An Improved Clearing and Mounting Solution to Replace Chloral Hydrate in Microscopic Applications

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Source: Applications in Plant Sciences, 1(5)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1300016

Applications in Plant Sciences

Applications in Plant Sciences 2013 1(5): 1300016

Protocol Note

An improved clearing and mounting solution to replace chloral hydrate in microscopic applications[1]

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- *Premise of the study:* This study presents Visikol™, a new proprietary formulation that can be used as an efficient replacement for chloral hydrate as a clearing agent for microscopic examination. In the United States, chloral hydrate is regulated and therefore difficult to acquire.
- *Methods and Results:* Fresh and dry samples of the following plants: ginger (*Zingiber officinale*), maté (*Ilex paraguariensis*), lime basil (*Ocimum americanum*), oregano (*Origanum vulgare*), and mouse-ear cress (*Arabidopsis thaliana*), were cleared using Visikol or chloral hydrate solution and compared using a light microscope.
- *Conclusions:* This new method can be used successfully to clear specimens, allowing identification of diagnostic characteristics for the identification of plant materials. Visikol is as effective as chloral hydrate in providing clarity and resolution of all tissues examined. Tissues become transparent, allowing observation of deeper layers of cells and making it effective in research, botanical and quality control, and for educational applications.

Key words: acidified chloral hydrate; botanical quality control; clearing; histology; microscopy; Visikol.

Light microscopic observation of whole plant tissues for anatomy, taxonomy, quality control, and species identification, as well as teaching, has been used since the discovery of the microscope. When light passes through intact, nonstained plant tissues or organelles (cytoplasm, vacuole, and cell walls), the different refractive indices of the materials contribute to spherical aberration, scattering of light, and thus a lack of clarity. Because most of the time tissues are opaque or semiopaque, they require a clearing procedure to improve visualization. A plant specimen is considered clear when some of its components are made visible at the expense of others, while the form of the material remains more or less undistorted (Gardner, 1975).

[1]Manuscript received 20 February 2013; revision accepted 22 April 2013.

The authors thank Élan Sudberg (Alkemists Laboratories, Costa Mesa, California, USA) for his encouragement to identify a suitable replacement for choral hydrate; Beth Lambert, Qingli Wu, Rodolfo Juliani, and Marshall Bergen for their suggestions; Jim White for use of his research microscope; Tom Leustek for providing *Arabidopsis* plantlets; and Lena Struwe and Edith Robbins for their excellent suggestions and review of our manuscript. Funds for this research were provided by the Rutgers New Use Agriculture and Natural Plant Products Program.

Disclosure statement: Visikol™ was invented by the authors and is patent pending (filed by Rutgers University). Rutgers University has granted an exclusive license to Phytosys LLC to commercially market Visikol. Phytosys LLC is co-owned by the authors.

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doi:10.3732/apps.1300016

Although there are different clearing solutions described for plant tissues, one of the most commonly used ones is acidified chloral hydrate (Lersten, 1967). Chloral hydrate is used as an aqueous solution, often added to glycerol to prevent crystallization of the reagent when used as a temporary mounting medium. The clearing ability of chloral hydrate has been known for nearly a century, and it has been widely included in various protocols to examine different plant structures (McBryde, 1936; Arnott, 1959; Lersten, 1967, 1986; Shobe and Lersten, 1967; Herr, 1971, 1993; Gardner, 1975; Jackson and Snowdon, 1990; Liang and Herr, 1994). As a result of the clearing treatment, tissues or plant materials become more transparent, which greatly reduces problems with light scattering and enables high-resolution images to be captured (Haseloff, 2003). Chloral hydrate solutions have a high refractive index (typically around 1.4280), which allows for a high degree of light to pass through the medium without refraction between the boundary of the glass and microscope. Clearing agents with high refraction indices therefore allow light to pass unobstructed through the medium, allowing more light to continue through the microscope to the observer. A high refractive index also allows for an increased depth of field, meaning that more vertical planes can be observed in the microscope in a particular focal plane; the depth of field is proportionate to the refractive index (Rost and Oldfield, 2000). Many pharmacopeias (such as the U.S. Pharmacopeia, American Herbal Pharmacopoeia, and World Health Organization) have published protocols for microscopic authentication analyses of herbal preparations using acidified chloral hydrate (Hertwig's solution) as clearing agent (World Health Organization, 1998; United States Pharmacopeia and National Formulary, 2005; Upton et al., 2011). Consequently,

chloral hydrate has become the industry standard and an important reagent required on a daily basis for many laboratories focused on quality assessment of herbal products.

