A Rapid Microwave Mediated Polyethyleneglycol Embedding Method Showing Retention of Intracellular Specialized Metabolites in Leaves of *Cinnamomum Tamala* (Buch-Ham.) T. Nees & Nees

Sulagna Saha
Indian Institute of Technology Kharagpur
Raktim Bhattacharya
Indian Institute of Technology Kharagpur
Adinpunya Mitra (adinpunya@gmail.com)
Indian Institute of Technology Kharagpur https://orcid.org/0000-0002-1740-8147

Technical Report

Keywords: Embedding, histochemistry, infiltration, microwave, polyethyleneglycol

Posted Date: September 30th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-82677/v1

Abstract

Polyethyleneglycol (PEG) is considered one of the most effective substitutions for paraffin in plant histochemistry as an embedding medium. A rapid and straightforward method of PEG embedding has been developed that resulted in a significant reduction of infiltration time than the traditional method of PEG embedding. The material used for PEG embedding was leaves of *Cinnamomum tamala*, a member of Lauraceae. Samples were put successively in aqueous solutions of PEG 6000 with increasing concentration for infiltration. A microwave oven was used as a mode of heating medium. The infiltration was completed within 2 h. After the completion of infiltration, the samples were embedded in PEG and solidified. Compared with the existing methods available for PEG infiltration and embedding, this microwave mediated PEG embedding method saves significant time; this also saves the tissue from long-term heat-induced damage. Retention of intracellular metabolites, which was not possible in earlier PEG embedded methods, has also demonstrated in the tissue.

Introduction

Polyethyleneglycol (PEG) is one of the most suitable substitutes for paraffin in animal histochemistry for a long time (Barka and Anderson 1963). The first reported use of PEG was in 1942 (Richards et al. 1942). PEG can be found in different molecular weights, and along with that, their consistency varies. The earlier use of PEG had been restricted in low molecular weight; one reason for this is the lack of cell walls in animal cells. The use of PEG as an embedding medium was popularized among histochemists only after investigations by Rinehart and Haj (1951). They demonstrated the localization of tissue lipids in samples dehydrated and embedded in PEG. However, for use in plant histochemistry, the high molecular weight PEG was preferred by the researchers (Ferreira et al. 2014, 2017); this may help overcome the cell wall barrier. Very few reports have been found in the use of PEG in plant histochemistry, so it can be deduced that the use of PEG in this field is not well explored. Researchers have used PEG 6000 in plant histochemistry, which gave a fair and consistent result.

Using heat as a form of microwave radiation was started in the field of histochemistry from the late 1990s (Schichnes et al. 1999). Apart from histochemistry, microwave-induced heat has been used extensively to extract plant essential oils (Ferhat et al. 2006; Iriti et al. 2006). Schichnes et al. (1999) first showed the use of microwave in plant histochemistry. Earlier, it had been used only for animal histochemical techniques. Paraffin had traditionally been used as an embedding medium for microwave technique. After the availability of microwave-mediated paraffin embedding method, few researchers have made effort to use plastic embedding medium such as, resin, in the microwave method, which led to satisfactory results (Giberson and Demaree 1999; Webster 2007; Zechmann et al. 2011). One significant advantage of the microwave method is the reduction in time. In the traditional method, the paraffin embedding of a plant tissue requires five to nine days, depending on the hardness (Ruzin 1999). On the other hand, the microwave method brings that down to only five to seven hours (Schichnes et al. 2001). This substantial reduction in time helped the researchers to work with the materials more efficiently than earlier.

One of the major advantages of using PEG over paraffin is that the water-soluble nature of PEG, and thus there is no need for dehydrating the tissue sample (Barka and Anderson 1963). This results in good preservation of internal tissue structure than that of paraffin embedding methods. As PEG has a higher infiltrating capability than paraffin, therefore the overall process can be completed within two to three days; this is a major advantage over the paraffin method as the heat-induced damage of the tissues can be easily avoided (Ferreira et al. 2014). While microwave-based methods of paraffin (Schichnes et al., 1999) and resin (Zechmann et al., 2011) embedding are well known as the case with PEG 6000 as a plant embedding medium (Ferreira et al., 2014), here we report for the first time a rapid protocol for the use of microwave technique with PEG 6000 as an embedding medium for plant histochemistry work. In this method, the overall time reduction comes down from five to seven hours of microwave-based paraffin embedding to one hour. Thus, the protocol presented here is unique in the sense that this is the first time PEG 6000 has been successfully attempted as an embedding medium under microwave-mediated heating. Further, this is the first report of intracellular retention of plant metabolites in microwave-mediated embedding of plant tissue. Additionally, heat-induced damage of the plant tissue and subsequent loss of intracellular metabolites can be avoided.

