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Identification of the pattern of heterochromatin distribution in *Passiflora* species with C-banding

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ABSTRACT. We tested four C-banding protocols to obtain heterochromatic bands in the passion fruit species *Passiflora edulis* and *P. cacaoensis* (Passifloraceae). Three of these protocols had been previously described. The three published protocols were not adequate to obtain C-bands in these species. An adapted protocol demonstrated heterochromatin distribution in metaphasic chromosomes of species of *Passiflora* for the first time. The differentiated coloration for C-bands was obtained with immersion of the slides in 99% ethanol, 45% acetic acid (additional step), 0.2 N hydrochloric acid, hydroxide of barium, 45% acetic acid, and 2X standard saline citrate at four different temperatures. The C-bands were observed in the satellites and in the telomere and centromere regions of all chromosomes, both in *P. edulis* and in *P. cacaoensis*.

Key words: Passion fruit; *Passiflora cacaoensis*; *Passiflora edulis*

INTRODUCTION

Giemsa C-banding is the most commonly used procedure to show the constitutive heterochromatin in the chromosomes and nuclei of plants (Fukui and Nakayama, 1996). By means of this technique, the pre-treatment with hydroxide of barium, followed by standard saline citrate (SSC), modifies the structure of the chromosome, resulting in the preferable extraction of proteins and DNA from the euchromatic regions, providing a characteristic pattern of bands (McKay, 1973).

The degree of polymorphism and the heterochromatin-staining properties observed in the chromosomes when submitted to Giemsa C-banding are determined by the type of heterochromatin, which is differentiated in facultative and constitutive heterochromatin according to the contents of the DNA satellite (Multani et al., 2001). The constitutive heterochromatin differs substantially from the euchromatin in the composition of its DNA bases and by the low or absent transcriptional activity. This condition is partly associated with the constantly condensed state of this type of chromatin (Guerra, 2000; Sumner, 2003).

The preferential occurrence of the constitutive heterochromatin is described in determined regions of the chromosomes, particularly in the centromeres or in their proximities (pericentromere or paracentromere), forming blocks of variable sizes, according to the group analyzed (Sumner, 2003). Species with small chromosomes, of less than 3 µm, generally present proximal bands (centromeres), and the bigger the chromosomes the higher the frequency of the telomeres and interstitial bands (Guerra, 2000).

Gill and Kimber (1974) developed a protocol to detect the heterochromatin in the chromosomes of rice. Since then many adaptations have been made with the purpose of obtaining larger number of C-bands in the plant chromosomes. Such modifications were mainly developed for grasses, where the technique presents better results (Chrzastek, 2003; Ellneskog-Staam et al., 2007). Giraldez et al. (1979) introduced adaptations in the protocol of Gill and Kimber (1974) to obtain the best solution in the C-banding pattern in the chromosomes of rice. Darvey and Gustafson (1975) adapted the protocol to hybrid lineages of rice and wheat. Although these protocols have presented efficient results for grasses, they had to be modified and adapted to obtain good results in other plant groups (Hoshi et al., 1998).

The distribution of the heterochromatin by C-banding has not yet been reported in species of the *Passiflora*. The study of the pattern of heterochromatin distribution in *Passiflora* is important for the evolutionary study of the strategies of improvement in this genus. Therefore, the chromosomal banding, together with the molecular genetics, may provide information to identify and characterize the chromosomes, aiding in the construction of physical maps. Due to the difficulty of obtaining and observing C-bands in chromosomes of species of *Passiflora*, this investigation aimed at applying four protocols to observe the C-banding, and obtain an efficient protocol to visualize bands of clearly differentiated heterochromatin in these species.

MATERIAL AND METHODS

The material was constituted from five specimens of *Passiflora edulis* f. *edulis* O. Deg. obtained from seeds donated by IAC (Instituto Agronômico, Campinas), UENF (Uni-

versidade Estadual do Norte Fluminense Darcy Ribeiro) and Embrapa Cerrados (Brazilian Agricultural Research Corporation), and five specimens of *P. cacaoensis* Bernacci & Souza collected in the Atlantic Forest of Serra Bonita, Camacan, State of Bahia, Brazil.

Root tips presenting around 1.0 cm in length were obtained from cuttings. The samples were treated with 2 mM 8-hydroxyquinoline for 1 h at room temperature and 21 h at 6°C, fixed in Carnoy 1 (Johansen, 1940) for 3 h at room temperature and kept in the freezer at -20°C for at least 24 h. Samples were washed twice in distilled water for 5 min each, dried on filter paper and immersed in a drop of 100% enzyme Ultrazym® 100G (Novozymes). The slide was incubated in a moist chamber at 37°C for 1 h. The enzyme was removed and 30 µL 45% acetic acid was added to the root tips, which were macerated with the help of needles and covered with a glass coverslip. The set slide-coverslip was firmly pressed between filter papers. The slides were frozen in liquid nitrogen for around 3 min and the glass coverslip was quickly removed with the help of a blade. The slides that were not immediately used were stored at -20°C.

