Male reproduction stability assessed through meiotic and post-meiotic cycles in bushy matgrass (*Lippia alba*) genotypes for use in breeding

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ABSTRACT

The aim of the present study is to analyze the meiotic and post-meiotic cycles of bushy matgrass genotypes (citral chemotype) in order to better understand their respective male reproduction stability. It is essential estimating such a stability when a non-domesticated species is selected. Therefore, the number of chromosomes of pollen-mother cells, and their ploidy were assessed; possible meiosis anomalies were assessed. Recombination index (RI) was estimated. The number of normal and abnormal post-meiotic products (PM's) was counted. Meiotic index (MI) and pollen grain viability (VIA) were estimated. These estimates were carried out per genotype and they were subjected to ANOVA and to average tests. All genotypes were diploid (2n = 30 chromosomes). Pairing was quite regular (RI = 44.33%). Meiotic cycle anomalies recorded very low frequency rates. Assumingly, chromosomal breaks resulted from micronuclei formation in PM's. Estimated MI was 94.48%, and estimated VIA was 94.32%. MI and Via did not statistically differ between genotypes. These outcomes point towards genotypes' high male reproduction stability and to the significant influence of genetic effects on MI and VIA.

Key words: chromosome number, ploidy level, meiotic index, pollen grain viability, genetic parameters.

INTRODUCTION

Bushy matgrass [*Lippia alba* (Mill.) N.E.Br. ex Britton & P. Wilson] is a bushy hermaphrodite species belonging to family Verbenaceae. It is distributed from South United States to North Argentina and Uruguay (Salimena and Múlgura 2015). Its economic importance mostly lies on its medicinal potential and on the production of its aromatic essential oils (Tareau et al. 2017). The species' genotypes are classified based on their chemotype, which is determined by the major substance in their respective essential oils.

Citral is one of the chemotypes mostly found in Brazil; it presents citrus aroma similar to that of lemon and is largely used in the chemical and cosmetics industries. Despite its high economic potential, nowadays, there are no recommended cultivars of it in Brazil for commercial production (Brasil 2021). Thus, implementing bushy matgrass genetic enhancement programs is essential to explore its chemical and medicinal potential. However, because this species remains non-domesticated, information about its gamete fertility and about its reproduction stability remains scarce. This stability is initially related to parents' ability to produce seeds to form new generations. This information is essential for the enhancement program to last long.

According to Pierre et al. (2011), species *L. alba* shows different ploidy levels, depending on the considered chemotype: 2n = 2x = 30 chromosomes (citral), 2n = 4x = 60 chromosomes (carvone) and 2n = 12-60 chromosomes (linalool), which is a mixoploid. Besides the existence of different ploidy levels between species' genotypes, which belong to different chemotypes, Viccini et al. (2014) and Lopes et al. (2020) also reported diploid and tetraploid individuals with citral chemotype. Crossing between parents with different ploidy levels carried out by a given enhancement program can generate

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progenies accounting for severe fertility issues – irregular pairing between chromosomes is the major issue.

Accordingly, the main aim of the present study was to infer on the male reproduction stability of bushy matgrass' genotypes (citral chemotype) to be used as parents in the species' genetic enhancement program carried out at Universidade Federal Rural do Rio de Janeiro. In order to do so, all genotypes were analyzed to assess the number of chromosomes, ploidy level and likely anomalies during and after the meiotic cycle. Based on such an information, meiotic indices and pollen grain viability were estimated, and their means were tested. Finally, the genetic parameters were found to get the magnitude of genetic and environmental influence on male reproduction stability in the assessed genotypes.

MATERIAL AND METHODS

Research site location and experimental conditions

The research was carried out in the Laboratory of Cytogenetics and Molecular Biology of Plants (LCBMP) of the Plant Science Department – Agronomy Institute – of Universidade Federal Rural do Rio de Janeiro (22º45' S; 43º 41' W).

Genotypes were grown in the field, they were spaced 1.2 x 1.2 m from each other, under randomized blocks design, with eight treatments (genotypes), and two repetitions (blocks) – 6 plants per plot, which totaled 12 plants per genotype.

