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ARTICLE

Morphological and cytogenetic characterization of new ornamental *Passiflora* hybrids (*P.* 'Vivis' and *P.* 'Jhovi')

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Abstract: *The present study was performed for the morphological and cytogenetic description of F_1 hybrids obtained from a cross between Passiflora coccinea Aubl. and Passiflora hatschbachii Cervi. Hybridization was confirmed by genomic in situ hybridization (GISH). Corona banding was the most relevant floral descriptor for separating the hybrids into two groups, the cultivars P. 'Vivis' and P. 'Jhovi', and the parents and hybrids had diploid chromosome number 2n = 18. The F_1 progenies exhibited normal meiotic behavior with normal tetrad production, which guaranteed high pollen viability (>70%) and fertile hybrids. Therefore, P. 'Vivis' and P. 'Jhovi' have the potential to be used for ornamental plants market.*

Keywords: *Interspecific hybrid, P. 'Vivis', P. 'Jhovi', pollen viability, GISH.*

INTRODUCTION

Passiflora hybridization has been conducted in many countries in Europe and North America (Abreu et al. 2009), and in Brazil, is mainly conducted for disease resistance and high productivity. However, many ornamental hybrids have been developed and registered by the Passiflora Society International, including *P.* 'Alva', *P.* 'Aninha', and *P.* 'Priscilla' (Santos et al. 2012), and *P.* 'Gabriela' and *P.* 'Bella' (Belo et al. 2018). In addition to backcrossed hybrids (BC1) produced from a cross between *P. sublanceolata* (Killip) J.M. MacDougal and *P. foetida* var. *foetida* L. (Melo et al. 2016).

Interspecific hybridization in *Passiflora* is generally considered to be a simple technique, because many species bloom all year round, produce abundant flowers, and are compatible. Therefore, species of this genus have been investigated in order to obtain hybrids with intermediate parental characteristics for the purpose of genetic improvement (Junqueira et al. 2005, Abreu et al. 2009). However, interspecific hybridization is often incorrectly performed, and many hybrids exhibit developmental problems, male sterility, susceptibility to disease, low pollen grain viability, poor flowering of certain crosses *etc.* (Meletti and Brückner 2001). Despite this, sexual hybridization allows the introgression of new genes of interest and greater genetic variability (Ulmer and MacDougal 2004).

The confirmation of hybrids from interspecific crosses is an essential step in genetic breeding programs, and can be performed based on genomic identification

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through cytogenetic techniques. Genomic *in situ* hybridization (GISH) has been widely used for this purpose in several hybrids (Lee et al. 2011, Silva and Souza 2013, Silva et al. 2018) and other plant species (Fu et al. 2004, Yao et al. 2010). In a cross between *P. gardneri* and *P. gibertii*, GISH identified a set of chromosomes from the parents in the genomes of the hybrid plants, confirming the hybridity of all plants of this progeny (Silva et al. 2018). In *Passiflora* species with ornamental potential, GISH has confirmed the hybrid status of backcrossed plants, and verified recombination in most BC_1 germplasm (Melo et al. 2016).

We selected *P. hatschbachii* and *P. coccinea* as parents for the production of new ornamental hybrids because they have ornamental characteristics. *P. hatschbachii* has white flowers that open at night (~2:00 am). *P. coccinea* produces striking and attractive red flowers throughout the year, and flowers during the day. This species is resistant to leaf virus and fruit and branch anthracnose (Junqueira et al. 2005). The primary objective of this study was to obtain hybrids with ornamental potential, *i.e.*, that flower throughout the year with intense coloration. In addition, we conducted a morphological and cytogenetic analysis of the F_1 obtained (*P.* 'Vivis' and *P.* 'Jhovi'), and confirmed the interspecific hybridization using GISH.

