Apomixis in *Brachiaria decumbens* Stapf

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Cover: *Brachiaria decumbens* in the field

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Chapter 1

Introduction

APOMIXIS

In angiosperms the sexual reproduction cycle, with the fusion of the male and female gametes, results in genetic recombination that produces species variability. However, some species developed the ability to pass sexuality and to produce seeds by means of asexual reproduction known as apomixis.

The term apomixis was first used to designate, in general, the asexual mode of reproduction in plants and animals (Winkler 1908). In this definition modes of asexual reproduction like vegetative propagation and vivipary were included. Nowadays, apomixis is used in the narrow sense meaning asexual seed formation or agamospermy (Bashaw 1980; Nogler,1984) and implies embryo formation without fusion of the male and female gametes. Apomictic seeds carry the genetic information to make a progeny identical to the mother plant. It is in other words a way to clone plants through seeds. Apomictic reproduction was found in more than 300 species of plants from 36 families and it is very common in Poaceae (Gramineae), Asteraceae (Compositae), Rosaceae e Rutaceae (Bashaw 1980, Hanna and Bashaw 1987).

Importance of apomixis

Apomixis is a trait with a single genetic control (reviewd in Savidan 2000). Therefore there is a possibility of being manipulated through the conventional techniques of plant genetic improvement as well as through genetic engineering. Because of the prospect of controlling apomictic reproduction, interest on this trait has increased, and the impact of apomixis discussed by many authors (Bashaw, 1980; Hanna and Bashaw, 1987; Asker and Jerling, 1992; Koltunow, 1993; do Valle and Savidan 1996). There are many advantages of the use of apomictic seeds in cultures where apomixis naturally does not occur. Some consequence of the controlled use of apomixis in agriculture are:

- Fixation of hybrid heterosis vigour through seed propagation
- The possibility of propagation, and storage by seeds of cultures that are now propagated vegetativelly
- Small farmers will be able to produce their own seed for uncounted number of generations.
- Simplification of commercial hybrid seed production with consequent reduction in cost.

- Simplification of breeding programs with consequent higher number of cultivars adapted to local environments.

Do Valle and Savidan (1996) also point to an adaptative advantage of apomixis with its capacity to restore fertility in plants with chromosomal abnormalities that would otherwise be sterile.

All put together shows that once the mechanism of apomixis could be dominated and transmitted to other plants, it will be of economic importance for agriculture and reflect in social area.

Apomixis and Amphimixis - types of reproduction

Apomixis is a process that occurs in the female part of the flower, in the ovary and more specific in the ovule. Apomixis has a strong connection with the sexual reproduction pathway. Therefore the comparison of both apomixis and sexuality is the obvious way to interpret this special phenomena.

Amphimixis

In amphimixis or sexual reproduction, meiotic divisions play a key role providing the reduction of chromosome number to form a reduced gametophyte and embryos are formed after the fusion of reduced female and male gametes getting a copy of both sets of chromosomes. Sexual reproduction in angiosperms has three main steps: the development of the male and female gametes inside differentiated structures, pollen and embryo sac, the gametophytes; the event of double fertilisation and the embryo development.

The female gametophyte or embryo sac is developed into the ovule, inside the ovary. The most common embryo sac in angiosperm is the Polygonum type (Willemse and van Went, 1984) (Fig. 1). In brief: this embryo sac originates from an unreduced (2n) sub epidermal cell of the ovule, the archesporial cell and turns into the megaspore mother cell. During the megasporogenesis this cell is surrounded by callose, and undergoes two meiotic divisions to form a linear tetrad of reduced cells, the megaspores (n). Three megaspores, except the chalazal one are surrounded by callose. The three megaspores close to the micropyle degenerate while the chalazal one survives as a functional megaspore. During the megagametogenesis the nucleus of the functional megaspore undergoes three successive mitoses to form an embryo sac with eight reduced nuclei (n). After celularization seven cells are formed: three antipodal cells at the chalazal pole; a central cell

containing two polar nuclei; two synergids and an egg cell at the micropylar region forming the egg apparatus. Later the polar nuclei will fuse to form a 2n central cell nucleus. This embryo sac is a meiotic embryo sac because it is a result of reduction of chromosome number through meiosis. During anthesis the double fertilisation occurs with one of the male gametes (n) fusing with the egg cell (n) to form the zygote nucleus (2n) and the other male gamete fusing with the polar nuclei (2n) to form the endosperm nucleus (3n). The embryo will develop from the zygote and the endosperm will be used as source of nutrients to support the embryo development and is essential to good quality of the seed. The progeny resulting from the sexual reproduction is genetically different from their progenitors.

Apomictic reproduction

While in sexual reproduction, meiotic divisions provides the reduction of chromosome number and embryos carry a copy of maternal and paternal sets of chromosomes in result of fertilisation, in apomictic reproduction meiosis is partially or completely avoided and fertilisation by male and female gamete fusion does not occur. Embryos that carry only the maternal set of chromosomes are formed from unreduced cells in one of the mechanisms described here.

Two different mechanisms of apomictic reproduction are described (Nogler 1984, Asker and Jerling 1992): adventitious embryony and "gametophytic apomixis", including diplospory and apospory (Fig. 1).

Adventitious embryony also known as nucellar embryony is a sporophytic form of agamospermy and involves direct formation of embryos from somatic cells of the nucellus or inner integument (Lakshmanan and Ambegaokar, 1984). In *Citrus*, adventitious embryos develop usually when the reduced embryo sac is already formed in mature ovules (Koltunow 1993). These embryos will use the endosperm formed after the fertilisation of the polar nucleus as a source of nutrients, establishing a competition with the zygotic embryo (Asker and Jerling 1992, Koltunow 1993).

"Gametophytic apomixis" is characterised by apomeiosis (Nogler 1984), that is, the structure of the gametophyte, an embryo sac, is formed without the complete meiosis reduction. Two steps are clear distinct from the sexual pathway, the development of unreduced embryo sacs from unreduced cells, followed by the development of embryo(s) without fertilisation. The endosperm development can be formed autonomously from the central cell and is independent of fertilisation. Or endosperm development needs a fusion

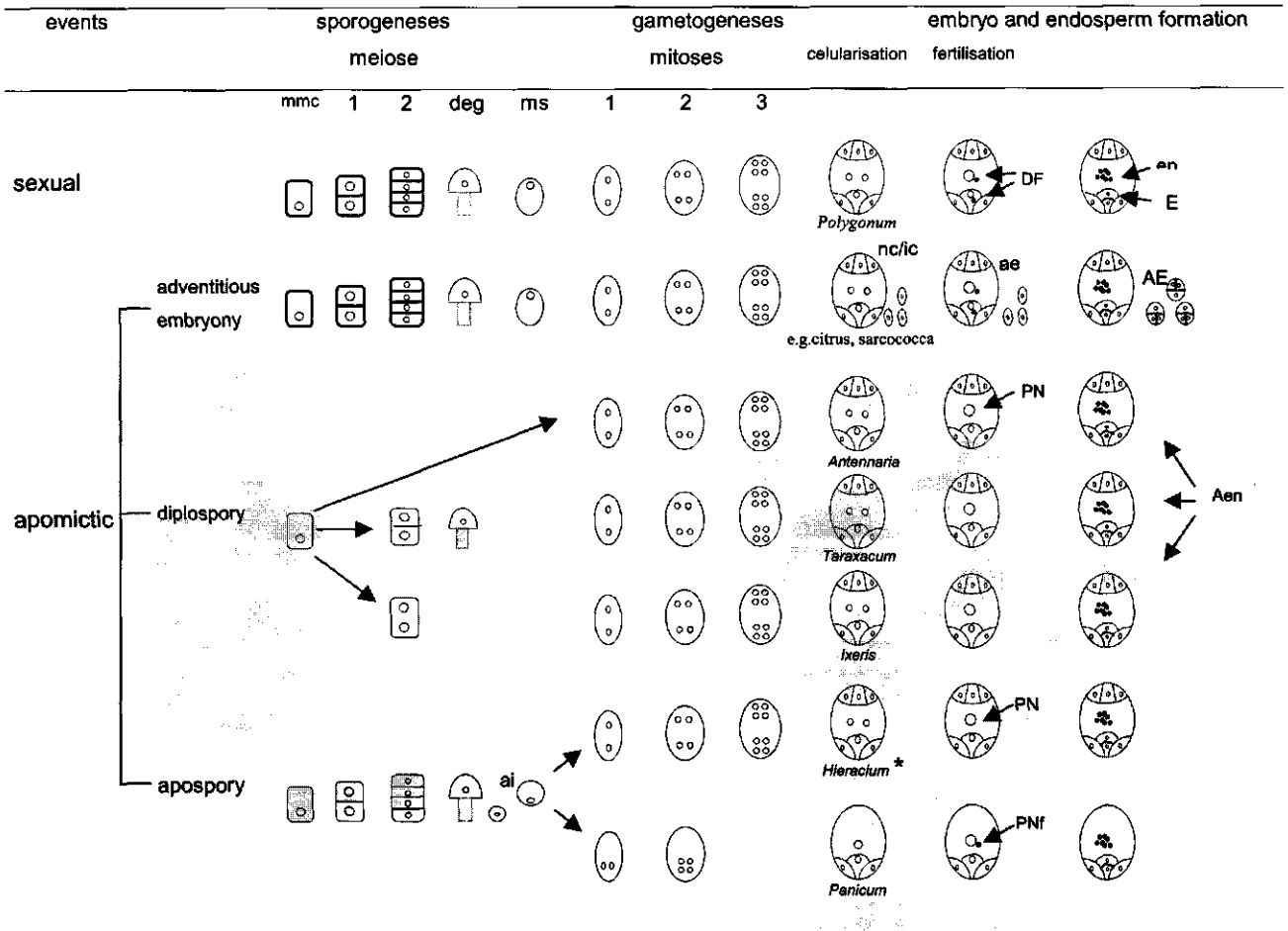
of sperm cell with the central cell nucleus, a fusion product of the two polar nuclei (Asker and Jerling, 1992).

"Gametophytic apomixis" is divided in two main types according to the origin of the unreduced embryo sac, diplospory and apospory (Nogler 1984, Asker and Jerling 1992). In diplospory, embryo sacs develop from the unreduced megaspore mother cell. This cell undergoes mitosis directly or enter meiosis but do not complete it and the nucleus remains unreduced. Three types of embryo sac containing eight nuclei can be formed: *Antennaria*, *Taraxacum* and *Ixeris*. Autonomous endosperm formation is common among diplosporous apomicts particularly in the Asteraceae (Compositae) (Koltunow 1993).

In apospory, unreduced embryo sacs originate from somatic cells of the ovule, generally cells of the nucellus. Embryo sacs developed by apospory are mainly of two types: the eight-nucleate bipolar embryo sac of the *Hieracium* type or the four-nucleate monopolar embryo sac of the Panicum type. Other numbers of nuclei were described like the five nucleated embryo sac in *Paspalum minus* (Bonilla and Quarin 1997). Generally endosperm formation needs fertilisation of the polar nucleus but *Hieracium* is autonome (Koltunow 1993). Many embryo sacs can be present in one ovule.

The aposporic embryo sac development of the Panicum type

In early stages of ovule development, some somatic cells of the nucellus begin to differentiate from the others. They can be distinguished from the other nucellar cells by their large cell size, large nuclear size, dense cytoplasm, vacuolation and thick cell wall (Nogler, 1984; Koltunow 1993, Naumova and Willemse, 1995). These cells are called aposporous initials or just apospore initials. After vacuolation, one or more of these cells undergo directly through two mitotic divisions to form the 4-nucleated unreduced embryo sacs of the Panicum type. The four nuclei are positioned in one side of the embryo sac, making easy to distinguish from the meiotic eight nucleated embryo sac of the Polygonum type. Commonly, in the ovule of the apomictic plant, embryo sac development is arrested by the time of meiosis or even just after meiosis. Sometimes a meiotic embryo sac can be developed and the egg cell fertilised. A progeny by means of sexual reproduction can be present next to the apomictic seed. Therefore the development of aposporic embryo sac can potentially coexist with sexual process (Nogler 1984, Asker and Jerling 1992).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e.g.citrus, sarcococca
apomictic
diplospory
Antennaria
PN
Taraxacum
Aen
Ixeris
apospory
ai
Hieracium *
PN
PNf
Panicum

The Panicum type of embryo sac is very common in the Poaceae family, which has economically important grasses .

Apomictic embryo development

Discussion on how the unreduced egg cell avoid being fertilised lead to different ideas that range from the fact that embryogenesis occurs before the arrival of the pollen tube (Nogler 1984), or the presence of a physical barrier in unreduced egg cell wall that inhibits fertilisation (Savidan 1989; Asker and Jerling 1992). Both ideas have support in only part of the species with diplospory or apospory. Fertilisation is known to happen in many species where the embryo development depends on polar nuclei fertilisation. The cell wall hypothesis could fit the system of some plants like *Pennisetum ciliare* (Vielle et al. 1995). But the hypothesis could not be applied to all plants as in *Pennisetum* hybrids (Chapman and Busri 1994), and *Panicum maximum* (Naumova and Willemse, 1995) of which the apomictic mature embryo sac egg apparatus is comparable to the sexual one. Some physiological or metabolic changes difficult to detect may exist leading to a change of egg apparatus structure and of early embryo formation. As suggested by Vielle et al. (1995), some activation process may be taking place before any change in cell wall.

Facultative apomixis

The majority of the apomicts are facultatives (Nogler 1984, Asker and Jerling 1992). Because they have unreduced embryo sacs and in some ovules also reduced embryo sacs, they are capable after fertilisation to sexual and apomictic modes of reproduction (Koltunow 1993). Nogler (1984) thinks that all apomicts have the potentiality to reproduce by means of the sexual mechanism once there is always some residual sexuality even in the species where apomixis seems to be obligate.

Figure 1. Comparison between the mechanisms of apomictic and sexual reproduction. Based on Nogler 1984, Asker and Jerling 1992 and Koltunow 1993. **mmc** megaspore mother cell **deg** cell degeneration; **ms** functional megaspore; **DF** double fertilisation; **en** endosperm; **E** embryo; **e** embryonic initials; **ai** apospore initials; **AE** adventitious embryos; **Aen** autonomous endosperm; **PNf** polar nuclei fertilisation. **PN** polar nuclei fusion only; (*) Although *Hieracium* has autonomous endosperm formation other species have polar nuclei fertilisation.

In case of apospory, where the mechanisms are distinct, the facultative character of apomixis is clear and both modes of reproduction can coexist even in the same ovule. In fact, the time of initiation of embryo sac is important. If there is a delay in differentiation of aposporous initials compared with the reduced embryo sac, both types of embryo sac coexist (Nogler 1984).

Aspects of environment and aspects of timing influencing apomixis

Sexual reproduction as well as apomixis is expressed in a facultative apomict. By definition (Nogler 1984) the degree of apomixis is the percentage of offspring due to apomixis and it is the final expression of apomixis. There are two main points of regulation during the reproductive pathway that will influence the degree of apomixis: 1-. the number of ovules with absence of meiosis and with a complete development of meiotic embryo sacs and, 2- the need of egg cell and / or central cell fertilisation.

In diplospory, the moment of apomixis induction is obviously interfering in the degree of apomixis once there is a switch of the sexual pathway to the apomictic one in the megaspore mother cell. In apospory, the effect of the moment of apomixis induction is less clear. Apospore initials can differentiate before, during or after the megaspore mother cell enter and complete meiosis. The competition between reduced and unreduced embryo sacs would be affected by this timing of aposporous induction as well as the occurrence of multiple induction resulting in multiple embryo sacs (Nogler, 1994). However it remains unclear if the differentiation of the apospore initials is the factor that inhibits meiotic embryo sacs development.

Timing is important regarding to the early maturation of the aposporic embryo sac compared to the sexual one, like in *Panicum maximum* (Savidan, 1982), that could lead, as a consequence, to failure in fertilisation, influencing apomixis degree in favour of apomixis.

The degree of apomixis can be influenced by external factors like environmental conditions (Bashaw 1980, Nogler 1984a, Asker and Jerling 1992), as climate factors or nutrient levels. Water stress, temperature and photoperiod are climate factors that affect the expression of apomixis in *Dichanthium aristatum* (Knox 1967). Nutrient levels (Cox and Ford 1987) and inorganic salts (Gounaris et al. 1991) also influence the degree of apomixis. Bell (1992) considers the flow of nutrients an important component of the parthenogenetic development of the unreduced egg cell. The nature of pollen source can be also related to higher or lower percentage of fertilised egg cells

and affect the degree of apomixis (Nogler 1984a). All those factors seem to be related to each other and a different production of nutrients can be expected under different climate and biotope conditions.

Aspects of ploidy in relation to diplospory and apospory development

Apomixis is often associated with polyploidy and tetraploidy is the commonest level of ploidy, while the related sexuals are mainly diploid (Asker and Jerling 1992). It seems that somehow expression of the genes that lead to apomictic development may be possible just under polyploidisation. In *Brachiaria ruziziensis,* chromosome doubling by autotetraploidisation of sexual diploid through colchicine treatment, result in obligately sexual tetraploid plants that did not have apomixis (Gobbe et al. 1981, Sween et al 1981). Same result was found to the colchicine induced tetraploid *B. brizantha* (Pozzobon et al. 1999, Pinheiro et al. 2000). However Quarin and Hanna (1980) obtained autotetraploid plant of *Paspalum hexastachyum* that has apospory. Evidence that apomixis is associated to induced polyploidy is not given.

Callose as a marker for apomictic development

In angiosperms, during the early stages of ovule and anther development callose, a β-1,3 glucan, is deposited as a wall around meiocytes, and during meiotic divisions around microspores and megaspores. Although its role is not fully understood, this callose wall provides a physical barrier isolating those cells from the neighbouring cells. In addition, monosaccharides are released after the digestion of the callose by calase and may be used for further development. In diplospory and apospory, callose deposition follows a different pattern during ovule development. Many apomicts like diplosporous *Elymus* (Carman et al. 1991) and *Tripsacum* (Peel et al. 1997) do not show callose or show just a micropylar cap of callose like aposporous *Poa* (Naumova et al. 1993), *Panicum maximum* (Naumova and Willemse 1995) and *Pennisetum squamulatum* and buffelgrass (Peel et al. 1997). Carman and co-authors (1991) consider that the lack of callose around megaspore mother cells in apomicts could be a consequence of expression of apomixis or even the factor that causes it.

Expression of apomixis on the male side

In apomicts pollen development can also be aberrant. A disturbance during male meiosis is frequent even in plants that need fertilisation of the polar nuclei in order to produce viable seeds (Asker and Jerling 1992). Meiotic anomalies with consequent abortion of microspores or pollen or decrease in pollen fertility may occur (do Valle and Savidan 1996).

Genetic basis of diplospory and apospory and regulation of apospory

Research on inheritance of apomixis through the analysis of the progeny resulting from crosses between apomictic and sexual plants, lead to the conclusion that both apospory and diplospory are controlled by few genes.

Some initial work suggested two genes controlling apomixis in diplosporous *Parthenium argentatum* (Powers, 1945) and in aposporous buffelgrass (Taliaferro and Bashaw, 1966).

Later, a model of one dominant gene regulating apospory was suggested by Savidan (1982) for *Panicum.* In this model, the proposed genotype for sexual diploids is aa, for the induced autotetraploids (sexual) is aaaa and for the apomictic autotetraploids is Aaaa. This model fits the inheritance observed in other aposporic grasses like the one from the genus *Brachiaria* (Ndikumana 1985, do Valle and Savidan 1996). Also the model of one dominant gene can be used for *Cenchrus ciliaris* (Sherwood et al 1994). Diplospory in *Tripsacum* seems to be controlled by a cluster of linked genes that fit the one-dominant-gene hypothesis (Grimanelli et al. 1996). Diplospory in *Taraxacum* seems to be controlled by a single locus (Mogie 1988).

Using Brachiaria as a model, Miles and do Valle (1996) hypothesise the existence of unknown number of segregating genes, which, to some degree or other, modify the expression of a single gene conferring the potential for apomictic reproduction.

Expression of apospory involves two major steps of regulation: the differentiation of the apospore initial and the autonomous development of the unreduced egg cell and endosperm development. Asker and Jerling (1992) considered that the regular inhibition of meiosis should be under genetic control while the egg cell development could be a consequence of physiological and developmental changes due to the expression of apomictic genes. In addition to the genetic control, there are two main questions to consider about apomictic genes as suggested by Koltunow 1993: proteins

resulting from expression of apomictic genes have a function absent in plants with sexual reproduction or more likely, these proteins initiate events in sexual reproduction and may have their activity modified or another spatial and temporal distribution during development.

Strategies and models for apomixis studies

There are many characteristics observed in plants that can indicate a possible apomictic reproduction. Among them one can consider high level of morphological polymorphism, polyploidy and some degree of pollen abortion and sterility (Czapik 1994). Nevertheless morphological and cytological characterisation are necessary to confirm the actual occurrence of apomixis in the species. For many years such characterisation has been carried out and today in more than 300 species of plants this mode of reproduction was identified (Bashaw 1980, Hanna and Bashaw 1987). Apart of the use of cytological techniques for detection of apomictic plants, observation of all steps in ovule development is important to detect timing and other characteristics of apomictic development. More recently, ultrastructural features of the embryo sac development organisation in gametophytic apomicts, has been observed and compared with the characteristics of the meiotic embryo sacs (Chapman and Busri, 1994, Naumova and Willemse, 1995, Araujo et al. 1997). This studies aim to present data that point changes in structure that could be a consequence or cause of apomixis. Until now, data is not uniform between species and certainly not conclusive. Recently, molecular markers linked to apomixis were identified as a consequence of the genetic manipulation of this trait (Grimanelli et al. 1996, Ozias-Akins et al. 1998, Pessino et al. 1999). Molecular markers can facilitate precocious and large scale detection of apomicts being very useful in analysis of hybrids that otherwise have to be characterised cytologically in a time consuming work.

As apomixis is a factor of single genetic control, there is a possibility of being manipulated through the conventional techniques of plant genetic improvement as well as through genetic engineering techniques.

By conventional genetic improvement two strategies are being followed. Search for natural occurrence in species that apomixis were not described, like in rice (Khush et al. 1994), and introgression of apomixis from apomictic wild relatives, like in maize (Savidan et al. 1994) and pearl millet (Ozias-Akins et al. 1993, 1998). The diplosporous *Tripsacum* is being used to transfer apomixis to maize (Savidan et al 1994, 1996, Grimanelli et al. 1996).

The wild relative of pearl millet, the aposporous *Pennisetum squamulatum* is being used as a pollen donor to introduce apomixis into pearl millet. Molecular markers used in these hybrids show one specific genomic region related to apospory, where genetic recombination was not detected (Ozias-Akins et al. 1998). Yet, untill now, this approach results in plants that can not be used agronomically.

Identification of apomictic related genes, its isolation and characterisation are today the main objective of the research on this area. The control of this trait would allow genetic transformation of plants and the introduction of this mode of reproduction to different species. The identification of genes related or involved with the apomictic reproduction process, has been searched by approaches that include cloning of genes through molecular markers, construction of cDNA libraries, mutagenesis and differential display technique. In *Arabidopsis thaliana,* a species that does not show natural occurrence of apomixis, genes responsible for "apomictic" development are being searched through mutations that modify the female gametophyte development (Ray, 1994; Chaudhary and Peacock, 1994, Vielle-Calzada et al. 1998). So far an apomictic mutant was not found. Some mutants were found that form seeds without fertilisation but endosperm and embryo development are arrested in an early stage (Chaudhury et al. 1997). Induction of apomixis by mutagenesis is also in the program to obtain apomictic plants in rice (Khush et al. 1994).

BRACHIARIA

Brachiaria (Trin.) Griseb. is a grass genus that belongs to tribe Paniceae subfamily: Panicoideae family Poaceae and contains about 100 species that occur mainly in Africa (Renvoize et al. 1996, Dahlgren et al 1985).

The importance of Brachiaria

Some grasses of the genus *Brachiaria* tolerate conditions of low fertility acid soils and they adapt to the poor soils of the savannas in South America (Lapoint and Miles 1992, Fisher and Kerridge 1996, Rao et al 1996). Commercially important cultivars, spread in tropical areas of America, belongs to species like *B. brizantha, B. dictyoneura, B. humidicola, B. ruziziensis* and

particularly *B. decumbens* (Hopkinson et al. 1996). In Brazil, an estimated area of 160 million hectares of grazed pastures raises a cattle population of about 160 million head (Santos Filho 1996). Brachiaria alone accounts for 85% of the cultivated pastures, covering an estimated area of 40 million hectares (Macedo, 1995). Brachiaria pastures have higher forage quality as compared to other tropical grasses (Lascano 1996) and shows higher production when compared with the native savanna (Lapoint and Miles, 1992).

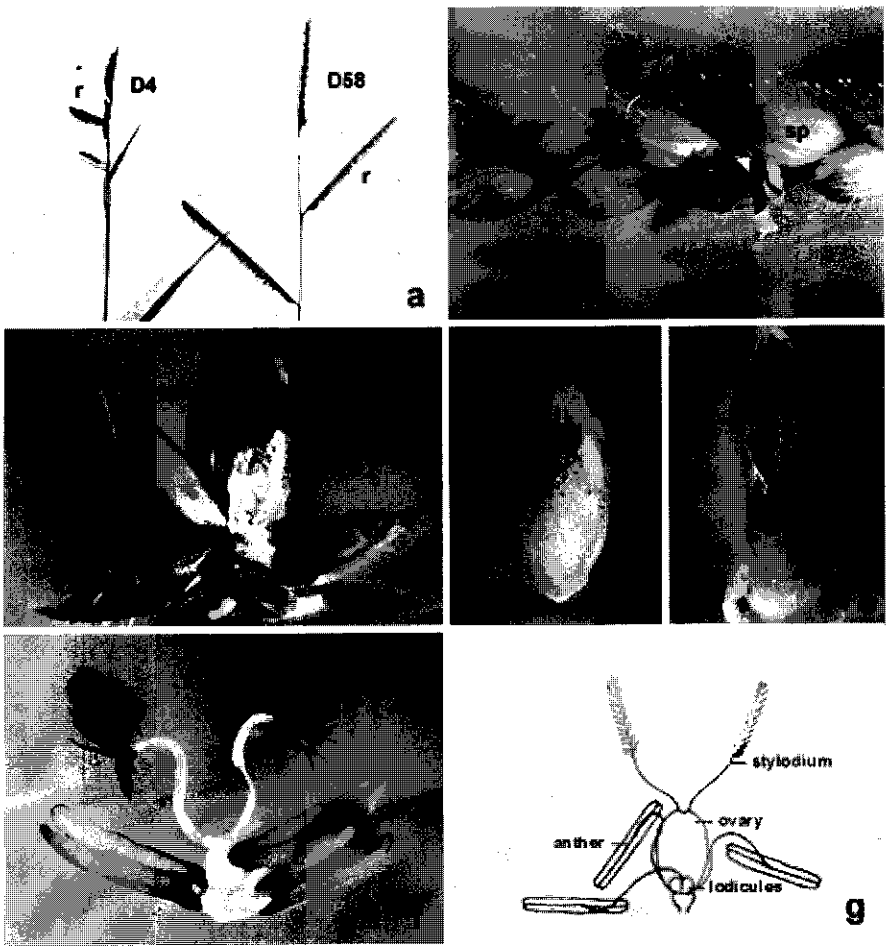

Figure 2. **a**, Inflorescence of *Brachiaria decumbens* sexual (D4) and apomictic (D58), **r** raceme; **b**, detail of the raceme at anthesis, **sp** spikelet; **c**, an open spikelet enclosing the two flowers, the hermaphodite flower (**hf**) and the male flower (**mf**); **d**, a hermaphodite flower closed and **e**, with the palea open; **f**, the flower without the bracteas showing the three anthers and the pistil and the lodicules as schematically represented in **g**.

Commercial propagation of species of the genus *Brachiaria* is by seeds that generally have good germination and plants cover the soil rapid (Hopkinson et al 1996).

Brazil produces annually almost 100,000 t of Brachiaria seeds, most of them being of two commercial species, *B. decumbens* and *B. brizantha* (Hopkinson et al. 1996).

Biology

The inflorescence of Brachiaria is a panicle with 2 to 5 racemes that support the spikelets placed in two series on the raceme (Fig. 2a,b). Each spikelet develops two flowers, a male flower and a hermaphrodite one (Skerman and Riveros 1989, Ndikumana 1985) (Fig. 2c,d,e). The male flower has three anthers and seems to develop normally releasing pollen grain during anthesis. The hermaphrodite flower has three anthers and one ovule (Fig. 2f). Figure 2g shows a schematic representation of the hermaphrodite flower of Brachiaria during anthesis.

The genus *Brachiaria* comprises species that have sexual and apomictic mode of reproduction. Plants are classified as sexual if they have meiotic embryo sacs in their ovaries and few empty and sterile ovaries. Apomictic plants have mainly aposporic embryo sacs, that could be single or multiple, and meiotic embryo sacs in variable degrees, indicating that apomixis is facultative (do Valle 1990). Meiotic embryo sacs are of the Polygonum type, that have eight nuclei distributed in two poles (Fig. 3a). At the end of the divisions, mature embryo sacs have higher number of cells due to the proliferation of the antipodals to 12 (Gobbe et al. 1982; Lutts et al. 1994). Aposporic embryo sacs are of the Panicum type and have four "diploid" (unreduced) nuclei distributed in one pole which cellularise in an egg apparatus and central cell (Fig. 3b).

The need of pollination for seeds development in apomictic Brachiaria was observed by Ngendahayo, 1988. This suggests that in aposporous Brachiaria the male gamete contributes genetically to seed viability only through the endosperm formation, but not to embryo formation and subsequently to the progeny of plants. To verify pollination dependence by direct methods, analysis of endosperm ploidy and morphological analysis of polar nucleus fertilisation should be done.

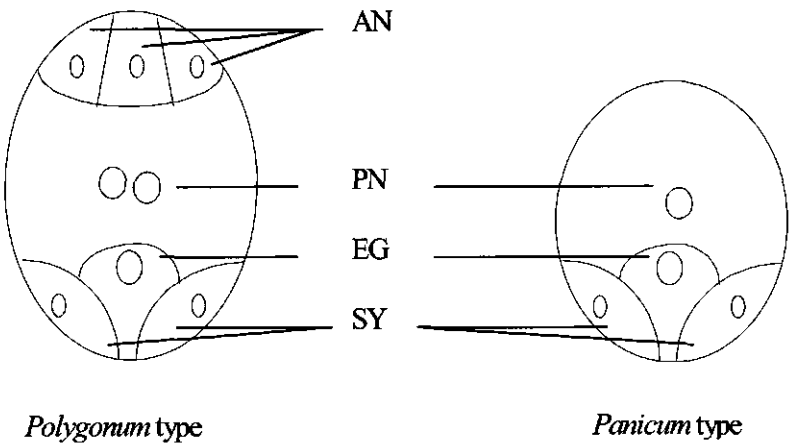

Figure 3. Schematic representation of an ovule showing a Polygonum type of embryo sac (left) and a Panicum type of embryo sac (right). **AN** antipodals, **PN** polar nucleus, **EG** egg cell, **SY** synergids.

The basic number of chromosomes for the genus *Brachiaria* is nine and there are poliploidy intra- and interspecific (do Valle, 1986). Apomictic reproduction predominates and most apomictic plants of Brachiaria sps. are tetraploids (2n=4x=36) while sexual are diploid (2n=2x=18) (do Valle 1991). In the genus *Brachiaria*, irregular meiosis and polyploidy are often associated with apomixis, while regular chromosome pairing, as usually happens in diploids, is associated with sexuality (do Vallle 1986). Facultative apomixis, as represented by percentage of sexuality expressed at the embryological level, is not necessarily equivalent to the field, observations by the presence of off-types (Miles and Valle 1991).

A reproductive calendar was described that relate male and female sporogenesis and gametogenesis in a diploid and an induced tetraploid ecotype of *Brachiaria ruziziensis* (Gobbe et al. 1982) and in a tetraploid apomictic *B. brizantha* and *B. decumbens* and their F1 hybrids with an artificially induced sexual tetraploid *B. ruziziensis*.

Aspects of breeding

Until few years ago improvement of Brachiaria consisted in the selection of superior genotypes among the naturally existing ones (Milles and Valle 1996). The cultivated Brachiaria corresponds to only very few well adapted varieties that are predominantly apomictic and therefore uniform, it has the characteristic of an extensive monoculture that implies in enormous economical risk. Therefore there is a need not only for forage quality improved cultivars but also for cultivars resistant to pest and diseases (Lapoint and Miles1992; Valério 1996;do Valle and Savidan 1996; Milles and Valle 1996).

Introduction of genetic variability in cultivated species in order to develop new varieties was only possible with the identification of sexual genotypes in species compatible with the cultivated ones (Valério 1996). In addition the possibility of genetic recombination trigged studies on inheritance of apomixis in Brachiaria (do Valle and Savidan 1996). Hybrids from crosses between sexual and apomictic plants are difficult to obtain due to the difference in ploidy number in plants with sexual and apomictic mode of reproduction (do Valle 1990). To solve this problem, breeders are using an artificially induced sexual tetraploid of *B. ruziziensis* (Gobbe et al. 1981; Sween et al 1981) as intermediate cross-parent to enhance variability in apomictic tetraploid *B. decumbens* and *B. brizantha* (do Valle 1990; Miles and do Valle 1996). Although this approach permits introduction of some degree of variability into commercial cultivars of Brachiaria, it is a long process. The knowledge about the mechanisms of apomixis and the possibility of controlling this system will extend the possibilities of introducing variability in Brachiaria and will maintain the apomictic character desirable for its propagation.

Inheritance of apomixis in Brachiaria

Just recently it was possible to study inheritance of apomixis in Brachiaria. For this purpose, the development of a sexual artificially induced tetraploid of *Brachiaria ruziziensis* (Sweene et al. 1981; Gobbe et al. 1981) was decisive. It allowed the production of genetic variation through crosses between sexual and apomictic plants at the same ploidy level. Artificially induced sexual tetraploid clones of *B. ruziziensis* were used in crosses with apomicic tetraploid *B. brizantha* cv. Marandu and to *B. decumbens* cv Basilisk and progeny hybrids resulted in a proportion near to 1:1 sexual and apomictic plants. A single gene model was suggested for inheritance of apomixis in

Brachiaria (Miles and Valle 1991; do Valle et al., 1994; do Valle and Savidan, 1996) based on those crosses. Hybrids from second-generation crosses fits this model of a single-gene responsible for apomixis. The recessive character of sexuality was confirmed by the sexual progeny resulting from crosses between sexual diploid or tetraploid plants (Miles and Valle 1991; do Valle and Savidan, 1996).

The use of Brachiaria to study apospory

Apart of the reason to studying Brachiaria reproduction due to its own economical importance and impact in agriculture of tropical countries, there are some characteristics that makes Brachiaria suitable for cytological and molecular studies on apomixis, these are:

- the natural occurrence of sexual and apomictic plants in the same species
- the morphological difference between embryo sacs of sexual and apomictic modes of reproduction that makes clear the distinction between both modes of reproduction.
- the availability of a large characterised germplasm collection and accessions where apomictic or sexual modes of reproduction were characterised.
- the control of apomixis as a single locus (do Valle et al., 1994).
- a plant regeneration protocol from mature embryos (Lenis, 1992) and from meristemas and immature panicles (Carneiro et al. 1995).
- the possibility of genetic transformation through a recently developed protocol based on particle acceleration (Lentini et al. 1999).
- the recent development of a colchicine induced tetraploid of *B. decumbens* and *B. brizantha* (Pinheiro et al. 2000) that will allow interspecific crosses to produce hybrids without the interference of genetic species-specific characters.

In addition to the previous characteristics, progress on studying apomixis in *Brachiaria* added knowledge and possibility of using other techniques that will support the further molecular work .

The aim and contents of this study

The aim of this study is to better understand the regulation of the apomixis reproduction mechanism and to provide more information about aposporous apomixis in *Brachiaria decumbens*. The study will add aspects of Brachiaria reproduction research for efficient breeding.

Chapter 2 describes ovule and pollen development in sexual and apomictic reproduction with respect to morphological markers. The data are represented in calendar and in the reference information for the other chapters. The calendar study was done to detect and to summarize the differences in ovules of aposporous and sexual plants and the development of the apospore initial cells to form an aposporic embryo sac.

Chapter 3 shows differences in aspects of carbohydrate metabolism during ovule development as reflected in the sucrose synthase and the invertase activity and the localisation of the enzymes. This study was performed as a consequence of observations on callose deposition described in chapter 2.

Chapter 4 describes an approach to study general regulation of apomixis based on spatial and temporal distribution of total RNA and poly(A)+ RNA and ribossomal RNA.

Chapter 5 describes a comparative analysis of gene expression in ovules in two stages of development, young and mature, and the spatial and temporal distribution of some of that sequences as observed by *in situ* hybridisations.

Chapter 6 describes expression of the *Arabidopsis* SERK gene during ovule development in sexual and apomictic *B. decumbens* in critical stages of reproductive development.

Chapter 7 presents a general discussion of the data with respect to the apomictic and sexual development and a conclusion.

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Chapter 2

Apomixis in *Brachiaria decumbens* Stapf.: gametophytic development and reproductive calendar

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Abstract

Brachiaria decumbens is a species of forage grasses widely cultivated in tropical areas. This apomictic species reproduces mainly by facultative apospory. A comparison of male and female gamete development between a diploid sexual ecotype and a tetraploid apomictic *B. decumbens* was made. Aspects of sporophytic and gametophytic development such as developmental stage, a number of morphological variations, and callose deposition during meiosis are compiled in a real time reproductive calendar that can be used to select plants as well as reproductive stages. Based on this calendar, part of the differences observed may be related to the tetraploidy. Apomictic embryo sacs are formed earlier than the sexual ones and callose deposition during meiosis follows a different pattern in sexual and apomictic plants. Effects of apomixis are expressed during both male and female development.

