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Brenda Brookes
Biosystematics Research Centre Agriculture Canada

Ernest Small
Biosystematics Research Centre Agriculture Canada

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ENHANCED FLORAL ANALYSIS BY LOW TEMPERATURE SCANNING ELECTRON MICROSCOPY

Brenda Brookes* and Ernest Small
Iliosystematics Research Centre
Agriculture Canada, Central Experimental Farm
Ottawa, Ont., Canada KIA OC6

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Abstract

Traditional methods of preparation of botanical specimens for scanning electron microscopy (SEM) have proven to induce artifacts in some specimens which often reduce quality of resulting images, and are highly misleading for taxonomic purposes. The advantages of low temperature SEM are illustrated by an investigation of freshly collected flowers, using a cryo-system interfaced to an SEM. This method overcame the deficiencies of traditional procedures to produce exceptional images of floral surfaces in their natural state. Depending on the nature of the material under investigation, the method may be indispensable (e.g., for accurate details of petal surfaces), preferable (e.g., glandular hairs are better shown than by critical point drying), inefficient (e.g., images of stigmas of equivalent clarity are obtainable by direct examination of unfixed uncoated material), or unrewarding (e.g., examination of dry herbarium specimens). This paper stresses previously unappreciated adaptive and taxonomic importance of microtopography of floral surfaces of several genera important as agricultural forage crops. Certain species have evolved adaptive petal surface microsculpturing that facilitates cross-pollination by their insect associates. In the cross-pollinating species examined, different petal areas appear to be specialized for the landing of pollinating insects, and perhaps also as tactile (braille-like) orientation guides to the hidden nectaries. By contrast, in related self-pollinating species and cultivars, and when flowers are clustered in a head that provides a landing platform for pollinators, these adaptations are reduced or absent.

Key Words: Scanning electron microscopy, low temperature microscopy, hydrated method, pollination, petal, alfalfa, Medicago.

Introduction

This paper has two purposes: to review some intriguing new observations of floral surfaces as revealed by scanning electron microscopy (SEM), and to describe the circumstances under which low temperature SEM best brings out these as well as other floral features.

Light microscopic studies conducted during the last century have shown that the petal surfaces of many species of plants are textured in characteristic ways (Schubert, 1925). Only recently has the SEM been applied to this subject. The most notable studies to date have been carried out by C.H. Stirton and P.G. Kevan and their associates. Kay et al. (1981) surveyed petal sculpturing in 60 angiosperm families, and Stirton (1981) did the same for the Leguminosae family. Two hypotheses to explain the surface features were suggested in these papers; (1) in some cases the features act as non-skid platforms or ramps to facilitate landing and movement of pollinating insects; (2) the roughened epidermis deflects incident light into the petals, and subsequently the light is reflected back out to provide visual guides for insects. Kevan and Lane (1985), in a study of sunflower petals and the ability of bees to detect through their antennae the microtexture of these petals, advanced a third hypothesis, namely that the microsculptural patterns serve as braille-like guides to the nectaries.

Low temperature analysis of specimens have been discussed by a number of researchers (cited in the following), some of whose general conclusions are relevant to the present analysis of the applicability of the method to flowers. We shall limit the discussion to external morphology, and exclude analytical and internal studies, since we are primarily concerned with the taxonomic significance of external microstructure. Good recent reviews of analytical and internal studies are: Robards (1985), and Echlin and Taylor (1986). Good general reviews, within which the preservation of external structure is discussed, are: Echlin (1978), Lott et al. (1985), and Sargent (1986a). A few examples of how low temperature SEM has been shown to be useful for observing fine morphological details follow. The early study of Parson et al. (1974) on leaf and petals of geranium (Pelargonium) by a variety of procedures concluded that low temperature SEM was promising but required further development. Blackmore and Barnes (1984) used this method on pollen of several species and concluded that the
technique would become increasingly important in palynology. Sargent (1986b) found that root morphology was greatly improved by using low temperature analysis. Plant taxonomists have almost completely ignored roots, and perhaps there are interesting characters to be discovered.

