Journal of General Microbiology (1979), **113**, 119–126. *Printed in Great Britain*

Laboratory Production of Ergot Alkaloids by Species of *Balansia*

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(*Received* 7 *November* 1978)

Four species of *Balansia* (clavicipitaceous systemic grass pathogens) isolated from pastures where cattle showed signs of ergot toxicity were grown in culture. *Balansia epichloë*, one isolate of *B. claviceps*, *B. henningsiana* and two isolates of *B. strangulans* produced conidia in submerged culture during the first stage of a two-stage fermentation procedure. When transferred to a glucose/sorbitol/inorganic salts medium during the second stage, these four species produced ergot alkaloids in stationary cultures. The transfer of fungi cultured in the first medium to the second medium was necessary for alkaloid biosynthesis. One isolate of *B. claviceps* did not produce alkaloids. *Balansia epichloë* produced chanoclavine (I), agroclavine, penniclavine, elymoclavine, ergonovine and ergonovinine. *Balansia claviceps* produced chanoclavine (I), ergonovine and ergonovinine. This is the first report of isolating ergonovine and ergonovinine, two lysergic acid derivatives, from fungi outside the genus *Claviceps*. Only chanoclavine (I) was identified from extracts of *B. strangulans* and *B. henningsiana*. Chanoclavine (I) and ergonovine were identified from smut grass (*Sporobolus poiretii*) parasitized by *B. epichloë*, indicating that this endophyte produces alkaloids both *in vivo* and *in vitro*.

INTRODUCTION

Several species of *Claviceps* are associated with toxic effects on cattle grazing on parasitized grasses (for review, see Mantle, 1969). However, many pasture toxicity syndromes of cattle, though resembling those caused by ergot alkaloids, are not caused by species of *Claviceps* (Yates, 1971; Porter *et al.*, 1975; Bacon *et al.*, 1975; Diener *et al.*, 1976; Mortimer, 1978). Other members of the family Clavicipitaceae causing systemic infections of grasses (*Epichloë typhina*, *Myriogenospora atramentosa* and species of *Balansia*) have also been implicated in the aetiology of ergot-like toxicity syndromes of cattle grazing on infected grasses (Nobindro, 1934; Maag & Tobiska, 1956; Walker, 1970; Bacon *et al.*, 1975; Bacon *et al.*, 1977; Luttrell & Bacon, 1977).

The involvement of systemic fungi in pasture toxicity syndromes is complicated by the existence of several biotypes of these grass parasites (Bacon *et al.*, 1977) and by possible seasonal interactions between host and fungus to produce toxic compounds. Moreover, the systemic nature of these fungi increases the difficulties of distinguishing, isolating and identifying toxic fungal compounds from suspect grasses. Nevertheless, the parasitic production of compounds structurally related to alkaloids from *Claviceps* by these endophytes is suggested by (i) clinical signs observed in cattle, (ii) earlier reports on toxicity of grasses without seedheads and thus no ergot sclerotia and (iii) isolation of indole alkaloids from cultures of these fungi (Porter *et al.*, 1977). In order to establish a definite relationship between pasture toxicities and these clavicipitaceous endophytes, a laboratory procedure is needed for their culture and for assessment of alkaloid production. With the exception of

0022-1287/79/0000-8441

Claviceps species, no critical attempts have been made to determine the ability of clavicipitaceous fungi to produce ergot alkaloids. We report here a procedure for production of ergot alkaloids by four species of *Balansia*.

METHODS

Organisms. Balansia epichloë (Weese) Diehl, parasitic on smut grass (*Sporobolus poiretii*), and *B. henningsiana* (Möller) Diehl, parasitic on *Panicum anceps*, were obtained from ascospores germinated on a semi-synthetic medium (Bacon *et al.*, 1975). Two isolates of *B. strangulans* (Montagne) Diehl, 233 and 257, parasitic on panic grass (*Panicum hians*), were obtained from ascospores germinated on corn meal/malt extract (CMM) agar (Bacon *et al.*, 1977). One isolate of *B. claviceps* Spegazzini, 219, was obtained from E. S. Luttrell, Department of Plant Pathology, University of Georgia, Athens, Ga, U.S.A., and the other isolate, 266, was obtained from conidia germinated on CMM; both isolates were obtained from *Chasmanthium laxum*. These endophytes were maintained on CMM agar slants at room temperature (24 to 28 °C). The viability of the fungi declined rapidly if they were stored under refrigeration (4 to 8 °C).

