Scientific Article

A novel source of cytoplasmic male sterility in *Calibrachoa pubescens*[1]

NOEMÍ COLOMBO[2]*, ANDREA COVIELLA[3], JUAN CARLOS HAGIWARA[3]

ABSTRACT

Calibrachoa pubescens is a species native from the south of Brazil, Uruguay and northeast Argentina. An accession identified as 7.3.1.1 was collected at San Martín Department in Corrientes Province, Argentina and is included in the calibrachoa breeding program at the Institute of Floriculture, INTA. This accession is male sterile and produces male-sterile progeny, characterized by the lack of pollen production. Male sterility may be controlled by nuclear or cytoplasmic genes with each type presenting a different mode of inheritance. The objective of this research was to present a novel source of cytoplasmic male sterility in *Calibrachoa pubescens.* Crosses were made in the greenhouse between the male-sterile line 7.3.1.1 as the female parent and seven male-fertile lines of diverse origin. F1s were backcrossed and self-pollinated. Individual plants of the progenies were classified as male-fertile or male-sterile according to pollen viability. Analyses of observed segregations showed that male sterility observed in 7.3.1.1 results from the interaction of a male sterile cytoplasm and nuclear restorer genes. A novel male sterile cytoplasm is now available for breeders. Main advantages of cytoplasmic male sterility in ornamentals breeding are: efficient hybrid production, increased flower longevity, avoidance of pollen allergens and control of plant invasiveness.

Keywords: *Calibrachoa pubescens*, cytoplasmic male sterility, fertility restoration, ornamental breeding.

RESUMO

Uma nova fonte de esterilidade masculina em *Calibrachoa pubescens*

Calibrachoa pubescens é uma espécie nativa do sul do Brasil, Uruguai e nordeste da Argentina. Um acesso identificado como 7.3.1.1 foi coletado no departamento de San Martín, no estado de Corrientes -Argentina e incluído no programa para o melhoramento genético de *Calibrachoa* no Instituto de Floricultura, INTA. Esse acesso é macho-estéril e produz progênies masculinos-estéreis, caracterizada pela falta de produção de pólen. A esterilidade masculina pode ser controlada por genes nucleares ou citoplasmáticos cada um dos quais apresenta diferentes mecanismos de herança. O objetivo desta pesquisa foi presentar um novo citoplasma masculino estéril em *Calibrachoa pubescens*. Os cruzamentos foram feitos em estufa entre a linhagem macho-estéril 7.3.1.1 como genitor feminino e sete linhagens masculinas e férteis de origem diversa. Os F1s foram recortados e auto-polinizados. Plantas individuais das progênies foram classificadas como masculinas férteis ou macho-estéril de acordo com a viabilidade do pólen. As análises das segregações observadas mostraram que a esterilidade masculina observada em 7.3.1.1 resulta da interação de um citoplasma estéril masculino e genes restauradores nucleares. Um novo citoplasma masculino estéril está agora disponível para melhoradores. As principais vantagens da esterilidade masculina citoplasmática no melhoramento de plantas ornamentais são: produção híbrida eficiente, aumento da longevidade das flores, ausência de alérgenos do pólen e controle da invasividade das plantas.

Palavras-chave: *Calibrachoa pubescens*, esterilidade masculina citoplasmática, restauração da fertilidade, melhoramento de plantas ornamentais.

1. INTRODUCTION

The cultivation of calibrachoa (million bells) as an ornamental plant is relatively recent. It begins with the launching of the first cultivars in the 1990s. However, today it is one of the most important bedding and balcony plants with global economic importance (KANAYA et al., 2010; JĘDRZEJUK et al., 2017). Calibrachoa cultivars are bred by interspecific hybridization using different wild species of *Calibrachoa* spp. Cerv. (Solanaceae) (KANAYA et al., 2010). At present Argentina grows 12 native species of *Calibrachoa* (GREPPI et al., 2013). The Institute of Floriculture of INTA carries out a breeding program of calibrachoa using native species of Argentina to create new vigorous and freely-flowering calibrachoa plants with unique and attractive flower coloration and good garden and summer performance.

