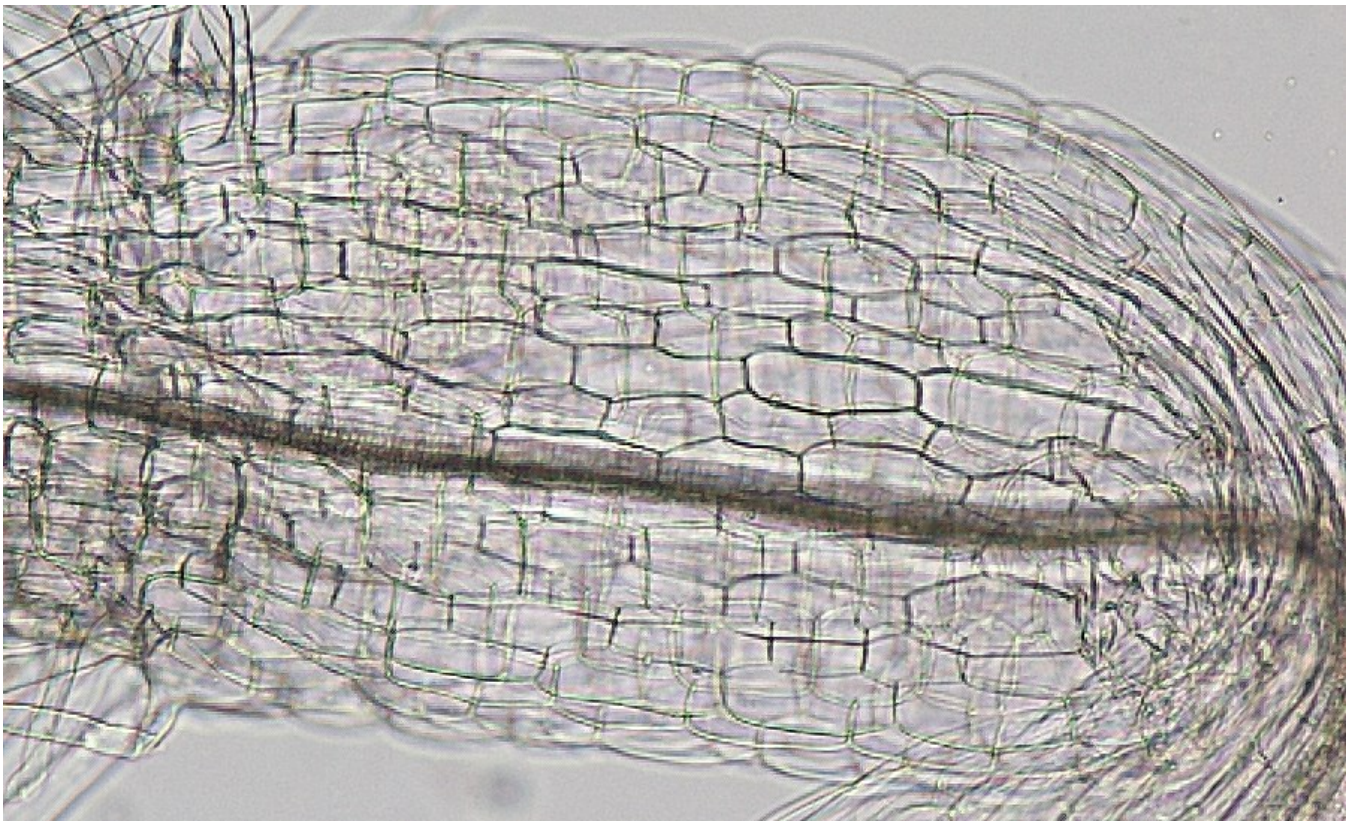
Visikol™ compared to Chloral Hydrate

White Paper



Arabidopsis thaliana, root

Summary: Visikol™ is a new formula designed to replace chloral hydrate in a wide variety of microscopic applications. Here, the superior clearing action of Visikol on *Arabidopsis thaliana* can be easily observed when directly compared to acidified chloral hydrate solution.

Introduction

Microscopic observation has been long used by scientists conducting biological research. In plant science, botanical pathology, and anatomy, some unique challenges are encountered when attempting to observe cells under the microscope. Most tissues contain pigments or other opaque structures, and so they require a clearing procedure to improve visualization. Also when light passes through optically clear tissues and organelles, the different refractive indices of the materials can scatter the light and severely reduce the clarity of the image, obscuring the internal structures of interest. So in order to get the best image possible, the tissue is treated with a clearing agent to both remove the internal pigmentation and improve the refractive indices of the different components of the tissue.

