

Frequency of the RAD51 (radiation sensitive 51) recombinase in species and hybrids of *Brachiaria* and relationship with genetic variability

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Abstract

This study identified and compared the frequency of homology and recombination hotspots in sexual diploid and tetraploid species, apomictic tetraploids and hybrids of *Brachiaria*, by immunolocalization of the radiation sensitive 51 (RAD51). Roots were fixed in 4% paraformaldehyde. We applied primary antibody (Rat anti-*Arabidopsis thaliana* RAD51) and detected with a secondary antibody (Goat anti-rat IgG FITC). The analysis of this protein showed diffuse signals at leptotene. In zygotene, signals of RAD51 were identified in unpaired chromosomes or partially paired and adjacent signals of RAD51 in paired chromosomes, confirming its close involvement with the search for chromosomal homology. During pachytene, RAD51 signals were located between chromosome axes in recombination hotspots. The mean number of recombination signals was greater in apomictic species of *Brachiaria* (*B. decumbens*, 85.80 and *B. brizantha*, 83.87), this higher frequency of signals can be related to the mode of reproduction of the species. The artificially tetraploidized *B. ruziziensis* showed a mean number of RAD51 signals similar the diploid *B. ruziziensis*, as a result of the polyploidization. The hybrids presented an overlap of the mean number of RAD51 signals compared to diploid and tetraploid of *B. ruziziensis*. This non-additivity of genomes is possibly an adaptation to genomic stress caused by hybridization and polyploidy.

Key words: Crossover; homology; immunolocalization; meiosis; recombination hotspots.

Abbreviations: AtRAD51_ *Arabidopsis thaliana* RAD51; BSA_bovine serum albumin; CO_crossovers; DAPI_4', 6-diamidino-2-phenylindole; DMC1_Disrupted Meiotic cDNA1; DSBs_double-strand break; FITC_Fluorescein isothiocyanate; IgG_immunoglobulin G; Mbp_megabase pair; MRE11_meiotic recombination 11 homolog A; NBS1_Nijmegen breakage syndrome 1; NCO_noncrossovers; PBS_phosphate buffer saline; RAD50_radiation sensitive 50; RAD51_radiation sensitive 51; RecA_bacterial recombination protein; SC_synaptonemal complex; SPO11_meiotic protein covalently bound to DSB homolog; TopoVIA_topoisomerase proteins.

Introduction

The genus *Brachiaria* (Trinius) Grisebach [(syn. *Urochloa* Hochst. Ex A.Rich.) R. D. Webster] is native to tropical Africa and comprises a large and diverse genus with approximately 100 species (Renvoize et al., 1996) of great economic importance for forage crops, as it makes possible the livestock farming in acid and poor soils (Souza Sobrinho, 2005; Araújo, 2008; Valle et al., 2009). In *Brachiaria* breeding programs, the main goal is to achieve persistent hybrids with desirable traits of two agronomically promising parents, such as adaptation to acid soils, high yield, good nutritional value and resistance to spittlebugs (Pereira et al., 2001). In this sense, the strategy consists of evaluating and selecting genotypes with superior traits (Pereira et al., 2001; Souza Sobrinho, 2005), and intra- and interspecific crosses involving the apomictic and usually polyploid species *Brachiaria brizantha* (Hochst. ex A. Rich.) Stapf, *Brachiaria decumbens* Stapf and the sexual, artificially tetraploidized species, *Brachiaria ruziziensis* Germain & Evrard. (Souza Sobrinho, 2005). In the early stages of a breeding program, the use of recombination is essential for the expansion of existing variability, when the different traits of the parents are combined into new cultivars (Wijnker and Jong, 2008). In

this context, genetic recombination allows the generation of new allele combinations with important implications for genome evolution (Schuermann et al., 2005; Massy, 2013). These reasons justify the interest of plant geneticists and breeders in understanding the mechanisms that control recombination and in determining its frequency. In some species (Baptista-Giacomelli et al., 2000; Souza and Pereira, 2011), the recombination frequency can be estimated by counting chiasmata in conventional meiotic analysis. However, in *Brachiaria*, the identification of chiasmata is hampered by the size of the chromosomes. The largest pair in the diploid species has 5.15µm and in the tetraploid species, 5.60µm (Nielen et al., 2010). An alternative is to estimate the recombination sites by immunolocalization of recombinase proteins (Phillips et al., 2013). Several proteins, part of the synaptonemal complex (SC), are directly or indirectly related to effective pairing and recombination (Osman et al., 2011). Recombination begins at zygotene with the DNA double-strand break (DSBs) by SPO11 proteins, homologous to TopoVIA (Massy, 2013), generating a single-stranded DNA protrusion, whose 3' end invades the duplex DNA of the homologous chromosome, forming a Holliday junction, and

