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Developmental stage-dependent response of *Pilobolus crystallinus* sporangiophores to gravitative and centrifugal stimulation

Tadashi Horie[1], Christine Schimek[1], Joe Fukui[1], Atsushi Miyazaki[1], Hitoshi Mihara[2], Toshisuke Tsuru[3], Maki Koseki[3] and Tamotsu Ootaki[1],*

[1] Institute of Genetic Ecology, Tohoku University, 2–1–1, Katahira, Aoba-ku, Sendai 980–8577, Japan
[2] Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606–8502, Japan
[3] Physics Laboratory, Faculty of Education, Yamagata University, 4–12, Koshirakawa, Yamagata 990–8560, Japan

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Gravitropic response of sporangiophores of *Pilobolus crystallinus* was studied by successive microscopic observation of the sporangiophores horizontally placed in the dark (red light) and by analysis of sporangiophore response to centrifugal stimulation. Negative tropism against the gravitative and also centrifugal stimulation was found only in mature sporangiophores after development of sporangium and after the resumption of elongation beneath the fully-developed subsporangial vesicle, but there was no response in younger sporangiophores, implying that the gravitative perception system of the sporangiophores is dependent on their developmental stages.

Key Words——centrifugal force; gravitropism; *Pilobolus* sporangiophore.

Pilobolus crystallinus (Wiggers) Tode, a fungus belonging to the Mucorales, develops unicellular multinucleate sporangiophores. Its development is divided into six stages, according to McVickar's (1942) classification based on morphological characteristics and the modification by Ootaki et al. (1993) based on the elongational and rotational behavior of the sporangiophores. At stage I, the sporangiophore initial elongates at the apex but does not rotate. The sporangiophore then develops a sporangium at the tip after cessation of elongation (stage II), and after the full development of the sporangium the sporangiophore ceases transiently any growth (stage III). In the next stage, a subsporangial vesicle expands beneath the sporangium (stage IV), and this is followed by spore maturation and resumption of elongation in the region beneath the subsporangial vesicle (stage V). This stage is characterized by clockwise rotation of the sporangiophore when viewed from above. The sporangiophore completely ceases its elongation about 1 h before the subsporangial vesicle bursts and ejects the sporangium into the air (stage VI).

The *Pilobolus* sporangiophore has been intensively investigated as a model organism for photobiology (Buller, 1934; Banbury, 1959; Page, 1962; Page and Curry, 1966; Kubo and Mihara, 1988, 1989, 1996). Like *Phycomyces*, *Pilobolus* shows a pronounced phototropism in response to unilateral light both in young sporangiophores which are elongating at the apex and in mature sporangiophores after the sporangium has developed. The direction of phototropism, positive or negative, depends on both the wavelength and fluence rate of light (Page and Curry, 1966; Kubo and Mihara, 1988, 1989), again like *Phycomyces* (reviewed by Galland and Lipson, 1987).

The phototropic response is strongly influenced by both the growth pattern and gravitropic response of the sporangiophores. In *Phycomyces*, the gravitropic response affects the maximal phototropic bending angles as a vectral balance (Pilet, 1956; Dennison, 1958, 1959; Dennison and Shropshire, 1984; Ootaki et al., 1991). In *Pilobolus*, however, gravitropism has not been as intensively studied as phototropism in this fungus or gravitropism in *Phycomyces*. Thus inconclusive and limited information is available on gravitropic behavior of *Pilobolus* sporangiophores, gravisensitive receptors, and the mechanisms involved. The effect of gravitropism on phototropism, as a result, has not been studied in *Pilobolus* sporangiophore. The sparsity of studies on gravitropism may be in part due to the long latency period and the slow velocity of the response to gravitative stimulation, compared with phototropism.

In the present study, we investigate the response of *Pilobolus* sporangiophores to gravitative and centrifugal stimulation, in order to elucidate the correlation between gravitropism and phototropism, the gravitative perception systems, and the mechanisms of tropic responses.

Materials and Methods

Strain and culture conditions *Pilobolus crystallinus*, wild-type strain IFO 8561, was obtained from the Institute for Fermentation, Osaka, Japan and cultured on

* To whom correspondence should be addressed.

MYC agar medium, consisting of 1% malt extract (Difco), 0.2% yeast extract (Difco), 0.2% casamino acids (Difco), 0.1% ammonium nitrate, 0.05% sodium acetate, and 1.2% agar (Koso Chem., Tokyo), according to Kubo and Mihara (1988). Petri plates (6 cm in diam) with several mycelial inocula were placed under continuous white fluorescent light at about 0.1 Wm^{-2} at mycelial level and maintained at 23°C until the spores or pieces of mycelia were used for the experiments.

