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INDUCTION AND IDENTIFICATION OF POLYPLOIDS IN *Cattleya intermedia* LINDL. (ORCHIDACEAE) BY *in vitro* TECHNIQUES[1]

INDUÇÃO E IDENTIFICAÇÃO DE POLIPLOIDES EM *Cattleya intermedia* LINDL. (ORCHIDACEAE) ATRAVÉS DE TÉCNICAS *in vitro*[1]

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RESUMO

*No presente trabalho, foi estudado o efeito da colchicina em corpos semelhantes a protocormos de **Cattleya intermedia** L. (**Orchidaceae**). Diferentes concentrações da droga (0,00; 0,05; 0,10 e 0,20%), bem como diferentes tempos de cultura (4 ou 8 dias) foram testados para determinar o melhor tratamento para a indução de plantas tetraplóides. Dos três níveis de colchicina aplicados, os tratamentos com 0,05 e 0,10% da droga parecem ser mais efetivos na produção de mixoplóides e tetraplóides, sendo que tais tratamentos podem ser utilizados em programas de melhoramento. Como critério para distinção entre diplóides e tetraplóides, foram realizadas medidas de área de estômatos e densidade de estômatos das folhas. Os resultados sugerem que as plantas tetraplóides podem ser identificadas com elevado grau de certeza quando a seleção é realizada com base na densidade de estômatos.*

Palavras-chave: ***Cattleya**, colchicina, número de cromossomos, melhoramento de orquídeas, poliploidia, análise de estômatos.*

SUMMAY

*The effect of colchicine on the protocorm-like bodies of **Cattleya intermedia** L. (**Orchidaceae**) cultured **in vitro** was studied. Different concentrations of the drug (0.00, 0.05, 0.10 and 0.20%) as well as two times of culture (4 or 8 days) were tested to determine the best treatment for the induction of tetraploid plants. Of the 3 colchicine levels applied, 0.05 and 0.10% of drug treatments appeared to be effective on the production of mixoploids and tetraploids and could be further used in breeding programs. The suitability of stomatal area and stomata density as criteria for the distinction between diploids and tetraploids was also tested. The results suggested that tetraploid plants could be identified with a fair amount of certainty when the screening was based on the density of stomata.*

Key words: ***Cattleya**, colchicine, chromosome number, orchid improvement, polyploidy, stomata analysis.*

INTRODUCTION

The artificially induced polyploidy has played an important role on the improvement of many plant species and hybrids. In particular, orchidculture has been benefited with the fertility restoration of hybrids with problems of chromosome pairing and assortment during meiosis. Besides increasing genetic variability, the induced polyploidy results in flowers which are usually larger in size, with rounder conformation, and greater substance than the diploids (WIMBER & WIMBER, 1967). DERMEN (1940) reported other changes, such as the intensification of color and fragrance. Because of these traits, breeders have selected and used polyploid forms as parents.

The polyploidy has been an important factor in the development of improved commercial varieties and hybrids of the ***Cattleya*** orchids (KAMEMOTO, 1950).

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Although methods using colchicine for induction of polyploids are common for a range of plant species, few reports for orchids on the ***in vitro*** application of colchicine in culture medium are available. The drug has been used into culture medium in low concentrations during prolonged times, which reduces its phytotoxic effect and enhances the production of solid tetraploid plants (VAJRABHAYA, 1983).

It has been demonstrated that protocorms of ***Cymbidium*** (WIMBER & VAN COTT, 1966), ***Dendrobium*** (SANGUTHAI ***et al.***, 1973), ***Phalaenopsis*** (GRIESBACH, 1981; 1985) and ***Paphiopedilum*** (WATROUS & WIMBER, 1988) can be induced to double their chromosome number in liquid culture medium containing colchicine and regenerate tetraploid plants. The concentration of the drug as well as the duration of treatment are important factors that should be determined for each type of material (DERMEN, 1940). Higher concentrations of drug or prolonged treatments may be lethal to sensitive plant tissue.

