

Methods of preparing flower stem samples for scanning electron microscopy

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ABSTRACT

Brazil has great capacity for expansion in the floriculture sector. Studies on postharvest cut flowers contribute to development of the sector, helping to maintain the quality of domestic production. Scanning electron microscopy (SEM) is a powerful tool that allows viewing of flower structures and also microorganisms. The aim of this study was to evaluate methods of preparing flower stem samples for viewing in SEM as a support for studies on postharvest cut flowers. Ways of cutting, fixing, and drying samples were tested. Cutting with a stainless steel blade and through freeze-fracture were tested; fixation was carried out without the use of osmium tetroxide (OsO₄); and drying of the samples was performed through freeze-drying and through critical point drying with CO₂. Cutting with a stainless steel blade proved to be a satisfactory method for stem samples, with low cost and simple application compared to freeze-fracturing. Good fixation and high image contrast were obtained without the use of osmium tetroxide, thus avoiding the use of this toxic compound. Freeze-drying allowed the structure and morphological composition to be viewed, while critical point drying with CO₂ preserved the microorganisms present in the samples.

Keywords: gerbera, stainless steel blade, freeze-fracturing, freeze-drying, critical point with CO₂.

RESUMO

Métodos de preparação de amostras de hastes florais para microscopia eletrônica de varredura

O Brasil possui grande capacidade de expansão na área da floricultura. Estudos em pós-colheita de flores de corte contribuem para o desenvolvimento do setor, auxiliando na manutenção da qualidade da produção nacional. A microscopia eletrônica de varredura (MEV) é uma ferramenta potente que permite a visualização das estruturas florais e também de microrganismos. Este trabalho objetivou avaliar métodos de preparação de amostras de hastes florais para visualização de imagens em MEV como suporte em estudos de pós-colheita de flores cortadas. Foram testadas formas de obtenção de corte, fixação e secagem das amostras. Os tipos de cortes testados foram por meio de lâmina de aço inoxidável e criofratura, a fixação foi realizada sem a utilização do tetróxido de ósmio (OsO₄) e a secagem das amostras foi realizada por liofilização e por ponto crítico de CO₂. O corte com a lâmina de aço inoxidável, demonstrou ser um método satisfatório para amostragem das hastes, de baixo custo e simples aplicação com relação a criofratura. Obteve-se uma boa fixação e alto contraste das imagens sem o uso do tetróxido de ósmio, desta forma, evitou-se a utilização deste composto tóxico. A secagem por liofilização permitiu a visualização da estrutura e composição morfológica, enquanto a secagem por ponto crítico de CO₂ conservou os microrganismos presentes nas amostras.

Palavras-chave: gérbera, lâmina de aço inoxidável, criofratura, liofilização, ponto crítico de CO₂.

1. INTRODUCTION

In Brazil, floriculture still has great potential for expansion, seeking to assert a more relevant position both in production and in exports (AGRIANUAL, 2014). In 2012, gross sales of the sector were R\$4.8 billion, with a planted area of 13,800 thousand ha (IBRAFLOR, 2014). The state of São Paulo has 74.5% of domestic production of flowers and ornamental plants (IBRAFLOR, 2013). Studies that involve maintaining the quality of ornamental plants in Brazil may assist in development of the sector.

Water is the main constituent of live cells and is

responsible for sustaining plant tissues (REICHARDT, 1985). Water balance determines the longevity of cut flowers. Loss of moisture to the environment without due replacement reduces vase life (KOHL, 1968; BOROCHOV et al., 1982). Flower stems take up water through the transport vessels of the vascular system. Water transport through these vessels is simple and shows little resistance, efficiently allowing displacement of large amounts of water (RASCHKE, 1975). Negative hydrostatic pressure created by transpiration drives water through the vessels and allows it to rise up to points far from the roots (KERBAUY, 2004). The cohesion-tension theory of ascent of sap explains the

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mechanism for water rising through the vessels, where the tension created comes from the evaporation that occurs on the flower surface, and cohesion is one of the properties of water (TAIZ and ZEIGER, 2009). Water imbalance leads to dehydration of flower tissues, which may be linked to occlusion of the transport vessels, making maintenance of the cohesion-tension dynamic in the vessels impossible (AHMAD et al., 2010).

The inability of cut flowers to take up water through vascular transport may be caused by embolism, physiological occlusion, or occlusion caused by microorganisms (VAN DOORN, 1999). Embolism is the entrance of air in the transport vessels after cutting. When the stems are cut and exposed to air, bubbles are formed throughout the transport vessels, which break the cohesion among the water molecules (VAN DOORN, 1999). Physiological occlusion occurs due to the presence of substances resulting from the plant metabolism itself (CLINE and NEELY, 1983), such as the activity of peroxidase (POD) and polyphenol oxidase (PPO) enzymes (VASLIER e VAN DOORN, 2003). The occlusion caused by the proliferation of microorganisms occurs due to their presence and also due to the production of their exudates. The microorganisms responsible for vascular occlusion include bacteria of the genus *Pseudomonas* and yeasts (ZAGORY e REID, 1986); VAN DOORN et al., 1995

Scanning electron microscopy (SEM) has been used in countless studies for observation of microbial proliferation in the transport vessels of cut flowers such as gerberas (ANTES et al., 2009) and roses (LI et al., 2012), and for observation of the effect of preservative solutions on the cells at the base of gerbera stems (DURIGAN et al., 2009). The scanning electron microscope is an instrument used for microstructural analysis of solid materials, and produces results that are easily interpreted. The image produced has a maximum magnification between the optical microscope (OM) and the transmission electron microscope (TEM). The advantage of the SEM in relation to the OM is its high depth of focus and its high resolution, around 2 to 5 nm, and its advantage in relation to the TEM is the ease of preparation of the samples (MALISKA, 2014).