Observation of gravitropism To observe successive gravitropism of sporangiophores through their development, a block (about 1 cm square) of nutrient agar covered with a mycelial mat was cut with a razor blade from the subculture plate and placed on the top of a glass shell vial (1 cm diam, 3 cm height) filled up with 3% plain agar. The vials were fixed in a horizontal position on a platform using double-sided tape, then covered with a box made of red methacrylate (Mitsubishi No. 102, Tokyo). Red light of low intensity does not induce a phototropic reaction. The successive gravitropic behavior was photographed using a microscopic camera (Nikon UFX-II, Nikon, Tokyo) connected to a time-lapse autotimer (Nikon CFMA). The sporangiophore was illuminated with red light from the front only during photography. The light originated from a halogen lamp and was passed through a heat absorption filter, a red Plexiglas filter (No. 2444; Rohm and Haas, Philadelphia), and a convex lens. Serial photographs were taken at 30-min intervals, and enlarged photocopies of the images were analyzed for length and gravitropic bending angle of the sporangiophores. The gravitropic bending angle is defined as the angle between the horizontal axis and the sporangiophore axis after exposure to the gravitational stimulation.

Centrifugation Several small pieces of mycelia were picked out of the subculture plate with tweezers, and reinoculated along the central line of the methacrylate

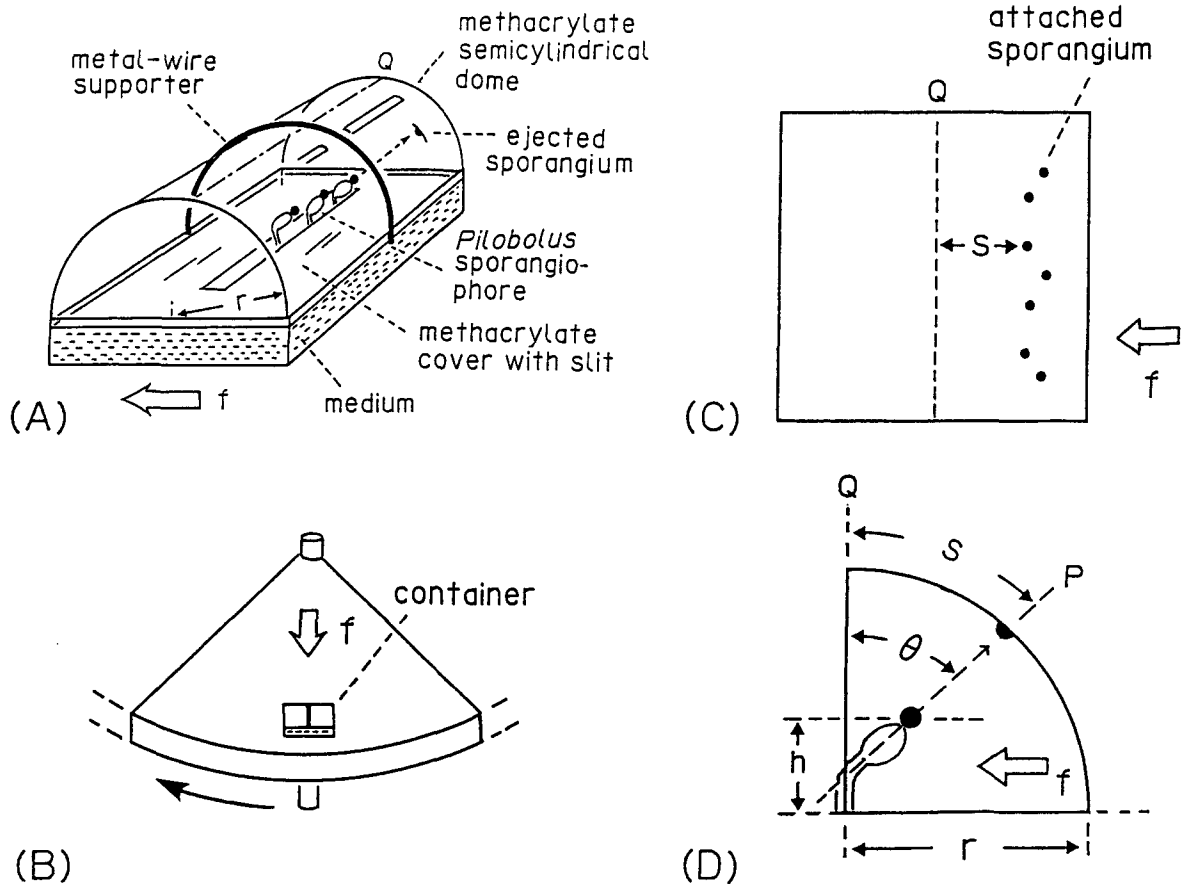

Fig. 1. Schematic diagrams of the experimental set up and bending angle determination used in the estimation of centrifugal response of *Pilobolus* sporangiophores.

