Identification of GA₃ in *Neurospora crassa* and Its Changes during Conidial Germination and Mycelial Growth

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GA₃ was identified as a major GA in *Neurospora crassa* by gas chromatography/selected ion monitoring (GC/SIM) and its content was measured at various stages (0~96 hr after inoculation) of conidial germination and mycelial growth. The GA₃ content in the fungus was 190 ng/g dry weight at the initial stage and then decreased rapidly; that per liter culture decreased soon after the inoculation, and then increased to 17.6 ng 96 hr after inoculation. The GA₃ concentration in the culture medium was around 10⁻¹¹ M throughout the 96-hr incubation. The physiological role of endogenous GA in *Neurospora crassa* is also discussed.

Exogenous GA₃ promotes the conidial germination and mycelial growth of *Neurospora crassa*.^{1~3)} We have reported the identification of endogenous GA₃ in the mycelia of *N. crassa*.⁴⁾

In this paper we report the details of the identification of GA₃ and the changes of its content in the conidia, mycelia, and culture media during the conidial germination and mycelial growth of *N. crassa*.

The conidial germination and mycelial growth of *N. crassa* are shown in Fig. 1. By 3 hr after inoculation, 50% of the conidia had germinated and by 6 hr, 90% had germinated. Both the ungerminated and germinated conidia are referred to as conidia unless otherwise specified. The dry weight of conidia and mycelia changed from 33 mg/liter to 3600 mg/liter during the 96-hr incubation.

The GA fractions in the extracts of mycelia and culture medium from the 96-hr incubation were investigated by gas chromatography/selected ion monitoring (GC/SIM) after several purification steps. The mycelia were extracted with 80% aqueous methanol using a blender. After removal of the methanol in

vacuo, the residual aqueous solution was extracted with ethyl acetate to give an acidic ethyl acetate (AE) fraction.⁵⁾ Culture medium was extracted with ethyl acetate and ethyl acetate extract was also fractionated in the same procedure to give an AE fraction. The AE fractions were purified by preparative thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) using a Nucleosil 5N(CH₃)₂ column to give GA₁- and GA₃-like fractions,⁶⁾ which are analyzed by GC/SIM after conversion to their trimethylsilyl ester trimethylsilyl ethers (TMS-TMS). As shown in Fig. 2B and 2C, in

FIG. 1. Conidial Germination and Dry Weight Changes during Incubation of *Neurospora crassa*.

FIG. 2. GC/SIM Profiles of the TMS-TMS Derivatives of the GA-Active Fractions from the Mycelia and Culture Medium of Wild-type Strain 74 A of *Neurospora crassa*.

The TMS-TMS of authentic GA_3 (1 ng) (A), and of the GA-active fractions from mycelia (320 mg dry weight) (B) and culture medium (90 ml) (C) were used for each scanning by a 2% OV-1 column (100 mm $\times$ 3 mm i.d.) at 215°C. Helium was used as carrier gas at a flow rate of 50 ml/min.

the monitoring of the fragment ions at m/z 562 (M^+), 547 and 445, the GA_3 fractions had three peaks with the same retention time and the same relative intensities as those of authentic GA_3 -TMS-TMS (Fig. 2A), thus confirming the presence of GA_3 in the mycelia and culture medium from the 96-hr incubation of *N. crassa*. The quantities of GA_3 in the mycelia and culture medium were calculated from a calibration curve made for a peak area of m/z 562 ion, those contents being 17.6 ng/liter and 4.7 ng/liter, respectively.

Except for *Gibberella fujikuroi*, a plant pathogen causing the rice disease "Bakanae-byo," the evidence for production of gibberellin by microorganisms is rather limited. In 1979, Rademacher and Graebe⁷⁾ reported the identification of GA_4 as a metabolite of *Sphaceloma manihoticola*, a plant pathogen of a superelongation disease of cassava. *N. crassa* is the third fungus which was proved definitely to produce gibberellin. It is also noteworthy that GA_3 was accumulated mainly in the mycelia of *N. crassa*, while other fungi excrete

FIG. 3. Changes in GA_3 Content in the Conidia, Mycelia, and Culture Media of *Neurospora crassa* during Incubation.