The procedure for C-banding followed four protocols: a) Protocol 1, proposed by Gill and Kimber (1974); b) Protocol 2, proposed by Darvey and Gustafson (1975); c) Protocol 3, proposed by Giraldez et al. (1979), and d) Protocol 4 (adapted), containing the steps of the Protocols 2 and 3, and one additional step (Table 1). The samples were observed and photographed with a digital camera (7.5 Megapixels) adapted to a light microscope.

Table 1. Steps of Protocol 4 (adapted) to obtaining C-bands in *Passiflora*.

Stages	Protocol 2 (Darvey and Gustafson, 1975)	Protocol 3 (Giraldez et al., 1979)	Additional step
Dehydration		Immersion of the slide in 99% ethanol for 12 h and drying in RT for 5 min.	
			Immersion in 45% acetic acid in RT for 30 min*.
		Immersion in 0.2 N hydrochloric acid at 60°C for 2.5 min*.	
Denaturation	Immersion in saturated solution newly prepared of hydroxide of barium in RT for 10 min. Washing in distilled H_2O. Immersion in 45% acetic acid and in distilled H_2O at 45°C for 2 min*.		
Renaturation	Immersion in saline solution of 2X SSC at RT for 15 min. The temperature was increased to 30°C for 10 min, later to 40°C for 10 min and then increased to 52°C for 60 min*.		
Staining		Staining of the slides with 4% Giemsa for 30 min*.	

*After this step, the slides were washed with distilled H_2O and dried with compressed air. RT = room temperature; SSC = standard saline citrate.

RESULTS

Protocol 1 was not able to produce differentiated coloration for C-bands in the chromosomes, and an intense and uniform staining along the chromosomes was observed (Figure 1a). On the other hand, by using Protocol 2, excessive extraction of DNA and a very weak staining along the chromosome were observed (Figure 1b). Some bands of heterochromatin

were obtained from Protocol 3. However, there was low contrast between the clear and dark bands, and a weak staining along the chromosome was observed.