Our studies have been concerned particularly with floral adaptations at the ultrastructure level of alfalfa (Medicago sativa L.) and other species of Medicago, and taxonomic relatives such as sweet clover (Melilotus) and clover (Trifolium). Therefore, a brief orientation to flowers is helpful. A diagrammatic representation of an alfalfa flower is shown in Fig. 1: intact in Fig. 1A, and dissected in Fig. 1B. Typical of the legume family, there are three kinds of petals: a large standard petal serving to attract pollinators, two fused keel petals (collectively constituting the keel which surrounds and protects the sexual column), and two wing petals which serve as landing platforms for visiting bees. The sexual column consists of a pistil, surrounded by a single free stamen and nine fused stamens. A diagrammatic longitudinal section through the alfalfa flower is shown in Fig. 2, in which a bee is shown foraging for nectar at the base of the flower.

Materials and Methods

Wing petals, stigmas and glandular-haired calyces from flowers of greenhouse-grown plants of various species of Medicago and allied genera (identified in legends to the photographs) were employed. Examinations were performed on an AMR 1000 SEM.

Analysis of frozen hydrated material

The floral parts were dissected from a fresh flower and rapidly mounted on the stub of a Hexland CT 1000 Cryotrans system, using a mixture of carbon and Tissu-Tek (1:1) as a mounting medium. This stub was immediately plunged into liquid nitrogen slush and transferred to the prechamber; once a vacuum of 0.1 torr was established the specimen was inserted onto the SEM cold stage. The uncoated

Fig. 1. Flower of alfalfa (Medicago sativa L.) A: intact flower; B: dissected flower.

Fig. 2. Longitudinal section of an alfalfa flower showing bee foraging for nectar at the base.
Fig. 3. Comparison of the microsculptural pattern on wing petals of alfalfa. A, B: frozen hydrated; C, D: critical point dried; and E, F: rehydrated. Papillate region is shown in photomicrographs on left, scale-like region is in photomicrographs on right.

specimen was examined at 5kV on the cold stage (at -160°C) in the SEM and when necessary the surface ice was sublimed by raising the stage temperature to -83°C. The temperature was lowered to -160°C and the specimen was withdrawn into the prechamber for sputter-coating with gold. The coated specimen was returned to the microscope cold stage and photomicrographs were taken.

Analysis of critical point dried material
Specimens were fixed in formalin: ethanol: acetic acid: water (2:10:1:7) for 24 hours, then dehydrated through a series of 50%, 70%, 80%, 95%, and 100% ethanol. The material was then critical point dried in a Tousimis Sandri PVT-3 apparatus
According to the instructions in the manual and coated with gold in a Technics Hummer-V sputter coater.

Analysis of fresh, unfixed uncoated material

The stigmas were dissected from fresh flowers and immediately mounted in partially-dried conductive silver paste on an aluminum stub, and viewed at 3kV in the SEM. Photomicrographs were taken before collapse of tissue became apparent.

Analysis of rehydrated material

Flowers from dried herbarium specimens were rehydrated in a solution of water and a surfactant (Anachemia brand aerosol) for 24 hours, and the frozen wing petals were observed on the cold stage in the SEM.

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Results and Discussion

Applicability of low temperature SEM to floral parts

Petals. The microsculptural patterns of frozen hydrated, critical point dried, and rehydrated wing petals are compared in Fig. 3, for two specialized regions with: a) papillate cells, or b) scale-like cells (discussed in detail later). As is apparent, a more "natural" appearance (without obvious distortions, as discussed below) has been preserved in frozen hydrated material (Figs. 3A, 3B). With critical point drying both the papillate and scale-like cells (Figs. 3C and 3D, respectively) are strikingly shrivelled and misshapen. In the rehydrated material, the papillate cells have become notably swollen (Fig. 3E), and the scale-like cells have become somewhat separated (Fig. 3F). These artifacts could be misleading taxonomically. It may be noted, however, that alternative methods of fixation combined with critical point drying might produce better results.

A comparison of preparative and overall time required to process the petals is shown in Table 1.

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Fig. 6. Microsculptural pattern on a wing petal of alfalfa, a typical outcrossing species. A: region of indistinct microtexture; B: region of apically-oriented papillate microtexture; and C: region of basally-oriented shingle-like microtexture.
As can be appreciated, low temperature SEM requires considerably less work time. The use of dried material is obviously undesirable, but in systematic studies may be unavoidable. Sometimes it is essential to ascertain the characteristics of a species for which only dried material is available. As can be seen from Figs. 3E and 3F there could be considerable difficulty in correctly interpreting such material.

