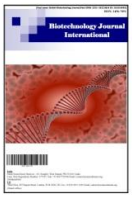

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In vitro Callogenesis and Organogenesis from Carolina Reaper (Syn. *Capsicum Chinense* Jacq.) and Chromosomal Analysis

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Authors' contributions

This work was carried out in collaboration among all authors. Authors BHG and RJOJ designed the study, performed the analysis of study, wrote the protocol and wrote the first draft of the manuscript. Author RJOJ improved the manuscript and authors FMO and APON managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this paper is to develop an *in vitro* organogenesis and callogenesis protocol for Carolina Reaper pepper, and to determine the karyotype and nucleoli of this cultivar.
Methodology: The MS medium with supplemented with indole-3-butyric (0, 1, 2 and 4 mg L^{-1}) and kinetin (0, 1, 2 and 4 mg L^{-1}) was used. The leaves and nodal segments of Carolina reaper was utilized for the callogenesis and organogenesis induction. The responses to growth regulators were evaluated 30 days of cultivation. The meristematic tissue was pre-treated with 0.05% (w/v) of colchicine for six hours at 18°C. The samples were fixed in Carnoy for 12 hours. Chromosomal observations were made with binocular optical microscope (Leica DM 750) and the cells in good condition for counting the chromosomes and karyotype assembly were photographed. Results were

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presented as mean ± standard deviation and were compared by the two-way Analysis of Variance. The means were separated according to Tukey test (P = 0.05).
Results: Calli were induced from both leaf and stem segments when indole-3-butyric 0 mg L^{-1} + kinetin 1 mg L^{-1} were used. Development of shoots in leaf and stem segment were obtained when indole-3-butyric 2 mg L^{-1} + kinetin 4 mg L^{-1} were used, and roots regenerated with indole-3-butyric 4 mg L^{-1} + kinetin 1 mg L^{-1}. It was found two nucleoli in every cell interphase, suggesting that two nucleolar organizer regions are expressing their ribosomal genes. Karyotype analysis indicated a chromosome number of 2n = 24, which is correlation with other Capsicum genus varieties. It was observed 1 or 2 nucleoli per nucleus of both types, homomorphic and heteromorphic. The results can help in programs of breeding and conservation of this cultivar and other species of pepper.
Conclusion: Using the concentrations of growth hormones indicated in the present report, it could be possible to regenerate leaves and nodal segments in vitro clones from the original genotype. We have also described the chromosome number and nucleolus number of *Carolina reaper*, generating a data that could help in programs of breeding, as in the generation of polyploid plants and conservation species of pepper.

Keywords: In vitro culture; chromosomes; capsicum chinense; growth regulators; cytogenetic.

1. INTRODUCTION

Capsicum chinense is a widely distributed and consumed pepper, playing an important role in agriculture and economy of many countries. Among *Capsicum chinense* cultivars, *Carolina reaper* variety has the highest level of pungency. *Carolina reaper* is a hybrid obtained from the crossing between the varieties 'Habanero' (*Capsicum chinense* Jacq) and 'Naga Bhut Jolokia' (hybrid between *Capsicum chinense* and *Capsicum frutescens*). Capsicum genus has over 30 species [1], five of which are domesticated: *Capsicum annuum, Capsicum bacccatum, Capsicum chinense, Capsicum frutescens*, and *Capsicum pubescens* [1]. The center of origin of peppers is southern Brazil, western Bolivia, and Paraguay, extending from northern to southern Argentina [2]. Peppers are important sources of vitamins A, C, E, carotenoids and, alkaloids, like the capsaicin with propriety that presents antioxidant, anti-inflammatory [3,4], antimicrobial [5-7], analgesic [8], anti-mutagenic and, anti-cancer [9,10].