Key words: Apomixis, *Brachiaria decumbens*, callose, gametophytic development, reproductive calendar.

Introduction

Brachiaria is a genus of forage grasses originating in Africa. Several species of this genus are now planted throughout tropical grazing areas thereby playing an important role in the economy of many countries. In Brazil, *Brachiaria* alone consists of 85% of the cultivated pastures, covering an estimated area of 40 million hectares (Macedo, 1995). Among the cultivated species, *Brachiaria decumbens* is the most important since it adapts well to the poor and acid soils prevailing in the majority of the savannahs in that country.

The common mode of reproduction of commercial cultivars of *Brachiaria* is by facultative apomixis. In these plants, as the megaspore mother cell is differentiating, some cells of the nucellus enlarge to become apospores. Subsequently these apospores will undergo two mitoses, leading to the formation of unreduced embryo sacs of the Panicum-type in which an embryo is formed without fertilisation. Since apomixis is generally facultative, the megaspore mother cell can continue its development into an embryo sac of the Polygonum-type, which is present alone or together with the aposporic ones in the same ovule. Sexual reproduction involves meiosis in the megaspore mother cell, producing four reduced cells, of which only one will

undergo three mitoses to produce an eight-nucleate embryo sac (Polygonum-type) with an egg cell capable of fertilisation.

Apomixis is not yet a well genetically understood process, although research efforts have increased significantly in recent years (Mogie, 1992; Leblanc and Savidan, 1994; Nogler, 1994; Koltunow et al., 1995; Vielle-Calzada et al., 1996; Pessino et al. 1997). A better understanding of the mechanisms that control apomixis will improve the possibility of using apomictic varieties in breeding programs and facilitate the introduction of apomixis gene(s) into cultivated crops. This may result in large benefits to agriculture, through the fixation of hybrid vigour or simplification of hybrid seed production followed by a reduction in overall costs.

Initial steps towards a better understanding of apomixis in *Brachiaria* started with Gobbe et al. (1982), who established a chronological correlation between the stage of the male and the female sporophyte and gametophyte development in a diploid and induced tetraploid ecotype of *B. ruziziensis*. Both plants had Polygonum-type meiotic embryo sacs. Later, Lutts et al. (1994) reported similar studies for tetraploid apomictic *B. brizantha* and *B. decumbens* and their apomictic F1 hybrids with artificially induced sexual tetraploid *B. ruziziensis*. Both studies presented data describing male and/or female gamete development, but provided no information about sexual *B. decumbens* and little information about other morphological characteristics that identify these stages.

Recently, studies on callose formation around meiocytes established its close relationship with apomixis. In *Poa pratensis*, apomictic megasporocyte development is accompanied by abnormal callose deposition, which is present as a micropylar cap only (Naumova et al., 1993). A similar behaviour was observed in aposporous *Panicum maximum* (Naumova and Willemse, 1995), *Pennisetum squamulatum* and buffelgrass (Peel et al., 1997). In *Brachiaria decumbens* a different pattern of callose deposition was observed by Naumova et al. (1995). These authors described the presence of a complete callose wall around the meiocyte, dyad and tetrad in sexual and apomictic plants. The implication of these and other aspects of apomictic development are not yet clear and further consideration is needed.

This paper describes developmental comparisons between a diploid sexual ecotype and a tetraploid apomictic one in *B. decumbens* with the objectives of establishing morphological and cytological markers for the process of apomixis in *Brachiaria* which could be related to stages in male and female gamete development.

Materials and methods

Plant material

Two accessions of *Brachiaria decumbens* from EMBRAPA/CNPGC were used in all experiments: BRA000191 (D58-cv. IPEAN), tetraploid apomictic with only 16% sexuality at the embryological level, and BRA004430 (D4), diploid sexual (Valle, 1990). Several plants were maintained in pots in a green house with temperatures ranging from 25 ^{0}C during the day to 20 ^{0}C at night, with a 16 h light and 8 h dark regime. Inflorescences from several stages of development were excised and placed in water for preparation in the laboratory.

Measurements

A calibrated ocular microscale was used for measurements of the flowers of different plants. Spikelet length (spl) was measured from the "scar", i.e. where the pedicel is attached to the lower glume, to the tip of the upper glume. Spikelet width (spw) was measured at the maximal spikelet width. Anther length (a) was taken along the long axis of the anther. In the last stages when the anther curves, the curvature was ignored. The length of the pistil (p) was taken from the basal part of the ovary to the tip of the stigma.

The pistils were divided into classes according to their length. Class one consisted of pistils which were less than or equal to 0.3 mm. Subsequent classes (2 to 9) were divided into intervals of 0.3 mm. In class 10 the interval increased to 0.6 mm and class 11 consisted of pistils that were greater than 3.3 mm.

Stür (1986) established that maturation of spikelets starts from the middle of the raceme toward the ends. Therefore, it was assumed that some flowers in the middle of the raceme were in almost the same stage of development. The maturation stage (m) refers to the morphology and colour of the stigma.

Timing

Nine inflorescences at three different stages of development (young, middle and old) were taken from the sexual plants and 14 inflorescences from the apomictic plants. From each of these inflorescences, one raceme was selected and marked. One spikelet was collected from the middle of each raceme. Pistils were excised and the length measured. Either four or seven days afterwards, for each raceme, the spikelet immediately below the one

sampled, in the same row, was also collected. Again the pistils were excised and their length measured. From this data, the duration of growth in pistil could be estimated. The duration of the complete pistil development, in days, was estimated by placing the subsequent pistil length in a time schedule, beginning with the size of the youngest pistil on the first measurement. Other measurements were aligned according to the size of the pistil of each raceme and at the first pistil measurement in relation to the growth of the immediate younger measured pistil. The average length of 2 - 3 pistils was calculated and plotted in a graph according to the time in days. A regression line was calculated making the regression line pass through the origin. Ten spikelets in stage of anthesis were collected from the sexual plants and 10 spikelets from the apomictic plants. Pistils were excised, their length measured and the average sizes of the pistils were calculated for both sexual and apomictic plants. The day of anthesis for both, apomictic and sexual, plants were inferred by plotting those average sizes of the pistils during anthesis on the regression line.

Cytological methods

The developmental stage of the ovule was determined by the following clearing method. Pistils, divided in classes as previously described, were fixed and cleared according to Young et al. (1979) with a few modifications. The pistils were fixed in FAA: 37% formalin, acetic acid, 96%ethanol and water at the ratio 3:3:40:14 for 24 hours, then transferred to 70% ethanol and stored at 4°C. These samples were dehydrated in an ethanol series to 100% ethanol and were then transferred to methyl salicylate (MS) in a ethanol:MS series of 1:1, then 1:3 ethanol:MS for 4h each step and finally, two changes in 100% MS. The material was mounted and observed with a Nikon optiphot differential interference contrast microscope to characterise ovule development and to observe the transition of the ovule to the anatrope position. A total of 128 pistils from the sexual plants and 196 pistils from the apomictic plants, distributed from class 1 to 11 were analysed by this method.

The following methods were used to distinguish between sexual and apomictic development:

1. Callose deposition was observed in young anthers (stages from pollen mother cell to young free microspore) and fresh ovaries by staining with a solution of 0.03% aniline blue (BDH chemicals Ltd., Poole, England) in 0.2% K_3PO_4 .$7H_2O$, pH ~ 8.0, and observed using a Nikon UV microscope. Apomictic ovaries that presented long meiocytes were cleared with glycerol

after staining with aniline blue. In total, 50 pistils from the sexual plants corresponding to classes 1 to 7 and 60 pistils from the apomictic plants corresponding to classes 1 to 8 were analysed by this method. Also 42 anthers (classes 1 to 3) from the sexual plants and 18 anthers from the apomictic plants (classes 1 to 4) were analysed.

2. The stage of microspore and pollen development in anthers was determined from each class of pistil length. Anthers were placed on a slide in a drop of water and gentle pressure was applied to release the pollen mother cell, microspores or pollen out of the loculi for microscopical analysis. Pollen mother cells till microspores were stained on the slide with a drop of 0.5 μg/ml of 4',6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co., St Louis, MO) to observe the nuclei with a UV microscope. A total of 145 anthers for the apomictic plants and 95 anthers for the sexual plants were analysed.

3. Pollen viability was calculated by counting viable cells in the tetrad stage or early microspore stage and pollen grains just before anthesis collected from several plants. This material was placed in an isotonic drop of 0.5mg/ml lissamine green (Gurr, Searle Scientific Services, High Wycombe, Bucks) added to a half salt concentrated B5 medium (Gamborg et al., 1968) enriched to 6.5% sucrose, then gently cut and spread out for microscopical observation. For the apomictic plants 393 tetrads from 4 plants, 289 microspores from 2 plants and 1169 pollen grains before anthesis from 5 plants were sampled. For the sexual plants 904 tetrads from 3 plants, 449 microspores from 2 plants and 1495 pollen grains before anthesis, from 5 plants, were sampled.

Results

Characteristics and timing

In order to make a reproductive calendar from early development until anthesis of the sexual and apomictic embryo sac development, several measurements were taken. The pistil length increased in a linear fashion, allowing for a division into 11 classes.

Figure 1 shows the length of the pistil according to the selected classes and also displays the length of the anther, of the spikelet and the width of the spikelet in relation to the classes of the pistil length. The anther and the spikelet show proportional growth only in the very beginning of sexual and apomictic flower development. Pistil length development for sexual and apomictic plants is represented in Figure 2 based on an estimate

of pistil growth and assuming that at time zero the size of pistil is zero. It can be seen in this plot that the pistil of the apomictic plant grows faster than the one from the sexual plant. The growth of the pistils tends to be linear as expressed by the regression lines. The regression lines were statistically significant (table 1). Using the regression line and assuming the growth per day is 0.27 mm for D4 and 0.33 mm for D58, then growth in each class of 0.3 mm for the sexual plant takes 1.1 days and for the apomictic plant it takes 0.9 days. The average length of the pistil at anthesis for D4 is 4.9 mm and for D58 is 6.0 mm, therefore, from the regression line it is possible to estimate that anthesis occurs around the 18^{th} day both for sexual and apomictic plants.

Table 1. Statistics analysis of the regression lines for pistil growth of sexual (D4) and apomictic (D58) plants.

	Plant	
Regression analysis	D4	D58
a) coefficient (day)	0.269	0.332
SE	0.011	0.015
p	< 0.01	< 0.01
b) confidence interval (95%)		
uper level	0.295	0.367
lower level	0.244	0.298
c) Intercept	0	0
d) Regression statistics		
R^2	0.924	0.929
SE	0.298	0.380

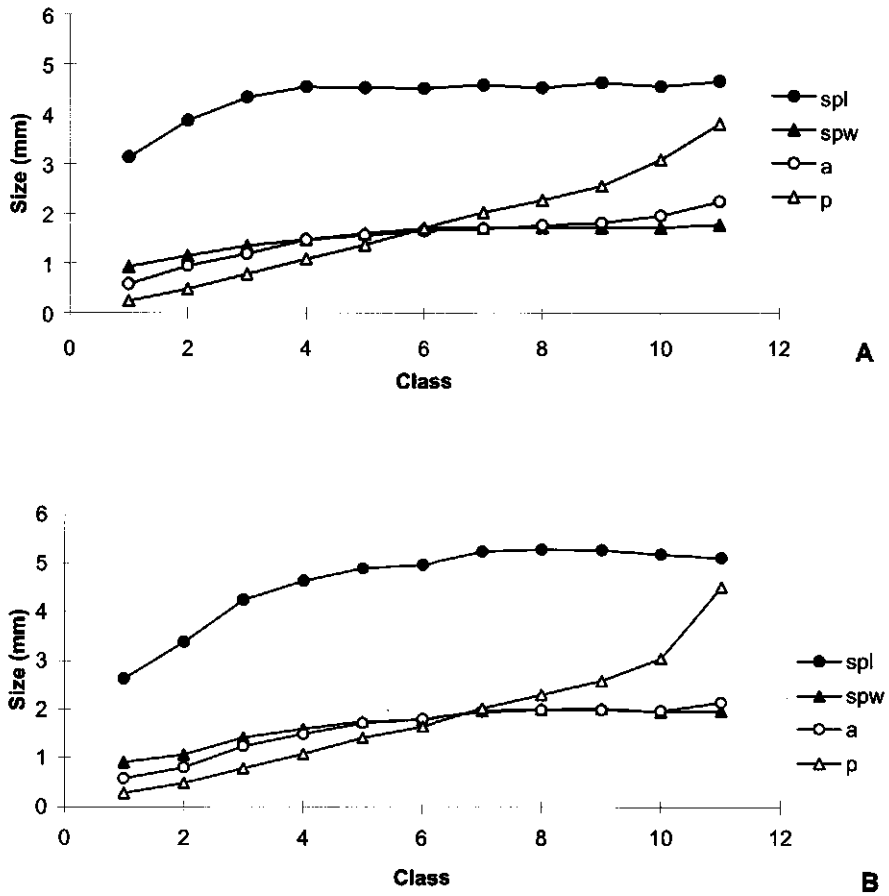


Figure 1. Correlation between means of spikelet length (**spl**), spikelet width (**spw**), anther length (**a**) and pistil length (**p**) and classes of pistil for sexual (**A**) and apomictic (**B**) *Brachiaria decumbens.*

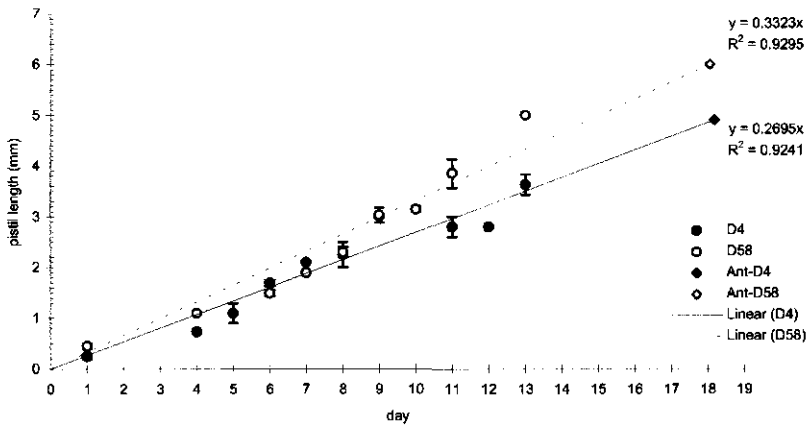


Figure 2. Growth curve of pistils of *Brachiaria decumbens* in mm per day. The day of anthesis was estimate from the regression equation. Bar is the standard error of the mean. Ant-D4 and Ant-D58: pistil lengths at the day of anthesis for sexual and apomictic plants respectively.

Pistil morphology

Changes in pistil morphology were subdivided into 5 stages: stage 1, where the stylodium was very small, about 0.01 mm; stage 2, where the stylodial axis was elongating; stage 3, when white stigmatic hairs started to grow and remained white, stage 4, when the stylodium was completely elongated and became tinted red; and stage 5, when the red colour darkened (Figure 3: column 3).

The pistils of apomictic and sexual plants differed in length. The apomictic pistil developed less intensive colour in stage 5 but the shape of the stylodium was the same.

Inside the ovary, the ovule shifted from the atropic to the anatropic position till class 5 in the apomictic plant, and till class 7 in the sexual plant, which was about three days later (Figure 3: column 4). The anatropic position was achieved during the vacuolated megaspore stage in sexual plants but by the first enlarged apospore stage in apomictic plants.

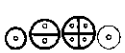
Class pistil lenght (mm)	Time (d) sex	Time (d) apo	Pistil morphology sex	Pistil morphology apo	Atrope/ anatrope movement sex	Atrope/ anatrope movement apo	Stage of female development: sexual	Stage of female development: apomictic	Stage of male development: sexual	Stage of male development: apomictic
							A m D T M 1C 2C 4C 8C ES	sex dev. A m T / apo dev. Al 1C 2C 4C ES	m D T 1m 2m %	m D T 1m 2m %
1 ≤ 0.3	1.1	0.9						 		
2 0.3-0.6	2.2	1.8						 		
3 0.6-0.9	3.3	2.7						 	 14	
4 0.9-1.2	4.4	3.6						 	 10	 35
5 1.2-1.5	5.5	4.5						 		 24
6 1.5-1.8	6.6	5.4								
7 1.8-2.1	7.7	6.3								
8 2.1-2.4	8.8	7.2								

Figure 3. Reproductive calendar of sexual and apomictic *Brachiaria decumbens*. All events are related with classes of pistil length (first column). Time in days was estimated by the regression equations (Fig. 2). Pistil morphology is divided in 5 stages according to the changes in size of pistil, presence, size and colour of stylodium that occur in pistil during development, the normal line indicate white colour, the dark lines represent the darkens in the reddish colour of the pistil. The lines inside the circle in atrope/anatrope movement show the most probable position of the micropylar side in relation to the vertical axis of pistil. The darker line in drawings in stage of development represents the callose wall. The dotted line represents the degeneration. The interrupted line represents vacuoles. Nuclei are round or oval filled in grey. Legend: sex = sexual, apo = apomictic, sex dev. and apo dev. = sexual and apomictic development respectively; A = archespore, m = meiocyte, D = diad, T= tetrad, M = megaspore, 1C, 2C, 4C, 8C = 1, 2, 4 and 8 nucleate coenocyte; AI = apospore initial or initial cell of the aposporous embryo sac; 1m = unicellular microspore, 2m = bicellular pollen; % = percentage of microspore abortion. ↓ see stage of development of sexual plant.

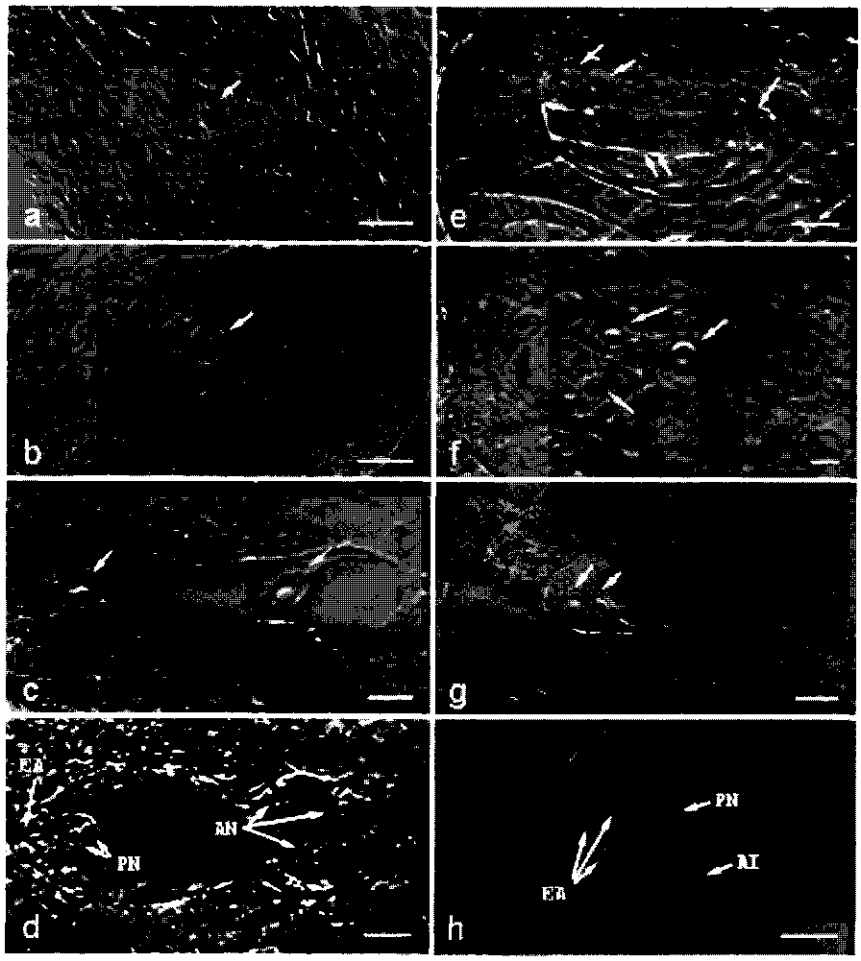


Figure 4. Megasporogenesis and megagametogenesis in sexual (**a-d**) and aposporous (**e-h**) *Brachiaria decumbens*. Bars, 20μm for all figures. Micropyle is positioned to the left side of the figures. Sexual *B. decumbens*: **a**, archespore showing enlarged nucleus (arrow). **b**, survival megaspore situated in the chalazal end of ovule. **c**, two-nucleate coenocyte. Note the two large vacuoles, one in the middle separating the two nuclei (arrows) and one in the chalazal end of the embryo sac. **d**, young complete sexual embryo sac showing egg apparatus (**EA**), two polar nuclei (**PN**) and enlarged antipodals (**AN**). Aposporous *B. decumbens*: **e**, young stage of development showing the simultaneous presence of an elongated meiocyte (double arrow), and apospore initials surrounding it (arrows). **f**, enlarging apospore initials, near micropylar side of ovule. **g**, an aposporous two nucleate coenocyte, note that the two nuclei (arrows) are in the same side of the embryo sac and the vacuole in the other side. **h**, aposporous embryo sac showing egg apparatus (**EA**), only one polar nucleus (**PN**) and another apospore (**AI**) in the same ovule.

Female gametophyte development

Sexual development of the female gametophyte followed the Polygonum-type. During the initial stage, a parietal cell could not be observed and the archespore (Fig. 4a) developed into a meiocyte. The late meiocyte has a complete callose wall with a thicker cap on the micropylar side and a fainter one on the chalazal side (Fig. 5a). During dyad and tetrad formation the transverse walls displayed a high amount of callose (Fig. 5b). After the breakdown of the callose walls around the tetrad, callose was observed in some big cells, the embellum cells (Bursi et al., 1993), positioned at the entrance of the ovule (Fig. 5c). The callose in these cells disappears before vacuolation of the megaspore. The degeneration of the three megaspores near the micropyle took some time and finally only the surviving megaspore could be detected (Fig. 4b). Megaspore vacuolation was observed at the micropylar side. Following the nuclear division, an elongated coenocyte was formed (Fig. 4c) and then a complete embryo sac was formed (Fig. 4d).

Female gametophyte development in the apomictic plant was of the Panicum-type. Apospore cells were observed in the nucellus together with the archespore. Around a meiocyte more apospores were present (Fig. 4e). A long complete callosic wall enveloped the meiocyte (Fig. 5d) which after elongation remains with the same pattern of callose (Fig. 5e). In a few cases, patterns like dyad, triad or tetrads were found. After some time, only a thick callosic cap at the micropylar side (Fig. 5f) was present. In general, the meiocyte degenerated or occasionally, a megaspore was formed and thereafter degenerated. Callose was not observed in any of the micropylar cells. After degeneration of the meiocyte or megaspores, only apospores were visible (Fig. 4f). Thereafter, vacuolation began in apospores and the formation of a two-nucleate coenocyte (Fig. 4g) preceded the formation of the apomictic embryo sac, a four-celled structure (Fig. 4h). Other apospores were formed and developed, but not all of them completed the formation of a four-celled embryo sac. Commonly, the aposporic embryo sac near the micropyle had all of its cells positioned on the micropylar side. Other embryo sacs in the same ovule had their cells positioned in any direction including the chalazal side.

Male gametophyte development.

In the sexual development of the male gametophyte, callose deposition first appeared in the inner longitudinal to transversal walls of the pollen mother cell (Fig. 6a). Later, the meiocyte was completely surrounded with

callose (Fig. 6b) and became round (Fig. 6c). The locular space was filled with a fluid indicating callose degradation. Callose remained around the meiocyte through the dyad stage, until the end of the tetrad stage in class 3 (Fig. 6d, e) in which the deposition of callose in the new formed walls appeared to be much more intense. The faintly fluorescent locular fluid surrounded the dyads and tetrads. The percentage of aborted microspores in the tetrad stage and as free microspores was 14% and 10%, respectively. Other microspores developed into a bi-cellate pollen, and were present from class 7 onwards. Finally, in the mature anther of class 11 only 4% pollen abortion was counted.

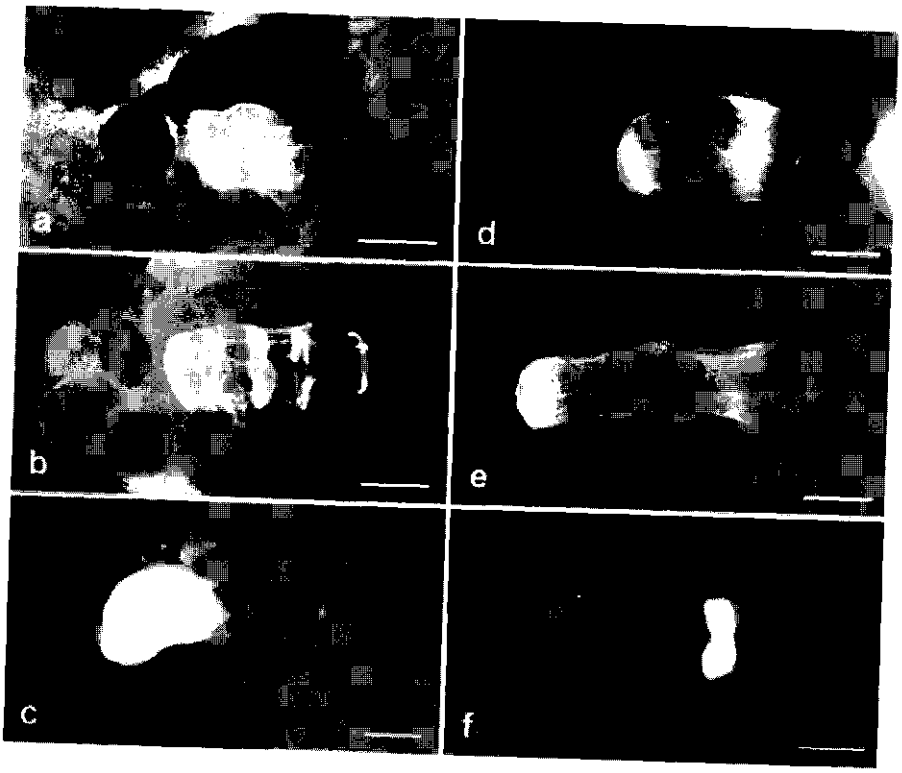


Figure 5. Callose deposition during megasporogeneses in sexual and aposporous *Brachiaria decumbens*. Bars, 20μm for all figures. Micropylar pole is oriented to the left side of the figures. Sexual *Brachiaria decumbens*: **a**, meiocyte completely surrounded by callose. **b**, tetrad showing callose deposition around megaspores. **c**, embellum cells showing high deposits of callose. Aposporous *B. decumbens*: **d**, meiocyte showing high callose deposition. **e**, elongated meiocyte present by the time that tetrad should already be formed showing thick callosic wall. **f**, cap of callose that remains for long time near micropyle.

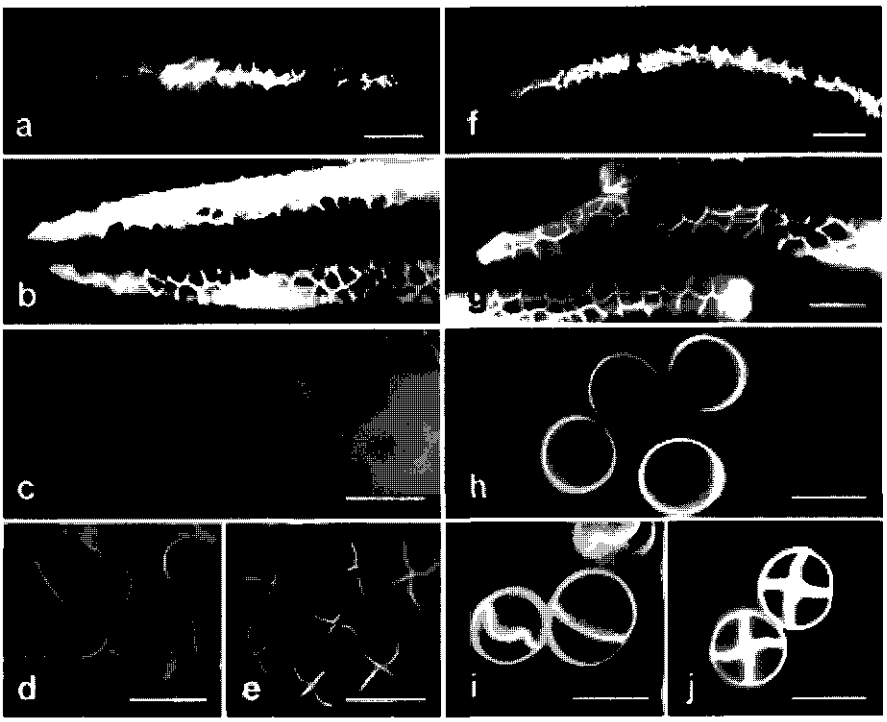


Figure 6. Callose deposition during microsporogeneses in sexual and aposporous *Brachiaria decumbens*. Bars for c, d, e, h, i, j, 30μm; a, b, f, g, 50μm. Sexual *Brachiaria decumbens*: **a**, early stage of microspore development, beginning of callose deposition in inner longitudinal walls of pollen mother cell (PMC) and in transversal walls to the outer walls of PMC. **b**, callose is completely surrounding PMC or meiocytes. Note the irregular shape of the cells. **c**, callose around the meiocytes and filling the intracellular space. Note that the shape of the cells is round. **d**, dyads and **e**, tetrads showing callose deposition around cells as well as in the newly formed walls. Note that the callose staining in the new walls is much more intense. Aposporous *B. decumbens*: **f**, early stage of microspore development showing callose deposition starting from inner longitudinal cell walls to outer walls. **g**, PMC completely surrounded by callose. Note the irregular shape of cells. **h**, round meiocytes showing thick callosic wall and intracellular fluid is absent. i, dyad and j, tetrad showing thick callose walls.

In apomictic plants, the onset of callose deposition in the male meiocyte followed the same pattern as in the sexual plant (Fig. 6f, g). The meiocytes were easily set free and the layer of callose around the meiocyte remained thick, even in class 4, when the dyad and tetrad were formed. (Fig. 6h, i, j). The locular fluid seemed to be scarcer than in the sexual anther, indicating less callose degradation. The abortion percentage in the tetrad and microspore stages was 35% and 24%, respectively. Free microspore development took place until class 10 in which microspore mitosis gave rise to bi-cellate pollen. At class 11, only 5% abortion could be observed.

In both sexual and apomictic plants pollen grain was filled with starch.

Discussion

Morphological differences between sexual and apomictic plants

The increase in pistil length was linear during the selected developmental periods and therefore the rate of growth could be determined. The data collected on the development of the pistil, ovule, male and female gametophytes in relation to pistil length, from archesporial differentiation to fertilisation was formatted into a reproductive calendar (Fig. 3). Other measurements, such as the length or width of the spikelet or anther length may be useful with the pistil length below 0.9 mm.

Louant and Longly (1981) reporting on timing of male and female gametogenesis in *Cichorium*, comented the difficulty of this approach due to the effects of environmental conditions on plant behaviour. The effect of climatic conditions may be minimised when data are collected at the same time from both sexual and apomictic plants, grown under uniform conditions. Indeed, without such a time relationship it becomes impossible to compare the two developmental processes.

This study also revealed differences between sexual and apomictic development. In the apomictic plants the pistil developed with a growth rate about 20% higher than the sexual plants and, at anthesis, the pistil length was about 1 mm longer. These two features may be related to the tetraploid character of the apomictic plants as was happen with enhancement in sizes found in induced tetraploid of *B. ruziziensis* (Gobbe et al. 1981).

Bending of the ovule, which reaches its anatropic position about 3 days earlier in the apomictic plants, coincides with the vacuolation of the apospore. In the sexual plants this anatropic position is related to vacuolation of the megaspore. Even though there is a 3-day difference in the onset of vacuolation, in both cases, the bending of the ovule is concomitant with vacuolation, which means the initiation of the sexual and apomictic embryo sac development. According to Gobbe *et al.* (1982) the induced tetraploid of *Brachiaria ruziziensis* reaches the anatrope position of the ovule earlier, i.e., at the time when a tetrad was present.

Pigmentation of the stylodium was completed before anthesis. In class 11 the apomictic stylodium had less intensive pigmentation. It is possible that this difference in pigmentation is due to the larger size of the apomictic stylodial cells.

The calendar developed can be easily utilised to select distinct stages of ovular develpment to study gene expression or some metabolic pathways.

Gametophytic difference between sexual and apomictic plants

Female development in *B. decumbens* exhibited a Polygonum-type and a Panicum-type embryo sac development. In the apomictic plants, the meiocyte was somewhat more elongated, as could be expected in a tetraploid plant. The meiotic stage in the apomictic plants was delayed to class 5. In addition, a thick callose wall enveloped the whole cell. This thicker callose wall points to a prolonged callose wall deposition phase around the meiocyte of the apomictic plants. However, the breakdown of the callose wall was incomplete (Fig. 5f) and a micropylar cap, as in *Poa* (Naumova *et al.*, 1993) remained visible in classes 5 to 7.

Although apospore initials were present in the nucellus from the archesporial stage, class 1, the number of apospore initials increased just after degeneration of the meiocyte, during class 6. At about that time, apospore vacuolation was observed, and in class 7 the first mitosis took place. The degeneration of the meiocyte with its callose wall coincided with the increase in apospore initials and apospore vacuolation. The degeneration of the callose wall contributes to a large carbohydrate source in the nucellus. The vacuolation of the megaspore in the sexual plants and the first mitosis took place in class 7, about 3 days later than in the apomictic plants. In the apomictic plants, embryo sac development was faster than that in the sexual plants and began just after degeneration of the meiocyte The embryo sac development of the sexual plants began after degeneration of the three megaspores of the tetrad and the embryo sac formation occurred here later. The development from vacuolated megaspore to embryo sac takes place during four classes, from class 7 to 11. Meanwhile occurs the development of the aposporous embryo sac, from class 6 to class 9. In *Brachiaria*, the early appearance of an apospore and early degeneration of the meiocyte results in fast development of the apomictic embryo sac.

The morphology of embryo sac development in *B. ruziziensis*, as described in detail by Gobbe *et al.* (1982), is very similar to that observed for the sexual *B. decumbens*. Our results show a different temporal relationship between male and female development from that described for sexual diploid and tetraploid *B. ruziziensis* (Gobbe *et al.*, 1982) and for the apomictic *B. brizantha* and *B. decumbens* and their hybrids with *B. ruziziensis* (Lutts *et al.*, 1994). For instance, in the previous study megaspore mother cell differentiation starts later than male meiosis. By the time a tetrad was formed in the ovule, microspores were already vacuolated in the anther, whereas in this study the tetrad stage in the male and female organs occurred

simultaneously in the same class. In the tetraploid apomict, the tetrad stage in the ovule was seldom observed during female development, and meiosis did not appear to reach completion thus making a correlation with male meiosis impossible. Nevertheless meiocytes were still observed in ovules at the same time that microspores were present in anthers. On the other hand in apomictic plants, high numbers of apospore initials differentiated from the nucellus and started vacuolization at the same time that in the anthers, microspores were vacuolated as was also observed by Lutts *et al.* (1994).

Peel et al (1997) observed callose deposition in the walls of the nucellar epidermis at the micropylar end of both aposporous and sexual *Pennisetum ciliare*. In *Brachiaria* only in sexual plants callose deposits were in the embellum cells, just after the disappearance of the callose around the tetrad. The micropyle was not yet fully developed because the micropylar cells continued to stretch. The micropyle of the apomictic plants was more elongated than that of the sexual plants but had a similar cellular composition.

Male development in apomictic plants commonly shows aberrations (Winkler, 1908). In the sexual plant pollen development took place from class 1 to class 8, in 8.8 days, which is about the same time as the apomictic pollen development, from class 1 to class 11, in at least 9 days. A thicker callose wall, also observed in the late meiocyte of female development, was present at the male tetrad stage. The presence of small amounts of locular fluid with some callose faintly marked by fluorescence, points to a low callase activity in the apomictic plant. The abortion percentage of microspores in the tetrads and of free microspores was two to three times higher in the apomictic plants compared to the sexual plants. This pattern of abortion confirms the connection between male and female development showing abortion coinciding with the beginning of embryo sac development. Thus, characteristics of apomixis are already present during the onset of the development to the megaspore and microspores. In the stages of the pollen grains the abortion percentage is low and equal for sexual and apomictic plants. This points to another, relatively mild but equal abortion factor during the development of the pollen grain.

The work presented here, as based on the calendar, indicates that in *Brachiaria*, apomixis is expressed in the very early stages of female development leading to a different type of embryo sac development and is reflected in male development by partial abortion of microspores and pollen.

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Chapter 3

Activity and localisation of sucrose synthase and invertase in ovules of sexual and apomictic plants of *Brachiaria decumbens*

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Abstract

Brachiaria decumbens plants have sexual and apomictic reproduction. Apomixis is facultative and of the aposporic type. In early stages of ovule development, differences in the pattern of callose deposition between sexual and apomictic plants were observed which points to possible differences in carbohydrate metabolism. Therefore, a comparative study on carbohydrate metabolism between a sexual diploid ecotype and an apomictic tetraploid *B. decumbens* was made. A histochemical determination of two enzymes responsible for sucrose degradation, sucrose synthase and invertase, was performed for all stages of ovule development. In addition, the concentrations of sucrose, glucose, and fructose were measured for each stage of ovule development, both for sexual and apomictic plants. The enzymes were localised by immunohistochemistry with heterologous antibodies. A distinct difference between sexual and apomictic plants was observed in the localisation of sucrose synthase activity as well as in the amount of activity, especially in the early stages of ovular development. Invertase activity localisation was comparable between ovules of the sexual and apomictic plants, but its activity is clearly higher in ovules of sexual plants. The localisation of the enzymes coincided with the place of activity. For both sexual and apomictic plants the amount of sucrose in the ovaries increased with the stage of ovule development. Differences in the amount of sucrose between sexual and apomictic plants in ovaries with ovules in comparable stages of development were detected. A delay in the onset of carbohydrate metabolism, during early stages of ovule development characterises the apomictic plant.

