A novel method to prepare slides for high resolution chromosome studies in *Musa* spp.

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Contrary to the importance of bananas and plantains both as a staple food and export commodity, the knowledge of their genome at the chromosomal level remains poor. It is surprising that most genotypes maintained in gene banks have unknown, a speculative or even a wrongly determined ploidy level (Jenny *et al.* 1997, Horry *et al.* 1998). Although ploidy can be now determined also by flow cytometry (Doležel *et al.* 1994, 1997) detailed microscopical analysis of karyotype cannot be replaced by other methods. In contrast with the situation in other economically important crops, the state of *Musa* karyology is rather poor. This is mainly due to problems with preparation of chromosome spreads and the small size of *Musa* chromosomes (1 - 2 μm, when observed at mitotic metaphase). Considering the nuclear genome size in *Musa* (Doležel *et al.* 1994) an average chromosome has only about 50 Mbp DNA.

Difficulties in preparing good metaphase spreads are mainly due to the rigid cell wall. The tissue can be softened by maceration in strong acids or by incubation in hydrolytic enzymes, and metaphase spreads can be obtained by squashing the softened tissue. In *Musa*, several authors used this technique and in some cases, metaphase spreads suitable for chromosome counting were obtained (Shepherd and Dos Santos 1996, Osuji *et al.* 1996). However, due to the presence of remnants of cell wall and cytoplasm, the squash technique is not suitable for high resolution chromosome analysis as the cytoplasm may be non-specifically stained. Furthermore, squash preparations are not optimal for physical mapping of DNA sequences using *in situ* hybridization. The presence of cellular remnants decreases the accessibility of chromosomes to molecular probes and may also cause their non-specific binding. Alternative procedures based on protoplast isolation and spreading were developed for some species to avoid this problem (Ma *et al.* 1996, Martin *et al.* 1994). This paper describes a protocol for the preparation of high quality metaphase spreads for chromosome analysis in *Musa*. The protocol is based on protoplasts isolation from root tips and was developed through many experiments in which each step of the procedure was optimized.

Materials and methods

All *Musa* species and clones used in this study (Table 1) were obtained from the INIBAP Transit Centre (Katholieke Universiteit Leuven, Belgium) as *in vitro* rooted plantlets. After transfer to soil, plants were maintained in a greenhouse.

Actively growing roots were cut about 1 cm from the root tip and collected in 50 mM phosphate buffer (pH 7.0) containing 0.2% β-mercaptoethanol. The roots were then transferred to 0.05% 8-hydroxyquinoline for three hours at room temperature to accumulate dividing cells in metaphase. Subsequently, the roots were fixed in 3:1 (ethanol: acetic acid) fixative at 4°C overnight. After two rinses in 70% ethanol, the roots were transferred to 70% ethanol and stored at 4°C for up to several months.

Prior to slide preparation, 5 to 15 roots were washed three times in a solution of 75 mM KCl and 7.5 mM EDTA (pH 4). Meristem tips were then cut to small pieces and digested for 60 min at 30 °C in 400 μl of enzyme mixture in a microcentrifuge tube. The enzyme mixture consisted of 1% pectinase (Sigma P-2401), 0.5% pectolyase (Sigma P-3026) and 0.5% cellulase (Serva 16419) made in 0.1M citrate buffer (pH 4.7). The suspension of released protoplasts was filtered through a 150 μm nylon mesh and pelleted at 1000 rpm. The pellet was resuspended in 400 μl of 75 mM KCl and 7.5 mM EDTA (pH 4) and incubated for 5 min at room temperature. After pelleting, the protoplasts were resuspended in 400 μl of ice-cold 70% ethanol, incubated for 5 min and pelleted again. After resuspending in ice-cold 70% ethanol, isolated protoplasts were stored at 4 °C for up to several months.

To prepare a slide, 7 μl of protoplast suspension in 70% ethanol was dropped onto a clean ice-cold microscope slide. The suspension was allowed to spread out and air-dry. This process was monitored under a microscope. Shortly before complete drying out, 7 μl of ice-cold 3:1 fixative was added to the drop to induce cell bursting. Shortly before complete drying the slide was briefly rinsed in 100% ethanol and air-dried at room temperature. For chromosome staining, the slides were immersed for 25 min at room temperature in 3% Giemsa solution made in 50 mM phosphate buffer. The stain was removed by a wash in distilled water. Air-dried slides were mounted in Euparal and observed under Olympus BX60 microscope using a 100x/1.35 oil immersion objective. Images were photographed

