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A procedure for SEM of complex shoot structures applied to the inflorescence of snapdragon (*Antirrhinum*)

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Summary. An indirect procedure for the scanning electron microscopy of living complex shoot structures, e.g., an inflorescence tip, employs the polymerization of a dental impression plastic. Application of the plastic to exposed surfaces during prolonged dissection minimizes desiccation. The resulting complex mould is everted so that recesses representing surface detail can be filled with molten epoxy polymer. The mould is then allowed to revert to its original configuration; it is now filled with epoxy. After the epoxy hardens, the resulting cast is sputter-coated and imaged. Intricate structures up to 1 mm in dimension can be imaged with all components, e.g., flowers on an inflorescence, in situ.

Keywords: *Antirrhum*; Flower structure; Inflorescence; Phyllotaxis; Plastochron; Scanning electron microscopy; Shoot morphology; Replicas.

Introduction

Scanning electron microscopy, SEM, because of its great depth of field, allows both overall morphology and tissue surface detail to be imaged together for a single specimen. In many shoot structures, successive primordia are initiated at regular intervals, called plastochrons (Erickson and Michelini 1957). Often a given shoot tip has equally-spaced successive stages of its appendages arranged in a geometrically regular array (Endress 1989). SEM of such a structure, after proper dissection, can offer great range, because many primordia are present. It can also offer great resolution because surface histology on each primordium can be observed.

While this combination can be obtained with freeze-fracture methods for cells and parts of organs (Beckett and Read 1986, Jeffree et al. 1987) there are difficulties in obtaining these features in large complex structures. SEM is best with fully turgid cells. If the dissected tissue is fixed, shrinkage detracts from quality. If cryo-stage or freezing-dehydration methods are used, shrinkage from a previous dissection can still be a problem.

We describe here an inexpensive procedure which minimizes shrinkage while still allowing very large and complex structures to be imaged. It is based on the mould-cast procedure of Williams and Green (1988). For single objects, the procedure is straight-forward as in Fig. 1. To image multiple structures on the same axis, a more involved procedure is required. Basically, a mould is made of successively younger turgid structures until the entire structure is covered. Coverage by the mould minimizes shrinkage during dissection. The compound mould is elastic and can be everted. While everted it is covered with viscous epoxy cement. Once reverted to its original configuration, the mould allows the now enclosed epoxy to harden into a faithful cast of the original structure. The cast is completely stable and can be trimmed to further expose surfaces of interest. Meanwhile, the original plant material is typically intact and can be studied by other techniques.

The method is applied here primarily to the wild-type inflorescence of snapdragon (*Antirrhinum majus*). This species has many interesting homeotic floral mutations. For both wild type and mutants, it is a great advantage to be able to follow the details of morphology and cell pattern in primordia of known age, measured in plastochrons. The information can aid in the staging of primordia seen in sections, e.g., in in situ hybridization

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studies. Staging requires that all the primordia, or bracts, between a given primordium and the apical dome of the inflorescence be visualized. This can be done, with all primordia in situ, by the present technique. The information allows a description of "standard" successive stages and also the calculation of the plastochron relative growth rate. This latter is the continuously compounded interest rate that would produce the observed increase in linear dimension between successive primordia, per plastochron.

Materials and methods

Plants

Wild-type strains of *Antirrhinum majus*, designated 75 and 98 at the John Innes Institute, grown in winter in the glasshouse, were studied in detail. Field grown plants, when available, in general were less susceptible to wilting. A commercial strain, 45, was also used. The method was also applied to tobacco.

Dissection

A Pasteur pipette was fused near its narrow end and nearly filled with water. The base of an inflorescence, about 8 cm long, was trimmed of lower leaves and bracts. It was lowered into the pipette which would hold it for dissection. It was important to prevent water from creeping up the plant surface into the flower region. A seal was made by first coating the hairy stem with super-glue (cyanoacrylic cement) or a silicone-rubber cement. To this coated region, epoxy gel (Devcon Corp. Danvers, MA, USA) was applied in abundance so that the base of the area of interest became affixed to the rim of the pipette. This anchored the tip of the organ, and kept it turgid (due to nearby water) but dry (due to the seal).

