The Journal of Animal & Plant Sciences, 29(5): 2019, Page: 1344-1351
ISSN: 1018-7081

FLOW CYTOMETRIC DETECTION OF HAPLOIDS, DIPLOIDS AND MIXOPLOIDS AMONG THE ANTHER-DERIVED PLANTS IN INDICA RICE (*ORYZA SATIVA* L.)

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ABSTRACT

Plants regenerated from anther culture of the Indica rice var. 'At 303' were analyzed using flow cytometry and morphological parameters were studied. Plant analysis carried out using flow cytometry confirmed that 40% of the plants were haploid, 50% were diploid and 10% were mixoploid (haploid/ diploid). Stomatal measurements (length, width and density) were obtained for plants regenerated from anther culture and compared with measurements from diploid plants derived from seeds which served as the standard reference material. Smaller cell size and higher cell density are indications of lower ploidy status. The data showed that the cell size was significantly lower ($p \leq 0.05$) in all anther-derived plants compared to diploid seed-grown plants. However, stomatal density was significantly higher ($p \leq 0.05$) in only two of the plants (plant nos. 11 & 12) regenerated from anther culture compared to seed-grown plants. Cluster analysis of the stomatal data as well as other morphological and reproductive trait measurements, indicated that only the latter two plants in this group were true haploids (33%). Thus, the ploidy estimates from stomatal and other morphological trait analyses corroborated the flow cytometric evidence. That is, both types of analyses determined that less than half of the total regenerants were haploid. Anther culture is performed with the intention of regenerating haploid or doubled haploid plants to develop homozygous lines. Therefore this study revealed that, it is important to improve further, the efficiency of regenerating haploids or dihaploids through anther culture in Indica rice for the technique to be practically viable.

Keywords: Anther culture, Flow cytometry, Haploids, Diploids, Mixoploids.

INTRODUCTION

Anther culture technique can be used to produce homozygous lines immediately, thereby shortening considerably the time required for inbred line development in plants by conventional methods. For the technique to be effective, plants that are regenerated from anther culture should be haploids or dihaploids (doubled haploids). Since regenerated plants do not always have the expected ploidy, an assessment of the level of ploidy of anther-derived plants is often required. A direct method of establishing ploidy is to obtain chromosome counts from regenerated plant tissue. However, preparation of metaphase chromosome spreads for chromosome counting from mitotic cell nuclei is ineffective due to the small size and poor staining of the chromosomes. Therefore, indirect methods such as cell size measurements that can be applied more easily have been employed to estimate ploidy in plant tissue, based on the general premise that there is an overall increase in cell size with increase in ploidy. Stomatal size (length /width), stomatal density (Aryavand *et al.* 2003; Glennon *et al.* 2007; Mohommadi *et al.* 2012; Yildiz 2013; Shrestha and Kang 2016), as well as chloroplast number in guard cells (Yuan *et al.* 2009) have been shown to correlate well with chromosome number or ploidy level of many different plant species such as *Aegilops neglecta*, *Phragmites australis*, *Brassica napus* L., *Beta vulgaris*, *Capsicum annuum* L. and *Brassica oleracea* L..

On the other hand flow cytometry, which determines the DNA content in individual cell nuclei with reference to a known standard, is a rapid and accurate method that is easily applied at individual cell level. This method is most useful in analyzing plants derived from *in vitro* culture because it allows detection of chimeras which is not an uncommon phenomenon in cell cultures. Therefore, indirect estimates of chromosome number in regenerated plants should be coupled with flow cytometry to obtain a comprehensive view of the ploidy level in plants that are regenerated from tissue culture.

In the present study, the ploidy status of anther-derived plants of Indica rice var. 'At 303' was examined using flow cytometry. The possibility of using indirect methods such as stomatal and other morphological trait measurements to discriminate different levels of ploidy was also examined.