Eight bushy matgrass (*L. alba*) genotypes defined as citral chemotype were used (Soares et al. 2019). All assessed genotypes were recorded in the Botanical Herbarium of UFRRJ's Biological and Health Sciences Institute: UFRRJ ECB001 (RBR00045072), UFRRJ ECB002 (RBR00045071), UFRRJ ECB005 (RBR00045070), UFRRJ ECB011 (RBR00045069), UFRRJ ECB015 (RBR00045068), UFRRJ ECB018 (RBR00045067), UFRRJ ECB019 (RBR00045066) and UFRRJ ECB020 (RBR00045065).

Vegetal material collection and fixation

Flower buds at different development stages were collected in the morning, between 7:00 and 8:00 h. They were randomly collected from all assessed genotypes for the analysis of meiosis phases. This mix of flower buds was stored in properly identified glass flasks filled with ethanol and acetic acid solution, at 3:1 ratio (v/v) (Carnoy solution) for their fixation. Subsequently, the flasks were stored in freezer until analysis time.

Buds were collected from each genotype, in the two blocks (repetitions), for meiotic index and pollen grain viability analyses. Next, they were stored in properly identified glass flasks filled with 70% ethanol. The flasks were kept in refrigerator, at 12 °C, until analysis time.

Analysis of meiotic cycle in protoplasts

In total protoplasts in 5 Eppendorf tubes were analyzed. Each tub had 100 anthers, which were extracted from flower buds presenting size ranging from 0.5 to 1.0 mm.

Initially, the used anthers were dried for 5min in distilled water, so the fixer could be removed for protoplasts' preparation. Subsequently, the anthers were deposited in Eppendorf tub filled with 1ml of distilled water; the tubes were centrifuged at 2,000 rpm, for 10 min. After centrifugation, the water was taken out of the tub and the anthers in it were left to air dry for 10 min. The aliquot of 200 µl of enzymatic solution (2% pectinase P4716-5KU SIGMA + 2% cellulase CELLULYSIN® Merck) was added to Eppendorf tube filled with 100 air dried anthers in order to obtain the protoplasts. Next, the anther mixes were heated in waterbath at 37 °C, for 3 h 30 min. Right after this procedure, the enzymatic solution was taken out of the tube, and 200 µl of distilled water was added to it. The obtained protoplasts were re-suspended and once more centrifuged at 2,000 rpm, for 10 min in Eppendorf tub filled with distilled water. Once again, the water was taken out of the tub and replaced by 100 µl of Carnoy solution for final protoplasts' fixation.

In total, 10 µl of protoplast suspension were deposited for slide assemblage, one drop of carmine-acetic was added to it after air drying. Subsequently, the slide/coverslip set was assembled. The slide was observed under the light field in optical microscope (Olympus BX43, USA).

Different meiosis phases and the likely anomalies in cell division were assessed. Images were taken with the aid of high-resolution digital camera (Olympus Q-Color3, USA). The number of chromosomes and ploidy level of the assessed species were also observed. Recombination index (RI) was calculated based on Darlington (1958), wherein: ; wherein *n* is the haploid number.

The number of ring-type chiasmas ("ring") and the number of rod types ("rod") in diploid cells were counted to calculate RI. The rod configuration only describes the occurrence of one chiasma, whereas the ring one describes two chiasmas (Senda et al. 2005).

Meiotic index analysis and estimate

Four (4) slides were prepared per genotype, per block. Each slide housed 20 anthers from the five flower buds (length ranging from 1.0 to 1.5 mm), based on the screening. The anthers were

sectioned and smashed in 1% carmine-acetic; they were observed under the light field in the optical microscope (Olympus BX43, USA) after the blade/coverslip set was assembled. The number of post-meiotic products (Monads, Dyads, Triads, Tetrads and Polyads) was recorded. Post-meiotic product images were captured with the aid of high-resolution digital camera (Olympus Q-Color3, USA).

Subsequently, meiotic index (MI) was estimated based on Love (1951), through the following estimator: . Mean meiotic index (MI) rates recorded for each genotype were subjected to statistical analysis.

Pollen grain viability analysis

Four (4) slides per genotype, per block, were prepared for this analysis. Anthers from two flower buds at pre-anthesis stage were used to assemble the slides. The anthers were sectioned and smashed in one drop of triple Alexander solution. The slide/coverslip set was assembled 1 h later. The slides were observed under the light field in optical microscope (Olympus BX43, USA).

