

Comparison of ploidy level screening methods in regenerants derived from anther culture of anthurium

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Abstract

Anther culture was successfully developed in anthurium. The anther culture resulted in morphology and growth response variation of regenerants. Different morphological variations indicated different ploidy level. Screening a convenient, rapid and reliable indirect method in ploidy level determination expected gave high benefit in estimation of ploidy level of anthurium practically. Three different ploidy levels of regenerants derived from anther culture were used in the study. Five indirect methods of chloroplast number in a stomatal guard cell, stomatal length and width ratio, stomatal density, ratio of length and width of leaves, and microspore number per anther were compared to chromosome counting as a direct method. Simple regression correlation analysis was applied to know level and direction of correlation of two methods compared. Results of the study indicate that chloroplast number in a stomatal guard cell was the most convenient and reliable indirect method in determination of ploidy level of regenerants derived from anther culture of anthurium. While number of microspores per anther was the second best method for the same purpose. Both methods was highly correlated to anthurium ploidy level with $r = 0.945$ and 0.813 ($p < 0.01$), respectively. Practically application of the methods was faster than chromosome counting method. Higher number of chloroplast and microspore, higher anthurium ploidy level. Application other indirect methods were not suggested in anthurium.

INTRODUCTION

Anthuriums are bisexual and protogynous, with the spadix first producing a female phase followed by, after about a month, a male phase. This reduces self-pollination, but increases cross-pollination frequency of the plants (Caroll, 2007). Therefore establishment of anther culture method for producing the homozygous lines in anthurium is important tool in strengthening anthurium agribusiness in Indonesia dealing with producing high qualified new hybrids and seeds.

Anther culture of anthurium has been initiated by Rachmawati (2005) and Winarto and Rachmawati (2007) since 2003 and improved simultaneously till now. The method resulted in morphological and ploidy level variations of regenerated

explants and/or plants both *in vitro* and *ex vitro* level. There were easy and difficult regenerated-calli, slow and fast growth of explants, easy and difficult-to-root of shoots, flowered and un-flowered plants derived from anther culture of anthurium. From the previous study, ratio of ploidy level from 12 explants tested was 9% of haploid, 76% of diploid and 15% of triploid based on chromosome counting method (Rachmawati, 2005)

Chromosome counting in mitotic cell of root tips is a classic and accurate method in determination of plant ploidy level (Sharma and Sharma, 1994). The method was successfully applied in many plants. However, when many plants are to be analyzed, this is a rather laborious method. Indirect methods such as chloroplast counting in a pair of stomata guard cell (Sari *et al.*, 1999, Yudanova *et al.*, 2002; Ho *et al.*, 2006); ratio of length and width of stomata, stomatal size and frequency (Beck *et al.*, 2003); size of leaves (Naiki and Nagamasu, 2004); pollen grain size (Singsit and Ozias-Akins, 1992) have been utilized as convenient, rapid and reliable methods in identifying ploidy level of many plant species.

In the present study, comparison of different ploidy level methods using chromosome counting, chloroplast number in a stomatal guard cell, stomatal length and width ratio, stomatal density, ratio of length and width of leaves, and microspore number per anther were studied.

MATERIALS AND METHODS

Variation of acclimatized-plants derived from anther culture of *Anthurium andreanum* Linden ex André cv. Tropical i.e. haploid, diploid and triploid plants were used in this study. Different ploidy level methods i.e. chromosome counting (as basic and direct method), chloroplast number in a stomatal guard cell, stomatal length and width ratio, stomatal frequency, ratio of length and width of leaves, and microspore number per anther (as indirect methods) were used to determine ploidy level of plants.

For mitotic chromosome counts, actively growing root tips from greenhouse-grown plants were harvested at 08.00 – 10.00 am, cut 0.5-1.0 cm and pretreated in 0,002 M hydroxyquinoline for 3-5 hours at $\pm 20^{\circ}\text{C}$. After the treatment, the root tips were treated in 45% acetic acid solution for 10 minutes and rinsed with distilled water. The roots were hydrolyzed in 1N HCl : acetic acid glacial (3:1, v/v) solution for 10 minutes in water bath at 60°C , and stained with 2% aceto-orcein solution for 15 minutes. Roots were then put on an object glass, cut ± 1.0 mm, added 1-3 drops of new aceto-orcein solution, covered with cover slip, and squashed to make a thin layer and spread out of the root tip cells. Chromosomes were counted from 5 cells and 1 to 2 root tips per sample. Total plants used in the experiment were 5-10 plants per type of ploidy.