MATERIALS AND METHODS

Anther culture in Indica rice var. 'At 303'

Plant material: Mother plants of the Indica rice var. 'At 303'were raised for about one and half months in a field at Regional Rice Research and Development Centre, Bombuwela, Sri Lanka, under standard agronomic

practices. When plants reached the booting stage they were uprooted and transplanted in pots with paddy soil and brought to the laboratory of Department of Plant Sciences, University of Colombo. The pots were kept outside the laboratory until panicles were ready for harvest. Immature panicles were harvested while panicles were still concealed within leaf sheath, to obtain anthers with microspores at the appropriate stage of maturity (late uni-nucleate) for culture (Mayakaduwa and Silva 2017). Panicles were collected from mother plants between 9.00 – 10.00 am.

Anther culture and callus induction: Harvested panicles were cold pre-treated at 10 ^{0}C for 7 – 10 days. Cold pre-treated panicles were surface disinfected according to the standard procedures (Silva and Ratnayake 2009). Anthers excised from spikelets were cultured on N_6 callus induction medium (Chu 1978), modified by replacing sucrose with maltose (60.0 g L^{-1}). Callus induction medium was supplemented with the growth regulators, 2,4-Dichlorophenoxyacetic acid (2,4-D) (0.5 mg l^{-1}), Naphthaleneacetic acid (NAA) (2.5 mg l^{-1}), and Kinetin (0.5 mg l^{-1}) and solidified with agar (8.0 g l^{-1}). Up to 200 anthers were placed on a single petri dish containing 15 ml of the culture medium. Culture plates were incubated in the dark at 27±2 ^{0}C for callus induction.

Plant regeneration and acclimatization: When calli reached 1-5 mm in diameter they were separated from parent anther tissue and transferred to petri dishes with MS (Murashige and Skoog 1962) medium solidified with agar (8.0 g l^{-1}). The regeneration medium was supplemented with NAA (1.0 mg l^{-1}), Kinetin (0.5 mg l^{-1}) and 1.0 mg l^{-1} of 6-Benzylaminopurine (BAP). Chemicals including agar used in the experiment were from HiMedia Laboratories Pvt. Ltd., Mumbai, India and growth regulators were from Sigma-Aldrich, St. Louis, MO. The cultures were maintained at 27±2 ^{0}C under continuous light (light intensity of 60 µmol m^{-2} s^{-1}) provided with cool white florescent tubes (36W) for shoot regeneration to occur. Regenerated shoots with minute roots were transferred to test tubes with half strength MS medium without growth regulators, for further shoot elongation and root development. Well developed plantlets (approximately 5 cm tall) were taken out from culture tubes, washed well to remove agar and planted in pots containing sand. Plants were maintained in a propagator till acclimatized and moved to pots with paddy soil. Acclimatized plants were grown to maturity in a glass house (Mayakaduwa and Silva, 2017).

Analysis of anther-derived rice plants

Flow cytometric analysis: Leaf samples of ten fully grown plants regenerated from anther culture were analyzed by flow cytometry to establish their ploidy level. Leaves of normal diploid plants of similar maturity, obtained from the same rice variety, were also analyzed and used as the standard for 2C DNA content. Samples were processed using Cystain UV Precise P Kit (Partec GmbH, Germany) and DAPI stained nuclei were analyzed on flow cytometer CyFlow Space (Partec GmbH, Germany).

Stomatal measurements: Stomatal measurements were obtained from six plants of rice var. 'At 303' regenerated from anther culture and grown to maturity. Epidermal peels were obtained from the two youngest leaves to study stomata. From each leaf 5 lower epidermal peels were observed under the light microscope and in each peel 5 stomatal units were selected randomly to obtain length (distance between the ends of the two subsidiary cells measured lengthwise) and width (horizontal distance across the two subsidiary cells at the widest point). Measurements were made under 100 magnification using an oil immersion lens. To determine the stomatal density in each epidermal peel, the number of stomata observable in a microscopic field under 10 magnification was counted first and converted to the number of stomata per unit area. Micrographs were taken using a light microscope (Carl Zeiss, Oberkochen, Germany) with a Axiocam digital camera (Carl Zeiss) and images were processed with Zen 2012 (blue edition) imaging software. The above measurements were repeated on the six diploid plants of the same rice var. ('At 303') raised *in vitro* from seed culture and grown to the same level of maturity for comparison (Mohommadi *et al.* 2012).

