Mycologia, 77(3), 1985, pp. 418–423.

A CHEMICALLY DEFINED MEDIUM FOR THE GROWTH AND SYNTHESIS OF ERGOT ALKALOIDS BY SPECIES OF *BALANSIA*

CHARLES W. BACON

Toxicology & Biological Constituents Research Unit,
R. B. Russell Agricultural Research Center,
U.S. Department of Agriculture, Agricultural Research Service,
P.O. Box 5677, Athens, Georgia 30613

ABSTRACT

A chemically defined medium was developed for the growth of and ergot alkaloid synthesis by five species of *Balansia* (Clavicipitaceae). It was determined that the nitrogen required for growth was not strict, but NH_4Cl (2.4 g/l) produced good growth and favored total ergot alkaloid synthesis. The fungi required thiamine, nicotinic acid, and pyridoxine for growth. Incorporating DL-tryptophan into the medium did not increase growth, but did cause a 2–4-fold increase in the yield of total alkaloid produced by the species tested.

Key Words: *Balansia* species, ergot alkaloids, systemic grass parasites, endophytes.

In a series of studies we demonstrated the *in vitro* and *in vivo* synthesis of ergot alkaloids by species of the Balansiae and defined their role in cattle toxicity syndromes (**1**, **3**, **7**). These studies indicated that the genetic potential for the biosynthesis of this class of mycotoxins was conserved during evolution by many members of the Clavicipitaceae. The *in vitro* demonstration of alkaloids by species of *Balansia* utilized a procedure consisting of a semisynthetic medium (SM) for the alkaloid production phase (**2**, **3**). In that SM medium yeast extract was used as an essential factor for growth and synthesis of ergot alkaloids. In this paper I examined the factors in yeast extract that favored growth and alkaloid synthesis and report on a completely defined medium that will support growth and produce higher yields of ergot alkaloids than earlier reported.

MATERIALS AND METHODS

The fungi.—Isolates of *Balansia epichloe* (Weese) Diehl parasitic on smutgrass (*Sporobolus poiretii*) (Roem. & Schult.) Hitchc., *B. henningsiana* (Moller) Diehl parasitic on broomsedge (*Andropogon virginicus* L.), *B. claviceps* Spegazzini parasitic on *Chasmanthium laxum* (L.) Yates, and *B. strangulans* (Montagne) Diehl parasitic on *Panicum hians* Ell. were obtained from ascospores germinated on corn-meal malt extract (CMM) agar following the procedure described earlier (**2**). *Balansia obtecta* Diehl parasitic on *Cenchrus echinatus* L. and *B. cyperi* Edgerton parasitic on *Cyperus virens* Michx. were received from K. Clay, Department of Botany, Louisiana State University, Baton Rouge, LA. Cultures of these two fungi were obtained by surface-sterilizing the ascomata with full strength commercial bleach, and plating on CMM agar, and their identities were established on the basis of their production of ephelidial conidia. The fungus inoculum used for growth and alkaloid studies was obtained by culturing the fungus in darkness for 10 da on a gyratory shaker (150 rev/min, 1 cm circular orbit) in 50 ml of sporulation medium M102 (**8**) at 25 C in 125 ml triple-baffled shake flasks.

Media and growth.—Ergot alkaloid biosynthesis and growth were studied on basal medium containing (per liter distilled water): sorbitol, 100 g; glucose, 40 g; succinic

TABLE I

MYCELIUM PRODUCTION (MG DRY WEIGHT/50 ML MEDIUM) AND TOTAL ERGOT ALKALOID SYNTHESIS BY *Balansia epichloe* GROWN IN BASAL LIQUID MEDIUM CONTAINING EQUIMOLAR CONCENTRATIONS OF NITROGEN