Chromosome counting in mitotic cells of root-tips is an accurate procedure to determine the ploidy, but it is time-consuming and requires much experience. Therefore, attempts have been made to find indirect methods for ploidy determination. The area of stomata has been used to differentiate diploid and tetraploid regenerants of orchids (WATROUS & WIMBER, 1988), rye-grasses (SPECKMANN ***et al.***, 1965), daylilies (ARISUMI, 1972; CHEN & GOEDEN-KALLEMEYN, 1979), barley (BORRINO & POWELL, 1988), and banana (HAMILL ***et al.***, 1992; VANDENHOUT ***et al.***, 1995; VAN DUREN ***et al.***, 1996). The number of chloroplasts per guard cell pair (JACOBS & YODER, 1989; SINGSIT & VEILLEUX, 1991; COMPTON ***et al.***, 1996) and differences in stomata density (VANDENHOUT ***et al.***, 1995; VAN DUREN ***et al.***, 1996) have also been used as criteria in the distinction between diploid and tetraploid plants.

The present study aimed to determine an effective concentration and duration of treatment of colchicine on inducing doubling of chromosome number in protocorm-like bodies of ***Cattleya intermedia***. The suitability of stomata size and density as criteria for the distinction between diploid and autotetraploid regenerant plants was also tested.

MATERIAL AND METHODS

Plant material

Two clones of diploid (2n = 40) ***Cattleya intermedia*** were obtained from meristem culture. The culture procedure was divided into induction, growth and multiplication stages. The explants were excised from young shoots and cultured in the induction liquid medium (VACIN & WENT, 1949) modified by adding 20% (v/v) coconut water (Sigma), 100mg/ℓ myo-Inositol, vitamins (0.5mg/ℓ nicotinic acid, 0.5mg/ℓ pyridoxine HCl, 0.5mg/ℓ thiamine HCl), aminoacids (2mg/ℓ glycine, 2mg/ℓ tryptone), and 20mg/ℓ sucrose. Cultures were incubated at 25±1ºC, under fluorescent light at intensity of 22.5μE/m^2/s^1 and 16/8h light/dark photoperiod. After 4 weeks, the explants were transferred to the growth medium whose composition was the above mentioned induction medium supplemented with 1mg/ℓ benzilaminopurine (BAP), and 0.01mg/l naphtaleneacetic acid (NAA). During these first two stages, the explants were agitated on a shaker (100rpm). After 8 weeks following culture initiation, the grown meristems were transferred to the multiplication solid medium containing half-strength MS salts (MURASHIGE & SKOOG, 1962), 0.6% agar, 2mg/ℓ BAP, 0.2mg/ℓ NAA, and the same vitamins and aminoacids added to the induction medium.

Colchicine treatment

When a sufficient number of clumps of protocorm-like bodies (plbs) became available, colchicine treatments were initiated.

The concentrations of colchicine used were 0.00, 0.05, 0.10, and 0.20%, and the duration of treatments was either 4 or 8 days. The colchicine (Plant Cell Culture Grade-Sigma) was added to the induction liquid medium through filter sterilization (0.45μm). Single plbs of 2 to 3mm in length (figure 1a) were isolated from actively growing cultures. A total number of 9 plbs was used per treatment (3 replications of 3plbs/flask). Flasks containing plbs in the several colchicine concentrations were placed on horizontal gyratory shakers and kept in dark at 25±1ºC for the treatment duration.

Following the colchicine treatment, the plbs were transferred to the induction liquid medium lacking the colchicine for two days to eliminate the effect of the drug. After that, the plbs were transferred onto the multiplication solid medium (previously described). Three subcultures to fresh medium resulted in the proliferation of a high number of new plbs and plantlets development. These plantlets were transferred to the rooting solid medium (mother flask medium with charcoal from G & B Orchid Laboratory) (figure 1b).