Morphological and reproductive traits: Measurements were obtained for plant height, leaf blade length and width, number of reproductive tillers, number of spikelets per panicle and panicle length. The data were recorded for the six anther-derived and six seed-grown plants separately.

Data analysis: Data of stomatal length, width and density were analyzed using one-way ANOVA with SAS; Release 9.0. Mean comparisons were carried out using Tukey's multiple comparison test. Hierarchical cluster analysis was performed with stomatal data as well as morphological and reproductive trait measurements using SPSS 16.0. Two separate dendrograms, one for stomatal measurements and the other for morphological data, were developed based on the similarity relationships.

RESULTS AND DISCUSSION

Anther culture in Indica rice var. 'At 303': Callus induction occurred from cultured anthers approximately 4 weeks after anther inoculation. Of 631 anthers cultured, 24 anthers responded (4.12%) by producing calli which is a reasonable rate of success for the rice var. 'At 303' (Mayakaduwa and Silva 2017). After transfer to the

regeneration medium 8 calli (33%) produced green shoots and all of these were moved to pots for acclimatization (Fig. 1a-b). The plants that survived the acclimatization were grown to maturity (Fig. 1 c). The mature plants were characterized by flow cytometry and analysis of stomatal data together with morphological and reproductive traits.

Fig. 1. Acclimatization of anther-derived plants of rice var. 'At 303': a- green shoots with roots regenerated from anther culture, b- plantlets in pots containing sand being acclimatized inside propagators, c- plantlets moved to pots with paddy soil and grown to maturity in a glass house (bar a = 3.0 cm, bar b = 5.0 cm, bar c = 15.0 cm)

Analysis of anther-derived rice plants

Flow cytometric analysis: Flow cytometric analysis of regenerated plants showed that they could be haploids, diploids or mixoploids, as illustrated by the representative histograms (Fig. 2). Of the 10 plants, 4 were determined to be haploid (1C), 5 diploid (2C) and 1 plant was a mixoploid (1C and 2C). Presence of haploid plants among the regenerants confirmed their microspore origin. In the diploids and mixoploid, cells with higher ploidy may have arisen from doubling of chromosomes *in vitro*. However, further confirmation will be required using molecular markers to establish that their origin was from haploid microspores and not from diploid somatic tissue of anther wall.

