

CENTENARY REVIEW

Gravimorphogenesis in agarics

DAVID MOORE¹, BERTOLD HOCK², JOHN P. GREENING¹, VOLKER D. KERN²,
LILYANN NOVAK FRAZER¹ AND JAN MONZER²

¹ School of Biological Sciences, 1.800 Stopford Building, The University of Manchester, Manchester M13 9PT, U.K.

² Lehrstuhl für Botanik, Technische Universität München at Weihenstephan, 85350 Freising, Germany

The shape changes which occur in agaric fruit bodies in response to change in the direction of gravity, usually referred to as gravitropism, are morphogenetic changes. Our interest in what we prefer to call gravimorphogenesis is to use it to examine morphogenesis experimentally. We are examining two agarics, *Coprinus cinereus* and *Flammulina velutipes*, and applying the best available technologies, including video analysis, all forms of electron microscopy, computer-aided image analysis and experiments in orbit in Spacelab.

Responses to gravity of the two organisms differ in ways which can be related to their ecological and structural adaptations. *C. cinereus* reacts extremely rapidly; its fruit body can regain the vertical within 3 h of being placed horizontal, whereas *F. velutipes* requires 12 h to bend through 90°. The fungi also differ in the bulk of tissue involved in the response. In *Coprinus*, a zone extending several cm down from the apex is normally involved in bending. In *Flammulina*, gravisensing is limited to a region just a few mm immediately below the cap, although curvature is performed in a zone of up to 2 cm below.

Flammulina cultures were flown on the Spacelab D-2 mission in 1993, and fruit body disorientation in orbit provides the first definitive proof that 'gravitropism' really is a response to the unidirectional gravity vector. Experiments with different clinostat rotation rates in *Flammulina* indicate that the perception threshold is about $10^{-4} \times g$. Analysis of different times of exposure to an altered gravity vector prior to clinorotation in *Coprinus* reveals that the perception time is 7 minutes and that continued response requires continued exposure.

Cell size determinations in *Coprinus* demonstrate that cells of the stem increase in length, not diameter, to produce the growth differential. In *Flammulina* a unique population of highly electron-transparent microvacuoles changes in distribution; decreasing in upper cells and increasing in the lower cells in a horizontal fruit body within a few minutes of disorientation. These are thought to contribute to vacuolar expansion which accompanies/drives cell elongation. Application of a variety of metabolic inhibitors indicates that the secondary messenger calcium is also involved in regulating the growth differentials of gravimorphogenesis but that gravity perception is unaffected by inhibitors of calcium signalling. In both *Flammulina* and *Coprinus*, gravity perception seems to be dependent on the actin cytoskeleton since cytochalasin treatment suppresses gravitropic curvature in *Flammulina* and, in *Coprinus*, significantly delays curvature without affecting stem extension. This, together with altered nuclear motility observed in living hyphae during reorientation suggests that gravity perception involves statoliths (possibly nuclei) acting on the actin cytoskeleton and triggering specific vesicle/microvacuole release from the endomembrane system.

There has been an active interest in the way in which fungi perceive and react to changes in their physical environment for much longer than the one hundred years we celebrate in this centennial year of the British Mycological Society. Sadly, fungi have been confused with plants for most of this century (and before), resulting in reduced effort on fungal research and to the expectation that what is known about plants should be applicable to fungi. The very late recognition that fungi belong to a completely separate Kingdom of organisms (Whittaker, 1969; Cavalier-Smith, 1981) and, ironically, recent evidence that fungi may be more closely related to animals than to plants (Baldauf & Palmer, 1993), has left many aspects of fungal biology under-developed and under-researched.

Nevertheless, research on tropisms and taxes has proceeded for many years (e.g. Schmitz, 1842; Hofmeister, 1860; Sachs,

1877) and members of the British Mycological Society have been among those who have contributed to its regular review and discussion (Hawker, 1950; Dennison, 1961; Banbury, 1962; Carlile, 1975; Gorovoj, Kasatkina & Klyushkina, 1987; Gorovoj, Kasatkina & Laurinavichius, 1989; Molitoris, 1990; Moore, 1991a, 1996; Kern & Hock, 1993). During the past 20 years, space research has influenced the focus of attention and more work has been done on the effect of gravitational disturbance on the behaviour of single cells. This has partly been to study their taxes and tropisms, but increasing emphasis has been given to study the overall gravitational biology of individual cells as well as multicellular systems (Moore & Cogoli, 1996). Most of this work will have some significance for manned space flight, either by making direct contribution to better understanding of the reaction of the

human organism to weightlessness or by revealing the reactions of other organisms, including fungi, which might be present in the space vehicle as commensals, pathogens, food, or components of 'closed ecological life support systems' (Hinghofer-Szalkay & Moore, 1996; Moore, Bie & Oser, 1996).

In this Centenary Review we will concentrate upon recent research on the gravitropic reactions of two agarics: *Coprinus cinereus* (Schaeff.: Fr.) S. F. Gray and *Flammulina velutipes* (M. A. Curtis: Fr.) Singer. The most obvious gravitropic reactions of fungal fruit bodies were described at the end of the last century (Schmitz, 1842, 1843; Hofmeister, 1860; Sachs, 1865, 1877) and in the early years of this century (Buller, 1905, 1909, 1922, 1924; Hasselbring, 1907; Streeter, 1909). These studies showed how the response to gravity is crucial for proper spore liberation from the fruit bodies of higher fungi and established the basic facts that agaric stems are negatively gravitropic and the gills positively gravitropic, but neither this nor later research (for reviews see Banbury, 1962; Moore, 1991a) enabled models of the cellular mechanisms involved to be established. In the 1990s, detailed study has been resumed after a gap of almost 25 years; there are two motives for this. Though interesting in its own right, the gravitropic response is a simple developmental pattern, it is a morphogenetic process. Its control requires signal perception and translocation system(s) and a coupling between the signal and the control of differential tissue growth. Study of gravitropism is therefore a natural, non-invasive means of generating, at the experimenter's convenience, a particular morphogenetic change in a specific location (hence the stress in our title on 'gravimorphogenesis' rather than the more mundane 'gravitropism'). Thus, in the most recent research, gravitational biology is being used as a tool to study fundamental aspects of cell and developmental biology in the belief that understanding the cell biology of gravity perception mechanisms could well contribute to a general understanding of sensory mechanisms, signal transduction and growth control processes.

The second motive is more pragmatic because both laboratories (School of Biological Sciences, University of Manchester and Lehrstuhl für Botanik, Technische Universität München at Weihenstephan) became involved in this topic following invitations to contribute experiments to space missions. In Manchester it was planned to fly *C. cinereus* on the Juno and IML-2 missions (Moore, 1990, 1991b), though lack of funding for research (of any sort) in orbit in the U.K. prevented fulfilment of this hope. In Weihenstephan, experiments with *F. velutipes* were developed and successfully flown on the German Space Agency's D-2 mission of the Shuttle-Spacelab complex (Hock *et al.*, 1993). On the ground, the two research programmes proceeded in parallel, and in ignorance of each other, over several years and we are pleased to have the opportunity now to present this review as the first jointly-authored account of our researches.

The attraction of gravity

Environmental stimuli programme and control the initiation and development, growth and behaviour of many organisms;

plants and animals as well as mushrooms and structurally simpler fungi. The stimuli include touch or contact with solid objects (hapto- or thigmotropism), chemicals (chemotropism), airflow (anemotropism), light (phototropism) and gravity (gravitropism). Tropic growth curvature usually results from differential growth on the two sides of the organism or structure. Growth towards the stimulus (positive tropism) results from the side closest to the stimulus growing least, in negative tropisms the region closest to the stimulus grows most and growth curvature causes the structure to bend away from the stimulus.

Tropic bending in response to light, wind, temperature or chemical stimuli can all be ascribed to the effects of differential exposure to a unilateral impulse, i.e. one side is more stimulated than the other. But gravity cannot be shaded and over the scale of living organisms there is no gravitational gradient. So all gravitational responses occur within the same, uniform, field and must depend on gravity establishing an asymmetric distribution of mass within the organism.

The gravitational force is vectorial, having both magnitude and direction. Mass is accelerated towards the centre of the Earth by gravity, defining what we know as the 'vertical' direction, and exerting a force on a restraint that we call weight (weight = mass \times acceleration due to gravity). Gravitational acceleration on Earth has an internationally agreed standard value of 9.80665 m s^{-2} , which, in biological terms, is essentially uniform over the planet's surface and has been a constant feature of the environment since before the origin of life.

Uniform? Did somebody say uniform? In fact, the acceleration due to gravity varies between 9.76 and 9.83 m s^{-2} over the surface of the Earth. At any one location it is influenced by elevation (gravity lessens as you move further from the centre of the planet), latitude (the Earth is not spherical so gravitational field strength at sea level varies with latitude by about 0.5%, being 'strongest' at the poles), local topography (the mass of a neighbouring mountain will affect your gravitational field), variations in the density of underlying rocks (amounting to about $10^{-5} \times g$) and tidal deformations. This last is caused by the influences of nearby celestial bodies, especially the Sun and Moon, and its periodicity results from the bodies orbiting each other. Whilst the first-mentioned variations are positional differences which do not change over biologically-important time scales, tidal effects have periodicities of about 12 or 24 h. This includes induced movements of 'the solid Earth' (the amplitude of which can be about 100 mm), although oceanic tides have the greatest biological influence through their effects on sea level and water movement on shorelines. Direct effects of these cyclical changes in gravity are unlikely at the biological level as the maximum variation (caused by the Moon at the equator) amounts to only $10^{-8} \times g$. Considering the enormous variations in other environmental factors (light intensity, temperature, humidity, etc.) which organisms cope with, in some cases on a minute-by-minute basis, it seems quite reasonable to describe the gravitational field as 'uniform'.