Removal of bracts, sepals, etc. was done with fine forceps or with a fine knife made as described in Williams and Green (1988). Once a region was exposed, it was quickly covered with polymer to make a mould and to minimize further drying. If tissue lost turgor, turgidity could be restored by keeping the holder and plant in a moist chamber for several hours.

Making the moulds

Many dental impression polymers can be used. Best results came from a mixture of resin from Kerr's Reflect and catalyst from Kerr's Mirror wash (Kerr UK Ltd, Peterborough, UK). Paired droplets of the two components were put on a glass slide for rapid mixing, with a tooth-pick. Mixed polymer was transferred to the primordium by very fine applicators. A supply of these was made from Pasteur pipettes by pulling the narrow part down to about 100 μm diameter in an alcohol flame. Further reduction in diameter (30 μm) required a second pulling near the blue base of the flame. The very slender tube was broken and a ball tip was then added by touching the tip to the flame. Fine applicators allowed the polymer to be spread into recesses of the object, without leaving bubbles.

Older primordia were exposed and covered, each making an independent simple mould. As the primordia became younger and smaller, adjacent moulds were fused to make a compound mould. It was important to keep the polymer of high enough viscosity so that it did not creep into upper, unexposed, regions prematurely. This would impair later dissection. Once the apical dome was reached, two large mounds of polymer were added at opposite sides of the base of the mould. These were in the position of ear flaps on a woollen helmet. Reinforcing polymer was also added to the tip. The individual moulds of the older basal flowers were then removed, inverted, and anchored to a glass slide with fresh polymer. A cast of each was made as in Fig. 1. The axis just below the compound mould was then severed and the polymer coated stem was removed.

Everting the complex mould

The complex mould, with tissue still in it, was then pinned down firmly at the edge of a composition board. The open end of the mould faced away from the board. The mould was horizontal, with the pins passing through the polymer in the "flaps" which were horizontal on the board (see Fig. 2). Once firmly anchored, the tip of a horizontal tooth-pick was used to push the tip of the mould outward (turn it inside out). This removed the plant material and put all of the detailed topography on the outside of the structure. This strained configuration was made stable by passing a pin through the horizontal tooth-pick, anchoring it to the board.

In this state, the original mould inner surface was stretched and had become the outer surface of a cone. The board was then made vertical, for making the cast.

Filling the everted complex mould, cast production

Equal amounts of "Two ton" (5 h) epoxy cement (Devcon Corp. Danvers, MA, USA), resin and catalyst were mixed carefully with a pin, so as to avoid bubbles. It was applied so as to fill all the depressions of the mould, trapping no air. Once the surface was covered with thick viscous epoxy, the horizontal tooth-pick was slowly released so the mould returned to its original configuration while becoming completely filled with epoxy (no bubbles during reversion). The base of the mould, filled with epoxy, was placed on a narrow strip from a glass slide. This could be turned over and inspected to confirm that no air had been trapped during reversion.