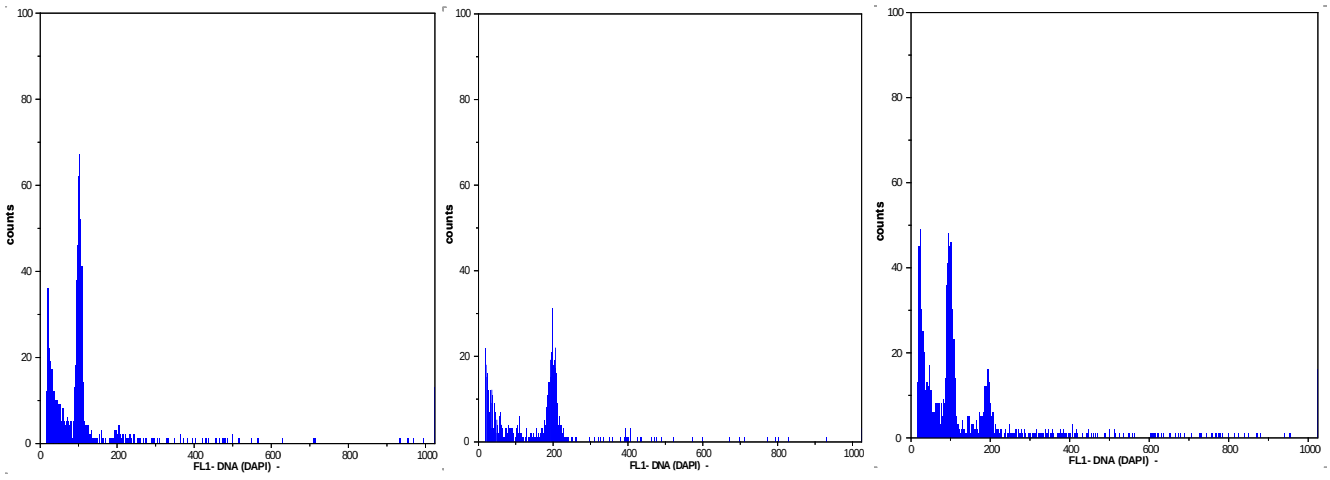

Fig. 2: Flow cytometric analysis of nuclear DNA (stained with DAPI) content in anther-derived plants against seed-grown normal diploid plants (var. 'At 303'): a- haploid plant with G1 DNA peak (1C) set at channel number 100, b-diploid plant with G1 DNA peak (2C) set at channel number 200, c-mixoploid plant with two G1 DNA peaks (1C and 2C) at channel numbers 100 and 200

Stomatal measurements: Stomatal size and density have been used as indirect measurements to determine ploidy of plants. In general plants with higher ploidy have larger cell sizes and lower stomatal densities compared to plants

with lower ploidy status (Dhooghe *et al.* 2011; Shrestha and Kang 2016).

Stomatal measurements of 12 plants of rice var. 'At 303', six grown *in vitro* from seeds and six regenerated from anther culture, are presented in Table 1. The mean width of stomata was very consistent and significantly higher ($p \leq 0.05$) in the seed-grown plants than in anther-derived plants (Table 1). The mean length of stomata, although more variable among individual plants, was also significantly higher ($p \leq 0.05$) in seed-grown plants compared to anther-derived plants. Therefore, the two groups of plants could be distinguished based on the size of stomata. However, stomatal density could not be used to distinguish clearly between the two plant groups. Among the anther-derived plants only two plants (plant nos. 11 & 12) showed significantly higher values for stomatal density whereas the other four plants were grouped together with normal seed-grown plants (Table 1; Fig. 3). The same two plants (plant nos. 11 & 12) displayed the lowest values for stomatal width and length also. Stomatal measurements had been proven for their suitability to successfully distinguish in between different ploidy levels due to the significantly different values reported across varying ploidy levels (Shrestha and Kang 2016).

Clustering based on the stomatal measurements produced two main groups. Seed-grown normal diploid plants and plant nos. 7, 8, and 10 of anther-derived plants formed a single distinct cluster (at a relative distance 0.97). Plant no. 9 (anther-derived) also showed a closer relationship to this group although at a relative distance 2.33. Plant nos. 11 and 12 separated out with the greatest distance from other phenotypes (at a relative distance 25) indicating their genetic uniqueness (Fig. 4). Therefore, it may be assumed that these two plants which had the smallest cell size and highest stomatal density are true haploids whereas the remaining anther-derived plants which clustered together with normal diploid plants are diploid or may even be mixoploid.

Table 1. Stomatal measurements of anther-derived plants and plants grown from seed (var. 'At 303')

Plant Number	Source of plant	*Mean stomatal width (µm)±SEM	*Mean stomatal length (µm)±SEM	*Mean stomatal density (mm^{-2})±SEM
1	seed-grown	17.669^{a}±0.228	26.978^{a} ± 0.156	571^{b}±30
2	seed-grown	17.482^{a}±0.293	26.270^{a} ± 0.225	571^{b}±26
3	seed-grown	17.137^{a}±0.217	26.107^{ab}±0.266	571^{b}±30
4	seed-grown	17.425^{a}±0.219	26.010^{ab}±0.178	571^{b}±30
5	seed-grown	17.239^{a}±0.196	25.918^{ab}±0.267	571^{b}±30
6	seed-grown	17.886^{a}±0.195	25.110^{b} ± 0.389	586^{b}±33
7	anther-derived	15.955^{b}±0.249	23.717^{c} ± 0.226	543^{b}±63
8	anther-derived	14.333^{c}±0.222	19.678^{d} ± 0.269	606^{b}±51
9	anther-derived	14.213^{c}±0.201	18.185^{e} ± 0.269	483^{b}±26
10	anther-derived	13.049^{d}±0.140	17.976^{e} ± 0.307	617^{b}±38
11	anther-derived	10.779^{e}±0.137	15.647^{f} ± 0.143	828^{a}±19
12	anther-derived	10.985^{e}±0.183	15.091^{f} ± 0.177	914^{a}±32