Media for alkaloid production. A two-stage fermentation procedure was used to produce alkaloids. First, the fungi were incubated in 50 ml of sporulation medium M102 (Bacon *et al.*, 1977) for 10 d on a gyratory shaker (150 rev. min^{-1}, 1 cm circular orbit) at 24 °C in 125 ml triple-baffled shake flasks. Then, for the second stage of fermentation, during which alkaloid biosynthesis occurred, the fungi were incubated in modified medium SM (Abe *et al.*, 1969) containing (per litre distilled water): sorbitol, 100 g; glucose, 40 g; succinic acid, 10 g; KH_2PO_4, 1·0 g; $MgSO_4.7H_2O$, 0·3 g; yeast extract, 1·0 g; $FeSO_4.7H_2O$, 0·1 mg; $CuSO_4.5H_2O$, 0·01 mg; $ZnSO_4.7H_2O$, 0·01 mg; $MnSO_4.H_2O$, 0·001 mg. The pH was adjusted to 5·6 with NH_4OH. Medium SM (100 ml) in 500 ml triple-baffled cotton-stoppered flasks was inoculated with 2 ml of sporulating culture from medium M102. The flasks were incubated at 24 °C for 10 d on a gyratory shaker and were then removed from the shaker and incubated as stationary cultures until harvested. Medium-scale production of alkaloids for isolation and chemical identification was accomplished using 500 ml medium SM in Pyrex carboys (19 l) stoppered with cotton plugs. The carboys were inoculated with 10 ml of spores from medium M102 and incubated for 7 weeks at 26 °C as stationary cultures. All cultures were incubated in the dark.

Analytical methods. Dry weights were determined on mycelium that had been separated from the culture filtrate by vacuum filtration through Whatman no. 4 filter paper, washed with distilled water and dried in a forced air drying oven at 90 °C for 24 h. Inorganic phosphate was assayed by the method of Martin & Doty (1949). Glucose was determined by the *o*-toluidine procedure (Hyvarinen & Nikkila, 1962). Sorbitol was measured as its trifluoroacetyl derivative by gas–liquid chromatography (Sullivan & Schewe, 1977) on columns containing 3 % OV-225 on Chromosorb W(HP), 80 to 100 mesh.

Alkaloid analysis. Alkaloids were determined by adding 0·2 ml 10 M-NaOH to 10 ml culture filtrate and extracting the alkaloids with 25 ml $CHCl_3$. The $CHCl_3$ extract was evaporated to dryness on a rotary evaporator (40 °C) and the residue was dissolved in 2 ml 2 % (w/v) tartaric acid. The tartaric acid extracts were diluted and the total alkaloid content was measured colorimetrically at 590 nm (Michelon & Kelleher, 1963) using a standard solution of ergonovine maleate as a reference.

Chemical separation and identification of alkaloids were done with larger volumes of media using the techniques described above, except that the pH of the tartaric acid extract was adjusted to between 8 and 9 with NaOH and this basic tartaric acid extract was then further extracted with $CHCl_3$. The $CHCl_3$ extract was evaporated to dryness on the rotary evaporator and the residue was taken up in 20 ml $CHCl_3$. The alkaloids were separated by thin-layer chromatography of the chloroform extract on silica gel GF254 developed with chloroform/methanol (90:10, v/v); they were viewed under ultraviolet light and sprayed with a *p*-dimethylaminobenzaldehyde solution (Stahl, 1969). The identity of the alkaloids was established by co-chromatography with reference standards, and by ultraviolet and low resolution mass spectral analyses (Porter *et al.*, 1978).