However, in the United States, chloral hydrate is a Federally Regulated Schedule IV substance, and thus a special permit is required to purchase, possess, and use it (Code of Federal Regulations, 1974 [Schedule IV Drugs, 21 C.F.R. Section 1308.14]). Compliance with this regulation requires yearly permit application fees and copious amounts of paperwork and documentation to ensure proper transfer and use. This level of regulation places chloral hydrate out of reach for the majority of scientists and technicians, and as such, microscopic analysis according to standard techniques is limited. Restriction of analytical techniques causes an inherent problem in quality control in industry, as well as in educational and research laboratories. Chloral hydrate is also a narcotic substance with addiction potential, and chronic exposure has been linked to a number of health issues (Daniel et al., 1992; Sing et al., 1996). We report here the use of a new solution, Visikol™, as a suitable, nonregulated proprietary substitute for chloral hydrate in microscopic applications for botanical and agricultural quality assessment, pathology, and histology, in both research and teaching. The Material Safety Data Sheet of Visikol (www.visikol.com) indicates that with proper handling use of this product should not present the toxicity issues found with chloral hydrate.

METHODS AND RESULTS

The control solution of acidified chloral hydrate–glycerol solution was prepared by dissolving 45 g chloral hydrate into a solution consisting of 25 mL 4.2% HCl (1 : 8, 38% HCl to H_2O) (Fisher Scientific, Pittsburgh, Pennsylvannia, USA; catalog no. A508-4) and 10 mL glycerol (Fisher Scientific; catalog no. G33-1) as in standard methods. The experimental solution Visikol™ (patent pending) was obtained and used without modification (Phytosys LLC, New Brunswick, New Jersey, USA; catalog no. 01-30). Visikol is based on a unique polychlorinated alcohol mixture that has been optimized for optical and supramolecular properties, allowing the solution to span cellular membranes and organelles and enabling penetration of the solution deep into tissues. Visikol also contains glycerol to increase viscosity of the solution and increase solubility.

The refractive index for both chemicals was determined using a temperature-controlled refractometer at 23°C (Fisher Scientific; model no. 334620). The refraction index of Visikol (1.4450) was higher than acidified chloral hydrate in glycerol, lactic acid, ethanol, and water (Table 1).

Using authenticated botanical materials, a few milligrams of fine powder of ginger rhizome (*Zingiber officinale* Roscoe, Zingiberaceae) or leaves of maté (*Ilex paraguariensis* A. St.-Hil., Aquifoliaceae) were spread on a microscope slide (Fisher Scientific; catalog no. 12-544-1, 3 in × 1 in × 1 mm) and mounted either with two drops acidified chloral hydrate solution (control) or with two drops of Visikol clearing solution, and a cover slip (Fisher Scientific; catalog no. 12-548-B, 22 × 22 × 0.17 mm) was put over each. Slides were then heated on a hot plate (60–80°C) for 30–60 s until just before boiling, when the air bubbles moved out to the edges of the slide. Each sample was replicated three or more times. All the microscopic image analyses were taken on a Nikon Eclipse 80i microscope, with NIS-Elements D 3.00 SP7 imaging software (Nikon, Tokyo, Japan). Differences or similarities in diagnostic features for each experimental sample and control were recorded.

TABLE 1. Refractive indices of common microscope media compared to Visikol.