Material And Methods

Cinnamomum tamala (Buch-Ham.) T. Nees & Nees plants were grown and maintained in the experimental garden of Agricultural Biotechnology laboratory at Agricultural and Food Engineering Department of the institute. Mature leaves of these plants were used as experimental material.

Fresh leaves were plucked from the plant and cut into small pieces (1 cm X 1 cm) with a sharp blade. Samples were fixed in the commonly used and cost-effective fixative reagent FAA (formalin: glacial acetic acid: ethanol: water in 1:1:9:9 ratio) (Buda et al. 2009). Fixation was performed under vacuum at room temperature until the samples settled on the bottom of the vials. The fixed samples were not dehydrated due to usage of aqueous polyethyleneglycol solutions (25%, 50%, and 90%) for preparation of samples. Samples were transferred in a vial containing a 25% aqueous PEG 6000 solution. The vial containing samples was subjected to kitchen microwave treatment (Panasonic NN-ST266B, 800W) for 5 min at warm mode (~ 40 °C). After decanting the residual 25% PEG solution, the same procedure was repeated for 50% and 90% aqueous PEG solutions. The samples were treated with 90% aqueous PEG solution for two times (5 min each) consecutively, with removal of used and replenishment of fresh 90% PEG. In each case, the volume of the PEG solution was 10 times more of the sample volume. Further utmost care was taken during sample preparation to avoid charring. Embedding was done using 100% aqueous PEG solution followed by storage of blocks at 4 °C until further processing (Table 1). The traditional PEG embedding method, as described by Ferreira et al. (2014), was also done to compare these two methods.

The blocks were stubbed with a wooden block-holder with the help of molten PEG, as described by Ferreira et al. (2014) before cutting. Transverse sections were obtained using a rotary microtome (Leica RM2125 RTS, Leica Biosystems, Germany), followed by treatment of sections in lukewarm water for dissolution of residual PEG. The thickness of the sections was kept 30 µm for the whole study. The

stained and unstained sections obtained from both traditional and microwave-mediated PEG embedding methods were viewed under Leica DM 2500 LED microscope and subsequently photographed by using a Leica DFC7000T camera with Leica LAS X software platform (Leica Microsystems, Germany).

Table 1
A concise overview of the microwave mediated PEG embedding procedure

Step	Temperature (˚C)	Time(min)
Fixation Fixation in FAA under vacuum	Room temperature	Until sample sinks to the bottom of vial
Infiltration	40	5
25% PEG	40	5
50% PEG	40	5 (X2)
90% PEG (X2)		
Embedding 100% PEG	25	Until PEG solution reaches semisolid stage
Storage Stored in cold and dry condition	4	Till further use (can be stored up to 1 month without cutting)
*The ratio of sample and solution volume should be 1:10. After each treatment, used solution should be changed.		

Results And Discussion

Sections obtained from both methods were viewed under bright field without any staining (Fig. 1A and B). A few unstained sections were also examined under LED fluorescence light (CoolLED pE-3000 White, CoolLED Ltd., UK) fitted with Leica DM 2500 LED microscope (Leica, Germany) using 365 nm excitation wavelength and triple-band filter (LED Tripe filter). The sections obtained from the microwave mediated sample showed autofluorescence of the oil present inside the oil cells (Fig. 2B) while such kind of fluorescence was not present in traditionally-prepared samples (Fig. 2A).

Sections obtained from both the methods were stained with toluidine blue O (Gahan 1984) for studying structural anatomy as seen in Figs. 1C and D. Sections were stained with ruthenium red (Ratamales and Scharaschkin 2014) and Schiff reagent (Lewinsohn et al. 1998) for visualizing the depositions of pectin and aldehyde, respectively. In both the cases, sections of traditional and microwave mediated samples showed red and pink colours, on the wall of the oil cells respectively, for ruthenium red (Fig. 1E and F) and Schiff reagent (Fig. 1G and H). Sections obtained from both traditional and microwave mediated samples

showed the presence of cuticular lipids as visualization of orange colour upon staining with sudan IV (Fig. 1I and J) (Buda et al. 2009).

