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THE ROLES OF MECHANICAL STRESS AND ETHYLENE IN CLINOSTAT-  
INDUCED LEAF EPINASTY AND GRAVITROPIC RESPONSE OF DICOT SHOOTS

by

Raymond M. Wheeler

A dissertation submitted in partial fulfillment  
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Plant Science Ecology

Approved:

UTAH STATE UNIVERSITY  
Logan, Utah

1981



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Raymond M. Wheeler

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## ABSTRACT

The Roles of Mechanical Stress and Ethylene in Clinostat-  
Induced Leaf Epinasty and Gravitropic Response of Dicot Shoots

by

Raymond M. Wheeler, Doctor of Philosophy

Utah State University, 1981

Major Professor: Dr. Frank B. Salisbury  
Department: Plant Science

Aminoethoxyvinyl glycine (AVG) and silver thiosulfate, antagonists of ethylene biosynthesis and action in plants, both delayed onset of leaf epinasty in *Xanthium strumarium* L. (cocklebur) plants rotated on horizontal clinostats. *Xanthium* plants mechanically stressed by continuous horizontal or vertical shaking, or continuous twisting back and forth, did not develop any significant epinasty, while plants inverted every 20 minutes (upside down half the time) did develop epinasty. From this it appears that clinostat-induced epinasty is a result of gravity compensation rather than mechanical stress.

Treatment of *Xanthium*, *Lycopersicon esculentum* Mill. (tomato), and *Ricinus communis* L. (castor bean) plants with inhibitors of ethylene biosynthesis, AVG and cobaltous ion, and inhibitors of ethylene action, silver ion and carbon dioxide, significantly delayed stem gravitropic response times. AVG and silver were dependably effective in delaying gravitropism, while carbon dioxide and cobalt were less effective.

Unilateral application of ethephon solution (1%) to the upper 10 centimeters of tomato stems, caused stems to deflect up to 80° toward



the side of application after 24 hours on a clinostat, while unilateral application of indole-3-acetic acid in lanolin paste (1%) to tomato and cocklebur stems caused up to 250° and 200° bending respectively, away from the side of application after 24 hours on a clinostat.

Cocklebur stems that are restricted from bending after placing them horizontally store bending energy as seen from the springing upward that occurs when the stems are released (up to an average of 150° after 40 hours of restriction). Most of these stems also showed a stored stimulus of gravitropic bending, continuing to bend for several hours after release and being placed upright, before straightening.

(196 pages)

## INTRODUCTION

Three years ago I concluded research for a master's degree dealing with responses of greenhouse tomato plants to mechanical stress. Since then, I have continued in the same laboratory under Dr. Frank B. Salisbury, my major professor. Our curiosity continued in the area of mechanical stress in plants, but we have since extended these interests, particularly with regard to a role for mechanical stress in plants rotated on horizontal clinostats.

Clinostats are devices capable of rotating plants slowly, usually about a horizontal axis, which is often the stem-root axis. If a potted plant is attached to the wheel of a clinostat and the device is turned so that the plant is in a horizontal position, slow constant rotation of the plant can be used to negate the unilateral influence of gravity. Gravity then affects the plant from all directions, and its total vectoral influence is zero. This is the basis for using clinostats as zero-gravity simulators. Hypogravity (0-1 g) can be simulated by rotating around an axis at some angle to the horizontal. This has also been called gravity compensation or gravity nullification (Brown et al., 1976 a).

Tibbitts and Hertzberg published a paper in 1978 discussing the mechanical effects of clinostating on *Tagetes patula* (marigold) plants. With the aid of several drawings, they showed how the leaves of these marigolds were continuously displaced during the rotation cycle; i.e., they "flopped" back and forth. The weight stress of the leaves caused continuously shifting strains in the petioles and blades during the rotation.

Clinostating typically causes leaf epinasty (a downward bending) in plants (Pfeffer, 1906), and such responses are known to be mediated by the plant hormone ethylene (Leather et al., 1972). Similarly, mechanical stress responses in plants are thought to be mediated by ethylene (Jaffe and Biro, 1979), and mechanical stress has on occasion been shown to cause mild leaf epinasty (Mitchell et al., 1975); therefore it seemed likely that mechanical stresses in the leaves of clinostated plants could cause ethylene evolution, which in turn might produce epinasty.

Over the past three years this laboratory has attempted to assess the role of mechanical stress in clinostat responses in plants, particularly leaf epinasty, through two basic experimental approaches. First, can mechanical stresses comparable to those of clinostating also produce leaf epinasty? And second, can elimination of mechanical stress prevent epinasty in clinostated plants?

Since clinostats are thought to negate gravity's influence, a logical follow-up of the preceeding studies would be an attempt to gravity compensate plants by some other means, while minimizing any mechanical stimulation. Then one could determine if leaf epinasty is truly a response to clinostat negation of gravitational effects. However, alternate methods available for gravity compensation are very limited. Satellite free-fall would be the best condition for such studies, but opportunities for such experiments have been available only rarely in the past. (Hopefully this will change soon with Space Shuttle flights.) Because of this limitation, periodically inverting plants was the only other method we employed for attempting gravity compensation.

Another area that I investigated involved the participation of the



plant hormone ethylene, both in responses to mechanical stresses including clinostating and in the general gravity responses of plant shoots. If clinostats were indeed found to cause the ethylene mediated epinasty through altering or disruption of gravity perception in plants, then these would seem appropriate adjunct studies.

The results of such investigations could provide evidence one way or the other on the reliability of clinostats as hypogravity simulators and shed further light on the complex and still unsolved mechanisms of gravitropic response in plants.

## LITERATURE REVIEW

## Gravitropism

Attempts to understand the response of plants to gravity continue to be one of the longest, on-going studies in the field of plant physiology. Frank introduced the word "geotropism" in 1868 (see Rawitscher, 1937), with early investigations being undertaken by such people as Ciesielski, Sachs, Pfeffer, Noll, Czapek, and Charles and Francies Darwin (see Rawitscher, 1937; Larsen, 1962). Several comprehensive reviews have been written on plant response to gravity in recent years, and I will draw largely on these papers for this literature review, restricting references to higher plants. These summaries include Larsen (1962), Anker (1962), Audus (1962), Hoshizaki (1973), Juniper (1976), Audus (1979), Wilkins (1966 and 1979), and Volkmann and Sievers (1979).

The following are terms commonly used in literature concerned with plant gravitropism: The word *gravitropism* is now preferred to *geotropism* in order to more clearly relate the growth response to gravitational force and not just the directed growth response to an acceleration of  $9.8 \text{ m s}^{-2}$  at our planet's surface (Sievers and Volkmann, 1979). The *liminal* direction is the normal orientation of an organ in a gravitational field; *orthotropic* growth describes an alignment of plant organs parallel to gravitational field lines (e.g., main shoot and tap root); *plagiotropic* (plagio-gravitropic growth describes all planes of growth other than parallel to the gravitational field lines (e.g., branches, leaves, and secondary roots); and *diagravitropic* growth is a special form of plagiotropic growth that is parallel to the surface of the ground or perpendicular to the gravitational field lines (e.g., rhizomes or stolons). Hori-



zontal growth has also been referred to as horizontal nutation, transverse gravitropism, or agravitropism (see: Larsen, 1962; Hoshizaki, 1973; and Abeles, 1973).

### Perception of gravity

Perception of gravity is generally looked upon as the first step in the sequence of events as plants reorient themselves with respect to gravity. This is followed by a transmission or transduction stem, with the reaction or response being the last event in the sequence (Hoshizaki, 1973; Volkmann and Sievers, 1979). The term (*minimum*) *presentation time* is commonly used in discussion of gravity perception in plants and refer to the shortest stimulation time sufficient to elicit a visible response (Brain, 1926). *Latent time* is the period from the beginning of the stimulation to the first visible response (Hawker, 1932).

Knight (1806) was the first to show the similarities between the effects of centrifugal force and gravity (see also Westing, 1964), but the force perception mechanisms were not well understood for many years after this. Noll (1892, 1900) first suggested the idea of statoliths or gravity perceiving particles in crustaceans. As the organism reoriented itself, the statoliths would settle on different parts of membranes of the perceiving cells or tissues (Rawitscher, 1937). Nemec (1901) and Haberlandt (1902) independently postulated that starch granules might serve as statoliths in plants. Starch grains are known to be contained within amyloplasts (Juniper, 1976), and it is now thought that amyloplasts serve as the statoliths proposed by Nemec and Haberlandt. Interestingly, barium sulfate crystals have recently been shown to act as statoliths in the large single-celled alga *Chara* (Sievers and Volkmann, 1979).

The amyloplast statolith theory (see Darwin, 1903) has been put to the test through the years and still appears to be the most popular theory for gravity perception in plants (Juniper, 1976), with most of the favorable evidence coming from experiments with roots and the few contrary arguments from shoots (Hoshizaki, 1973).

Tests for the starch-statoliths have involved direct observation, removing of perceptive tissue, chemical removal of starch, and mutant strains of plants without starch (Volkman and Sievers, 1979).

Hawker (1932) observed a close correlation between the amount of statenchyma (starch statolith containing tissue) in roots, hypocotyls, and epicotyls, and gravitropic sensitivity. But, even more convincing evidence for the statolith theory has come from root cap studies. The root cap clearly appears to be the graviperception center in roots. The cells of the root cap in *Zea mays* seedlings contain numerous amyloplasts, while the meristematic and elongation tissues do not, although these tissues appear to be the sites of reaction (Juniper, 1976). Removal of the caps in *Zea* seedlings destroys all gravitational response in the roots, without affecting elongation (Juniper et al., 1966). But Pickard and Thimann (1966) observed delayed response in *Avena* coleoptiles persisting after starch had apparently been removed in the tissue following treatment with a gibberellin-kinetin mixture (sulfur dioxide, cold, and dark treatments have also been used to remove a plant's starch, but with mixed results as to effects upon gravitropism; see Haberlandt, 1902 and Wilkins, 1966). However, Iversen repeated these experiments (1969, 1974) and contends, after close scrutiny with the electron microscope, that Pickard and Thimann probably did not dissolve all the amyloplast starch.

With further refinement of their techniques, however, Iversen was able to totally rid the amyloplasts of starch after which the gravitropic response in the treated plants was destroyed.

The role of amyloplasts as statoliths has also been examined in tissues around the nodes (i.e., the leaf sheath base or pulvinus) of several grass species. The amyloplasts of *Avena* nodes are confined to parenchyma cells around vascular bundles and have been observed sedimenting toward gravity within 10-15 minutes of gravistimulation (Dayanandan et al., 1976). Osborne and Wright (1977) have observed amyloplasts settling in highly vacuolate statocyte cells within 2.5 min after inverting *Echinochloa colonum* plants.

Griffiths and Audus (1964) observed some displacement of dictyosomes in gravistimulated root-tip cells of *Vicia faba*, but concluded that amyloplasts were the only organelles to show marked sedimentation. Shen-Miller and colleagues have examined the settling movements of mitochondria, golgi apparatus, and dictyosomes, and observed increased dictyosome number and activity in the lower region of cells in the apex of gravistimulated oat coleoptiles; they concluded that dictyosomes or at least an interaction of dictyosomes and amyloplasts may be the gravity perceiving mechanism (Shen-Miller and Miller, 1972a, b; Shen-Miller and Hinchman, 1974). However, Volkmann and Sievers (1979) reported the densities of amyloplasts, mitochondria, and dictyosomes as 1.5, 1.2, and 1.125 g.cm<sup>-3</sup>, respectively, and concluded that neither mitochondria nor dictyosomes could be the primary sedimenting particles, because of their lower densities. The possibility of dictyosomes being the major sedimenting particles has since fallen from favor, and their apparent increase on



the bottom side of cells with gravitational stimulation might be an early step in the response mechanism (Audus, 1979).

Thus, the only serious evidence against the statolith theory, Pickard and Thimann's experiments, has been refuted (Iversen, 1969, 1974). Nonetheless, several discrepancies still exist with amyloplast-statolith perception of gravity. Organs such as the perianth of *Clivia nobilis* and the aerial roots of the epiphytic orchid *Laelia anceps*, both respond to gravity, but no amyloplasts have been observed in these plant tissues (Wilkins, 1966). Other evidence against the statolith theory comes from the works of Iwami and Masuda (1974) and Firn and Digby (1977, 1980), in which peeled segments of *Helianthus* and *Curcubita* hypocotyls did not respond to gravity, pointing to the peripheral layers as the perceptive tissue in these organs. Since sedimenting amyloplasts have not yet been found in the epidermis of these hypocotyls, some other perception system could be at work (Audus, 1979; Wilkins, 1979). But removal of the epidermis could upset steps in the response other than perception.

#### Mechanisms of action

One of the main questions in understanding gravity responses in plants is the transformation of the physical signal caused by the gravity into a physiological signal; the problem is particularly unique with gravity, which acts on all cells the same way (Volkman and Sievers, 1979). Juniper (1976) lists eight possible mechanisms of action for statolith system gravity perception: (1) motion of the statolith within the statocyte (perceiving cell); (2) asymmetrical distribution of, or pressure on endoplasmic reticulum membranes by the statoliths; (3) displacement of weight within the root cap; (4) displacement of and/or asymmetrical function of the dictyosomes; (5) asymmetrical pressure on

the plasmalemma; (6) asymmetrical distribution of enzyme activities on the statocytes; (7) statoliths as transport barriers; and (8) interaction between statoliths and plasmodesmata operating a valve-like mechanism of the endoplasmic reticulum and the plasmodesmata for some chemical signal or hormone flow.

Juniper (1976) favors the last of these mechanisms in which the ER membranes are connected between adjacent cells by desmotubules. Then, as amyloplasts would settle on ER membranes, they would close the plasmodesmata below them to intercellular exchange. Audus (1979) has tested this model with computer simulation and found that it works well in theory with one exception: Juniper's model cannot account for theoretical hormone flows if the plants are inverted prior to being placed horizontally, thereby forcing the amyloplasts to the proximal end of the cells (normally the upward end in roots). If such inverted roots are turned horizontally, they grow downward, but the model, according to Iversen (1974) and Audus (1979), predicts a reverse curvature.

Volkman and Sievers (1979) strongly favor an ER-amyloplast pressure interaction as a mechanism for gravitational response (see also Juniper and French, 1973). They contend that such a mechanism is the only one able to account for presentation times as low as 12 s in *Lepidium* roots (see also Johnsson and Pickard, 1979). Amyloplast movement within cytoplasm is far too slow to account for such observations, if settling upon the plasmalemma or even partial transit within the statocyte is the mechanism of action for statoliths. When *Lepidium* roots are stressed with treatments known to upset gravitational equilibrium such as horizontal clinostating, electric fields, centrifugation, ABA treatment, or forced to grow upward in capillary tubes, aside from the amyloplasts being

distributed throughout cells after clinostating, the only consistent difference caused by these treatments is a redistribution of the normally distally located endoplasmic reticulum membranes. Such results suggest a crucial role for these endoplasmic reticulum cisternae in gravity perception (Volkmann and Sievers, 1979).

#### Links between graviperception and responses

Cholodny (1927) and Went (1928) independently proposed that auxin emanating from the apex of a plant organ moved basipetally (away from the apex) toward the elongating tissue and deflected laterally toward the lower side of the organ, depending on its inclination from the vertical (see also Went and Thimann, 1937). In shoots, the excess auxin would cause more elongation on the lower sides, thereby causing the organ to curve upward; whereas in roots, the excess auxin would inhibit elongation of cells on the lower side and cause the root to curve downward (Wilkins, 1979).

Dolk's experiments (1930), using agar receiver blocks and excised tips, indicated that auxin did indeed move basipetally from the apices of *Avena* coleoptiles. His works also provided strong evidence for lateral transport of auxin to the lower sides of the coleoptiles, although this process was not demonstrated unequivocally until the labeling studies of Gillespie and Thimann (1963) and Goldsmith and Wilkins (1964), using  $^{14}\text{C}$ -IAA applied to *Avena* and *Zea* coleoptiles.

Brauner and Hager's paper in 1957 also indicated the essentiality of auxin in gravitropism in green dicot shoots and hypocotyls. They decapitated *Helianthus* hypocotyls and kept them dark for four days, in an attempt to deplete the plants of their auxin supply, after which they placed the "auxin-depleted" plants on their sides. No curvature resulted



from this treatment unless IAA was added to the hypocotyls stumps. This curvature developed even if the IAA were added 12 h after the plants had been turned upright, indicating a memory or "mneme" of the event (Brauner and Hager, 1957). Wilkins (1966) suggests three possible explanations for this observations; (1) a lateral movement of a cofactor of auxin; (2) direct sensitivity enhancement of the cells on the lower sides to auxin; (3) or induction of an auxin lateral transport system that persists even after the stems are placed upright.

However, Brauner and Hager's tests also pointed to some discrepancies in the Cholodny-Went theory for dicot shoots. If decapitated *Helianthus* hypocotyls were immediately placed on their sides, they would gravitrope nearly as fast as intact plants. These results were later confirmed by Iwami and Masuda (1974) and Digby and Firn (1979 b), and indicate that the apex does not necessarily serve as the source of auxin (or other hormone). Upon further analysis of others' results, Digby and Firn (1976) concluded that auxin movement was too slow and concentrations were too low to produce the observed responses in coleoptiles and dicot shoots. They found that by removing the peripheral layers of *Helianthus* hypocotyls, the gravitropic response could be destroyed (without affecting growth), thereby indicating an "on-site" perception and response to gravity.

Findings by Ganot and Reinhold (1970) indicated gravitropic curvature could be induced in dark-grown, auxin-starved *Helianthus* hypocotyl segments by placing them in low pH buffer solutions (e.g., pH 3.4), while added auxin had no effect. They suggested that a general physiological asymmetry may occur, rather than the traditionally proposed auxin or auxin cofactor asymmetry.

Differential translocation of substances other than IAA during tropic response has also been observed. Arslan-Cerim (1966) observed accumulation of  $^{45}\text{Ca}$  in the upper halves of gravistimulated *Helianthus* hypocotyls, and noted that this process was metabolically dependent. Goswami and Audus (1976) confirmed the accumulation of calcium on the convex sides of bending *Helianthus* and *Zea* seedlings, but concluded that this was neither the cause nor the result of curvature, and that it is some way linked to auxin gradients in the tissue. Electrical potentials can develop between the top and bottom of horizontal *Zea* and *Helianthus* seedlings (this has been called the "geoelectric effect"), but this too has been explained as a result of IAA gradients (Wilkins and Woodcock, 1965).

The involvement of other hormones in dicot shoot gravitropism has also weakened the Cholodny-Went theory. Railton and Phillips (1973) found four times more gibberellins in the lower (compared to upper) agar receiver blocks with excised *Zea* coleoptiles. Phillips and Hartung (1976) found that application of  $\text{GA}_3$  to decapitated *Helianthus* seedlings restored elongation to the internodal region, which was suppressed in controls, while application of IAA had no effect. Even when lateral transport has been observed for labeled IAA in dicot shoot segments, it was greatly reduced compared to coleoptile (Wilkins, 1979), and no lateral transport of externally applied IAA has been observed in dicot shoot internodes (Wilkins, 1979). Similarly, no lateral transport of IAA occurs in leaf sheath bases of gravistimulated grasses (Dayanandan et al., 1976; Osborne and Wright, 1977).

Zobel (1973, 1974) noted that ethylene might play an essential role in gravity responses of a mutant, diagravitropic (horizontal growing)



tomato. Applying  $5 \text{ nl l}^{-1}$  of ethylene to this prostrate growing tomato normalized upright growth, thereby implying a "positive" role for ethylene in normal shoot gravitropic response of this tomato. This was in contrast to earlier findings by Neljubov (1901) and Knight et al. (1910) with ethylene and its diagravitropic effect upon pea shoots (i.e., it induced prostrate growth in shoots). Zobel proposed that ethylene in very small quantities might be required for normal upright growth and that quantities used in previous experiments with other species were probably supraoptimal.

In roots, the Cholodny-Went theory predicts that inhibitory concentrations of auxin move basipetally from the apex into the elongation region, causing the root to curve toward the side of accumulation. Indeed, some inhibitor does appear to move from the cap into the expansion zone (Gibbons and Wilkins, 1970), but it cannot be auxin, because the transport of IAA is highly polarized toward root tips (i.e., acropetal), rather than away (Scott and Wilkins, 1968). Note though, that auxin does appear to be required in some fashion for normal root bending in some species, since Katekar (1976) and Katekar and Geissler (1977) have shown that auxin transport inhibitors such as 1-pyrenoylbenzoic acid (PBA) abolish root gravitropism in *Lepidium sativum*. The inhibitor involved does undergo lateral transport preferentially toward the lower sides (Shaw et al., 1973; Pilet, 1973, 1974), and at the present, it appears to be ABA or a structural analogue (Kundu and Audus, 1974; Pilet, 1974; Rivier et al., 1977). It therefore appears that the Cholodny-Went theory still holds in principal for roots, but was wrong with respect to the inhibitor involved. However, some arguments still continue to be raised. Most of these are based on the failures to observe

accelerated growth when the root caps have been removed (i.e., when there is no inhibitor moving into the elongation region; Firn and Digby, 1980).

### Links between gravitropism and ethylene

Neljubov (1901) first described the triple response in *Pisum* shoots, (i.e., shorter stems, thicker stems, and diageotropic or horizontal growth) to illuminating gas and particularly ethylene. The ethylene (at sufficiently high concentrations) did not simply destroy or disorient shoot growth, but completely redirected it in a horizontal fashion, regardless of the original orientation of the shoot (Neljubov, 1911; Abeles, 1973). That is, if etiolated pea seedlings were inverted or turned on their sides, and then subjected to ethylene gas, the shoots always grew perpendicular to the gravitational force vector (Abeles, 1973). Knight et al. (1910) and Knight and Crocker (1913) did further experiments, confirming the triple response observed by Neljubov in pea shoots.

Felix Rawitscher (1923) was one of the first to correlate leaf epinasty (downward leaf bending), a well-known symptom of treatment with ethylene gas (Doubt, 1917), to gravity, and developed the connection further in a gravitropism review in 1937. Crocker et al. (1932) noted that when tomato plants were inverted and exposed to ethylene, very little epinasty developed compared to upright, gassed plants. They also commented on the similarity between the night position of tomato leaves and ethylene-induced epinasty.

Denny (1936) showed that by placing tomatoes horizontally, epinasty would result in a potato leaf bioassay, much as the test leaves would respond to low concentrations of ethylene; in other words, the horizontal tomatoes appeared to evolve ethylene. Flowering in pineapples can be

controlled by ethylene, and van Overbeek and Cruzado (1948) were able to induce pineapple plants to flower by placing them horizontally, indicating that an ethylene producing mechanism was affected by the plant's position with respect to gravity. Abeles and Gahagan (1968) observed that *Coleus* placed on their sides showed accelerated leaf abscission and related this to the increased rate of ethylene production.

Horizontal clinostating studies have provided further clues linking ethylene and gravitropic response through clinostat-induced leaf epinasty. This is based on the assumption that clinostats do indeed affect the gravity response mechanism in plants. (Many of the data from the Biosatellite II study by NASA indicate that horizontal clinostats do, at least in some ways, compensate for gravity and simulate weightlessness; see Conrad, 1968; Gray and Edwards, 1968; Johnson and Tibbitts, 1968; and Lyon, 1968 a.) Leather et al. (1972) showed that  $CO_2$ , a known inhibitor of ethylene action (Abeles, 1973) blocked clinostat-induced epinasty in tomato. Lyon found that both exogenously applied ethylene and horizontal clinostating were identical in their effects on auxin transport in pea roots (1972). Palmer found that transient petiole epinasty in clinostated *Helianthus* plants was likewise caused by ethylene evolution (1973). Salisbury et al. (1979) reported that silver nitrate, a powerful inhibitor of ethylene action (Beyer, 1976 a, b), slowed clinostat-induced epinasty in *Xanthium strumarium* plants.

In so far as auxin has been implicated in the gravitropic response (Wilkins, 1979), any interactions that occur between auxin and ethylene might also imply a role for ethylene in gravitropism. Abeles and Rubenstein (1964) showed that ethylene evolution correlated very closely with endogenous auxin levels applied in *Phaseolus vulgaris*, and that



asymmetrical auxin distribution in gravitropically and phototropically stimulated hypocotyls is paralleled by an asymmetrical ethylene evolution. Ethylene has in turn been found to affect auxin transport in plants (Morgan and Gausman, 1966; Lyon, 1970). Kang (1979) suggests that in events such as epinasty, an interaction of IAA and ethylene seems likely, since auxins in general stimulate ethylene, while ethylene affects IAA transport. It would appear difficult to separate auxin and ethylene in some plant responses; aside from growth promotion and allied responses, many auxin events appear to be mediated through an ethylene step (Burg and Burg, 1967 a).

Finally, several cases of direct involvement of ethylene in gravitropism have been reported. Chadwick and Burg (1967, 1970) and Burg and Burg (1967 a) concluded that ethylene participated in root gravitropism as an inhibitor, resulting from IAA-dependent production. They observed that CO<sub>2</sub> reduced gravitropic response in pea roots without retarding the overall elongation rate. They were unable to observe any CO<sub>2</sub> effects in excised pea shoots, however, concluding that ethylene's participation in gravitropism was probably restricted to roots.

As mentioned previously, Zobel (1973, 1974) showed that ethylene was essential for upright growth in a normally prostrate growing tomato mutant (i.e., without ethylene). Later studies of this mutant strain have shown that the plant's response system to ethylene is deficient, rather than its ability to produce ethylene (Jackson, 1979). IAA enhancement of ethylene production also appears to be lacking in this mutant (Bradford and Yang, 1980). Similarly, ethylene has been shown to be essential for upright growth in strawberry clover (*Tritifolium fragiferum* L.), which normally is prostrate (diagravitropic). Treating

the prostrate stolons of this clover with ethephon or ethylene caused rapid stem elongation and erect curvature within 2 h, while IAA treatment showed little effect (Hansen and Bendixen, 1974; Bushnell, 1976).

Wright et al. (1978) observed increases in ethylene evolution in the lower halves of *Echinochloa* nodes which were preceded by increased levels of extractable IAA, but they observed no effects from addition or depletion of ambient ethylene or addition of rhizobitoxine analogue (an ethylene antagonist) on the normal gravitropic response. They concluded that the rise in ethylene evolution was symptomatic rather than causal. Similarly, Kaufman et al. (1980) have observed ethylene evolution in horizontal *Avena* leaf sheath bases (pulvini).

Osborne (1975) has theorized that ethylene may exert inhibitory effects on the elongation of the top cells in horizontal grass stems. Auxin build-up in the lower halves of gravistimulated stems (nodes) would cause ethylene production in these bottom tissues, but the ethylene's effects would be countered by the high auxin concentration. The ethylene could then passively diffuse to the auxin-depleted top cells and exert an inhibitory influence.

### The Clinostat

In 1904 Ganong described the clinostat as "the most important piece of apparatus in the laboratory of plant physiology," and indeed it has been a useful tool for plant physiologists for over 100 years. A clinostat is a device capable of rotating an object at various angles of inclination (Larsen, 1962), and has traditionally been used to rotate plants or other organisms about a horizontal axis in an attempt to negate the unidirectional influence of gravity. Obviously, clinostats

do not extinguish the earth's gravitational field for the plant; rather, they compensate or nullify gravity (Dedolph et al., 1966; Brown et al., 1976 a), creating a multilateral stimulus (Volkman and Sievers, 1979).

Julius von Sachs was the first to build a clinostat and described its invention as follows: "If, then, it is gravitation, which we suppose to be situated in the center of gravity of the earth, so to speak, and the action of which takes place in the direction of the earth's radius, or, what is the same thing, in the vertical line, it must be possible to nullify this action by compelling growing plants to continually alter their direction with respect to the vertical, in such a manner that the gravitation acts on the symmetrically opposite sides of a growing part of a plant for equal periods in opposite directions. Starting from this reflection I constructed an apparatus which I called the Klinostat. This apparatus, which may be constructed in very different ways, has essentially the one object of slowly rotating, by means of clockwork or other motive power, a solid rod of wood or metal which must be exactly horizontal, and this so that a rotation is completed in 15-20 minutes. On this rod growing plants, e.g., seedlings may be so fixed that they participate in the rotation of the rod without hindrance to their further growth. It matters not in what direction the growing organs are fastened on the rotating axis as long as the rotation is equable, so that every growing part of the plant turns the same side upwards as well as downwards during equal periods of time, so that the influence proceeding from the center of gravity of the earth must act on the growing portions of the plant during equal periods in exactly contrary directions. If this occurs, no action of gravitation whatever can make itself effective on the direction of growth, since a longer or shorter time is



necessary for this, and before the part of the plant has had time to make a curvature downwards or upwards, it finds itself already, in consequence of the rotation, again in a position which would necessitate its making the exactly contrary curvature, and thus no curvature at all is accomplished: it goes on growing in exactly the direction arbitrarily given to it when it was fastened to the axis" (Sachs, 1887).

Sachs was not the first to use the clinostat principal for testing plants, however. Larsen (1962) and Hoshizaki (1973) cite one work by J. Hunter, dated 1794, in which he used a water driven clinostat. Other early works with clinostats include attaching plants to clock hands (Wigand, 1854), while Francis Darwin also used clockwork drives (as did Sachs; see Hoshizaki, 1973). Pfeffer used a spring-driven device (1881, see Plate 1), and Goldschmidt (1896) drove a motor with hot air escaping from a kerosene lamp. Newcombe (1897) was one of the first to use an electrically driven clinostat motor. Van Harreveld's paper, "The inadequacy of present clinostats for physiological stimulus investigations" in 1906 reviewed the problems of clinostat development up until the development of the synchronous-induction electric motor around the turn of the century (Hoshizaki, 1973).

Elfving (1884) noted that normally quiescent grass nodes began to grow when rotated horizontally, much as they would if they were displaced from the normal vertical position, but growth during clinostating was even on all sides as opposed to more growth on the bottom sides of stationary nodes displaced from the vertical. Vöchting (1882, cited in Jost, 1907) noted that gravitropically bent stems straightened when placed on a clinostat. These results led Fitting (1905) and others (see Pfeffer, 1906 and Jost, 1907) to conclude that only bending was

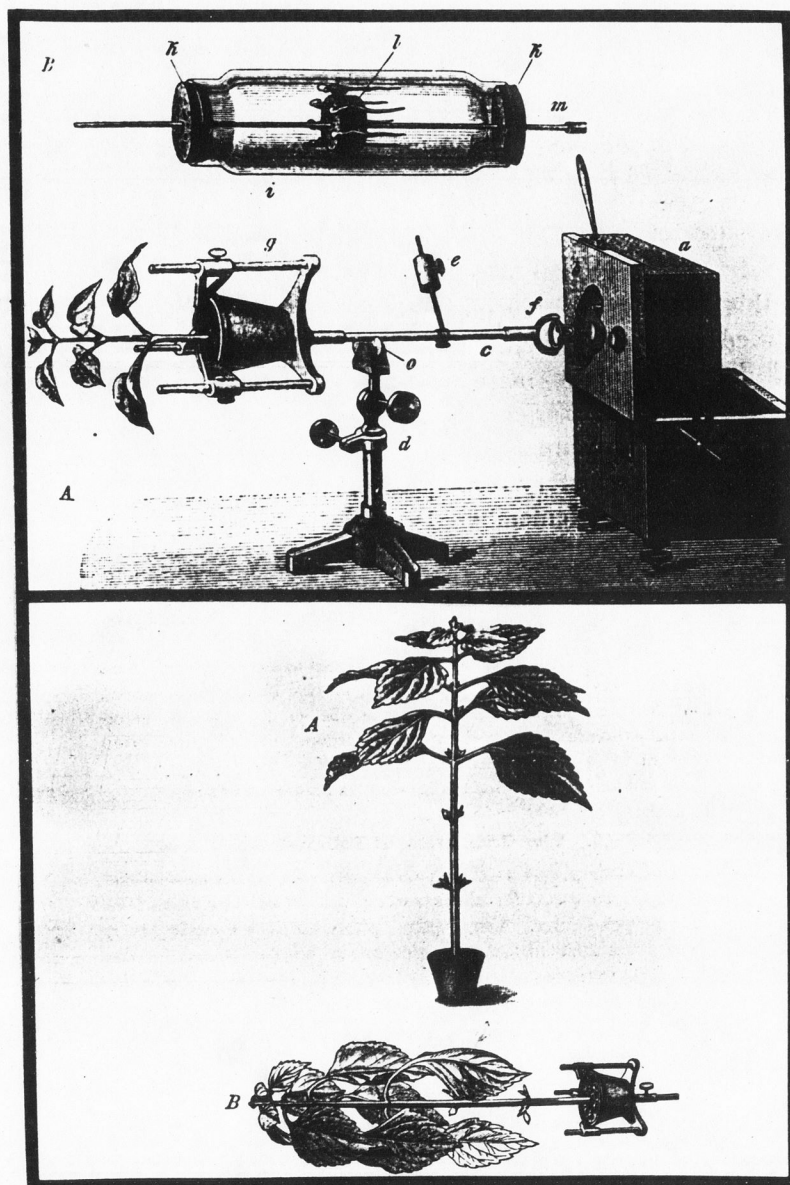


Plate 1. Wilhelm Pfeffer's clinostat (klinostat) as shown in his text, *The Physiology of Plants* (1906). Leaf epinasty was a well known response to horizontal clinostating, as can be seen in the bottom picture of a *Coleus* plant before and after clinostating (also taken from Pfeffer, 1906).



impossible on a clinostat, and that perception of gravity was still possible; that is, the individual stimuli were cancelling one another rather than no stimulus being perceived. Because of such results, the usefulness of clinostats to negate gravitational influence was questioned.

Newcombe summarized many of the capabilities and shortcomings of the clinostat for scientific research in 1904. Among other things, he recommended rotational rates between 1 rpm and 2 rph in order to avoid centrifugal effects or gravitational curvatures. He observed that rotating plants end over end (about a horizontal axis) was equally as effective, if not better, than the traditional rotation about the horizontally positioned growth axis. (As also noted by Sachs in the above quotation.) This was confirmed by Hoshizaki et al. (1966) and Lyon (1967) many years later. Newcombe (1904) also warned against using clinostats with their axis of rotation set at oblique angles and against using clinostats in studies of plant autotropism, as clinostats were ineffective at negating this process (see Firn and Digby, 1979).

Dedolph et al. (1967) noted that rotation of plants on horizontal clinostats causes a more uniform distribution of protoplasmic inclusions, as opposed to a noticeable settling tendency of cellular particles when plants were not subjected to gravity nullification (clinostating). Salisbury (1969) developed this idea further by relating clinostat-caused cellular organelle suspension to the expected responses to satellite free-fall. One of the more obvious differences would be the "churning" or "stirring" of the clinostated plants' organelles as they continually tried to settle. This would not be expected in true weightlessness (Salisbury, 1969). Dedolph and Dipert (1971) and Silver (1976) independently described the movements of intracellular particles subjected

to clinostating in which the organelles would continually settle through the period of rotation, tracing quasi-circular or elliptical paths through cytoplasm. These paths would be functions of the cytoplasmic viscosity, particle size and density, and the rotation rate, with its resulting centrifugal force (the latter also being dependent upon the distance of the particle from the axis of rotation; Dedolph and Dipert, 1971). This then is the basis for gravity nullification or compensation by clinostat rotation (Dedolph and Dipert, 1971).

In recent years, the addition of a second, simultaneously rotating axis has been tested (Hoshizaki et al., 1966; Hoshizaki and Hamner, 1970; Dedolph and Dipert, 1971; see also Newcombe, 1904), but the results from either single or double-axis devices were found to be equally reliable (Lyon, 1967; Hoshizaki, 1973). However, some phototropic responses have been observed in clinostat studies performed in the light with single-axis clinostats (Hoshizaki and Hamner, 1970).

The responses of plants to clinostating have been varied and diverse. Brain (1935) observed increases in growth rates of *Asplenium bulbiferum* and *Narcissus pseudo-narcissus* leaves on a clinostat, while *Lupinus albus*, *Helianthus annuus*, and *Avena sativa* showed decreased radicle growth. *Avena* coleoptile growth was unaffected, but leaf growth was slowed. Cell-sap pH was unaffected in *Lupinus albus*, while clinostated plants' hypocotyls showed cell "suction pressure" or 4.4 atm compared to 5.5 atm in upright plants (Brain, 1942). Hendricks (1940) observed a cessation of twining in morningglory plants when clinostat angles were greater than 75°. Larsen (1953, 1957) reported that *Artemisia absinthium* roots would curve irregularly on the clinostat when rotation rates were between 4 and 0.5 rpm.

Dedolph et al. (1965) found increased growth in *Avena* in response to horizontal clinostating, while Audus and Brownbridge (1957) found no effect upon *Pisum sativum* root growth. *Avena* coleoptile growth was not affected by horizontal axis rotation, according to Dedolph et al. (1965), but Shen-Miller and Gordon (1967) observed decreased growth for *Avena* coleoptiles grown for 24 h in red light and then transferred to the dark for the rest of the experiment. Hoshizaki et al. (1966) observed increased growth in *Hordeum vulgare* coleoptiles grown on a 2-axis clinostat for both continuous light and dark conditions. *Helianthus annuus* hypocotyls grown on horizontal clinostats showed increased growth (Brain, 1935, 1942), while *Xanthium pensylvanicum* (*strumarium*) rotated horizontally showed decreased growth (Hoshizaki et al., 1964). Leaf growth in *Xanthium* was also reduced.

Flowering response was decreased in horizontally clinostated *Xanthium* plants (Hoshizaki and Hamner, 1962); although GA<sub>3</sub> treatment increased growth compared to that of controls, the expected GA<sub>3</sub> enhancement of flowering did not occur in clinostated *Xanthium* plants (Hoshizaki et al., 1964).

Increased gravitropic sensitivity was observed in clinostated *Helianthus annuus* seedlings (Härtling, 1964), while von Bismarck (1959) reported delayed gravitropic responses in several *Sphagnum* species rotated horizontally. Shen-Miller (1970) concluded that gravitropic sensitivity was unaffected by horizontal clinostating. Placing *Xanthium* plants along the perimeter of a large, horizontally rotating wheel caused stem growth to deflect into the direction of rotation (Hoshizaki and Hamner, 1962).

Härtling (1964) observed increased phototropic sensitivity in clino-



Table I. Some of the species used for experiments involving clinostats. Some of the earlier investigators are listed for each of the species, but many of the species were used in succeeding years by others.

Species	Investigator and Date
<i>Phycomyces</i> spp. . . . .	Sachs, 1887
<i>Phaseolus vulgaris</i> . . . . .	Fischer, 1890
<i>Phaseolus multiflorus</i> . . . . .	"
<i>Lupinus albus</i> . . . . .	"
<i>Desmodium gyrans</i> . . . . .	"
<i>Taraxacum dens-leunis</i> (officinale) . . . . .	Darwin, F. and Pertz, 1892
<i>Beta</i> sp . . . . .	Czapek, 1899
<i>Avena sativa</i> . . . . .	"
<i>Phalaris canariensis</i> . . . . .	"
<i>Biophytum sensitivum</i> . . . . .	Pfeffer, 1906
<i>Trifolium pratense</i> . . . . .	"
<i>Acacia laphantha</i> . . . . .	"
<i>Helianthus annuus</i> . . . . .	"
<i>Coleus blumei</i> . . . . .	"
<i>Chenopodium</i> . . . . .	"
<i>Amicia</i> sp . . . . .	"
<i>Cassia marylandica</i> . . . . .	"
<i>Lepidium sativum</i> . . . . .	Zimmerman, 1927
<i>Lupinus polyphyllus</i> . . . . .	Brain, 1926
<i>Curcubita pepo</i> . . . . .	"
<i>Asplenium bulbiferum</i> . . . . .	Brain, 1935
<i>Narcissus pseudo-narcissus</i> . . . . .	"
<i>Zea mays</i> . . . . .	"
<i>Ipomea purpurea</i> . . . . .	Hendricks, 1940
<i>Humulus lupulus</i> . . . . .	"
<i>Tiniaria convolvulus</i> . . . . .	"
<i>Artemisia absinthium</i> . . . . .	Larsen, 1953
<i>Sphagnum</i> spp. . . . .	von Bismarck, 1959
<i>Xanthium strumarium</i> . . . . .	Hoshizaki and Hamner, 1962
<i>Torenia fournieri</i> . . . . .	Lyon, 1963, 1965 a, b
<i>Lycopersicon esculentum</i> . . . . .	"
<i>Brassica oleracea</i> . . . . .	"
<i>Hordeum vulgare</i> . . . . .	Lyon and Yokoyama, 1966
<i>Secale cereale</i> . . . . .	"
<i>Triticum vulgare</i> (aestivum) . . . . .	Lyon, 1968 b
<i>Capsicum annuum</i> . . . . .	Jonson and Tibbitts, 1968
<i>Pteris longifolia</i> . . . . .	Conrad and Yokoyama, 1971
<i>Pinus strobus</i> . . . . .	Wilson, 1973
<i>Arabidopsis thaliana</i> . . . . .	Brown et al., 1976 a, b
<i>Tagetes patula</i> . . . . .	Tibbitts and Hertzberg, 1978

stated *Helianthus annuus* seedlings, and Shen-Miller and Gordon (1967) observed a similar response in *Avena sativa* seedlings.

Clinostating has been shown to eliminate leaf "sleep" movements in some species, while it had no effect in others. Pfeffer (1906) reported decreased leaf movements in *Cassia marylandica* on a clinostat, but *Desmodium gyrans* continues its leaf movements (Fischer, 1890). *Phaseolus multiflorus* plants with their petioles perpendicular to the axis of rotation lost their sleep movements, while plants whose petioles were parallel to the rotational axis maintained their movements (Stoppel, 1916). Clinostating stopped primary leaf movements in *Canavalia ensiformis* (Kleinhoonte, 1931), but the plants resumed the rhythmic movements, completely in phase, after being taken off the clinostat. Fischer (1896, cited in Hoshizaki, 1973) described plants whose leaf movements were dependent upon gravity as being geoncyctinastic, and those independent of gravity as autoncyctinastic. *Phaseolus vulgaris*, *Phaseolus multiflorus*, and *Lupinus albus* were all geoncyctitropic, while *Acacia lophantha*, *Trifolium pratense*, and *Biophytum sensitivum* were examples of autoncyctotropic plants (Hoshizaki, 1973).

Lyon (1961) concluded that  $0.04 \text{ m s}^{-2}$  centrifugal acceleration provided threshold force for the gravitropic response in corn seedlings grown in weak light. Horizontal clinostating induced epinastic curvatures in the branches of *Coleus blumei* within several hours. If the branches were debudded or defoliated, epinastic curvatures could be restored by supplying the branches with 1% IAA in lanolin. Applying labeled IAA ( $^{14}\text{C}$ ), showed an excess accumulation of the auxin in the upper sides of the branches at a ratio of ca. 9:5 (Lyon, 1963). Lyon also observed similar asymmetries of labeled IAA in the curved stem

growth resulting from clinostating (i.e., more auxin on the convex side of curved organs). Tomato and *Torenia fournieri* plants grown on clinostats for six weeks showed reduced internodal lengths and shoot weights (Lyon, 1965 b).

Lyon and Yokoyama (1966) reported that horizontal clinostating disturbed the precise positive gravitropism of primary wheat roots. Epinasty was observed in lateral roots, and the coleoptiles developed curvatures in three dimensions.

Rates of 0.3 to 4 rpm for horizontal clinostats produced maximal epinastic curvatures of leaves and branches in *Coleus blumei*, with curvatures reduced at rates of 15 min per revolution or slower. Rotation rates of 1 to 3 min were found acceptable for most plants (Lyon, 1970).

*Pisum sativum* plants grown on clinostats showed a 3-fold decrease in IAA-5-<sup>3</sup>H transported into lateral roots, while the auxin that did move in these roots accumulated to an excess of 20% in the upper halves. Treating roots with 6.4 to 9.1 ppm of ethylene instead of clinostating caused similar effects (Lyon, 1972). He concluded that the clinostat and ethylene were identical in their effects on auxin transport in lateral roots.

### Biosatellite II

The Biosatellite II experiments are the only major United States studies of plant responses to weightlessness to date. One group of experiments involved wheat (*Triticum vulgare*, GA. Exp. Sta. #1123) seedlings, with the effects of weightlessness on growth physiology, morphogenesis, histochemistry, and biochemical changes being the major concerns. Simultaneously, wheat seedlings were grown under similar conditions on



earth, and a third set of seedlings were grown on horizontal clinostats on earth. Lyon (1968 a, b) observed no effects on growth rates or external morphology of wheat roots or coleoptiles grown in space. Similarly he observed no differences in auxin distribution and concluded that horizontal clinostats were reliable devices for studying gravitropism (Lyon, 1968 a).

Gray and Edwards (1968) also were not able to detect any outward differences in wheat seedling morphology between space germinated and terrestrially germinated plants. No differences in germination rates were observed between the earth and space experiments (Gray and Edwards, 1968). The space seedlings had higher shoot height to root length ratios than did earth plants, while the coleoptiles of the flight seedlings were taller than erect earth controls (Gray and Edwards, 1968). Both the space-grown and the clinostat seedlings had more starch-containing amyloplasts (assumed to be statoliths) than erect earth plants, and these statoliths were distributed randomly in the cells of the space and clinostated plants; in contrast, the statoliths were grouped in the bottom sides of erect control plants' cells (Gray and Edwards, 1968). Gray and Edwards concluded that the clinostated seedlings resembled the space-grown seedlings more closely than the erect earth control plants.

Conrad (1968) observed some differences in glucose-6-phosphate dehydrogenase activity between space-grown seedlings and both erect and clinostated terrestrial plants. He observed no significant differences in Michaelis constants or maximum velocities of reactions involving glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, malic dehydrogenase, transaminase, cytochrome c reductase, or peroxidase in any of the groups of seedlings; therefore, Conrad concluded that

weightlessness had not effect upon enzyme affinity for substrates.

S.J. Johnson (1968) reported no significant differences in carbohydrates, amino acids, or nitrogen fractions between space-grown wheat seedlings and erect or clinostated earth plants.

A second set of experiments on Biosatellite II, conducted by S.P. Johnson and T.W. Tibbitts, were directed at observing the effects of weightlessness on the angles of the leaves and petioles of pepper plants (*Capsicum annuum* cv. Yolo Wonder). Weightlessness produced a downward curvature in leaves, similar to that produced on the horizontal clinostat on earth (i.e., epinasty resulted in each case; Johnson and Tibbitts, 1968). Similarly, mobilization of carbohydrates and amino acids occurred in the leaves of both clinostated and space-grown plants.

Due to the untimely death of S.P. Johnson, complete analyses of these data were delayed until Brown et al. (1974) pointed out that significant differences did exist between the petiole angles of the space-grown and clinostated plants.

In the 1970's clinostats have been used in a variety of studies including: estimates of gravitropic sensitivity and presentation times of *Avena* (Shen-Miller, 1970); the effects upon IAA transport in roots, (Iversen et al., 1971); estimating thresholds of epinastic response by altering the angle of inclination (Conrad and Yokoyama, 1971); observations on the areas of deposition of compression wood in *Pinus strobus* trees (Wilson, 1973); and the effects of fast rotation (organ centered on rotation axis) on amyloplast distribution and response thresholds in *Lepidium* roots (Sobick and Sievers, 1979).