subsequently effecting gene exchange (Osman et al., 2011). During this process, a protein complex containing RAD50, MRE11 and NBS1 acts on DSBs to remove SPO11 (Waterworth et al., 2007; Osman et al., 2011). The repair of DSBs is facilitated by two recombinases, the radiation sensitive 51 (RAD51) and Disrupted Meiotic cDNA1 (DMC1) that promote the invasion in the homologous double strand (Osman et al., 2011). DNA synthesis followed by its linkage can be processed in crossovers (CO) or noncrossovers (NCO) (Hamant et al., 2006). Numerous studies (Drouaud et al., 2006; Mezard, 2006; Paigen and Petkov, 2010; Massy, 2013) have demonstrated that the meiotic recombination occurs preferentially in recombination hotspots whose distribution is not random. These recombination hotspots can be expressed as early and late recombination nodules, which appear, respectively, at zygotene and pachytene and are high in recombinase proteins (RAD51, DMC1) (Pawlowski and Cande, 2005; Osman, et al. 2011). The RAD51 protein is highly conserved in eukaryotes and is homologous to the bacterial recombination protein RecA (Shinohara et al., 1992). Among eukaryotes, such as *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (Smith and Nicholas, 1998) e *Arabidopsis* Heynh. (Kurzbaue et al., 2012; Pradillo et al., 2012), the role of RAD51 is well established in meiotic recombination. However, the presence of RAD51 signals immunolocalized in unpaired or partially paired chromosomes in *Lilium longiflorum* Thunb. (Terasawa et al., 1995; Anderson et al., 1997), rats (*Mus musculus*) (Ashley et al., 1995), humans (*Homo sapiens*) (Barlow et al., 1997) and maize (*Zea mays* L.) (Franklin et al., 1999) has indicated that this protein may perform a second function during meiotic prophase, that is, indirectly assist in the identification/detection of chromosome homology. Also, foci related to homology are not necessarily associated with meiotic recombination, since the signals observed in zygotene (homology) disappear after formation of the SC and others may be formed in pachytene (recombination) (Franklin et al., 1999; Moens et al., 2002). Besides these aspects, it has been observed that some factors can affect the frequency of recombination, such as genetic background and morphological and developmental differences (Wijnker and Jong, 2008), size, morphology of chromosomes and the AT/GC ratio (Jensen-Seaman et al., 2004; Borodin et al., 2008) as well as external factors, like environmental influences by temperature (Francis et al., 2007; Wijnker and Jong, 2008), chemical treatment or physical stress (Preuss and Copenhaver, 2000; Wijnker and Jong, 2008). Thus, quantifying the signals of the RAD51 protein at pachytene enables the identification of genotypes with high frequency of signals of homology and recombination. This information is important for the early stages of breeding programs, as it can probably be reflected in the genetic variability of progenies. Similarly, at final stages, it can assist in the indication of genotypes with low recombination frequency, i.e., which are more homogeneous and desirable for the release as new cultivars. Therefore, it is also possible to determine the influence of certain parental genotypes on the recombination rate of their hybrids. The aim of this study was to identify and compare the frequency of homology and recombination hotspots in sexual diploid and tetraploid species, apomictic tetraploid species and interspecific hybrids of *Brachiaria*, by immunolocalization of the RAD51 protein.

Results

With the exception of observations in leptotene, differences were found in the visualization of the signals of RAD51 for

apomictic species (*B. decumbens* and *B. brizantha*) compared with the other species and hybrids.

