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IN VITRO AUXIN PRODUCTION BY *BALANSIA EPICHLOË*

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Key Word Index—*Balansia epichloë*; Clavicipitaceae; systemic phytopathogens; 3-indole acetic acid; 3-indole ethanol; 3-indole acetamide; methyl 3-indole carboxylate.

Abstract—*Balansia epichloë*, a systemic plant pathogen isolated from *Sporobolus poiretii*, was shown to produce the plant growth regulators 3-indole acetic acid, 3-indole ethanol, 3-indole acetamide and methyl-3-indole carboxylate when grown on a medium containing tryptophan. When grown on a tryptophan deficient medium, 3-substituted indole derivatives were not detected. However, extracts of the medium in lower doses increased and in higher doses inhibited the growth of wheat coleoptiles.

INTRODUCTION

Investigations on systemic members of Clavicipitaceae (*Balansia* sp., *Epichloë typhina* and *Myriogenospora atramentosa*) have suggested the operation of stress mechanisms by these fungi on their respective host grasses [1–3]. The observed effects of these systemic fungi on their host's morphology include dwarfing, faciation and, occasionally, deformation, especially of the flag leaf and developing inflorescence. The dwarfed and faciated conditions of the infected grasses may result from the production of either plant growth inhibitors or altered metabolites of plant growth regulators. Plant inhibitors or altered hormone metabolism are likely possibilities when it is considered that hyperauxing in parasitized plants is well established [4] and that most of the major groups of plant hormones have been isolated from pathogenic fungi [5].

Previous studies [6, 7] have demonstrated that *Balansia epichloë* (Weese) Diehl in culture produced precursors of plant auxins (e.g. 3-indole glycerols). We now report on the *in vitro* syntheses of 3-indole acetic acid, 3-indole ethanol, 3-indole acetamide and methyl-3-indole carboxylate by *B. epichloë* isolated from *Sporobolus poiretii* (Roem. and Schult.) Hitchc.

RESULTS

Fractions 1 and 2 isolated from *B. epichloë* media containing tryptophan (TM) (cf. Experimental) were dissolved in acetone and aliquots screened for plant growth activity using the wheat coleoptile bioassay system. Fraction 1, assayed at the equivalents of 3 ml fungal culture (120 μg crude material), gave 160% stimulation of the wheat coleoptile (equivalent to 10^{-5} M indole acetic acid). Fraction 2 was devoid of activity. It contained the ergot alkaloids previously described [8–10].

Fractions 3 and 4, isolated from the fungus cultured on tryptophan deficient medium (TDM), were tested in the wheat coleoptile assay. Fraction 4 was devoid of any activity up to the equivalent of 10 ml fungal culture. This fraction contained the ergot alkaloids previously described [8–10]. Fraction 3, at the equivalent of 1 ml fungal culture, resulted in a 38.5% stimulation for the 8-week-old culture, 63.5% stimulation for the 10-week-old culture and 48.1% stimulation for the 12-week-old culture. Simple 3-substituted indoles could not be detected in these fractions using *p*-dimethylaminobenzaldehyde (PDAB) analyses [11]. Aliquots of the above fractions (8-, 10- and 12-week-old fraction 3) equivalent to 10 ml fungal culture completely inhibited growth of the wheat coleoptiles.

Fraction 1 was subjected to prep. TLC in solvent system 'a' which resulted in four uniform dark blue bands when the plates were observed under UV radiation (254 nm). Each band was scored, collected in glass funnels (fritted disk) and eluted from silica gel with chloroform–methanol (1:1). A longitudinal guide section, left on the side of the plates, was sprayed with PDAB. Equivalent areas on the plates were sectioned into the following fractions: (A) (R_f 0.25–0.39), grey reaction to PDAB; (B) (R_f 0.46–0.54), violet reaction; (C) (R_f 0.54–0.61), yellow-green reaction; (D) (R_f 0.65–0.68), yellow-green reaction. The eluates were concentrated under nitrogen and aliquots subjected to UV and mass spectrometry (EIMS, probe, 70 eV, from 25 to 200° at 20°/min) analyses. Co-chromatography (TLC) was performed with authentic standards in solvent systems a–c. In addition, the R_ts (GC) of the TMSi derivatives of the isolated compounds were compared with those made from the standards. Spectral and chromatographic data showed that: fraction A was 3-indole acetic acid [12]: UV λ_{max}^{MeOH} nm: 221, 273, 281, 289; EIMS m/z (rel. int.): 175 $[M]^+$ (35), 130 $[M-CO_2H]^+$ (100); R_t (TMSi): 10.9 min; fraction B corresponded to 3-indole acetamide [12]: UV λ_{max}^{MeOH} nm: 219, 267, 270, 277, 280; EIMS m/z (rel. int.): 174 $[M]^+$, (18), 130 $[M-CONH_2]^+$ (100); R_t (TMSi): 11.9 min; and fraction C corresponded to 3-indole ethanol [12] UV λ_{max}^{MeOH} nm: 221, 273, 281, 289; EIMS m/z (rel. int.): 161 $[M]^+$ (19), 130 $[M-CH_2OH]^+$ (100); R_t (TMSi): 10.2 min. After additional prep. TLC in chloroform–methanol (9:1; relative humidity 26%; temp. 23°; R_f 0.69–0.61), fraction D yielded spectral and R_t data identical to methyl-3-indole carboxylate [12, 13]:

UV λ_{max}^{MeOH} nm: 225, 276, 280, 286; EIMS m/z (rel. int.): 175 $[M]^+$ (58), 144 $[M-OMe]^+$ (100), 116 $[144-CO]^+$ (50), 89 $[116-HCN]^+$ (75); R_f 0.63 (a), 0.47 (c), 0.51 (d); R_t (TMSi): 11.1 min.

Due to decomposition of the indoles on work-up (photolytic and air oxidation) no attempt was made to quantitate the individual compounds isolated. However, visual observation of the TLC plates (UV and PDAB reaction) indicated 3-indole acetic acid as the major compound.

DISCUSSION

When grown on a tryptophan medium (TM), *B. epichloë* produced *in vitro* auxins identified as 3-indole acetic acid, 3-indole ethanol (tryptophol), 3-indole acetamide and methyl-3-indole carboxylate. The isolation and identification of the indole auxins from *B. epichloë*, along with the systemic nature of the fungus suggest this fungus may catabolize endogenous tryptophan to regulate plant growth. Simple 3-substituted indoles were not detected in the active fraction (fraction 3) when *B. epichloë* was cultured on a tryptophan deficient medium (TDM) and suggests that compounds other than the catabolic products of tryptophan may be involved in the regulation of plant growth by this fungus. The isolation and identification of these compounds are under investigation. Nevertheless, an auxin involvement is suggested as the differences in activities obtained at the different concentrations (1 ml = stimulation vs 10 ml = inhibition) parallel auxin activity observed in normal tissues. Plant development is controlled by an endogenous concentration gradient of auxins throughout the vascular systems of the plants, i.e. high concentrations of auxin inhibit rather than stimulate growth [13]. In addition to growth, flowering in infected grasses is affected. Generally, *Balansia* infected grasses are sterile and, since flowers are never produced, sterility might be maintained by qualitative or quantitative levels of plant hormones. The *in vitro* production of auxins by the fungus suggests that the fungus could augment the levels of at least this class of compound in the *in vivo* situation. While data are lacking, the *in situ* levels in infected and non-infected plants should strengthen this generalization.

Studies have shown that *B. epichloë* can produce ergot alkaloids both *in vivo* and *in vitro* [9, 10]. In addition, *erythro*- and *threo*-3-indolyl glycerols have been isolated from cultures of this fungus [1, 6, 7]. Biosynthesis of both the ergot and simple 3-substituted indole alkaloids is dependent on tryptophan metabolism. Tryptophan metabolism is directly associated to *erythro*-3-indolyl glycerol phosphate [14–16]. Whether *B. epichloë* and/or other systemic plant pathogens of the Clavicipitaceae regulate plant growth through catabolism of host's tryptophan or by shunting *erythro*- (to *threo*-)3-indolyl glycerol phosphate by an 'epimerase' is currently unknown. This indeed would be a novel way for these fungi to shift or alter the equilibrium of hormonal homeostasis and regulate plant growth.

EXPERIMENTAL

Organism. *Balansia epichloë* (RRC 313) isolated from *Sporobolus poiretii* as previously described [8] was cultured according to the two-stage fermentation procedure [9]. For the second stage of fermentation, the fungus was incubated in a medium (TM) containing (per l. H_2O): sorbitol, 100 g; sucrose, 40 g; succinic acid, 10 g; NH_4Cl, 2.4 g; D,L-tryptophan, 0.8 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; citric acid, 0.5 mg; $ZnSO_4 \cdot 7H_2O$, 0.5 mg; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.1 mg; $MnSO_4 \cdot H_2O$, 0.05 mg; H_3BO_3, 0.05 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.05 mg; $CuSO_4 \cdot 5H_2O$, 0.025 mg; thiamine·HCl, 0.4 µg; nicotinic acid, 2.8 µg; and pyridoxine, 0.4 µg. The pH was adjusted to 5.6 with NaOH. The fungus was incubated for 4 weeks at 26° in stationary culture (final pH 5.8). *Balansia epichloë* was also cultured on a medium as above without tryptophan (TDM) for 8, 10 and 12 weeks (final pH 5.0).