A.H. Brown and coworkers have observed growth of *Arabidopsis* seedlings on clinostats as compared to plants grown in the normal 1-g upright

position or plants grown on centrifuge, with acceleration (forces) up to 15 times g. The centrifuged plants' characteristics were extrapolated to zero and compared with those grown on horizontal clinostats (Brown et al., 1976 a). The results of the two methods were not in agreement. However, the horizontally clinostated plants were morphologically different from stationary upright controls and vertically clinostated plants (which were similar to the stationary upright plants; Brown et al., 1976 a). These incongruous results were tested further by placing clinostats on a large centrifuge and observing plant growth under these conditions (a concept that was attempted several years earlier by J.C. Finn and O. D.R. Brown, 1961; see also Salisbury and Ross, 1969). If clinostats were totally effective at compensating for gravity, the centrifugation should have been ineffective on altering growth. But this did not occur, and several morphological characteristics varied depending upon the applied g-force. The authors concluded that clinostat gravity compensation was incomplete (Brown et al., 1976 b).

The usefulness of horizontal clinostats for nullifying gravity was further questioned by Tibbitts and Hertzberg (1978), after they observed leaf movements and bending on plants placed on horizontal clinostats. They proposed that clinostat-induced epinasty might be a result of such uncontrollable leaf movements (i.e., mechanical stresses). This was a logical suggestion, since mechanical stress effects in plants are thought to be mediated by ethylene (Jaffe and Biro, 1979).

#### Mechanical Stress

Possibly the earliest reference to plant response to mechanical stress is in Darwin's book, *The Power of Movement in Plants* (1896). He



reported a sensitivity in *Pisum sativum* radicles to contact; that is, by attaching a small square of paper with shellac to the apex of the radicle, he was able to induce deflected growth away from the direction of attachment. Darwin also measured a sensitivity of the cotyledons of *Cassia tora* to mechanical stimulation. Lightly tapping the cotyledons with a twig for 3 min caused them to rise from approximately 45°, forming an angle of about 90° between them. Similarly, hitting the cotyledons with a light stream of water from a syringe would also induce a rise (Darwin, 1896).

The response of plants to tactile stimuli, such as the folding of the leaves of *Mimosa pudica* or the closing of the trap mechanism in *Dionaea muscipula*, have been known for a long time, and such responses are reviewed by Sibaoka (1969).

Differences in growth caused by wind-induced motion have been detected by several researchers. Venning (1949) measured thicker collenchyma tissue in celery petioles subjected to wind-induced motion. Similarly, Jacobs (1954) measured narrower radial growth at the base of *Pinus radiata* supported from swaying by wires when compared to non-supported, free-swaying trees after ten years of growth. Whitehead and Luti (1962) and Whitehead (1962) reported reduced shoot dry weights in *Zea mays* and *Helianthus annuus* subjected to constant wind speeds up to 33 miles per hour.

Other wind tests with *Larix laricina* trees resulted in reduced heights for free-swaying trees, which could be partially offset by staying the trees (Larson, 1965). Pelton (1967) reported increased yields of 24 to 43% in test fields of "Chinook" wheat where snow fence wind breaks had been erected each spring.

Walker (1960) observed increased collenchyma cell wall thickening in *Datura stramonium* plants placed on a mechanical agitator for 9 h a day for 40 days. Neel and Harris also tested direct mechanical shaking with *Liquidambar styraciflua* (sweet gum trees) and reported that moderate shaking of the trees for 30 s each day reduced height growth 20 to 30% (1971). They also noted that most of the shaken trees set terminal buds within three weeks, while none of the control trees did.

Many of these preceding papers concluded that water stress was the main reason for the observed reduction in growth of such mechanically disturbed plants, and Parkhurst and Pearman (1972) suggested this for the results obtained in the *Liquidambar* tests, proposing cavitation in the trunks of the shaken trees as a possible explanation. But Neel and Harris (1972) responded to this with evidence supporting an independent hormonal response mechanism to mechanical stimulation. They reported that daily shaking of *Zea mays* plants reduced heights up to 50%, but that the shaken plants would return to normal growth rates within 3 days after shaking, thereby ruling out cavitation as a possible cause for the reduction. Growth reductions have also been reported in *Cucurbita melopepo* (Turgeon and Webb, 1971) and wheat (*Triticum aestivum*) in response to daily handling or shaking (Steucek and Gordon, 1975).

Goeschl et al. (1966) reported that a nonwounding-physical stress applied to pea epicotyls increased ethylene evolution, resulting in decreased elongation and increased diameter. This effect could be duplicated by treatment with ethylene, and they surmised that this might be a natural hardening response in the epicotyl insuring proper emergence.

Jaffe (1973, 1976 a) noted that young plants of *Hordeum vulgare*, *Bryonia dioica*, *Cucumis sativus*, *Phaseolus vulgaris*, *Mimosa pudica*, and

*Ricinus communis* responded to gentle rubbing of the internodes for 10 s each day with reduced internodal elongation. He coined the term "thigmomorphogenesis" to describe this response of plants to touching or contact and proposed that this might be an adaptation to natural sources of stress, such as wind or animal movements.

Hammer, Mitchell, and Weiler (1974) and Beyl and Mitchell (1977 a, b) observed significant reductions in the heights of greenhouse chrysanthemums when plants were subjected to daily shaking (30 s per day), and proposed that mechanical stress may be a convenient way to reduce the stem length of chrysanthemums without the use of chemical growth retardants. Mitchell et al. (1975) measured reduced growth in tomato and pea by mechanical stress from shaking, flexing or rubbing of the plant axis, and proposed the term "seismomorphogenesis" to describe the effects of vibrational stress on plant growth. Wheeler (1978) and Wheeler and Salisbury (1979) showed that shaking induced by a gentle water spray was capable of reducing heights of greenhouse tomato up to 40%, while yields were reduced from 7 to 15%. Other earlier reports on mechanical stress side effects include reduced internodal lengths, numbers of nodes, and heights of cotton plants subjected to handling (Frizell et al., 1960). Salisbury (1963) reported up to 30% reduced size of *Xanthium* leaves as a result of daily handling and measurement during a photoperiod study.

Various other responses have been correlated to mechanical stress in plants. Pickard was able to detect asynchronous action potentials in pea epicotyls following mechanical stimulation (1971). Leopold et al. (1972) measured increases in ethylene content of white pine, apple and peach wood stressed by tying branches in arcs, while Saltveit et al. (1979) made similar measurements with bent poinsettia petioles. Irving



Table 2. Some of the species used for experiments involving mechanical stress. Earlier investigators are listed for each species, but many of the species were used in succeeding years by others.

Species	Investigator and Date
<i>Pinus radiata</i> . . . . .	Jacobs, 1954
<i>Apium graveolens</i> (celery). . . . .	Walker, 1957, 1960
<i>Datura stramonium</i> . . . . .	"
<i>Larix decidua</i> . . . . .	Larson, 1965
<i>Pisum sativum</i> . . . . .	Goeschl et al., 1966
<i>Bryonia dioica</i> . . . . .	Boyer, 1967
<i>Curcubita melopepo</i> . . . . .	Turgeon and Webb, 1971
<i>Liquidambar styraciflus</i> (sweetgum) . . . . .	Neel and Harris, 1971
<i>Hordeum vulgare</i> . . . . .	Jaffe, 1973
<i>Cucumis sativus</i> . . . . .	"
<i>Phaseolus vulgaris</i> . . . . .	"
<i>Mimosa pudica</i> . . . . .	"
<i>Ricinus communis</i> . . . . .	"
<i>Chrysanthemum morifolium</i> . . . . .	Hammer et al., 1974
<i>Lilium longiflorum</i> . . . . .	Hiriaki and Ota, 1975
<i>Triticum aestivum</i> . . . . .	Steucek and Gordon, 1975
<i>Lycopersicon esculentum</i> . . . . .	Mitchell et al., 1975
<i>Pinus resinosa</i> (red pine). . . . .	Quirk and Freese, 1976
<i>Pinus sylvestris</i> (Scotch pine) . . . . .	Fayle, 1976
<i>Pseudotsuga menziesii</i> (Douglas fir). . . . .	Kellogg and Steucek, 1977
<i>Euphorbia pulcherrima</i> (poinsettia) . . . . .	Salveit et al., 1979
<i>Juglans nigra</i> (black walnut) . . . . .	Ashby et al., 1979
<i>Acer saccharinum</i> (silver maple). . . . .	"

and Osborne (1973) noted that handling peas had effects similar to ethylene treatment with respect to (1-<sup>14</sup>C) glycerol incorporation. Jaffe (1973) and Mitchell et al. (1975) reported mild leaf epinasty in tomato plants subjected to mechanical stress, implicating ethylene indirectly.

Jaffe (1976 b) reported a decrease in electrical resistance of the stem tissue of *Phaseolus vulgaris* immediately after mechanical stimulation. Mitchell et al. (1977) measured increased leaf diffusive resistance and decreased transpiration in mechanically stressed tomato plants; he noted that these responses were only transitory and concluded that they were probably not responsible for the typical reduction in dry weight gain from mechanically stressed plants. Suge (1978) reported that a daily mechanical stress of stroking the top of bean plants for 1 min reduced gibberellin production. Mitchell (1977) observed that hood removal, ethylene pretreatment, or thigmo-mechanical stress yielded similar results on the growth of etiolated pea seedlings, and he proposed that an auxin-ethylene interaction might be involved in mechanical stress growth reduction. Erner et al. (1980) have shown a translocatable thigmomorphogenetic factor in grafting experiments with *Phaseolus vulgaris*, and that this factor is not ethylene. Boyer et al. (1979) have observed that pretreatment of *Bryonia* plants with lithium (LiCl) prevents inhibition of elongation normally resulting from rubbing.

Jaffe and Biro (1979) summarized many of the current findings on the mechanism of thigmomorphogenesis in kidney bean by the following: an increase in cell membrane permeability (decrease in cell resistance) occurs immediately after stimulation; this lasts for about 3 min, during which time, the growth rate accelerates slightly, but then stops once the prestimulus resistance level is restored, about 30 min after mechan-

ical perturbation; the amount of ethylene produced by the tissue increases, and growth concomitantly reduces to about one half of the prestimulus rate; ethylene production returns to normal about 3 h after stimulation, but the reduced growth continues for about 2 days and accelerates to the normal rate after 3 days; and cuticular thickening and radial cell division are increased sometime during the first days after mechanical stimulation.

### Ethylene

The role of ethylene in plant physiology is reviewed quite thoroughly in F.B. Abeles's book, *Ethylene in Plant Biology* (1973), and most of my remarks on this plant hormone will be taken from his book.

Neljubov was probably the first to identify ethylene as the physiologically active component in illuminating gas (1901). Crocker and Knight (1908) and Harvey (1915) pursued investigations of ethylene further, while Denny (1924) was the first to discover that ethylene was involved in fruit ripening. Not until 1934 did Gane unequivocally prove that ethylene evolved from plants, however.

A great deal of the knowledge of ethylene's effects on plants comes from the works by A.W. Crocker, F.E. Denny, A.E. Hitchcock, F. Wilcoxon, and P.W. Zimmerman at the Boyce Thompson Institute during the 1930's (Abeles, 1973). Crocker et al. (1932) tested numerous plant species and their responses to ethylene and various other hydrocarbon gases. Of 202 species tested, 89 showed ethylene-induced leaf epinasty and 113 did not. Of the gases tested, ethylene was by far the most active physiologically, while any others showing some activity were lighter carbon gases with unsaturated bonds. Zimmerman and Wilcoxon (1935) and Crocker



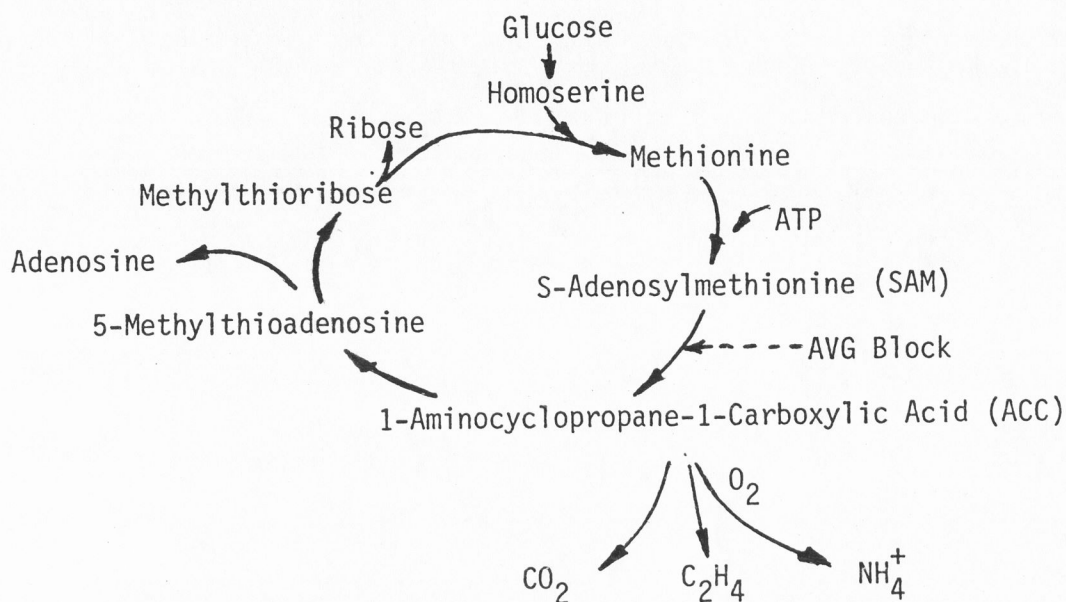
et al. (1935) proposed that some of the effects observed from auxin treatment could be caused by ethylene action. But Michener (1938), in a review, concluded that ethylene did not influence production or transport of auxin, although it might increase sensitivity of the plants to auxin, and that ethylene does not resemble auxin in its action on stem elongation. Went and Thimann (1937) agreed with Michener's conclusions, and ethylene's prominence as a plant hormone faded considerably (see also Abeles, 1973).

It was not until Morgan and Hall (1964) and Abeles and Rubenstein (1964) showed again that auxin stimulated ethylene production, that the auxin-ethylene interaction conflict resurfaced. Burg and Burg (1966 a, 1967 a) then revitalized Zimmerman and Wilcoxon's idea that, aside from growth promotion, many auxin events might be mediated via ethylene. However, findings since then have shown that this is not completely true (Ridge and Osborne, 1969; Ridge, 1975).

Ethylene's roles as a plant hormone are numerous, although it is generally perceived as an inhibitory and senescent hormone, as well as being linked with many types of physiological and physical stress (Abeles, 1973). Ethylene is also known for its fruit ripening effects (Burg and Burg, 1965), its ability to cause leaf abscission (Abeles, 1973), as well as being the controlling hormone in plant organ epinasty (Kang, 1979); recently, it has also been implicated in mechanical stress responses, as mentioned previously (Jaffe and Biro, 1979). However, ethylene is unique among plant hormones in that its molecules are very small in size (molecular weight of 28), it is soluble in both the aqueous and lipid phases of the cells, and due to its size, it is highly diffusive (Leopold, 1972). Ethylene appears to be produced in the same cells or tissue that it acts

upon, so transport is not really an important aspect of ethylene physiology (Leopold, 1972; Abeles, 1973).

If now appears that the primary precursor of ethylene production in higher plants is methione (Lieberman, 1979), with the present scheme appearing as follows:



For the above ethylene biosynthesis scheme, see: Adams and Yang (1977, 1979). Yeong-Biau and Yang (1979), Yu and Yang (1980), and Lieberman (1979).

Formerly, it was thought that no detoxification systems existed for ethylene (Leopold, 1972), but Beyer (1975, 1977, 1978) has shown that ethylene does undergo incorporation and oxidation, although the modes of action and fate of ethylene are still a moot issue (Lieberman, 1979).

### Ethylene antagonists

Mack (1927) was one of the first to observe the inhibitory effects of CO<sub>2</sub> gas on ethylene actions, and CO<sub>2</sub> had been the primary inhibitory

agent used in ethylene experiments up until the mid 1970's. The  $\text{CO}_2$  molecule competitively inhibits ethylene's action (Burg and Burg, 1967 b).

More recently, silver ion has been found to be an even stronger antagonist of ethylene's action (Beyer, 1976 a, b; 1979). Kofranek and Paul (1972) observed that cut carnation stems dipped in  $\text{AgNO}_3$  solutions showed a longer vase-life; they thought that this was due to the bactericidal action of silver, but Beyer showed that the silver blocks ethylene's action at the receptor site, possibly in a noncompetitive fashion (1975 a, b). Since then silver, usually in the form of a thiosulfate solution (to create a more mobile anion for movement within plants; Veen and van de Geijn, 1978) has been used in floral perservation experiments (Veen, 1979), to delay the ripening of fruits (Salveit et al., 1978), and to induce staminate flowers in gynecious cucumbers (Tolla and Peterson, 1979). It has recently been pointed out that the silver itself actually stimulates some ethylene production, probably due to heavy metal toxicity, but that its effectiveness as an action inhibitor overpowers this (Walker et al., 1978, 1979; Aharoni et al., 1979).

The use of ethylene biosynthesis inhibitors is probably the most effective method for prevention of ethylene events in plants. Owens et al. (1971) first described the effects of rhizobitoxine on plant ethylene biosynthesis. Rhizobitoxine is a phytotoxin produced by *Rhizobium japonicum*, which has powerful inhibitory properties on ethylene production. It and other enol-ether amino acids such as aminoethoxyvinyl glycine (see Appendix A) or AVG (from *Streptomyces* spp.) and methoxyvinyl glycine or MVG (from *Pseudomonas aeruginosa*)



are some of the most potent of these synthesis inhibitors (Lieberman et al., 1975; Baker et al., 1978; Lieberman, 1979). AVG and MVG have also been shown to inhibit protein synthesis in some tissues, when concentrations are greater than  $10^{-3}$  M (Matoo et al., 1979). Other tests have shown AVG effective at blocking fruit ripening (Ness and Romani, 1980) and induction of staminate flowers in gynecious cucumbers (Owens et al., 1980).

Cobaltous ion ( $\text{Co}^{2+}$ ) has also been shown to block ethylene synthesis in some plant tissues (Lau and Yang, 1976), and since then has been used to extend the vase-life of cut roses (Venkatarayappa et al., 1980).

Other compounds such as metal chelating agents EDTA, DIECA, and KCN have been effective at reducing ethylene production, thereby implicating metal cofactors in ethylene synthesis (Lieberman, 1979). Similarly, 2,4-dinitrophenol (DNP), a classical respiration uncoupler, also inhibits ethylene production in some tissues, implicating an essential role for ATP (Yu et al., 1980). Lowering the  $\text{O}_2$  concentration around plant tissue has been commonly used to reduce ethylene production (e.g., in fruit storage, Abeles, 1973). Hypobaric conditions have also been used to prevent ethylene action, presumably by removing the gas rapidly from tissue (Burg and Burg, 1966b). This might not only reduce the effects of the already existent gas but also reduce further ethylene synthesis thanks to ethylene's autocatalytic properties (Leopold, 1972).

A substituted benzothiadiazole has been shown to block ethylene action (Simmons and Dilley, 1971), while two O-substituted hydroxylamine derivatives, N-benzyloxycarbonyl-L-(a)-aminooxy-propionic acid and (a)-aminooxyacetic acid, have also recently been shown to be powerful

inhibitors of ethylene synthesis in plant tissue (Amrhein and Wenter, 1979; Amrhein and Schneebeck, 1980).

### Epinasty

The term epinasty was first introduced by De Vries (1872) and is defined as curvature resulting from differential growth in a plant organ, where the inner or adaxial side grows more rapidly than the outer or abaxial (Kang, 1979). In leaves, this essentially amounts to a downward curvature. Epinasty in contrast to most other tropisms or nastic movements is thought to be an autonomous event; that is, not induced or oriented by certain external factors (Kang, 1979). Ethylene appears to be the major regulatory hormone involved in epinasty (Harvey, 1915), but tests with both auxin and ethylene suggest that an interaction of these two hormones may occur; that is, auxin may induce ethylene formation, and ethylene may in turn affect auxin transport, thereby causing asymmetrical distributions (Kang, 1979). However, some evidence suggests a direct stimulatory role for ethylene (Palmer, 1972, 1976).

Epinasty can be induced in plants by a variety of treatments, particularly with ethylene producing compounds such as herbicides, auxins, or unstable compounds that degrade into ethylene, such as 2-chloroethylphosphonic acid (ethephon; Kang, 1979).

There also appears to be an intimate relationship between gravity (gravitational orientation) and epinasty in plants (Rawitscher, 1923). Crocker et al. (1932) noted reduced epinastic responses in tomatoes treated with ethylene when plants were in an inverted position, while Denny (1936) observed increased epinasty producing volatiles (i.e.,

ethylene) in tomatoes turned horizontally. Numerous examples can be cited for epinasty resulting from horizontal clinostating, including: Pfeffer (1906), Finn and Brown (1961), Lyon (1963, 1965 a, 1967, 1970, and 1972), Johnson and Tibbitts (1968), Conrad and Yokoyama (1971), Leather et al. (1972), Palmer (1973), Hoshizaki (1973), Brown et al. (1974), and Tibbitts and Hertzberg (1978). The series of pepper plant experiments with Biosatellite II dealt primarily with the epinasty resulting from weightlessness and horizontal clinostating, clearly implying a connection between gravity and epinasty (Johnson and Tibbitts, 1968; Brown et al., 1974). Hayes and Lippincott (1976) and Hayes (1977) have observed that auxin-induced hyponasty in bean leaves is also sensitive to gravitational orientation, as it can be reduced by inversion or horizontal rotation.

A variety of methods have been used for measuring leaf epinasty, including liminal or axillary angles (Crocker et al., 1932), or angles formed by permanent markers on the leaves and the vertical (Lyon, 1963; see also Palmer, 1972). Palmer estimated the radius of curvature formed by the mid region of the leaf by matching a set of premeasured arcs to fit the leaf curvature most closely (1972). These radii of curvature were then plotted as reciprocals, yielding an increasing value with an increase in epinasty. The plotting of reciprocals damps out the large changes in radius of curvature occurring when the leaf is nearly straight and increases in sensitivity as the radii or curvature decrease (Palmer, 1973 and personal communication). Salisbury and Denney (1971) plotted the heights of leaf tips of *Xanthium* as a system for tracking leaf movements, as have others in the past (see Darwin, 1896).



## METHODS AND MATERIALS

### Theoretical Background

Many gaps remain in the explanation of the "clinostat problem"; that is, the basis of the assumed gravity compensation caused by clinostating, and the observed effects of clinostating. With Brown et al.'s (1974) reworking of the epinasty data obtained from the Biosattellite II (Johnson and Tibbitts, 1968), it was shown that true satellite free-fall and clinostating are statistically different in their effects. But the epinasty trends on the clinostated plants and the space plants were very similar, nonetheless. Likewise, clinostats have been shown to suspend cellular organelles, that is, prevent statoliths (amyloplasts) from settling (Dedolph et al., 1967; Dedolph and Dipert, 1971; Volkmann and Sievers, 1979), a result that has also been seen in satellite free-fall (Gray and Edwards, 1968). Is the clinostat then capable of partially achieving or simulating 0-gravity conditions? And, if it is only incomplete gravity compensation, what accounts for this incompleteness?

Ironically, and simply enough, the basic difference between the two environments is the pull of gravity. Horizontal clinostats, stationary on the earth's surface, can not possibly eliminate gravity. Every plant organ, cell, and cellular organelle is subjected to a constant  $9.8 \text{ m s}^{-2}$  acceleration toward the center of the earth, whereas in a satellite orbiting the earth in outer space, this is not true; the accelerational forces upon such structures are practically zero. The clinostat negates gravity in theory, by exposing all parts of the plant to gravity from all sides; nonetheless, gravity still establishes stresses and consequent strains in all plant parts, if only instantaneously from

any given direction.

Suspension of cellular organelles on clinostats must be accomplished by rotating the plants faster than the time period required for settling or transit through the cytoplasm, and its subsequent response (minimum presentation time). By rotating the plants on clinostats at sufficiently fast speeds (which can have complexing side effects of centrifugal acceleration if rotation is too fast), these organelles are caused to churn about or orbit in small elliptical paths within the cytoplasm (Dedolph and Dipert, 1971; Silver, 1976). What effects this churning has on cellular respiration and metabolism or hormonal or other biochemical exchanges between cells as opposed to free-fall suspension, are not completely known. Clearly, solving such a problem would involve a continuation of space flight experiments such as those of Biosatellite II.

The report by Tibbitts and Hertzberg (1978) presents a more testable hypothesis, however. During close observation of marigold (*Tagetes petula*) plants growing on clinostats, they noted a constant shifting or flopping of the leaves as the plants progressed through their rotation. This can be easily envisioned for petioles (which are not completely rigid) as they approach the zenith of their rotation from one side, pass over this apex, and progress down the other side (see Plate 4). The direction of applied force (the stress) changes 180° in this half circle, causing a deformation (strain) in the petioles: the flopping. Even if very little flopping occurs, however, as might be expected with more rigid leaves, shifting of stresses within the petiole still occurs. From such observations, Tibbitts and Hertzberg made the logical proposal that such uncontrolled leaf movements could be a source of mechanical stress ("stress" in a biological sense), which was causing the

clinostat-induced responses such as epinasty. The role of ethylene in both clinostating and mechanical stress as discussed previously supports such a hypothesis.

To test this problem, two possible approaches might be invoked: (1) subjection of plants to mechanical stress to see if comparable epinasty will develop; or (2) elimination of mechanical stresses during clinostating or gravity compensation to see if epinasty can be reduced. Various other morphological results might also be compared, including stem growth, dry weight gain, stem width, or internodal length (all of which are affected by mechanical stress, Mitchall et al., 1975), but leaf epinasty is clearly one of the first responses evident from clinostating. Written in the form of a hypothesis, one could propose: *Leaf epinasty induced by horizontal clinostating is caused by mechanical stresses to the plants.*

Other information on the problem could come from alternate ways of suspending statoliths within plants (i.e., gravity compensation) while minimizing any mechanical agitation: for example, periodic inversion of plants; that is, turning the plants upside down for set intervals of time, alternated with equal intervals in the upright position. This approach could have a convenient control of inverting and immediate returning to the upright position, while leaf flopping should be greatly reduced as compared to clinostating. If such a process could cause epinasty like a clinostat, one could make firmer statements as to the role of statolith suspension as the possible cause of leaf epinasty.

With the apparent involvement of ethylene in the clinostat response, and a clinostat's connection with the gravity perception mechanism in



plants, it also seemed worthwhile to investigate possible roles for the hormone ethylene in gravitropism. Clues linking ethylene and gravity are abundant in the literature, yet current reviews on shoot gravitropism continue to explain plant response mechanisms in terms of auxin (IAA) gradients, and more recently gibberellins (Wilkins, 1979; Phillips and Hartung, 1974). Inhibitors affecting the upper side of horizontally placed stems are frequently mentioned as a possible mechanism for causing curvatures, but actual testing seems to be habitually avoided. A consistent observation in gravitroping dicot shoots is a slowing or cessation of elongation of cells on the top side of the stems, as opposed to a normal or often accelerated rate of growth on the bottoms (Digby and Firn, 1979 a). Digby and Firn (1979 a) state that the cessation of growth on the top is totally responsible for curvatures observed in cucumber hypocotyls. Could such a cessation of growth be solely caused by migration of growth promoter out of the upper cells? Or could an inhibitor be functioning in the shoot response mechanism, much as it has been shown for roots?

The second major problem that I have approached, then, is whether ethylene is involved in gravitropism of plant shoots. Or perhaps a more testable null hypothesis might be stated: *Ethylene is not essential for shoot gravitropic response*. Refuting such a statement could prove enlightening in view of the weakened state of the Cholodny-Went Theory today.

#### Preparation of Plant Materials

Most of the experiments conducted used cocklebur (*Xanthium strumarium* L. Chicago Strain) plants, with frequent repetitions with tomato (*Lycopersicon*

*persicon esculentum* Mill., cv. Bonny Best or Rutgers), pepper (*Capsicum annuum* L. cv. Yolo Wonder), and castor bean (*Ricinus communis* L.).

Tomatoes have been used commonly, as reported in both the clinostat and mechanical stress literature, and cockleburs have been used previously in clinostat studies. The strong epinastic response to clinostating of all four species make them particularly good subjects for studying this response.

All plants were grown in the Utah State University Plant Science Department's T. E. Building greenhouse. The east-west greenhouse is covered with corrugated fiberglass. The north wall of the greenhouse is the southside of the wooden T. E. Building. Cooling in the summer was accomplished with a vertical, excelsior drip pad located on the western wall, and an exhaust fan on the eastern end. Daily temperature limits were set at 24 C and 32 C, with 3-5 C variance on exceedingly hot days or cold nights. Supplemental lighting from Sylvania 'cool white' fluorescent lights provided an 18-h daylength for maintaining cocklebur plants in a vegetative state.

Cocklebur plants were germinated in sand flats and transferred to 10-cm square plastic pots after emergence and greening of the cotyledons. Tomato, pepper, and castor bean plants were germinated in vermiculite and generally transplanted in the cotyledon stage as well. All mature plants were grown in a soil-sand mix (3:1 vol.), with some earlier experiments conducted using soil-sand-peat mix (3:1:1 vol.). The soil used was a silty clay loam, pH 7.9 and  $EC_e$  0.6. Soil phosphorus and potassium ratings were 8.3 and 146 ppm, respectively. Approximately 0.5 g of Osmocote 14-14-14 slow-release fertilizer and 0.5 g of phosphate 0-20-0 were mixed into the soil of each pot prior to transplanting seedlings.

Periodically (ca. every 2 or 3 weeks) about 5 to 10 pellets (ca. 0.1 g) of ammonium nitrate 34-0-0 were added to each pot. Waterings were generally required each day.

Lower leaves and axillary buds were pruned from all cocklebur plants, leaving three to four fully expanded, healthy leaves for growth. The lower leaves of tomato and castor bean plants were also removed as they became necrotic. For clinostat experiments, plants were pruned to two or three fully expanded leaves, while for most gravitropism tests, plants were pruned to one 1/2-3/4 fully expanded leaf. Plants used ranged in age from 25 to 90 days old, although most tests used the younger, more vigorous plants (ca. 40 days).

Other cultural practices included monthly application of Temik, a systemic insecticide for aphids and white flies, and periodic application (by dipping the shoots) of Karathane fungicide, for powdery mildew on cocklebur plants.

As with most investigative endeavors, many preliminary tests were conducted prior to more refined experiments; therefore, I shall only report what I consider the most important of my experiments. Most experiments were repeated two or three times.

#### Antiethylene treatments

Four different ethylene antagonists were used in experiments. The most useful of these was aminoethoxyvinyl glycine (AVG, see Appendix A). AVG is soluble in water, and 1.0 mM and 0.1 mM solutions were used for blocking ethylene synthesis in plants for both clinostat and gravitropism tests. One drop of 'Tween 20' surfactant per 100 ml was added to all AVG solutions, as well as to all solutions to be discussed later. For application, plants' shoots were either dipped in, or misted until



"dripping wet" with the desired solutions.

Cobaltous ion ( $\text{Co}^{2+}$ ) is also known to block ethylene synthesis in some plant tissue, and 1.0, 2.0 and 10.0 mM solutions (using  $\text{CoCl}_2$ ) were tested in shoot gravitropism experiments with cockleburs. Control salt solutions at the same concentrations of  $\text{NaCl}$ ,  $\text{CaCl}_2$ , and  $\text{MgCl}_2$  were used for comparison in cobalt tests. The  $\text{NaCl}$  and  $\text{MgCl}_2$  solutions were tested independently against untreated controls to determine if they had any effects.

Silver ions ( $\text{Ag}^+$ ) are known to be powerful inhibitors of ethylene action in plants. Silver nitrate has traditionally been used as the source for silver in plant experiments, and several of my tests were conducted using  $\text{AgNO}_3$  solutions. But silver's solubility in such solutions is readily disrupted, resulting in a reduced  $\text{Ag}^0$  precipitate; this renders the solutions ineffective. Similarly, transport of the  $\text{Ag}^+$  ion once it has gotten into plant tissue might be expected to proceed slowly, due to negatively charged sites frequently found in plant structural tissue (e.g., glucuronic and galacturonic acids, Salisbury and Ross, 1978). Because of this, Veen and Geijn (1978) applied a solution of silver complexed with sodium thiosulfate (as commonly used in photographic processing), creating a more mobile anion:  $\text{Ag}(\text{S}_2\text{O}_3)_2^{3-}$ . These more stable solutions were found to be equally effective in their ethylene blocking ability. Therefore, most of my experiments used such solutions, formed either by pouring 1.0 mM  $\text{AgNO}_3$  solutions into an equal volume of 4.0 mM  $\text{Na}_2\text{S}_2\text{O}_3$ , or a 2.0 mM  $\text{AgNO}_3$  solution into 8.0 mM  $\text{Na}_2\text{S}_2\text{O}_3$ . The silver solution had to be added to the sodium thiosulfate to avoid precipitation of reduced silver. Silver thiosulfate

solutions were used for both clinostat and gravitropic tests.

Control solutions of  $\text{Na}_2\text{S}_2\text{O}_3$  (4.0 or 8.0 mM) were used for silver tests, while in one experiment I used both  $\text{Na}_2\text{S}_2\text{O}_3$  (8.0 mM) and  $\text{NaNO}_3$  (2.0 mM). Salt solutions of  $\text{Na}_2\text{S}_2\text{O}_3$  were also tested independently against untreated controls.

$\text{CO}_2$ -enriched air was also used to treat clinostated plants. For treatment, plants were enclosed in plexiglas chambers built to fit over the clinostat wheels (to be described later; see Plate 10), and subjected to a constant flowing stream (100–200 ml min<sup>-1</sup>) of  $\text{CO}_2$  (5%),  $\text{O}_2$  (20%), and  $\text{N}_2$  (75%). A 20% level of oxygen is preferred in order to avoid respiratory complications of treated plants (Dilley, 1979, personal communication).

The plexiglas chambers were also used for gravitropic experiments, with the pots fixed to the stationary wheels of the clinostat. In both gravitropic and clinostat experiments, plants were subjected to a flowing  $\text{CO}_2$ -enriched atmosphere for 30 min in an upright position prior to turning horizontally. Controls were sealed in chambers and maintained in a constant-flowing air stream (100–200 ml min<sup>-1</sup>), supplied by an oilless Neptune "Dyna Pump". Three  $\text{CO}_2$ -treated and three controls could be tested at once, using a gas manifold delivery system (to be described later).

In clinostat- $\text{CO}_2$  experiments, leaf epinasty could only be measured at the beginning and end of the experiment with visual estimates in between, but continuous measurements were made on stem bending during gravitropic tests by viewing through the clear plexiglas cylinders to simulate the stem angle with a pair of dividers, held on the opposite side of the chamber. During dark experiments, a light colored back-

ground, reflecting dim green light, was positioned on one side to facilitate measurements.

Since difficulties arise in maintaining large plants on clinostats for long periods of time (because of watering stops, soil slippage, and yellowing of leaves during prolonged dark tests), tests were usually restricted to 24 h. Because of this, measurements of traditionally observed responses to mechanical stress (which take several days or weeks to develop) such as increased stem diameter, reduced shoot elongation, and reduced internodal length were only taken once, in a week-long, clinostat-mechanical shaker comparison carried out in the greenhouse. In this and all other experiments, epinasty was the principal parameter for comparison. Leaf epinasty, a classical symptom of clinostating (Pfeffer, 1906; see Plate 1) is often very noticeable after 6 h on a clinostat, thereby providing a morphological trait that could easily be compared in 24-h experiments.

To accomplish this comparison, a very sensitive system for measuring leaf epinasty was required. Initially, I recorded changes in petiole angles, but Palmer (1972) states that such angle measurements are not sensitive for detecting events occurring strictly in the lamina portion or the upper part of the petioles. His radius of curvature method produced a much more integrated view of the events of the overall leaf, with accuracies using radius templates varying from  $\pm 0.5$  cm to  $\pm 2$  cm, depending on the size of the radius.

Therefore, for my measurements of epinasty, the radius of curvature of leaves was also used, but it was calculated very precisely ( $\pm 1$  mm) from three linear measurements taken on the leaves. The distances between (1) the base of the petiole and the petiole-blade junction, (2)



the petiole-blade junction and the tip of the blade, and (3) the tip of the blade and the base of the petioles were measured in millimeters. These three lines form a triangle, about which one, and only one circle can be drawn. If the perpendicular bisectors are drawn for each side of the triangle, they will intersect at the center of the circle circumscribing the triangle. The radius of this circle (which will be called the *radius of curvature* of the leaf) can be found by determining the distance between the center of the circle and any of the points of the triangle, on the circle's perimeter (Fig. 1). India ink marks (dots) were made at the base of the petiole and at the petiole-blade connection for exact measurement references over 24-h experiments.

Using trigonometric theorems and the law of cosines, the three linear measurements from each leaf can be used to calculate the radius of curvature with an equation derived as follows:

Given: 1).  $2 R = 2 \text{ (Radius)} = \frac{a}{\sin A} = \frac{b}{\sin B} = \frac{c}{\sin C}$  (for any circumscribed triangle)

2). Law of Cosines:  $\cos B = \frac{a^2 + c^2 - b^2}{2ac}$

3).  $\cos^2 B + \sin^2 B = 1$

Then: 1)  $\sin^2 B = 1 - \left( \frac{a^2 + c^2 - b^2}{2ac} \right)^2$

2)  $\sin B = \left[ 1 - \left( \frac{a^2 + c^2 - b^2}{2ac} \right)^2 \right]^{\frac{1}{2}}$

3)  $2 R = \frac{b}{\sin B} = \frac{b}{\left[ 1 - \left( \frac{a^2 + c^2 - b^2}{2ac} \right)^2 \right]^{\frac{1}{2}}}$

4)  $R = \frac{b}{2 \left[ 1 - \left( \frac{a^2 + c^2 - b^2}{2ac} \right)^2 \right]^{\frac{1}{2}}}$

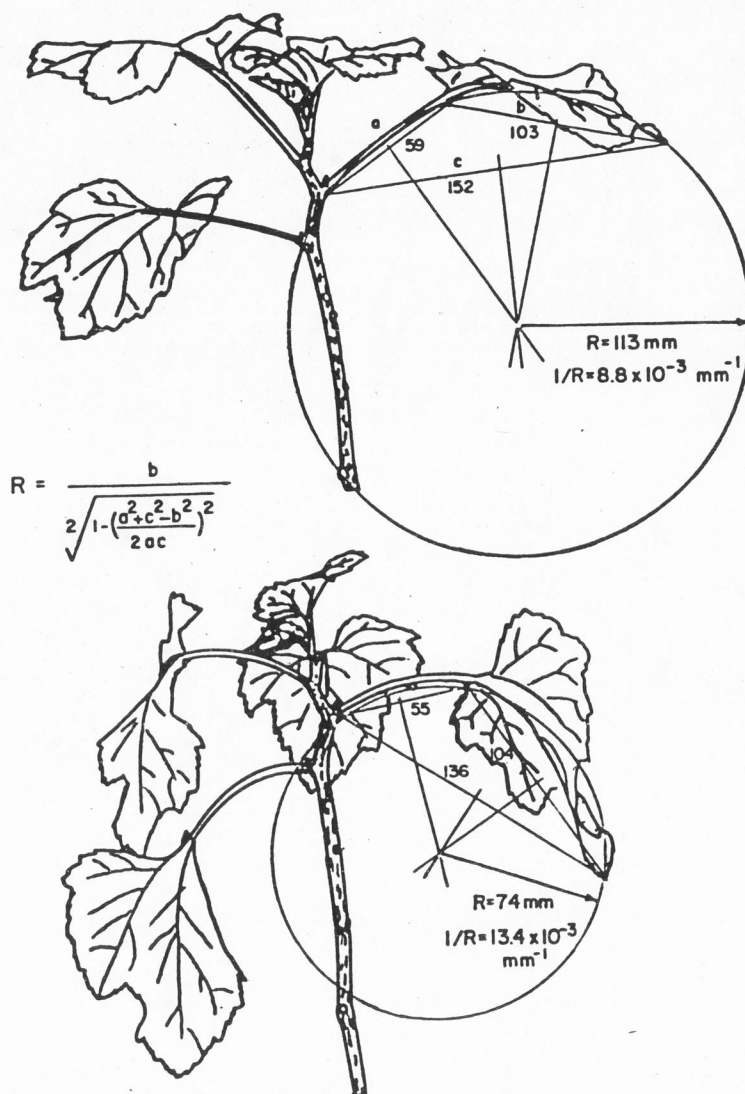


Figure 1. Illustration of epinastic curvature in cocklebur plants and the formula for calculating the radius of curvature of leaves. The sketches were made from a cocklebur plant with its leaves at normal daylight positions (top) and from the same plant immediately after 24 h of horizontal clinostating (bottom). The radius of the measured leaf decreased nearly 40 mm, while the inverse of the radius increased 3.6 reciprocal units in response to the clinostating. Reciprocal radii were used to track epinasty during clinostat experiments.

### The Clinostat

Clinostat experiments were conducted in both the greenhouse, with its lighting cycle, or in dark, temperature controlled rooms in the T. E. Building. Temperatures in these rooms were usually maintained near 25 C, with fluctuations as low as 20 C and as high as 28 C in one of the rooms.

A six-wheeled, belt-driven, variable speed clinostat (Plates 2 a, b, and c) was used for most of the experiments (built by USU Technical Services). Rotation speeds were controlled with a "zero-max" reduction gear box connected to a 1/25 horsepower electric motor. Speeds up to 44 rpm could be generated on the clinostat wheels. Speeds of rotation were usually kept between 0.25 and 0.33 rpm, however, since rotation rates in this range were found to be acceptable for plant experiments (Newcombe, 1904; Lyon, 1970). The clinostat could be set to any angle between the vertical and horizontal for achieving partial gravity compensation (Anker, 1962), but only vertical and horizontal tests were conducted.

Soil surfaces of the pots were covered with foam sponge pads (1.0 cm thick), cut to the middle to fit around plant stems, after which the pads were fastened to the pots with masking tape. This was done to prevent soil from falling out of the pot during rotation on the clinostat. Pots were fastened to the clinostat wheels with four turnbuckles hooked into holes on the wheels, and over the edges of the pots. The belt driving the clinostat alternated between the wheels causing the four wheels at the corners to rotate counterclockwise and the two inner wheels clockwise (looking down on them).

In a typical experiment, mature cocklebur plants were either dipped



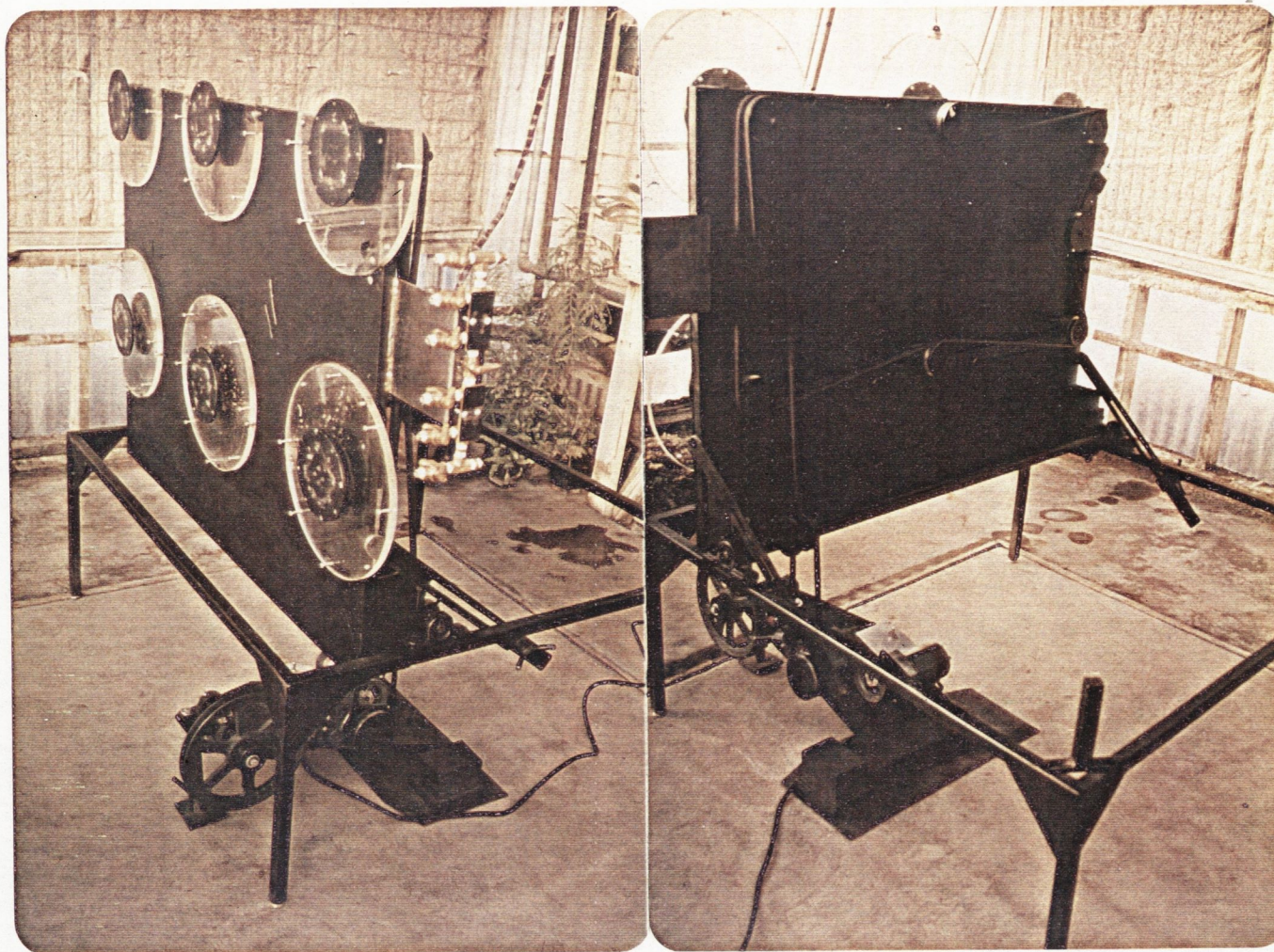


Plate 2 a. Six-wheeled, variable speed clinostat used for epinasty studies. The clinostat is shown in the upright position for rotating plants horizontally (i.e., the horizontal clinostat). The picture on the left shows a front view of the clinostat platform and the six wheels, while the right picture shows the back of the clinostat and the belt to drive the turntables. The motor is shown at the bottom of each.