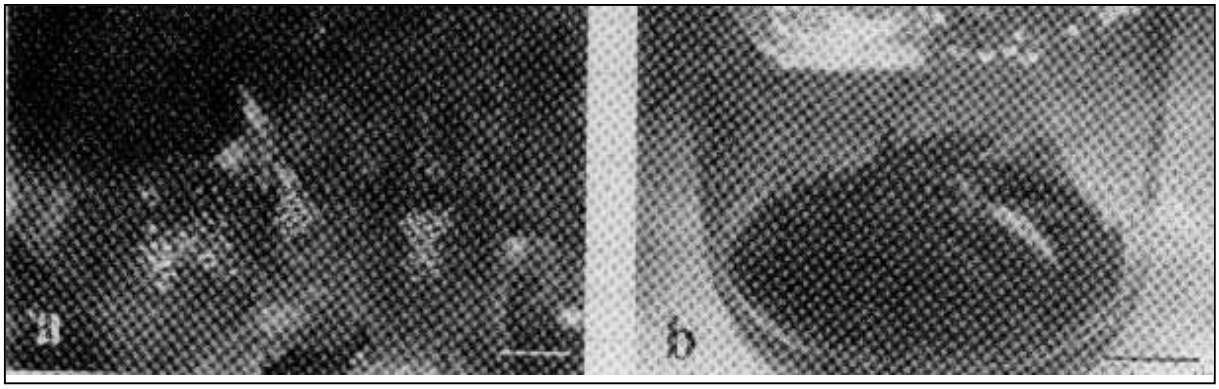

Figure 1 - (a) Aspect of the protocorm-like bodies utilized on treatment. Bar = 1mm. (b) Plantlet detail in rooting medium. Bar = 10mm.

Estimation of ploidy level

The ploidy level of the plants regenerated from treated plbs was estimated by chromosome counting in root-tips and by stomatal guard-cells measurements. Then 4 to 5-month-old plantlets were randomly sampled from each treatment. The root-tips were pretreated for 24 hours in iced water (0ºC), fixed in acetic acid - chloroform - 95% ethanol (1:3:6). Hydrolysis was performed in 1N HCl for 10 minutes at 60ºC, before staining with Shiff solution (Feulgen reaction) and squashing in 0.6% propionic carmim. Microscope slides were examined at 1000x magnification and the plants were classified according to the chromosome number.

Young leaves of the same plants were fixed in formaldehyde - acetic acid - 50% ethanol (1:1:18) (JOHANSEN, 1940). The stomata were measured from an imprint of the lower leaf surface, obtained by painting clear finger-nail polish on the leaf, allowing it to dry and peeling it off (HAMILL ***et al.***, 1992). The imprint was laid on a microscope slide with a drop of water to allow the analysis of the guard cells. The stomata were measured at 200x magnification under a Zeiss Axiophot microscope equipped with AVT-HORN color video chamber. In order to obtain the mean stomatal area, ten stomata per plant were measured. The measurements were recorded using pixel unit (1 pixel = 1.25μm). The stomata density average was calculated for each plant by counting stomata in 10 fields of view ($0.12mm^2$). The stomatal area and density were evaluated using the Global Lab Image V. 3.0 software by Data Translation.

Statistical analysis

The treated explants mortality data was analyzed using the Chi-Square with the Yates continuity correction or the Fisher's Exact Test for R x C tables. The latter was also employed in the comparison among different concentrations of colchicine in relation to the production of non-diploids. A combine p-value was obtained by the Fisher's method (SOKAL & ROHLF, 1981:779) for colchicine concentrations effect.

The relationship between ploidy and stomata density or stomatal area was assessed by means of one-way Analysis of Variance. Both stomata variables were log-transformed because the distributions were skewed and the variances increased with increasing averages. Heteroscedasticity tests done after the transformation yielded p-values larger than 0.70. The subsequent multiple comparisons of means were done by the Student-Newman-Keuls (SNK) test. The Pearson product-moment correlation coefficient was calculated between the two log-transformed stomata variables.

RESULTS AND DISCUSSION

A fraction of the colchicine-treated plbs transferred to the multiplication solid medium presented a dark green color indicating the explant death. The mortality range was 0 to 44% in clone 114 and 0 to 33% in clone 121 (table 1). Although an increase in the mortality was expected with increasing concentrations of colchicine (SANGUTHAI ***et al.***, 1973), no significant differences were found among concentrations when the four combinations of clones and duration of treatment were considered separately (Fisher's Exact Test - FET: p values ranging from 0.163 to 0.623). The mortality after 4 day-treatment and 8 day-treatment did not differ either in clone 114 (FET: p = 0.778) or in clone 121 (FET: p = 1.000). However, a χ^2 test between the total mortality in clone 114 (16/72) and in clone 121 (7/72) reached almost a significant result (χ^2_{calc}=3.31; p = 0.069), suggesting a possible genotype effect on this characteristic. OWEN ***et al.*** (1988) demonstrated that internal

Table 1. Number of dead protocorm-like bodies out of nine plbs treated with one of four different concentrations of colchicine for four or eight days.