MATERIAL AND METHODS

Plant material and interspecific crosses

P. coccinea (Figure 1A, 1B) and *P. hatschbachii* (Figure 1C, 1D) were used as female and male parents, respectively, and maintained in a collection of Passifloras at the Universidade Estadual de Santa Cruz (lat 14° 39' 15'' S, long 39° 10' 59'' W, alt 78 m asl), Ilhéus, Bahia, Brazil. Interspecific crosses were performed in the early hours of the day. On the previous day, pre-anthesis flower buds were protected with paper bags to avoid contamination by exogenous pollen. Using tweezers, *P. hatschbachii* pollen grains were carefully deposited on the stigmatic papilla of *P. coccinea*, which had already been emasculated. The fruits produced from the crosses were protected against falling until they were ripe (Brückner and Otoni 1999), after which, the seeds were removed, dried at room temperature, stored in paper bags, and preserved at 10 °C.

Seed germination and cultivation conditions

Hybrid seed arils were removed, and sowing was conducted in Styrofoam trays containing the substrate (soil) in a greenhouse. After the emergence of true leaves, the seedlings were placed in 0.5 L black polyethylene bags. After 3 months, the mother plants were transferred to 45 L pots containing soil.

The parents and hybrids were propagated by taking cuttings from the mother plants. Cuttings were taken from the middle of the branches, and were prepared and standardized with two nodes and two leaves that had been reduced to half their area, beveled, and immersed in synthetic auxin [acid butyric indole-3 (1000 mg L^{-1})]. The cuttings were placed in 1.5 L black polyethylene bags containing rooting sand and watered daily. When the plants reached ~1 m in height, they were transplanted into 25 L pots containing soil and transferred to the experimental area in a randomized block design. The hybrid plants were placed in a vertical spillway system with 2.5 m high posts and 1.8 m high wire. There were 1 m gaps between plants and 2 m gaps between rows. Pruning was performed monthly and fertilizer was applied every 15 days, based on the soil analysis (38.33 mg of purified monoammonium phosphate (MAP), 45.18 mg of urea, and 55.6 mg of manganese sulphate for 6 months). Subsequently, 38.33 mg of purified MAP, 41.38 mg of potassium chloride, 99.53 mg of urea, and 111.1 mg of manganese sulphate were applied.

Morphological description of the hybrids and parents

The morphological description was based on traits included in the official descriptors of ornamental *Passiflora* (MAPA 2008) and in the EMBRAPA handbook (Jesus et al. 2015). The following morphological traits were assessed: flower diameter, corona diameter, width, length, and color of petals and sepals, size and color of the first and second corona filaments, floral peduncle length, anther, stamen, pollen grain, stiletto, and stigma color, leaf length, width, color, and shape, stem diameter, main branch internode length, and corona banding (present or absent). The morphological characterization relating to leaf color was based on the Munsell color chart for plant tissues (Munsell 1977), and colors were identified according to a code containing letters and numbers.

Conventional cytogenetics

Metaphase chromosomes were prepared to ascertain the chromosome numbers of the parental and hybrid plants. Radicles were collected from cuttings of mature branches and pretreated with 0.002 M 8-hydroxyquinoline for 1 h at room temperature (RT) and then at 8 °C for 23 h. The roots were fixed in Carnoy's solution [ethanol-glacial acetic acid (3:1, v/v)] (Johansen 1940) for 24 h at RT and stored at -20 °C until slide preparation, according to the protocol of Guerra and Souza (2002). The roots were digested in 2% cellulose (Sigma®) and 20% pectinase (Sigma®) (w/v) at 37°C for 1h and 20 min. The root meristem was macerated in 45% acetic acid and placed on a slide. Slides were observed under an optical microscope (Olympus BX41), and chromosome staining was conducted according to the Guerra and Souza (2002) protocol, with a slight modification regarding the dye concentration (4% Giemsa).

Meiosis analysis

Flower buds at different stages of development were collected in the morning to analyze their meiotic behavior. The buds were fixed in Carnoy's solution [ethanol-glacial acetic acid (3:1, v/v)] at RT (Johansen 1940). Temporary slides were prepared by the squashing and staining technique using 2% aceto-carmine (Fluka 22000) (Souza and Pereira 2011). The meiotic phases were observed under an Olympus BX41 optical microscope, and photodocumentation was performed using the Olympus BX41 microscope equipped with a 5M Olympus DP25 digital camera and the DP2-BSW software system.