Smut grass leaves (*S. poiretii*) parasitized by *B. epichloë* were collected and lyophilized; the black stromata of the fungus located on the adaxial leaf surfaces were used to indicate infection. Alkaloids were extracted from the lyophilized grass (250 g) according to the procedure of Stoll *et al.* (1954) and analysed as described above.

RESULTS

Sporulation by *Balansia epichloë*, *B. claviceps* 219, *B. strangulans* and *B. henningsiana* began after 7 d incubation on medium M102 and reached a maximum after 10 d; the average spore concentration for these four species was 10^6 ml^{-1}. *Balansia claviceps* 266 did not sporulate on medium M102 or on a variety of other media.

The fermentation procedure used demonstrated that the isolates of the four species of

Table 1. *Alkaloid production by species of Balansia in culture*

Fungi were incubated according to the two-stage fermentation procedure and harvested when total alkaloids were maximum. Alkaloids were extracted and identified as described in Methods.

Fungus	Incubation time (d)	Alkaloids: Chanoclavine (I)	Ergonovine	Ergonovinine	Agroclavine	Maximum total alkaloids (mg l^{-1})
B. epichloë	28	+	+	+	+	390·00
B. henningsiana	36	+*	–	–	–	6·95
B. strangulans 233	38	+*	–	–	–	85·00
B. strangulans 257	38	+*	–	–	–	158·00
B. claviceps 266	28	–	–	–	–	0·00
B. claviceps 219	28	+	+	+	–	170·25

+, Present; – not detected.
* Additional clavine-type alkaloids were detected but their structures were not determined.

Table 2. *Alkaloid production by Balansia epichloë in single- and two-stage laboratory cultures*

In single-stage culture (M102; SM), the fungus was incubated in triplicate flasks containing 100 ml medium M102 or SM for 28 d. In two-stage culture (SM to M102; M102 to SM), the fungus was incubated for 10 d in either SM or M102 and then 1 ml inocula were transferred to triplicate flasks containing 100 ml of the other medium for 28 d.

Culture medium	Mycelial dry wt (mg)	Mean alkaloid yield (mg l^{-1})
M102	210	0·00
SM	161	0·25
SM to M102	197	0·00
M102 to SM	176	394·50

Balansia tested varied in their ability to produce alkaloids (Table 1). Not only did *Balansia epichloë* produce the highest total amount of alkaloids, but it also produced the greatest variety of ergot alkaloids. Medium-scale production of alkaloids from *B. epichloë* resulted in the isolation and identification of two more minor ergot alkaloids – elymoclavine and penniclavine – and another unidentified clavine alkaloid. In addition to chanoclavine (I), the total alkaloid fraction of *B. henningsiana* and *B. strangulans* contained several minor but unidentified clavine-type alkaloids. Smut grass parasitized by *B. epichloë* contained 17 mg total alkaloids kg^{-1}. The major alkaloids identified from the grass were chanoclavine (I) (16·0 mg kg^{-1}) and ergonovine (0·48 mg kg^{-1}). Several minor alkaloids extracted from the grass were not identified.

Since *B. epichloë* produced the largest amount of alkaloids, the results of studies of some of the requirements for alkaloid biosynthesis on medium SM are presented for this species and references to the other species are made only when different results were obtained. The use of medium M102 followed by medium SM was essential for high yields of alkaloids from *B. epichloë* (Table 2) and for alkaloid production by the other species of *Balansia*. Alkaloids were not produced when the fungi were cultured for 10 d on medium M102 and then transferred to fresh M102; nor were they produced when this type of experiment was done using only medium SM. When medium M38 (Bacon *et al.*, 1975) and a soluble starch medium (Porter *et al.*, 1977) were used instead of M102 or SM in various combinations, alkaloids were not produced. The yield of alkaloids was greatly reduced (60 to 80 %) when the fungi were incubated as submerged cultures in medium SM (data not shown). Incubation on the gyratory shaker for 10 d increased the rate of mycelial growth, thus reducing the time before maximum alkaloid production.