Plant tissue culture techniques are the most frequently used biotechnological tools for basic and applied purposes ranging from the investigation on plant developmental processes, functional gene studies, commercial plant micropropagation, generation of transgenic plants with specific industrial and agronomical traits, plant breeding and crop improvement, virus elimination from infected materials to render high-quality healthy plant material, preservation and conservation of germplasm of vegetative propagated plant crops, and rescue of threatened or endangered plant species [11]. Tissue culture involves many biotechnological processes including organ culture, callus induction, cell isolation, protoplasts cultures, anther, and embryos cultures [12]. The organogenic process occurs by physiological mechanisms controlled by growth regulators called plant hormones [13]. The major classes of growth regulators used in tissue culture *in vitro* are auxins and cytokinins [14]. It is believed that the effects of these substances applied to the culture medium act by altering auxin and cytokinin endogenous balance in plant cells [15,16]. This process leads to the formation of roots, shoots, and callus from a previously selected plant tissue [17,16]. Defining the ideal hormonal dose is an important step in establishing *in vitro* protocols. Over the years, *in vitro* culture of capsicum has been developed [18] related to the determination of growth regulators [19] and explants [20,21].

Another important tool in plant research is cytogenetics, which enables karyotype analysis, an important parameter to follow the genetic stability of offspring and can be used in plant breeding. These studies allow us to seek information causally related to ontogeny and phylogeny of living beings by analyzing the chromosomes [22] and is divided into classical and molecular cytogenetics [23]. Here, we describe a tissue culture protocol *in vitro* regeneration of Carolina reaper (*Capsicum chinense* Jacq.) Using different sources of explants (leaves and stem segments) to verify how the variety behaves in regeneration processes; and we characterized the karyotype of this hybrid for the first time, for futures research such as physical maps and nucleolar organization.

2. MATERIALS AND METHODS

2.1 Culture Conditions and Plant Materials

Full strength MS [24] medium supplemented with 0.7% agar-agar and 3.0% sucrose. The pH of the culture medium was 5.8, and was autoclaved at 1.13 kg cm^{-2} pressure and 120°C for 20 min. The plant material used was *Capsicum chinense* seeds (variety Carolina reaper), and plantlets from these seeds germinated *in vitro* in the laboratory. All cultures were incubated at 25 ± 2°C under a 16-h light and 8-h dark cycle with a light intensity of 25.2 µM/s irradiance in a controlled environment chamber.

2.2 Effect of Indole-3-Butyric Acid and Kinetin Effects on Callus Induction and Organogenesis

The MS medium with supplemented with indole-3-butyric (0, 1, 2 and,4 mg L^{-1}) and kinetin (0, 1, 2 and, 4 mgL^{-1}) was used. The leaves and nodal segments of Carolina reaper plats were utilized for the morphogenesis and organogenesis induction. The responses to growth regulators, calluses, roots, and shoots were evaluated 30 d of cultivation. Code for the presence (1) and for absence (0) was used for the variable percentage of calluses formed. The number of shoots and roots were calculated using the average repetitions for each treatment to determine those with a greater number of shoots and roots. The experiment was conducted in a completely randomized design in a factorial 4 x 4 ways, with 16 treatments, each treatment with 3 repetitions. In each repetition, there were six explants from each source. The repetitions consisted of Petri dishes containing 20 mL of MS medium.

2.3 Chromosomal and Nucleoli Analysis

The material for chromosomes analysis was obtained from roots and meristem of seeds germinated *in vitro*. The meristematic tissue was pre-treated with 0.05% (w/v) of colchicine for six hours at 18°C. The samples were fixed in Carnoy (absolute methanol and glacial acetic acid, 3:1 v/v) for 12 hours. For the preparation of the slides, the roots were washed with distilled water and dipped in HCl 2M at 37°C for 20 minutes for acid hydrolysis. After hydrolysis, they were dissected in acetic acid (45% v/v) and crushed. The microscope slide was stained with Giemsa 10% for 10 minutes. Chromosomal observations were made with a binocular optical microscope (Leica DM 750), and the cells in good condition for counting the chromosomes and karyotype assembly were photographed. For the detection of nucleoli, cells were impregnated with silver nitrate [25]. Firstly, it was dripped on the slide 25 µL of colloidal gelatin solution at 2% (with formic acid in the proportion of 1.0 ml per 100 ml solution). After, it was added 50 µL of aqueous solution of silver nitrate at 50% (v/v) and 25 µL of deionized water over the blade. The slide was incubated in an incubator at 60°C for 5 minutes. Nucleoli analysis was performed in 505 interphasic nuclei, counting the number of nucleoli per nuclei and characterizing its morphology by visual analysis of format (regular and unregular).

