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Embryo Sac Analysis and Crossing Procedure for Breeding Apomictic Guineagrass (*Panicum maximum* Jacq.)

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Abstract

In Japan, the hybridization method for breeding or apomictic guineagrass has been adopted only after the isolation of an obligately sexual accession in the collections introduced from East Africa by a joint plant collection mission of the Tropical Agriculture Research Center and the National Grassland Research Institute in 1971. The breeding of apomictic species, such as guineagrass, requires identification of reproduction mechanism and establishment of efficient crossing procedures. Two embryo sac analyses, i.e. a paraffin sectioning method and two cleaning methods, were applied to guineagrass for comparison. An efficient Procedure for hybridization in the breeding program of apomictic guineagrass is proposed. The results of the present study indicate that the paraffin sectioning method is available for histological studies on development of embryo sacs, through it requires a skilled technique and much time, while the cleaning methods are quicker and simpler than the paraffin sectioning method. There are no significant differences between the two methods in identifying reproduction methods. A plastic bag method can be effectively used for emasculation and hybridization of guineagrass. An advantage of using apomixes in the breeding program is to provide an efficient method for fixing desirable genotypes of F1 hybrids and simplifying commercial seed production. For practical use of apomictic guineagrass, it may be necessary to mix a few apomictic lines with diverse cytoplasms and genetic backgrounds for protection from pathogens infecting specific cytoplasms.

Discipline: Grassland
Additional key words: apomixes, embryology, hybridization, reproduction, method, sexuality

Introduction

Guineagrass (*Panicum maximum* Jacq.) is one of the most important tropical forage grasses native to tropical Africa and cultivated widely in tropical, subtropical, and even in temperate regions. Its reproduction method is based on aposporous apomixis with pseudogamy[18]. It produces progenies genetically the same as their mother plants, though their intraspecific variation is greater than colored guineagrass (*Panicum coloratum* L.)[9], which reproduces sexually[8]. As a result, breeding of guineagrass was more difficult than that of colored guineagrass because of the difficulty in hybridization. Sexual plants or facultative apomicts with high sexuality were found[2,4,17] and the role of sexual embryo sacs in the evolution of guineagrass through diversification and adaptation to new environments was suggested[15].

In Japan, exploration for introduction of tropical grasses was made by a plant collecting mission of the Tropical Agriculture Research Center and the National Grassland Research Institute in Ethiopia, Kenya, Nigeria, Tanzania, and Uganda during the period 1971 to 1973, when about 140 accessions of guineagrass were collected[6]. Among them, one diploid obligate sexual strain, GR–297, was isolated from the collection[10]. This finding opened the way to utilize apomictic gene(s) in breeding programs of guineagrass in Japan.

Apomixis, an asexual method of reproduction,

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provides a method for cloning plants through the seed. It can be used for fixing desirable genotypes as F_1 hybrids and simplifying commercial hybrid seed production[5]. Therefore, efforts are being made to produce apomictic cultivars not only in apomictic grasses but also in various crops such as rice, wheat, maize, sorghum, and pearl millet by introducing apomictic gene(s) from their wild apomictic relatives or other apomictic species.

One of the most important steps to be taken for utilizing apomictic gene(s) is to establish a simple method for detecting reproduction methods of plants. For this purpose, progeny tests and embryo sac analyses are most commonly used. The progeny test allows one to evaluate differences in morphological characteristics, iso-enzymic patterns etc. between a mother plant and its self-, open-, cross-pollinated progenies. Embryo sac analyses allow one to identify the morphology of embryo sacs by a cytological method and to know the real ratio of sexual to apomictic embryo sacs. These two methods are complementary and identification of sexuality requires both of these methods. Another important issue to be considered in the breeding of this forage grass relates to the procedures of emasculation and pollination. Florets of guineagrass is very small and manual emasculation is difficult.

This report describes two improved techniques of embryo sac analyses and a crossing procedure. These techniques could be efficiently used in breeding programs of apomictic guineagrass.

Reproduction method of guineagrass

Broad definition of apomixis includes reproduction through purely vegetative organs. However, this term in the present paper is restricted to asexual seed formation which includes apospory, diplospory, adventitious embryony, and parthenogenesis after the classification by Bashaw[1].

