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Meiotic irregularities associated to cytomixis in *Buddleja iresinoides* (Griseb.) Hosseus. (Buddlejaceae) and *Castilleja arvensis* Schltdl. & Cham. (Orobanchaceae)

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Abstract. The current paper analyzes the male meiotic behavior in wild populations of *Buddleja iresinoides* and *Castilleja arvensis* from Piedmont areas of the Northwest Region of Argentina. *Castilleja arvensis* showed tetraploid number of chromosome of $2n = 24$. Our results are not in agreement with the previously reported base number $x = 19$ for *Buddleja* and the chromosome number $n = 28$ found for *B. iresinoides* is atypical in the genus. Around 7 % pollen mother cells were aneuploid as they showed meiotic chromosome count of n = 20-21 bivalents. Possible origin for such atypical chromosome number has been discussed in this paper. During the cytological studies we also came across pollen mother cells showing meiotic abnormalities such as cytomixis, chromatin stickiness and anaphase bridges with lagging chromatin. Consequently microsporogenesis was also irregular showing dyads and triads. However, the percentage of these irregularities during meiosis and microsporogenesis was not higher, and pollen fertility was not affected to a great extent. Cytomixis and other meiotic abnormalities in these species are reported here for the first time.

Keywords. *Buddleja iresinoides*, *Castilleja arvensis,* Chromatin, pollen mother cells, cytomixis, chromosome numbers.

INTRODUCTION

The migration of chromatin from the nucleus of one pollen mother cell (PMC) through specialized channels (named cytomictic channels) into an adjacent PMC was observed by Gates (1911) who called it cytomixis. Subsequently, Risueno *et al.* (1969) during their investigations noticed that these intercellular channels were sufficiently large to permit the migration of chro-

Figure 1. Morphological overview of the studied plants, A) General appearance of *Buddleja iresinoides*, and B) inflorescence detail; C) General appearance of *Castilleja arvensis*.

matin/chromsomes and other cytoplasmic organelles. In addition, the studies of Mursalimov et al. (2018) have established that plastids can pass into another cell through cytomictic channels.

Cytomictic connections were observed for the first time Körnicke (1901) in PMCs of *Crocus sativus*, but as the cytogenetic studies in plants advanced this phenomenon was also reported in meristematic, tapetal, integumental, nucellar and ovary cells in both Angiosperms and Gymnosperms (Cooper, 1952; Koul, 1990; Guzicka & Wozny, 2005; Wang *et al*., 2004; Oliveira-Pierre & Sousa, 2011; Kumar et al., 2015; Kumar & Chaudhary, 2016; Kumar & Singhal, 2016; Reis et al., 2016; Mursalimov & Deineko, 2017; Mursalimov & Deineko, 2018). As the cytogenetic analysis in higher plants expanded, cases of cytomixis were observed more frequently in accessions of cultured or natural plants populations. During our previous investigations, we observed the presence of cytomixis in different families of Angiosperms such as Pipperaceae, Cuscutaceae, Ranunculaceae and Cactaceae from the Northwest of Argentina (NOA) (Andrada *et al*., 2009; Lozzia *et al*., 2009; Páez *et al*., 2013 a, b). We also investigated *Buddleja iresinoides* (Griseb.) Hosseus. (Buddlejaceae) and *Castilleja arvensis* Schltdl. & Cham. (Orobanchaceae) for male meiosis and pollen fertility and we observed cytomixis and meiotic irregularities.

The genus *Buddleja* consist of ca. 100 species and cultivars that occur in warm, tropical, and subtropical climates from the Americas, Africa and Asia (Tallent-Halsell & Watt, 2009). *Buddleja iresinoides* is a shrubby plant, native to South America, distributed from Bolivia to the Northwest of Argentina were it is found in Catamarca, Jujuy, Salta and Tucumán provinces. It is a dioecious plant with quadrangular stems and ovate-lanceolate leaves, tomentose flowers with a bell-shaped calyx and corolla, the latter of white or yellow color (Fig.1A and B) (Carrizo & Isasmendi, 1994).