Escaping gravity. The gravitational field strength cannot be

reduced below $1 \times g$ under terrestrial conditions, which means that true sensitivity thresholds cannot be obtained for even the simplest phenomena on Earth. Furthermore, the gravitational field is such a natural part of the environment we all experience that it is difficult to convey a proper appreciation of how important gravity is to cell biology. Centrifuges enable experiments at higher acceleration levels than terrestrial gravity (hypergravity), and a response can be predicted by extrapolation for conditions of lower acceleration than $1 \times g$ (hypogravity) but, except for fleeting phenomena, there is no way of testing any resultant predictions on Earth. Very low gravity exposure, or microgravity, is only possible in a state of free fall towards the Earth. True zero gravity is unattainable even in orbital vehicles and the phrase is little used. To describe something in orbit as being 'weightless' is acceptable, but the preferred descriptive term is 'microgravity'. The reason for this is that orbital spacecraft experience accelerations from atmospheric drag, centrifugal forces, crew movements and manoeuvring. The largest component of this residual acceleration spectrum provides accelerations in the order of 10^{-6} of the normal gravitation experienced on the Earth's surface. Hence, *microgravity*. A recent illustration is provided by the accelerometer measurements made in the EURECA satellite which showed that the major component of the microgravity acceleration spectrum was at 0.8 Hz and was caused by movements of the wing-like solar panels on the outside of the satellite (Minster, 1993).

Since routine research using orbital facilities is simply not possible, techniques for simulating some of the effects of microgravity must be used for terrestrial research. A variety of facilities are available that allow microgravity to be attained for periods of a few to several seconds (drop towers and parabolic aircraft flight profiles) or even several minutes – in sounding rockets. Cheapest, and most accessible, of all is the clinostat, which provides uniform circular rotation around an axis which is usually placed perpendicular to the normal gravity vector. A specimen mounted on the clinostat is subject to a constant change in the direction of the gravity vector. Importantly, the specimen is not removed from the effects of gravity. The clinostat produces a similar effect to microgravity by arresting sedimentation of particles, but density driven phenomena are still present. Whereas any gravity sensors are deprived of stimulus in true microgravity, the clinostat can be regarded as a means of confusing the sensor by constantly varying stimulus direction. Unfortunately, the rotation rate cannot be optimised for more than one combination of sedimenting particle + suspending fluid at a time so the multitude of components within a cell experience a wide range of conditions as the clinostat rotates. This contrasts with orbital space flight where everything is subjected simultaneously to the same acceleration environment. Nevertheless, there is often at least broad agreement between clinostat and spaceflight studies, with the former acting as a useful means of preparing for the latter (Block, Briegleb & Wohlfarth-Bottermann, 1986; Lorenzi & Perbal, 1990; R. Moore, 1990; D. Moore, 1991a; Hatton & Moore, 1992).

Gravity sensing. Examples of the use of gravity as a cue for directional orientation can be found all around us and involve

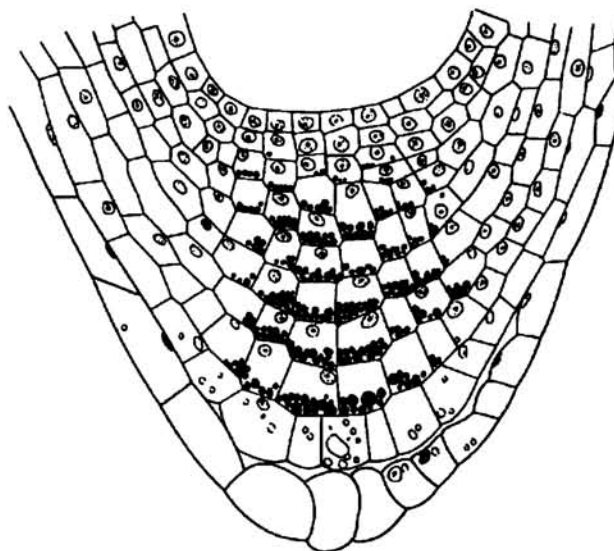


Fig. 1. An early illustration of amyloplast (statolith) sedimentation in plants. In this illustration of a median longitudinal section of a vertical root cap of *Rorippa amphibia*, the amyloplasts in cells of the columella are shown as black blobs. (Modified after Nèmec, 1900).

all the major Kingdoms of organisms. Those with which we are most familiar are the directional growth of plants and balance in animals. For the past hundred years it has been known (e.g. Haberlandt, 1900; Nèmec, 1900) that in plants particles called amyloplasts within particular cells of the stem or root are displaced (Fig. 1) and set up a chain of reactions which results in an uneven distribution of growth hormones which guarantees that shoots grow upwards and roots grow downwards. In animals, the movement of mineral grains on sensory hairs triggers nerve impulses which cause muscle activity to compensate for change in orientation (Fig. 2) and there is a palaeontological case for suggesting that the statocyst of coelenterates may have been the first true sensory organ in animals (Hyman, 1940; Bullock & Horridge, 1965).

Gravity-sensing organs in animals detect change in shear, usually the distortion of filaments. The biophysical advantages of this strategy are discussed by Moore & Cogoli (1996), but the essence is that shear force increases with tilting angle and is directional whereas the pressure of a mass on a surface declines with increasing tilt and is not directional. Although the detection system in plants has for many years been thought to depend on the pressure of amyloplasts (or other statoliths in lower plants) on stacks of endoplasmic reticulum membranes, there is now a growing body of opinion that statoliths are suspended on actin microfilaments, so shear forces may be used to sense the gravitational vector in plant cells too (Hejnowicz & Sievers, 1981; Sievers *et al.*, 1991; Volkmann *et al.*, 1991; Buchen *et al.*, 1993).

In addition to these specialised sensory cells in animals and plants, altered gravitational accelerations also affect cells with no obvious structural specialisations to sense gravity, implying some direct effect of gravity on cellular functions. The influence of gravity extends to the biochemical, and even molecular, levels of metabolic control and is not simply limited to the mechanical relationships between parts of organisms. The influence of gravity on the function and

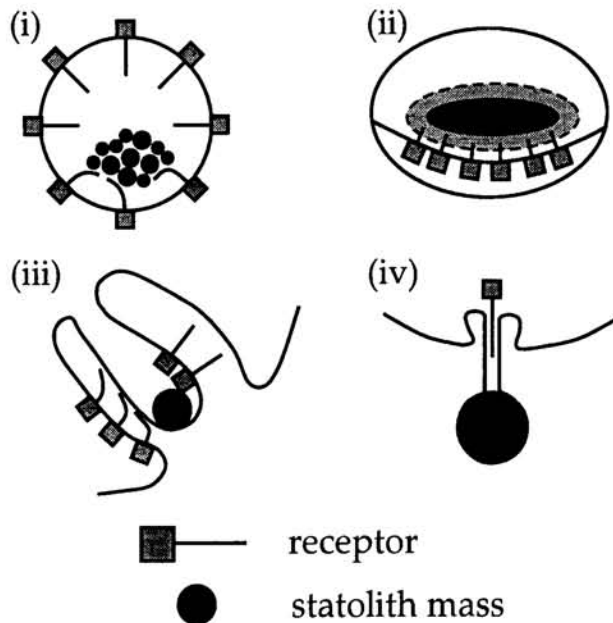


Fig. 2. Gravity perception systems in animals. Schematic illustrations (redrawn after Platt, 1984) of sections showing relationship of receptors and the dense mass (the statolith) whose movement is detected. (i) The statocyst, found in many invertebrates, consists of a fluid-filled sac, lined with sensory hairs, which contains granules of calcium carbonate, sand, etc. (statoliths). As the animal moves, the statoliths stimulate the receptors on the lowest side, giving a sense of the position of the body. (ii) Otocysts are similar to (i) but statolith (otolith) displacement is limited and mechanically coupled to the sensory hairs by a gelatinous membrane (indicated by the dashed line). The semicircular canals in the ears of vertebrates act on the same principle (but detect fluid shifts) and have a similar function. (iii) In a pendant statolith organ deflection of the pendant mass stimulates surrounding receptors or receptors on the pendulum itself. (iv) The tricholith organ also depends on a pendant mass, but in this case the stalk of the pendulum has an internal receptor which is stimulated as the pendulum stalk flexes.

structure of living things has been discussed by numerous authors from a variety of standpoints (Siegel, 1979; Brown, 1983; Cogoli, Tschopp & Fuchs-Bislin, 1984; Ross, 1984; Langbein, 1986; Cogoli & Gmünder, 1991; Mesland, 1992).

The *potential* effect of gravity on cell biology is a worthwhile study because of the *observations* which have been made in experiments conducted in orbit. Altered metabolism has been observed in cultures of *Physarum polycephalum*, *Neurospora*, *Paramecium*, *Euglena*, bacteria and many mammalian cell lines which have been transported into microgravity (Moore & Cogoli, 1996). Although individual experimental programmes have frequently been relatively simple because of the paucity of background knowledge, and often poorly replicated because of accommodation and other engineering constraints in the vehicles, research using microgravity facilities has revealed quite categorically that the normal unilateral gravity vector is **not** required to establish embryonic axes in animals, plants or fungi, nor circumnutation or plumule hook formation in plants. Altered gravitational acceleration **does** cause changes in basic cellular processes, including:

- energy metabolism and intermediary metabolism;
- cell proliferation rates;

- nuclear division;
- membrane composition and function (especially of the plasmalemma);
- cell and tissue differentiation;
- early events in ontogeny;
- rate of aging.

These different effects occur in animals and plants alike and can also be traced in fungi and microorganisms including prokaryotes. The overall conclusion must be that the fundamental cellular machinery is dependent on the normal unilateral gravity vector for some of its functioning and is consequently disturbed when exposed to microgravity.

Any effect of gravity must be exercised through its influence on the physical world; gravity determines many aspects of our normal environment. The effects of weight and mechanical loading on macroscopic structures are obvious and need no discussion. Where density differences exist between organelles (or other intracellular structures) and the surrounding cytosol the particles may either sediment or float (these processes are identical in physical terms, apart from the sign of the vector). A specific distribution of intracellular masses may be an important aspect of cellular anatomy; if disturbed, cellular function and behaviour may be adversely affected. The weight of cell structures, even the cytosol itself, exerts forces on membranes and cytoskeletal elements. Consequential configurational changes may be used for gravisensing. Even a minor change in membrane properties could lead to major alterations in metabolism because of the importance of membranes in regulating activities in the compartments they enclose. At cellular dimensions, surface tension forces are relatively strong in comparison to the effects of gravity (specifically weight), so it has been argued that gravity dependent phenomena in membranes are unlikely candidates as gravity sensing systems (Audus, 1962). The argument is usually based on consideration of pressure differentials, and as turgor pressure within a plant cell is approximately 10^6 -fold greater than the calculated hydrostatic pressure difference caused by mass redistribution, it is considered unlikely that such a small difference in hydrostatic pressure could be sensed against such a large background (Björkman, 1988).