The presence of viable/fertile pollen grains was highlighted by red and purple shades, whereas inviable pollen grains have presented greenish/blueish shade. In total, 500 pollen grains were counted per slide. Viable and inviable pollen grain images were taken with the aid of high-resolution digital camera (Olympus Q-Color3, USA).

Mean pollen grain viability rates (VIA) recorded for each genotype were subjected to statistical analyses.

Statistical analyses

At first, variables "meiotic index (MI)" and "pollen grain viability (VIA)" were subjected to Lilliefors test in order to assess their normality. Subsequently, they were subjected to analysis of variance (ANOVA) – fixed model adjusted to random block design, with 8 treatments (genotypes) and two repetitions (blocks). Next, mean MI and VIA recorded for each genotype were subjected to Tukey test, at 5% probability level.

The following genetic MI and VIA parameters were estimated based on estimates found through analysis of variance: experimental variation coefficient; genotypic variation coefficient; genotypic determination coefficient and genetic variation index.

All statistical analyses were carried out in GENES software, version 1990.2018.71 (Cruz 2016).

RESULTS AND DISCUSSION

Based on the results, the assessed genotypes were diploid (2n = 2x = 30 chromosomes). Figure 1A shows a cell at pachytene stage; it has chromosomes showing regular pairing. Figures 1B and 1C evidenced the presence of bivalent 15 in cells, and this finding confirms the diploid nature of the herein assessed genotypes (citral chemotype). These same outcomes were also found by Brandão et al. (2005), Brandão et al. (2007), and Sousa et al. (2009).

The analysis applied to number of chromosomes and ploidy level in potential bushy matgrass parents is quite important, because the literature also informs differences in the number of chromosomes and in ploidy level in plants belonging to this species (Pierre et al. 2011; Reis et al. 2014). There are cases when the literature highlights that these differences can be even observed between genotypes belonging to the same chemotype, in representatives of this species (Viccini et al. 2014; Lopes et al. 2020). It is important pointing out that crossing between genotypes presenting different chromosome number and ploidy levels can cause, among other abnormalities, aneuploid genotypes and reduced fertility in progenies that are mainly obtained from irregular pairing. In other words, this process can lead to cytologically unstable genotypes and, consequently, to reduced reproduction stability.

The occurrence of aneuploidy in non-domesticated species, such as the herein analyzed one (Malallah et al. 2001; Singhal and Kumar 2008), and other anomalies, can be quite common to find (Damasceno Junior et al. 2010). Therefore, it is crucial assessing the meiotic cycle to detect anomalies. Overall, the meiotic cycle of the assessed genotypes was quite regular, but, yet, some anomalies were detected at very low frequency. Figure 1B evidences a diplotene presenting regions with chiasmas, where one can observe 1 pair of rod chromosomes, and other 14 ring pairs. The recombination index (RI) between genotypes was estimated (44.33%) based on such a configuration. The magnitude of this estimate can point out high regularity degree in chromosome pairing during meiosis.

Still, with respect to the meiotic cycle, Figures 1D and 1E point out cells at metaphase I stage and regular analysis I. Figure 1F points out one normal telophase I, whereas Figure 1G indicates abnormal telophase II. It is possible noticing the presence of a small DNA fragment out spread in the cell (Figure 1G).

Mean meiotic index (MI) was high; estimates reached 94.48% (Table 1). According to Love (1951), plants presenting meiotic level lower than 90% can be indicative of cytological instability. The generation of fertile progenies by these plants can be a problem. The herein recorded MI estimate likely derives from high meiotic cycle in the assessed genotypes. This

