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Improvements in cytological preparations for fluorescent *in situ* hybridization in *Passiflora*

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ABSTRACT. Cytological preparations for the fluorescent *in situ* hybridization (FISH) technique require cytoplasm-free metaphases, with well-spread chromosomes, for the localization of DNA sequences and chromosome mapping. We tested various procedures for FISH analysis of *Passiflora cacaoensis*, *P. gardneri* and hybrid F_1 progeny of *P. gardneri* x *P. gibertii*. Two treatments with four enzymes and three incubation times were compared. The material was treated with 1.0 M HCl before enzymatic digestion. The following criteria were used to determine the quality of the metaphases: a) lack or presence of cytoplasm; b) well-spread chromosomes or with overlap; c) complete or incomplete chromosome number (2*n*). The enzyme Pectinex® SP ULTRA gave the best performance, with the shortest incubation time. The best results were observed after 30 min of incubation; more than 70% of the metaphases did not have large amounts of cytoplasm or overlapping chromosomes, and about 75% maintained the chromosome number. FISH was carried out using a 45S rDNA probe (*p*Ta71) labeled with biotin and detected with fluorescein isothiocyanate. Sites with strong staining and without

nonspecific signals were observed. Our methodological adaptations allowed the preparation of metaphase slides of high quality for the FISH technique, with less time required for the preparation of samples.

Key words: Passion flower; Cytogenetic; Pectinex; FISH

INTRODUCTION

Cytological preparations to be used in *in situ* hybridization method must have metaphases with adequate chromosome spread, free of cytoplasm. The FISH (fluorescent *in situ* hybridization) technique is based on the *in situ* localization of specific DNA sequences in somatic metaphases, interphase nuclei and meiosis, using complementary nucleic acid probes labeled with a fluorochrome. In the GISH (genomic *in situ* hybridization) technique, the whole genome is labeled so that it can be distinguished from another genitor genome in a hybrid (Sumner, 2003). The localization of these sequences or of the labeled genomes may be examined by microscopy, leading to the physical mapping of DNA sequences (Pedrosa-Harand et al., 2009).

In FISH, the probe may be indirectly labeled using biotin (vitamin H) or digoxigenin (a steroid from *Digitalis purpurea*, foxglove). These molecules are commercially available linked to nucleotides, and are incorporated into the DNA probe using standard molecular labeling techniques (Schwarzacher and Haslop-Harrison, 2000). The probe hybridizes with the denatured target DNA and must be detected allowing visualization of the probe-target hybrids formed. Fluorescent detection is often used for DNA targets. Probes labeled with biotin or digoxigenin need to be detected by a fluorophore conjugated to avidin or antibodies, forming complexes that can be visualized (Schwarzacher and Haslop-Harrison, 2000; Sumner, 2003). The labeling of the probe may also be direct, using nucleotides directly connected to the probe (Schwarzacher and Haslop-Harrison, 2000).

The presence of cytoplasm in the preparation is often a physical barrier to hybridization, preventing the probe from reaching the target DNA or hampering the visualization of signal in the chromosomes. Dense cytoplasm between nuclei, distorted chromosomes and dark and contrasty interphase nuclei are not likely to provide good targets for hybridization (Schwarzacher and Haslop-Harrison, 2000). Another problem in cytological preparations is agglomeration or overlapping of chromosomes, frequently disabling a reliable analysis of signals in the metaphases. Slides with a large number of metaphases and well-spread chromosomes are essential for obtaining unequivocal results and making the technique economically viable (Guerra, 2004). Enzyme treatment in the cytological preparation that dissolves the cell wall is often employed for obtaining well-spread metaphases, but excess cytoplasm may prevent the spreading of the chromosomes, even when enzymes are used.

Cellulase and pectinase, in different concentrations, are the most used enzymes (Belyayev et al., 2001; Cuco et al., 2003; De Melo and Guerra, 2003). However, they require long incubation periods, up to 2 h or more with some species (De Melo and Guerra, 2003; Marcon et al., 2005). Aiming to achieve a faster or more efficient results, other enzymes have been used, such as macerozyme (Fukui and Nakayama, 1996), pectolyase and cellulase Onozuka RS (Danilova and Birchler, 2008; Yuan and Tomita, 2009), Ultrazym (Almeida and Carvalho, 2004), and Pectinex (Viccini et al., 2006; Guimarães et al., 2009), among others.

