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Evidence of programmed cell death during microsporogenesis in an interspecific *Brachiaria* (Poaceae: Panicoideae: Paniceae) hybrid

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ABSTRACT. Morphological changes have been investigated during plant programmed cell death (PCD) in the last few years due to the new interest in a possible apoptotic-like phenomenon existing in plants. Although PCD has been reported in several tissues and specialized cells in plants, there have been few reports of its occurrence during microsporogenesis. The present study reports a typical process of PCD during meiosis in an interspecific *Brachiaria* hybrid leading to male sterility. In this hybrid, some inflorescences initiated meiosis but it was arrested in zygotene/pachytene. From this stage, meiocytes underwent a severe alteration in shape showing substantial membrane blebbing; the cytoplasm became denser at the periphery; the cell nucleus entered a progressive stage of chromatin disintegration, and then the nucleolus disintegrated, and the cytoplasm condensed and shrunk. The oldest flowers of the raceme showed only the callose wall in the anthers showing obvious signs of complete sterility.

Key words: Apoptosis, *Brachiaria* hybrid, Chromatin fragmentation, Meiosis, Male sterility, Programmed cell death

INTRODUCTION

Programmed cell death (PCD) occurs during the development of animals and plants and is responsible for the selective elimination of unwanted cells (Obara et al., 2001). It consists of the activation of highly regulated physiological mechanisms leading to cell suicide (Jones, 2001). The importance and occurrence of PCD during the life cycle of plants is well established (Greenberg, 1996) and can occur on a local or large scale (Pennell and Lamb, 1997). The best known examples of PCD in plants happens in male gametogenesis where after microspore release from the tetrad, tapetal cells begin to degenerate providing nutrients for microspore growth (Wu and Cheung, 2000). Other examples occur during xylem differentiation (Lam et al., 1999), degeneration of the suspensor during embryo development, synergid degeneration to allow egg cell fertilization, elimination of the three haploid megaspores at the end of megasporogenesis, and anther dehiscence (Beers, 1997). According to Wu and Cheung (2000), reproductive development is a rich arena to showcase PCD in plants.

The grass subfamily Panicoideae includes approximately 208 genera grouped in several tribes; among these, Paniceae, with more than 110 genera, and Andropogoneae, with 85 genera, are the largest and most important ones (Watson and Dallwitz, 1992). Comparative studies of floral development in Andropogoneae demonstrated that the formation of unisexual spikelets is uniform. All florets initiate both pistil and stamen primordia. In florets destined to be male, cell death occurs in the subepidermal layers of the gynoecium after the formation of a gynoecial ridge. In florets destined to be female, there is no apparent cell death in stamens, but growth ceases after anther formation (Le Roux and Kellogg, 1999; Malcomber and Kellogg, 2006).

In unisexual flowers, sex is determined by the selective repression of growth or the abortion of either male or female reproductive organs by PCD (Beers, 1997). But, the mechanism by which this process is controlled is still poorly understood (Kater et al., 2001). Pollen abortion after normal meiosis was reported by Kawanabe et al. (2006) as being due to suppression of a gene that control normal PCD in the tapetum cells. The results demonstrated that the PCD signal commences at the tetrad stage and that the proper timing of PCD in the tapetum is essential for normal microsporogenesis. Although genetically PCD occurs in some specific plant cells, including the formation of unisexual flowers in several plant species (Beers, 1997), there is little documentation of this phenomenon occurring during meiosis in hermaphrodites because this process impairs the primordial function of creating sperm cells to generate new life, thus operating against evolution. PCD during meiosis will cause cell degeneration and sterility. This paper reports the evidence of PCD during meiosis in an interspecific *Brachiaria* hybrid.

MATERIAL AND METHODS

Cytological studies were carried out in an interspecific hybrid (BS 05). The female parent is an artificially tetraploidized sexual accession of *B. ruziziensis* (2n = 4x = 36) and the male parent is *B. brizantha* cv. Marandu (2n = 4x = 36). This hybrid was produced by controlled pollination at the Embrapa Beef Cattle Center (Campo Grande, State of Mato Grosso do Sul, Brazil), and reproduces sexually. Due to positive attributes it is being used in crosses in the breeding program. Site characteristics of cultivation in the field in Brazil were: climate type Aw: tropical humid savanna; average annual precipitation = 1526 mm; average temperature = 22°C;

altitude = 520 m; latitude = 20° 28' S; longitude = 55° 40' W; poor dark red latossol soil (59% sand; 8% silt; 33% clay; pH = 4.2).