Immunofluorescence with the AtRAD51 antibody revealed rare or diffuse signals of RAD51 at leptotene. This observation was similar in all species and hybrids (Fig. 1). Signals of RAD51 were distributed throughout the nuclear area, at zygotene (Fig. 2). A constant characteristic observed throughout this subphase was the presence of individual signals in unpaired partially paired chromosomes or (Fig. 2A) and two adjacent signals of RAD51 in paired chromosomes (Fig. 2B). In pachytene, when homologous chromosomes are paired in all their length, signals of RAD51 were located between chromosome axes, in the area occupied by the SC and supposedly in recombination nodules (Fig. 3), showing their influence on the meiotic recombination. After the crossover at pachytene, the diplotene is characterized by the dissolution of the synaptonemal complex between homologous chromosomes. At this and in the later stages, no signal of RAD51 was evident (data not shown). The mean and the number of RAD51 signals in zygotene and pachytene of species and hybrids of *Brachiaria* are presented in Table 1. There was a significant difference in RAD51 signals, at 95% level, between apomictic plants (*B. decumbens* and *B. brizantha*) and the other species and hybrids evaluated during zygotene and pachytene (Fig. 4, Table 1). During zygotene, whose signal possibly corresponds to the homology hotspots, the mean number of signals was higher in apomictic plants (53.87 in *B. decumbens* and 60.13 in *B. brizantha*). Nonetheless, we cannot affirm that the two species are different to each other. The mean number of signals of RAD51 in hybrid plants overlapped that of *B. ruziziensis* (diploid and tetraploid), which means that their means are equal. *B. decumbens* and *B. brizantha* also had the highest mean values of RAD51 signals at pachytene, when meiotic recombination occurs. These data suggest that the apomictic tetraploid species evaluated herein have a greater number of recombination sites (*B. decumbens*, 85.80 and *B. brizantha*, 83.87) when compared with the sexual species (*B. ruziziensis*), both at diploid and tetraploid levels. The sexual species *B. ruziziensis*, diploid and tetraploid, and the interspecific hybrids evaluated showed similar means for RAD51 sites.

Discussion

Using immunolocalization to investigate the behavior and the frequency of the RAD51 recombinase during prophase I of meiosis in species and hybrids of *Brachiaria*, we observed the presence of the protein in leptotene, zygotene and pachytene, confirming its different roles in different meiosis stages. The signals are rare and diffuse at leptotene, when the chromosomes begin to condense and pair. Similar results were reported in rat spermatocytes (Barlow et al., 1997; Sharan and Kuznetsov, 2007) and maize (Franklin et al., 1999; 2003; Pawlowski et al., 2003). During zygotene, we observed that signals of RAD51 are closely coordinated with the search for homology and the pairing of homologous chromosomes in *Brachiaria* spp. and hybrids analyzed. The signals identified in paired and unpaired chromosomes correspond to the sites of comparison of homologous DNA sequences, while the adjacent signals identified only in paired chromosomes continued until the synapse, after the formation of the synaptonemal complex, confirming the observations in maize by Franklin et al. (1999) and Pawlowski et al. (2003). The genome of most plants contain large amounts of repetitive sequences, thus the search for homology should restrict pairing of truly homologous sequences, preventing

Table 1. Variation range and mean number of signals of RAD51 during meiotic prophase in species and hybrids of *Brachiaria*.

Species	Variation range and mean of signals of RAD51	
	Zygotene	Pachytene
<i>B. ruziziensis</i> 2x	22 $\overline{36.93}$ 53 c	35 $\overline{43.53}$ 63 b
<i>B. ruziziensis</i> 4x	35 $\overline{38.73}$ 44 bc	38 $\overline{47.33}$ 61 b
<i>B. decumbens</i>	40 $\overline{53.87}$ 74 a	69 $\overline{85.80}$ 103 a
<i>B. brizantha</i>	48 $\overline{60.13}$ 76 a	70 $\overline{83.87}$ 98 a
H1 Hybrid (<i>B. ruziziensis</i> x <i>B. decumbens</i>)	36 $\overline{44.73}$ 59 b	36 $\overline{47.20}$ 63 b
H2 Hybrid (<i>B. ruziziensis</i> x <i>B. brizantha</i>)	36 $\overline{39.13}$ 47 bc	37 $\overline{43.73}$ 57 b
H3 Hybrid (<i>B. ruziziensis</i> x <i>B. brizantha</i>)	36 $\overline{41.93}$ 50 bc	47 $\overline{51.47}$ 57 b

*Means followed by different letters in the same column are significantly different by LSD-Bonferroni test at 95% confidence.