Castilleja Mutis ex L. f. comprises approximately 200 species native from western North to South America (González, 2013). *Castilleja arvensis* is an annual hemiparasitic herb, growing on humid soils from Mexico to the central region of Argentina. This species is characterized by its erect, simple, hispid, leafy stems (Fig. 1C). The leaves at the top of the stem are bract-like, gradually become smaller than the lower ones, and generally are red or purple colored (Botta & Cabrera, 1993).

For *Buddleja* chromosome numbers of 18 species are listed in IPCN (Index to plant chromosome numbers) (Goldblatt & Johnson, 1979+). The basic chromosome number $x = 19$ is accepted and the majority of species present this number or higher ploidy levels as gametophytic number (Norman, 2000; Tallent-Halsell & Watt, 2009).

Chromosome numbers of 54 species are listed in IPCN for the genus *Castilleja* (Goldblatt & Johnson, *op. cit*.). Based on the published literature the basic chromosome number of x = 12 and one or more polyploid levels have been suggested (Heckard, 1968; Heckard & Chuang, 1977; Chuang & Heckard, 1982; Tank & Olmstead, 2008; Tank *et al*., 2009).

The aim of the current paper is to analyze the male meiotic behavior in wild populations of *B. iresinoides* and *C. arvensis* in order to establish that meiotic irregularities are related to the phenomenon of cytomixis and, furthmore, to evaluate if they influence pollen fertility.

MATERIALS AND METHODS

Analyzed materials

All the material studied in this investigation was collected from natural populations of *Buddleja iresinoides* (Figure 1A-B) and *Castilleja arvensis* (Figure 1C) in Tucumán province. Voucher samples were deposited at the phanerogamic herbarium of Miguel Lillo Foundation (LIL).

Buddleja iresinoides: ARGENTINA, Prov. Tucumán, Dpto. Concepción, Loc. Cochuna, 27°10'20" S, 65°55'39" W, alt. 1160 m, *Andrada R.* S/N (LIL 610862).

Castilleja arvensis: ARGENTINA, Prov. Tucumán, Dpto. Tafí Viejo, Loc. Camino a la Toma, 26°43'05,122" S, 65°17'45.53" W, alt. 878 m, 29-lX-2007, *Andrada R.* S/N (LIL610759).

Analysis of meiosis

The material used consisted of flower buds from 5 randomly selected plants which were fixed in Farmer solution (3 ethanol : 1 glacial acetic acid) for one day, immediately transferred to 70% ethanol and stored at 4 °C. Anthers were first hydrolyzed in 1 N HCl at 60 °C for 20 minutes and then washed in distilled water. Pollen mother cells were prepared by the squash technique and stained with a drop of hematoxylin propionic with ferric citrate (Sáez, 1960; Núnez, 1968). 100 PMCs at each stage of the meiosis were observed.

Size and fertility of pollen grains

Fixed flowers immediately after anthesis were selected in order to estimate pollen fertility rates. At least 100 pollen grains of each species were measured to determine the typical pollen size range. Pollen grains were stained using Müntzing solution (glycerin-acetic carmin 1:1) (Sharma & Sharma, 1965). Well-filled pollen grains with uniformly stained cytoplasm were scored as apparently fertile/viable while the shrivelled/flaccid ones with unstained or poorly stained cytoplasm were counted as apparently sterile/unviable. At least 1000 pollen grains were analyzed for each taxon.

Photomicrographs were taken using a Nikon Eclipse E-200 microscope equipped with a Moticam 1000 digital camera (1.3 MP). The graphics were designed with the software CorelDRAW X3.