Inflorescences for meiotic study were collected and fixed in a mixture of 95% ethanol, chloroform and propionic acid (6:3:2) for 24 h, transferred to 70% alcohol and stored under refrigeration until use. Microsporocytes were prepared by squashing and staining with 0.5% propionic carmine and analyzed under light microscopy. Photomicrographs were made with a Wild Leitz microscope using Kodak Imagelink - HQ, ISO 25 black and white film.

RESULTS

Microsporogenesis was analyzed in 18 inflorescences of the hybrid. Meiotic abnormalities related to irregular chromosome segregation were found in low frequency in almost all of the materials examined of flowers and anthers with normal morphology. In these flowers, microsporocytes showed the normal spherical shape with cytoplasm homogeneously stained, and meiosis progressed normally untill haploid microspore formation (Figure 1). Two inflorescences, however, showed typical features of sterility: flowers were light and smaller than the normal ones as also were their anthers. In these flowers, meiosis progressed until zygotene/pachytene and pollen grains were not found. Dissection of flowers located in the basal portion of the racemes showed only cells in leptotene demonstrating a diffuse nucleus and a dense nucleolus located near the nuclear envelope (Figure 2a). From this phase on, meiocytes underwent an increased and severe alteration in shape. Initially, they showed some alterations in cell shape at the membrane level, forming substantial blebbing, which increased sharply along the maturation of anthers in the raceme (Figure 2b-f). In these cells, the inner portion of the cytoplasm was less dense than the outer, and meiosis stopped in zygotene/pachytene. In the middle portion of the raceme, the cell nucleus entered a progressive stage of chromatin disintegration (Figure 2g) and later disappeared completely. In this stage, only the nucleolus was intact and strongly stained (Figure 2h). From this stage on, along the racemes, the degenerative process of meiocytes increased which was visible by the continuous change in cell shape (Figure 2h,i). The blebbing then disappeared. The cytoplasm condensed and shrunk, becoming intensely stained (Figure 2i,j). A space was formed between the cytoplasm and the cell wall, and the nucleolus could no longer be observed any more (Figure 2k). Meiocytes acquired a hexagonal shape. Flowers located in the terminal portion of the raceme showed only a callose wall inside the anthers (Figure 2l), an evident sign of complete sterility.

DISCUSSION

In the interspecific BS 05 hybrid (2n = 4x = 36), polyploidy predispose it to abnormalities during meiosis. Multiple chromosome pairing, precocious chromosome migration to the poles, laggard chromosomes, and a micronucleus were frequently found during both meiotic divisions. These abnormalities were also reported in other interspecific *Brachiaria* hybrids recently analyzed (Risso-Pascotto et al., 2005). However, the typical feature of sterility observed in the two inflorescences of BS 05 has never been recorded among the *Brachiaria* hybrids studied here. The progress of cell degeneration inside the anthers in these inflorescences suggests the occurrence of PCD. Plant cells and tissues undergo various types of PCD. Each type processes and removes dead cells differently. According to Lee and Chen (2002), microtuning probably cre-