A: Container constructed for the culture of *Pilobolus* sporangiophores under conditions favoring bending angle measurement. Only the sporangiophores emerging through a narrow central slit of the methacrylate cover slip placed on the medium are able to project the sporangia to the inner surface of the semicylindrical dome after the response to centrifugal stimulation. The letter Q represents the top line of the dome, and r is the radius of the dome. B: Part of a horizontal rotor disk of centrifuge carrying the container wrapped with aluminum foil for keeping high humidity and darkness. C: Flattened and enlarged image of semicylindrical methacrylate dome with black spots of attached sporangia. D: A sporangiophore bending centripetally with a bending angle θ, which was estimated from the length of arc (S) after correction for the sporangiophore height (h). White arrows show the direction of centrifugal force.

container (6.4 × 3.4 × 1 cm) filled with MYC medium (Fig. 1A). Several such containers were placed in a large box (25 × 18 × 8 cm) under highly humid conditions, covered with a transparent glass, and kept under continuous white light (0.1 Wm^{-2}) for further 2 d until the sporangiophore initials emerged. A thin methacrylate cover slip with a central window slit (3 mm × 25 mm) was then placed on the medium to allow the elongation of only the sporangiophores emerging on the longitudinal axis of the container (Fig. 1A). The rims and edges of the cover slip were sealed with water agar to prevent the emergence of sporangiophores in these regions during the experimental duration, and also to prevent accidental shift of the cover slip during centrifugation.

When the sporangiophore initials emerged, each container was covered with a semicylindrical dome of flexible methacrylate supported by a wire frame to prevent distortion during centrifugation (Fig. 1A). The whole container was wrapped with aluminum foil to maintain high humidity and darkness during the experiment.

A large rotor disk (90 cm in diam), made up of thick (5 cm) Styrofoam, was horizontally installed on a speed-variable motor (Fig. 1B). Mechanical fluctuation of the rotation level during revolution was minimized by three all-round wheels installed below the table. The centrifuge was placed in an air-conditioned dark room at 23°C, and diverse intensities of centrifugal force were obtained by adjusting either the revolution speed or the distance of the methacrylate container from the central axis.

Measurement of bending angles of centrifuged sporangiophores At the end of the experiment, the semicylindrical methacrylate covers were removed, flattened out, and enlarged images were produced with a standard copy machine. These images showed many small spots, which represent the ejected sporangia attached to the inner surface of the semicylindrical methacrylate cover (dome; Fig. 1C). The maximal bending angle of the centrifuged sporangiophores were estimated by measuring the distance of these spots from the middle axis (top) of the methacrylate dome, because the sporangiophores collapsed by recoil immediately after the ejection of sporangia. The angles measured were corrected by consideration of the sporangiophore height.

To do so, we consider a sporangiophore of length (h), for which the bending angle (θ) is calculated as follows (Figs. 1D, 2):

$$S = r\alpha \quad (1)$$

$$AB = r\cos\alpha - h \quad (2)$$

$$BP = r\sin\alpha \quad (3)$$

$$\tan\theta = BP/AB = r\sin\alpha / r\cos\alpha - h \quad (4)$$

$$\theta = \tan^{-1}(r\sin\alpha / r\cos\alpha - h) \quad (5)$$

since $\alpha = s/r$,

$$\theta = \tan^{-1}[r\sin(S/r)/r\cos(S/r) - h] \quad (6)$$

where r is the radius of the semicylindrical dome, S is the arc QP, P is the point of sporangium attachiment, and $\theta \leqq 90°$.

Figure 2 shows the relationship between the arc length (S) and bending angle (θ) of sporangiophores with diverse heights (h). In the practical experiments,

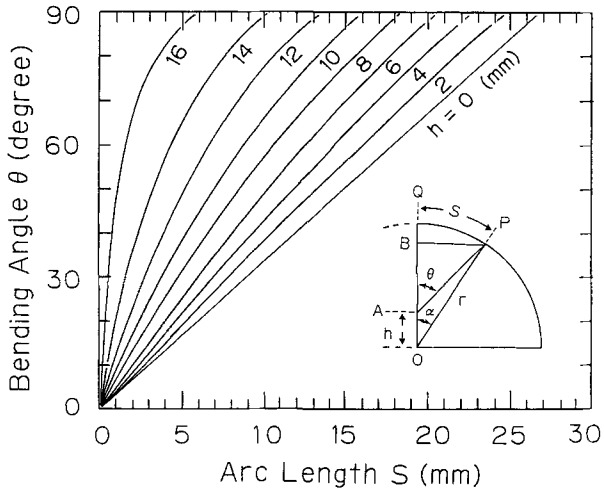

Fig. 2. Theoretical relationship among arc length (S), bending angle (θ), and sporangiophore height (h) in mm.
Inset: Schematic diagram of a half of the semicylindrical dome in which a sporangiophore with a height h was placed on the center (O) and ejected its sporangium at a bending angle θ to the point P. r is the radius of the dome and S is the arc QP.