C. pubescens (Spreng.) Stehmann is a species native from the south of Brazil, Uruguay and northeast Argentina (GREPPI et al., 2013). An accession identified as 7.3.1.1 was collected from a small population located at Tres Cerros, San Martín Department in Corrientes Province, Argentina, and it was included in the calibrachoa breeding program (Figure 1).

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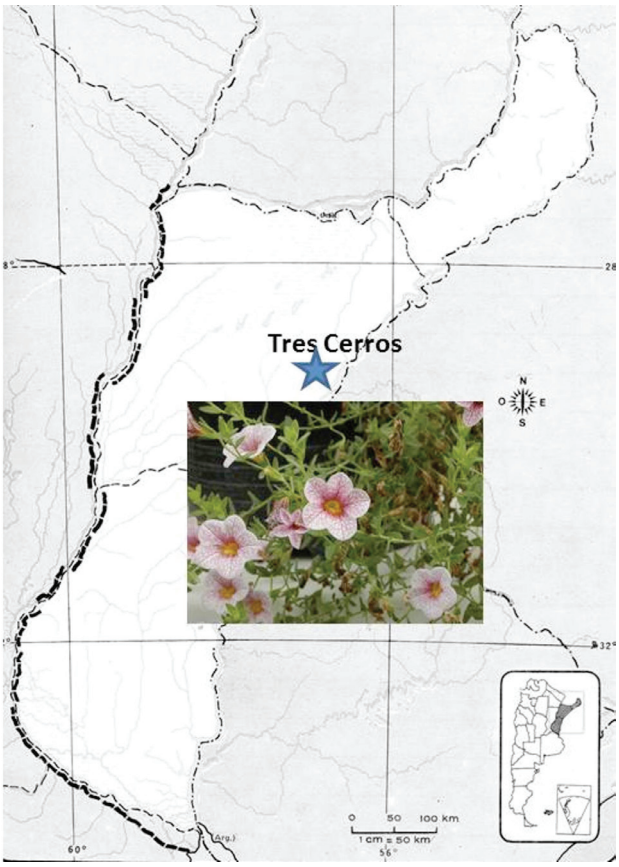

Figure 1. Accession 7.3.1.1 of *Calibrachoa pubescens* and geographic localization of the population from which it was collected.

This accession is male sterile and produces male-sterile progeny, characterized by the lack of pollen production. Female fertility, on the other hand is normal. Male sterility may be controlled by nuclear or cytoplasmic genes, with each type presenting a different mode of inheritance. Moreover, cytoplasmic male sterility can be restored by the effect of nuclear genes, representing a good example of the interaction between the mitochondrial and nuclear genomes (CHEN and LIU, 2014). Male sterility is a desirable trait for breeding purposes in ornamental crops to produce hybrid seed, to increase flowering duration (SMITH et al., 2004), to avoid pollen allergens (SINGH et al., 2012) and to control invasiveness (ANDERSON et al., 2006). The objective of this research was to present a novel source of cytoplasmic male sterility in *Calibrachoa pubescens.*

2. MATERIAL AND METHODS

Eight genotypes from five natural species and three commercial cultivars of calibrachoa were used in this study (Table 1 and 2).

Table 1. Accessions list of natural species of calibrachoa used in this study.

