J. Amer. Soc. Hort. Sci. 134(5):553–559. 2009.

An Oryzalin-induced Autoallooctoploid of *Hibiscus acetosella* 'Panama Red'

Ryan N. Contreras[1,3] and John M. Ruter[2]
Department of Horticulture, The University of Georgia, Tifton Campus, Tifton, GA 31793-0748

Wayne W. Hanna[2]
Department of Crop and Soil Science, The University of Georgia, Tifton Campus, Tifton, GA 31793-0748

Additional index words. cytochimera, cytology, flow cytometry, polyploidy, sterility

Abstract. ***Hibiscus acetosella*** **Welw. ex Hiern. 'Panama Red' PP20,121 (Malvaceae) has generated public and grower interest due to its attractive red foliage and vigorous growth, however, a horticultural goal is to develop more compact forms. Even though organs of induced polyploids are often larger than the wild type, whole plants are often shorter in stature. Three studies were conducted to induce polyploidy and to evaluate the growth and reproductive potential of the resulting polyploids. In study 1, seeds were soaked for 24 hours in aqueous solutions of 0%, 0.2%, 0.4%, or 0.5% colchicine (w/v) plus 0.5% dimethyl sulfoxide. In studies 2 and 3, apical meristems of seedlings at the cotyledon stage were treated for 1 or 3 days with 0, 50, 100, or 150 μM oryzalin solidified with 0.8% agar. Visual observations and measurement of guard cells were used to identify plants that potentially had their chromosome number doubled. Flow cytometry of nuclei stained with DAPI was used for confirmation of polyploidy. No induced polyploidy was observed following seed treatment with colchicine at the rates and duration used in this study. One-time application of 50 μM oryzalin resulted in a single mixoploid (4*x* + 8*x*) in which the ploidy of the L-I, L-II, and L-III histogenic layers were identified as a 4–4-4 + 8, respectively. Three-day applications with 100 and 150 μM oryzalin resulted in an octoploid (8*x*) and a mixoploid (4*x* + 8*x*), respectively. The mixoploid from the 3-day treatment stabilized at the 8*x* level before flowering, but was identified as a 4 + 8-*x*-4 cytochimera. Plant height was reduced, leaves were smaller, internodes were shorter, and canopy volume was reduced in the octoploid (8*x*) form compared with the tetraploid (4*x*) form. Furthermore, in contrast to the tetraploid, the octoploid produced no self-pollinated seed and performed poorly as a staminate and pistillate parent in controlled crosses. This represents the first time oryzalin has been reported to induce polyploidy in *Hibiscus* L. section *Furcaria* DC. *H. acetosella* is an allotetraploid species with the genome composition AABB. The resulting autoallooctoploid (AAAABBBB) form of 'Panama Red' exhibits a more compact habit and reduced production of seed.**

The genus *Hibiscus* (tribe Hibisceae) is comprised of ≈200 (Fryxell, 1988; Hochreutiner, 1900) to 250 (Bates, 1965) annual and perennial species in 10 sections (Fryxell, 1988). Section *Furcaria* is a circumglobal tropical and subtropical (rarely temperate) group (Wilson and Menzel, 1964) comprised of more than 100 species (Wilson, 1994). This group includes important fiber, food, and medicinal plants such as kenaf (*Hibiscus cannabinus* L.) and roselle (*Hibiscus sabdariffa* L.). Many species in this section have been used as ornamentals for their large, showy flowers (Wilson, 1994). The base chromosome number in section *Furcaria* is commonly regarded as x = 18 (Menzel and Wilson, 1961, 1963a, 1963b; Skovsted, 1941), but x = 9 has also been proposed (Sanyal and Kundu, 1959). Most species in section *Furcaria* are allopolyploids ranging from $2n = 4x = 72$ to $2n = 10x = 180$, with representative species on all continents with tropical or subtropical regions (Menzel et al., 1986).