Keywords: apomixis; sucrose synthase; *Brachiaria decumbens*; carbohydrates; invertase; ovule.

Abbreviations: MMC megaspore mother cell; MC meiocyte; MS megaspore; AI apospore initial; CO coenocyte; MES mature embryo sac; OA ovule after anthesis; SuSy sucrose synthase; INV invertase; BMM butyl-methyl methacrylate; DTT dithiothreitol; DAPI 4',6'-diamidino-2-phenylindole; PBS.phosphate-buffered saline.

Introduction

Brachiaria is a genus of forage grass widely cultivated in tropical areas. *Brachiaria decumbens* is the most common *Brachiaria* species planted in Brazil (Macedo 1995). Like other grasses, members of *Brachiaria* have sexual and apomictic reproduction (do Valle and Savidan 1996). Apomixis, an asexual reproduction with embryo formation without fertilisation of an unreduced egg cell (Czapik 1994, do Valle and Savidan 1996) is the prevailing mode of reproduction in the cultivated genotypes. As apomixis is facultative, a reduced embryo sac can develop and produce a progeny by the normal sexual process (Do Valle and Savidan 1996). The sexual plants of *B. decumbens* are diploid (2n = 2x = 18) and have a Polygonum type of embryo sac development. Apomictic plants are tetraploid (2n = 4x = 36) with an aposporic embryo sac development of the Panicum type. In both types, it is possible to describe ovular development in six stages on the basis of embryo sac development.

In sexual plants the archespore enlargement characterises the megaspore mother cell stage and points to the beginning of the embryo sac development. The meiocyte stage is characterised by the two meiotic divisions with elongation of the megaspore mother cell and deposition of callose in the meiocyte wall and in the dyad and tetrad. The megaspore stage is characterised by the degeneration of the tetrad and the survival and enlargement of the chalazal megaspore. This megaspore undergoes mitoses and a two-, four-, and eight-nucleated embryo sac is formed during the coenocyte stage. Finally, cellularisation takes place and the Polygonum-type embryo sac enters the mature-embryo-sac stage that ends at anthesis. In the last stage, ovules after anthesis, the stylodium is covered with pollen grains and dries out, and fertilisation may occur.

In apomictic plants the first stage is characterised by the presence of the megaspore mother cell and is also called the megaspore mother cell stage. From the beginning, in the nucellus early apospore initials can be observed (Dusi and Willemse 1999). The meiocyte stage is characterised by the elongation of the megaspore mother cell and callose deposition, but commonly the meiosis is arrested and only an elongated meiocyte is formed. In the apospore initial stage, the meiocyte degenerates and by this time many apospore initials are present. This stage is comparable with the megaspore stage in the sexual plant. The apospores enlarge and, during the coenocyte stage, undergo two mitoses; a four-nucleated embryo sac is formed.

Cellularisation occurs and one or more Panicum type embryo sacs are formed in one ovule in the mature-embryo-sac stage. Just before anthesis in these ovules embryos can be observed on the chalazal side. About one day later, in the stage of ovules after anthesis, fertilisation of the central cell may occur also in the apomictic plants, since *B. decumbens* is a pseudogamous species (Ngendahayo 1988) and therefore needs fertilisation of the central cell in order to form the endosperm that will support the embryo development.

On the basis of the data presented in the reproductive calendar of *B. decumbens* (Dusi and Willemse 1999), and also on reports for many other apomictic grasses (Naumova et al. 1993, Naumova and Willemse 1995, Peel et al. 1997) the deposition pattern of callose in members of *Brachiaria* differs between sexual and apomictic plants. When meiosis is arrested in the ovule of the apomictic plant, callose is present around the meiocyte. In the sexual plant, callose is deposited in the cell walls of the meiocyte, dyad and tetrad. The callose deposition in transversal walls during the development of meiocytes or dyads and before the tetrad stage is absent in the apomictic plant. The callose around the meiocyte degenerates, but a callose cap at the micropyle remains for some time. In ovules of the sexual plant callose is formed in some enlarged cells at the micropyle, the embellum cells (Busri et al. 1993) just after tetrad degeneration, but this is absent in the apomictic plant.

The research on the metabolism of carbohydrates presented here has been based on these observations on callose deposition. Apomixis as a facultative process can be influenced by environmental factors as photoperiodicity, heat, and dry conditions (Nogler 1984, Bell 1992). Also the supply of sucrose to enrich *in vitro* media can induce an asexual regeneration from gametophytic fern tissues (Bell 1992). Water stress during meiosis in pollen mother cells of wheat was found to affect the activity of acid invertase and sucrose synthase, and that affects all carbohydrate metabolism (Dorion et al. 1996). Genes such as the sucrose synthase genes *SuS1* and *Sh1* from maize (Koch et al. 1992) are differentially expressed, modulated by the level of sugar in the tissue.

The localisation of activity of invertase (INV) and sucrose synthase (SuSy), enzymes both involved in sucrose degradation, has been observed in maize kernels (Doehlert and Felker 1987, Wittich and Vreugdenhil 1998). These methods allowed a localisation of enzyme activity and provide a good approach for comparative studies, also in early stages of ovule development. Research on sugar metabolism has been done during seed development

from the early stages of embryo formation (Weber et al. 1997, Wittich 1998). Studies on the determination of INV and SuSy enzyme activities and the localisation of the same enzymes were done with ovules of *Gasteria verrucosa* (Wittich 1998). Little attention, however, was paid to the carbohydrate metabolism during the initial steps of ovule development. Therefore, a comparative analysis was done, based on sucrose synthase and invertase enzymes activity, the immunohistochemical localisation of these enzymes and the levels of sucrose, fructose, and glucose during early ovule development of sexual and apomictic *B. decumbens.*

Material and methods

Plants of *Brachiaria decumbens*, accessions BRA000191 (tetraploid D58, cv. IPEAN) and BRA004430 (diploid D4), from the Brazilian Agricultural Research Corporation, Embrapa Beef Cattle, CNPGC were used. The plants were grown in a greenhouse with temperatures ranging from 25°C during the day to 20°C at night, with a 16h light, and 8h dark light regime. Inflorescences were collected and the spikelets from several stages of development were removed from the middle of the racemes. Most of the stages were selected on the basis of a reproductive calendar determined by pistil length (Dusi and Willemse 1999). The following stages were distinguished: (1) megaspore mother cell stage (MMC), pistils with less than or equal to 0.3 mm; (2) meiocyte stage (MC), pistils with 0.3-0.9 mm; (3) megaspore or apospore initial stage (MS-AI), pistils with 1.2 - 1.8 mm; (4) coenocytic stage (CO), pistils with 2.1 - 2.7 mm for the sexual plant and 1.8-2.4 mm for the apomictic plant; (5) mature embryo sac stage (MES), pistils with more than 3.3 mm; (6) flower after anthesis with pistils collected from flowers with drying stigma already covered with pollen.

Staining of monosaccharides

Monosaccharides, especially glucose, were stained according to Okamoto et al. (1946) cited by Gabe (1976). This method was developed to stain glucose but was found to stain other monosaccharides as well (Wittich 1998).

Determination of enzyme activity

For enzyme histochemistry, flowers were separated from the spikelets and sectioned longitudinally in a way that the pistils were divided in two parts.

For the determination of both invertase and sucrose synthase activity, the methods described by Doehlert and Felker (1987) and by Wittich and Vreugdenhil (1998) were used. Both methods are based on a sequence of reactions in which nitro blue tetrazolium precipitates into a blue formazan salt in the presence of enzyme activity. The duration of these reactions varied from 45 min to 1 h. Control sections were incubated in a reaction mixture either without sucrose for the invertase activity assay or without phosphoglucomutase, glucose 1,6-P_2, and pyrophosphate (PPi) for the sucrose synthase activity assay. After incubation and washing, the pistils were separated from the rest of the flower, cleared, mounted in glycerol, and visualised with a Nikon differential interference contrast microscope.

Immunohistochemistry

Pistils were removed from the spikelets and fixed in 4% paraformaldehyde, 0.25% glutaraldehyde and 0.1 M NaCl in 0.01 M phosphate buffer, pH 7.2 for 1 h under vacuum. The fixative was refreshed and samples stayed overnight without vacuum. Then they were washed in buffer and dehydrated in an ethanol series to 100% ethanol. To the absolute ethanol 10 mM of dithiothreitol (DTT) was added. The samples were embedded at 4 °C in butyl-methyl methacrylate (Gubler 1989, Baskin et al. 1992) through a series of 5 : 1, 5 : 2, 5 : 3, 5 : 4, 1 : 1, 4 : 5, 3 : 5, 2 : 5, 1 : 5 (v/v) ethanol : butyl-methyl methacrylate (BMM) and then through two changes of 100% BMM. DTT was added in all steps of embedding to a final concentration of 10 mM. Incubation steps took at least 4h and the samples were kept at 4 °C. The material was placed in gelatine or plastic capsules containing a fresh BMM mixture and polymerisation was done under UV irradiation for 48 h at –20 °C.

Sections (2–3 μm) were cut and placed on drops of water under glass slides, stretched with chloroform vapour and placed on a 60 °C plate for 1 h. BMM was removed following a 15 min wash in acetone. The sections were washed two times for 10 min in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 7 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3), blocked in 0.1M hydroxylammoniumchloride for 5 min, washed again in PBS for 5 min, blocked in 1% bovine serum albumin (BSA) in PBS and washed in PBS supplemented with 0.01% acetylated BSA (BSAc). Sections were incubated overnight at 4 °C with the primary antibody diluted in 0.01% BSAc in PBS. Thereafter they were washed in the same buffer, incubated for 2 h at room temperature with the second antibody diluted in PBS, washed in PBS,

incubated 10 min in 1 µg of 4',6'-diamidino-2-phenylindole (DAPI) per ml, washed in PBS, and mounted in Citifluor.

Two different primary antibodies were used for invertase enzyme localisation. An antibody for a soluble grape invertase (Ruffner et al. 1995), raised in chicken, was used with a dilution of 1 : 20. The procedure was combined with a second antibody (rabbit anti chicken), with a dilution of 1 : 64, labelled with FITC. An antibody for cell wall invertase from carrot (*Laurière et al. 1988*), raised in rabbit (polyclonal antibody), was used with the dilution of 1 : 100, combined with a second antibody (goat anti-rabbit) labelled with fluorochrome Cy3 with a dilution of 1 : 200.

For sucrose synthase as primary antibody, an antibody for *Vicia faba* native protein SS1 (Ross and Davies 1992) raised in rabbit was used in a dilution of 1 : 100. The second antibody was the same used for invertase labelled with Cy-3 at the dilution of 1 : 200. Observations were made with a Nikon fluorescence microscope with FITC (fluorescein isothiocyanate) excitation filter for wavelengths of 450-490 nm, dichroic mirror (DM) for 510 nm, and barrier filters (BA) for 520 nm, FITC excitation filter for 470-490 nm, DM for 510 nm and BA for 520-560 nm, and TRITC (tetramethylrodamin isothiocyanate) excitation filter for 546 10 nm DM for 580 nm and BA for 590 nm. To observe the nuclei, the sections with the nuclei stained with DAPI were observed with a UV excitation filter for 365 10 nm, DM for 400 nm, BA for 420 nm.

Quantitative analysis of sugars

Pistils, divided in six stages, 3 pistils at the megaspore mother cell stage, 3 pistils at the meiocyte stage, 2 pistils at the megaspore or apospore initial stage, 2 pistils at the coenocyte stage, 1 pistil at the mature embryo sac stage and 1 pistil at the ovules after anthesis stage from both sexual and apomictic plants were placed in Eppendorf tubes containing 50 µl of 80% methanol in demineralized water. The tubes were placed at 76°C for 15 min. After centrifugation for 10 min at 11500 g, the supernatant was then collected and dried in speed vacuum. Pellets were resuspended in 60 µl of demineralized water, centrifuged for another 10 min and then the supernatant was collected and stored at 20 °C until use. Sugar analysis was performed with a Dionex HPLC-system (DX500 Chromatography System, Dionex Corp., Sunnyvale, Calif, U.S.A.) equipped with a CarboPac PA1 column (4 by 250 mm) preceded by a similar guard column (4 by 50 mm) to separate the neutral sugars. Sugars were detected by pulsed amperometric detection

(PAD) with an ED40 electrochemical detector. Samples were eluted at a flow rate of 1 ml / min, by an isocratic 50 mM sodium hydroxide elution profile.

Results

Invertase activity

The blue stain caused by the precipitated formazan salt marked the sites of INV activity; the pink colour reflects the partial reaction in places where the activity is very low (Lewis 1977). In an overview of pistils in different stages of development, the blue stain of the ovules of the sexual plant compared with that of the apomictic plant is clearly more intense (Fig. 1).

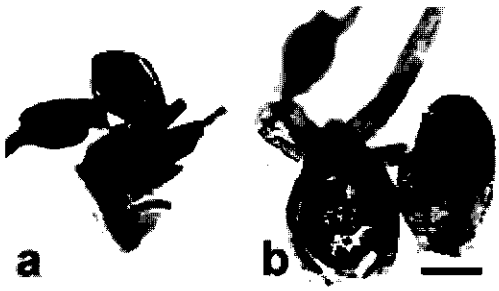

Figure1. Localisation of invertase activity in sections of pistils in three stages of development of sexual (**a**) and apomictic (**b**) *B. decumbens*. Note that the colour is stronger in the tissues of the sexual plant as compared to those of the apomictic plant. Bar: 320 μm.

In more detailed observations, this difference could be related to structural components of the ovule. In the sexual plant the ovules showed a more intensive activity from the MMC stage up to the MS stage (Fig. 2a, c, and e versus b, d, and f). Thereafter, from the CO stage up to the MES stage, both sexual and apomictic plants had a comparable intensity of the blue colour (Figs. 2g, h, and 5e, f). In an ovule after anthesis there was essentially no INV activity (Fig. 2i, j).

In the MMC stage, the whole ovule of the sexual plant stained positive but the tips of the developing integuments and epidermal nucellar tissue had a higher activity (Fig. 2a). The ovule of the apomictic plant showed a comparable pattern with a weaker reaction (Fig. 2b).

In the MC stage the whole ovule of the sexual plant had a stronger reaction compared to the apomictic ovule and there was a greater activity in the meiocyte (Fig. 2c). The ovule of the apomictic plant first showed activity in

the growing tips of the integument and nucellus, but the meiocyte had little activity comparable with the surrounding nucellar cells (Fig. 2d).

In the MS stage the ovule of the sexual plant had a high activity in nucellus and integuments; the functional megaspore was positive as well as the degenerating megaspores (Fig. 2e). In the ovule of the apomictic plants, at the AI stage at which apospore initials appear, the activity increased, especially in the growing integuments and around the centre of the nucellus. The apospores had a low activity (Fig. 2f). Remnants of meiocytes or megaspores were collapsed.

In the CO stage the activity in sexual plant seemed to be lower in the nucellus, but this could be due to cell stretching. The coenocyte reacted positive (Fig. 5e). In the apomictic plant the activity was high and comparable with the ovule of the sexual plant. The apospores were clearly positive (Fig. 5f).

In the MES stage, nearly the whole ovule of the sexual plant was positive, but in the region of the micropylar nucellus, the activity was decreasing. The antipodals were positive but the egg apparatus was stained very faintly (Fig. 2g). In the apomictic plant the ovule had the same level of activity as in the sexual one, but the Panicum type of embryo sac had a positive reaction in the egg apparatus and in the embryo (Fig. 2h).

By anthesis both the ovules of the sexual and the apomictic plant showed no INV reaction (Fig. 2i, j) and the controls appeared similar to the negative controls for the INV reaction (Fig. 2s, t).

A schematic representation of the INV activity during ovular development in sexual and apomictic plants is presented in Fig. 3.

Sucrose synthase activity

The reaction showing the SuSy activity is also marked by a blue precipitation of the nitroblue tetrazolium salt but resulted in a weaker reaction compared with the INV and a pink colour was also associated with the reaction producing a faint background.

In sections a difference between the sexual and apomictic plant was observed; the ovules of the sexual plant showed a more intensive activity up to the MC stage (Fig. 2k, m) compared with the ovules of the apomictic plant (Fig. 2l, n). Thereafter, from the MS–AI stage to the MES stage, both sexual and apomictic plants had a comparable intensity of the blue colour (Fig. 2o-r). Following more detailed observations, this difference could be related to the structural components of the ovule. In the MMC stage, the whole ovule of the

sexual plant stained weakly positive (Fig. 2k). The ovule of the apomictic plant had a weaker signal (Fig. 2l). In the MC stage the ovule of the sexual plant had a stronger reaction and the meiocyte reacted positive (Fig. 2m). However, the ovule of the apomictic plant had a very low activity, including the meiocyte (Fig. 2n).

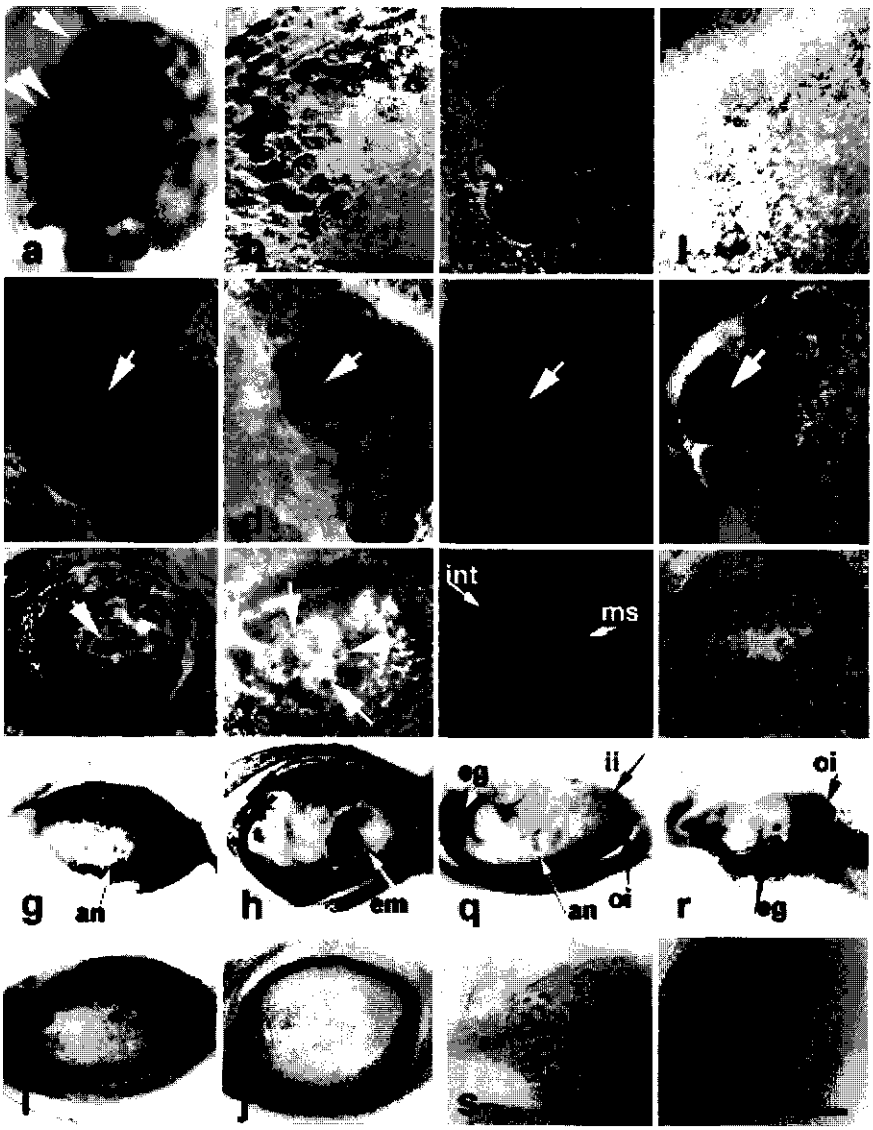

Figure 2 a-t. (color print in page 163) Localisation of invertase activity and sucrose synthase activity in thick sections of ovules of sexual and apomictic *B. decumbens* after clearing with glycerol. **a-j** INV activity localisation. **a** Ovary in the MMC stage from the sexual plant; note the darker blue colour in epidermal cells (double arrows) as well as in the tip of the integuments (arrow). **b** Ovary in the MMC stage of the apomictic plant showing less staining in general but with the same pattern as the sexual ovary. **c** Ovary in the MC stage in the sexual plant which shows overall staining and a very high stain in the meiocyte (arrow). **d** Ovary in the apomictic plant which shows less staining in all cells including the meiocyte (arrow). **e** Ovary in the MS stage in the sexual plant; megaspore (arrow) with high activity. **f** Ovary in the AI stage in the apomictic plant; apospore initials (arrows) with some, but few staining. **g** Ovary during MES stage in the sexual plant; note that the activity is decreasing from the micropylar part of the ovary to the chalazal part. Antipodal cells (arrow) are present with high stain. **h** Ovary in the MES stage in the apomictic plant; note the higher activity in this stage. The embryo (em) in the chalazal part of the embryo sac is totally stained in strong blue. i and **j** Ovaries in the ovule after anthesis in sexual (**i**) and in apomictic plant (**j**) do not show blue formazan precipitation. **k-r** SuSy activity localisation: **k** Ovaries in the MMC stage in sexual plant, show a low level of staining in all tissues. **l** in the apomictic plant at MMC stage there is very few staining. **m** and **n** Ovaries in the MC stage in sexual and apomictic plant. Note the high staining in the meiocyte of the sexual plant (**m**, arrow) in contrast with that of the apomictic plant that shows no special staining in meiocyte (**n**, arrow). **o** Ovary during the MS stage in the sexual plant with no special activity in the developing surviving megaspore (ms) and more high activity in the integuments (int) **p** Ovary during AI stage in the apomictic plant showing no activity in AI. **q** Ovaries during MES stage in the sexual plant. The egg apparatus (eg), antipodal cells (an) and outer integument (oi) show activity but the inner integument (ii) shows no activity. **r** Ovary in the MES stage in the apomictic plant; note the high staining in the egg apparatus (eg) especially in the chalazal area. In the chalazal position the inner integument (ii, arrow) shows no activity while the outer integument (oi) has activity. **s** and **t** Controls without sucrose for the invertase activity localisation reaction; ovaries in MS stage in the sexual plant (**s**) and in the CO stage in the apomictic plant (**t**). Note that there is no blue staining in the controls. Bar: for **a-d**, **k-p** and **s**, 25 μm; **e** and **f**, 40 μm; **g-j**, **q** and **r**, 160 μm; **t**, 50 μm.

In the MS stage the ovule of the sexual plant had a lower activity as the MC stage but the activity was more localised in the now stretching integuments. The functional megaspore was negative (Fig. 2o). In the AI stage the ovular activity was still comparable with the MC stage. The apospores were negative (Fig. 2p), as well as the degenerating meiocytes or megaspores.

In the CO stage the stretching nucellus, the outer integument and the coenocyte of the sexual plant had activity. In the apomictic plant at the CO stage SuSy activity increased and the apospores were positive.

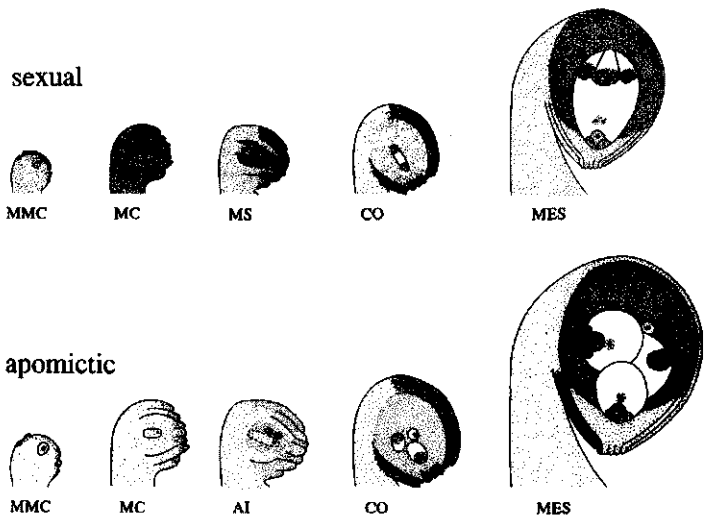

Figure 3. Schematic representation of the distribution of invertase activity during ovule development in sexual and apomictic *B. decumbens*. The pattern of enzyme distribution is comparable between the two plants but the sexual plant shows a higher intensity of reaction than the apomictic plant in the three initial stages.

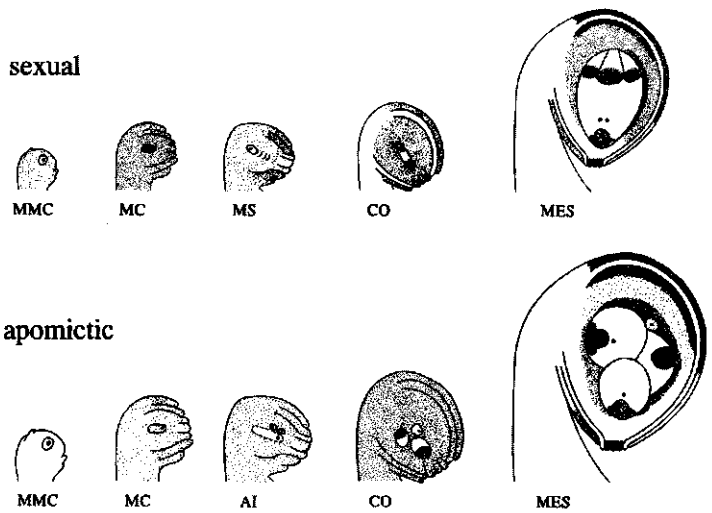

Figure 4. Schematic representation of the distribution of sucrose synthase activity during ovule development in sexual and apomictic *B. decumbens*. The apomictic plant seems to have a delay in expression of enzyme activity in the first stages of ovule development, but from the CO stage the activity seems to be comparable to that in the sexual plant.

In the MES stage the ovule of the sexual and the apomictic plant, the SuSy activity faded out from the micropylar part of the ovule and ovary carpels. In the sexual plant there was a strong reaction in the outer integument at the chalazal side of the ovule but the inner integument was negative. Also the lateral side of the nucellus against the funiculus and at the chalazal nucellus showed layers of high activity. The egg apparatus and antipodals were positive (Fig. 2q). In the apomictic ovule the pattern of activity in the nucellus as well as in the integument was comparable with the sexual one. The Panicum-type embryo sac had a positive reaction in the egg apparatus (Fig. 2r) and in the embryo, when it was present. The controls of the SuSy reaction were negative.

A schematic representation of the SuSy activity during ovular development in sexual and apomictic plants is presented in Fig. 4.

Immunohistochemistry of invertase and sucrose synthase

Anti-invertase from *Vitis vinifera* (grape) and from *Daucus carota* (carrot) and anti-sucrose synthase from *Vicia faba* were used to localise the enzymes INV and SuSy in apomictic and sexual *B. decumbens*.

In the early MMC and MC stages the anti-INV from grape reacted with protein in the cytoplasm of all ovular cells of the sexual plant (Fig. 5a). In the apomictic plant the label was less clear and positioned in the periphery of the cytoplasm (Fig. 5b) reflecting the enzyme activity observed previously (Fig. 3). In the later CO stage the immunological reaction in the ovule was stronger in both plants, and the cytoplasm from the coenocyte of the sexual plant reacts with anti-invertase (Fig. 5g) as did mainly the peripheral cytoplasm of the apospores in the apomictic plant (Fig. 5h, i) .

In the early MMC and MC stages the anti-SuSy reacted in the nucellus and integuments and marked the peripheral cytoplasm of the meiocyte in the sexual plant (Fig. 5c). In the apomictic plant the immunological reaction was very faint and essentially negative in the meiocyte cytoplasm (Fig. 5d).

Immunohistology of invertase and sucrose synthase in the embellum cells

Nucellar epidermal and subepidermal cells round up and elongate as a gland, which can be related to pollen tube guidance in some members of the family *Gramineae*, these cells are called the embellum cells (Busri et al. 1993). They occur in *B. decumbens* and we found that during the MS stage they are marked by callose production only in the sexual plant (Dusi and Willemse 1999).

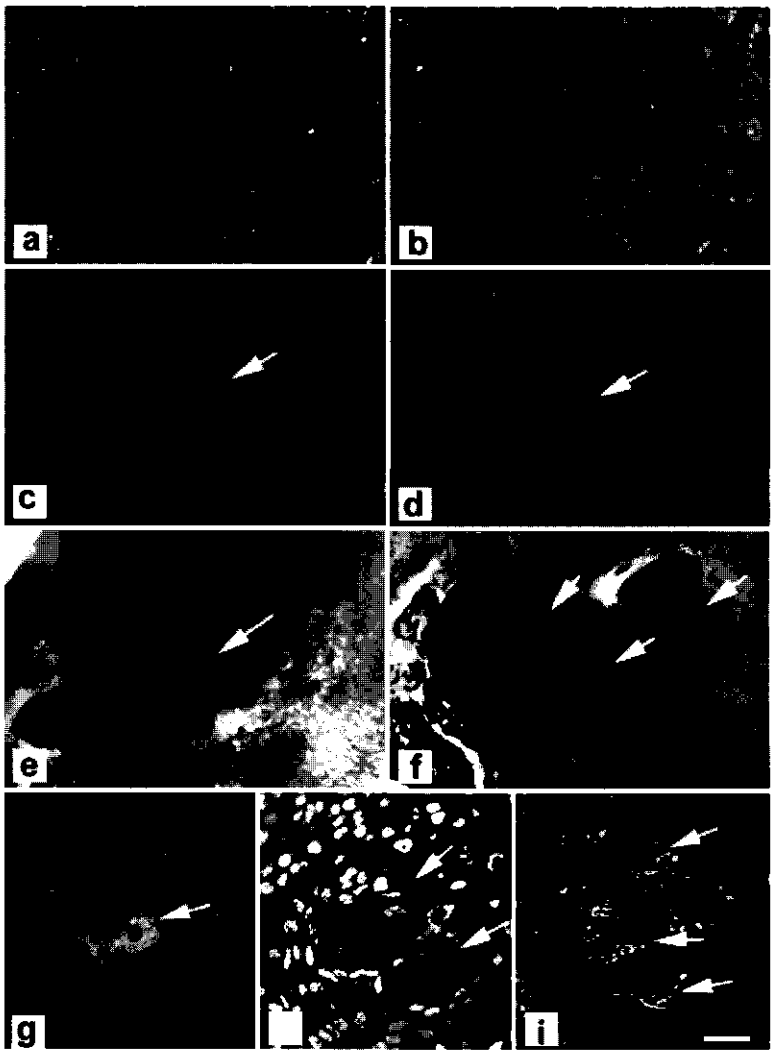

Figure 5. a-i Immunolocalization of invertase and sucrose synthase in sections of ovules from sexual and apomictic *B. decumbens* plants. a and b FITC fluorescence label resulted from the reaction with anti-soluble invertase from grape in ovules in the MC stage. Note the different intensity between the sexual plant (**a**) and the apomictic plant (**b**). c and d MC stage of ovule development showing TRITC fluorescence label after the reaction with anti-SuSy from *Vicia faba* native protein. **c** Sexual plant with reaction in the meiocyte (arrow). **d** Apomictic plant without reaction in the meiocyte (arrow). e and f Cleared thick sections of ovules in CO stage after reaction for invertase enzyme activity localisation. Note the high activity in the embryo sacs (arrows) the sexual (**e**) and the apomictic plant (**f**). g-i CO stage of the sexual plant (**g**) with FITC label resulting from the reaction with anti-soluble invertase from grape, and the apomictic plant (**h**) with UV fluorescence nuclei label resulting from DAPI staining. **i** FITC labelled apospores of **h**. **Int** integument, **nu** nucellus. Bar: for **a-d**, 17 μm; for **e-i**, 20 μm.

In the MS stage the anti-INV made against a cell wall invertase from carrot reacted with the embellum cell wall of the sexual plant (Fig. 6a, b), but this reaction was not present in the apomictic plant (Fig. 6c, d). Later, from the CO stage, both the sexual plant and the apomict showed a positive reaction in the embellum cells with anti-invertase from grape (soluble invertase) (Fig. 6e, f).

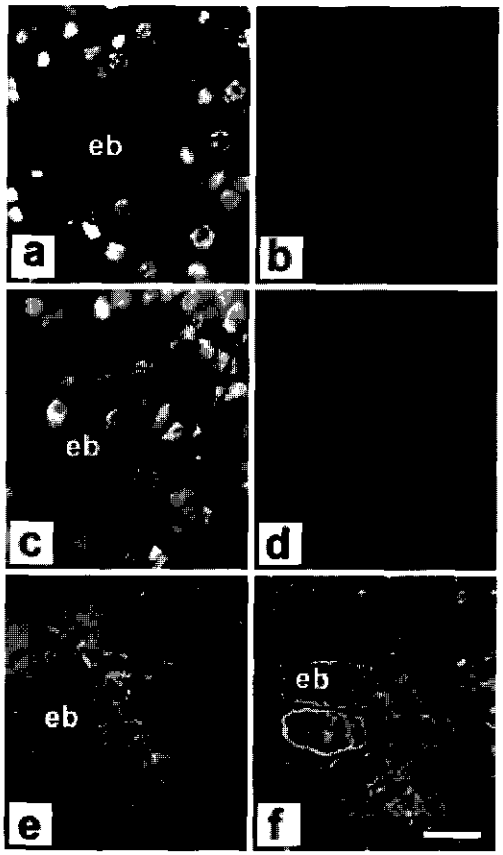

Figure 6a-f Immunolocalisation of invertase activity in the micropylar part of an ovary in sections of sexual and apomictic plants. eb Embellum cells. **a** UV fluorescent label in sections of ovule of the sexual plant during MS stage showing nuclei stained with DAPI. **b** The same section as in **a** showing Cy3 labelling resulting from the reaction with anti-cell wall invertase from carrot in the embellum cells. **c** UV fluorescent label of DAPI in sections of ovules from the apomictic plant during AI stage. **d** The same section as in **c** showing some Cy3 label resulting from the reaction with anti-cell wall invertase from carrot in the embellum cells. **e** FITC fluorescent label in the embellum cells from an ovule in the MES stage of the sexual plant as a result of the reaction with anti-soluble invertase from grape. **f** The same in the ovule of the apomictic plant. Bar: for **a-d**,16 μm; **e** and **f**, 20 μm.

During the MS-AI stage the anti-SuSy reaction in the embellum cells of the sexual plant was more intense than in the apomictic plant (Fig. 7a, b versus c, d). From the MS-AI stages a clear reaction in the nucellus occurred in the sexual (Fig. 7a, b) as well as in the apomictic plant (Fig. 7d). In these stages the reaction in the inner integument of the apomictic plant was more positive than in the sexual plant (Fig. 7b, d).

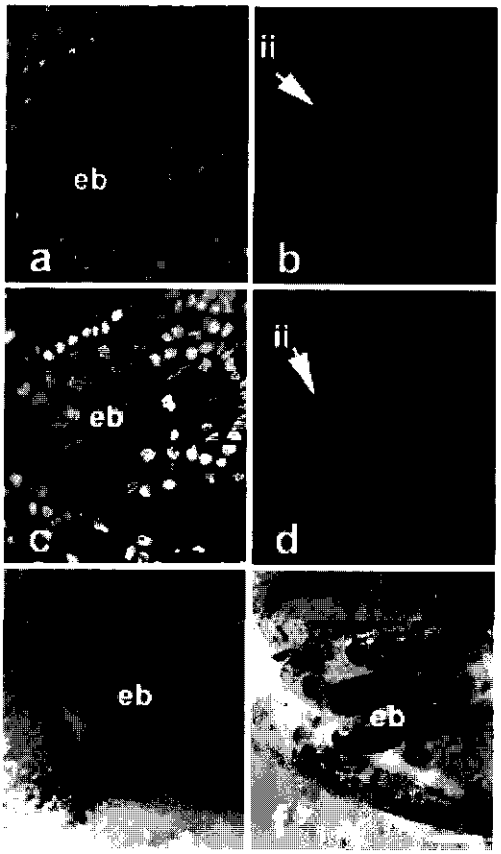

Figure 7. a UV fluorescent label of DAPI in ovules of the sexual plant during the MS stage. **b** The same section showing Cy3 fluorescent label in embellum cells resulting from the immunolabelling with anti-sucrose synthase from *Vicia faba* native protein. **c** UV fluorescent label of DAPI in ovules of the apomictic plant in the AI stage. **d** The same sections showing faint Cy3 label in embellum cells of the apomictic plant. e and f Sexual (**e**) and apomictic (**f**) plant ovules in early MES stage showing SuSy activity in the embellum cells of both ovules. Arrows point to inner integument (ii). eb Embellum cells. Bar: for **a-f**, 20 μm.

During the MS-AI stage INV activity of the embellum cells was observed in both the sexual and the apomictic plant, but in the apomict the reaction was weak. During the early MES stage there was SuSy activity in the embellum cells of both the sexual and the apomictic plant (Fig. 7e, f). This SuSy activity was evident for a longer period in the sexual plant.