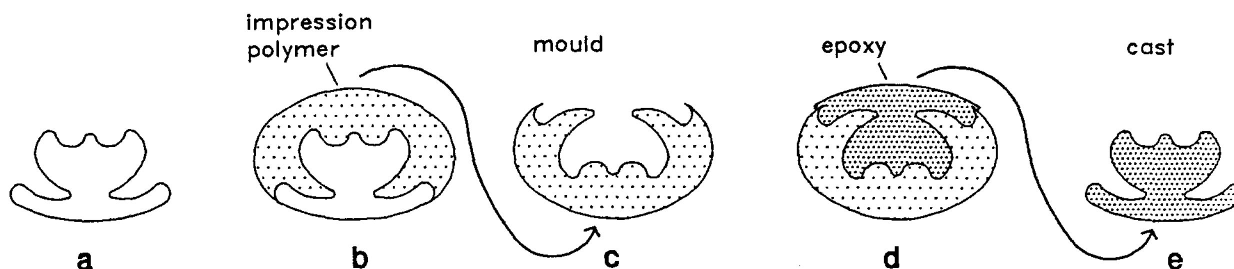


Fig. 1. Procedures for a simple mould and cast

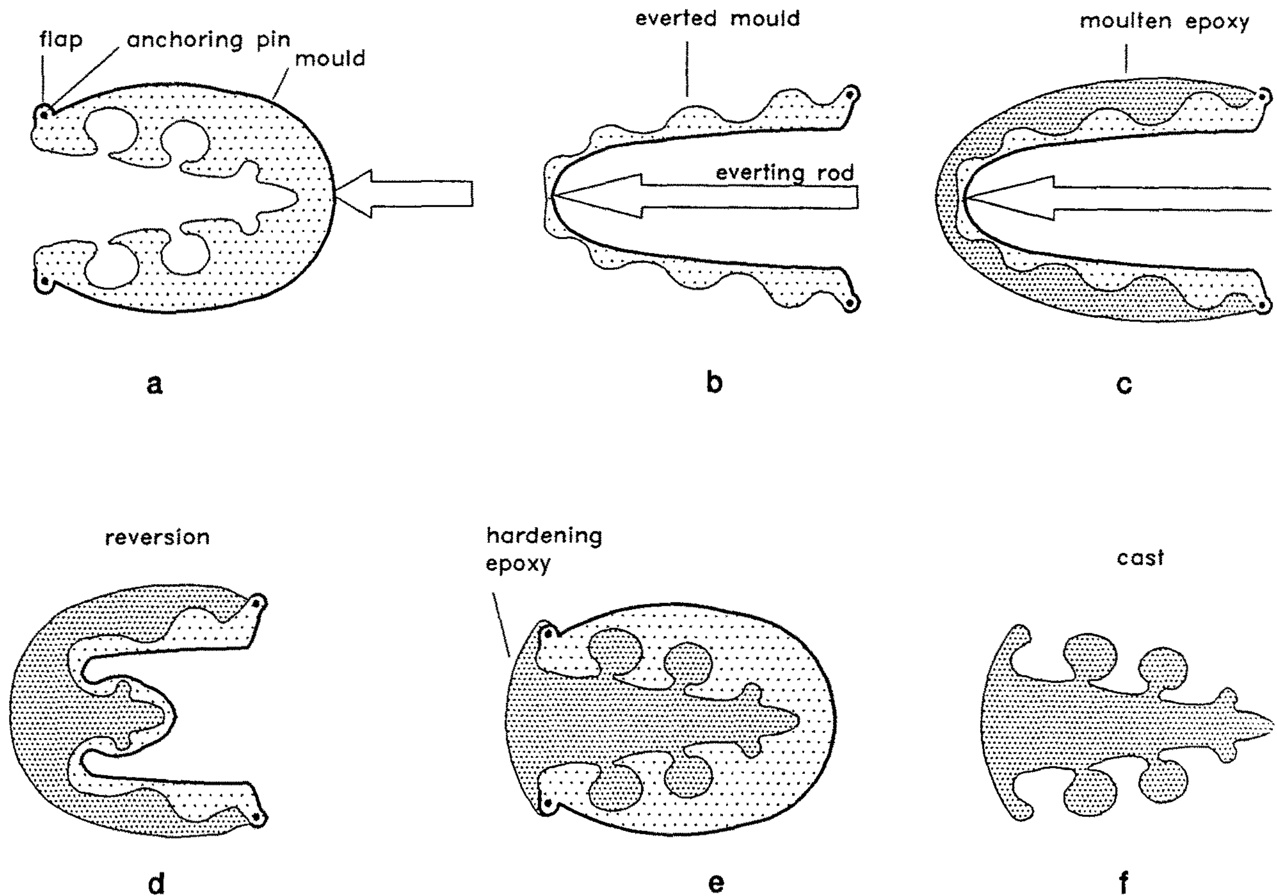


Fig. 2. Procedure for a compound mould and cast. The mould has two pins, normal to the page, which anchor it during eversion. The everting rod, e.g., a toothpick, needs to be anchored during the addition of epoxy cement because the everted state is often mechanically unstable. Both the addition of epoxy cement and the reversion process should be carried out with no formation of bubbles