There are a number of species in section *Furcaria* with ornamental potential. One that is of interest and was used in the current research is *Hibiscus acetosella. H. acetosella* is a tetraploid [$2n = 4x = 72$ (Akpan, 2000; Menzel and Wilson, 1961)] of African origin known only in cultivation (Siemonsma and Piluek, 1993; Wilson, 1994). Based on the prevalence of bivalents at metaphase I (MI) (Akpan, 2000; Kuwada, 1977) and genome studies (Menzel and Wilson, 1961), *H. acetosella* has been determined to be an allotetraploid with the genome composition AABB (Menzel and Wilson, 1961). It is widely grown as an ornamental in tropical and subtropical regions (Wilson, 1994). It is an annual or perennial herb or undershrub, typically with red foliage. Solitary flowers of wine-red, rarely yellow, are formed in the axils (van Borssum Waalkes, 1966). A number of selections have been released over the years, including a recent release from The University of Georgia, known as *H. acetosella* 'Panama Red' PP20,121. This release has generated interest in the green industry due to its attractive foliage and vigorous growth; however, evaluator and consumer comments have indicated that 'Panama Red' could be improved by developing more compact forms.

Induced polyploidy often results in the gigas effect of individual organs; particularly those with determinate growth such as sepals, petals, fruits, and seeds (Stebbins, 1950). However, in the case of induced autopolyploids, the growth rate of whole plants is often slower (Stebbins, 1950). Kumar Sen and Chheda (1958) found that induced tetraploids of black

Received for publication 30 July 2009. Accepted for publication 22 Sept. 2009. We thank Bruce Tucker, Nancy Hand, and Brandon Coker for their technical assistance. We also thank Peggy Ozias-Akins, David Knauft, and Brian Schwartz for discussion and critical review of the manuscript.
From a dissertation submitted by R.N.C. as partial fulfillment of the requirements for the Ph.D. degree.
[1]Graduate Research Assistant.
[2]Professor.
[3]Corresponding author. E-mail: rncontre@uga.edu.

gram (*Phaseolus mungo* L.) were stunted and exhibited reduced fertility. Induced polyploids of oil palm (*Elaeis guineensis* Jacq.) were also reported to be shorter than diploids (Madon et al., 2005). The current research was conducted to take advantage of the reduction in plant size and vigor often associated with induced polyploidy. The principle objectives were to induce polyploidy in seedlings from *H. acetosella* 'Panama Red' and to evaluate the effects on growth, morphology, and fecundity. Upon discovering that treatments induced two cytochimeras, ploidy of the histogenic layers of these plants was investigated.

Materials and Methods

Plant material

Seeds from autonomously self-pollinated flowers were collected from *H. acetosella* 'Panama Red' plants grown in a glasshouse. Plant material was previously described as a hybrid between *H. acetosella* × *Hibiscus radiatus* Cav. (Contreras and Ruter, 2009), however, the seed source was later correctly identified as 'Panama Red' based on morphology (personal observation). Plants resulting from self-pollination of 'Panama Red' are uniform and are nearly identical to the cultivar.

Chromosome doubling studies

Seed treatment with colchicine. Seeds were pretreated by soaking in an aqueous solution of 0.1% (v/v) Triton® X-100 (Integra Chemical, Renton, WA) for 24 h on a rotary shaker (model G-33; New Brunswick Scientific, Edison, NJ) at 150 rpm. Following pretreatment, seeds were transferred to solutions containing 0%, 0.2%, 0.4%, or 0.6% (w/v) colchicine (Sigma-Aldrich, St. Louis), plus 0.5% (v/v) dimethyl sulfoxide [DMSO (Sigma-Aldrich)] as an adjuvant. Treatments (75 seeds/treatment) were applied for 24 h on the rotary shaker at 150 rpm. Following treatment, seeds were rinsed under running tap water for 15 min and sown in 200-cell polystyrene tobacco float trays containing Pro-Mix BX with Biofungicide (Premier Tech, Quakertown, PA). Trays were placed in a glasshouse with natural daylength and set day/night temperatures of 27/20 °C.

One-day shoot tip treatment with oryzalin. Seeds were pretreated by soaking in water and surfactant, and were then germinated as described above. Seedlings were then transferred to 0.2-L containers filled with Pro-Mix BX with Biofungicide. Treatments consisted of 0, 50, 100, or 150 μM oryzalin solutions (supplied as Surflan® AS; United Phosphorus, Trenton, NJ) solidified with 0.8% (w/v) agar (Becton, Dickinson; Sparks, MD). About 25 μL of each solution was added while still liquid and allowed to solidify. Each treatment was applied to 25 seedlings. A single application was made in the laboratory to the shoot tip before emergence of the first true leaves. Twenty-four hours after treatment, the seedlings were moved to a glasshouse.

Three-day shoot tip treatment with oryzalin. Seedlings were prepared and treated as in study 2; however, three applications were applied on consecutive days. A total of 52 seedlings was treated, with 13 seedlings per treatment.

