na_2SO_4, 0.21; KH_2PO_4, 0.6; K_2CO_3, 0.4; $MgSO_4$ $7H_2O$, 0.85; $FeSO_4$ $7H_2O$, 0.086; $ZnSO_4$ $7H_2O$, 0.041; $MnSO_4$ H_2O, 0.0085; $CaCl_2$ $2H_2O$, 0.0147. Nitrogen was incorporated into the media at the constant level of 1 g/l (i.e. g/l: $NaNO_3$, 6; NH_4Cl, 4; NH_4NO_3, 3). The pH was adjusted to 5.4 before autoclaving at 120°C for 20 min. After autoclaving, the pH was 5.3.

Calcium-deficient media were prepared following a method previously reported [5].

Cultures were grown as surface cultures in 250-ml erlenmeyer flasks each containing 130 ml of liquid medium. Solid media were obtained by the incorporation of 20 g/l of agar (Difco).

Liquid media were inoculated with young colonies of *Sphaerostilbe repens* grown for 48 h on solid media, according to a method described previously [5].

Growth of the colonies on liquid media was determined by weighing after desiccation in a ventilated oven at 80°C. Rhizomorphs were severed and weighed separately from the rest of the thallus comprising vegetative mycelium and coremia. The results are based on a sample of six individual colonies.

Detection of aggregated units from thalli grown in solid media was by the following technique: the central aggregated region was taken out with a cork borer, the cylinders were mounted on a hand microtome and cut horizontally with a razor blade to separate agar and rhizomorphs from overlying mycelium and coremia. The aggregated units were thus distinguishable by the rhizomorph cross-sections which were counted under the binocular lens.

3.2. Extraction and analysis of amino acids

5 mg of freeze-dried material reduced to powder were homogenized in a mortar with 5 ml of a methanol–chloroform–water mixture (12 : 5 : 3) and centrifuged at $20000 \times g$ for 20 min. The extraction was repeated 5 times with this solvent and 2 times with 80% ethanol. All the operations were carried out at 4°C. The supernatants were pooled and evaporated at 30°C in a rotary evaporator under vacuum. The residues were redissolved in a mixture containing 2 ml distilled water and 0.5 ml chloroform, the latter being used to eliminate the lipids. The suspension was centrifuged at $30000 \times g$ for 20 min. The aqueous upper layer containing the amino acids was saved and evaporated as before, the residues being resuspended in 600 µl of 180 mM lithium citrate buffer at pH 2.2. A Biotronic LC 6000 autoanalyser was used to identify and assay amino acids.

4. RESULTS AND DISCUSSION

4.1. Growth and differentiation of the thallus

In the presence of calcium, ammonium nitrate was found to be the most favourable to differentiation as well as growth of the aggregated organs, especially rhizomorphs (Table 1). With ammonium chloride, aggregated structures were fewer and rhizomorph development was considerably reduced although mycelial production was not affected, the latter being even better than with ammonium nitrate. With sodium nitrate, only a few aggregated organs differentiated and rhizomorphs grew to a small extent.

In the absence of calcium, rhizomorph and coremium differentiation was suppressed; mycelium grew slowly and appeared as a thick uniform crust. Nevertheless, with ammonium nitrate a few rudimentary aggregated units were formed after eight days of culture but rhizomorphs hardly ever developed. Mycelium was not as differentiated and developed with sodium nitrate as with the other nitrogen sources (Table 1).

After 24 days of culture, dry weights were approximately doubled although the ratios between the values remained constant (not reported).

These results are consistent with observations that nitrate is often a poor source of nitrogen for differentiation and growth of fungi [8–11]. Moreover, whatever the nitrogen source, *Sphaerostilbe repens* requires calcium for the formation of

Table 1

Influence of calcium and nitrogen sources on growth and aggregated organ production in *Sphaerostilbe repens*

Results were determined on 8 day-old colonies.

		Calcium-containing medium			Calcium-deficient medium		
		$NaNO_3$	NH_4Cl	NH_4NO_3	$NaNO_3$	NH_4Cl	NH_4NO_3
Dry weight (mg)	Mycelium	72	103	71	50	70	69
	Rhizomorphs	100	450	984	0	0.1	1.6
Number of aggregated units on a colony		5	70	80	0	0.2	0.2

coremia and rhizomorphs, which confirms previous data obtained with slightly different culture media [5].

4.2. *Amino acid composition*

Alanine was the major component (44% molar basis) in the *Sphaerostilbe repens* free amino acid pool (Tables 2, 3 and 4). Concentrations of glutamine, glutamic acid, serine and aspartic acid were respectively 14, 10, 7 and 6% of the total amino acid pool. A substantial amount of γ-aminobutyric acid, arginine and threonine was also present (4, 3 and 2%, respectively). These 8 amino acids accounted for 90% of the total free amino acid content. Proline and tryptophan were gener-

Table 2

Free amino acid composition of *Sphaerostilbe repens* grown in the presence of sodium nitrate

Results expressed as μmol/g of dry material.

