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HIGH-RESOLUTION LEAF X-RADIOGRAPHY IN SYSTEMATICS AND PALEOBOTANY[1]

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The foliar vein nets of many seed plants and ferns display systematically informative characters. These venation characters traditionally have been observed by chemically clearing and staining leaves, a process that is slow, involves toxic chemicals, and yields delicate, glass-mounted specimens that require long-term maintenance to prevent or correct bubbling or crystallization of the mounting medium. A technique that uses X-rays and photographic film to produce images of leaf venation consistently shows veins that are 50–100 μm thick. Although the X-ray images are slightly less detailed than the best cleared and stained leaves, the images can be made much more quickly, are more easily stored and reproduced, and do not require permanent alteration of the original herbarium specimen. The technique should facilitate the use of vein characters in systematics, and the identification and systematic analysis of fossil leaves.

The vascularization of angiosperm leaves generally is complex, and can provide characters that are useful in systematic analyses at a variety of hierarchical levels (e.g., Hickey and Wolfe, 1975; Merrill, 1978; Tanai, 1978; Hickey, 1979; Wolfe, 1989; Hickey and Taylor, 1991; Todzia and Keating, 1991). Leaf architecture has become particularly important in identifying fossil dicot leaves (Hickey, 1973; Dilcher, 1974). Vein characters are also significant in many groups of ferns (Stolze, 1976; Mitsuta, 1984). One impediment to a better understanding of vein characters has been the difficulty of preparing and storing chemically cleared and stained leaves in which the venation can be observed.

There is a long history in biology and medicine of using contact X-ray microscopy of the type described here (e.g., Coslett, Engstrom, and Pattee, 1957; Engstrom, 1962; Parsons, 1980). It has also been known for some time that leaves can be X-radiographed to create an image of their venation (e.g., Richards, 1986). The leaf is simply placed between an X-ray source and a piece of X-ray-sensitive film. The vascular tissue of the leaf is more opaque to X-radiation than most of the other tissues, producing an image of the veins as a pattern of lighter (less exposed) lines against a darker (more exposed) ground. In spite of the relative ease and long history of this technique, it has not been widely used in systematic or comparative studies. This may be because the X-ray images generally have revealed only the primary and secondary veins (leaf architectural terminology follows Hickey, 1979).

Here I describe a technique for making X-radiographs of dicot leaves that is much faster and less expensive than chemical clearing, produces negatives that can be stored indefinitely without maintenance, and consistently resolves 4th and higher vein orders. This X-ray technique should make it easier to assemble large collections of comparative material for use in identifying fossils, and generally facilitate use of vein characters in systematic analyses.

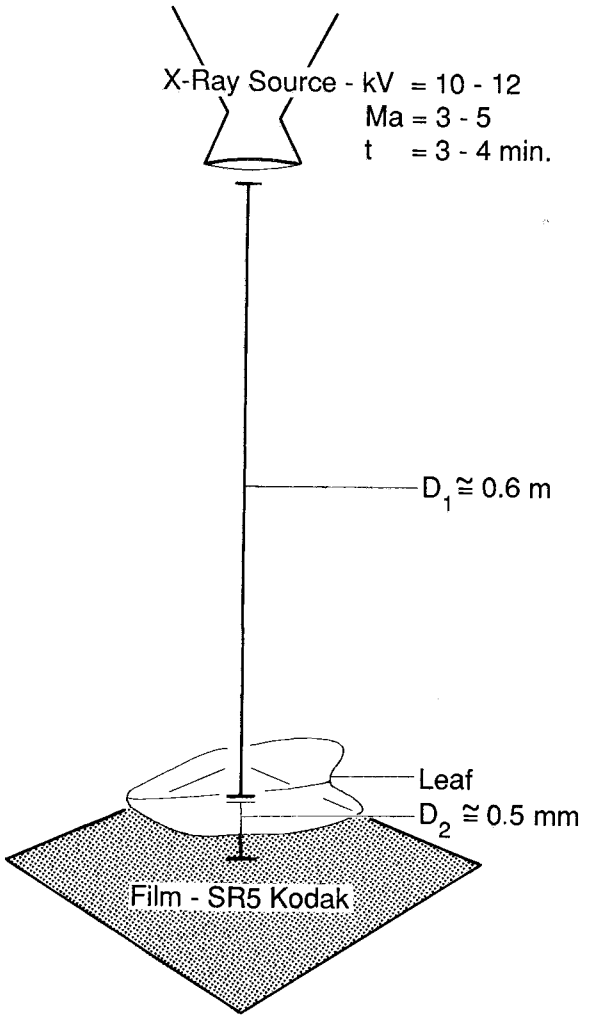

Fig. 1. Sketch of factors that can be varied in making leaf X-radiographs. kV = kilovoltage, Ma = milliamperage, t = exposure time, D_1 = distance from X-ray to specimen, and D_2 = distance from specimen to film.