RESULTS

Analysis of meiosis:

Buddleja iresinoides: Generally the meiosis at prometaphase I started totally normal (97%) with the presence of 28 bivalents at diakinesis (Fig. 2A and B). Cytomixis was a common phenomenon in different stages of meiosis. About 2% of the PMCs of telophase I (TI) showed simple cytomictic channels indicating transfer of chromatin and cytoplasmic material among proximate PMCs (Fig. 2C), simple cytomictic channels connecting two or more cells were observed in 25% of MII (Fig. 2D). Furthermore, at TII cytomixis consisting of 1-2 channels between two cells were found in 45% of PMCs (Fig. 2E). Different kinds of irregularities were observed (Table 1). At diakinesis, 7% of PMCs were aneuploid, and showed 20-21 bivalents. At metaphase I (MI), irregularities such as out of plate bivalents were observed in 9% of the PMCs (Fig. 2F). In addition, 8% of the PMCs at TI stage were found to show anaphase bridges with lagging chromatin between two nuclei (Fig. 2G). At MII and AII, respectively 6% and 4% of PMCs exhibited chromatin stickiness between contiguous nuclei. (Figs. 2H-I). At the end of meiosis, abnormal sporads such as dyads and triads were present. (Fig. 2J). The mean diameter for *B. iresinoides* pollen, as determined by light microscopy, was 13.3 µm (range of 12.9 to 13.6 µm) (Table 1). Pollen viability rates in *B. iresinoides* was 89 % (Fig. 4A).

Castilleja arvensis: Chromosome numbers in PMCs were not constant. The diakinesis showed 78% regular PMCs with a gametophytic number of $n = 12$ (Fig. 3A). Cytomixis was revealed to be a very frequent phenomenon during pachytene, and 92% of the PMCs were con-

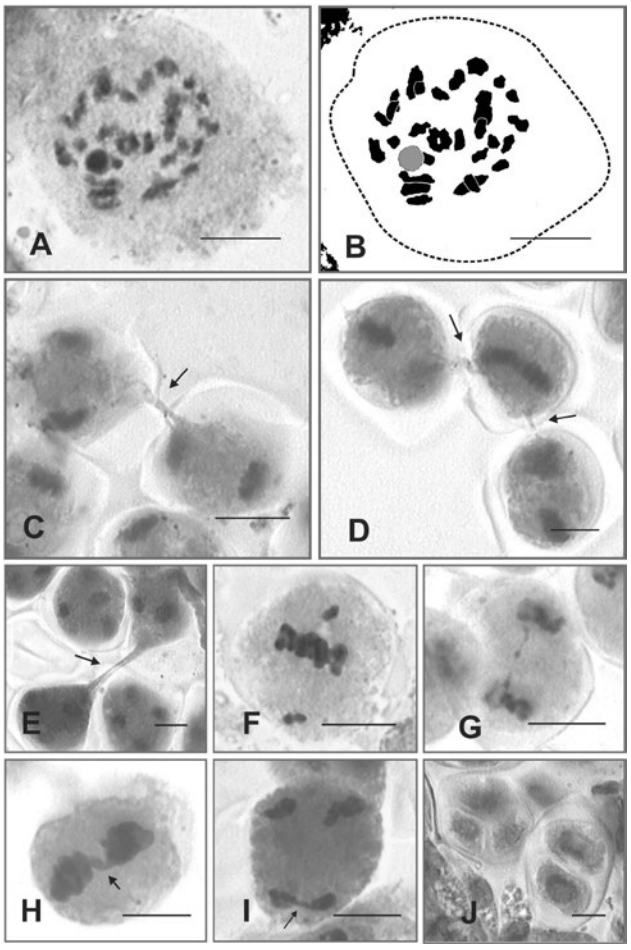

Figure 2. Meiosis in *Buddleja iresinoides*. A) Diakinesis with $n = 28$, B) Graphic representation of figure A; C) cytomictic channel in TI connecting 2 cells; D) MII with cytomictic channels connecting 3 cells; E) Cytomixis between tetrads; F) MI with two chromosomes away from the equatorial plate; G) TI showing anaphase bridges with lagging chromatin; H) MII with chromatin stickiness between two equatorial plates; I) AII with chromatin stickiness connecting 2 neighbour poles; J) Dyad and triad. Scale = 10 μm.