The reproduction method of guineagrass is apospory. In apospory, the embryo and endosperm develop in unreduced embryo sacs derived from somatic cells at various locations in the ovule. Usually, a megaspore or a young sexual embryo sac aborts and one or more 4-nucleated aposporous embryo sacs develop. In guineagrass, ovules with only one sexual embryo sac or with some apomictic and one sexual embryo sacs occasionally appear in an apomictic plant, which is called facultative apomict. The ratio in appearance of sexual embryo sacs differs in various apomictic lines and obligately sexual plants also exist in nature. This 4-nucleated aposporous embryo sac can be easily distinguished from 8-nucleated sexual one by the paraffin sectioning method or ovule clearing methods.

Paraffin sectioning method

A procedure of the paraffin sectioning method is explained hereafter. Inflorescences are fixed at anthesis in FAA solution (70% ethanol: acetic acid: formaldehide = 90 : 5 : 5) for at least 24 hr. Pistils are dissected from the inflorescences under a binocular stereo microscope, placed in a specimen carrier, and kept in 70% ethanol saturated with erythiosin B. The pistils contained in the specimen carriers are dehydrated with tertiary butyl alcohol and transferred to pure paraffin kept at 58°C. The pistils are aligned in mold and cooled on ice cubes. The cooled paraffin blocks are sectioned at 10–12 microns and stained. The specimen is stained with safranin — fast green and mounted in Permount (solid resin dissolved in oil of turpentine).

Plates 1A and 1B show a sexual and an apomictic embryo sacs prepared by this method, respectively.

Clearing methods

Clearing methods are to make ovules transparent by the use of clearing solutions and observe nuclei and tissues under a phase contrast microscope. Several techniques have been proposed for clearing plant tissues. Among them, the following two clearing techniques have given a good result with guineagrass:

(1) Florets at anthesis are fixed in Navashin's solution for 24 hr. The florets are transferred to 70% ethanol and stored until use. They are transferred to a clearing solution; i.e. lactic acid : chloral hydrate : phenol : eugenol : xylene = 10 : 10 : 10 : 10 : 5 (by weight) for 48 hr[13].

(2) Whole inflorescences at anthesis are fixed in FAA solution (70% ethanol : acetic acid : formaldehyde = 90 : 5 : 5) for at least 24 hr. The inflorescences are dehydrated and cleared by the procedures of Young et al.[19]