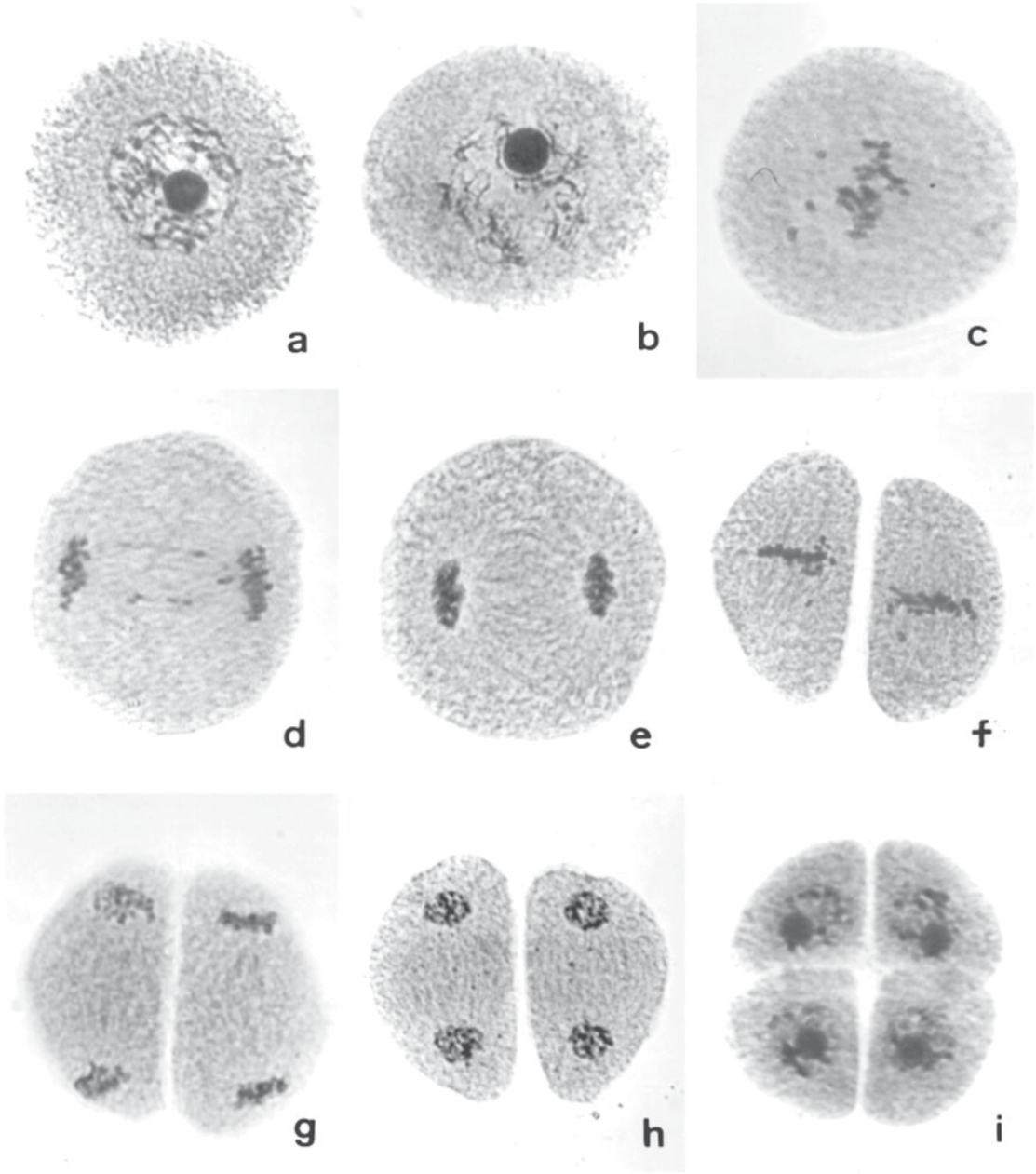

Figure 1. Aspects of normal microsporogenesis in fertile flowers of the hybrid. Notice in all the meiotic stages, the spherical shape of meiocytes, homogeneous stainability of cytoplasm, and the progress of meiotic division untill the tetrad stage. a, b) Different stages of pachytene. c) Metaphase I with precocious chromosome migration to the pole. d) Anaphase I with laggard chromosomes. e) Telophase I. f) Metaphase II with precocious chromosome migration to the poles. g) Late anaphase II. h) Telophase II. i) Tetrad of microspores. Magnification: 400X.

