Phycologia (1981) Volume 20 (4), 392–396

Use of clearing and fluorescence techniques in anatomical studies of the sporophyte of *Macrocystis* (Phaeophyceae, Laminariales)

by H.L. BARRALES, R.L. PETERSON, D.J. GRENVILLE AND J.F. GERRATH *Department of Botany and Genetics, University of Guelph, Guelph, Ontario, Canada N1G 2W1*

Dried herbarium specimens of *Macrocystis pyrifera* were rehydrated, cleared by removing chlorophyll and other pigments with hot ethanol or acetone and then treating with hot alkali, and stained by a number of techniques. The distribution and structure of mucilage canals and associated glands were studied in material stained by safranin, basic fuchsin, or alcian blue. Sieve tubes were stained with aniline blue and the fluorescence induced by either ultraviolet or blue light was examined.

INTRODUCTION

Clearing techniques are used almost exclusively for the investigation of vascular plant morphology. In tissue clearing, various treatments render some tissue components more visible while removing or damaging others. Foster (1955) initiated a renewed interest in the use of clearing methods in plant anatomy. More recently, Lersten (1967) and Gardner (1975) have reviewed various aspects of the clearing procedure. Clearing techniques have been used mostly for studies of xylem development and leaf venation patterns (Lersten, 1967). However, the modifications explored by Arnott (1959), Herr (1971, 1972), Peterson & Fletcher (1973), Gardner (1975), and Thomasson (1978) reveal the great versatility and applicability of clearing as a basic tool in botanical research. A few studies using clearing methods in the investigation of phloem development have been published. La Motte & Jacobs (1962) studied quantitative phloem regeneration in dissected tissue cleared with lactic acid and stained with aniline blue while Bisalputra & Esau (1964) used sodium hydroxide and chloral hydrate for clearing and polarized light to study phloem differentiation in embryos of *Chenopodium album*. In a series of studies on the factors involved in the control of differentiation (Aloni & Sachs, 1973; Aloni & Jacobs, 1977; Aloni, 1978, 1979) tissue was cleared for examination. A method involving clearing and fluorescence microscopy has been developed to study phloem in various systems (Peterson & Fletcher, 1973).

In studies with algae, the only comparable technique involves the clearing of the growing apex of the calcareous charophyte *Nitella* (Blackburn & Christophel, 1976). In studies of large brown algae (kelps) it has been difficult to follow the differentiation and development of tissue systems, particularly the conducting system, from sectioned material alone. In our studies with *Macrocystis*, a simple method that combines clearing with fluorescence microscopy has been developed to complement standard sectioning techniques.

MATERIALS AND METHODS

Tissue samples were obtained from herbarium specimens of plants collected in the locality of Riachuelo (Province of Chubut, Argentina). Both young and adult sporophytes with mature sporophylls were examined. Techniques used for clearing were modifications of those in the literature (e.g. O'Brien & von Teichman, 1974; Gardner, 1975; Fuchs, 1963). In detail, the techniques used for rehydrating, clearing and staining were as follows:

 0031–8884/81/1200–0392 $02.00

Rehydration and clearing

(1) A piece of wet cheese cloth or filter paper was placed over the dried tissue until the tissue could be easily detached from the herbarium sheet.

(2) Tissue was cut into pieces and allowed to stand in water for 20–30 min or until fully hydrated.

(3) Pigments were extracted in either 80% ethanol or 80% acetone by boiling in a water bath. Young tissue was cleared of pigments in a few minutes but older tissue required 15–45 min boiling.

(4) Tissue was cleared in 2–5% KOH or NaOH at 50–60 °C for 5 to 20 min. Prolonged treatment with alkali should be avoided since it will cause the tissue to blacken due to phenolic oxidation (Gardner, 1975). Mild treatment with alkali is recommended as a means to control excessive depolymerization of the structural alginates (Percival & McDowell, 1967) and to prevent excessive tissue degradation (Gardner, 1975). After clearing, the tissue is rinsed several times with distilled water to remove the alkali. Tissue is now ready for dissection or staining.

(5) If dissection is required, tissue is placed in a Syracuse watch glass and covered with water. Working under the stereomicroscope, and using fine forceps and a scalpel, the meristoderm layers are separated and removed. The medulla can also be dissected and in difficult specimens this can be facilitated by placing the tissue in 1% KOH or NaOH during dissection and then washing with distilled water after the dissection is completed.

Staining

A variety of staining techniques can be used on the cleared tissue, depending on the structural elements to be studied. To observe the distribution and structure of canals and glands the following techniques have been useful:

(1) Tissue was stained in 0·5% basic fuchsin (modified from Fuchs, 1963) in 70% ethanol for 30–60 sec, rinsed in 70% ethanol, 95% ethanol, absolute ethanol-xylene (1:1), followed by two changes in xylene. Tissue pieces were mounted in Eukitt mounting medium (O. Kindler, Freiburg, West-Germany, Silberbachstraße 25).