Identification of polyploids. Phenotypic observations such as thicker leaves and stems and increased guard cell length were used to identify plants that potentially had a doubled chromosome number. Guard cell lengths were measured by applying ink to the abaxial side of leaves using a felt tip marker, pressing with clear tape, and then placing the tape on a microscope slide. Slides were then observed under a light microscope equipped with an ocular micrometer, and the ink outline of guard cells was measured at ×400 magnification. Three replicates (leaves) and 10 subsamples (guard cells) per taxon were measured.

Putatively doubled plants were then screened using flow cytometry. About 1 cm^2 of newly expanded leaf tissue was finely chopped with a razor blade in a petri dish with 400 μL of nuclei extraction buffer (CyStain ultraviolet Precise P Nuclei Extraction Buffer; Partec, Münster, Germany). The solution was filtered using Partec CellTrics™ disposable filters with a pore size of 50 μm to remove leaf tissue. Nuclei were stained with 1.6 mL of 4',6-diamidino-2-phenylindole (DAPI) staining buffer (CyStain ultraviolet Precise P Staining Buffer, Partec) and were incubated for 1 to 2 min at ≈25 °C. The suspension was analyzed using a flow cytometer (Partec PAS-III) to determine mean relative DNA fluorescence [mean relative fluorescence (MRF)]. Ploidy and genome size were determined by comparing the MRF of each sample with the 2C peak of diploids and an internal standard of known genome size. *Pisum sativum* L. 'Ctirad', with a genome size of 8.76 pg (Greilhuber et al., 2007), was used as an internal standard to calculate nuclear DNA content [2C DNA content of sample in picograms = 8.76 pg × (MRF sample/MRF standard)]. For analysis of leaf tissue the CV percentage (CV%) was ≤2.00, and at least 2500 nuclei were analyzed with the exception of the untreated control (1393 nuclei). For analysis of root tissue, CV% was ≤3.50, and 5000 nuclei were analyzed.

Cytological analysis. Cuttings were taken from tetraploid *H. acetosella* 'Panama Red' and placed in rooting substrate composed of 1 pine bark:2 perlite (by volume) under intermittent mist at a rate of 4 s every 15 min. Rooted cuttings were transplanted into 3.8-L containers and grown in trays containing vermiculite. Roots were allowed to grow out of containers into vermiculite for collection. Actively growing, healthy root tips were collected on sunny mornings before 1000 HR and were pretreated for 1 to 2 h in an aqueous solution of 2 mM 8-hydroxyquinoline (Fisher Scientific, Suwanee, GA) + 0.24 mM cycloheximide (Acros Organics, Morris Plains, NJ) at 4 °C. Following pretreatment, roots were transferred to Carnoy's solution [6 100% EtOH:3 chloroform:1 glacial acetic acid (by volume)] and fixed overnight at 25 °C. Roots were rinsed with deionized water and transferred to 70% EtOH (v/v) and stored at 4 °C until observation. Roots were hydrolyzed for 30 to 45 s in an aqueous solution containing 1 concentrated (12 N) HCl:3 100% EtOH (by volume). Root tips were removed with a razor blade and cells were spread by pressing under a cover slip; chromosomes stained using modified carbol fuchsin (Kao, 1975). Chromosomes from five cells were counted.

Ploidy of histogenic layers of cytochimeras. Plants that were identified as cytochimeras, also referred to as mixoploids, using flow cytometry on leaf material were further investigated to determine which histogenic layer(s) were doubled. L-I (Dermen, 1960) was evaluated by measuring guard cell length as above. Utility of guard cell length as a tool to discern 4*x* from 8*x* in the L-I histogenic layer was confirmed by comparing the untreated control to 8*x*. Ploidy of the L-III histogenic layer (Dermen, 1960) was evaluated by flow cytometry of root tissue, as described above for leaf tissue. Pollen was measured in an attempt to determine the ploidy of L-II (Dermen, 1960), but the

diameter of pollen from 4*x* and 8*x* plants was not different. Therefore, L-II was evaluated by germinating seeds from mixoploids and measuring the ploidy of their progeny. It has been previously established that the epidermis is derived from L-I, the germ line is derived from L-II, and adventitious roots from stem cuttings are derived from L-III (Dermen, 1960). Data were subjected to analysis of variance, and mean separation was conducted using Duncan's multiple range test.