However, independently of hydrostatic pressure, the weight of the cytosol on the plasma membrane could stress the inner face of the membrane more than the outer and thereby affect the activity and/or distribution of membrane proteins in a localised manner, effectively acting as a gravity sensing system. Ion channel currents have been observed to be about 20% higher in horizontally oriented membranes than in vertical membranes, possibly as a result of mechanically-altered geometry of the membrane bilayer in the two planes (Schatz *et al.*, 1992).

The idea has also been developed (in recent literature dealing with animal cells) that the cytoskeleton responds to stress as a single *tensegrity* (= tensionally-integrated) structure: so that in the normal circumstance the shape and form of a cell depends on the ordered interplay of tensile and stiff components of its cytoskeletal architecture all of which are mechanically coupled (Ingber, 1993; Wang, Butler & Ingber, 1993; Todd & Klaus, 1996). This would mean that the

gravitational field is a crucial element in determining cell shape, by causing the weight differentials and density differentials which the architectural components balance. Conversely, distortion of any part of the cytoskeleton could be mechanically coupled to movement, orientation or 'gravity' sensors.

Another important consequence of the Earth's gravitational field is that almost all physical phenomena involving fluid mixing and mass transport have a gravitational component. In mixtures of fluids with solids or other (immiscible) fluids, the denser constituents sediment at $1 \times g$. Obviously, this encompasses sedimentation of solids, from falling leaves to sinking sand, but buoyant convection in fluids is entirely driven by differences in density. This applies to everything on Earth, including rock, but in common experience is most evident in flames where convective in-rush of air is entirely responsible for transporting oxygen to the burning fuel (Williams, 1990). This type of density difference would be lost in microgravity, so particles remain in suspension and convective flow is halted. This is not just a macroscopic effect, in the absence of buoyancy driven convection the osmotic process is modified considerably (Slezak & Dworecki, 1984; Papazian & Mason, 1989) and electrolytes may accumulate near the membrane surfaces as a result of electrostatic interactions (Schatz & Linke-Holmes, 1989).

Convective currents caused by concentration differentials, which are known as Marangoni flows (Scriven & Sterlino, 1960; Napolitano, 1984; Schwabe *et al.*, 1990), are obscured by buoyant convection under normal circumstances, but become important as the only flows able to redistribute materials in mixtures of fluids which do not differ in density (Grunditz, Wozniak & Mathes, 1990). One example will suffice: concentration differentials at the sites of metabolite transport would be dissipated by Marangoni convection. So, as the only physical process contributing to removal of metabolite from the sink in the absence of gravity, Marangoni convection may become an important regulator of transport of molecules through membrane channels (Langbein, 1986). In orbit, this is a demonstrable consequence of the lack of density differentials due to the microgravity environment. In cells on Earth the circumstance might arise if density differentials were avoided by, for example, co-ordinated transport of solutes and water into/out of a membrane-bound compartment. The point is that phenomena which are normally masked can become highly significant in the absence of the environmental conditions generated by the gravitational field.

This brief catalogue of the direct effects of gravity and the phenomena which become evident in microgravity illustrates the wide range of processes which *might* be involved in gravity perception in fungi. This is the background against which our study of agaric responses has been conducted.

Tropic reactions of *Phycomyces*. The most information we have to date about tropisms in fungi comes from research done on *Phycomyces* sporangiophores, which exhibit a variety of interacting tropisms and avoidance growth responses (Dennison, 1961; Varjú, Edgar & Delbrück, 1961; Johnson & Gamow, 1971; Lafay, Matricon & Bodère, 1975; Gamow & Böttger, 1982; Gyure, Böttger & Gamow, 1984; Dennison &

Shropshire, 1984; Galland & Russo, 1985). Dennison (1961) found two sensory systems for gravitational acceleration: a transient extracellular mechanical reaction, later determined to be the response to activation of a stretch receptor (Dennison & Roth, 1967), and the longer term gravitropic response which he concluded was the result of redistribution of growth resources following an intracellular gravity detection event. Dennison & Shropshire (1984) argued that the sensory mechanism would involve particles and/or liquid phases with differing densities within the sporangiophore and identified the main cell vacuole as a floating orientation detector. They suggested that reorientation caused the vacuole to float upwards, causing protoplasmic asymmetry. The thicker layer of protoplasm below the vacuole was postulated to generate more wall growth on that side and thereby produce the differential growth responsible for the negative gravitropism of the sporangiophore. Until recently, this was the only published suggestion for a gravireceptor mechanism in fungi, and the relatively low power light micrographs which Dennison & Shropshire (1984) claimed to show the protoplasmic disturbance as the vacuole was displaced were the full extent of published cytological observations of fungal gravitropism.

Tropic reactions of *hymenomycetes*. Hymenomycetes exhibit a number of tropisms during morphogenesis. The youngest fruit body initials grow perpendicularly away from the surface on which they arise independently of the direction of light or gravitational signals (Buller, 1905, 1909; Plunkett, 1961; Schwantes & Barsuhn, 1971). This might be analogous to the avoidance reactions of *Phycomyces* sporangiophores (Johnson & Gamow, 1971; Lafay *et al.*, 1975) and may be a reaction to water activity (Gamow & Böttger, 1982).

As development proceeds the stems of agarics seem generally to be non-phototropic but show a marked negative gravitropism whereas lignicolous and coprophilous hymenomycetes are often both phototropic and gravitropic (Plunkett, 1961); anemotropism has also been demonstrated (Badham, 1982).

Generally, an initial phototropism is followed by negative gravitropism, described by Buller (1909, p. 48) as 'a remarkable change ... in the physiological properties of the stem'. Plunkett (1961) showed that though several tropisms may be expressed during fruit body development of *Polyporus brumalis* one usually predominates at any given stage but if the effect of the predominant tropism is experimentally diminished the weaker reactions begin to show. The stems of *P. brumalis* fruit bodies whose caps had not developed showed negative gravitropism, but when exposed to unilateral illumination they turned towards the light source; when shaded, gravitropism again predominated. Plunkett (1961) concluded that gravitropism is '... dominant under conditions of low light intensity when ... the phototropic mechanism is understimulated'. Plunkett (1961) sought to extend this interpretation to *Coprinus* species but this cannot be so as gravitropism *replaces* phototropism in *Coprinus* (Buller, 1909; and see below).

Hymenomycete spore-bearing tissue (the hymenophore) is positively gravitropic. Buller (1922, [p. 151]) states that spines (= teeth) of *Hydnum* are positively gravitropic, but detailed

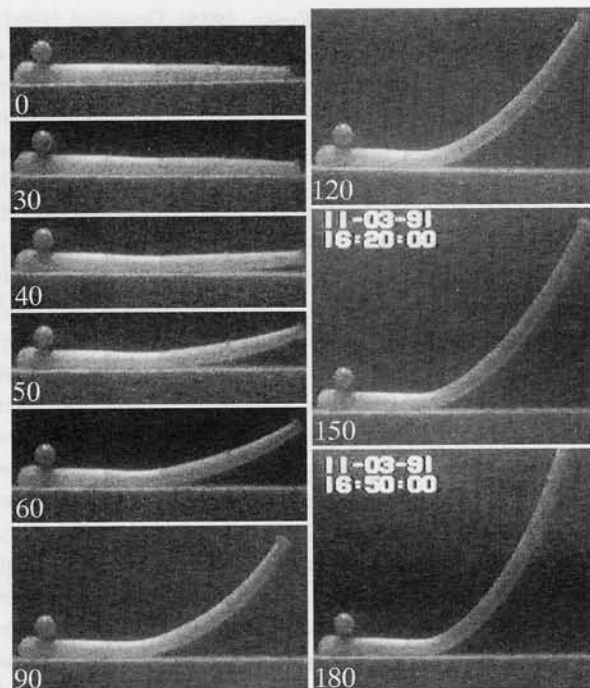


Fig. 3. The gravitropic reaction of a stem of *Coprinus cinereus* in air, shown in a panel of stills from a video recording. The stem was 64 mm long at the start of the experiment, and was pinned through its basal bulb (on the left) to a horizontal balsa wood support using a map pin with a 5 mm diameter head (= size marker). Numerals show elapsed time in minutes. From Kher *et al.* (1992).

attention has been restricted to the gravitropism of gills of *Agaricus campestris* (Buller, 1909 [pp. 51–52]) and pores of *Polyporus brumalis* and *Phellinus contiguus* (Plunkett, 1961; Butler & Wood, 1988).

In summary, the extent of knowledge of gravitropism as it existed when our studies commenced was that the stems of both agaric and aphylophoralean fruit bodies were known to be negatively gravitropic and their hymenophores positively gravitropic. The impression is that these responses can be quite rapid, but the only reports of anything relating to kinetics are from Buller (1909, 1922) who detected the gravitropic adjustment of the gills of *Agaricus bisporus* within an hour and states: 'If one turns a [*Coprinus plicatilloides*] fruit-body from a vertical to a horizontal position, the stem begins to turn up the cap within about three minutes after first receiving the geotropic stimulus' (Buller, 1909 [pp. 65–74]). Given the paucity of detailed information which had accrued over the previous 150 years, the emphasis of the research undertaken in both Manchester and Weihenstephan was to establish categorically (i) the basic kinetics of the gravitropic response; (ii) the mechanics, cell biology and physiology of gravitropism in agarics; (iii) the location of the gravity sensitive zone; and (iv) enough information about the mechanism of perception to permit modelling of processes at the intracellular level to guide further experimentation.

Gravitropism in agaric fruit bodies

Basic kinetics. We used video recording and computer-based video-image analysis to complete the first kinetic analysis of

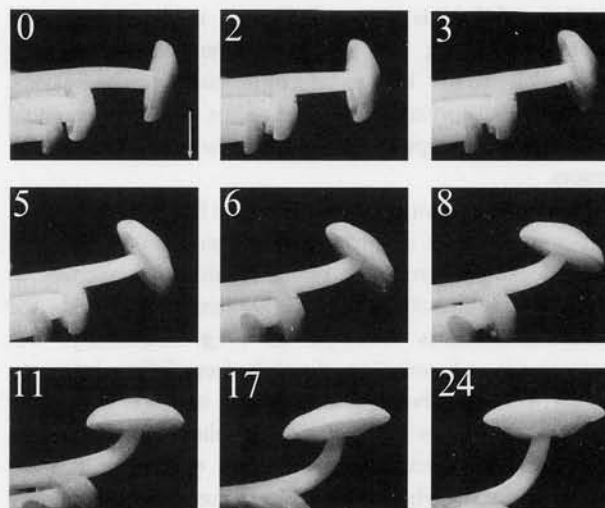


Fig. 4. Gravitropism of *Flammulina velutipes* fruiting bodies after being placed in a horizontal position at time 0. Numerals show elapsed time in hours. From Kern & Hock (1994).

gravitropism of the stems of an agaric, with fruit bodies of *Coprinus cinereus* (Kher *et al.*, 1992) and very similar observations have been made on *Flammulina* using serial photographic records (Monzer *et al.*, 1994). The standard assay in *Coprinus* involves removal of the cap followed by video observation of the stem secured on a horizontal platform (Fig. 3). In studies with *Flammulina*, the cap has sometimes been left in place, though stems were trimmed to a uniform 25 mm length and then secured, cantilevered, to the top of a pin for observation (Fig. 4); some experiments were done with stem segments without caps. In all cases, low-intensity red light has been used for illumination to avoid phototropic effects. The approaches used in the two laboratories cover the major experimental variables; namely with or without cap and with or without initial horizontal support.