however, we assumed, for simplicity of calculation, that the sporangiophore height (h) is 1.5 mm on average, based on our preliminary observations, and we ignored the change of sporangiophore height caused by bending. We also assumed that each ejected sporangium flew straight to the methacrylate dome, because in *Pilobolus* the sporangium can be ejected up to 1.8–2.4 mm height (Buller, 1934; Page, 1962).

For the young sporangiophores before the sporangium development, the bending directions and angles were determined on photographs taken through a dissecting microscope. Variations in the bending angles were given as standard errors (SE).

Results

When the sporangiophore initials were placed horizontally, the sporangiophores elongated straight and laterally without bending, until the sporangiophores developed the sporangia (Figs. 3, 4). Negative gravitropism was found only after the sporangium developed, and in particular it was distinct after the subsporangial vesicles fully developed and after active elongation resumed at the newly-established growth zone beneath the subsporangial vesicle (stages V–VI). If the sporangiophores could remain intact for sufficient time before the sporangium ejection, the sporangiophores would bend upwards until the maximum angle 90° was reached (Figs. 4, 5). These results imply that the stage-I sporangiophores, unlike the stage-V sporangiophores, lack the perception systems for gravitative stimulation or stage-I sporangiophore's sensitivity is too low to detect the Earth's gravitation.

When higher magnitudes of stimulation than the Earth's gravitation were applied by centrifugation,

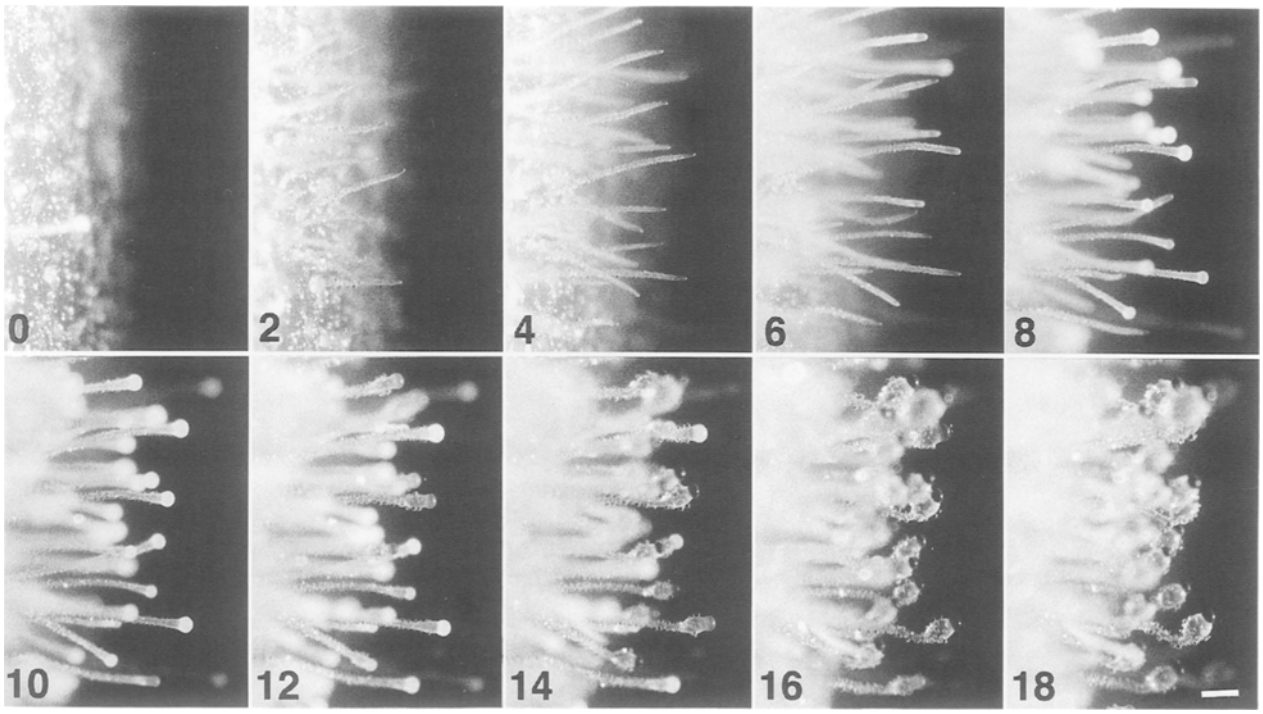

Fig. 3. Successive photographic records of the development and gravitropic bending of horizontally-placed sporangiophores of *Pilobolus crystallinus*, representing no gravitropism at the early stage before and during sporangium development but a negative gravitropism at a later stage after full development of sporangia.
The numbers on each photograph show time (h) after the start of the experiment. The scale bar represents 300 μm.