The epoxy then hardened, affixing the new cast to the glass strip. The strip served as a handle for later removing the mould with forceps. The strip was scored so it would break near the hardened cast. The cast, now on a small glass chip, was positioned on a drop of epoxy gel on a cover slip. This was glued to a stub for observation in the SEM. In cases where the compound mould could not be everted (e.g., the mould wall was too thick), it could be slit in half. Each half could then be filled as if it were a simple mould. Detail is well-shown, with the cast emerging as "bas relief" from the cut surface of the mould (Fig. 10).

Figs. 3–6. *Antirrhinum majus* Stock 75, wild type, unless noted otherwise

Figs. 3 and **4.** Steropair of the tip of the inflorescence. Numbers show a bract or bract plus primordium of increasing age in plastochrons. Bar = 300 μm

Fig. 5. Side view of a cast showing three primordia on a parastichy (helical line) along which consecutive flowers differ in age by 3 plastochrons. Bar = 300 μm

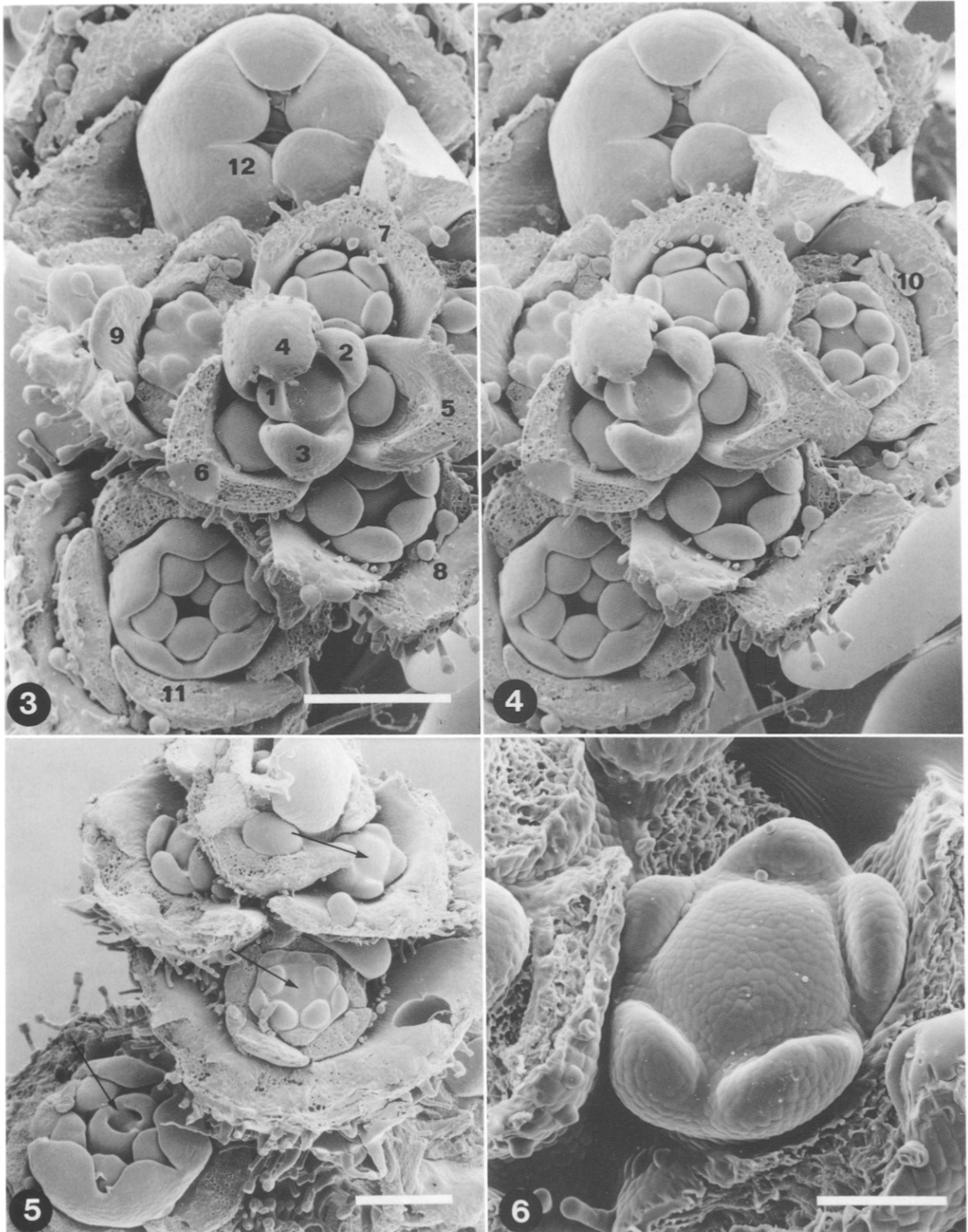
Fig. 6. Flower primordium at the stage of sepal formation. Note detail. Bar = 100 μm