Pollen viability

To perform the Alexander solution test (Alexander 1969), open flowers were collected in the morning and a dehiscent anther was delicately placed over a drop of dye to release the pollen grains. Four slides per hybrid were prepared, resulting in a total of 2000 pollen grains per hybrid (500 per slide). Alexander's solution dye is based on triple staining: orange G (intensifier), basic fuchsin (stains cytoplasm red), and malachite green (stains pollen grain walls green). This dye exploits the reactivity of the wall and cytoplasm. Pollen grains with intact cytoplasm were considered viable and those with absent or contracted cytoplasm were considered inviable (Souza et al. 2004). Pollen viability was measured as the percentage of viable pollen grains.

GISH Application

GISH was performed to confirm interspecific hybridization. The genomic DNA of the parents was extracted using the protocol by Doyle and Doyle (1990), with modifications for the GISH (Melo et al. 2015). Labeling of the maternal probe (*P. coccinea*) was performed by Nick translation with digoxigenin-11-dUTP (Roche Diagnostics), while biotin-16-dUTP (Roche Diagnostics) was used for the paternal probe (*P. hatschbachii*) at a final concentration of 33 ng μL^{-1} of cleaved DNA, following the manufacturer's protocol.

Slide preparation was performed in the same manner as chromosome number determination, but the slides were not stained. Shortly after this procedure, the slides were observed under a light microscope with phase contrast and stored at -20 °C until the GISH. Slide preparation followed the protocol for fluorescent *in situ* hybridization proposed by Schwarzacher and Heslop-Harrison (2000) and Souza et al. (2010), with minor modifications (Melo et al. 2015). Slides containing the mitotic metaphases were dried at 37 °C for at least 1h. After adding 50 µL of a solution containing 1 µg mL^{-1} RNase (Sigma-Aldrich) in 2 × saline-sodium citrate (SSC) buffer (0.3 M sodium chloride and 0.03 M sodium citrate, Sigma-Aldrich), the slides were incubated in a humid chamber at 37 °C for 1 h. In order to identify the hybrids, 33 ng of each DNA probe (representing the two parents) were added to the hybridization mixture (50% formamide, 10% dextran sulfate, 2 × SSC, and 0.13% sodium dodecyl sulfate). The biotin-labeled probe was detected using 0.7 µL of avidin-fluorescein isothiocyanate (FITC; Vector), and the digoxigenin-labeled probe was detected using 0.7 µL of anti-digoxigenin-rhodamine plus 18.6 µL of 5% bovine serum albumin (Sigma-Aldrich) per slide. Slides containing the antibody for detection were incubated in a humid chamber at 37 °C for 1h, before being mounted and counterstained with 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI)/VECTASHIELD® (H-1200) and stored at 8–10 °C until analysis.

Metaphases were observed and photo documented using the Olympus BX41 epifluorescence microscope equipped with a 5 MP Olympus DP25 digital camera and DP2-BSW software system. Hybridizations detected using avidin-FITC were visualized using an U-MWB filter (excitation at 450–480 nm, dichroic cut-off at 500 nm, and emission at >515 nm), and hybridizations detected using anti-digoxigenin-rhodamine were visualized using an U-MWG filter (excitation

at 510–550 nm, dichroic cut-off at 570 nm, and emission at >590 nm). The SC5 Photoshop software system was used to prepare the FITC/rhodamine overlays.

RESULTS

Interspecific crosses and morphological description of the hybrids and parents

The cross between *P. coccinea* and *P. hatschbachii* resulted in one fruit with 248 seeds, 75.91% of which were viable. The floral and vegetative morphological descriptors revealed differences between the hybrids in shape, size, and color (Table 1). Corona banding, a characteristic that was absent in the parents, was the most significant floral descriptor that distinguished the hybrids into two groups, and gave rise to *P.* 'Vivis' (Figure 1E and 1F) and *P.* 'Jhovi' (Figure 1G and 1H), which will be registered with the Passiflora Society International (www.passiflorasociety.org) and the Brazilian Ministry of Agriculture, Livestock, and Supply. Hybrids of the group *P.* 'Vivis' had a banded corona with the first filaments (external) having alternating red and white bands, while hybrids of the group *P.* 'Jhovi' had an unbanded corona with the first filaments (external) being red and white. The parents, *P. coccinea* and *P. hatschbachii*, had coronas with the first filaments (external) being red and white, respectively (Table 1).