Stigmas. A comparison of frozen hydrated and fresh unfixed uncoated stigmas is shown in Figs. 4A and 4B, respectively. For most of the species we have studied, the stigmas at maturity exude a viscous secretion; critical point drying is unsuitable because the solvents remove the exudate, an important taxonomic character (Heslop-Harrison, 1981). Both the methods used produced excellent results, the former being slightly superior but comparatively time-consuming, and therefore, less efficient. An extensive survey of stigma morphology was successfully carried out by direct examination of fresh unfixed uncoated material (Small and Brookes, 1983).

Glandular hairs. Glandular trichomes often protect various plant parts against insects (Small and Brookes, 1986). Frozen hydrated and critical point dried samples of sepals covered by glandular hairs are compared in Figs. 5A and 5B. The former is superior in that there is considerably less collapse of the delicate material. However, because of shrinkage, cellular outlines are much more apparent in the latter material. Curiously then, as in this circumstance, some artifacts may facilitate taxonomic comparisons. However, one should always establish the morphology of the characters in their natural state.

In the following, we present some studies of petal microstructure which we found exceptionally well demonstrated by low temperature SEM. The petal microtopography of interest is due to the pattern of overlap of epidermal cells. Ridging of individual epidermal cells also contributes to "microtexture", but is not of concern here.

Previously unappreciated petal microtexture

The pattern in outcrossing species. In the species we examined, epidermal patterning was found to be mostly confined to the outer (abaxial) surface of the wing petals. On the wing surfaces one often observes what appear to be patterns of scales which overlap in various ways. These "scales" are epidermal cells or cell extensions, and the patterns they make differ depending on species and part of petal examined. The patterns in different regions of the...
Fig. 8. Lack of typical overlapping epidermal cell microsculptural pattern on a wing petal of an inbreeding cultivar of alfalfa, 'Canuto'. Compare regions A, B, C with Fig. 6.

Table 1. Times required to complete tasks in preparation of critical point dried (CPD) and frozen hydrated (FH) material.

<table>
<thead>
<tr>
<th>Sample Preparation (minutes)</th>
<th>CPD Material*</th>
<th>FH Material**</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Collection</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>b) Fixation</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>work time</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>elapsed time</td>
<td>1440</td>
<td>-</td>
</tr>
<tr>
<td>c) Dehydration</td>
<td>120</td>
<td>-</td>
</tr>
<tr>
<td>d) CPD</td>
<td>90</td>
<td>20</td>
</tr>
<tr>
<td>e) Coating</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>f) Sublimation of ice</td>
<td>-</td>
<td>120</td>
</tr>
</tbody>
</table>

Total work time
- per 6 specimens: 260 minutes (167 minutes for FH)
- per specimen: 43 minutes (28 minutes for FH)

Total elapsed time
- per 6 specimens: 1685 minutes (167 minutes for FH)
- per specimen: 280 minutes (28 minutes for FH)

*6 samples concurrently **6 samples sequentially.

wing petal of alfalfa, a typical outcrossing species, are shown in Fig. 6. A petal may be divided into the claw and the limb (Fig. 1B). Patterning is confined to the limb. At the far end of the limb the papillate cell extensions point toward the apex (Fig. 6B). Towards the base of the limb, on the side nearest to the standard petal, is another distinctive region, where cells appear to overlap, and the direction of overlapping is towards the base (Fig. 6C). Patterning on the remainder of the petal, if present, is much less obvious (Fig. 6A), so that the two regions are prominently different.

Three hypotheses mentioned to explain petal microtexture were: anti-skid surfaces for the "feet" of visiting pollinators, tactile guides to the basal nectary, and to orient light as a visual guide to the nectary. It is unclear which hypothesis is most likely correct. Our preferred interpretations are that the apical portion is an anti-skid surface, since the direction of overlapping papillae is suited for this; and the distinct region at the base of the blade serves as a kind of braille nectar guide, since there is no need for an anti-skid surface here, and the flower is well supplied with color guides making the light guide hypothesis less likely as an explanation.
The pattern in an inbreeding species. The pattern, or more accurately the lack of a pattern, in a typical inbreeding species is shown in Fig. 7. The conclusion seems inescapable that there has been an evolutionary loss of petal microsculpturing, because the advent of autogamy would have made it unnecessary to accommodate insect pollinators.

