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Kinetics of stem gravitropism in *Coprinus cinereus*: Determination of presentation time and 'dosage-response' relationships using clinostats

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1. SUMMARY

The sensitivity to gravitational stimulation of excised stems of the mushroom fruit body of *Coprinus cinereus* was determined using clinostat rotation to remove partially-stimulated stems from the normal unidirectional gravitational field. For the strain and conditions tested, the presentation time (the minimum time of stimulation required to elicit a gravitropic reaction) was determined to be 9.6 min. This is the first time the presentation time has been determined for a fungal gravitropic response. Constructional details are given of the clinostats employed in the research and their further use is discussed.

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2. INTRODUCTION

Many cells respond to altered gravitational acceleration [1,2] but gravitational reactions of cells not obviously specialised for detecting acceleration is a poorly-explored area of cellular sensory physiology. In particular, no gravity-sensing apparatus has been identified in fungi, although higher basidiomycete fruit bodies (mushrooms and toadstools) are able to maintain the precise vertical orientation which is crucial to their spore distribution.

The basic features of gravitropism in mushrooms and toadstools were established during the 19th century [3–6]. More detailed experimental work was done in the early part of this century [7–13] and there have been a number of studies and reviews at regular intervals since [14–20]. The total amount of research done is extremely small and detailed knowledge consequently meagre. In particular, no attempt has ever been made

to determine the kinetics of the gravitropic response to clinostat treatment. This is the subject of this report.

We have recently completed the first kinetic analysis of mushroom stem gravitropism [20] and found that stem bending first occurred 25.4 ± 13.1 min ($n = 18$) after being placed horizontal. By analogy with gravitropic responses in plants, this value is the reaction time, which is the time from first reorientation of the organ to the appearance of the tropic growth curvature. In plants this can vary between about 10 min and many hours and it covers all of the processes involved in gravitropism from initial detection of disorientation to final production of the growth response which generates the gravitropic curvature. Of more significance is the presentation time which is the minimum time of stimulation required to provoke a gravitropic response. The presentation time can be as low as 10 to 15 s in some plants, but an average value is 4 min. The presentation time can be used as the basis of calculations aimed at identifying cell organelles which might be involved in gravity perception (e.g. [21–23]). As far as we can establish, there is no report in the literature of any attempt to determine the presentation time of any fungal tissue.

Determination of the presentation time requires clinostat treatment. This valuable tool has a long history of use but is still of enormous value as it remains the most practical way for earth-bound laboratories to investigate 'weightlessness' over periods greater than the few tens of seconds available in drop towers and aircraft on parabolic flight profiles and at much less cost than orbital experiments. A clinostat provides circular rotation at uniform speed around the horizontal axis. This does not remove the subject from the effects of gravity and care must be exercised to use descriptive terms which do not carry unwarranted implications. A test subject mounted on the clinostat experiences altered vector direction; the normal gravity vector sweeps through 360° in each revolution, so in comparison with a stationary subject which is placed horizontally (and thereby experiences a unilateral gravity stimulus) the clinostat subject experiences a continuously shifting omnilateral stimulation. The crucial point, though, is that the effect of the treatment is relative. It depends on the rate of rotation, on the mass of the object considered, on its size and density and on the viscosity and density (specific gravity) of the surrounding fluid; it is inevitable that different components of a living object on a clinostat experience different conditions. This is in contrast to the situation on an orbiting space craft in which all components experience the same microgravity environment. Nevertheless, results of experiments using the two approaches are broadly similar in fungi [19].

Although the clinostat is a useful device and has been in use for a long time, it is a remarkable fact that constructional details are almost impossible to find. Such details are included in this paper.

3. MATERIALS AND METHODS

3.1. Organism and culture conditions

All experiments were done with the 'Meathop' dikaryon of *Coprinus cinereus* (Schaeff.: Fr.) S.F. Gray; this was originally isolated from a dung heap in Lower Meathop Hill farm in Cumbria. The Meathop vegetative dikaryon was grown on complete medium [24] in 9-cm Petri dishes in the dark at 37°C. Vegetative cultures of the dikaryon were maintained by serial transfer. Fruit bodies were produced by inoculating YMG medium [24] with pieces of dikaryon taken from the Petri dish cultures. These cultures were incubated in the dark for 3 days at 37°C to allow the mycelium to establish itself before being transferred to a 27°C incubator with a 16 h light/8 h dark photoperiod to induce production of fruit bodies. Illumination was provided by white fluorescent lights which gave an average illuminance of 800 lux.