For studying the lipids, sections derived from both the methods were stained with sudan black B for total lipids (Gahan 1984) and with sudan III for neutral lipids (Gahan 1984; Laboratory solution preparation 2020). In both the cases, only oil cells present in the microwave mediated sample sections were found to be stained, showing blackish-blue for sudan black B and orange-red for sudan III (Fig. 2D and F) while the other remain unstained (Fig. 2C and E). Phloroglucinol-HCl was used to stain lignins present in oil cell walls (Laboratory solution preparation 2020), and only sections from microwave-treated sample took light pink colouration (Fig. 2H), which remained absent in the sections obtained by traditional method (Fig. 2G). Nadi staining was done for observation of terpenes (David and Carde 1964), and only the oil cells from the sections of microwave mediated samples showed purple colour (Fig. 2J) while oil cells from the traditionally prepared sections remained unstained (Fig. 2I).

From our study, it has been found that the metabolite retention is only possible in the microwave mediated method. In this method, with the help of different staining we have proposed that our method is better than the traditional PEG embedding method in terms of intracellular metabolite preservation. When stained with toluidine blue O, ruthenium red, Schiff's reagent, and sudan IV, the microwave-based method showed similar results to the traditional PEG embedding method. Bright field sections (without any staining) also showed a similar type of results as compared to the traditional method. However, when compared with the results obtained from histochemical stainings done with sudan black B, sudan III, phloroglucinol-HCl, and nadi, along with results obtained after excitation under ultraviolet wavelength (at 365 nm), the microwave-based methods show better results than the traditional PEG embedding methods. Intracellular metabolite retention was achieved in microwave-based PEG embedding method as evidenced from the specific colour produced upon reacting with different stains, which remained absent in the traditional PEG embedding method. Since our work aimed to check the metabolite status at the intracellular level, from that viewpoint, the microwave-based method is more suitable than the traditional method.

The significant advantage of this proposed method is its rapidness and can be completed within one hour. Since the process is straightforward and quick, long-term heat-induced damage is less than the classical paraffin embedding method or traditional PEG embedding method. Another major advantage is the avoidance of tissue dehydration because of the usage of aqueous PEG solutions. As a result, enhanced metabolite retention with reduced cellular distortion can be visualized in the tissue under microscopic examination, which otherwise is impaired upon dehydration processes. Apart from all these advantages, this method is a low cost, since we have used only a kitchen microwave as a mode of heating medium and a simple rotary microtome. The use of the FAA as a fixative medium has also made it more affordable, as this is a low cost and most readily available fixative. However, there are few limitations that exist with this microwave-mediated PEG embedding method. On a few occasions, microwave-induced heat damage was observed, leading to the secretion of phenolic compounds from the neighboring cells. When compared to other methods of embedding, our method does not show a

significant improvement in anatomical preservation, and in a few cases, various morphological distortions have also been observed. The last point of difficulty we have noted was about the storability of the PEG blocks; these blocks can be stored in 4□C for a good amount of time (up to one month, only). However, once a block is cut, it cannot be stored for further study. Thus, a prepared PEG block once being started cutting, has to be used within 24 hours. This is because since the embedded tissue samples are not dehydrated at the time of preparation, and thus, once cut, the embedded tissue starts to lose water rapidly even at 4□C. Further, these blocks cannot be stored properly at room temperature (25□C). Nevertheless, this problem is not unique for this new method, as similar problems have been encountered in the traditional PEG embedding method too. This problem can only be overcome by paraffin embedding method. In our opinion, the less preservation of finer anatomical details should not hamper the overall process, as our goal has been to target the intercellular metabolites, which we achieved with ease by our method. We suggest here that for anatomical observation it is always better to go for classical paraffin or resin-based embedding methods, as those are well known for obtaining finer anatomical details. The microwave-based PEG embedding method can be used along with traditional paraffin or resin-based method, which will enable the researcher to study both the anatomical structure as well as the localization of intracellular metabolites in finer details. The embedding procedure reported here is based on our chosen sample i.e., leaf of *C. tamala*. However, the finer details of the procedure such as infiltration timing, repetition of infiltration dosages may need modifications depending on the hardness and the type of the tissue sample.

