Ploidy Determination in *Musa* Germplasm Using Pollen and Chloroplast Characteristics

A. Tenkouano¹, J.H. Crouch, H.K. Crouch, and D. Vuylsteke

Plantain and Banana Improvement Program, International Institute of Tropical Agriculture, P.M.B. 5320, Oyo Road, Ibadan, Nigeria

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Abstract. We attempted to determine ploidy level in the gametophyte and the sporophyte of *Musa* using pollen and chloroplast characteristics, respectively. In the gametophyte, interploidy differences accounted for 63.8% of the genetic variance for pollen diameter and 87.5% for pollen stainability, the remainder being attributable to intraploidy differences among clones. While pollen count and stainability effectively separated triploid accessions from diploids or tetraploids, they did not discriminate between diploids and tetraploids. In the sporophyte, the relative contributions of interploidy and intraploidy differences to genetic variation in the number of chloroplasts in stomatal guard cells were 70.8% and 29.2%, respectively. Although pollen diameter and chloroplast number increased with ploidy, the use of the sporophytic parameter appears to provide a more satisfactory means of estimating ploidy status in *Musa*.

Genetic enhancement of *Musa* germplasm has been achieved through interspecific crosses that are facilitated by compatibility between *Musa* species (for a review, see Ortiz and Vuylsteke, 1996). However, genome size is variable within and across generations without any obvious morphological indicator of ploidy state, i.e., euploid clones have from two to four times the basic set of 11 chromosomes (Ortiz and Vuylsteke, 1994). Differences between morphological traits are often greater within each ploidy level than between ploidy levels (Dessauw, 1988). Moreover, intraclonal differences are observed between mother plant and ramets. Therefore, ploidy determination cannot be based upon morphological criteria alone.

Ploidy estimation is traditionally determined by chromosome counting (Hamill et al., 1992; Osuji et al., 1996). However, this procedure is not suitable for large-scale screening of breeding materials. While easier than chromosome counting, ploidy determination based on stomatal size and density has not proven reliable in *Musa* (Vandenhout et al., 1995) and many other species (Blanke et al., 1994). Flow cytometry remains the most accurate tool for ploidy determination (van Duren et al., 1996). In his approach, sample analysis is inexpensive, but the initial expenses are high.

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¹To whom reprint requests should be addressed. International mailing address: IITA c/o L.W. Lambourn & Co., Carolyn House, 26 Dingwall Road, Croydon CR9 3EE, England. E-mail: IITAOnne@Satmail.bt.com.

Pollen derived from normal microsporogenesis should contain chromosome numbers that reflect the ploidy state of the sporophyte. If this hypothesis is true, the average typical pollen diameter should be a function of genome size (Dessauw, 1988), since both the nucleus and the cytoplasm are expected to increase as chromosome number increases. Chloroplast density in stomatal guard cells of the sporophyte could provide another means for rapid determination of ploidy state based on the postulate that chloroplast number in guard cells should increase as cell size increases in response to increased ploidy state. This technique has been used to differentiate between diploid and tetraploid accessions of several species, including potato (Cardi et al., 1993) and watermelon (Compton et al., 1996; McCuiston and Elmstrom, 1993).

This study was undertaken to assess the ploidy discrimination potentials of pollen and chloroplast characteristics of *Musa* germplasm.

Materials and Methods

Ploidy determination in the gametophyte.

Pollen was collected from 12 diploid (AA) and 20 triploid (AAA, AAB, ABB) accessions and from 9 tetraploid (AAB × AA, ABB × AA) hybrids. Plants were grown at the Onne (4°51'N, 7°03'E; southeastern Nigeria) research station of the International Institute of Tropical Agriculture (IITA). Male flowers were excised from the male bud between 0730 and 1030 hr and pollen grains were dislodged from the stamen, spread on a microslide, and stained with acetocarmine glycerol as described previously (Dumpe and Ortiz, 1996). Pollen was collected from five plants per accession and slide preparations were made from two random samples per plant. The preparations were observed under bright field illumination (one to two field views) using a Leitz Diaplan (Leitz Wetzlar, Germany) binocular micro-

scope at 400× magnification. The numbers of stained and unstained pollen cells were recorded and the diameter of 10 stained cells was measured using a calibrated reticle.