3.2. Preparation of specimens for clinostat treatment

Fruiting bodies approx. 50–70 mm tall were excised from the medium with a scalpel in the dark room under red light. The cap was removed and the stems pinned at the base to a balsa support wrapped in PVC tape, then placed in the clinostat container. Throughout all of these

preparative operations, the stem was kept vertical. The stem was then placed horizontal and attached to the clinostat. The arrangement was kept in this position for various lengths of time to provide gravistimulation, then the clinostat rotation was initiated at 2 rpm which was continued for the duration of the experiment. The control experiments were identical except that the stem was subjected to constant gravistimulation (i.e. no clinostating). In both cases the entire operation from initial orientation was videotaped under low intensity red light.

3.3. Recording and measurement of responses

Stem morphology was determined from images in freeze frames of the video which were traced onto acetate film. Lengths and angles were determined from these tracings using a digitising tablet interfaced to a PC running the AutoCAD program.

4. RESULTS AND DISCUSSION

4.1. Clinostat design and construction

Those who use animal cells for their research are currently most likely to rely on the 'fast-rotating' clinostat although the original reference to which this is usually ascribed [25] referred specifically to the human organism and a speed of rotation at the "... psycho-physiological optimum for disengaging his vestibular apparatus from an effective pull by gravity...". Preference for such a device over the conventional one for animal cells would clearly need justification other than the psychological. Fortunately, the basic theory upon which clinostat operation depends is well represented in the literature, very similar detailed mathematical treatments being published by botanists [26–28], mycologists [29] and a zoologist [30].

The fundamental purpose of the clinostat is to equalise the effects of gravity on a particle through its circular rotation with uniform speed about a horizontal axis. For any particle there is a theoretical optimum rotation rate at which the rotation sweeps the gravity vector around the particle too swiftly for any sedimentation to occur. Both higher and lower speeds result in the particle executing circular motions; in the former case because of the centripetal accelerations due to centrifugal forces, and in the latter case because of sedimentation during the slow sweep of the gravity vector. A major problem in interpreting clinostat experiments is that the cell must contain many particles exhibiting a variety of density differentials with their suspending medium with a consequent potential for a variety of responses to any given clinostat treatment. However, the clinostat can be used in an analytical manner. Theoretically, any quantifiable effect produced by clinostat treatment can be optimised at a specific rotation rate and the physical characteristics of the particle(s) deduced from the experimental circumstances which achieve this. Apart from the early work on plant organs [21,31] this experimental approach has been applied rarely, most experimenters being content to work at one speed of rotation. Dissimilar responses to different speeds of rotation have been noted. Increased fresh weight of cell cultures of *Haplopappus gracilis* occurred when they were cultivated on a 50 rpm clinostat, but not when rotated at 2 rpm [32] and a decrease in cell division in rapeseed protoplast cultures was recorded on a 2 rpm clinostat, but not on a 50 rpm clinostat [33]. Lyon [34] used nine clinostat rotation rates to study root and coleoptile development in wheat seedlings (*Triticum aestivum*) and curvatures of leaves and branches of *Coleus blumei*. The former showed the same effect over the whole range of rotation rates tested, while curvatures in *Coleus* were maximal at 0.3 to 1 rpm. Evidently, gravity perception in different organisms, and perhaps even separate phenotypes in the same organism, depends upon particles with diverse physical characteristics so provision for experiment at different rates of rotation is a crucial aspect of clinostat design.

The basic functional demands are that the clinostat must have sufficient power to rotate the experimental objects smoothly and reliably at constant speed. For fungal (and similar) cultures contained in standard culture vessels (9-cm crystallising and Petri dishes, Beatson jars, plastic McCartney bottles, multi-well tissue culture/as-

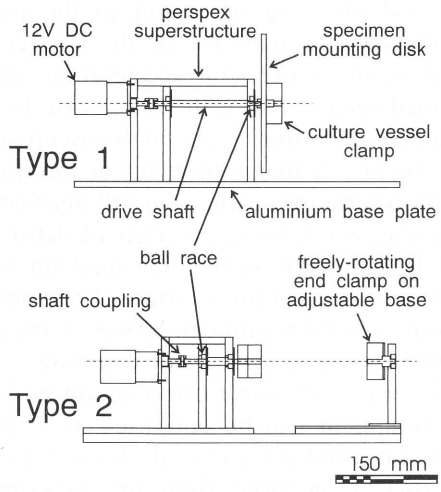

Fig. 1. Line diagrams (to scale) of the two types of clinostat designed for this research.