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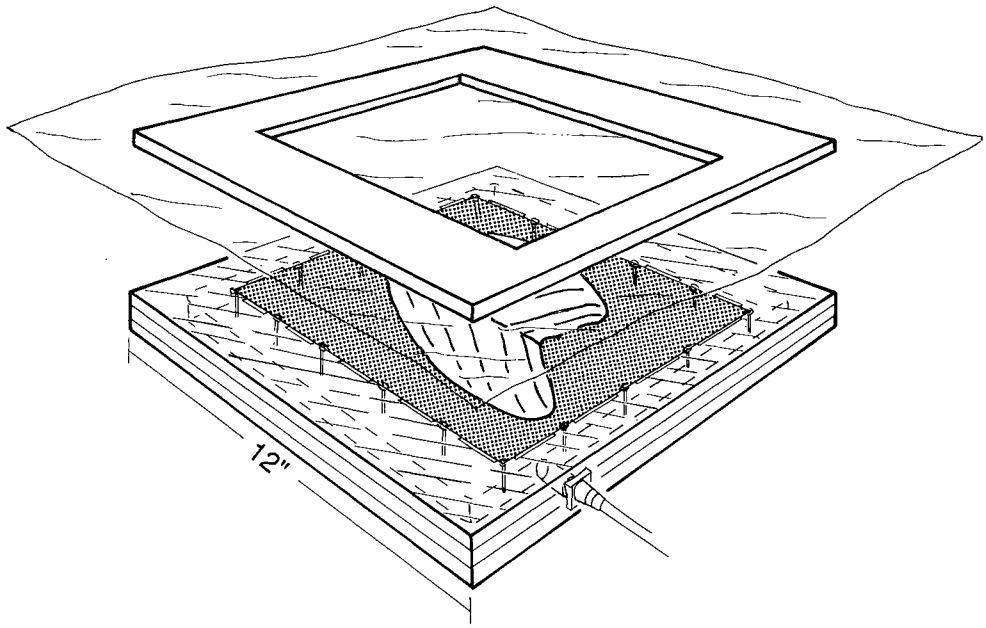

Fig. 2. Sketch of vacuum press used to reduce the distance from leaf to film. The base consists of three pieces of ½″ plexiglass that have been glued together; the center of the middle sheet has been removed to create an airspace to which a vacuum is applied through the valve on the right. The small holes in the top of the base pull air from around the X-ray negative and suck the 4-mil polyethylene sheet against the leaf and negative. The top of the press helps maintain a good seal between the base and the plastic sheet. This device does not require a strong vacuum source.

MATERIALS AND METHODS

The basic equipment for X-radiography of leaves consists of an X-ray source and X-ray-sensitive film, with the leaf being placed between the source and the film. All the experiments described here were carried out using a circa 1950 Picker brand X-ray machine with a tungsten anode; the type of radiation produced by this machine can be produced by any commonly available medical X-ray. Six basic factors were varied in trying to produce the most detailed X-radiographs: kilovoltage (kV), milliamperage (Ma), exposure time (t), distance from X-ray to specimen (D_1), distance from specimen to film (D_2), and film type (Fig. 1). The first two factors describe the X-radiation: kV is a measure of energy and is directly proportional to the penetrating power of the radiation. Ma is a measure of current, the number of waves emitted per unit time.

The best results have been obtained with the following settings: 10–12 kV, 3–5 Ma, 3–4 min exposure time, 60 cm D_1, and 0.5 mm D_2, and 8″ × 10″ sheets of Kodak SR5 film. Kodak SR5 film is a relatively slow but fine-grained film that produces much more detailed images than the faster medical films, which have emulsion on both sides. The use of low kV X-rays decreases the penetrating power of the X-rays, reducing the number that reach the film through the smaller-diameter, less lignified veinlets. When the kV setting was less than 10–12, exposure time had to be increased to between 5 and 10 min, but there was little increase in the visibility of fine veins. Ma was kept at the highest possible level in order to minimize exposure time. Long exposure times do not affect the sharpness of the image, but obviously reduce the efficiency of the process. Three to 4 min was the shortest exposure time that gave good results at the kV and Ma settings described.