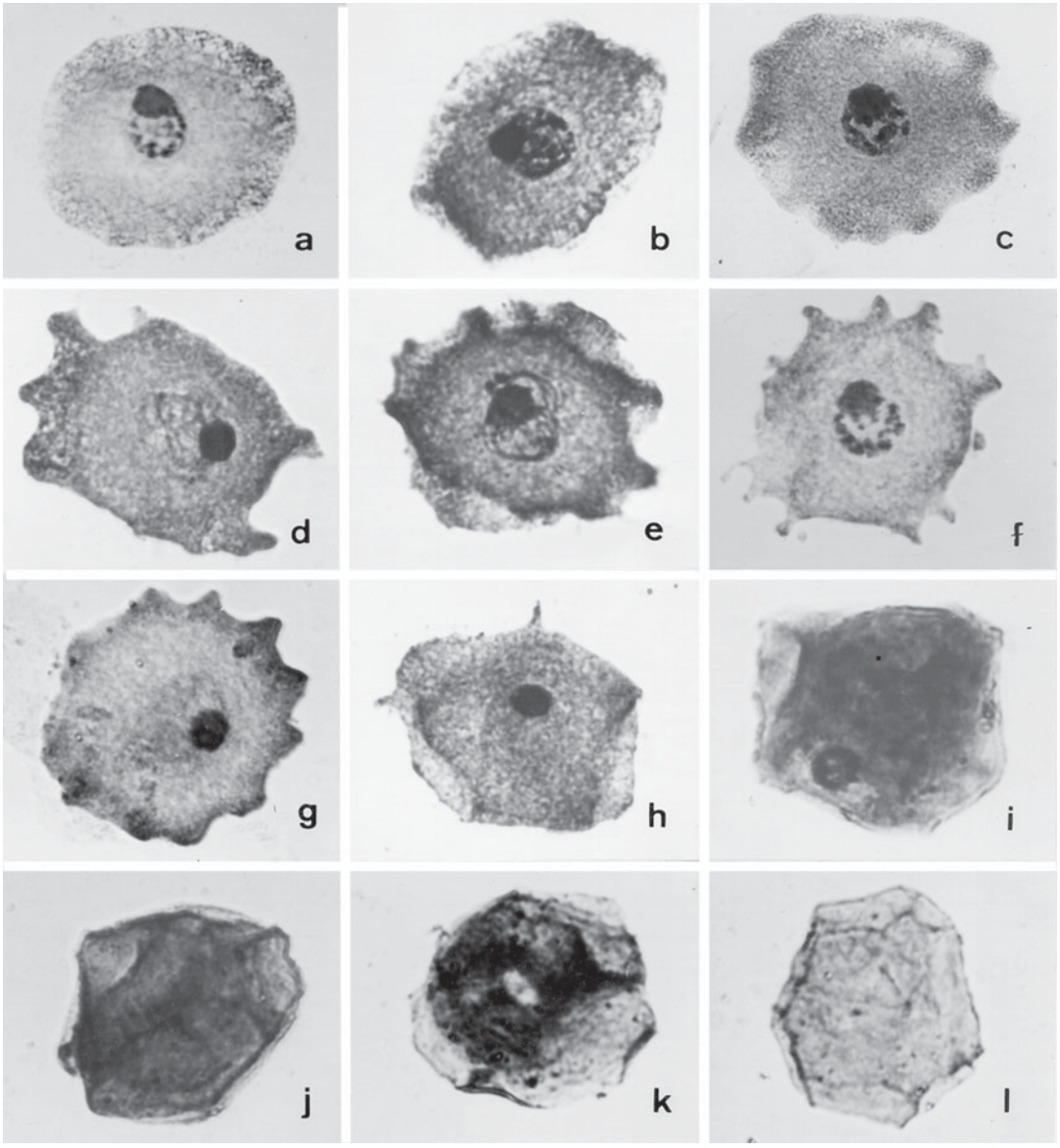

Figure 2. Aspects of programmed cell death during microsporogenesis in the hybrid under light microscopy. a) Meiocyte in leptotene. b to f) Meiocytes in leptotene (b, c) and pachytene (d, e) showing increased morphological alteration in cell shape by membrane blebbing. Observe that initially the membrane undergoes little deformation (b) which increases sharply (c to f) before chromatin disintegration. Note also that during cell shape alteration, the cytoplasm is more condensed in the periphery. g) Chromatin disintegration in a meiocyte with blebbing. Notice that nucleus contour is not well evidenced. h) A more advanced stage of chromatin disintegration. Note that blebbing disappeared, nucleolus remains intensely stained, and cell shape became altered. i) Meiocyte showing only the nucleolus and cytoplasm intensely stained. j) Meiocyte completely devoid of chromatin and nucleolus. k, l) Meiocytes in advanced stage of degeneration showing progressive lack of stainability (k) to complete absence of content (l). In l, only the callose wall is observed. Magnification: 400X.