Conclusion

This study showed that the quality of tissue preservation in both traditional PEG embedding method and microwave-based PEG embedding method is identical. Moreover, in microwave-based embedding, the cells retain some of their intracellular metabolite, which is not possible in the traditional method. These encouraging results obtained from this very low-cost and time-saving method can lead to further research on this microwave mediated tissue embedding method and popularize PEG as an embedding medium. From our study, it is evident that this new method can further be utilized for studying a wide range of plant materials.

Abbreviations

PEG
polyethyleneglycol; FAA:formalin- acetic acid- alcohol; HCl:hydrochloric acid

Declarations

Acknowledgments

One of us (A Mitra) acknowledges the institute for the award of a competitive infrastructure challenge grant (IIT/SRIC/AGFE/EPI/2014-15/154) to build up the fluorescence microscopy and microtome

facilities used in this study. S Saha thanks the institute for a joint MTech-PhD assistantship award.

Author Contributions

SS conceived and conducted the microwave embedding experiment, analysed and interpreted the data, and wrote the draft manuscript; RB conducted the traditional embedding experiment and contributed in writing the manuscript too; AM supervised the research and finalized the manuscript. All the authors are in agreement with the results obtained and approved the final version of the manuscript.

Availability of data and materials

Please contact the corresponding author for data availability.

Competing interests

The authors declare that they have no competing interests.

Author details

Natural Product Biotechnology Group, Agricultural and Food Engineering Department, Indian Institute of Technology Kharagpur, Kharagpur, India 721302

Funding

This work was supported by a grant (BT/PR24702/NER/95/821/2017; Agri/2017/20 to A Mitra) from the Department of Biotechnology (DBT, www.dbtindia.nic.in), Government of India, under Twinning Programme for North Eastern Region.

References

1. T Barka, PJ Anderson, Histochemistry: theory, practice and bibliography (Harper & Row, Inc., New York, 1963)
2. GJ Buda, T Isaacson, AJ Matas, DJ Paolillo, JKC Rose, Three-dimensional imaging of plant cuticle architecture using confocal laser microscopy. Plant J. 60, 378-385 (2009)
3. R David, JP Carde, Coloration différentielle des inclusions lipidiques et terpéniques des pseudophylles du Pin maritime au moyen du réactif nadi. C. R. Acad. Sci. 258, 1338–1340 (1964)
4. MA Ferhat, BY Meklati, J Smadja, F Chemat, An improved microwave clevenger apparatus for distillation of essential oils from orange peel. J. Chromatogr. A 1112, 121-126 (2006)
5. BG Ferreira, CT Teixeira, RMS Isaias, Efficiency of the polyethylene-glycol (PEG) embedding medium for plant histochemistry. J. Histochem. Cytochem. 62, 577-583 (2014)
6. BG Ferreira, R Falcioni, LM Guedes, SC Avritzer, WC Antunes, LA Souza, RMS Isaia, Preventing false negatives for histochemical detection of phenolics and lignins in PEG-embedded plant tissues. J. Histochem. Cytochem. 65, 105-116 (2017)

7. PB Gahan, Plant histochemistry and cytochemistry (Academic Press, London, United Kingdom, 1984)
8. RT Giberson, RS Demaree, in Microwave processing techniques for electron microscopy: a *four-hour protocol*, ed. by N Hajibagheri, Electron microscopy methods and protocols. Methods in Molecular Biology, vol 117 (Humana Press, 1999), p. 145-158
9. M Iriti, G Colnaghi, F Chemat, J Smadja, F Faoro, FA Visinoni, Histo-chemistry and scanning electron microscopy of lavender glandular trichomes following conventional and microwave assisted hydrodistillation of essential oils: a comparative study. Flavour Fragr. J 21, 704-712 (2006)
10. Laboratory solution preparation. https://www.flinnsci.com/api/library/Download/18ce587821c24fb3b0ad7d878bd6a3d9. Accessed 11 September, 2020
11. E Lewinsohn, N Dudai, Y Tadmor, I Katzir, U Ravid, E Putievsky, DM Joel, Histochemical localization of citral accumulation in lemongrass leaves (*Cymbopogon citratus* (DC.) Stapf., Poaceae). Ann. Bot. 81, 35-39 (1998)
12. AG Richards, THF Anderson, RT Hance. A microtome sectioning technique for electron microscopy illustrated with sections of striated muscle. Proc. Soc. Exp. Biol. Med. 51, 148-152 (1942)
13. JF Rinehart, A Haj, Histological demonstration of lipids in tissue after dehydration and embedding in polyethyleneglycol. A.M.A. Arch. Pathol. 51, 666-669 (1951)
14. SE Ruzin, Plant microtechnique and microscopy (Oxford University press, New York, 1999)
15. D Schichnes, J Nemson, L Sohlberg, SE Ruzin, Microwave protocols for paraffin microtechnique and in situ localizations in plants. Microsc. Microanal. 4, 491-496 (1999)
16. D Schichnes, JA Nemson, SE Ruzin, in Microwave paraffin techniques for botanical tissues, ed. by R.T. Giberson, R.S. Demaree Jr. Microwave techniques and protocols (Humana Press Inc., Totowa, NJ, 2001), p. 181-189
17. P Webster, in Microwave-assisted processing and embedding for transmission electron microscopy, ed. by J Kuo. Electron microscopy: methods and protocols, 2nd edn. (Humana Press Inc., Totowa, NJ, 2007), p. 47-65
18. B Zechmann, G Graggaber, G Zellnig, Microwave assisted rapid diagnosis of plant virus diseases by transmission electron microscopy. JOVE-J Vis Exp 56, e 2950 (2011)