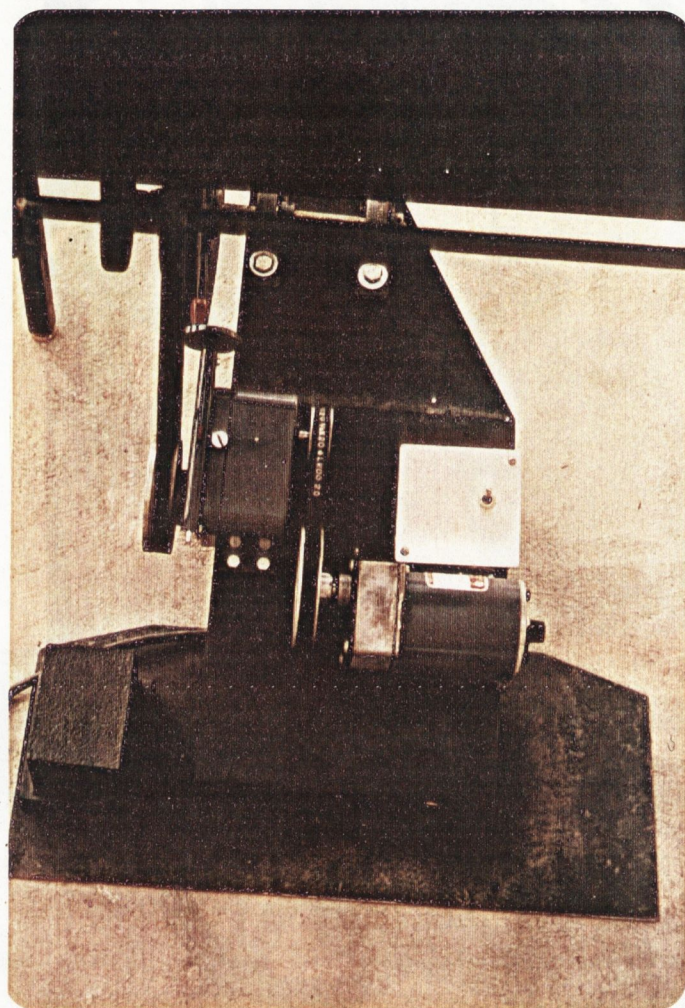


Plate 2 b. Motor-drive system for the six-wheeled clinostat. The 1/25 horsepower motor is the gray, cylindrical object at the lower right. The motor is connected by a small belt to the Zero-max reduction gear box at the middle left of the picture. The drive is transmitted from the Zero-max to the turntables by the belt in the upper left. The speed of the Zero-max could be adjusted with the red-handled crank in the upper left. The shiny box at the middle right is an on-off switch box. The weight of the entire system provided sufficient pressure downward to prevent slippage between the drive sheave and the belt. This was possible because of a hinged connection between the motor plate and a basal plate. The black object at the lower left is a weight welded to the basal plate.



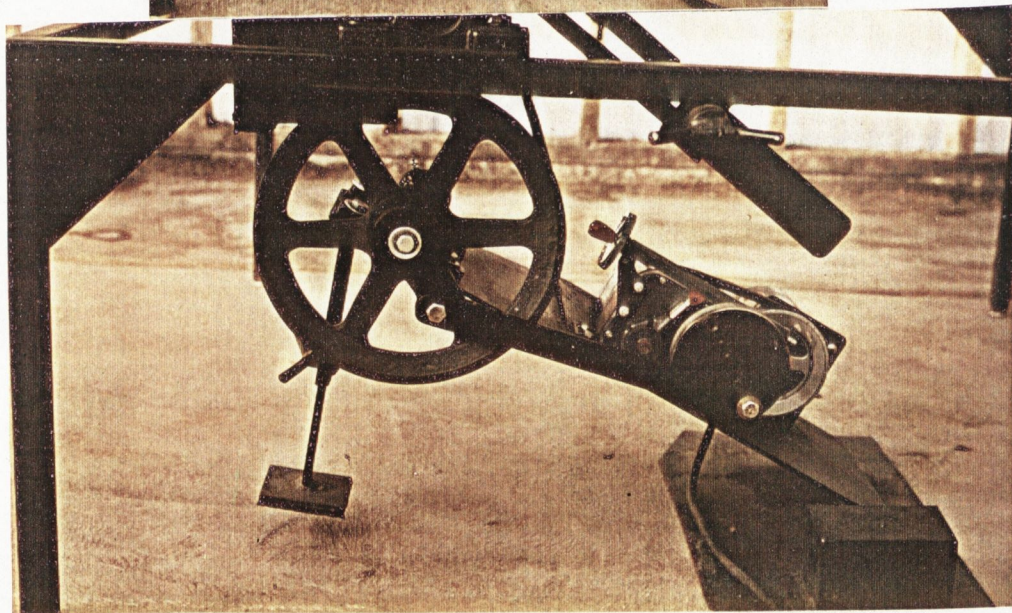
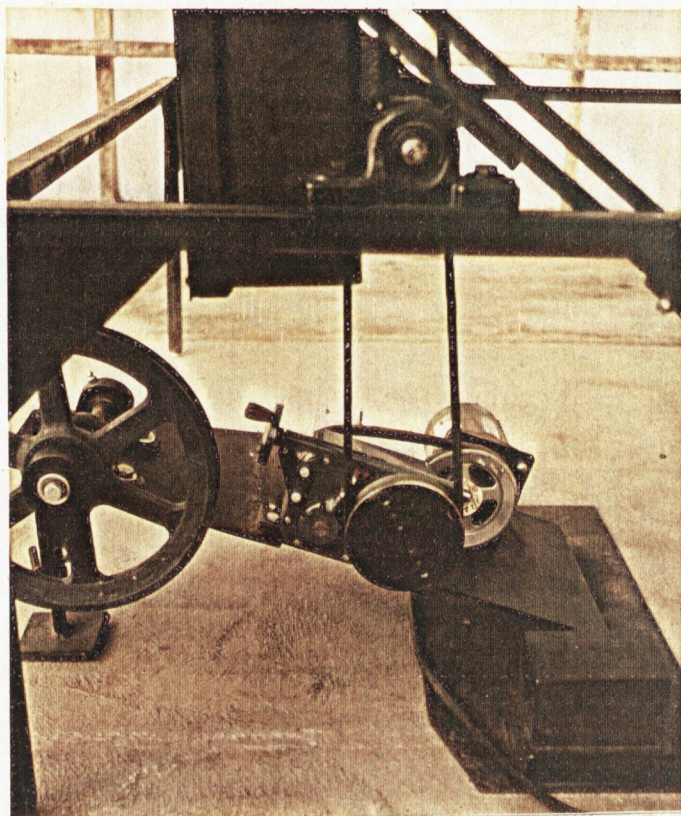


Plate 2 c. Drive connections for normal clinostating or twisting mechanical stress. The upper picture shows the belt connecting the "Zero-max" gear box and the clinostat, while the lower picture shows the crank-arm connecting the Zero-max to a larger wheel which in turn connects to a belt driving the clinostat. The drive sheave of the Zero-max and crank arm can not cause a complete rotation of the larger wheel, thereby causing a continuous back and forth motion of the entire system. This translates into a twisting motion for plants on the wheels of the clinostat.



in or sprayed with  $\text{AgNO}_3$  solutions until dripping wet, approximately 30 min prior to attachment to the clinostat. Spraying was used almost exclusively in later tests. The clinostat could accommodate six plants at one time, so tests with  $\text{AgNO}_3$  used three treated and three untreated control plants, and would run for at least 24 h. In later experiments with cocklebur, the epinastic leaf movements of stationary upright plants were also tracked simultaneously with clinostated plants.

### Mechanical Stress

#### Shaking

Plants were mechanically stimulated by a variety of methods, the first being manual shaking. This involved clasping the mid-section of the stem between the thumb and index finger and shaking the plant back and forth, up to approximately 200 times per minute for "mild shaking", and up to approximately 300 times per minute for "severe shaking". Shaking was also applied in a more precise manner by placing plants on an Eberbach laboratory mechanical shaker. The shaker's platform moved back and forth at either 90 or 132 cycles per minute, with a table displacement of 3.7 cm. The shaker was used in a horizontal position, accommodating six plants at once (usually no more in order to avoid leaf contact between adjacent plants), or could be turned on end, for shaking in an up and down fashion (max. two plants at once, see Plates 3 a and b). Vertical shaking was also used as a possible means of suspending statoliths within perceptive cells, although results indicated that it was ineffective in this regard.

A one-week experiment comparing the effects of shaking and clinostating on cocklebur plants was conducted. Treatment included six



untreated control plants measured during the experiment, six controls measured only at the beginning and end (to avoid any effects of agitation during measurement), six plants shaken vigorously by hand each day for 120 s, six plants shaken each day with the mechanical shaker for 120 s (shaker on low speed), six plants shaken for 8 h daily with the mechanical shaker (low speed), and three plants on the horizontal clinostat. Each of the above treatments was duplicated, but plants were sprayed daily (for 7 days) with a mixture of 2.0 mM  $\text{AgNO}_3$  and 8.0 mM  $\text{Na}_2\text{S}_2\text{O}_3$ . The first set was sprayed daily with just 8.0 mM  $\text{Na}_2\text{S}_2\text{O}_3$ .

Increases in height, internodal length and width, and changes in leaf epinasty of the first and second fully expanded leaves were measured during the experiment. All length measurements were made with a clear plastic millimeter ruler, while width measurements were made with vernier calipers.

### Twisting

To more closely simulate the stresses and strains a plant might experience on a clinostat, the clinostat was modified to create a twisting motion. To accomplish this, a crank arm was fastened between the drive sheave (ca. 8 cm diameter) and a larger wheel (ca. 22 cm diameter). The belt normally attached to the drive sheave on the zero-max gear box was switched to the larger wheel, which only cycled through a partial turn, thereby reversing the direction of pull repeatedly (up to 76 times per minute, or 38 cycles per minute max.). The zero-max could be used to adjust the speed of the twisting, the same as for the clinostat (see Plate 2 b).

The twisting motion caused the plants to spin back and forth (Plate 3 c) through approximately  $180^\circ$  of rotation (this could also be



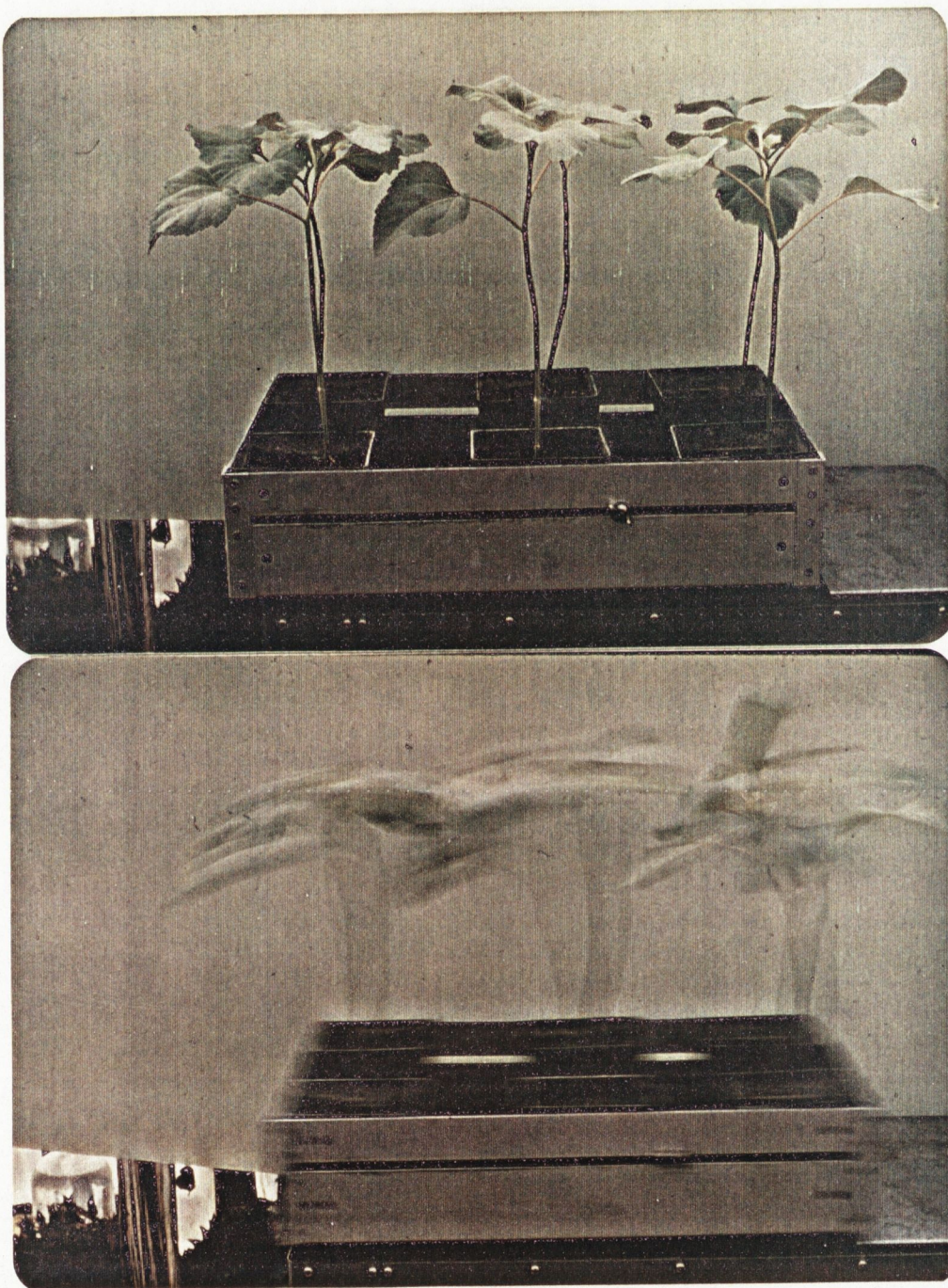


Plate 3 a. Cocklebur plants on an Eberbach laboratory shaker (top) and being shaken horizontally (bottom).



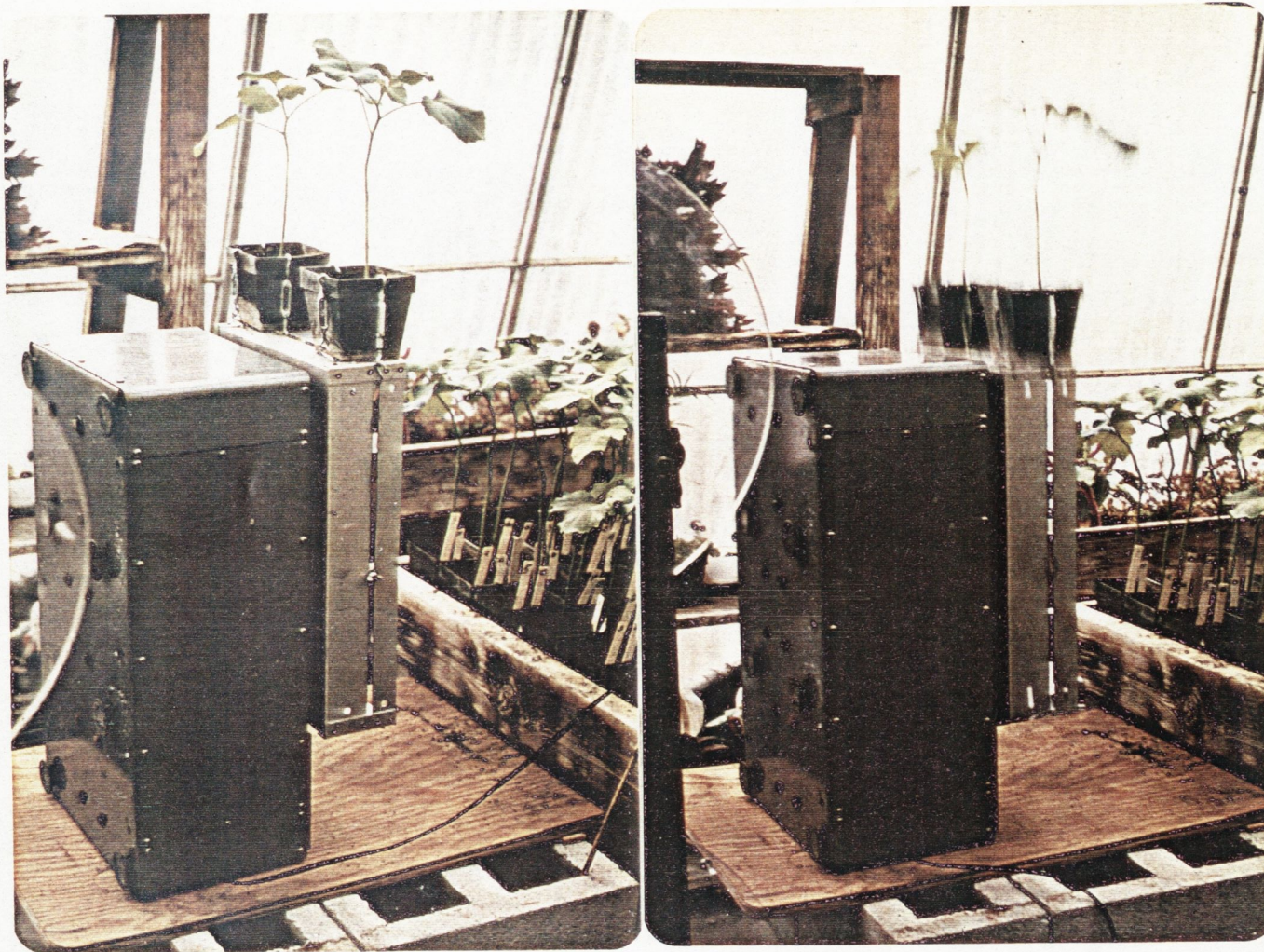


Plate 3 b. Cocklebur plants on an Eberbach laboratory shaker which has been turned on its side for vertically shaking plants.



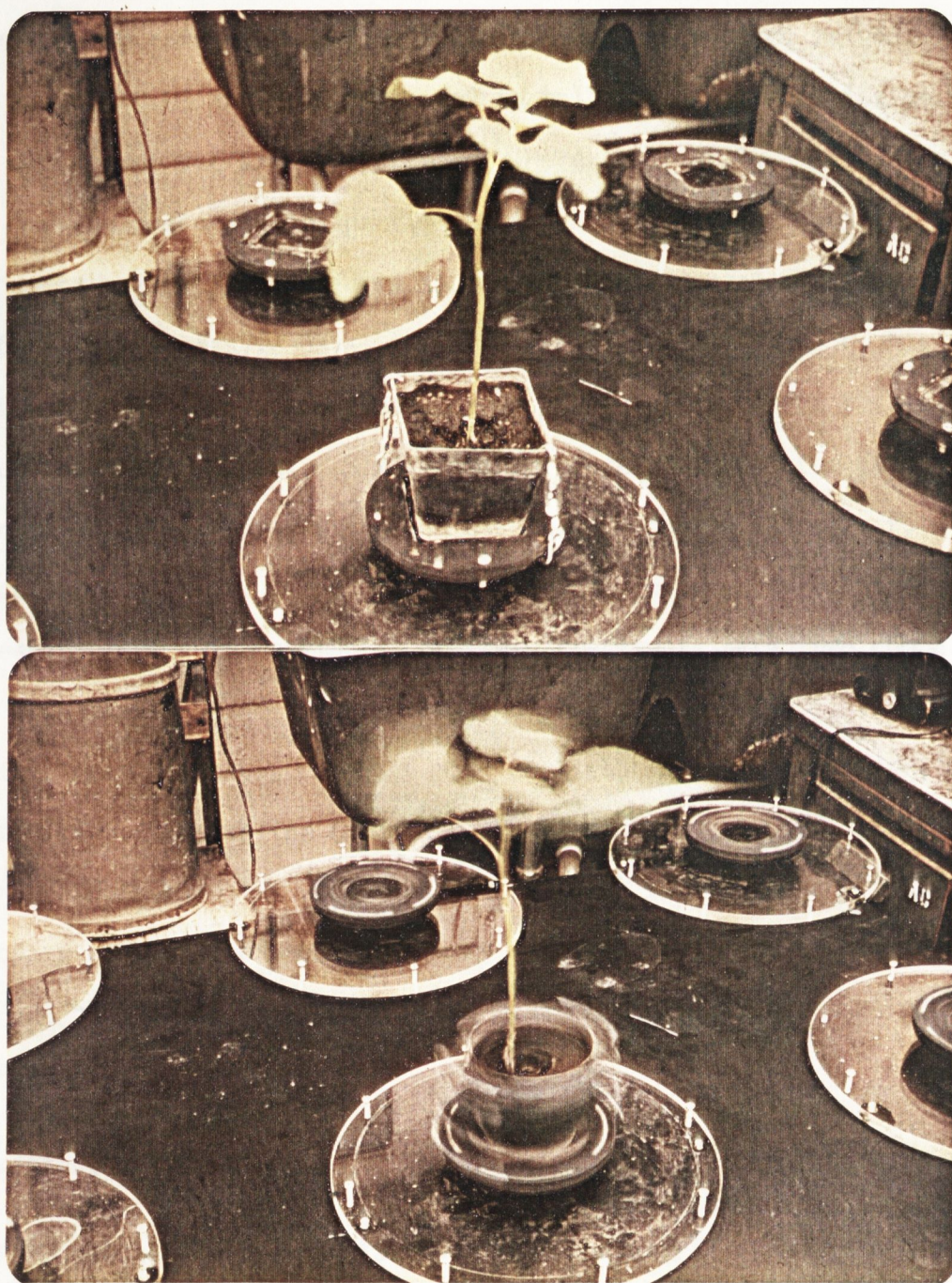


Plate 3 c. Cocklebur plants mounted on the clinostat in a vertical position (top) for application of a twisting form of mechanical stress (bottom), using the specially built crank arm to connect the motor to the belt-drive of the clinostat (see Plate 2). The plants were twisted back and forth through  $180^\circ$  at 38 cycles per minute.



varied, by shifting position of the crank arm) with all tests being run at the maximum rate of 38 cycles per min. Such a twisting motion would apply between 0.03 and 0.08 g of centripetal force for leaf tips between 6 and 16 cm from the axis of rotation. More importantly, the motion would induce torsional strains similar (although more frequent) to those experienced by the leaf petioles subjected to horizontal clinostating.

On three successive days, the effects of vertical shaking, horizontal shaking, the twisting on leaf epinasty were observed for the same individual cocklebur plants. Six plants were used for both the twisting and horizontal shaking tests, while two plants were chosen randomly for the vertical shaking test. (The shaker was set on low gear for these tests.) The results (epinasty movements) of these treatments were then compared to three plants subsequently subjected to clinostating on the following day.

This comparison was repeated with cocklebur plants, but this time synchronizing the starting time for each treatment, thereby closely matching the endogenous daily leaf movements of each test. In each test, stationary control leaf movements were also tracked. Twisting and vertical and horizontal shaking tests were also conducted with tomato plants (cv. Rutgers).

#### Intermittent clinostating

Perhaps the closest simulation to the mechanical stresses a plant experiences can be applied by intermittent horizontal clinostating, as suggested by Dr. Frank B. Salisbury. Although the term "intermittent klinostat" has been used in the past by Darwin and Pertz (1892, 1903; see also Larsen, 1962), referring to a horizontal clinostat that could be stopped for various periods of time to apply a desired gravitrimulus,



my use of the term refers to rapid, periodic rotation of plants while in the horizontal position, and then returning the plants to an upright position for a much longer period of time. For example, a typical rotation rate for constantly clinostated plants might be 1 rotation per 4 min (0.25 rpm). If plants could be tilted to the horizontal position and forced to go through one rapid rotation (e.g., 10 s), they could then be turned back upright for the remainder of the 4 min, or about 3 min and 40 s, taking into account about 10 s required to move the clinostat between position. In such a treatment, the plants would be subjected to the same overall horizontal rotation in 4 min as would constantly clinostated plants, but they would spend most of their time in an upright position, thereby avoiding possible suspension of their statoliths (i.e., gravity compensation).

One major test comparing the effects of intermittent clinostating with normal horizontal clinostating was conducted with cocklebur. Six plants were attached to the large, six-wheeled clinostat and turned horizontally and rotated once, lasting approximately 10 s, after which they were immediately returned upright (the entire process lasting about 20 s). This continued once every 4 min, for 12 h. These plants were compared to six stationary control plants and one plant rotated constantly at 0.25 rpm on a smaller clinostat. Similar experiments, lasting 10 and 12 h were conducted with tomato and castor bean plants, respectively.

### Inversion

In an attempt to achieve statolith suspension and its resultant gravity compensation, plants were inverted for set intervals of time,



after which they were returned to an upright position for an equal unit of time, and the process was repeated continuously. In effect, this is a crude form of clinostating, although inverted plants would experience much less leaf flopping than plants continuously clinostated and hopefully, then, less mechanical stress. Inversion experiments have been conducted by others in the past (see Pfeffer, 1906; Crocker et al., 1932; Little and Goldsmith, 1967), but usually leaving plants inverted for prolonged intervals to observe such things as leaf position or movements. By comparing inverted plants to controls inverted and then immediately returned to an upright position (controlling for the slight leaf movements), one might be able to assess more accurately the role of statolith displacement as a cause of leaf epinasty.

This was done by placing the inverted plants on notched boards (stem in the notch when inverted) for 20 min intervals, with alternate intervals spent in the upright position on top of the boards. Intervals of 10 and 30 min were also tested.

In several other inversion tests, four plants were clamped between two specially cut boards that were tightened together by bolts and wing-nuts, thereby permitting four plants to be flipped at once. The clamped sets of plants were supported on specially built wooden racks (clamps and racks built by Dr. Frank Salisbury).

One major experiment, comparing the epinasty of four periodically inverted (every 20 min) cockleburs, four plants inverted and immediately returned upright, four stationary control plants, and four plants horizontally clinostated, was conducted. Along with this, each of these treatments was duplicated, but all plants were pretreated with 0.1 mM AVG (clinostat-AVG treatment only used three plants). The experiment



was conducted in the greenhouse.

The epinastic effects of periodic inversion and clinostating were also compared for castor bean, tomato and pepper plants, but in the dark.

Periodic inversion in theory would appear to be a good method for gravity compensation, with controls inverted and then immediately returned, being subjected to the same movements (in fact twice as many) as the controls; but, such controls are not exposed to entirely the same mechanical stress regimes. The petioles and leaf blades of the inverted plants are exposed to a constant 1 g force in the upside-down position for alternating 20 min periods, whereas the controls are only exposed to this for approximately 1 to 2 s every 20 min. This might be overcome by attempting to force the leaves of the controls upward for alternating 20 min periods. For example, control plants might be lowered into a tube or sleeve, sufficiently narrow to force the leaves upwards a measured amount, or perhaps supports or strings could be used to hold the petioles up every other 20-min period.

Because of this difference in prolonged stresses, one inversion test was conducted in which bamboo put stakes were used to support petioles of control plants during periods when treated plants were in an inverted position. The stakes were originally cut to lengths to raise the petiole-blade junction of the leaf 1 cm, when placed directly underneath; but, due to daily leaf movements, the supports bracing the leaf petioles had to be adjusted at various angles to raise the petiole-blade junction 1 cm (visually estimated). The 1-cm displacement was chosen after photographically observing the movements of mature cocklebur leaves during inversion (see Plate 8). Treatments of the experiment included: four plants inverted every 20 min, four plants inverted and immediately



returned upright every 20 min, four stationary controls, four plants inverted and immediately returned upright every 20 min and with their petioles (two on each plant) propped up every other 20 min, and four stationary plants whose petioles were propped upward every other 20 min. Each time the inverted treatment's plants were upside-down, the props or supports were positioned under the petioles of the appropriate plants. The experiment lasted 24 h.

In this inversion experiment, as well as all other inversion or clinostat experiments, epinasty measurements were taken at 2, 3, or 6 h intervals using the first fully expanded leaf and the next leaf above.

#### Prevention of Mechanical Movements

The second general approach to the clinostat problem would be elimination of mechanical stress in clinostated plants. This might be done by supporting the petioles with something more rigid, such as wooden splints; but, torsional strains would still occur at the petiole-stem junction, as well as the blade-petiole junction. Further, using such supports, even if each one were built to conform to an individual leaf, would involve some form of attachment to the stem which in itself might cause a "contact stress" (thigmomorphogenesis). Nonetheless, I attempted one experiment in which wire supports were fastened to petioles. These supports were shaped to the original angle of the unstressed petiole. Approximately 2 cm of the wire ran parallel to the stem and was attached to the stem just below the petiole. Supports were attached with strips of tape. Measurements were taken at 6-h intervals and compared to non-supported clinostated plants, supported and nonsupported upright, sta-



tionary controls. The entire experiment lasted 24 h, while three leaves of clinostated plants were released as 12 and 24 h. Epinasty measurements were taken before release and 10 min after release of supported petioles.

A second approach to this would be preventing the petioles from flopping back and forth during the rotation of positioning supports such as pot stakes on either side of the petiole, allowing up and down movement but restricting lateral movements. One clinostat experiment using this method was conducted in which three plants each had two leaves supported to prevent lateral movement and were compared to nonsupported controls. Bamboo pot stakes (bound together by masking tape) were stuck into the soil of the pots and positioned on each side of two petioles of each of the three supported plants. Shorter bamboo stakes (which did not reach the leaves) were stuck in the soil of the control pots to equalize any root damage.

In another attempt to support clinostated plants' leaves, the plants were placed in rigid plastic (PVC) cylinders (with one end permanently closed), 40 cm in length, and 30 or 38 cm in diameter, and packed around the stem and leaves with vermiculite. With the volume of the cylinders taken up by the vermiculite surrounding the plants, the remaining open end could then be sealed and the cylinders attached to the clinostat. Mounting was done by attaching the cylinders with wing nuts and bolts to circular wooden forms bolted to the clinostat wheels. Control tests were conducted in the upright position (vertical clinostat). Problems were encountered initially due to the tumbling action of the vermiculite within the rotating cylinders, but this was overcome by placing 10- or 8-cm wide plastic baffles on opposite sides of the cylinders, and repacking the cylinders after a short period of rotation to fill any gaps.



Similar experiments were conducted with inverted plants. That is, placing plants in 18.9 l (5 gal) plastic buckets, and then firmly packing vermiculite all around plants, and sealing the buckets. The plants could then be inverted every 20 min, with controls inverted and immediately returned to the upright position, as in other inversion experiments. Compression and settling of the vermiculite were often a problem in these experiments also, so gaps in buckets were refilled during the first several hours of the experiments. Many of the inversion and intermittent clinostat experiments were conducted with the aid of Dr. Frank Salisbury, Julianne Sliwinski, Wesley Mueller, and Mary Jo Hansen taking shifts at flipping the plants every 4 or 20 min during the 24 or 48 h tests.

Another method that I attempted for eliminating mechanical stress in clinostated plants' petioles was by lightening the weight load. This was done by excising the lamina of the leaf. The epinastic effects of deblading, along with decapitation, were observed for clinostated cockleburs. This test was followed with a deblading test in which IAA (1%) in lanolin paste was added to the petiole stumps of three of the plants on the clinostat. This test was subsequently followed by observing the effects of IAA (1%) on the bending of debladed petioles of upright plants on the greenhouse bench.

#### Gravitropism Experiments

Shoot gravitropism experiments were usually carried out in the dark (with a few in the greenhouse). Dark experiments were conducted in a temperature controlled room, with most experiments run at 25-27 C. For placing plants in a horizontal position, pots were fastened to a board by placing wooden slats on each side of the stems of a row of



five plants, and binding the slats to the board (max. ten at once), after which the entire board was suspended vertically, with the stems in a horizontal position. In later experiments, pots were placed horizontally on specially built wooden racks (built by Dr. Frank B. Salisbury) which held the tops of the pots in a nearly vertical position ( $\pm 2$  or  $3^\circ$ ). The racks could accomodate more than 50 plants.

As plant shoots curved in response to gravity, stem angle measurements were made by shaping a pair of drafting dividers to the angle formed between the apex or terminal bud region (ca. upper 0.5 cm of cocklebur and pepper stems and the upper 1.0 cm of castor bean and tomato stems) and the lower, nonelongating region of the stems. The opening of the dividers was then measured with a protractor. Dark experiments' measurements were made in green safelight (25-W incandescent light filtered through a layer of green and a layer of blue plexiglas). Several other experiments used photographed silhouettes against a dim green background.

The change in the stem angle as the plants responded to gravity could be recorded as the actual angle formed between the stem and the terminal; that is, starting at  $180^\circ$  or straight, and bending upward toward the vertical of  $90^\circ$ , or as the supplement of this angle, calling the starting position  $0^\circ$ , and the final vertical position  $90^\circ$  (see Fig. 17).

The effects on stem gravitropic bending of the four previously mentioned ethylene antagonists (AVG,  $\text{Ag}^+$ ,  $\text{CO}_2$  and  $\text{Co}^{2+}$ ) were tested. Controls for 2.0 mM  $\text{AgNO}_3$ -8.0 mM  $\text{Na}_2\text{S}_2\text{O}_3$  tests were treated with 8.0 mM  $\text{Na}_2\text{S}_2\text{O}_3$ , while controls in cobalt tests ( $\text{CoCl}_2$ ) were treated with  $\text{NaCl}$  or  $\text{MgCl}_2$  at equivalent concentrations. Salt solutions of  $\text{Na}_2\text{S}_2\text{O}_3$  (8.0

mM), NaCl (1.0 mM), and  $\text{MgCl}_2$  (1.0 mM) were also tested independently against untreated plants. The chambers and flow system used for  $\text{CO}_2$  inhibition of clinostat epinasty and gravitropic response are shown in Plate 10. The clinostat wheels were kept stationary during gravitropic tests, while three chambers were treated with constant-flowing  $\text{CO}_2$ -enriched air and the other three simply had air pumped through at an equivalent rate (ca.  $100\text{--}200\text{ ml min}^{-1}$ ). Inhibitors were administered approximately 30 min prior to placing plants horizontally by either dipping or spraying the plants, while  $\text{CO}_2$ -enriched air was run through chamber at a more rapid rate for 30 min prior to turning them horizontally.

Treatments with the ethylene inhibitors were found to delay shoot gravitropic bending (see Results Section), so several tests were conducted using a pretreatment of AVG, followed by restoring ethylene to the plant. Note, AVG blocks ethylene synthesis in plants, but not the action of exogenous ethylene.

In one experiment, six cocklebur plants were sealed in plexiglas chambers (35.3 l), while four of these were pretreated with AVG (1.0 mM), and two were untreated controls. Ethylene was added to two of the AVG treated plants' chambers, bringing the internal concentration up to  $100\text{ nl l}^{-1}$  (ppb), and all were turned horizontally for stem bending measurements. Higher concentrations of both AVG (10.0 mM) and ethylene ( $10\text{ ul l}^{-1}$ ; ppm) were also tested.

Ethylene was also applied to AVG-treated (1.0 mM) plants in the form of an ethephon solution (0.1%). The ethephon was swabbed onto the epidermis of either the top or bottom sides (with regard to a horizontal stem) of AVG treated cocklebur stems, and their bending was compared



to AVG treated check plants and untreated control plants. Five plants were used in each treatment.

In order to more accurately interpret the results from the preceding experiments, the effects of ethylene on non-AVG treated, gravitroping cocklebur plants was also observed. Plants in plexiglas chambers were treated with concentrations of 10, 1, and 0.1  $\mu\text{l l}^{-1}$  of ethylene, and their stem bending was observed along with controls similarly sealed in chambers, but with no ethylene added. Three plants were used in each treatment.

#### Decapitation-Defoliation Experiments

Several experiments were conducted to observe the effects of removal of the leaves or the leaves and apical bud on the gravitropic bending response in cocklebur stems. Pretreating plants with AVG (1.0 mM) followed by decapitation and defoliation treatments was also tried. All experiments of this type were carried out in the dark.

#### Mechanical Stress Effects on Gravitropism

Jaffe and Biro (1979) reported that mechanically stressed plants respond slower gravitropically than unstimulated controls. Experiments were carried out testing this observation by vigorously shaking cocklebur plants by hand for 120 s prior to placing them horizontally. In another test, cocklebur plants were attached in a horizontal position to the platform of a mechanical shaker (horizontal mode) and constantly shaken (90 cycles  $\text{min}^{-1}$ ) as they responded gravitropically. Interestingly, Haberlandt (1903) subjected plants to shaking prior to laying them horizontally in an attempt to sensitize them to gravity by stirring stato-

liths in gravity perceiving cells.

### Hormonal Deflection Experiments

Since preliminary evidence indicated a possible essential role for ethylene in shoot gravitropism of cocklebur, tomato, and castor bean, several tests were conducted to directly observe effects on bending (if any) of exogenously applied ethylene and other growth regulators.

In the case of ethylene, ethephon solutions (1%) formed by mixing 'Florel' (3.9% ethephon) with water were applied with cotton-tipped applicator sticks to one side of the apical 10 cm of cocklebur, tomato, and pepper stems. The side of application was marked with a spot of India ink, and the plants were fastened to the clinostat and rotated horizontally. Plants were placed on the clinostat in an attempt to overcome natural gravitropic tendencies that might dominate in upright plants, thereby accentuating any hormonal-induced bending. Six plants could be clinostated at once; therefore, three ethephon treated and three untreated plants were observed in each test. Untreated controls are particularly important in these tests, due to the deflected stem growth sometimes reported for clinostated cockleburs. (Note, however, that these observations were recorded using plants attached to the perimeter of a large wooden wheel that slowly rotated; Hoshizaki and Hamner, 1962.) The degree and direction of bending were measured for each plant using a pair of dividers to form the angle of the stem and a protractor to measure the dividers.

IAA (crystals mixed into lanolin paste heated to 50 C in a water bath) was applied at 0.01, 0.1, and 1.0% (wt) concentrations to one side of tomato and cocklebur stems, after which plants were attached



to the clinostat. Lanolin pastes of naphthalene acetic acid (NAA, 1%), gibberellic acid ( $GA_3$ , 1%), kinetin (1%), and abscissic acid (ABA, 1%) were also used in unilateral application tests with cocklebur and tomato, and stem deflection was observed. In each case, control plants treated only with lanolin were observed simultaneously. Pastes were applied with wooden applicator sticks.

Auxins are known to be powerful ethylene stimulants, so application of IAA and NAA 1% pastes were crossed with pretreatment of half of the plants with 1.0 mM AVG. The auxins (known to stimulate cell elongation) would be expected to cause deflection away from the side of application, while ethylene (known to inhibit cell elongation) would be expected to cause deflection toward the side of application. The AVG might therefore enhance the auxin-induced bending, since AVG is known to block auxin-stimulated ethylene (Yu and Yang, 1980). Similarly, ethylene is known to be autocatalytic, therefore AVG pretreatments were coupled with ethephon applications to see if ethylene-induced bending could be affected. In contrast to expected results above, the AVG treatment in this case might be expected to have an abating effect upon deflection; that is, the straight ethephon application might lead to more ethylene than that supplied by the ethephon, thanks to autocatalysis, while the AVG pretreatment might supply only the ethylene released by the ethephon. This is speculative however, since AVG's effects on endogenous ethylene autocatalysis have not been tested.

#### Ethylene Measurement

In an attempt to correlate more closely the basic physiological responses between mechanically stressed and clinostated plants, a system



was set up to measure the ethylene evolved from *intact plants*, enclosed in plexiglas cylinders, under constant gas flow conditions. The system was modeled after one described by Bassi and Spencer (1979), in which plants were placed in ethylene-free cuvettes and maintained under constant gas flow conditions. The air from the chambers was then cycled through a silica gel cold-trap in order to absorb any ethylene (and other hydrocarbons) from the plant or plant organ within the cuvette.

Past endeavors to measure ethylene evolution related to clinostating have used excised organs or tissue (Leather et al., 1972; Palmer, 1973), but such procedures are subject to wound ethylene pollution, as well as to the autocatalytic enhancement of ethylene of its own production. Even though time curves have been calculated for the drop off of wound ethylene, measurements of evolution rates of minute quantities of ethylene still might be clouded by tissue wounding (Bassi, personal communication, 1979). Along with this, excising the tissue for enclosure prior to the measurement removes the treated tissue from the stress treatment, which also presents problems in trying to correlate measurements to the treatment. In this regard then, a constant-flow system of measurement on intact plant tissue is highly desirable.

In construction of the system, except for the plexiglas chambers, all gas conducting components between the plant and the cold trap were teflon, glass, or copper. Eastwell et al. (1978) have shown that these three materials (along with other metal tubing, e.g., stainless steel) are all safe with respect to ethylene pollution, providing the customary oil film, lining the metal tubing, has been scrubbed out or washed clean.

Six cylindrical chambers, one end open and one end covered, made of 0.6 cm plexiglas, were built (USU Technical Services) to fit over



the clinostat wheels. Each cylinder was 30 cm in diameter and 50 cm in height (see Plate 10). A plexiglas ring, 2.5 cm wide and 1.3 cm thick, was glued to the bottom (open end) of each cylinder, forming a circular flange by which the chambers could be attached to the clinostat. Attachment was done by drilling eight holes in each circular flange which could be slipped over eight screws mounted on a piece of plexiglas backing, permanently attached around the shaft of each clinostat wheel. A partial turn of the cylinders would force the narrower end of the holes in the flange against the screws, which could then be tightened down into pretapped holes in the plexiglas basal piece. Airtight sealing was provided by a rubber tubing "O" ring gasket, partially sunken into a groove running the entire circumference underneath each circular flange.

Air was drawn through the cylinders from a basal port, to which a flexible piece of teflon tubing (0.5 cm) was attached. The teflon tubing led on to a gas-bubble wash tube, in which the air stream bubbled through a saturated KOH solution for removal of  $\text{CO}_2$  and some of the water, prior to the cold trap. This was required to avoid clogging the trap with frozen  $\text{CO}_2$  and ice. Short sections of copper tubing (8-10 cm) were fused to the glass inlets and outlets of the gas-bubble wash tubes. The incoming teflon line could then be attached to the metal tubing section with "Swagelok" gas fittings.

A section of flexible teflon tubing was attached to the outlet of the bubble tube, on which the cold traps could be attached. Traps consisted of 0.6 cm ( $\frac{1}{4}$  inch) copper tubing, bent into "u" shapes, with Swagelok fittings at each end. The traps could then easily be attached and removed from the flexible teflon line leading from the bubble tube.

Later, I found it convenient to divide the trap and the teflon line with a brass needle valve for finer control of flow rates. Each trap contained about 0.5 g of white silica gel (60-80 mesh), held in place by small wads of glass wool stuffed into each end.

For trapping the ethylene, "U" tubes were submerged in a dry-ice-acetone slurry (tem.  $-78.5^{\circ}\text{C}$ ) held in a pyrex beaker, surrounded by three styrofoam cups. The cups were supported on an adjustable ring-stand, permitting easy lowering of the cups from below the traps.

The other end of the trap was connected with a length of tygon tubing to a Matheson (#603) flow meter, which was in turn connected with another tygon section leading to an electric, diaphragm gas pump (Neptune Dyna Pump, #3). The pump was used in a suction capacity to draw air through the entire system. Flow rates could be controlled with the needle valve connecting the teflon line, and also with the needle valve control on the flow meter.

Flow rates were usually set at  $600\text{ ml min}^{-1}$  or less, with maximum rates for the whole system being restricted by the silica gel packing of the trap. Maximum rates had to be carefully adjusted to avoid blowing the silica gel grains out of the traps.

Once the trapping was complete, the "U" tubes were disconnected and sealed with rubber serum stoppers, after which cooling was no longer essential. A single trapping run usually lasted 15 to 20 min. It was difficult to go much longer than this because of ice formation in the traps. Traps could then be transferred for analysis, using a Hewlett Packard 5080 A gas chromatograph. Ethylene was released from the silica gel absorbant by submerging the sealed traps in hot water (ca.  $80^{\circ}\text{C}$ ) for several minutes, and then withdrawing syringe samples of the internal



gas for G. C. injection. Six individual traps were used, ranging in size from 5.25 to 5.60 ml in volume. The silica gel (0.5 g) occupied approximately 0.8 ml of the trap volume.

The gas chromatograph was equipped with a flame ionization detector, with the oven temperature set at 50 C, and injection ports and detectors at 225 C. A 61 cm Poropak column (100 mesh) was used, with nitrogen carrier gas. Standard samples of 1%, 1000 and 100 ppm ethylene were obtained from Alltech Associates, and injected prior to each sampling run.

During trapping, background ethylene pollution (from the laboratory or greenhouse) could be removed by first passing the air stream over a heated platinum catalyst (800 C), thereby oxidizing all hydrocarbon pollutants to CO<sub>2</sub>. This was accomplished by packing a 1.2 cm stainless steel pipe with platinum-coated asbestos wool. The temperature of the pipe could then be controlled by placing it in a tube furnace (Lindberg, max 1010 C). Operating temperatures of 800 C are sufficient to purify the air of hydrocarbons (Eastwell et al., 1978).

The furnace was connected to the clinostat chambers via a copper coil (to permit cooling and mobility for moving the clinostat between vertical and horizontal positions), which led to a brass manifold, welded to the side of the clinostat platform. Six separate copper tubing lines could connect the manifold to any of the six chambers, with the gas flow of each line being controlled by an "on-off" brass valve mounted on the manifold (see Plate 10). The manifold could also separate incoming flow between the three upper and the three lower sets of valves with one dividing valve between the two sets. This separation of flows between the three upper and three lower valves was also used for CO<sub>2</sub>-enriched



gas flow treatments in both clinostat and gravitropic studies (as described above).

Several possible sources of error exist in such a constant flow system. First, leaks around the seal of the shaft driving the clinostat might reduce the amount of air coming from the heated platinum catalyst purifier. However, if the background ethylene were sufficiently low, the purification system could be bypassed, and such a leak would not matter in the remaining open system. Since this system is driven by suction, other minor leaks would also be tolerable, providing one can still measure the amount of air coming from the chamber. That is, if too many leaks exist between the chamber and the trap, one will underestimate the evolution rate of ethylene, even though the total amount of ethylene being drawn from the chamber will pass through the trap. Minor leaks were tolerable as long as the system remained unchanged between different treatments, but any statements as to the absolute evolution rate would require accurate measurement of the air flowing from the chamber. Note though, gas fittings were used at all couplings of tubing sections, and in addition, all male couplings were wrapped with teflon tape, and every connection, except for the immediate connection between the trap and the incoming flow lines, was wrapped around the outside with teflon tape.

A second source of error might arise from "other" sources of ethylene. Plexiglas exposed to intense light can evolve minute quantities of ethylene (Bassi, personal communication, 1979). The grease sealant around the bearings of the shaft driving the clinostat wheel enclosed in the chamber, might also be a source of some pollution. All other valves were sealed with silicon vacuum grease, which is relatively safe with



regard to ethylene pollution. The rubber septa used to seal the traps after trapping runs might also evolve small amounts of ethylene (Eastwell et al., 1978). However, if all of these factors are kept constant between runs, their presence can be subtracted out by conducting appropriate control tests. Perhaps the single largest source of ethylene pollution in studies of plant shoots using this system might come from the soil and roots in the pot enclosed in the chamber. However, the difficulty in designing a moveable seal around the plant stem necessitated enclosure of the entire plant and pot for this approach. But here again, suitable controls tests could be conducted to determine the contributions of the soil to the ethylene count.