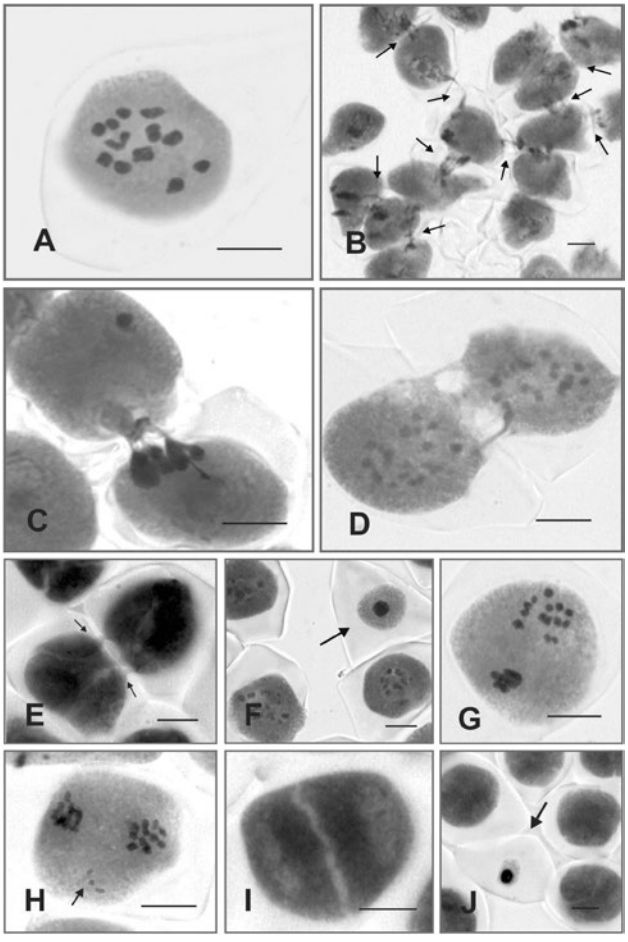

Figure 3. Meiosis in *Castilleja arvensis*. A) Diakinesis with 12 bivalents; B) Cells with multiple cytomictic channels in pachytene; C) Donor PMC transferring almost all the chromatin to a neighbor PMC; D) Two cells in diakinesis connected by 3 cytomictic channels; E) Cytomictic channels between tetrads; F) Small-sized meiocytes with a small nucleus during the division I; G) MII showing a plate with 14 chromosomes; H) MII showing chromosomes disconnected from equatorial plate; I) Dyad; J) small-sized meiocytes with a small nucleus at the end of the division II. Scale = 10 μm.

nected by 1-5 cytomictic channels linking two or more adjacent meiocytes (Fig. 3B). To a great extent, these channels were filled by chromatin strands indicaticating material transfer from one PMC to another. The donor cell sometimes transferred almost the whole of its chromosome complement to a recipient meiocyte leaving only a chromosome-like heteropycnotic body beside the nucleolus; the recipient meiocytes had bigger agglomerations of chromatin material (Fig. 3C). At diakinesis, 22% meiocytes showed 1-5 cytomictic channels (Fig. 3D). In 8.5% of PMCs, 1 or 2 cytomictic channels were found between the neighbour tetrads at the end of second division (Fig. 3E). Irregularities observed in this species occurred in different stages (Table 1). During diakinesis there were present hyperploid PMCs with up to $n = 20$ (Fig. 3D). During this stage, small-sized meiocytes with only a small nucleus were found. These small sized cells were covered by thick callose walls giving them an aspect of monads (Fig. 3F). Subsequent stages of first meiotic division (MI, AI and TI) were not observed in the preserved material. In MII, up to 7% of meiocytes were found to possess the hyperploid chromosome number of 14 at one pole (Fig. 3G). In 5% PMCs at metaphase II, it was found that chromosomes do not align on the metaphase plate and tend to lie towards the periphery of the cell wall (Fig. 3H). Dyads were also observed in 3%