say plates) we have found small electric motors (torque = 600 mN m) to be entirely satisfactory (steel geared, medium duty 8 rpm DC motor, item ref. 341–676, RS Components Ltd., PO Box 99, Corby, Northants, UK). We have favoured 12 V DC motors for the ease and safety of supplying power to clinostat rigs in environmental chambers and because the voltage supplied to the motor can be reliably varied with appropriate transformers to vary the rate of rotation (regulated mains adaptor, 300 mA output with taps at 3, 4.5, 6, 7.5, 9 and 12 V (item ref. 593–502, RS Components Ltd.) or 0–15 V, 1 A bench variable power supply (item ref. 148–583, Farnell Electronic Components Limited, Canal Road, Leeds, UK).

The devices are illustrated in Figs. 1–3. The base plate is 8 mm thick aluminium but all other parts of the superstructure are constructed from 10 mm perspex sheet. The design features a 150 mm aluminium drive shaft mounted in ball races and independently coupled (shaft couplings, item ref. 319–477, fitted with torque disks, item ref. 319–506, RS Components Ltd.) to the motor at one end and specimen plate at the other. Though demanding precise alignment between the rotational axis of the motor and drive shaft, this arrangement isolates the specimen from the motor vibration and magnetic field [35,36].

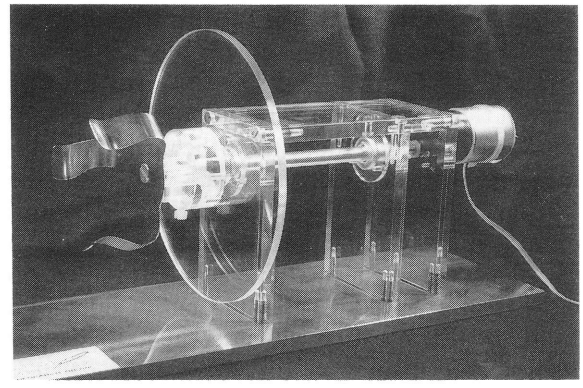

Fig. 2. The Type 1 clinostat.

The specimen plate (and the height of the superstructure) differ according to experimental demands. One design is a 170-mm diameter disk of 5 mm thick perspex with a central block to which can be fitted clamps appropriate to the culture vessels in use. This design permits specimens to be attached directly to the clamp for rotation on the drive axis or to the surface of the disk for rotation at right angles to the main axis. In another variation (used for the experiments reported here), the specimens are mounted immediately around the axis of rotation, being contained in a perspex enclosure connected to the (now 70 mm) drive shaft at one end and to a third ball race in an adjustable end-plate. The motor/gearbox/supply voltage combinations of the components described here permit experimentation at rotation rates between approx 0.5 rpm and 8

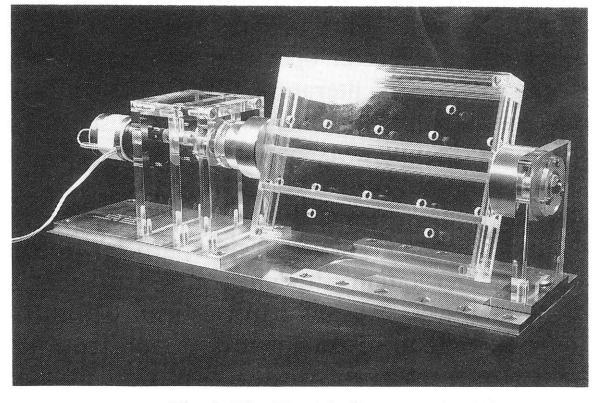

Fig. 3. The Type 2 clinostat.

rpm. Other motors with the same casing design (so that they are easily interchangeable on the rigs) are available rated at speeds up to 130 rpm (RS Components Ltd., item numbers 341–660, 330–799, 330–783 and 330–777).