Colchicine concentrations (%)	Duration of treatment			
	4 days		8 days	
	Clone 114	Clone 121	Clone 114	Clone 121
0.00	2/9	1/9	3/9	0/9
0.05	1/9	0/9	0/9	3/9
0.10	3/9	0/9	4/9	1/9
0.20	1/9	2/9	2/9	0/9
FET among concentrations	p = 0.623	p = 0.592	p = 0.220	p = 0.163

genetic factors and external culture factors may affect the process of endopolyploidization.

All the regenerated plants that were treated with colchicine showed vigorous growth and much more intense color than the untreated controls, regardless the chromosome number. This phenomena could be explained by assuming that colchicine could have an effect similar to the cytokinins' effect (WEBSTER & DAVIDSON, 1969). According to RUIZ & VÁZQUEZ (1982), the colchine added to the media could, to a certain extent, modify the auxin/cytokinin relation and therefore change the growth of the cell population in culture.

A total of 134 plantlets was examined for chromosome number: 85 were diploid, 16 mixoploid, and 33 tetraploid. The diploid chromosome number was 40, as already observed by KAMEMOTO (1950). Mixoploid plantlets presented different combinations of diploid and tetraploid cells in root tips. The distribution of plantlets with different ploidy is presented for each treatment in table 2.

The effect of colchicine concentration was evaluated for each clone in separated analysis for each duration of treatment. Assuming that mixoploids and tetraploids resulted from colchicine treatment, the data relative to these classes were pooled. With four days of treatment, different concentrations yielded different percentages of non-diploid plants in clone 114 (FET: p = 0.013), the higher proportion (71%) resulting from the 0.05% treatment. A similar effect was observed in clone 121 (FET: p = 0.053), but in this clone the non-diploid plantlets were more frequently obtained (55%) with the 0.10% colchicine concentration.

After 8-day-treatment, the effect of colchicine concentration on non-diploid plantlet production was statistically non significant (FET: p = 0.153 for clone 114 and p = 0.263 for clone 121).

The fact that it was not possible to detect significant differences among the colchicine concentrations in this experiment could be partially accounted for the unexpected high frequency of mixoploid and tetraploid plantlets in the control groups. It has been demonstrated that tissue culture itself is a potential tool for obtaining polyploid plants as reported in tomato (COMPTON & VEILLEUX, 1991), melon (FASSULIOTIS & NELSON, 1992), potato (CARDI ***et al.***, 1992), and watermelon (COMPTON ***et al.***, 1996). Thus, the arising of plants with duplicated chromosomes in

Table 2 - Percentage of diploid, mixoploid and tetraploid plantlets derived from colchicine-treated protocorm-like bodies of two ***Cattleya intermedia*** clones, for four concentrations and two duration of treatments.

Duration of treatment	Colchicine concentration. (%)	Clone 114				Clone 121			
		Nº of plants	Regenerates (%)			Nº of plants	Regenerates (%)		
			Diploid	Mixopl	Tetrapl		Diploid	Mixopl	Tetrapl
4 days	0.00	9	100	0	0	9	100	0	0
	0.05	7	29	42	29	9	78	11	11
	0.10	10	60	20	20	9	45	33	22
	0.20	7	43	14	43	6	83	0	17
8 days	0.00	10	60	0	40	7	43	43	14
	0.05	8	25	0	75	10	70	20	10
	0.10	10	40	0	60	5	60	0	40
	0.20	9	78	0	22	9	89	11	0
Total		70	55	9	36	64	71	16	13

non-treated groups could be attributed to the tissue culture procedure itself.

In spite of the non significant results obtained in the 8-day experiment, the data seem to show the same tendency of 0.05 and 0.10% concentrations, producing more non-diploid plantlets in clones 114 and 121, respectively. As a matter of fact, combining the p-values obtained in the 4-day and 8-day-treatment for clone 114 using a technique developed by R.A. Fisher (SOKAL & ROHLF, 1981:779), the overall p assigned to the differences among concentrations is 0.014. For clone 121, the combined p-values is 0.074. The small number of the analyzed plants did not allow us to attribute directly our results to different susceptibility between clones. Further analysis of larger samples could provide more conclusive results.