response of sporangiophores again depended on the developmental stage: stage-V sporangiophores responded but stage-I sporangiophores did not. During exposure to centrifugal stimulation, the sporangiophore initials elongated, developed sporangia, and projected them to the inner surface of the semicylindrical dome. Figure 6 shows the bending angles (θ) estimated from the positions of the sporangia attached to the dome as a function of the centrifugal force (f).

A *Pilobolus* sporangiophore exposed to centrifugal force (f) will perceive the resultant force (F) as a combined vector with the Earth's gravitational stimulus (g) (Fig. 6, insert). Thus, the sporangiophore perceives a higher magnitude of force from a direction with angle θ than the centrifugal forces actually given. Angle θ varies with F. If the sporangiophore exhibits negative tropism against F, its maximum bending angle will equal θ. The sporangiophore thus responds with different bending angles corresponding to either the magnitude of the stimulation force F, the direction of F, or both.

The positions of the black spots represented the negative tropism of the stage-V sporangiophores against the centrifugal force, but the bending angles were smaller than those theoretically expected, in particular in the region of high magnitudes of stimulation (Fig. 6). These smaller resultant angles may be due to a mechanical centrifugal leaning of the sporangiophore stems or due to ejection of some sporangia before full bending angle is reached. Rather large statistical deviations may support this speculation.

Bending angles of young sporangiophores before sporangium maturation are shown in Fig. 7 and Table 1. At both centrifugal forces examined, 3 and $5 \times g$ respectively, no significant bending was observed either in stage-I sporangiophores elongating at the apex or in stage-II–III sporangiophores expanding at the sporangium.

Discussion

It is well known that *Pilobolus* sporangiophores at both stage I and V are positively phototropic when illuminated with unilateral light (Buller, 1934; Banbury, 1959; Page, 1962; Page and Curry, 1966; Kubo and Mihara, 1988, 1989, 1996). Similar phototropic phenomena are observed in *Phycomyces,* which shows a pronounced positive phototropism at stage I (elongation in the apical region) and at stage IVb (elongation in the newly-established growth zone beneath the sporangium). The present study, however, revealed that *Pilobolus* sporangiophores were gravitropic only at stage V, unlike *Phycomyces,* where negative gravitropism occurred at both stages I and IVb (reviewed by Shropshire and Lafay, 1987). The present results quantitatively confirmed the statements that a recognizable gravitropic bending is detectable only after the sporangiophores have been allowed to elongate in the dark for several days (Page, 1962) and that young sporangiophores showed no gravitropism when placed horizontally (Kubo and Mihara, 1988).