2.4 Statistical Analysis

The graphical and statistical analyses were performed using GraphPad Prism 7 software and R software package (http://cran.rproject.org). Results were presented as mean ± standard deviation and were compared by the two-way Analysis of Variance (two-way ANOVA). The means were separated according to the Tukey test (P = 0.05).

3. RESULTS AND DISCUSSION

3.1 Indole-3-Butyric Acid and Kinetin Effects in the Callus Induction and Organogenesis

It was observed that Carolina reaper *in vitro* regeneration depends strongly on the balance of auxin and cytokinin. These growth factors were essential for the formation of calluses, shoots, or roots in both sources of explants (Fig. 1). The increase of auxin induces the root formation, while the increased cytokinin induces the formation of adventitious shoots, and the callus is induced when there is an equal balance of both phytohormones [17,25].

Stem and leaf explants of Carolina reaper were evaluated after 14 days of explants inoculation in the culture medium at different concentrations of these growth factors (Fig. 1). We can check that the combinations of IBA and KIN were effective for callus formation using stem segments (Fig. 2a) or leaf explants (Fig. 2b). They were efficient in the following combinations: 0 mg L^{-1} IBA + 1 mg L^{-1} KIN; 2 mg L^{-1} IBA + 1 KIN mg L^{-1}; 2 mg L^{-}

^{1} IBA + 2 mg L^{-1} KIN and 2 mg L^{-1} IBA + 4 mg L^{-1} KIN, showing callus formation in 100 % of samples from both source of explants. Combinations of 4 mg L^{-1} IBA + 2 mg L^{-1} KIN and 4 mg L^{-1} IBA + 4 mg L^{-1} KIN were effective to induce callus on 100% of the leaf explants, however, these same concentrations showed only 40% of callus formation when explants source is stem segments.

Fig. 1. Callogenesis and organogenesis from reaper Carolina (*C. chinense* Jacq.) explant, after 14 days of cultivation on MS medium supplemented with different concentrations of indole-3-butyric acid (IBA) and kinetin (KIN). The arrows show the roots

