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A New Method for Study of Shoot Apices using Epi-illumination Light Microscopy

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ABSTRACT: For study of plant organogenesis, epi-illumination light microscopy (ELM) is considered as an efficient procedure due to its simplicity and low cost feature. In this study, the advantage of digital ELM use in conjunction with new staining method was demonstrated for study of shoot apex ontogeny in *Tanasetum baltisma*. Anilline blue stained materials revealed good image contrast and quality in compare to previous methods. On the other hands, the new staining method in this study was very fast and simple. Using digital ELM, we were capable to study in detail of the first stages of organ initiation as well as the developmental patterns of shoot apices. Upon our findings, we suggest implementation of the digital ELM as a rapid and low cost technique for plant science investigations.

Keyword: Organogenesis, floral initiation, epi-illumination, SEM, aniline blue, depth-of-focus

INTRODUCTION

Plant development involves morphological and physiological pathways which in first steps is revealed as changes in the shoot apex form and geometry (Albrechtova *et al.,* 2004; Jaeger *et al.,* 2006; Kwiatkowska, 2006). Study of developmental patterns in the course of apex activity has an economical and scientific importance in respect to botanical and agricultural investigations (Dennis *et al.*, 2006; Esumi *et al.,* 2007; Teeri *et al.,* 2006). Clearly, such studies offer leading insights into the mechanisms of manipulating the flowering and fruit loading (Teeri *et al.,* 2006; Tromp, 2000; Valiente and Albrigo, 2004). Further, manipulation of plant growth and development depends on our knowledge about the process of organogenesis in the apex and the time of organ formation (Oukabli *et al.,* 2003; Reig *et al.,* 2006; Schmidt *et al.,* 2006). For this purpose, application of appropriate microscopic techniques would be essential at which any change in apex becomes possible (Foster *et al.,* 2003; Kurokura *et al.,* 2005; Kwiatkowska, 2006).

Amongst various methodology, three dimensional microscopic techniques have been successfully applied to study of the apex ontogeny, because they provides relevant information on topography of the organs in organogenesis (Fleming, 2006; Green *et al.,* 1991; Kwiatkowska, 2004). Accordingly, the scanning electron microscopy (SEM), in particular cryo-SEM and environmental-SEM, is a highly demanded technique (Albrechtova *et al.,* 2004). However, utilizing of SEM has been limited because it is expensive and time consuming technique.

Epi-illumination microscopy (ELM) was first introduced to study of floral development (Sattler, 1968) and became widely used thereafter for many developmental studies in plant biology (Charlton, 2004; Valipour *et al.,* 2007). In contrast to other microscopic methods, the ELM is a low cost and rapid technique, at which the prepared samples for can be reused for other purposes (Posluszny *et al.,* 1980; Bartlett *et al.,* 2008). Taking all these together, many attempts have been accomplished for improvement of this methodology (Charlton *et al.,* 1989; Lacroix and Macintyre, 1995a). Briefly, many investigators attempted to enhance the staining procedures for improvement of image contrast (Charlton *et al.,* 1989) where as, the other works had been focused on the methods by which the depth of field could be increased (Dadpour *et al.,* 2008; Posluszny *et al.,* 1980; Wilson *et al.,* 2006). Nevertheless, it should be evoked that staining of plant materials play a key role for achieving high quality images. Therefore, in this current investigation, attempt was accomplished to introduce a rapid, simple and high contrast staining methodology for microscopic study of shoot apical meristems.

MATERIAL AND METHODS

An experiment was conducted for study of effectiveness of several staining procedures combined with digital version of epi-illumination in the Plant Organogenesis and Morphogenesis Laboratory at University of Tabriz, Tabriz, Iran. An herbaceous drug plant (*Tanasetum baltisma*) was used as the source of material in this study.

The shoot apices were collected at various developmental stages and were then fixed in FAA (formalin-acid acetic-ethanol) according to Posluszny *et al.* (1980). The prepared samples were dissected under a SZX 9 stereomicroscope (Olympus, Tokyo, Japan) and stained with several traditional methods according to a previous report (Charlton *et al.*, 1989).