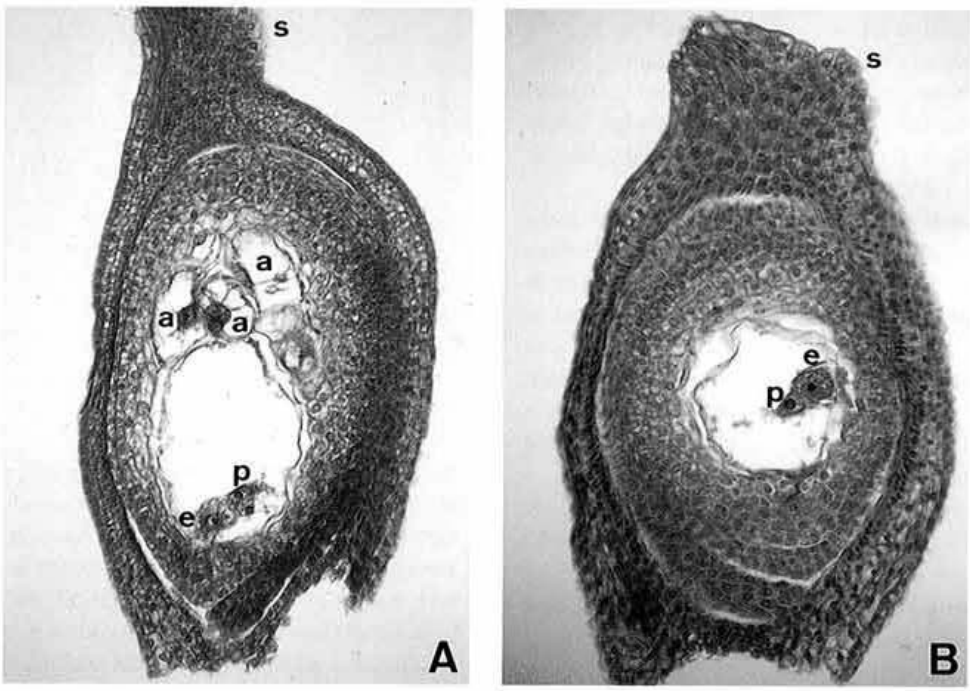

Plate 1. A sexual embryo sac (A) and an apomictic embryo sac (B) of guineagrass observed by a paraffin sectioning method
s: a part of stigma, e: an egg, p: polar nuclei, a: an antipodal cell.

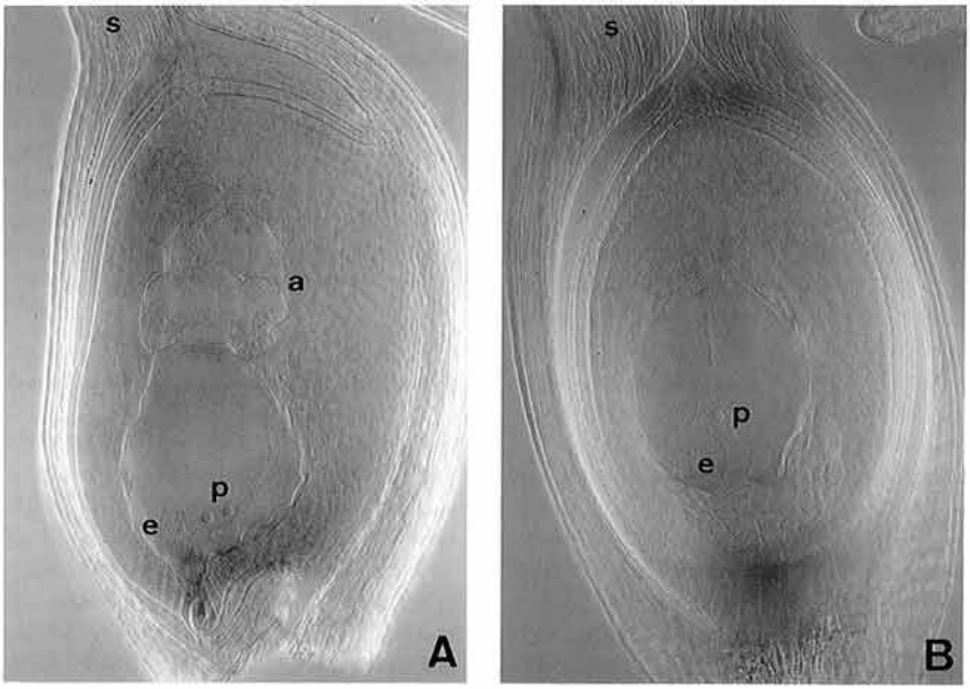

Plate 2. A sexual embryo sac (A) and an apomictic embryo sac (B) of guineagrass cleared with methyl salicylate and observed with a differential interference contrast microscope
s: a part of stigma, e: an egg, p: polar nuclei, a: antipodal cells.

with some modifications. The specimens are placed successively for at least 2 hr in 50% ethanol; 70% ethanol; 85% ethanol; 100% ethanol (two changes); ethanol : methyl salicylate = 1 : 1; ethanol : methyl salicylate = 1 : 3; ethanol : methyl salicylate = 15 : 85; 100% methyl salicylate.

The cleared pistils are dissected from florets under a binocular stereo microscope and aligned between two 18 × 18 mm cover-slips stuck upon a slide at an interval of ca. 12 mm. Another cover-slip is placed on the two cover-slips to make a bridge and the clearing solution is slowly poured to the space (Plate 3). The cleared pistils are observed with a differential interference contrast microscope (Nomarski).

Plates 2A and 2B show a sexual and an apomictic embryo sacs observed by this method, respectively.