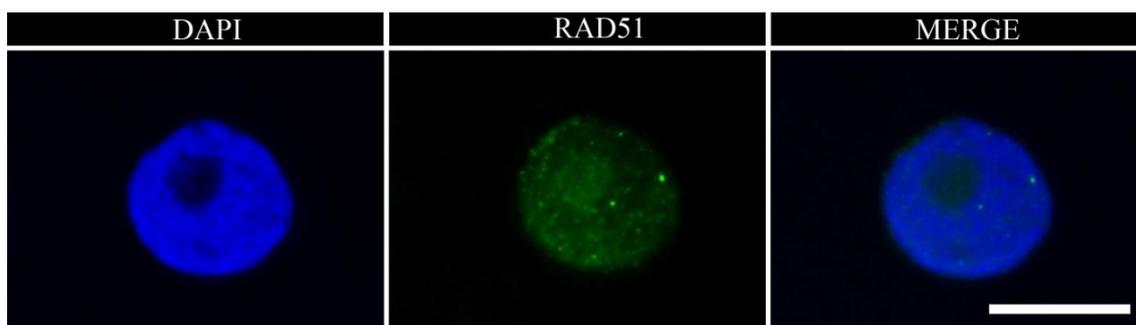


Fig 1. Immunolocalization of RAD51 with diffuse signals at leptotene in tetraploidized *Brachiaria ruziziensis*. The scale bar represents 10 μ m.

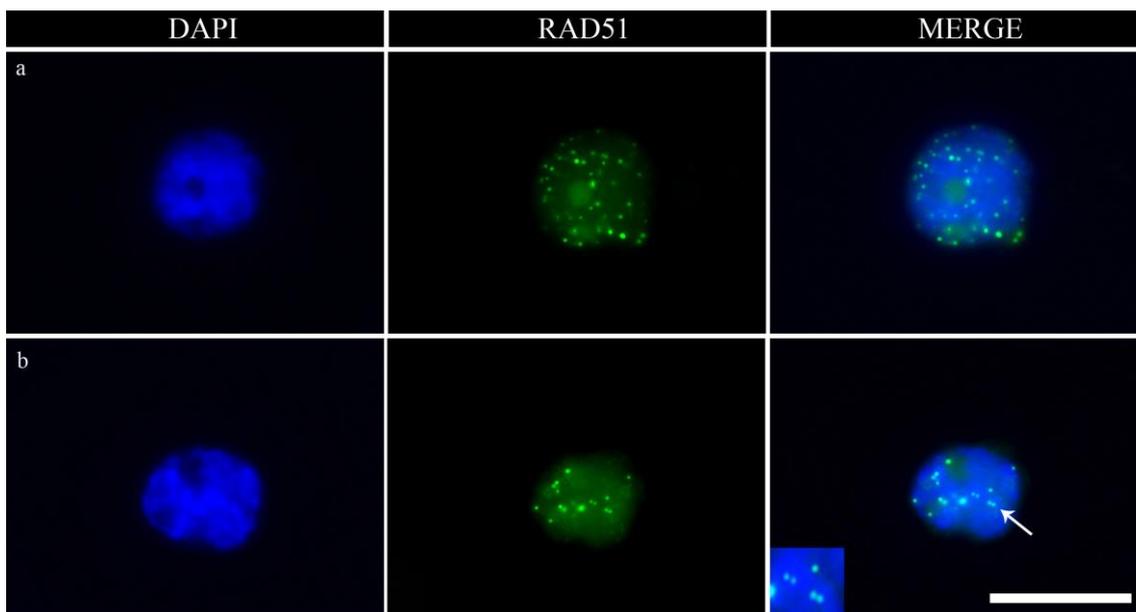


Fig 2. Distribution of the RAD51 signals at zygotene in tetraploidized *Brachiaria ruziziensis*. a) Individual signals of RAD51 in unpaired or partially paired chromosomes. b) Two adjacent signals of RAD51 in paired chromosomes (Arrow and in detail). The scale bar represents 10 μ m.