There has been growing interest in the use of Pectinex in cytogenetic procedures. This product is a mixture of enzymes based on pectinases, containing a variation of hemicellulytic

activities, primarily used for the treatment of fruits, for the purpose of increasing the production of fruit juices (Tanriseven and Aslan, 2005; Vendrúsculo and Quadri, 2008), and it is stable at room temperature. The use of Pectinex for enzymatic treatment of chromosomes has been successful; however, the cytological preparations still show many metaphases with dense cytoplasm, and auxiliary treatments are often necessary for softening root-tips (Feitosa, 2009).

Aiming to obtain metaphases of *Passiflora* with adequate chromosome spreading and free of cytoplasm, different enzymes, most used in species of this genus, were used to optimize the procedures for obtaining cytological preparations for use in *in situ* hybridization.

MATERIAL AND METHODS

The species *Passiflora cacaoensis* Bernacci and Souza (2*n* = 18; Souza et al., 2005), *P. gardneri* Mast. (2*n* = 18; Seger and Souza, 2009) and interspecific hybrids of *P. gardneri* Mast. x *P. gibertii* N.E. Brown, progeny HD15 (2*n* = 18; Seger and Souza, 2009) were used. The species and hybrid genotypes are kept at the Bank of Active Germplasm (BAG-Passifloras) of Universidade Estadual da Santa Cruz (UESC), Ilhéus, BA, Brazil, located between coordinates 14° 39' S, 39° 10' W and at 78 m asl.

The cytological analyses were carried out at Laboratório de Biossistemática, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil. Samples were prepared from the root tips of up to 1.0 cm, obtained from cuttings cultivated in washed sand and kept in 50% shade. They were pretreated with 2 mM 8-hydroxyquinoline for 1 h at room temperature (RT) and up to 22 h at 6-8°C. After washing twice for 5 min each, the root tips were fixed in Carnoy I (ethanol-glacial acetic acid, 3:1; Johansen, 1940) and kept at -20°C. The root tips were washed twice for 5 min each, with mixing (120 rpm), transferred to a chambered slide and kept in 1.0 M HCl for 20 min at 37°C in a humid chamber (Andras et al., 1999). After washing twice, the root tips were transferred to slides and treated with the enzymes: a) 100% Pectinex SP ULTRA®; b) 2% cellulase + 20% pectinase +1% macerozyme. To each root tip, 15 µL enzyme was added. The slides were kept at 37°C in a humid chamber for different incubation times: 20, 30, and 40 min. The material still on the slide was washed to remove the enzyme. After drying with filter paper, 6 µL 60% glacial acetic acid was added to each slide, aiming to spread the cells under an 18 x 18-mm coverslip. The slides were prepared using the squash technique and immersed into liquid nitrogen until frozen and the coverslips were flicked off with a razor blade, air-dried and kept at -20°C. To test the effects of the enzymes on the cytological preparations with *Passiflora*, the slides were stained with 2 µg/mL DAPI (4',6-diamidino-2-phenylindole/Vectashield® (1:99, v/v) and examined with a fluorescence microscope equipped with 340/380 excitation and 425/470 emission filters. All metaphases in each slide were analyzed for the evaluation, using a completely randomized statistical design with five replications (slides). Metaphases were counted: a) with or without cytoplasm; b) with well-spread or overlapping chromosomes, and c) complete or incomplete chromosome number (2*n*).