Increasing the ratio D_1/D_2 can greatly sharpen the final image. If D_1 is approximately 2 m or more the X-rays are nearly parallel when they reach the leaf, and the sharpness of the image is good even if the leaf is simply placed on top of the film (D_2 = approximately 1 to 2 mm). However, the intensity of the X-rays diminishes as $1/D_1^2$, so a twofold increase in D_1 requires a fourfold increase in exposure time. This limits the usefulness of increasing D_1 to obtain sharper images. If $D_1 = 0.6$–1.0 m, the image of the fine venation on the negative will be fuzzy unless D_2 is extremely small (<0.5 mm). In order to make D_2 as small as possible I developed a press that uses a vacuum and a 3-mil sheet of polyethylene plastic to hold the leaf against the film (Fig. 2). This is necessary because the low kV X-rays that give the best resolution of fine veins do not penetrate even thin plates of glass or plastic that could be used to hold the leaf against the film.

RESULTS

The technique yields consistent results within species, but reveals a fairly large range of variation in the visibility of small veins across species. This variation probably

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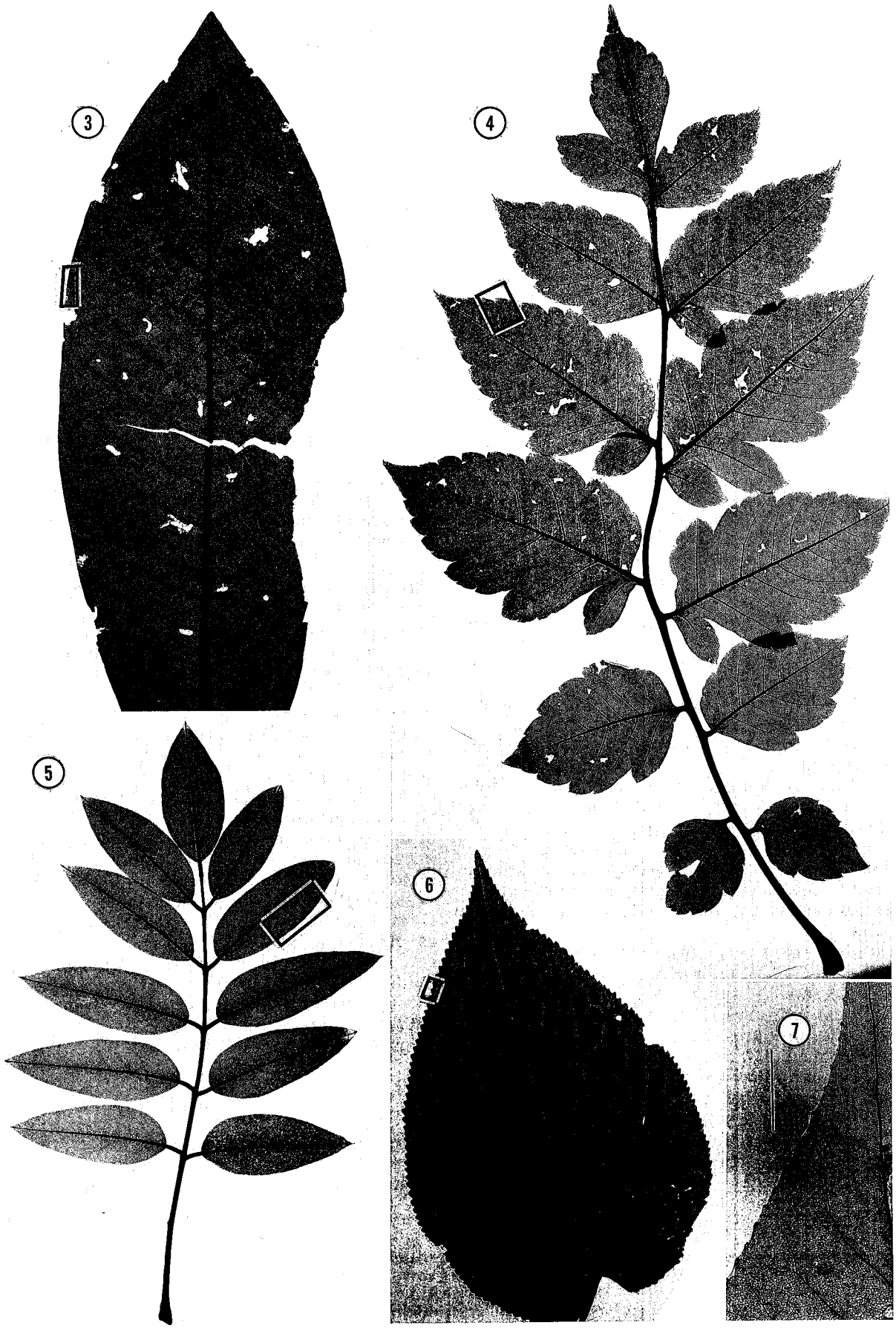
Figs. 3–7. Direct prints of X-ray negatives, all ×0.65, except for 7. **3.** *Guatteria cardoniana* R. E. Fries; rectangle 10.5 mm long shows area enlarged in Fig. 10. **4.** *Kolreuteria paniculata* Laxm.; rectangle 11 mm long shows area enlarged in Fig. 9. **5.** *Sophora japonica* L.; rectangle 16 mm long shows area enlarged in Fig. 14. **6.** *Tetracentron sinense* Oliv.; rectangle 5.5 mm long shows area enlarged in Fig. 11. **7.** Direct enlargement (×2) of X-ray negative of *Populus mexicana dimorpha* (Brandegee) Eckenwalder. Bar = 10 mm.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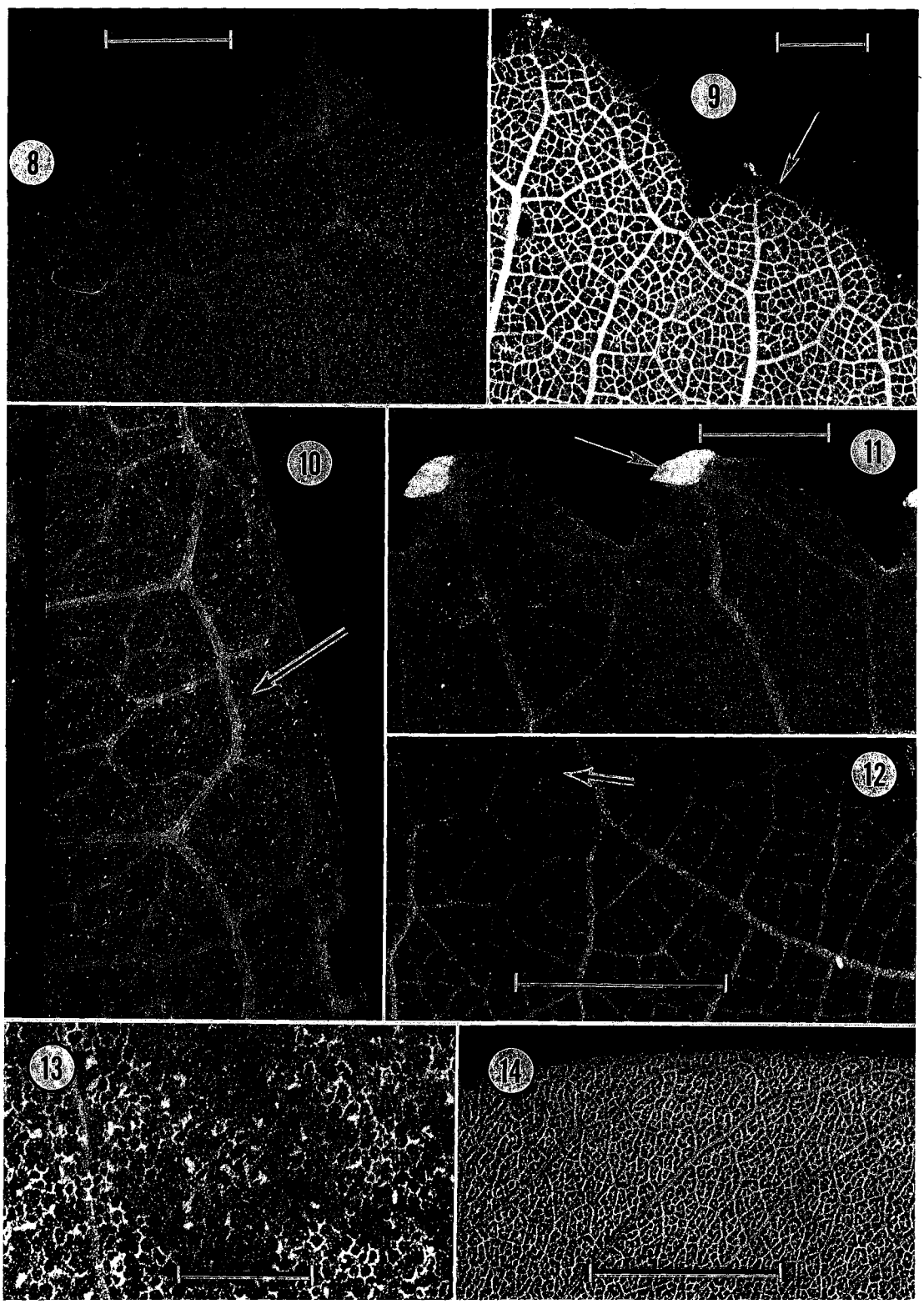