Accessions code	Botanical name	Collection locality
25I2	*Calibrachoa caesia* (Sendtn) Wijsman Phil.	Teyu Cuare, San Ignacio, Prov. Misiones, Argentina
2.5.8.1	*Calibrachoa caesia* (Sendtn) Wijsman Phil.	Dos de Mayo, Cainguas, Prov. Misiones, Argentina
24H1	*Calibrachoa excellens* (R.E.Fr.) Wijsman Phil.	Garruchos, Santo Tomé, Prov. Corrientes, Argentina
9346	*Calibrachoa humilis* (R.E.Fr.) Stehmann & Semir	Paraje Santa Juana, Mercedes, Prov. Corrientes, Argentina
7311	*Calibrachoa pubescens* (Spreng) Stehmann	Tres Cerros, San Martín, Prov. Corrientes, Argentina
D2	*Calibrachoa pubescens* (Spreng) Stehmann	Tres Cerros, San Martín, Prov. Corrientes, Argentina
17B1	*Calibrachoa thymifolia* (A. St.-Hil.) Stehmann & Semir	Santa Ana, Federación, Prov. Entre Rios, Argentina
2.1.9.1	*Calibrachoa thymifolia* (A. St.- Hil.) Stehmann & Semir	Nueva Escocia, Concordia, Prov.EntreRios, Argentina

Table 2. List of calibrachoa cultivars used in this study.

Code	Name of cultivar	Breeder / Company
Dark Blue	Callie Dark Blue	Goldsmith Seeds
Scarlet	Callie Scarlet	Goldsmith Seeds
Sunrise	Callie Sunrise	Goldsmith Seeds

Six interspecific crosses were made in the greenhouse to obtain F1s between the male-sterile line 7.3.1.1 of *C. pubescens* as the female parent and six male-fertile lines of diverse origin, i.e. 1) 07-531#4; 2) 08-217#3; 3) 06-53N#1; 4) Dark Blue; 5) 9.3.4.6 ; 6) 08-236#1 (Table 3).

Table 3. List of male-fertile lines of calibrachoa used in this study and their origins.

Male fertile line	Origin
07-531#4	[(25I2[a] x 24H1[a]) x Callie Dark Blue[b]][2]
08-217#3	25I2 x Callie Sunrise[b]
06-53N1	2.1.9.1[a] x Callie Scarlet[b]
Dark Blue	Callie Dark Blue
9.3.4.6[a]	*Calibrachoa humilis*[c]
08-236#1	2.5.8.1 x 17B1[a]

[a] Accession code of natural species (Table 1). [b] Cultivar name (Table 2). [c] Natural species (Table 1).

The backcross (BC1) between F1 (7.3.1.1 x Dark Blue) x Dark Blue was also obtained. An intraspecific cross was made in the greenhouse between *C. pubescens* 7.3.1.1 and the male-fertile accession D2 collected from the same population (Table 2). F1s were backcrossed (BC1s) to the male fertile parent and self-pollinated to produce F2s.

Pollen viability was evaluated using fluorescein-diacetate (FDA) / propidium iodide (PI) and epifluorescence microscopy with UV light (GREISSL, 1989) using an OLYMPUS BX50 fluorescence microscope. About 500-1000 pollen grains were scored per plant as viable (green) or non-viable (red). Plants were classified as Sterile (≥ 90% non-viable pollen); Partially fertile (90 % › % non-viable pollen › % fertile parent); Fertile (% non-viable pollen ≤ % fertile parent). Mean percentages of viable pollen were estimated for each generation and for the male fertile parents. Frequencies distributions of pollen viability were determined for each analyzed generation.

3. RESULTS AND DISCUSSION

Pollen viability of plants from different generations and from the pollen donors of the crosses was determined using fluorescein-diacetate (FDA) / propidium iodide (PI) and epifluorescence microscopy with UV light (Figure 2). This method allows differentiating bright green viable pollen grains from death pollen grains stained in red. FDA is considered a vital staining dye, which is hydrolyzed by cellular esterases and originates the accumulation and easy detection of fluorescein. On the other hand, PI cannot penetrate intact, living cellular membranes, so the observed red fluorescence is an indicator of non-viable cells or damaged membranes (GREISSL, 1989). A total of 246.764 pollen grains were observed.