Fig. 7. Flower of w.t. strain 45 showing six petals at the periphery. Stamens just inside, and the pair of carpels in the interior. Bar = 300 μm

Fig. 8. Flower of w.t. strain 45, sepals removed. Two artefacts are evident (arrows). In the recess at the top of center, the mould polymer failed to penetrate a cavity and hence replicated a bubble. Below the recess at bottom, the epoxy cast material failed to penetrate the mould of the petal, giving the appearance of a "bite" being taken out. Bar = 1 mm

Fig. 9. Stigmoid surface of the fused carpels in the mutant *deficiens.* Recessed surface at left (arrow) is a bubble. Bar = 100 μm

Fig. 10. The inflorescence of tobacco. The original mould was slit in half prior to filling its recesses with epoxy cement. The rough background surface at right is the cut mould. Four consecutive flower primordia can be recognized. Note the asynchronous development of sepals. Bar = 100 μm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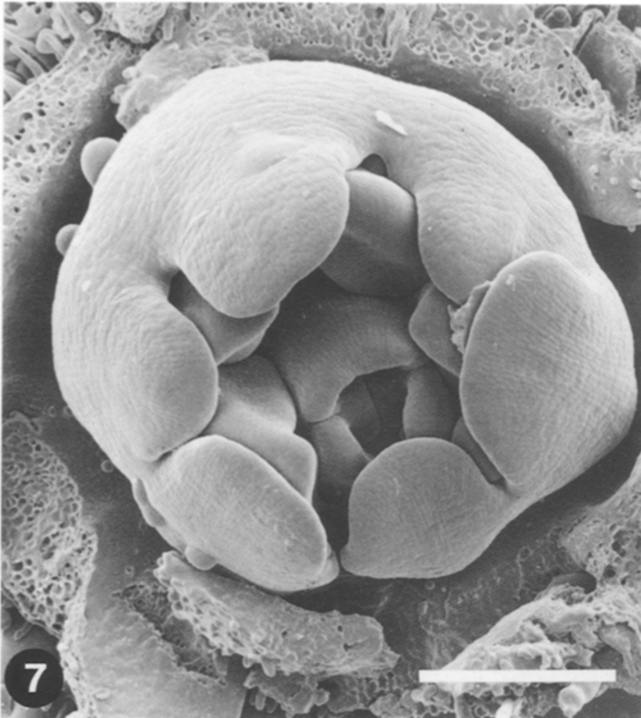
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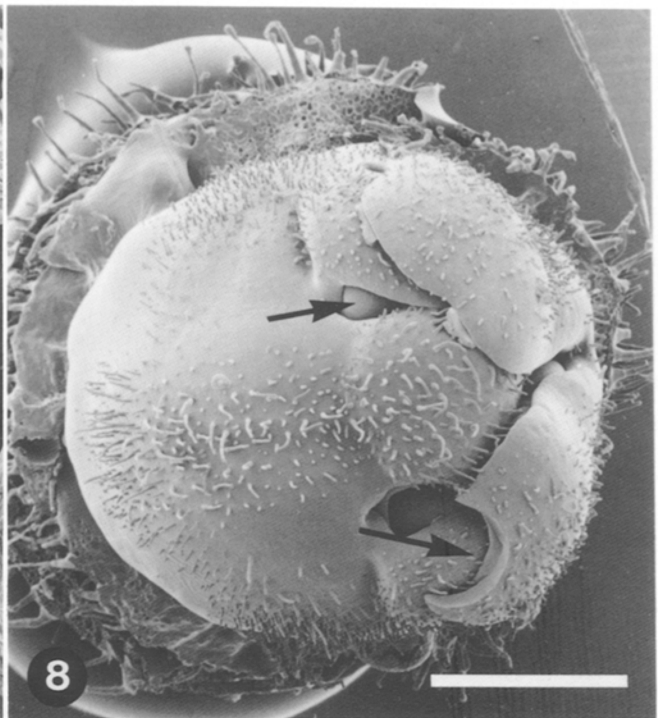
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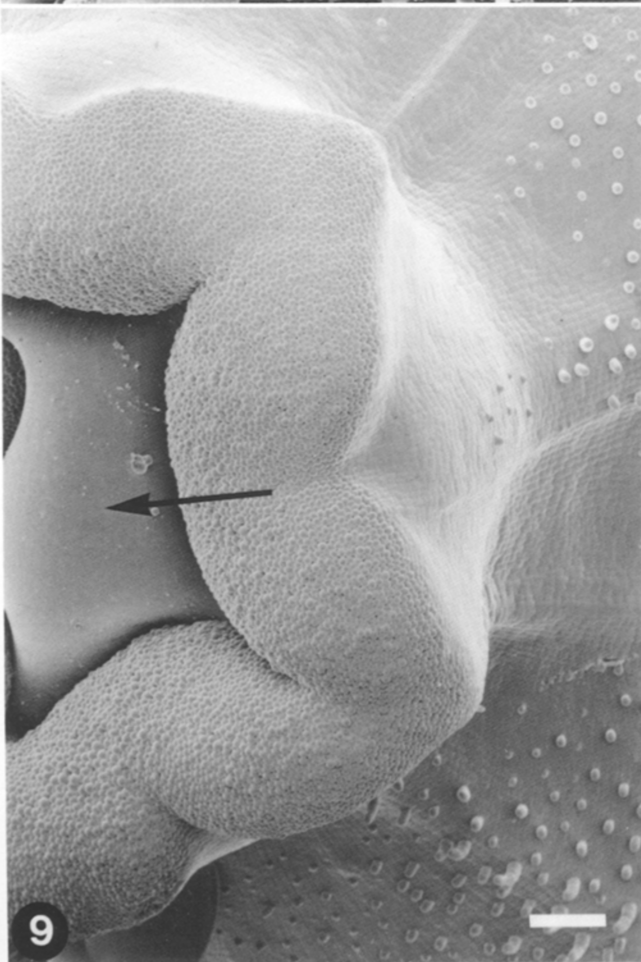
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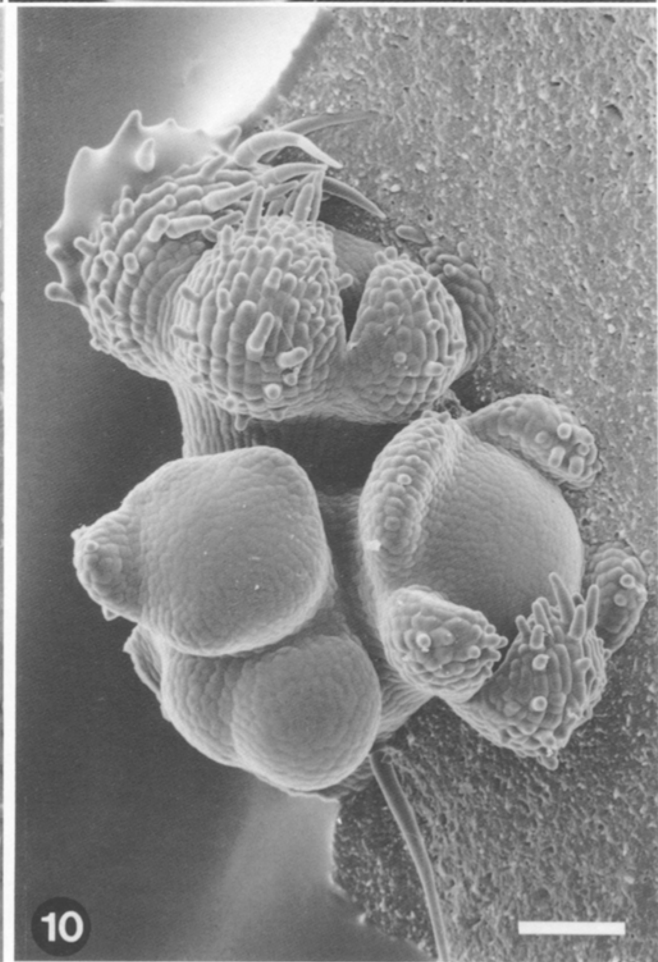
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The simple moulds were filled, also with no bubbles, and the epoxy allowed to harden. These were also affixed to a cover slip with epoxy gel for the SEM. The same epoxy gel could be used to write, with a forceps tip, key data on the cover slip (e.g., the sequence of the simple casts). This could be read both directly by eye and in the SEM.