The pattern in inbreeding cultivars. Alfalfa is primarily an outbreeder and most cultivars do not tolerate inbreeding. It is difficult to obtain a seed crop in many locations in North America using outcrossing cultivars, because natural pollinators have decreased as a result of environmental destruction (Ivanochko, 1979). One of the few inbreeding cultivars is 'Canuto'. The pattern, or once again more accurately, the lack of a microtextural pattern of epidermal cell overlap on the wing petal, is shown in Fig. 8. Again the conclusion seems that there has been an evolutionary loss of an adaptive pattern because it is no longer required.

Clustered versus separated flowers. There is one further circumstance in which we have discovered a lack of overlapping epidermal cell microsculpturing. In the species studied, we have consistently found that flowers taken from head-like inflorescences have petals lacking the kind of microsculpturing discussed in the preceding. For example, this is the case in the clover (Trifolium) shown in Fig. 9. The reason for this may be that an anti-slip surface is not required because visiting bees can conveniently land on the crowded inflorescence.

Summary

Low temperature SEM is the technique of choice for the petals and glandular trichomes examined, but is more time-consuming than direct examination of fresh unfixed uncoated stigmas, and when only dried material is available for taxonomic study, has no advantage. Clearly, circumstances and peculiarities of the material being studied determine the usefulness of low temperature SEM.

The pattern of petal microsculpturing observed, and indeed the presence of petal microsculpturing, appears to be intimately associated with relationships with pollinating insects.

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References


Discussion with Reviewers

H.P. Rasmussen: Are insects known to land only on the wing petals? If so why? Usually bees are not so selective.

Authors: Many pollinators are "generalists" and are not behaviorally and structurally adapted to particular flowers. For example, honey bees are not well adapted to alfalfa flowers. Indeed, honey bees often do not even enter the throat of alfalfa flowers but push their heads through the sides of the flower to obtain nectar. In the legume family generally bee pollinators that are closely adapted to particular plant species usually land particularly on the wing petals. One evidence of this is that the outer part of the wings petals, much more so than any other part of the flower, has surface microtopography seemingly constructed to facilitate landing.

H.P. Rasmussen: Is sublimation by raising the temperature of the cold stage superior to removal of surface ice by etching with the electron beam at cold temperatures? Could one use a combination approach for faster results?

Authors: Surfaces can be etched with an ion beam but not with an electron beam. For surface sublimation of ice, radiant heat can be used as an alternative or in addition to heating the specimen. However, the best method to achieve precise control of sublimation rate is by careful control of the specimen temperature.

H.P. Rasmussen: What did you use as a control in the CPD process? Did the fixation process cause introduction of artifact?

Authors: In this study we regard the use of frozen hydrated material as providing the control. We did not examine what stage of our CPD process was responsible for the artifacts observed, although the fixation process was likely involved. It has been pointed out to us that glutaraldehyde provides superior fixation to the mixture we have used. Unfortunately, glutaraldehyde is very difficult to use under many of our collecting conditions in the field.

H.P. Rasmussen: Many fresh tissues expand under the electron beam before collapse. How do you know you were not observing such artifacts?

Authors: The fresh, unfixed, uncoated tissues we observed were almost identical to the frozen hydrated material observed, so that such artifacts were not evident. However, the point is well taken, and many tissues cannot be examined so reliably.

H.P. Rasmussen: What specific sequence did you follow in the CPD process? You indicated you followed the directions. Many tissues require severe modification of standard procedure to prevent distortion and artifact.

Authors: Samples in stainless steel mesh baskets in 100% ethanol were placed in the chamber of the critical point dryer. The chamber was flushed with liquid CO₂ for 15 min. to allow complete exchange of ethanol to CO₂ in the tissues and to completely clear ethanol from the chamber. The chamber was then warmed to 40°C and the CO₂ gas slowly released from the chamber over a period of 30 min.

H.P. Rasmussen: There seems to be excessive swelling of the stigmatic surface in Figure 4B. This may also lead to errors in taxonomic character
identification. What did you do to insure swelling was not occurring?
Authors: We also observed fresh stigmas under the light microscope. In addition to considerable natural variation, one finds differences in appearance depending on the stage of development. The stigma shown in Figure 4B is quite natural in appearance.

H.P. Rasmussen: Is it appropriate to consider fixation time in critical point drying as much as prior fixation is not necessary for good specimen preparation in all types of tissue?
Authors: We concede the point. The estimates provided in Table 1 are for circumstances for which fixation is necessary.

