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Determination of Ploidy Levels and Breeding of *Ruellia*

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***Ruellia* consists of approximately 300 species found mostly in tropical and subtropical areas. *Ruellia simplex* (Mexican petunia) is an economically important ornamental in the southern United States due to its low maintenance, prolific flowering, and overall performance. However, since its introduction to Florida in the 1940s, *R. simplex* has naturalized in disturbed ecosystems of seven southern U.S. states, Hawaii, Puerto Rico, and the U.S. Virgin Islands. It is considered a Category I invasive by the Florida Exotic Pest Plant Council because it displaces native plant communities, disrupts ecological functions and can hybridize with native species. For these reasons, a breeding program was initiated at the University of Florida to develop cultivars with reduced fertility and with different flower colors, growth habits and ornamental traits. Breeding methods used include artificial hybridizations and ploidy manipulations. Tetraploid individuals are developed utilizing oryzalin and then intercrossed with related species to obtain hybrids with reduced female and male fertilities and no invasiveness by seed production. To date, *Ruellia* 'Mayan Purple', 'Mayan White', 'Mayan Pink', and 'Mayan Compact Purple' have been released and patented. The objectives of this research were to determine an optimal oryzalin treatment resulting in polyploidization of *R. simplex* and develop tools to efficiently determine ploidy levels. Individuals were screened for ploidy level by counting chloroplasts on the stomata and measuring stomata length, and presumed polyploids were confirmed by flow cytometry.**

Ruellia simplex Wright (commonly known as Mexican petunia, Mexican bluebell or Britton's petunia) has low maintenance requirements, great pest and environmental stress resistance, and prolific flowering. It has become a very popular landscape plant in the southern United States (Gilman, 1999) since its introduction to Florida sometime in the 1940s (Hupp et al., 2009). *Ruellia simplex* has naturalized in disturbed uplands and wetlands of seven southern U.S. states (from Florida and South Carolina, west to Texas), plus the U.S. Virgin Islands, Puerto Rico, and Hawaii (USDA-NRCS, 2013). Since 2001, the Florida Exotic Pest Plant Council has considered Mexican petunia as a Category I invasive plant, described as "plants that are altering native plant communities by displacing native species, changing community structures or ecological functions, or hybridizing with natives" (Florida Exotic Pest Plant Council, 2015). The Institute for Food and Agricultural Science (IFAS) Assessment of the Status of Non-Native Plants in Florida's Natural Areas also considers *R. simplex* as an invasive species, and does not recommend its use in Florida (IFAS, 2015).

A survey conducted in 2002 including 946 active nurseries in Florida indicated that 15.9% of them grew or sold *R. simplex*, and total reported annual sales for this plant was estimated at approximately $12 million (Wirth et al., 2004). Until recently, *R. simplex* cultivars commercially available included tall ('Purple Showers', 'Chi Chi' and 'Snow White') and dwarf plants ('Katie' and 'Southern Star' series) in purple, pink and white flower colors. These cultivars are vegetatively propagated, except for the 'Southern Star' series, which is propagated by seed (PanAmerican Seed Co., Chicago IL). All of them set fruit and are potentially invasive, with the exception of 'Purple Showers' (Wilson and Mecca, 2003).

In 2007, a *Ruellia* breeding program was initiated at the University of Florida, Institute of Food and Agricultural Sciences (UF/IFAS) in Gainesville, FL, directed specifically towards creating new cultivars with reduced weed potential for the landscape plant industry. To reduce the spread of *R. simplex* by seed, cultivars are bred with reduced female fertility (fruitlessness or lack of seed viability) and reduced male fertility (Freyre et al., 2012a). So far, cultivars 'Mayan Purple' (R10-102, PP24422), 'Mayan White' (R10-108, PP25156) (Freyre et al., 2012b), 'Mayan Pink' (R10-105-Q54, PP26063) (Freyre et al., 2014) and most recently 'Mayan Compact Purple' (Freyre et al., 2016) have been introduced. These four cultivars were assessed by the IFAS Invasive Plants Task Force for invasive potential, and their landscape use was approved.