Figs. 8–14. Enlargements made by photographing X-ray negatives through a photomacroscope with 35 mm black and white film. **8.** Spinose teeth of *Ilex opaca* Aiton and 4th order veins. Bar = 2 mm. **9.** Rosoid teeth in *Kolreuteria paniculata* Laxm. Arrow points to area near margin that lacks vascular tissue. Bar = 1 mm. **10.** *Guatteria cardoniana* R. E. Fries. Arrow indicates freely ending veinlets. Scale marks = 1 mm. **11.** *Tetracentron sinensis* Oliv. Arrow indicates glandular apex of Chloranthoid type tooth. Bar = 1 mm. **12.** *Ephedranthus parviflorus* S. Moore. Arrow points to one of many fuzzy white patches that are probably laminar resin glands. Bar = 1 mm. **13.** *Ilex opaca* Aiton, cellular pattern is probably created by palisade tissue. Bar = 1 mm. **14.** *Sophora japonica* L. showing higher vein orders. Bar = 5 mm.

eorresponds to differences in the thickness of leaves and the degree of lignification of their veins. Leaves with thick cuticle and mesophyll may pass fewer X-rays; thus the venation does not show well, particularly if the veins are thin. However, almost all species X-rayed (including representatives of Annonaceae, Aquifoliaceae, Cercidiphyllaceae, Eucommiaceae, Lauraceae, Leguminosae, Myristicaceae, Magnoliaceae, Salicaceae, and Tetracentraceae) have revealed 4th or higher order veins (Figs. 3–14). The smallest, freely ending veinlets are visible in about half of the images. Veins generally can be seen as well in the X-ray negatives as they can in leaves from the same families that have been cleared with NaOH and chloral hydrate and stained with safranin.

In addition to displaying vascular tissue, X-radiographs commonly show crystals, resin bodies, and indistinct outlines of cell walls (Figs. 8, 12, 13). In some X-radiographs helical thickenings on the walls of the tracheids can be seen.

X-radiographs can be duplicated either by contact printing (Figs. 3–6), by direct enlargement of the X-ray negative (Fig. 7), or by photographing a small part of the X-ray negative at high magnification through a stereomicroscope (Figs. 8–14). Some detail is lost in all processes, but direct enlargement of the X-ray minimizes intermediate steps.

DISCUSSION

In many angiosperm groups fine veins display characters of systematic importance. The high resolution provided by the X-ray technique described here is a prerequisite for useful images. However, the greatest value of the technique is its speed, which makes studies of intraspecific variation and/or diverse higher taxa feasible. With deep-tank manual developing of the X-ray plates, 20–40 negatives a day can be produced, and with an automated developing system this rate could probably be tripled. Other advantages of the X-ray technique over standard chemical clearing include the relative ease with which large leaves and compound leaves can be processed, the lack of exposure to toxic or controlled substances such as xylene and chloral hydrate, the compactness and durability of the images, and the nondestructive nature of the process, which leaves the original specimen available for anatomical study or return to the herbarium sheet. X-radiographs are nearly ideal for automated digitization because they have high contrast and a dark, evenly exposed background. The major disadvantage of X-radiography is that the negatives do not show cellular-level detail or nonvascular tissue to the degree that cleared specimens can.

Further refinements of X-ray techniques are no doubt possible. Such refinements probably would depend either on varying or "tuning" the X-ray source to enhance the production of radiation that discriminates best between different plant tissue types, or on preferentially staining the vascular tissue with an X-ray-dense substance. This latter approach might be achieved most easily in living plants by introducing an X-ray-dense compound into the water supply.

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