Laboratory Techniques for Determining Ploidy in Plants

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SUMMARY. Determination of ploidy is an essential plant breeding technique. Laboratory exercises for teaching students how to determine ploidy in plant tissues using various techniques are described for geranium and onion. The different methods include root tip squashes, pollen mother cell squashes, pollen grain size and germinal pore counts, stomata size and density determination, and gross morphology.

The determination of ploidy level is an essential technique in plant breeding and genetics. Many crops represent one species in a polyploid series; others are ploidy chimeras. Ploidy determination is used extensively on plants regenerated from tissue culture as ploidy variation is common. Chromosome loss can occur often after interspecific hybridization. The objective of this laboratory is to teach students basic techniques for determining ploidy in plants. Not all of the mentioned techniques may be successful for determining plant ploidy of every crop. Therefore several techniques are presented in order to allow the student to select the technique that is most appropriate for a given species.

Laboratory setup

PLANT MATERIAL. Geranium (*Pelargonium ×hortorum* Bailey) will be used for pollen mother cell squashes, pollen grain size and germinal pore counts, stomata size and density and macroscopic morphological comparisons. Onion (*Allium cepa* L.) will be used for root tip squashes. Both diploid and tetraploid forms of geranium can be easily purchased or grown. Diploid geraniums are sometimes referred to as seedling geraniums and are sold as bedding plants. Tetraploid geraniums are propagated primarily by cuttings and are often sold as potted plants. Macroscopic morphological differences between the two ploidy forms make them easily distinguished. Diploid and tetraploid plants should be used when flowering in order to obtain pollen and pollen mother cells. Onions purchased from a supermarket are suitable.

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They can provide large, thick roots with chromosomes that are easy to stain and to observe. The basal plates of the dormant onion bulbs should be soaked in water for several days before the laboratory to induce fresh root growth.

OTHER MATERIALS. For each exercise, forceps, scalpels, glass slides and cover slips, and a light microscope will be needed. An ocular micrometer, that is mounted on the microscope, is needed for stomata and pollen grain measurements. The stains required for the various experiments include cotton blue, acetoorcein and acetocarmine (Darlington and LaCour, 1976; Sharma and Sharma, 1980). Each stain can be purchased from most chemical companies. Both carmine and orcein must be dissolved in hot, glacial acetic acid in order to obtain an aqueous solution (Darlington and LaCour, 1976; Sharma and Sharma, 1980). Both of the chromosome experiments will require the use of an alcohol lamp. Additional items include a metric ruler, 1 N hydrochloric acid (HCl), clear fingernail polish, and a ceramic crucible.

Laboratory period

The exercises in this laboratory are organized sequentially from macroscopic to microscopic ploidy assessment.

MORPHOLOGY. Students should compare diploid and tetraploid plants for morphological differences. Diploid and tetraploid geraniums exhibit morphological differences in leaf size, leaf thickness, stem thickness, petal size, and growth rate (Cramer, 1991). Petals and leaves of tetraploid plants tend to be larger than those of diploid plants. In addition, leaves and stems of tetraploid plants tend to be thicker than those of diploid plants. Students should take at least ten independent measurements of each of these features and use a *t* test to determine if there is a significant difference (Steel and Torrie, 1980).

STOMATA. Leaves are removed from both diploid and tetraploid plants. Clear fingernail polish is applied to the abaxial side of the leaf for a 2×2 cm square. Allow the polish to dry. While this is drying, students can move on to another technique. After the polish has dried, students should carefully remove the dried polish with a pair of fine-tip forceps. The polish strips are mounted on a dry microscope slide. The ocular micrometer is used to measure the largest diameter of 10 stomata at 125× (Table 1). In addition, students should count the number of stomata in 10 different fields of vision. They can calculate an average density for both the diploid and tetraploid plants (Table 1). Diploid plants should possess more dense, smaller diameter stomata than tetraploid plants.

POLLEN GRAINS. Mature anthers are collected from both diploid and tetraploid plants (Cramer, 1991). The anthers are dipped in a drop of cotton blue stain on a slide. The anthers should be macerated to release pollen grains. The anther debris should be removed and the stain with extruded pollen covered with a cover slip. After 15 min, the slide is viewed under a light microscope at 125× and the pollen diameter of 10 pollen grains is measured using an ocular micrometer (Table 1). Tetraploid plants typically have larger pollen grains than diploid plants (Cramer, 1991; Philippi, 1961). Students should also count the number of germinal pores per grain for 10 pollen grains from both diploid and tetraploid plants (Table 1). Pollen grains from tetraploid plants should have more pores than those from diploid plants (Cramer, 1991; Philippi, 1961).

POLLEN MOTHER CELLS. Flower buds are removed from both diploid and tetraploid plants. A range of buds with different levels of development should be collected. The range should be from small green to enlarged and slightly colored buds. From the range of bud development stages, students should be able to determine which stage is optimum for

Table 1. Sample stomata and pollen size counts, stomata density determinations, and germinal pore counts for diploid and tetraploid geranium plants.