Growth and foliar measurement of tetraploid and octoploid. Cuttings from tetraploid and octoploid *H. acetosella* 'Panama Red' were collected and rooted as described above at the same time. Rooted cuttings were then transferred into #1 containers (2.8 L) containing 8 pine bark:1 sand amended with 0.91 kg of dolomitic lime and 0.45 kg of Micromax (Scotts, Marysville, OH) and were fertilized with 15 g of Osmocote Plus 15–3.96–9.13 (Scotts) and grown for 2 months. Plants were pruned to ≈25 cm at the time of field planting to provide uniform material. Two and three plants of tetraploid and octoploid 'Panama Red', respectively, were transplanted to the field on 14 May 2009 at The University of Georgia, Tifton. On 15 July 2009, plant height, plant widths taken perpendicular to each other (width 1 and width 2), internode lengths, and leaf lengths and widths were measured. Plant height was measured a second time on 3 Sept. 2009. Crown volume was calculated by multiplying plant height × width 1 × width 2 and is expressed in cubic meters. Internodes on three stems were measured between the sixth and eleventh (five internodes per stem) nodes basal from the shoot tip. Leaves six through 10 (five leaves per stem) basal from the shoot tip were measured on three stems. Data were analyzed using a *t* test for pairwise comparison.

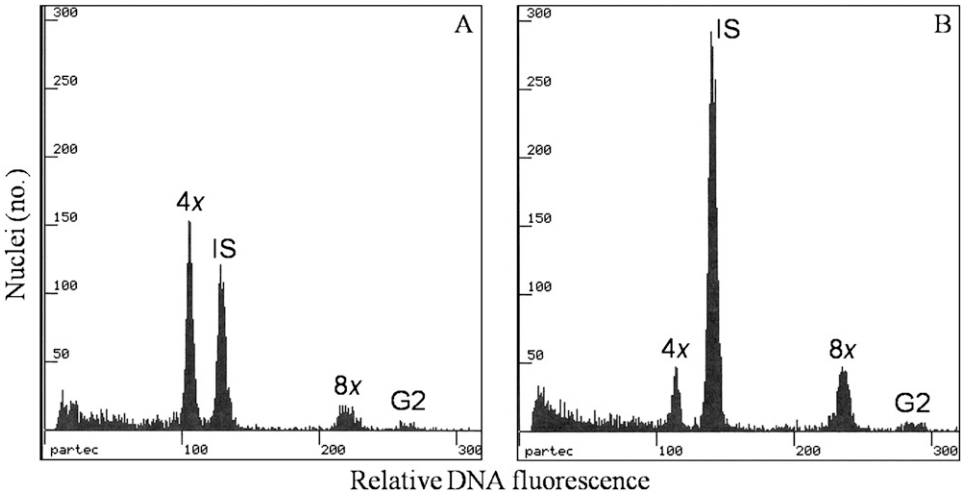

Fig. 1. Histograms generated using flow cytometry on leaves from the two cytochimeras (HIB-33 and HIB-41) of *Hibiscus acetosella* 'Panama Red' PP20,121 composed of tetraploid (4*x*) and octoploid (8*x*) cells. (**A**) HIB-33; 4*x* mean relative fluorescence (MRF) = 106, IS MRF = 128, 8*x* MRF = 216; and (**B**) HIB-41; 4*x* MRF = 114.5; IS MRF = 140.5; 8*x* MRF = 235.5. (IS = *Pisum sativum* 'Ctirad') and its G2 peak is present in **A** and **B**.

Results

Chromosome doubling studies

Seed treatment with colchicine. No induced polyploidy was observed with the rate of colchicine tested. Germination was uniform and percentage was unaffected by treatment; all treatments germinated at 100%. Seedlings exhibited no phenotypic evidence of induced polyploidy.

One-day shoot tip treatment with oryzalin. No mortality was observed in any of the single applications of oryzalin. A single plant treated with 50 μM showed altered morphology typical of induced polyploidy. Flow cytometric analysis suggested that it was a 4*x* + 8*x* cytochimera (Fig. 1A). This plant hereafter will be referred to as HIB-33.