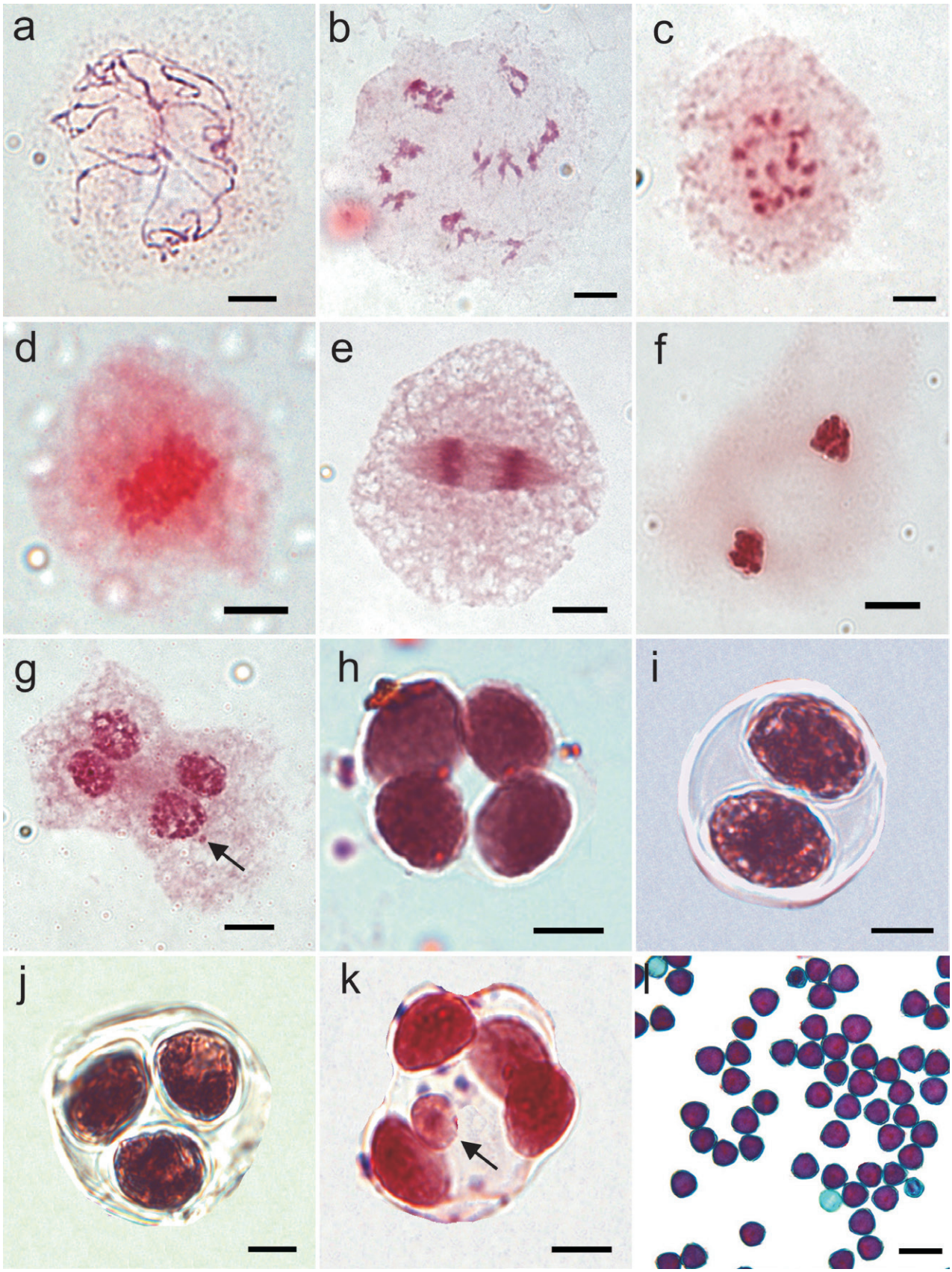

Figure 1. Meiosis observed in bushy matgrass. (A) Pachytene, (B) Diplotene with chiasma regions and 15Π, (C) 15Π in diakinesis, (D) Metaphase I, (E) Anaphase I, (F) Telophase I, (G) Telophase II evidencing DNA fragment (arrow), (H) Tetrad, (I) Dyad, (J) Triad, (K) Polyad with highlighted micronuclei (arrow), (L) Viable (purple) and inviable (green) pollen grains. Bars: A to G = 10 µm, H to K = 25 µm, L = 50 µm.

stability, in its turn, will contribute to the production of normal tetrads (Figure 1H). However, anomalies were also detected among post-meiotic products (Figures 1I, 1J and 1K). These Figures depict dyads, triads and polyads. Figure 1K evidences the presence of micronuclei. The presence of Polyads was expected, since DNA fragments were found in cell at telophase II phase in the meiotic cycle (Figure 1G). According to Zamariola et al. (2014), the presence of abnormal post-meiotic products evidences likely issues related to chromosomal break and issues concerning chromosomal break during meiosis.