In the alkaloid-producing medium SM, maximum alkaloid yields from *B. claviceps* (190 mg l^{-1}) and *B. epichloë* (390 mg l^{-1}) were obtained after 28 d incubation (Table 1 and

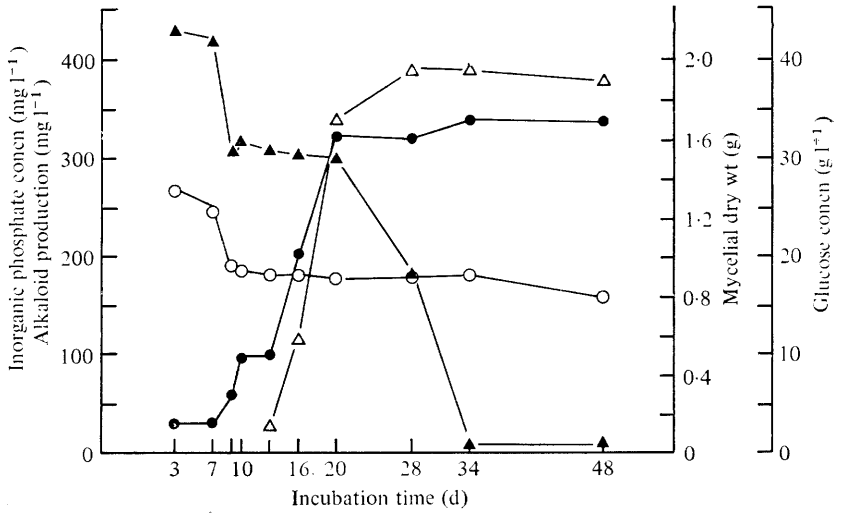

Fig. 1. Changes in mycelial dry weight (●), production of alkaloids (△), glucose concentration (▲) and inorganic phosphate concentration (○) during growth of *Balansia epichloë* in 100 ml medium SM in 500 ml flasks; the fungus was grown in submerged culture during the first 10 d.

Table 3. *Effect of substituting various carbon sources in medium SM on growth and alkaloid production by B. epichloë*

Cultures were analysed after 28 d of two-stage culture in 100 ml medium SM.

Carbon source	Concn ($g\ l^{-1}$)	Mycelial dry wt (mg)	Total alkaloids ($mg\ l^{-1}$)
Glucose/sorbitol	50:100	450·80	403·0
Glucose	50	458·80	ND
Glucose	100	335·08	ND
Sorbitol	100	58·27	1·6
Glucose/mannitol	50:100	156·24	397·0
Mannitol	100	61·37	ND
Sucrose	100	118·44	3·0
Sucrose/sorbitol	100:100	294·27	5·0

ND, Not detected.

Fig. 1). Maximum alkaloid production (Table 1) occurred within 38 and 36 d for *B. strangulans* and *B. henningsiana*, respectively. Accumulation of alkaloids paralleled the growth phase, and production levelled off when the concentration of glucose in the medium began its second decline (Fig. 1). High concentrations of inorganic phosphate and sorbitol remained in the medium at the end of fermentation for all species. Although sorbitol was not used during fermentation, its presence along with glucose was necessary for high levels of alkaloid production; it could be replaced by mannitol, but this was accompanied by a substantial growth reduction (Table 3). When sorbitol was the sole source of carbon, 20% was used after 28 d incubation and alkaloids were not produced. Negative results were also obtained when mannitol was the sole carbon source. No alkaloids were produced during the 28 d incubation period when glucose at either normal or twice normal concentration was used as the sole carbon source (Table 3). In a separate experiment (data not shown) in which glucose ($50\ g\ l^{-1}$) was the sole carbon source, the glucose was depleted after 8 d incubation, rather than after 34 d as it was when both glucose ($40\ g\ l^{-1}$) and sorbitol ($100\ g\ l^{-1}$) were present (Fig. 1). However, reduced yields of alkaloids ($70\ mg\ l^{-1}$) were obtained in media containing glucose as the only carbon source when the incubation period

Table 4. *Effect of inorganic phosphate on alkaloid production by B. epichloë*

Triplicate cultures were analysed after 28 d growth in 100 ml medium SM containing different concentrations of KH_2PO_4.