Ploidy determination in the sporophyte.

Leaf samples were collected from 44 accessions, including 14 diploids, 21 triploids, and 9 tetraploids. The procedure of Compton et al. (1996) was used for sample preparation, with a minor modification: the lower epidermis was removed with forceps, transferred to a microscope slide, and immersed in one drop of iodine solution (2%). A coverglass was placed on the preparation, which was allowed to stain for 5 min prior to observation under bright field illumination using a Leitz Diaplan binocular microscope at 400×. Ploidy was estimated by counting the number of chloroplasts per guard cell pair for 10 stomata of each of four leaf sections evenly and alternately distributed along the mid-rib, from the distal to the proximal ends of the leaf. The youngest two fully expanded leaves were observed for each of five plants per clone, giving a total of 400 cell pairs per clone. In addition to chloroplast counts, leaf length and width were also measured.

Statistical analysis. All calculations were carried out with SAS (SAS Institute, 1988). Data were subjected to the analysis of variance and mean separation using a hierarchical linear procedure with combinations of ploidy and clones serving as independent variables. Variance components were also calculated based on expected mean squares, using the REML option in SAS. This allowed for determination of the magnitude of interploidy variation relatively to intraploidy variation for pollen stainability and diameter, chloroplast number, and leaf length : width ratio.

Results and Discussion

Ploidy determination in the gametophyte.

Variation in pollen abundance was observed among and within accessions. Triploid accessions produced less pollen than the diploid and tetraploid accessions, as expected (Dumpe and Ortiz, 1996). Significant differences were found among and within ploidy levels for the stainability and diameter of pollen grains. Diploids and tetraploids produced >90% pollen with high stainability. In contrast, triploid accessions produced <50% pollen grains that stained when treated with acetocarmine glycerol (Table 1). The lower stainability of pollen from triploid accessions is expected because of meiotic irregularities, such as poor chromosome synapsis, that lead to unbalanced gametes. This should considerably reduce the percentage of viable pollen compared with a normal disomic segregation. In this study, pollen stainability was 49.1% in triploids vs. 90.1% in diploids and 90.0% in tetraploids (Table 1). Whether pollen stainability can be used for ploidy assessment is uncertain. While this characteristic appeared to separate the triploid accessions from those with an even number of chromosomes, it failed to discriminate between diploids and tetraploids (Table 1). Furthermore, pollen production and stain-

Table 1. Effect of ploidy on pollen and leaf characteristics of *Musa* accessions.

Ploidy class	Pollen stainability (%)	Pollen diam (μ)	Leaf length : width ratio	No. chloroplasts in stomatal guard cells
Diploids	90.8 $\pm$ 1.5	100 $\pm$ 1	2.73 $\pm$ 0.04	10.1 $\pm$ 0.2
Triploids	49.1 $\pm$ 3.3	112 $\pm$ 1	2.54 $\pm$ 0.07	13.1 $\pm$ 0.2
Tetraploids	90.0 $\pm$ 1.1	135 $\pm$ 2	2.39 $\pm$ 0.04	15.5 $\pm$ 0.3
F-test				
Inter-ploidy	** (77.0)	** (44.6)	* (17.0)	** (58.8)
Intra-ploidy	** (11.0)	** (25.3)	** (53.1)	** (24.3)

*,**Significant at $P = 0.05$ and 0.01 , respectively by ANOVA. Numbers in parentheses represent relative proportion (%) of phenotype accounted for by variance component.

ability, like seed set, may be influenced by unspecified environmental factors (Ortiz and Vuylsteke, 1995).

Assuming a spherical shape for pollen grains and a 1.5-fold increase in the volume of pollen from triploid sporophytes, then cell diameter should increase by ≈ 1.14 . Similarly, the diameter of pollen from tetraploid sporophytes should be 1.26 times the diameter of pollen from diploids. Our results appear to match theoretical expectations (Table 1), although there was considerable variation in pollen size. Moreover, when phenotypic variance was partitioned, the specific contribution of inter-ploidy differences was only 44.6%, leaving more than 50% of phenotypic variance not accounted for. For example, intra-ploidy clonal differences accounted for 25.3% of the total variation. Dumpe and Ortiz (1996) also reported only slight increases in mean pollen diameter as ploidy level increased. In contrast, Dessauw (1988) considered that pollen diameter was the only morphological criterion of those tested that allowed for definite discrimination of diploid and tetraploid *M. acuminata* Colla and *M. balbisiana* Colla accessions. However, the current study suggests that pollen size determination should not be used as an indicator of ploidy level in *Musa*.