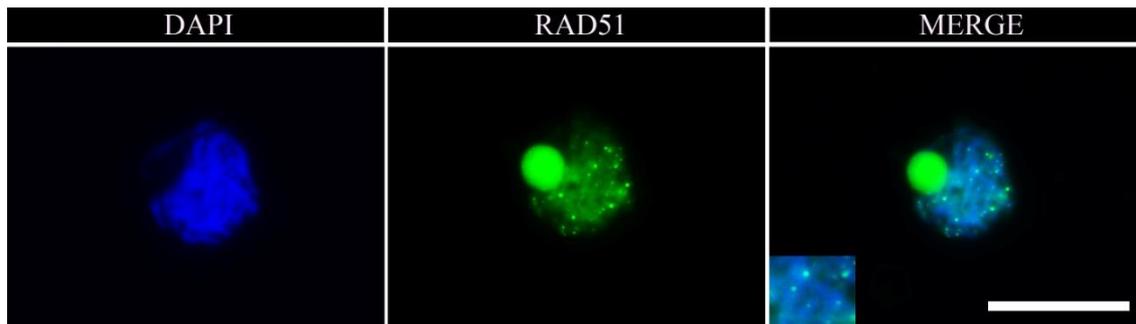


Fig 3. Distribution of the RAD51 signals at pachytene in the *Brachiaria* H1 hybrid. The scale bar represents 10 μ m.

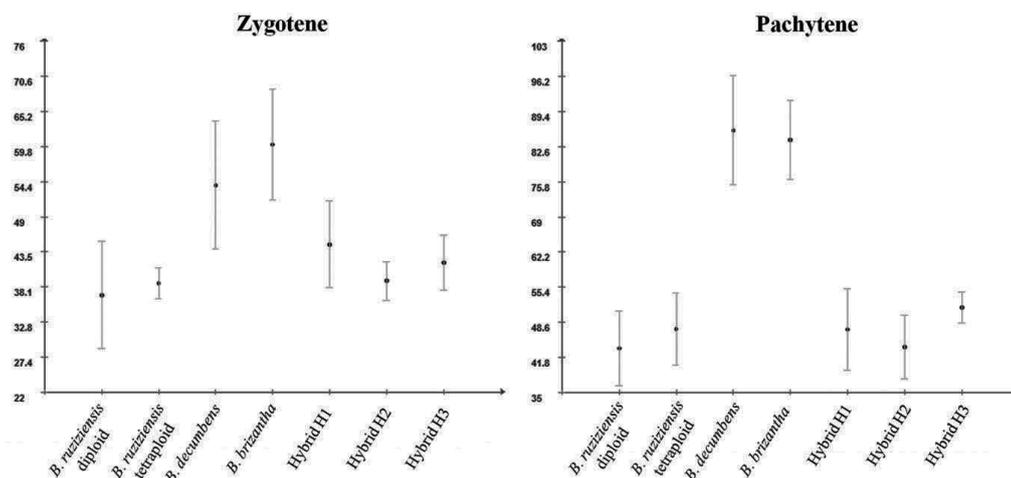


Fig 4. Mean and standard deviation (black symbol and gray bar) of RAD51 signals in species and hybrids of *Brachiaria* evaluated at zygotene and pachytene.

the pairing between non-homologous chromosomes. Studies on mutant species indicated that RAD51 is required for efficient chromosome pairing and its absence results in pairing and synapsis of non-homologous chromosomes. Works with double mutants of maize (*Zea mays rad51*) evidenced changes in meiosis, such as reduced homologous pairing, synapsis of non-homologous chromosomes and reduced in bivalents at diakinesis (Li et al., 2007). Similarly, *Arabidopsis* rad51 mutant plants exhibit univalent rather than bivalent chromosomes at metaphase I, due to defective pairing and absence of chiasmata (Li et al., 2004). In addition to these aspects, a number of other independent recombination mechanisms (telomeres, centromeres, euchromatic/heterochromatic regions, epigenetic mechanisms/modifications of chromatin and non-coding RNAs) work together to facilitate the recognition of homology and the pairing of homologous chromosomes (Da Ines et al., 2014). The highest mean number of signals of RAD51 in tetraploid and apomictic species, both at zygotene and pachytene, can be correlated with genome size or chromosome length. Among the species studied, the diploid *B. ruziziensis* has a genome size estimated at 615 Mbp C⁻¹ and is considered much smaller than the tetraploid species *B. decumbens* and *B. brizantha*, which have genome with 1633 and 1404 Mbp C⁻¹, respectively (Ishigaki et al., 2010). The size of the haploid set also varies among species, the diploid *B. ruziziensis* presents 18.22 μ m, *B. brizantha*, 36.52 μ m and *B. decumbens*, 36.49 μ m (Bernini and Marin-Morales, 2001).