Throughout the scientific literature, one of the most frequently used clearing agents used is acidified chloral hydrate (Lersten, 1967). Chloral hydrate is used as an aqueous solution, often along with glycerol to prevent crystallization when used in a temporary mount to examine a wider variety of plant structures (see page 4). With advances in optical imaging, the utilization of clearing agents has allowed scientists to capture incredibly detailed high-resolution images (Haseloff, 2003).

Chloral hydrate is ideal because of its high refractive index (around 1.4280) and its ability to penetrate and clear a wide variety of tissues, which allows for light to pass through the medium and on to the microscope observer without refraction between the boundary of the specimen and the cover glass. An additional benefit of the refractive index is an increased depth of field; meaning that more vertical depth is in focus and can be observed in the same image without adjusting the focus controls of the microscope (Rost and Oldfield, 2000).

Pharmacopeias like the US Pharmacopeia, American Herbal Pharmacopoeia, and the WHO have published procedures for microscopic authentication of herbal preparations using acidified chloral hydrate as clearing agent (also known as Hertwig's solution). And consequently, chloral hydrate has become the industry standard used daily in laboratories focused on the quality assessment of herbal products.

However not everyone can obtain chloral hydrate. Since, it also has powerful narcotic effects and its use as a date rate drug, access to chloral hydrate is strictly regulated by governmental authorities. In the U.S., chloral hydrate is a Schedule IV substance, controlled by the Drug Enforcement Administration. In addition to high yearly permit fees, the DEA requires detailed documentation of every transfer and use to ensure no material is illegally diverted. The compliance cost of these regulations makes chloral hydrate impractical for the large majority of users in the field, and as such, their ability to perform most accepted standard techniques is limited. This restriction leads to substandard quality control in industry, and constricts both research and educational opportunities.

Visikol™ has been reported in the scientific literature as a suitable, non-regulated substitute for chloral hydrate in microscopic applications for botanical and agricultural quality assessment, pathology and histology, research as well as for teaching (Villani, 2013).

Materials and Methods

The control solution of acidified chloral hydrate-glycerol solution was prepared by dissolving 45g chloral hydrate into a solution consisting of 25mL 4.2% HCl (1:8,38% HCl to H_2O) (Fisher Scientific, Pittsburgh, PA, cat. no A508-4) and 10mL glycerol (Fisher Scientific, Pittsburgh, PA, cat. no G33-1) as in standard methods.

The refractive index for each chemical was determined using a temperature controlled refractometer at 23°C (Fisher Scientific Model #: 334620). The refraction index of Visikol™ (1.4450) was higher than acidified chloral hydrate in glycerol, lactic acid, ethanol and water (Table 1).

Table 1. Table of Media by Refractive Index

Medium	Refractive Index
Water	1.3330
Ethanol	1.3550
Acidified chloral hydrate	1.4280
Lactic Acid	1.4255
Visikol™	1.4450

Seven day old, dried *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) seedlings were submerged in Visikol™, acidified chloral hydrate solution, or water for 30 minutes. Specimens were placed on a microscope slide (Fisher Scientific, Cat No. 12-544-1, 3”x1”x1mm) and mounted either with two drops of Visikol, acidified chloral hydrate solution (control), or water and a cover slip (Fisher Scientific, Cat. No. 12-548-B, 22x22-1, 0.17 mm thickness) was put over each. Slides were then heated on a hot plate (60-80°C) for 30-60 seconds 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 D 3.00 SP7 imaging software (Nikon, Tokyo, Japan).

Results are shown below in Figure 1.

Conclusions

The images presented here show that Visikol can be effectively used as a direct replacement of chloral hydrate in botanical microscopy. Visikol yields high quality microscopic images and can be used to clear herbal products for research, quality assessment and botanical authentication. Treatment with Visikol clears tissues and, due to the increased depth of field over chloral hydrate, it allows different layers of internal structures as well as surface details of the specimen to be simultaneously identified, without the need for sectioning or remounting. As these results show, *Visikol is the superior, non-regulated alternative to chloral hydrate for use in research, education and quality control.*