Both assays have provided extensive data sets to analyze response kinetics and the two species differ considerably in the rate with which they react (Fig. 5). The reaction time (time to first visible response after being placed horizontal) in *C. cinereus* stems is typically about 30 minutes (Kher *et al.*, 1992) and the apex of the stem attains the vertical within 3 to 4 hours. On the other hand, stem segments of *F. velutipes* fruit bodies required 24 to 36 h to reorient to the vertical, though intact fruit bodies re-orient within 12 h (Monzer *et al.*, 1994). Note that Fig. 5 shows that the major difference between *Coprinus* and *Flammulina* is a 6-fold difference in the initial *rate* of response (51° h^{-1} in *Coprinus*, 9° h^{-1} in *Flammulina*). Other aspects of their gravitropic reactions are quite comparable: reaction times (estimated from the illustrated data as the intercepts on the *x*-axis) of 20 min in *Coprinus* and 42 min in *Flammulina* are similar, and both organisms overshoot the vertical by about the same amount before re-adjusting to 90° .

Different mechanical processes seem to be used by the two organisms to generate stem bending. During gravitropic bending of *Flammulina velutipes* fruit bodies the elongation rates of the upper and lower sides of the stem not only differed from one another (the lower side elongating more

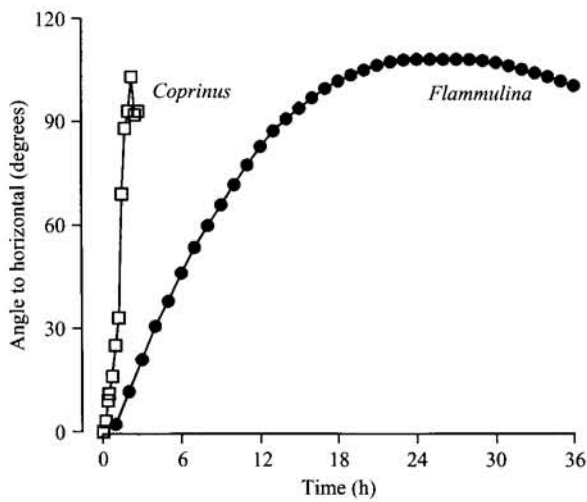


Fig. 5. Kinetics of gravitropic curvature after reorientation to the horizontal at $1 \times g$ in *Coprinus cinereus* (open squares) and *Flammulina velutipes* (closed circles).

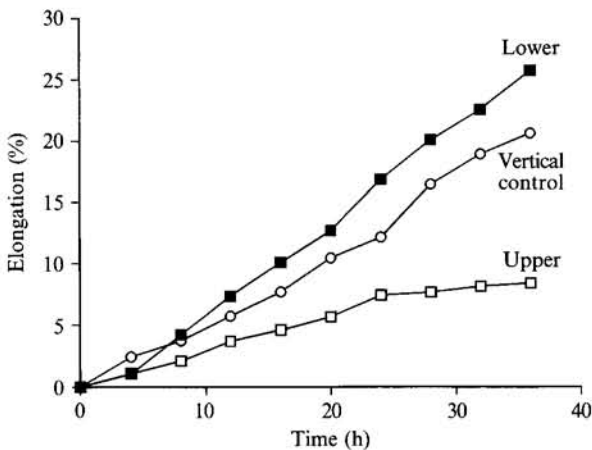


Fig. 6. Elongation of *Flammulina velutipes* fruiting body stems during 36 h of gravitropic reorientation, measurements made at 4 h intervals. Comparison of elongation of vertical controls (open circles) with the elongation of the upper (open squares) and lower (closed squares) sides of horizontal stems. Regression analysis of these data shows that the rate of elongation of the lower side of horizontal stems ($y = 0.75x - 1.37$) is about 30% greater than that of the vertical controls ($y = 0.59x - 0.72$); whilst the rate of elongation on the upper side ($y = 0.25x + 0.38$) is about 60% less than the vertical controls. Redrawn from Fig. 1e in Monzer *et al.* (1994).

rapidly, of course, to make the stem bend upwards) but elongation rate of the upper side was reduced to 40% of the rate of elongation of vertical controls, whereas elongation rate of the lower side was about 30% higher than the vertical control (Fig. 6).

Comparable observations of *C. cinereus* (Greening & Moore, 1996) showed that the upper and lower sides of the bending stem eventually elongated at about the same rate (which was higher than the rate of elongation at the time of reorientation); what caused bending was that the lower surface accelerated to that rate almost immediately, whilst the upper surface accelerated to the maximum rate over a period of 2 h (Fig. 7

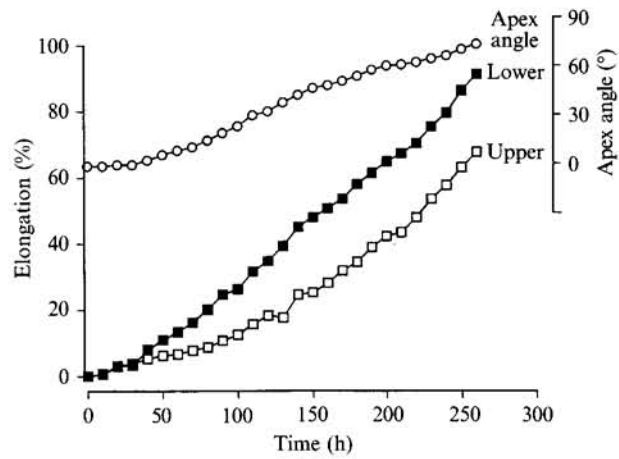


Fig. 7. Elongation of *Coprinus cinereus* fruiting body stems during 4.5 h of gravitropic reorientation, measurements made at 10 min intervals. Comparison of elongation of the upper (open squares) and lower (closed squares) sides of horizontal stems. The angle of the stem apex to the horizontal is also shown. Note that the most rapid phase of curvature (as reflected in increasing angle of the stem apex) occurs while the upper side of the stem is slowly accelerating towards the elongation rate which the lower side achieved immediately the reaction time (typically 30 min) had elapsed (see also Table 1). Redrawn from Fig. 2 in Greening & Moore (1996).

Table 1. Differential elongation rates of the upper and lower sides of a horizontal stem of *Coprinus cinereus*

	Time interval (min)			
	0-30	50-100	150-200	210-260
Upper side	0.14*	0.13	0.34	0.49
Lower side	0.12	0.33	0.34	0.49

* Entries are the slopes of linear regressions calculated over the indicated time intervals for the data displayed in Fig. 7

and Table 1). The most rapid phase of increase in the apex angle coincided with the time interval over which the elongation rate of the lower side was 2 to 3-fold higher than that of the upper side (note that this 1:3 differential was also seen in *Flammulina*, Fig. 6). Eventually, both upper and lower sides reached the same rate of elongation in the segment of stem under study, but by this time the position of the bend had moved towards the stem base (see Fig. 8) and further bending was the responsibility of a more distal stem segment.

It is very easy to study response kinetics in *Coprinus* because the timescale over which gravitropism operates is within the scope of domestic video recorders and tape cassettes. We have, therefore, been able to accumulate a great many observations and large amounts of data. These large data sets have revealed details in the response which are otherwise difficult to visualise, including: (i) there was no correlation between response time and stem length or rate of response and stem length, which together suggest that the stems were equally competent over the whole range of sizes from 20 to 64 mm (and to 80 mm in later experiments); (ii) there was no correlation between rate of response and response time, suggesting that these two features are

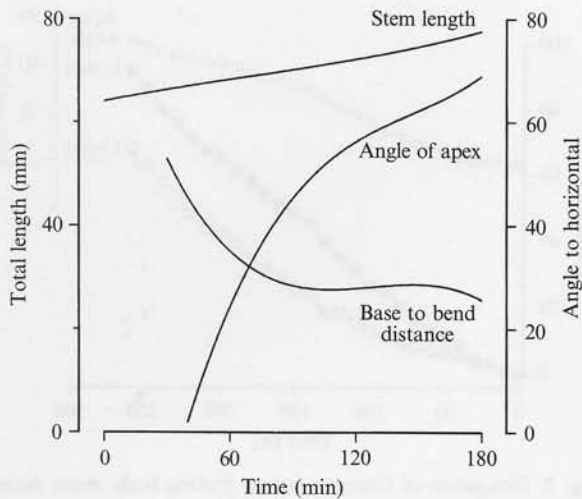


Fig. 8. Idealised plot showing summary of parameters for a 'typical' *Coprinus* stem (original data derived from the stem shown in Fig. 3 above). The angle of the stem apex to the horizontal is a measure of the progress of gravitropic curvature. The stem increases steadily in length as it bends (i.e. bending and elongation occur simultaneously, the former does not supplant the latter). The distance between the stem base and the position of the bend steadily decreases, illustrating how the bend moves down the length of the stem towards its base. Note that rate of bend movement and rate of change in apex angle are greatest in the first hour or so of the response. When the angle of the stem apex reaches about 30 to 40° to the horizontal the kinetics change. This is thought to be a reflection of the activation of the bend compensation phenomenon. Based on Fig. 3 in Kher *et al.* (1992).

independent of one another (i.e. the factor that determines how soon the stem will bend is independent of that which determines how fast it bends); (iii) bending first appeared towards the apex of the stem, on average within the apical 15% of the length of the stem; (iv) all stems increased in length during the bending process; the mean increase in length was 25.6% (range 11 to 47%) but there was no correlation between initial stem length and percentage increase. Data supporting these statements can be found in Kher *et al.*, 1992.