Three-day shoot tip treatment with oryzalin. No mortality was observed in any of the treatments following application of oryzalin on 3 consecutive days. Two polyploids were identified. One plant each from the 100 and 150 μM treatments were determined to be 8*x* (Figs. 2, B and C, and 3) and 4*x* + 8*x* (Fig. 1B), respectively. Genome sizes were calculated to be 2C = 7.27 pg and 2C = 14.85 pg for tetraploid (4*x*) and octoploid (8*x*) *H. acetosella* 'Panama Red' (Fig. 2C); a ratio of 2.05. The cytochimera induced in this study will hereafter be referred to as HIB-41.

Cytological analysis. Chromosome spreads from root tips showed that the untreated control was a tetraploid [(2*n* = 4*x* = 72) (Fig. 3)]. This was used as a basis to set the fluorescence channel for the tetraploid at 100 and allowed us to determine that tissue that had twice the fluorescence was octoploid.

Ploidy of histogenic layers of cytochimeras. HIB-33 was a cytochimera in which the L-I, L-II, and L-III histogenic layers were, 4—4-4 + 8, respectively. The L-I histogenic layer was not different from the control (Table 1) and the root tissue was found to be mixoploid (Fig. 4A). Thirty-two seedlings resulting from self-pollination of HIB-33 were found to be tetraploid using flow cytometry (data not shown). HIB-41 was a 4 + 8-*x*-4 cytochimera. Mean guard cell length of HIB-41 was intermediate between tetraploid and octoploid means (Table 1) and root tissue contained only tetraploid cells (Fig. 4B). Before flowering, HIB-41 stabilized at the 8*x* level with no evidence of chimerism (data not shown), therefore, the L-II could not be identified.

Growth and foliar measurement of tetraploid and octoploid. In morphological comparison of field-grown material, the octoploid was shorter on both measurement dates, had a smaller canopy volume, shorter internodes, and smaller leaves than tetraploid *H. acetosella* 'Panama Red' (Table 2, Fig. 5).

Discussion

Cytological analysis confirmed that *H. acetosella* 'Panama Red' is a tetraploid (2*n* = 4*x* = 72), which allowed for accurate identification of induced polyploids with flow cytometry when 'Panama Red' was used as a standard. Even though only five cells provided unambiguous counts due to the high number of chromosomes, flow cytometric data showed no evidence of chimerism in the tetraploid (Fig. 2A). The genome size calculated for the tetraploid in the current study is slightly

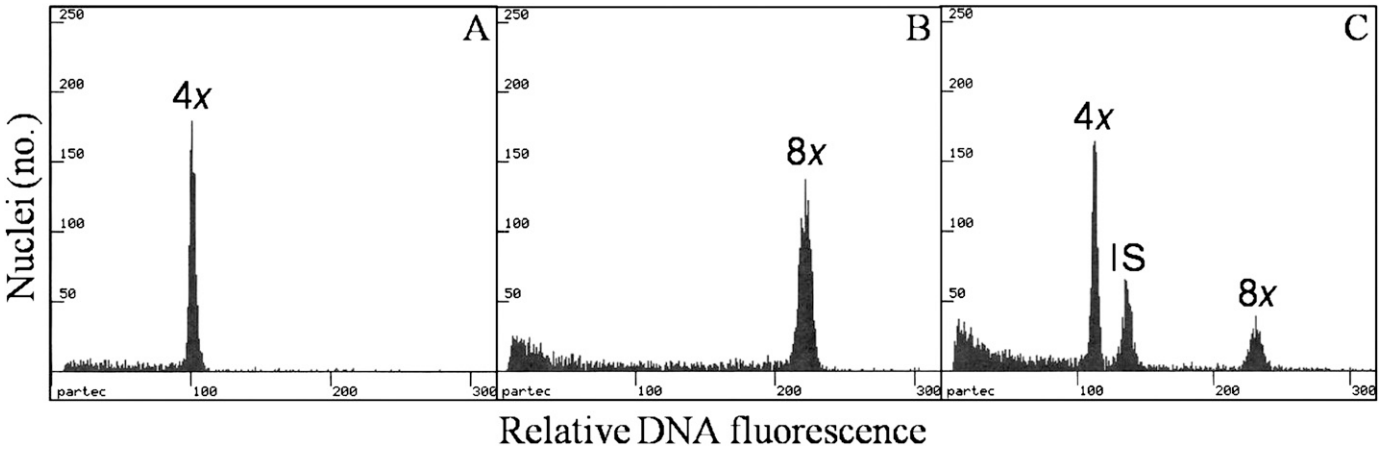

Fig. 2. Histograms generated using flow cytometry from leaves of (**A**) untreated tetraploid (4*x*) with a mean relative fluorescence (MRF) = 101.5, (**B**) successfully doubled octoploid (8*x*) with a MRF = 222, and (**C**) combination of leaf tissue from an untreated tetraploid (4*x*, MRF = 113), an internal standard (IS = *Pisum sativum* 'Ctirad'; 8.76 pg; MRF = 136.5), and the octoploid (8*x*; MRF = 231.5). In **C**, the ratio of the MRF of 8*x*:4*x* was 2.05, and genome sizes were calculated as 7.27 and 14.85 pg for 4*x* and 8*x*, respectively.