#### Restricted Gravitropism

Several exploratory experiments were conducted on plant reactions in a restricted gravitropic situation; that is, restricting a horizontal shoot rigidly in a straight position to prevent it from bending upward.

Restriction of bending has been observed by several researchers. In one of the earlier references, Anna Bateson and Francis Darwin (1888) pinned *Plantago lanceolata* inflorescences to a piece of board and measured the springing action upon release. They refer to earlier works by Sachs and stated that this springing action after release of restricted flower stalks was a "well-known" result.

Two methods of shoot restriction were attempted in my studies. Firstly, cocklebur plants were placed in 18.9 l (5 gal) plastic food buckets and packed securely with vermiculite on all sides before sealing. The buckets were then placed on their sides, and plants were released (unpacked) at 2-h intervals to observe the immediate springing effects

(*stored bending energy*) upon release. Several plants were set upright after unpacking and measured again after 2 more hours to observe the *stored bending stimulus*.

In a second experiment, two wooden dowels 46 cm (18 in), braced with tape to a cross-piece of wood 9 cm by 15 cm at one end, were forced into the soil on each side of a cocklebur stem. The lower ends of the dowels to be driven into the soil were sharpened in a pencil sharpener to facilitate positioning. Strips of masking tape were attached between each dowel, with the nonstick side held taut against the stem. A total of five or six tape strips were usually required, with each tape strip alternated between sides of the stem, thereby bracing the stem against any movement perpendicular to the plane formed by the two dowels and the stem. Alternating strips also prevented a tendency of downward pushing beneath an upper restriction surface.

One half of the plants used in the taping experiment were sprayed with 1.0 mM AVG. At 2-h intervals, beginning at 4 h, three controls and three AVG plants were removed from the dark room and released from restriction by rapidly cutting away the masking tape supports with a razor blade. The angle immediately formed upon release was measured, then plants were set upright on the greenhouse bench for 2 more h, after which they were again measured to record the stored bending stimulus. Concurrently, taped plants were also positioned horizontally in the greenhouse with its normal 18-h light cycle. Three control and three AVG plants from the greenhouse were released at 14, 26, 38, and 50 h to compare restricted responses between light and dark conditions. A set of unrestricted control and AVG plants (three of each) were also tracked during the experiment, for both light and dark conditions.



Both experiments were conducted with the aid of Dr. Frank B. Salisbury, Wesley Mueller, Julianne Sliwinski, and Mary Jo Hansen in standing shifts for releasing and measuring of plants. This group also aided in setting up the other vermiculite tests.

### Statistical Analyses

For all gravitropism experiments treatment means were calculated and plotted. Standard deviations were also calculated, after which each was divided by  $n^{\frac{1}{2}}$  ( $n$  = the number of plants) to figure a standard error of the mean. Bars representing plus or minus the standard error value for several means along each curve were drawn (usually at 6 h intervals), and the degree of statistical differences was estimated visually from these. If the bars of two curves overlapped, they were considered statistically the same, and if they did not overlap, they were considered statistically (significantly) different.

Results from certain crucial leaf epinasty experiments were tested using an analysis of variance program (HSCF, UCLA) in the Utah State University STATPAC program series. This included: (1) the experiment comparing the effects of ethylene inhibitors on cocklebur leaf epinasty (see Fig. 4); (2) the intermittent clinostat experiment with cocklebur (see Fig. 9); (3) the comparison between inversion, clinostating, and AVG treatment (see Fig. 14); and (4) a second experiment comparing inversion and clinostating along with the effects of simulating the leaf displacements of inverted plants (see Fig. 16).

The analyses of variance run on all the data from a given experiment would yield a calculated "F" value and give two degrees of freedom values from which a second F value could be determined in an F distribu-

tion table. If significant differences were found after comparing these values, the means could then be ranked in a Duncan's multiple range test and the treatments compared to one another at any time into the experiments. The Duncan's test yields critical values of the Studentized range for comparing means that are numerically ranked. If two means are separated by more than this critical value, they are significantly different. Fisher's Least Significant Difference (LSD) values were also calculated for the above experiments using the AOV results (see Ott, 1977).



## RESULTS

### Clinostat Responses

Figure 2 shows the typical epinastic response through time of three cocklebur plants subjected to clinostating (controls). The epinasty is plotted as an increase in the reciprocal of the results of curvature. (The absolute radius is also shown as a decreasing scale on the right side of the graph.) The response of three other clinostated cockleburs, dipped in 2.0 mM  $\text{AgNO}_3$  just prior to attachment to the clinostat is also shown. Silver nitrate treatment is known to block ethylene action in plants, and in this case is temporarily delayed the onset of leaf epinasty. Figure 3 shows the results from subjecting tomato plants to clinostating. In this case, the silver delay is not observable, but plants treated with AVG (ethylene synthesis inhibitor) show a marked delay in epinasty.

A clearer comparison of epinasty in clinostated cockleburs can be seen in Figure 4. In this case, the absolute radius scale had been eliminated, and all treatments have been normalized to zero at the beginning by subtracting the zero-time measurement from all subsequent readings for a given plant. This experiment was conducted in the greenhouse, with its normal 18-h day lighting pattern, and the delaying action of silver and AVG on the epinastic response can be seen. Stationary control plants are also compared to the clinostated ones, to give an indication of the magnitude of normal daily movements of the leaves.

### Ethylene antagonists

The essentiality of ethylene in clinostat-induced epinasty is shown

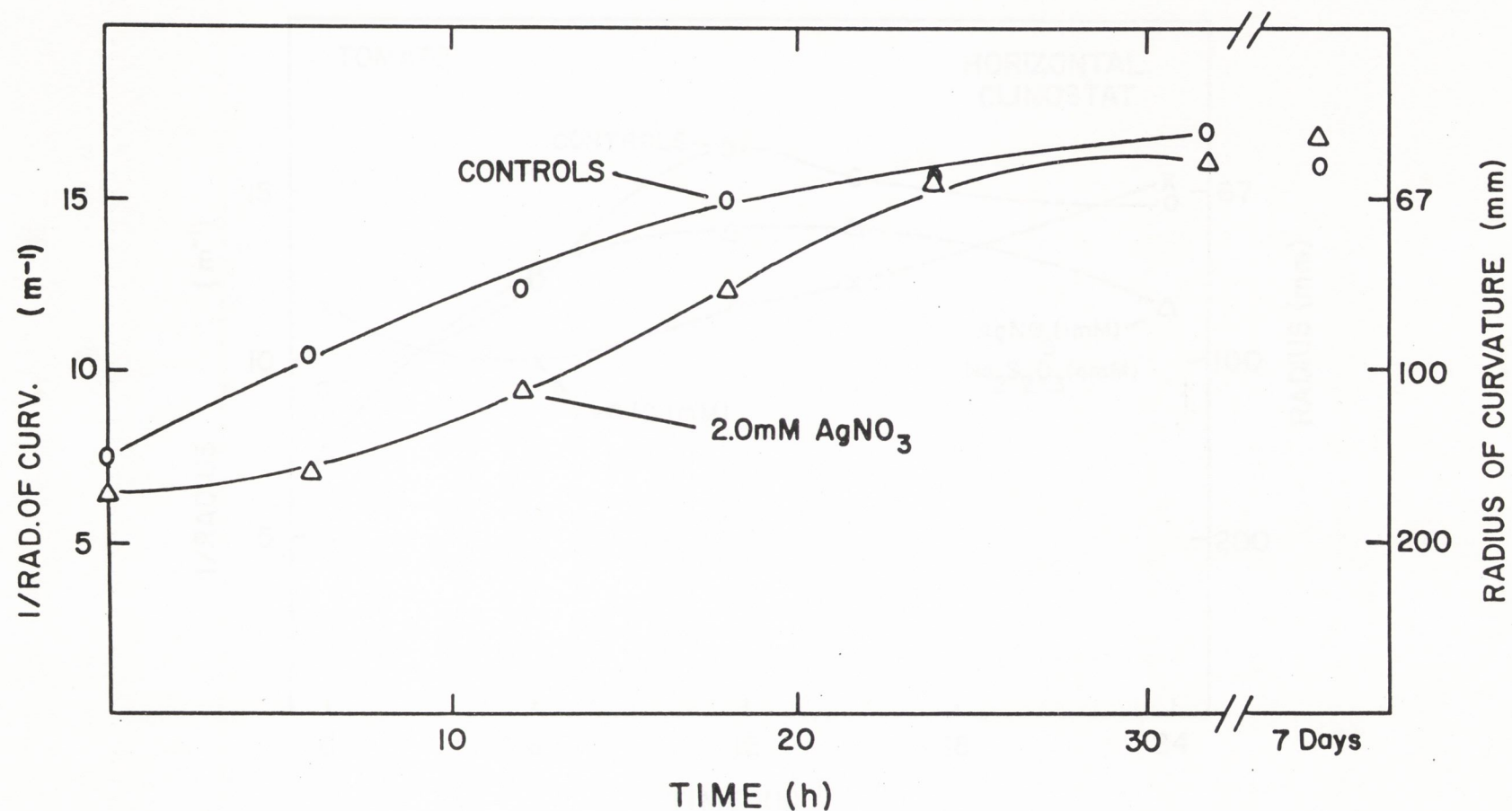


Figure 2. Plot of the change in the inverse radius of curvature of cocklebur leaves caused by clinostating through time. Three plants dipped in 2.0 mM AgNO<sub>3</sub>, an inhibitor of ethylene action, showed a delayed epinastic response. Each point represents the average of six leaves from three different plants.



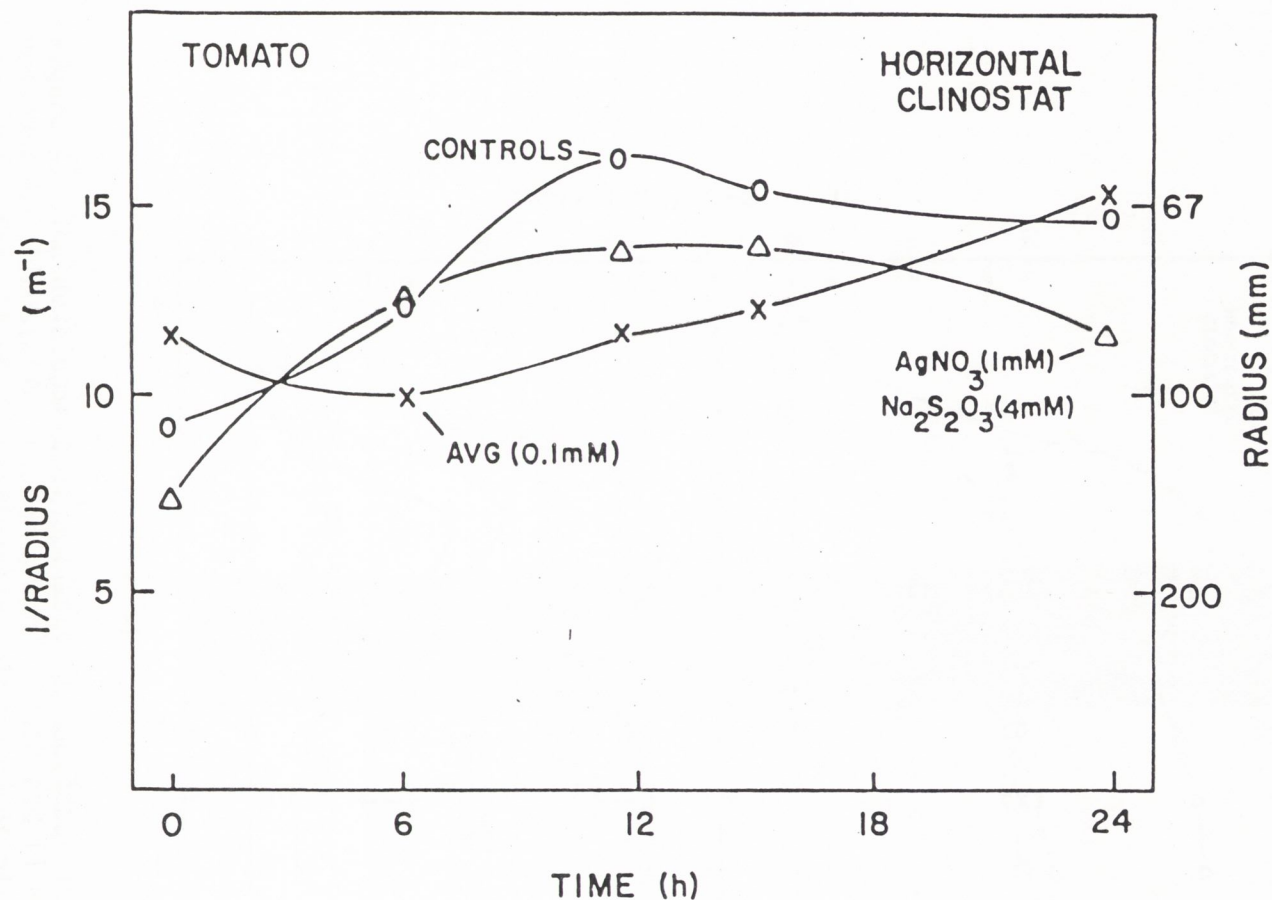


Figure 3. Inverse radius-of-curvature plot of tomato leaves subjected to clinostating. Two plants were dipped in 1 mM silver thiosulfate (an inhibitor of ethylene action), two in 1 mM AVG (an inhibitor of ethylene synthesis), and two plants were untreated controls. The AVG delayed the onset of epinasty, while the silver had little effect in this experiment. Each treatment's data are averages of four leaves from two plants.

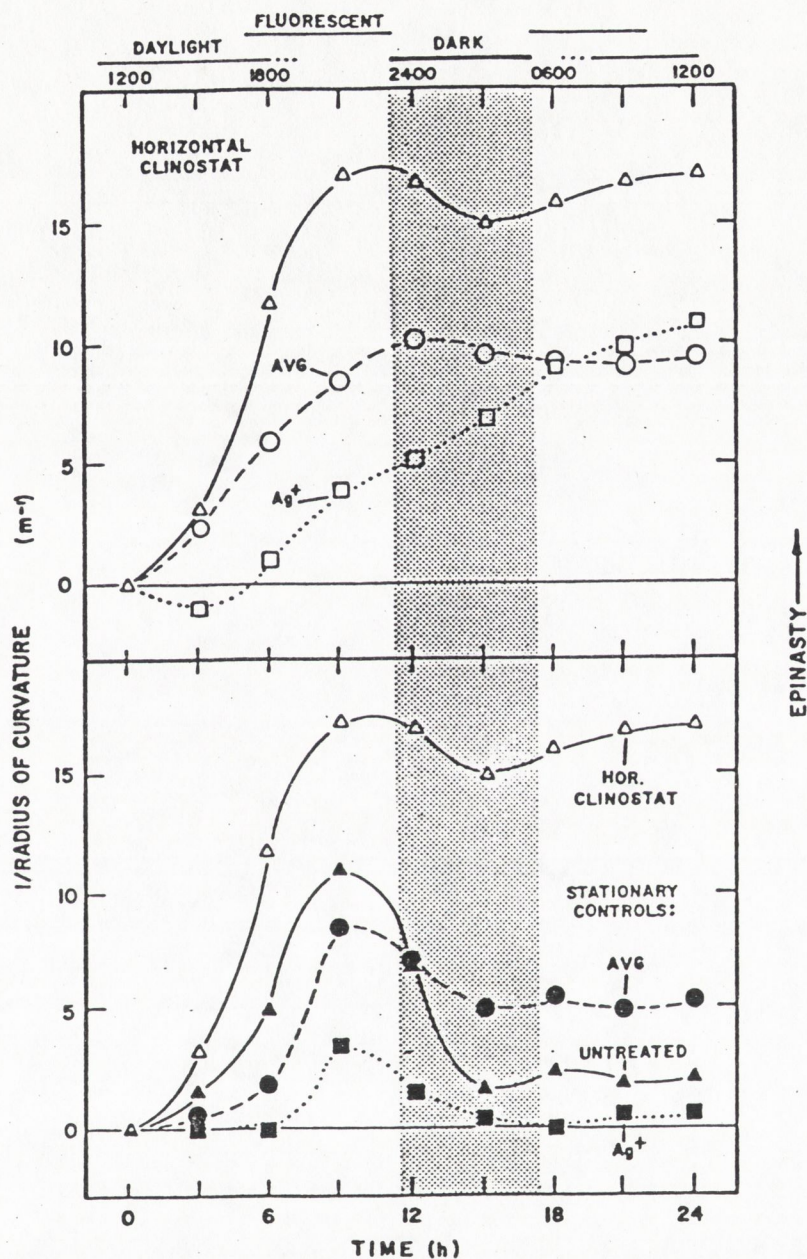


Figure 4. The development of epinasty in cocklebur leaves in response to clinostating (upper graph). Both AVG (1 mM) and silver thiosulfate (1 mM) significantly delayed onset of epinasty. The bottom graph shows the changes in epinasty of stationary plants throughout the same day. AVG and silver damped the daily leaf movements. Note that leaves became epianstic at dusk and straightened during the dark period. Each point is the average of four leaves from two plants. In contrast to the previous graphs, the absolute radius scale has been eliminated, and all inverse radii have been normalized to a zero starting point.  $LSD_{0.05}=3.94$  (see Appendix B for analysis of variance and Duncan's test).



clearly from the results in Figures 2, 3, and 4. Generally, both the silver and the AVG delay onset of epinasty and become less effective through time, that is, they cannot completely block the epinasty. Use of concentrations higher than the 1.0 mM range often have other damaging or lethal effects, such as severe chlorosis (AVG), aberrant growth (AVG), or leaf burn ( $\text{Ag}^+$ ). The AVG is consistently effective at slowing epinasty, while the silver delay is not always as marked. Frequently near the end of clinostat experiments, silver-treatment plants show slightly more epinasty than the untreated controls. This may be due to silver's capacity to induce ethylene evolution, possibly through a heavy metal toxicity or wounding action. However, initially its ethylene action inhibition overrides this (see below). AVG is also highly effective at slowing clinostat-induced epinasty in tomato and pepper.

### Mechanical Stress

#### Simulation of mechanical stresses.

A comprehensive comparison of the effects of one week of mechanical shaking treatments, either by hand (a form of thigmomorphogenesis) or by placing pots on a mechanical shaker (seismomorphogenesis), and clinostating are shown in Table 3. Each treatment combination represents the average results from six cocklebur plants, with each of the clinostat treatments being averages of three plants.

Several result trends can be seen. The vigorous hand-shaking ( $120 \text{ s day}^{-1}$ ) and the clinostating reduced growth the most, and in all but the hand-shaking, the silver thiosulfate reduced shoot growth compared to similar mechanically treated plants without silver (sprayed with sodium thiosulfate).

Table 3. Comparison of effects of clinostatting and mechanical stress responses of *Xanthium strumarium* L. (cocklebur) after a one week experiment in the greenhouse.

Treatment	Ave. Stem Growth ± S.D. (mm)	5th Internode Growth (mm) Length Diam.		4th Internode Growth (mm) Length Diam.		Ave. Change in 1/Radius of Curva. ± S.D. (m <sup>-1</sup> )
Hor Clinostat	26.3 ±5.5	0.0	0.4	10.7	0.7	8.6 ± 3.6
Hor. Clino. + Ag <sup>+</sup>	21.0 ±2.6	1.7	0.4	4.5	1.0	0.9 ± 2.2
Controls (Measured Daily)	38.8 ±5.8	3.4	0.2	11.0	0.6	0.4 ± 3.4
Controls (Measured Daily) + Ag <sup>+</sup>	37.0 ±4.6	1.8	0.35	10.4	0.55	3.1 ± 2.2
Shaker 8 h·day <sup>-1</sup>	44.5	2.4	0.55	11.8	0.8	1.1 ± 1.4
Shaker 8 h·day <sup>-1</sup> + Ag <sup>+</sup>	39.2 ±7.4	2.0	0.65	9.5	0.75	2.9 ± 2.4
Shaker 120 s·day <sup>-1</sup>	45.3 ±12.5	6.4	0.4	13.2	0.75	2.2 ± 3.3
Shaker 120 s·day <sup>-1</sup> + Ag <sup>+</sup>	30.5 ±4.6	1.1	0.4	5.7	0.5	3.5 ± 3.1
Shaken by Hand 120 s·day <sup>-1</sup>	24.0 ±3.9	1.1	0.2	5.7	0.5	1.7 ± 2.2
Shaken by Hand 120 s·day <sup>-1</sup> + Ag <sup>+</sup>	27.0 ±4.8	0.0	0.5	5.0	0.55	4.3 ± 2.0
Controls (Measured Beg. and End)	40.0 ±6.2	1.5	0.6	10.8	0.6	0.2 ± 1.7
Controls (Beg. and End) + Ag <sup>+</sup>	32.0 ±6.4	3.0	0.35	10.0	0.6	1.0 ± 1.3



Silver has the capacity to induce ethylene evolution in plants, probably through tissue wounding, and this could possibly explain the growth reduction in daily silver-treated plants. No clear trends appear in the comparison of internodal elongation or diameter growth. Clinostating clearly induced much more epinasty than any of the mechanical stress (shaking) treatments. The shaking appears to have little, if any effect upon epinasty. The silver-sprayed plants in each type of shaking treatment show more epinasty than the comparable shaken sodium thiosulfate-sprayed plants. The silver plants of all shaking treatments increased epinasty by about 4 units (reciprocal radius units, see the reciprocal radius plots of epinasty), while the sodium thiosulfate controls changed by an average of 2 units. Thus, daily spraying of cocklebur plants with 2 mM silver solution caused some mild epinasty itself, again, a result not surprising in light of silver's capacity to stimulate ethylene production.

Figures 5 a and b present the epinasty data for the mechanical stress-clinostat experiment in a graphic form. If the starting points are normalized to zero, it is easier to see the difference between the clinostated plants, and the other treatments (Fig. 6). Horizontal clinostating was the only treatment to cause noticeable epinasty after 12 h and this difference persisted after 7 days of continuous treatment (Figs. 5 a, b, and 6).

The shaker used for mechanically stimulating plants is shown in Plate 3 a. It could be operated in a horizontal or vertical fashion (Plate 3 a and b). Plate 3 c shows a cocklebur being twisted when the modifying crank arm connects the clinostat to its motor.

The results of a cocklebur experiment comparing the epinasty induced

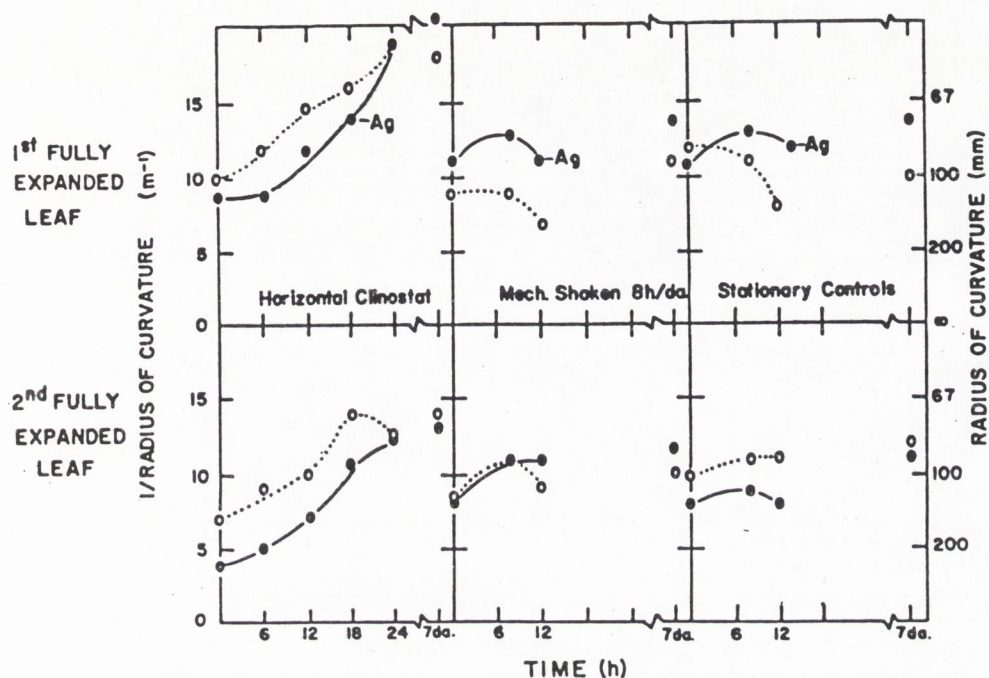


Figure 5 a. The development of epinasty in cocklebur leaves in response to clinostating or mechanical shaking (8 h per day) as compared to stationary plants. Only the clinostated plants showed epinastic response, while silver (2 mM) delayed the onset of this epinasty. Points of the clinostat curve represent averages of three leaves and all other points are averages for six leaves.



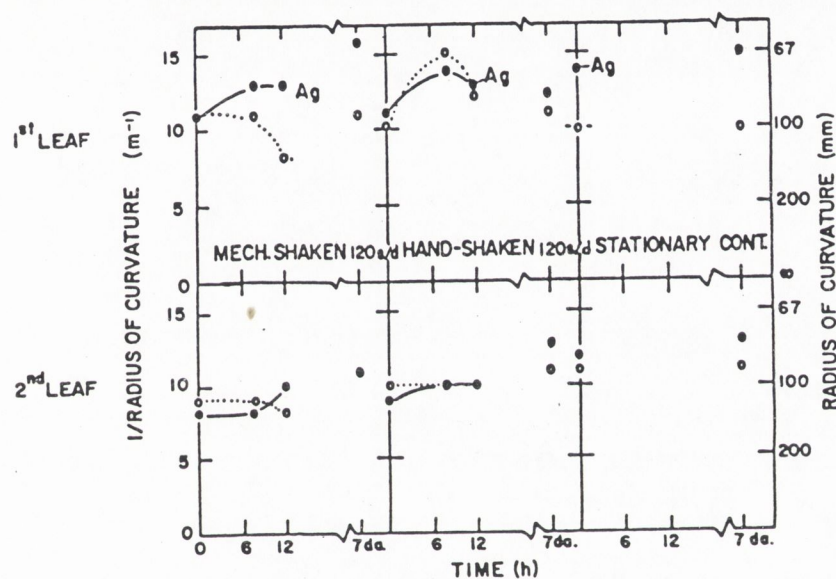


Figure 5 b. The development of epinasty in cocklebur leaves in response to mechanical shaking or vigorous shaking by hand for 120 sec per day as compared to stationary plants measured only at the beginning and end of the 7 day test. Only the leaves shaken by hand showed any epinasty (upper middle), and this was only slight. Each point is the average of 12 leaf radii.

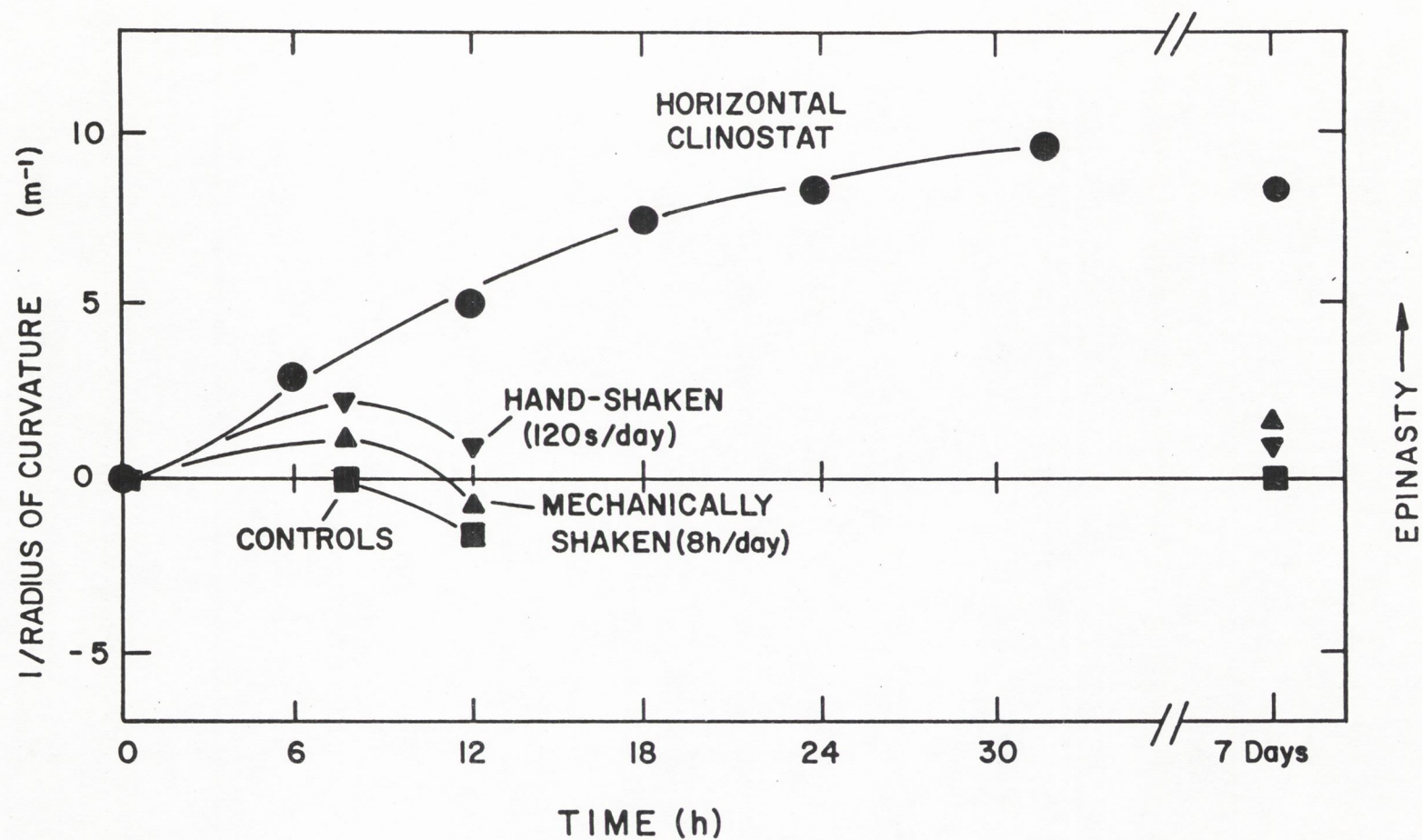


Figure 6. Comparison of epinasty in cocklebur resulting from clinostating, shaking by hand or machine, and stationary control plants. The inverse radii have all been normalized to a zero starting point. Each point is the average of 12 leaves.



by clinostating, twisting, horizontal and vertical shaking are presented in Fig. 7. Unfortunately, the component tests for the Figure 7 graph could not be started at the same times, and the vertical shaking results appear slightly out of phase due to the different starting leaf positions. Nonetheless, none of the attempts to mechanically stress the plants were capable of inducing epinasty to the degree that clinostating does. In a repeat experiment, the starting times for these tests were synchronized; the epinasty comparison between treatments can be seen more clearly in this test (Fig. 8).

Subjecting tomato plants to twisting, and to vertical and horizontal shaking likewise had very little effect upon leaf epinasty (not shown). The mechanically treated plants were slightly more epinastic than stationary controls after 12 h (ca. 1.5-2.0 units), while the clinostated plants were nearly 5 units more epinastic at 12 h. At 6 h the clinostated plants were nearly 8 units more epinastic than controls or mechanically stressed plants.

Figure 9 shows the results of an intermittent clinostat experiment with cocklebur. The intermittently-clinostated plants show no epinasty when compared to the continuously-clinostated plants. The intermittent curves differ insignificantly from the stationary control curve, shown to compare the normal leaf movements of untreated plants. A similar set of results were obtained for both tomato (Fig. 10) and castor bean (not shown).

#### Elimination of mechanical stresses

Another approach to assessing the role of mechanical stress in clinostat-induced epinasty would be to eliminate leaf movement in clinostated plants.

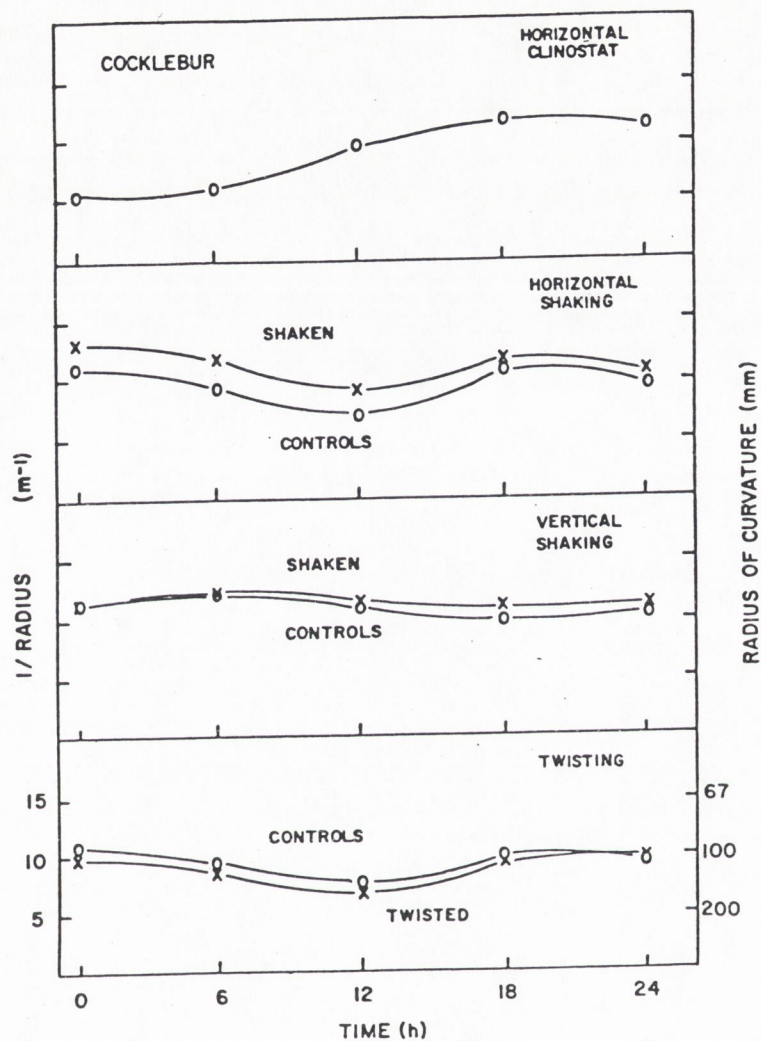


Figure 7. The development of epinasty in cocklebur leaves as a result of clinostating (six-leaf averages), horizontal shaking (12-leaf averages), vertical shaking (four-leaf averages), and twisting the plants back and forth (12-leaf averages). The controls on the lower three graphs represent stationary plants. No difference can be seen between any of the mechanical stresses and stationary controls with regard to epinasty.



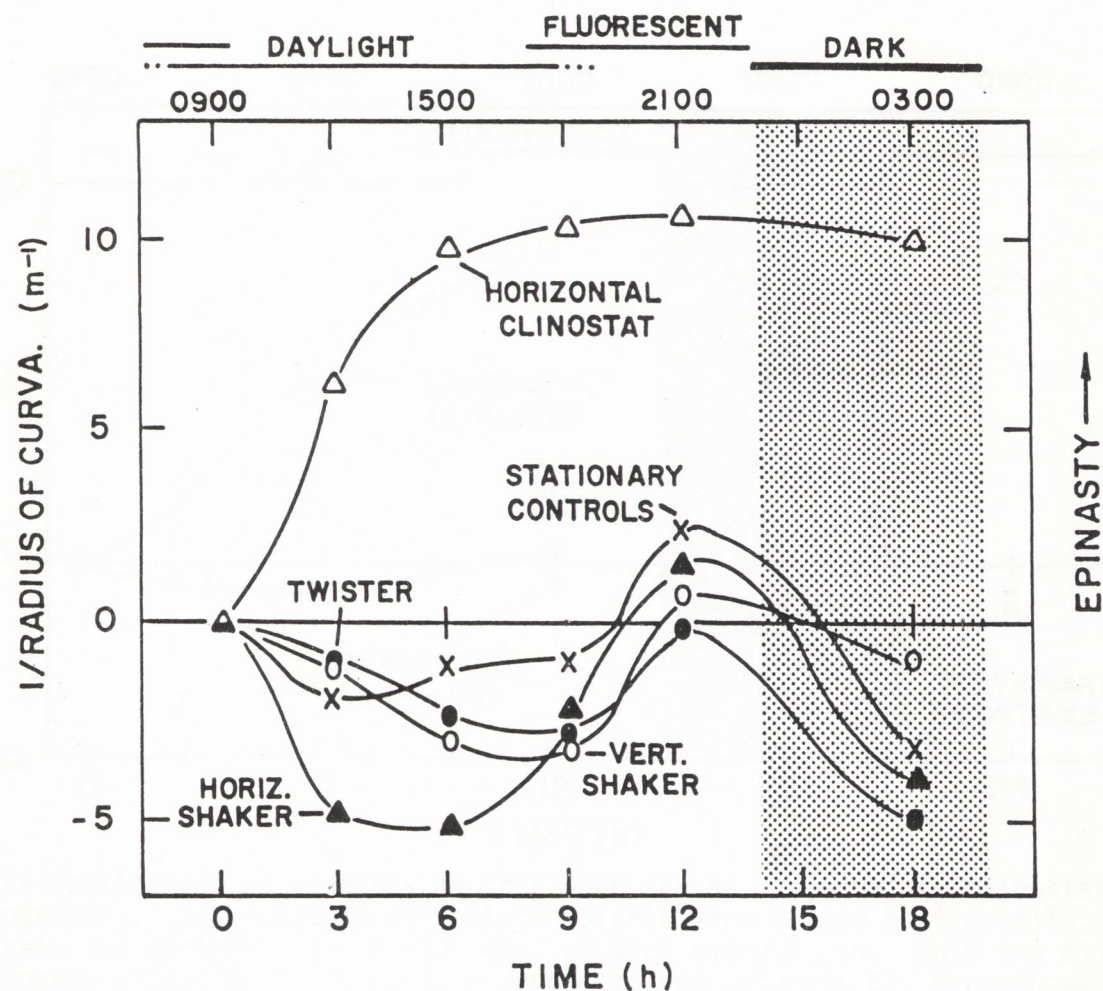


Figure 8. The development of epinasty in cocklebur leaves comparing three forms of mechanical stress, horizontal clinostating, and stationary controls. Little difference can be seen between the forms of mechanical stress and stationary plants, while the clinostated plant showed marked epinasty. Note the slight epinasty at dusk, followed by the typical straightening at night. All points are six-plant leaf averages, except the vertical shaker curve, which represents four-leaf averages.

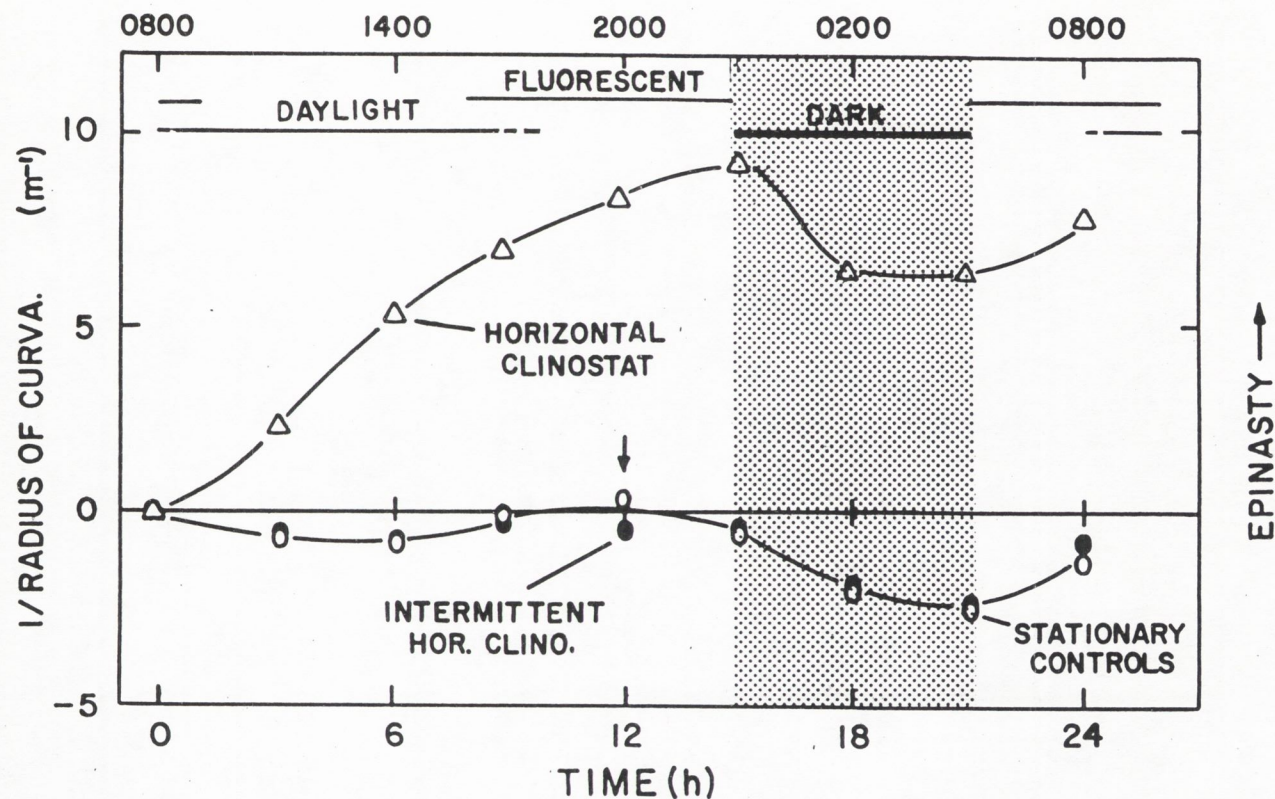


Figure 9. The development of epinasty in cocklebur leaves subjected to clinostating or intermittent clinostating. Intermittent clinostated plants were turned horizontally, rotated rapidly (10 s), and returned upright. The cycle was repeated every 4 min. Only the clinostated plants developed epinasty (significant at 0.01 in Duncan's, see Appendix C), even though the intermittently clinostated plants were subjected to the same mechanical stresses. Points are averages of twelve leaves, except for the clinostated plant, which represents a two-leaf average. The intermittent clinostating was halted at 12 h (arrow), but tracking of epinasty was continued.  $LSD_{0.05} = 1.29$ .



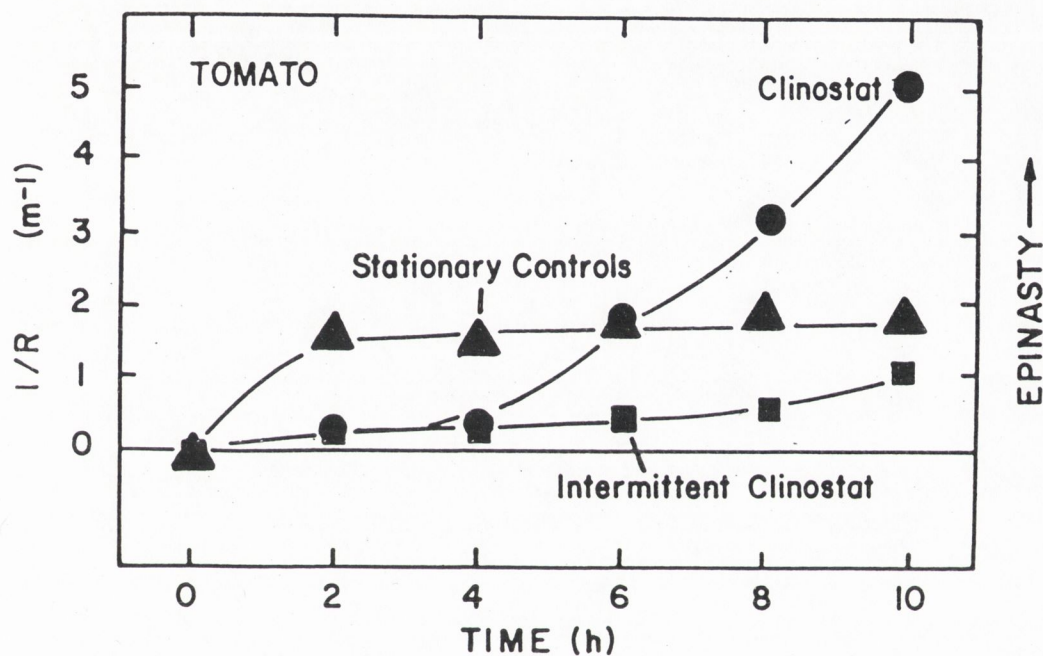


Figure 10. The development of epinasty in tomato leaves in response to clinostating, or intermittent clinostating. As with cocklebur (Fig. 8), only the clinostated plants developed epinasty. Points are averages nine leaves, while the clinostat points are averaged from three leaves.

As a mature cocklebur plant rotates on a clinostat, the leaves shift or flop noticeably as they pass over the apex of their rotation. An example of this can be seen in the series of photographs in Plate 4. The photographs were taken at successive 90° intervals as the plant rotated on the clinostat. Each photo is rotated 90° to keep the plant in the same apparent position for ease of comparison of the leaf movements.

When cocklebur plants were placed in PVC cylinders and packed with vermiculite and then rotated on a clinostat, no marked leaf epinasty developed. After this treatment the leaves appeared quite wrinkled and frequently were bent slightly from contact with the vermiculite, so these results had to be interpreted with some caution. It would also seem likely that this treatment might allow a buildup of gases such as ethylene and CO<sub>2</sub> which would normally be vented.

Bamboo stakes positioned on each side of a leaf petiole by sticking them into the soil of the pot prevent some of the shifting of leaves during rotation. Plate 5 shows a series of photographs similar to those in Plate 4, but in this case two of the petioles were braced against flopping by bamboo pot stakes. The shifting can be reduced by such supports; therefore, if stress caused epinasty, one might expect these plants to undergo less mechanical stress than unsupported plants.

The results of an experiment comparing plants whose petioles were supported by opposing bamboo stakes with completely unsupported plants is shown in Figure 11. There was very little difference in the epinastic movements of both treatments through 24 h of clinostating, although the nonsupported plants appeared slightly more epinastic (ca. 2 units after 24 h).





Plate 4. Cocklebur plant on a clinostat showing the "flopping" experienced by leaves as the plant rotates. Photographs were turned  $90^\circ$  to keep the leaves in the same relative position, and two individual leaves (A and B) have been labeled in all the pictures to easily follow their motion. This leaf flopping causes a strain in the leaf petioles and was thought to produce the resultant epinasty typical of clinostating (see text).