Quantitative analysis of sucrose, glucose and fructose during ovary development of sexual and apomictic plants

In the presence of sucrose, INV activity produces glucose and fructose, whereas SuSy mostly leads to the formation of UDP glucose and fructose. To compare the quantitative distribution of sucrose, glucose and fructose in ovaries of the sexual and the apomictic plant during development, ovaries were extracted and sugar concentration were determined. The results are represented in Fig. 8.

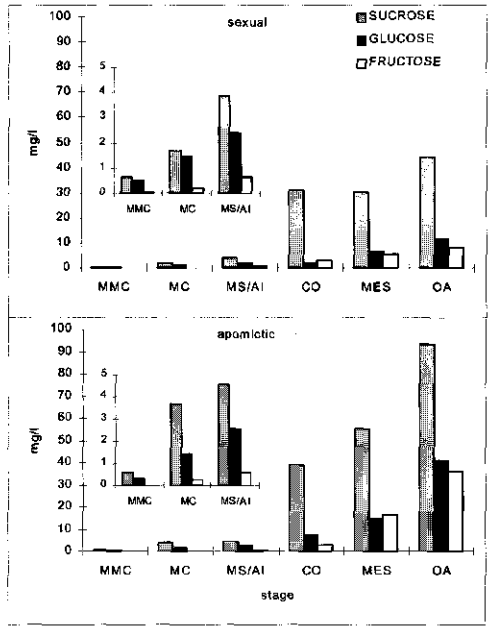

Figure 8. Concentrations of sucrose, glucose and fructose (mg.l^{-1} pistil) in pistils from different stages of sexual and apomictic *B. decumbens*. Inserts show the first three stages of development in at a 20fold larger scale

In the apomictic and the sexual plant during the MMC, MC, and MS-AI stages, the quantity of sucrose, glucose and fructose per pistil was relatively lower than that observed in the following stages. The OA stage had the highest level. Compared with the sexual plant, the apomictic plant had a higher quantity of these carbohydrates especially after the MS-AI stage. In the ovule after anthesis the quantity of glucose and fructose was about 4 times higher in the apomictic plant, and that of sucrose was about 2 times higher. By contrast, during the MMC stage a lower quantity of particularly glucose, and also fructose and sucrose, was observed in the apomictic pistils.

Discussion

Carbohydrate metabolism is important for cell and organism maintenance, tissue development and storage products like starch. Both the invertase and sucrose synthase pathways are in particular directly related to the growth process (Hawker et al. 1991, Weber et al. 1997). The sucrose synthase pathway with the formation of UDP-glucose can be related to cell wall formation and starch production (Doehlert 1990, Delmer et al. 1993, Amor et al. 1995).

From the results it becomes clear that a difference exists in the carbohydrate metabolism between the sexual and the apomictic plant of *B. decumbens*. These differences are expressed in differences in the activity and localisation of INV and SuSy as well as in the quantities of sucrose, glucose, and fructose during the development of the ovary.

Wittich (1998) has shown that there is no INV reaction from the MC stage to the MES stage in *Gasteria verrucosa* plants. In the MES stage the nucellus has the highest activity, which partly co-localizes with the anti-invertase. Activity of SuSy is in the archespore of the MMC stage. Until the MES stage the ovule has a weak SuSy activity in all tissues as well as the presence of the enzyme. The activity increases before fertilisation and is of a higher level present in the integuments and in an associated ovular structure, the arillus. In comparison with *Gasteria verrucosa, B. decumbens* has a higher level of INV and SuSy activity and a more distinct localisation of these enzymes from the beginning of the ovular development.

Invertase activity and localisation

The activity of INV in the sexual plant was present from the MMC up to the OA stage. From the MMC to the CO stage there was a higher and more localised activity, like in meiocyte and embellum cells of MC stage, in the sexual plant as compared with the apomictic plant. The INV activity was more expressed in the growing area of the nucellus and integument. The megaspore had a lower level of activity. In the MMC and MS stages immunological localisation of higher amounts of INV in meiocyte, in the nucellus, and integuments of the sexual plant compared to the apomictic one follow the same pattern observed for enzyme activity.

In the CO and MES stages the pattern of INV activity was nearly the same between the sexual and apomictic plant; the embellum cells had INV activity also in the apomictic plant. In the CO stage the INV activity marked the still growing integuments. In the MES stage the nucellus around the developing reduced and unreduced embryo sacs or the apospore initials and aposporic embryo sacs was highly active, converting the sucrose source into glucose and fructose for the growth. The egg apparatus of the sexual embryo sac had INV activity. The remarkable activity of the antipodal cells is a sign of involvement in glucose and fructose production. In the ovule from the apomictic plant, with multiple embryo sacs, the egg apparatus near the micropyle had a lower activity than the ones near the chalaza, where in some cases embryo formation had already started before fertilisation. The chalazal side therefore is a preferential place for further apomictic development, probably due to the import of nutrients via the funiculus. In the sexual and apomictic ovule the INV activity started to diminish from the micropylar part into a very low activity in the ovule after anthesis. The high activity in the nucellus during the MES stage prepares the ovule after anthesis and probably the carbohydrate sources for seed development just after fertilisation. The difference between sexual and apomictic plants was expressed in the very beginning of ovular development. The apomictic plant is marked by the low INV activity that is confirmed by the absence of the INV enzyme in meiocyte and embellum cells.

Sucrose synthase activity and localisation

The activity of SuSy in the sexual plant is present from the MMC up to the OA stage. The MMC and MC stages have a higher activity in the sexual plant compared with the apomictic plant. From the MS stage in the sexual

plant and from the MC stage in the apomictic plant the SuSy activity becomes more localised in the growing area of the nucellus and integuments.

In the MC stage the meiocyte had a distinct SuSy activity in the sexual plant but it was weak in the apomict. This activity may be related to the formation of the callose wall since UDP-glucose could be used as a substrate (Delmer et al. 1993, Amor et al. 1995). The callose wall around the meiocyte in the apomictic plant is less elaborated (Dusi and Willemse 1999) which is in accordance with the low SuSy activity observed. Embellum cells had no distinct SuSy activity in the apomictic plant but appeared especially in the sexual plant in the MS stage just before the formation of callose. The enzyme localisation during the MMC and MC stages in the ovule with anti-SuSy confirms the pattern of the SuSy activity.

In the CO and MES stages the pattern of SuSy activity was comparable between the sexual and apomictic plants. In the apomictic plant, even the embellum cells had SuSy activity, which, in contrast with the sexual plant, did not produce callose in an earlier stage in these cells. The effect of the absence of callose and enzyme activity in the embellum cells probably should not affect the capacity of pollen tube guidance of these cells since in the apomictic plant the fertilisation of the polar nucleus is necessary and seed set takes place (Ngendahayo 1988). In the CO stage the activity marked still the growing integuments of the apomictic plant, but in the sexual plant the inner integument had no SuSy activity. The same occurs in the MES stage in both plants. The reason for the loss of SuSy activity is not clear. As part of the future seed coat this layer will degenerate.

In the MES stage the pattern and intensity of the SuSy activity was nearly the same of that of the INV activity. The lateral outer integument and nucellus around the developing embryo sac or the AI and aposporic embryo sacs were highly active, converting the sucrose source in UDP-glucose. The egg apparatus of the sexual embryo sac had SuSy activity as did the apomictic one. The remarkable activity of the antipodal cells suggests involvement of UDP-glucose production. In the apomictic embryo sac the egg apparatus near the micropyle had a lower activity than the one near the chalaza. This supports the idea that there is a preferential place for nutrient import for further apomictic development. As postulated for other plants (Erdelska 1975, Mestre and Vannerean 1980, Mogensen 1981, Willemse 1981) the nutrient flow during the MES stage is directed from around the embryo sac to mainly near the micropylar side. The pathway from the vascular bundle into the funiculus gives a preferential import at the chalazal

side of the ovule. Depending on the presence and composition of a hypostase the chalazal part of the embryo sac may have a preferential position for import. The lateral pathways around the embryo sac may have a preference for the ad-funicular side, as can be observed from autoradiographic studies (Chamberlin and Horner 1993). In the sexual and apomictic ovules the SuSy activity started to diminish from the micropylar part towards the chalazal part and finally there is a very low activity in the ovule after anthesis, probably because the carbohydrate sources for seed development after fertilisation were already prepared.

The difference between sexual and apomictic plant is expressed in the very beginning of ovular development. The apomictic plant is marked by the low SuSy activity, followed by the absence of the SuSy enzyme in the meiocyte. A comparable conclusion is drawn from the INV activity and localisation.

In conclusion, the higher and earlier INV and SuSy presence and activity in ovules of the sexual plant from the MMC up to the CO or MS-AI stages respectively, marks the difference with the apomictic plant. This higher and earlier activity can be due to the presence of a higher quantity of enzymes as well as to the presence and quantity of sucrose available for breakdown.

The quantitative analysis during the MMC stage of ovary development of sucrose, glucose, and fructose reveals the lower level of these carbohydrates in the apomictic plant. This carbohydrate shortage is quickly restored in the apomict after the MMC stage and in the MC and MS stages the levels of the carbohydrates are comparable with the sexual plant. From the CO stage, the apomictic plant gets a higher level of these carbohydrates than the sexual plant. From the CO stage, the quantity of sucrose is converted by invertase into high amounts of glucose and fructose. Although there is a backlog in the apomictic plant in the MMC stage, this is quickly corrected during ovary development to a level that is higher, maybe due to the tetraploid character of the apomictic plant. The tetraploidy of the apomictic plant is a factor that enhances its development and this means a relative higher overall investment in the ovary compared with the sexual plant.

In the sexual plant, during the MMC stage there exists a higher quantity of enzymes as well as of sucrose available for breakdown. In the MMC stage the apomictic plant has a delay in its carbohydrate metabolism. This delay is also expressed by the weak INV and SuSy activity and presence in the meiocyte and in the embellum cells, and may be related to callose formation.

It may include also the late breakdown of the callose wall, visible by a cap around the megameiocyte as has been observed in other apomictic species (Naumova et al. 1993).

The delayed carbohydrate metabolism characterises the apomictic plant. In addition, the presence of the apospore initial from the MMC stage also points to an early onset of apospory. That the carbohydrate delay in the early stages evokes the early induction of apospory cannot be stated from the collected data but may be considered as a potential apomixis-promoting condition. The continuous shortday photoperiod on *Dichanthium annulatum*, a shortday grass, which enhances the degree of apospory (Knox and Heslop-Harrison 1963) also points to a condition in which the carbohydrate metabolism is involved. A difference in timing of the floral induction genes with regard to environmental and phenological stimuli is an element in the duplicate-gene asynchrony hypothesis to explain the cause of apomixis (Carman 1997)

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Chapter 4

Patterns of RNA distribution during embryo sac development in sexual and apomictic plants of *Brachiaria decumbens*.

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Abstract

Temporal and spatial distribution of total RNA and poly(A)+ RNA was followed during the sequential stages of meiotic and aposporic embryo sac development in two accessions of *Brachiaria decumbens*. Some significant differences in amount and distribution of total RNA and mRNA were found after histochemical staining and *in situ* hybridisation with oligo-dT probe. The distribution pattern of RNA shows a comparable development of the selected megaspore and the apospore initials. In the apomictic plant many ribosomes were present in some nucellar cells. These nucellar cells with large nucleus are believed to be apospore initials in early beginning of differentiation, before vacuolation and closure of plasmodesmata.

Abbreviations: MMC, megaspore mother cell; MC, meiocyte; MS, megaspore; AI, apospore initial; CO, coenocyte; MES, mature embryo sac; BMM, butyl-methyl methacrylate; DTT, dithiothreitol; mRNA, messenger RNA; rRNA, ribossomic RNA; DIG, digoxigenin: AO, acridine orange.

Introduction

Apomixis is an asexual mode of reproduction followed by seed formation. Two main types of apomixis are distinguished: adventitious embryony and gametophytic apomixis (Nogler 1984; Asker and Jerling 1992). In adventitious embryony the embryo is formed direct from the somatic cells of the nucellus or integument. Gametophytic apomixis implies formation of unreduced embryo sac and could be diplospory, if the embryo sac develops from a unreduced spore; or apospory, if the embryo sacs are originated from somatic cells of the ovule. Apospory is often accompanied by the arrest of meiosis during megaspore mother cell development.

In sexual and apomictic plants, an ordered sequence of gene expression governs the development of the ovule and the embryo sac. The morphological and cytological analysis of the sequential steps of the reduced and unreduced embryo sac development and the development and differentiation of the surrounding nucellus and integuments offers the information on the differences between sexual and apomictic plants. In apomictic plants, in contrast with the sexual ones, some nucellar cells develop to unreduced embryo sacs, changing to a reproductive development.

Differences between the sexual and apomictic plants could also be reflected by the pattern of total RNA distribution. By localising the total mRNA

during ovular development by *in situ* hybridisation with oligo-dT probes, the presence or absence of mRNA can be traced. This approach localises the places where RNA could be translated into proteins or may point, in some cases, to the places of storage of mRNA for future development. The difference in timing or quantity of the total RNA and mRNA patterns may offer some insight in some aspects of regulation of ovular development for both, the sexual and apomictic plants. In a developing tissue the number of ribosomes or polysomes and their association with the endoplasmatic reticulum in the cell can be related to protein synthesis (Lewin, 1994).

Plants of the genus *Brachiaria* have apospory as apomictic mode of reproduction. Progeny analysis from crosses between sexual and apomictic plants of *Brachiaria* suggest that the apomictic character is regulated by one or more genes that are inherited as a single gene (do Valle and Savidan, 1996). Therefore, it is likely that some differences in RNA expression between ovules of sexual and apomictic plants exist. Another fact is the difference in ploidy, observed also in other *Brachiaria* species. The sexual plants of *B. decumbens* are diploid and the apomictic plants are tetraploids (do Valle and Savidan 1996).

In members of the genus *Brachiaria*, the embryo sac development of the sexual and apomictic plant is well known (Gobbe *et al.* 1982, do Valle and Savidan, 1996, Dusi and Willemse, 1999b). The sexual plant follows the Polygonum-type embryo sac development and the apomictic plant follows the Panicum-type. In the ovule of the apomictic plants, in contrast with that of the sexual ones, some cells of the nucellus enlarge and differentiate into apospore initials that will form after two mitoses an aposporic embryo sac. In apomictic plants meiosis may occur and a reduced embryo sac will develop side by side with the aposporic ones with the potentiality to produce a progeny by the normal sexual reproduction. This feature occurs in a percentage that varies from 0 to 100% according to different species and accessions within species (do Valle 1991).

It is possible to group ovular development in stages based on embryo sac development (Dusi and Willemse, 1999a). In sexual and apomictic plants the archespore enlargement characterises the megaspore mother cell (MMC) stage and is the begin of the embryo sac development. At this stage, in the nucellus of the apomictic plant, apospore initials can be observed (Dusi and Willemse, 1999b). The meiocyte (MC) stage is characterised by the two meiotic divisions with elongation of the megaspore mother cell and deposition of callose in the wall of the meiocyte, the dyad and tetrad. At this stage, in the

apomictic plant, meiosis is often arrested and a tetrad is rarely formed, only an elongated meiocyte with callose wall is visible. The megaspore (MS) stage is characterised by the selection and enlargement of the chalazal megaspore. In the apomictic plant this stage corresponds with the apospore initial stage (AI) because then the meiocyte degenerates and many apospore initials are present. In the sexual plant the selected megaspore undergoes three mitoses and a two-, four- and eight-nucleated embryo sac is formed during the coenocyte (CO) stage. In the apomictic plant, the apospores enlarge and after two mitoses a four-nucleated embryo sac is formed. At the mature embryo sac (MES) stage, cellularisation takes place. In the sexual plant one Polygonum-type embryo sac is formed and in the apomictic plant one or more Panicum-type embryo sacs are formed. The MES stage ends at anthesis.

Most mature mRNAs of plants and animals contain a polyadenylic acid sequence. Demonstrating the distribution of poly(A)+RNA by means of this poly(A) sequences is used to localise mRNAs. Using *in situ* hybridisation with oligo-dT as a probe, the site of poly(A)+RNA can be localised and the total mRNA concentration and distribution can be studied, (Chandre *et al.* 1992, Bimal *et al.* 1995, Bimal and Willemse 1996, Masunaga *et al.* 1996). Differences in mRNA concentration in early stages of ovule development suggest an essential step in the MMC stage leading to embryo sac formation (Bimal *et al.* 1995).

Cytological aspects of apospory during early stages of embryo sac development were observed by ultrastructure study of ovule development in *Panicum maximum* (Naumova and Willemse, 1995) and recently was investigated in *Brachiaria brizantha* (Ana Cláudia G. Araujo personal communication). In *Panicum*, the vacuolated apospore initial is comparable with a vacuolated functional megaspore. Meiotic and aposporic mature embryo sacs of *Penisetum* were compared but no special difference could be observed in both egg apparatus (Chapman and Busri, 1994). Different observations were reported for *Penisetum ciliare* where synergids from the aposporous embryo sac start degeneration prior to the synergids from the meiotic embryo sac (Vielle *et al.* 1995).

Based on the calendar and structural events during the embryo sac development of sexual and apomictic *Brachiaria decumbens* plants, a study on the total RNA, mRNA and ribosome / polysome population was done to elucidate possible differences in the development of sexual and apomictic plants.

Material and methods.

Plant Material

Plants of *Brachiaria decumbens*, accessions BRA000191 (tetraploid D58- cv. IPEAN 2n=4x=36) and BRA004430 (diploid D4, 2n=2x=18), from the Brazilian Agricultural Research Corporation, Embrapa, were used. The plants were grown in the greenhouse with temperatures ranging from 25 °C during the day to 20 °C at night, with a 16 / 8 h light / dark light regime. Inflorescences were collected and the spikelets from several stages of development were removed. Most of the stages of ovule development were selected based on a reproductive calendar determined by pistil length (Dusi and Willemse, 1999b). The following stages were distinguished: 1. Megaspore mother cell (MMC) stage: pistils with less than or equal to 0.3 mm. 2. Meiocyte (MC) stage: pistils with 0.3-0.9 mm. 3. Megaspore (MS) or apospore initial (AI) stage, pistils with 1.2-1.8 mm. 4. Coenocyte (CO) stage, pistils with 2.1-2.7 mm for the sexual plant and 1.8 - 2.4 mm for the apomictic plant. 5. Mature embryo sac (MES) stage, pistils with more than 3.3 mm.

Total RNA and mRNA detection

Tissue preparation:

Pistils were removed from the spikelets and fixed in 4% paraformaldehyde, 0.25% glutaraldehyde and 0.1 M NaCl in 0.01M phosphate buffer, pH 7.2 for 1 h in vacuum at room temperature. The fixative was refreshed and samples stayed overnight without vacuum. They were then washed in buffer and dehydrated in an ethanol series (10, 30, 50, 70, 85, 96, 100% ethanol), 20 min each. At 100% ethanol, 10 mM of dithiothreitol (DTT) was added to prevent oxydation. The samples were embedded at 4°C in butyl-methyl methacrylate, BMM (Gubler 1989, Baskin *et al.* 1992, Kronenberger *et al.* 1993) through an ethanol:BMM (with 10 mM DTT) series of 5 : 1, 5 : 2, 5 : 3, 5 : 4, 1 : 1, 4 : 5, 3 : 5, 2 : 5, 1 : 5 (v/v) and two changes of 100% BMM. Incubation steps took at least 4h each and the samples were kept at 4 °C. The material was placed in gelatine or plastic capsules containing a fresh BMM mixture and polymerisation was done with 8 watt/h UV irradiation for 48 h at -20 °C. After polymerisation, the samples were kept at 4 °C. Sections of 2 to 4 μm were cut and placed on drops of water on glass slides, stretched with chloroform vapour and placed on a 60 °C plate for 1 h. To get a good control of tissue and RNA preservation, sections were placed alternated on two slides in a way that one section could be used for total RNA

(AO) stain and the other for DIG labelled oligo-dT or poly(A) *in situ* hybridisation. BMM was removed by two washes of 10 min in acetone, one wash in a 1:1 mixture of acetone : water and finally in water.

Paraplast embedding: Pistils were fixed in 4% paraformaldehyde, 0.25% glutaraldehyde in 0.01M phosphate buffer pH 7.2 for 4 hours. Then they were washed in the same buffer and dehydrated in an ethanol series. The samples were transfered to tertiary buthyl alcohol (TBA) through an series of 3:1, 1:1, 1:3 ethanol:TBA, for 1h each and then to pure TBA overnight. The samples were stained in a solution of 1% eosine in TBA for 15 min at 37 °C, washed in TBA. After changing the TBA the samples were placed at 60 °C for 1h. Blocks of paraplast were added from time to time. After 1 day, the TBA evaporation was started and finally the samples were mounted in paraplast. Paraplast sections of approximately 7μm were collected on a drop of water on glass slides covered with organosilane or poly-L-lysine, stretched and dryed at 42 °C overnight. Paraplast was removed with 2 times 15 min in xylene, 10 min in xylene:hybridisation buffer (1:1) and 10 min in hybridisation buffer.

Total RNA staining:

To observe total RNA the method described by Bimal and Willemse (1996) was used. Sections were rinsed with 0.2M acetate buffer pH 2.1 and stained with 0.05% acridine orange (AO) in 0.2M acetate buffer pH 2.1 for 30 min and then washed 15 min in veronal-acetate buffer pH 7.8. These sections were examined with UV or FITC light. RNA fluoresces orange or red while DNA fluoresces green or yellow.

In situ hybridisation:

To detect poly(A)+ containing RNA, probes and *in situ* hybridisation protocol were performed as described by Bimal *et al.*(1995) that label a short oligo-dT with a tail of DIG. Using DIG labelled oligo-dT probes (Boehringer), mostly mRNA can be labelled. After the final washes, air drying the slides with the sections and mounting them in Depex (BDH, Brunschwig Chemie), crystals are formed in places where hybridisation occur. A DIG 46bp oligo-dT made from a tail of poly A (Kindly provided by Dr. Ed Schmidt) was used as an alternative probe to hybridisation. In this case, slides were dried in an ethanol series instead of air dried before mounting so crystals were not developed and positive reaction was observed only by the blue colour formed.

As negative controls, sections in each stage of development mentioned here were hybridised with DIG labelled poly(A) probe (Bimal *et al.*, 1995). In addition, sections where total RNA was degraded during manipulation were used as control. To control if AO would influence the site of oligo-dT binding, sections treated with AO were washed in hybridisation buffer and used for *in situ* hybridisation with DIG labelled oligo-dT probe.

Transmission electron microscopy

Epon embedding:

Pistils were fixed in a solution of 3.5% glutaraldehyde in 0.1M phosphate buffer pH 7.2 to 7.4 at room temperature for 4h in vacuum and agitation. Fixative was renewed and fixation was overnight at 4 °C without vacuum. Samples were washed in buffer three times for 10 min each. They were then fixed in 2% osmium tetroxide in 0.1 M phosphate buffer, washed in the same buffer for 10 min and dehydrated in an ethanol series to 100% ethanol and two more times in ethanol, then through a series of 3:1, 1:1, 1:3 ethanol:propyleenoxide and three times in propyleenoxide for 20 min each step. Samples were transferred to a 10% epon in propylene oxide for 4h at room temperature. The propylene was evaporated overnight and the epon solution renewed. Polymerizaton was at 60 °C. Semithin sections were cut, stained with toluidine blue and examined using a light microscope. Ultrathin sections were stained for 1h with 2% uracyl acetate at room temperature or with uracyl acetate and lead citrate in a LKB ultrastainer . Sections were observed with a Jeol TEM 1200 EX II or with an EM 912 Omega zeiss 80 kV and photographed. Sections containing ovules in appropriate MC stage were selected. Ribosome and polysome populations were estimated. Random sampling was made using areas of $1\mu m^2$ from different areas of the megaspore mother cell (MMC) during meiocyte stage, from nucellar cell from sexual and apomictic as well as the differentiating cell from the apomictic and the electron dense cells from the sexual, positioned in the chalazal side of the MMC cell. Data were compared by a t-test.

Results

Control reactions

The sections hybridised with a dig poly A probe (Fig. 1a) as well as RNA degraded sections as observed with AO staining, showed no reaction. Except for a negligible background, NBT crystals were not visible in any stage of development. When paraplast sections were used, hybridisation with oligo-dT was possible (Fig. 1b) but finally BMM embedding was preferred because the tissue infiltration and consequently preservation, was more efficient with BMM than with paraplast. Therefore, all sections showed here for *in situ* hybridisations are BMM sections. When sections were treated before hybridisation with AO (Fig. 1c), washed in buffer and then hybridised with oligo-dT, mRNA detection remained possible (Fig. 1d).

Total RNA, ribosomes and mRNA pattern during sexual and apomictic megaspore or apospore initial development

Sexual development

All ovular cells of the sexual plant showed strong total RNA staining, during early MMC stage (Fig. 1e). The cytoplasm of the ovular cells was labelled with the dig oligo-dT probe. The enlarged archespore cell had label spread over the whole cytoplasm but few label in nucleus (Fig. 1f). The integuments had less label for mRNA than the nucellar cells. In the apomictic plant the total RNA staining showed a pattern of less dense cytoplasm in nucellar cells and

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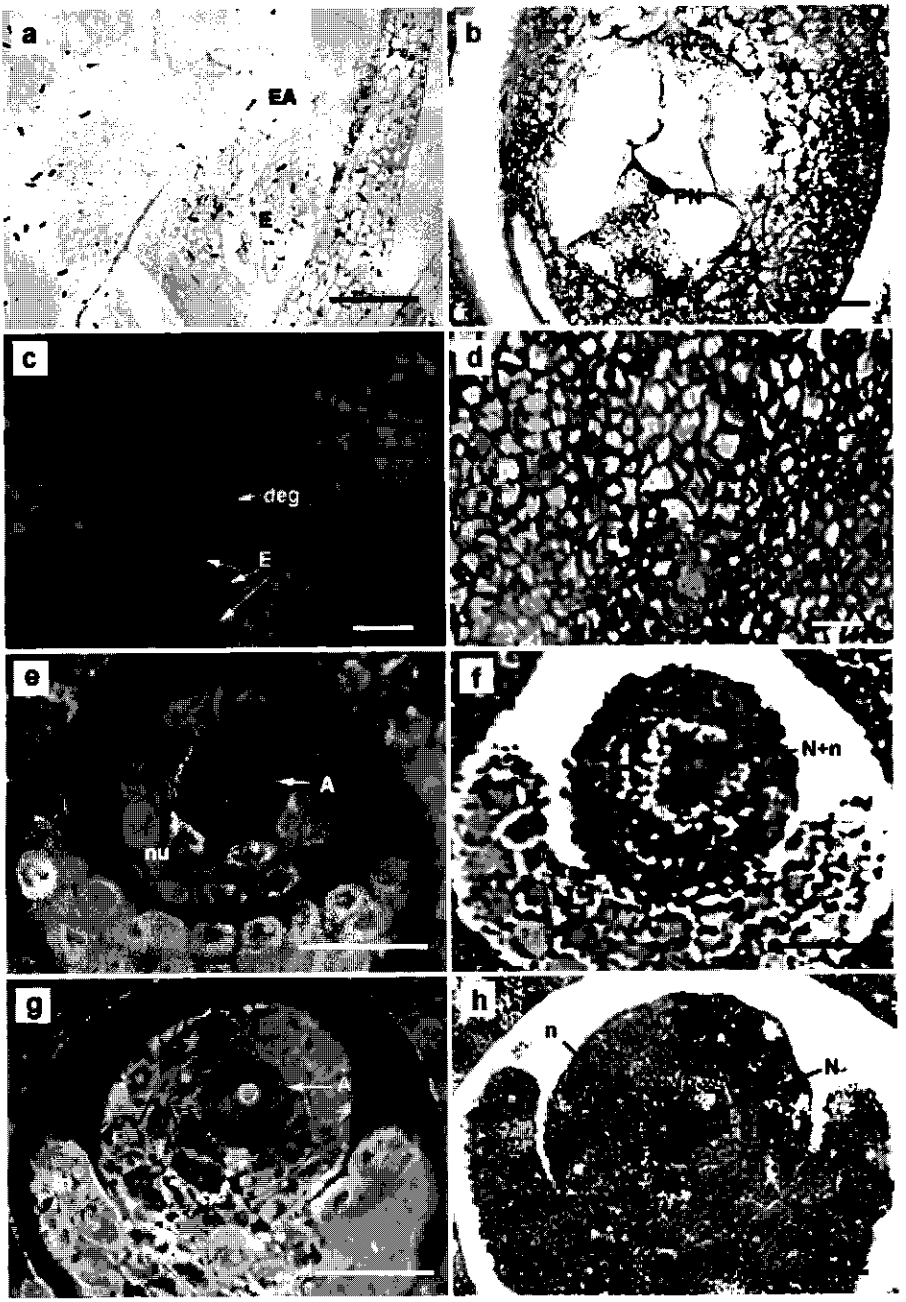
Figure1. Histological staining of total RNA and *in situ* hybridisation of digoxigenin (DIG) - labelled poly(A) and dig-labelled oligo dT to poly(A)+ RNA in sections of sexual and apomictic *Brachiaria decumbens*. **a** Longitudinal section of a mature aposporic embryo sac showing egg apparatus after hybridisation with the sense probe, DIG-labelled poly(A) as a control, which is negative. Bar: 50 μm. **b** Section from a paraplast-embedded ovule from the apomictic plant with embryo sac in MES stage showing labelling for poly(A)+ RNA. Note that FA and PN are not labelled. Bar: 50 μm. **c** AO staining of a paraplast section from an ovule of the sexual plant during megaspores degeneration, showing high total RNA staining in degenerating megaspore and in embellum cells. Bar: 20 μm. **d** Same section as in "c" after hybridisation for poly(A)+ RNA. Note that most of the cells are labelled except the degenerating megaspore and the embellum cells. Bar: 20 μm. **e** AO staining in ovule of the sexual plant during mmc stage. Note the high staining for total RNA in all cells of the ovule, including the archespore cell, nucellus and integuments. Bar: 20 μm. **f** Label of poly(A)+ RNA in the same ovule as in "e" of the sexual plant during mmc stage. Note that integuments show less label than nucellus. Bar: 20 μm. **g** AO staining in ovule of the apomictic plant during the archespore stage. Total RNA is stained in archespore cell in same intensity as in nucellar cells and less than in the integuments. Bar: 20 μm. **h** Label of poly(A)+ RNA in same ovule as g, all cells have a high label also in the integuments. Bar: 20 μm. **A** archespore cell, **deg** Degenerating megaspore, **E** embellum cells, **FA** filiforme apparatus, **in** Integuments, **nu** Nucellus, **PN** polar nucleus.



archespore cell than in the integuments (Fig. 1g). Nevertheless the poly(A)+ RNA label was intense over the whole ovule specially nucellus and archespore. The cytoplasm of the archespore cell was labelled as well as the large nucleus, with exception of the nucleolus (Fig. 1h).

At the MC stage the faint colour in total RNA staining in the sexual ovule showed a decrease in cytoplasm density by increase of volume and vacuolation in the nucellar cells as well as in the meiocyte (Fig. 2a). At this stage, mRNA label was homogeneous in cytoplasm of all nucellar cells and was comparable to the meiocyte. Nuclei and nucleoli were not labelled (Fig. 2b). To get an impression about the changes in the ribosome and polysome population in the young nucellus with meiocyte and apospore initial, an ultrastructural analysis was done. At the MC stage the meiocyte of the sexual plant was elongated in the chalazal-micropylar direction. The large irregular nucleus with a nucleolus was positioned in the centre of the cell at zygotene stage in prophase of meiosis I (Fig. 2c, d). The surrounding cytoplasm was electron transparent in comparison with the nucellus cells and ribosomes and some polysomes are present (Fig. 2e, f). The cell wall that was bordering the neighbouring nucellar cells did not show plasmodesmata in any direction (Fig. 2e) except at the chalazal border where very few plasmodesmata could be observed (Fig. 2f). Nucellar cells surrounding the meiocyte had more ribosomes than the meiocyte. Bordering the meiocyte at the chalazal side of the ovule, two cells were very electron dense (Fig. 2c,d). Those cells had higher number of ribosomes in cytoplasm when comparing with the other nucellar cells. The mean number of ribosomes and polysomes and its relation per μm^2 is shown in table1.

Figure 2. Histological staining of total RNA and *in situ* hybridisation of DIG-labelled oligo dT to poly(A)+ RNA in sections of sexual *Brachiaria decumbens.* Transmission electron photomicrograph of sexual *Brachiaria decumbens* ovules. **a** AO staining of an ovule from the sexual plant at MC stage. Total RNA is all over the cytoplasm (arrow) of meiocyte. Bar: 10 µm. **b** Label of poly(A)+ RNA in cytoplasm (arrow) of the meiocyte and nucellar cells in ovule of the sexual plant during MC stage. Nucleus and nucleolus of the meiocyte are not labelled. Bar: 10µm. **c** Survey of a young ovule of the sexual plant during MMC profase showing nucleus and nucleolus of the meiocyte and electron dense cells at chalazal position. Bar: 5µm. **d** Detail of the meiocyte and of the two electron dense cytoplasm cells at chalazal position. Bar: 2 µm. **e** Detail of the cell wall that divides the meiocyte and the nucellar cell. Note the absence of plasmodesmata in the cell wall and the higher amount of ribosomes in the nucellar cells compared to the meiocyte. Bar: 100 nm. **f** Detail of the cell wall of the MMC showing one of the few plasmodesmata observed at the border with the chalazal cell with ribosomes rich cytoplasm (the degenerating cell). Bar: 100 nm. **CW** cell wall, **ED** electron dense cells, **int** integument, **MC** meiocyte, **N** nucleus, **n** nucleolus, **nu** nucellar cells, **pl** plasmodesmata.

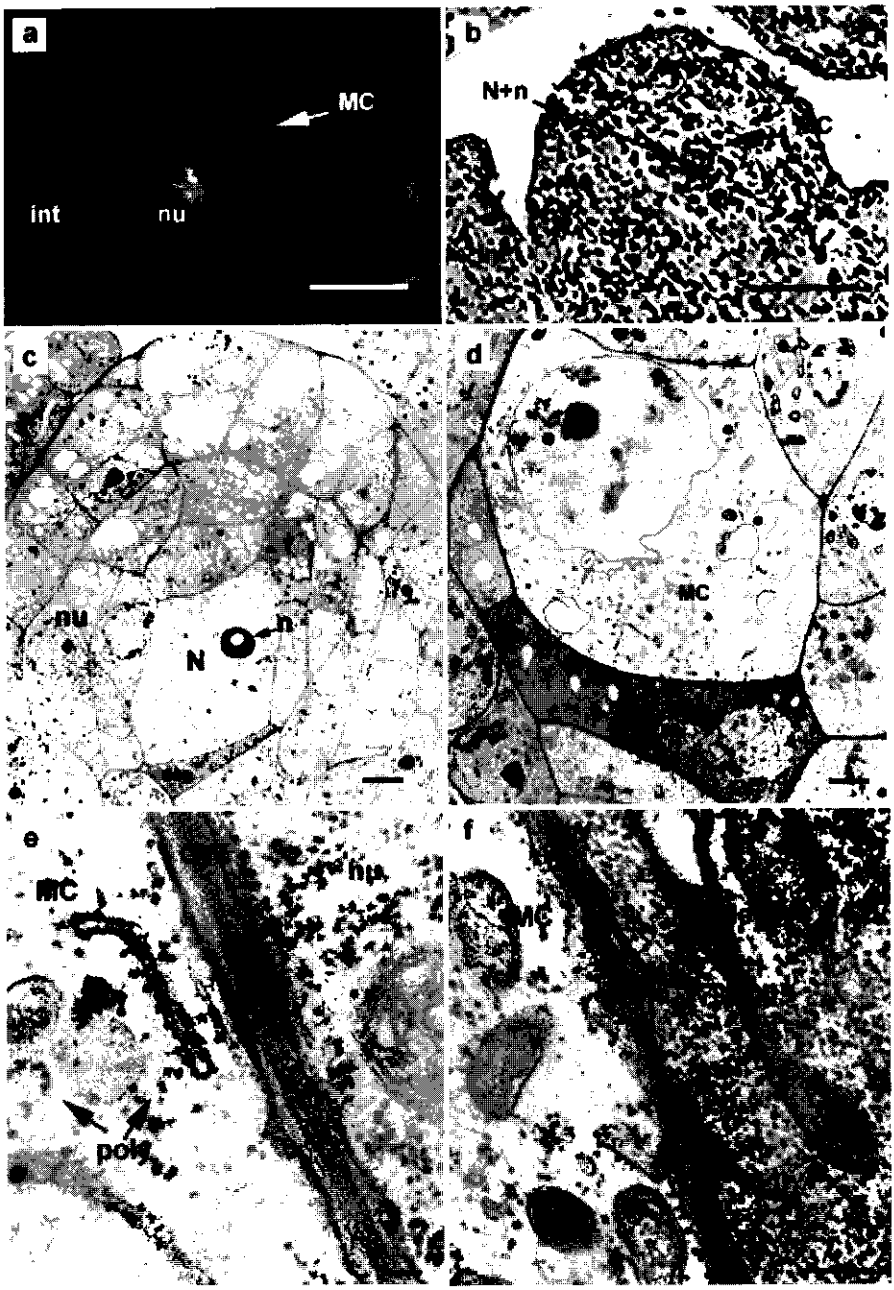

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Table 1. Average number of ribosomes, polysomes and ribosome / polysome ratio, in cells of MC stage of apomictic and sexual plants (mean ± SEM). **n** number of observations in 1μm^2; **R** number of ribosomes; **P** number of polysomes; **R / P** number of ribosomes / number of polysomes; **S** sexual genotype D4; **A** apomictic genotype D58; **p** probability of the t-test; **AI** apospore initial cell for D58 and ED, electron dense chalazal cell for D4.