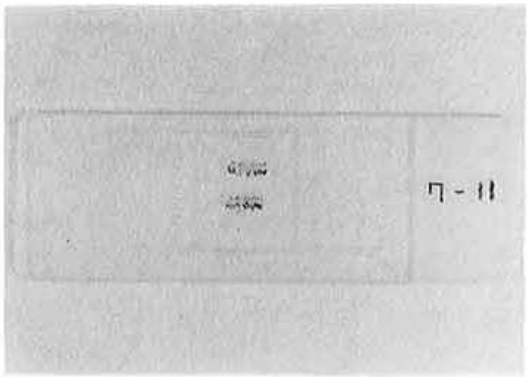

Plate 3. A preparation of pistils aligned on a slide

Comparison of the two clearing methods and the paraffin sectioning method

The above-mentioned two clearing solutions have essentially the same effect, thereby the nuclei and cells are clearly identified. The pistils cleared in methyl salicylate, however, are more fragile than those cleared in the other clearing mixture[7]. On the other hand, the clearing mixture is very toxic and the dissection must be done in a draft room. The advantage of clearing technique by methyl salicylate is that samples can be preserved at any step of the procedure and a large number of samples, e.g. more than 500 samples, could be cleared, preserved for a long time, and subjected to investigation at any time, though the clearing requires many steps. On the contrary, the other clearing mixture turns dark quickly in color and the cleared samples have to be observed within a few days, though the clearing procedure is very simple. The methyl salicylate method is preferable to the other method when more than 100 samples have to be observed.

Advantages and disadvantages of the two methods, i.e. the paraffin sectioning method and the clearing method, have been discussed by a number of authors. It is generally concluded that the clearing method is technically easier than the sectioning method for routine examination. That is, more than 1,000 pistils can be treated by the clearing technique without serious difficulties since an average of 5–6 min is only needed for the preparation of each pistil, while the sectioning technique requires an average of approximately 40–60 min per pistil[19]. As suggested by Young et al.[19], however, users should be familier with nature of the sectioned materials, prior to the first application of clearing method to any species. Histological and morphological studies on nuclei usually require treatments by paraffin sectioning and staining. For example, cytoplasms and fibers are stained green or blue, and nuclei and lignfied or dead tissues are stained red with the safranin — fast green (Plates A1 and A2). Therefore, the paraffin sectioning is indispensable for the study of origin and development of embryo sacs in ovule of sexual and apomictic plants. However, there are no significant differences between the two methods in identifying reproduction methods (Plates 1A, 1B, 2A, and 2B). Both methods should be used in the study, accordingly, depending on the purpose. Recently, another clearing mixture has been developed for the study on megasporogenesis of *Elymus*[3].

Morphological differences between sexual and apomictic embryo sacs in guineagrass

As in the case of buffelgrass (*Cenchrus ciliaris* L.) and dallisgrass (*Paspalum dilatatum* Poir.) in which apospory is easy to distinguish from sexual development because of the absence of antipodal cells present in sexual embryo sacs[1], aposporous sacs in guineagrass have no antipodal cells[4] (Plates 1B and 2B) and identified easily. During the development of maturing embryo sacs, there are eight nuclei, viz. one egg, two synergids', two polar, and three antipodal

cells' nuclei. However, matured embryo sacs have one egg, two polar nuclei, three or more antipodal cells, and no synergids' nuclei (Plates 1A and 2A). On the other hand, matured apomictic sacs have one egg and one polar nucleus (Plates 1B and 2B), and occasionally two or more embryo sacs are observed in one ovule, which is called polyembryony.

Procedures of artificial hybridazation

There are two methods for artificial hybridization of guineagrass: one with emasculation and the other without emasculation. Usually, sexual plants are mostly self-sterile and exceptional self-fertilized progenies are morphologically distinguished from hybrids[14]. Therefore, Savidan[14] bagged inflorescences of a sexual female and an apomictic plant together and collected the seed only from the inflorescences of the sexual female plant. Nakajima and Mochizuki[11] placed a sexual female plant among vegetatively propagated plants of a male parent in a separated greenhouse and collected the seed from the sexual one. Hanna et al.[4] modified a plastic bag method that had been improved for emasculation of sorghum[16]. This emasculation method is as follows: (1) an inflorescence is put in a plastic bag sealed with a paper clip before anthesis (Plate 4A); (2) the plastic bag is removed when all the florets finish anthesis; and (3) the inflorescence is pollinated with a glassine bag which contains collected pollens (Plate 4B). This method is very simple but completely controls dehiscences by heat and moisture. One problem of this technique is that early flowered florets become aged and may get fungi infected when the last floret finishes anthesis because it takes more than a week for all the florets in one inflorescence to finish anthesis. Since this method prevents self-pollination and all the seeds obtained are the hybrids, this method is preferable to the other methods for hybridization of guineagrass.