All of the experiments described here were performed with the clinostat rotating at 2 rpm. Stems were gravistimulated on the stationary clinostat for a defined time after which clinostat rotation was started to nullify further stimulation. The results of a typical experiment are shown in Fig. 4. An important point is that all the stems extended during the experiments; in the case of the stem illustrated in Fig. 4, from 60 to 93 mm in approx 3 h. This stem was fixed on the rotational axis of the stationary clinostat (and thus horizontal) for 45 min before rotation was started. Since the reaction time is less than 30 min this stem began to bend before clinostating, reaching an angle of 36° to the rotational axis before the clinostat was switched on. After the start of clinostating it bent further, reaching an angle of 48° but then, in the absence of further unidirectional gravistimulation the tip angle declined to 9° when the clinostat was switched off 180 min after the start of the experiment. Continued observation of the stationary (horizontal) stem showed that it immediately expressed negative gravitropism,

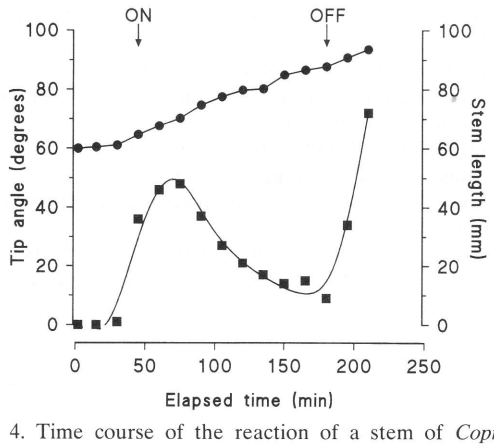

Fig. 4. Time course of the reaction of a stem of *Coprinus cinereus* to 45 min horizontal gravistimulation. The plots show the total length of the stem (circles) and the angle of the tip to the (horizontal) axis of rotation (squares). The stem was held stationary for the first 45 min and then the clinostat was switched on; it was switched off after 180 min.

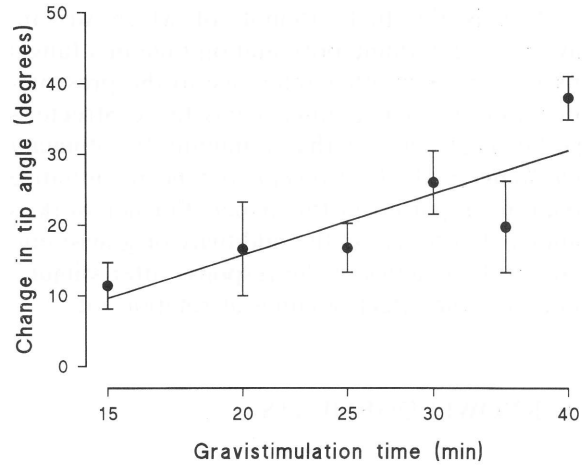

Fig. 5. Dosage-response relationship in terms of the maximum tip angle achieved during clinostat rotation compared with the length of time the stem was held stationary (horizontal) before starting the clinostat. Data plotted are the means of four to nine replicates. The *x*-axis is logarithmic.

reaching an angle of 72° to the horizontal within 30 min of the clinostat being stopped. These observations are representative of many others. Generalising: (a) the degree of bending achieved by the stem depended on the 'dose' of gravistimulation (Fig. 5); (b) most stems reached a maximum angle of bend and then relaxed back towards the rotational axis (their original horizontal); and (c) stopping the clinostat resulted in immediate expression of the usual negative gravitropic response. Relaxation back towards the rotational axis was unexpected as the stems have no external gravitational reference during clinostat rotation. It may be an artifact of the single direction of observation used. Further experiments are planned with simultaneous lateral and axial video recording so that the vector of the stem apex can be established.

Combining results from experiments involving different gravistimulation times permits determination of the presentation time. The usual method is to plot extent of reaction against log stimulation time [37,38] (Fig. 5). Although the data are variable the plot is essentially linear ($r = 0.83$) so there is no evidence for more than one sensitivity threshold. The intercept on the time axis of this plot (= 9.6 min) is the presentation time.

This is the first attempt, of which we are aware, to determine presentation time in a fungal tissue. The only other reference to the presentation time in higher fungi seems to be Streeter's [13] estimate of 'less than a minute' for *Amanita phalloides* and *A. crenulata*, but no quantitative data are reported in this paper. Further work is anticipated to assess the additivity of gravistimulation, the kinetics of the response after stimulation, and the effect of clinostat rotation rate.

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