Extraction and isolation of indole alkaloids. The medium (TM) was separated from the mycelium by filtration through cheesecloth and extracted with 3 vols CH_2Cl_2–*iso*-PrOH (3:1). The mycelium and aq. phase were stored at $-20°$ for future studies. The combined organic extracts were dried over Na_2SO_4, filtered and concd *in vacuo* ($\leqslant 30°$). The crude residue (1.07 g) was dissolved in 20 ml 2% tartaric acid (pH 3) and the aq. acid soln extracted with 3 vols EtOAc. The EtOAc extracts were combined and worked-up as above to yield 144 mg crude material (fraction 1). The aq. tartaric acid soln was adjusted to pH 10 (NH_4OH) and the resulting soln extracted with EtOAc as above. This extract provided 254 mg crude material (fraction 2). The aq. phase, after extraction, was discarded. Fractions 1 and 2 were divided, one part was used for the isolation procedure and one part for the bioassay. Each fraction was treated with Me_2CO and the Me_2CO solubles tested at the equivalent of 3.0 ml fungal culture for plant growth activity using the wheat coleoptile bioassay.

Extraction of B. epichloë *grown on medium TDM*. The medium was separated from the mycelium as above, adjusted to pH 4.0 with HCl and the resulting soln (500 ml) extracted with 3 vols EtOAc. The extracts were combined, dried (Na_2SO_4), taken to dryness and the resulting residues treated ($\times 4$) with 5 ml Me_2CO. The Me_2CO solubles (acids) (fraction 3) contained: (a) 8-week-old culture, 1.0 g; (b) 10-week-old culture, 0.82 g; (c) 12-week-old culture, 0.76 g crude material.

The aq. medium, after removal of the 'acids', was adjusted to pH 10 with NH_4OH and extracted with EtOAc. The organic extracts were combined and treated as described above for fraction 3. The resulting Me_2CO solubles (bases) (fraction 4) contained: (a) 8-week-old culture, 46 mg; (b) 10-week-old culture, 38 mg; (c) 12-week-old culture, 23 mg. Fractions 3 and 4 were bioassayed (wheat coleoptile) at the equivalent of 1 and 10 ml fungal culture

Chromatography. All solvents were analytical reagent grade and were not further purified. Preparative TLC was performed on 20 × 20 cm glass plates coated with 0.75 mm silica gel GF 254 (Brinkman). The developing solvents were: (a) $CHCl_3$–MeOH (4:1); (b) $CHCl_3$–MeOH–NH_4OH (94:5:1); (c) $CHCl_3$–Et_2NH (9:1); (d) $CHCl_3$–*iso*-PrOH (23:2). Compound application was performed using a Desaga-Autoliner (Brinkman) with N_2. The alkaloids were observed under UV radiation (254 and/or 366 nm) and after spraying the edge of the plates with PDAB reagent [11].

FID–GC: glass column (183 × 0.32 cm) packed with 3% SP-2250 on 100/120 Suplecon AW-DMCS (Supleco), He 30 ml/min, column temp. from 100 to 250° at 16°/min with a 4 sec delay to program, injector and FID temps 275°. GC R_t values are for the trimethylsilyl derivatives [17] which were synthesized using *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce Chemical Co.) according to ref. [18].

Standards. 3-Indole acetic acid, 3-indole acetamide and 3-indole ethanol (tryptophol) were obtained from Aldrich Chemical Co. Methyl-3-indole carboxylate was obtained by reported synthesis [19] with modifications in procedure and isolation. Dichlorodicyanobenzoquinone (DDQ) and 3-indole acetonitrile used in the synthesis were obtained from Aldrich Chemical Co.

Dichlorodicyanobenzoquinone, 1.0 mmol, in 2 ml MeOH was added drop-wise to a stirred (magnetic) soln (25°) of 0.5 mmol 3-indole acetonitrile in 5 ml MeOH (temp. increased to 27°). After addition, the soln was stirred for 4.5 hr at room temp. and then for 1 hr at 40°. The mixture was cooled (room temp.) and concd *in vacuo* (≤ 30°). The magma was dissolved in 30 ml CH_2Cl_2 and filtered through Whatman No. 1 filter paper and then through Florisil (1 g) in a sintered glass funnel. The Florisil was washed (× 3) with 10 ml CH_2Cl_2 and the combined filtrate and washings were concd to 10 ml The soln was subjected to prep. TLC (relative humidity 39%, temp. 23°) in solvent system c. An area equivalent to the blue band observed at 254 nm and giving a yellow reaction to PDAB (R_f 0.46–0.6) was cut from the plate, eluted from the gel with CH_2Cl_2 and subjected to prep. chromatography in solvent system a (relative humidity 45%, temp. 23°) (R_f 0.49–0.59). Rechromatography, in the same solvent system, by developing the plate twice and air drying at room temp. between runs (final R_f 0.51–0.61) resulted in pure methyl-3-indole carboxylate: EIMS m/z, UV and mp as reported in refs [12, 19].

Wheat coleoptile bioassay. Wheat seed (*Triticum aestivum* L., cv. Wakeland) was germinated on moist sand for 4 days and used for plant growth bioassay as in ref. [20].

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