In past years, *R. simplex* was listed as *R. tweediana* Griseb (or *R. brittoniana* Leonard) while *R. coerulea* Morong from subtropical South America was considered closely related to North American species *R. malacosperma* Greenm and *R. brittoniana* (Ezcurra, 1993). More recently, taxonomists have grouped both *R. tweediana* and *R. coerulea* under the name *R. simplex*. This is the name used to describe a species from Cuba in 1870, and since it is the oldest name in record, it has taxonomic priority and reduces other names to synonyms (Ezcurra and Daniel, 2007). Chromosome numbers of $2n = 34$ for 27 taxa of *Ruellia* have been reported (Grant, 1955) as well as a few rare counts of $n = 16$ and $n = 18$ (Federov, 1969). Furthermore, chromosome numbers in several *Ruellia* species from Central and North America were all found to be $n = 17$. Thus, this chromosome number appears

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to be widespread in this large and variable genus from various parts of the world (Daniel and Chuang, 1993; Daniel et al., 1984, 1990). *R. simplex* (as *R. tweediana*) was also reported as $2n = 34$ (Piovano and Bernadello, 1991).

Taxonomic considerations are important in breeding efforts, since hybridizations between what were previously known as *R. tweediana* and *R. coerulea* have produced sterile hybrids. *Ruellia coerulea* individuals have similar growth habit to *R. tweediana*, but have lanceolate leaves rather than elongated. In our breeding populations *R. coerulea* individuals have either purple or white flowers, while *R. tweediana* shows more diversity, with individuals with purple, pink, white, white with purple throat, and white with pink throat flower colors (Freyre et al., 2015). We have also found a difference in ploidy between *R. tweediana* and *R. coerulea*. So far, our attempts at counting meiotic or mitotic chromosomes have not been successful. However, our flow cytometry data suggests that *R. coerulea* plants are tetraploid, while *R. tweediana* plants are diploid. The crosses (4x *R. coerulea* x 2x *R. tweediana*) have not resulted in sterile triploids as expected. However, we discovered that the crosses (4x *R. tweediana* x 4x *R. coerulea*) resulted in tetraploid hybrids with reduced female and male fertilities. In order to effectively produce these hybrids, efficient polyploidization techniques must be in place for the polyploidization of *R. tweediana* individuals, as well as efficient screening methods for their identification. *Ruellia* polyploidization treatments at our laboratory generally used 50 µM of oryzalin, and no mortality or phytotoxic effects were observed. In this trial, the effects of higher concentrations of oryzalin were studied, as well as determining if the addition of dimethyl sulfoxide (DMSO) as a solvent had an effect on its efficacy as a polyploid inducer. The objective was to find a protocol which would be effective in producing stable polyploids while minimizing seedling mortality.

For several years, flow cytometry analysis of our plants was conducted by a colleague at the University of Florida. Besides the high cost involved, the turnaround for these analyses was sometimes slow and results were not always accurate. Additionally, to reduce the number of individuals that were analyzed by flow cytometry, populations were screened based on phenotypic differences (i.e. flower and leaf size) but this was not necessarily accurate. Therefore, we attempted to conduct the flow cytometry analyses in-house. Initially, we tested several protocols to stain suspended nuclei prepared from fresh plant tissue (Galbraith et al., 1983). We found that modified protocols on the "Determination of Nuclear DNA Content of Plants by Flow Cytometry" by Arumuganathan and Earle (1991) as described in "Plant Cytogenetics", 2nd Ed. by Ram J. Singh worked best. Data obtained indicated differences in ploidy levels, and also identified ploidy chimeras. However, this process was still time-consuming and costly for large number of individuals. Therefore, our objective was to find a screening method to reduce the number of plants analyzed through flow cytometry. This method needed to be efficient and low-cost, while providing reliable information in a short amount of time. Methods for counting of stomata chloroplast and measurements of stomata length have been used to identify polyploids in crops such as buddleia, potato, salvia, watermelon and *Ziziphus*, among others (Dunn et al., 2007; Gu et al., 2005; Grouh et al., 2011; Sari et al., 1999; Singsit et al., 1991)

For this research we conducted a polyploidization experiment on seeds of *Ruellia* 'Southern Star' series (PanAmerican Seed Co., Chicago IL), which are representative of *R. tweediana* and have stable diploid ploidy. We tested the effectiveness of different concentrations of oryzalin and the addition of dimethyl sulfoxide (DMSO) as a solvent in polyploidizing. Treated plants were screened to determine potential tetraploids by staining and counting chloroplast in stomata guard cells, and by measuring the length of stomata. Plants were subsequently analyzed by flow cytometry. The results, ease of replication, and accuracy between screening methods were compared.