Ploidy	Counts	Mean
	Stomata size (μM)	
Diploid	2.34. 2.40, 2.46, 2.60, 2.59, 2.53, 2.47, 2.32, 2.51, 2.42	2.46
Tetraploid	3.74, 3.84, 2.91, 3.42, 3.07, 3.26, 3.46, 3.31, 3.33, 3.21	3.36
	Stomata density (stomates / mm^2)	
Diploid	29, 29, 27, 34, 31, 32, 27, 28, 32, 31	30.0
Tetraploid	12, 9, 9, 11, 11, 10, 11, 9, 13, 10	10.5
	Pollen size (μM)	
Diploid	4.45, 4.80, 4.22, 4.80, 4.67, 4.61, 3.65, 4.26, 4.10, 4.51	4.41
Tetraploid	6.34, 5.76, 6.21, 6.27, 5.98, 6.37, 5.12, 5.12, 5.86, 5.92	5.90
	Germinal pores (no. / grain)	
Diploid	2, 3, 2, 2, 3, 3, 1, 2, 2, 1	2.1
Tetraploid	4, 4, 3, 4, 5, 4, 3, 3, 5, 3	3.8

observation of meiosis in pollen mother cells. The flower buds should be cut with a scalpel and the anthers removed with forceps from the bud. The anther is placed on a glass slide with a drop of acetocarmine stain. The anther is cut at one end with a scalpel. The uncut end of the anther should be pressed to squeeze pollen mother cells out of the anther through the cut end. The anther debris should be removed from the slide and a glass cover slip placed over the drop. The slide should be heated but not boiled by passing the slide through the flame of an alcohol lamp several times. This heating will help stain the chromosomes darker. The slide should be viewed under a light microscope at 1250× and the number of chromosomes counted from five cells of both diploid and tetraploid plants. From those cells, students should estimate the chromosome number of diploid (2n = 2x = 18) and tetraploid (2n = 4x = 36) geraniums. Students should also be able to observe chromosomes in the different stages of meiosis. If students are unable to observe chromosomes, the incorrect stage of bud development was chosen and another bud should be selected.

ROOT TIPS. Small root tips (≈5 mm in size) from actively growing roots are cut from the onion bulb (Sundberg, 1981) and placed in a crucible containing a 9:1 solution of acetoorcein and 1 N hydrochloric acid. The crucible is heated over an alcohol lamp four times for 10 s each time. Root tips are removed with forceps and placed on a glass slide. A drop of acetoorcein stain is placed over the root tip. A cover slip is placed over the root tip and gentle pressure is applied to the cover slip with a pencil eraser to smear the root tip. Cells will separate from each other and spread out in a single layer. The root tip smear technique may take some practice before a single layer of cells is obtained. Chromosomes in cells undergoing mitosis can be observed under the microscope. Students should count the number of chromosomes observed in five cells at metaphase to determine the chromosome number of onion. In addition, students should be able to observe the different stages of mitosis. The visibility of chromosomes will be related to the quality of the smearing and the degree of staining. The fixation and staining technique mentioned here is fast, relatively simple and quite effective. Other techniques such as Feulgen or Giemsa stain may give better chromosome definition; however, most of these techniques require additional fixation and more preparation time (Darlington and LaCour, 1976; Sharma and Sharma, 1980). The benefit of this procedure is for students to be able to isolate and stain somatic chromosomes in order to count chromosome number. Students should understand that the size of onion chromosomes is much larger than most plant chromosomes. The same techniques may not provide sufficient distinction of chromosomes in many species.

Followup

Instructors should request a laboratory report that summarizes the results from the experiments. From these experiments, students should be able to determine the ploidy level of a plant at its three histogen layers. The stomata size and density experiments allow students to determine the ploidy of the L-I histogen layer, while the pollen grain experiments determine the ploidy of the L-II histogen layer (Cramer, 1991). Root tips are commonly associated with the L-III histogen layer.

The time required to set up the laboratory is 30 to 60 min granted that microscopes and stains are available. Additional time should be allowed if stains need to be made. The laboratory was designed to run from 2 to 3 h. Some procedures may need to be repeated before the desired results are obtained. Using these techniques, students should be able to determine the ploidy of most plants. Advanced techniques, such as flow cytometry (Faure and Nougarede, 1993; Michaelson et al., 1991; Ormerod, 1994; Shapiro, 1995; Watson, 1991), or microspectrophotometry (Faure and Nougarede, 1993; Michaelson et al., 1991) require expensive equipment but may be necessary or efficient if many samples need to be processed. Partec (Partec GmbH, Münster, Germany) sells an instrument called a Ploidy Analyzer for automatic ploidy determination in plants.

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