	Calcium-containing medium				Calcium-deficient medium	
	Rhizomorphs		Mycelium		Mycelium	
	8-day-old colonies	24-day-old colonies	8-day-old colonies	24-day-old colonies	8-day-old colonies	24-day-old colonies
Aspartic acid	4.3	29.2	0.8	41.8	18.4	45.5
Threonine	7.2	17.6	4.3	8.7	5	6
Serine	15.2	60.9	12.3	27.7	10.6	17.2
Glutamic acid	45	47.8	29.2	48.4	23.3	38
Glutamine	73	85.2	37.7	72.8	23.6	39.4
Proline	traces	traces	traces	traces	traces	traces
Glycine	7.6	2.3	5.3	6.5	5.3	5.7
Alanine	97.1	377.9	80.6	93	75.4	90
Valine	2.4	12.7	1.9	2.9	2.7	3.1
Cystine	1.1	1.1	1.1	1.3	1.6	1.4
Methionine	1.9	0.9	0.9	1.3	1.1	0.9
Isoleucine	2.1	4.6	1	1.9	1.7	1.7
Leucine	2.1	4.6	1.7	1.7	1.5	1.5
Tyrosine	1.5	3.9	2.5	2.5	2.2	2.7
Phenylalanine	1.5	1.7	1.2	1.2	1	1.2
β-Alanine	2.2	3.5	3.2	2.9	2.5	2.9
γ-Aminobutyric acid	8.3	12.8	7.8	8.3	7.8	4.1
Ornithine	0.4	1.3	0.4	0.8	0.4	0.4
Lysine	1.7	12	2.1	5.3	1.7	2.1
Histidine	1.8	6.8	2	4.8	1.6	2.4
Tryptophan	traces	traces	traces	traces	traces	traces
Arginine	3.3	22.5	4.2	9.1	3.6	4.2

ally detected only in a trace amount; this was also so for asparagine which in certain cases, could not be properly separated and thus is not mentioned in the results. The amino acid content expressed on a molar basis does not accurately represent the quantity of nitrogen stored in each amino acid. When concentrations are expressed on a nitrogen molar basis, alanine, glutamine, arginine and glutamic acid comprised 34, 22, 10 and 7% of the nitrogen in the free amino acids. This emphasizes the key role of these amino acids in the metabolism and storage of nitrogen in this fungus.

The present data are in good agreement with those obtained for several fungi including: *Neurospora crassa* [12], *Agaricus bisporus* [13], *Aspergillus flavus* [14] and *Phymatotrichum omnivorum* [15].

Lea and Miflin [16] have indicated that the synthesis of glutamate is the primary mechanism of ammonia assimilation in plant cells. From glutamate, nitrogen can be incorporated in alanine by the action of aminotransferase as observed from ^{15}N labelling in *Cenococcum graniforme* [17] and ^{15}N nuclear magnetic resonance studies in *N. crassa* [18]. However, it has also been indicated that the reductive amination of pyruvate may be an alternative mechanism of alanine biosynthesis resulting from ammonia assimilation [19–21]. Whether the synthesis of alanine as well as glutamic acid is a primarily pathway by which amino compounds are formed from inorganic nitrogen remains to be investigated.

4.2.1. Amino acid composition as affected by the nitrogen sources

The constitution of the free amino acid pool was not changed by the form of nitrogen although

Table 3

Free amino acid composition of *Sphaerostilbe repens* grown in the presence of ammonium chloride

Results expressed as μmol/g of dry material.