Figures

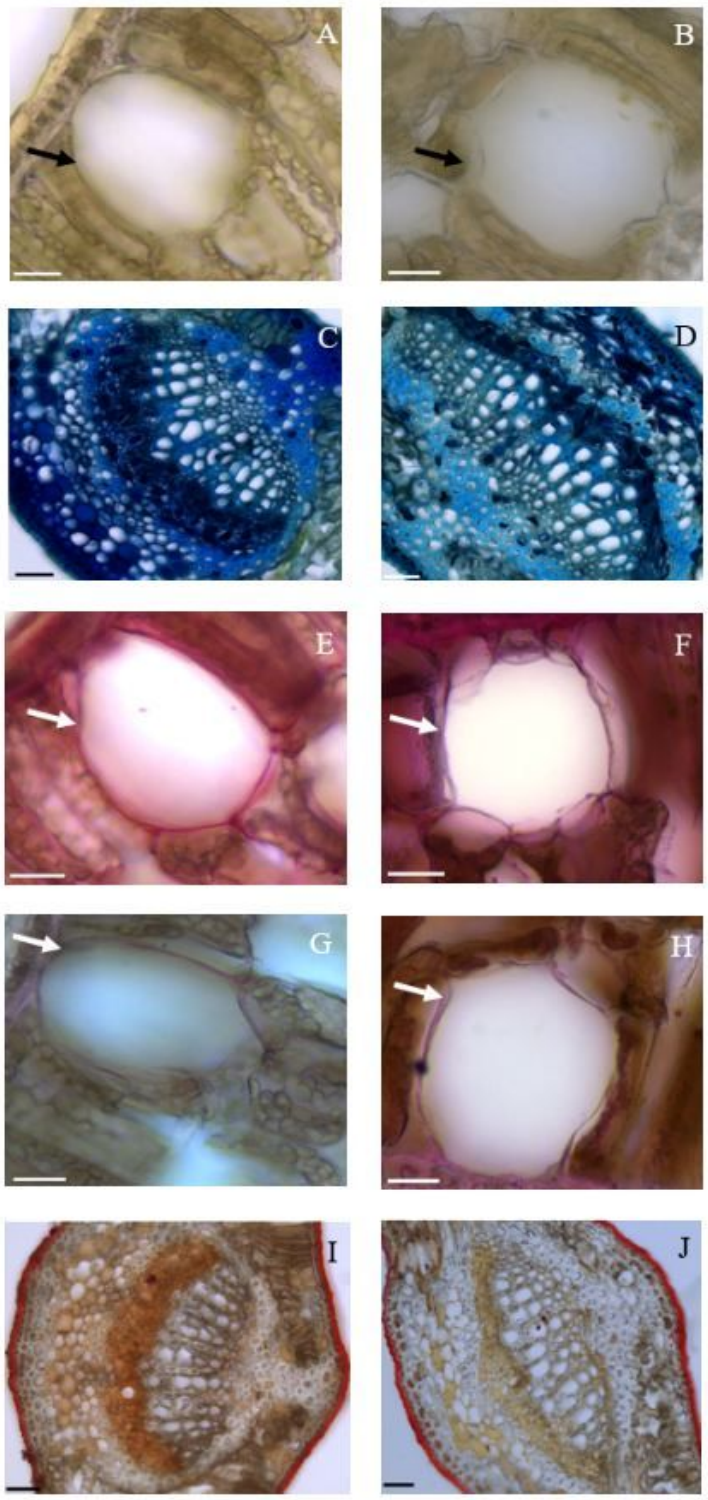

Figure 1

General morphology and anatomy of the oil cells and the leaf tissue in traditional PEG embedding method (A, C, E, G, I) and microwave mediated embedding method (B, D, F, H, J), unstained samples viewed under bright field (A, B), stained with toluidine blue O (C, D), ruthenium red (E, F), Schiff's reagent (G, H), sudan IV (I, J). A-H, scale bars represent 25 µm; I, J, scale bars represent 50 µm.