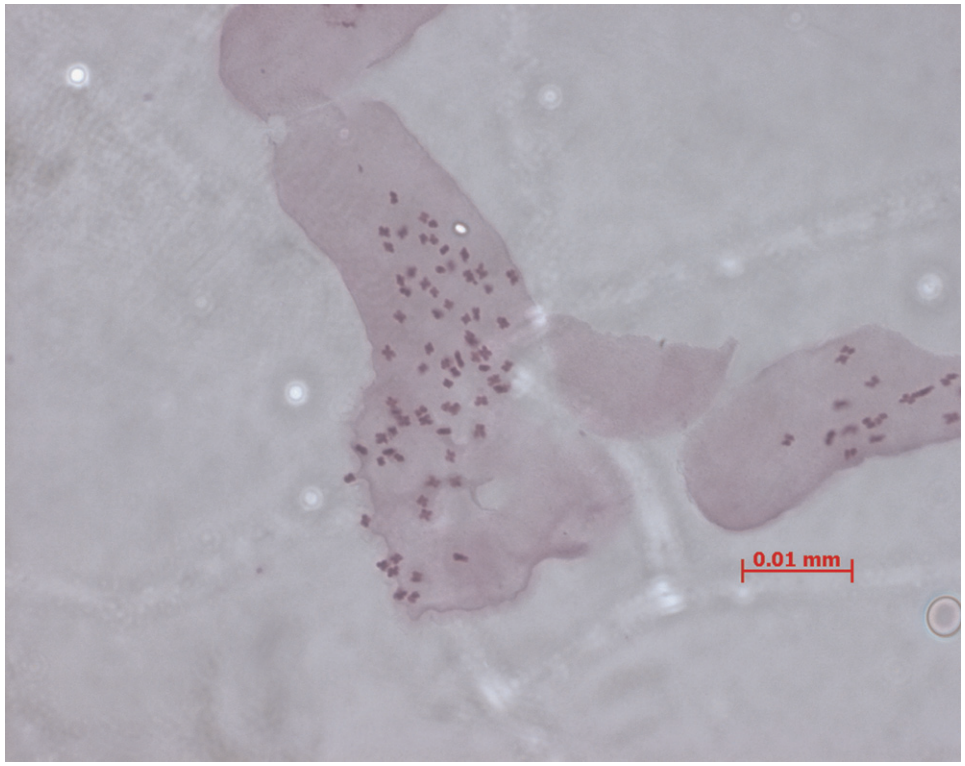

Fig. 3. Photomicrograph of a root tip squash of $2n = 4x = 72$ *Hibiscus acetosella* 'Panama Red' PP20,121 stained using a modified carbol fuchin technique and taken at ×1000 magnification. Chromosomes of five unambiguous cells were counted.

larger (7.27 pg), but similar to the 4C = 6.1 pg calculated for *H. cannabinus* by Bennett et al. (2000); a difference of less than 20%. The discrepancy could be attributed to interspecific variation. For example, in the related genus *Gossypium* L., 25 records compiled for 1C DNA ranged from 1.20 pg to 3.23 pg (Bennett and Leitch, 2005). Differences may also be attributed to technique. Our study was conducted using DAPI, which binds preferentially to AT, while Bennett et al. (2000) used propidium iodide (PI), a DNA intercalator that binds uniformly. DAPI has the potential to overestimate genome sizes because many families and genera have greater than 50% AT base composition (Meister and Barow, 2007).

Colchicine treatment of seeds of *H. acetosella* 'Panama Red' was unsuccessful in inducing chromosome doubling in the current study. Treating seeds with similar rates of colchicine has been successful in related diploid species *Gossypium herbaceum* L. and *Gossypium arboreum* L. (Omran and Mohammad, 2008). With a 16-h treatment of 0.2%, 0.4%, and 0.6% colchicine, Omran and Mohammad (2008) reported 1.0%, 6.6%, and 92.5% of seedlings had tetraploid cells.