Medium	Refractive index (n_{D20})
Water	1.3330
Ethanol	1.3550
Acidified chloral hydrate in glycerol	1.4280
Lactic acid	1.4255
Visikol	1.4450

Visikol clearing solution proved to be an effective clearing agent (i.e., resulting in transparent tissues) in all samples tested, and similar results as chloral hydrate were observed (Figs. 1 and 2). Visikol was originally intended for quality assessment of commercial herbal products. Here, we found it was useful for clearing whole mounted fresh and dried materials. Characteristic fragments of parenchyma cells, fibers accompanied by vessels, and abundant starch grains with sharp edges in fresh ginger rhizome were visible under a light microscope and could be demonstrated (Fig. 1A–F). In fresh maté leaves, clear details of the upper epidermis composed of polygonal cells with unevenly thickened walls and lower epidermis with stomata and well-marked cuticular striations were identifiable (Fig. 1G–J). Whole fresh leaves of lime basil (*Ocimum americanum* L., Lamiaceae), oregano (*Origanum vulgare* L., Lamiaceae), and seven-day-old, dried *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) seedlings were submerged in Visikol until they were transparent, usually taking 20–30 min depending on the thickness of the material. We expect that larger samples may require up to 2–3 d. Once the material was cleared, it was mounted on a microscope slide with one or two drops of Visikol, and a cover slip was added. Oregano or basil leaves cleared with Visikol solution allowed the visualization of deeper layers of tissues without losing clarity. For example, in basil, the oil glands, epidermis with stomata, and underlying palisade cells could be observed (Fig. 2A, B). In oregano, the epidermis over the vein with covering trichomes, capitate, and peltate oil glands was distinguished (Fig. 2C–F). Details of the cellular organization of the root apical meristem in *A. thaliana* can be observed after clearing with Visikol (Fig. 2G, H). In addition, a number of other herbs and spices (dry samples and whole tissues) were analyzed subsequently using Visikol as clearing reagent with comparable results.

This study will have a substantial impact on many laboratories that rely on routine microscopic analyses, based on standard techniques that use chloral hydrate as the main clearing and mounting agent. Modern legal regulations place chloral hydrate out of reach of many scientists, for regulatory and availability reasons. For a variety of dried and fresh plant samples, Visikol has been shown to be a viable substitute for chloral hydrate in microscopic preparations. Visikol is easy to obtain, is available without regulation, and does not require any special permit or paperwork. This reagent was found to be as effective as chloral hydrate yet can be used without the taxing chore of managing Drug Enforcement Administration regulation compliance, thus saving time, reducing risks to health and public safety, and reducing administrative costs.

CONCLUSIONS

This is the first report of a new clearing and mounting agent for microscopy that can substitute or replace choral hydrate. Results demonstrated that the new clearing agent Visikol can be effectively used as a replacement of chloral hydrate in botanical microscopy. Visikol can thus be used for clearing herbal products for quality assessment and yielding high-quality images. Visikol, like chloral hydrate, penetrates into tissues and renders them more transparent. After treatment with Visikol, tissues are cleared, enabling internal as well as surface details to be clearly identified. This feature is most significant when Visikol is used with whole mount tissues in which different layers of the transparent tissues are observed without the need of sectioning or remounting. Clear tissues also allow for staining techniques to more effectively highlight diagnostic features.

Visikol has a higher refractive index than the chloral hydrate control solution, and it clears samples in short periods of time. Given the clarity obtained, Visikol may also have potential applications for use in confocal microscopy or fluorescence microscopy, which would allow highly detailed models of internal structures to be obtained. Visikol clearing solution was also effective to clear other nonplant species such insects, fungi, and protists. Thus, this new method has potential applications when seeking to examine the internal morphology of other small organisms (data not shown).