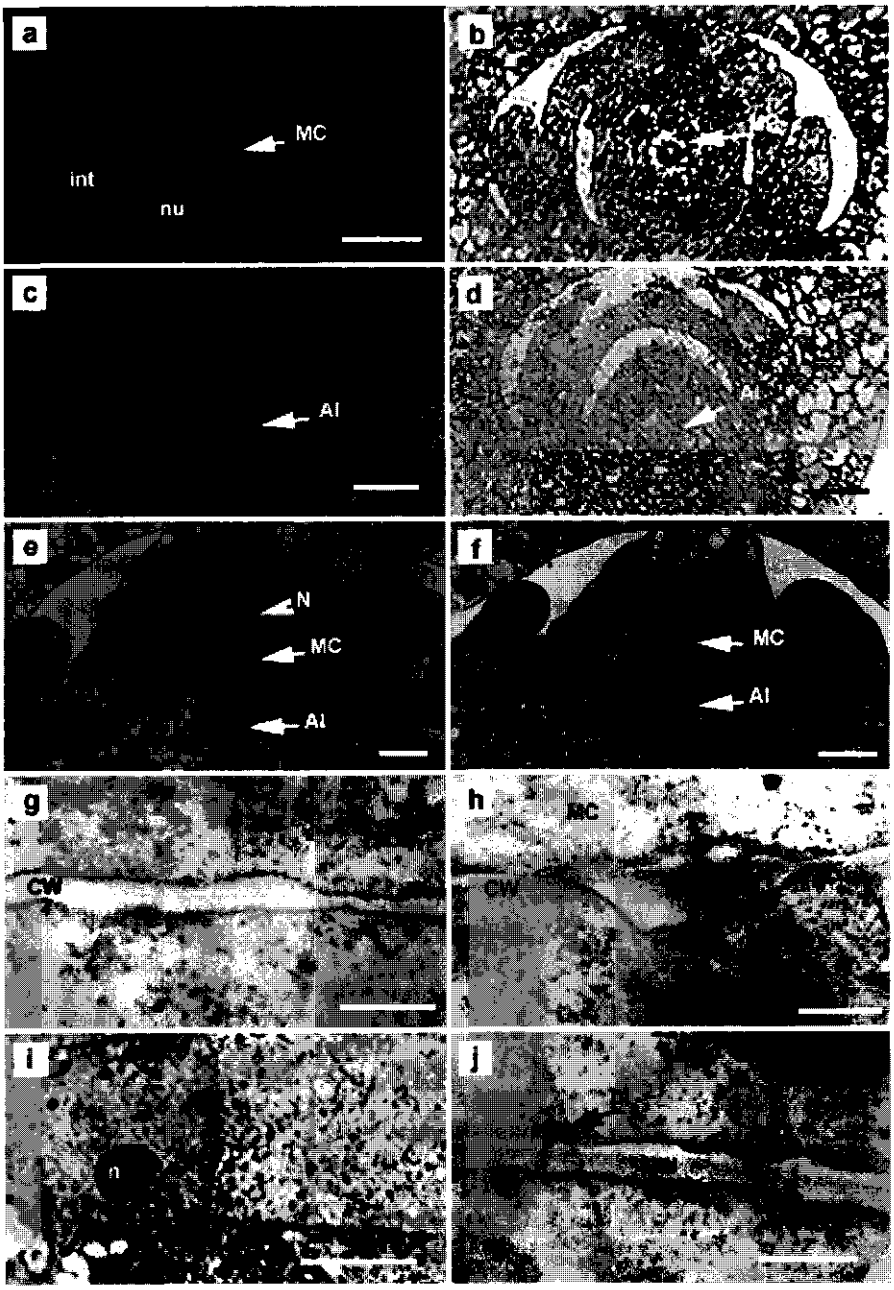
	n		R			P			R/P		
cell type	S	A	S	A	p	S	A	p	S	A	p
MC	24	15	68.4 ± 6.39	51.2 ± 4.64	0.061	19.3 ± 1.80	39.1 ± 1.93	<0.001	4.0 ± 0.34	1.4 ± 0.18	<0.001
NC	19	7	30.4 ± 4.09	75.8 ± 9.42	<0.001	32.1 ± 2.91	17.3 ± 2.56	=0.007	0.9 ± 0.07	2.1 ± 0.17	<0.001
ED / AI	14	12	280.6 ± 5.51	80.2 ± 6.36	<0.001	12.6 ± 3.28	23.8 ± 2.57	=0.019	112.3 ± 32.07	4.1 ± 0.65	0.012

When the orthogonal contrast of t-test was applied, comparison between different cells of the same ovule is possible. The result of this test is resumed in tables 2 and 3.

Table 2. Relative number of ribosomes (R), polysomes (P) and R / P ratio in D4 sexual genotype. Difference between cell types are significant at p = 0.05 by t-test using orthogonal contrast. ED = electron dense chalazal cell, MC = meiocyte; NC = nucellar cell, representing the means in table 1.

R	ED > MC > NC
P	NC > MC > ED
R/P	ED > MC > NC

Figure 3. Histological staining of total RNA and *in situ* hybridisation of DIG-labelled oligo dT to poly(A)+ RNA in sections of apomictic *Brachiaria decumbens*. Transmission electron photomicrograph of apomictic *Brachiaria decumbens* ovules. **a** AO staining of a longitudinal section of an ovule from the apomictic plant at MC stage. Bar: 20 μm. **b** Label of poly(A)+ RNA in other section of the same ovule of the apomictic plant during MC stage. Bar: 20 μm. **c** AO staining of other section of the same ovule as "a" and "b" from the apomictic plant at meiocyte stage showing a chalazal apospore initial with dense cytoplasm marked by high amount of total RNA. Bar: 20 μm. **d** Label of poly(A)+ RNA in another section of the same ovule of the apomictic plant during MC stage showing the apospore initial cell with label comparable with the other nucellar cells. Bar: 20 μm. **e** Light microscopy from a toluidine blue stained semithin longitudinal section of a young ovule showing cytoplasm and nucleus of the meiocyte and part of the chalazal apospore initial. Bar: 2.4 μm. **f** Light microscopy from a toluidine blue stained semithin section from the same young ovule showing the apospore initial with nucleus near the chalazal end of MC. Bar: 3.2 μm. **g** Detail of the MC cell wall in connection to the neighbouring nucellus showing no plasmodesmata. Note a similar ribosome pattern of both cells. Bar: 242 nm. **h** Detail of the cell wall of AI in division with MMC showing plasmodesmata with the nucellar cell. Note the similar ribosome pattern. Bar: 302 nm. **i** Detail of the apospore initial showing nucleus and nucleolus. Bar: 6 μm. **j** Cell wall of the apospore initial in connection with nucellar cell showing plasmodesmata. Note the equal ribosomal population in both cells. Bar: 242 nm. **AI** apospore initial, **CW** cell wall, **int** integument, **MC** meiocyte, **N** nucleus, **n** nucellus, **int** itegument, **pl** plasmodesmata.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Apomictic development

In the apomictic plant the total RNA staining showed, compared to the sexual plant, a less dense cytoplasm in most of the nucellar cells (Fig. 3a). There ws an uniform pattern of mRNA distribution in this stage and the cytoplasm and nucleus of meiocyte also shows some label (Fig. 3b). During MMC stage and MC stage, in the apomictic plant an enlarged nucellus cell, just near to the meiocyte could be observed. This enlarged cell was differentiating from the other nucellar cells and had a more dense cytoplasm as could be seen by the strong staining for total RNA. This cell was thought to be an apospore initial cell (Fig. 3c). After *in situ* hybridisation with oligo-dT probe, no special label could be seen in such cell (Fig. 3d). In the MC stage the meiocyte of the apomictic plant had a large nucleus positioned in the micropylar side of the cell (Fig. 3e). At the chalazal end, as usual, a nucellar cell was enlarging. This special nucellar cell was in the position of a very young apospore initial and had large nucleus and nucleoli and a somewhat dense cytoplasm (Fig. 3f, i). The wall surrounding the meiocyte had no plasmodesmata (Fig. 3g), except for the chalazal wall that makes connection with a remarkable nucellar cell (Fig. 3h). Between the special cell and the other nucellar cells plasmodesmata were observed (Fig. 3j) as between all nucellar cells.

The mean number of ribosomes, polysomes and its relation for the MC cell, normal nucellar cell and AI cell is added in table1. Comparing the cells, as in table 2, there was a significant difference between them that is presented in table 3.

Table 3. Relative number of ribosomes (R), polysomes (P) and R / P ratio in D58 apomictic genotype. Difference between cell types are significant at p = 0.05 by t-test using orthogonal contrast. AI = apospore initial cell, MC = meiocyte; NC = nucellar cell, representing the means in table 1.

R	AI > NC > MC
P	MC > AI > NC
R/P	NC > AI > MC

Total RNA and mRNA patterns during sexual and apomictic embryo sac development

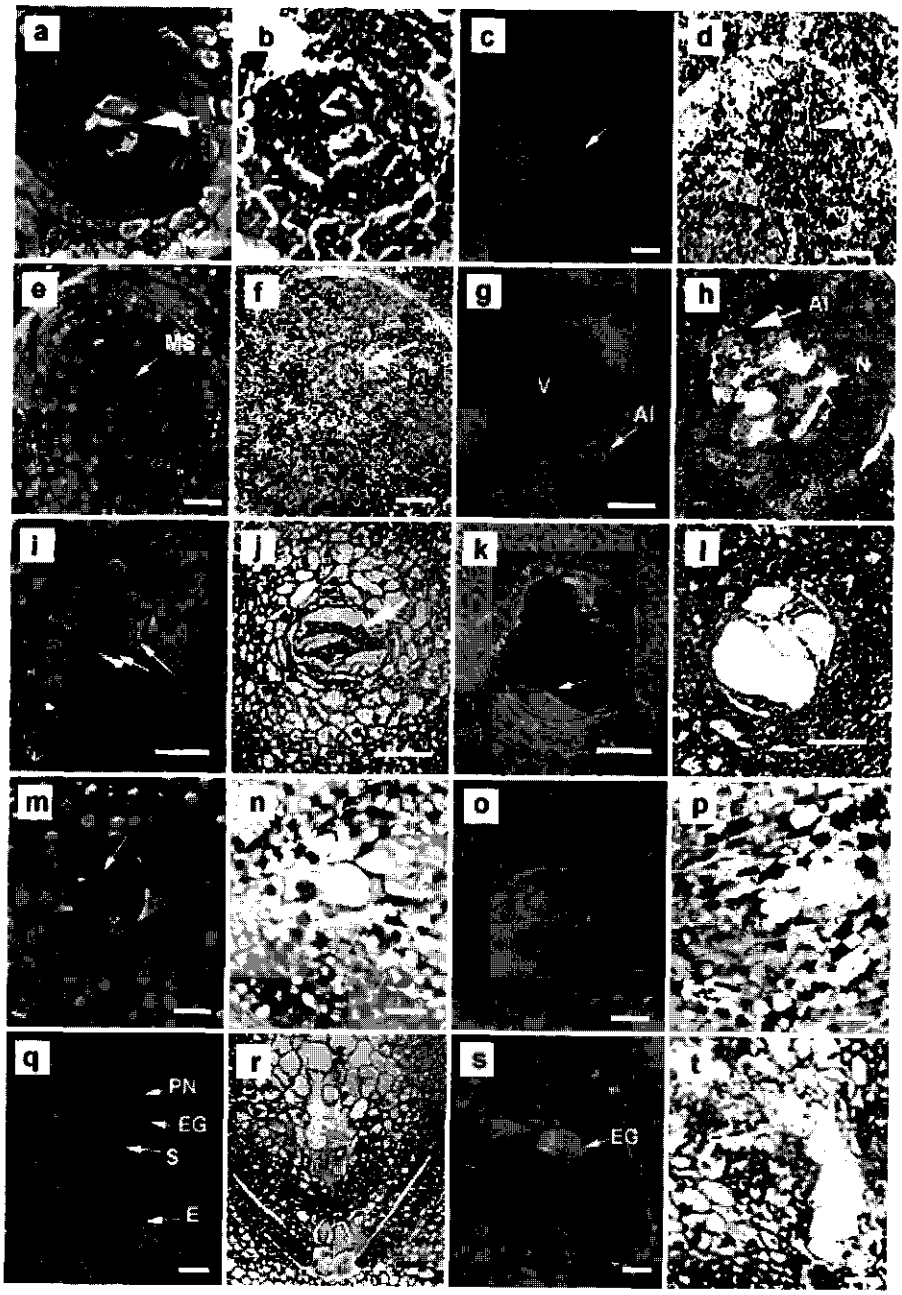
In the sexual plant, the meiosis lead to tetrads formation which had a linear row of megaspores with high cytoplasm density as shown by the total RNA staining with AO (Fig. 4a). The distribution of mRNA was clearly concentrated on the cytoplasm of the tetrads and nucellar cells, integuments show less label (Fig. 4b). At this stage in the ovule of the apomictic plant the meiosis is already arrested and generally tetrads are not formed. Total RNA staining (Fig. 4c) was more intense in integuments than in nucellar cells and meiocyte. In the nucellus there was an equally distributed pattern of mRNA label which was more intense than in the integuments (Fig. 4d).

At the MS stage, in the sexual plant, the survival megaspore could be easily observed after staining for total RNA and showed a dense cytoplasm with high amount of RNA (Fig. 4e). For mRNA an equal label marks the nucellus and megaspore (Fig. 4f). The degenerated megaspores had also high amount of total RNA but no label for mRNA was observed as shown earlier in Figures 1c and d. At the AI stage in the apomictic plant, there were some vacuolated apospore initials and young apospore initials with very strong staining for total RNA (Fig. 4g). Those apospores had a high level of cytoplasmic mRNA (Fig. 4h).

At the CO stage, the cells from the ovule of the sexual plant were very vacuolated. They had high total RNA staining in all cells with somewhat more staining on the cytoplasm around the nuclei of the coenocyte (Fig. 4i). The mRNA cytoplasm label followed a comparable pattern (Fig. 4j). In the apomictic plant, the CO stage hasd the same pattern as in the sexual plant with staining and high label in cytoplasm (Figs. 4k,l). When using the 46bp oligo-dT probe, as in CO stage, the results were similar to that observed using the tailered probe but there were more background making it more difficult to distinguish the level of labelling. Figures 4m,n,o,p summarise the observations.

At the MES stage of the sexual plant, the nucellar and embryo sac cells were quite vacuolated. A strong staining for total RNA was found in the egg apparatus as well as in the embellum cells (Fig. 4q). mRNA label was seen over the cytoplasm of all cells and of the egg cell cytoplasm but the embellum cells were negative (Fig. 4r). In the apomictic plant, egg apparatus cytoplasm was marked with high total RNA staining (Fig. 4s) and egg cell had label for poly(A)+ RNA (Fig. 4t).

Figure 4. (color print in page 166) Histological staining of total RNA and *in situ* hybridisation of DIG-labelled oligo-dT to poly(A)+ RNA in sections of sexual and apomictic *Brachiaria decumbens* from the tetrad stage. **a** AO staining in a oblique section of an ovule from the sexual plant at tetrad stage. Arrow points to megaspores of the tetrad. Bar: 10 μm. **b** Label of poly(A)+ RNA in other section of the same ovule as "a", of the sexual plant during tetrad stage, shows label in the cytoplasm of the nucellar and tetrad cells. Bar: 10 μm. **c** AO staining in a longitudinal section of an ovule from the apomictic plant at late MC stage that corresponds to tetrad stage. Arrow points to the meiocyte. Bar: 20 μm. **d** Label of poly(A)+ RNA in a transversal section of the same ovule as "c", of the apomictic plant during late MC stage corresponding to tetrad stage. Bar: 20 μm. **e** AO staining in a transversal section of an ovule from the sexual plant at the MS stage showing the survival megaspore with strong staining for total RNA. Bar: 20 μm. **f** Label of poly(A)+ RNA in another section of the same ovule, as in "e", of the sexual plant during the MS stage showing that megaspore has same pattern of label as other nucellar cells. Bar: 20 μm. **g** AO staining in a transversal section of an ovule from the apomictic plant at AI stage showing young (arrow) and vacuolated apospores with strong staining for total RNA. Bar: 20 μm. **h** Label of poly(A)+ RNA in a transversal section of an ovule of the apomictic plant during AI stage showing high but uniform label in cytoplasm of apospore initials (AI) and other nucellar cells. AI's have big nucleus and are vacuolating. N arrow points to a nucleus of a vacuolated apospore initial. Bar: 20 μm. **i** AO staining in a transversal section of an ovule from the sexual plant at CO stage showing two chalazal nuclei of the coenocyte (arrows) that are surrounded by a dense cytoplasm with strong staining for total RNA. Bar: 20 μm. **j** Label of poly(A)+ RNA in other transversal section of the same ovule, as in "i", at CO stage showing label on cytoplasm from the embryo sac (arrow). **k** AO staining in a transversal section of an ovule from the apomictic plant at CO stage showing one nucleus (arrow) surrounded by a cytoplasm strongly stained for total RNA. Bar: 20 μm. **l** Label of poly(A)+ RNA in longitudinal section of an ovule of the apomictic plant during CO stage showing high cytoplasmic label in apospores and other nucellar cells. Bar: 20 μm. **m** AO staining in longitudinal section of an ovule from the sexual plant at CO stage showing cytoplasm total RNA staining. Bar: 20 μm. **n** Label of poly(A)+ RNA using the 46bp probe in section of the same ovule, as in "m", of the sexual plant at CO stage showing uniform label in nucellar tissue and embryo sac cells. Bar: 20 μm. **o** AO staining in longitudinal section of an ovule from the apomictic plant at CO stage showing high cytoplasm staining for total RNA in embryo sac. Bar: 20 μm. **p** Label of poly(A)+ RNA using the 46bp probe in section of the same ovule, as in "o", of the apomictic plant at CO stage showing label comparable with nucellar tissue. **q** AO staining in longitudinal section of an ovule from the sexual plant at young MES stage showing the micropylar part of ovule with strong staining for total RNA in cytoplasm of the cells of the egg apparatus and the embellum cells. Bar: 20 μm. **r** Label of poly(A)+ RNA in other section of the same ovule, as in "q", of the sexual plant at young MES stage showing micropylar part of ovule with label all over it, including egg apparatus, but without label in embellum cells. Cells can be localised by the previous one. Bar: 20 μm. **s** AO staining in a longitudinal section of an ovule from the apomictic plant at MES stage showing at the micropylar side of ovule an aposporic embryo sac with cytoplasm from egg apparatus highly stained for total RNA. Bar: 20 μm. **t** Label of poly(A)+ RNA in a section of the same ovule of the apomictic plant at MES stage showing micropylar side of embryo sac with label in egg cell cytoplasm. Cells can be localised by previous section. Bar: 50 μm. **AI** apospore initial, **E** embellum, **EG** egg cell, **PN** polar nuclei, **S** synergid, **V** vacuole.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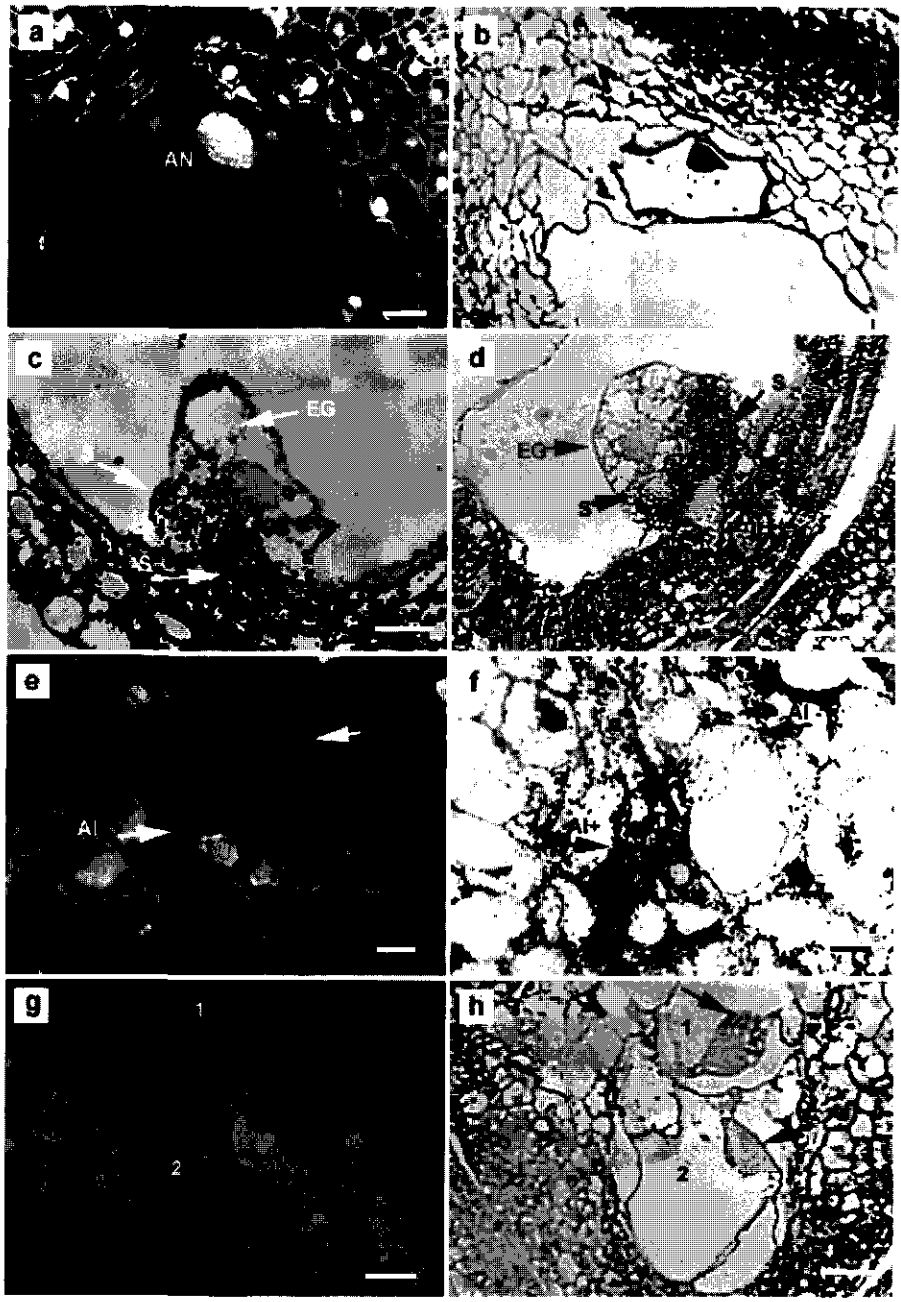
Antipodal cells showed some total RNA around the nucleus and, in cytoplasm, near the cell wall (Fig. 5a), but there was no mRNA label (Fig. 5b). The synergids of the sexual plant had a very high level of mRNA poly(A)+ label in the cytoplasm (Fig. 5c). The synergids of the aposporic embryo sac also had a strong label (Fig. 5d). At the MES stage in the apomictic ovule near the embryo sac, several apospores develop, each with an intensive total RNA staining (Fig. 5e), and strong mRNA label, however, some apospore initials lacked this label (Fig. 5f). Some embryo sacs from apomictic plant in mature stage that did not follow the *Polygonum* nor the *Panicum* characteristics and in which differentiated cells could not be identified, were found to have total RNA (Fig. 5g) but lack mRNA label (Fig. 5h).

Figure 5. Histological staining of total RNA and *in situ* hybridisation of DIG-labelled oligo-dT to poly(A)+ RNA in sections of sexual and apomictic *Brachiaria decumbens* embryo sac stages. **a** AO staining in a longitudinal section of an ovule from the sexual plant at MES stage showing a vacuolated antipodal cell with very few staining for total RNA. Bar: 20 μm. **b** Label of poly(A)+ RNA in a longitudinal section of an ovule of the sexual plant showing the lack of label in a vacuolated antipodal cell. Bar: 20 μm. **c** Label of poly(A)+ RNA in a longitudinal section of an ovule of the sexual plant at MES stage showing egg apparatus and high label in cytoplasm of synergids. Bar: 10 μm. **d** Label of poly(A)+ RNA in a longitudinal section of an ovule of the apomictic plant at MES stage showing an aposporic embryo sac with high label in synergids. Bar: 20 μm. **e** AO staining in a longitudinal section of an ovule from the apomictic plant at MES stage showing the chalazal side of ovule with young apospores (arrows) with cytoplasm stained for total RNA. Bar: 10 μm. **f** Label of poly(A)+ RNA in longitudinal sections of an ovule of the apomictic plant at MES stage showing chalazal side of ovule with apospores, some of them labelled (arrow AI+) and others without any label (arrow AI-). Bar: 10 μm. **g** AO staining in longitudinal section of an ovule from the apomictic plant at MES stage showing two embryo sacs. In the chalazal one (1) this section shows only one of the cells from egg apparatus. The micropylar embryo sac (2) is an embryo sac with just two cells with stained cytoplasm. Bar: 20 μm. **h** Label of poly(A)+ RNA in other section of the same ovule, as in "g", of the apomictic plant at MES stage showing label in the embryo sac 1 (arrow) while embryo sac 2 has no label (arrow). Bar: 20 μm. **AI** apospore initial; **AN** antipodal cell; **EG** egg cell; **FA** filiform apparatus; **S** synergid.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Discussion

Differences in the label of in situ hybridisation on cytological staining were observed within one ovule and between the sexual and apomictic plant. However, the effect of polyploidy in these labels had to be considered before a further analysis of the RNA patterns. Transcriptional and translational activities increases proportionally as the genome doubles (cf. Brodsky and Uryvaeva 1977, Nagl 1978 cited by D'Amato 1984) and polyploidy could be related to the high rates of RNA and protein synthesis (Scharpe and van Parijs 1973, Syamasundar and Panchaksharappa 1995 cited by D'Amato). In the accessions of *B. decumbens* used here, the cell size had also some relation to the ploidy (Dusi and Willemse, 1999b). Compared to the diploid sexual plant, the tetraploid apomict had an large ovule with increased cell volume. As a consequence, although the quantity of cytoplasm is higher in the apomictic plant, the ratio cytoplasm / area may be about the same, allowing a comparison between sexual and apomictic plants based on the localisation and amount of label resulting from *in situ* hybridisation and cytological staining.

The number of ribosomes in the cell is related to the possibility to synthesise protein. Generally, ribosomes performing translation are not free in the cell. Instead, polysomes are associated with cellular structures as the endoplasmic reticulum (Lewin, 1994). The mRNA concentration is though to be related to the level of protein synthesis as well. By detecting total RNA and mRNA, and by observing the number of ribosomes / polysomes, it is possible to detect changes in activities with respect to the cell metabolism and cell differentiation.

In all stages of ovule development the cytoplasm and nuclei were marked by AO showing the total RNA. AO staining is a useful method to study and apospore initials in any stage of ovule development. AO did not influence the site of poly(A) binding and can be used if necessary before *in situ* hybridisation.

Apomictic *B. decumbens* nucellus cell or cells marked by a strong RNA reaction were located in the chalazal end near the enlarged archespore or megaspore mother cell. They are considered to differentiate into apospore initials because of their large nuclei and nucleoli and they had the same RNA pattern (strong RNA reaction) as the apospore initials that were differentiating later during MS and AI stages. Although in *Brachiaria decumbens* first apospore initial is located at the chalazal end of the meiocyte, other AI

develop in a position along the whole meiocyte. This pattern differs from the only chalazal AI's that were observed in other Gramineae like *Panicum maximum* (Naumova and Willemse, 1995). The frequent observation of apospores developing prior in the chalazal part of the ovule could be the reason why the first embryos were visible in embryo sacs located in the chalazal part of the ovules before anthesis (Dusi and Willemse, 1999b).

The amount of total RNA detectable by AO staining was not in direct correlation with amount of poly(A)+ RNA as detected by *in situ* hybridisation. This is due to low concentration of mRNA as part of the total RNA always present in the cell. Cytoplasmic rRNA is the most abundant RNA and can be present in the eucariotic cell in a concentration 4 to 23 times higher than tRNA and mRNA respectively (Alberts *et al.* 1994).

During ovular development most of the cells were labelled for poly(A)+ RNA with the oligo-dT probe, but the level of label differed. The few data concerning poly(A)+ RNA labelling in developing ovules, represent differences during the early MMC stages. In *Capsella bursa-pastoris* in the MMC stage, poly(A)+ RNA is absent in megasporocyte, as compared to the rest of nucellar cells (Raghavan 1990) but in *Medicago sativa* (Bimal *et al.* 1995) megasporocyte and the functional megaspore acumulate Poly(A)+ RNA, indicating that the data can not be generalised and should be restricted to the species or even to the genotypes within species. In sexual species like *Stellaria media* (Pritchard, 1964), *Argemone mexicana* (Bhandari *et al.* 1980) and *Brachiaria decumbens* also a relatively high concentration of RNA in the archesporial cells or megasporocytes was observed. In *Brachiaria*, there was few difference in localised amount of poly(A)+ RNA in sexual and apomict in the early stages of development.

At the MMC stage, the archespore cell of the apomictic plant of *Brachiaria*, in contrast with what was observed for the sexual plant, showed a nucleus with high amount of mRNA. This high amount of mRNA in the nucleus could be a consequence of the active mRNA synthesis in the nucleus and delayed in transport to the cytoplasm, which could be related to a retarded or blocked meiotic embryo sac development in the apomict. At the MC stage, the amount of mRNA in the meiocyte of sexual and apomict, as observed by the hybridisations, was comparable with the amount in the nucellar tissue and integuments.

The AO staining allowed a comparison in ribosome population between MMC stage and MC stage between sexual and apomict. While there was a drop in total RNA staining in ovules of the sexual plant, from archespore to

meiocyte, in the apomictic plant this staining seems to be similar, and low, in both archespore and meiocyte. Such drop in ribosome population was observed at ultrastructural level and is quite common during sexual development (Hotta and Stern, 1963). It means that, in that cell, there was a transition to a sexual development steps. Our observations at ultrastructure level showed that the cytoplasm of the MC from the apomict was more electron dense than the MC from the sexual. The counting of ribosome and polysome population shows that this difference must be the consequence of the higher polysome population in the cytoplasm of MC from the apomict when compared to the sexual. While the meiocyte in the apomict showed few staining for acridine orange, in the cytoplasm of the young apospore initial, the stain on total RNA was clear and more intense compared to the surrounding nucellar cells. The AI also had more ribosomes than that observed for meiocyte and nucellar cells.

An apospore initial before vacuolation had an increase in total RNA that was comparable to that of the functional megaspore indicating the switch in the development pathway of the nucellar cell to form an embryo sac. In the ovule of the apomictic plant, the MC cell wall at the micropylar side lacked plasmodesmata while the chalazal portion that borders the apospore initial in *Brachiaria* had many plasmodesmata, like in *Panicum* (Naumova and Willemse, 1995). This form of connection guarantees the symplastic transport between meiocyte and nucellar cells and may be a factor that promotes the growth of the chalazal nucellar cell even when meiocyte degenerates. At the MC stage in sexual, plasmodesmata were not visualised in meiocyte cell wall. Some cells with high ribosome population and low number of polysomes could be visualised at the chalazal side of meiocyte. These cells, not always present, showed very few plasmodesmata in cell wall connecting to the meiocyte indicating a low transport of nutrients cell to cell and were considered to degenerate. Maybe the ribosome rich cells can be considered as an apospore initial-like cells. By exception some nucellar cells in ovules of the sexual plant had strong acridine orange staining at meiocyte stage (data not shown). If these cells were apospore intitials, in contrary to apospore intitials, they did not develop to an aposporic embryo sac. The occurrence of aposporic embryo sacs in ovules of the sexual plant were never observed in any stage of ovule development in our plants, in contrary of what was observed by Naumova *et al.* (1999). The cells will remain and function as nucellar cell or will die.

At the AI stage, vacuolated apospores could be seen together with very young apospores before vacuolation, both showed a more intense total RNA label. The AI stage was comparable to the survival megaspore in the sexual plant during megaspore stage. The high amount of total RNA in the apospore may prepare the change from nucellus cell into an gametophyte as the megaspore of the sexual plant. The mRNA label from the AI's was not enhanced and was similar to the neighbouring nucellar cells. The change to a diploid gametophyte did not affect radically the amount of mRNA.

At the coenocyte stage, mRNA was present in high amounts in the cytoplasm of both sexual and apomictic plants. This high amount of total RNA remains untill the end of gametophyte formation when cellularisation occurs to form a sexual and aposporous embryo sacs. This points to a consistent population of ribossomal RNA necessary for the translation of the mRNAs in proteins while embryo sac cells are passing through mitoses divisions. High amounts of total RNA was also found in degenerating megaspores and apospores at the end of ovule development. In these cells mRNA was not enhanced and this marks the degeneration of the apospores at the end of development.

During the MES stage the high level of mRNA label in the young synergids of *Brachiaria*, also observed in *Medicago* (Bimal *et al.* 1995) and in *Gasteria verrucosa* (Bimal and Willemse 1996) is related to the events that take place before and after fertilisation and seed formation. Synergids are very active cells and their cytoplasm is rich in RNA and proteins (Willemse and van Went, 1984). In *Arabidopsis* (Belostotsky and Meagher, 1996) the PAB5 gene encoding one of the poly(A) binding proteins (PABPs) is though to be expressed in synergids. The high amount of poly(A)+ RNA found in the synergids may reflect a special function of this cell within the embryo sac.

The highly vacuolated antipodals had very few RNA and mRNA accumulation. Although they may be already differentiated, its dispersed cytoplasm easily gives an impression of a low poly(A)+ RNA level.

Differences in carbohydrate metabolism in ovules of sexual and apomictic plants during the initial stages of embryo sac development suggested a variable and lower nutrient condition at the beginning of apomict development (Dusi and Willemse, 1999a). A difference was also observed in the amount and distribution of total RNA and mRNA in the MMC, high total RNA in the sexual and low in the apomict, mRNA concentrated in nucleus in apomictic and not in the sexual. In *Brachiaria* the induction of the AI may take place in a very early stage of development. Ovular cells from the apomictic

plant ovule that will undergo differentiation, have to get their machinery ready for transcription. The pattern of total RNA distribution, showed that apospore initials were comparable to functional megaspores. However there should be a reason for the close proximity of apospores to the MMC or to the degenerating meiocyte and that could be the possible role of the megaspore mother cell, which will degenerate later, as a source of nutrients or even as a source of proteins necessary to apospore development. In contrast to the functional megaspores, apospore initials are unreduced cells that does not seem to need to change from diploid to haploid state before enter gametophytic development. The ultrastructure of a chalazal located apospore initial showed a dense cytoplasm and high ribosomic population to be expected in cells with high amount of total RNA, as observed by acridine orange staining. During vacuolation ultrastructural similarities between apospore initial and vacuolated functional megaspore, were observed in *Panicum* (Naumova and Willemse 1995). Like in *Panicum*, in the ovule of the apomictic plant, the MMC cell wall at the micropylar side lacked plasmodesmata while the chalazal portion that borders the apospore initial in *Brachiaria* had many plasmodesmata. This form of connection guarantees the symplastic transport between cells and may be a factor that promotes the growth and differentiation of the chalazal nucellar cell when meiocyte will soon degenerate.

The evidence for a single genetic control of apomixis in *Brachiaria* (Miles and Valle 1991; do Valle *et al.*, 1994; do Valle and Savidan, 1996) suggests that, as happens during cell differentiation in multicellular organisms, the apospore initials diverge from other cells of the nucellus as a consequence of differentiatiation in the RNA and protein synthesis.

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Chapter 5

Pattern of gene expression in ovaries of sexual and apomictic plants of *Brachiaria decumbens*. Cloning of a differentially expressed cDNA.

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Abstract

A differential display RT-PCR approach was used to compare gene expression in ovaries of sexual and apomictic plants of *Brachiaria decumbens*. Most of the differentially displayed cDNAs were found in ovaries with young ovules. *In situ* hybridisation analysis showed that clone number 7-21 is expressed in ovaries of sexual and apomictic plants, while clone number 4-29 is expressed during early stage in ovules of both plants but in mature embryo sacs and young embryos only in the apomictic plant. The results are discussed with respect to the autonomous embryo formation of the apomict.

Introduction

Brachiaria (Trin.) Griseb is a genus of forage grasses that contains about 100 species (Renvoize *et al.* 1996, Dahlgren et al 1985). *Brachiaria decumbens* is the widely cultivated species in Brazil, having great economical importance (Hopkinson 1996). As other cultivated species of *Brachiaria*, *B. decumbens* reproduces predominantly by apomixis although sexual plants were identified in this species (do Valle 1990; do Valle 1991). Apomictic plants of the different *Brachiaria* species are polyploids while sexual plants are diploids. Apomixis in *Brachiaria* is apospory, which implies unreduced embryo sac formation and embryo development without fertilisation of the egg cell.

The inflorescence of members of *Brachiaria* is a panicle with 2 to 5 racemes that support the spikelets placed in two series on the raceme. Each spikelet develops two flowers, a male and a hermaphrodite flower (Skerman and Riveros 1990, Ndikumana 1985). The hermaphrodite flower has three anthers and one ovule. The megasporogenesis and megagametogenesis in sexual and apomictic *B. decumbens* was analysed in detail (Dusi and Willemse, 1999b). The sexual plant follows the Polygonum-type embryo sac development. In ovules of the sexual plant the archespore enlarges and enters meiosis with elongation of the megaspore mother cell and deposition of callose in the meiocyte wall, in the dyad and tetrad. From the tetrad only the chalazal megaspore survives. This functional megaspore undergoes three mitoses and an eight-nucleated coenocyte is formed. After cellularisation a Polygonum-type embryo sac is formed that has an egg apparatus with two synergids and the egg cell, two polar nuclei and three antipodals, which are able to divide until anthesis. After anthesis, the stylodium covered with pollen grains dries out. After double fertilisation, a zygote and the endosperm is formed.