Of particular interest is the fact that the location of the bend moved towards the base of the stem (Fig. 8). This observation in *Coprinus* is paralleled by observations that although gravisensing is limited to a region just a few mm immediately below the cap in *Flammulina*, curvature is performed in a zone of up to 2 cm below the cap (see Fig. 4). Basipetal movement of the bend clearly implies baseward translocation of whatever impulse or signal prompts the differential elongation which causes stem curvature. But there is another implication which is that if all that happens is that bending occurs initially close to the stem apex and then the location of the bend moves towards the base, then the stem would bend into a circle. Yet, in fact, when the apical regions attain the vertical they are straight (see Fig. 3). This shows that more bending occurs than is necessary to restore the stem apex to the vertical and there is a *second process*, which we have called curvature compensation (Kher *et al.*, 1992) which 'unbends' the already curved upper regions of the stem. Bending raises the apex, and as this approaches an angle of about 35° to the horizontal,

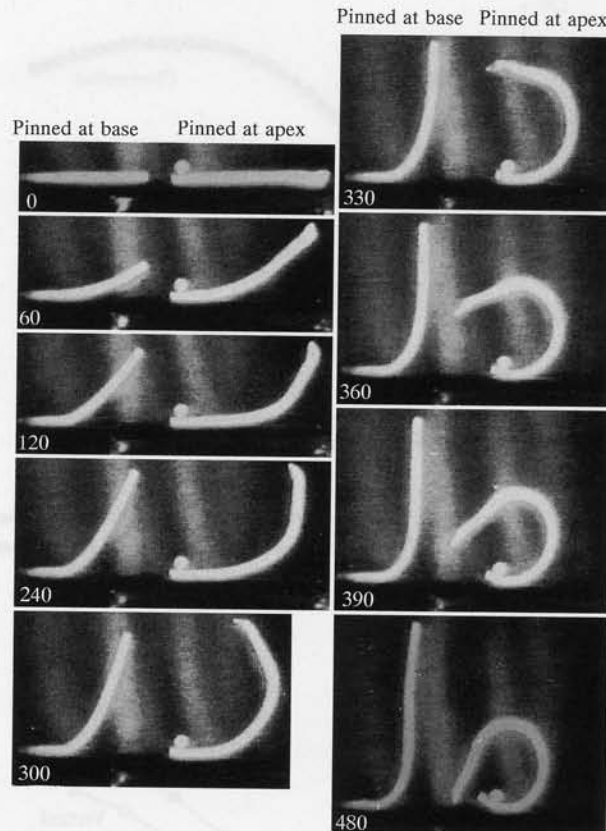


Fig. 9. Panel of stills from a video recording of an experiment comparing the gravitropic reactions of a stem of *Coprinus cinereus* pinned through the basal bulb (left) and one pinned through its apex (right). Numerals show elapsed time in minutes. From Kher *et al.* (1992).

curvature compensation begins to adjust the degree of bending so that the apex can be brought exactly vertical.

This is an extremely important, but previously unknown aspect of the gravitropic process. Calculation suggests that 90% of the initial bend was compensated as the stem brought its apex accurately upright (Kher *et al.*, 1992). The apex had to be free to move for curvature compensation to occur. When the apex of *Coprinus* stems is restrained to the horizontal, the stem can bend into a complete circle (Fig. 9). The reaction of *Flammulina velutipes* was similar, free movement of the cap being essential for adjustment back to the vertical (Fig. 10).

The role of the apex. The apex of the fruit body evidently exerts crucial control over gravimorphogenesis. The question now is this: what constitutes 'the apex'? In *C. cinereus* it is well established that the fruit body cap influences stem development during normal vertical growth. Hammad *et al.* (1993) have shown that the phase of rapid stem elongation is correlated with the ending of meiosis. Indeed, expansion of the different cell types in the cap as well as inflation of cells of the stem began immediately post-meiotically. These authors suggested that the coordination of different parts of the fruit body may be achieved by a signalling system that 'reports' the end of meiosis to spatially distant parts of the basidiome. The route such a signal might take is not clear, but since primary gills are attached to the stem in *Coprinus*, with their

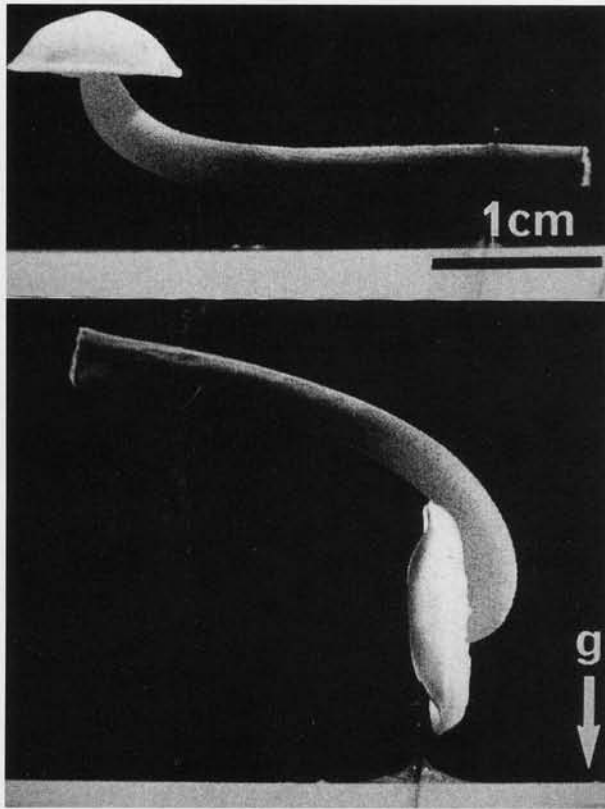


Fig. 10. Gravitropic curvature in relation to specimen attachment in *Flammulina* fruit bodies. Samples attached at the stem (top) show the common graviresponse pattern. Specimens attached at the cap (bottom) also feature negative gravitropic curvatures, but they overshoot the vertical (illustrations show state reached after 72 h of gravistimulation). From Monzer *et al.* (1994).

tramal regions in full hyphal contact with stem tissues (Reijnders, 1963, 1979; Moore, 1987), the connection between tissues undergoing meiosis and the upper (most reactive) regions of the stem may be fairly direct. This indication of the profound influence of the cap does not necessarily conflict with Gooday's observation (Gooday, 1974) that stem elongation in *C. cinereus* had no requirement for connection either with the cap or the parental mycelium. Although the fruit bodies which Gooday used were at fairly late stages in development, Cox & Niederpruem (1975) confirmed and extended the observation by showing that primordia about 5 mm in height (equivalent to 5–10% of final size) were able to elongate after excision and decapitation. Hammad *et al.* (1993) demonstrated another aspect of this cap/stem interplay by showing that whilst the stem may not be *dependent* on the cap, its elongation certainly benefits considerably from the presence of the cap: intact fruit bodies elongated about 25% more than decapitated ones (amounting to 2 to 3 cm greater length). Elongation of *Flammulina* stems is more dependent on the cap; Gruen (1982) showed that *F. velutipes* has only a limited potential for stem growth after removal of the cap and we have confirmed this.

Meiosis is highly synchronised in *C. cinereus*, and it has been demonstrated that only fruit bodies which had completed meiosis (average height 16 mm [$n = 7$]) before being placed horizontal were graviresponsive (Kher *et al.*, 1992). Meiosis is

Table 2. Dependence of the gravitropic response on the developmental stage in *Flammulina velutipes* fruiting bodies

Hymenium stage of development	Stem curvature (°)
No basidia	10.1 ± 2.3
Basidial precursors	15.0 ± 4.8
Mature basidia	73.6 ± 8.5

Data extracted from Fig. 4a in Monzer *et al.*, 1994. Means (±SD) of 6 or 7 replicates.

not synchronised in *Flammulina* but a clear dependence of the gravitropic responses on developmental stage has been demonstrated; significant gravitropic curvatures were not seen until maturation of basidia (Monzer *et al.*, 1994; see Table 2).

The relationship between cap and stem with regard to gravimorphogenesis seems to be that gravitropic competence is a post-meiotic event, therefore the stem requires a signal indicating that meiosis has been sufficiently advanced. Subsequent gravitropic curvature may benefit from the presence of the cap but does not *require* its presence (note that the *Coprinus* standard assay uses decapitated stems). So the cap is not required to promote curvature. Even the upper regions of the *Coprinus* stem can be removed, leaving the remnants of the stem with the ability to show both gravitropic bending and curvature compensation to adjust to the vertical. Tolerance of this sort of treatment differed ten-fold between the two organisms. Apical segments 10, 20 and 30 mm long have been removed from 31–80 mm *Coprinus* stems prior to their being laid horizontal, and even when more than half of the stem was removed the remaining segment usually responded gravitropically and usually adjusted to the vertical; i.e. curvature compensation, as well as gravitropic bending, occurred normally in these specimens (Greening, Holden & Moore, 1993). There was a very clear positive correlation between the amount of material removed and the time elapsed before the remnant reacted: the greater the portion of stem removed, the longer it took for a response to gravity to be observed (Fig. 11). Very similar observations have been made with *F. velutipes*, but removal of just a few mm of the apex was sufficient to reduce curvature drastically in this species. Whereas controls curved through $76 \pm 4.5^\circ$ over a 72 h period, removal of the 2–3 mm apical 'transition zone' (where the cap and stem hyphae interconnect) reduced the curvature to $32 \pm 4.3^\circ$, removal of a further 5 mm from the top of the stem reduced curvature to $21 \pm 1.7^\circ$ and a further 5 mm excision reduced curvature to $5 \pm 1.5^\circ$ in 72 h (Monzer *et al.*, 1994).