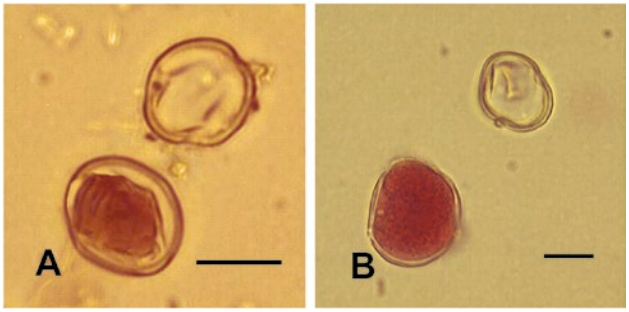

Figure 4. A-J: Fertile/stained and Sterile/unstained pollen grains in; A) *Buddleja iresinoides* and, B) *Castilleja arvensis*. Scale = 10 µm.

cases (Fig. 3I). Interestingly, 2% of PMCs were of small sizes and small nuclei (Fig. 3J).

Pollen size of *Castilleja arvensis* was observed to range from 20.1 µm to 20.8 µm (the mean diameter was 20.5 µm) (Table 1). Pollen viability rates in *Castilleja arvensis* was 95%, (Fig. 4B).

DISCUSSION

Our results are not in agreement with the base number $x = 19$ previously reported for *Buddleja* (Norman, 2000; Tallent-Halsell & Watt, 2009) and the chromosome number $n = 28$ found for *B. iresinoides* is atypical in the genus. However, Gadella (1980) suggested that $x = 19$ may have been derived from ancestral hybridization between two basic stocks with $x = 12$ and 7 (Norman, 2000; Oxelman *et al*., 2004). Our results suggested that *B. iresinoides* could be an octoploid with a putative basic number $x = 7$ or its chromosome number, $n = 28$ may have derived through secondary aneuploidy from a diploid parent having $n = 36$.

Another hypothesis is that the unusual chromosome number $n = 28$ ($2n = 56$) in this population may have derived by fusion of an unreduced gamete $n = 36$ from a putative parent and another normal gamete $n = 19$ (total $2n = 55$) followed by a chromosome gain (e.g. through cytomixis) to reach 2n = 56. Similarly, the rest of irregular gametes found $n = 20$-21 could have increased their chromosome number by cytomixis; this is after normal gametes $n = 19$ "acted" like recipient cells increasing their chromosome complement in 1 or 2 additional chromosomes.

The chromosome number of $x = 12$ had been suggested for the genus *Castilleja* (Heckard, 1968; Heckard & Chuang, 1977; Chuang & Heckard, 1982; Tank & Olmstead, 2008; Tank *et al*., 2009) and *C. arvensis* showed tetraploid number of chromosome of $2n = 24$.

The phenomenon of cytomixis as well as dyads and chromosomes that didn't attach to the equatorial plate were observed in both analyzed species. These latter kinds of meiotic irregularities could be caused by the cytomixis (Kumar, 2010; Kumar *et al*., 2010; Kumar *et al*., 2013).

The origin of cytomixis is still not clear and different opinions exist with respect to its causes and permanence during meiosis. Oliveira-Pierre & Sousa (2011) concluded that the cytomixis could have multiple origins. However, these authors have cited relatively recent investigations which show that cytomictic channels always structurally occur in the same way: 1) in the beginning, plasmodesms loss their connections with smooth endoplasmic reticulum (desmotubules) and then starts the intrusion of cytoplasmic material into the plasmodesms that inncrease their size forming cytomictic channels (Wei-cheng *et al*.,1988; Oliveira-Pierre & Sousa, *op. cit*.); 2) During the cytomixis process both cellulase and pectinase enzymes are presented as playing a role in digesting the cell walls of PMCs involved in this phenomenon (Wang *et al*.,1998); 3) in cells of germinal tissues of anthers during callose depositions that should block up the plamodesms occur disturbances, the connector channels increase their size and in this way facilitate the formation of cytomictic channels (Falistocco *et al*., 1995; Sheidai & Fadaei, 2005; Sheidai *et al*. 2006; Sidorchuk *et al*., 2007).