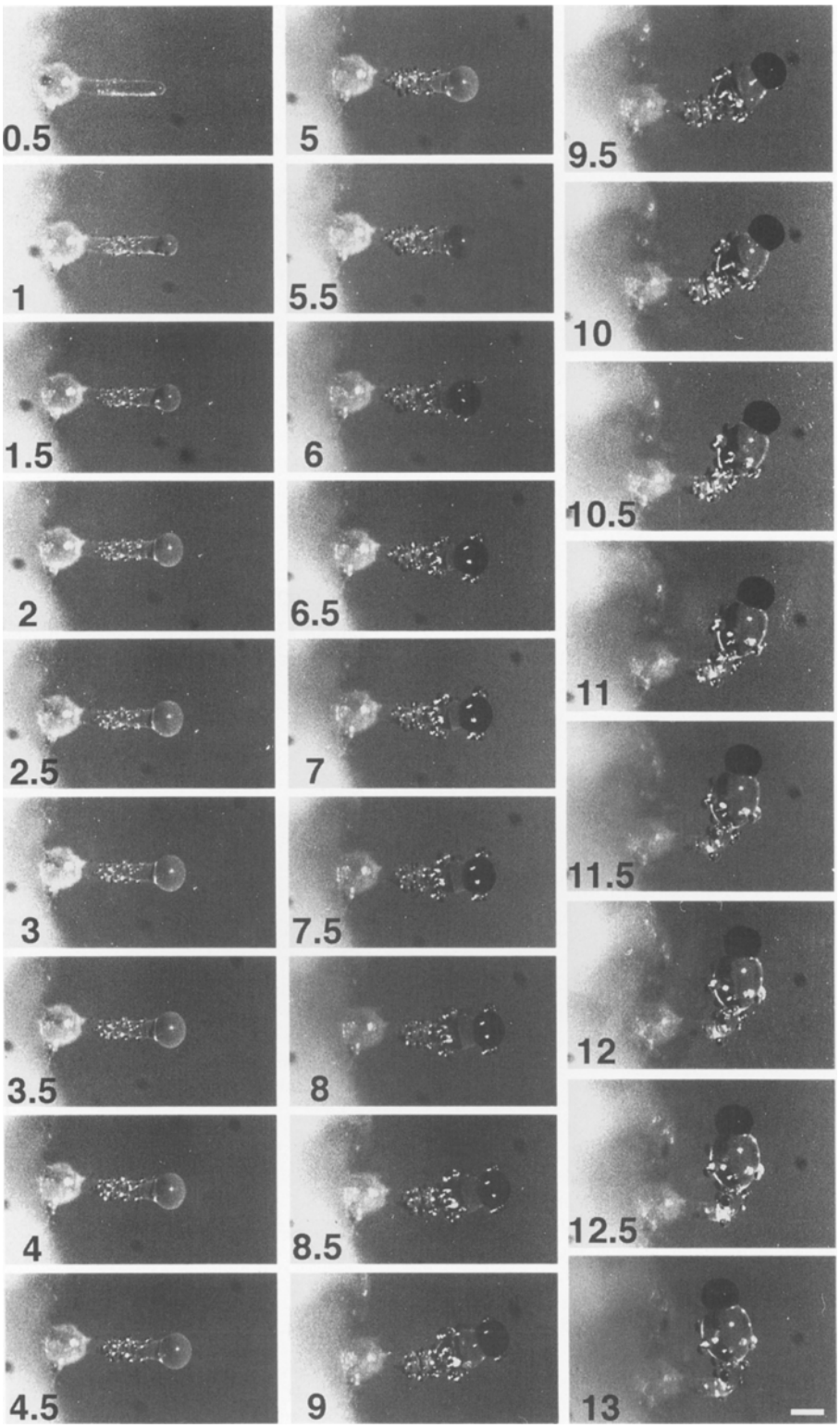

Fig. 4. Successive photographic records of gravitropism of a horizontally-placed sporangiophore for growth analysis. The numbers on each photograph show time (h) after the start of the experiment. The scale bar represents 300 μm.

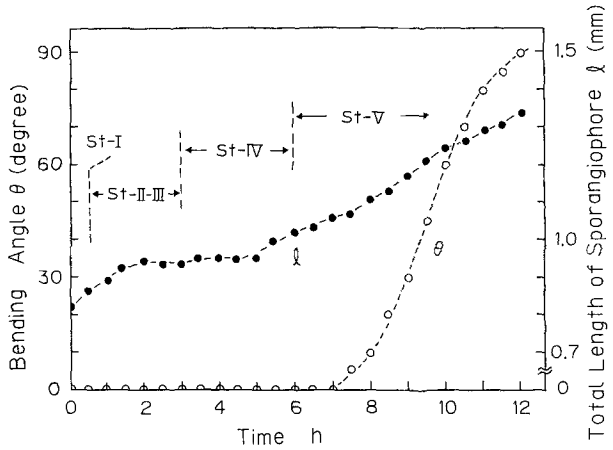

Fig. 5. Growth analysis of the horizontally placed sporangiophore in Fig. 4.
The total length (ℓ) of the sporangiophore slightly increased during expansion of sporangium and subsporangial vesicle, but no elongation occurred in the stalk region beneath the sporangium or subsporangial vesicle. Bending with angle θ occurred only at stage V. The times indicated on abscissa coincide with those on photographs in Fig. 4.

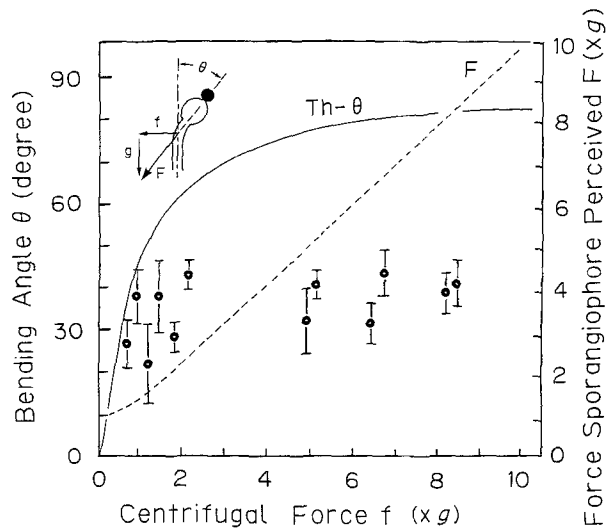

Fig. 6. Tropic responses of *Pilobolus* sporangiophores with bending angle θ at stage V as a function of applied centrifugal force (f).
Broken line (F) is the combined vectral force of the centrifugal force (f) and the Earth's gravity (g), which the sporangiophores theoretically perceived. Solid line (Th-θ) represents the theoretical maximal bending angle of sporangiophores if the sporangiophores bend away from the resultant vector F. Each point represents an average bending angle with SE of 10–150 sporangia ejected in a container.