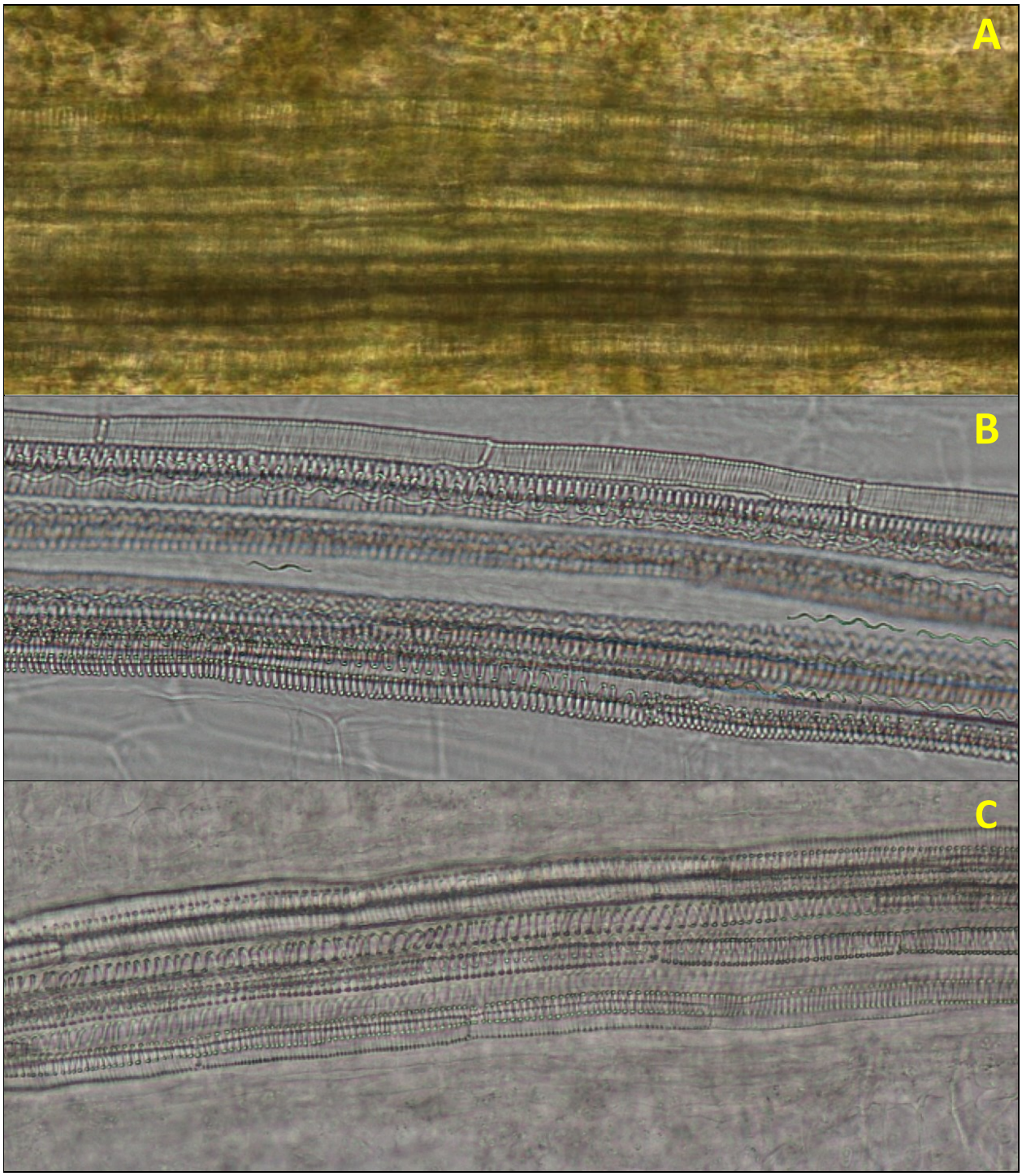


Figure 1. A) *Arabidopsis thaliana* leaf treated with water; B) *Arabidopsis thaliana* leaf treated with Acidified Chloral Hydrate solution; C) *Arabidopsis thaliana* leaf treated with Visikol™

Table 2. Overview of literature which utilizes chloral hydrate to clear specimens