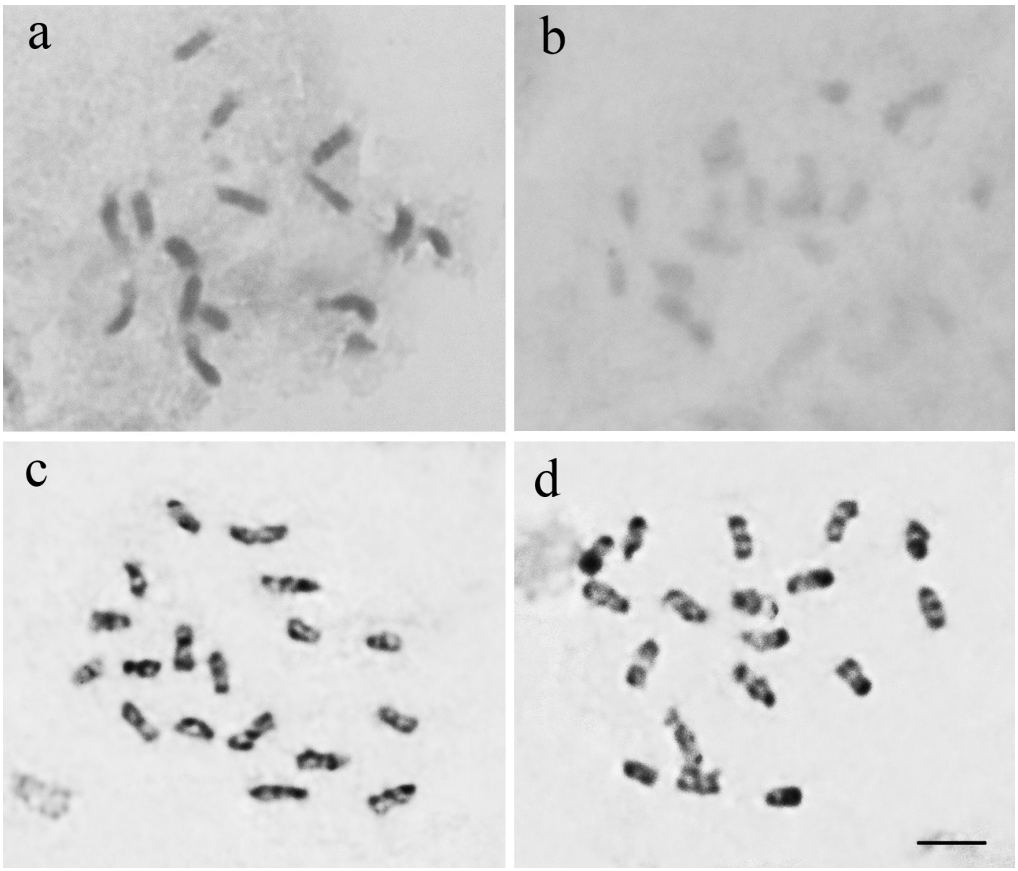

Figure 1. C-banding on the metaphase chromosomes of *Passiflora* species. **a.** Treatment with Protocol 1 in *P. cacaoensis*. **b.** Treatment with Protocol 2 in *P. cacaoensis*. **c.** Treatment with Protocol 4 (adapted) in *P. edulis*. **d.** Treatment with Protocol 4 (adapted) in *P. cacaoensis*. Bar = 5 µm.

Protocol 4 (adapted), resulting from adjustments to Protocols 2 and 3, was the protocol that provided the best results, showing the chromatin present in the satellites, in the telomere and centromere regions of all the chromosomes, both for *P. edulis* and *P. cacaoensis* (Figure 1c and d). *P. cacaoensis* presented a higher amount of heterochromatin in its chromosomes. In this species, chromosomes 3 and 9 were constituted, in great part, by heterochromatin. In *P. edulis*, the distribution of heterochromatin was more uniform, except for the chromosome 6, which presented a short arm that was practically heterochromatic.

DISCUSSION

Protocol 1 was not efficient in denaturing the chromatin probably because the expo-

sure time to the hydroxide of barium was insufficient for alkaline denaturation (Fukui and Nakayama, 1996). Protocol 2 is routinely used for chromosomes of cereals (Maria, 1996; Chrzastek, 2003); however, for other plant groups some modifications in the exposure time and temperature for hydroxide of barium were recommended for obtaining C-bands (Fukui and Nakayama, 1996). For species of passifloras, it was not able to obtain C-bands. On the other hand, Protocol 3 has been used for different plant groups, such as *Dasypyrum villosum* (Friebe et al., 1987), *Secale cereale* (Cuñado et al., 1986), *Bromus riparius* (Tuna et al., 2001), and *Alstroemeria ligtu* (Ishikawa and Ishikava, 2002). However, it adjusts better to chromosomes of grasses.

In the adapted protocol, the combination of treatment of the chromosomes with 45% acetic acid followed by treatment with 0.2 N chloridic acid before the alkaline denaturation resulted in the best contrasts between the bands. The chloridic acid removes purine-type bases from the chromosomal DNA (Devanter and Von Hoff, 1990) leaving its structure more fragile. The acetic acid acts on the proteins associated with the DNA (Davie, 2003) by destroying them and, in this way, facilitating alkaline denaturation by hydroxide of barium. Such adaptation favored the preferential extraction of non-heterochromatic DNA, which is usually less condensed than the DNA of the heterochromatin (Guerra, 2000). The denatured and fragmented DNA is extracted during incubation in 2X SSC (Holmquist, 1979).

The exact mechanism of the action of the C-banding was initially proposed by Gagne et al. (1971), who alleged that the occurrence of the more strongly stained bands by Giemsa after treatment with hydroxide of barium and 2X SSC corresponded to differentiated association of the repetitive DNA. The most commonly explanation is that the more strongly stained bands by Giemsa are, in fact, found from the preferential extraction of chromatin, which is more susceptible to the action of alkaline denaturation (McKay, 1973; Comings, 1978).

Fernández et al. (2002) developed a new method of obtaining C-bands using formamide instead of hydroxide of barium. The authors suggest that the bands resulted from the differentiated re-association of highly repetitive DNA. The authors mention the study of Comings et al. (1973) as a basis for their affirmation, in which it was demonstrated that the re-association of the heterochromatic DNA occurs within 20 s after denaturation, while the remaining DNA lasts from 3 to 5 min. However, the preferential extraction of euchromatin described by Comings (1978) and McKay (1973) is a more reliable hypothesis and the one that explains the results found in our investigation, as long as the lengthy exposure time of the chromosomes to 2X SSC, more than 1 h, is enough to promote the renaturation of all chromosomal DNA. If the DNA was only denatured, the bands would not be produced, because the chromosomes would be entirely stained.

With the adaptations made in the methodologies of Darvey and Gustafson (1975) and Giraldez et al. (1979), it was possible to obtain a protocol for C-banding in species of *Passiflora* that was considered to be very efficient for visualizing the clearly differentiated heterochromatic bands.

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