Table 1. Quantitative and qualitative floral and vegetative morphological descriptors of the parents and their hybrids of *Passiflora*

Descriptors (cm)	***P. coccinea***	***P. hatschbachii***	***P.* 'Vivis'**	***P.* 'Jhovi'**
Stem diameter	3.12	4.39	1.10-2.22	0.81-1.97
Internode length	4.38	8.00	2.50-5.50	2.67-5.50
Leaf length	12.12	12.10	8.42-12.83	8.90-12.32
Leaf width	7.54	14.34	6.43-11.12	6.67-11.42
Peduncle length	5.59	17.94	5.55-12.90	5.85-12.58
Flower diameter	13.62	8.87	8.80-10.40	8.67-10.90
Petal length	6.3	3.87	3.79-4.66	3.73-4.92
Petal width	1.37	0.91	0.90-1.16	0.90-1.19
Sepal length	6.27	3.80	4.02-4.60	3.70-4.73
Sepal width	1.54	1.30	0.95-1.22	0.95-1.53
Length of first corona filaments	1.97	2.16	2.40-3.20	2.47-3.00
Length of second corona filaments	1.32	0.60	0.43-0.65	0.45-1.11
Leaf Type	Oblong	Departure and trilobada	Departure and trilobada	Departure and trilobada
Leaf Color	Green	Green	Green ranging from dark to light green (6/8 7.5GY – 3/4 7.5GY)	Green ranging from dark to light green (6/8 7.5GY – 3/4 7.5GY)
Flower Color	Red	White	Red	Red
Petal Color	Red	White	Red	Red
Sepal Color	Red	White	Red	Red
Corona First Filament Color	Red	White	Red and white alternating bands (corona banding)	Red and white (not corona banding)
Corona Second Filament Color	White	White	White	White
Anther Color	Green	Light green	Light green	Light green
Stamen Color	Red	Light green	Light green	Light green
Pollen Color	Yellow	Yellow	Dark yellow	Dark yellow
Stiletto Color	Red	Light green	Light green with red scores	Light green with red scores
Stigma Color	Green	Light green	Light green	Light green
Flowering	Higher flower production from October to December	Higher flower production from October to December	Higher flower production from October to December	Higher flower production from October to December
Leaf Type	Oblong	Departure and trilobada	Departure and trilobada	Departure and trilobada
Leaf Color	Green	Green	Green, ranging from dark to light green (6/8 7.5GY – 3/4 7.5GY)	Green, ranging from dark to light green (6/8 7.5GY – 3/4 7.5GY)

In general, the hybrids had intermediate values for the vegetative characteristics (stem diameter, internode and peduncle lengths, and leaf length and width) when compared to the parents; however, the paternal parent had wider leaves and longer peduncles than the maternal parent (Table 1). Regarding floral characteristics, the maternal parent had higher values of flower diameter, petal and sepal length and width, and second corona filament length than the paternal parent. Hybrids of the groups *P.* 'Vivis' and *P.* 'Jhovi' had intermediate floral characteristics to those of the parents, as was the case for the vegetative characteristics; however, the paternal parent had a longer first corona filament than the maternal parent. The hybrids had higher values than the parents (Table 1). Because quantitative characteristics can be affected by the environment, the large variations observed in the values of the two hybrid groups may be evidence that these characteristics are not easily inherited. However, the hybrids exhibited some similarities to the parents: leaf shape (broken and trilobed) and stamen and stigma color (light green) were inherited from the paternal parent (*P. hatschbachii*), and flower, petal, and sepal color (red) were inherited from the maternal parent (*P. coccinea*) (Table 1).