These factors may partially explain the observed variation in the number of signals between species and hybrids analyzed. The relationship of the apomictic reproduction mode with the recombination should also be considered in these evaluations. Apomixis described in *Brachiaria* is aposporic, *Panicum*-type and facultative (Valle et al., 2009), so that, eventually, some flowers can use the sexual reproduction mode. For the development of viable seed in these plants, pollination is necessary to for endosperm development, characterizing this way, pseudogamy (Carneiro and Dusi, 2002). Therefore, the higher frequency of observed recombination signals and probably the greatest variability is not transmitted to the apomictic embryo unless the plant uses sexual mode of reproduction. A previous study (Ambiel et al., 2008) had already shown, using molecular analysis, a greater genetic variability within facultative apomictic populations of *B. brizantha*. This variability in apomictic tetraploid plants can be important when used in the interspecific crosses with the sexual species *B. ruziziensis*, artificially tetraploidized. Thus, it is possible to produce new genetic combinations and incorporate desirable traits in interspecific hybrids selected by the breeder.

One explanation for the similarity in the number of recombination foci between the diploid sexual and tetraploid plants of *B. ruziziensis* may be the synthetic origin of tetraploid plants. The induction of chromosome duplication is performed to facilitate its use in interspecific hybridization

with *B. brizantha* and *B. decumbens* (Ishigaki et al., 2009). To this end, antimitotic substances are used, such as colchicine, which is widely used in forage (Pereira et al., 2012). Polyploidization is usually accompanied by genetic and epigenetic changes that lead to an extensive restructuring of the genome at all levels, for example, chromosomal repatterning, gene silencing, new patterns of gene expression and transposon action (Schifino-Wittmann, 2004). Therefore, the frequency of recombination of tetraploidized *B. ruziziensis* plants may have been affected by the recent process of artificial polyploidization and its consequences. Another factor to consider is the complexity of chromosome pairing in polyploid species. In autotetraploid species, pairing is characterized by multiple forms of chromosomal association with frequent formation of multivalents (Hamant et al., 2006). Risso-Pascotto et al. (2005) and Pagliarini et al. (2008) reported the occurrence of univalent to tetravalent at diakinesis in artificially tetraploidized species of *B. ruziziensis*, which can explain the lower number of signals of RAD51 when compared to the diploid species of *B. ruziziensis*. Additionally, the overlap of mean values for the RAD51 signals of hybrid plants and diploid and tetraploid plants of *B. ruziziensis* means that hybrids have lower mean of recombination sites than expected, if there were an additivity of genomes. According to Ozkan et al. (2003), the non-additive change occurring during allopolyploidization may be an adaptation to genomic stress caused by hybridization and polyploidy. Some works with different species have reported the effects of hybridization, including loss of DNA content and genomic rearrangements (Kashkush et al., 2002; Leitch and Bennett, 2004; Nunes et al., 2013). *Brachiaria* hybrids evaluated cytologically showed evidence of genomic elimination during microsporogenesis. Likewise, Risso-Pascotto et al. (2004) identified in triploid hybrid of *B. brizantha* x *B. ruziziensis* that the genome of *B. ruziziensis* was eliminated by asynchrony during meiosis. Similar behavior was reported by Mendes-Bonato et al. (2006) in 29.7% of the pollen mother cells in hybrid tetraploids of *B. brizantha* x *B. ruziziensis*. And thus, considering the possibility of genetic and epigenetic rearrangements, after hybridization, as a result of genomic conflicts according to the combination of two different parental genomes within a single nucleus (Riddle and Birchler, 2003), it is possible that some genetic rearrangement has influenced the recombination frequency in hybrid plants, resulting in a number of signals of RAD51 equal to the *B. ruziziensis*, used as the female parent in the interspecific crosses. Our study is an initiative to map the recombination landscape in *Brachiaria* species, by assessing the number and distribution of proteins located in recombination nodules, associated with CO. Still, it is essential to evaluate other proteins, since the RAD51 tends to mark all the breaks (DBSs), which will not necessarily be effective in CO. Alternatively, it should be evaluated proteins required for the formation of the class I CO (Osman et al., 2011; Phillips et al., 2013).