	Calcium-containing medium				Calcium-deficient medium	
	Rhizomorphs		Mycelium		Mycelium	
	8-day-old colonies	24-day-old colonies	8-day-old colonies	24-day-old colonies	8-day-old colonies	24-day-old colonies
Aspartic acid	6.4	16.6	8.6	19.5	21.6	12.9
Threonine	7.2	9.6	2.4	7.2	4.8	4.8
Serine	21.9	26.6	16.4	20.8	16.4	10.9
Glutamic acid	5.8	19.6	7.8	18.8	21.5	17.6
Glutamine	21.3	15.5	39.4	29.6	31.5	23.6
Proline	traces	traces	traces	traces	traces	traces
Glycine	7.6	7.2	3.8	6.5	6.9	3.8
Alanine	103.5	270	80.9	84.1	132.7	90.6
Valine	2.4	4.9	2.4	4.9	4.9	2.4
Cystine	1.1	1.4	1.1	1.5	2.3	2.3
Methionine	1.3	0.9	0.9	1.1	1.7	1.1
Isoleucine	1.9	2.1	1.7	2.1	2.6	2.1
Leucine	1.5	3.5	1.5	2.1	2.6	2.1
Tyrosine	1.5	4.7	1.5	3.9	3.9	3.1
Phenylalanine	0.6	2.4	0.8	1.5	2.2	1.7
β-Alanine	1.9	4.1	1.2	3.8	5.1	3.2
γ-Aminobutyric acid	25.1	12.8	25.1	27.9	11.1	16.7
Ornithine	1.3	0.8	0.8	0.8	0.4	0.6
Lysine	1.9	11.2	1.9	10.2	3.7	3.9
Histidine	1.8	4.2	1.8	5.1	2.4	3.7
Tryptophan	traces	traces	traces	traces	traces	traces
Arginine	8.2	16.5	11.5	19.8	12.9	14.9

the concentrations of some were a little affected. Thus, glutamic acid levels were low in rhizomorphs and mycelia cultured on ammonium chloride in the presence of calcium (Table 3). In this case there was a substantial decrease in the aspartic acid and alanine contents, while γ-aminobutyric acid showed a pronounced increase.

It is likely that in the presence of nitrates, glutamate is converted mainly to alanine and aspartate probably by transamination; indeed, glutamate-oxaloacetate aminotransferase and glutamate-pyruvate aminotransferase have both been found in *Sphaerostilbe repens* (Botton, B., unpublished). In the presence of ammonium, glutamate is predominantly decarboxylated to γ-aminobutyric acid. This latter pathway has already been demonstrated by using ^{15}N-ammonium salts in the Ascomycete *Cenococcum graniforme* [22].

Although significant morphological changes were induced in the thallus, exclusively quantitative variations of the free amino acids occurred. Similar results have already been obtained with other fungi when they were grown on different nitrogen sources [15,23].

4.2.2. Amino acid composition as affected by the developmental stages of the fungus

Contents of most of the amino acids increased during growth, almost doubling, on average, between the first and the third week of culture. Thus, alanine was present in relatively large amounts after 24 days of incubation, reaching as much as 3.3% of the dry rhizomorphs (Table 2).

Sulphur amino acids (cystine, methionine) were the exceptions. Their levels were nearly constant in both the stages of growth (8 and 24 days), tending to decrease in the older cells. The most significant decrease of cystine was found in rhizomorphs cul-

Table 4

Free amino acid composition of *Sphaerostilbe repens* grown in the presence of ammonium nitrate

Results expressed as μmol/g of dry material.

	Calcium-containing medium				Calcium-deficient medium	
	Rhizomorphs		Mycelium		Mycelium	
	8-day-old colonies	24-day-old colonies	8-day-old colonies	24-day-old colonies	8-day-old colonies	24-day-old colonies
Aspartic acid	27.7	8.8	19.3	21.9	14.5	15.5
Threonine	14.1	14.1	6	9.5	3.9	5
Serine	36.8	39.3	17.6	20.1	9.3	10.1
Glutamic acid	33.5	36.9	31.7	34.1	21.4	16.4
Glutamine	20.9	20.6	52.6	102.4	23.9	22.8
Proline	traces	traces	traces	traces	traces	traces
Glycine	6.6	9.9	3.9	4.4	2.6	2.6
Alanine	127.1	129.5	32.3	312.9	66.4	65.3
Valine	14.2	12.2	6.6	3.8	2.7	1.9
Cystine	5.2	0.7	0.8	0.9	0.9	0.8
Methionine	0.5	0.1	0.5	0.6	0.5	0.6
Isoleucine	1.9	2.9	2.6	2.1	1.1	1
Leucine	1.7	3.6	4.6	1.8	1.3	1.5
Tyrosine	0.9	1.6	2.9	1.5	1.2	2.5
Phenylalanine	0.7	0.9	1.9	0.6	0.6	1.2
β-Alanine	6.1	0.6	0.3	0.6	0.5	0.7
γ-Aminobutyric acid	8.5	15.4	4.8	7	5.1	4
Ornithine	0.4	0.9	0.8	1.5	0.3	0.2
Lysine	0.3	7.2	2.5	6.7	2.2	2.4
Histidine	1.9	4.7	2.1	5.7	1.4	2.5
Tryptophan	traces	0.4	traces	traces	traces	traces
Arginine	5.1	15.3	6.8	13.6	0.1	traces

tured on ammonium nitrate, that favoured the growth of the rhizomorphs (Table 4). The depletion of free cystine in the cell might be due to its mobilization in the cell walls when aggregation of the hyphae takes place, because organ differentiation in this fungus appears to be a disulphide group-dependent phenomenon [24]. In mycelium and in the thalli which developed in the absence of calcium, concentrations of these amino acids remained constant.