Some authors argued that cytomixis is a process that occur in the early stages of meiotic division (at prophase I generally) supporting the idea that after passage of chromatin from one PMC to another, cells which acquired or donated chromatinic material tend to degenerate. This kind of result was obtained by Koul (1990) through investigations carried out in *Alopecurus rundinaceus* Poir. On the other hand, there are authors that state that cytomixis could develop in all stages of meiosis (Basavaiah & Murthy, 1987 in *Urochloa panicoides* P. Beauv.; Bellucci *et al*., 2003 in *Medicago sativa* L.; Malallah & Attia, 2003 in *Diplotaxis harra* Boiss.; Singhal & Kumar, 2008 in *Meconopsis aculeata* Royle; Singhal *et al*., 2009 in *Anemone rivularis* Buch.-Ham. ex DC.). Authors have different positions regarding the transfer of cell components through cytomixis. During cell division, Heslop-Harrison (1966) suggested that intercellular connections occur to foment the synchrony between meiocytes allowing homogeneity of organelles and cytoplasmic components among them. However, Guanq-Qin & Gou-Chang (2004) refused this hypothesis because they considered it inconsistent, being that in plants the tapetal cells are responsible for providing nutrients to the PMCs (not the passage from one PMC to another),

between the last cells never has hitherto been observed cytoplasmic connections together the meiocytes. These authors attributed to cytomixis a more general function such as the mechanism that allows share regulatory and structural genetic products (e.g., mRNAs, oragnelles, etc.) between connected cells, favoring thus a necessary homogenization of cytoplasmatic restructural events occurred during prophase I which could cause heterogeneity in the meiocytes and consequently could lead to loss of their quality (generating more abnormal but less normal gametes).

During the pachytene stage, we have never observed cytomixis in PMCs of *B. iresinoides* but these started from telophase I. Our observations are not in agreement with Koul's hypothesis (1990) according to which the cytomixis occurs in first stages of meiotic division. However, in *C. arvensis* cytomictic channels were present in prophase I in most of the meiocytes (92% of PMCs) suggesting that absence or presence of cytomixis does not essentially depend on the stage of cell division. Participation of other factors that may play some role in cytomixis is still not clear. It is evident that the cytomixis can occur in different stages of meiosis from prophase I to tetrad formation as revealed in our results. Our findings agree with Guanq-Qin & Gou-Chang (2004) who reported that cytomictic channels always are formed among cells at the same division stage.

Nevertheless, the above cited authors mentioned that cytomixis promote homogeneity between meiocytes, observations that contradict our results because we observed in *C. arvensis* pachytene the transfer of almost all the chromatin from donor cell to recipient cell and the presence of hypoploid and hyperploid PMCs. Altogether, these irregularities (heterogeneity), probably produced by cytomixis, made up more than 30% of the observed cells.

According to morphological characteristics, the small-sized meiocytes with a little nucleus observed in both the division I and the division II correspond to apoptotic cells similar to the ones cited by different authors in both plants and animals (Fuzinatto et al., 2007; Kravets, 2013; Andrada et al., 2016). The abnormal cells could be degraded by means of apoptosis, thus explaining the high percentage of pollen grains viability observed in *C. arvensis*. In this species this phenomenon occured two times: after pachytene at diakinesis and between the tetrads at the end of TII (both in division I and division II after or during the two stages with major percentage of cytomixis). Although this kind of abnormal cell was not observed in *B. iresinoides* it is possible that some similar mechanism could occur and the abnormal cells would be eliminated. By removing the abnormal pollen grains these plants ensure that gametes transferred being viable.

In *B. iresinoides* transfer of chromatin from a donor cell to a recipient cell is not limited only to neighbouring meiocytes but also occurs between PMCs at same stage of division. According to Ortíz *et al.* (2006) and Andrada & Páez (2014), this kind of connections could disturb the normal development of the phragmoplast during cytokinesis causing irregularities which could finish as unbalanced gametes and give rise to dyads as it was observed in *B. iresinoides*. Although in *C. arvensis* connections among meiocytes from the same PMC was not observed, these were present in dyads once meiosis had been completed.