However, direct viewing of the live material in a conventional high vacuum SEM is not possible, due to water present in the tissue. For this technique, the sample must be extremely dehydrated. Thus, preparation of the biological samples involves the following steps: obtaining the sample, fixation, dehydration, drying, set up on a metallic support, and metallization, described as follows.

Obtaining the segment of interest of the stem under analysis must preserve the condition of the plant tissue in its hydrated form to the greatest extent. For that reason, the biological samples are frequently frozen with liquid nitrogen and then fractured to obtain segments of the sample (RATNAYAKE et al., 2012).

The fixation process is an essential step for the visual quality of the structures present in the sample. The samples are stabilized by chemical fixation. Chemical fixation is responsible for the integral structure of the sample, ensuring maintenance of the original format of the plant

matter obtained in the sample (CASTRO, 2002). In this step, there is immobilization of all the molecules that make up the material, which, after drying, allows an appearance as near as possible to the *in vivo* state of the material.

During drying of the plant material, water is removed so as not to damage the sample. There are some alternatives for the drying step, as shown in Ratnayake et al. (2012). Among them, critical point drying with CO₂ is one of the most-used methods, which allows preservation of microorganisms present in the flower stems. At the critical point with CO₂, the sample is dried at surface tension equal to zero through control of temperature and pressure (DEDAVID et al., 2007). Freeze-drying, i.e., dehydration through sublimation, is an interesting alternative in the drying process since the cost requirements of equipment are lower in relation to the critical point with CO₂, and it can be performed more rapidly (RATNAYAKE et al., 2012). For some types of less sensitive materials, it offers an alternative to equipment for critical point drying with CO₂.

After the processes described above, the samples are fastened on brass stubs and metallized with gold for viewing in SEM to be possible. SEM is a powerful tool for investigation in postharvest of flowers, where the sampling directly affects the surface to be examined (RATNAYAKE et al. 2012).

The aim of this study is to evaluate methods of preparation of flower stem samples for viewing of images in SEM as a support for postharvest studies of cut flowers.

2. METHODOLOGY

Sampling

Gerberas (*Gerbera jamesonii* ‘Stanza’) were acquired in São Carlos, SP, Brazil, and taken to the Postharvest Laboratory of Embrapa Instrumentation. In the laboratory, after cutting under water, the flower stems were selected for elimination of defects and rehydrated for 2 h. Stem segments (~2 mm) from the base were removed as samples, obtained by freeze-fracturing and cutting with stainless steel blades. In freeze-fracturing, the stems were treated with a 10% glycerin solution (1h), 20% solution (1h), and 30% solution (2h) to protect the cells from the cold, and then immersed in liquid nitrogen until they were totally frozen. The samples were removed from the liquid nitrogen and fractured. Obtaining the samples through cutting was carried out by direct use of new blades, which were duly sharpened and sanitized with 70% ethanol.

Fixation

The samples were placed in 2% paraformaldehyde and 2.5% glutaraldehyde fixative in a buffer solution of 0.05 M cacodylate and 0.001 M CaCl₂, pH 7.2 (without addition of osmium tetroxide - OsO₄) for 1 h in a volume 10 times greater than the volume of the sample. After that, the gases remaining in the cells were removed with the aid of a vacuum pump for 5 min, allowing more efficient entry of the fixative in the sample. The samples were kept under refrigeration until drying.

Drying

Drying was through critical point drying with CO₂ or freeze-drying. The samples dried to the critical point were previously prepared by washing them three times with 0.05 M cacodylate buffer (pH 7.2) and dehydrated in 30% acetone (10 min), 50% acetone (10 min), 70% acetone (10 min), 90% acetone (10 min), and three times for 10 minutes in 100% acetone. The critical point drying equipment used was Baltec CPD 030, following the manufacturer's instructions. After drying, the samples were kept in a desiccator until their set-up on stubs and metallization.

In freeze-drying, the gerbera stem samples were frozen at -30 °C and placed in the L101 Liotop freeze-dryer under vacuum pressure at -40 °C for 2 h. After freeze-drying, the samples were stored in a desiccator until their set-up on stubs and metallization.

Metallization

The samples were fastened with adhesive tape on brass stubs and then gold coated in the metallizer Sputter Coater SCD050 / LEICA.

Viewing the flower stem samples.

The samples were viewed in a scanning electron microscope JSM-6510 / JEOL.