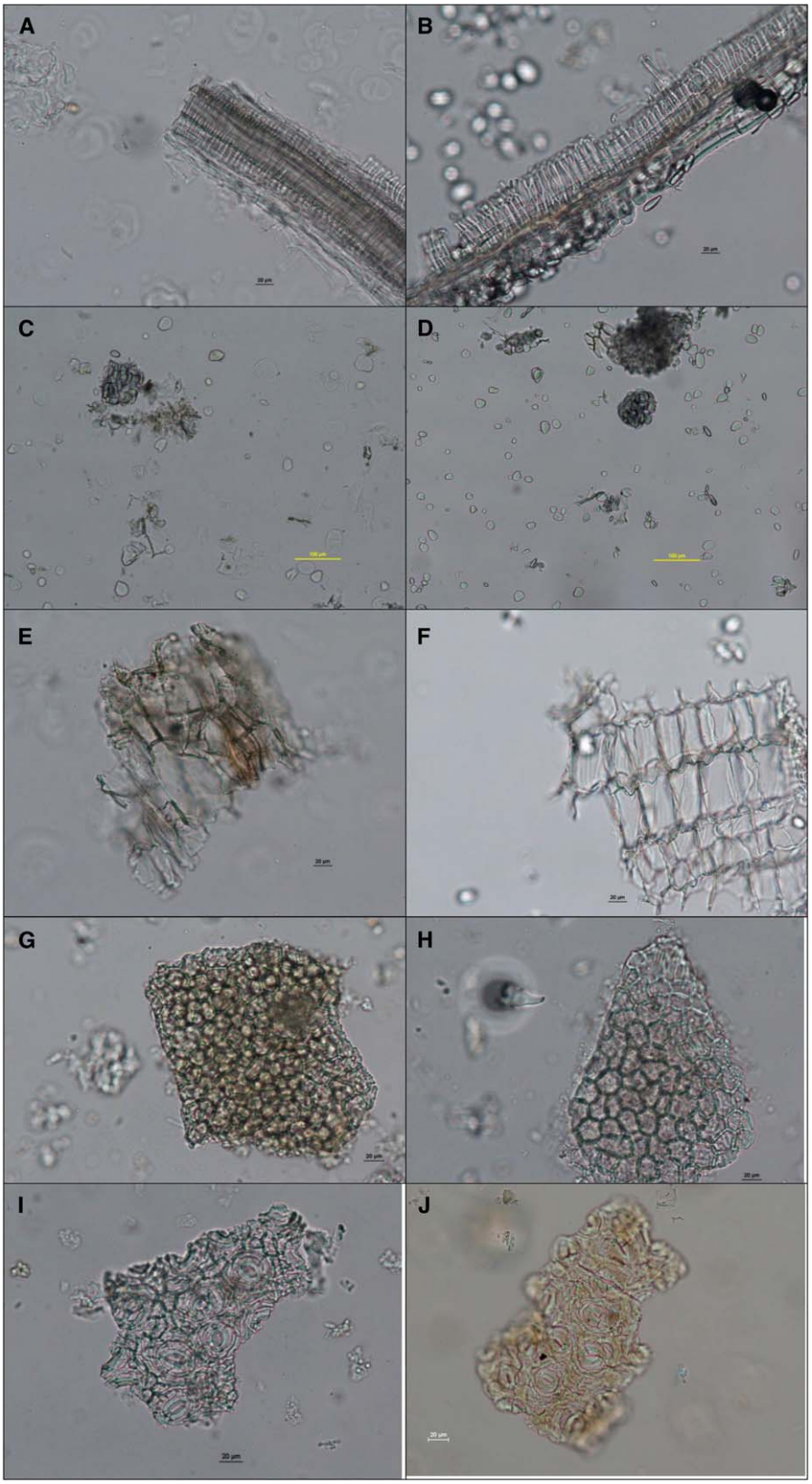

Fig. 1. Light micrographs of dry and powdered botanical specimens cleared with chloral hydrate (left column) and with Visikol (right column). (A–F) Ginger rhizome. Annular vessel element and fibers (A, B); abundant starch grains in rhizome (C, D); thin-walled parenchyma cells (E, F). (G–J) Maté leaves. Leaves, upper epidermis with underlying palisade cells, large and closely packed (G, H); lower epidermis surface showing anomocytic stomata and circular cuticular striations (I, J).