An alternative staining method was carried out to preparation of apices with some modifications (Dadpour et al., 2011). First, dehydrated materials were immersed in glacial acetic acid for 15 min. They were then stained by means of saturated aniline blue solved in methyl cellosolve (2-methoxy-1-ethanol) for at least 24 h.

The stained samples were viewed through an Olympus BX61 (Olympus optical Co., Ltd. Tokyo, Japan) research reflected microscope equipped with catadioptric objectives UMPlanFL-BDP. Contrast-enhancing interference filters (red, green and yellow) were used based on staining methods (Table 1). Photomicrography was done by means of Olympus DP70 high resolution digital camera at two steps, i.e., z-stack acquisition and z-stack composition (Dadpour *et al.*, 2011). At the first step, consecutive image series from different focal plates of the apex were acquired and converted to z-stack or image stack. At the second step, the created z-stack was processed for automatically improving depth of focus by means of Image J 1.37 software (freely available from http://rsb.info.nih/ij/). Outputs from the z-stacks processing were saved as TIFF format images (1280× 1024 pixels).

(Table 1).

Table 1: Combination of staining methods and contrast-enhancing filters for image acquisition.

Stainning method	Contrast-enhancing filters			
	No filter	Red filter	Green filter	Yellow filter
Acid fuchsin	+		+	
Fast green	+	+		
Nigrosin	+			+
Anillin blue	+			+

RESULTS AND DISCUSSION

Primary developmental stages of shoot apices in studied plant, which have been taken by the digital ELM, were evaluated for clarity and contrast (Fig. 1-8). These images had different qualities according to staining method and utilizing contrast-enhancing filters. All images resulted in clear details in terms of the depth of focus for the apices and incepted organs. The required time for preparation of plant materials was considered as the simplicity of selected method. Amongst applied methods, staining with Anillin blue was very fast and simple. Indeed, Anillin blue stained materials were properly prepared within 48 h in contrast to other staining methods which need several weeks for setting up.

Image quality and contrast was evaluated according to detectable protodermal cells and clarity in terms of cellular division patterns. This study revealed that materials which had been stained with Anilline blue, demonstrated highest contrast and quality in compare to other traditional methods (Fig. 1-8). The results obtained in this study, show that application of contrast-enhancing filters play a crucial role for increasing the image contrast and quality.

Depth-of-focus is basically considered as limiting factor for epi-illumination light microscopy. The nature of light beams is responsible for such weakness of ELM and could not be fixed using instrumental methods (Posluszny *et al.*, 1980). Therefore many researchers preferred SEM to other optical techniques in study of plant organs, because it presents excellent details of apex (Manakasem and Goodwin, 1998). Introduction of complex-wavelets algorithms offers promising procedure for improving the depth-of-focus by means of image processing softwares (Forster et al., 2004; Valdecasas *et al.*, 2001). Automatic processing of image stack for improving the depth-of-focus seems to be easy, quick and accurate (Dadpour *et al.*, 2008; Dadpour *et al.*, 2011). Indeed, clarity of the images in this study is comparable with the presented SEM micrographs in previous works at which the time of floral initiation and developmental patterns of flower organogenesis was determined (Diaz *et al.*, 1981; Evans and Dickinson, 2005). In addition, some researchers mentioned that epi-illumination specimens can be reused by other techniques such as serial sectioning (Posluszny *et al.*, 1980). On the other hand, some other workers determined the quality of prepared materials for SEM by means of ELM (Lacroix and Macintyre, 1995b). Contrary to SEM, prepared material for ELM could be preserved several years for further study. Take consideration to flexibility, along with low cost and time saving feature and perfect clarity of image, the digital ELM may be comparable to SEM in aspect of plant developmental morphology. In this study, further simplification of digital version of epi-illumination light microscopy was accomplished. Using modification which was mentioned in this study, ELM could be considered as valuable and flexible standard in the field of plant development researches.