KH_2PO_4 concn ($g\ l^{-1}$)	Mycelial dry wt (mg)	Total alkaloids ($mg\ l^{-1}$)
0·025	219·27	4·72
0·500	410·72	67·34
1·000 (control)	439·62	380·42
2·000	484·71	376·91

was extended to a total of 45 d. Sucrose supported growth and low production of alkaloids when used alone or in combination with sorbitol. When succinic acid was omitted from medium SM, alkaloids were not produced, whereas mycelial yields were comparable to those of controls. Maximum amounts of alkaloids were produced by all species with succinic acid at $10\ g\ l^{-1}$. These fungi did not produce alkaloids when succinic acid was replaced with equimolar concentrations of glutamic acid or when succinic acid was added to medium M102 and extracted for alkaloids after a 28 d single-stage fermentation as described in Table 2.

The concentration of inorganic phosphate in medium SM was varied to determine its effect on alkaloid yield (Table 4). With KH_2PO_4 at $2\ g\ l^{-1}$, alkaloid production was no different from normal (i.e. with $1\ g\ KH_2PO_4\ l^{-1}$). Alkaloid yields were reduced at concentrations of phosphate below the control level. Thin-layer chromatography of the alkaloid fractions from media low in phosphate showed that only chanoclavine (I) was produced. Alkaloid fractions from media containing normal and high levels of phosphate consisted of the ergot alkaloids described for *B. epichloë* (Table 1). Addition of tryptophan ($0{\cdot}001\ g\ l^{-1}$) to medium SM with normal, high ($2\ g\ l^{-1}$) or low ($0{\cdot}025\ g\ l^{-1}$) concentrations of KH_2PO_4 led to a decrease in total alkaloid yield ($130\ mg\ l^{-1}$) in each case. Alkaloids were not produced when tryptophan was added at 0·01 or $0{\cdot}1\ g\ l^{-1}$ to medium SM with normal, high or low inorganic phosphate concentrations. The effects of tryptophan on alkaloid production by *B. henningsiana*, *B. claviceps* and *B. strangulans* were not determined.

DISCUSSION

We isolated chanoclavine (I), agroclavine, penniclavine and elymoclavine, all clavine-type ergot alkaloids commonly found in species of *Claviceps*, from cultures of *B. epichloë*. These alkaloids were also isolated by other workers from cultures of *Aspergillus fumigatus* (Spilsbury & Wilkinson, 1961), *Penicillium concavo-rugulosum* (Abe *et al.*, 1969) and *P. chermesinum* (Agurell, 1964). Chanoclavine (I) was also produced by *B. claviceps*, *B. henningsiana* and *B. strangulans*. Additional minor alkaloids produced by these species were not completely identified, but mass spectral analysis indicated that they were of the clavine type. The isolation of ergonovine and ergonovinine from cultures of *B. claviceps* and *B. epichloë* is the first demonstration of lysergic acid derivatives in fungi outside the genus *Claviceps*. It suggests that the ability to synthesize these lysergic acid derivatives resides more generally within the family Clavicipitaceae, not just in the genus *Claviceps*. Peptide-type alkaloids were not detected in culture extracts of *Balansia*. These species of *Balansia* might therefore be placed under the second biochemical grouping established for ergot fungi – the *C. paspali* type that produce the clavine alkaloids and simple lysergic acid derivatives but no peptide alkaloids (Gröger, 1972). Interesting in this respect is the concept that, taxonomically, the *C. paspali* group is intermediate between the *Balansia* and the *C. purpurea* groups, but is more closely related to the *Balansia* group (Gröger, 1972).

Production of ergot alkaloids in culture by species of *Balansia* has several requirements.