We previously found that the sporangiophores of *P. crystallinus* rotate at stage V but not at stage I (Ootaki et al., 1993), unlike *Phycomyces* sporangiophores, which rotate at both stages I and IVb. We speculated that the absence of rotation in the *Pilobolus* stage-I sporangiophore may be due to restriction of the growth zone to the extreme tip of the sporangiophore, where wall materials will be produced only at the extreme tip and, as a result, reorientation of the microfibrils, which is believed to be a cause of rotation in *Phycomyces* (Ahlquist and Gamow, 1973; Ortega and Gamow, 1974; Ortega et al., 1974; Gamow and Böttger, 1979), will not occur during elongation. In *Phycomyces*, on the other hand, the growth zone of the stage-I sporangiophore is rather broad (down to 3 mm from the tip; Castle, 1958) and, as a result, the reorientation of microfibrils will occur during elongation.

The above speculation was based on the long-held belief that the phototropism of the stage-I sporangiophores of *P. crystallinus* is a consequence of the displacement of the growth point from the extreme tip to the proximal side, and that it involves neither lens effect nor differential growth between the proximal and distal sides in the growth zone (Page, 1962; McVickar, 1942). In *Phycomyces* sporangiophores, on the other hand, the phototropism has been believed to be a consequence of the differential growth caused by the lens effect (Castle,

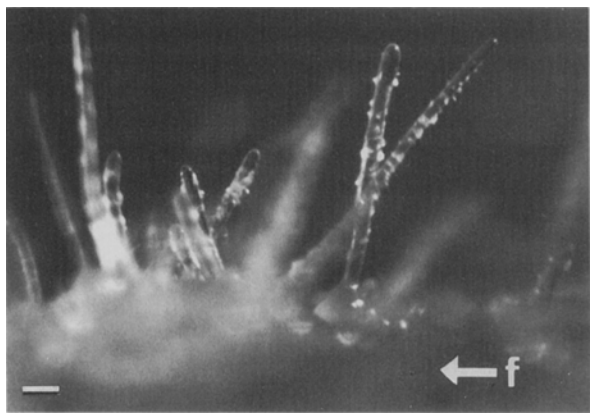

Fig. 7. Stereomicroscopic photograph of stage-I sporangiophores of *Pilobolus* emerged and elongated in random directions without showing tropism for the duration of exposure to centrifugal stimulation (f) at $5\times g$.
The scale bar represents 200 μm.

Table 1. Tropism of young sporangiophores of *Pilobolus* to centrifugal stimulation.

Developmental stages	Bending angles (θ)	
	$3\times g$	$5\times g$
Stage I	2.5±5.9 (21)	4.7±3.2 (52)
Stages II–III	0.4±1.1 (31)	0.2±2.4 (18)

Bending angles were measured 4 d after the start of centrifugation and designated as the angles between the longitudinal axis and the sporangiophore bending towards the centripetal end. The values are means±SE, representing no tropic response to the centrifugal stimulation. The magnitudes of centrifugation forces shown are the actual forces (f) applied to the sporangiophores. Numbers of sporangiophores measured are recorded in parentheses.

1959, 1961; Dennison, 1965). Kubo and Mihara (1996), however, revealed by microbeam irradiation experiments of *P. crystallinus* that the growth zone exists at the apex of stage-I sporangiophore, and phototropic bending is accomplished by differential growth caused by the lens effect, as reported in *Pilobolus kleinii* (Page and Curry, 1966).

If the findings of Kubo and Mihara (1996) are valid, the lack of gravitropism in stage-I sporangiophores of *P. crystallinus* cannot be due to the lack of differential growth of the sporangiophores, though the growth zone of the apex is small (0.3 mm; Kubo and Mihara, 1996) compared with that of *Phycomyces* (3 mm; Castle, 1958). The lack of gravitropism of stage-I sporangiophores, therefore, may be due to the lack of gravitative perception systems. A lack of improvement in the tropic response of *Pilobolus* stage-I sporangiophores upon centrifugation with higher magnitudes of stimulation than the Earth's gravity may support this speculation. At present we have no information on gravireceptor(s) of *Pilobolus* sporangiophores, but comparative analysis with *Phycomyces* sporangiophores may contribute to the solution, because the stage-I sporangiophores of *Phycomyces* are capable of responding to gravitative and centrifugal stimulation.