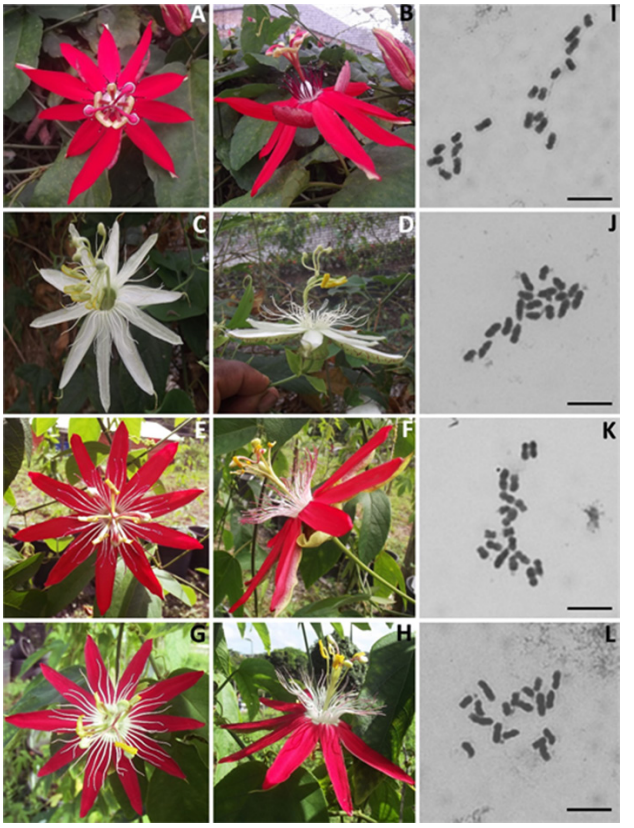

Figure 1. **(A-H)** Parents and F_1 hybrids obtained from the cross. **(A, B)** Maternal form - *Passiflora coccinea*; **(C, D)** Paternal form - *P. hatschbachii*; **(E, F)** *P.* 'Vivis'; **(G, H)** *P.* 'Jhovi'; **(I-L)** Mitotic metaphase chromosomes of the parents and the interspecific hybrids of *Passiflora* (2*n* = 18). **A)** *P. coccinea*; **B)** *P. hatschbachii*; **C)** *P.* 'Vivis'; **D)** *P.* 'Jhovi'. Bar = 10µm.

Cytogenetic analysis and pollen viability

A chromosomal number of 2*n* = 18 was observed in the parents (Figure 1I and 1J) and hybrids of the *P.* 'Vivis' and *P.* 'Jhovi' groups (Figure 1K and 1L), in which normal meiosis was observed (Figure 2). During metaphases I and II, the chromosomes were arranged in the equatorial plane (Figure 2B and 2F), and normal segregation occurred in anaphases I and II (Figure 2C and 2G). The genetic constitution of four haploid nuclei was observed in telophase II (Figure 2H). Meiotic behavior was normal in most cells observed, with few meiotic irregularities (early or delayed chromosomes in metaphase I, asynchrony during cell division, or one chromosome group in metaphase II and another in anaphase II). In most cells, tetrad formation was not prevented, resulting in normal post-meiotic products (Figure 2I). Alexander's solution was effective in determining pollen viability, because pollen grains from both hybrids reacted positively (Figure 2). Viable pollen grains were observed in most cells in the hybrid progeny (Figure 2 J and 2K), with pollen viability above 75%. Inviable pollen grains had no cytoplasm and had contracted (Figure 2K).

Gish

GISH was performed on the *P.* 'Vivis' and *P.* 'Jhovi' hybrids to identify the genomes of the parents in the F_1 hybrid progeny using the genomic DNA of both parents as probes (Figure 2L and 2M). Nine chromosomes with hybridization signals corresponding to the *P. coccinea* genome and nine chromosomes with hybridization signals corresponding to the *P. hatschbachii* genome were identified.