(2) Tissue was stained as above but counterstained with light green. After the 95% alcohol rinse, the material was placed in a 0·5% solution of light green in 95% ethanol for 30 sec, rinsed in 95% ethanol, absolute ethanol, absolute ethanol-xylene (1:1), and placed in two changes of xylene before mounting in Eukitt.

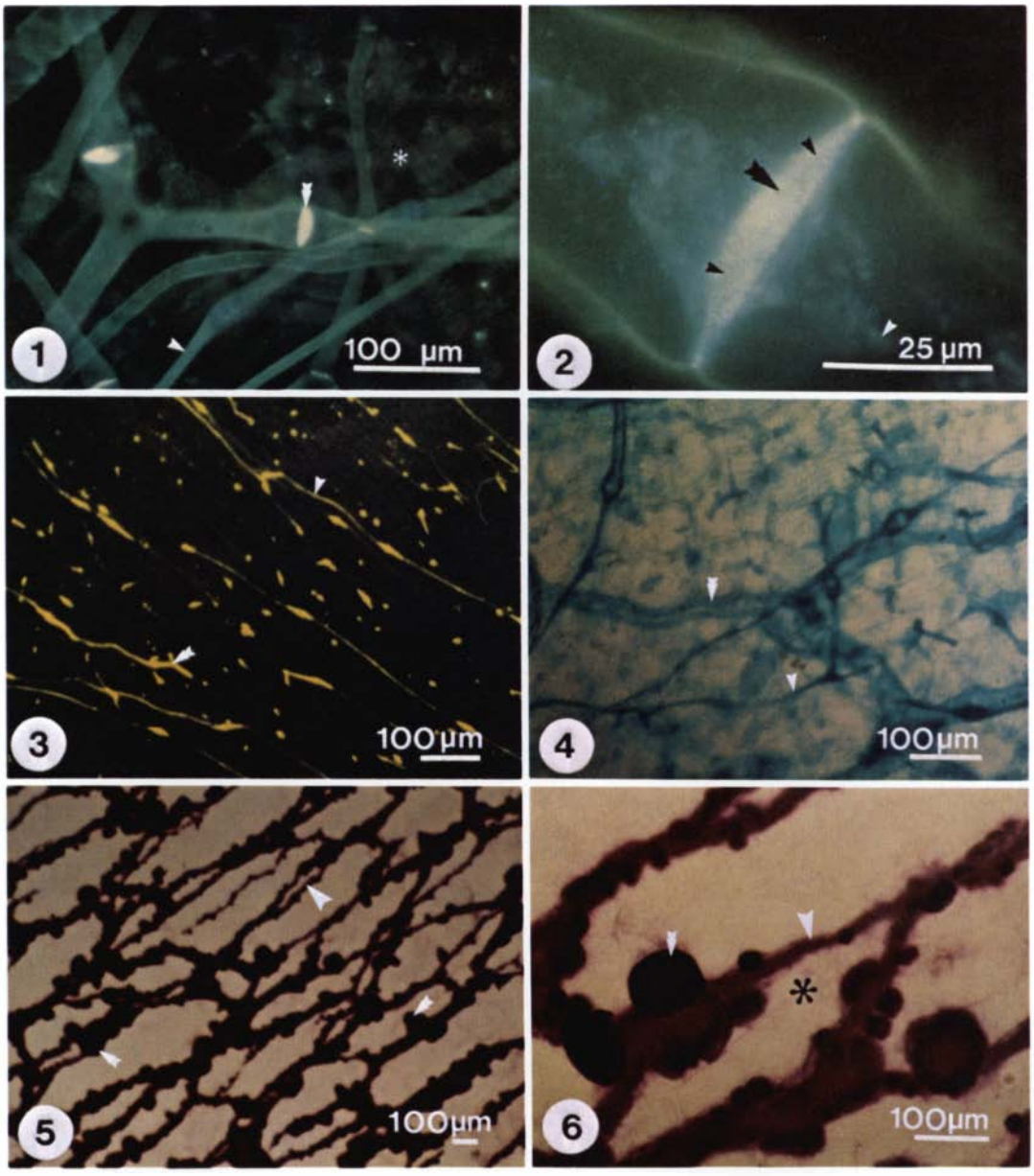
(3) Tissue was stained in 0·5% safranin O in 70% ethanol, dehydrated in 95% ethanol, absolute ethanol, absolute ethanol-xylene (1:1), followed by two changes of xylene. Tissue was again mounted in Eukitt mounting medium.

(4) Tissue was stained in freshly prepared 0·1% alcian blue in 3% acetic acid from 5–15 min, rinsed in distilled water and observed without dehydration. This technique is good for mucopolysaccharides (Pearse, 1968).

(5) For the study of the sieve elements the following technique has been especially useful. Tissue was stained on 0·005% solution of water soluble aniline blue in 0·067 M KH_2PO_4 buffer (Currier, 1957), a stain used for callose. Stained tissue was examined while mounted in the dye using a Leitz SM-Lux epifluorescence microscope with a mercury vapour HBO 50 lamp and using either the broad band blue filter system (exciter filter BG12 and barrier filter TK510/K515) or the broad band ultraviolet filter system (exciter filter UG1/TK and barrier filter 400/K 436).