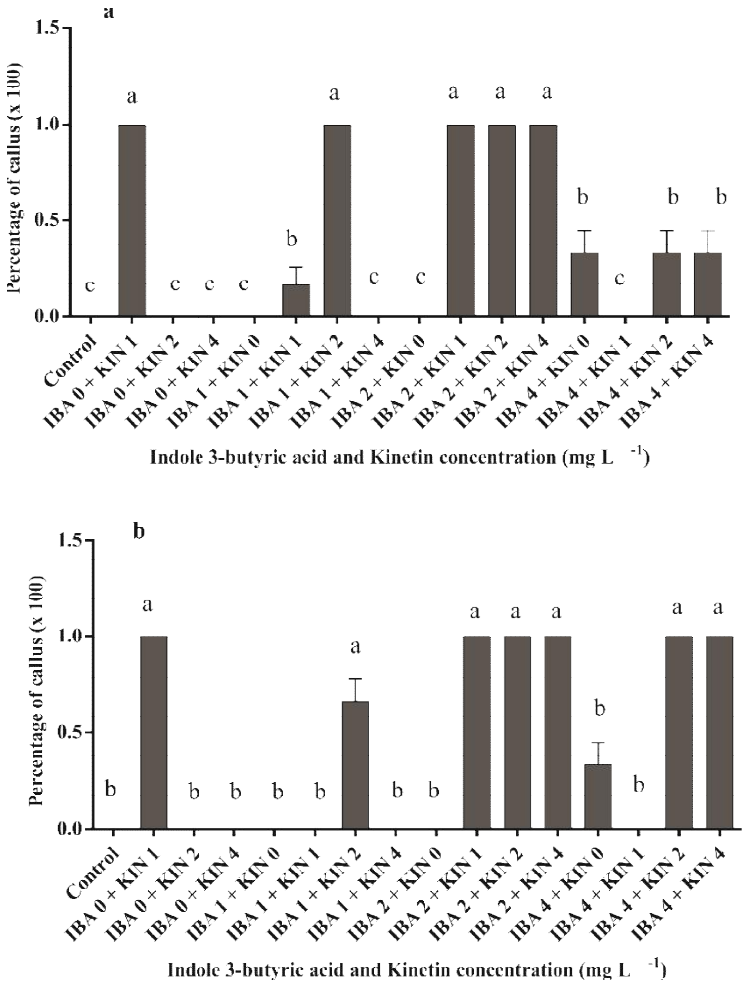

Fig. 2. Effect of different concentrations of indole 3-butyric acid in combination with kinetin for the induction of callus from stem segments (a) and leaves discs (b) of Carolina reaper (*C. chinense* Jacq.). Means with different letters are significantly different, by Tukey's test at *p* = 0.05

It was found that callus formation was induced in different combinations of IBA and KIN concentrations, even when there were not equimolar concentrations of these growth regulators. These results indicate that there are differences in the sensitivity of the explants to growth regulators, and differences in the endogenous hormones level in each explant can influence the interactions between them, as described by some researchers [26,27]. Khan et al. [19]. evaluating the *in vitro* organogenesis of *C. annuum* obtained optimal responses in the development of callus from nodal segments on MS medium supplemented with 10 mM of 2.4-D and 2.0 mM of BAP. This result, like ours, shows that callus induction is not always dependent on an equimolar balance of auxin and cytokinin.

The combinations of 1 mg L^{-1} IBA + 4 mg L^{-1} KIN and 2 mg L^{-1} IBA + 4 mg L^{-1} KIN were effective for shoot induction on stem segments as shown in Fig. 3a. When leaf explants were used, the best combinations for shoot induction were 1 mg L^{-1} IBA + 2 mg L^{-1} KIN and 2 mg L^{-1} IBA + 4 mg L^{-1} KIN as shown in Fig. 3b.

In this work, the inductions of shoots in both sources of explants were more efficient when the culture medium was supplemented with a higher concentration of cytokinin (KIN) in relation to auxin (IBA). The regeneration processes of species of the Capsicum genus investigated by some researchers showed the critical effect of cytokinin, cytokinin-cytokinin or cytokinin-auxin ratio on the *in vitro* regeneration of different explants [28,29]. Induction of shoots using two cytokinins (BAP and KIN) alone or in combination was obtained in *C. frutescens* with a maximum of 5.6 shoot buds from explants cultured on medium containing 22.2 mM BAP in combination with 4.6 mM of kinetin [29]. Sanatombi & Sharma [30]. assessing the *in vitro* propagation of *C. chinense* cv. 'Umoro' and achieved a maximum number of shoots through the induction with 91.2 mM of Zeatin or 31.1μM BAP with 4.7 μM KIN. In the cultivar of pepper 'Bhut Jolokia', also *C. chinense*, the inoculation of cotyledon explants in MS medium supplemented with 35 μM BAP and 15 μM KIN was efficient to induce the formation of shoots with an average of 4 shoots per explant [31]. The formation of shoots from internodes (stem) of *C. annuum* was observed when using BAP and TDZ at a concentration of 5.0 μM BAP with 2.5 μM TDZ [19]. Altogether, these data as well as our work highlight the importance of adding cytokinins in the medium to induce shoots in species from genus Capsicum.