	Title	Specimens
McBryde, 1936	A Method of Demonstrating Rust Hyphae and Haustoria in Unsectioned Leaf Tissue	Garden beans, corn, mayapple, and barberry
Arnott, 1959	Leaf Clearings	*Syringa sp., Crossosoma parviflorum, Thea sinensis*
Lersten, 1967	An Annotated Bibliography of Botanical Clearing Methods	Bryophytes; "all plant parts, including pollen;" Dalea, *Lemna minor*,
Shobe and Lersten, 1967	A Technique for Clearing and Staining Gymnosperm Leaves	Gymnosperms, *Metasequoia glyptostroboides*
Herr, 1971	A New Clearing-Squash Technique for the Study of Ovule Development in Angiosperms	Angiosperms, *Cassia abbreviata*, *Ludwigia uruguayens,*
Gardner 1975	An Overview of Botanical Clearing Techniques	Review paper
Lersten, 1986	Modified Clearing Method to Show Sieve Tubes in Minor Veins of Leaves	Soybeans and other dicotyledonous species
Jackson and Snowdon, 1990	Atlas of Microscopy of Medicinal Plants, Herbs, and Spices	>100 common herbs and spices
Herr, 1993	Clearing Techniques for the Study of Vascular Plant Tissues in Whole Structures and Thick Sections	*Wisteria sinensis, Selaginella apoda, Abelia grandiflora, Nymphaea odorata*
Liang and Herr, 1994	Use of the Four-and-a-Half Clearing Technique to Study Gymnosperm Embyrology: *Cunninghamia lanceolata*	*Cunninghamia lanceolata*

Work Cited

Arnott, H.J. 1959. Leaf clearing. *Tutox News* 37: 192-194.
Daniel, F. B., A. B. DeAngelo, J. A. Stober, G. R. Olson, and N.P. Page. 1992. Hepatocarcinogenicity of chloral hydrate, 2-chloroacetaldehyde, and dichloroacetic acid in the male B6C3F1 mouse. *Fundamental and Applied Toxicology*, 19: 159-168.
Gardner, R.O. 1975. An overview of botanical clearing techniques. *Stain Technology*. 50:99-105.
Haseloff, J. 2003. Old botanical techniques for new microscopes. *BioTechniques* 34: 1174-1182.
Herr, J. M, JR. 1971. A new clearing-squash technique for the study of ovule development in angiosperms. *American Journal of Botany* 58: 785-790.
Herr, J. M. Jr. 1993. Clearing techniques for the study of vascular plant tissues in whole structures and thick sections. *In* C.A. Goldman, P.L. Hauta, M.A. O'Donnell, S.E. Andrews, and R. van der Heiden, Editors. Tested studies for laboratory teaching. Volume 5,63-84. Proceedings of the 5th Workshop/Conference of the Association for Biology Laboratory Education (ABLE).
Jackson, B.P. and D. W. Snowdon. 1990. Atlas of microscopy of medicinal plants, culinary herbs and spices. Belhaven Press, London.
Lersten, N.R. 1967. An annotated bibliography of botanical clearing methods. *Iowa State Journal of Science* 41: 481-486.
Lersten, N.R. 1986. Modified clearing method to show sieve tubes in minor veins of leaves. *Stain Technology* 61:231-234.
Liang, D. and J. M. Herr, Jr. 1994. Use of the four-and-a-half clearing technique to study Gymnospermem bryoIogy: *Cunninghamia lanceolata*. *Biotechnic and Histochemistry* 69: 279-282.
McBryde, MC. 1936. A method of demonstrating rust hyphae and haustoria in unsectioned leaf tissue. *American Journal of Botany*10:686-688.
Rost, F. and R. Oldfield. 2000. Photography with a Microscope. United Kingdom: Cambridge University Press.
Ruzin, Steven E. 1999. *Plant microtechnique and microscopy*. Vol. 198. New York: Oxford University Press
Schedule IV Drugs, 21 C.F.R. Section 1308.14
Shobe, W.R. and N.R. Lersten 1967. A technique for clearing and staining gymnosperm leaves. *Botanical Gazette* 128:150-152.
Sing, K., T. Erickson, Y. Amitai, and D. Hryhorczuk. 1996. *Chloral hydrate toxicity from oral and intravenous administration. Clinical Toxicology* 34: 101-106.
The United States Pharmacopeia 28/The National Formulary 23; The United States Pharmacopeial Convention, Inc.: Rockville, MD, 2005.
Upton, R., A. Graff, G. Jolliffe, R. Langerand and E. Williamson. 2011. American herbal Pharmacopoeia. Botanical pharmacognosy. Microscopic Characterization of Botanical Medicines. CRC Press. Taylor & Francis Group.
World Health Organization Geneva. 1998. Quality control methods for medicinal plant materials. WHO Library Cataloguing in Publication Data

