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Environmental factors affecting development of *Aspergillus nidulans*

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***Aspergillus nidulans*, a homothalic ascomycete, has a complete sexual reproductive cycle as well as an asexual one. Both sexual and asexual development are known to be genetically programmed, but are also strongly affected by environmental factors including nutrients, light, temperature and osmolarity. We have examined these factors to define favored conditions for fruiting body (cleistothecium) formation. In general, fruiting body formation was enhanced where carbon and nitrogen sources were sufficient. Limitation of C-source caused predominant asexual development while inhibiting sexual development. When higher concentrations of glucose were supplied, more cleistothecia were formed. Other carbon sources including lactose, galactose and glycerol made the fungus develop cleistothecia very well, whereas acetate caused asexual sporulation only. Organic nitrogen sources like casein hydrolysate and glycine, and an increase in nitrate or ammonium concentration also enhanced sexual development. In addition to nutrient effects, low levels of aerobic respiration, caused either by plate-sealing or treatment with various chemicals, favored sexual development. Carbon limitation, light exposure and a high concentration of salts promoted asexual development preferentially, suggesting that stress conditions may drive the cell to develop asexual sporulation while comfortable and well-nourished growth conditions favored sexual development.**

***Key words*:** *Aspergillus nidulans,* sexual and asexual development, environmental stresses

A homothallic ascomycete, *Aspergillus nidulans*, has two major reproductive cycles-sexual and asexual. A germinated spore grows for nearly 20 h to form a thallus that acquires competence for developing conidiophores (Axelrod, 1973). After competence is acquired, induction of sporulation occurs through intracellularly programmed events, which include the processes of signaling and signal transduction. Then morphologically distinct cells are developed sequentially to produce a large number of conidia. Many genes have been identified as involved in the processes and the regulatory pathway or a network among the genes is now well established (for review see Adams *et al.*, 1998). As for sexual reproduction, similar differentiation processes are expected to occur; however, only a few genes are known that are involved in the process or in the regulation of sexual development. Both sexual and asexual sporulation require FlbA function which antagonizes FadA-mediated growth signaling (Yu *et al.*, 1996; Han *et al.*, 2001). After vegetative growth is stopped by the FlbA function, induction of development takes place. The induction of sporulation is largely dependent on the environmental conditions. Conidium development in *A. nidulans* is affected by starvation (Skromne *et al.*, 1995), light (Moony and Yager, 1990) and salt such as KCl (Song *et al.*, 2001). Development of cleistothecia is also affected by nutrition (Zonneveld, 1977) and temperature (Champe *et al.*, 1981).

A lot of mutants defective in sexual development have been screened and analyzed (Han *et al.*, 1990). They were grouped into three categories. Some mutants (NSD: never in sexual development) did not produce any sexual organ, some (BSD: block in sexual development) produced immature sexual organs and some (ASD: abnormal in sexual development) produced fully matured sexual organs but differed in the rate of formation of sexual organs. Several NSD mutants have been analyzed for their genetic and morphogenic characteristics (Han *et al.*, 1994b; 1998; 2001). Cleistothecia are not formed under standard culture conditions, while plenty of conidia develop. This phenotype is not apparently affected by some environmental factors in some NSD mutants (Han *et al.*, 1994b; 2001). Overexpression of *nsdD* resulted in the development of cleistothecia in the presence of 1M KCl which normally inhibited sexual development (Han

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et al., 2001). There may be some genes including *nsdD* that control development in response to those environmental factors. Thus, it is necessary to clarify first how environmental factors affect development to study the detailed function of these genes. Several factors including visible light are known to affect asexual sporulation (Mooney and Yager, 1990; Lee and Adams, 1994a; Han *et al.*, 1994; J. H. Yu, personal communication). These factors also affect sexual development, usually in the apposite way. In previous work (Han *et al.*, 1994a), the effect of carbon sources on sexual development was examined. As an extension of the preliminary data, here we examined in more detail how the nutritional, physical or chemical factors affect the development, alone or in combination, to establish the basis of conditions under which sexual or asexual sporulation is preferred.