Fig. 4a shows the interaction between tested hormones for the induction of roots when used stems segments from Carolina reaper. It can be noticed that higher concentrations of IBA induced a larger number of roots. Low concentrations of IBA were not effective in root induction. Similar behavior was observed when using stem segments for roots induction. The best combinations for roots induction using stem segments were 4 mg L^{-1} IBA + 0 mg L^{-1} KIN, 4 mg L^{-1} IBA + 1 mg L^{-1} KIN and, 4 mg L^{-1} IBA + 2 mg L^{-1} KIN. It can be clearly seen that the best combination of hormones for root formation was 4 mg L^{-1} IBA + 1 mg L^{-1} KIN in both explants sources.

When using leaf explants for rooting induction (Fig. 4b), it was noticed that the best concentrations for this purpose were 4 mg L^{-1} IBA + 0 mg L^{-1} KIN, 4 mg L^{-1} IBA + 1 mg L^{-1} KIN and 4 mg L^{-1} IBA + 2 mg L^{-1} KIN. The average number of roots obtained in leaf explants was higher than that obtained in the stem explants.

In relation to root formation leaf and stem explants of Carolina reaper also demanded the use of growth regulators. Rhizogenesis is a crucial stage for the development of plants propagated *in vitro*. In our research, the inductions of roots were more efficient when the culture medium was supplemented with a higher concentration of auxin in relation to cytokinin. Peddaboina et al. [32]. observed 72-94% of rooting of shoots from Capsicum species cultivated *in vitro* by using 5.71 μM IAA. Khan et al. [19]. have obtained the best results for root formation from shoots explants of Capsicum annuum using 1.0 μM IBA in MS medium and Orlińska & Nowaczyk [33] assessing the *in vitro* regeneration of Capsicum genotypes obtained the highest levels of rhizogenesis using MS medium supplemented with 1.1 mg L^{-1} IAA. *In vitro* rooting of the cultivar 'Bhut Jolokia' (*C. chinense*) using cotyledon explants was found that supplementation with 5 μM IBA was sufficient for the formation of roots regenerated *in vitro* from shoots explants [30]. Corroborating with data of the authors previously cited, we obtained rhizogenesis using MS medium supplement just with auxin (IBA), but we also obtained equal significant levels of rhizogenesis with IBA associated with KIN, predominantly in the medium with higher concentrations of IBA. This result indicates that the presence of auxin in the medium is a determinant factor to induce rhizogenesis in *Capsicum chinense* cv. Carolina Reaper.

3.2 Nucleoli and Chromosomal Analysis

The chromosome number in Capsicum has revealed diploid karyotypes based on x = 12 and x = 13 [1]. There are two hypotheses regarding the direction of chromosome base number change: 1) karyotype x = 13, more asymmetrical, is derived from x = 12 by Moscone et al. [1]. and x = 13 is the ancestral basic number describe by Pozzobon et al. [33]. Reported chromosome numbers allow us to distinguish two species groups: One with 2n=2x=24 (13 species) and another with 2n=2x=26 (10 species). Active nucleolar organizing regions vary in number from one (several species) to four pairs (*C. baccatum*). Karyological analyses provide valuable diagnostic features for taxonomic identification at the species level in the cultivated peppers, particularly in the *C. annuum, C. chinense* and *C. frutescens* [1].

The analysis of metaphasic cells obtained from the root meristem region stained with Giemsa allowed the analysis of Carolina Reaper (*C. chinense*) chromosomes (Fig. 5a). The analysis of metaphases (Fig. 5b) revealed a chromosome number of 2n = 24, with a karyotypic formula of 11 metacentric chromosomes and 1 submetacentric.