ates different cell death mechanisms for specific purposes in plants, dictating its own specific set of morphological and biological variations. The occurrence of typical animal apoptosis in plant PCD remains undetected. Apoptotic cell death is considered rare during plant PCD since the presence of cell walls precludes the absorption of apoptotic bodies by neighboring cells. According to Danon et al. (2000), instead of phagocytosis, the process in plants may involve autolysis. The occurrence of blebbing in meiocytes as observed in this *Brachiaria* hybrid could be facilitated by the absence of cell wall in these cells. The callose wall is incipient and flexible in young meiocytes, allowing plasma membrane expansion.

Apoptosis in animal cells is characterized by chromatin condensation and fragmentation, cytoplasmic condensation and vacuolization, cytoplasmic membrane blebbing and disassembly of the cell into apoptotic bodies that are rapidly engulfed by phagocytes or neighboring cells (Wyllie et al., 1980). It is usually associated with the activation of nucleases that degrade the chromosomal DNA first into large fragments (50-300 kb) and subsequently into multiple fragments corresponding to the internucleosomal spacing of about 180 bp (Walker and Sikorska, 1994). The occurrence of oligonucleosomal DNA cleavage has been also reported in some plant tissues undergoing PCD (Kirnos et al., 1997; Marubashi et al., 1999; Kawai and Uchimiya, 2000; Coimbra et al., 2004). In the *Marsilea*, megaspore death involves chromatin pycnosis (Bell, 1996), which, however, is a morphological marker for both apoptotic and nonapoptotic PCD (Schwartz, 1992). In the inflorescences analyzed here, chromatin pycnosis did not occur but was replaced by DNA disintegration.

Evidence of DNA fragmentation in plants through light microscopy shown here has been reported during pathogen-induced PCD (Ryerson and Heath, 1996), also during tracheary element differentiation (Mittler et al., 1995) and root-cap cell shedding (Wang et al., 1996). According to the literature, PCD in plants could also be caused by defense mechanisms in response to pathogens, stress and developmental cues (Danon et al., 2000; Beers and McDowell, 2001; Zaina et al., 2003). *Brachiaria* inflorescences are susceptible to at least pathogens known to seriously compromise seed production: ergot diseases, caused by *Claviceps sulcata* (Fernandes et al., 1995), which reduces seed production late in the season and *Ustilago operta*, which has recently been reported to cause severe flower damage in *Brachiaria brizantha* (Verzignassi et al., 2004). These fungi are known to produce alkaloids that affect the metabolism of cells although there is no evidence of this effect in the present study. In the present hybrid, meiocyte death shows morphological changes characteristic of apoptosis in animal cells suggesting that PCD was responsible for male sterility. Considering the structural changes observed, such as chromatin disintegration and the occurrence of blebbing, meiocyte cell death observed here is only slightly different from apoptosis in animal cells as reported for carrot suspension cells (McCabe et al., 1997) and *Actinidia* female flowers (Coimbra et al., 2004).

Due to increased interest in the PCD phenomenon in plants, great efforts have been made in this field. From an application perspective, the ability to regulate cell death in plants may have important uses in agriculture and post-harvest industries. Suppression of PCD induced by biotrophic pathogens could minimize disease symptoms, and inhibition of cell death during senescence could extend the shelf-life of crops and vegetables (Lam et al., 1999). Another important aspect of PCD is related to the mechanism of reproductive isolation of species through hybrid lethality. Marubashi et al. (1999) showed apoptotic features in cells of interspecific hybrid seedlings of *Nicotiana glutinosa vs N. repanda*, where condensation of chromatin and fragmentation of nuclei were the main apoptotic characteristics. In the present interspecific

Brachiaria hybrid, reproductive isolation is not the cause of apoptotic symptoms because only two of several inflorescences examined showed complete sterility caused by PCD.