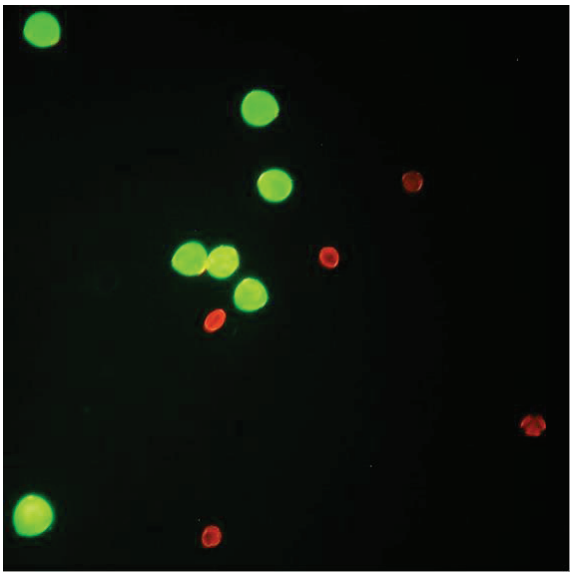

Figure 2. Pollen viability determined with fluorescein-diacetate (FDA) / propidium iodide (PI) and epifluorescence microscopy with UV light.

Plants were classified as fertile, partially fertile or sterile according to the percentage of non-viable pollen. When considering the interspecific crosses, no male fertile plants were observed in the F1s between the male sterile parent 7.3.1.1 and the pollen donors 07-531#4 and Dark Blue. However, fertile plants were found in the other F1s. All the F1 populations showed a reduction in mean pollen viability compared to the male fertile parent (Table 4). In BC1s plants from the crosses F1 (7.3.1.1 x Dark Blue) x Dark Blue, no fertile plants were observed and the mean pollen viability values were drastically reduced (Table 4). The segregation patterns of these BC1s reveal that the male sterility observed in the accession 7.3.1.1 is maternally inherited and determined by cytoplasmic factors. Analysis of the intraspecific cross between 7.3.1.1 and D2 showed no male sterile plants in the F1. The F2 and BC1 were obtained using two different F1 plants as male parents, each of them showing a different degree of male fertility. In both generations, male sterile plants were observed and the mean pollen viability decreased as compared to the F1 generation (Table 4).

Table 4. Observed segregations and means pollen viability.

Generation	Fertile	Partially fertile	Sterile	Mean % of viable pollen of the male parent	Mean % of viable pollen of progenies
F1 (7.3.1.1 x 07-531#4)	0	4	12	44	10
F1 (7.3.1.1 x Dark Blue)	0	5	11	58	9
F1 (7.3.1.1 x 08-217#3)	2	6	8	39	17
F1 (7.3.1.1 x 08-236#1)	5	7	4	40	29
F1 (7.3.1.1 x 06-53N1)	5	7	5	37	23
F1 (7.3.1.1 x 9.3.4.6)	7	4	5	37	29
F1 (7.3.1.1 x D2)	11	24	0	65	54
BC1 (7.3.1.1 x Dark Blue) # 1 x Dark Blue	0	1	6	45	5
BC1 (7.3.1.1 x Dark Blue) #2 x Dark Blue	0	1	19	45	7
BC1 (7.3.1.1 x Dark Blue) #-3 x Dark Blue	0	4	10	45	5
BC1 (7.3.1.1 x D2) #7 x D2	5	12	5	70	37
F2 (7.3.1.1 x D2) # 6@	15	9	7	28	32

Frequency distributions of pollen viability in F1s plants from interspecific crosses were represented (Figure 3). In crosses between the male sterile 7.3.1.1 and the pollen donors Dark Blue and 07-531#4 only plants showing high percentages of non-viable pollen were observed. All the other F1s displayed a wider range of categories corresponding to different grades of non-viable pollen. In the BC1s derived from Dark Blue only plants presenting high percentage of non-viable pollen were observed (Figure 4). Interestingly, in the intraspecific cross between 7.3.1.1 and D2 an increase in the frequency of plants with non-viable pollen was evident both in F2 and BC1 generations compared with F1 (Figure 5). Taken together, these data indicate the presence of restorer genes in the pollen donors 08-217#3, 06-53N#1, 9.3.4.6, 08-236#1 and D2, but not in Dark Blue and 07-531#4.