SEM procedures

Specimen casts were sputter-coated with gold and examined in a CamScan Mk4 scanning electron microscope (Cambridge Scanning Co. Ltd., Cambridge, UK). The microscope was operated at 16 kV. A final aperture of 200 µm was used and a working distance of between 20 mm and 40 mm. Photographs were taken on Ilford FP4 film developed in FX-1 High Acutance Developer (British Journal Photography Annual 1972) for 10 min at 20 °C.

Results and discussion

The method can provide stereo pairs of large objects (Figs. 3 and 4). Stages separated by a 3 plastochron interval, i.e., lying on a parastichy, can be recognized and compared (Fig. 5). The resolution is well illustrated in Figs. 6 and 7. It is comparable to that of cryopreparations.

There are two major sources of artefact. First, the impression polymer may fail to reach the extreme recesses of the object. Once air is trapped, it cannot be displaced. The mould thus makes a replica of a "bubble". This is readily recognized in the cast by its lack of surface features (Figs. 8 and 9). A related problem appears when the epoxy cement fails to reach the recesses of the mould. Such regions correspond to "extremities" of the object. Hence an appendage appears to be cut off (Fig. 8 at right). Because the mould can be used repeatedly, an improved cast can usually be obtained to remedy this problem.

The compound mould procedure has been applied here (Figs. 3 and 4) in a preliminary way to study the entire inflorescence of *Antirrhinum*. Individual flowers have been well imaged by Sommer et al. (1990). A semi-logarithmic plot of consecutive primordium diameter versus plastochron age in preparation of the type in Figs. 3 and 4, has a slope of about 0.154 per plastochron for strains 75 and 98. This means that growth in linear dimensions occurred at about 15%/plastochron, continuously compounded. A commercial strain, 45, gave a value of about 0.23 per plastochron. In brief, the method can produce equally-spaced "standard stages" which can provide quantitative data on expansion rates as well as details of form and tissue surface pattern.

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