RESULTS AND DISCUSSION

Tissues from young and mature sporophytes of *Macrocystis pyrifera* were studied using the clearing and staining techniques described. Clearing of young tissue was accomplished more readily and completely than with more mature tissue and therefore gave excellent results. The medullary region dissected from a fully developed young sporophyll shows considerable detail (Fig. 1). Sieve tube elements shown in the foreground exhibit well-developed sieve plates with a slight and uniform deposition of aniline blue-positive material. Although this substance might be callose, other substances do react with aniline blue (Smith & McCully, 1978). It appears that very early in sieve tube differentiation the end walls become flared and the sieve tube elements acquire the typical trumpet shape. The sieve tube elements and filaments seen in this clearing form a part of the complex and unique phloem system in the giant kelps. The cell wall of the phloem element in the left hand corner of Fig. 1 shows a somewhat irregular type of thickening. Cell walls similar to this were frequently seen in cleared material. Sieve plates in fully developed sieve tube elements from the medulla



Figs 1–6. Conducting elements, mucilage canals, and secretory glands in cleared sporophytes of *Macrocystis pyrifera*.

Fig. 1. Young sieve tube elements (single arrow) with sieve plates (double arrow) from the medullary region of a young blade. Aniline blue-positive substance is present at two of the sieve plates (double arrow). Hyphal filaments (asterisk) are interspersed among the sieve elements. Tissue stained with aniline blue and the fluorescence induced with ultraviolet light was observed.

of young sporophylls often show pores surrounded by deposits of aniline blue-positive substances (Fig. 2). The pores are numerous and uniformly distributed. The peripheral constriction of the cell wall around the sieve plate is characteristic of mature trumpet hyphae. The presence of apparently unobstructed pores in the sieve plate would suggest that the sieve elements in Fig. 2 are functional.

At low magnification, the complex array of conducting elements in the lamina of a very young, single blade stage sporophyte is apparent in cleared tissue (Fig. 3). The occurrence of numerous longitudinal files of sieve tubes as well as abundant connections between them is evidence that a well-developed conducting system develops early in blade ontogeny. However, the presence of an abundance of a brightly fluorescent aniline blue-positive substance in the area of the sieve plates and within the lumen of the sieve element suggests that either some of the conducting elements are no longer functional or that wound substances form during tissue drying.

Clearing techniques are also very useful to show the spatial relationship between the conducting tissue and the mucilage canal system (Fig. 4). The sieve tube elements shown in the foreground are embedded in the medullary region while the mucilage canals are present in the cortex. The meristoderm tissue is apparent beyond the mucilage canals. This particular tissue which was dissected from the lamina of an older sporophyll, shows sieve elements with unobstructed sieve plates. Staining with alcian blue reveals the presence of acidic polysaccharides both in the mucilage canals and in the sieve elements. Additional views of the mucilage canal system and associated secretory glands in cleared tissue from laminae of single blade sporophytes are illustrated in Figs 5 and 6. Figure 5 shows an overall view of the mucilage canal system stained with basic fuchsin. Anastomosing of the larger canals is quite common while some of the minor ones have blind endings. In Fig. 6, the relationship of the secretory glands to the canals is seen quite clearly. Staining of these systems with either fuchsin or safranin gave excellent results. This technique should be useful in determining the variability in distribution of mucilage canals in *Macrocystis* and other kelps. Since dried herbarium materials can be rehydrated and used for the clearing and staining techniques, the problem of fixing samples at the collection site is avoided. Although the technique has not been used with other kelps, it should be applicable to them.

ACKNOWLEDGEMENTS

The authors wish to thank the staff at the Instituto Nacional de Investigaciones y Desarrollo Pesquero (Argentina) for their financial assistance and the Centro Nacional Patagónico (Argentina) for their help in collecting samples. We

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Fig. 2. Young sieve tube elements showing a sieve plate (double arrow) with a uniform deposition of aniline blue-positive substance around pores (single arrows). Typical flared walls at the sieve plate are evident. Aniline blue-positive substances are present within the sieve elements (white arrow). Tissue stained with aniline blue and observed with ultraviolet light.

Fig. 3. Sieve tube elements (single arrow) and connections (double arrow) from the medullary region of the lamina of a single blade sporophyte. Deposition of aniline blue-positive substances occurs within the sieve elements and at the sieve plates. Material stained with aniline blue and the fluorescence induced with blue light was observed.

Fig. 4. Sieve tube elements (single arrow) and mucilage canals (double arrow) from the medullary and cortical regions of a young blade stained with alcian blue. Contents of the sieve tube elements and canals are alcian blue-positive.

Fig. 5. Mucilage canals (single arrow) and secretory cells (double arrows) found immediately beneath the meristoderm. Tissue stained with safranin O.

Fig. 6. Secretory cells (double arrow) develop as clusters along the mucilage canals (single arrow). The meristoderm (*) is apparent. Tissue stained with basic fuchsin.

thank the Research Advisory Board, University of Guelph, for financial assistance.

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Accepted 16 *April* 1981