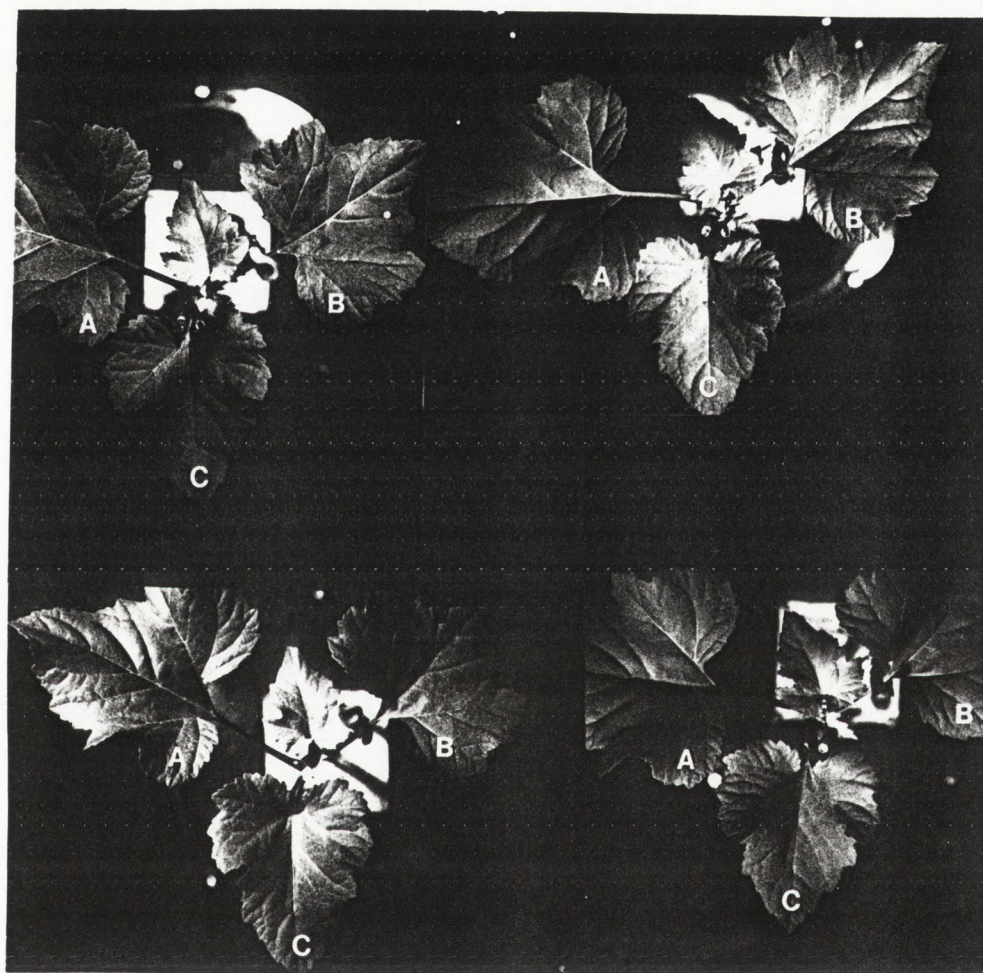


Plate 5. Cocklebur plant on a clinostat showing reduced leaf flopping after the petioles (B and C) have been braced against lateral movement by bamboo pot stakes. Two bamboo stakes held together with tape were driven into the soil and positioned on each side of a petiole to prevent lateral strains, but still allow epinastic curvature. An unbraced leaf (A) is also shown for comparison. Such prevention of "mechanical stress" in the leaves did not prevent epinasty.



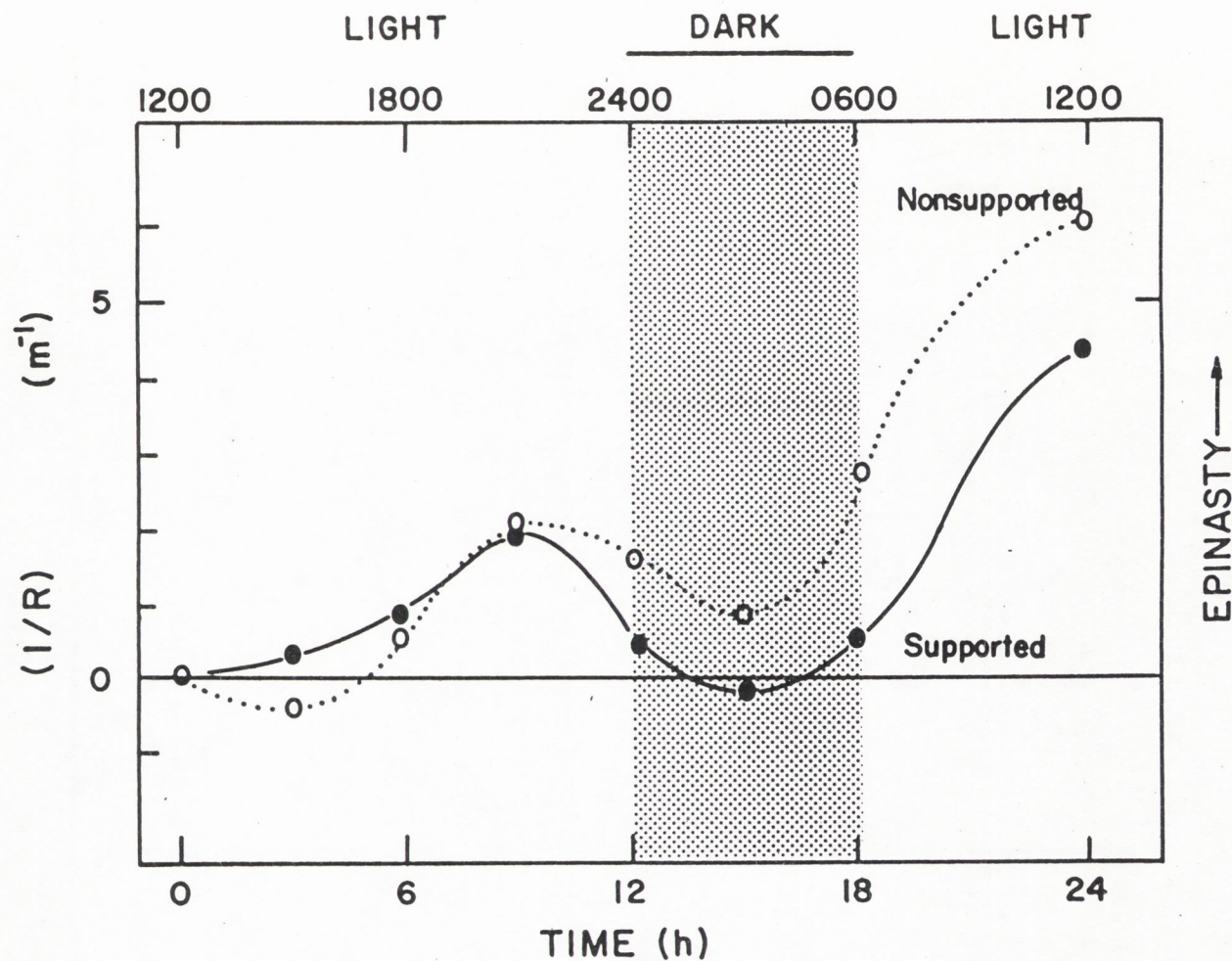


Figure 11. The development of epinasty in cocklebur leaves with their petioles supported from lateral movement or flopping during rotation by wooden stakes bracing each side, as compared to nonsupported plants. The nonsupported plants were slightly more epinastic. Each point is the average of six leaves.

In a similar experiment, wire supports shaped to the starting curvature of the petiole were fastened to the stem and petioles of measured leaves prior to placing on the clinostat. After 24 h, the supports were removed, and the epinasty was recorded. Measurements were again taken, 10 min after release to observe if any storage of epinastic stimulus or energy occurred. Although released petioles became slightly more epinastic (0.3 to 2.3 units) 10 min after release, the supported and nonsupported were identical in their development of epinasty, and both were approximately 9 units more epinastic than stationary controls after 24 h.

If the lamina or blade of a leaf is removed, a lightened stress load would be expected in the leaf petiole. Figure 12 shows the results of a test in which the blades were excised, and the curvature of the petioles was compared to the petiole curvature of intact controls. The controls with the blades show a much more marked epinastic response than the debladed plants. Such experiments have been conducted in the past, with similar results, but the epinasty could be restored by adding IAA to the stump (Lyon, 1963, 1965 a with *Coleus blumei*). Therefore, a similar test was conducted by applying IAA to the stumps of cocklebur petioles. Figure 13 shows the epinastic response patterns for debladed cocklebur plants, debladed with IAA (1%) in lanolin added to the stumps, and intact control plants. It can be seen that adding IAA to the debladed petiole stumps allowed epinasty to develop during horizontal clinostating, whereas the deblading by itself reduced epinasty (see Plate 6). It appears that IAA from the leaf blade is required by the petiole for epinastic curvature, and that the weight of the blade is not adding to the mechanical strains in the petiole, with respect to epinasty.



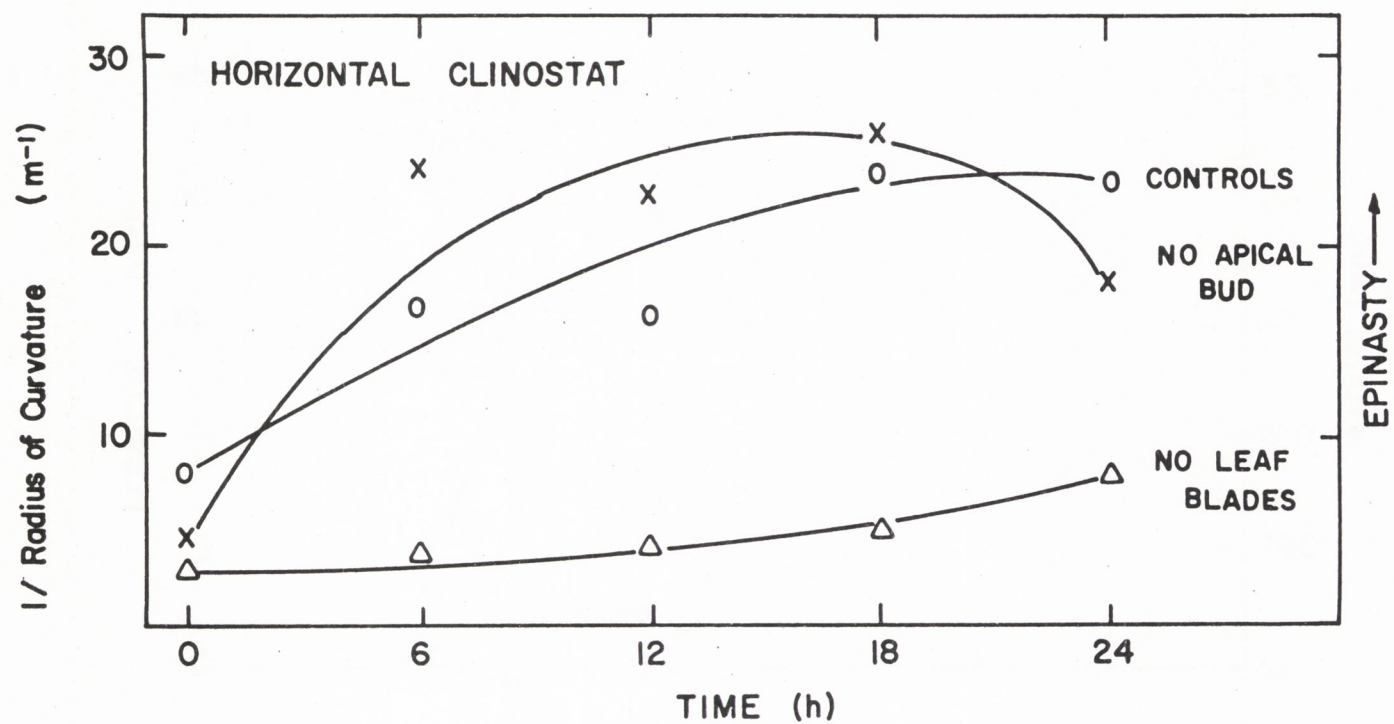


Figure 12. The development of epinasty in clinostated cocklebur leaf petioles after blades or apical buds were removed. The deblading prevented epinasty. Points are averages of four petioles.

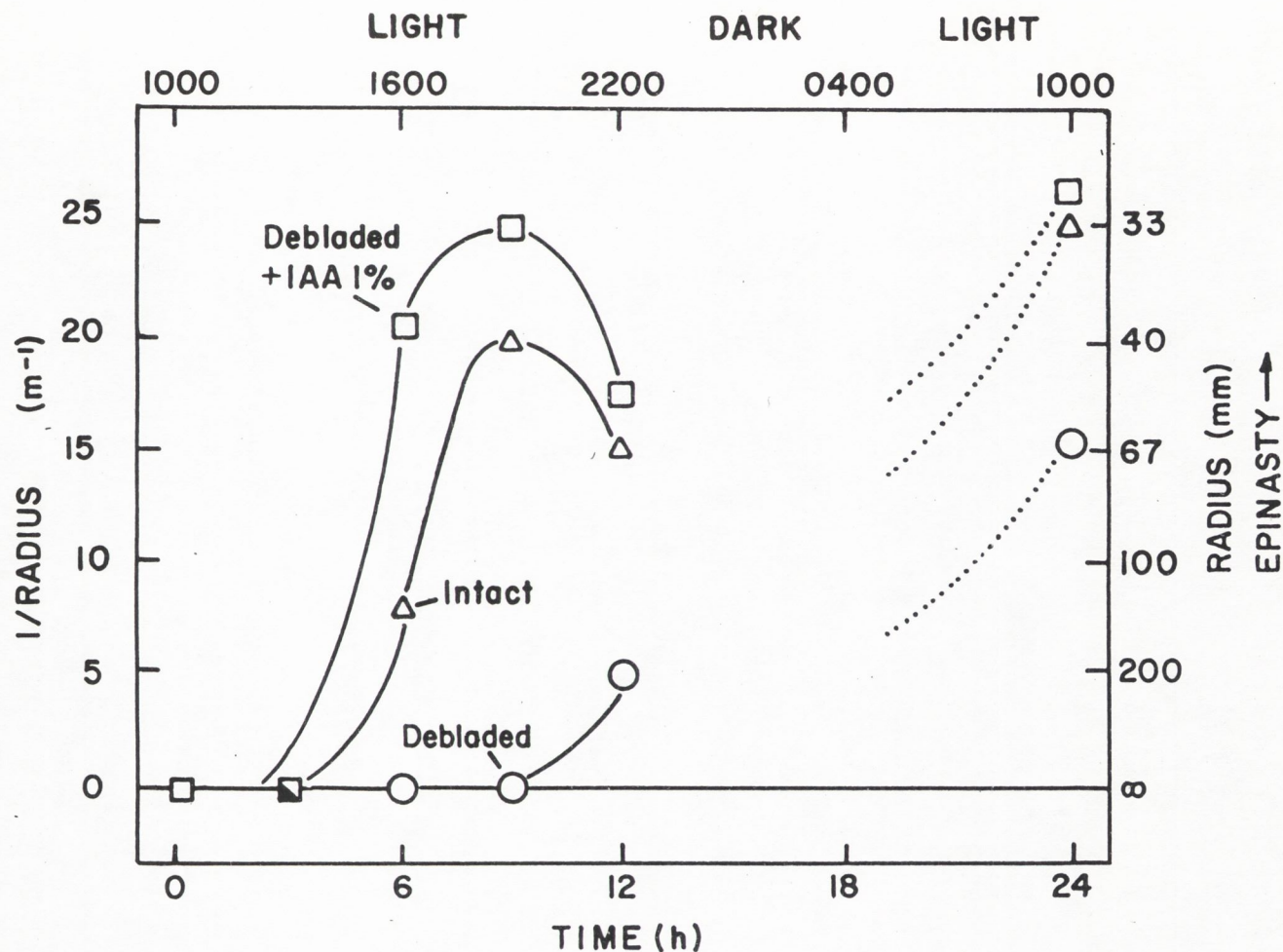


Figure 13. The development of epinasty in clinostated cocklebur petioles after blades were removed and IAA (1%) in lanolin was applied to the stumps of half of these. As before (Fig 12), deblading resulted in greatly reduced epinasty, but adding IAA in lanolin to the stumps permitted complete epinastic development. Each point is the average of four leaf petioles. IAA was also added to debladed upright plants (not shown) and found to have no effect on petiole epinasty (see text).



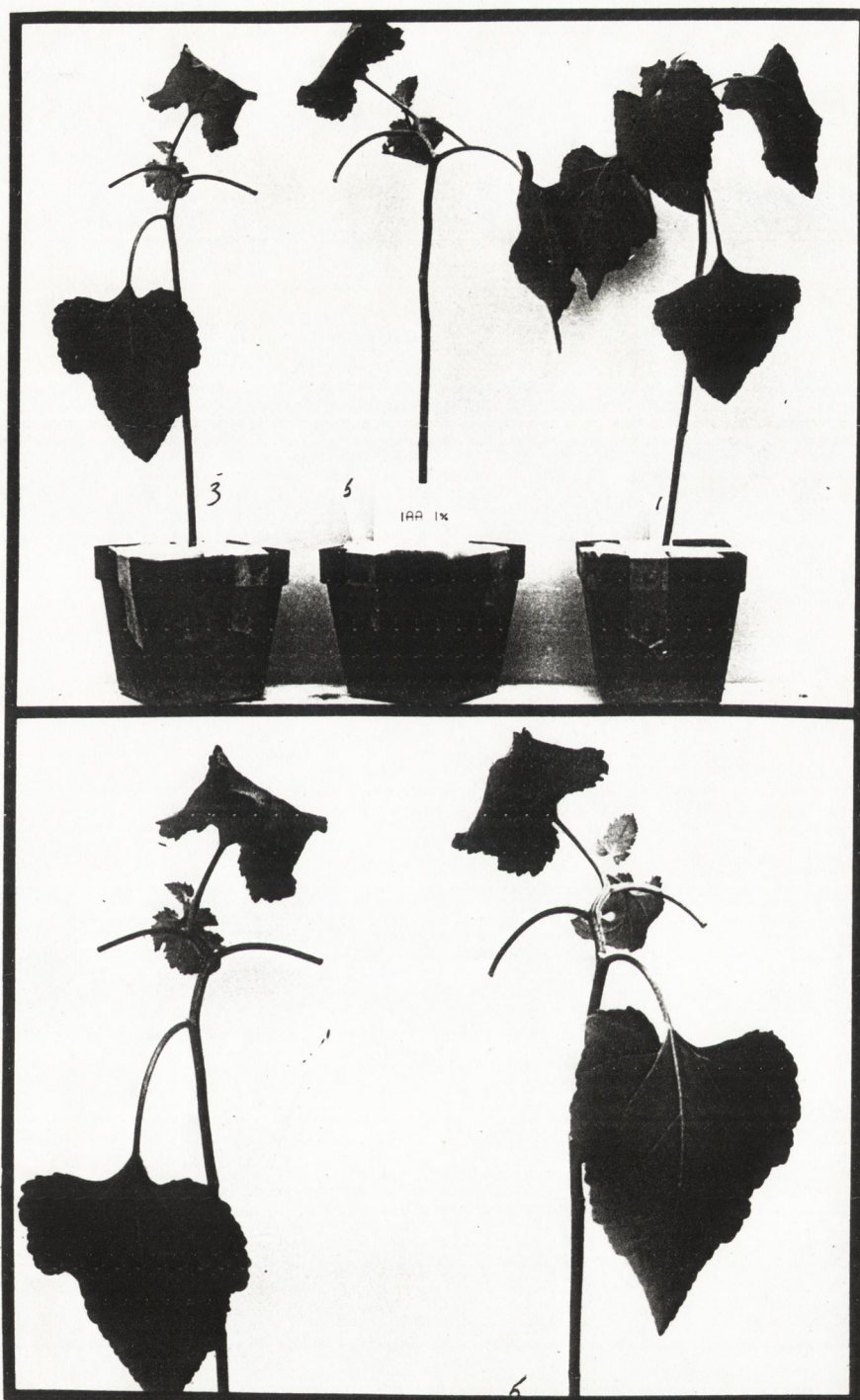


Plate 6. Clinostat-induced epinasty in cocklebur leaf petioles after deblading and deblading plus IAA (1%) in lanolin to petiole stumps. The top picture shows (left to right) a cocklebur with two leaf blades removed, a cocklebur with two leaf blades removed and IAA added to the stumps, and an intact plant. All plants were horizontally clinostated for 24 h. The bottom picture compares the debladed plants. The petioles to which IAA had been added (right) became epinastic, while the debladed petioles without IAA did not (left).



Stationary upright plants were treated in a fashion similar to the three groups of clinostated plants (above); that is, two intact controls, two debladed plants (with lanolin added to the petiole stumps), and two debladed plants with IAA (1%) in lanolin added to the petiole stumps. No significant difference was observed between the daily leaf movements of any of these plants, although the intact and IAA plants showed a very slight petiole curvature, while the debladed, no-IAA-plants' petioles remained perfectly straight. No change in the radius of curvature using the "3-point" measurement method was detected for any of the plants; therefore, it appears that very little of the daily leaf movement occurs as bending in the petioles of cocklebur. In other words, much of the total daily leaf movement probably occurs as bending within the blade.

These results show that the petioles of the debladed plants on the clinostat were capable of perceiving gravity. Rotation on a clinostat caused epinasty in these petioles when treated with IAA. It appears that the leaf blades are not essential for gravity perception in cocklebur, providing of course that clinostat-induced epinasty is a manifestation of gravity compensation. The apical meristem also does not appear to be essential for clinostat epinasty (Fig. 12). Meristematic tissue such as in root caps or coleoptile tips has generally been viewed as the most likely site for gravity perception (Wilkins, 1979); therefore, mature dicot shoots may have some other system of perception at work (as others have proposed; see Iwami and Masuda, 1974; Digby and Firn, 1979 b).

#### Gravity Compensation by Inversion

When cocklebur plants were inverted every 20 min, they developed an



epinasty closely similar to that of clinostated plants (Fig. 14). In contrast, plants inverted and immediately returned to the upright position did not become epinastic. These inverted and returned plants received mechanical agitation similar to the inverted plants but failed to develop any epinasty and, in fact, closely followed the leaf movements of upright controls (Fig. 14). The bottom of Figure 14 shows that AVG (0.1 mM) slowed the development of epinasty in both the clinostated and inverted plants, as well as damping out the leaf sleep movements in all treatments and controls. This reduction of endogenous leaf movements by AVG implies an essential role for ethylene in cocklebur nyctinasty.

Figure 15 shows the resultant epinastic development in castor bean, pepper, and tomato plants subjected to inversion every 20 min. In each case, the epinasty caused by inversion closely approximates that caused by horizontal clinostating, while control plants, inverted and immediately turned back upright every 20 min did not become nearly so epinastic. The progression of epinasty can be seen in the series of photographs in Plates 7 a, b, and c. Unlike the preceding cocklebur test, this experiment was conducted in the dark, and photos were taken against a background of green light.

Plate 8 shows a cocklebur plant against a fixed (with respect to the plant) background grid, in both an upright and inverted position. Leaves were displaced substantially because of inversion (anywhere from 30 to 137 mm), while the petioles at the lamina junction moved from 6 to 30 mm against the background. The lower, larger leaves were displaced the most by inversion, but the leaves used to measure epinasty (i.e., the first fully expanded, and the next leaf above) were displaced

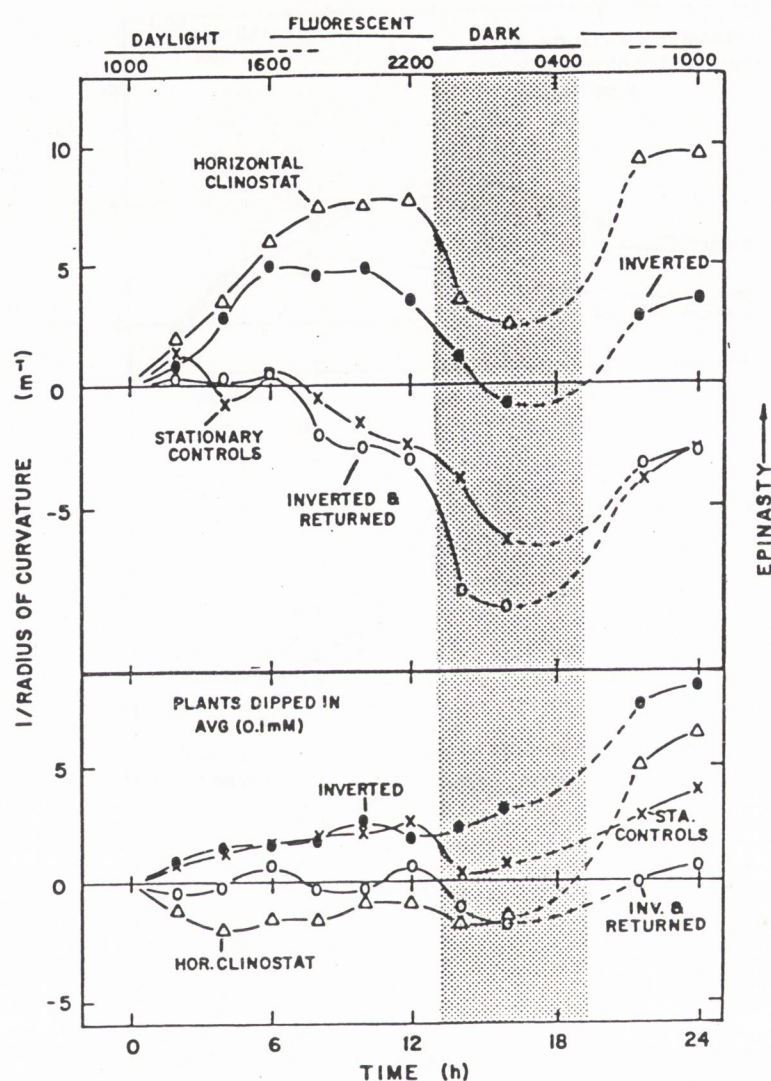


Figure 14. The development of epinasty in the leaves of cocklebur plants subjected to clinostating, inversion every 20 min, or inversion followed by immediately returning the plants upright. Epinasty was always more significant in the clinostated and inverted treatments than in the stationary and inverted and returned plants (Duncan's test, 0.01; see Appendix D). The lower graph shows the same treatments except all plants were pretreated with 0.1 mM AVG, an inhibitor of ethylene synthesis in plants. The AVG damped all leaf movements and onset of epinasty in the clinostated and inverted plants for about 24 h. Points are averages of eight leaves.  $LSD_{0.05} = 2.11$ .



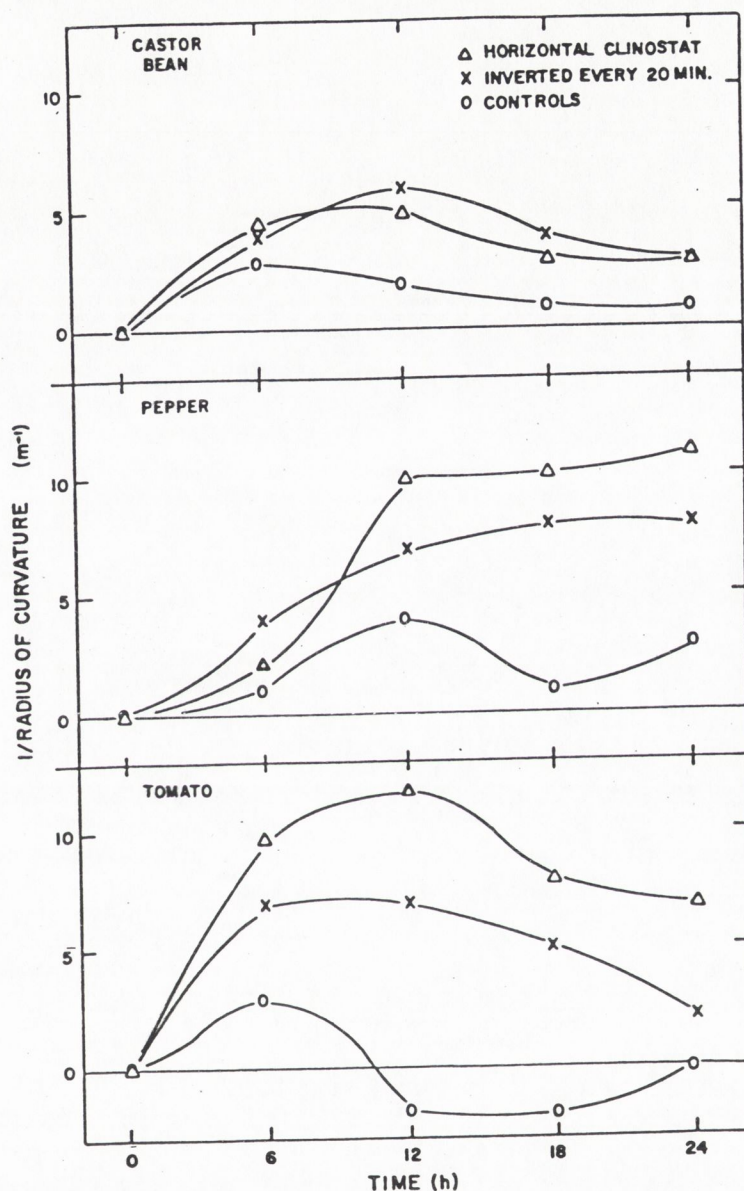


Figure 15. Development of leaf epinasty in castor bean, pepper, and tomato plants subjected to clinostating or inversion every 20 min. As with cocklebur, both clinostating and inversion caused epinasty when compared to controls inverted and immediately returned to an upright position. Clinostat points are two-leaf averages, while all other points are four-leaf averages. See Plates 7 a, 7 b, and 7 c for pictures of this same experiment.



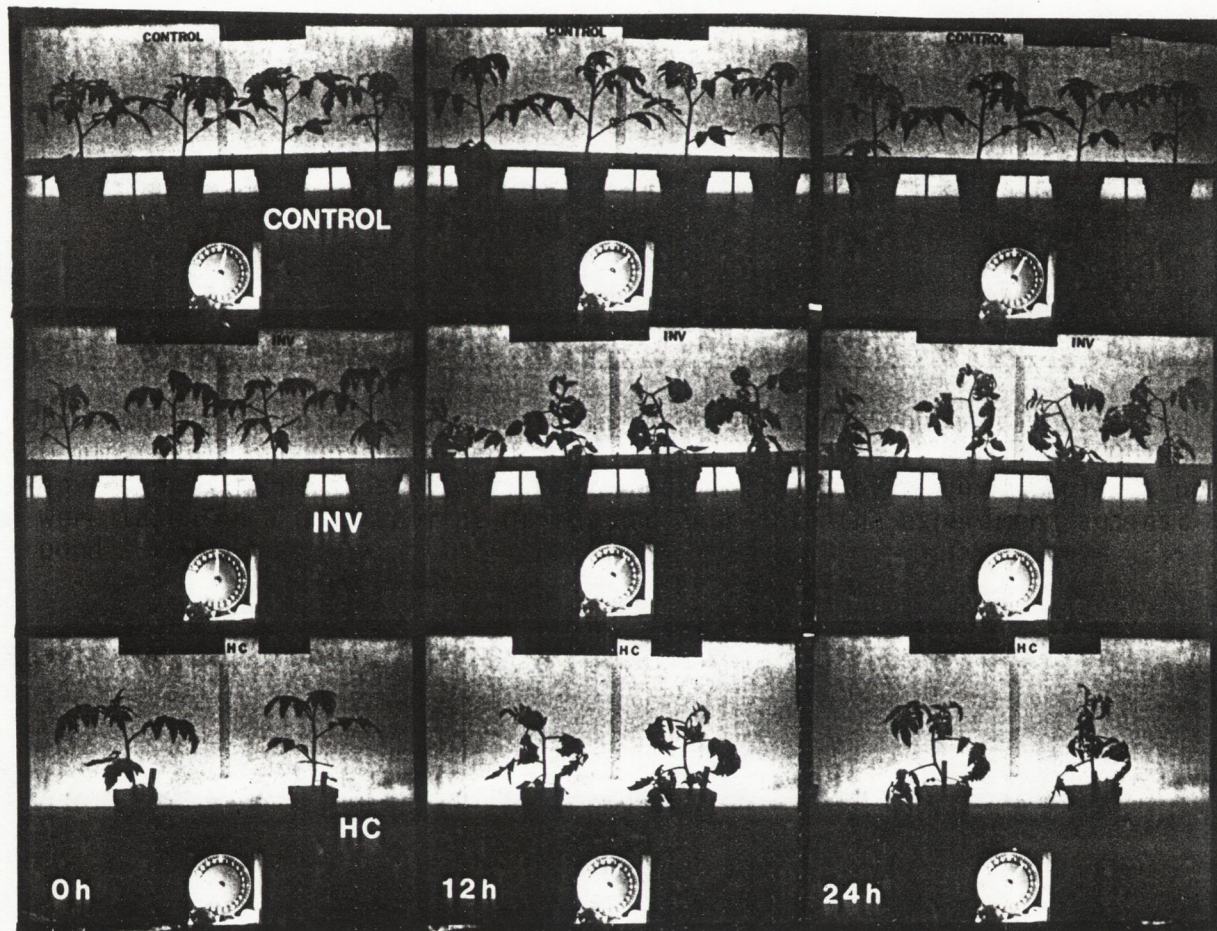


Plate 7 a. Development of epinasty in tomato in response to horizontal clinostating (HC), inversion every 20 min (INV), and inversion and turning back upright every 20 min (CONTROL). Both clinostating and inversion caused epinasty, while inversion followed by returning plants upright did not. Photographs were taken in a dark room at 0, 12, and 24 h into the experiment against a dim green light background.



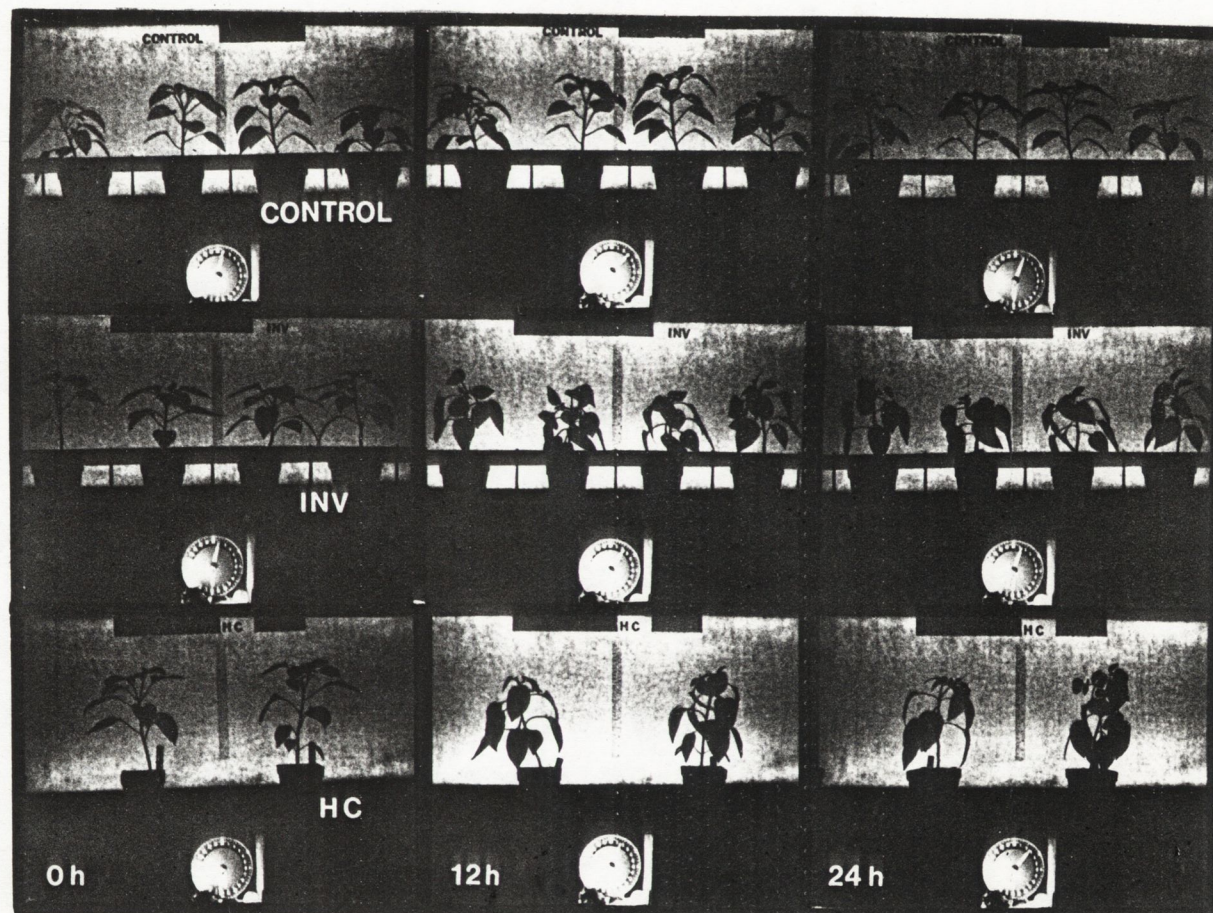


Plate 7 b. Development of epinasty in pepper in response to clinostating (HC), inversion every 20 min (INV), and inversion and turning back upright every 20 min (CONTROL). Both clinostating and inversion caused epinasty, while inversion followed by returning plants upright did not. Photographs were taken in a dark room at 0, 12, and 24 h into the experiment against a dim green light background.



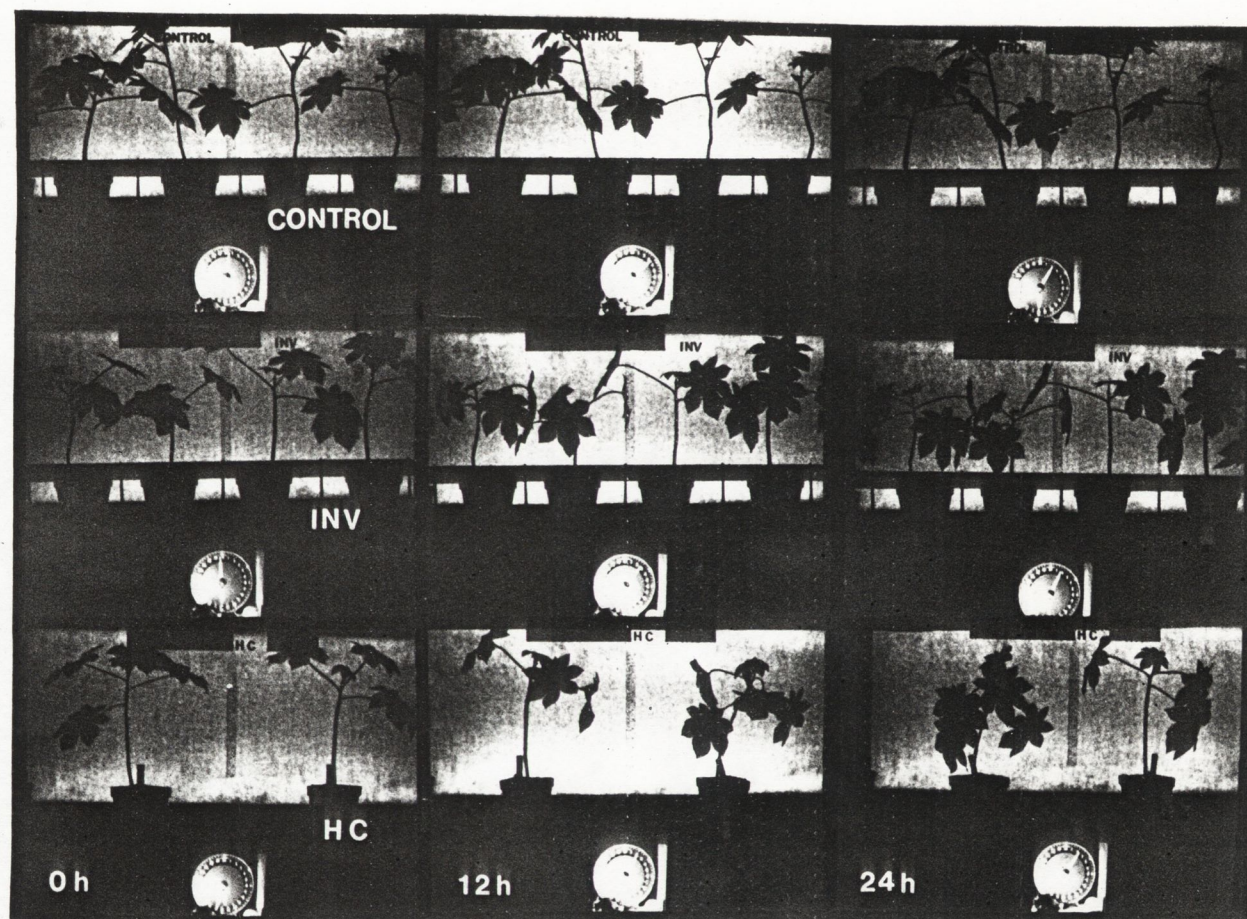


Plate 7 c. Development of epinasty in castor bean in response to clinostating (HC), inversion every 20 min (INV), and inversion and turning back upright every 20 min (CONTROL). Both clinostating and inversion caused epinasty, while inversion followed by returning plants upright did not. Photographs were taken in a dark room at 0, 12, and 24 h into the experiment against a dim green light background.



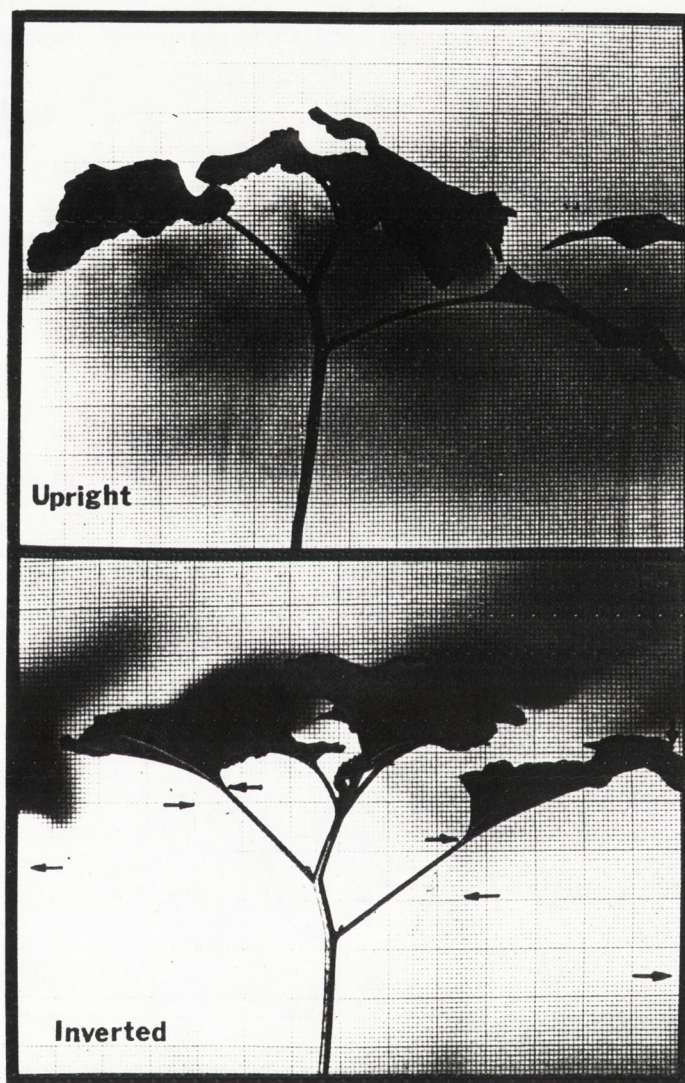


Plate 8. Leaf displacement of a cocklebur plant during inversion. Both prints are of the same plant with the top picture showing the leaves in the normal upright position, while the lower picture shows the leaves in an inverted position (the picture has been rotated 180° for easier comparison). The background is fixed with respect to the plant.



less. An average displacement for the petiole-blade junction of these leaves seemed to be on the order of a centimeter.

Since the inverted plants were subjected to this sort of distortion for alternating 20 min intervals while the controls were not, one might argue that this is the source of stress inducing the epinasty.

To test this, wood supports, cut to a length to raise the petiole-blade junction 10 mm were placed under the leaves of stationary plants at alternating 20 min intervals. The results of the propping experiment are shown in Figure 16. The displacement of the leaves with bamboo crutches appears to have no effect on epinasty in cocklebur.

As with clinostating, plants were placed in plastic buckets and packed securely in vermiculite and subjected to periodic inversion, to see if their leaves could be supported from shifting, thereby minimizing mechanical stress. As before, no obvious epinasty developed in either the controls or the inverted plants.

#### Ethylene in Shoot Gravitropism

##### Silver treatment

If the shoots of mature cocklebur plants are treated by dipping or spraying with silver nitrate, or silver nitrate-sodium thiosulfate solution, a clear slowing of the negative gravitropic response occurs (Plate 9; Fig. 17). Concentrations of 2 mM  $\text{Ag}^+$  delay the time required to reach  $60^\circ$  up to 8-10 h in some tests, particularly with older, slower growing plants, while delays might be as little as 2 h in tests with younger, faster growing plants. Figure 17 shows a slight delay in the initiation of bending, but silver, as with all the other ethylene inhibitor tests to be described, appears to slow the rate of shoot bending, rather than the



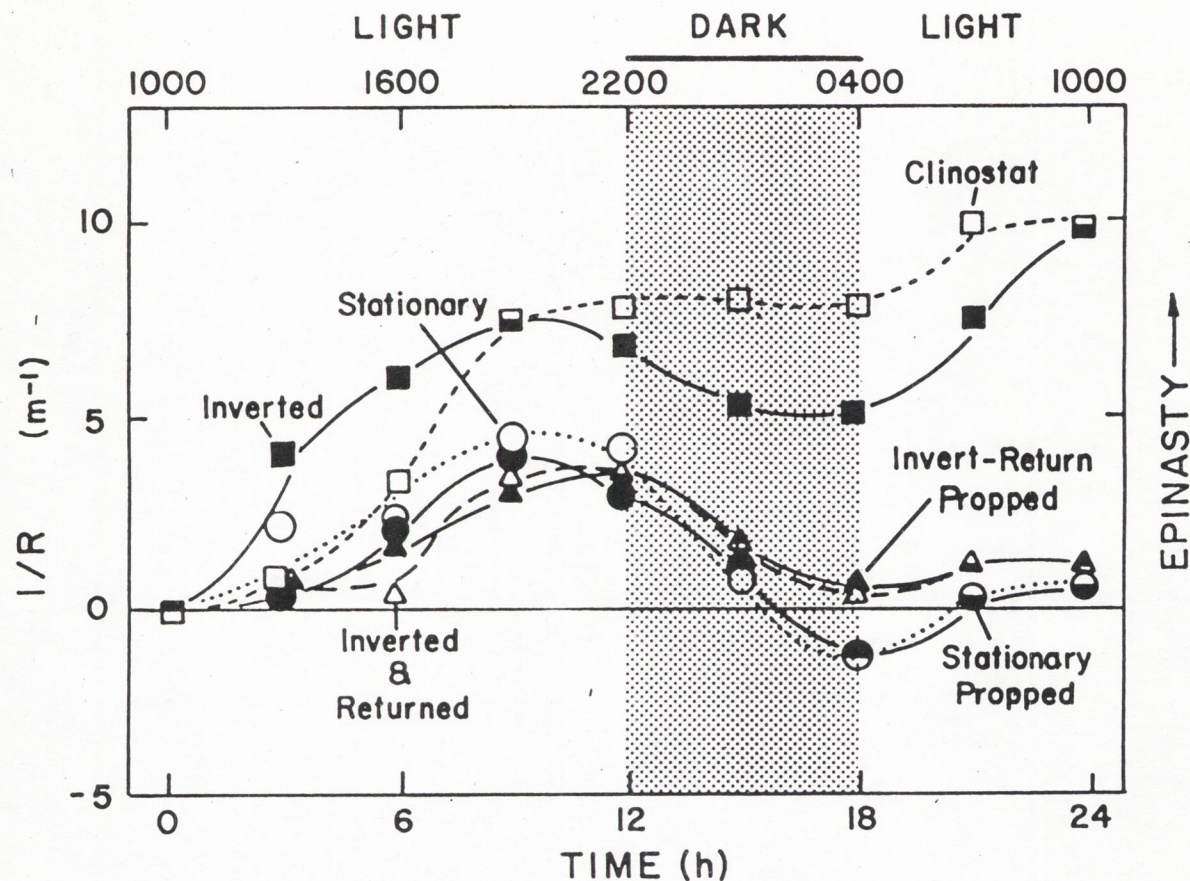


Figure 16. Development of epinasty in cocklebur plants subjected to clinostating, inversion every 20 min, or propping up of the leaf petioles every other 20 min with wooden supports. The propping of the petioles was attempted to more closely simulate the prolonged (alternating 20 min periods) displacement of leaves on plants periodically inverted. Inversion followed by immediately returning to an upright position could not provide such a stress. Wooden supports were positioned to raise the petiole-blade junction of the leaf approximately 1 cm. Only the clinostated and inverted plants showed noticeable epinasty (significant at 0.01, Duncan's; see Appendix E). All points are eight-leaf averages from four plants.  $LSD_{0.05} = 1.34$ .