To test the efficiency of the cytological preparation in the use of FISH, the technique was carried out according to Schwarzacher and Haslop-Harrison (2000) with modifications. Slide preparations were pretreated with 100 µg/mL RNase in 2X SSC (0.3 M sodium chloride plus 0.03 M sodium citrate) incubated at 37°C for 1 h in a humid chamber and rinsed in 2X SSC, twice for 5 min each. Slides were immersed in 4% paraformaldehyde at RT for 10 min, rinsed in 2X SSC, twice for 5 min each, and dehydrated in 70 and 96% ethanol at RT for 5 min each. Plasmid

45S rDNA (*p*Ta71) was labeled with biotin-16-dUTP using Biotin-Nick Translation Mix (Roche 11745824910). The hybridization mixture contained 7.5 µL 100% formamide, 3.0 µL 50% dextran sulfate, 1.5 µL 20X SSC, 0.2 µL 10% SDS (sodium dodecyl sulfate) and 2.8 µL 50-200 ng probe DNA per slide. The mixture was heated at 75°C for 10 min (thermocycler) and cooled in ice for 5 min, at least. Slides with hybridization mixture (15 µL/slide) were heated at 75°C for 5 min (thermocycler). Hybridization was carried out at 37°C in a humid chamber overnight. The slides were then washed in 2X SSC at RT for 5 min, 2X SSC at 42°C, twice for 5 min each, 0.1X SSC at 42°C, twice for 5 min each, and subsequently washed in 2X SSC at 42°C, twice for 5 min each, and in 4X SSC/0.2% Tween 20 at RT for 5 min. Slide preparations were treated with 50 µL 50% BSA (bovine serum albumin, fraction V) at RT for 10 min in a humid chamber. Biotin-labeled 45S rDNA was detected with 0.5 µL avidin-FITC (fluorescein isothiocyanate) plus 19.5 µL 5% BSA per slide. Detection was carried out at 37°C in a humid chamber for 1 h. The slides were washed in 4X SSC/0.2% Tween 20 at RT, 3X for 5 min each and quickly immersed in 2X SSC. All washing steps with SSC were carried out with mixing (120 rpm). Chromosomes were counterstained and mounted with DAPI/Vectashield®. FITC signals were visualized with a fluorescence microscope equipped with 499-nm excitation and 518-nm emission filters.

RESULTS AND DISCUSSION

In the genotypes studied, the enzyme Pectinex was the one that best digested the cell wall, and in less time. After 20-min treatment with Pectinex, more than 70% of the metaphases did not show significant presence of cytoplasm associated with chromosomes, whereas after 40-min treatment with a mixture of enzymes (cellulase, pectinase and macerozyme), the metaphases still showed chromosomes covered by dense cytoplasmic material in all genotypes analyzed. Thus, the cytological analyses were carried out only with metaphases treated with Pectinex due to the better performance of this enzyme in *Passiflora* meristematic cells.

Although the use of Pectinex for a 40-min incubation provided the better mean values for obtaining cytoplasm-free metaphases and without overlap, almost 50% of metaphases showed an incomplete chromosome number (2*n*). The best incubation time for the use of Pectinex in *Passiflora* was 30 min; around 75% of the metaphases analyzed maintained chromosome number, and more than 70% did not show a significant presence of cytoplasm or overlapping chromosomes (Table 1).

Table 1. Mean values (%) obtained for *Passiflora* metaphases with regard to presence of cytoplasm, chromosome spreading and integrity of chromosome number (2*n*) in samples treated with Pectinex® for different incubation times.

Genotype	Time (min)	Metaphases			Mean number of metaphases analyzed
		Without cytoplasm	Without overlap	Complete (2*n*)	
P. cacaoensis	20	22 (70.8)	16 (51.6)	28 (90.3)	31
	30	23 (79.3)	21 (72.0)	22 (75.8)	29
	40	25 (92.0)	24 (88.9)	15 (55.6)	27
P. gardneri	20	25 (71.4)	20 (57.1)	29 (82.8)	35
	30	31 (86.0)	26 (72.2)	28 (77.7)	36
	40	30 (88.2)	28 (82.0)	20 (58.8)	34
Hybrids HD15	20	24 (70.5)	20 (58.8)	29 (85.3)	34
	30	22 (70.9)	24 (77.4)	22 (70.9)	31
	40	26 (86.6)	28 (93.3)	16 (53.2)	30

Results are means of five replications.