When stained with silver nitrate, the cells of Carolina reaper root meristem evidenced one or two nucleoli marks (Fig. 6), suggesting that the species is a simple NOR carrier. This methodology allowed us to analyze the activity of NORs according to the number and morphology of nucleoli. Despite the efforts, it was not possible to obtain a pattern of chromosomal staining showing the NOR carrier chromosomes. We analyzed 505 interphases cells in which the occurrences of single nucleoli (69.5%) was predominant in relation to two nucleoli occurrence (30.5%). The morphology of nucleoli was also analyzed, showing a higher frequency of heteromorphic nucleoli (72%) in relation to homomorphic (28%).

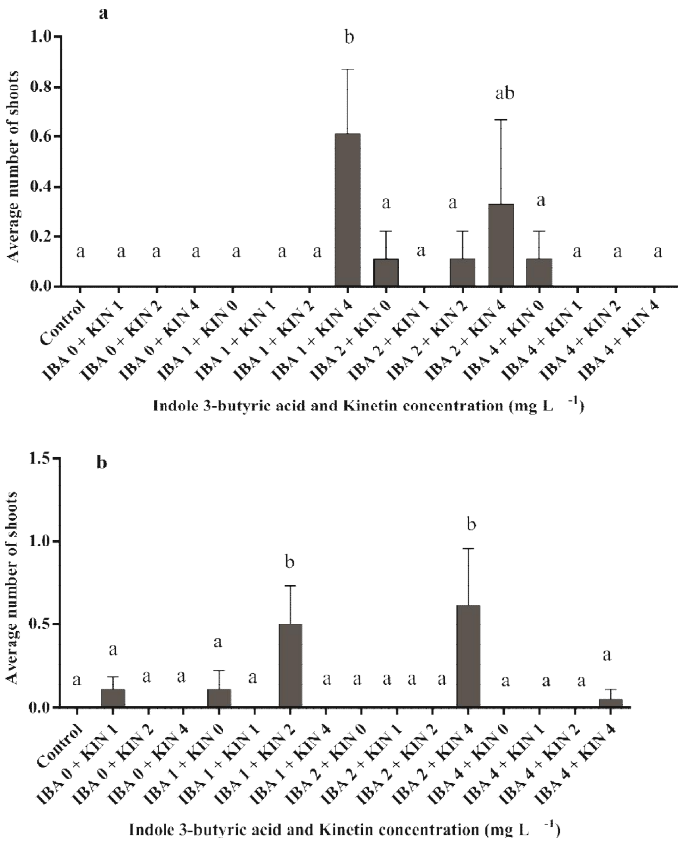

Fig. 3. Effect of different concentrations of indole-3-butyric acid in combination with kinetin for the induction of shoots from stem segments (a) and leaf discs (b) of Carolina reaper (*C. chinense* Jacq.). Means with different letters are significantly different, by Tukey's test at *p* = 0.05