Furthermore, nondisjunction of chromatids following normal equational division of cross-over products may lead to the inclusion of sister chromatids in the same gamete, thereby producing gametes with the sporophytic chromosome number (Stanford, 1951). Pollen with the sporophytic number of chromosomes is occasionally produced by a variety of diploid species as a result of first or second division restitution (Barcaccia et al., 1997; Chen et al., 1997; Ortiz et al., 1988). Occurrence of $2n$ pollen was once thought to be rare in *Musa* (Dodds and Simmonds, 1946), although this appears to be affected by weather conditions. Recently, Ortiz (1997) reported $2n$ pollen frequency of 22% in diploid *M. acuminata* (AA) accessions. In triploid germplasm, the frequency of $2n$ pollen was 14% in ABB accessions, 17% in AAB accessions, and 56% in AAA accessions (Ortiz, 1997). This may account for the large contribution of intraploidy effects to variation in pollen size in this study.

Ploidy determination in the sporophyte. Significant differences ($P < 0.05$) were found among and within ploidy levels for leaf length : width ratio (LWR) and for the number of chloroplasts per pair of stomatal guard cells. There was a linear decrease in LWR as ploidy level increased (Table 1). The reduced LWR in triploids and tetraploids was due to their

broader leaves with little change in leaf length (data not shown). However, partition of variation in LWR into its components showed that 76% of genetic variance was accounted for by intraploidy differences, leaving only 24% of the genetic variance attributable to interploidy differences. Therefore, LWR was not a reliable indicator of ploidy status in this study. This contrasts with the results of Compton et al. (1996) who used the LWR parameter and chloroplast density to distinguish ploidy in watermelon germplasm.

Data from Vandenhout et al. (1995) indicated that the average stomatal size in triploid and tetraploid *Musa* germplasm was, respectively, 1.22 and 1.47 times as great as that in diploid accessions. Assuming that the size of the chloroplasts does not vary according to ploidy level, then the number of chloroplasts observed in a transverse section of a pair of stomatal guard cells should also increase in a similar pattern. In this study, the average number of chloroplasts in triploids and tetraploids was, respectively, 1.30 and 1.53 times as great as that in diploids (Table 1). The relative contributions of interploidy and intraploidy differences to genetic variation in the number of chloroplasts were 70.8% and 29.2%, respectively. Thus, ploidy in *Musa* germplasm can be determined by chloroplast number in stomatal guard cells.

Gametophyte vs. sporophyte. In the gametophyte, interploidy differences accounted for 63.8% of the genetic variance for pollen diameter, and 87.5% for pollen stainability. However, while pollen count and stainability effectively separated triploid accessions from diploids or tetraploids, they did not discriminate between diploids and tetraploids. In the sporophyte, interploidy and intraploidy differences accounted for 70.8% and 29.2%, respectively, of the genetic variation in the number of chloroplast in stomata guard cells. Thus, while pollen diameter and chloroplast number increased with ploidy, the use of the sporophytic parameter appears to provide a more satisfactory means of estimating ploidy status in *Musa*. Whereas the number of chloroplasts is a direct indicator of the status of the somatic cells of the sporophyte, pollen diameter and other pollen characteristics only indicate the ploidy status of the gametophyte itself. For *Musa*, this may be a remote indicator of the ploidy level of pollen source, since irregular meiotic events will often produce gametes that do not relate in a simple way to the genomic constitution of the sporophyte (Ortiz, 1997). Paradoxically, it is most useful for the breeder to be able to estimate the most common gametic ploidy

level for each accession in order to design effective breeding schemes.

This study suggests that parameters describing meiotic products should not be considered for inferences on the ploidy status of the sporophyte. It is also of practical importance for *Musa* breeders to determine the ploidy status of clones before they are established in field evaluation plots. This could be readily achieved using chloroplast profiles from nursery seedlings. In contrast, pollen size determination would require waiting until anthesis, which typically requires from 9 to 12 months after field establishment.