Similar to the meiotic index, pollen grain mean viability (VIA) was high; estimates reached 94.32% (Table 1) - Figure 1I well expresses this rate. Damasceno Junior et al. (2010) mentioned that the low viability recorded for *Vasconcellea monoica* (Desf.) A. DC. (70.93%) can be explained by the occurrence of a series of anomalies in its meiotic cycle, whose occurrence is quite common, given the fact that it is a non-domesticated species.

Analysis of variance (Table 1) and average test (Table 2) results, and results recorded for pollen grain viability (VIA) have shown statistical differences between genotypes. The estimated variation indices (VI = 2.32 and 1.29, recorded for MI and VIA, respectively) (Table 1) have also confirmed genetic diversity between genotypes. Despite these differences, all means were high in all genotype averages (Table 2). These estimates (means) confirm genotypes' male cytology stability, which was previously introduced and discussed.

Table 1. Summary of the analysis of variance and estimates of genetic parameters recorded for meiotic index features (MI) and for pollen grain viability (VIA), which were estimated for bushy matgrass genotypes (citral chemotype) grown in Seropédica, Brazil.

FV	GL	Means Squares	
		MI	VIA
Block	1	37.0530	0.6865
Genotype	7	3.4877 **	34.1170 *
Residue	7	0.2963	7.9245
Mean		94.48	94.32
Maximum		97.63	97.90
Minimum		90.23	81.94
$VC_{(\%)}$		0.58	2.98
$VC_{g(\%)}$		1.34	3.84
$IV_{(\%)}$		2.32	1.29
S^2_g		1.5957	13.0963
S^2_e		0.2963	7.9245
$H^2_{(\%)}$		91.50	76.77

** e * significant at 1% and 5% probability level, respectively, in the F test; $VC_{\%}$ = experimental variation coefficient; $VC_{g(\%)}$ = genotype variation coefficient; IV = genetic variation index; S^2_g = genetic variance; S^2_e = environmental variance; $H^2_{(\%)}$ =genotype determination coefficient.

Table 2. Average tests between bushy matgrass genotypes (citral chemotype) grown in Seropédica, Brazil, by taking into account the meiotic index (MI) and pollen grain viability (VIA) estimates.

Genotypes	MI (%)	VIA (%)
UFRRJ ECB001	93.63^{ab}	97.31^{ab}
UFRRJ ECB002	91.86^{b}	96.31^{ab}
UFRRJ ECB005	93.75^{ab}	97.61^{a}
UFRRJ ECB011	94.85^{a}	96.34^{ab}
UFRRJ ECB015	95.45^{a}	85.85^{b}
UFRRJ ECB018	95.49^{a}	93.27^{ab}
UFRRJ ECB019	94.99^{a}	97.00^{ab}
UFRRJ ECB020	95.79^{a}	90.91^{ab}
Means	94.48	94.33

Means in the columns followed by the same letter did not statistically differ in the Tukey test, at 5% significance level.

The cytological stability of the analyzed genotypes can be substantiated by genotype determination coefficient estimates (H^2) (91.50% and 76.77% for MI and VIA, respectively) (Table 1). These estimates point out that the cytological stability was much more influenced by genetic effects than by the environment.

Based on such outcomes, one can observe that the assessed parents can generate fertile progenies. This fertility, in its turn, can be kept throughout the selection cycle, since the genetic effects were quite stronger than the environmental ones. However, it is also recommended to monitor the reproduction stability of future parents, since although reduced, environmental effects still have some influence on the meiotic cycle of bushy matgrass citral chemotype.

CONCLUSIONS

Based on the recorded results, it is possible concluding that the assessed genotypes presented high male reproduction stability; they were diploid (2n = 2x = 30 chromosomes). It was also possible inferring that the assessed genotypes showed low frequency of anomalies during and after the meiotic cycle, and that genetic control at male gamete production mostly results from genetic factors.

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AUTHORS' CONTRIBUTION

All authors contributed to the conception and design of the study. Material preparation, data collection, and analysis were performed by all authors. The first draft of the manuscript was written by T. O. Pinto and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

DECLARATION OF CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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