Material and Methods

Seeds of all three 'Southern Star' flower colors (purple, pink and white) were purchased from PanAmerican Seeds. A total of 60 seeds (20 of each color) were germinated on seedling trays. Seeds were sowed on a peat : perlite (60% : 40%, by volume) substrate and placed in a growth chamber with a 12-h photoperiod at a constant 22 °C (72 °F).

Polyploidization of seedlings. Polyploidization treatments were applied when seedlings had fully expanded cotyledons, but prior to the expansion of first true leaves. The polyploidization agent used was oryzalin, which was mixed with agar and applied as a droplet directly onto each meristem. Three oryzalin treatments were used with five replicates for each of the three flower colors. The first treatment received agar droplets with 50 µM oryzalin dissolved in a minimum amount of DMSO. The second and third treatments received agar droplets with 100 µM oryzalin. For the second treatment deionized water (DIW) was used to vortex the chemical until suspended, and in the third treatment DMSO was used as a solvent. Additionally, there was one control group for each flower color which received agar droplets containing DMSO without oryzalin.

The agar mix was prepared by mixing a 0.75% agar solution (150 mg agar powder thoroughly mixed in 20 mL of DIW), which was then autoclaved in autoclave-safe bottles. Pure oryzalin powder was measured and dissolved using one to two drops of DMSO. One agar solution bottle per oryzalin treatment was prepared, and the chemical was added after autoclaving to avoid degradation. Once the oryzalin was thoroughly mixed the solution was taken up with a disposable dropper. The dropper was then placed under cold water for 20–30 seconds to cool the solution and make its consistency thicker so a drop could be placed on the seedlings. All treated seedlings were placed in a growth chamber set at 23 °C (73 °F) with a 24-h photoperiod. Following a 24-h contact time, the agar on each seedling was removed using tweezers and its meristem was rinsed with water. Seedlings were kept in the growth chamber for an additional week, and then moved to the greenhouse. Once the seedlings had filled their cells, they were transplanted into 10-cm (4 inch) containers.

Flow cytometry. Two samples for each treatment replicate were analyzed through flow cytometry to assess their effectiveness to create tetraploids or ploidy chimeras. Survival rates and phytotoxic effects were documented for each treatment. For the determination and comparison of ploidy, suspensions of intact nuclei were prepared by chopping 40 mg of young plant tissue (Galbraith et al., 1983) and stained with propidium iodide (PI) (Arumuganathan et al., 1991). This protocol was modified by using leaf tissue from the lamina rather than from the mid-rib and lateral veins, so as to obtain more suspended nuclei. *Ruellia tweediana* 'RU2' and 'RU4', which are confirmed diploid and tetraploid individuals, respectively, were used as external controls. The relative fluorescence of nuclei was measured with a BD Accuri™ C6 Cytometer (BD Biosciences, San Jose, CA).

Stomata size and chloroplast number in stomata guard cells. The size and number of chloroplasts in stomata guard cells were determined to screen for potential polyploids. Healthy leaves were taken from the top four nodes of the dominant, treated apical stem, rinsed with DIW and then placed inside a growth chamber at 27 °C (80 °F) for up to one day. Leaves were placed in a water container to maintain them hydrated until sample preparation.

We found that it was difficult to peel Ruellia cuticles to prepare them for staining. Instead, better results were obtained by sanding the leaf adaxial surface with wet 3000 grade sandpaper until the area was transparent when viewed under light. Then a disk-shaped portion was cut using a hole-puncher and placed on plexiglas with the adaxial surface facing down. A roller was used to spread the tissue, and then each sample was transferred onto a microscope slide. Two to three drops of 1% Lugol's solution was added and allowed to stain for 2–3 minutes. The stain was then removed and replaced by DIW. A cover slip was placed on top of the sample and pressed to remove excess liquid. Each slide was viewed using a Zeiss Primo Star light microscope (Zeiss, Thornwood, NY) at 400X magnification. Stained chloroplasts within the guard cells were counted and the length of each stoma was recorded using a crossline eye piece micrometer. Two samples for each plant were prepared using different leaves, and data were recorded for 25 stomata per sample.