**Means within a column with the same letter are not significantly different ($p \leq 0.05$)*

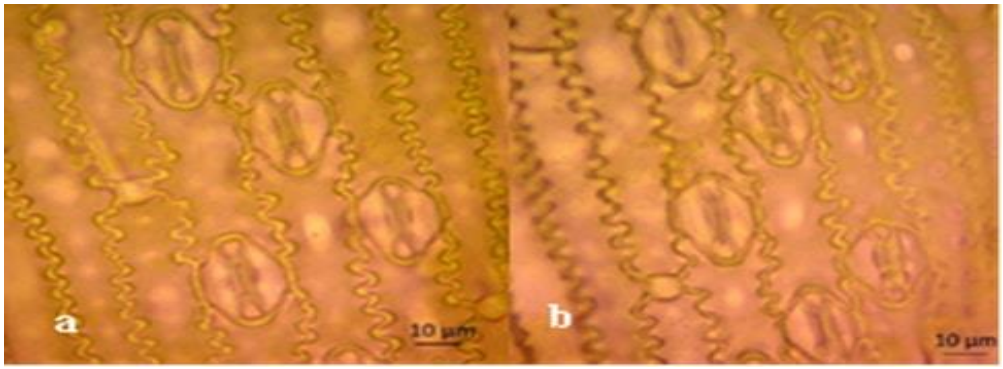

Fig. 3. Stomata observed in epidermal peels of rice var. 'At 303': a- four stomata seen on epidermal peel of diploid seed-grown plant (plant no. 5), b-six stomata seen on epidermal peel of an anther-derived plant (plant no. 12) in a single microscopic field

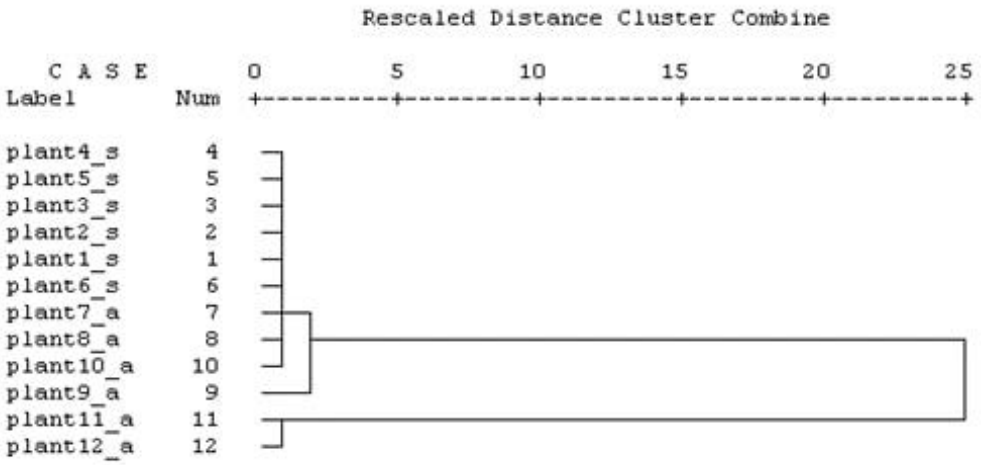

Fig. 4. Dendrogram showing clustered relationships of the seed-grown and anther- derived plants of the var. 'At 303', analysed based on stomatal measurements; s=seed-grown plants; a=anther-derived plants