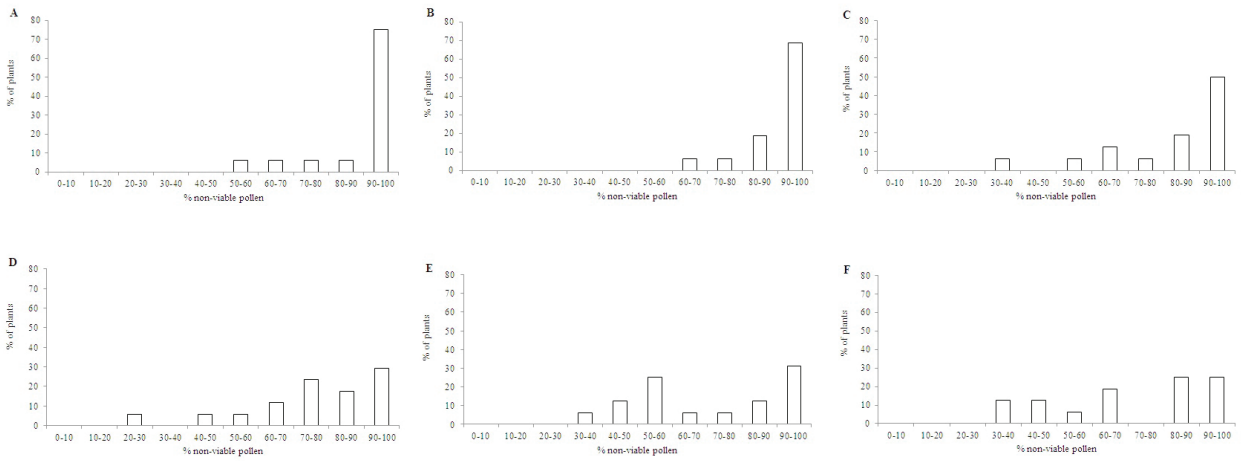

Figure 3. Frequency distribution of pollen viability in F1s plants from interspecific crosses. A: F1 (7.3.1.1 x 07-531#4); B: F1 (7.3.1.1 x Dark Blue); C: F1 (7.3.1.1 x 08-217#3); D: F1 (7.3.1.1 x 08-236#1); E: F1 (7.3.1.1 x 06-53N1); F: F1 (7.3.1.1 x 9.3.4.6).

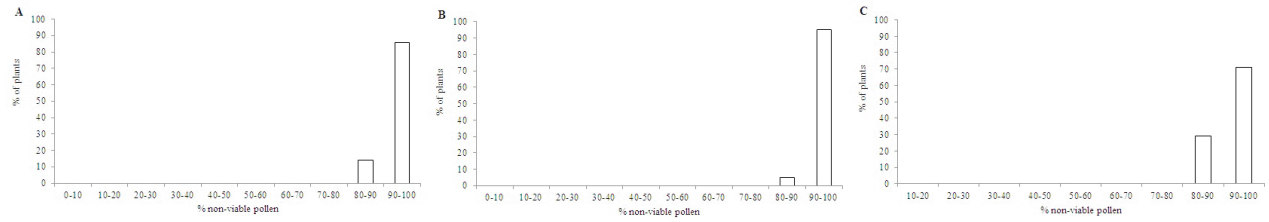

Figure 4. Frequency distributions of pollen viability in BC1s plants derived from Dark Blue. A: BC1 (7.3.1.1 x Dark Blue) # 1 x Dark Blue; B: BC1 (7.3.1.1 x Dark Blue) #2 x Dark Blue; C: BC1 (7.3.1.1 x Dark Blue) #-3 x Dark Blue.