Results and Discussion

Polyploidization. Treatments 100 µM oryzalin and 50 µM oryzalin + DMSO did not cause seedling mortality. Seedlings treated with 100 µM oryzalin + DMSO were deteriorated and showed phytotoxic effects such as chlorosis and deformed foliage. Cultivars exhibited different levels of susceptibility depending on their flower color. It was observed that seedlings with white flowers were more susceptible to the treatments, while purple and pink flowered seedlings were less affected. For example, only three out of five white flowered seedlings survived the treatment with 100 µM of oryzalin + DMSO (60% mortality). All pink and purple flowered seedlings under this treatment survived, although they appeared deteriorated in terms of deformed foliage and reduced vigor. This indicates that cultivar specific susceptibility must be considered when working with high concentrations of oryzalin and when DMSO is added as a solvent.

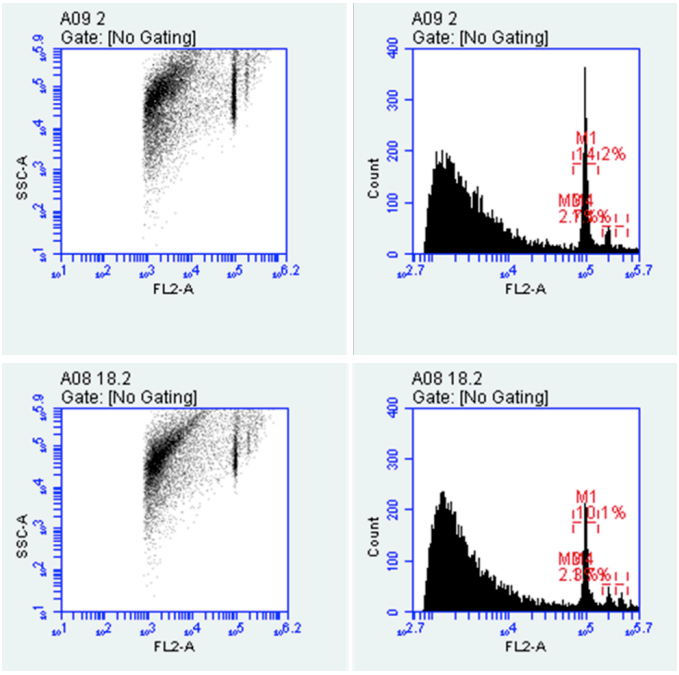

Fig. 1. Treatment of *Ruellia simplex* with 50 µM oryzalin + DMSO. Pink flowered control seedling with large diploid level peak and smaller tetraploid peak (top); pink flowered seedling with diploid and tetraploid peaks and an additional hexaploid peak (bottom).

Flow cytometry indicated that peaks in all control seedlings coincided with those from the diploid control 'RU2'. Diploid curves include a large peak, along with a smaller tetraploid peak due to dividing cells in new or expanding leaves. The tetraploid peak is sometimes less noticeable when tissue is extracted from older leaves. With the 50 µM oryzalin + DMSO treatment, most seedlings had very similar flow cytometry curves to the treatment controls and the diploid control. Only two of the 15 seedlings treated (13%) showed ploidy differences: one pink flowered seedling had a small peak of hexaploid cells in addition to the diploid and tetraploid peaks (Fig. 1); one white flowered seedling had a shift of the diploid peak toward the triploid level.

Three of the 15 seedlings (20%) treated with 100 µM of oryzalin (and no DMSO) had ploidy differences that were more noticeable than in the previous treatment. One purple flowered seedling had a clear dominant tetraploid peak, with the diploid peak being almost completely absent. A second purple flowered seedling had a triploid peak, and one white flowered seedling had both tetraploid and hexaploid peaks (Fig. 2). With this treatment, seedlings exhibited reduced vigor compared to those treated with 50 µM oryzalin + DMSO, and most of the flow cytometry curves coincided with the treatment controls and the diploid control. However, the higher concentration of oryzalin resulted in more effective ploidy increases, even without the use of a solvent.