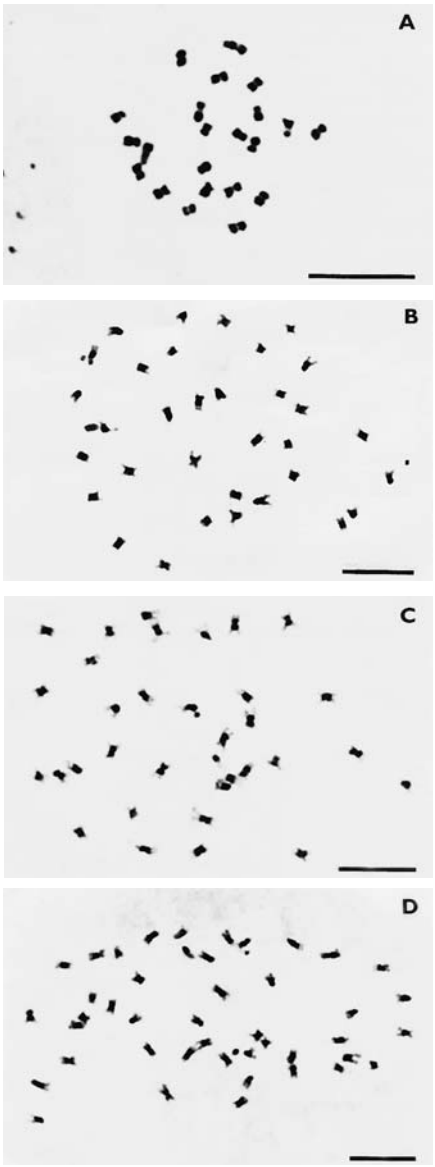

Figure 1. *Mitotic metaphase plates of selected* Musa *genotypes:*
*• **A** 'Niyarma Yik'(ITC0269): 2n=2x=22 chromosomes; • **B** 'Balonkawe'(ITC0473): 2n=3x=33 chromosomes; • **C** 'Pisang Jambe' (ITC0694): 2n=3x=33 chromosomes; • **D** '(Kluai) Ngoen'(ITC0286): 2n=4x=44 chromosomes. Bar = 10 μm.*

on Ilford PAN F 50 film using a green optical filter.

Results and discussion

After putting on a slide, the protoplast suspension in 70% ethanol remains in a drop without significant spreading. When the fixative (3:1) is added, the drop spreads on the glass surface and begins to evaporate rapidly. As the mixture evaporates, the surface tension makes the protoplasts wider from side to side and eventually causes the protoplasts to break. At this moment the metaphase plate is released from the protoplast, the cytoplasm is dispersed and chromosomes spread. We have found that the quality of metaphase spread depends critically on the rate of drying. If the drying is too fast, the protoplasts dry without sufficient spreading and metaphases are clumped. On the other hand, very slow drying may lead to broken metaphases. Optimal conditions for drying have to be found empirically. The rate of drying can be controlled by the temperature of the microscope slide, the fixative and (to a certain extent) by room temperature.

Although the method described here is more laborious than traditionally used procedures, it has important advantages. Pre-treated roots and even isolated protoplasts can be stored in a freezer and used to prepare fresh slides when needed. From a single isolation procedure, ten to twenty slides may be prepared in a very short time. Under optimal conditions, the method results in chromosome spreads whose quality surpasses all currently used protocols for karyological studies in *Musa*. Chromosomes are not covered by a cell wall; they are well separated and intensively stained. Furthermore, the staining of cytoplasmic remnants underlying chromosomes is very weak and usually completely absent (Figure 1). This makes chromosome counting and the analysis of chromosome morphology at metaphase or prometaphase very easy. We have found that slides prepared according to this method are suitable for physical mapping of DNA sequences on *Musa* chromosomes using *in situ* hybridization (Doležel 1996, Doleželová *et al.* 1997).

The method has been tested on a random selection of genotypes representing *Musa acuminata, M. balbisiana*, a range of triploid clones and one tetraploid clone (Table 1). In all cases, chromosome numbers could be unambiguously determined. Our results confirmed the reclassification of 'Kluai Tiparot'as a triploid clone (Jenny *et al.* 1997). Contrary to other reports indicating the occurrence of chromosome number instability in *Musa* (Sandoval *et al.* 1996, Shepherd and Da Silva 1996), our results obtained on a limited number of plants did not indicate a wide-spread occurrence of aneuploidy or mixoploidy in these genotypes. Furthermore, high quality of metaphase spreads permitted evaluation of chromosome morphology. To conclude, the procedure described here opens a way for reliable chromosome counting in *Musa*, for high resolution studies of their morphology and for physical mapping of *Musa* genome using *in situ* hybridization.

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Table 1. Determination of chromosome number in selected *Musa* species and landraces.

Accession name	ITC Code*	Species/ Group	Subspecies/ Sub-group	Chromosome number	Ploidy
Higa	0428	*acuminata*	*banksii*	22	2x
Pisang Mas	0653	AA	Sucrier	22	2x
Pa (Rayong)	0672	*acuminata*	*siamea*	22	2x
Niyarma Yik	0269	AA	*banksii* -derivative	22	2x
M. balbisiana	0094	*balbisiana*		22	2x
Cameroun	0246	*balbisiana*		22	2x
Singapuri	0248	*balbisiana*		22	2x
Tani	1120	*balbisiana*		22	2x
Yangambi km5	1123	AAA	Ibota	33	3x
Obino l'Ewai	0109	AAB	Plantain (French)	33	3x
Agbagba	0111	AAB	Plantain (False Horn)	33	3x
Saba	1138	ABB	Saba	33	3x
Kluai Tiparot	0652	?	Kluai Teparod	33	3x
Balonkawe	0473	?	Kluai Teparod	33	3x
Pisang Jambe	0694	?		33	3x
(Kluai) Ngoen	0286	AABB		44	4x

*Code assigned by the INIBAP Transit Centre (Leuven)

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