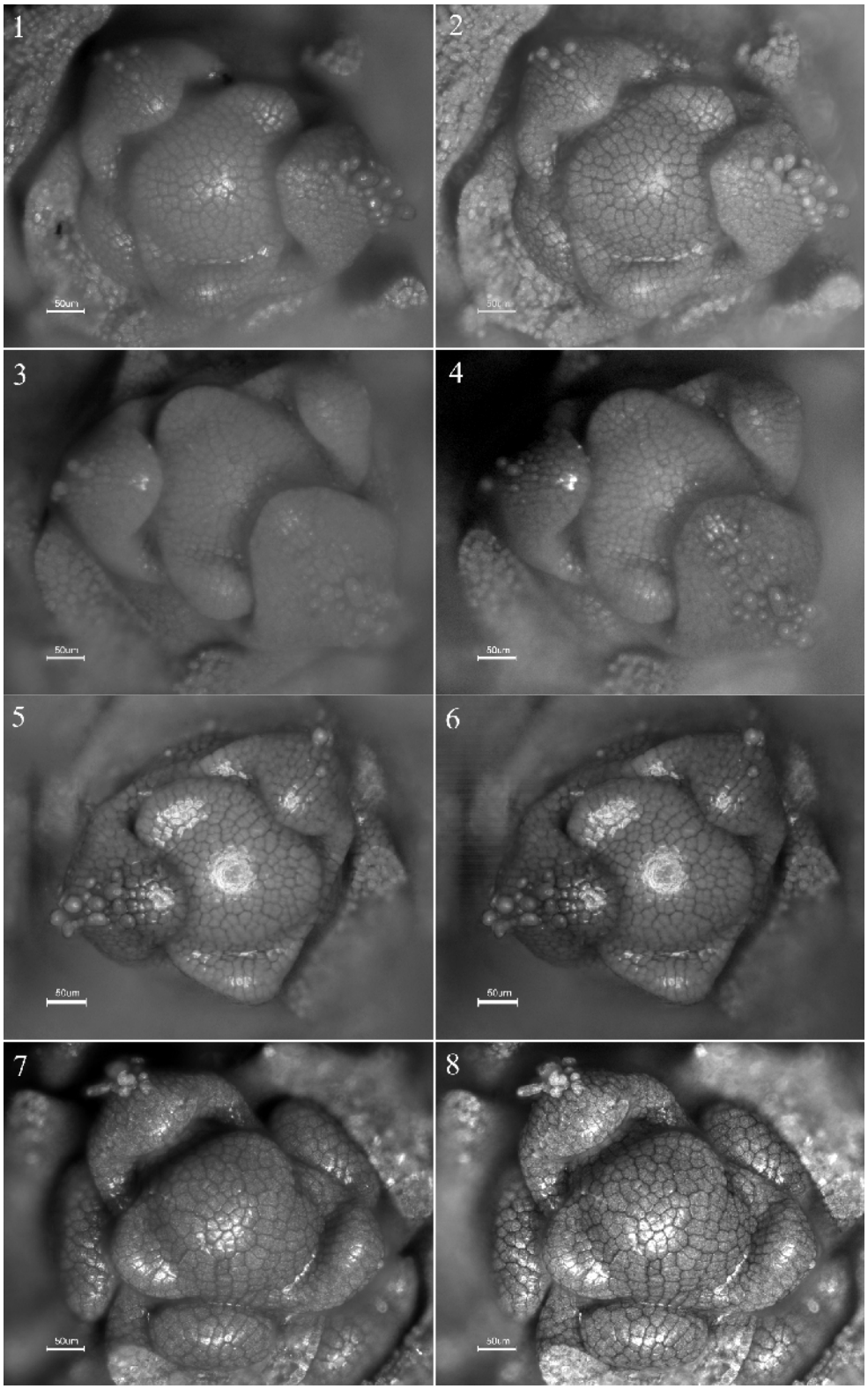

Fig. 1. Developing apices of *Tanasetum baltisma.* (1) Stained with Acid fushcin and photographed without using contrast-enhancing filter, (2) Stained with Acid fushcin and photographed using green filter, (3) Stained with Fast green and photographed without using contrast-enhancing filter, (4) Stained with Fast green and photographed using red filter, (5) Stained with Nigrosin and photographed without using contrast-enhancing filter, (6) Stained with Nigrosin and photographed using yellow filter, (7) Stained with Anillin blue and photographed without using contrast-enhancing filter, (8) Stained with Anillin blue and photographed using yellow filter.

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