Number of chloroplasts per pair of guard cells was counted from leaf samples prepared by removing lower epidermis, staining the epidermis with 10% of Iodid solution (Betadine) for 5 minutes and covering with cover slip. Chloroplasts were counted from ten guard cell pairs per type of ploidy and replicated three times under stereo microscope in 400x magnification. The similar prepared-leaf samples were also used to measure length and width of stomata and calculate number of stomata per 1 mm^2 of leaf epidermis. The length and width of stomata was measured with calibrated-micrometer. Ten stomata and ten times of stomatal counting and 5-10 plants per type of ploidy were used for the purposes.

All leaves in one plant and 5-10 plants per type of ploidy were used in calculating length and width leaf ratios. Leaf length was measured from the tip to end of leaf. While leaf width was measured from the widest part of leaf. Whereas number of microspores per anther was counted from microspore solution. The solution was prepared by isolating 3 anthers, putting in small glass tube containing 0.5 mm MMS liquid medium, grinding gently with glass rod to break anther wall and release all microspores, and shaking the microspore solution to homogenize it. Twenty five microliter of microspore solution were pipetted, put on haemocytometer, covered with cover slip, and observed under stereo microscope in 100-400x magnifications. Number of microspores per anther was calculated by summing number of microspores from 5 big squares of haemocytometer sampled randomly times by 6000 divided by 3 anthers. Ten times of sampling per spadix and 5-10 spadixes per type of ploidy were carried out for the microspore counting purpose.

All data from chloroplast number in a stomatal guard cell, stomatal length and width ratio, stomatal density, ratio of length and width of leaves, and microspore number per anther method were analyzed and compared to basic method using simple regression-correlation analysis of SAS Release Window 6.12. The analysis was carried out to know level and direction of correlation between two methods compared. Interval coefficient of 0.00 – 0.199 is the lowest correlation, 0.20 – 0.399 is low, 0.40 – 0.599 is moderate, 0.60 – 0.799 is high, and 0.80 – 1.000 is the highest correlation.

RESULTS

Different indirect methods in determination of ploidy level gave different level and direction of correlation. Statistically, most of them gave significant differences among three types of ploidy in regenerants derived from anther culture of anthurium. From five indirect methods investigated, chloroplast number in a stomatal guard cell and number of microspores per anther were convenient and reliable methods in determination of ploidy level in anthurium. Stomatal length and width ratio and stomatal density were less convenient methods, while leaf length and width ratio was the most un-convenient method in estimation of ploidy level in the plant.

The chloroplast counting in a guard cell of stomata was the most convenient and reliable method in determination of ploidy level for anthurium. The method was highly correlated ($r=0.945$, $p<0.01$) with the ploidy of the androgenic anthurium plants (Figure 1). Haploid individuals were characterized by bigger size and less number of chloroplasts, diploid with moderate size and number of chloroplast, and triploid with smaller size and high number of chloroplasts (Figure 2). Number of chromosomes and chloroplasts were 17.13 ± 1.81 and 17.38 ± 1.56 for haploid plants, 30.1 ± 2.02 and 30.4 ± 1.63 for diploid, and 51.5 ± 3.03 and 50.2 ± 2.15 for triploid respectively.

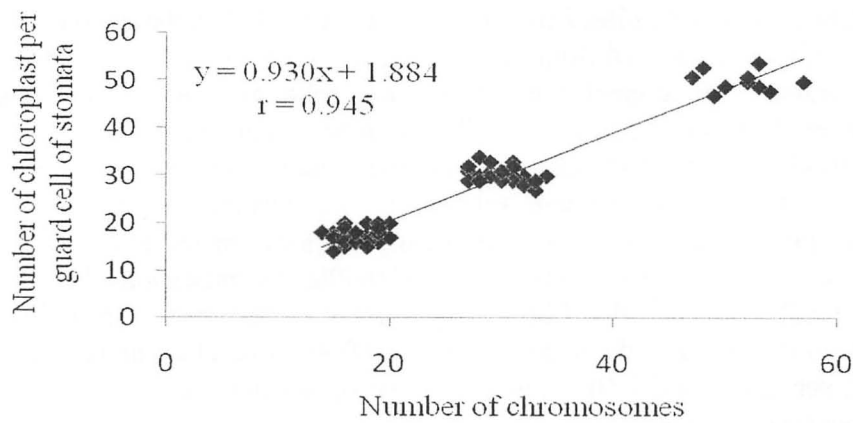


Figure 1. Distribution of data, regression and correlation between chromosome and chloroplast counting methods.