3. RESULTS AND DISCUSSION

Sampling

The samples obtained after fracturing in liquid nitrogen

showed excellent preservation of the flower stem cells; however, they did not allow viewing of the whole sample in a cross section, as a result of damage caused by freezing in liquid nitrogen (Figure 1). This type of sectioning is important for studies on plant anatomy and on the condition of the stem structures when subjected to treatments with flower preservatives, and in evaluation of the number of occluded transport vessels in the vascular system. Irregular surfaces, like those observed, are common in this type of sampling and vary according to the species studied. The format of the fracture depends on its fragility and also on factors such as temperature, force, and the presence of small discontinuities (ECHLIN, 2009). In contrast, plant morphology was well preserved, offering a good view of the cell ultrastructure, preserving inner details of the cells and their anatomy, demonstrating the effectiveness of this method, depending on the objective of the study (Figure 2 and 3).

Cutting with the stainless steel blade preserved the structural part of the sample in a satisfactory manner, and it was possible to obtain regular, standardized surfaces that were easily identified, for each tissue to be analyzed (Figure 4 and 5). The advantage of removing complete samples and not just small segments also resides in the ease of finding the structures of interest at the time of viewing. Small deformations of the walls of the transport vessels in the region of the cut were observed; however, this may be minimized through continual training of the sample taker and through the use of highly sharpened blades (ECHLIN, 2009) (Figure 6). This method had the advantages of being

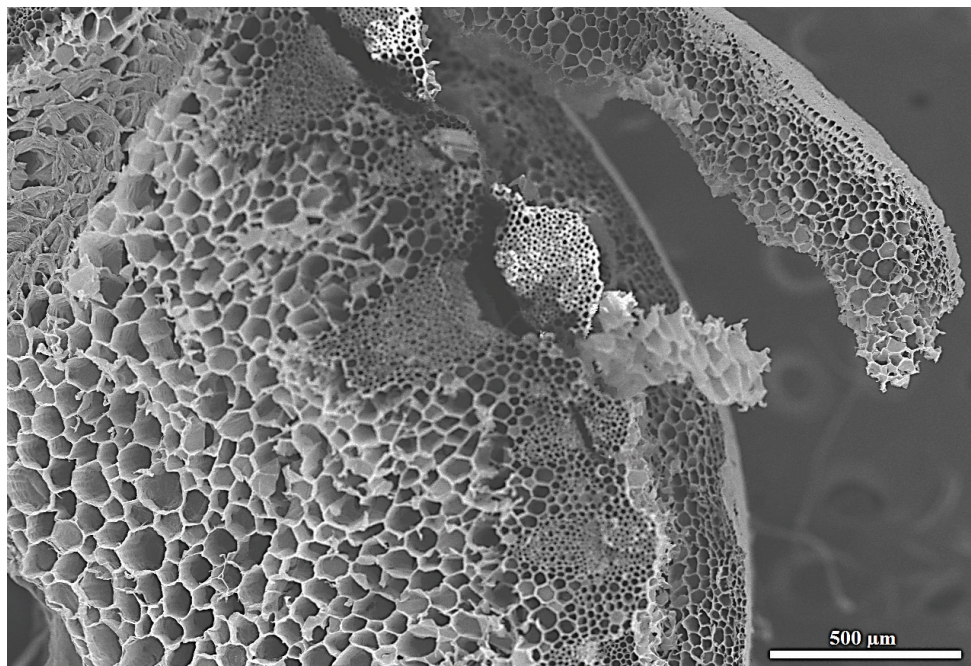


Figure 1. Micrograph from electron microscopy of gerbera flower stems fractured in liquid nitrogen. The circled area indicates the xylem vessel region. Bar – 500µm.

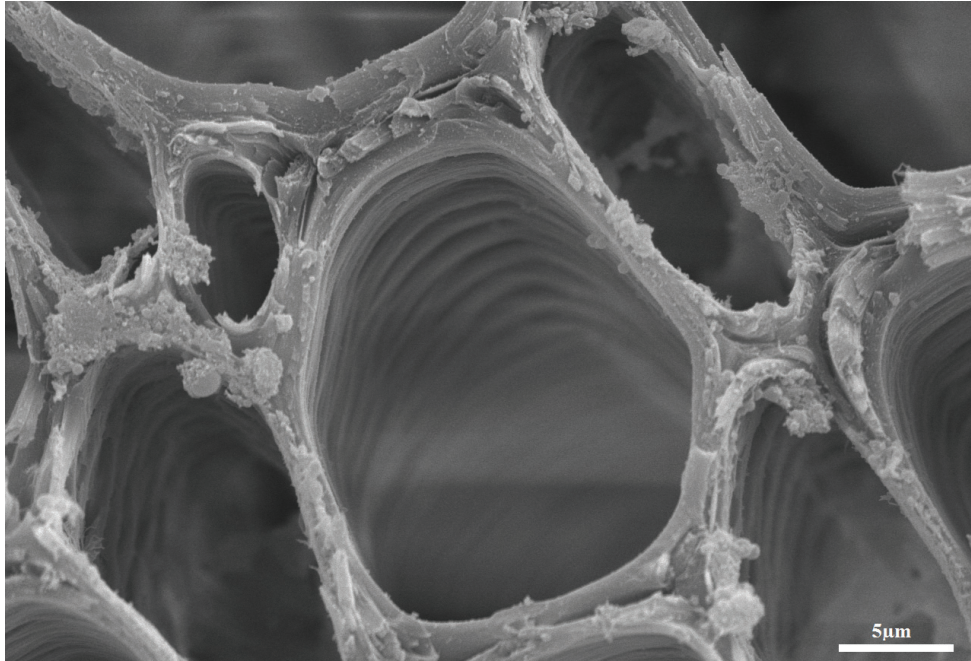


Figure 2. Micrograph from electron microscopy of gerbera flower stems fractured in liquid nitrogen (xylem vessels). Bar – 5 μ m.