DISCUSSION

Two new interspecific *Passiflora* hybrids were developed (*P.* 'Vivis' and *P.* 'Jhovi') that have attractive characteristics for the ornamental plant market: bright red flowers that open for ~14 h periods, a long flowering period between October and December, and corona banding, which was an important characteristic in classifying the hybrid progenies into two

groups. In addition to these characteristics, the hybrids exhibited diurnal anthesis, and opened their flowers around 8:00 am. Red petals and sepals were inherited from the maternal parent, *P. coccinea*, and leaf shape (broken and trilobed) and color of the stamen and stigma (light green) were inherited from the paternal parent (*P. hatschbachii*). The hybrids' qualitative characteristics were more heritable than their quantitative characteristics, making it possible to select promising genotypes because there is little effect of environmental factors (Freitas et al. 2016). Quantitative characteristics, which are inherited less than qualitative ones, reflect environmental effects on phenotypic expression, and so are not recommended for selection.

Interspecific hybridizations in *Passiflora* are generally performed to increase their ornamental value, and in most cases, the results are positive. Hybridizations can be performed between plants of the same species or between different species (Santos et al. 2012), but they are usually conducted among phylogenetically close species (Silva and Souza 2020), *i.e.*, the parent species of *P. coccinea* and *P. hatschbachii* both belong to the same taxonomic section (*Granadillastrum*) of the subgenus *Passiflora* (Feuillet and MacDougal 2004). Closely related species are easier to cross because they have weak reproductive barriers (Melleti 2005), but have sufficient genomic and cytogenetic compatibility to produce successful progenies, as observed in this and other studies (Santos et al. 2012, Belo et al. 2018). A study on commercial and wild *Passiflora* species found that there was high compatibility in some hybrid combinations, which is promising for breeding programs (Ocampo et al. 2016).

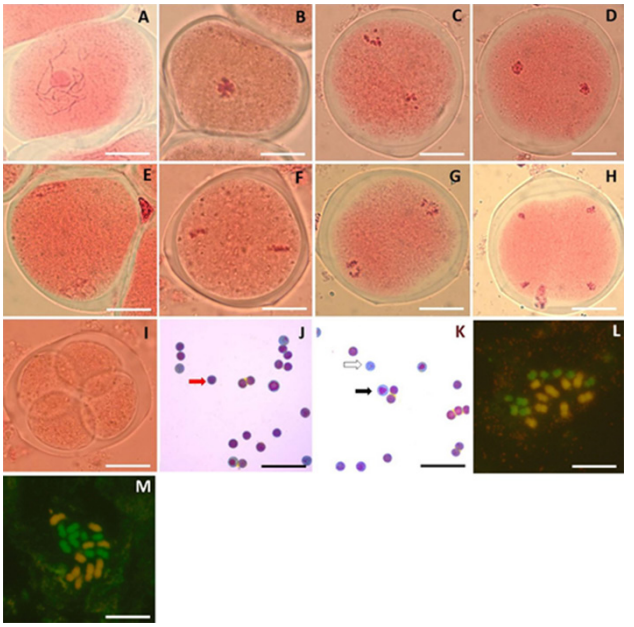

Figure 2. (**A-I**) Meiotic behavior in the F_1 interspecific hybrids obtained from the crosses between *Passiflora coccinea* and *P. hatschbachii* (2*n* = 18). (**A-D**) Meiosis I: **A)** Pachytene; **B)** Metaphase I; **C)** Anaphase I; **D)** Telophase I; (**E-I**) Meiosis II: **E)** Prophase II; **F)** Metaphase II; **G)** Anaphase II; **H)** Telophase II; **I)** Tetrad of microspore. Bar = 10 µm. (**J-K**) Pollen viability using Alexander's solution. (**J**) *P.* 'Vivis': Viable pollen grain (red arrow); (**K**) *P*. 'Jhovi': Empty and inviable pollen grains (white arrow) and shrunk one (black arrow). Bar = 100 µm. (**L-M**) Genomic *in situ* hybridization (GISH) in mitotic metaphases in interspecific hybrids of *Passiflora* (2*n* = 18). **L)** *P.* 'Vivis'; **M)** *P.* 'Jhovi'. Red and green signals show the chromosomes hybridized with the genomic DNAs of *P. coccinea* and *P. hatschbachii*, respectively. Bar = 10 µm.