The apomictic plant follows the Panicum-type embryo sac development. In ovules of the apomictic plant as in the sexual ones the megaspore mother cell enters meiosis. But, in contrast to ovules of the sexual plants, some cells of the nucellus, starting from a chalazal one, enlarge and differentiate into apospore initials that will form, after two mitoses, an aposporic embryo sac (Dusi and Willemse 1999a). The meiocyte is characterised by the elongation of the megaspore mother cell and partly callose deposition, but commonly meiosis is arrested and only an elongated meiocyte is formed. By the time that the meiocyte is degenerating more apospore initials are formed around it. The apospores enlarge and undergo two mitoses to form a four nucleated coenocyte. After cellularisation one or more Panicum type embryo sacs are formed in one ovule. In these ovules, just before anthesis, embryos can be observed (Dusi and Willemse 1999a). About one day later, after anthesis, fertilisation of the central cell may occur also in the apomictic plants. *B. decumbens* is a pseudogamous species (Ngendahayo 1988) and therefore needs fertilisation of the central cell in order to form the endosperm that will support embryo development. Eventually, in apomictic plants meiosis may occur and a reduced embryo sac can develop side by side with the aposporic ones. Therefore, it has the potential to produce seeds by the normal sexual process.

There are to consider two main points of regulation in the apomictic pathway of embryo sac development. One, at the very beginning of embryo sac differentiation, when a signal in nucellar cells induces the growth of apospore initials. The other point is at a late stage of development of the embryo sac, before anthesis, when embryos are formed autonomously in embryo sac of apomictic plants independent of fertilisation.

The trigger as well as the development of the ovule require gene products that control this process (Nadeau *et al.* 1996). The different pattern of differentiation of cells in the young ovule between sexual and apomictic modes of reproduction could be a consequence of changes in gene expression. But, few is known to date about the expression of genes required for normal development of the female gametophyte (reviewed in Drews *et al.* 1998) and even less about apomictic development.

There are many different approaches aiming at the identification and cloning of genes related to apomixis. They go from identification of molecular markers linked to apomixis to strategies of mutagenesis and differential display (reviewed in Pessino *et al.* 1999, Savidan 2000). Mutagenesis induction in *Arabidopsis thaliana*, produced the mutants fertilisation

independent endosperm, *fie* (Ohad *et al.* 1996, 1999) and fertilisation independent seeds, *fis1, fis2 and fis3* (Chaudhury *et al.* 1997). Both types of mutants develop endosperm in the absence of pollination. Mutants *fis3* and *fie* produce only endosperm at free nuclear stage. Mutants *fis* 1 and *fis* 2 produce autonomous endosperm up to the celularized stage and, in some seeds, also proembryos up to the globular stage are formed. None of the mutant seeds maturate and develop plant. Recently two models for action of the *FIS* genes were proposed (Luo *et al.* 1999). In one model, *FIS*, a transcription factor, is responsible to repress activity of seed development genes (SDG) while *FIS1*, *FIS2* and *FIS3* keep them repressed. In normal sexual reproducing plants, after pollination and fertilisation, derepressed SDG genes may be introduced to initiate development of the endosperm and at some degree the embryo. The other model is a sequential regulatory model. In this model the product of *FIS2* regulates *FIS1* gene positively, *FIS1* gene than represses SDG. *FIS3* may also be controlled by *FIS2* or may be in a complex with *FIS1*. The role of *FIS* genes in the apomictic reproduction may include a modified regulatory model in apomictic plants (Chaudhury *et al.* 1997, Luo *et al.* 1999).

For *Brachiaria*, it was proposed that apospory is inherited as a single dominant factor (Miles and Valle 1991; do Valle *et al.*, 1994; do Valle and Savidan, 1996). Molecular analysis of gene expression during ovule development will reveal whether there are specific genes that confer the apomictic character or whether this mode of reproduction is due to a change "in the spatial and temporal expression of key regulatory genes" that are related to sexual reproduction (Koltunow 1993).

Polyploidy is often associated to apomicts. Tetraploidy is the commonest level of ploidy of the apomicts, while their related sexuals are mainly diploid (Asker and Jerling 1992). In some cases, making autotetraploids from diploid plants lead to expression of apospory as in *Paspalum hexastachyum* (Quarin and Hanna 1980). If polyploidy allow the expression of genes that control the apomictic development, but are silent in the diploid genome, the comparison of gene expression of diploid sexual and tetraploid apomicts is a good choice to detect apomixis related genes.

Differential display, a method first described by Liang and Pardee (1992) is widely used to detect and characterise altered gene expression in eukaryotic cells. Because it uses only small amounts of total RNA, it is a good choice when the amount of tissues and RNA are limited. That is the case with the very small ovaries of *Brachiaria* at the initial steps of ovule development, when the differentiation to the archespore starts. Therefore this method was

chosen to compare expression of ovaries of the apomictic and sexual plants. Two stages of development were selected. The first stage corresponds to the period of sporogenesis, at the moment when in the ovule the archespore cell is developing into a meiocyte, and in the apomictic plant, the first apospore initial is differentiating. At this stage it is expected that the differences in gene expression that lead to a different type of embryo sac formation could be detected. The second stage correspond to the period of gametogenesis at the moment of the end of embryo sac formation, just before anthesis, when in the aposporic embryo sac the development of the embryo is triggered.

Material and methods

Plant material

Plants of *Brachiaria decumbens*, accessions BRA000191 (facultative apomictic, tetraploid, D58- cv. IPEAN) and BRA004430 (obligate sexual, diploid D4), from the Brazilian Agricultural Research Corporation, EMBRAPA - Embrapa Beef Cattle, CNPGC were used. The plants were grown in a greenhouse with temperatures ranging from 25 °C during the day to 20 °C at night, with a 16 h light, and 8 h dark light regime. Inflorescences were collected of young (megaspore mother cell or meiocyte stage) and mature stages (differentiated embryo sac before anthesis). The selection was based on a reproductive calendar determined by pistil length (Dusi and Willemse 1999b). Because of the small size of the ovules, RNA was extracted from the complete pistils.

For each type of plant, sexual or apomictic, five categories of plant material were used. 1. young flowers containing pistils of less than 0.6 mm in length; 2. pistils of less than 0.6 mm; 3. ovaries at mature stage; 4. stylodia from pistils in mature stage from flowers before anthesis; 5. flowers before anthesis without pistil. After collecting, the material was immediately frozen in liquid nitrogen.

RNA isolation

The frozen plant material was grinded and RNA was extracted using the Qiagen RNAeasy kit. DNA present in the RNA samples was eliminated using the Message Clean^{TM} Kit (GenHunter Corporation).

Differential display

Differential display of mRNA (DDRT-PCR) experiments were carried out using the kit RNA map™ - mRNA Differential Display * system from GenHunter Corporation. First strand cDNA was synthesised using 5'-T_{12}MA-3' primers in a 20 µl reaction mixture containing: 0.2 µg of total RNA, 10 µM of the anchor primer, 250 µM of dNTP, in reverse transcriptase buffer. The mixtures were subsequently incubated at 65°C, 5 min, at 37°C, 60 min, at 95°C, 5min and then stored at 4°C. After incubation for 10 minutes at 37 °C, 1µl (100 u/ml) of MMLV reverse transcriptase (Superscript, Gibco-BRL) was added.

AP-6 primer was used in a PCR reaction containing: 2 µl of the reverse transcription mix, 25 µM dNTP, 2 µM AP-6, 10 µM T_{12}MA, 0.2 µl Ampli Taq and 1 µl (pmol) α ^{33}P-dATP (>1,000 Ci/mmol) in PCR buffer. PCR was carried out for 40 cycles as follows: 94 °C for 30 s for denaturing; 40 °C for 2 min for annealing; and 72 °C for 30 s for extension, followed by 1cycle for extension at 72 °C for 5 min and hold at 4 °C. An aliquot of 3.5 µl of each radioactive sample was mixed with 2 µl of loading dye. Eletrophoresis in 6% polyacrylamide DNA sequencing gel was performed for about 4h at 60 W constant power. After vacuum drying at 80 °C for 1 h, the gel was exposed to a X-ray film for 18 h.

DNA fragments from about 200 bp and more were selected and excised from the dried gel using the autoradiogram as reference. DNA was eluted by rehydrating in 100 µl of sterile water and placed on ice for at least 10 min, kept overnight at 4 °C, boiled for 15 min and centrifuged for 2 min. The supernatant was transferred to a new microfuge tube and 10% 3M sodium acetate was added. DNA was precipitated in ethanol and 50 µg of glycogen were added. The clean DNA was dissolved in 10 µl of H_2O. cDNA was amplified using the two flanking primers, AP-6 and T_{12}MA.

Cloning and sequencing

Reamplified cDNA products were eletrophorised in a 1% agarose gel and the corresponding bands were cloned into the following plasmids according to their manufacturing instruction manual: pMOSBlue (pMOSBlue bluntended cloning kit - Amersham Life Science) or pCR-Script ™ (pCR-Script Amp SK(+) cloning kit - Stratagene). DNA was sequenced using a ABI PRISM model 310 sequencer from Perkin Elmer.

In situ hybridisations

Inflorescences of plants grown in the greenhouse or in the field were used. Pistils were removed from the spikelets and fixed in 4% paraformaldehyde, 0.25% glutaraldehyde and 0.1 M NaCl in 0.01M phosphate buffer, pH 7.2 for 1 h in vacuum at room temperature. The fixative was refreshed and samples were kept overnight without vacuum. After washing with the same buffer, they were dehydrated in an ethanol series (10, 30, 50, 70, 85, 96, 100% ethanol), 20 min each. At 100% ethanol, 10 mM of dithiothreitol (DTT) was added. The samples were embedded at 4°C in butyl-methyl methacrylate - BMM (Gubler 1989, Baskin *et al.* 1992, Kronenberger *et al.* 1993) through an ethanol:BMM series of 5:1, 5:2, 5:3, 5:4, 5:5, 4:5, 3:5, 2:5, 1:5 (v/v) and two changes of 100% BMM. DTT was added in all steps of embedding to a final concentration of 10 mM. Incubation steps took at least 4h each and the samples were kept at 4 °C. The material was placed in gelatine or plastic capsules containing a fresh BMM mixture, closed with a lid, and polymerisation was done with Philips 8 watt/h UV irradiation for 48 h at -20 °C. After polymerisation, the samples were kept at 4 °C. Sections of 2 to 4 μm were cut and placed on drops of water on glass slides, stretched with chloroform vapour and placed on a 60 °C plate for 1 h. BMM was removed by two 10 min washes in acetone, one wash in a mixture of acetone : water 1:1 and finally in water. For a good control of tissue and RNA preservation, some sections were stained for total RNA with acridine orange (AO). Sections were rinsed with 0.2M acetate buffer pH 2.1 and stained with 0.05% acridine orange (AO) in 0.2M acetate buffer pH 2.1 for 30 min and then washed 15 min in veronal-acetate buffer pH 7.8 (Bimal and Willemse, 1996). These sections were examined with either UV or FITC light. RNA fluoresces orange or red while DNA fluoresces green or yellow respectively. After control only the material with intact RNA was used.

A whole mount method was used: flowers were separated from the spikelets, placed in Petri dish with fixative (described above) and with the use of a stereoscope microscope and sectioned longitudinally with a RNAse free knife, in a way that the pistils were divided in two parts. They were then incubated overnight in microfuge tubes with fixative. Tissue was washed in a buffer identical to the fixation buffer but without the fixative and in hybridisation buffer. No prehybridisation treatment was performed for those samples. After each wash or change of solutions, a brief centrifugation was made to collect the specimens.

RNA probes were prepared using the DIG RNA labelling kit (Boehringer Mannhein) according to the manufacturer's instructions. For clones 7-21 and 4-29, the pCR Script plasmids were linearized by digestion with EcoRI and T7 polymerase was used to synthesise sense probes. When the digestion was done with SSTI, T3 polymerase was used to generate anti-sense probes of 586 and 430 nucleotides in length for 7-21 and 4-29 respectivelly. Sequence 4-29 was also cloned in the pMOS plasmid, that was linearized by digestion with Eco RI and T7 polymerase used to make the anti-sense probe of 430 nucleotides in length.

Prehybridisation treatments: The slides were incubated in 100 mM Tris-HCl pH 7.5; 50 mM EDTA buffer containing 1µg ml^{-1} of proteinase K and washed 2 times in DEPC treated water for 10 min.

Sections were hybridised overnight at 42 °C with 600 ng ml^{-1} of digoxigenin-labelled probe in 100 µl of 50% deionised formamide, 1 x Denhardt's, 250 ng ml^{-1} tRNA, 300 mM NaCl, 10mM Tris-HCl (pH 7.5), mM, 1mM EDTA pH 8.0 in water.

After hybridisation the sections were washed at 37 °C in 4x SSC, 2x SSC, 1x SSC, 0.5x SSC (1x SSC = 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7), 30 min each. 5 min in detection buffer 1 (0.1 M Tris-HCl pH 7.5; 0.15 M NaCl). Sections were incubated in detection buffer 2 (1% Blocking Reagent - Boehringer Mannhein or 2% BSA -Bovine Serum Albumin) subsequently in detection buffer 1 for 1 h and washed 5 min in detection buffer 1. Sections were then incubated with Anti-Digoxigenin-AP Fab fragments (Boehringer Mannhein) diluted 1: 1000 in detection buffer 1, for 1h. Washed 2 times, 15 min each, in detection buffer 1 and one time, 5 min, in detection buffer 3 (0.1 M Tris-HCl pH 7.5; 0.1 M NaCl; 0.05 M $MgCl_2$; final pH adjusted to 9.5). Sections were incubated in staining solution 3.5 µl BCIP (0.05 g ml^{-1}) and 4.5 µl NBT (0.05 g ml^{-1}) in 1 ml detection buffer 3 for 1 to 3h. The colour reaction was stopped by incubating with detection buffer 4 (0.01 M Tris-HCl pH 8.0; 1 mM EDTA).

After the final washes and air drying the slides with the sections, they were mounted in Depex (BDH, Brunschwig Chemie) or in Permount SP15 - 500 (Fisher chemicals - Fisher Scientific) and observed with a Zeiss Axiophot light microscope. The tissue from the whole mount hybridisation were clarified with glycerol, mounted on glass slides, cover with coverslip and observed with a Nikon optiphot or Zeiss Axiophot differential interference contrast microscope.

Results

The reproductive calendar of *Brachiaria decumbens* shows morphological and cytological differences and timing of ovule development in sexual and apomictic plants, allowing a selection of stages based on pistil size (Dusi and Willemse 1999b). From the initial steps of ovule development to anthesis, the apomictic and sexual characteristics were distinctly visible. Differential display RT-PCR was used to detect different expression patterns in ovules of the sexual and apomictic plant and stage specific transcripts. mRNA from flowers and ovaries containing the archesporial or megaspore mother cells and cellularized embryo sacs and embryo sacs before anthesis were used for screening the differences. Figure 1 shows the pattern of expression of mRNA in ovaries and flowers of sexual and apomictic *Brachiaria decumbens* genotypes. The control lanes, corresponding to cDNAs from flowers that had their pistils removed and to cDNAs from stylodia, indicate the differences due to the background of genotypes. Some fragments were present in all lanes of one genotype but not in the other. Most likely, these fragments are specific for each genotype rather than being specific for apomixis or sexual reproduction. Some bands were present only in one stage of development, like young pistils from apomictic or sexual plants. With the primer combination 5'-T_{12}MA-3' and AP6, we identified differential expression of genes between sexual and apomictic plants (Table 1): seven different bands in young pistils; two fragments that were present in young pistils as well as young flowers; one fragment in young flowers only; one fragment in old ovaries and stylodia. Less fragments were identified specific for the sexual plant: two fragments from young pistils; one present in young pistils and old ovaries; two in old ovaries; one in stylodia; one in ovaries and stylodia; and one in stylodia and flowers.

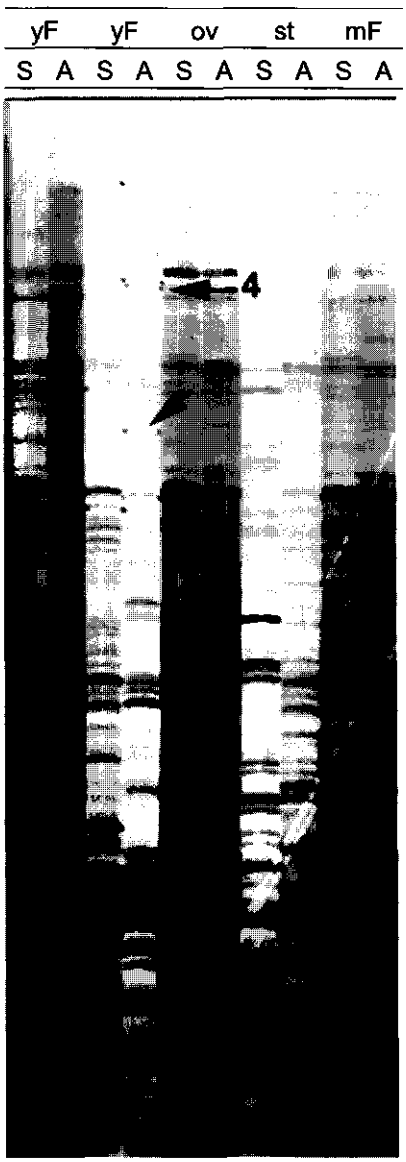


Figure 1. Pattern of mRNA in ovaries and flowers of sexual (**S**) and apomictic (**A**) *Brachiaria decumbens* genotypes. A poly(A)$^+$ -anchored primer ($T_{12}MA$) was used in combination to the AP6 primer from GenHunter Corporation. **A** apomictic; **S** sexual; **yF** young flowers containing a pistil with less than 0.6 mm; **yP** young pistils with less than 0.6 mm; **ov** ovaries in mature stage; **st** stylodia from pistils in mature stage from flowers before anthesis; **mF** flowers before anthesis without pistils. **4,7** isolated clones.

Table 1. Number of bands (n.) specifically for either apomictic or sexual plants. RNA was extracted from young flowers (yF), young pistils (yP), mature ovaries (ov), stylodia from pistils in mature stage (st), flowers that had their pistil removed (fl).

Apomictic					Sexual					
n.	yF	yP	ov	st	n.	yF	yP	ov	st	fl
7		x			2		x			
2	x	x			1		x	x		
1	x				2			x		
1			x	x	1				x	
					1			x	x	
					1				x	x

These differential bands were isolated, re-amplified and cloned in vectors. For the re-amplification, they were used as template with the same primers as were used for the DDPCR reaction. We observed, by 1% agarose gel eletrophoresis that some of them were not properly re-amplified, and others showed more than one band. In this latter case, the most abundant band was chosen to be cloned. Sequencing revealed that most of the clones had the AP6 primer on both sides of the fragment. Some clones have the same sequence although they were isolated as different bands. Finally 7 different sequences were obtained.

To confirm whether these bands reflect a true difference in expression pattern between apomictic to sexual development, *in situ* hybridisations were done. The patterns of expression of two of the cloned sequences during ovule development are shown.

Clone 7-21 was initially identified in young pistils and young flowers of the apomictic plant. Comparison of amino acid sequence using the data bases of NCBI showed that sequence 7-21 has similarity to glucosamine-6-P isomerase from Bacillus subtilis. Figure 2 shows comparison of the deduced amino acid sequence of clone 7-21 and glucosamine-6-P isomerase (*Bacillus subtilis*).

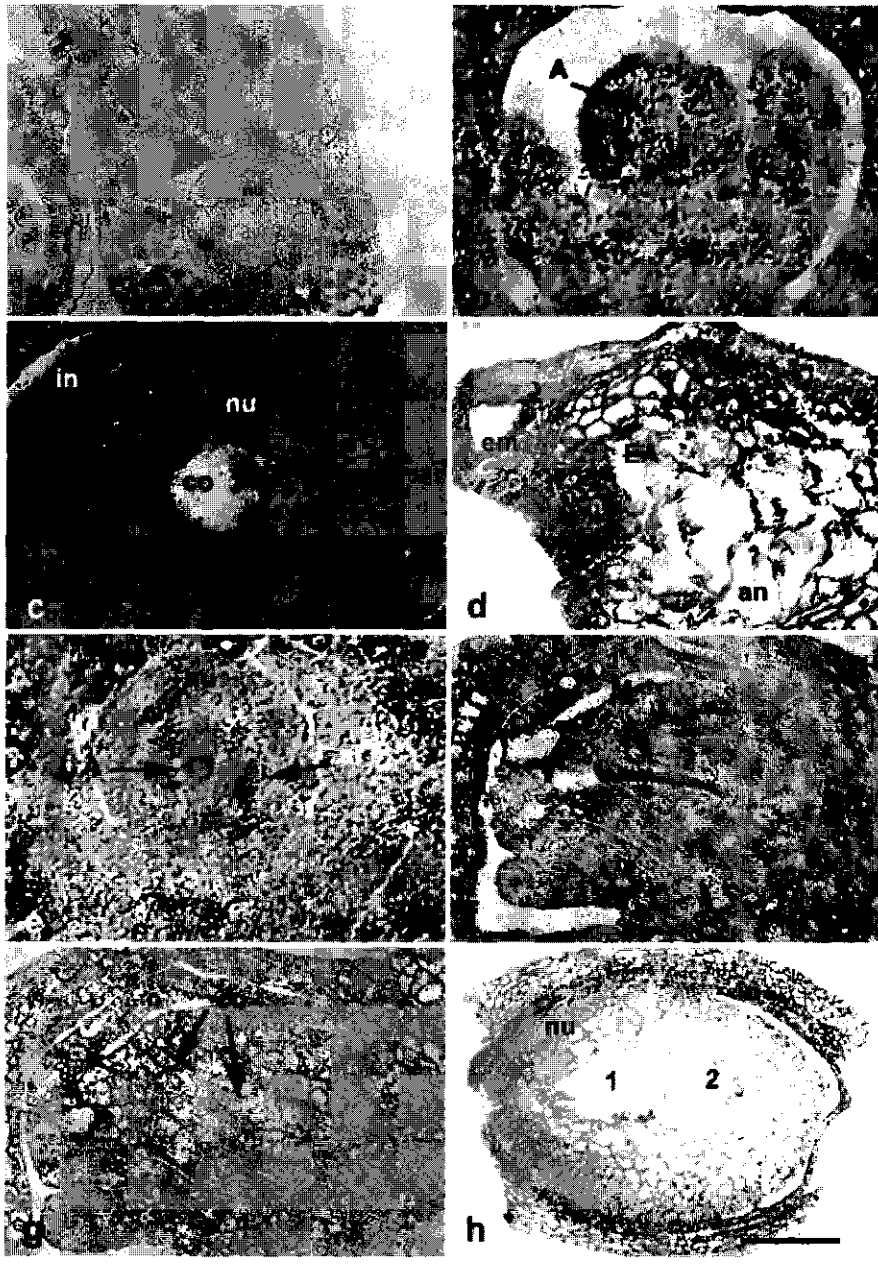
```
7-27:              7 DVQILGIGENGHIGFNEPGTSFDAKTALVDLTDSTIQANKRYFESEADVPRQAYSMGIAS 186
identity             D+Q+LGIG NGHIGFNEPGTSF ++T +V L + T QAN RYF S VP++A +MGI +
gb|AAC67284.1: 127 DIQLLGIGRNGHIGFNEPGTSFKSRTHVVTLNEQTRQANARYFPSIDSVPKKALTMGIQT 186

7-27:            187 IMKSKKIILMAFGKNKANAIKQLMAGEVTTDXPATVLINHPDVAVILDEDAASL 348
identity             I+ SK+I+L+ GK+KA A+++L+ G ++ D PA+ L H DV V++D +AASL
gb|AAC67284.1    187 ILSSKRILLLISGKSKAEAVRKLLEGNISEDFPASALHLHSDVTVLIDREAASL 240
```

Figure 2. Comparison of the deduced amino acid sequence of clone 7-21 and glucosamine-6-P isomerase (*Bacillus subtilis*) gb|AAC67284.1. Conserved amino acids are shown in the middle lane. (+) equivalent amino acids.

Figure 3 shows the result of *in situ* hybridisation of ovules at different stages of development in sexual and apomictic *Brachiaria decumbens* with probe 7-21. The sense probe gave a good negative control because it did not give any signal (Fig. 3a). Using the anti-sense probe the expression pattern was weak and similar for tissues form sexual and apomictic plant at all stages (Fig. 3 b-h). In general, the hybridisation as observed by the faint colour present in all cells of the ovule shows a weak and overall pattern of expression of 7-21. Lack of expression of 7-21 was only observed in integuments of ovules of sexual plant at mature stage, near the anthesis (not shown).

Figure 3. *In situ* localisation of 7-21 RNA in longitudinal sections of developing ovules of sexual and apomictic plants of *Brachiaria*. **a** A young ovule of a sexual plant hybridised with the sense probe. Bar: 70 μm. **b** a young ovule of the sexual plant during archespore stage showing regular pattern of 7-21 expression in nucellus, integument and archespore. Bar: 25 μm. **c** an ovule of sexual plant at coenocyte stage Bar: 50 μm. **d** an ovule of sexual plant at mature stage. Bar: 100 μm. **e** an ovule of apomictic plant at archespore stage, showing also the presence of apospore initial. Bar: 25 μm. **f** an ovule of apomictic plant at meiocyte stage, the meiocyte is starting degeneration. Bar: 50 μm. **g** an ovule of apomictic plant at coenocyte stage. Bar: 50 μm. **h** an ovule of apomictic plant with two embryo sacs (1 and 2) at mature stage. Bar: 200 μm. **A** archespore; **ai** apospore initial; **an** antipodals; **CO** coenocyte; **EA** egg apparatus; **em** embellum; **in** integuments; **M** meiocyte; **nu** nucellus.


A
in
nu
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Clone 4-29 was cloned from an isolated band that was present in young pistils of the apomictic plant only. Amplification of the original band revealed two different PCR fragments from which the largest fragment of 430 bp was cloned. Comparison of the amino acid sequence to the data base of NCBI revealled similarities to hypothetical proteins (*Bacillus subtilis*); to a *Streptococcus pneumoniae* putative membrane protein; to a conserved protein (*Methanobacterium thermoautotrophicum*) and to a putative permease Perm (*Escherichia coli*).

When sections of the sexual ovaries were hybridised with a 4-29 sense probe, low background staining was observed at all stages of sexual and apomictic development as shown in young pistils of the sexual plant (Fig. 4a). Using the anti-sense probe, at young stages, the epidermal cells of the nucellus, that will grow to form the micropylar channel as well as the meiocyte, hybridise slightly stronger than the other nucellar cells (Fig. 4b). During coenocyte stage, there is comparable expression in coenocyte and surrounding nucellar tissue (Fig. 4c). Later, at a mature stage, when embryo sac is cellularised, there is no hybridisation at all detectable (Fig. 4d). After anthesis, when egg apparatus is still recognisable (Fig. 4e) as well as when an proembryo like structure can be seen also no label was detected (Fig. 4f).

In ovaries of the apomictic plant , the expression of 4-29 anti-sense was also observed. At the early stages of development, young pistils had label that was not uniform for all cells. In fact some central positioned cells of the nucellus showed more label than the others (Fig. 5a). This reaction was also observed in some other cells of the pistil. During ovule development, embryo sacs in different stages of development were present in the ovule, with a different pattern of label. The nucellar tissue was highly labelled during coenocyte stage, whereas the young coenocyte does not show a hybridising signal (Fig. 5b). In the same stage, with a highly labelled nucellus cells, an embryo sac had label in the cytoplasm of the egg apparatus cells (Fig. 5c).

In a more advanced stage of development, the label was weak in nucellar tissue and only cells from the embryo sac showed label (not shown). Before anthesis, in apomictic ovaries, proembryo was heavily labelled (Fig. 5d). After anthesis, more than one embryo were observed in the same ovule, all of them labelled while the nucellar tissue had no label at all (Fig. 5e). When embryo sacs were isolated from the ovule it becomes clear that the label is spread in the cytoplasm from all cells of the embryo sac (Fig. 5f). Proembryos at this stage had label (Fig. 5f-h).

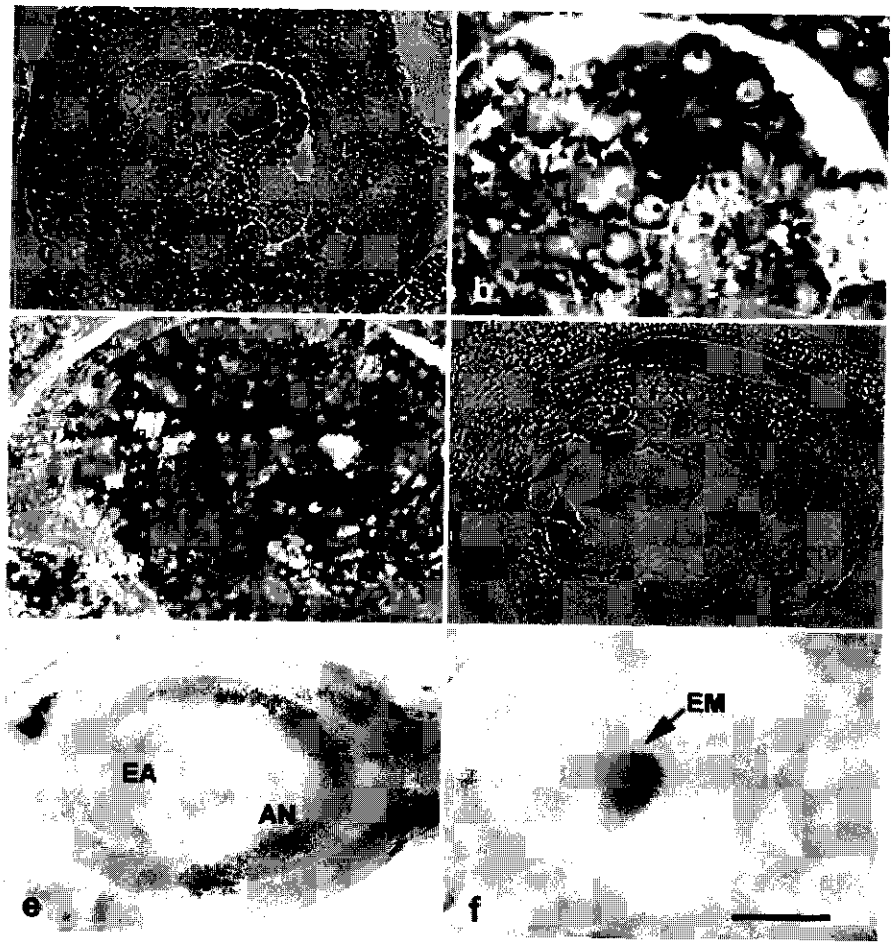


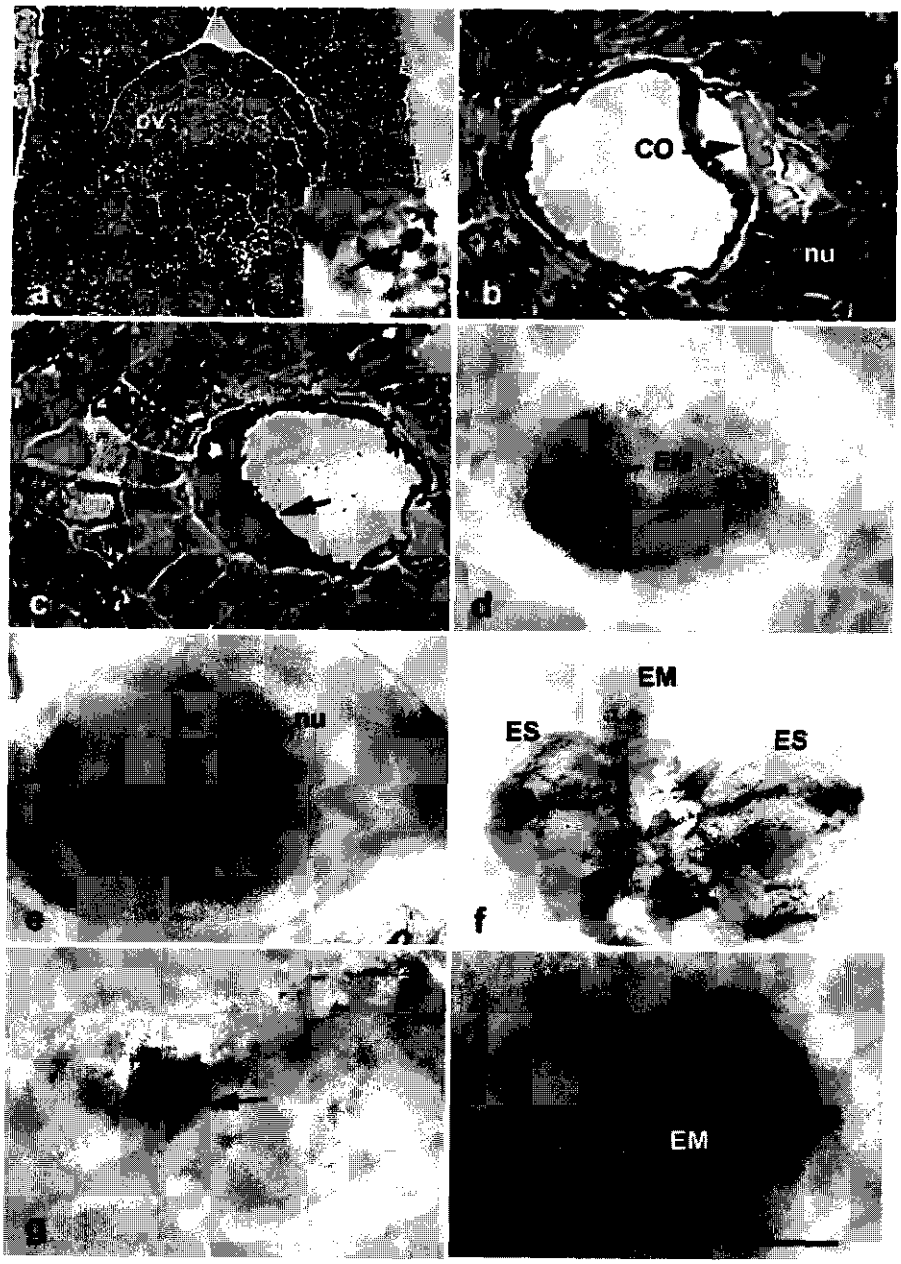
Figure 4. (color print in page 167) Expression of 4-29 sense (**a**) and anti-sense (**b-f**) probe in ovules of the sexual plant. **a** Young ovary in stage of meiocyte (dyad) hybridised with the sense probe. Bar: 40 μm. **b** closer view of the ovule in meiocyte stage showing that some cells of the nucellus show more label. Bar: 20 μm. **c** ovule in coenocyte stage showing expression in coenocyte, nucellar tissue and integuments. Bar: 50 μmμm. **d** ovule in mature stage, before anthesis showing no hybridisation. Bar: 110 μm. **e** cleared thick section of ovule just after anthesis, when proembryo like structure is not visible yet, showing no hybridisation. Bar: 140 μm. **f** thick section of cleared ovule after anthesis, showing the proembryo structure with no hybridisation. Bar: 90 μm. **AN** antipodal cells; **CO** coenocyte; **EA** egg apparatus; **EM** proembryo; **M** meiocyte; **nu** nucellus; **ov** ovule; **PN** polar nuclei.

Discusssion

Differential display of mRNA (DDRT-PCR), first described by Liang and Pardee (1992) is a method that has been used successfully for identifying and cloning differentially expressed genes. This technique is particularly attractive due to the small amount of total RNA that is sufficient to screen a large part of the mRNA population (Liang and Pardee, 1992). Although some authors discussed the lower sensitivity of DDRT-PCR to less abundant RNAs (Bertioli *et al.* 1995), it is sensitive enough to identify changes on RNA levels (Wan *et al.*, 1996). In addition, the use of specific tissues, would in some degree minimise the problem with low abundant RNAs.

Some sequences related to sexual or apomictic reproduction were obtained from mature ovaries in *Pennisetum ciliare* (Vielle-Calzada 1996) and interspecific hybrids of *Brachiaria brizantha* and *B. ruziziensis* (Leblanc et al 1997). Because this method can generate high numbers of false positives, rescreening of the differential displayed band is necessary. There are many methods available to determine whether a differentially expressed cDNA is a false positive. When large amounts of RNA are available, a Northern blot analysis or a differential screen described by Zhang and colleagues (1996), would help. If not, as in the case of *Brachiaria* ovaries, the choice could be a method that combines differential screening with amplified cDNA (Poirier *et al.* 1997) or *in situ* hybridisation. Although being a laborious technique, *in situ* hybridisation is very useful to analyse gene expression during early stages of ovule development. There were two main reasons that led us to choose *in situ* hybridisation to analyse the clones from the differential display:

Figure 5. Expression of 4-29 anti-sense in ovules of apomictic plant. **a** young pistil showing hybridisation in ovary with more label in the central part of the nucellus. Bar: 40 μm. Insert detail shows, in a cleared ovule after whole mount hybridisation, that some cells from the nucellus, around the meiocyte of the apomictic plant, shows more label. Bar: 70 μm. **b** section showing a chalazal coenocyte (arrow) and nucellar tissue of the ovule. Bar: 32 μm. **c** In the same ovule as in b, an embryo sac during cellularisation showing egg apparatus where strong label can be seen (arrow). Bar: 32 μm. **d** Cleared thick section of an mature ovule before anthesis showing proembryo like structure. Bar: 140 μm. **e** Cleared thick section of ovary showing many embryo sacs (1, 2 and 3) and nucellar tissue. Bar: 180 μm. **f** isolated embryo sacs and proembryo of the previous ovary. Bar: 70 μm. **g** Thick section of ovary after anthesis showing embryo like structure. Bar: 140 μm. **h** detail of this embryo. Bar: 93 μm. **CO** coenocyte; **EM** proembryo; **ES** embryo sac; **nu** nucellus; **ov** ovule.


ov
a
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EM

First, the small size of young pistils limits obtaining enough RNA to perform a Northern analysis that would confirm the expression of the genes in a determined stage of development. Second, genes of interest could be expressed in only a few cells of the ovary or very temporally, at a special stage of development, and could differ in the level of expression from one stage to another and between apomictic and sexual plants.