Nitrogen source	Concentrations g/l	Dry weight[a]	Total ergot alkaloid mg/l
Yeast extract	5.63	275.30 ± 1.2	311.4 ± 3.2
KNO_3	4.56	121.90 ± 1.3	4.2 ± 3.6
NH_4NO_3	3.28	82.40 ± 1.3	182.3 ± 2.4
NH_4Cl	2.39	116.12 ± 1.3	376.2 ± 3.6
Asparagine	3.08	136.73 ± 2.3	51.2 ± 5.6
DL-Glutamic acid	6.0	182.23 ± 2.1	10.3 ± 5.1
DL-Methionine	6.12	42.76 ± 2.2	ND
Urea	2.7	121.68 ± 1.3	4.2 ± 5.0
Tryptophan	8.37	10.21 ± 3.2	ND

[a] Dry weights were determined after 10 da of shake culture, alkaloids were determined after 21 additional da of stationary culture. ND = not detected. Average (±SD) from 6 different analyses.

acid, 10 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.30 g; and trace element solution, 5 ml. The trace element solution consisted of (g per 100 ml): citric acid, 5; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 100; $ZnSO_4 \cdot 7H_2O$, 5; $MnSO_4 \cdot H_2O$, 0.05; H_3BO_3, 0.05; $Na_2MoO_4 \cdot 2H_2O$, 0.05; and $CuSO_4 \cdot 5H_2O$, 0.25. The final medium was adjusted to pH 5.6 with a saturated solution of NaOH. Under experimental conditions designed to determine the nitrogen requirement, yeast extract (0.1 g/l) was used and various equimolar concentrations of nitrogen sources were added to the medium. Yeast extract was substituted with NH_4Cl (2.24 g/l) in all experiments designed to determine vitamin requirements and all vitamin solutions were filter-sterilized before adding to autoclaved medium. The three vitamins that gave a growth response were heat-stable and in subsequent experiments these vitamins were autoclaved along with the other ingredients. Either 100 ml or 50 ml of medium was placed in 500 ml or 125 ml triple-baffled cotton-stoppered flasks, respectively, and autoclaved for 15 min at 121 C. The medium was inoculated with 2 ml of 10-da-old cultures prepared from M102. The cultures were incubated in the dark at 24 C for 10 to 14 da on the gyratory shaker, removed from the shaker, and for ergot alkaloid synthesis incubated as stationary cultures until harvested (3–4 wk). For comparison and as a control, fungi were also grown on the SM medium already described (2) following the above procedure. Briefly the SM medium consisted of essentially the same ingredients on the basal medium, but it also contained yeast extracts (0.1 g/l).

Dry weights of fungus grown in liquid media were determined after 10 to 14 da of culture on the gyratory shaker. The mycelium was separated by filter paper, washed, and dried in a forced air drying oven at 90 C for 24 h. Dry weights of mycelium grown on agar were determined by steaming the agar until it melted, the mycelial mats were collected on mericloth, and washed 4 times with hot distilled water. The mats were dried and weighed as above.

Alkaloid analysis.—Alkaloids were determined by adding a saturated NaOH solution to 100 ml of culture filtrate until a pH of 10 was reached. The alkaloids were extracted from the alkaline medium with two 50 ml portions of $CHCl_3$. The $CHCl_3$ extracts were combined and evaporated to dryness on a rotary evaporator (40 C) and the residue was dissolved in 5 ml 2% (w/v) tartaric acid. The total alkaloid content of the solution was quantitated on 2 ml of the tartaric acid solution

TABLE II

MYCELIUM PRODUCTION (MG DRY WEIGHT/50 ML MEDIUM) BY *Balansia epichloe* GROWN FOR 14 DA IN SHAKE CULTURE IN A MEDIUM OF NH_4Cl (2.39 G/L) WITH AND WITHOUT GROWTH FACTORS