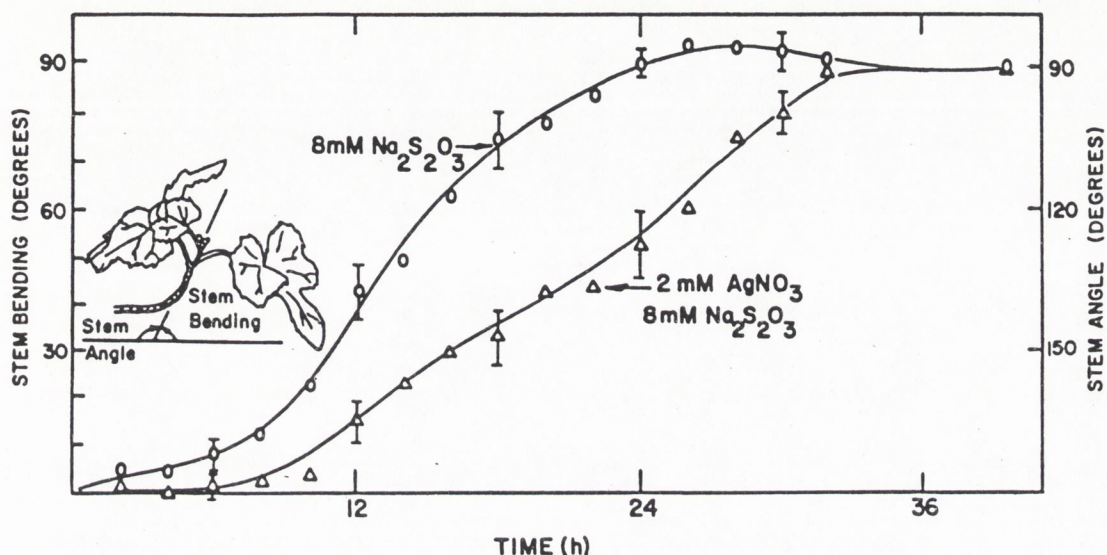


Figure 17. Progression of gravitropic bending of cocklebur shoots treated by dipping in 2 mM silver nitrate-8 mM sodium thiosulfate, or dipping in a control solution of 8 mM sodium thiosulfate. The angle of bending as formed between the apical bud and the nonelongating lower stem region can be expressed by starting with a straight stem ( $180^\circ$ ), and progressing toward a vertical position ( $90^\circ$ , stem angle), or by using the supplementary angle (stem bending) and starting at  $0^\circ$  proceeding upward to a vertical position ( $90^\circ$ , see the insert sketch). The silver markedly delayed shoot gravitropic response. Points are averages of five plants, with standard error of the means shown.



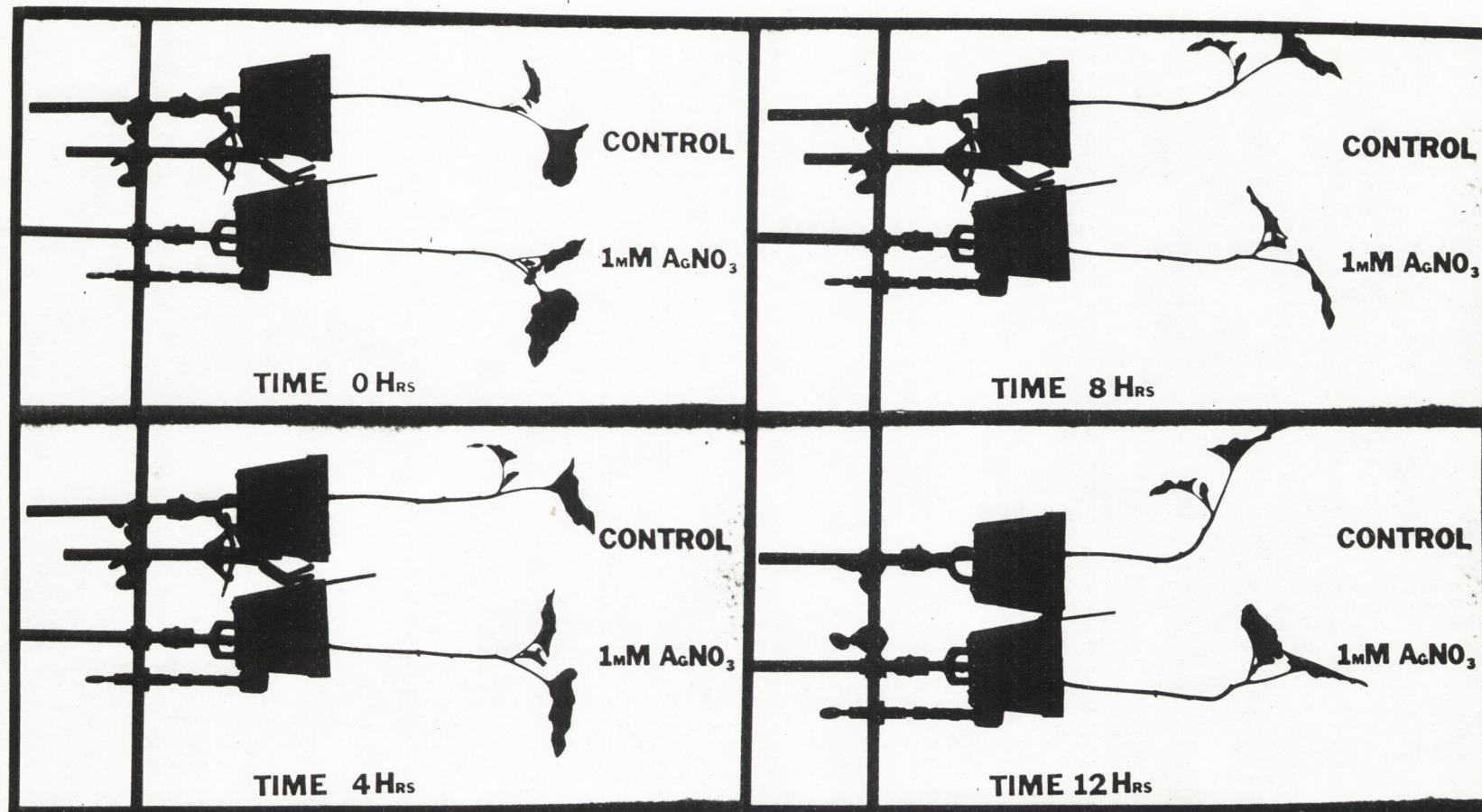


Plate 9. Series of gravitroping cocklebur stems showing the delaying action of silver ( $\text{AgNO}_3$ ), an inhibitor of ethylene action in plants. Pictures were taken at 4 h intervals in the dark, using a dim green light source for a background.



initiation of bending.

Silver nitrate-sodium thiosulfate solutions were used in preference to straight silver nitrate in later tests, in order to apply the silver in a more mobile, yet equally effective form. Therefore, the sodium thiosulfate solutions were tested independently and found to have no effect upon shoot gravitropic response (Fig. 18). In another test, a sodium nitrate solution was used as a control against silver nitrate, and silver again delayed significantly (Fig. 19).

#### Carbon dioxide

CO<sub>2</sub>, like silver, is known to block ethylene action in plants, and when cocklebur were sealed in plexiglas chambers and subjected to a constant flowing, CO<sub>2</sub>-enriched (5%) atmosphere, shoot gravitropism was again delayed (Plate 10; Fig. 20). However, the CO<sub>2</sub> delay was not nearly as marked as the silver delay, with the maximum lag time to reach 60° being 6 h, although other CO<sub>2</sub> tests usually showed less delay in bending than this. Tomato and castor bean shoot gravity response can similarly be delayed with CO<sub>2</sub> treatment.

#### Aminoethoxyvinyl glycine (AVG)

AVG (1 mM), an inhibitor of ethylene synthesis in plants, proved to be a very effective treatment for delaying shoot gravitropism in cocklebur (Fig. 21). Concentrations of 0.1 mM were also effective (Fig. 22), but increasing the concentrations up to 10 mM added little lag beyond the 1.0 mM treatment (results not shown). Concentrations up to 10 mM AVG treatment often caused severe chlorosis and an aberrant leaf growth in young leaves several days after treatment. Lag times up to 12 h to reach 60° were observed in one experiment (see Fig. 29).



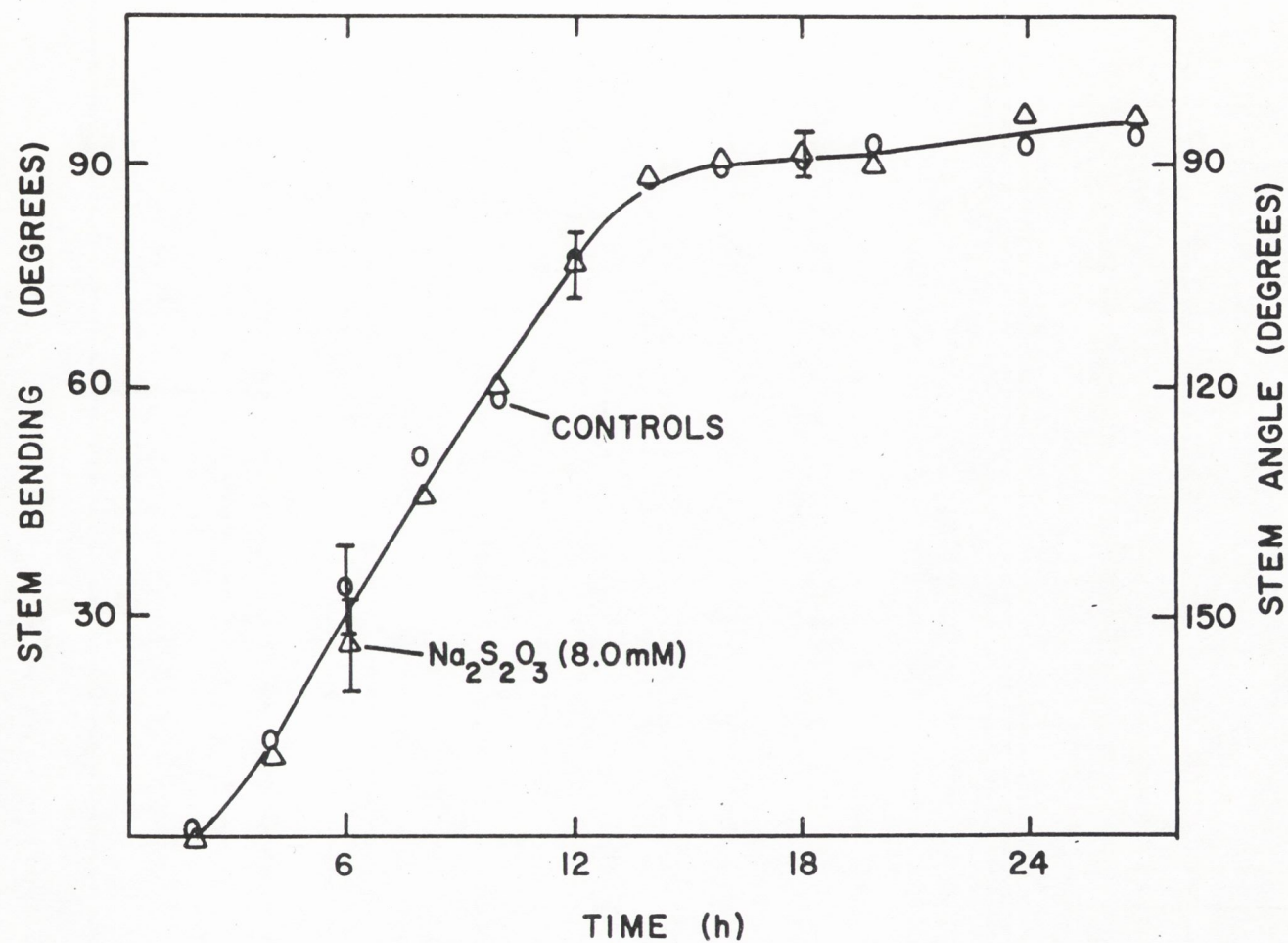


Figure 18. Effect of 8 mM sodium thiosulfate on shoot gravitropic response in cocklebur. Points are averages of five plants with standard errors of the mean shown.

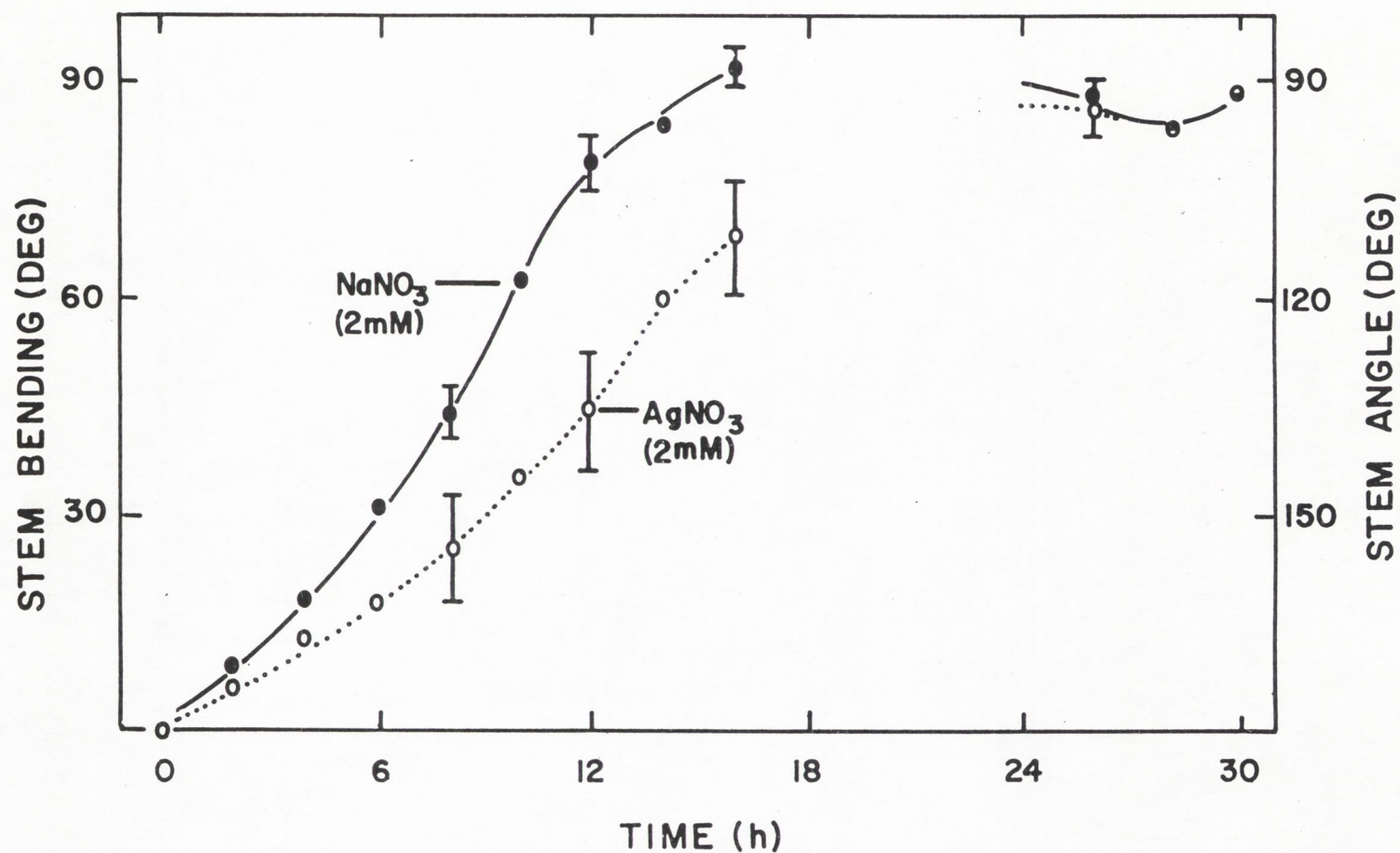


Figure 19. Effect of silver nitrate (2 mM) on gravitropic response of cocklebur shoots, compared to plants treated with sodium nitrate (2 mM). As before (Fig. 17), the silver treated plants bent significantly slower than the salt control plants. Points are averages of five plants with standard error of mean bars shown.



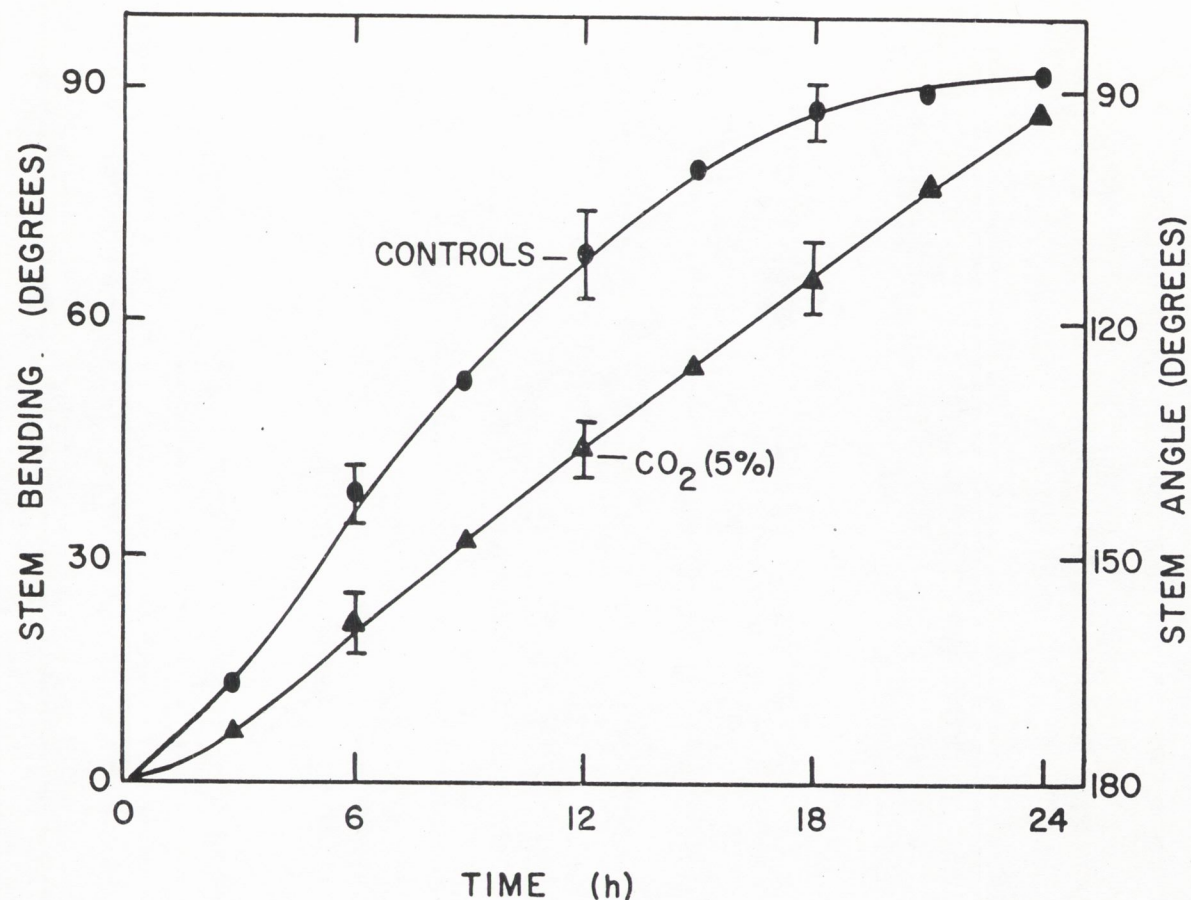


Figure 20. Gravitropic response of cocklebur shoots subjected to a constant-flowing (100-200 ml min<sup>-1</sup>) CO<sub>2</sub>-enriched atmosphere (5% CO<sub>2</sub>, 75% N<sub>2</sub>, and 20% O<sub>2</sub>). CO<sub>2</sub> is known to inhibit ethylene action in plants, and it significantly delayed gravitropic bending in cocklebur. Control plants were similarly enclosed in plexi-glas chambers but treated with constant-flowing air (100-200 ml min<sup>-1</sup>). Points are three-plant averages, with standard errors shown.



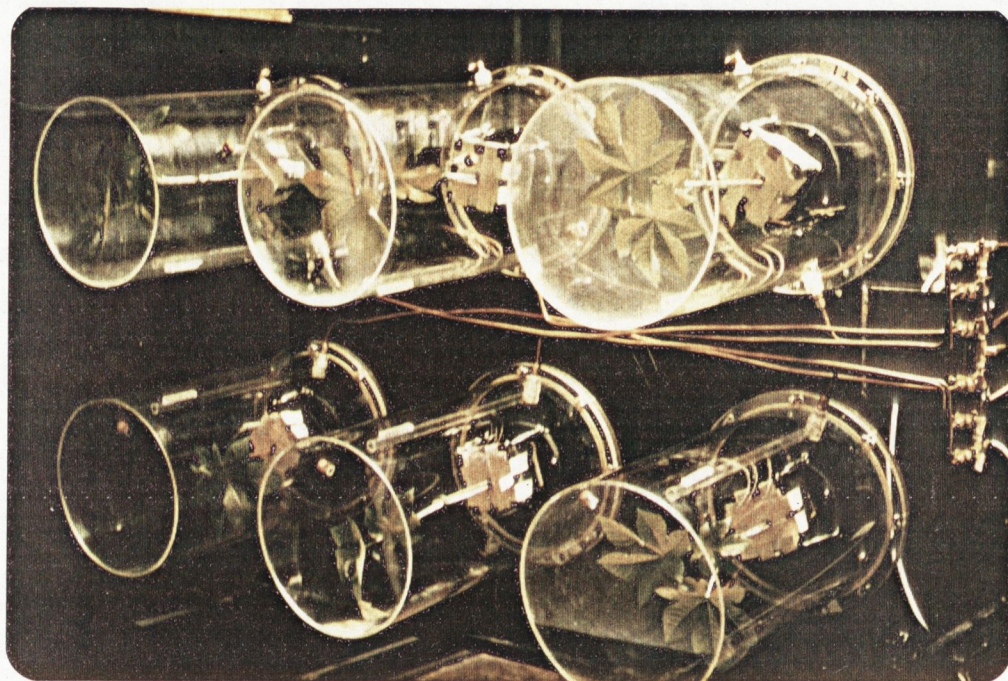


Plate 10 a. Plexiglas cylinders (35 l) used for enclosing plants in  $\text{CO}_2$ -enriched air for inhibition of clinostat-induced epinasty and gravitropic bending. The  $\text{CO}_2$  mixture or air from a diaphragm pump could be forced through the manifold at the right and into the copper lines leading to each cylinder and then expelled through a port on the opposite side. For gravitropism experiments, the clinostat wheels remained stationary. Castor bean plants are shown in this picture.



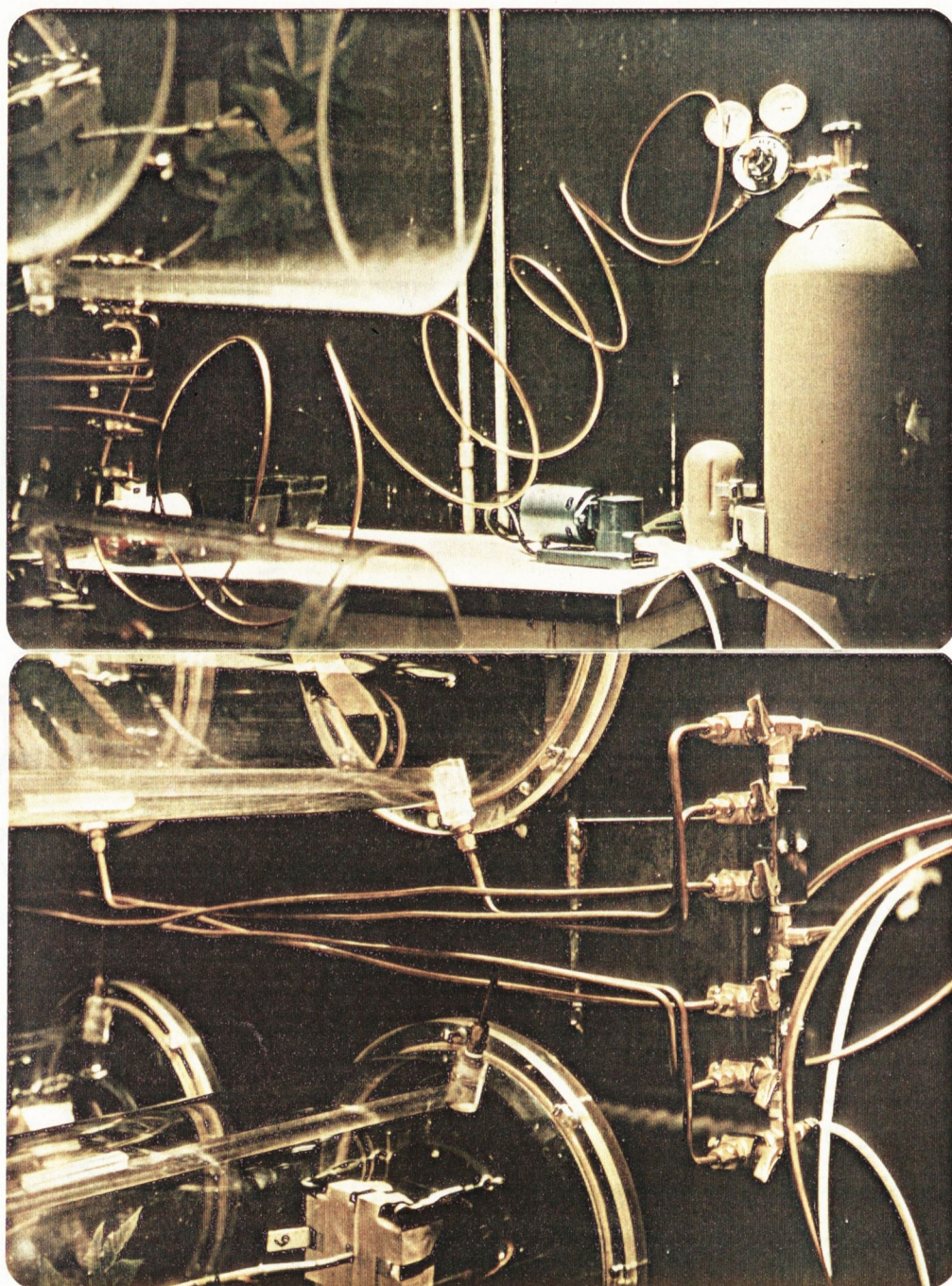


Plate 10 b. Gas cylinder, pump, and manifold system used for CO<sub>2</sub> inhibition experiments. The upper picture shows the compressed gas cylinder (CO<sub>2</sub>, 5%; N<sub>2</sub>, 75%, and O<sub>2</sub>, 20%) connected to the manifold by a copper coil and a Neptune 'Dyna' Pump' connected to the manifold by a teflon line. The lower picture shows the manifold with six individual valves leading to each cylinder and one main divider valve which separated the CO<sub>2</sub> mixture and the air from the pump. In this case, the CO<sub>2</sub>-enriched mixture is coming from the copper line at the top and preceeding on to the three upper cylinders, while the air is coming from the teflon line at the bottom and going on to the three lower cylinders.



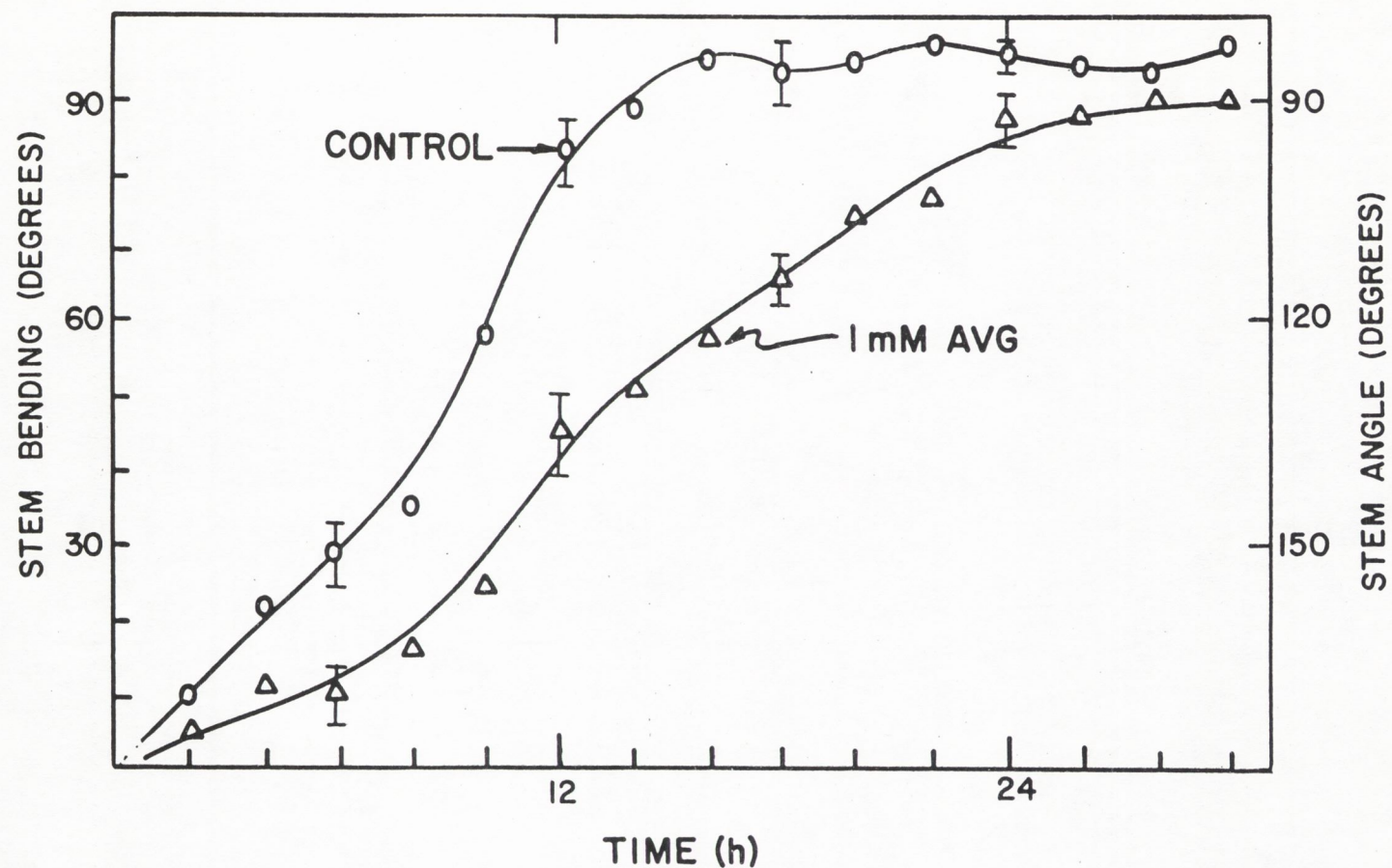


Figure 21. Effect of AVG (1 mM) on shoot gravitropic response of cocklebur. AVG inhibits ethylene synthesis in plants and, as shown above, significantly delayed gravitropic response. All points are five-plant averages, with standard error bars shown. Note the nutational wobbles in the control curve, synchronized among the five plants.



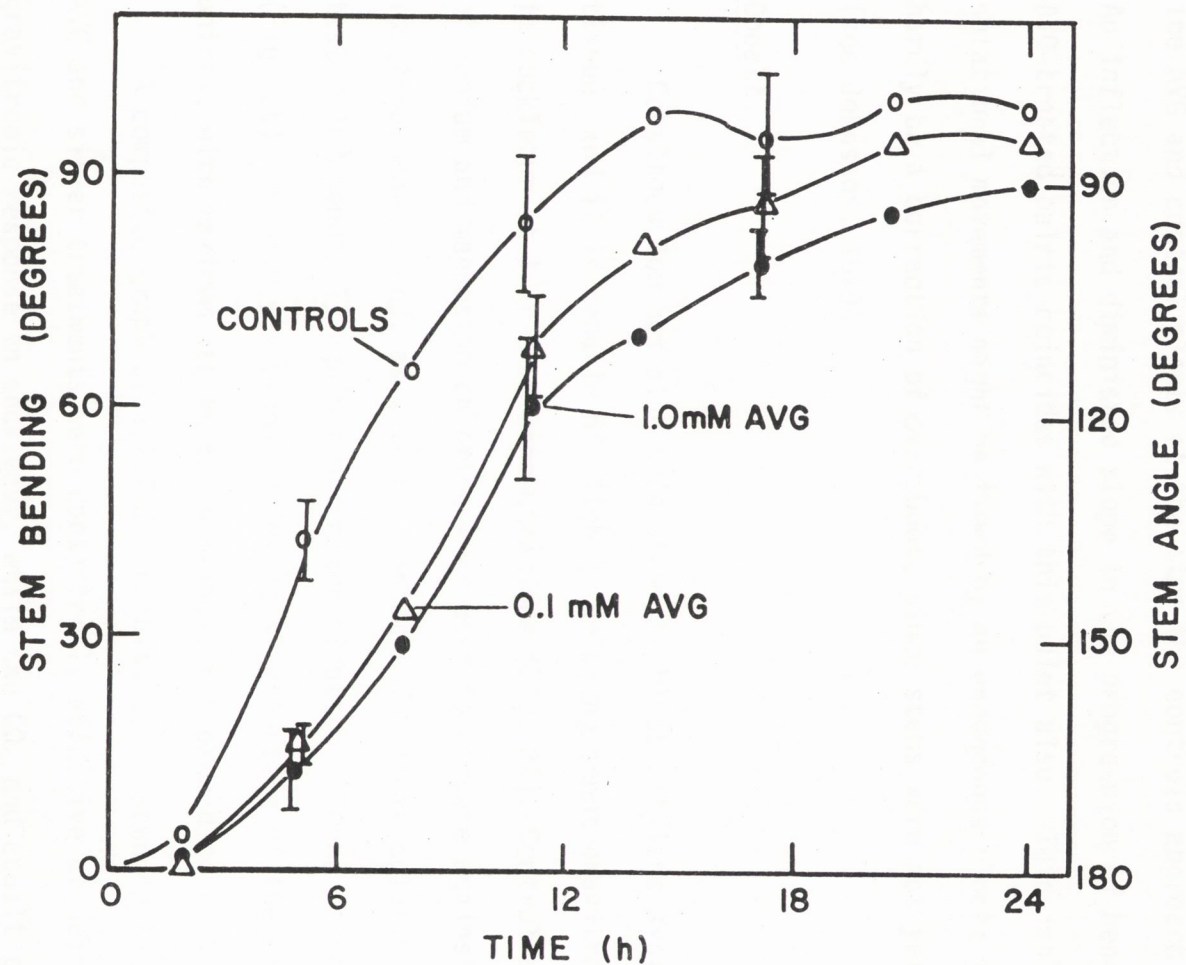


Figure 22. Effects of AVG (1 mM and 0.1 mM) on gravitropic response of cocklebur shoots. All points are five-plant averages, with standard error bars shown.

AVG also effectively delays shoot gravitropism in tomato and castor bean (Fig. 23). This figure shows nutational wobbling (as do many others) that seems to be well synchronized for an averaged-point curve. It is also interesting that the nutational movements become clearly visible for both the AVG and control plants at the time the controls approach the vertical. An inflection and diminished slope in the progression of bending of the AVG-treated plants coincides with this point also. This implies that the nutational movements might be timed by an endogenous clock; they could hardly be a correction of overshoot, since stems were not yet vertical (see Johnsson, 1979).

### Cobalt

Cobaltous ion has also been shown to block ethylene synthesis in plant tissue, and it is capable of slightly delaying shoot gravitropic response in cocklebur at 1 or 2 mM concentrations (Fig. 24). Control salt solutions of sodium and magnesium chloride were used to compare against cobalt chloride treatments. And, as with the sodium thiosulfate solution, each was tested independently against untreated plants and found to have no effect (Fig. 25). Cobalt was probably the least effective of the ethylene antagonists, with maximum lag at 60° generally not exceeding 2 h.

A composite graph of all four inhibitors is shown in Figure 26. The AVG and silver treatments were consistently effective at delaying stem gravitropic response in cocklebur, while the CO<sub>2</sub> and cobalt treatments were significant in some tests but barely detectable in others.

### Mechanical Stress and Shoot Gravitropism

Mechanical stress is reportedly capable of slowing plant shoot gravitropic response (Jaffe and Biro, 1979), and since by initial results



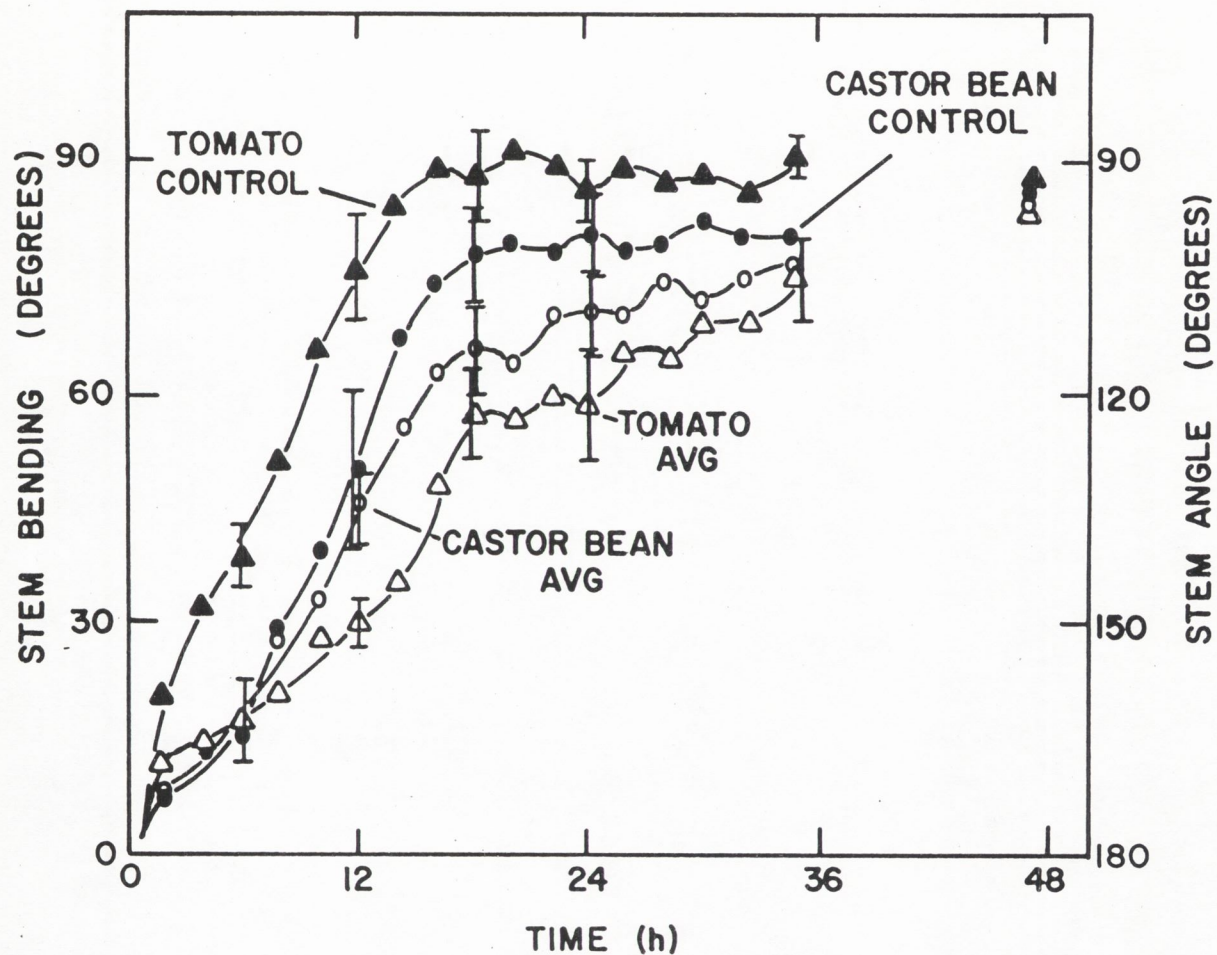


Figure 23. Effect of AVG (1 mM) on shoot gravitropic response in castor bean and tomato. As with cocklebur, AVG significantly delayed the bending. Again, note nutational waves in the bending curves, particularly as the plants neared the vertical position. Castor bean data are six-plant averages, while tomato data are five-plant averages. Bars represent standard error of the mean.

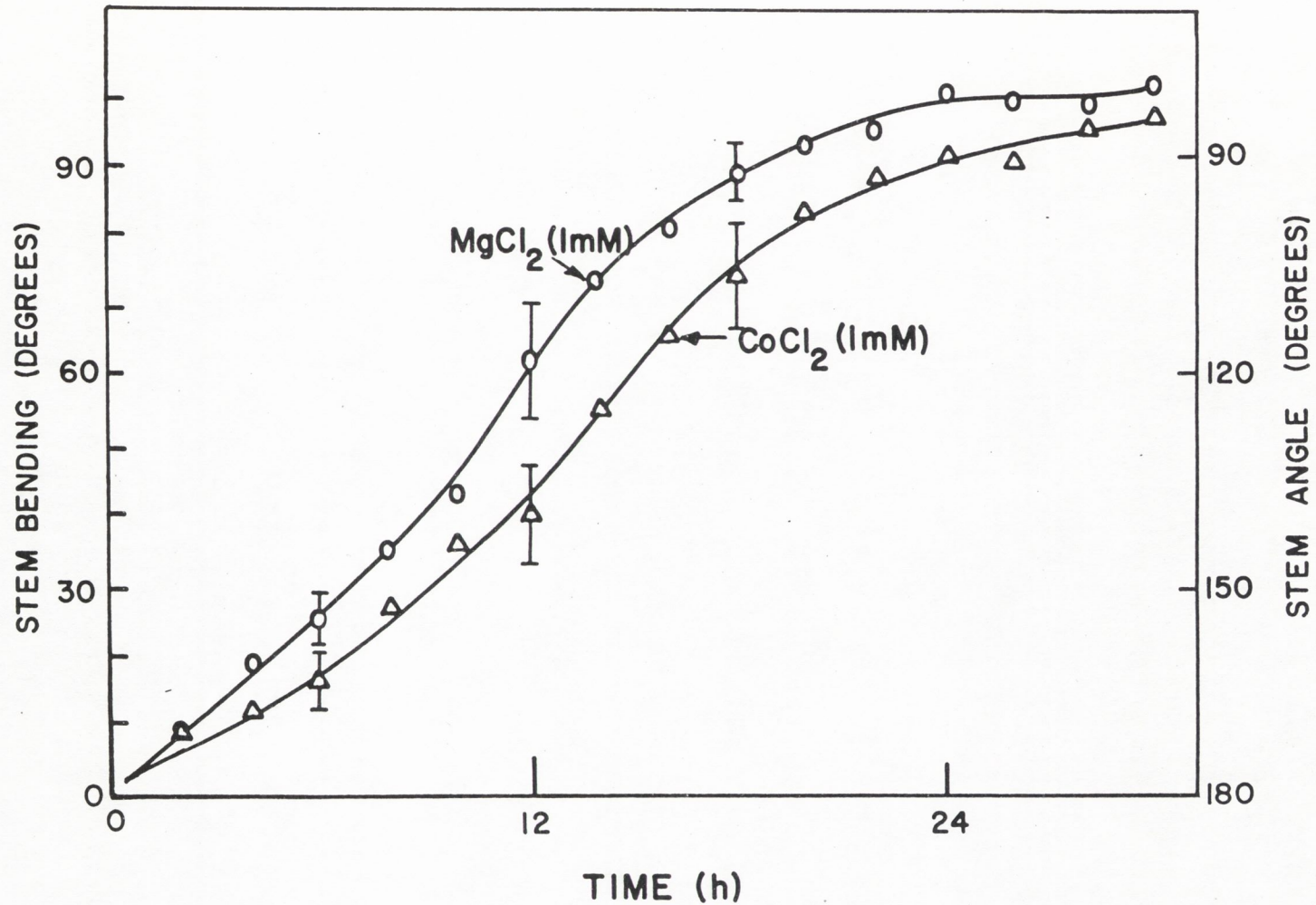


Figure 24. Effect of cobaltous chloride (2 mM), an inhibitor of ethylene synthesis in plants, on cocklebur shoot gravitropism, as compared to plants treated with magnesium chloride (2 mM). The cobalt delayed the bending response slightly, but significantly, compared to the salt control plants. All points are five-plant averages, with standard error bars shown.