One way of accounting for these observations might be to suppose that the proportion of gravity detecting (gravi-perceptive) cells is successively reduced in zones further from the true apex. Such an interpretation implies that gravi-perception extends over a considerable proportion of the stem in *Coprinus*, certainly several cm down from the apex. On the other hand, gravi-perception in *F. velutipes* is effectively localised to the few mm which makes up the transition zone. This localisation makes *Flammulina* a far better subject for studies with the microscope (light or electron), whilst the speed of reaction and extent of stem involved in the response

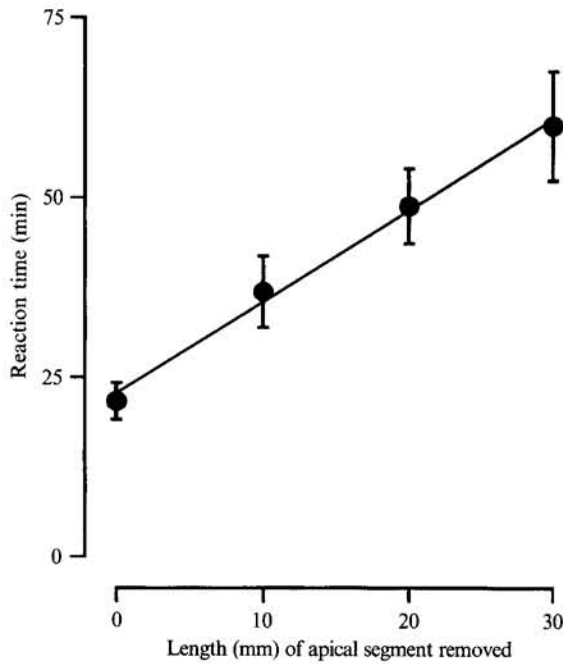


Fig. 11. Relation between reaction time of stems of *Coprinus cinereus* and amount of the apical part which had been removed prior to reorientation. Linear regression correlation coefficient, $r = 0.997$. Revised, with additional data, from Fig 2 in Greening *et al.* (1993).

suits *Coprinus* to kinetic studies. If the rate of response in each species is, indeed, related to the bulk of tissue able to react, it is significant that the difference between the genera in terms of the amount of tissue involved also correlates with major differences in rate of gravitropic response (Fig. 5). This may reflect a general rule that rate of response in agarics is determined by the bulk of gravitropically competent tissue.

A microscopic study we have completed in *C. cinereus* is a morphometric analysis of the cell patterning which achieves the gravitropic curvature (Greening & Moore, 1996). Earlier

studies showed that *Coprinus* stems contain two populations of hyphae: narrow and inflated. During normal vertical growth, inflated hyphae inflate further and the proportion of narrow hyphae declines as the stem grows from 45 to 70 mm, indicating that normal vertical stem extension involves both an increase in cross-sectional area of inflated hyphae and recruitment of narrow hyphae into the inflated population (Hammad, Watling & Moore, 1993). Similar analyses of the upper and lower regions of gravitropically-responding stems at the point of maximum curvature show that neither cell cross-sectional area nor cell-size population structure changes during bending, there is a change only in cell length (Table 3). This suggests strongly that the growth mechanism which causes gravistimulated stems to bend is different from that which generates normal vertical extension growth. The observations also provide a contrast with plant stems where increases have been recorded in both length and diameter during gravitropic bending (Sliwinski & Salisbury, 1984).

Sensitivity. At 10.50h on 26 April, 1993 the Space Shuttle Columbia was launched carrying cultures of *F. velutipes* as part of the FUNGI experiment on the D-2 Spacelab mission. Cultures grown in weightlessness for 165 h during this mission showed random orientation of their fruit bodies in contrast to the normal vertical orientation (Fig. 12). This, for the first time, demonstrated that gravimorphogenesis really does require a gravitational vector.

Experiments with centrifuge and clinostats to determine the sensitivity of *F. velutipes* indicate that directional growth of the fruit bodies occurs in response to a gravitational field about 10^{-4} of normal Earth-gravity (Kern, 1994). No attempt has been made yet to determine sensitivity of *C. cinereus*, but the species was used in the first attempt to use a clinostat to estimate the presentation time (the minimum gravistimulation time needed to evoke a response) for any fungal gravitropism (Hatton & Moore, 1992). Continuation of this research has

Table 3. Cell morphometric analysis of sections of gravitropically responding stems of *Coprinus cinereus* at the point of maximum curvature.

	Lower region of the bend		Upper region of the bend	
	n	mean \pm S.E.M.	n	mean \pm S.E.M.
Mean cross sectional area of narrow hyphae (μm^2)	626	9.82 \pm 0.6	881	9.43 \pm 0.4
	664	9.01 \pm 0.7	625	8.95 \pm 0.4
	775	9.15 \pm 0.8	474	9.67 \pm 0.8
Mean cross sectional area of inflated hyphae (μm^2)	1442	176 \pm 6.5	1442	183 \pm 7.6
	1140	186 \pm 9.0	1202	179 \pm 9.4
	1195	184 \pm 11.7	1194	190 \pm 15.4
% narrow hyphae	3 stems	30.5–39.1 (range)	3 stems	28.8–41.5 (range)
Packing density (expressed as % of transect area occupied by free space)	18	0.44 \pm 0.02	18	0.47 \pm 0.28
Cell length of inflated hyphae in upper and lower sides of three different stems	34	542 \pm 35.0	34	116 \pm 7.4
	34	534 \pm 37.9	34	170 \pm 12.4
	34	698 \pm 50.6	34	107 \pm 5.7

Stems were fixed 3 h after reorientation and embedded in glycol methacrylate. Sections were cut 2 to 4 μm thick with glass knives on an ultra-microtome and observed by light microscopy, video images being processed by computer-aided image analysis. Means represent values of 6 replicate sections, three stems were measured, the values shown represent the upper and lower regions of corresponding stems. Data from Greening & Moore (1996) and J. P. Greening (unpublished).

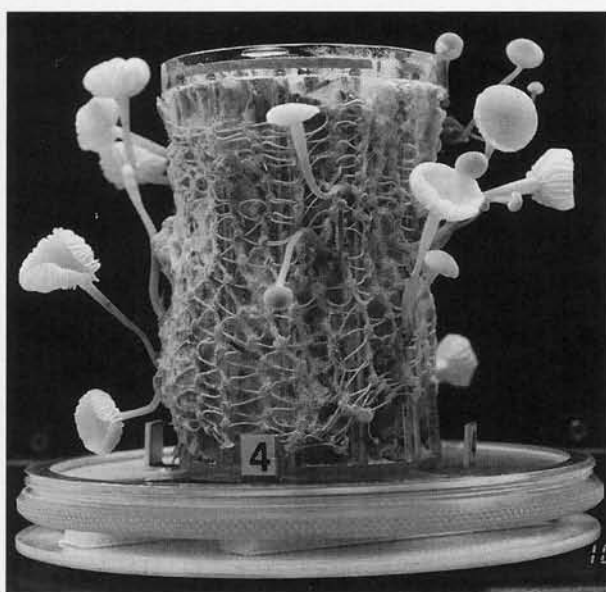


Fig. 12. Top: gravitropic orientation of *Flammulina velutipes* fruit bodies grown for 5 days on Earth as a reference for experiments carried out in orbit. Bottom: a similar culture grown for 165 h in microgravity in Spacelab during the D-2 mission (26 April to 6 May, 1993), note the random orientation of the fruit bodies. From Kern & Hock (1996).

shown the presentation time to be 7 minutes and that the extent of the gravitropic response, measured as the angle of the stem apex at maximum curvature, was dependent upon the gravitational exposure time (Hatton & Moore, 1994). The reaction time did not depend on exposure time and exposures were not additive. An unexpected feature of clinostat experiments with *C. cinereus* fruit bodies is that stems placed on the clinostat after various gravity exposure times 'relaxed' by 5° immediately after reaching maximum curvature. This relaxation process has parallels in the 'tropic reversal' reported in *Phycomyces* sporangiophores (Galland & Russo, 1985), 'springback' in plant roots (Leopold & Wettlaufer, 1989), 'spatial memory' of maize coleoptiles (Nick, Sailer & Schäfer, 1990) and 'autotropisms' of seedlings (Heathcote, 1987;

Chapman *et al.*, 1994). Perhaps in all these otherwise very different cases, gravitropic bending has an initial, reversible, phase of plastic bending which is followed by a 'fixation' phase if the tropic stimulus has been maintained but which relaxes if the tropic stimulus has been removed. Overall, clinostat experiments reveal:

- that the gravitropic impulse is an 'all-or-nothing' signal in *C. cinereus*;
- that sustained exposure to the unidirectional gravity vector is necessary for the normal gravitropic response;
- that perception and response probably occur in the same tissue regions;
- that gravitropic bending is a two-stage process with an initial, reversible, phase of plastic bending.

As explained above, perception of gravity is potentially quite different from perception of other stimuli because there is no gravitational gradient. The perception system must depend on gravity establishing an asymmetric distribution of mass within the organism. Gravity-induced asymmetries might include (i) differences in hydrostatic pressure between 'top' and 'bottom' due to different mass distribution; (ii) differences in forces of compression or extension between the 'top' and 'bottom' of rigid structures due to different mass distribution; (iii) changes caused by movement of extracellular or intracellular structures relative to immobile parts of the cell or tissue.

Reaction to changed mass distribution seems to be excluded by experiments in which lateral loads were applied to vertical stems of *C. cinereus* which were secured at their base. No reaction at all was obtained until the stem was loaded with enough weight to bend it sufficiently from the vertical to trigger the gravitropic response. This required application of weights between 10 and 20 g, depending upon the stature of the specimen. Such loading was followed by an apparently normal gravitropic reaction. The stem apex returned to the vertical and vertical growth was sustained, despite the continued 10–20 g lateral load (Greening *et al.*, 1993). Detection of altered distribution of extracellular mass and/or mechanical stress is unlikely to be a component of the control of gravitropic bending in *C. cinereus* stems. Reaction kinetics were unaffected by submergence of *C. cinereus* stems in water, which again indicates that mechanical processes do not contribute to control of gravitropism (Kher *et al.*, 1992), but submerged stems bent first at the base rather than at the apex.

This last observation was interpreted as an indication that translocation of the regulatory signal(s) was altered under water; perhaps the putative growth factor is diluted or dispersed. The view that water immersion disperses a growth control substance is endorsed by comparable experiments with *F. velutipes* (Haindl & Monzer, 1994). Immersion of *Flammulina* explants in water caused almost complete loss of gravitropic curvature without serious adverse effect on elongation. Addition of a 400 mg weight to compensate for buoyancy did not restore gravitropism, but even less curvature was seen in stems split longitudinally. The favoured conclusion is that water immersion dissipates a chemical signal which normally diffuses through the extracellular spaces to control gravitropic curvature (Haindl & Monzer, 1994). Longitudinally split *C. cinereus* stems react identically to *F. velutipes*; the split

segments do not bend gravitropically under water even after 6 h (L. Novak Frazer, unpublished).