The GA_3 content was estimated by GC/SIM. The GA_3 contents per liter culture in the conidia and mycelia (■) and in the culture media (▨). Inset: The GA_3 content per g dry weight in the conidia and mycelia.

gibberellin into culture media.

By the same procedure GA_3 was also identified and quantified as the major GA in the conidia, mycelia, and culture media from various stages of conidial germination and mycelial growth of *N. crassa*.

As shown in Fig. 3, the GA_3 content in the conidia and mycelia started decreasing immediately after inoculation and then increased gradually to 17.6 ng/liter culture after 96 hr, while the amount per g dry weight (Fig. 3, inset), which was 190 ng at the initial stage, also decreased rapidly after inoculation to reach the lowest level after 48 hr, and then slightly increased during the further incubation. The GA_3 concentration in the culture medium was approximately 10^{-11} M throughout the 96-hr incubation.

The changes in gibberellin activity (data not shown), measured by the micro-drop bioassay⁸⁾ using seedlings of dwarf rice (cv. Tan-ginbozu), were similar to those in GA_3 content (Fig. 3).

As previously reported, exogenous GA_3 promoted the conidial germination rate and the elongation of germ tubes and young hyphae in *N. crassa*.^{1,3)} These facts together with the higher concentration of GA_3 in the conidia

at the initial stage and its rapid decrease after inoculation strongly suggest that GA₃ acts as an endogenous regulator of the process of conidial germination in this fungus. And further, since the concentration of endogenous GA₃ in the fungus (about 10^{-8} M to 10^{-9} M) was much less than that of the exogenous GA₃ we used, it seems that the exogenous GA₃ may act in modifying the endogenous level of GA₃.

The effect of GA₃ on the growth of mature hyphae (48 and 96 hr incubation) is not defined. GA₃, however, showed little promoting effect on the mycelial growth when it was given alone, while exogenous GA₃ overcame the inhibitory effect of AMO1618 on the dry weight of mycelia (48-hr incubation) completely.²⁾ Although the endogenous level of GA₃ (per g dry weight) in the mycelia was much lower than that in the conidia (Fig. 3, inset), the level in the mycelia after a 48-hr incubation may be enough to regulate the growth of mature hyphae.

Comparison of GA₃ contents between 48-hr and 96-hr incubated mycelia clearly indicated that GA₃ was being increased in the mycelia 96 hr after inoculation (Fig. 3). After 96 hr of incubation, the mycelial growth was almost in the stationary phase and new conidia formation began. On the other hand, as the conidia contained the GA₃ at a higher level (Fig. 3, inset) increased GA₃ in the mycelia may be due to the newly produced conidia.

These results suggest that GA₃ may act as a growth regulator in this fungus. The endogenous level of GA₃ during conidia formation is under study.

EXPERIMENTAL

Instrumental analysis. HPLC was done with a Jasco UVIDEK-100-IV with a packed column (100 mm × 6 mm i.d., Nucleosil 5N(CH₃)₂), which was eluted with 0.05% acetic acid in methanol (flow rate, 2 ml/min) at room temperature. The eluent was monitored with a UV detector at 210 nm.

Samples to be submitted to GC/SIM analysis were converted to the TMS-TMS derivatives with pyridine-*N,O*-bis-(trimethylsilyl)acetamide-trimethylchlorosilane (1:2:1, v/v/v).

GC/SIM was done with a Hitachi 80 A with a glass

column (100 mm × 3 mm i.d.) packed with 2% OV-1 on Chromosorb W; oven temperature, 215°C; carrier gas, He; flow rate, 50 ml/min. The quantity of GA₃ was calculated from a calibration curve made for a peak area of the strongest ion.

Organisms and culture conditions. The wild-type strain 74 A of *N. crassa* was used. Conidia, produced on a glycerol complete agar medium⁹⁾ after 7 days of incubation of mycelia, were collected by suspending in distilled water, and washed twice by distilled water. These were referred to as conidia at the initial stage. The conidia were inoculated at a cell density of about 2×10^6 conidia/ml into a 500 ml Sakaguchi flask containing 300 ml of Fries minimal medium⁹⁾ with 1.5% sucrose, and cultured at 26°C with gentle reciprocal shaking (120 rpm).