Evidence of PCD during microsporogenesis in the *Brachiaria* genus has been presented by Mendes-Vieira et al. (2005) in one tetraploid accession (2n = 4x = 36) of *B. brizantha* where meiosis was complete but severe abnormalities were observed during the process and no viable pollen was produced. After irregular chromosome orientation at metaphase plates and absence of anaphases, chromosomes were rejoined into pycnotic nuclei in telophases with abnormal disposition in the cytoplasm. Microspores initiated degeneration as soon as they were released from the tetrad, acquiring irregular shapes, and becoming intensely stained. The nucleus degenerated and microspores gave rise to an amorphous mass. Pollen grains were not found in the affected inflorescences.

The genus *Brachiaria*, native to the African tropical savannas, has achieved a great significance as a pasture grass in many tropical and subtropical countries, especially in Brazil. To increase genetic variability in the genus, an extensive breeding program based on intra- and interspecies hybridization has been underway at the Embrapa Beef Cattle Center since 1988. Hybridization in the genus *Brachiaria* is not easy primarily due to ploidy differences among accessions and species, and to reproduction by apomixis (Valle and Savidan, 1996; Miles et al., 2004). However, interspecific hybrids with agronomic potential have been identified (Miles et al., 2004; Pereira et al., 2005). These must produce a good amount of viable seeds to be widely adopted as cultivated pastures, thus the absolute need for production of fertile pollen grains. This hybrid, selected due to some positive attributes, belongs to the sexual crossing block to produce superior mother plants at Embrapa Beef Cattle. Seed production has never been outstanding, but the behavior described in this paper, if proven frequent, shall hamper its future use as a genitor in the program. Pollen viability as well as seed production will need to be closely monitored in order to ascertain its value as a mother plant.

REFERENCES

Beers EP (1997). Programmed cell death during plant growth and development. *Cell Death Differ.* 4: 649-661.

Beers EP and McDowell JM (2001). Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues. *Curr. Opin. Plant Biol.* 4: 561-567.

Bell RP (1996). Megaspore abortion: a consequence of selective apoptosis? *Int. J. Plant Sci.* 157: 1-6.

Coimbra S, Torrao L and Abreu I (2004). Programmed cell death induces male sterility in *Actinidia deliciosa* female flowers. *Plant Physiol. Biochem.* 42: 537-541.

Danon A, Delorme V, Mailhac N and Gallois P (2000). Plant programmed cell death: a common way to die. *Plant Physiol. Biochem.* 38: 647-655.

Fernandes CD, Fernandes ATF and Bezerra JL (1995). "Mela": uma nova doença em sementes de *Brachiaria* spp, no Brasil. *Fitopatol. Bras.* 20: 501-503.

Greenberg JT (1996). Programmed cell death: a way of life for plants. *Proc. Natl. Acad. Sci. USA* 93: 12094-12097.

Jones AM (2001). Programmed cell death in development and defense. *Plant Physiol.* 125: 94-97.

Kater MM, Franken J, Carney KJ, Colombo L, et al. (2001). Sex determination in the monoecious species cucumber is confined to specific floral whorls. *Plant Cell* 13: 481-493.

Kawai M and Uchimiya H (2000). Coleoptile senescence in rice (*Oryza sativa* L.). *Ann. Bot.* 86: 405-414.

Kawanabe T, Ariizumi T, Kawai-Yamada M, Uchimiya H, et al. (2006). Abolition of the tapetum suicide program ruins microsporogenesis. *Plant Cell Physiol.* 47: 784-787.

Kirnos MD, Alexandrushkina NI and Vanyushin BF (1997). Apoptosis in cells of the initial leaf and coleoptile of wheat seedlings: internucleosomal fragmentation of genome and synthesis of 'heavy' oligonucleosome-size DNA fragments. *Biochem.* 62: 864-869.