In general, gravitropism is less effective than phototropism, in terms of the latency period and the velocity of response. The gravitropism of *Pilobolus* sporangiophore required about 5 h from the onset of bending to reach its maximum (Fig. 5), whereas phototropism required 3 h (Page, 1962). This is also the case in *Phycomyces*, which required about 20 h until the gravitropic bending reaches its maximum, but 10 h is sufficient for phototropism (Ootaki et al., 1995). The reasons for these differences remain unsolved.

The bending angles obtained with mature sporangiophores were smaller than the theoretical angles, in particular when a high magnitude of centrifugal force was applied (Fig. 6). Centrifugation of sporangiophores on a rotating table at high speed may cause some physical effects such as the Coriolis' force, which acts in the rotating direction. If the Coriolis' force is too big, the sporangiophore does not bend exactly in the plane of the central pole of the rotator disk (aiming error). When the aiming error is too big, it might cause considerable errors in the angle measurement of the sporangiophores, which could not be neglected.

To estimate the Coriolis' force, we consider a sporangiophore which is placed at a point (R_0, 0) on the X axis of a disk rotating at angular velocity ω and projects its sporangium at an angle θ to the vertical axis (z) towards the center (O) with a muzzle velocity V_0 (Fig. 8). The position of the ejected sporangium after t sec on the rotating axes (X, Y) can be estimated as follows, when the air resistance is neglected:

$$X = R\cos\omega t + R_0\omega t\sin\omega t \quad (7)$$

$$Y = R\sin\omega t - R_0\omega t\cos\omega t \quad (8)$$

where $R = R_0 - V_0 t\sin\theta$. If $\omega t \ll 1$, these formula may be closely rewritten as follows:

$$X = R + R_0(\omega t)^2 \quad (9)$$

$$Y = R\omega t - R_0\omega t \quad (10)$$

If our *Pilobolus* sporangiophore is capable of projecting its sporangium to a height of 1.8 m (Buller, 1934; Page, 1962) with the resultant muzzle velocity ($V_0 = 4.2$ msec^{-1}) from a position of radius ($R_0 = 0.38$ m), as the farthest position where the sporangiophore was placed, on the disk rotating at 150 rpm at the maximum ($\omega_{max} = 16$ (rad sec^{-1})), then the ejected sporangium is able to reach the inner surface of the semicylindrical methacrylate dome with a radius of 1.7 cm at $t = 4\times10^{-3}$ sec, if air resistance is neglected. When these parameters are applied to the above formula, the values of X and Y are respectively estimated as follows:

$$X = 0.3695 \text{ m} \quad (11)$$

$$Y = -7.7\times10^{-4} \text{ m} \quad (12)$$

When the disk is not rotating, the coordinates (X, Y) of the position of the ejected sporangium after time t is estimated as (X, Y) = (R, 0) = (0.368, 0). The differences between these values and the values of (11) and (12) imply the aiming error caused by the Coriolis' force. The resultant aiming errors are thus estimated to be 1.5 mm to the X axis and 0.77 mm to the −Y axis at the maximum, and these are small enough to be neglected in the present experiments.

Other presumable effects on the bending angle determination are due to mechano-stimulation such as stretch during centrifugation. We neglected these effects in the present study, however, because available information on these parameters is limited.

Though available physiological and cytological information on the tropism of *Pilobolus* and *Phycomyces* sporangiophores is limited, comparative analysis of tropic

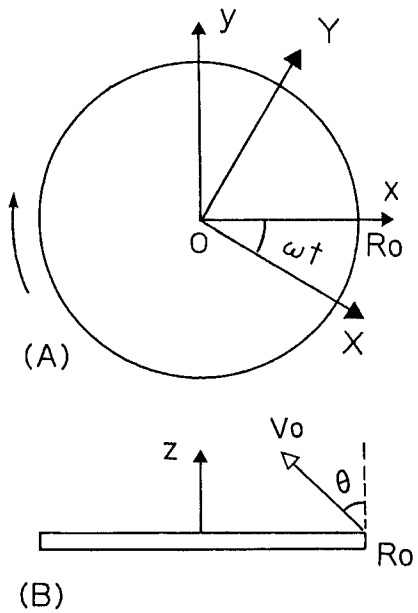

Fig. 8. Schematic diagram of the top (A) and side (B) views of the centrifuge disk rotating at an angular velocity ω, representing the displacement of coordinates after time t (A) and projection of sporangium at angle θ and muzzle velocity V_0 towards the centripetal end (B).

responses and biological characteristics of these closely related but, in a sense, differently behaved fungi is strategically useful for elucidation of the mechanisms of the perception, transduction, and response of the fungi to external stimuli.

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