Apomixis breeding of guineagrass

Apomixis breeding of guineagrass requires collection and improvement of sexual plants, crossings between selected sexual plants and apomictic plants with promising characteristics, selections of progenies, and ditection of their reproduction methods.

Pernès et al.[12] proposed a breeding scheme of guineagrass. Basically, his scheme is acceptable for practical use. However, diversity of characteristics in diploid and tetraploid sexual plants in nature is generally smaller than that in apomictic tetraploids. Therefore, it might be more effective to undertake a chromosome doubling of diploid sexuals to develop tetraploid sexuals by colchicine treatment and to produce new sexual lines by hybridization.

One potential problem in apomixis breeding is

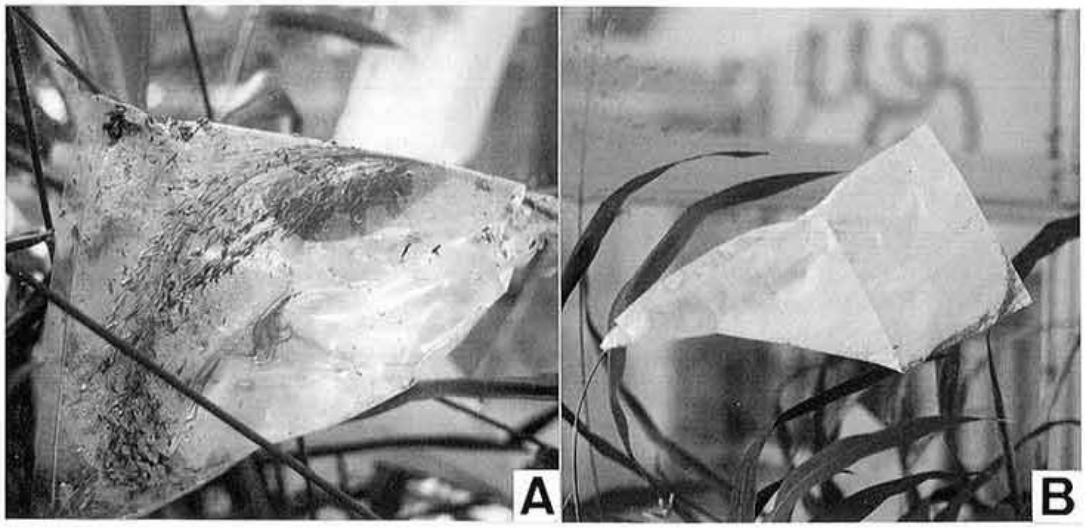

Plate 4. A: Emasculation of guineagrass by a plastic bag method
B: Hybridization of guineagrass with a glassine bag after the emasculation

similar to that encountered when male-sterile cytoplasms are used. As the progenies have the same cytoplasm of their mother plant, pathogens that infect the cytoplasm may destroy all the plants with this cytoplasm in the field. Furthermore, the situation might be more serious in the case of apomixis breeding, because not only cytoplasms but also genetic backgrounds of plants in a cultivar are completely the same. Such a serious problem has not taken place so far with an apomictic cultivar in Japan. However, in the breeding program of guineagrass, it may be necessary to mix two or more apomictic lines with diverse cytoplasms and genetic backgrounds.

The breeding program of apomictic guineagrass has just started in Japan and studies are required to elucidate cytological and genetical facets of the apomixis. But, it is clear that various kinds of progenies with new genotypes will be obtained from the crosses between sexual and apomictic plants. Therefore, additional information on heritability, genetic correlaion, and combining ability of those materials will have to be accumulated in the future.

A number of researchers are engaging in the manipulation of apomictic gene(s) by biotechnology in not only apomictic species but also rice, wheat, maize and other crops. However, it would be most important to identify genetic and cytological mechanism of apomixis for better use of apomixis before its manipulation.

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