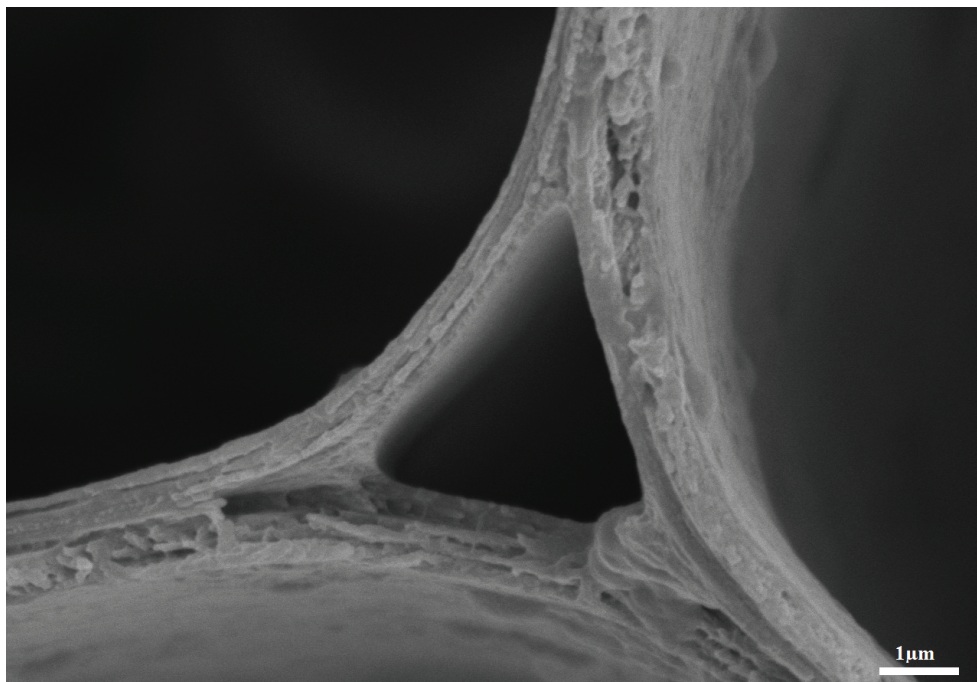


Figure 3. Micrograph from electron microscopy of gerbera flower stems fractured in liquid nitrogen (1. Cell wall; 2. Intercellular space) . Bar – 1 μ m.

simple and fast since the use of liquid nitrogen is not necessary, and neither is keeping the material in glycerin solution for hours before cutting. The samples obtained through the use of only the stainless steel blade are also of low cost since large investments are not necessary to replace the blades.

Drying without fixation may cause deformation of the tissues through evaporation of the water, which has high surface tension. For analyses in SEM, non-coagulative chemical fixatives are used, the most common being glutaraldehyde and osmium tetroxide (OsO_4). Osmium tetroxide has the feature of leaving the materials with high

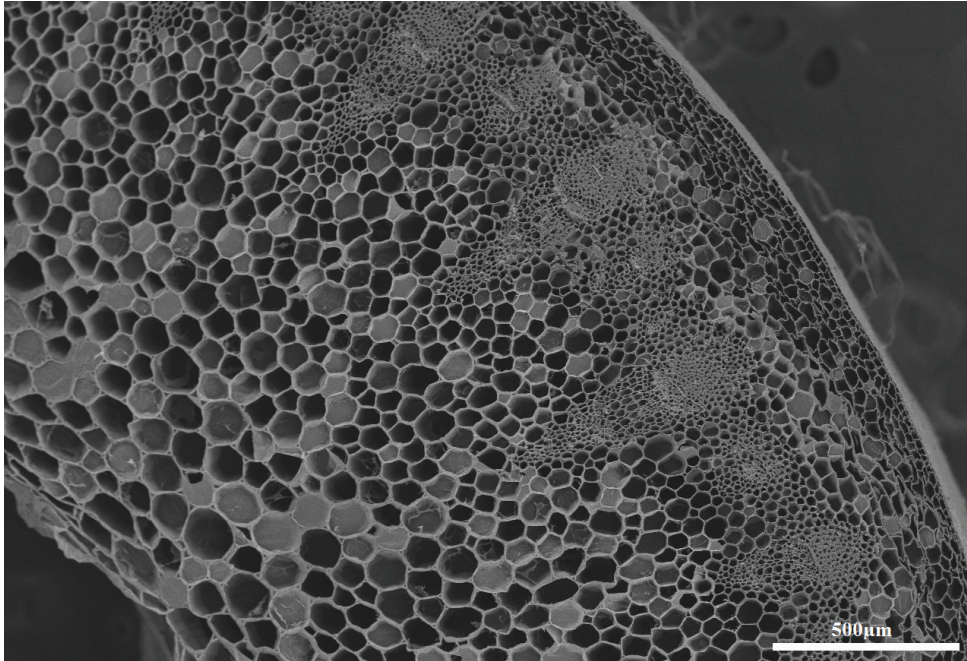


Figure 4. Micrograph from electron microscopy of gerbera flower stems cut using a stainless steel blade. The circled area indicates the xylem vessel region. (Pf: fundamental parenchyma). Bar – 500 μm .