The reactions of intact stems of *C. cinereus* and *F. velutipes* seem to be different under water but are the same when longitudinally split stems are submerged. Reactions of the two organisms are not so different, therefore; perceived differences can be explained simply by the different mode and/or timing of the response in each. The difference in the amount of tissue which reacts to gravity (more than half the *Coprinus* stem, only a few mm at the apex of the *Flammulina* stem; see 'apex excision' experiments described above) could account for their different reactions to water immersion when intact. Signalling chemicals are presumably much more readily dissipated in *Flammulina* because they are produced in a more localised region than in *Coprinus*, and need to translocate some distance (and/or persist for a longer time) to exert their effect. When split longitudinally, the growth factors are dissipated easily in both organisms and the stems of both fail to react gravitropically.

Perception. If extracellular mechanical events are excluded, attention is concentrated on the intracellular environment as a site for the gravity perception mechanism. Very few observations have been recorded in the mycological literature. Borriss (1934) described '... particles of plasma, ... situated in about the centre of the cell...' in *Coprinus lagopus* (= *C. cinereus*?). Gooday (1985) states that 'The cytology of geotropism of stems of *Coprinus cinereus* was investigated by the late G. H. Banbury (personal communication) ... light and electron microscopy ... showed that when horizontal, the distribution of cell contents was displaced so that the vacuole occupied most of the upper part, and the cytoplasm ... the lower part.' These are the only reports of any cytological examination of gravitropism in mushrooms prior to 1990 and we have not been able to substantiate either of them despite careful examination of many sections of *C. cinereus*. As we have indicated above, the problem with *Coprinus* is that of identifying meaningful intracellular differences among the enormous number of cell profiles seen in stem sections and within the large volume of tissue which might be involved. The situation is different in *F. velutipes* where attention can be focused on the transition zone.

Gravity perception in *F. velutipes* was found to be related to the actin cytoskeleton. Monzer & Haindl (1994) and Monzer (1995) demonstrated that the actin-depolymerising drug cytochalasin D caused up to 80% loss of graviresponse at a concentration of 10^{-4} M. Actin microfilaments in the gravisensitive tissue were positionally correlated with the nuclei (Fig. 13), with spindle shaped actin aggregates around individual nuclei, whereas microtubules were mainly oriented longitudinally and located at the periphery of the cell. Nuclear motility in living hyphae was reduced by treatment with 10^{-4} M cytochalasin D and the agent dissipated the actin microfilament 'cage' which normally surrounds the nuclei of stem cells.

These results immediately suggest a plausible mechanism for gravity perception in agarics (Monzer, 1995), with the nuclei acting as statoliths and exerting tension on the actin filament system. This proposal is in accord with the most recent

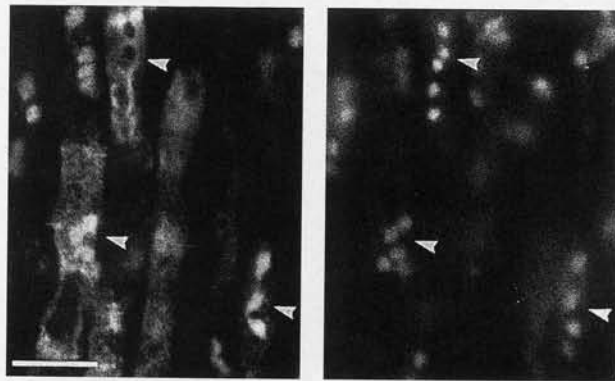


Fig. 13. Immunolocalisation of F-actin (left hand panel) and bisbenzimidazole staining of nuclei (right hand panel) in the transition zone of the stem of *Flammulina velutipes*. Note that actin fluorescence is concentrated in spindle-shaped aggregates enclosing the nuclei; bar = 10 μ m. From Monzer (1995).

interpretations that plant gravisensing depends on statolith interaction with the actin microfilaments of the cytoskeleton (Sievers *et al.*, 1991), and that animal cells also use the cytoskeleton to detect mechanical stress (Ingber, 1993; Wang *et al.*, 1993).

Similar conclusions have been reached from inhibition experiments with *C. cinereus*. Treatment of *C. cinereus* stems with cytochalasin B or D caused diminished gravitropic bending and significantly delayed the response, a result not observed with any other inhibitors. Treatment of *C. cinereus* stems with agents disrupting proton gradients, inhibitors of stretch-activated ion channels or inhibitors of microtubule polymerisation had no effect on gravitropism (Novak Frazer & Moore, 1996). This last result is particularly significant in view of the important part played by microtubules in determining the positioning of nuclei in cells of the vegetative dikaryon of *C. cinereus* (Kamada, Hirai & Fujii, 1993). Our observations indicate that even if microtubules have a role in determining the position of nuclei in stem cells, it is the cytoskeletal microfilaments which are involved directly in gravity perception.

Signal transduction. In plants, control of cell calcium accumulation has been implicated in the gravitropic mechanism. We have examined the role of Ca^{2+} in *Coprinus* using inhibitors of calcium signalling at concentrations and using treatment regimes which are known to eliminate gravitropism and other tropisms in plant organs. We recognise the hazards of this approach, key among which are the lack of knowledge of the specificity (and, even, identity) of the targets of these inhibitors in fungi and the difficulties of guaranteeing adequate penetration and delivery to their target. Such problems must be kept in mind when interpreting these results.

The experiments were done by exposing *C. cinereus* stems to a Ca^{2+} channel blocker, verapamil [1 mM]; a Ca^{2+} ionophore, A23187 [1 μ M]; a Ca^{2+} chelator, BAPTA [10 mM]; or calmidazolium [100 μ M], an inhibitor of calmodulin-mediated Ca^{2+} uptake (Novak Frazer & Moore, 1993). These inhibitors had no effect on gravity perception but the ionophore (which enhanced stem extension), the chelator and calmidazolium (which had no effect on stem extension) all significantly

diminished the gravitropic response. The ionophore, A23187, enhanced stem extension growth rate (by 30%) but decreased the rate of gravitropic curvature by 43%. This suggests that tropic bending may be different from normal (vertical) extension growth, not a simple redistribution of normal growth potential of the stem. A similar conclusion was reached from the cell size measurements described above.

All of the calcium signalling inhibitors affected gravitropic curvature; but curvature *did* occur in their presence. Thus, it is concluded that Ca^{2+} is not involved in gravity perception, but may be part of the signal transduction process, regulating gravitropic growth differentials via accumulation of Ca^{2+} within a membrane-bound compartment. The best evidence for the role of Ca^{2+} in gravitropism would be derived from direct observation of Ca^{2+} concentrations in gravitropically-stimulated material. The problem with such an approach is that gravitropism, as shown above, is a phenomenon that involves a community of cells which are difficult to visualise with any detail or clarity using standard microscopic techniques. Confocal microscopy and Ca^{2+} -binding fluorescent dyes are now being applied successfully to filamentous hyphae (Knight, Trewavas & Read, 1993) so it is to be hoped that these techniques can be adapted for use with multihyphal systems in the near future.

Early cellular responses. The earliest response visible in electron micrographs of the transition zone of *F. velutipes* is a changed distribution of vesicles or microvacuoles (Kern, 1994; Kern & Hock, 1994, 1996). However, this is not evident as vacuole or droplet flotation, a mechanism suggested to perceive change in the gravity vector in *Phycomyces* sporangiophores (Dennison & Shropshire, 1984). Rather, within about 1 h after orientation to the horizontal, hyphae of the upper side of the transition zone have only a few microvacuoles visible, whilst hyphae of the lower side exhibit large accumulations of microvacuoles or vesicles (Fig. 14).

The redistribution of these microvacuoles after reorientation to the horizontal is a very obvious feature, but their function is unknown. Some, at least, tend to cluster around the main cell vacuole and may fuse with it, so they could be contributing to vacuolar enlargement as the cell volume increases to drive elongation of the lower side of the stem. Provision of cell wall synthetic capacity is another obvious potential function, but as yet we have no evidence on these points other than electron micrographs (Kern, 1994).

Although these microvacuole/vesicle redistributions are evident extremely soon after reorientation, they must still be part of the response apparatus, rather than the perception mechanism.

Conclusions

As indicated above, a plausible mechanism for gravity perception in agarics is that nuclei act as statoliths, their displacement within the cytoskeleton surrounding them being communicated by some of those actin microfilaments to the endomembrane system, and maybe by similar means directly to the plasma membrane (Kern, 1994; Kern & Hock, 1994, 1996; Monzer, 1995). An important point is that the initial

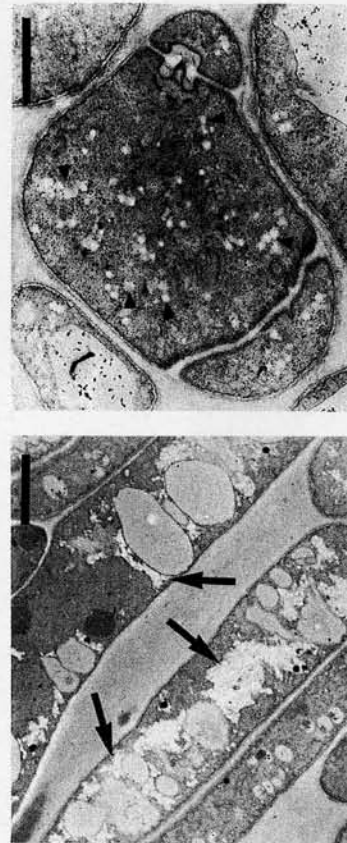


Fig. 14. TEM sections of hyphae in the transition zone of *Flammulina velutipes* fruit bodies. Top: transverse section of a hypha on the upper side of a horizontal stem fixed 1 h after reorientation to the horizontal. Only a few 'light vesicles' (arrow heads) can be observed. Bar = 1 μ m. Bottom: longitudinal section of hyphae in the lower side of the horizontal stem (again, 1 h after reorientation), note accumulation of light vesicles surrounding the main vacuoles (arrows). Bar = 2 μ m. From Kern & Hock (1996).

event does not need to be a major displacement. The whole point of having a signal transduction chain is to provide for amplification of the primary input. The work described above has been based on standard assays in which stems were placed horizontal; i.e. at 90° to normal orientation. However, in nature the system must be constantly monitoring orientation and correcting small disturbances to maintain vertical growth. In these circumstances statolith movement may be extremely restricted.