The normal meiotic process produces viable gametes and normal post-meiotic cells, and, consequently, higher plant fertility (Pagliarini 2000). Therefore, meiotic behavior should be studied in detail in plant breeding programs in which inter- and intraspecific hybridizations are required, because it is possible to check for homeologous chromosome pairing, meiotic irregularities, and gamete viability (Kiihl et al. 2011). Knowledge of chromosome pairing is important for selecting species that are genetically close for interspecific crosses (Techio and Davide 2007). During meiosis in interspecific F_1 hybrids, there may be no pairing between the genomes because there are different genomes in the cell nucleus (female and male parents), which can affect the fertility of the hybrids (Belo et al. 2018).

In interspecific hybrids, it is possible to observe homeologous chromosome pairing during normal diakinesis, and bivalents are maintained until metaphase I because of the presence of chiasmas. These events ensure genetic stability and the normal segregation of chromosomes in anaphase (Sybenga 1992). Chromosome paring was not observed during diakinesis in this study, because it was not possible to visualize this sub-phase (a phase that is part of prophase I). However, based on observations of anaphases I and II, there was normal segregation of chromosomes into cell poles and the presence of four haploid nuclei in telophase II indicate that normal meiosis occurred in hybrids, because normal post-meiotic products (tetrads) were found in most cells and, as a result, viable pollen grains were produced. Therefore, the F_1 hybrid progeny had high pollen viability, because >70% is considered a high value (Moreira and Gurgel 1940). High pollen viability was found in *P. subrotunda*, species of ornamental value (Souza et al. 2018), and in other species (Tolomeotti et al. 2018, Tedesco et al. 2019, Krycki et al. 2020), including tetraploid plants (Krycki et al. 2016). Normal meiotic behavior has been reported in interspecific F_1 hybrids obtained from a cross between *P. sublanceolata* and *P. foetida* var. *foetida*, in which >90% the cells had bivalents in diplotene and diakinesis, while >87% of the cells in anaphases I and II exhibited normal segregation (Santos et al. 2012). The hybrids *P*. 'Gabriela' and *P*. 'Bella' were obtained

from a cross between *P. gibertii* N.E. Brown and *P. gardneri* Mast., and they also exhibited normal meiosis with normal segregation in anaphases I and II, and the formation of four haploid nuclei in telophase II (Belo et al. 2018). Our results suggest that these hybrids can be used in breeding programs to obtain future generations (F_2) and in backcrosses with possible genetic segregation, resulting in greater diversity and ornamentation. Backcrossing as a method of introgressing agronomic characteristics of ornamental interest (greater quantitative and qualitative variability) has been conducted in *Passiflora* BC_1 backcrossed hybrids, with positive results (Melo et al. 2016).

GISH is a very effective tool to confirm that cross hybridization has occurred (Silva and Souza 2013), and in this study, GISH was used to identify the genomes of the parents of the F_1 hybrid progenies. Hybridism in these individuals was confirmed by the visualization of nine *P. coccinea* chromosomes and nine *P. hatschbachii* chromosomes in the hybrid plants. The ability to differentiate parent genomes in hybrids using this technique is affected by the degree of homology between species, and by stringency conditions (Silva et al. 2018). It was inferred that the parents had a close genetic relationship and shared many repetitive sequences, so it was necessary to use the probes of both parents to ensure specificity. Similar results have been obtained in F_1 and backcrossed *Passiflora* hybrids (Melo et al. 2015, Silva et al. 2018), and in orchid hybrids (Lee et al. 2011).

CONCLUSIONS

We produced two new *Passiflora* cultivars (*P.* 'Vivis' and *P.* 'Jhovi') from a cross between *P. coccinea* and *P. hatschbachii*, both of which are highly attractive ornamental plants. The complex actinomorphic flowers of *Passiflora* and the specific floral characteristics of the novel cultivars give these hybrids great potential in the ornamental market, in formal or informal horticultural planting schemes, and for decorating different environments. The meiotic behavior of the F_1 progeny was normal, with normal tetrad production, guaranteed high pollen viability (>70%), and fertile hybrids. GISH confirmed the hybrid character of the plants, and is an important cytogenetic tool that should be used in future Passifloraceae breeding programs for the identification of interspecific hybrids in other plant families.

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