The treatment 100 µM oryzalin + DMSO had the most effect in polyploidizing seedlings. Eight of the 15 seedlings treated (53%) had differences in ploidy. Two purple flowered seedlings had differences between samples, where one had diploid and the other a tetraploid peak, indicating diploid-tetraploid chimerism (Fig. 3). Another purple flowered seedling had a triploid peak. There were three seedlings (one pink and two white flowered) which had tetraploid peaks, and one pink flowered seedling had both tetraploid and hexaploid peaks.

Results indicate that high concentrations of oryzalin have a higher success rate in polyploidizing *Ruellia*, and that the use of proper solvents such as DMSO can further improve ploidy conversions. Doubling the concentration of oryzalin to 100 µM and using DMSO increased polyploidization rates by 2.5 fold.

Stomata size and chloroplast number. Considerations to select ploidy screening methods involves ease of sample preparation and data collection, as well as time and inputs needed. Most importantly, they must highly correlate with flow cytometry results. Diploid seedlings had an average of 12 chloroplasts per stoma, while tetraploids averaged between 20–24 chloroplasts per stoma. Average stoma length varied from 22.5 µM for diploid individuals to 27.5–30.0 µM for tetraploids, but no difference was found in average stomata lengths between the control seedlings and those treated with oryzalin (Table 1).

The preparation of samples to count chloroplasts or to measure stomata length was the same; however, it took considerably more time to take stomata length measurements. About 48 h were needed to collect data of chloroplast counts and stomata length for 60 plants. This is much faster than flow cytometry analysis,

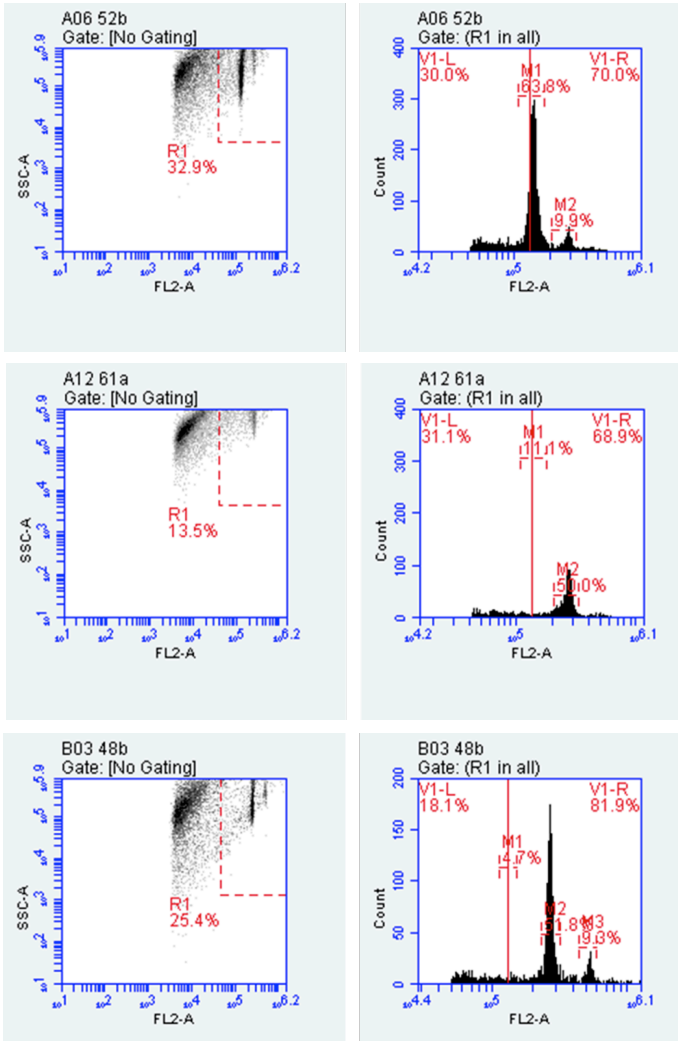

Fig. 2. Treatment of *Ruellia simplex* with 100 µM of oryzalin (without DMSO). Purple flowered control seedling with diploid peak and smaller tetraploid peak (**top**); purple flowered seedling with dominant tetraploid peak (**middle**); white flowered seedling with tetraploid peak and additional hexaploid peak (**bottom**).