Materials and Methods

Plant material

The immunolocalization technique was applied to sexual plants of *Brachiaria ruziziensis* (cultivar Kennedy, $2n = 2x = 18$ and tetraploidized population obtained by Embrapa Dairy Cattle, Juiz de Fora, Minas Gerais State, Brazil, $2n = 4x = 36$), to apomictic *Brachiaria brizantha* (cultivar Marandu, $2n = 4x = 36$) and *B. decumbens* (cultivar Basilisk, $2n = 4x = 36$) and interspecific hybrids from the crossing *B. ruziziensis* x *B.*

decumbens (identification: H1, $2n = 4x = 36$) and *B. ruziziensis* x *B. brizantha* (identification: H2 and H3, $2n = 4x = 36$). In crossings, the artificially tetraploidized sexual species *B. ruziziensis* was used as female parent and apomictic species *B. brizantha* and *B. decumbens*, as male parents. The hybrid certification was performed by using molecular markers.

Cytological procedures

Slide preparation was performed according to Armstrong and Osman (2013) with some modifications. Preselected anthers at early stage (leptotene, zygotene and pachytene) were incubated in 60 μ L of enzyme digestion mixture (0.1g cytohelicase, 0.375g sucrose, 0.25 g of polyvinylpyrrolidone) at 33°C for 20 minutes in moist chamber. Next, the anthers were ground on a slide coated with poly-L-lysine, to which we added 10 μ L 0.2% Triton. The slide was again incubated at 33°C and, after 8 minutes, we added 20 μ L 4% paraformaldehyde. The slide was air dried and kept at room temperature until the application of immunolocalization technique.

Immunolocalization

Slides were washed (PBS + 0.1% Triton) and incubated with 100 μ L blocking buffer (PBS + 3% BSA - bovine serum albumin) for 45 minutes in moist chamber at room temperature. Subsequently, we applied on the slides 25 μ L/slide of the primary antibody (Rat anti-*Arabidopsis thaliana* RAD51) at a dilution of 1:100, according to Kurzbauer et al. (2012). Slides were kept in moist chamber for at least 16 hours at 4°C, washed in PBS and detected with the secondary antibody (Goat anti-rat IgG FITC) at 1:100 dilution. Slides were kept in moist chamber for 1 hour at 37°C under aphotic conditions. After washing in PBS, slides were counterstained and mounted in solution of DAPI (4', 6-diamidino-2-phenylindole)/Vectashield H-1000 (1: 100).

Image capture and processing and statistical analysis

Slides were analyzed with an epifluorescence microscope Nikon Eclipse E400 at excitation/emission wavelengths of 358/461nm for DAPI and 495/515nm for FITC. Images were captured using NIS-Elements BR software and processed in Adobe Photoshop CS3. Signals of the RAD51 recombinase were quantified with the aid of the count tool of Image Tool 3.0. For each phase (zygotene and pachytene) of the species/hybrids, we counted 15 meiocytes. Data obtained in the evaluation were subjected to analysis of variance. Means of population were grouped by LSD-Bonferroni test (Ramalho et al., 2012).

Conclusion

We conclude that, also in the species and hybrids of *Brachiaria* studied, the RAD51 protein is related to the search for chromosomal homology, at zygotene, and genetic recombination, at pachytene. The apomictic species (*B. decumbens* and *B. brizantha*) have higher mean number of signals of RAD51 at zygotene and pachytene when compared to other species and hybrids. The artificially tetraploidized *B. ruziziensis* showed a mean number of signals of RAD51 similar to the diploid species *B. ruziziensis*, because of the polyploidization effect. In hybrids, we observed mean equal to that of *B. ruziziensis*, used as female parent in crosses.

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