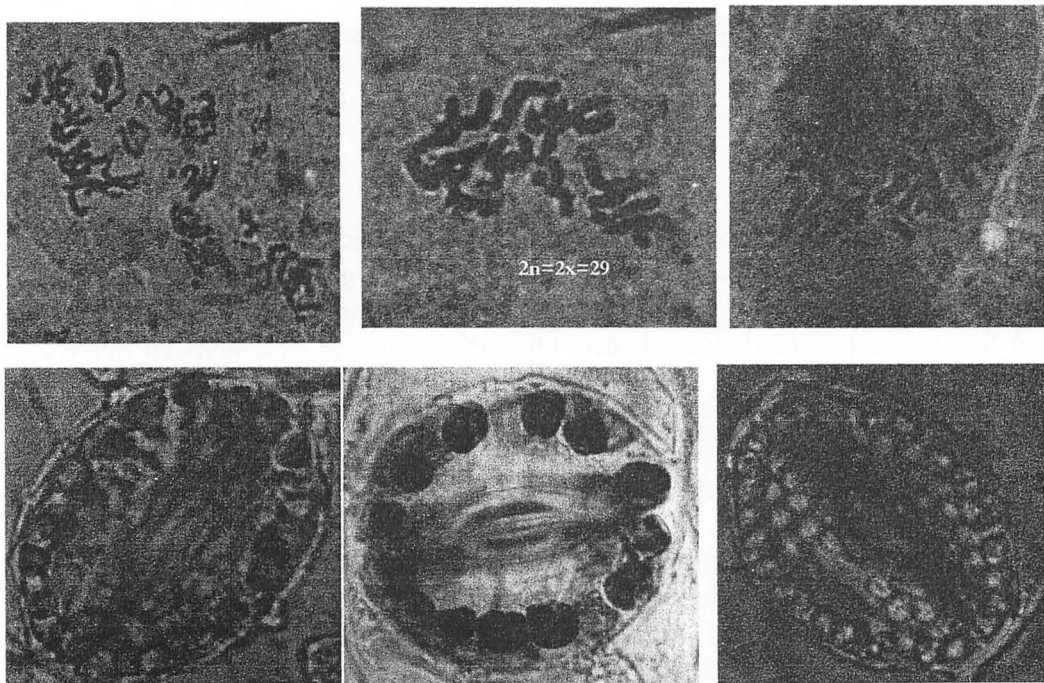


Figure 2. Comparison between number of chromosomes and chloroplast in different ploidy level

Second best indirect method in ploidy level estimation in anthurium was microspore counting per anther. The method was high correlated ($r=0.813$, $p<0.01$) with ploidy level of the plant. Higher ploidy level higher number of microspores per anther (Figure 3). Number microspores per anther in haploid individuals was $6.259 \pm 542,43$ microspores, $10.545,2 \pm 3931,56$ microspores for diploid and $27.576,1 \pm$

2.189,37 microspores for triploid. Ratio of stomatal length and width and stomatal density per mm^2 of leaf epidermis was less convenient method for ploidy level determination on anthurium. The methods were low correlated with $r = 0.435$ and 0.341 ($p < 0.01$), respectively. The ratio of stomatal length and width was 1.17 ± 0.08 for haploid, 1.31 ± 0.11 for diploid, and 1.43 ± 0.11 for triploid plants. The stomatal density was 7.5 ± 1.86 for haploid, 7.7 ± 1.31 for diploid, and 4.4 ± 0.93 for triploid plants.

Ratio of leaf length and width was not suitable for ploidy level determination in anthurium. The method was the lowest correlated ($r = 0.023$, $p < 0.01$) with ploidy level of androgenic plants derived from anther culture of anthurium. Although leaf shapes were visually difference among three types of plant ploidy level (Figure 4), there was no differences in leaf length and width ratio. The ratio of length and width leaf of haploid, diploid and triploid individuals was 1.6 ± 0.11 , 1.6 ± 0.12 , and 1.6 ± 0.14 respectively.

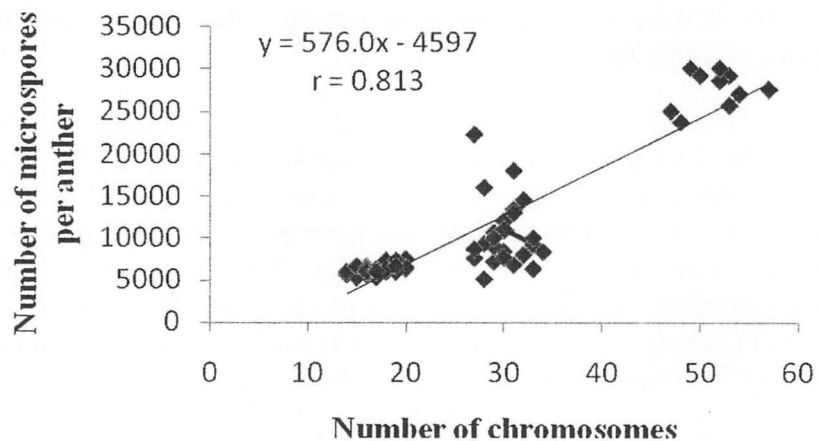


Figure 3. Distribution of data, regression and correlation between chromosome and microspore counting methods.