On basis of this preliminary investigation, for the practical use of colchicine to induce polyploidy in ***C. intermedia*** we recommended concentrations between 0.05 and 0.10% (added through filter sterilization) for 4 days treatment. This result is comparable to previous reports obtained with other orchid genera. SANGUTHAI ***et al.*** (1973) obtained high production of hexaploids and mixoploids plants by treating ***Dendrobium*** protocorm-like bodies with 0.05, 0.10, and 0.15% of colchicine, the most effective concentration being 0.10%. In ***Paphiopedilum***, treating protocorms with 0.05% of colchicine produced more than 50% of tetraploid plants (WATROUS & WIMBER, 1988). Both reports recommended short periods of treatment, not exceeding 10 days. GRIESBACH (1981) also obtained 50% of tetraploid ***Phalaenopsis*** using 0.05% of colchicine, however using a more prolonged treatment (10-14 days).

The suitability of ploidy determination using stomatal measurements, namely area and density of stomata (figure 2), was evaluated by associating these traits with chromosome counts (table 3). Significant differences among levels of ploidy were found both for the first ($F = 57.5$; $p < 0.001$) and the second variable ($F = 120.2$; $p < 0.001$). Diploid plants presented the lowest stomatal area (1269) while tetraploid showed the highest one (1916), mixoploid presenting intermediate values (1441). The association between the density of stomata and the ploidy level was negative, where lower density values were obtained in tetraploid plants (4.7). This finding is explained by the strong negative correlation observed between these two stomata log-transformed measurements ($r = - 0.78$; $df = 170$; $p < 0.001$).

The frequency distributions of stomata density in 42 tetraploids, 24 mixoploids, and 106 diploid plants (total = 172) are shown in figure 3. Although there is an overlap of the tetraploid and mixoploid distributions, a stomata density average less than 6.0 would allow the identification of 38 out of 42 (90%) solid known tetraploids. Because of the overlapping, 8 mixoploids (33%) and 4 diploids (4%) would be misclassified as tetraploids. The differential manifestation of the mosaicism in different parts of plant could explain discrepant results in the same plant. GILISSEN ***et al.*** (1994) found diploids, tetraploids and mixoploid plants in tobacco, using flow cytometric analysis. However, when root-tips were analyzed by conventional cytology, only diploids and tetraploids were identified. According to VAN DUREN ***et al.*** (1996), mosaicism can present several levels, with different ploidy number observed in different locations of the same leaf. WATROUS & WIMBER (1988) identified some plants presenting stomatal areas in agreement with the estimated for diploid plants

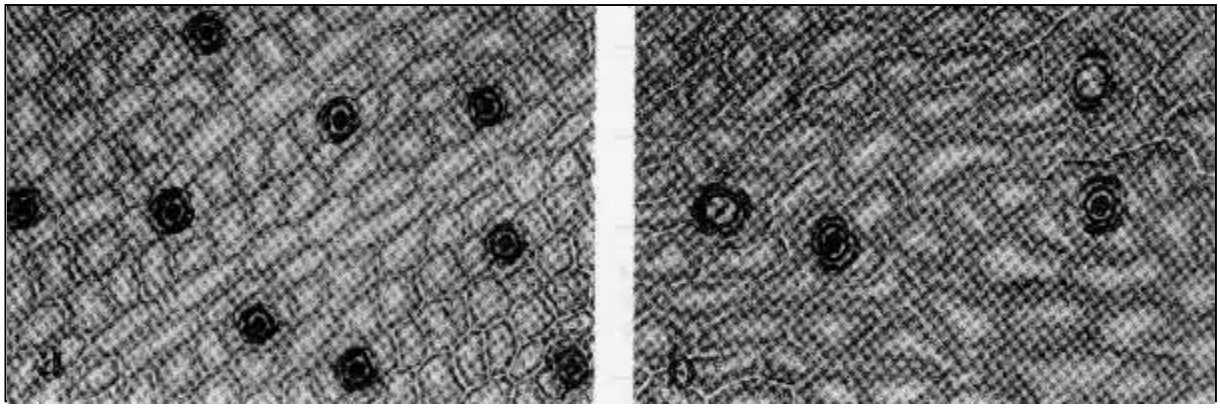

Figure 2 - (a) Imprint of leaf showing stomata in a diploid plant and (b) in a tetraploid plant. Both photographs were taken at the 200X magnification.

although they had tetraploid root-tip chromosome counts, suggesting that these might be other mosaic forms. Thus, to confirm ploidy level, root-tip chromosome counts should be obtained.