Apomixis is often associated to polyploidy. Apparently, the difference in ploidy is not interfering in the use of this method. Difference in genetic background of the plants can become apparent in expression pattern in the ovule. But the use of more than one stage of ovule development as well as tissue from other parts of the plant besides the ovule can minimise this problem.

One single combination of primers was used to detected 20 differential display bands between sexual and apomictic plants. Most of them were present in the young pistil of the apomictic plant suggesting that at this stage some genes are being expressed only in apomictic plants and could be related to the expression of apomixis. The selected sequences however do not give such expected expression. Our previous results discussed here, showed that possibly those bands could just reflect a difference in degree of expression of the corresponding genes and will be further analysed.

In situ hybridisation revealed the expression pattern of the two clones described here. The expression of both clones showed that the differential bands were in reality not exclusively expressed in one stage and one type of reproductive development. Clone 7-21, similar to a glucosamine-6-P isomerase of *Bacillus subtilis*, was expressed at low levels in ovules of sexual and apomictic plants from the beginning of ovule development, when only archespore and apospore initials were present in the nucellus, to the end of embryo sac maturation. Glucosamine in its N-Acetyl form is found, among others, in organisms like bacteria, fungi and higher plants (Collins 1987). Glucosamine-6-P isomerase converts fructose 6-phosphate to glucosamine 6-phosphate and its reverse and can be a common reaction in the cell metabolism. Two genes encoding a putative *Giardia lamblia* 6-phosphate isomerase were cloned but only one was expressed and is under transcriptional control (Keulen *et al.* 1998). In our *in situ* hybridisation experiments it seems that there is no transcriptional control of expression of this clone 7-21 during ovule development. A quantitative analysis of the expression of 7-21 could give more information about the possible differences in expression and establish a connection of the corresponding enzyme and

the carbohydrate metabolism observed during ovule development in *Brachiaria decumbens* (Dusi and Willemse 1999a).

Clone 4-29 had a pattern of expression that differs in ovules of sexual and apomictic plants and during the stages of ovule development. Clone 4-29 was expressed in tissues where cells are carrying products and exchanging metabolites as are nucellus cells of young ovaries (Bouman 1984, Willemse and van Went 1984) and embryos (Vijayaraghavan and Prabhakar 1984). Expression of 4-29 during meiocyte stage was higher in some nucellar cells than in others. In the sexual plant the meiocyte as well as the epidermal cells that will form the micropylar channel were strongly labelled. Both types of cells are elongating. In a similar way, some nucellar cells surrounding the archespore had very strong label in the apomictic plant. But other elongating cells of the pistil had also strong label and this makes the pattern not specific to nucellar cells. The expression of 4-29 in the apomictic ovules is from the beginning of ovule development at meiocyte stage to the end of development with the proembryo formation. The expression is long in time and becomes more localised with the maturation of the ovule. The nucellus for example shows expression at early stages but in mature ovules nucellar tissue has a faint expression. At this stage the nucellar tissue is not growing and has low nutritive function (Willemse and van Went 1984). By contrast in sexual plants the expression is interrupted after coenocyte stage and mature embryo sacs do not show expression neither in nucellar tissue nor in egg apparatus or proembryos. The contrast of expression observed in cellularized embryo sacs of sexual and apomictic plants reflects a difference in timing of expression probably due to the function of the resting period that the ovule containing a meiotic embryo sac normally pass before fertilisation. The nucellar tissue in ovules of apomictic plants, with more than one embryo sac present in different stages of development, including the early embryogenesis, lacks such resting period and has a heavy label. In contrast to the sexual plant in which the embryo does not show label, particularly interesting is the high expression of this probe in proembryos of the apomictic plant before and after anthesis. The high expression in egg cell and proembryos of the apomictic plant could be a reflection of its capacity to develop autonomously, without fertilisation of the egg cell. Few is known about autonomous embryo development in apomictic plants. A hypothesis of a physical barrier in the unreduced egg cell that would obstruct the penetration of the spermatic nucleus into the egg cell and by that preventing fertilisation (Savidan 1989) has some support in plants of *Pennisetum ciliare* (Vielle *et al.*, 1995). However it can not be applied to other

species like *Panicum maximum* (Naumova and Willemse, 1995) and F2 of hybrids of *Pennisetum glaucum* and *P. squamulatum* (Chapman & Busri, 1994). Another suggestion for the absence of egg cell fertilisation would be its activation before fertilisation like in *Poa*, *Phartenium*, *Taraxacum* and *Tripsacum*, where the development of the embryo starts before anthesis (Nogler, 1984a). Such "activation" would be the case of *Brachiaria decumbens* and *B. brizantha* which develop embryos before anthesis (Dusi and Willemse 1999a, Araújo pers. inform.).

Cellular and morphological development of embryo was extensively studied (reviewed in Natesh and Rau 1984). Recently, interest has been paid to elucidate the molecular mechanism and signal transduction pathway of embryo development (Goldberg *et al.* 1994, Laux and Jügens 1997, Schmidt *et al.* 1994, 1997, Walker 1994). Membrane proteins like permeases are carrier proteins that bind to a specific molecule, and pass to conformational changes to transfer it across the membrane (Alberts *et al.* 1994). The similarity of 4-29 to a membrane protein, and to a permease and the observation that its expression is regulated during the ovule and embryo development, indicates that 4-29 may be part of a pathway of signal transduction that participate in the autonomous embryo development.

Cytologically the mechanisms by which the development of the zygotic and autonomous embryos begin are completely different. While the zygotic embryo needs the stimulus of pollination and fertilisation of the reduced egg cell to develop into an embryo, autonomous embryo formation in the aposporic embryo sac from unreduced egg cells is allowed to develop without the need of fertilisation. Asker and Jerling (1992) suggested that autonomous embryo development is a phenomena inherent to egg cells, reduced or not, and that its occurrence would depend on both the moment of its induction, and the competition of embryo sacs and moment of pollination for the species. If this is so, the mechanism that let pass the fertilisation could be the same for all groups of plants, and only the timing of its expression would be different, before or after anthesis and pollination. Whether this common mechanism exist or not, is not yet confirmed. But the difference in expression of clone 4-29 observed in zygotic and autonomous embryos of *Brachiaria decumbens*, shows a point where both development diverge in a molecular pathway that is coincident to the maturation of the sexual derived ovule and to the early autonomous embryogenesis of the apomict .

When studying apomixis one should consider the possibility that apomixis is not only a result of the existence of different genes in apomictic

plants, as first observed in inheritance studies, but a result of differential expression of genes. Whether a primary stimulus is enough to enable all the subsequent developmental steps, including the differentiation of the egg cell into an embryo, it is not known.

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Chapter 6

Expression pattern of the *Arabidopsis thaliana* somatic embryogenesis receptor-like kinase (AtSERK1) gene in ovules of sexual and apomictic plants of *Brachiaria decumbens*.

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Abstract

The pattern of expression of the *Arabidopsis thaliana* AtSERK1 gene (AtSERK1) was observed during ovule development, from archespore to mature embryo sac, before and after anthesis using a sexual and an apomictic accession of *Brachiaria decumbens*. *In situ* hybridisation showed expression of the AtSERK1 gene in the sexual plant in all cells of the meiotic embryo sac after anthesis, including the proembryo. In the apomictic plant, some expression was observed before anthesis in the egg apparatus and after anthesis in the synergids. The results show differences in AtSERK1 expression in zygotic and autonomous embryo development in *Brachiaria decumbens*.

Introduction

Embryogenesis in angiosperms is part of a sexual reproduction process that initiates after the fusion of the male and female gametes. It includes the development of the zygote by a sequence of cellular and morphological changes leading to the formation of a mature embryo (West and Harada 1993). However, some species produce embryos autonomously, without fertilisation of the egg cell, by asexual reproduction known as apomixis (Nogler 1984, Asker and Jerling 1992). In addition, some species are able to produce embryos by asexual process of somatic embryogenesis (Zimmerman 1993).

Apospory, one mode of apomixis, implies that unreduced cells form embryos (Nogler, 1984). With amphimixis, including zygotic embryo formation, apospory has in common that a gametophytic structure, i.e. the embryo sac, is differentiated during the process. The aposporic embryo sac has a cell with the characteristics of an egg cell, though unreduced and not receptive to fertilisation, which is the origin of the embryo.

In an attempt to understand the molecular events of embryogenesis, genes that are specifically expressed during early embryogenesis are being searched during zygotic and somatic embryogenesis processes (reviewed by Drews et al. 1998, Goldberg et al. 1994, Thomas 1993).

The somatic embryogenesis receptor-like kinase (SERK) genes were isolated from carrot , *Arabidopsis* and rice, from cells in the process of somatic embryogenesis and are highly conserved in monocots and dicots (Schmidt et al. 1997). Studies on expression of SERK gene in carrot show that it is expressed not only in embryogenic cell cultures but also transiently in somatic

and zygotic embryos (Schmidt et al. 1997). During somatic embryo formation, SERK is expressed to a high level in globular somatic embryos of up to about 100 cells only and not in later stages of embryo development. During carrot seed development, SERK is expressed in early stages up to the globular stage of the embryos, and in no other stage or tissues. SERK expression in zygotic embryos could be detected first in flowers at 3 days after pollination when fertilisation occurred and endosperm is already developing.

Brachiaria decumbens, a grass species of the family Poaceae, has sexual and apomictic modes of reproduction (do Valle 1991). In *Brachiaria*, apospory with ovules containing one or more aposporic embryo sacs is the mode of reproduction.

Embryo sac development of sexual and apomictic plants of *Brachiaria decumbens* as described in detail (Dusi and Willemse 1999b). Sexual plants of *Brachiaria* are diploid (2n=18) (do Valle 1991) and follow the common Polygonum-type of embryo sac development. In these plants, a megaspore mother cell gives origin after mitosis of four megaspores. One of them, the chalazal one, develops after 3 mitoses in the eight nucleated coenocyte. After cellularisation seven cells and the mature embryo sac are formed: two synergids and one egg cell at the micropylar side, forming the egg apparatus, a central cell with two polar nuclei, and three antipodal cells at the chalazal side of the ovule, characteristic of the Polygonum-type embryo sac. Antipodal cells continue to multiply. Near to the anthesis, the two polar nuclei will fuse in one diploid nucleus (2n). After anthesis double fertilisation is needed for development of the zygotic embryo and the endosperm.

Apomictic plants of *Brachiaria decumbens* are tetraploids (2n=4x=36) and follow the Panicum type of embryo sac development (do Valle 1991). The initial stages of development are similar to the ovules of the sexual plant except that in the nucellus, an aposporous initial cell is often differentiating near the archespore or meiocyte. The meiocyte degenerates during meiosis and the number of apospore initials rises by this time. The apospore initial (2n) will directly undergo two mitoses to form the coenocyte with four nuclei. All nuclei are unreduced (2n=4x) and are distributed at the same pole, not necessarily the micropylar pole. After cellularisation, four cells can be distinguished: two synergids and one egg cell forming the egg apparatus, and a central cell with one polar nucleus. More aposporic embryo sacs with different stages of development, can be observed in an ovule. The unreduced egg cell develops autonomously and structures like proembryos are visible before anthesis (Dusi and Willemse 1999a). After anthesis fertilisation of

central cell nucleus is necessary for endosperm formation and seed viability (Ngendahayo 1988). Recent studies in the apomictic *Brachiaria brizantha* cv. Marandu indicate that fertilisation occurs from ten hours after anthesis (Alves 2000).

These characteristics make *Brachiaria* an interesting system to study gene expression during autonomous embryo formation and to compare it with the development of zygotic embryo.

Because the SERK gene is expressed only in embryogenic cells for just a period of time that coincides to the beginning of embryogenesis (Schmidt et al. 1997) , it could also be expressed during embryogenesis in a system that has similarities with both somatic as well as zygotic embryogenesis.

In this study, the *in situ* expression of *Arabidopsis thaliana* SERK gene (AtSERK1) was followed during ovule development, from archespore to mature embryo sac before and after anthesis using a sexual and an apomictic accession of *Brachiaria decumbens*.

Material and methods

Plant material

Plants of *Brachiaria decumbens*, accessions BRA000191 (facultative apomictic, tetraploid, D58- cv. IPEAN) and BRA004430 (obligate sexual, diploid D4), from the Brazilian Agricultural Research Corporation, EMBRAPA - Embrapa Beef Cattle, were used. The plants were grown either in a greenhouse with temperatures ranging from 25 °C during the day to 20 °C at night, with a 16 h light, and 8 h dark light regime or in the field in Brasília, Brasil. Inflorescences were collected and placed in water in the laboratory.

In situ hybridisations

Semithin sections on glass slides or whole mount *in situ* hybridisations were done.

For semithin sections, pistils were removed from the spikelets and fixed in 4% paraformaldehyde, 0.25% glutaraldehyde and 0.1 M NaCl in 0.01M phosphate buffer, pH 7.2 for 1 h in vacuum at room temperature. The fixative was refreshed and samples were kept overnight without vacuum. After washing with the same buffer, they were dehydrated in an ethanol series (10, 30, 50, 70, 85, 96, 100% ethanol), 20 min each. At 100% ethanol, 10 mM of dithiothreitol (DTT) was added. The samples were embedded at 4°C in butyl-methyl methacrylate - BMM (Gubler 1989, Baskin *et al.* 1992, Kronenberger *et*

al. 1993) through an ethanol:BMM series of 5 : 1, 5 : 2, 5 : 3, 5 : 4, 5 : 5, 4 : 5, 3 : 5, 2 : 5, 1 : 5 (v/v) and two changes of 100% BMM. DTT was added in all steps of embedding to a final concentration of 10 mM. Incubation steps took at least 4h each and the samples were kept at 4 °C. The material was placed in gelatine or plastic capsules containing a fresh BMM mixture, closed with a lid, and polymerisation was done with Philips 8 watt/h UV irradiation for 48 h at -20 °C. After polymerisation, the samples were kept at 4 °C. Sections of 2 to 4 µm were cut and placed on drops of water on glass slides, stretched with chloroform vapour and placed on a 60 °C plate for 1 h. BMM was removed by two 10 min washes in acetone, one wash in a mixture of acetone : water 1 : 1 and finally in water. For a good control of tissue and RNA preservation, some sections were stained for total RNA with acridine orange (AO). Sections were rinsed with 0.2M acetate buffer pH 2.1 and stained with 0.05% acridine orange (AO) in 0.2M acetate buffer pH 2.1 for 30 min and then washed 15 min in veronal-acetate buffer pH 7.8 (Bimal and Willemse, 1996). These sections were examined with either UV or FITC light. RNA fluoresces orange or red while DNA fluoresces green or yellow respectively. After control only the material with intact RNA was used.

For the whole mount method flowers were separated from the spikelets, placed in Petri dish with fixative (described above) and with the use of a stereoscope microscope and sectioned longitudinally with a RNAse free knife, in a way that the pistils were divided in two parts. They were then incubated overnight in microfuge tubes with fixative. Tissue was washed in a buffer identical to the fixation buffer but without the fixative and in hybridisation buffer. No prehybridisation treatment was performed for those samples. After each wash or change of solutions, a brief centrifugation was made to collect the specimens.

RNA probes were prepared using the DIG RNA labelling kit (Boehringer Mannheim) according to the instructions. Plasmid pSAC 61 that contains AtSERK1 sequence was digested with Sma I (for anti-sense) or Kpn I (for sense) enzymes. For anti-sense and for sense probe T7 and T3 polymerases respectively were used. Probes were hydrolised to a final length of 200 bp.

Prehybridisation treatments for thin sections: The slides were incubated in buffer (100 mM Tris-HCl pH 7.5; 50 mM EDTA) containing 1µg ml^{-1} of proteinase K, washed in DEPC treated water 2 times, 10 min.

Sections were hybridised overnight at 42 °C with 600 ng ml^{-1} of digoxigenin-labelled probe in 100 µl of 50% deionised formamide, 1 x

Denhardt's, 250 ng ml^{-1} tRNA, 300 mM NaCl, 10mM Tris-HCl (pH 7.5), mM, 1mM EDTA pH 8.0 in water.

After hybridisation the sections were washed at 37 °C in 4x SSC, 2x SSC, 1x SSC, 0.5x SSC (1x SSC = 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7), 30 min each. 5 min in detection buffer 1 (0.1 M Tris-HCl pH 7.5; 0.15 M NaCl). Sections were incubated in detection buffer 2 (1% Blocking Reagent - Boehringer Mannhein or 2% BSA -Bovine Serum Albumin) subsequently in detection buffer 1 for 1 h and washed 5 min in detection buffer 1. Sections were then incubated with Anti-Digoxigenin-AP Fab fragments (Boehringer Mannhein) diluted 1: 1000 in detection buffer 1, for 1h. Washed 2 times, 15 min each, in detection buffer 1 and one time, 5 min, in detection buffer 3 (0.1 M Tris-HCl pH 7.5; 0.1 M NaCl; 0.05 M $MgCl_2$; final pH adjusted to 9.5). Sections were incubated in staining solution 3.5 µl BCIP (0.05 g ml^{-1}) and 4.5 µl NBT (0.05 g ml^{-1}) in 1 ml detection buffer 3 for 1 to 3h. The colour reaction was stopped by incubating with detection buffer 4 (0.01 M Tris-HCl pH 8.0; 1 mM EDTA).

After the final washes and air drying the slides with the sections, they were mounted in Depex (BDH, Brunschwig Chemie) or in Permount SP15 - 500 (Fisher chemicals - Fisher Scientific) and observed with a Zeiss Axiophot light microscope. The whole mount hybridisation samples were clarified with glycerol, mounted in glass slides, cover with coverslip and observed with a Nikon optiphot or Zeiss Axiophot differential interference contrast microscope.

Results

The expression pattern of AtSERK1 in *Brachiaria decumbens* ovules in different stages of development was determined by *in situ* hybridisation. When a sense probe was used no label was detectable in sexual nor in apomictic ovules (Fig. 1a,b). *In situ* hybridisation showed that the AtSERK1 gene was not expressed in young ovules from the archespore to coenocyte stages in both sexual and apomictic plants (Fig 1c-f).

During ovule development in the sexual plant, expression of AtSERK1 was not detected before anthesis in any stage of development. (Fig. 1c,e; Fig. 2a,b). After anthesis, synergids were strongly labelled (Fig. 2c and 2d) while cytoplasm around the polar nucleus were weakly labelled. The egg cell cytoplasm was also labelled (Fig. 2d). Antipodal cells in ovaries after anthesis also showed strong label (Fig. 2e) as well as the proembryo (Fig. 2f).

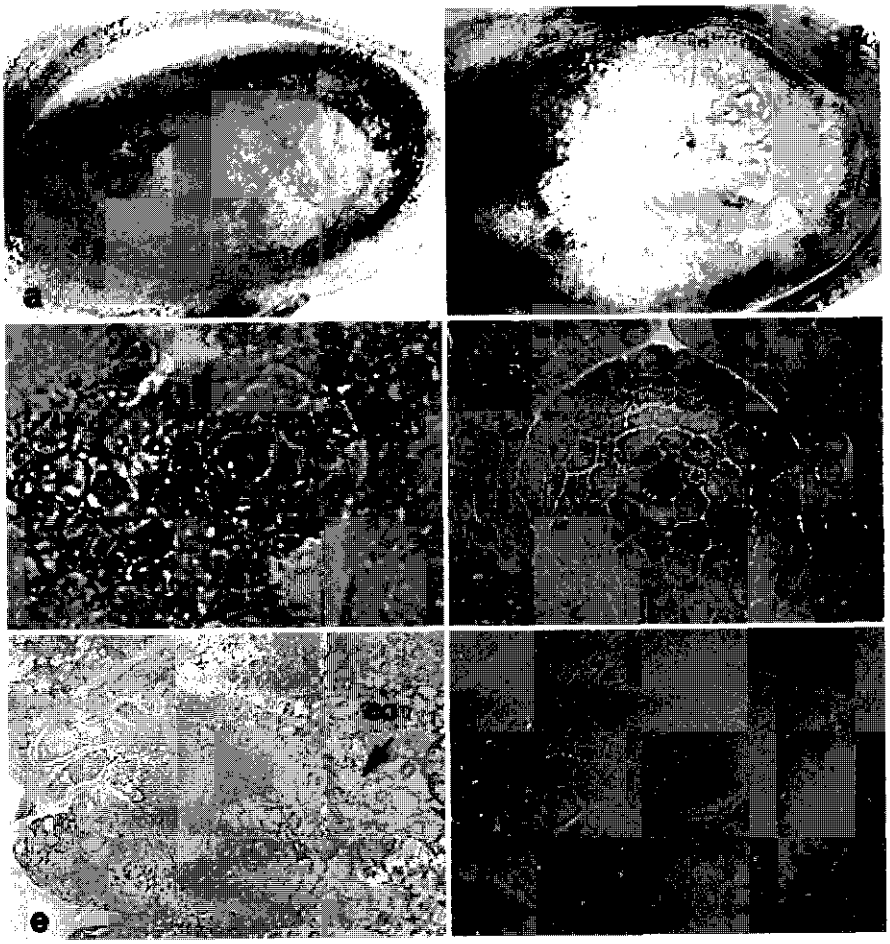

Figure 1. Expression of AtSERK1 in ovules of sexual and apomictic plant. **a-b** Thick section of ovary after anthesis after whole mount *in situ* hybridisation with AtSERK1 sense showing no label. **a.** sexual plant. Bar: 170 μm. **b.** apomictic plant. Bar: 170 μm. **c-f** Semi-thin sections of ovaries hybridised with AtSERK1 anti sense probe. **c.** Ovule of sexual plant during archespore stage without expression. Bar: 20 μm. **d.** ovule of apomictic plant during apospore initial stage with no expression. Bar: 35 μm. **e.** Ovule of sexual plant during coenocyte stage. Bar: 50 μm, and **f:** ovule of apomictic plant during coenocyte stage, both without expression of AtSERK1. Bar: 50 μm. **CO** coenocyte.

During the development of the apomictic plant ovule, from archespore to coenocyte no label could be detected (Fig. 1d,f). In ovules in mature stage, two patterns of expression were obtained. Either there was no hybridisation (Fig. 3a) or the egg apparatus (not shown) including the egg cell (Fig. 3b) showed some label. Before anthesis a proembryo did not show any label (Fig.

3c). After anthesis synergids showed strong label (Fig. 3d) while proembryos either shows a residual expression (Fig. 3e) or no expression at all (Fig. 3f).

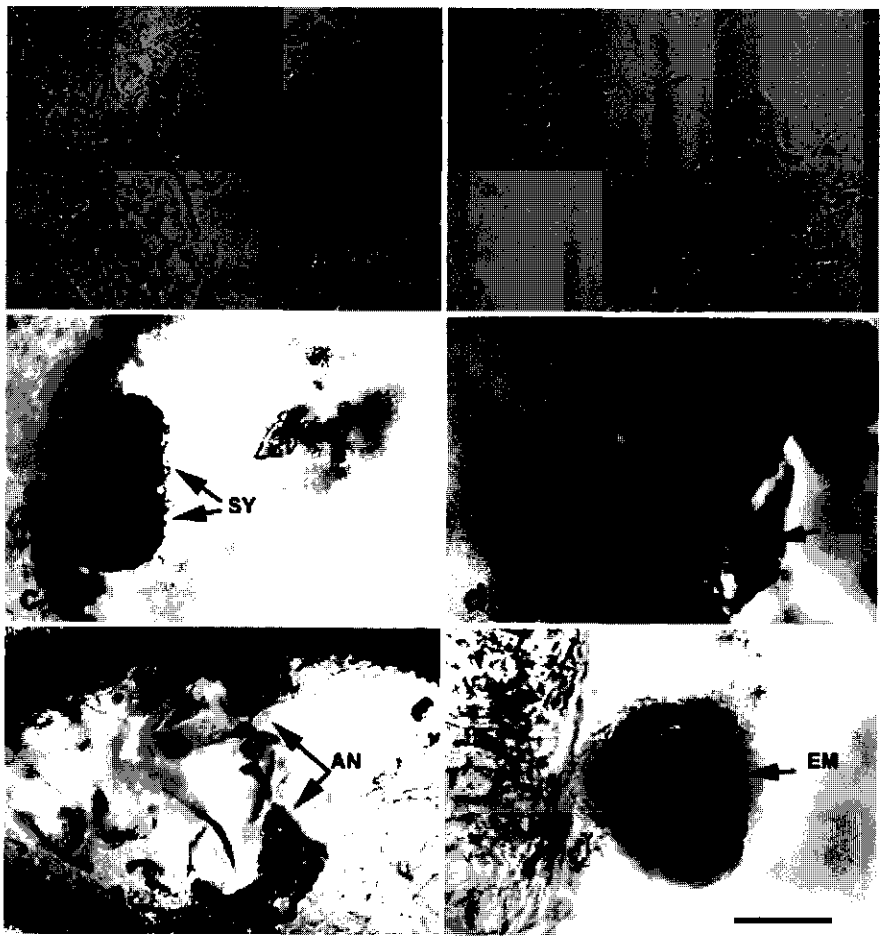

Figure 2. AtSERK1 gene expression as detected by *in situ* localisation in thin and thick longitudinal sections of the sexual plant of *Brachiaria decumbens* after clearing. **a.** micropylar side of a mature ovule before anthesis showing egg cell without label. Bar: 50 μm. **b.** chalazal side of the previous ovule showing the antipodals with no label. Bar: 50 μm. **c.** micropylar part of an ovule after anthesis showing strong expression in synergids. Bar: 40 μm. **d.** an ovule after anthesis focused in the labelled egg cell behind the two synergids. Bar: 25 μm. **e.** an ovule after anthesis showing antipodal cells with strong expression. Bar: 82 μm. **f.** an ovule after anthesis with proembryo showing strong expression of AtSERK1. Bar: 20 μm.

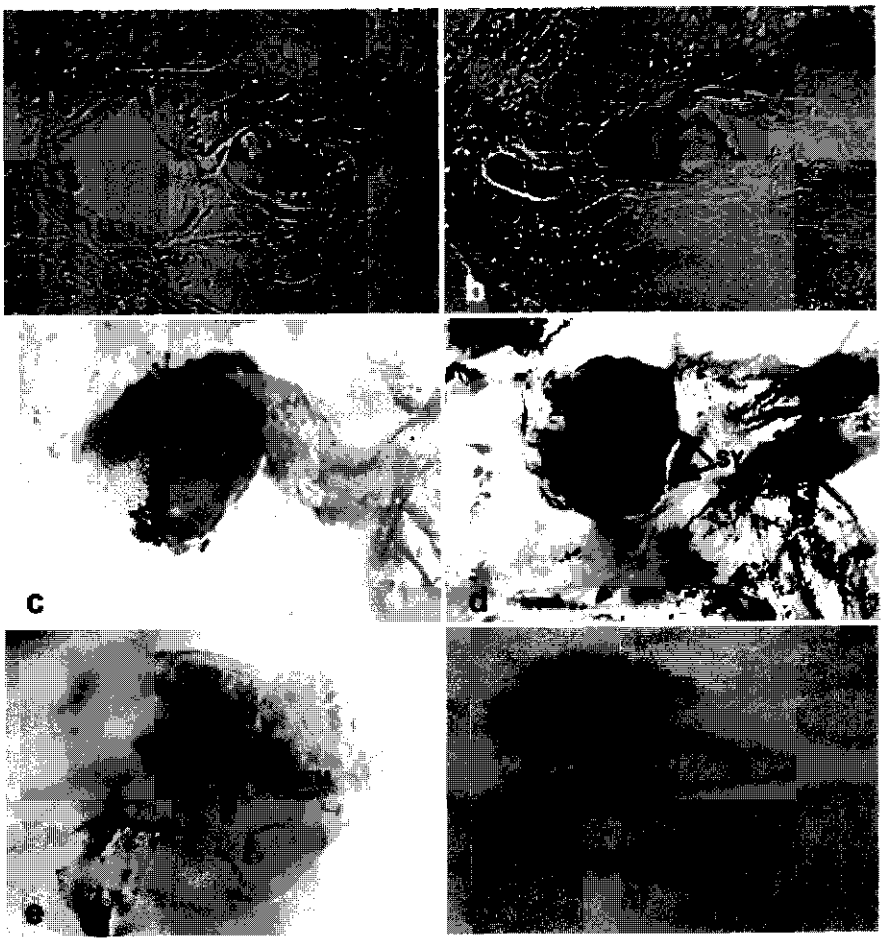

Figure 3. AtSERK1 gene expression as detected by *in situ* localisation in thick longitudinal sections of the apomictic plant of *Brachiaria decumbens*. **a.** semi-thin section of ovule with embryo sacs before anthesis showing no expression. Bar: 35 μm. **b.** semi-thin section of mature ovule before anthesis showing an egg cell with some cytoplasmic label. Bar: 50 μm. **c.** proembryo in ovule before anthesis with no label. Bar: 50 μm. **d.** synergids of an ovule without embryo after anthesis showing high expression of AtSERK1. Bar: 50 μm. **e.** embryo and remnants of synergids in ovule after anthesis showing some spots of AtSERK1 label. Bar: 40 μm. **f.** globular embryo after anthesis showing no AtSERK1 gene expression. Bar: 35 μm. **ov** ovule; **nu** nucellus; **EG** egg cell; **EM** proembryo.

Discussion

In plants of *Brachiaria decumbens* the expression pattern of the *Arabidopsis thaliana* AtSERK1 gene is transient and differs between sexual and aposporic plants during ovule development.

Before anthesis, in ovules of the sexual plants AtSERK1 expression was not found. In the apomict, a faint AtSERK1 expression detected in egg apparatus before anthesis may be a consequence of differences in stage of development of the aposporic embryo sacs or to a low level of expression.

After anthesis, the egg cell in the sexual *B. decumbens* as well as proembryo showed expression of SERK as observed in carrots (Schmidt et al. 1997). Unexpectedly AtSERK1 expression in ovules was not restricted to embryonic cells. Antipodals and synergids in the meiotic embryo sac were strongly labelled. Also in the aposporic embryo sac the synergids were labelled after anthesis. The expression of the AtSERK1 gene in the synergids of both types of plants suggests that the pollination, also a condition for the apomictic plant to develop a seed, may have a connection with the AtSERK1 gene expression. In *B. decumbens* embryogenesis in the apomictic plant was observed frequently before anthesis in mature embryo sac (Dusi and Willemse, 1999a). Therefore, in relation to autonomous embryogenesis in the apomictic plant, the expression in synergids of the apomict is late when comparing to its expression in the sexual and cannot be related to the formation of the autonomous embryo.

After anthesis, in the apomict with autonomous embryo formation, the embryos show no AtSERK1 gene expression. Sometimes there is a not distinct labelling pattern observed in some embryos that could be due to a remnant of the closely connected synergids. It seems that AtSERK1 expression after anthesis is connected with pollination and fertilisation of the reduced embryo sac leading to a normal embryo development. In *Brachiaria*, the autonomously-developed embryo lacks ATSERK1 gene expression but apomictic plants maintain their capacity to develop a reduced embryo sac and therefore a zygotic embryo. Absence or low level of AtSERK1 expression in the egg cell, and the absence of expression in the proembryo give the impression that the AtSERK1 gene product is not involved in the autonomous embryogenesis and therefore is not related to the state of the unreduced egg cell. If this is true, the genes involved in the autonomous development of the embryo could be different from the ones involved in zygotic embryogenesis. However, it is still possible that the AtSERK1 is involved just in a specific step

of development, in initial transition of egg cell to embryo, stage that was not possible to be detected in our experiments. Thus, it is still possible that the genes involved in zygotic and autonomous embryogenesis, are the same but have a distinct timing of expression. Because very little is known about embryo development in *Brachiaria*, to establish a precise correlation of the expression of AtSERK1 and stage of embryo development more fundamental cytological studies have to be done. In *B. decumbens* sexual plants, the suppression of the AtSERK1 gene could also give an insight in the process. In any case, the different pattern of expression of the AtSERK1 gene points to differences in embryogenesis during zygotic and autonomous embryo formation in *Brachiaria decumbens.*

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Chapter 7

Apomixis in *Brachiaria*

1. Apospory and male and female sporogenesis and gametogenesis

In this study special attention was paid to the sporogenesis and gametogenesis in the sexual and aposporic *Brachiaria decumbens* plants. The process of plant sexual reproduction, consists of sporogenesis, gametogenesis and fertilisation. During the sporogenesis the sporogenous cell goes into meiosis and finalises the division in four haploid spores. In the anther, four microspores are formed. In the ovule, four megaspores are formed.

Comparing sporogenesis in a sexual and aposporic *Brachiaria decumbens*, in the apomict, during microsporogenesis, a thick callose wall isolated the meiocytes and there was a high level of abortion of microspores in tetrads. These features suggest that early expression of apospory in the female part has effect in steps of the male sporogenesis. These effects on male side, is also observed in other genera (reviewed in Asker and Jerling 1992), and imply that some genes that are related to apomixis, expressed in ovaries, could be expressed in the anther as well. The assumption that there is a close relationship in genes involved in megasporogenesis and microsporogenesis have support in data collected from mutants affecting male meiosis that were found to affect also female meiosis (Gohibovskaya *et al.* 1992). After the formation of microspores the abortion of pollen continues, at the same proportion, in sexual and apomictic plants. The microgametogenesis seems not to be influenced.

During sporogenesis, in the ovule of the apomict, the partial development of the callose wall and the ribosome pattern in the meiocyte indicate that the initiated megasporogenesis is arrested. In addition, a very early aposporic initial was observed. Therefore, genes leading to apospory, are being expressed in the early stages of development. Apospory illustrates that the process of female sporogenesis is passed and only the gametophytogenesis with the aposporic embryo sac formation, takes place. Therefore, the sporogenesis should be considered as a distinct developmental process, but in sexual plants, strongly connected with the gametophytogenesis. In apomixis, the induction of apospory means a direct selection of an unreduced spore. The high number of differential bands in young ovules of the apomict showed by the differential display, points to a higher variability of RNA expression between sexual and aposporic genotypes in young ovules during the sporogenic phase. But this method displays also some false positive bands as shown by the clone 7-21, similar to a

glucosamine 6-P isomerase that is expressed in both sexual and aposporic ovules.

During the megagametophytogenesis or embryo sac formation, the apomictic process differs from the sexual one. In the first place, in the apomict there is a higher number of "spores" or the apospore initials. Subsequently there is a higher number of aposporic embryo sacs, of which, some will degenerate. Finally, in the apomict the formation of the embryo is autonomous, independent of pollination. The capacity of the nucellus to develop more than one archespore is expressed in some genera with the formation of more than one embryo sac. In fact, an ovule is a macrosporangium and, in some species as *Potentilla*, more nucellar cells convert more or less synchronous in sporogenic cells (Rutishauser 1969). Apospore initials are continuously formed in aposporic plants.

During the formation of a meiotic embryo sac, in sexual plants, a lot of variations can occur. The number of the mitosis leading to different types of embryo sacs can be variable even in one plant (Toke 1980). Besides, the number synchrony of the mitoses can be disturbed. In that respect, the aposporic embryo sac of the Panicum type can be easily compared with a sexual one as develops in the *Oenothera* type and *Podostemon*. In aposporic plants embryo sac degeneration takes place, which is uncommon in sexual plants. The main difference between aposporic and sexual ovules is the absence of resting period in the ovule as a whole structure and the autonomous embryo formation, and no fusion of the egg cell with a sperm cell as in sexual plants. The development to an aposporic embryo sac seems to follow the sequential steps of the process of sexual embryo sac formation with a reduced number of mitosis. The expression patterns of the clones 7-21, 4-29 and AtSERK1 gene, observed in the sexual and aposporic *Brachiaria* support the idea that the development of the apomictic embryo sac is similar to the sexual one, but that differences are concentrated in only some steps like embryogenesis.

Clone 4-29 and AtSERK1 gene show differences in expression between both plants. The pattern of expression of clone 4-29 that is not interrupted in the embryo sac and is expressed in embryos only of the apomictic plant may be related to its autonomous embryo development. SERK protein might have connection with the plasma membrane and might be part of a signal transduction cascade that lead to the formation of somatic and zygotic embryos (Schimidt *et al.* 1997). Clone 4-29 product is also a membrane protein, maybe a permease. Although clone 4-29 is not specifically

expressed in embryogenic cells and its function is not known, its expression pattern suggest that it could also make part of a signal transduction pathway that lead to the autonomous formation of embryos. Probably, the developmental pathway to an autonomous embryo and to a zygotic embryo diverge. The brief AtSERK1 expression in the apomict gives the impression that this gene is not involved, or involved just in a specific stage, in the autonomous embryogenesis, and that the expression after the anthesis in the synergids of the apomictic embryo sac may be remnant of the normal function of the synergids in the sexual development of the embryo. In sexual and pseudogamous apomictic plants, the pollination is necessary to produce seeds. In sexual plants, pollination is a stimulus and includes an activation of the ovule before the pollen tube reach the ovule (Deurenberg 1977, Willemse 1996). Such regulation by stimulation points to a function of double fertilisation followed by an independent development of the embryo and of the endosperm. In *Brachiaria* it is clear that the pollination stimulus, which activates the ovule, is not present since the embryogenesis can start before pollination. In *Brachiaria* the pollination is only required for the development of the endosperm. The differences in expression of AtSERK1 gene in *Brachiaria* around the moment of anthesis, which commonly includes pollination, may be dependent on the stimulus to activate the ovule. Both AtSERK1 and clone 4-29 are expressed in the synergids and this place of expression coincides with the high level of total mRNA in the synergids. These observations add information that converge to point the synergids as real nurse cells for the further development of the zygote and endosperm.