Materials and Methods

Strains and growth conditions

FGSC4, the Glasgow wild type isolate of *A. nidulans*, was used as a *veA*$^{+}$ wild type. Complex medium (CM) and standard minimal medium (MM) were prepared with some minor modification on the basis of Pontecorovo *et al.*, (1953) and Käfer (1977). Minimal salt solution (MS) was pre-made as 20X and added to the medium after sterilization. The ingredients of MS were almost identical to those of Käfer (1977) except some trace elements (boric acid and cobalt chloride were not included). CM was used for strain maintenance, and MM for the development assay (Han *et al.*, 1990; 1994a; 1994b; 2001). The pH of all media was adjusted to 6.0 before autoclaving. Strains were cultured at 37°C, if not otherwise noted.

Light illumination

Light illumination was carried out in a temperature-controlled plant growth chamber (Vision Co., Korea) equipped with 5 white fluorescent lamps and 5 SP lamps (Toshiba, Japan) emitting red light. To protect from UV light, the culture plates were covered with 1 cm-thick acryl plates. The temperature was maintained at 37°C. Illumination intensity was measured with a light meter (Extech Ins., Taiwan).

Measurement of growth rate, conidial and cleistothecial development

The radial growth was measured every 24 h after inoculation at the center of the plate. The number of conidia was measured with a haemacytometer by a light microscope. The conidium sample was prepared by suspending and vortexing a 1 cm diameter culture block removed from a 3 day-cultured plate in 1 ml of 0.08% Tween80. The number of cleistothecia was directly scored under a dissecting microscope.

Results and Discussion

Sexual reproduction is favored under well-nourished conditions

To examine the effects of environmental factors on development, it is necessary to set a standard culture condition. We had previously established a standard culture condition for medium volume (30 ml), inoculum size (10^5/plate), incubation temperature (37°C) and medium type (Minimal medium) (Han *et al.*, 1990). As shown in Table 1, both sexual and asexual development are largely dependent on carbon (C) sources. On 1% glucose, which is one of the favored C sources, both sexual and asexual organs developed with 0.1% sodium nitrate as the sole nitrogen source. As the concentration of glucose was increased, sexual organs increasingly developed, but the cleistothecial development was restricted at concentrations lower than 0.5% (Han *et al.*, 1994a), indicating that a certain level of carbon was required for the induction or completion of sexual development. At concentrations higher than 6%, few cleistothecia were observed. However, the inhib-

Table 1. Effect of carbon sources and light on development of conidia and cleistothecia

Strains	Genotype	Carbon sources	Dark		Light (3,000-3,500 Lux)	
			Conidia[a]	Cleistothecia[b]	Conidia	Cleistothecia
FGSC4	*veA*$^{+}$	Glucose (0.5%)	++	-	++	-
		Glucose (1%)	+	++	+++	-
		Glucose (3%)	-	+++	+++	-
		Glucose (6%)	+++	-	+++	-
		Lactose (2%)	-	+++	++	+++
		Acetate (2%)	+++	-	+++	-
		Lactose + Acetate	+++	+++	+++	+++
		Glucose (1%: sealing)	-	+++	++	+++

Nitrogen source in all media was 0.1% sodium nitrate.

[a]The number of conidia was examined by removing an agar block with a cork borer to Tween80 (1 ml)-containing vials and scoring them with a haemacytometer after vigorous shaking. The numbers of conidia per ml from at least 3 agar blocks were averaged. –, <10^4; +, 10^4–10^5; ++, 10^5–5×10^6; +++, >5×10^6.

[b]Average number of mature cleistothecia per cm^2 of 5 different areas of a plate. –, <1; +, 1–10; ++, 10–50; +++, 50–100.

itory effect of a high concentration of glucose was relieved by increased nitrate concentration or the addition of organic nitrogen (N) sources such as casein hydrolysate. The result supports the idea that a balanced C:N ratio is important for the induction of sexual development (Zonneveld, 1977). On moderately favored C sources including glycerol, lactose, acetate and galactose, the balanced development as on glucose was not found. Although the growth rates on the C sources were almost the same (Han *et al.*, 1994a), the developmental pattern on acetate was very different from those on the other C sources (Table 1). Cleistothecia developed predominantly while only a few conidiphores developed on the medium with glycerol, galactose or lactose as the sole C source. On acetate, however, the pattern was completely reversed. No cleistothecia or Hülle cells ever were formed and only conidia developed (Table 1). The contrasting effects of these C sources raised the possibility that the energy metabolic pathway may be a significant factor that affects the determination of reproductive cycles. Acetate could be utilized only via aerobic respiration and active mitochondrial activity is required. It is likely that asexual sporulation is favored when cellular energy is provided mainly by aerobic respiration. When lactose and acetate, which showed contradictory effects, were co-supplied as carbon sources, both sexual and asexual developments were induced (Table 1), indicating that neither signal generated during the metabolism of lactose and acetate predominates over the other.