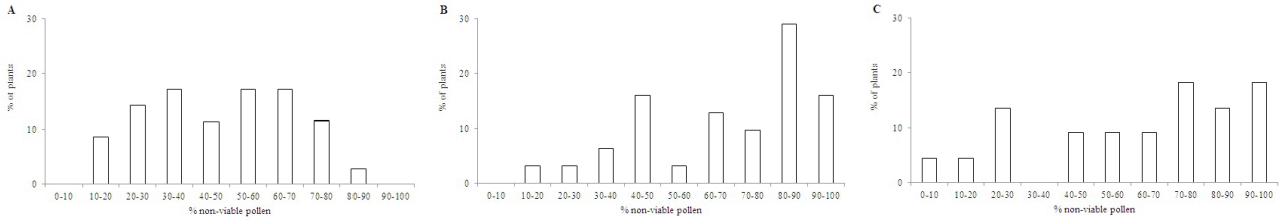

Figure 5. Frequency distributions of pollen viability in F1 (A), F2 (B) and BC1 (C) plants from the intraspecific cross (7.3.1.1 x D2).

Male sterile phenotype in the accession 7.3.3.1 is characterized by the lack of pollen. However, male sterile plants obtained in our crosses presented from complete absence of pollen to variable degree of pollen production. We hypothesize that microsporogenesis seems to be affected at different stages depending on the nuclear background. Similar results were described in cytoplasmic male sterility in petunia, where a wide variation of male sterile phenotypes expressed at different times of the breakdown of microsporogenesis was reported (IZHAR, 1977). Recently, the effect of nuclear background on the expression of male sterile cytoplasms has been reported in chicories (HABARUGIRA et al., 2015) and leaf mustard (WAN et al., 2014). Further research is needed in order to characterize microsporogenesis in 7.3.3.1 and in the male sterile plants originating from different crosses.

Cytoplasmic male sterility can arise spontaneously or result from intraspecific, interspecific or intergeneric crosses (KAUL, 1988). This trait has been reported in several ornamental plants: aquilegia (SKALINSKA, 1928; KAPPERT, 1943), begonia (VILLERTS, 1942), godetia (HIORTH, 1948), petunia (DUVICK, 1959; EDWARSON AND WARMKE, 1967; NIVISON and HANSON, 1989; BENTOLILA et al., 2002), sunflower (LECLERQ, 1969; ECHEVERRÍA et al., 2003; LIU et al., 2014) and *Eustoma* (MORI et al., 2016).

It should be mentioned that both male sterile and hermaphrodite plants coexist in the original population of *C. pubescens*, thus representing a case of gynodioecy (MC CAULEY and BAILEY, 2009).The use of genetic and ecological approaches would be of help to determine the mechanisms involved in the maintenance of gynodioecy in this population.

4. CONCLUSIONS

To our knowledge this is the first report of a cytoplasmic male sterile cytoplasm in calibrachoa. Due to maternal inheritance of the sterile cytoplasm it will be easy to get its introgression in different backgrounds with breeding purposes. This novel resource will reduce the labor demanded for making crosses by eliminating the need of manual emasculation and will allow hybrid production more efficiently. Besides, cytoplasmic male sterility may be of interest for increasing flower longevity and for controlling the dispersion of invasive species. Although we have not tested the effect of different environmental conditions on cytoplasmic male sterility and fertility restoration, it is likely that they play a role on the expression of these characters, as it has been shown in several species (VAN MARREWIJK, 1969; IZHAR, 1975; DHALL, 2010; DE STORME and GEELEN, 2014; BUECKMANN et al., 2016). Future research should consider this aspect to better describe the expression of the male sterile cytoplasm of calibrachoa.

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AUTHORS CONTRIBUTIONS

N.C. and **J.C.H.** planned the experiments, obtained and analyzed the data and wrote the article; **AC**: helped to obtain the data and provided technical support

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