The fungi must first be cultured in a nutrient medium in which they grow and sporulate, and then transferred to a different growth medium in which they produce alkaloids. These species of *Balansia* produced spores on medium M38 (Bacon *et al.*, 1975) which were morphologically identical to those produced on medium M102, but subsequent transfer to medium SM did not result in alkaloid production. Thus the process of sporulation is not essential for alkaloid biosynthesis. The nature of the differences between the fungi grown on these two media is unknown. Large quantities of sorbitol remained in the medium after fermentation and it may be used as a growth promoter, as is inositol in many fungi. If it does function as a growth promoter, the small amount utilized could not be determined by our method of analysis. Glucose is the main source of carbon for these fungi in medium SM. The requirement for sorbitol or mannitol, the continued increase in mycelial dry weight even though glucose utilization is reduced, and the delay of maximum alkaloid synthesis until glucose enters a second period of rapid utilization characterized by no further increase in mycelial dry weight suggest that alkaloid production is controlled by a growth-linked suppression mechanism (Bu'Lock, 1965), which is described as regulated by catabolite repression in *Claviceps*, *Cephalosporium* and other fungi by Drew & Demain (1977). Additional evidence needed to support this generality (other means of measuring growth, and the nature of the limiting nutrient) was not obtained in this study. The relationship of inorganic phosphate concentration to alkaloid synthesis in *B. epichloë* is the reverse of that in *Claviceps* species. In submerged cultures of *Claviceps*, uptake of inorganic phosphate was rapid and complete (Arcamone *et al.*, 1970; Banks *et al.*, 1974). High concentrations of phosphate depressed alkaloid biosynthesis, the effect being related to the concentration of tryptophan (Robbers *et al.*, 1972). The rapid drop in phosphate concentration seen in our study during the first 10 d fermentation, when the fungi were cultured submerged, indicates that the submerged culture of *Balansia* leads to an initial uptake of phosphates. Tryptophan was reported to increase the yield of alkaloids in certain species of *Claviceps* (Robbers *et al.*, 1972). The addition of tryptophan to medium SM did not increase alkaloid yield and any relationships of tryptophan to phosphate utilization and alkaloid biosynthesis in these *Balansia* species are unknown.

Variations between two isolates of *B. strangulans* and two of *B. claviceps* when each species was obtained from identical grass species from the same location indicate that the distribution of ergot alkaloids within a pasture might not be uniform and, indeed, that some of the grass might not be toxic. Thus, the presence of endophytes would not necessarily indicate toxicity. A more extensive survey of isolates of endophytes from individual pastures would determine the degree of variability. The isolation of ergonovine and chanoclavine (I) from randomly collected *B. epichloë*-infested smut grass indicated that at least two of the alkaloids are produced *in vivo* and established a relationship between the systemic species on this grass and pasture toxicity. Since the parasitized smut grass was collected during the time when black stromata were visible on the leaves (end of the growing season), we have no indication as to the amount and types of alkaloids present in the grass throughout the growing season. Since each species of *Balansia* parasitizes several grass species, a comparative survey of isolates from different hosts would indicate whether the host influences alkaloid production, as reported in *Claviceps* (Gröger, 1972). The biological activities of the ergot alkaloids produced by these *Balansia* species are known. The clavine alkaloids, depending on the concentration, produce hyperpnoea, mydriasis, anxiety (Yui & Takeo, 1958), prolactin inhibition (Cassady & Floss, 1977), nidation and death in animals (Mantle, 1969). The toxicity of the lysergic acid derivatives to domestic animals has been reviewed by Burfening (1973) and Mantle (1969). The present results, therefore, indicate that the production of the ergot alkaloids by *B. epichloë*, *B. claviceps*, *B. henningsiana* and *B. strangulans* may be involved in the many ergot-type cattle toxicity syndromes in pastures where species of *Claviceps* are absent.

We thank F. W. Gould and W. H. Duncan for identifying the grasses; E. S. Luttrell, University of Georgia, for supplying *B. claviceps* 219; R. M. Bennett, J. Robbins and C. Groover for technical assistance; and R. W. Roncadori, University of Georgia, for use of greenhouse facilities to grow parasitized grasses.

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