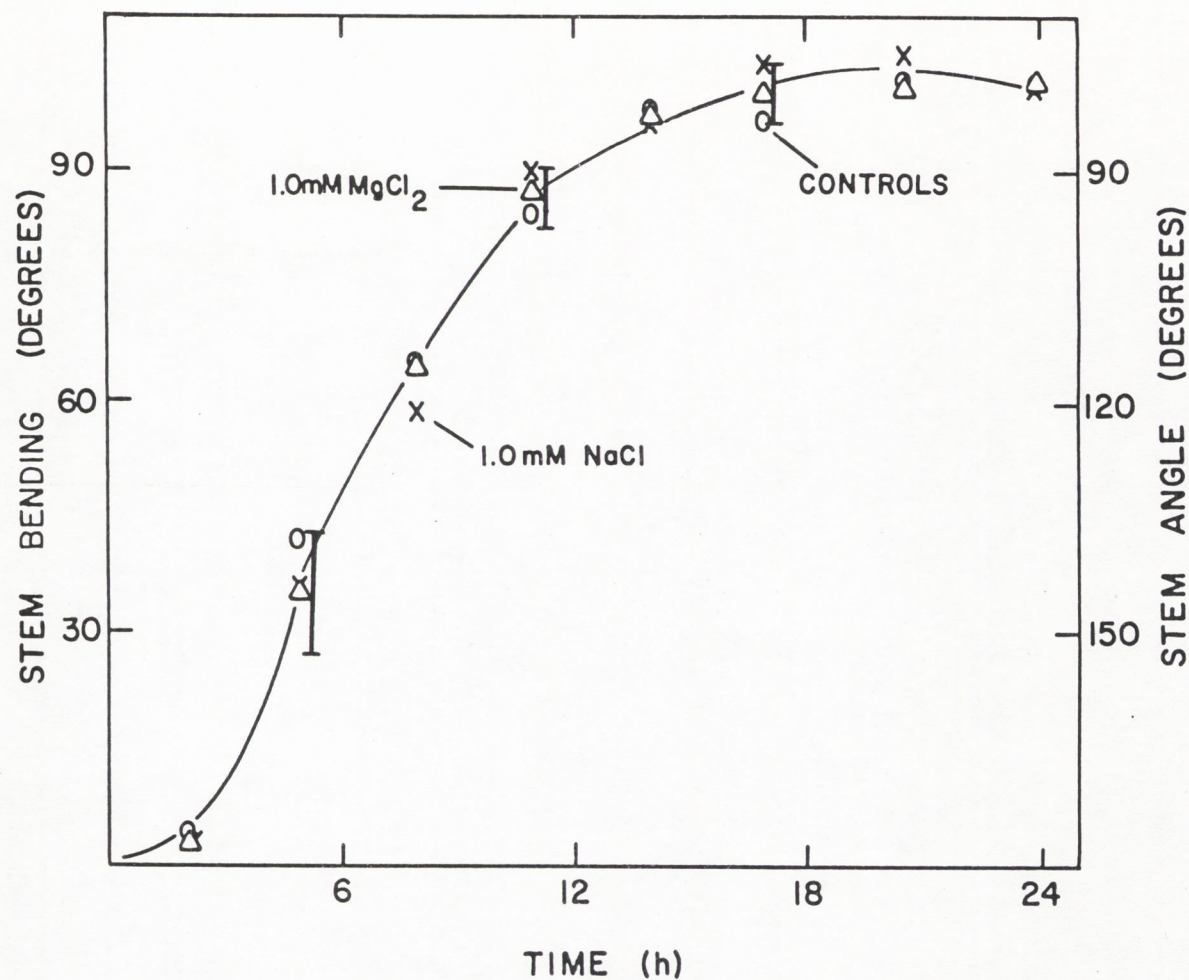


Figure 25. Effect of magnesium chloride (1 mM) and sodium chloride (1 mM) on shoot gravitropism of cocklebur. No significant effect appeared from such treatment when compared with untreated control plants. All points are five-plant averages, with the standard error of the magnesium treated plants shown.

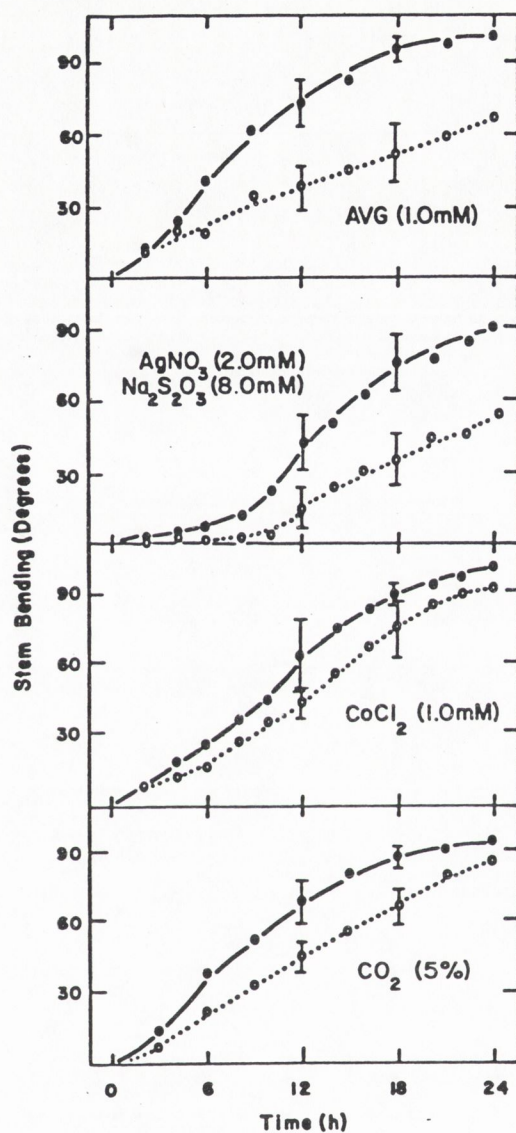


Figure 26. Composite graph showing the delaying effects of four ethylene antagonists on shoot gravitropic response of cocklebur. The AVG and silverwere consistently effective, while the delaying by cobalt and CO<sub>2</sub> treatments wereless dramatic. Standard error bars are shown.



indicated a possible role for ethylene in shoot gravitropism, several tests observing the gravitropic response of mechanically stimulated plants were conducted.

Figure 27 shows the results of cocklebur plants shaken vigorously by hand for 120 s prior to turning them horizontally as compared to unshaken plants. Pretreatment of AVG (1 mM) was crossed into the shaking treatment. Shaking seemed to cause an initial delay in the shoot curvature, but it was not significant and diminished with time. The AVG as usual caused a delay compared to the non-AVG plants.

If cocklebur plants are shaken continuously during their gravitropic response by fastening them in a horizontal position on the mechanical shaker (horizontal position), no difference could be observed (Fig. 28). As before, the AVG plants were significantly delayed, but much later into the response than normally seen. Unfortunately, no unshaken AVG treated plants were measured during this test, so this unusual delay may simply be due to the plants used.

#### Decapitation-Defoliation Tests

If cocklebur plants are completely defoliated, their initial gravitropic response appeared to be as fast, or even faster, than intact control plants (Fig. 29). Some slowing could be seen toward the end of the experiment, and this delay was significant in another test (Fig. 30). However, in each test, the defoliated stems reached or nearly reached the vertical.

If the apical bud was removed (decapitated) along with the leaves, again the initial response appeared unimpeded (Fig. 29), although the delay became more noticeable later in the experiment. This delay can be

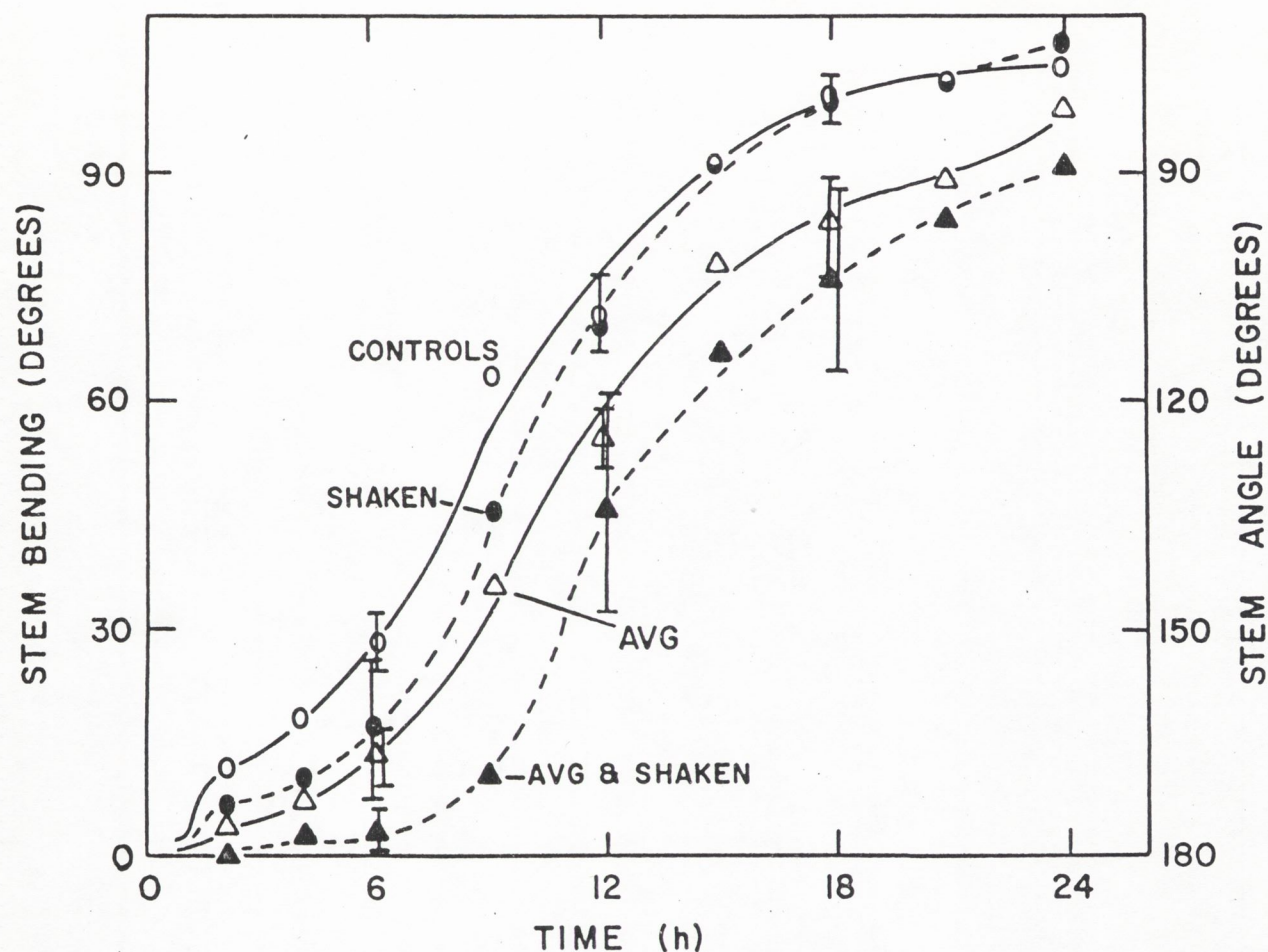


Figure 27. Effects of vigorous hand shaking-mechanical stress on the gravitropic response in cocklebur shoots. Shaking (120 s) was administered immediately prior to placing plant horizontal. Half of the plants of both the shaken and nonshaken groups were pretreated with AVG (0.1 mM). The shaking appeared to cause a slight initial delay in bending, but it diminished as the plants approached the vertical. The AVG delayed bending in both the shaken and nonshaken plants. All points are four-plant averages, with standard error bars shown.



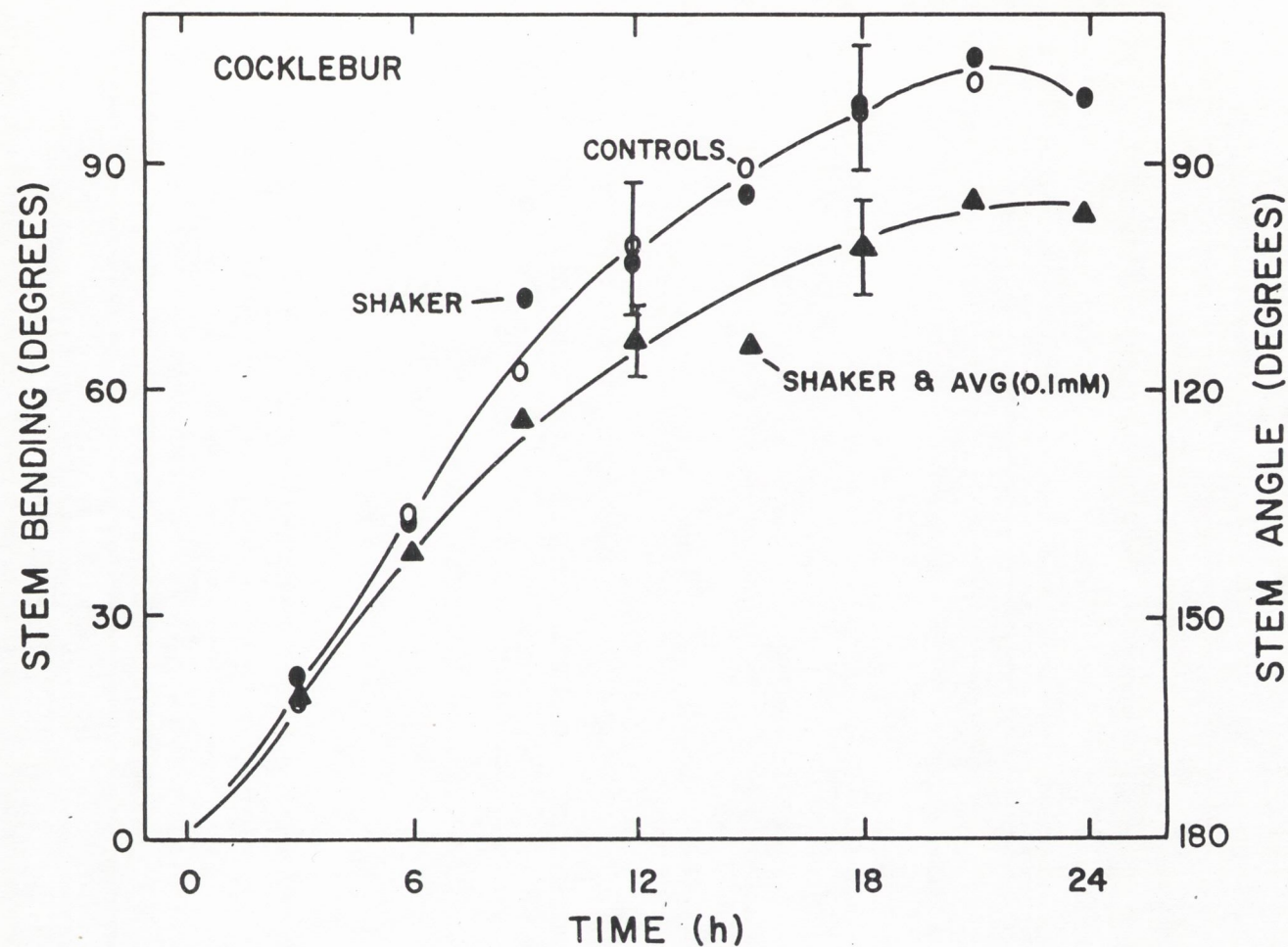


Figure 28. Effect of continuous mechanical shaking (applied with a mechanical shaker) on gravitropic response of cocklebur shoots. As with the previous hand-administered shaking, no overall effect could be seen for gravitropic response. AVG again slowed the response for both shaken and nonshaken plants (not shown). Points are four-plant averages with standard error bars shown.

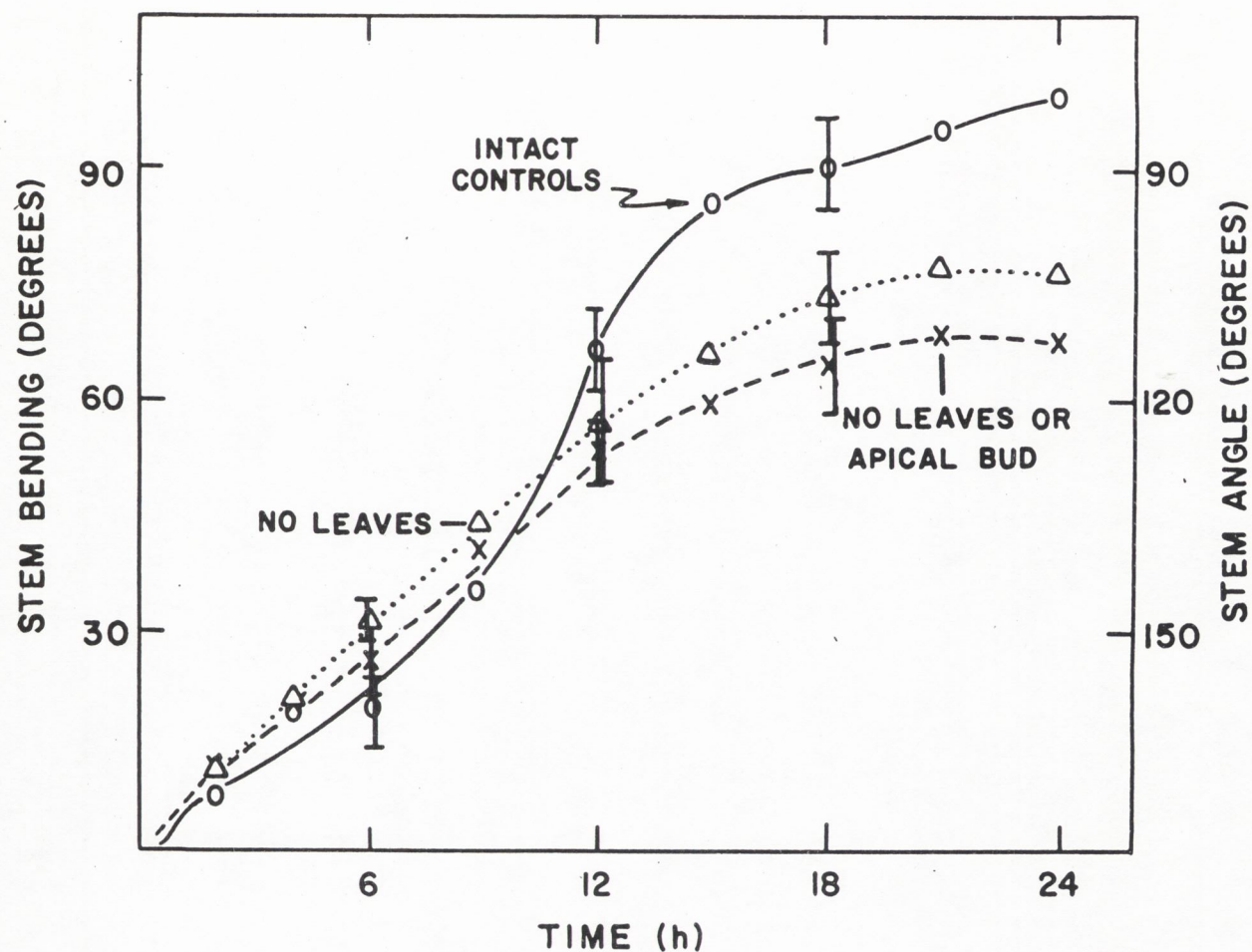


Figure 29. Effect of decapitation and defoliation on gravitropism in cocklebur shoots. Both defoliated and decapitated plants showed slightly faster bending initially than did intact controls, but in this case, both treatments stopped short of the vertical whereas controls do not. All points are four-plant averages, with standard errors indicated.



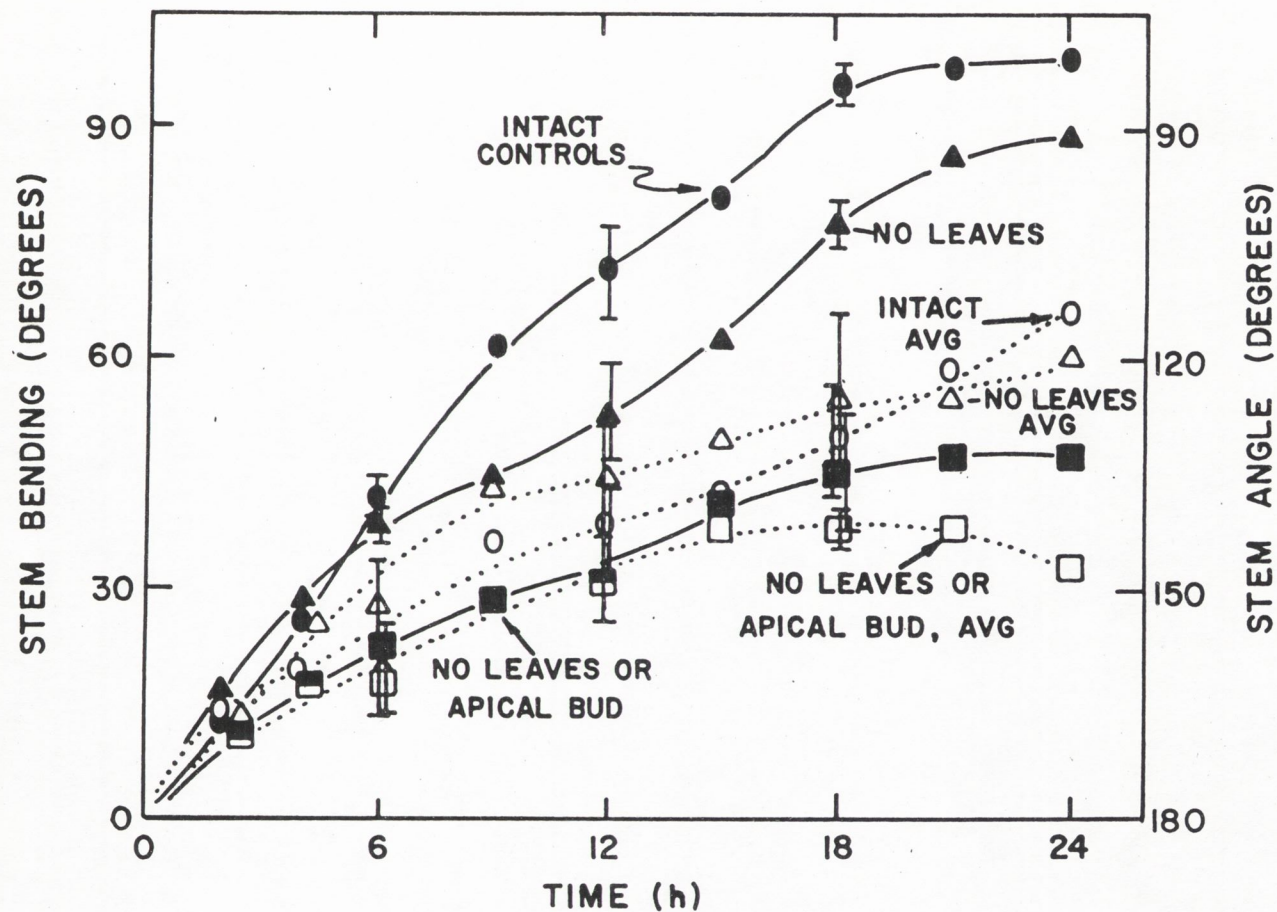


Figure 30. Effect of decapitation, defoliation, and AVG (1 mM) on shoot gravitropic response of cocklebur. As before, the defoliated plants responded slightly faster initially than did intact controls, but later they bent more slowly. Decapitated-defoliated plants' bending was markedly delayed and incomplete. AVG pretreatment slowed bending beyond each of the other treatments. All points are three-plant averages, with standard error bars shown.

seen more clearly in Figure 30. In other experiments with very fast responding plants (i.e., healthier cockleburs), neither defoliation nor decapitation-defoliation treatments appeared to have any effects. Thus, if the plant is vigorous and growing rapidly, it appears that the leaves and apical bud are not essential for gravitropic response, but they may aid in completing the upward bending in slower growing plants.

In one preliminary experiments, IAA (1%) in lanolin paste added to the apical stumps of decapitated-defoliated cocklebur and castor bean plants speeded the gravitropic response over decapitated-defoliated plants with just lanolin added to the stumps. This experiment used large, slow-growing plants; therefore the apical meristem appears to supply some IAA needed for gravitropic bending in such older plants.

#### Hormonal Deflection of Stems

The ethylene antagonist effects upon shoot bending indicate an essential role for this hormone in gravitropism. To ascertain this, however, it would be useful to know if ethylene can directly cause stem bending. If an ethephon solution (1%) is applied to the apical 10 cm of one side of a tomato shoot, ethylene (released from the ethephon) causes a deflection of the stem toward the side of application (Fig. 31; Plate 11 a). Ethylene is known to inhibit cell elongation, so this result is not surprising. Deflections up to  $83^\circ$  toward the side of application were observed in these tomato plants, placed on a horizontal clinostat immediately after treatment (Fig. 31). Few consistent stem deflections were observed for ethephon application to cocklebur stems (Fig. 33). However, indirect evidence discussed below strongly indicates ethylene may be participating directly in hormonal deflection of cockle-



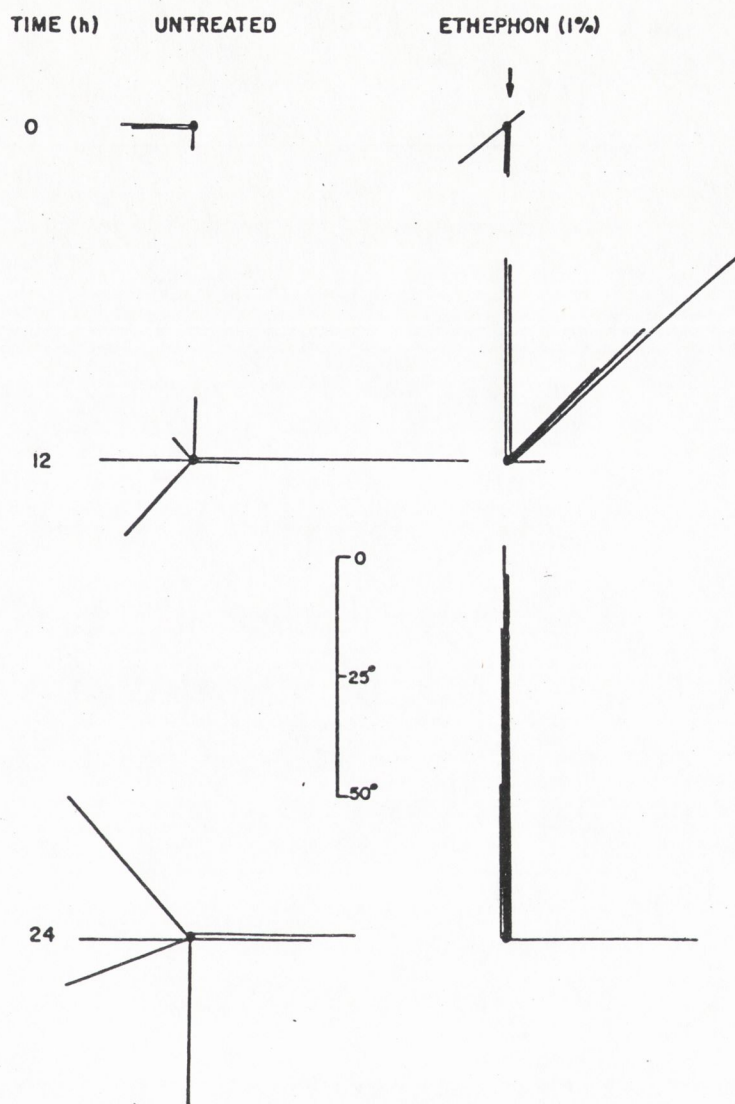


Figure 31. Effect of a unilateral application of 1% ethephon (an ethylene releasing compound ) to the apical 10 cm of tomato stems. Each line represents the amount and direction of bending occurring for an individual plant. In this graph, as with all the other vector diagrams to follow, the plant growth regulator was added from the top side of the page as they are shown (see the arrow). The bending of six untreated controls and six ethephon treated plants were measured 0, 12, and 24 h after attachment to a clinostat (to free plants from natural gravitropic tendencies). The ethephon caused a highly directed deflection toward the side of application, whereas control plants bent randomly.





Plate 11 a. Deflected growth in tomato in response to an application of ethephon (1%) to one side of the apical 10 cm of the stem. The plant on the left was treated with ethephon on the right side of the stem, while the plant on the right was an untreated control. The ethephon caused a deflection toward the side of application. Both plants were rotated on a horizontal clinostat for 24 h after application of ethephon.



bur stems.

Auxin (IAA) has long been considered the primary hormone involved in plant organ bending or tropism. If tomato stems are unilaterally treated with IAA in lanolin paste and then attached to a horizontal clinostat, dramatic stem deflections can be observed (Fig. 32; Plate 11 b). In the case of auxin, the deflections occur away from the side of application, up to  $200^{\circ}$  in 24 h with 1% concentrations in the plant shown in Figure 32. In another experiment, a tomato plant bent  $360^{\circ}$  away from the side of IAA (1%) application in 48 h, while in a 48 h experiment with cocklebur, one plant bent  $441^{\circ}$  (see Plate 11 c and d). Pepper plants deflected up to  $119^{\circ}$  from IAA (1%) application after 24 h.

The synthetic auxin NAA is also capable of deflecting growth of cocklebur stems. I have observed up to  $95^{\circ}$  bending away from the side of NAA (1%) application after 24 h of horizontal rotation.

Both IAA and NAA are known to be powerful stimulants of ethylene production in plant tissue, and thus far, it has been shown that auxins cause bending away from the side of application, while ethylene causes bending toward the treated side. Therefore, one might expect that when auxins are applied, the resultant bending might be somewhat decreased because of antagonistic effects of auxin-stimulated ethylene on the same side. When cockleburs are pretreated with AVG (1 mM) to block auxin-stimulated ethylene production, an enhanced bending can indeed be observed for both IAA and NAA treatments (Fig. 33).

Ethylene is known to be autocatalytic, so ethephon treatment may not only release ethylene from the degradation of the 2-chloroethylphosphonic acid, but it might also stimulate endogenous ethylene production. If cocklebur stems are pretreated with AVG (1 mM) before ethephon treat-

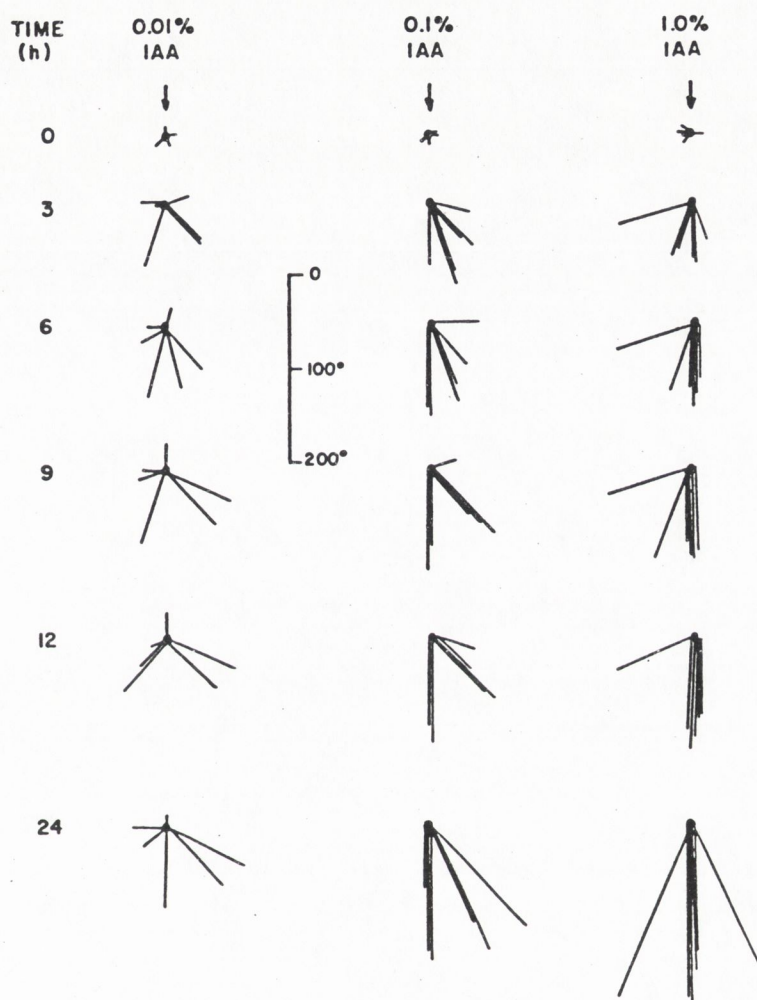


Figure 32. Effect of unilateral application of IAA in lanolin paste to the upper 10 cm of tomato stems. The IAA strongly directed stem deflection away from the side of application, and as can be seen from the untreated control plants of Fig. 31, bending was greatly enhanced (note scales). As with the ethephon test, six plants were used for each treatment, and all plants were placed on the clinostat after IAA application.



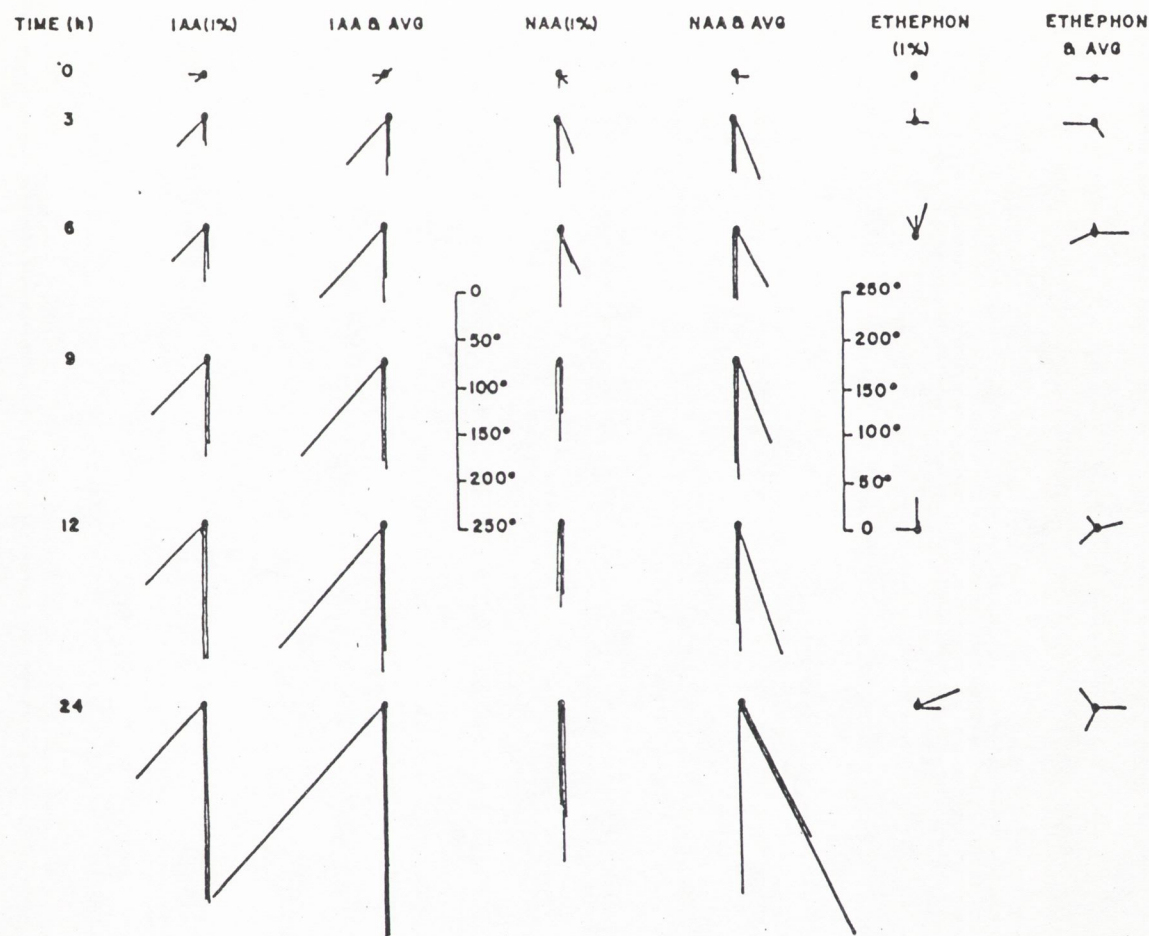


Figure 33. Effects of IAA, NAA, and ethephon on cocklebur stem deflection. Both IAA (1%) and NAA (1%) in lanolin caused marked deflection away from the side of application. But, unlike tomato, only a slight directional trend of deflection can be seen from unilateral application of ethephon. However, the role of ethylene in cocklebur stem can be seen indirectly in the tests with AVG (1 mM) pretreatment, in which treatment of stems with IAA and NAA plus AVG showed enhanced bending away from the side of auxin application. Auxins are known to stimulate ethylene production, so with the blocking of ethylene production on the side of auxin application with AVG, this result is not totally unexpected.





Plate 11 b. Deflected growth in tomato in response to an application of IAA (1%) in lanolin to one side of the apical 10 cm of the stem. The plant on the right was treated with IAA on the right side of the stem, while the plant on the left was treated with simple lanolin paste on the left side. The IAA caused deflected growth away from the side of application. Both plants were rotated for 48 h on a horizontal clinostat after application of the IAA.



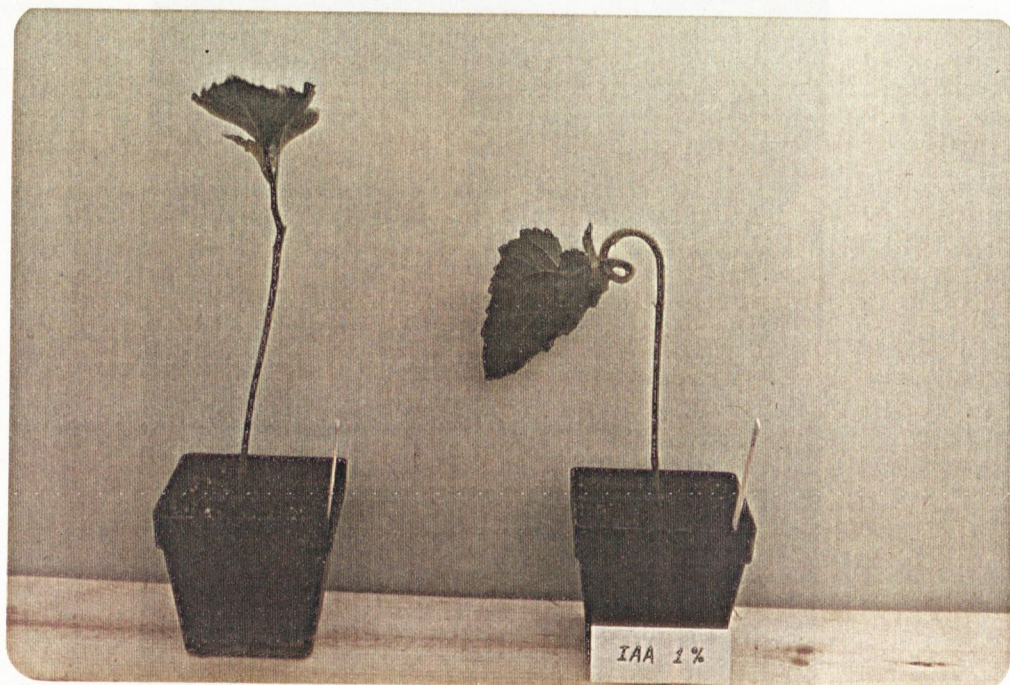


Plate 11 c. Deflected growth in cocklebur in response to an application of IAA (1%) in lanolin to one side of the apical 10 cm of the stem. The plant on the right was treated with IAA on the right side of the stem, while the plant on the left was treated with simple lanolin paste on the right side. The IAA caused a deflected growth away from the side of application. Both plants were rotated on a horizontal clinostat for 48 h after application of the IAA.





Plate 11 d. Close-up picture of a cocklebur stem showing deflected growth in response to a unilateral application of IAA (1%) in lanolin paste to the upper 10 cm of the stem (added to the right side). This plant bent nearly  $450^\circ$  away from the side of IAA application after 48 h of rotation on a horizontal clinostat. Note the swollen stem in the region of the paste application; this is probably a response to auxin-stimulated ethylene production in the plant.



ment, a more directed bending is observed in the non-AVG treated plants (Fig. 33). These results then, in an indirect manner, indicate a potential for ethylene to affect stem bending in cocklebur.

The effects of unilateral applications of several other plant hormones, including gibberellic acid ( $GA_3$ ), kinetin (a cytokinin), and abscissic acid (ABA) were also tested with tomato (Fig. 34),  $GA_3$  (1%) in lanolin treatment resulted in mildly enhanced bending with a slight trend of direction away from the side of application for cocklebur (results not shown). No clear effects on bending were detected in kinetin or ABA tests (Fig. 34).

IAA appeared to be the plant hormone most capable of inducing bending in the intact dicot stems tested, while ethylene (ethephon) also appeared capable, but only 1/3 as effective as IAA, and in an opposite direction. Auxin caused deflection away from the side of application, while ethylene caused deflection toward the side of application.

AVG has been shown to significantly delay gravitropic stem bending. If cocklebur stems are pretreated with AVG (1 mM) and then placed into an atmosphere supplemented with ethylene ( $100 \text{ nl l}^{-1}$ ), a gravitropic response intermediate to untreated control plants and plants treated just with AVG can be seen (Fig. 35). Note that AVG only blocks ethylene synthesis and not ethylene action in plants. AVG-treated plants reached  $60^\circ$  approximately 6 h after the controls, while AVG plus ethylene plants were only delayed about 2 h.

The effects of ethylene gas on gravitropic response of untreated (no AVG) cocklebur stems were also observed (Fig. 36). Ethylene at  $100 \text{ nl l}^{-1}$  concentrations showed no effects on shoot gravitropic response, but concentrations of 1 and  $10 \text{ ul l}^{-1}$  delayed the response significantly

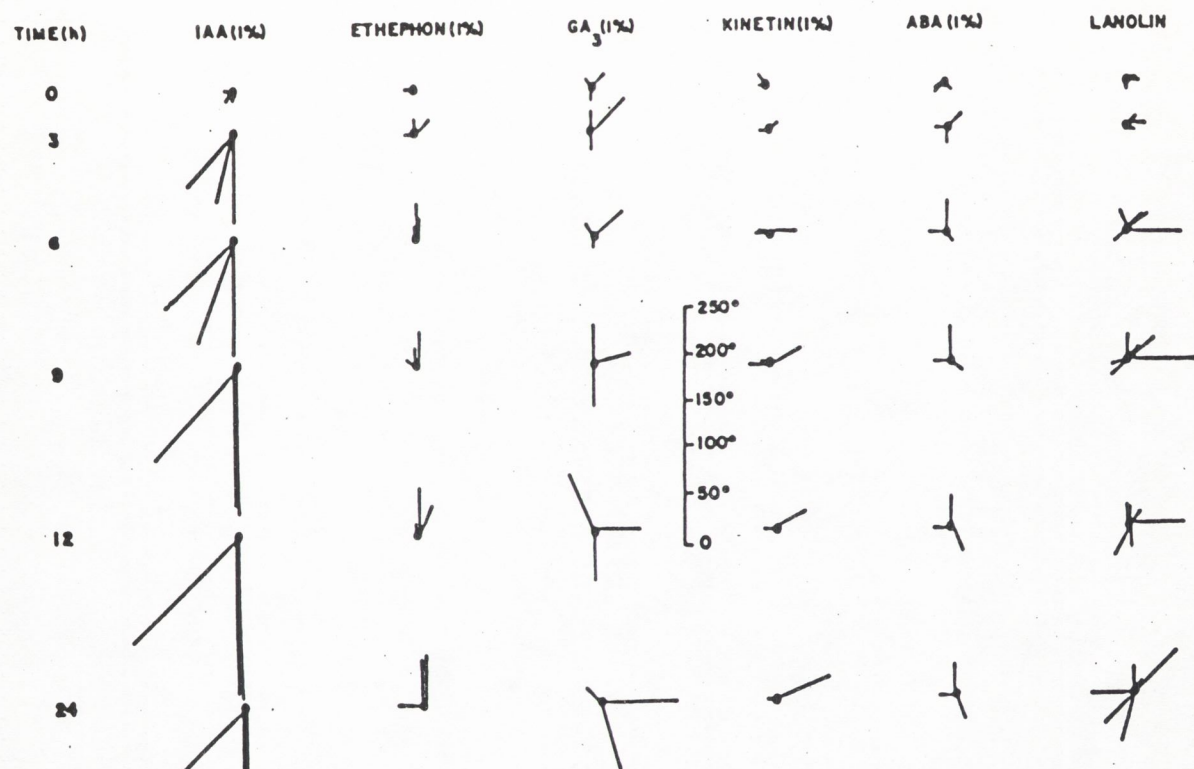


Figure 34. Effects of unilateral application of five different plant growth regulators on tomato stem deflection. IAA (1%) in lanolin caused the most bending, in a direction away from the side of application. Etkephon (1%) appears to direct bending toward the side of application, but no enhancement was apparent. Both kinetin (1%) and ABA (1%) in lanolin showed no clear deflection patterns when compared with the lanolin controls.  $GA_3$  (1%) in lanolin might enhance bending slightly, but no direction of deflection was apparent in this test.



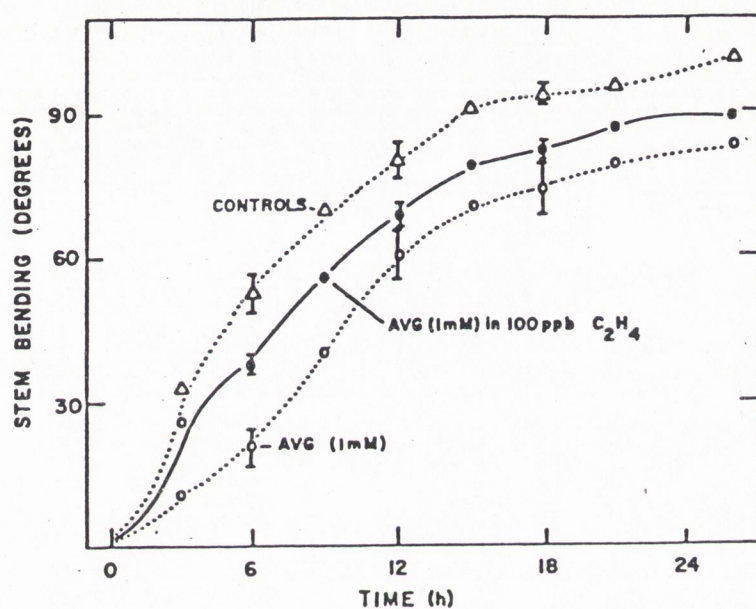


Figure 35. Gravitropic response of cocklebur stems treated with AVG (1 mM) or AVG and ethylene (100 nl l<sup>-1</sup>). AVG treatment as usual delayed response significantly, but if AVG-treated plants were enclosed in plexiglas chambers to which ethylene had been added, an accelerated bending response occurred (note, AVG only blocks ethylene synthesis in plants). The response still lagged behind a control response. All points are averages of two plants, with standard error bars shown.