Vitamin	Factors concentration[a] μg/ml	Dry weight
Vitamin mixture		685.70 ± 2.1
P-aminobenzoic acid	0.4	36.18 ± 4.2
Biotin	0.2	23.73 ± 4.1
Inositol	5.0	83.18 ± 2.3
Nicotinic acid	2.8	284.17 ± 1.1
Pyridoxine	0.4	282.91 ± 1.2
Riboflavin	0.4	21.21 ± 1.5
Thiamine	0.4	273.24 ± 1.6
Yeast extract		775.30 ± 1.4
Without vitamin mixture		23.75 ± 4.3
Without yeast extract		29.37 ± 4.3
Nicotinic acid, pyridoxine, and thiamine		698.73 ± 3.6

[a] Mixture consisted of the 7 vitamins in the concentrations indicated; yeast extract, 0.1 g/l; the mixture of nicotinic acid, pyridoxine, and thiamine was used in concentrations specified for the individual vitamin; average (±SD) from 6 analyses.

following the colorimetric procedure of Michelon and Kelleher (**6**) using a standard solution of ergonovine maleate as a reference. Initially, the chemical identity of the alkaloids was established by ultraviolet and low resolution mass spectral analyses (**7**). For routine use, the identity of the alkaloids was established by co-chromatography with reference standards on thin-layer chromatograms prepared from silica gel GF-254 and developed with chloroform/methanol (80:20, v/v). The alkaloids were visualized after spraying the chromatograms with *p*-dimethyl-aminobenzaldehyde solution (**9**).

RESULTS AND DISCUSSION

While there were other nitrogen sources that gave comparable growth of *B. epichloe,* NH_4Cl (2.39 g/l) was the compound that stimulated both growth and ergot alkaloid synthesis comparable with the yeast extract medium (TABLE I). Experiments designed to determine the vitamin requirement indicated that there was no growth when NH_4Cl was substituted for yeast extract (TABLE II). Further, this experiment indicated that of the seven vitamins tested, pyridoxine, nicotinic acid, and thiamine stimulated growth when used alone. A combination of the vitamins produced growth comparable with that when yeast extract was used. Various concentrations of these vitamins had no effect on the yield of ergot alkaloids. These data indicated that for growth this species required three vitamins and a nitrogen source, and that these substances were present in the yeast extract. This defined medium, BS, established for *B. epichloe,* also supported the growth and alkaloid synthesis of five other species of the Balansiae (TABLE III).

Incorporating tryptophan into the medium (BST) had no effect on the growth of the five species of *Balansia* tested, but a two- to three-fold increase in the yield of ergot alkaloids was achieved when fungi were cultured on BST medium as opposed to SM (TABLE III). Tryptophan had no effect on those species that were nonproducers of ergot alkaloids, i.e., *B. obtecta* and *B. cyperi.* The final yield of total alkaloid depended on the concentration of tryptophan (TABLE IV). The effects of this amino acid were demonstrated when we compared the yields obtained in

TABLE III

MYCELIA PRODUCTION AND ERGOT ALKALOID SYNTHESIS BY SPECIES OF *Balansia* GROWN IN 100 ML OF BST (WITH TRYPTOPHAN), AND SM

	BST						SM[b]	
	Alkaloids[a]							
Fungus	Chanoclavine (L)	Ergonovine	Ergonovinine	Agroclavine	Total alkaloids (mg/l)	Dry wt (mg)	Total alkaloids (mg/l)	Dry wt (mg/100 ml)
Balansia claviceps	+	+	+	−	387.5 ± 1.3	876 ± 1.1	141.3 ± 1.3	885 ± 1.5
B. epichloe	+	+	+	+	708.0 ± 1.2	942 ± 2.0	387.3 ± 1.4	915 ± 2.1
B. henningsiana	+	−	−	−	78.5 ± 3.4	731 ± 1.4	19.2 ± 4.5	695 ± 3.6
B. obtecta	−	−	−	−	−	986 ± 1.3	−	929 ± 1.9
B. strangulans	+	−	−	−	342.1 ± 2.1	991 ± 1.0	91.2 ± 4.2	974 ± 1.3
B. cyperi	−	−	−	−	−	893 ± 1.2	−	882 ± 1.3

[a] These were the alkaloids that were produced in high concentrations and could be routinely analyzed by thin-layer chromatography; +, present; −, not detected.