A possible model is illustrated in Fig. 15. We would envisage that statolith-activation of microfilament membrane connections could prompt export of a signalling molecule through the plasma membrane, and/or positional-dependent amplification of vesicle/microvacuole production by the endomembrane systems. Though very speculative, this model may describe the initial events in an integrated process which has been summarised in flow-chart form (Fig. 16; Moore *et al.*, 1994). Taken together, Figs 15 (derived largely from cytological and cytochemical observations of *Flammulina*) and 16 (derived largely from kinetic and morphometric observations of *Coprinus*) provide what is currently the most succinct overview of agaric gravitational biology. Clearly, it is our view that the two sets of data *can* be merged to create a

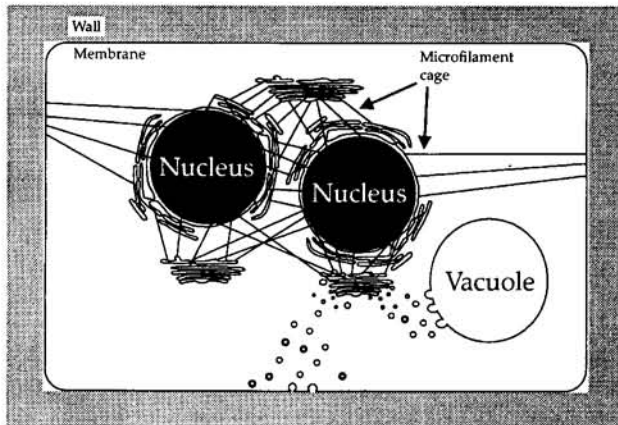
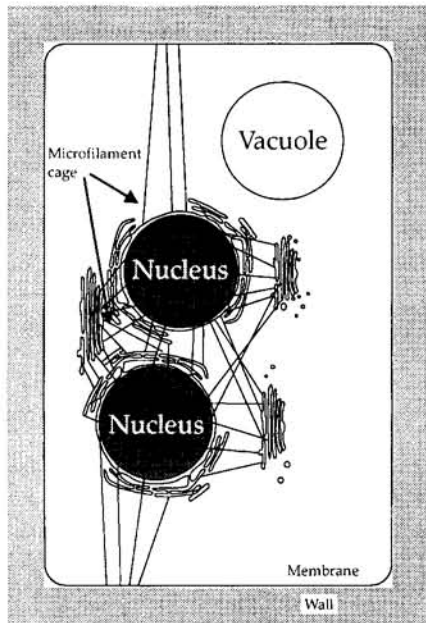


Fig. 15. Diagrammatic interpretation of gravity perception in agarics. Vertical (top) and horizontal (bottom) orientation of a 'typical' cell. Movement of nuclei within the 'cage' of actin microfilaments is assumed to stress connections to the endomembrane system and generate directional production of vesicles and/or microvacuoles. Some of these seem to be targeted to the vacuole(s) and others are targeted to the plasma membrane to externalise their contents. Materials exported immediately disorientation is perceived are probably the growth factors which co-ordinate and regulate neighbouring hyphae. Subsequently, components required for wall growth and vacuole enlargement will be exported.

unified overview. Differences between the two organisms we have studied must reflect contrasts in their ecology and habit; *C. cinereus* fruit bodies being delicate, short-lived and produced singly or in small numbers, compared to the more persistent, usually clustered, fruit bodies of *F. velutipes* which are slow-growing and able to withstand the extremes of winter conditions. However, the only major dissimilarities we encountered were that much more tissue was involved in the gravitropic reaction in *C. cinereus* and this species exhibited a much higher rate of response. The latter seems likely to be a consequence of the former, and other minor differences (e.g. in the mechanics of bending (Figs 6 & 7, above), or in reaction to water immersion) might also be related simply to the

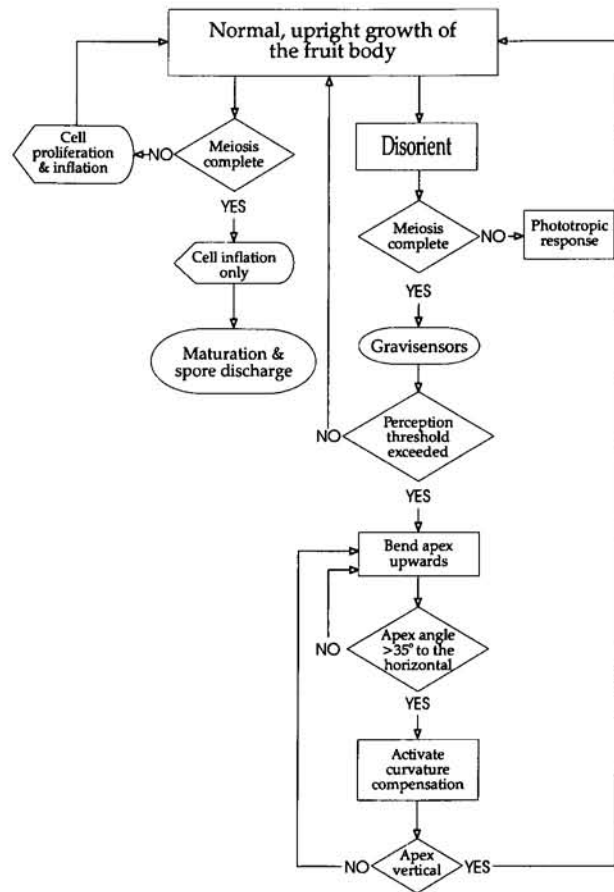


Fig. 16. Flow-chart describing the gravitropic response in stems of *Coprinus cinereus*. Redrawn after Moore *et al.* (1994).

quantity of tissue which is able to react and consequential mechanical or concentration effects.

Many of the steps in Fig. 16 require hypha-to-hypha signalling. The information we have about hormones or growth factors in agarics is sparse and unsatisfactory (discussion and references in Moore, 1991a). Many experiments have been reported which imply the presence of hormones (e.g. Hagimoto & Konishi, 1959, 1960; Hagimoto, 1963; Gruen, 1963, 1969, 1982), but none of the putative growth control chemicals have been isolated. Indeed, most of the published experiments are fundamentally flawed by allowing cut tissues to disgorge their innumerable contents into agar blocks which are themselves made from plant extracts (malt agar, potato-dextrose agar, etc.), the 'extracts' then being subjected to bioassay over unrealistic time scales (several days being most usual). Lilian Hawker's statement of 1950 is, unfortunately, still true: 'It is desirable that research should be directed towards an interpretation of tropisms in fungi based on the study of growth-regulators. At present nothing is known of any mechanism in fungi comparable to the redistribution of auxins in the higher plants.'

Jeffreys & Greulach (1956) concluded that their '... results suggest, not only that auxin is not involved, but also that no other hormone is involved. It seems likely that each hyphal strand responds individually to environmental factors. Because the strands are aggregated, this results in a unit action by the stipe'. Banbury (1962) and Gorovoj *et al.* (1989) also make

statements which seem to show their conviction that the apparently coordinated expression of gravitropic response is in truth a common but independent response by the individual component hyphae of the structure concerned. We do not agree with these views. We have demonstrated concerted action in longitudinally dissected stems of *F. velutipes* (Haindl & Monzer, 1994) and *C. cinereus* (L. Novak Frazer, unpublished) and have observed cytological responses which differ between the upper and lower parts of horizontal stems in *F. velutipes* (Kern, 1994; Kern & Hock, 1994, 1996) and *C. cinereus* (Greening & Moore, 1996; J. P. Greening, unpublished). In a horizontal stem ALL hyphae experience the same gravitational field. Having received the same gravitational impulse, the hyphae which make up a stem cannot show a spatially co-ordinated differential response unless some additional component (hormone, growth factor) orchestrates it. Thus, beyond the problem of the mechanism of perception, there is the problem of coordination: the nature of the co-ordinating signal, its transduction, how the gradient across the diameter of the stem is achieved and the manner in which the target hyphae are distinguished.

This putative chemical signal may be constitutive in the extracellular matrix of the stem. Perhaps a general growth hormone secreted into the extracellular matrix to promote normal elongation. Differential elongation growth (to cause tropic bending of the stem) might then result from a differential distribution of hormone receptors on the plasma membrane of cells across the diameter of the stem. A credible sequence of events could be: nuclei displaced by disorientation → compression/tension on actin microfilaments → activation of plasma membrane receptors for extracellular signal molecules on lower side of hyphae → binding of signal molecules (mostly on 'lower' side) causing an increase in cytoplasmic Ca^{2+} (either from extracellular matrix or intracellular Ca^{2+} -rich compartment) → sequestration of Ca^{2+} in vacuole or endomembrane system → export of wall-building materials in microvesicles to the plasma membrane → increase in wall extension.

Alternatively, detection of disorientation might establish a gradient of the growth factor itself across the stem diameter (either by *de novo* synthesis or selective destruction/inactivation of an all-pervading growth hormone). A possible sequence of events in this case might be: nuclei displaced → compression/tension on actin microfilaments → activation of endomembranes and plasma membrane causing production and secretion of extracellular signal on lower side of hyphae → establishment of gradient of extracellular signal molecules → differential binding of extracellular signal to plasma membrane receptors causing an increase in cytoplasmic Ca^{2+} (either from extracellular matrix or intracellular Ca^{2+} -rich compartment) → sequestration of Ca^{2+} in vacuole or endomembrane system → export of wall-building materials in microvesicles to the plasma membrane (mostly on 'lower' side) → increase in wall extension. In view of the indications in some of our data that bending growth is different from normal (vertical) extension growth, we favour this speculation, rather than that outlined in the previous paragraph.

Proof of our belief in the involvement of 'hormones', and of the other components of our model of the agaric

gravimorphogenetic system (Figs 15 and 16) will depend on further basic research on relationships between nucleus, cytoskeleton, membrane complexes, vacuolar systems and growth factors. This will require major efforts, but study of fungal gravitational biology is far from esoteric. It will make a significant contribution to improved understanding of the workings of the fungal cell both individually and in organised communities.

We express our thanks to the many people who have contributed to the progress of this research, but we offer special thanks to Kerstin Dressel, Eva Haindl, Jason Hatton, Judith Holden and Kavita Kher. The work has been supported by the British Mycological Society, DARA (Deutsche Agentur für Raumfahrtangelegenheiten) within the D-2 Spacelab mission programme, Deutsche Forschungsgemeinschaft, Hulme Hall Trust, the Natural Sciences and Engineering Research Council of Canada, Nuffield Foundation, the Science and Engineering Research Council, and the University of Manchester Research Support Fund. This review was first presented as a spoken paper at the BMS meeting *Genetics and Cellular Biology of Basidiomycetes III* held at King's College London 17–19 June, 1995. We thank Professor D. A. Wood and Dr C. F. Thurston for the invitation to contribute to that meeting.

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