4.2.3. Amino acid composition in the different parts of the thallus

Almost all the amino acids were more concentrated in rhizomorphs than in vegetative mycelia. This could be related to the fact that rhizomorphs grow more rapidly than individual filaments and consequently must have a very active metabolism. However, some major amino acids, viz., aspartic acid, glutamine, alanine were more abundant in the young mycelium grown on ammonium (Table 3).

4.2.4. Amino acid composition as affected by calcium

Calcium deficiency generally induced a decrease in the free amino acid contents of the cells. This is particularly true for arginine and to a lesser extent for ornithine, especially when the fungus was grown on ammonium nitrate (Table 4). This drop in these amino acid contents appeared to be correlated with the presence of less free urea in the cells [6]. These observations are interesting since urea is a byproduct of the ornithine-cycle when arginine is reduced [25]. Thus, the presence of arginine, ornithine and the pattern of free urea production could be an indication that the ornithine-cycle is operative in *Sphaerostilbe repens.* Further investigations are needed to know the role of calcium in this pathway.

Aspartic acid and alanine were, however, more abundant in mycelia cultured in the absence of calcium on nitrate and ammonium, respectively (Tables 2 and 3).

Finally, calcium seems to have a more pronounced effect than the forms of nitrogen. In the absence of the cation, the decrease in the pool of free amino acids is probably correlated with a less intense metabolic activity, as shown by reduced growth and differentiation.

ACKNOWLEDGEMENTS

We thank Mr. G. Durand and Mr. J. Banvoy for their technical assistance. A financial aid from the Direction de la Recherche, Ministère de l'Education Nationale is gratefully acknowledged.

REFERENCES

[1] Guillaumin, J.J. (1970) Cah. Orstom. Sér. Biol. 12, 51–64.
[2] Botton, B. (1983) Protoplasma 116, 91–98.
[3] Botton, B. (1983) Protoplasma 116, 99–114.
[4] Botton, B. (1980) 'Doctorat d'Etat' Thesis, University of Nancy I.
[5] Botton, B. (1978) Canad. J. Microbiol. 24, 1039–1047.
[6] Es-Sgaouri, A. (1984) 'Third cycle' Thesis, University of Nancy I.
[7] Botton, B. and Msatef, Y. (1983) Physiol. Plant. 59, 438–444.
[8] Garrett, S.D. (1953) Ann. Bot. 65, 63–79.
[9] Azevedo, Natalina F. Dos Santos de. (1963) Trans. Br. Mycol. Soc. 46, 281–284.
[10] Jacques-Felix, M. (1968) Bull. Soc. Mycol. Fr. 84, 161–307.
[11] Harris, J.L. and Taber, W.A. (1970) Mycologia 62, 152–170.
[12] Debusk, B.G. and Debusk, A.G. (1967) Neurospora Newslett. 11, 3.
[13] Kissmeyer-Nielsen, E., McClendon, J.H. and Woodmansee, C.W. (1966) J. Agr. Food Chem. 14, 633–636.
[14] Pillai, N.C. and Srinivasan, K.S. (1956) J. Gen. Microbiol. 14, 248–255.
[15] Gunasekaran, M. and Weber, D.J. (1979) Mycopathologia 67, 95–100.
[16] Lea, P.J. and Miflin, B.J. (1974) Nature 251, 614–616.
[17] Genetet, I., Martin, F. and Stewart, G.R. (1984) Plant Physiol. 76, 395–399.
[18] Kanamori, K., Legerton, T.L., Weiss, R.L. and Roberts, J.D. (1982) J. Biol. Chem. 257, 14168–14172.
[19] Sims, A.P. and Folkes, B.F. (1964) Proc. Roy. Soc. Lond. B 159, 479–502.
[20] Bryan, J.K. (1976) Amino acid biosynthesis and its regulation, Plant Biochemistry (Bonner, J. and Varner, J.E., Eds.), pp. 525–560. Academic Press, New York.
[21] Booker, C.E. (1980) Mycologia 72, 868–881.
[22] Martin, F. (Personal communication).
[23] Verona, O., Nuti, M.P. and Anelli, G. (1973) Experientia 29, 1162–1164.
[24] Botton, B. and Bonaly, R. (1982) Arch. Microbiol. 131, 291–297.
[25] Pateman, J.A. and Kinghorn, J.R. (1976) Nitrogen metabolism, in The Filamentous Fungi, Vol. 2: Biosynthesis and Metabolism (Smith, J.E. and Berry, D.R., Eds.), pp. 159–237. Edward Arnold, London.