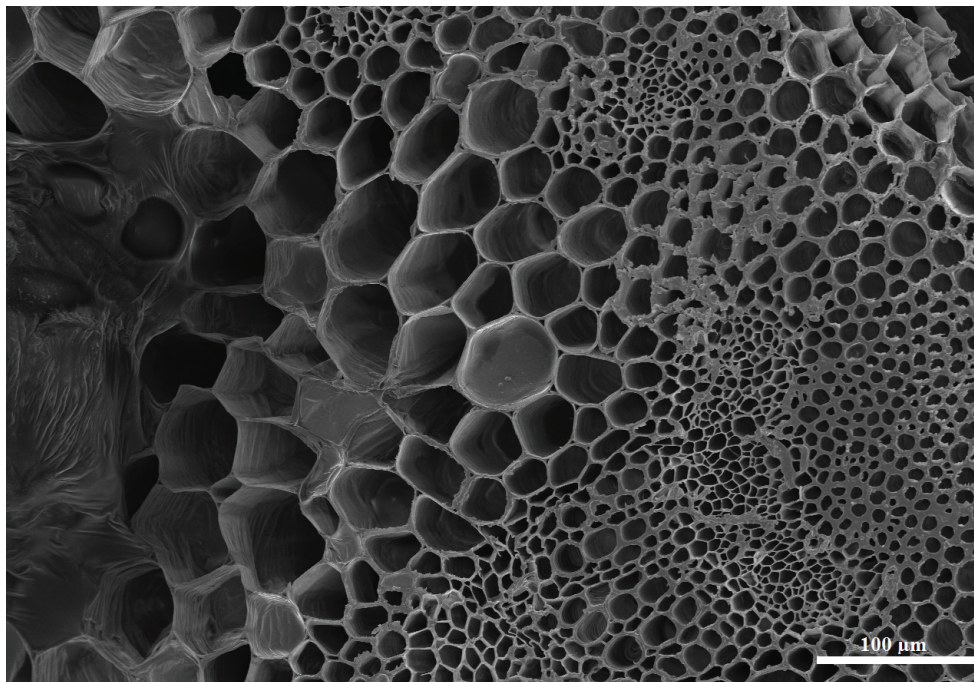


Figure 5. Micrograph from electron microscopy of gerbera flower stems cut using a stainless steel blade. The circled area indicates the xylem vessel region. Bar – 100 μm .

contrast and it is widely used; however, it is an expensive and highly toxic substance and may cause damage to human health and to the environment (MCLAUGHLIN, 1946; KOBAYASHI et al., 2001).

Fixation with 2% paraformaldehyde and 2.5% glutaraldehyde in gerbera stems was effective in preservation of the sample, and it was not necessary to

use osmium tetroxide. The OsO_4 acts as a contrast of biological samples and also as a fixative; however, it is highly toxic and volatile. This chemical compound may be handled only by trained personnel according to strict safety precautions and, also for that reason, the recommendation for preparation of samples in alternative manners is important (DEDAVID et al., 2007). In electron

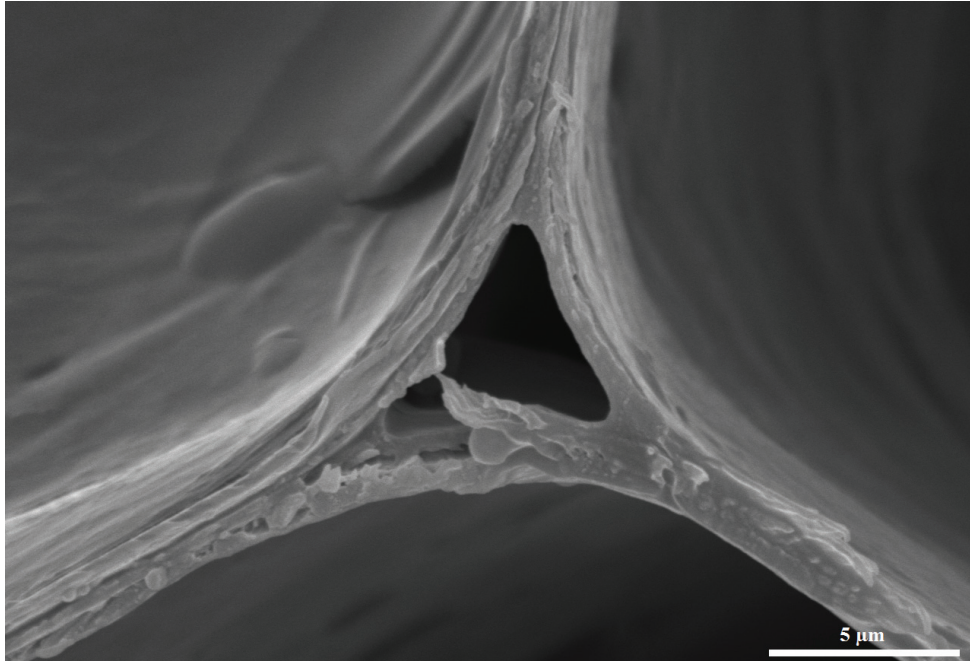


Figure 6. Micrograph from electron microscopy of gerbera flower stems cut using a stainless steel blade. (1. Cell wall; 2. Intercellular space) Bar – 5 μm .