All of the effects of C sources on development were observed under the condition where 0.1% sodium nitrate was supplied as the sole nitrogen source. For the normal development of sexual spores, a certain level of nitrogen was required. When less than 0.05% sodium nitrate was supplied as the sole nitrogen source, very few cleistothecia were developed. On ammonium tartrate as the sole N source, asexual spores increased, while the formation of sexual spores was not significantly affected in comparison with sodium nitrate. However, the difference was not so distinct as in the case of C sources. Sexual development was preferentially induced and asexual sporulation reduced on organic N sources such as casein hydrolysate and glycine. Increases in nitrate or ammonium concentration also resulted in a slight increase in the ratio of sexual to asexual development. This trait of *A. nidulans* is in contrast to those of other ascomycetes such as *Neurospora crassa*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* which require nitrogen limitation for mating or sexual sporulation (Glass and Lorimer 1991). All of these results suggested that sexual development of *A. nidulans* is preferentially induced under rather well-nourished conditions, while the asexual development is favored by nutritional limitation.

Limitation of aerobic respiration results in preferential sexual development

When the culture plate was sealed with parafilm just after inoculation and cultured for more than 20 h under standard culture conditions, neither asexual nor sexual development took place. If the seal was removed and incubated further, the mycelia developed only sexually (Han *et al.*, 1990; Table 2). The same result was observed when the 20 h submerged-culture mycelia were laid on the MM agar plate, moistened with MM broth and cultured for more 20 h with a parafilm-seal. Plate-sealing may result in the limitation of oxygen, increase of carbon dioxide and humidity as hyphae grow. These conditions, separately or in combination, may be responsible for the irreversible determination of sexual development. Instead of plate sealing, we treated 2, 4-dinitrophenol, an electron transport chain uncoupler, and sodium azide, a blocker, to mycelia cultured in the same way. These chemical treatments resulted in the preferential development of cleistothecia (Table 3), which strongly implied that the limitation of oxygen which eventually caused the blockage of the electron transport system is a major factor responsible for the irreversible determination of sexual development. Sodium oxalate, an inhibitor of various TCA cycle

Table 2. Effect of nitrogen sources on development of conidia and cleistothecia in the presence or absence of visible light

Strains	Genotype	Nitrogen sources	Dark		Light (3,000-3,500 Lux)	
			Conidia[a]	Cleistothecia[b]	Conidia	Cleistothecia
FGSC4	*veA*$^{+}$	Sodium Nitrate (0.2%)	+	+++	+++	-
		Ammonium tatrate (0.2%)	++	+++	+++	-
		Glutamine (0.1%)	-	+++	+++	+
		Casein hydrolysate (0.2%)	-	+++	ND	ND
		Yeast extract (0.2%)	-	+++	+++	++
		Glycine (10mM)	+	+++	+++	+

Carbon source in all media was 1% glucose.

[a]The number of conidia was examined by removing an agar block with a cork borer to Tween80 (1ml)-containing vials and scoring them with a haemacytometer after vigorous shaking. The numbers of conidia per ml from at least 3 agar blocks were averaged. –, $<10^4$; +, 10^4–10^5; ++, 10^5–5×10^6; +++, $>5\times 10^6$.

[b]Average number of mature cleistothecia per cm^2 of 5 different areas of a plate. -, <1; +, 1–10; ++, 10–50; +++, 50–100.

ND : Not determined.

Table 3. Effect of several factors that inhibit aerobic respiration on development

Inhibitors	Conc. (mM)	FGSC4 (*veA*$^-$)		VAJ1 (*veA1*)	
		Conidia[d]	Cleistothecia[e]	Conidia	Cleistothecia
None	0	+++	+	+++	-
Sodium azide[a]	0.1	+++	+	+++	-
	0.5	++	++	+++	-
	1	+	+++	++	+
2,4-dinitro-phenol[a]	0.1	+++	+	+++	-
	0.2	++	+	+++	-
	0.5	-	+++	++	+
Sodium oxalate[c]	10	+++	++	+++	-
	20	+	+++	+++	-
	50	-	+++	+++	-
Plate sealing[b]		-	+++	++	++

[a]The mycelia in a submerged culture with vigorous shaking for 16 h were laid on the MM agar plate, moistened with MM broth containing the drug and incubated for another 20 h. The mycelia were washed and induced to differentiate. The number of conidia or cleistothecia was scored after incubation for two additional days.