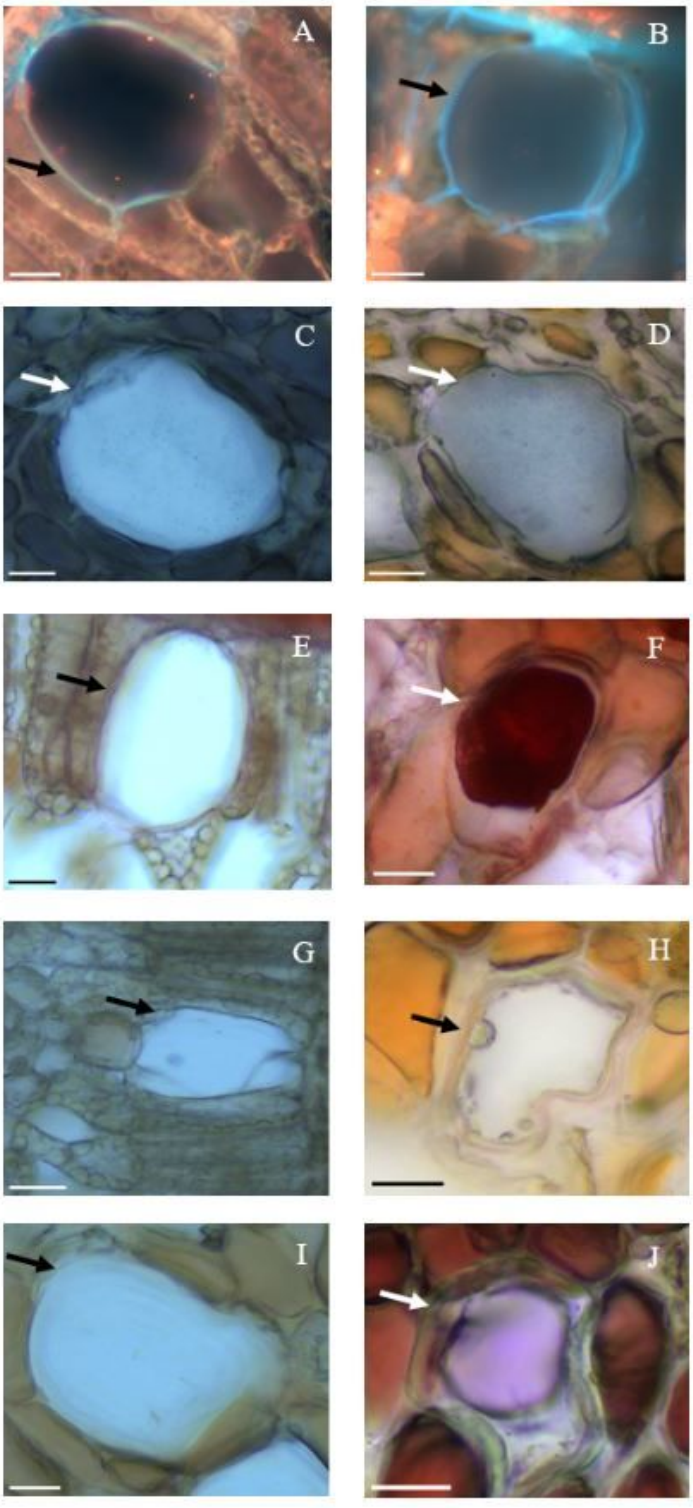

Figure 2

Metabolite status of the oil cells in traditional PEG embedding method (A, C, E, G, I) and microwave mediated embedding method (B, D, F, H, J); unstained samples when excited with 365nm wavelength (A, B), after staining with sudan black B (C, D), sudan III (E, F), phloroglucinol-HCl (G, H), nadi reaction (I, J). Scale bars represent 25 µm.