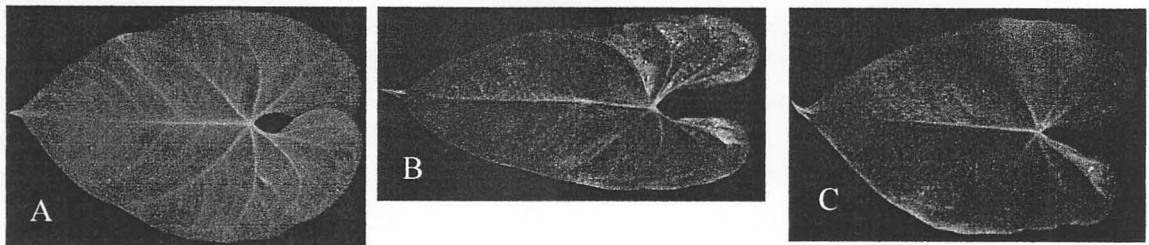


Figure 4. Differences of leaf morphology between haploid, diploid and triploid plants observed visually. A. Haploid leaf, B. Diploid leaf, and C. Triploid leaf

DISCUSSION

The highest correlation ($r = 0.945$, $p < 0.01$) chloroplast number with chromosomes number in the study indicated that chloroplast number in a guard cell represents a more convenient, reliable indicator in determination of plant ploidy level for anthurium. Satisfactory results in application of the method were also reported in potato (Singsit and Veilleux, 1991), *Arachis* (Singsit and Ozias-Akins, 1992), water melon (Sari *et al.*, 1999), banana (Ganga *et al.*, 2002), and maize (Ho *et al.*, 2006). This means that variability in chloroplast number was almost similar to variability in chromosome number. According to Yudanova *et al.* (2002) the variability of the number of chloroplasts in epidermal cells (Stomata guard cells) is genetically related to their variability in meristem cells. By origin, epidermis is a primary cover tissue, because it develops directly from upper layer of apical meristem (protoderm). Guard cells differ from common epidermal cells in having chloroplasts. One of protoderm cells becomes maternal and divides into two daughter cells, which develop as guard cells. Epidermal cells inherit the properties of the protodermal maternal cells: sizes, ploidy, and the number of intracellular organelles. Therefore, the variability of the number of protoplasts in stomata guard cells exactly matches its variability in the protodermal (meristem) cells of the leaf apex (Yudanova *et al.*, 2002). The fact was also observed in number of chloroplast in a guard cells of regenerants derived from anther culture of anthurium.

Number of microspores per anther was the second best indirect method in ploidy level estimation in anthurium. Increasing ploidy level, increasing microspore number, however microspore size was almost similar in all ploidy levels. Different results in application the method was reported in oat and *Cordalys* (Fukuhara, 2005a and b). Pollen grain size was positively correlated with ploidy level, while number of pollen grain was not different between ploidy levels. Higher ploidy level larger pollen grain (Fukuhara, 2005b). Positive correlation between pollen grain size with ploidy level was also reported in *Arachis* (Singsit and Ozias-Akins, 1992).

Varied-results were reported in application of stomatal length and width ratio and stomatal density in determination of ploidy level. Both methods gave in-convenient results in determination ploidy level of anthurium with low correlation. Although the two methods was significantly affected by plant genotype in banana (Vandenhout *et al.*, 1995) and high correlated with ploidy level in *Phragmites australis* (Saltonstall *et al.*, 2007) and *Buddleja macrostachya* (Chen *et al.*, 2009), the method was less suitable for anthurium as also reported in banana (Ganga *et al.*, 2002). Successfully application of leaf size measurement as reported in *Phragmites australis* (Pauă-Comănescu *et al.*, 1999), *Damnacanthus* (Naiki and Nagamasu, 2004), *Buddleja macrostachya* (Chen *et al.*, 2009) was not recorded in anthurium. Leaf length and width ratio was the worst indirect method in determination of plant ploidy level for regenerants derived from anther culture of anthurium.

CONCLUSION

Chloroplast number in a stomatal guard cell was the most convenient and reliable indirect method in determination of ploidy level of regenerants derived from anther culture of anthurium. While number of microspores per anther was the second best method for the purpose in anthurium. Both methods was highly correlated to

anthurium ploidy level with $r = 0.945$ and 0.813 ($p < 0.01$), respectively. Practically application of the methods was faster than chromosome counting method. Higher number of chloroplast and microspore, higher anthurium ploidy level. Application other indirect methods were not suggested in anthurium.

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