[b] The four predominate specific alkaloids comprising the total alkaloid fraction produced on this medium were the same as those indicated under BST; average (±SD) from six analyses.

its absence with that obtained in its presence from two strains of *B. epichloe,* one consistently low (97 mg/l) and the other higher (387 mg/l) for total alkaloid yield. Results from such an experiment established that when tryptophan (0.80 g/l) was added to the medium, the alkaloid yields from the low strain increased to 320 mg/l, and that from the high strain the yield increased to 738 mg/l. The addition of tryptophan to the medium produced only quantitative results, because the number of individual alkaloids remained the same. Indeed, isolates from a species that synthesized only one alkaloid produced more of that one compound when tryptophan was added (TABLE III). Inositol was the only other compound tested that had an effect on the synthesis of alkaloids (data not shown). Unlike the effects on growth, the stimulation achieved with inositol was significant but strain specific. For example, the addition of inositol to one strain of *B. epichloe* produced 860 mg/l total alkaloids, as opposed to 708 mg/l total alkaloids on BST without this compound, but another strain of this species produced statistically the same amount with or without inositol.

Incorporating agar (1.5% w/v) into this medium resulted in growth comparable to that achieved on SM or corn meal–malt extract agar media (TABLE V). However, conidiation was achieved only when fresh isolates were placed on BST agar or SM agar plates, whereas on corn meal–malt extract agar medium almost all isolates

TABLE IV

EFFECT OF TRYPTOPHAN ON YIELD OF ERGOT ALKALOIDS FROM *Balansia epichloe*

Concentration DL-tryptophan (g/l)	Total alkaloid (mg/l)[a]
0.10	386.5 ± 4.5
0.40	429.4 ± 3.2
0.60	584.3 ± 2.4
0.80	715.8 ± 1.4
1.00	700.3 ± 1.2

[a] Alkaloid analysis determined after 31 da incubation. Average (±SD) from 6 different experiments.

TABLE V

COMPARISON OF MYCELIUM PRODUCTION (MG DRY WEIGHT/20 ML AGAR MEDIA) ON AGAR MEDIA BY SPECIES AND STRAINS OF *Balansia*

Fungus	Corn meal–malt[a]	BST	SM
Balansia claviceps	92.1 ± 8.1	89.2 ± 9.9	94.2 ± 2.1
B. epichloe	31.2 ± 12.1	30.2 ± 11.6	34.1 ± 9.4
B. henningsiana	62.4 ± 12.2	60.1 ± 9.7	61.2 ± 9.3
B. obtecta	63.6 ± 8.4	54.1 ± 11.9	61.2 ± 9.0
B. strangulans	74.7 ± 8.9	68.2 ± 11.6	64.5 ± 12.1
B. cyperi	52.1 ± 9.7	41.5 ± 11.3	49.2 ± 9.1

[a] All media contained 1.5% Noble purified agar; 0.1 ml of a distilled water washed inoculum (2–2.4 mg dry weight) of each fungus was placed on the agar and incubated for 21 da; average (±SD) from 6 analyses.

conidiated to varying degrees regardless of the age of the isolate. The conidiation of these fungi on BST (agar or liquid) was species-specific; those species that formed many conidia during subculture continued to conidiate on BST. For example, *B. epichloe* rapidly lost its ability to conidiate after several subcultures were made, and on BST conidia were never produced after subculturing this fungus.