[b]The mycelia in submerged culture with vigorous shaking for 16 h were laid on the MM agar plate, moistened with MM broth and incubated for more 20 h with parafilm seal. The number of conidia or cleistothecia was scored after incubation for two additional days.

[c]Sodium oxalate was added to agar standard medium.

[d]The number of conidia was examined by removing an agar block with a cork borer to Tween80-containing vials and scoring them with a haemacytometer after vigorous shaking. The numbers of conidia from at least 3 agar blocks were averaged. -, $<10^4$; +, 10^4–10^5 ; ++, 10^5–5×10^6 ; +++, >5 x10^6

[e]Average number of mature cleistothecia per cm^2 of 5 different areas of a plate. –, <1; +, 1-10; ++, 10–50; +++, 50–100; ++++, > 100

enzymes (Zollner, 1989), also induced sexual development preferentially. All of these results, together with the acetate effect, strongly suggested that sexual development preferentially occurs under conditions where the rate of aerobic respiration is reduced. However, under conditions where aerobic respiration is the major energy-yielding metabolism, sexual development hardly takes place, but instead asexual development occurs. There have been many reports presenting the essential role of the oxidative metabolism for normal conidiation of ascomycetes (Ng *et al.*, 1973; Galbraith and Smith, 1969; Urey, 1971). Acetate is mainly utilized via the TCA cycle (Hondmann and Visser, 1994) and requires more oxygen for being metabolized than other carbon sources. High rate of oxidative metabolism generates an oxidative stress and this may be the real factor that is favored for the asexual rather than the sexual cycle.

Visible light inhibits sexual development.

Light is a significant factor in affecting the development of a wide variety of fungal species (Tan, 1978). Mooney and Yager (1990) first reported the effect of light on the development of *A. nidulans*. They proposed that light, especially long wave light, is required for the development of asexual spores. It is not clear, however, whether light is a real inducing factor of asexual sporulation or an inhibitor of sexual development. We examined the effect of light in combination with some other conditions under which sexual development preferentially occurred while asexual development was almost completely inhibited. The results are summarized in Table 1. As expected, under the standard condition, asexual spores were developed extensively in the presence, but weakly in the absence, of light. Darkness resulted in prolific formation of cleistothecia bearing abundant mature ascospores. Our result was somewhat different from that of Mooney and Yager (1990) in some respects. Instead of continuous vegetative growth in the dark, a mass of sexual organs was formed as well as a small but significant amount of conidia was developed. It may be due to the difference of medium used. Complete medium where yeast extract was supplied in excess (0.5%) was used by Mooney and Yager (1990), while minimal medium was used in this work. If yeast extract was added to minimal medium at a concentration higher than 0.1%, conidiation in the dark was almost completely inhibited.

Table 1 shows that the asexual sporulation was greatly induced by light even under conditions where asexual development was selectively inhibited, such as on 2% lactose or 3% glucose. Few conidiophores developed when cultured on plates supplied with lactose or glycerol as a C source or sealed with parafilm just after inoculation in the dark (Han *et al.*, 1994; Table 1). Exposure to light relieved the inhibitory effects of such conditions on asexual sporulation. The result implies that the induction of asexual sporulation by visible light occurs and is possibly independent of the inhibition of sexual development. Apical growth rate was reduced as the intensity of illumination increased (Table 5). Development as well as growth was significantly inhibited by doses above 5,000 Lux (approximately 20-25 W/m^2; Table 5), indicating that intense light may be a source of general stress to the fungus.