Identification of GA₃ in the mycelia and culture medium from the 96-hr incubation.

a) Extraction and fractionation. After 96 hr of incubation, mycelia and culture medium were separated by passing the culture suspension (600 ml) through filter papers (Toyo, 5C). Mycelia (about 45 g fresh weight) was homogenized in 2 liters of cold 80% aqueous methanol using a blender, and the homogenate was kept overnight at 4°C and filtered. After removing the methanol *in vacuo*, the residual aqueous solution (400 ml) was acidified (pH 3.0) and fractionated by solvents as usual⁵⁾ to give an AE fraction, which was then concentrated to an acidic gum (29 mg). The culture filtrate (600 ml) was also acidified (pH 3.0) and fractionated in the same way to give an AE

FIG. 4. Histograms of TLC Fractions of Mycelia and Culture Medium from the 96-hr Incubation of *Neurospora crassa*.

The AE fractions of mycelia (A) and culture medium (B) were chromatographed on a silica gel plate (Kiesel-gel 60F₂₅₄, Merck, 200 mm × 200 mm, 0.5 mm thick) developed with a solvent system of ethyl acetate-chloroform-acetic acid (15:5:1, v/v/v). GA activity was measured by the micro-drop method using dwarf rice (cv. Tan-ginbozu) seedlings.

fraction which was then concentrated (20 mg).

b) *Purification by TLC.* The AE fractions were dissolved in small amounts of ethyl acetate and purified by preparative TLC to give GA-active fractions. TLC was done with a precoated Kiesel-gel 60F₂₅₄ (Merck, 200 mm × 200 mm, 0.5 mm thick) developed with a solvent system of ethyl acetate–chloroform–acetic acid (15:5:1, v/v/v). GA activity was measured by the micro-drop bioassay⁸⁾ using seedlings of dwarf rice (cv. Tan-ginbozu). Since biological activity of GA-like substances appeared in the regions of authentic GA₃ (Fig. 4), the zone from *R*_f 0.34 to 0.50 was scraped off, eluted with water-saturated ethyl acetate, dried over anhydrous sodium sulfate, and then concentrated *in vacuo*.

c) *Purification by Nucleosil 5N(CH₃)₂.* The GA fractions were dissolved in small amounts of methanol and injected onto a packed column (100 mm × 6 mm i.d., Nucleosil 5N(CH₃)₂),⁶⁾ which was eluted with 0.05% acetic acid in methanol (flow rate, 2 ml/min) at room temperature. The eluent was monitored with a UV detector at 210 nm. GA₃ was eluted at *t*_R 14–16 min.

Semiquantitative analysis of GA₃ in conidia, mycelia, and culture media during conidial germination and mycelial growth.

a) *Extraction and fractionation.* Conidia (about 200 mg fresh weight, at 0-hr incubation; about 240 mg fresh weight, after 3 hr of incubation; about 1500 mg fresh weight, after 6 h of incubation), collected by passing the culture suspensions (600 ml) through membrane filters (Millipore, 0.45 μm), were homogenized in 200 ml of 80% aqueous methanol using an ultrasonic disintegrator (Toyo, UD-2N-100). Mycelia (about 45 g fresh weight, after 48 hr of incubation; about 45 g fresh weight, after 96 hr of incubation), collected by passing the culture suspensions (600 ml) through filter papers (Toyo, 5C), were homogenized in 2 liters of 80% aqueous methanol using a blender. The homogenates were kept overnight at 4°C and filtered, and evaporation of the solvent *in vacuo* gave the residual aqueous solutions. The aqueous solutions from conidia, mycelia and culture filtrates were extracted with ethyl

acetate at pH 3.0 and the AE fractions were obtained.

b) *Purification and quantification.* The fractions were purified by TLC and Nucleosil 5N(CH₃)₂ and analyzed by GC/SIM as the samples from the 96-hr incubation.

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