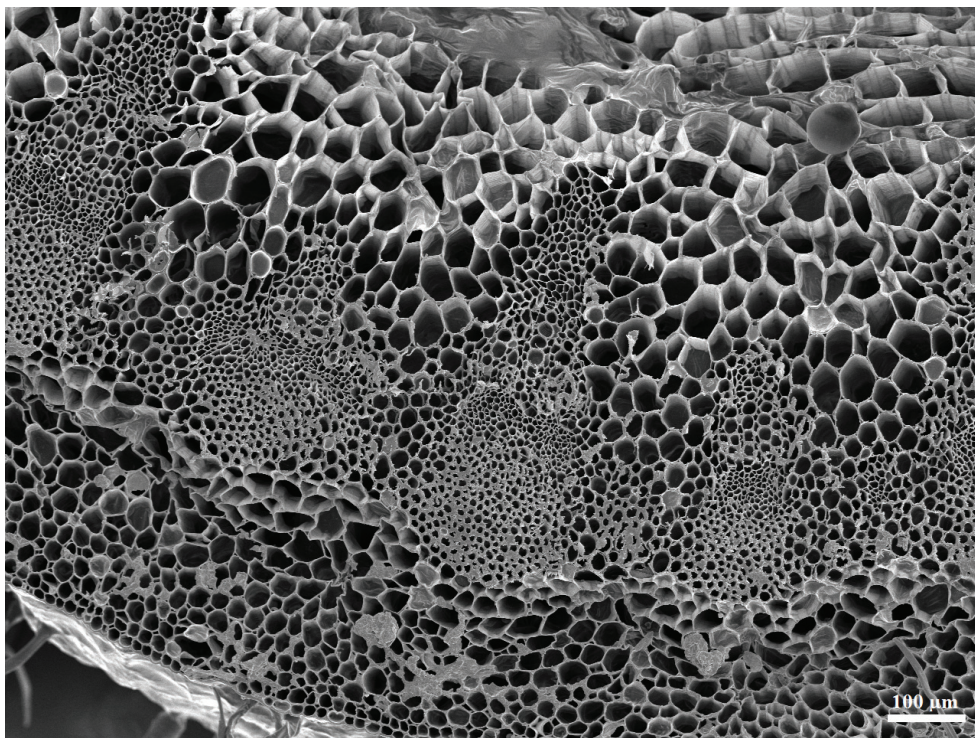


Figure 7. Micrograph from electron microscopy of gerbera flower stems cut using a stainless steel blade and freeze-dried. The circled area indicates the xylem region. Bar – 100 μm .

microscopy, adequate contrast is desirable so that image acquisition is satisfactory. However, the results obtained in this study indicate that the use of OsO_4 may be dispensable (DEDAVID et al., 2007).

In addition to this study, other authors also dispensed with the use of OsO_4 in flower stems, as in acacia fixed with 3% glutaraldehyde followed by critical point drying

with CO_2 , which was effective in preserving bacteria and exopolysaccharides and allowed good contrast for images (RATNAYAKE et al., 2012).

Drying

Drying through freeze-drying preserved the morphology of the gerbera stem, allowing good viewing