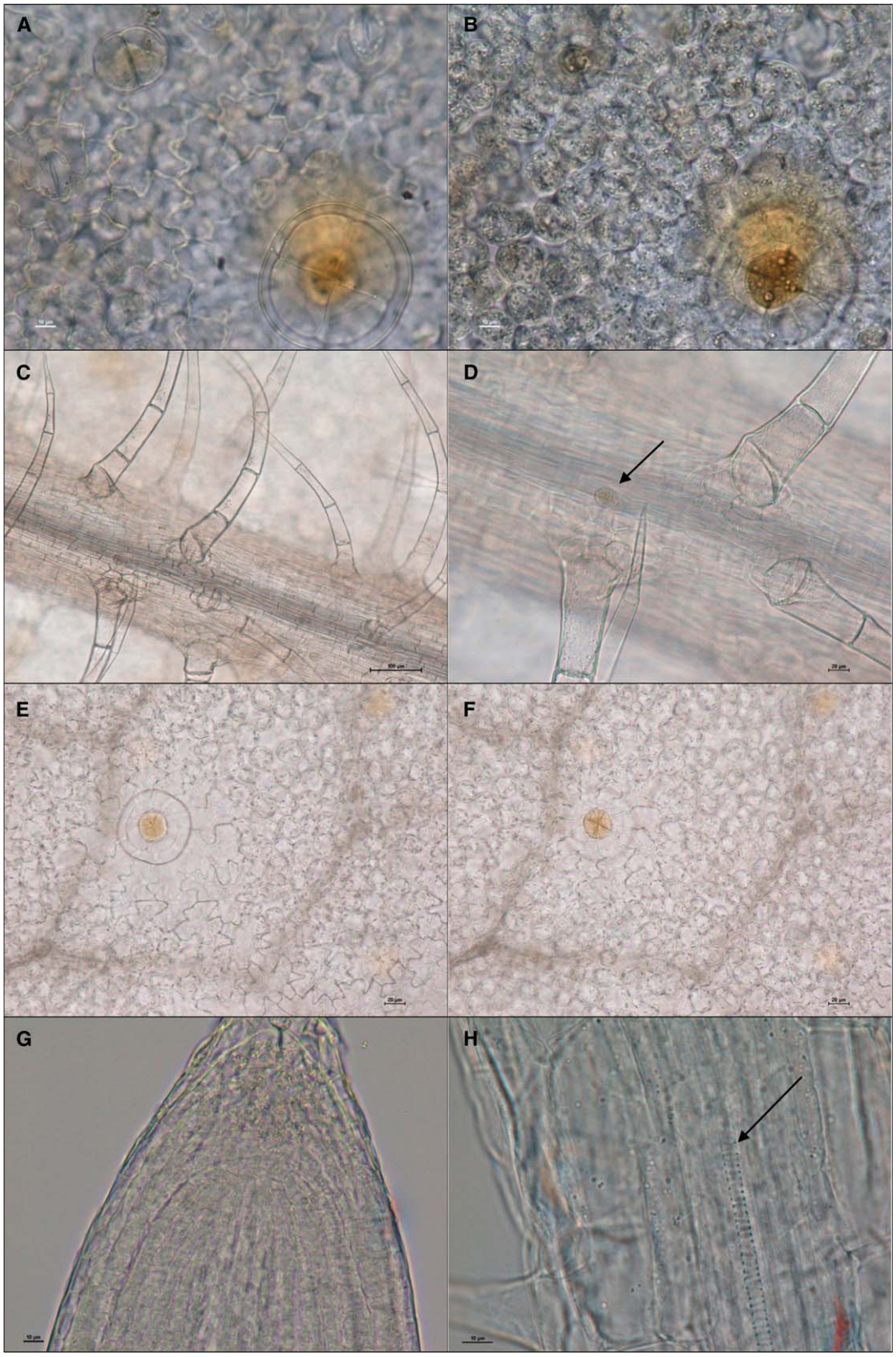

Fig. 2. Light micrographs of fresh, whole-mounted specimens cleared with Visikol. (A, B) Basil leaf. Epidermis with diacytic stomata, capitate and peltate glands (A); mesophyll cells with chloroplasts (B). (C–F) Oregano leaf. Covering trichomes with thick cell walls over the vein and capitate glands (C); close-up capitate glands (arrow) (D); epidermis and peltate oil gland (E); mesophyll cells (F). (G, H) *Arabidopsis thaliana* root. Root tip cellular differentiation (G); xylem differentiation in root (arrow) (H).

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