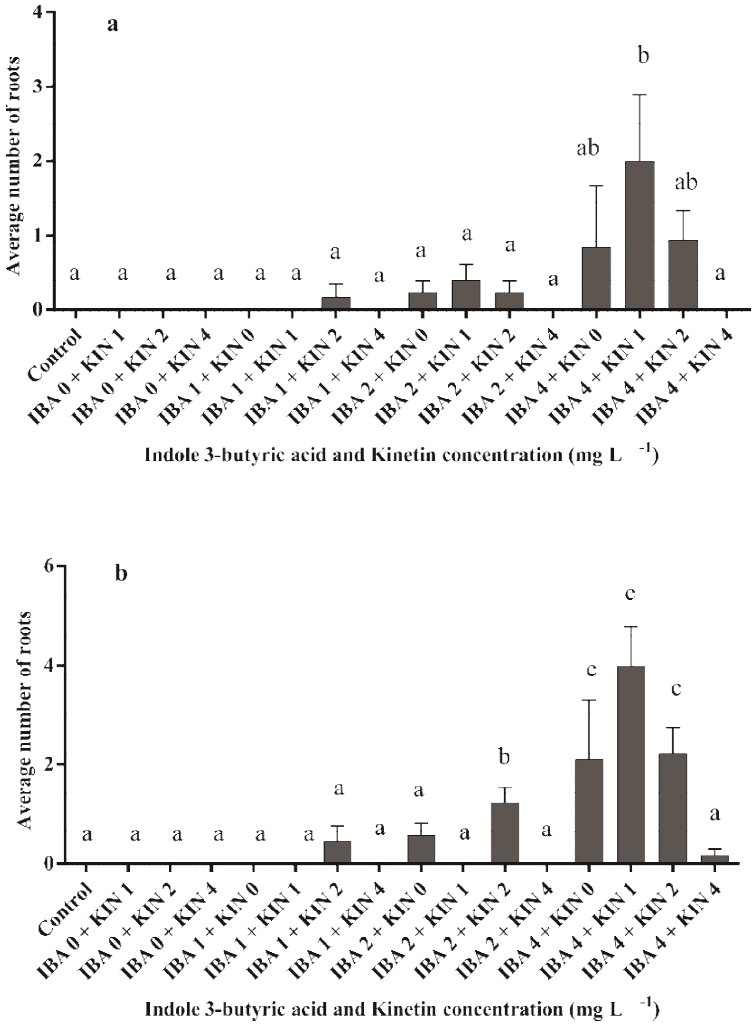

Fig. 4. Effect of different concentrations of indole-3-butyric acid in combination with kinetin for the induction of roots from stem segments (a) and leaf discs (b) of Carolina reaper (*C. chinense* Jacq.). Means with different letters are significantly different, by Tukey's test at *p* = 0.05

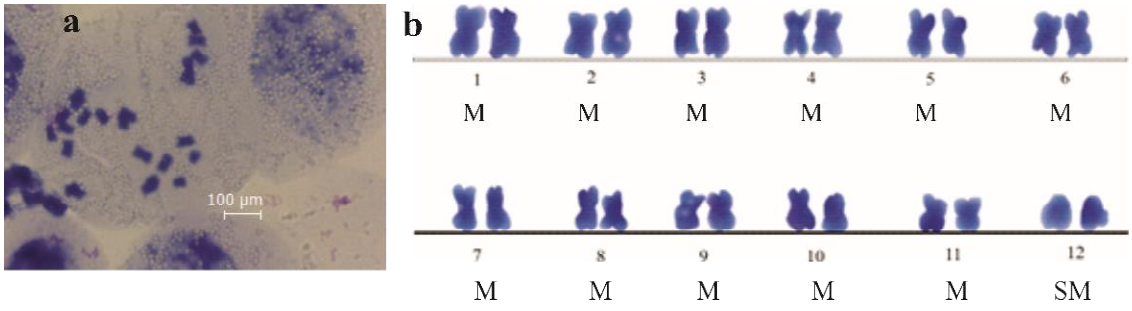

Fig. 5. Chromosomes of carolina reaper (*C. chinense* Jacq.). a. Metaphase chromosomes Carolina Reaper (*C. chinense* Jacq.), obtained from meristematic tissue treated with colchicine 0.05%, for 6 h at 18°C, subjected to conventional staining with Giemsa 10%. b. Cariogram representative diploid karyotype Carolina reaper (*C. chinense* Jacq.). M: Metacentric, SM: Sub metacentric