Many aposporic embryo sacs develop in one ovule and some of them degenerate. Degeneration of the embryo sacs is expressed in the absence of the mRNA in the whole embryo sac. In the sexual plant, this effect exceptionally occur only if more than one megaspore developed.

In sexual reproduction, sporogenesis, gametogenesis, followed by, after pollination, embryogenesis and endosperm formation, will lead to a seed. This sequence is a gene-regulated process during which some variations can occur. The facultative character of apospory in *Brachiaria* is a sign that both sexual and apomictic reproduction are not strictly separated process. To add on sexual and apomictic reproduction is the influence of external factors on flowering (Sedgly and Griffin 1989) and also the influence of environmental conditions in the expression of apomixis (Knox 1967).

2. The timing of the apospory in *Brachiaria*

In the reproductive calendar, some differences in timing between the sexual and the apomict could be noted. The aposporic *Brachiaria* is a tetraploid plant and some of the data collected could not be exclusively related to the character of polyploidy or apospory. Nevertheless, the aposporic plant reaches earlier isolation of the sporogenic cells in the anther. The anatropic position of the ovule is somehow achieved faster and the apomictic embryo sac development is earlier completed.

In the aposporic plant, the digestion of the callose wall around the microspores is delayed and there is no callose formed in the embellum cells of the apomict. The development of the pollen and pistil and the carbohydrate metabolism, at the beginning of ovule development, are slower in the apomictic than in the sexual plant.

The differences in callose and carbohydrate metabolism are related to the sink-source balance of the plant. In fact, it is an internal factor depending on external conditions influencing the process of reproduction. Other influences on apospore development are the products that are set free in the apoplast of the nucellar tissue, as consequence of the degeneration of the meiocyte, and can be used as nutrients, such as the breakdown product of the callose wall. The high amount of plasmodesmata connecting the meiocyte with a chalazal differentiated cell, only observed in the apomictic plant, might provide a way to transfer molecules and nutrients from the meiocyte directly to only one cell, before the meiocyte degenerates.

Especially the differences in the calendar, as the carbohydrate metabolism, leads to the opinion that influences from outside, such as the plant biotope, the condition of the organism, the cellular environment around the apospore, are influencing the process of apomixis. In the literature, such examples are known in a grass of the Andropogonae (Knox and Heslop-Harrison 1963) or in apple (Sedgly & Griffin 1989). Nogler (1984) points to the possibility that physiological factors, produced during the induction of the sexual process, invade the surrounding nucellar tissues and induce the formation of an aposporic embryo sac. That idea has some support in our observations in *Brachiaria*: a connection through many plasmodesmata of the meiocyte to a chalazal cell, believed to be the first apospore initial, that is observed in the apomict ovary of *Brachiaria*.

External influences combined with a genetic base finally lead to the plasticity of the phenotype. This combination between genes and environment

is also expressed in the theories of the origin of apomixis and in some models for inheritance of apomixis (Mogie 1992, Martinez *et al.* 1999).

3. Origin and inheritance of apomixis

Winkler (1907), followed by Rutishauser (1969) and Nogler (1984) are confronted with the high level of instability and variety of apomixis. Nevertheless Rutishauser (1969) postulates that there are apomictic genes with a quantitative effect control of apomixis. Attempts to find a Mendelian genetic basis for the control of gametophytic apomixis are scarce and difficult. The difference in ploidy number of apomicts and their related sexuals makes difficult to obtain hybrids that would allow characterisation of the genetic bases of apomixis. However, the most recent data obtained from many apomictic genus, including *Brachiaria*, suggest that this complex trait is controlled by one dominant Mendelian factor. The existence of a single locus controlling apomeiosis was suggested for diplosporic *Taraxacum* (Mogie 1988) and *Tripsacum* (Grimanelli *et al.* 1996) and the aposporic *Panicum* (Savidan 1982, 1989), *Ranunculus* (Nogler 1984b), *Brachiaria* (do Valle *et al.* 1994, do Valle and Savidan, 1996) *Pennisetum* (Sherwood et al 1994) and *Hieracium* (Bicknell *et al.* 2000). Whether there exist a dominant allele at one single locus or a group of tightly linked genes is being discussed (Savidan 2000).

Nogler (1984a) discusses the possibility that mutation could originate the basic component of gametophytic apomixis but the complexity of the trait should point to other considerations as polyploidy, environmental effects and latent tendencies within the embryo sac of normal sexual development.

The model of Mogie (1992) for apomixis consider the inheritance of incompletely penetrant alleles and the occurrence of mutation to originate apomixis. A dosage effect achieved in the polyploids would permit the expression of apomixis. The diploids would always be sexual due to the inviability of the homozygotes for the mutant allele. It also assumes that the dosage effect have some plasticity and therefore can be modelled. This model contemplates the effect of the environment in the somatic cells of some ovules that lead to a preferential expression of the dominant wild-type gene even at polyploid level where the mutant allele is present in more than one copy. That takes in account the occurrence of facultative apomixis in many degrees and also explain why environmental effects, external and internal, have drastic effect, on the degree of apomixis.

A hypothesis for the origin of some apomict tetraploids include the occurrence of autoploidy (Quarin 1992). Autoploidy is supported by data obtained in apomictic species of *Paspalum* (Quarin 1992, Quarin and Hanna 1980) and species of *Tripsacum* (Grimanelli et al 1998) and *Pennisetum* (Ozyas Akins *et al.* 1998). Martinez and co-authors (1998) hypothesised that apomixis in *Panicum* is a result of "*two doses of the recessive allele together with a single copy of the wild type allele and homozygous recessive genotypes would be non-viable*". It fits the model proposed by Mogie (1992). An alternative model would be the one dominant gene with lethal effect on gametes containing two copies of the gene (Martínez *et al.* 1999).

Carman (1997) hypothesised that apomixis is the result of asynchronously expressed duplicate genes that control female gametogenesis. This hypothesis assumes that polyploids displaying apomeiosis were consequence of interspecific hybridisation of different genomes of plants with very different timing in megasporogenesis and megagametogenesis leading to polyploid apomicts. Therefore apomixis would be associated to alloploidy. Because duplicate genes resulted from polyploidy, diploids are excluded from apomicts. This hypothesis consider that mutations have a secondary role in the evolution of apomixis and exclude the possibility of specific genes regulating apomictic development. The asynchronously expressed duplicate genes hypothesis does not fit all the inheritance data available that points to a dominant Mendelian factor.

Our studies did not aim to add information about inheritance of apomixis that involve hybrid analysis. In contrary, it was the study of two natural stabilised genotypes. Despite of the real origin of apomixis, occurrence of apomixis is a result of differential expression of genes in tissues and time. In *Brachiaria*, aspects of gene expression, as well as timing, were found, both connected with the apospory. The genetic regulation is influenced by exogenic factors that finally leads to the expression of apomixis. The origin may be genetically controlled but from the beginning there is some interaction with the capability to sexual reproduction.

4. Future prospects

The information in this thesis, and the recent results in other species of *Brachiaria* (Carneiro and Alves 2000) show that *Brachiaria* can provide a good system to study apomixis, i.e. apospory, on cytological and molecular levels. Methods such as differential display and *in situ* hybridisation can be used to

monitor gene expression in any stage of ovule development. It is an example of the potential of comparative studies that are now possible due to availability of the ovule development characterisation. In addition, comparative studies at any level of biology are useful to study the divergences among the different reproductions systems. Such studies provide insight in the different strategies used by plants to achieve reproduction. Information in *Brachiaria* development can be improved by using material provided by other systems as happens with the sucrose synthase and invertase antibodies that were isolated for other plants, and the AtSERK1 gene that was isolated from *Arabidopsis thaliana*.

The need for variability in *Brachiaria* cultures leads the geneticists to make artificial tetraploids from sexual diploids. This is an attempt to develop sexual tetraploids that can cross with the cultivated species that are tetraploid apomicts. Such crosses will allow to compare intraspecific hybrids at cellular and molecular levels. Today the hybrids available for *Brachiaria* are all interspecific.

Cellular totipotency has been exploited mainly in microspore or pollen embryogenesis system to produce haploid plants (Reynolds 1997). The effect of apomixis on the development of the pollen grain was observed in several species. The possibility of rescuing diploid microspores or pollen of those plants should be investigated. The effect of apomixis by passing meiosis to form an embryo, if expressed in anther, could provide a natural way to obtain pollen embryogenesis at the unreduced level. That could render an efficient system with the opportunity to make artificial seeds.

In a short term approach to proceed our experiments, the isolated clone 4-29 should be used to clone the correspondent gene that will be further analysed to establish its direct effect on autonomous embryo development. It could be a candidate for genetic transformation using antisense approach in the aposporic *Brachiaria* to observe whether it blocks the autonomous embryo development or not.

The possibility of apomixis being controlled not by one but by many closed linked genes, make cloning apomictic genes and their introduction in crops of interest more difficult. In *Brachiaria*, where apomixis is facultative and the need of variability is an urgent question, the breakdown of apomixis would improve the material for breeders to work and increase variability in the field. Although still there is a long way to go before the control of apomixis could be achieved, all the knowledge obtained from the work on *Brachiaria* and other apomictic plants, as well as from amphimictic plants will be very useful. The

introduction of apomixis in amphimictic plants can be very difficult due to the many factors involved in reproduction, such as on genetic dosage effects, ratio of the ploidy of endosperm and embryo, the possible existence of many tightly closed genes, the polyploidy dependence to complete and maintain the whole process of apomixis. However, the possibility to control the trait in apomictic cultures by silencing only one of the genes that could (or not) be the trigger, but that is essential for the final result, might be much more feasible.

5- Actual research on apomixis

Many strategies have been used to study apomixis (reviewed in Pessino *et al.* 1999, Savidan 2000). Molecular markers linked to diplospory were found to belong to the long arm of chromosome 6 in hybrids of *Tripsacum dactyloides* and maize (Leblanc 1995). Two molecular markers, UGT197 and OPC-04 were found linked to apospory in hybrids of *Pennisetum* (Ozias-Akins *et al.* 1993,1998). In *Brachiaria* hybrids, OPC-04 was also found to be linked to apospory (Pessino *et al.* 1997). Efforts continue to find a marker closer to the apomictic locus.

The two main points of the apomictic reproduction are the bypass of meiosis and the autonomous embryo formation. Molecular analysis and cloning of transcripts related to sexual and apomictic ovule development has been used to identify key genes involved in one of these points of the process of sexual and apomictic reproduction. Differential display of mRNA method has been used to compare expression in mature ovules of hybrids of sexual and apomictic plants (Vielle-Calzada *et al.* 1996b, Leblanc *et al.* 1997) and during ovule development in sexual and apomictic genotypes of *Brachiaria decumbens* (chapter 5 of this thesis) and B. *brizantha* (Vera Carneiro, personnal communication). In *Brachiaria*, a genetic transformation system is available (Lentini *et al.* 1999). The isolation of genes related with sexual and apomictic reproduction should take the advantage of this system to study gene function.

Mutagenesis is another strategy used to study genes that have an important function during the ovule development (reviewed in Drews *et al.* 1998, Grossniklaus *et al.* 1998). Mutants affected in functions that lead to sexual development are being studied in a hope to find genes that could lead to the apomictic development (Vielle-Calzada *et al.* 1998). This approach is supported by some indication that apomixis could be present at the diploid level, through the observation in nature that few diploid plants show at some

degree apomeiosis, and that apomixis could be a result of mutation (Nogler 1984a, 1994, Mogie 1992). Mutation in plants that have amphimictic mode of reproduction, rescue mutants that lack some function in a specific stage of ovule development (reviewed in Drews *et al.* 1998, Dornelas 2000). Therefore, mutants for early development of the ovule, that carry mutations affecting formation of ovule primodia and growth of the integuments, were described. During sporogenesis, mutants that affect megaspore mother cell differentiation, polarity during meiosis and cytokinese were described. During gametogenesis many mutants with embryo sac development blocked in one of the nucleate stages of coenocyte were described. Of particular interest are the mutants of *Arabidopsis thaliana* fertilization-independent seed, *fis* (Chaudhury *et al.* 1997) and fertilisation-independent endosperm, *fie* (Ohad *et al.* 1996, 1999) that are able to develop endosperm and sometimes embryos *fis1 and fis2* without the stimulus of pollination and the event of fertilisation. Whether mutations will be able to establish apomictic mode of reproduction in amphymictic diploid plants is still unknown. But the mutants are very useful to study the function of genes expressed during the normal sexual reproduction in diploid level. Those genes could also have some part in the apomictic reproduction.

All apomictic reproduction have in common the autonomous embryo formation, and therefore the search for genes expressed during embryo development could add knowledge to the process. Somatic embryogenesis provides a good system to isolated key genes related to the process of somatic embryogenesis. Some of them, like SERK, are not only related to somatic embryogenesis but also have a role in the zygotic embryogenesis. It is possible that genes involved in the origin of somatic embryogenesis also take part in the autonomous development of the embryo observed in apomictic plants.

6- Disadvantages of apomixis

Some authors are concerned about the environmental risk that would come with the introduction of the apomictic trait in sexual plants (van Dijk and van Damme 2000). They predict that the controlled introduction of apomixis in diploid cultures will provide a faster spread of genes from transgenic apomictic plants to other sexual plants compromising the variability of other species. In nature, the mechanisms of apospory and nucellar embryony are facultative. Diplospory may result in some degree of recombination and excludes the

occurrence of sexual reproduction in the same ovule, but in an adjacent ovule, sexual reproduction may occur. Therefore plants reproducing by diplospory have the potential for sexual reproduction. Once apomixis can be controlled, it will be possible that the degree of sexuality can be maintained at a desirable low level.

Natural apomicts are able to stabilise their genome but remaining dependent on the plant condition and environment, and have some variability. The reason and restore of variability will need more attention.

Nowadays the scientists are dealing with the use of transgenic crops, that leads to the research on the spread of such genes in other population of plants. This studies are in discussion but will be more advanced and therefore can serve as a basis for the scientists to use appropriately the so called apomixis technology.

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Summary of the thesis

In this thesis a comparison of gametophyte development between a sexual diploid and an apomictic tetraploid genotype of *Brachiaria decumbens* was made. Apomixis is asexual reproduction leading to a seed.

An introduction is given on apomixis in general and in particular of *Brachiaria*. The work started with morphological and cytological observations of sporophyte and gametophyte development to detect the moments in which the sexual and apomictic development differ and therefore some points of regulation could be identified. Metabolic and molecular studies were added to detect the differences in the sexual and apomictic plants, to analyse the regulation of the two types of reproduction.

Some aspects of apomixis in *Brachiaria decumbens*

A calendar made based on the development in time was made to distinguish sexual and apomictic development. The aposporic embryo sac of the Panicum type grows faster than the Polygonum meiotic one. The atrope to anatrope ovular movement is faster in apomictic plant ovules and is consistent with the stage of embryo sac development. The apospore initials in *Brachiaria decumbens* were observed very early in the ovule, next to archespore or to the meiocyte. Tetrads were not commonly observed in the apomictic ovule and the meiocyte generally degenerates before cell division when the number of apospore initials is higher. During meiocyte development, callose was deposited only in the wall of meiocyte in the apomict and rarely there was callose in transversal walls of dyad or tetrad in contrast with what happens in the sexual plant. The embellum cells never showed callose in the apomictic ovules as it did in the sexual plant. Only in the apomict a cap of callose at the micropylar position persists for a long time. The microspores of the apomict plant had an enhanced callose formation that seemed to be due to the delay of the breakdown of the callose by callase.

The distribution of the nuclei, positioned in one pole in the aposporic embryo sac allowed to differentiate them from the bipolar distribution of the nuclei observed in meiotic embryo sacs of the sexual plants. Cellularisation took place early in aposporic embryo sac and by the time of anthesis embryogenesis had already started in the apomict. In ovules of apomictic plants many embryo sacs developed.

Finally, there was some synchrony between male and female development in sexual and apomictic plants as observed by the stage of meiocyte development. Apomixis expressed in the female side, had also an influence on the male side. During microspore formation, in stage of tetrad and young microspore, the abortion in the apomict was much higher than in the sexual.

Metabolism of carbohydrates

One of the primary stage of divergence of sexual and apomictic development was during female meiocyte development. As observed and pointed in the calendar, differences in callose deposition during meiosis indicated a distinct balance in carbohydrate source and use between sexual and apomictic developments.

Sucrose is the main source for the glucose and fructose production used in the cell. Invertase and sucrose synthase are the two enzymes mostly related to sucrose degradation and its activity can be used to evaluate the carbohydrate metabolism. Invertase converts sucrose in glucose and fructose. This enzyme can be present in the cytoplasm as well as in the wall and even in liquids outside the cell. Sucrose synthase converts the sucrose in glucose-1-phosphate and fructose. This enzyme can be membrane bound and cytoplasmatic. Histochemical tests developed to localise the *in situ* enzyme activity were used during ovule development in an apomictic and a sexual genotype of *Brachiaria decumbens.* A delay of activity of both enzymes was observed in apomictic compared to the sexual development. This delay was also reflected by the amount of carbohydrates detected in a HPLC assay. Furthermore antibodies were used to localise the enzyme in the cell. The higher level of carbohydrates detected in the early stages of development in the sexual plant is in line with the amount of enzyme and its activity. The retarded metabolism of carbohydrate in the apomict could be related to the entrance of the apospore directly in gametophytic pathway leading to a faster embryo sac development.

Pattern of total RNA and mRNA's and ribosomes

Total mRNA pattern as observed by labelling poly A tails did not detect the differences in expression in sexual and apomictic plant ovules. But the difference found in the *in situ* amount of total RNA showed a change in

metabolism of cells that are differentiating from nucellar cells in the chalazal side of the archespore or meiocyte during aposporic development. Such cells the first initial apospores also showed ribosome population that differ from other nucellar cells. In the sexual plant, the presence of some particular cells in the nucellus at chalazal side of the meiocyte suggested a silent aposporic capacity of this tissue. If such cells could develop into apospore initials under other conditions, like polyploidisation, is not known. In our observations the diploid plants never developed apospores in any stage of ovule development. Only studies with artificial polyploids could give a clue to these questions.

Molecular studies

Few is known about the genes that are expressed during female gametophyte development and even less during apomictic reproduction. In an attempt to fulfil this lack of information, the system of comparison of sexual and apomictic natural occurring genotypes of *Brachiaria decumbens* were used.

Differential display of mRNA was used for identifying and cloning differentially expressed genes related to apomictic and sexual ovule development. RNA was extracted from two key stages of ovule development: the archeporial - meiocyte stage and the mature embryo sac stage. Both stages were before anthesis so that an effect of pollination could not interfere in gene expression. A initial stage when only archespore, meiocyte, and apospore initials were present and a more advanced stage when embryo sac were mature and proembryos could be present in the apomictic plant ovule. Appropriate control lines were used to discard sequences expressed constitutively in floral organs.

The *in situ* pattern of expression of two selected sequences showed that the differential bands were in reality not exclusively expressed in one stage and one type of reproductive development. Yet, one of the sequences (4-29), similar to a membrane protein, had a pattern of expression that differs in ovules of sexual and apomictic plants and during the stages of ovule development. An important point is its very high expression in some cells of the nucellus and in proembryos of the apomictic plant, indicating that it could be necessary during gametophytic and autonomous embryo development.

The use of genes isolated in other systems to study apomixis

An *in situ* pattern of expression of a somatic embryogenesis receptor-like kinase gene isolated from *Arabidopsis thaliana* (AtSERK) is presented on the sexual and apomictic plant. This gene was expressed in early stages of somatic and zygotic embryogenesis. It is though to play important function in cells with embryonic capacity. In an apomictic and sexual genotypes of *Brachiaria decumbens*, distinct pattern of expression of this gene was observed in mature ovules before and after anthesis. The occasional expression of this gene before anthesis in the egg apparatus of the apomict pointed to briefly and transiently expression of this gene, that in contrary might be necessary for longer time in the development of the zygotic embryo.

Conclusion and prospects

Finally, the data found in the sexual and apomictic plant of *Brachiaria* was compiled to characterise the reproduction processes. The existing theories about the origin of apomixis are summarised and some consideration was made based on the data observed in *Brachiaria*.

Samenvatting

In deze thesis wordt de ontwikkeling van de embryo zak van een sexueel diploid met een apomictisch tetraploid genotype van *Brachiaria decumbens* vergeleken.

Apomixis is het proces van ongeslachtelijke voortplanting via zaadvorming.

In de inleiding is een algemeen overzicht gegeven van apomixis en van de apomixis in *Brachiaria decumbens* in het bijzonder.

De studie begint met morfologische en cytologische waarnemingen van de moederplant en de embryozakontwikkeling, om het verschil tussen de sexuele en apomichtishe ontwikkeling te identificeren en vandaaruit enkele momenten van regulatie te ontdekken. Metabolische en moleculaire studies zijn toegevoegd om verschillen tussen sexuele en apomictische planten te vinden, om de regulatie van de twee wijzen van reproductie te analyseren.

Enkele aspecten van apomixis in *Brachiaria decumbens*

Om de sexuele van de apomictische ontwikkeling te onderscheiden is een kalender op basis van de ontwikkeling in de tijd gemaakt.

De apospore embryozak van het Panicum type aanwezig in de apomict groeit sneller dan het Polygonum type, aanwezig in de sexuele plant die na meiose ontstaat. De beweging van atrope naar anatrope positie van het ovulum gaat sneller bij de apomictische ovuli en komt overeen met de stadia van embryozakontwikkeling. De jonge aposporen worden zeer vroeg in *Bracharia decumbens* waargenomen en liggen naast de archespore of de meiocyt. Tetraden worden niet zo vaak waargenomen in het ovulum van de apomict en de meiocyt degenereert in het algemeen vóór de celdeling wanneer het aantal jonge aposporen is toegenomen. Tijdens de ontwikkeling van de meiocyt in de apomict wordt callose alleen in de celwand van de meiocyt afgezet en zelden zit er callose in de dwarswanden van de dyade of tetrade, dit in tegenstelling met wat gebeurt tijdens de ontwikkeling van de meiocyt in de sexuele plant. De embellumcellen in het ovulum van de apomict hebben geen callose maar dit is wel aanwezig in de sexuele plant. Alleen in de apomict blijft de callosekap aan de micropylaire kant van de meiocyt langere tijd aanwezig. De microsporen van de apomict hebben een sterkere callosevorming, dit schijnt een gevolg te zijn van het vertragen van de calloseafbraak door callase.

De verdeling van de kernen, gelegen aan één kant van de apospore embryozak, maakte het mogelijk dit type te onderscheiden van de meiotische embryozak in de sexuele plant, waar de kernen aan twee kanten van de embryozak liggen. Cellularisatie in de embryozak van de apomict heeft vroeg plaats en op het moment van anthesis is de embryogenese in de apomict al begonnen. In ovuli van de apomict ontwikkelen zich meerdere embryozakken. Tenslotte was er enige gelijkenis tussen de mannelijke en vrouwelijke ontwikkeling in de sexuele en apomictische planten zoals waargenomen is tijdens de ontwikkeling van de meiocyt. Apomixis die tot uitdrukking komt aan de vrouwelijke zijde had ook een invloed op de mannelijke kant. Gedurende microsporevorming tijdens het stadium van de tetrade en de jonge microspore is de abortie in de apomict hoger dan in de sexuele plant.

Carbohydraat metabolisme

Een van de eerste stadia waar een afwijking tussen sexuele en apomictische plant te zien is, vindt plaats tijdens de ontwikkeling van de vrouwelijke meiocyt. Zoals waargenomen en aangegeven in de kalender, wijzen de verschillen in callosevorming tijdens meiose op een duidelijk evenwicht in de carbohydraatbron en het carbohydraatgebruik tijdens de sexuele en apomictische ontwikkeling.

Sucrose is de voornaamste bron voor de productie van glucose en fructose ten behoeve van de cel. Invertase en sucrosesynthetase zijn de twee enzymen die zeer direct betrokken zijn bij de sucroseafbraak en hun activiteit kan worden gebruikt om het carbohydraatmetabolisme te markeren. Invertase zet sucrose om in glucose en fructose. Dit enzym kan voorkomen in het cytoplasma alsook in de celwand en zelfs in vloeistoffen buiten de cel. Sucrosesynthetase zet sucrose om in glucose-1-fosfaat en fructose. Dit enzym kan membraangebonden zijn en in het cytoplasma van de cel aanwezig zijn. Histochemische testen om de emzymactiviteit *in situ* te localiseren werden gebruikt tijdens de ovulumontwikkeling van een apomictisch en sexueel genotype van *Brachiaria decumbens*. In vergelijking met de sexuele ontwikkeling werd een vertraging in activiteit van beide enzymen waargenomen in de apomict. Deze vertraging was ook te zien in de hoeveelheid carbohydraten, chromatografisch aangetoond met de HPLC methode. Ook werden antilichamen gebruikt om de enzymen in de cel te localiseren. De grotere hoeveelheid van carbohydraten gevonden tijdens de vroege stadia van ontwikkeling in de sexuele plant is in lijn met de

hoeveelheid enzym en zijn activiteit. Het vertraagde metabolisme van de carbohydraten in de apomict kon in verband gebracht worden met het direct doorlopen van de apospore in het proces van de embryozak vorming die leidt tot een snellere embryozak ontwikkeling.

Patroon van totaal RNA en mRNA's en ribosomen

Het totaal mRNA patroon zoals waargnomen door de labeling van poly-A staarten van het mRNA liet geen verschil in de expressie zien in ovuli van sexuele en apomictische planten. Tijdens de ontwikkeling van de apospore is er een verschil gevonden in de *in situ* aangetoonde hoeveelheid totaal RNA, die liet een verandering in metabolisme van cellen zien die differentieren vanuit nucelluscellen aan de chalazale kant van de archespore of van de meiocyt. Dergelijke cellen, de jonge aposporen, hebben ook een populatie van ribosomen die verschilt van nucelluscellen. In de sexuele plant getuigen de aanwezigheid van enkele speciale nucelluscellen aan de chalazale zijde van de meiocyt van een onderdrukt vermogen tot apospore vorming van dit weefsel. Of dergelijke cellen kunnen ontwikkelen tot beginnende apospore onder andere condities, zoals polyploidisatie, is niet bekend. In onze waarnemingen ontwikkelde de diploide plant in geen van de ontwikkelingsstadia ooit aposporen. Alleen studies met kunstmatige polyploiden geven een sleutel tot een antwoord op deze vraag.

Moleculaire studies

Weinig is bekend over genen die tot expressie komen tijdens de ontwikkeling van de vrouwelijke gametofyt en dit geldt deste meer tijdens de apomictische reproductie. In een poging dit gebrek aan informatie op te vullen, werd het systeem van vergelijking van sexuele en apomictische van nature aanwezige genotypen van *Brachiaria decumbens* gebruikt.

De methode van differential display van mRNA werd gebruikt om de differentiële genexpressie met betrekking tot de apomictische en sexuele ovulumontwikkeling te identificeren en kloneren. RNA werd geextraheerd van twee sleutel stadia van ovulum ontwikkeling: het archespore-meiocyt stadium en het rijpe embryozak stadium. Beide stadia liggen vóór het opengaan van de bloem, zodat een effect van bestuiving niet kan interfereren met de genexpressie. Een begin stadium waarin alleen archespore, meiocyt en beginnende aposporen aanwezig zijn en een verdergevorderd stadium waarin

de embryozak afrijpt en de proembryo's al aanwezig kunnen zijn in de apomict. Gebruikelijke controles werden gebruikt om de sequenties die doorgaans tot uitdrukking komen in de bloemorganen uit te sluiten.

Het *in situ* expressiepatroon van twee geselecteerde sequenties liet zien dat de differentiele banden in werkelijkheid niet exclusief binnen één stadium tot expressie kwamen en in één type van reproductieve ontwikkeling. Echter, een van de sequenties (4-29), gelijkend op een membraaneiwit, liet een expressiepatroon zien dat verschilde tussen de ovuli van sexuele en van apomitische planten én tijdens stadia van ovulum ontwikkeling. Een belangrijk punt is de erg hoge expressie in enkele nucelluscellen en in de proembryo's van de apomict, hetgeen wijst dat het gen noodzakelijk is tijdens de gametofytontwikkeling en autonome ontwikkeling van het embryo.

Het gebruik van genen geisoleerd in andere systemen om apomixis te bestuderen.

Een *in situ* expressiepatroon van een somatisch embryogenese receptorachtig kinase gen geisoleerd uit *Arabidopsis thaliana* (AtSERK) is uitgevoerd op de sexuele en apomictische plant. Dit gen komt tot expressie in vroege stadia van embryo ontwikkeling. Men denkt dat het een belangrijke functie heeft in cellen met een embryogeen vermogen. De in de apomictische en sexuele genotypen van *Brachiaria decumbens* onderscheiden expressiepatronen van dit gen werden waargenomen in rijpe ovuli vóór en na het opengaan van de bloem. De nu en dan voorkomende genexpressie vóór bloemopening in het eiapparaat van de apomict wijst op een facultatief karakter of een onderdrukking van deze genexpressie, in tegenstelling hiermee, zou deze genexpressie noodzakelijk moeten zijn voor de ontwikkeling van het zygotisch embryo.

Conclusie en toekomstige aspecten.

Tenslotte zijn de gevonden gegevens in de sexuele en apomictische planten van *Brachiaria decumbens* samengevat om de reproductieprocessen te karakteriseren. De bestaande theorien over het ontstaan van apomixis zijn samengevat en enkele beschouwingen worden gepresenteerd op basis van de gegevens gevonden in *Brachiaria.*

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CURRICULUM VITAE

Diva Maria de Alencar Dusi, was born in Viçosa, Brazil, on July 10, 1960. I obtained my degree in Agronomy in the Federal University of Viçosa in 1985. From 1985 to 1986 I attended to specialization courses in cell and molecular biology. In 1987 I joined the staff of Embrapa Genetic Resources and Biotechnology, where I still work. In 1991 I obtained the M.Sc. degree in Molecular Biology at the University of Brasília, under the supervision of Dr. Eugen S. Gander and Linda S. Caldas, with the thesis entitled "Regeneration and transformation of ramie (*Boehmeria nivea* Gaud.)". From 1991 to 1994 I worked in projects on genetic transformation of plants. In 1995 I started my Ph.D. research at the Laboratory of Plant Cytology and Morphology, at Wageningen University, having as my promoter Prof. Dr. Michiel T. M. Willemse.

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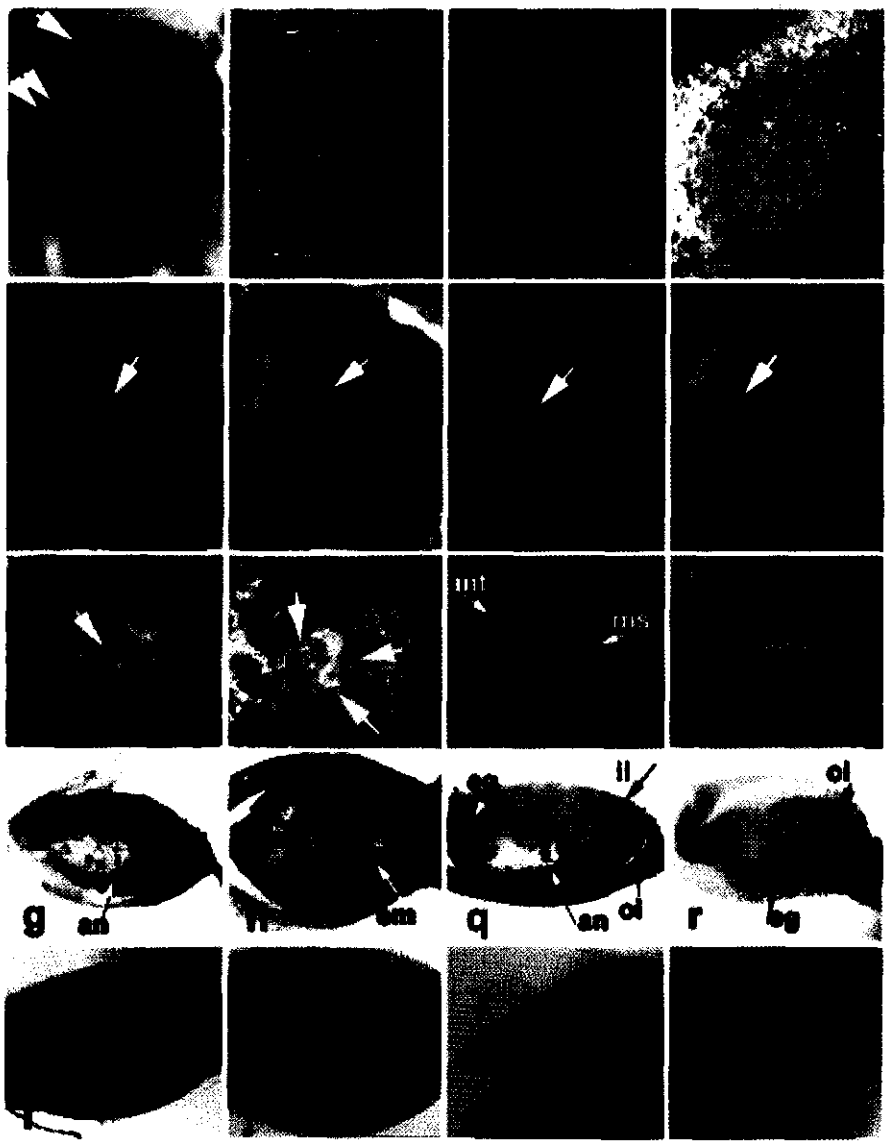
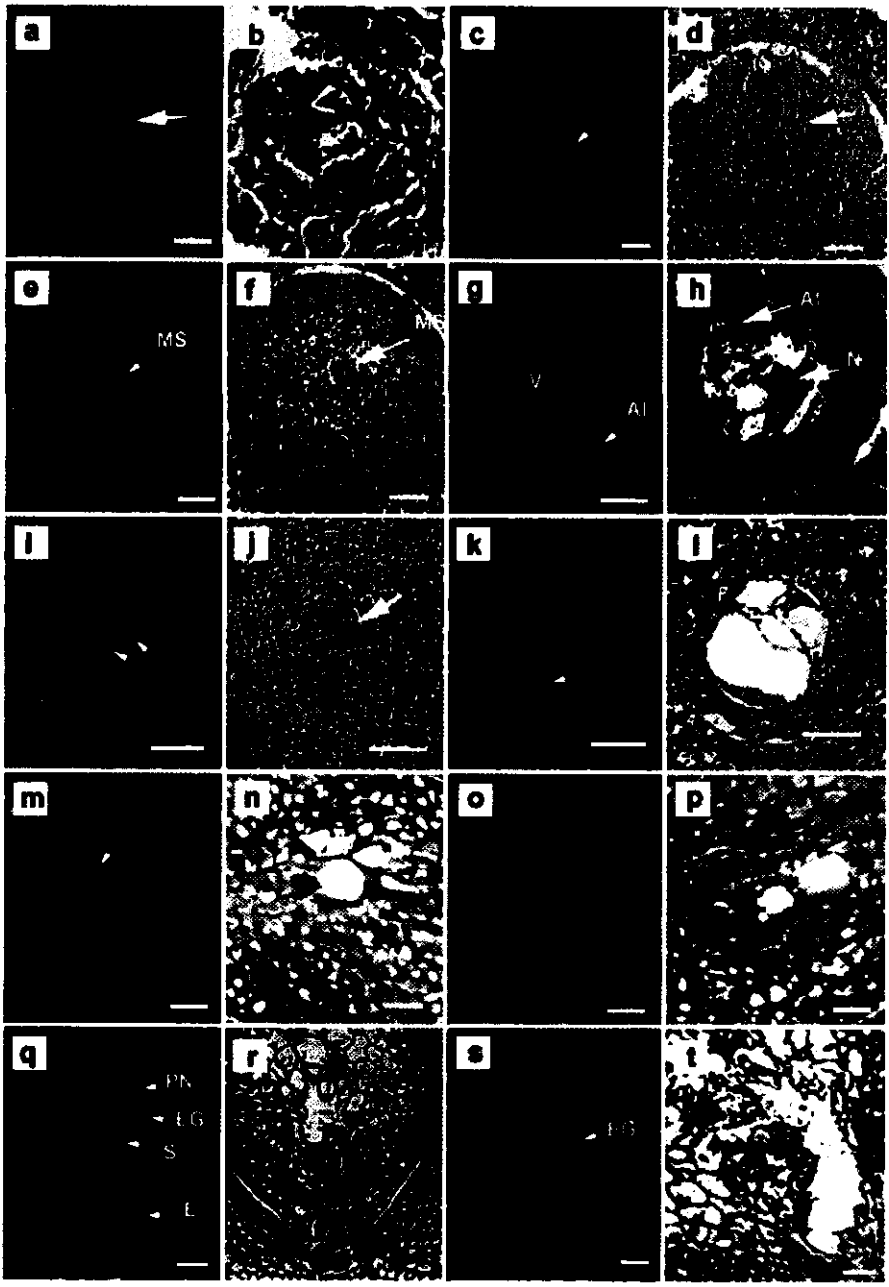
Annex

Chapter 3 figure 2.



Chapter 4 figure 4



Chapter 5 figure 4