High osmolarity selectively inhibits sexual development

One of the environmental factors that induce asexual development is a high concentration of a salt such as sodium chloride or potassium chloride (T. Adams, personal communication; Song *et al.*, 2001). It is thought to be the high osmolarity that is responsible for the preferential development of asexual spores (Lee and Adams 1994). In order to define whether the high osmolarity is a real factor that controls the developmental balance, effects of several salts and sorbitol at various concentrations were examined. As shown in Table 4, only asexual spores were developed and sexual development was completely inhibited by addition of 1 M KCl, 1 M NaCl or 0.5 M $MgCl_2$. The effect of salts on developmental

Table 4. Effect of osmolarity on development

Osmo-compounds	Conc. (M)[a]	FGSC4 (*veA*$^+$)		VAJ1 (*veA1*)	
		Conidia[b]	Cleistothecia[c]	Conidia	Cleistothecia
None	0	+	++	+++	+
KCl	0.5	+++	+/-	+++	-
	1	+++	-	+++	-
	1.5	+++	-	+++	-
NaCl	0.5	+++	+/-	+++	-
	1	+++	-	+++	-
	1.5	+++	-	+++	-
$MgCl_2$	0.5	+++	-	+++	-
	1	+++	-	+++	-
$MgSO_4$	0.5	+++	-	+++	-
	1	+++	-	+++	-
KH_2PO_4	1	+++	-	+++	-
	1.5	+++	-	+++	-
Sorbitol	1	+++	-	+++	-
	2	+++	-	+++	-
Glycerol	1	+++	+	+++	-
	2	+++	-	+++	-

[a]The concentrations of salts are additional to medium supplement.
[b]The number of conidia was examined by removing an agar block with a cork borer to Tween80-containing vials and scoring them with a haemacytometer after vigorous shaking. The numbers of conidia from at least 3 agar blocks were averaged. -, $<10^4$; +, 10^4–10^5 ; ++, 10^5–5 x10^6 ; +++, >5 x10^6
[c]Average number of mature cleistothecia per cm^2 of 5 different areas of a plate. -, <1; +, 1-10; ++, 10-50; +++, 50-100; ++++, > 100

Table 5. Effect of visible light and salts on the radial growth of FGSC4 (*veA*$^+$)

Agents	Dose or Concentration	%. Colony diameter treated vs. untreated		
		3 days	4 days	5 days
None[a]		100	100	100
Light	2500± 200 Lux[b]	96± 4	97± 5	98± 5
	3500± 200	86± 5	86± 4	88± 2
	4500± 200	83± 5	72± 3	65± 5
	5500± 200	75± 2	72± 5	63± 3
KCl	0.5 M	140± 6	140± 4	134± 5
	1 M	126± 3	125± 3	122± 5
	1.5 M	78± 3	80± 2	88± 4
	2 M	29± 1	35± 2	38± 2
$MgCl_2$	0.5 M	106± 5	115± 4	115± 5
	1 M	36± 2	45± 4	45± 2
	1.5 M	7± 1	1± 0.5	11± 2
$MgSO_4$	1 M	43± 3	46± 3	50± 4
	1.5 M	15± 1	21± 1	23± 2
Glycerol	1 M	140± 10	135± 5	130± 8
	2 M	82± 3	73± 2	75± 3

[a]Growth control on standard culture condition.
[b]Light intensity is represented as an intensity of illumination. The plates were placed below the white fluorescent lamps.

balance was also dose-dependent below these concentrations. As the concentration increased above these levels, the total amount of asexual sporulation also gradually decreased and the growth of aerial mycelia was inhibited at higher concentrations than 2 M $MgSO_4$ and KH_2PO_4 showed a similar effect on growth and developmental patterns, indicating that the effect was not due to the chloride ion. A similar pattern of development was observed when a high concentration of sorbitol or glycerol was added. This result strongly suggested that high osomolarity favors asexual rather than sexual development. Addition of compounds up to a certain concentration usually increased the growth rate, e.g. 1 M KCl, 1 M glycerol or 0.5 M $MgCl_2$ (Table 5). However, at higher concentrations growth was inhibited. Like stresses such as starvation and exposure to a high dose of light, the high osmolarity promoted asexual development below the inhibitory dose. The fact that the concentrations of KCl and $MgCl_2$ affecting the growth or development pattern were different from each other implied that the effect of salts was not only due to the osmolarity but also to physiological effects of individual cations.

Asexual sporulation takes place in a relatively short period, but produces a large amount of spores in comparison with sexual sporulation. Thus, the asexual reproductive cycle is more suitable for the purpose of propagation. The sexual reproductive cycle takes more time and various types of cells were developed during the cycle. Thus, enough nutrients and favorable culture conditions seem to be required for the induction of sexual development. It seems a reasonable deduction that the growing mycelia recognize the presence of an environmental stress that in excess can affect growth and are induced to develop into conidiophores, otherwise they form profuse aerial hyphae which later differentiate into sexual cells.

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