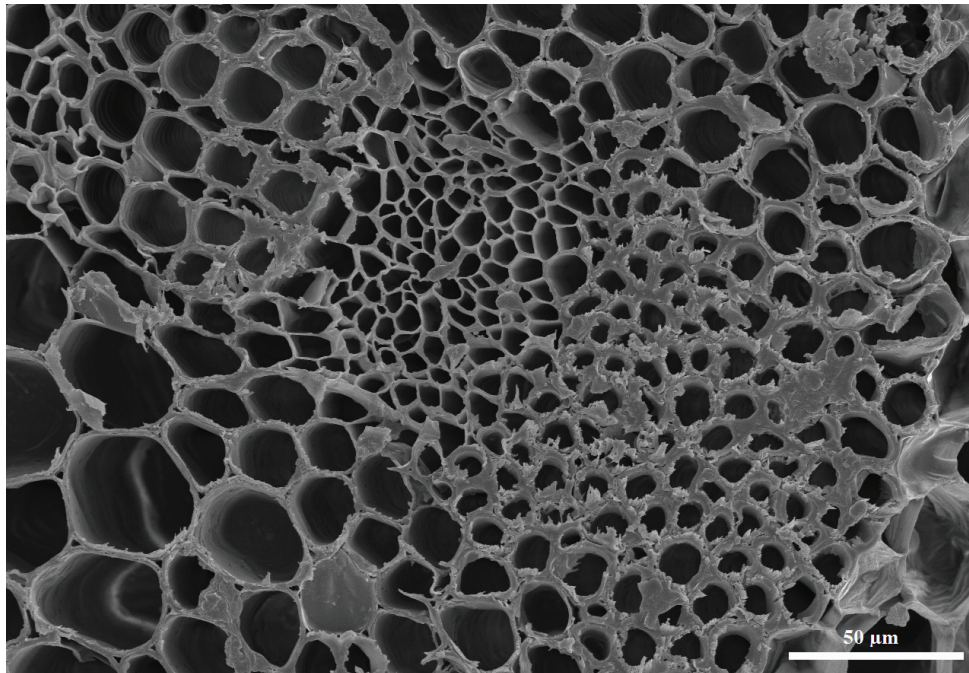


Figure 8. Micrograph from electron microscopy of gerbera flower stems cut using a stainless steel blade and freeze-dried. The circled area indicates the xylem region. Bar – 50 μm .

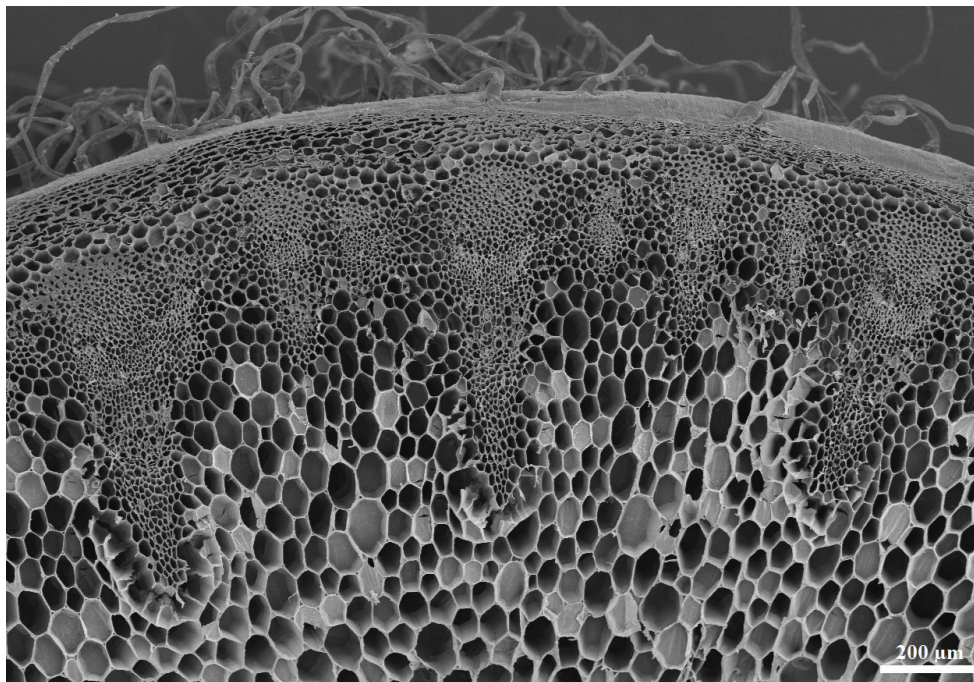


Figure 9. Micrograph from electron microscopy of gerbera flower stems cut using a stainless steel blade and critical point drying with CO_2 . The circled area indicates the xylem region. Bar – 200 μm .

and identification of the structures present, as may be observed in Figure 7 and 8. Preservation of quality in this method depends, among other factors, on the mechanical stability and size of the sample, and on the cooling rate (ENSIKAT et al., 2010). For preparation of leaf samples of *Salvinia oblongifolia*, the results of use of freeze-drying were also considered good, and sometimes excellent

(ENSIKAT et al., 2010). However, freeze-drying was not effective in preservation of microorganisms within the transport vessels, biofilms, or any substance secreted by them. Nevertheless, since it is a simple method, it may be an alternative when the objective of documentation is only observation of the anatomy and distribution of the constituent elements of the stem.