The genotypes displayed significant differences ($P < 0.01$) by the F-test (Table 2) in relation to the presence of cytoplasm only for the 30- and 40-min incubation times. As regards the other metaphase characteristics, chromosome overlap and the integrity of chromosome number, there was a significant difference between the genotypes for all incubation times tested. Such analyses indicate the need to optimize the incubation time of Pectinex for each species, which varies from 20 to 40 min. The FISH technique was successfully employed in *Passiflora* genotypes, with the 45S rDNA site well visualized and without non-specific signals (Figure 1).

Table 2. ANOVA summary for three incubation times using Pectinex®: presence of cytoplasm, chromosome spreading and integrity of chromosome number (2*n*) among *Passiflora* genotypes.

Source of variation	d.f.	Mean squares		
		20 min	30 min	40 min
Metaphases without cytoplasm				
Genotypes	2	1,086^{NS}	287,786**	38,394**
Error	12	1,182	4,985	2,179
CV (%)		1,532	2,834	1,659
Metaphases without overlap				
Genotypes	2	70,816**	42,722**	163,792**
Error	12	1,946	4,297	4,162
CV (%)		2,498	2,800	2,316
Metaphases with complete chromosome number (2*n*)				
Genotypes	2	73,086**	60,872*	39,760**
Error	12	11,518	12,633	5,210
CV (%)		3,940	4,751	4,085

d.f. = degrees of freedom; CV = coefficient of variation; Mean squares based on mean of five measurements; *Significance at the 5% ($P < 0.05$) probability level and **significance at the 1% ($P < 0.01$) probability level by the F-test; ^{NS}Not significant.

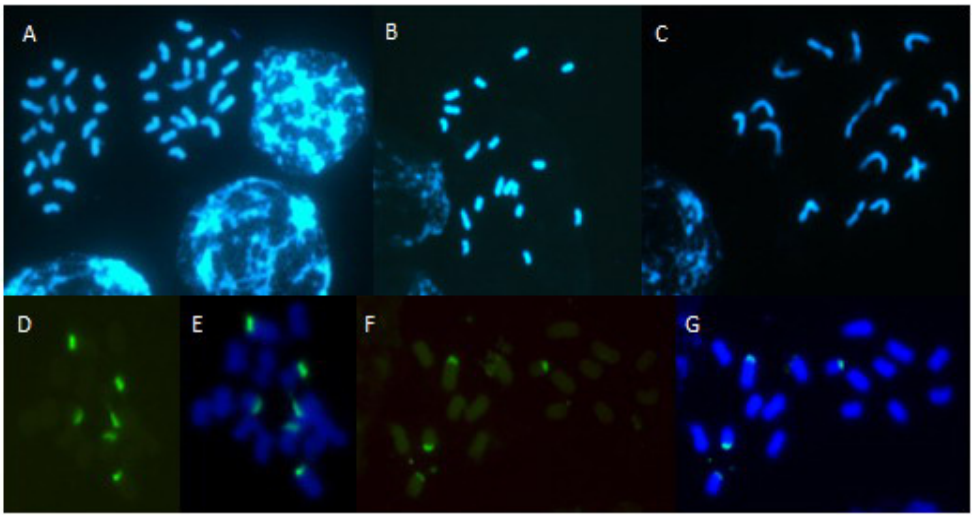

Figure 1. Metaphases in *Passiflora* (2*n* = 18) from cells pretreated with 1.0 M HCl and digested with Pectinex® in a humid chamber. A-C) Preparations stained with DAPI. **A.** F_1 interspecific hybrid HD15-110 after 20 min of incubation. **B.** *P. cacaoensis* after 30 min of incubation. **C.** HD15-110 after 40 min of incubation. D-G) FISH preparations with 45S rDNA probe. **D.** *P. gardneri* after 20 min of incubation and FISH with FITC detection, showing six sites. **E.** Metaphase (Figure D) counterstained with DAPI. **F.** HD15-101 after 30 min of incubation and FISH with FITC detection, showing five sites. **G.** Metaphase (Figure F) counterstained with DAPI.