The carbohydrate and major nutrient requirements for growth and alkaloid synthesis in this group of fungi have been reported (**2**), and served as the bases for the development of BST, a completely defined medium. The final medium (BST) arrived at consisted of (per liter of distilled water): sorbitol, 100 g; glucose, 40 g; succinic acid, 10 g; NH_4Cl, 2.39 g; KH_2PO_4, 1 g; DL-tryptophan, 0.8 g; $MgSO_4 \cdot 7H_2O$, 0.30 g; trace element solution, 5 ml, and vitamin solution, 2 ml. The trace element solution was that indicated in the Materials and Methods section. The vitamin solution consisted of (mg per 100 ml): thiamine·HCl, 20; nicotinic acid, 140; and pyridoxine, 20. The medium was adjusted to pH 5.6 with a saturated solution of NaOH and autoclaved for 15 min at 121 C.

Heretofore, this group of systemic parasites of pasture grasses were considered impossible (**5**) or difficult to culture (**10**), requiring complex media upon which very little growth was achieved. Germination of ascospores of these fungi still requires a complex medium, CMM (**1**), but once germination is achieved, isolates can be cultured on BST as described. This study demonstrates that the Balansiae are auxoheterotrophic, requiring nicotinic acid, pyridoxine, and thiamine in combination. The nitrogen requirement of these fungi for growth is not strict, but NH_4Cl results in increased yields of ergot alkaloids. The addition of tryptophan to this defined medium is required for optimum synthesis of alkaloids which is also required by strains of *Claviceps* (**4**, **11**) where it has been demonstrated to play a rather complicated regulatory role during the fermentation of ergot alkaloids. The relationship of tryptophan to alkaloid synthesis might be complex since the addition of it to an earlier used semisynthetic medium, SM, that contained yeast extract caused a decrease in the synthesis of ergot alkaloids (**2**). On that medium the total concentration of tryptophan and its relations with other ingredients present in yeast extract, while unknown, may be contributing factors. The role of this amino acid in the biosynthesis of alkaloids by the Balansiae is being investigated.

ACKNOWLEDGMENT

I thank R. M. Bennett for excellent technical assistance.

LITERATURE CITED

1. **Bacon, C. W., J. K. Porter, and J. D. Robbins.** 1975. Toxicity and occurrence of *Balansia* on grasses from toxic fescue pastures. *Appl. Microbiol.* **29**: 553–556.
2. ———, ———, **and** ———. 1979. Laboratory production of ergot alkaloids by species of *Balansia*. *J. Gen. Microbiol.* **113**: 119–126.
3. ———, ———, **and** ———. 1981. Ergot alkaloid biosynthesis by isolates of *Balansia epichloe* and *B. henningsiana*. *Canad. J. Bot.* **59**: 2534–2538.
4. **Bulock, J. D., and J. G. Barr.** 1968. A regulation mechanism linking tryptophan uptake and synthesis with ergot alkaloid synthesis in *Claviceps*. *Lloydia* **31**: 342–354.
5. **Diehl, W. W.** 1950. *Balansia* and the Balansiae in America. *Agriculture Monograph* **4**: 1–82.
6. **Michelon, L. E., and W. J. Kelleher.** 1963. The spectrophotometric determination of ergot alkaloids. A modified procedure employing paradimethylaminobenzaldehyde. *Lloydia* **26**: 192–201.
7. **Porter, J. K., C. W. Bacon, J. D. Robbins, and D. Betowski.** 1981. Ergot alkaloid identification in Clavicipitaceae systemic fungi of pasture grasses. *J. Agric. Food Chem.* **29**: 653–657.
8. **Rykard, D. M., E. S. Luttrell, and C. W. Bacon.** 1982. Development of the conidial state of *Myriogenospora atramentosa*. *Mycologia* **74**: 648–654.
9. **Stahl, E.** 1969. *Thin layer chromatography, a laboratory handbook.* Stain No. 4, p. 689. Springer Verlag, Inc., New York.
10. **Ullasa, B. A.** 1969. *Balansia claviceps* in artificial culture. *Mycologia* **61**: 572–579.
11. **Vining, L. C.** 1970. Effect of tryptophan on alkaloid biosynthesis in cultures of a *Claviceps* species. *Canad. J. Microbiol.* **16**: 473–480.

Accepted for publication November 6, 1984