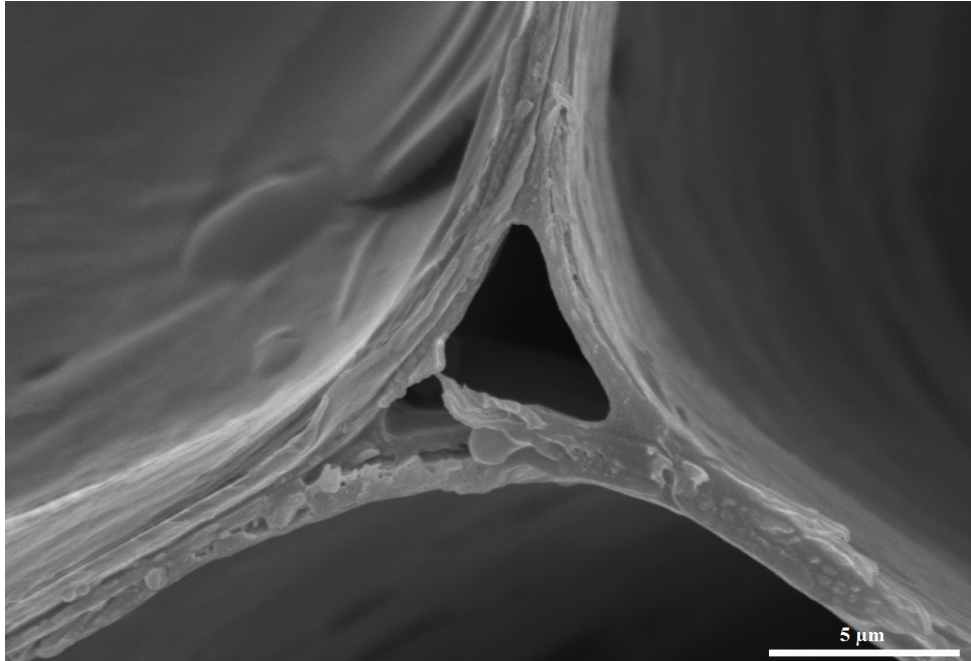


Figure 10. Micrograph from electron microscopy of gerbera flower stems cut using a stainless steel blade and critical point drying with CO₂. (1. Bacteria; 2. Occluded xylem vessel) Bar – 2 μm.

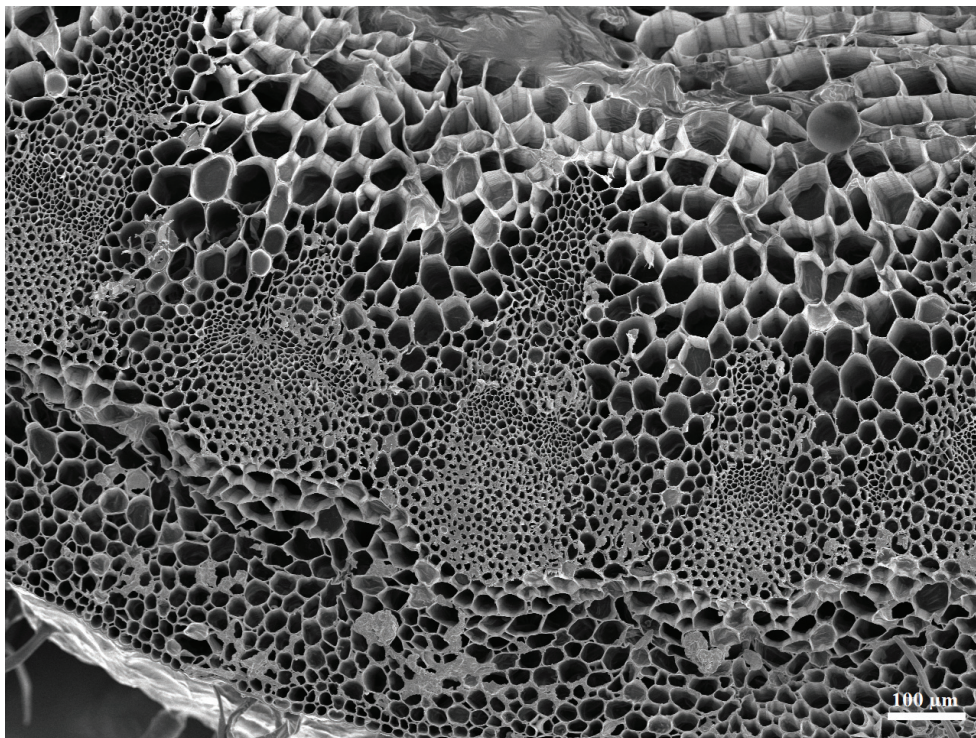


Figure 11. Micrograph from electron microscopy of gerbera flower stems fractured in liquid nitrogen. The circled area indicates the xylem vessel region. Bar – 500μm.

Critical point drying with CO₂ is the most common method of tissue dehydration for preparation of samples for microscopy (PATHAN et al., 2008). Critical point drying with CO₂ proved to be adequate in preservation of both the structures of the gerbera stems (Figure 9) and the microorganisms and exopolysaccharides of bacteria present in the samples (Figure 10 and 11). This same result with the use of critical point drying with CO₂ is in agreement with published studies on stems of *Acaccia holosericea* (RATNAYAKE et al., 2012), gerberas (ANTES et al., 2009), and roses (LI et al., 2012). Although the method is not completely problem-free, for it may cause some significant contractions in tissues (BOZZOLA et al., 1999), it is an effective manner for viewing colonization by microorganisms when the objective is to observe whether there is occlusion of the transport vessels. According to Ratnayake et al. (2012), the observation of extracellular bacterial polysaccharides in their original state is important for understanding the true nature of vascular occlusion caused by bacteria.

4. CONCLUSIONS

The use of a stainless steel blade proved to be a viable alternative for sectioning of the samples of flower stems since it showed satisfactory results, low cost, and simple application in relation to fracturing in liquid nitrogen. Fixation without the use of osmium tetroxide (OsO₄) did not affect the quality of the image contrast. Critical point drying with CO₂ allowed acquisition of images of both the structural part of the samples and of the microorganisms and their exopolysaccharides. However, drying by freeze-drying is also an alternative when the objective of the study is viewing the structural part and morphological conformation of the sample.

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