The enzyme treatment time for the merismatic material determines whether the cell wall is degraded, influencing the release and digestion of its cytoplasmatic material and, consequently, the spreading of the chromosomes. Remains of cytoplasm and other cellular and cell wall material will reduce the *in situ* hybridization signal and produce a high level of background (Schwarzacher and Haslop-Harrison, 2000). FISH cytological preparations in wild *Passiflora* have been carried out with the enzymes cellulase and pectinase, requiring up to 2 h of treatment (De Melo and Guerra, 2003). The enzyme Flaxzyme (Novo Ferment™) was used in *Passiflora edulis* f. *flavicarpa* O. Deg., with the incubation time reduced to 1 h 30 min (Miranda et al., 2008). Further shortening of the incubation time was obtained in *Passiflora* and interspecific hybrids by pretreating with cycloheximide plus 8-hydroxyquinoline to obtain good spreading (Cuco et al., 2005), making the procedure more expensive.

Pectinex enzyme has been used in *Passiflora* (Abreu, 2008; Amorim, 2009; Viana, 2009) due to its fast action in the digestion of the cell wall, when compared to other enzymes usually employed. However, its incubation time was still prolonged, longer than 40 min for some species (Viana, 2009). The use of the treatment with 1.0 M HCl combined with Pectinex substantially reduced the enzyme incubation time and increased the quality of the cytological preparations, without interfering with the binding and detection of the probes used in the FISH technique, according to Andras et al. (1999). The use of hydrochloric acid is usually avoided in chromosomal preparations, because it is deleterious to chromosome structure and since it extracts histones from the chromatin and denatures and depurinates DNA (Ma et al., 1996). The HCl treatment was optimized at 1.0 M concentration and incubation at 37°C for 20 min, which softens the meristematic tissue and hydrolyzes the cytoplasm without damage to the chromosomes, allowing a better dispersion of cytoplasm fragments on the slide (Andras et al., 1999).

The first step for the successful use of the *in situ* hybridization technique is to obtain excellent preparations. In *Passiflora*, although *P. edulis* is an agronomically valuable species with regard to exports and generating income for Brazil, few studies have been carried out exploring this technique. Although relevant, only 45S and 5S rDNA sequences have been detected (De Melo and Guerra, 2003; Cuco et al., 2005; Miranda et al., 2008). The number and localization of 45S and 5S rDNA sites analyzed in 20 species of *Passiflora* were consistent with the hypothesis of a probable ancestral genome $x = 6$ for the genus (De Melo and Guerra, 2003). Species $2n = 12$ (diploid) possess two 5S rDNA sites and two or four 45S rDNA sites, whereas in polyploid species the number of sites is proportional to the increase in ploidy, as observed in *P. suberosa*, a tetraploid with ten 45S rDNA sites and four 5S rDNA sites, and in *P. misera*, a hexaploid cytotype with twelve 45S rDNA sites and six 5S rDNA sites. In somatic *P. edulis* + *P. amethystina* ($2n = 4x = 36$) hybrids, a variation in the number of 45S rDNA sites was observed, with 10 to 12 detected, indicating the probable loss of DNA sequences and transpositions of polyploid hybrid genotypes (Cuco et al., 2005).

The FISH technique has been used in different species for chromosome mapping, both for repetitive DNA (Zhou et al., 2003) and unique sequences (Ohmido et al., 1998). With the construction of a genetic linkage map in *Passiflora* (Carneiro et al., 2002; Oliveira et al., 2008), genetic sequences of agronomic importance could be located on the chromosomes by the FISH technique, resulting in the integration of the cytogenetic and genetic linkage map, as noted in *Lotus japonicus* (Ohmido et al., 2010). With the utilization of *Passiflora* interspecific hybrids in the ornamental plant market (Abreu et al., 2009), the use of the GISH technique, still unpublished for this genus, is promising with regard to the identification of introgression of chromosomes or chromosomal fragments. If this technique is integrated with the analysis

of morphological characteristics, this could direct new crossings for obtaining progeny with characteristics of interest to this market.

Both FISH and GISH techniques in *Passiflora* may be favored by the methodological adaptation proposed in this study, allowing the preparation of metaphases with adequate chromosome spreading and practically free of cytoplasm, in a shorter period of time, when compared to the methods used in previous studies.

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