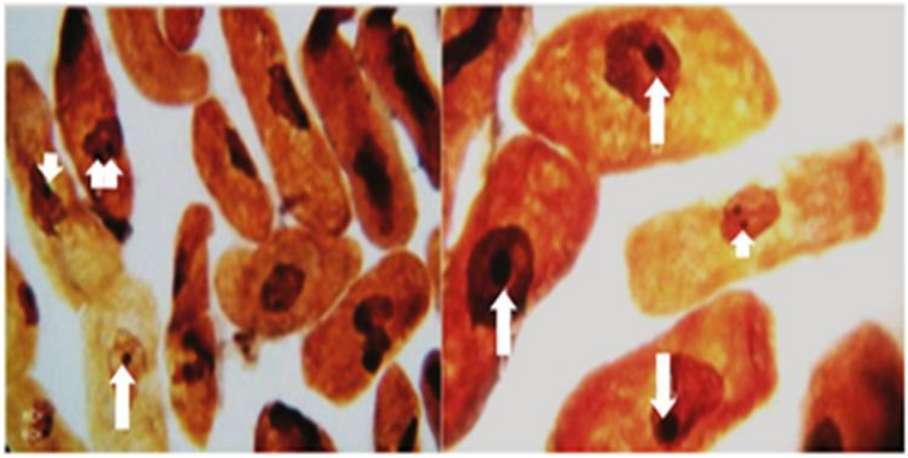

Fig. 6. Carolina reaper cells (*C. chinense* Jacq.) stained with silver nitrate to visualize the nucleoli (dark spots in cells indicated by arrows

The chromosome number can provide important information on the affinities of one species with another and, together with other cytological characteristics, contributes to the understanding of genetic variations involved in the evolution of the group. Thus, the cytogenetic analysis may bring contributions to increase the effectiveness of conservation strategies and even contributes to breeding programs of the species. The analysis of Carolina reaper metaphases revealed a chromosome number of 2n = 24, a number widely described in the literature for many species of Capsicum genus [1,34,35]. The karyotype formula found in Carolina reaper specimens was 11 metacentric and 1 submetacentric chromosomes (Fig. 5b), corroborating with another study with this specie [35,36]. Other studies with species of the genus Capsicum reported a variable karyotype formula, with just 12 metacentric chromosomes [37]. 11 metacentric and, 1 acrocentric [34]. and, 11 metacentric and 1 subtelocentric [35,37].

Cytogenetic studies of chromosome number and meiotic behavior involving cultivated species of Capsicum genus are important, the number of chromosomes of a species becomes constant it can be a useful feature in the taxonomy [38]. The emergence of polymorphism at the chromosomal level in individuals of a population can change the cytotype, thus creating chromosomally different variants, which could directly influence the phenotype of these individuals. The cultivated species of the Capsicum genus and some wild species have 2n = 2x = 24 chromosomes, however, in some wild species, such as C. *buforum, C. capylopodium* and, *C.cornutum*, 2n = 2x = 26 chromosomes [33]. However, just one cultivar of *Capsicum annuum* L. with 2n = 48 chromosomes has been previously reported by few researchers [39,40]. Differences in morphology or number of chromosomes may occur in populations of the same species or interspecific taxa and, according to Moscone et al. [1] are considered common within the Capsicum genus.

In this study, it was found two nucleoli in every cell interphase, suggesting that two NOR are expressing their ribosomal genes. Differences in the nucleolar size was also found, which can be explained by the fact that only a small part of the rRNA genes are actively transcribed and the relative activity of each region of the nucleolus reflects the volume of the corresponding nucleolus [41]. Analyzing the number of nucleoli in different species of the genus Capsicum, Moscone et al. [42]. found that the maximum number of nucleoli per species varied between 2 to 6, corroborating with data obtained in the present study with Carolina reaper.

4. CONCLUSION

A method developed for the in vitro regeneration of Carolina reaper (*C. chinense*), that could be applicable to the other varieties of peppers to obtain uniform plants and in great quantity possible with a short period of time. Using the concentrations of disinfection reagent and growth hormones indicated in the present study, it could be possible to regenerate leaves and nodal

segments in vitro from this cultivar, producing clones from the original genotype. further, this study also described the chromosome number and nucleolus number of Carolina reaper, generating a data that can help in programs of breeding, as in the generation of polyploid plants, and conservation species of pepper.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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