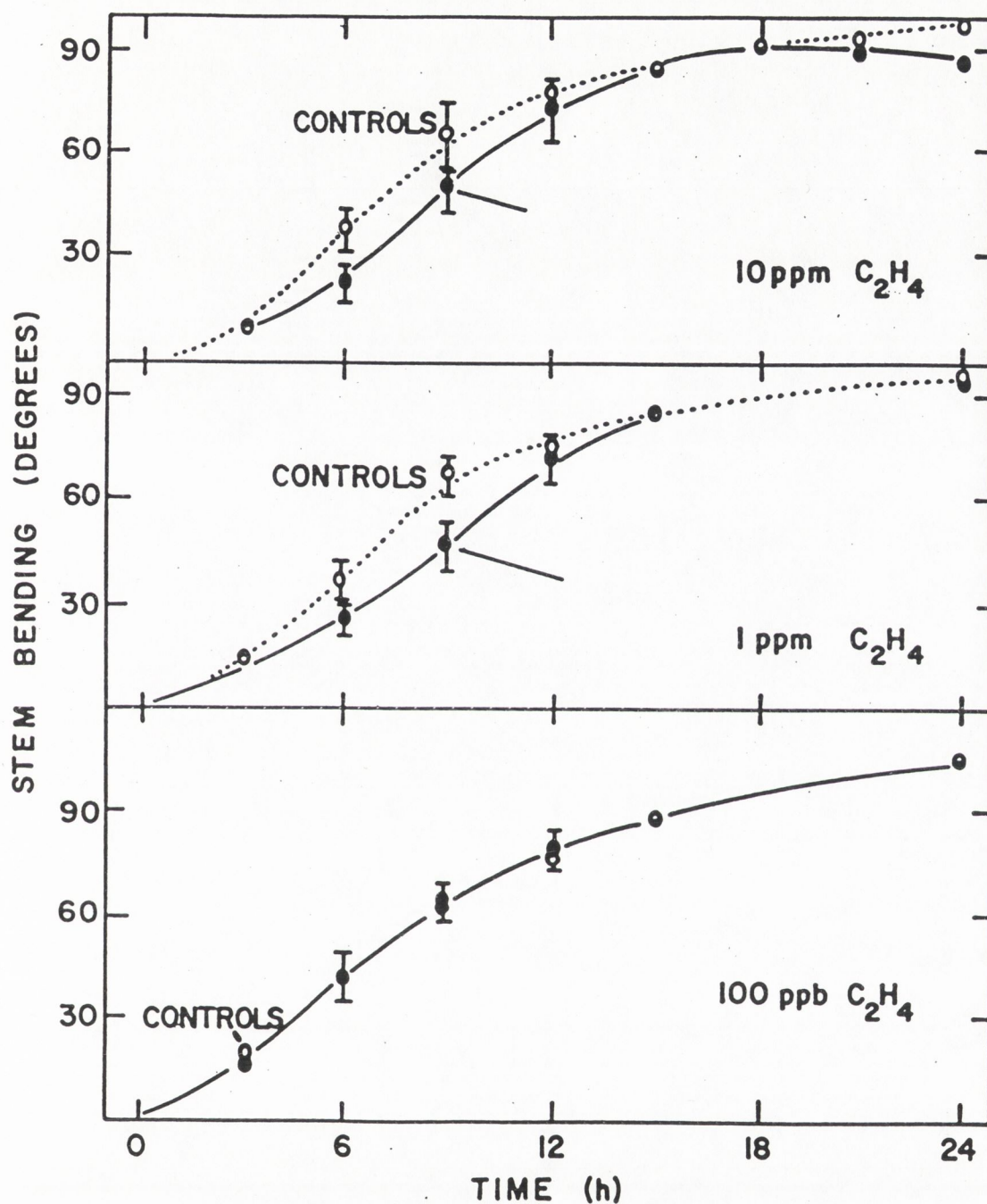


Figure 36. Effect of ethylene on gravitropic response of cocklebur stems. Plants were enclosed in plexiglas chambers into which quantities of ethylene were added. Concentrations of 10 and 1  $\mu l^{-1}$  slowed the rate of response significantly in the midregion of the curves. Ethylene at 100  $\mu l^{-1}$  showed no effect on the response. All points are three-plant averages with standard error bars shown.



in the mid-region of the response curve (Fig. 36). Both of these treatments appeared to reach the vertical at the same time.

Another method for treating AVG dipped plants with ethylene was application of an ethephon solution to the stem. Ethephon solution (0.1%) was swabbed onto either the bottom or top sides of horizontal cocklebur stems. Both top and bottom applications of 0.1% ethephon to AVG-treated plants accelerated gravitropic response somewhat, compared to AVG-treated plants (Fig. 37). But, as with tests directly adding ethylene to the surrounding air, the ethylene supplement was not enough to restore bending to the control rate. AVG-treated plants were delayed about 4.5 h in reaching  $60^\circ$  compared to controls, while AVG with bottom-applied ethephon were delayed only 1.5 h, and AVG with top-applied ethephon were delayed 2.0 h. In light of ethylene's tendency to inhibit cell elongation, it is somewhat surprising that the bottom-applied ethephon plants responded slightly faster than the top-applied plants. One might expect the faster response with the top-applied ethephon treatment, if inhibition of cell elongation on the tops of horizontal stems is in part responsible for gravitropic bending.

#### Restricted Gravitropism

When cocklebur plants were packed securely with vermiculite in plastic buckets and then placed horizontally, the stems were prevented from bending upward by the vermiculite. Figure 38 shows the results of such an experiment in which vermiculite-restricted plants were periodically unpacked and allowed to spring into a bent position. This springing upward of the released plants would occur immediately (within seconds) of unpacking. The restricted plants sprung to an average angle of  $90^\circ$

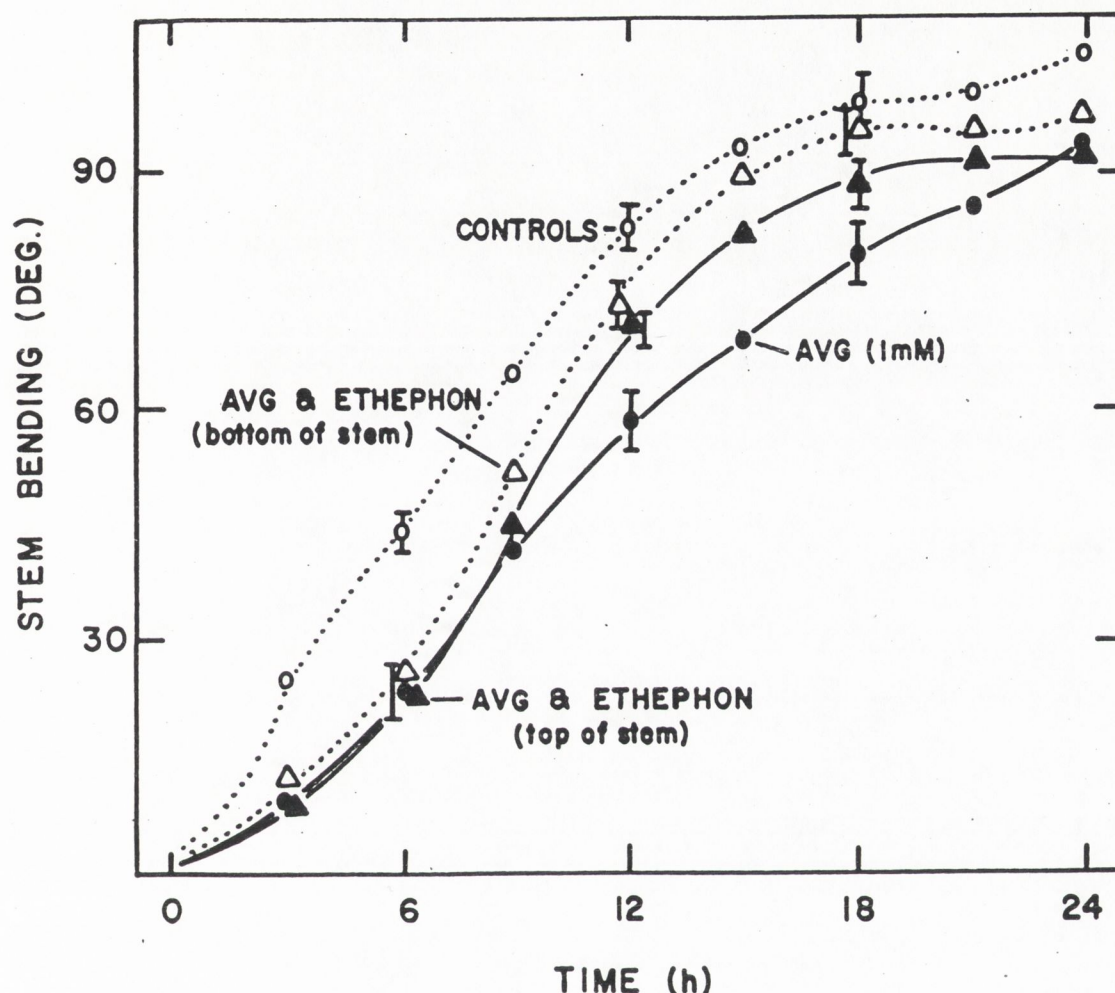


Figure 37. Effect of AVG and unilateral application of ethephon (0.1%) on the gravitropic response of cocklebur stems. If plants are pre-treated with AVG and then swabbed with 0.1% ethephon on either the top or bottom sides of their stems (horizontally placed), an intermediate response results. Both top or bottom applications of ethephon to AVG pretreated plants speed the response compared to plants just treated with AVG, but neither treatment responds as quickly as controls. In this test, bottom applications of ethephon appeared slightly more effective than top applications. All points are five-plant averages, with standard errors shown.



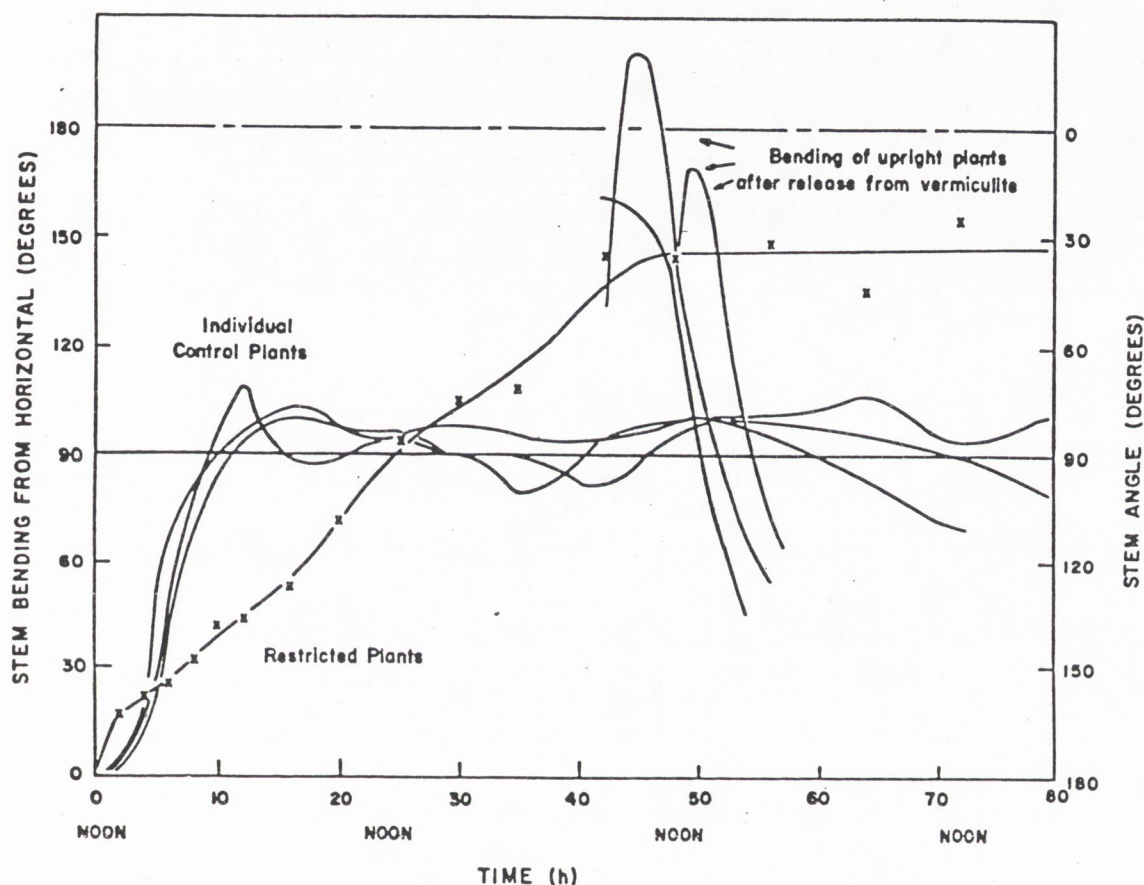


Figure 38. Gravitropic bending response of horizontal cocklebur plants restricted from bending by packing them securely with vermiculite in plastic buckets. Three individual plants were unpacked periodically, and the angle of bending to which the plants sprung upon release was measured. Stems would spring immediately upon release (ca. 5 s), indicating a storage of bending energy in the restricted state. This bending energy storage was sufficient to exceed  $90^\circ$  after 24 h (x-curve), and continued upward to about  $150^\circ$  at 40 h. The bending curves of three individual unrestricted plants are also shown without points. The bending of three restricted plants placed upright on the greenhouse bench after release was also tracked for several more hours. Two of these plants showed a continued increase in bending in the upright position before straightening, while the third plant began straightening immediately after release.

after 25 h into the experiment, approximately 12-14 h later than unrestricted control plants (Fig. 38). But the angle of springing upon release exceeded  $90^\circ$  after this time, progressing steadily upward until 42 h into the experiment, at which time a leveling off occurred. The leveling of the springing response approximated  $140^\circ$  up to 80 h of restriction. Thus, the restricted cocklebur stems showed a "stored bending energy" with their ability to spring toward the vertical upon release.

If the bending of a released plant was tracked for several hours after the release, a "stored bending stimulus" could be observed. Figure 38 traces the bending of three such plants after they were released and placed upright on the greenhouse bench. One plant sprung immediately to an angle of  $131^\circ$  and then continued bending in an upright position to a maximum angle of  $202^\circ$  2.5 h later, after which it began straightening. A second plant shown in Figure 38 shows a similar stored bending stimulus, while a third shows no stored stimulus; it began straightening immediately after release. Of six plants tracked after release, five showed stored bending stimulus, in the upright position, reaching a maximum angle approximately 2 h after release. The average increase in bending of six plants measured was  $26^\circ$ .

If horizontally placed cocklebur plants are restricted with pieces of masking tape fastened to wooden dowels on opposite sides of the stem, the tape can be cut quickly with a razor blade, allowing the stem to spring upward. The results of such an experiment are shown in Figure 39. In this case, a lag of only 4-8 h occurred between restricted plants reaching the vertical and unrestricted plants. As with the vermiculite experiment, the tape-restricted plants did not stop at the vertical and stored bending energy enough to cause springing greater than  $90^\circ$  upon



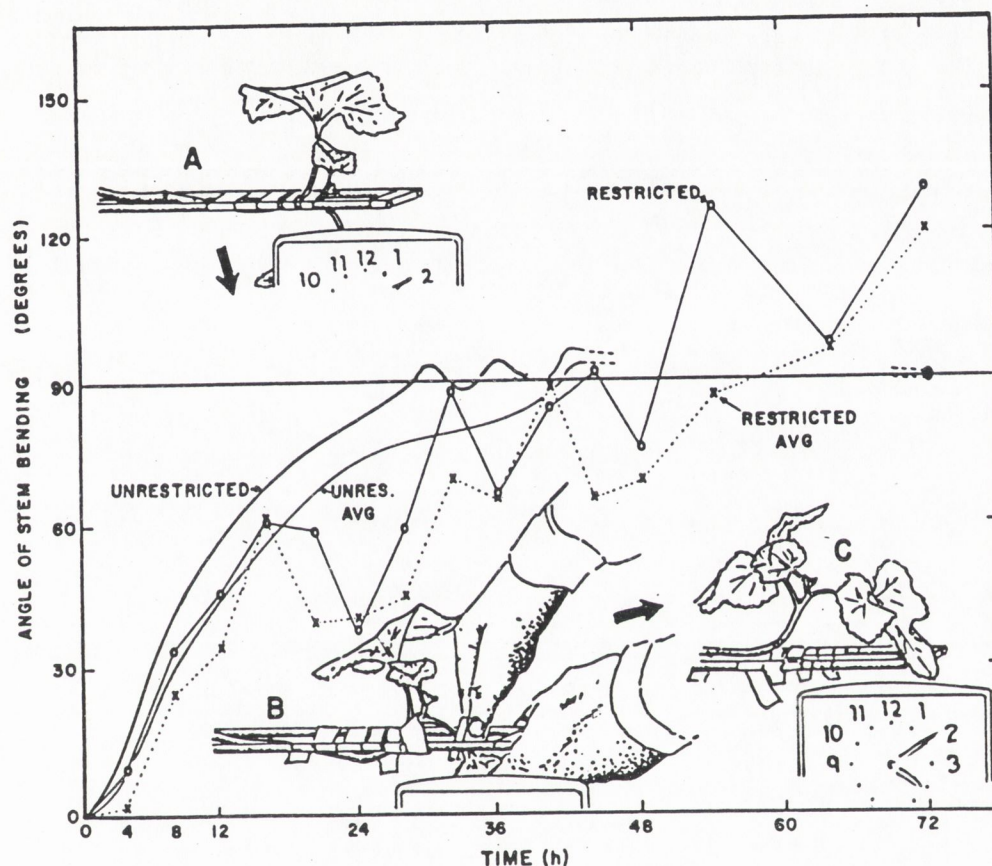


Figure 39. Gravitropic response of cocklebur stems restricted from bending by strips of tape stretched between two wooden dowels on opposite sides of the stem (A). Plants were released by quickly cutting the tape with a razor blade (B), and the angle of springing was measured (C). As with vermiculite restriction, the restricted plants stored bending energy sufficient to surpass the vertical (but at 54 h in this case). Half of all the plants were pretreated with AVG (1 mM). AVG delayed bending of unrestricted plants, and appears to delay the initial springing of restricted plants slightly. Unrestricted curves and all points are three-plant averages. Insert sketches traced from slides by Dr. Frank Salisbury.

release. An average of  $130^\circ$  was recorded after 72 h of restriction (i.e.,  $40^\circ$  overshoot). As with the vermiculite test, the taped plants showed stored bending stimulus by continuing to bend in an upright position after release. The average stored stimulus after 2 h in the upright position was  $12.4^\circ$ .

In the experiment of Figure 39, half the plants were pretreated with 1 mM AVG. The AVG slowed the response of unrestricted plants, as usual, and appeared to slow the response of the restricted plants as well. Because three different plants had to be averaged for each point, the data are considerably scattered. The average stored bending stimulus at 2 h after release was  $12.5^\circ$ , almost exactly the same for non-AVG treated plants; therefore, the apparent delay caused by AVG treatment among restricted plants had to be accounted for by a reduction of the initial bending upon release; that is, AVG appeared to cause a decrease in bending energy storage.



## DISCUSSION

## Clinostat Responses

The results of these clinostat tests clearly show a role for ethylene in the typical resultant epinasty. Leather et al. (1972) and Palmer (1973) have also shown this with CO<sub>2</sub> inhibition of clinostat-induced epinasty, but to my knowledge it had not been shown before with AVG or silver inhibition (Fig. 3, 13). This verification seems worthwhile, considering the complications of respiration and photosynthesis that can occur in enriched-CO<sub>2</sub> experiments.

It is still not clear how ethylene mediates epinastic leaf curvature. The response is apparently dependent some way on gravity. Crocker et al. (1932) showed this by reducing epinastic response to ethylene, when tomato plants were maintained in an inverted position. This would also seem apparent from the observation that exposing a plant and its leaves to an ethylene-enriched atmosphere (i.e., from all sides) consistently causes the directionally oriented growth response of epinasty. There are few environmental factors other than gravity to be cued on for such a response, unless epinasty develops in accordance with existent structural or morphological patterns in the plant organs.

One consistent observation in my records shows that epinasty from clinostating, ethylene, and auxin treatment, is always most pronounced in the youngest, fastest growing leaves. This would imply that epinasty caused by these treatments is a result of differences in true growth rather than temporary turgor pressure changes.

In so far as much still remains unknown about orthogravitropic reactions in plants (i.e., alignment of organs parallel to the direction



of gravity), one can only speculate on the physiological mechanisms controlling plagiotropic growth (i.e., angles away from the vertical, including epinastic growth), which is even less understood. For example, many plagiotropic organs will right themselves when inverted or displaced (e.g., leaves), while others show no response (e.g., some rhizomes; Elfving, 1884). The involvement of ethylene in epinasty of leaves and the diageotropic (horizontal) response of certain dicot shoots (e.g., etiolated peas), however, indicates that this hormone might be a good starting place for plant plagiotropic investigations.

#### Comparisons of Clinostat and Mechanical-Stress Responses

In the experiments that I have reported, *the severe epinasty caused by horizontal clinostating was never duplicated by mechanical stress*. All attempts to mechanically stress the plants, including horizontal shaking, vertical shaking, continuous twisting, and intermittent clinostating failed to produce any discernible epinasty. The intermittent clinostating exactly duplicated the motions experienced by a horizontally clinostated plant (although the stresses were not prolonged as in clinostating), and yet these plants' leaves responded almost identically to the leaves of stationary controls (Fig. 8, 9). Even the mechanically traumatic event of continuous twisting appears to cause no significant epinasty.

Only very vigorous hand-administered shaking caused cocklebur plants to show any epinasty, and this was barely half as intense as in simultaneously clinostated plants. Curiously, daily spraying of plants with silver thiosulfate also induced mild epinasty. This was somewhat surprising, because of silver's ability to block ethylene action in plants,



but not totally unexpected in light of silver's chemical reactivity and deleterious effects upon living tissue. As mentioned previously, silver has been shown to cause wound ethylene, although its ability to block the action of ethylene normally outweighs this enhanced evolution.

Although ethylene has clearly been implicated in both clinostat epinasty and mechanical-stress responses in plants, the concentration, origin, and active sites of ethylene for these two events might differ. Ethylene's effects on plant growth and development are amazingly diverse and widespread. Some have even proposed that, along with all of its normally associated responses, ethylene might also ultimately control most auxin events within plants (Burg and Burg, 1967 a). Recent discoveries have shown ethylene's effects ranging even into photosynthesis (Kays and Pallas, 1980) and phototropism (Humphrey, 1980).

Because of this diversity then, and from my results, it seems entirely possible that ethylene involvement in mechanical stress and clinostat responses may somehow differ in origin and/or effects. There is some evidence in the literature that responses typical of mechanical stress and gravity-related responses (in which I include epinasty) may differ by one or two orders of magnitude in concentration (Abeles, 1973). In addition, one might classify mechanical stress ethylene evolution as a form of wound ethylene, which probably evolves via methionine as does other ethylene production, but these systems might be under different controls (Lieberman, 1979).

Unfortunately, exact comparison of ethylene evolution between clinostated and mechanically stressed plants has not been successful so far. However, even though actual concentration measurements would add insight, I am not sure that they would be conclusive, for the reasons cited above.

For example, mechanical stresses might affect cell plasmalemmae (one possible source of ethylene), while disruption of gravity perception systems might affect plastids, or endoplasmic reticula (another possible source of ethylene). This could be important because of ethylene's tendency to have physiological effects near its source of production (Abeles, 1973).

From all of this, then, I feel confident in rejecting the initial hypothesis. Therefore, in conclusion, *clinostat-induced leaf epinasty is not simply a result of mechanical stress.*

### Inversion

In essence, periodically inverting plants approaches horizontal clinostating. Inversion cancel the gravity vector influence upon the plant the same as a clinostat, although it does not transform the gravity vector into a multilateral stimulus. Inversion should suspend cellular organelles and thereby gravity-compensate a plant the same as free-fall would. Ideally, the period of inversion should be less than the time required for directed growth to occur. Unfortunately, I have never measured the minimum presentation time for cocklebur (i.e., the shortest amount of stimulation time required to elicit a gravitropic response), but this would most probably vary somewhat, depending on the growth vigor of the individual plants. (From my results of gravitropic experiments with cocklebur, a 10- to 30-min period of displacement would not appear to cause any major bending responses.) The inversion cycle of 20 min was chosen as a compromise between a time long enough to minimize mechanical agitation and a period short enough to prevent gross oscillating growth responses. The closer attention required for shorter



inversion periods over 24-h experiments also influenced the decision! Ultimately, the small differences in the one experiment comparing 10, 20, and 30 min inversion periods were the determining factors, but this is something that deserves repeating and closer scrutiny.

Figures 14 and 16 show similar epinasty response patterns for both clinostated and inverted plants. Plants subjected to either clinostating or inversion become significantly more epinastic than stationary plants or plants inverted and immediately returned upright. But, Figure 14 also shows that the clinostated plants sometimes become a bit more epinastic than inverted plants. In speculating on why inverted plants were slightly less epinastic, one might attribute differences to environmental conditions such as lighting. For example, the clinostated plants were continuously exposed to light from all directions (except the bottom), while the inverted plants were alternately exposed on the top and the bottom, with one side slightly shaded each time from the shelf holding the plants. But the dark experiments (Fig. 15) also resulted in slight differences, so lighting was probably not crucial.

Slight differences in epinastic response might also be explained as differences in events on the subcellular level. The clinostat probably causes a continuous stirring of cellular organelles in elliptical paths as they constantly settle. The amount of stirring would be dependent on such factors as organelle size, cytoplasmic viscosity, and rotation rate (see Dedolph and Dipert, 1971). In contrast, periodic inversion would cause a reversing linear displacement of cellular organelles and, in all probability, some settling of these organelles during the 20 min.

A third possible difference might be caused by subtle differences



in mechanical stresses upon inverted and clinostated plants. But the overall results of these experiments thus far indicate that these would have very little effect. This is also evinced from the results of the test propping petioles upward of inverted but immediately returned upright (Fig. 16). Despite these small differences, the inversions tests indicate that *clinostat-induced epinasty is probably a result of gravity compensation.*

This finding brings forth an interesting question with regard to plant growth under satellite free-fall (i.e.,  $g = 0$ ). If epinasty is a result of gravity compensation, and ethylene antagonists such as AVG and silver block the development of this epinasty, it follows then, that gravity compensation probably causes endogenous ethylene production. Ethylene in high concentrations, or continuous exposure of plants to ethylene, often causes many aberrant and sometimes deleterious effects. In fact, ethylene toxicity has been cited as a possible mode of action for some auxin-like herbicides (Baur and Morgan, 1969). It would seem to be crucial, therefore, to strive for a more thorough understanding of the effects of gravity compensation (or weightlessness) on ethylene biosynthesis in plants. The grim possibility that many plants might not be able to develop normally or even survive under weightlessness surfaces from this. Cordeyev (1979) reported that the onions and peas grown on Salyut 4 and Salyut 6 in fact did die after 2 or 3 weeks of space flight. Prolonged clinostat studies may contribute to an answer of this problem. Palmer (1973) has shown that *Helianthus* plants are able to adjust somewhat to clinostating in that leaf epinasty was only a transient symptom in long term experiments. Others have also conducted long experiments in which plants apparently survive, including Lyon (1965 b) who clinostated tomato plants for 6 weeks and Wilson (1973) who grew



white pine (*Pinus strobus*) trees for 8 weeks on clinostats. As before, the ultimate answer will come from further space-flight tests.

#### Elimination of Mechanical Stress on the Clinostat

The results from the experiments supporting the petioles from flopping show slightly less epinasty than unsupported petioles (Fig. 11); this trend reoccurred, although the differences were less in a repeat of this experiment. This may indicate that mechanical stress might play a minor role in the responses to clinostating. This is indirectly supported by the fact that periodic inversion of plants does not cause quite as intense an epinastic response as does clinostating (Fig. 14, 16). One would expect that plants inverted at 20-min intervals would be gravity compensated much as those on a horizontal clinostat; yet the responses are not identical, albeit they are very close.

Evidence indicating that the responses caused by clinostating are not totally accounted for by gravity compensation can be seen from the Biosatellite II studies with pepper plants (note, however, there were only four plants used for the space-flight calculations). The plants taken into satellite free-fall showed significantly more epinasty (ca.  $10^\circ$  of petiole angle) than did terrestrially clinostated plants after 15 h into the experiment; they remained more epinastic until the conclusion of the flight (reentry began at 44  $\frac{3}{4}$  h; Brown et al., 1974). The remaining results from Biosatellite II showed little difference from ground controls, so the reasons for these differences can only be speculated about (see Salisbury, 1969).

In contrast to these preceding thoughts, however, the fact that any epinasty developed in outer space indicates mechanical stress is



probably not a major factor, since mechanical forces causing strains on plant organs would be absent in satellite free-fall.

Initially the results from clinostat experiments using plants with debladed petioles indicated that the weight of the blade might affect epinastic growth, but this was later shown to be incorrect by restoring IAA to the petiole stumps (Figs. 12, 13). This has also been shown by others (Lyon, 1963). These results bring to light the fact that the leaf blades and the apical bud are not required to perceive gravity. The debladed petioles provided with auxin became just as epinastic from clinostatating as did the petioles of intact leaves. (Auxin had no effect on debladed upright plants.) Providing, then that a clinostat does cause epinasty, at least in part, due to gravity compensation, the gravity perception for the petioles appears to be occurring "on site", near where the response occurs.

Plants rotated horizontally on clinostats are clearly mechanically agitated (see Plate 4), but it appears the role of this agitation in producing leaf epinasty is minor; therefore, the major cause of the epinasty is probably the gravity nullification or compensation effect of the clinostat, and this might be a result of statolith suspension within the gravity perceiving cells. A role for amyloplasts as statoliths has not yet been proven for dicot stems, however.

### Sleep Movements

Cockleburs typically straighten their leaves (become hyponastic) during the dark period each day. This seems particularly true for the youngest leaves of vigorous plants and for seedling cotyledons. Cocklebur leaves also typically become slightly epinastic prior to and into



the first hours of the dark period; that is, at dusk. The stationary control curves of Figures 7 and 8 show these movements.

Figures 3 and 14 show that the ethylene antagonists AVG and silver clearly damp these sleep movements in cocklebur. This implies an essential role for ethylene in nyctinasty of cocklebur.

Although the movements of pulvinus-controlled leaves have been studied (Satter and Galston, 1973), discussion of nonpulvinus-leaf mechanisms in the literature seem limited. Ethylene has been shown to alter leaf movements in *Prosopis juliflora* (mesquite) and *Acacia farnesiana* (huisache; Morgan and Baur, 1970), and Morgan and Baur (1970) proposed that ethylene may be involved in the natural leaf movements of these species (although Satter et al., 1972, were unable to observe any effects of ethylene in *Albizia*). Unlike cocklebur, however, all of these species' leaf movements are controlled by pulvini. Because of the lack of mechanistic explanations for movements in nonpulvinus leaves, this area could contain some potential physiological research, particularly with such powerful tools as hormone inhibitors.

#### Gravitropism Tests

Inhibitors of ethylene biosynthesis and ethylene action in plants significantly slow stem bending in response to gravity in cocklebur, tomato and castor bean. *This implies an essential role for ethylene in gravitropism of mature dicot shoots.*

The ethylene could be acting directly upon the growth of cells. It might some way be directed to stimulate growth of cells on the lower sides of horizontal stems (ethylene has been shown to promote growth in some plants or tissues; Ku et al., 1970; Osborne, 1977), or more



likely, it might directly cause inhibition of elongation of cells on the top side of stems. This would be in agreement with Digby and Firn's (1979 a) findings with cucumber hypocotyls, and it also appears to fit well with preliminary measurements from this laboratory on restricted cocklebur stems that are released and allowed to spring upward. These tests indicate that some cell contraction on the top sides of horizontal stems is occurring (Mueller and Salisbury, 1980; Sliwinski and Salisbury, 1980). If ethylene were inhibiting cell elongation of the top cells, it would have to be released in these cells or some way directed to the upper half of the stem. How such differential evolution or migration might occur is not known.

A third possibility of direct control could involve some cofactor that might increase the susceptibility of the top cells to ethylene's effects, or conversely protect the lower cells from its effects (assuming that ethylene is acting in an inhibitory capacity). If this were the case, then gravitational disorientation might cause ethylene evolution throughout the stem tissues, but only certain cells would respond. There is some evidence for the differential migration of nonhormonal substances (cofactors?). For example, Roux (1980) reports up to four times as much  $^{45}\text{Ca}$  per unit dry weight on the concave side of curved *Zea mays* coleoptile segments as on the convex side. This calcium builds up on the upper sides of horizontal coleoptiles before any discernible bending occurs and may in some way be associated with the calcium binding protein calmodulin and its movements.

Finally, ethylene might directly alter translocation of auxin in horizontal stems. Ethylene has been shown to do this in some other plant responses (Morgan and Gausman, 1966). In a similar light, ethylene might



also alter cell sensitivity to auxin (Michener, 1938).

A second general explanation for ethylene's role in shoot gravitropism would be through secondary or indirect effects. For example, if auxin were to accumulate in certain cells, this might cause higher ethylene evolution, thereby affecting growth through a combination of auxin and ethylene. Osborne (1975) postulates that auxin-stimulated ethylene from the lower halves of horizontal grass nodes might passively migrate (diffuse) to cells of the top where it could exert an inhibiting effect on elongation. This ethylene would also be present in the lower cells, where it is formed, but its growth regulating effects would be overridden by the auxin also present in those lower cells. In such a mechanism, auxin could determine the extent of cell elongating, while ethylene could regulate the orientation of expansion (Osborne, 1975). Also, if auxin were to migrate from certain tissues, this might reduce ethylene production in these cells, resulting in either reduced or enhanced elongation. However, these mechanisms would depend on rapid production or extensive migration of auxin, and this is precisely where complaints have arisen about the traditional hormonal explanations of gravitropism; namely, auxin apparently does not move fast enough, or in sufficient quantities, to cause the observed bending in some plant stems (Digby and Firn, 1976).

Clearly, more definitive studies are required before further speculation. For example, split stem studies might lend insight to the problem. If stem sections were cut longitudinally, with several pieces placed upward in an enclosed chamber, while an equal number were placed in the opposite orientation, down, in another chamber, ethylene measurements could be taken comparing the two treatments. Such studies have



been done with grass nodes by Wright et al. (1978).

A better approach might involve dividing the upper and lower sides of horizontal stems into separate, constant-flow cuvettes for ethylene trapping. This method would circumvent tissue wounding and ethylene autocatalysis. However, the equipment for such an endeavor might be difficult to assemble, and from my experience, the ethylene trapping system would have to be highly efficient. A particularly touchy problem would be the type of sealant between the cuvette and the stem.

Continuation of "restricted bending" experiments, on the other hand, might prove highly rewarding. Precise measurements of the anatomical patterns and events of restricted stems will surely shed light on the overall process. Also, if restricted stems could somehow be split, or if one side's cells could be killed, the side most responsible for the bending could be determined; that is, the top cells and the bottom cells could be compared as to their relative roles in the overall bending process. If the top cells were contributing significantly through contraction (after release from restriction), as it now appears, ethylene inhibiting cell elongation might fit into a mechanism explaining this.

Finally, research on the role of ethylene in gravitropism of monocots and other plant groups might also be useful, thereby indicating the extent of ethylene's importance in this growth response. Similarly, treatment with ethylene inhibitors in phototropic studies might provide interesting findings.



### Decapitated-Gravitropic Response

Only a few tests were conducted with decapitated (i.e., apical bud removed) and defoliated stems. The tests showed that rapidly growing, vigorous decapitated and/or defoliated plants could bend fully to the vertical just as fast as intact plants, very often with faster initial bending rates. But if the plants were a slower growing group (older), the defoliated plants would stop at a still smaller angle (ca. 70°, Fig. 28). Because of this variety of results (which also appears in the literature; see Wilkins, 1979) decapitation experiments should probably be interpreted cautiously. Nonetheless, these tests do show that neither the leaves nor the apical bud appear to be essential for perception of gravity or initiation of the bending response.

### Hormonal Deflection of Stems

Unilateral application of ethephon solutions (which release ethylene) to tomato stems caused distinct deflections toward the side of application. These applications were not as effective with cocklebur stems, but ethylene participation in stem bending was shown in cocklebur with the indirect approaches described in the results. These tests show that ethylene is indeed capable of causing stem bending in these species. The most potent promoter of stem deflection in these tests was IAA, which caused deflection away from the side of application. Therefore, IAA would seem to be a good starting place when investigating hormonal mechanisms in stem bending.



### Restricted Gravitropism

When the stems of cocklebur are restricted to a horizontal position for 48 h or more and then suddenly released, they spring upward within 10 s, frequently beyond  $90^\circ$ , up to a maximum of  $150^\circ$  in our tests. These plants are somehow able to store a bending energy, either in a tendency for cells on the bottom of these stems to elongate and/or a tendency for the cells on the top side to contract. The springing is apparently a combination of these two events (unpublished results of Wesley J. Mueller and Julianne Sliwinski).

The mechanism for this springing would appear to involve water potentials within the cells, I say this intuitively but also from observing that the springing is reduced when plants are water stressed; a wilted plant will obviously show no upward springing (or bending, for that matter). If it is indeed a combination of bottom cells "wanting" to stretch and top cells "wanting" to contract, then it would follow that the bottom cells are restricted from elongation, that is, compressed, while the top cells are stretched beyond their "desired" length. In other words, the bottom cells are signaled to elongate but can not, while the top cells are physically forced to elongate against their control signal.

More definitive research of this phenomenon would certainly involve actual quantification of the bending and observations of the actual cells before and after release from a restrained state. Electron microscopy of the cell wall microfibril patterns and densities would also shed light on these events, for apparently the walls of the top cells are able to contract elastically while the bottom cells can expand or



stretch. If water potential (i.e., turgor pressure) is the driving force for this, then one would expect little change in the total cellular volumes in 10 s or less; only the dimensions should change. Here also, direct measurements would add much insight, although water potential measurements on the cellular level would be very difficult. Perhaps a microscopic, capillary manometer tube could be used to penetrate epidermal cells (see Zimmerman, 1978), or aphids could be positioned on both sides of the stem to quantify the exudate (although most aphids appear to exclusively seek out phloem cells). Such information could add a great deal to the present knowledge of cellular growth and development mechanism.

The roles of hormones, that is, ethylene and auxin, should also be investigated more closely with regard to cell wall development and shifting in release of restricted stems. Auxin is known to cause loosening of cell walls (presumably through hydrogen ion release; Cleland, 1971), thereby allowing elongation driven by water pressure, while others have shown that ethylene might cause microfibril arrangement favoring transverse cell expansion and restricting longitudinal growth (Abelbaum and Burg, 1971; Sargent et al., 1973). Both of these hormones could be fit into speculative mechanisms explaining the restricted gravitropic responses, but how these hormones might be selectively translocated or released, or how cells might be differentially sensitized to their effects, remains a mystery to me. Here, as with past tropic growth studies, radioactive labeling might be a good initial approach.



## Summary

1. Mechanical stresses in horizontally clinostated plants are insignificant in causing the typically observed leaf epinasty. Mechanical stresses may contribute slightly to the overall clinostat response, but it appears that gravity compensation, probably via suspension of gravity perceiving organelles within the cells, is responsible for clinostat-induced epinasty.

2. Inhibitors of ethylene biosynthesis and action in plant tissues significantly slow gravitropic response in mature *Xanthium strumarium* L. (cocklebur), *Lycopersicon esculentum* Mill. (tomato), and *Ricinus communis* L. (castor bean) stems. Therefore, ethylene is apparently essential in low quantities, probably less than 1 ppm, for normal gravitropic response in these species and possibly other mature dicots.

3. Ethylene appears to be essential for the daily leaf movements of cocklebur, since inhibitors of ethylene action and synthesis damp these movements.

4. Ethephon solution (1%), applied exogenously to the apical 10 cm of one side of tomato stems causes the stems to deflect up to 80° toward the side of application after 24 h of horizontal clinostating (to overcome natural tendencies to grow straight). IAA (1%) and NAA (1%) lanolin pastes applied to one side of the apical 10 cm of tomato and cocklebur stems cause deflections away from the side of application. IAA deflections up to 250° for tomato and 270° for cocklebur were observed after 24 h of clinostating, while NAA deflections up to 150° were observed for cocklebur. ABA (1%), GA<sub>3</sub> (1%), and kinetin (1%) lanolin pastes applied unilaterally to the top 10 cm of tomato stems



showed no consistent results.

5. Cocklebur stems placed horizontally and restricted from any bending for over 12 h abruptly spring upward when released. Plants have sprung up to  $90^\circ$  after 24 h, and up to  $150^\circ$  after 48 h. This springing usually occurs within 10 s of release. The method of restriction and the vigor of plants used in the tests affected the springing somewhat. Many of the restricted plants would continue to bend for about 2 h after the initial springing action, sometimes reaching total bending angles of over  $200^\circ$ , indicating a storage of the gravitational stimulus.

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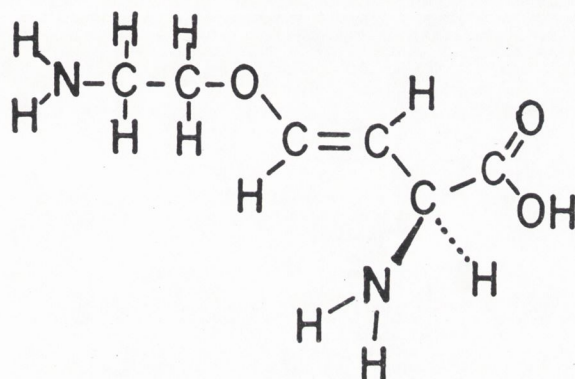
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## APPENDICES

## Appendix A

**AVG**

(Aminoethoxyvinyl glycine)

Chemical structure of aminoethoxyvinyl glycine, a powerful inhibitor of ethylene biosynthesis in plants.



Appendix B. Analysis of variance results and Duncan Multiple Range Comparison for data from experiment comparing effects of ethylene inhibitors on clinostat-induced epinasty (see Fig. 4).

Source	df	SS	MS	F
Treatment Over Time	45	1455.58	32.35	4.16 *
Error	54	419.79	7.77	

\* Significant at P 0.001.

Duncan's Table:

r	(2)	(3)	(4)	(5)	(6)
$q_{0.01(r,v)}^{**}$	3.76	3.92	4.03	4.12	4.17
$W_r^{***}$	5.22	5.44	5.59	5.72	5.79

\*\* This is a tabular value from a Duncan's percentage point listing using an value of 0.01 and 54 degrees of freedom (v).

\*\*\*  $W_r = q' (MS \text{ Error}/n)^{\frac{1}{2}}$  where  $n = 4$  for this experiment.

Ranking of Means: (Inverse radii of curvature values are given)

Time	A	B	D	E	C	F	
	(11.7)	(5.4)	(4.7)	(1.7)	(1.1)	(0.0)	
6 h							
12 h	A	B	E	D	C	F	A-Clinostat
	(16.7)	(10.2)	(6.7)	(6.5)	(5.2)	(1.4)	B-Clinostat + AVG
24 h	A	C	B	E	D	F	C-Clinostat + Ag <sup>+</sup>
	(17.0)	(11.0)	(9.5)	(4.3)	(1.0)	(0.6)	D-Stationary
30 h	A	B	C	E	D	F	E-Stat. + AVG
	(18.4)	(11.2)	(11.2)	(5.3)	(2.2)	(0.6)	F-Stat. + Ag <sup>+</sup>

Note: Underlining indicates that the means are not significantly different.

Appendix C. Analysis of variance results and Duncan Multiple Range Comparison for data from the intermittent clinostat experiment (see Fig. 9).

Source	df	SS	MS	F
Treatment Over Time	16	665.00	41.56	16.28 *
Error	120	306.59	2.55	

\* Significant at P 0.001

Duncan's Table:

r	(2)	(3)
$q_{0.01}^1(r,v)^{**}$	3.71	3.86
$W_r^{***}$	1.71	1.78

\*\* v = 120

\*\*\* n = 12

Ranking of Means: (Inverse radii of curvature given)

Time	A	C	B	
3 h	(2.50)	(-0.25)	(-0.42)	
6 h	(5.50)	(-0.42)	(-0.50)	A - Clinostat
12 h	(8.50)	(0.33)	(-0.04)	B - Stationary
24 h	(8.00)	(-0.58)	(-0.79)	C - Intermittent Clinostat

Note: Underlining indicates that the means are not significantly different.



Appendix D. Analysis of variance results and Duncan Multiple Range Comparison for data from experiment comparing inversion, clinostating, inverting and returning upright, and stationary plants (see Fig. 14).

Source	df	SS	MS	F
Treatment Over Time	70	2692.55	38.45	8.31 *
Error	240	1110.77	4.63	

\* Significant at P 0.001.

Duncan's Table:

r	(2)	(3)	(4)	(5)	(6)	(7)	(8)
$q_{0.01}^1(r,v)^{**}$	3.76	3.92	4.03	4.12	4.17	4.23	4.27
$W_r^{***}$	2.86	2.98	3.07	3.13	3.17	3.22	3.25

\*\* v = 70

\*\*\* n = 8

Ranking of Means: (Inverse radii of curvature values are given)

Time	D	C	E	G	F	A	B	H	
6 h	(6.1)	(4.9)	(1.7)	(1.6)	(0.8)	(0.7)	(0.1)	(-1.5)	A-Stationary
									B-Invert and Return
									C-Invert Every 20 min
12 h	(7.0)	(3.5)	(3.1)	(2.2)	(0.6)	(-0.7)	(-1.7)	(-2.9)	D-Clinostat
									E-Stat. + AVG
									F-Inv. and Ret. + AVG
24 h	(9.8)	(9.0)	(8.1)	(3.8)	(3.5)	(0.4)	(-2.5)	(-2.9)	G-Invert + AVG
									H-Clinostat + AVG

Note: Underlining indicates that the means are not significantly different.

Appendix E. Analysis of variance results and Duncan Multiple Range Comparison for data from experiment comparing clinostating, inversion, inversion and returning upright, stationary controls, and plants whose petioles were propped upward for alternating 20 min periods (see Fig. 16).

Source	df	SS	MS	F
Treatment Over Time	40	1037.09	25.93	14.7 *
Error	144	265.32	1.84	

\* Significant at P 0.001

Ducans Table:

r	(2)	(3)	(4)	(5)	(6)
$q'_{0.01}(r,v)^{**}$	3.71	3.86	3.93	4.06	4.11
$W_r^{***}$	1.78	1.85	1.88	1.95	1.97

\*\* v = 144

\*\*\* n = 8

Ranking of Means: (Inverse radii of curvature values are given)

Time	A	D	C	F	E	B	
6h	(6.3)	(3.5)	(3.2)	(1.7)	(1.7)	(0.4)	A = Invert Every 20 min B - Invert and Return
							C - Stationary Controls
12 h	(7.6)	(7.5)	(3.6)	(3.5)	(3.5)	(3.05)	D - Clinostat
							E - Invert, Return, and Prop Every Other 20 min
24 h	(9.9)	(9.8)	(1.4)	(1.2)	(1.0)	(0.5)	F - Stationary, Prop Every Other 20 min

Note: Underlining indicates that the means are not significantly different