H.P. Rasmussen: The trichome in Figure 5A appears to be extended to about the same extent that the critical point dried trichome is shrunken. How long after initial impact of the electron beam was the photograph taken?
Authors: The frozen hydrated trichome (Figure 5A) experienced about 15 min. of electron beam (much of this time required for sublimation of ice); the trichome prepared by critical point drying was observed after about 5 min. In these cases it seems clear that the differences in appearance cannot be attributed to artifacts induced by the electron beam.

H.P. Rasmussen: What is the angle of tilt of the specimen? If it is rotated on the stage do you observe the same overlapping direction?
Authors: Direction of overlap is easily ascertained from any angle of tilt and, of course, does not change when viewed from different perspectives. Generally, we photograph specimens at approximately a perpendicular perspective.

H.P. Rasmussen: Since the same bee may visit numerous species, the three hypotheses suggested infer that all flowers should have similar characteristics for guiding the bee. If that is not true, how can the bee interpret such a myriad of flower architecture?
Authors: Insect pollinators and plant species co-exist in various relationships. Pollinators may be narrowly adapted to particular flowers, or to many flowers. Conversely, flowers may be adapted to many insects or to only one. Of course, one may expect that the adaxial-surface of one pollinator is associated with very few or only one plant species, the structure of both is specialized in an adaptive fashion. The three hypotheses, however, suggest general ways flowers may be adapted to insects. A great deal of work remains.

H.P. Rasmussen: There is longitudinal orientation in all three samples as well as surface architecture sufficient to qualify as an anti-skid surface. How can you be sure that the cells in Figure 7 are not enlarged due to the interaction of the electron beam with the ice, thus forming gaseous water and thereby masking cellular architecture?
Authors: The material in Figure 7 was treated identically to that shown in Figures 6, 8, and 9 and so the absence of comparable structure is due to inherent differences in the material.

H.P. Rasmussen: The texture of the cells seems to be much more rough than those in Figures 6 and 7, there is also the appearance of overlapping of cells in Figure 8C. How can these be explained?
Authors: Figure 7 is another species which obviously differs in surface petal features from those shown for Medicago sativa (Figures 6 and 8). The magnification is greater in Figure 8 than in Figure 6 and tends to exaggerate the roughness. The petal of the selfing cultivar of alfalfa, shown in Figure 8, retains some microstructure (overlapping cells) but much less so than for the outcrossing cultivar shown in Figure 6, presumably because this is adaptive to pollinators as discussed in the text.

H.P. Rasmussen: Why were the areas of observation so vastly different between petals? It would seem the valid comparison would be made from tissue in very similar locations on the petal.
Authors: As explained in the text microstructure of the wing petals examined is characteristic for three regions. These same regions were examined in all comparisons, and obviously differ depending on species, and other considerations discussed in the text.

J.N.A. Lott: It presumably does not take a dry flower from a herbarium sheet 24 hours to rehydrate. Why was this length of time selected and do you know if the damage would have been less if a shorter time was used?
Authors: Herbarium sheets differ considerably in age, some being centuries old. Older specimens seem to require a longer period of rehydration. Most damage is induced in the killing phase and we do not regard the rehydration time as very important. We selected the time for convenience.

P.G. Kevan: Kevan and Lane (1985) used two species of Compositae, Helianthus annuus and Xylorhiza wrightii in their experiments and not only showed that honeybees could detect microstructural features on the petals of flowers, but also demonstrated the ability of these bees to discriminate between the textures of petals of different species, to learn the characteristic features and to orientate themselves with respect to gradations of the texture. They also showed that the adaxial (under) surface of the petals they studies were comparatively featureless, as Kevan (unpublished) has found for other species. The difference between the abaxial and adaxial surfaces of petals parallels that noted for outcrossers and in-breeder of alfalfa and relatives in this paper. Perhaps the explanation for the differences is similar. The preparation used by Kevan and Lane (1985) for training honeybees were merely dried and coated with gold. Although we recognize that critical point drying of fixed material would produce better, 'more natural', preparations for such experiments, I would like to know if the frozen hydrated and gold coated preparations produce specimens which are sufficiently robust to retain their characters when allowed to equilibrate with air at standard temperature and pressure (STP) conditions, and if so, could bee walk about on them without causing damage?
Authors: No, once removed from the cold stage and returned to ambient conditions, considerable deterioration occurs.