Literature Cited

- Barcaccia, G., S. Tavoletti, M. Falcinelli, and F. Veronesi. 1997. Environmental influences on the frequency and viability of meiotic and apomeiotic cells of a diploid mutant of alfalfa. *Crop Sci.* 37:70–76.
- Blanke, M.M., M. Hofer, and R.J. Pring. 1994. Stomata and structure of tetraploid apple leaves cultured *in vitro*. *Ann. Bot.* 73:651–654.
- Cardi, T., V. Iannamico, F. D'Ambrosio, E. Filipone, and P.F. Lurquin. 1993. *In vitro* regeneration and cytological characterization of shoots from leaf explants of three accessions of *Solanum commersonii*. *Plant Cell Tissue and Organ Culture* 34:107–114.
- Chen, C., D.A. Sleper, S. Chao, G.S. Johal, and C.P. West. 1997. RFLP detection of $2n$ pollen formation by first and second division restitution in perennial ryegrass. *Crop Sci.* 37:76–80.
- Compton, M.E., D.J. Gray, and G.W. Elmsstrom. 1996. Identification of tetraploid regenerants from cotyledons of diploid watermelon cultured *in vitro*. *Euphytica* 87:165–172.
- Dessauw, D. 1988. Etude des facteurs de la stérilité du bananier (*Musa* spp.) et des relations cytotoxiques entre *M. acuminata* et *M. balbisiana* Colla. *Fruits* 43:539–558, 615–638, 685–700.
- Dodds, K.S. and N.W. Simmonds. 1946. Genetical and cytological studies of *Musa*. VIII. The formation of polyploid spores. *J. Genet.* 47:223–241.
- Dumpe, B. and R. Ortiz. 1996. Apparent male fertility in *Musa* germplasm. *HortScience* 31:1019–1022.
- Hamill, S.D., M.K. Smith, and W.A. Dodd. 1992. *In vitro* induction of banana autotetraploids by colchicine treatment of micropropagated diploids. *Austral. J. Bot.* 40:887–896.
- McCusiston, F. and G.W. Elmsstrom. 1993. Identifying polyploids of various cucurbits. *Proc. Fla. State Hort. Soc.* 106:155–157.
- Ortiz, R. 1997. Occurrence and inheritance of $2n$ pollen in *Musa*. *Ann. Bot.* 79:449–453.
- Ortiz, R., M. Iwanaga, and H.A. Mendoza. 1988. Combining ability and parental effects in $4x-2x$ crosses for potato breeding. *Potato Res.* 31:643–650.
- Ortiz, R. and D. Vuylsteke. 1994. Inheritance of black sigatoka disease resistance in plantain–banana (*Musa* spp.) hybrids. *Theor. Appl. Genet.* 89:146–152.
- Ortiz, R. and D. Vuylsteke. 1995. Factors influencing seed set in triploid *Musa* spp. L. and production of euploid hybrids. *Ann. Bot.* 75:151–155.
- Ortiz, R. and D. Vuylsteke. 1996. Recent advances in *Musa* genetics, breeding and biotechnology. *Plant Breeding Abstr.* 66:1355–1363.
- Osuji, J.O., B.E. Okoli, and R. Ortiz. 1996. An improved procedure for mitotic studies of the *Eumusa* section of the genus *Musa* L. (Musaceae). *InfoMusa* 5:12–14.
- SAS Institute. 1988. SAS/STAT user's guide, release 6.03. SAS Inst., Cary, N.C.
- Stanford, E.H. 1951. Tetrasomic inheritance in alfalfa. *Agron. J.* 43:222–225.
- Vandenhout, H., R. Ortiz, D. Vuylsteke, R. Swennen, and K.V. Bai. 1995. Effect of ploidy on stomatal and other quantitative traits in plantain and banana hybrids. *Euphytica* 83:117–122.
- van Duren, M., R. Morpurgo, J. Dolezel, and R. Afza. 1996. Induction and verification of autotetraploids in diploid banana (*Musa acuminata*) by *in vitro* techniques. *Euphytica* 88:25–34.