Morphological and reproductive traits: The seed-grown plants, on average scored higher values in all the measured morphological parameters except in plant height (Table 2). The two anther-derived plants 9 and 10 were the taller (79.8 cm and 69.8 cm respectively) than the normal seed-grown plants (62.1 cm – 68.5 cm) while two other anther-derived plants 11 and 12 were distinctly shorter (45.2 cm and 46.8 cm respectively) than all others. The latter two plants scored lower values for all other parameters including leaf blade length and width, panicle length and number of spikelets per panicle. The clustering pattern in the dendrogram developed using morphological data was similar to the one observed with stomatal data. However, morphological data produced three clusters. All seed-grown plants were contained in one cluster. Anther-derived plants separated into two clusters, one that included plants 7, 8, 9 & 10, and another distinctly separate cluster with plants 11 and 12 at relative distance 25 (Fig. 5). The greatest distance displayed by plants 11 and 12 coupled with significant differences observed with Tukey's mean comparison tests on stomatal measurements further strengthen the suggestion that these two plants have a haploid genome. The other anther-derived plants which showed a closer affinity to seed grown normal diploids maybe diploids or mixoploids. Similarly, haploid plants had exhibited distinctly low values for many morphological traits allowing the anther-derived regenerants to be characterized (Luitel *et al.* 2012).

Table 2. Morphological and reproductive trait measurements of anther-derived plants and seed-grown plants at the panicle emergence maturity (var. 'At 303')

Plant Number	Plant type	Plant height (cm)	Leaf blade length (cm)	Leaf blade width (cm)	Number of reproductive tillers/plant	Panicle length (cm)	Number of spikelets/ panicle
1	seed-grown	68.5	34.2	1.7	2	14.5	62
2	seed-grown	65.4	33.2	1.5	2	14.0	70
3	seed-grown	63.0	28.6	1.5	1	15.4	58
4	seed-grown	62.1	29.8	1.5	1	15.1	61
5	seed-grown	62.3	34.0	1.4	2	14.8	54
6	seed-grown	64.5	37.8	1.5	2	15.6	50
7	anther-derived	66.2	19.0	1.2	1	8.0	15
8	anther-derived	58.1	21.0	1.1	1	11.0	18
9	anther-derived	79.8	20.0	1.1	1	3.6	16
10	anther-derived	69.8	21.3	1.1	1	7.5	18
11	anther-derived	45.2	15.5	1.0	1	4.8	11
12	anther-derived	46.8	16.8	0.9	1	5.1	10

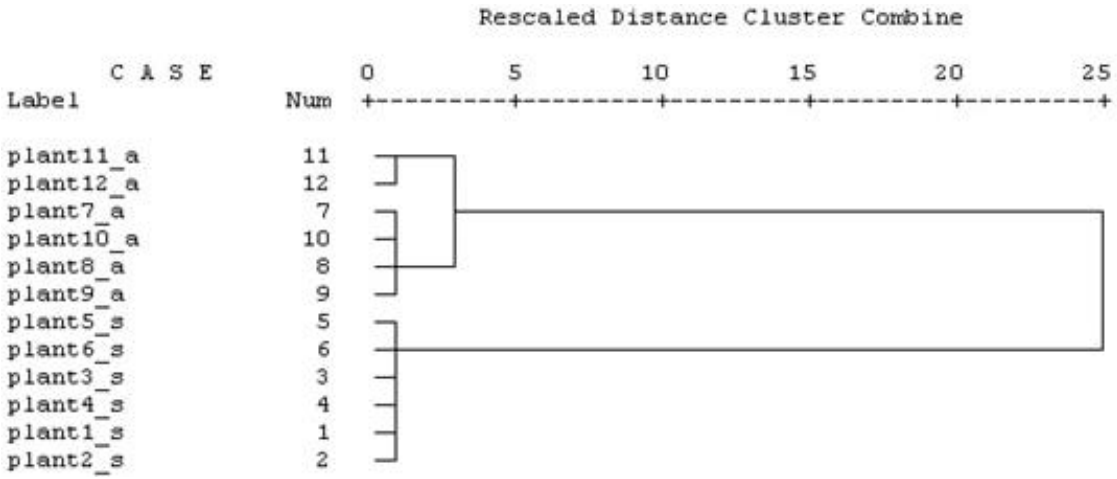

Fig. 5. Dendrogram showing clustered relationships of the seed-grown and anther- derived plants of the rice var. 'At 303', analysed based on morphological trait measurements; s=seed-grown plants; a=anther-derived plants

Flow cytometric analysis revealed that plants regenerated from anther culture of Indica rice var. 'At 303' could be haploid, diploid or mixoploid. Stomatal measurements and other morphological data also corroborated the flow cytometric evidence. In the plants subjected to flow cytometry, only 4 of 10 plants (40%) were haploid. Analysis of six regenerated plants using stomata and other morphological parameters indicated that a similar proportion (33%) were likely to be haploid. Thus it would appear that of the total regenerants less than half are haploids. Anther culture is performed with the intention of regenerating haploid or doubled haploid plants. Therefore in a system where majority of the regenerants are non-haploids, it is necessary to understand the cellular origin of these plants.