Our results indicate that stomata density is a useful tool for rapid pre-screening of solid tetraploids and to reduce the population size to be maintained after ***in vitro*** step. Stomata density is not as influenced as the stomatal area by external factors such as temperature and water content of the plant tissue and the counting method does not demand sophisticated equipment and skills. If a standard microscope is available the ploidy level of several dozens of plants can be estimated, yielding reliable results.

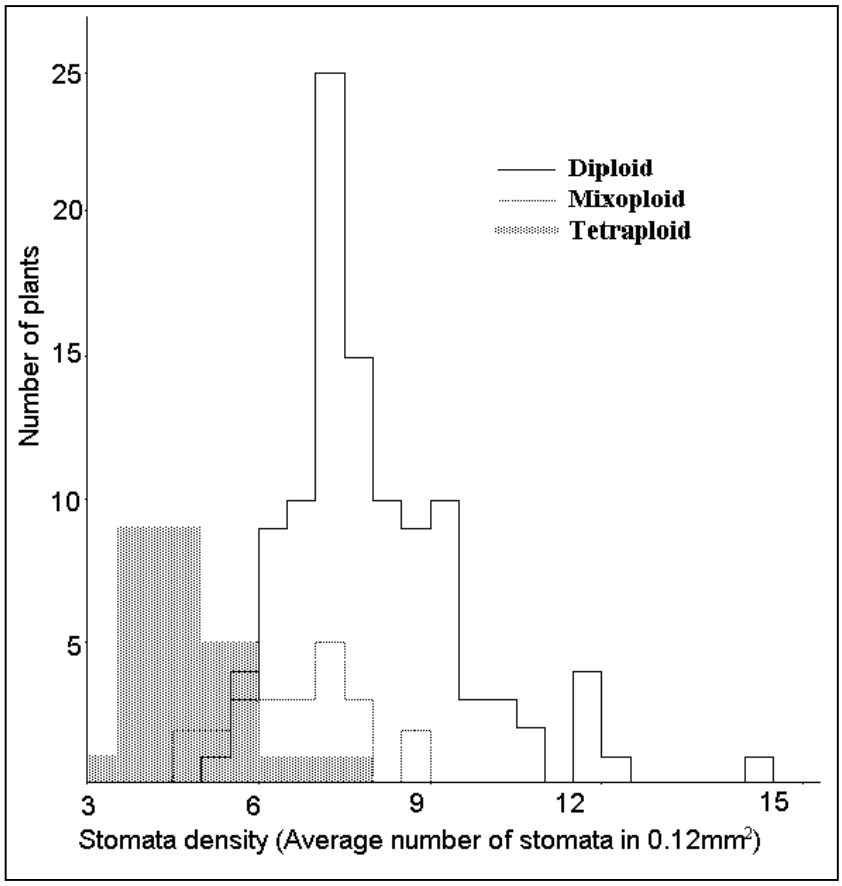

Figura 3 - Histograms of the stomata density for diploid, mixoploid, and tetraploid plantlets obtained after treatment with colchicine.

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Tabela 3 - Mean and standard deviation (SD) for stomatal area and density in regenerated plants of different ploidy levels.

Ploidy level	N	Stomatal area (in pixels)		Stomata density	
		Mean ± SD	Range	Mean ± SD	Range
Diploids	106	1269 ± 254 a	848 - 2038	8.0 ± 1.6 a	5.4 - 14.4
Mixoploids	24	1441 ± 318 b	934 - 1959	6.6 ± 1.1 b	4.5 - 8.9
Tetraploids	42	1916 ± 399 c	978 - 2708	4.7 ± 1.0 c	3.0 - 7.7

Stomatal area: F=57.5; df=2 and 169; p<0.001. Stomata density: F=120.2; df=2 and 169; p<0.001. Means within columns followed by different letters differ at the 0.025 (stomatal area) and 0.001 (stomata density) significance levels by the SNK test.

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