Table 1. Average and standard deviation for chloroplast number and average stomata length in *Ruellia simplex* for control and oryzalin treatments.

Treatment for each flower color	Average chloroplast no. ± SD	Average stomata length (µM)
Control White	12.1 ± 0.1	0.9
Control Pink	12.0 ± 0.1	1.0
Control Purple	12.3 ± 0.1	0.9
Average	12.2 ± 0.2	0.9
50 µM + DMSO White	13.1 ± 2.3	1.0
50 µM + DMSO Pink	17.6 ± 4.2	1.0
50 µM + DMSO Purple	13.4 ± 0.9	1.0
Average	14.8 ± 3.5	1.0
100 µM White	20.7 ± 5.9	1.2
100 µM Pink	14.6 ± 2.0	1.0
100 µM Purple	16.0 ± 4.0	1.1
Average	17.4 ± 5.1	1.1
100 µM + DMSO White	15.1 ± 3.3	1.0
100 µM + DMSO Pink	16.8 ± 4.0	1.1
100 µM + DMSO Purple	15.3 ± 2.4	1.0
Average	15.9 ± 3.4	1.0

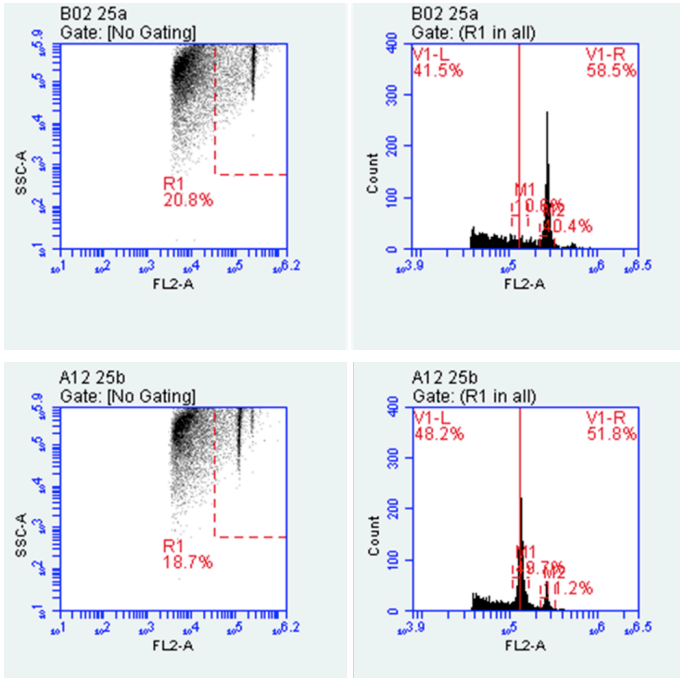

Fig. 3 Treatment of *Ruellia simplex* with 100 µM oryzalin + DMSO. Purple colored seedling displaying diploid-tetraploid chimerism: different samples collected from the same plant show either a tetraploid peak (**top**) or diploid peak (**bottom**).

where about 12 h were needed for 12 samples (five days for 60 plants). In regards to accuracy, counting chloroplasts in stomata guard cells gave more consistent data than measurements of stomata length, and correlated well with flow cytometry results. For these reasons, screening of large number of plants prior to flow cytometry analysis increases efficiency and reduces cost.

Conclusion

In this research we evaluated different concentrations of oryzalin as a polyploidization agent, finding that 100 µM was most effective. Additionally, using DMSO as a solvent for oryzalin increases its effect, even though it results in phytotoxicity. Counting the number of chloroplasts in the stomata guard cells is an efficient method to do a preliminary screen of polyploids in large *Ruellia* plant populations, giving consistent results that correlate well with flow cytometry data. It is easy to prepare samples using low inputs and short time.

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