In both species chromosomes which did not align to metaphase plate and stood near cell wall were found but in different stages in the two species examined. In *B. iresinoides* they occured during MI, whereas in *C. arvensis* this kind of irregularite was observed at MII even at the hyperploid cells. These chromosomes could occur due to transfer from a PMC to other neighbouring PMC through cytomixis channels during the early metaphase.

In *Buddleja iresinoides*, during the división II the 57% of PMCs showed irregularities (stickness, cytomixis and bad debelop phragmoplast that finished in dyads and triads). This percentage is far to the 11% of irregular and inviable pollen grains revealed by Münting's stain, however, before start the division II the regulatory mechanism that controls the normal course of the meiosis or the method to eliminate irregular PMCs still remains unknown. In addition, in *B. iresinoides* it is evident that the cytomixis (reaching the maximum value of 45% at TII) does not have a large impact on the development of non-viable pollen grains. Gernand *et al.* analyzed the mechanisms underlying selective elimination of the paternal chromosomes during the development of wheat × pearl millet hybrid embryos and found that chromosome elimination frequently took place during meiosis. These cytological observations showed that parental genomes were spatially separated within the hybrid nucleus, and the pearl millet chromatin destined for elimination occupied peripheral positions. A similar phenomenon was found in this study; chromosomes were spatially separated within the PMCs where the chromatin occupied a predominantly peripheral position at metaphase I from *B. iresinoides* and at pachitene and MII from *C. arvensis*. In addition, given that the *B. iresinoides* and *B. stachyoides* Cham. & Schltdl. (chromosome number unknown) grow together, it is likely that the taxon studied contain chromosomes from *B. stachyoides*. This would have given rise to populations of hybrids with this atypical gametophytic number (n = 28).

In *Castilleja arvensis*, among the frequent abnormalities (as stickness, cytomixis and bad debelop phragmoplast that finished in dyads and triads) the cytomictic channels at pachitene (Table 1 and Figure 3B) were salient. However, the cytomixis does not seem to be the main cause of pollen inviability. In this species, the apoptosis which was observed in both prophase and TII where occur the most of irregularities could regulate the number of abnormal PMCs during the meiosis and the non viable pollen grains would be obtained when simply some PMCs with different type of irregularities add together up to reach 5%.

Buddleja iresinoides and Castilleja arvensis have high percentages of pollen grains stained (viables) close to 90% and small ranges of variation of size close to 0,7 µm. This fact suggest that polyploid cells (produced through of dyads and triads) which should develop giant pollen grains or "Jumbo grains" were eliminated during the last steps of microsporogenesis. On the other hand, the limit size values of stained pollen grains may mask hyperploids and hypoploid cells as apparently normal and fertile pollen grains. Future studies related to germinabaility of pollen grains could clarify the strange behavior of these species that show high percentages of irregularities during the meiosis and low production of sterile pollen grains.

CONCLUSIONS

Castilleja arvensis is a diploid taxon with $n = 12$ while the unusual number $n = 28$ from *B. iresinoides* suggest that the basic chromosome number for this genus could be less than $x = 19$. However, that this atypical number could have originated through passage of additional chromosomes from a donor cell to recipient cell by cytomixis or through hybridization process is possible too. In the present study, we have found that cytomixis is a process which is not stage specific and its frequency may vary from species to species which is evident from our results in *B. iresinoides* where maximum percentage of cytomixis occur during TII, whereas in *C. arvensis* it is more frequent in pachytene. In addition, this process could cause numerous irregularities that would result in (at the end of meiosis) genetically unbalanced gametes. Furthermore depending upon the severity of meiotic irregularities it may hamper the reproductive success of species. Cytomixis has been reported here for the first time from both Buddlejaceae and Orobanchaceae families.

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