Flow cytometric analysis of the regenerated plants determined that 50% of plants were diploid. It could be that these were doubled haploids. Endoreduplication of chromosomes leading to spontaneous chromosome doubling is a relatively common phenomenon in *in vitro* culture systems (Ochatt *et al.* 2011) and some systems rely on it to produce doubled haploids or homozygous lines. Further, doubled haploids could also be arisen through nuclear divisions that take place during the induced androgenic pathway. Following the first pollen mitosis, further symmetric divisions would take place to form haploid nuclei. Later, two of such nuclei could fuse again and form doubled haploid cells (Segui-Simarro and Nuez 2008). The origin of doubled haploids needs to be substantiated by analyzing genetic loci for homozygosity using molecular methods since diploids can also arise from regeneration of maternal anther tissue. In several studies, SSR markers have been used to characterize plants produced through anther culture in rice (Sah and Niroula 2007; Luitel *et al.* 2012; Rout *et al.* 2016). However, molecular characterization was not attempted in the present analysis due to various constraints. Thus, further determination of the origin of the diploid plants cannot be concluded.

A small proportion (10%) of plants analyzed by flow cytometry was mixoploid. It was confirmed that mixoploids contained haploid and diploid cells but no higher ploidy levels. Presence of haploid cell lines in mixoploids can be considered as lending support to the hypothesis that plants originated from microspores but during the initial phase of cell division there was endoreduplication of chromosomes leading to some cell lines becoming diploid. Presence of mixoploids in plants regenerated through anther culture has been reported earlier in rice (Cha-um *et al*. 2009). Nevertheless, mixoploids are undesirable in a system of dihaploid production because they would be partially sterile and genetically unstable.

In recent studies on anther culture of Indica rice, ploidy of regenerated plants have been determined on the basis of visual observations on morphology and fertility (Sah and Niroula 2007; Mishra *et al*. 2015). Haploid plants were identified by being small and sterile compared to diploids that showed normal growth and fertility. Similar observations were made in the present study with regard to morphological data. In addition to such morphological traits, indirect methods to establish ploidy, especially the use of stomatal measurements (length, width and density) were examined. These cell measurements have been accepted as being useful in estimating the ploidy in many different plant species (Kadota and Niimi 2004; Sun *et al*. 2009; Yan *et al*. 2016). Although these cell measurements were sufficiently robust in discriminating haploids from diploids, the use of stomatal measurements may not allow adequate separation between diploids and mixoploids. Thus it could be suggested that while stomatal size and

density measurements could be used to provide a reasonable guide to ploidy determination of plants regenerated from Indica rice anther culture, the precise determination of ploidy will have to rely on chromosome counting or flow cytometry.

Conclusions: Determination of ploidy of regenerated plants derived from anther culture can be considered as essential as the anther culture technique is performed specifically for the production of homozygous lines. This study reveals that different levels of ploidy existed among the regenerated plants from anther culture, as detected by flow cytometry. Further, the ploidy estimates from stomatal, morphological and reproductive traits analyses highly corroborated the flow cytometric evidence. Therefore this study validates the use of flow cytometry combined with stomatal measurements, morphological and reproductive traits for successful characterization of ploidy status of anther-derived plants in Indica rice. Further, this suggests the importance of improving the efficiency of regenerating haploids or dihaploids through anther culture in Indica, in order to apply the anther culture technique as a practically viable breeding tool.

Acknowledgements: Funding by the National Science Foundation (NSF) of Sri Lanka under the research grant number: RG/2011/BT/10 is gratefully acknowledged. Assistance provided by the Regional Rice Research and Development Centre, Bombuwela, Kalutara, Sri Lanka is also appreciated.

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