Lam E, Pontier D and del Pozo O (1999). Die and let live - programmed cell death in plants. *Curr. Opin. Plant Biol.* 2: 502-507.
Le Roux LG and Kellogg EA (1999). Floral development and the formation of unisexual spikelets in the Andropogoneae (Poaceae). *Am. J. Bot.* 86: 354.
Lee RH and Chen SCG (2002). Programmed cell death during rice leaf senescence is nonapoptotic. *New Phytol.* 155: 25-32.
Malcomber ST and Kellogg EA (2006). Evolution of unisexual flowers in grasses (Poaceae) and the putative sex-determination gene, TASSELSEED2 (TS2). *New Phytol.* 170: 885-899.
Marubashi W, Yamada T and Niwa M (1999). Apoptosis detected in hybrids between *Nicotiana glutinosa* and *N. repanda* expressing lethality. *Planta* 210: 168-171.
McCabe PF, Levine A, Meijer PJ, Tapon NA, et al. (1997). A programmed cell death pathway activated in carrot cells cultured at low density. *Plant J.* 12: 267-280.
Mendes-Vieira D, Mendes-Bonato AB, Pagliarini MS and Valle CB (2005). Abnormal meiotic behavior in *Brachiaria brizantha* (Poaceae) leading to microspore degeneration. *Caryologia* 58: 396-402.
Miles JW, Valle CB, Rao IM and Euclides VPB (2004). *Brachiaria* - grasses. In: Warm-Season (C4) Grasses (Sollenberger L, Moser L and Burson B, eds.). ASA, CSSA, SSSA, Agronomy Monograph, Madison, 745-783.
Mittler R, Shulaev V and Lam E (1995). Coordinated activities of programmed cell death and defense mechanisms in transgenic tobacco plants expressing a bacterial proton pump. *Plant Cell* 7: 29-42.
Obara K, Kuriyama H and Fukuda H (2001). Direct evidence of active and rapid nuclear degradation triggered by vacuole rupture during programmed cell death in *Zinnia*. *Plant Physiol.* 125: 615-626.
Pennell RI and Lamb C (1997). Programmed cell death in plants. *Plant Cell* 9: 1157-1168.
Pereira AV, Valle CB, Souza Sobrinho F, Ledo FJS, et al. (2005). Selection of interspecific *Brachiaria* hybrids to intensify milk production on pastures. *Crop Breed. Appl. Biotechnol.* 5: 99-104.
Risso-Pascotto C, Pagliarini MS and Valle CB (2005). Meiotic behavior in interspecific hybrids between *Brachiaria ruziziensis* and *Brachiaria brizantha* (Poaceae). *Euphytica* 145: 155-159.
Ryerson DE and Heath MC (1996). Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. *Plant Cell* 8: 393-402.
Schwartz LM (1992). Insect muscle as a model for programmed cell death. *J. Neurobiol.* 23: 1312-1326.
Valle CB and Savidan Y (1996). Genetics, cytogenetics, and reproductive biology of *Brachiaria*. In: Brachiaria: biology, agronomy, and improvement (Miles JW, Maass BL and Valle CB, eds.). CIAT/ EMBRAPA, Cali, 147-163.
Verzignassi JR, Urben AF, Fernandes CD and Valle CB (2004). Ocorrência de *Ustilago operta* em sementes de *Brachiaria brizantha* no Brasil. *Fitopatol. Bras.* 26: 423.
Walker PR and Sikorska M (1994). Endonuclease activities, chromatin structure, and DNA degradation in apoptosis. *Biochem. Cell Biol.* 72: 615-623.
Wang H, Li J, Bostock RM and Gilchrist DG (1996). Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. *Plant Cell* 8: 375-391.
Watson L and Dallwitz MJ (1992). The grass genera of the world. CAB International, Wallingford.
Wu H and Cheung AY (2000). Programmed cell death in plant reproduction. *Plant Mol. Biol.* 44: 267-281.
Wyllie AH, Kerr JF and Currie AR (1980). Cell death: the significance of apoptosis. *Int. Rev. Cytol.* 68: 251-306.
Zaina G, Morassutti C, De Amicis F, Fogher C, et al. (2003). Endonuclease genes up-regulated in tissues undergoing programmed cell death are expressed during male gametogenesis in barley. *Gene* 315: 43-50.