Furthermore, in their study, viable seeds were reduced to 60.8% after treatment with 0.6% colchicine for 16 h, while in our study, seeds treated at the same concentration for 24 h germinated at 100%.

A single application of oryzalin yielded one cytochimera, which resulted from the 50 μM treatment. Application of oryzalin on 3 d (consecutive) yielded one octoploid and one cytochimera from the 100 and 150 μM treatments, respectively. Van Laere et al. (2006) treated seedlings of *Hibiscus syriacus* L. 'Oiseau Blue' and 'Woodbride' for 10 d (consecutive) with 0.2% colchicine + 2% DMSO to obtain 24.5% and 41.8% doubling in the two cultivars, respectively. The greater success obtained in the study by Van Laere et al. (2006) may be attributed to the longer treatment duration. Furthermore, a lower concentration of agar and preventing the agar droplet from desiccating may have allowed greater uptake of oryzalin. Jones et al. (2008) achieved as high as 41% induction of stabile tetraploids of *Rhododendron* L. 'Summer Lyric' by treating seedlings with 50 μM oryzalin solidified with 0.55% agar and maintaining seedlings in a humid chamber (100% humidity) to maintain the integrity of the agar droplet.

The length, width, and chloroplast number in guard cells have been positively correlated to ploidy in *Hibiscus schizopetalus* Hook.f., *Hibiscus mutabilis* L., and *Hibiscus rosa-sinensis* L. (Zhuang and Song, 2005). Colchiploids of *H. syriacus* also exhibit longer guard cells (Lee and Kim, 1976). A similar correlation was found in the current study between guard cell length and ploidy (Table 1), indicating that measurement of guard cell length is a useful tool in preliminary screening for polyploids. However, the time taken to prepare slides is similar to that for preparation of flow cytometry samples. Therefore, if a flow cytometer is readily available, the utility of measuring guard cells is in identification of the ploidy of L-I histogenic layer. Guard cell length for the tetraploid was statistically different from the octoploid. Mean guard cell length of HIB-33 was not statistically different from the tetraploid, which indicates that the L-I histogenic layer of HIB-33 is tetraploid. However, mean guard cell length of HIB-41 was intermediate and statistically different from the tetraploid and octoploid. The L-I histogenic layer of HIB-41 appears to be a composite of tetraploid (smaller) and octoploid (larger) cells, resulting in an intermediate mean size.

Table 1. Results of measurements of guard cells and pollen grains of tetraploid (4*x*), octoploid (8*x*), and cytochimeric (4*x* + 8*x*) *Hibiscus acetosella* 'Panama Red' PP20,121 plants using an ocular micrometer.

Ploidy	Pollen diam (μm)[z]	Guard cell length (μm)[z]
4*x*	173.9 a[y]	25.1 c
8*x*	169.5 a	36.1 a
4*x* + 8*x* (HIB-33)	173.2 a	24.4 c
4*x* + 8*x* (HIB-41)	175.5 a	27.3 b

[z]Mean pollen diameter, including the projections of the exine. Data are presented as means of three replicates of 25 and 10 subsamples for pollen diameter and guard cell length, respectively.
[y]Different letters within a column indicated statistical difference based on Duncan's multiple range test at $P < 0.05$.

In contrast to the findings of Zhuang and Song (2005) and Lee and Kim (1976), our research found no correlation between pollen size and ploidy. The lack of correlation between pollen size and ploidy precluded using pollen size to determine ploidy of L-II, leading to the evaluation of progeny resulting from self-pollination. Inference about the ploidy of the L-II histogenic layer of cytochimeras has been drawn by evaluating the ploidy of self-pollinated progeny. Olsen et al. (2006) reported that the L-II was tetraploid in cytochimeras of ×*Chitalpa tashkentensis* Elias & Wisura 'Pink Dawn' after flow cytometric analysis of progeny revealed that they were tetraploid. Identification of the ploidy of L-III in cytochimeras was previously accomplished by mitotic chromosome counts of root meristematic cells in the genus *Arachis* L. (Singsit and Ozias-Akins, 1992). Our study used flow cytometry to determine ploidy of the L-III (Fig. 4), which provides a more rapid method.

Plant height was reduced, leaves were smaller, internodes were shorter, and canopy volume was reduced in the octoploid form of *H. acetosella* 'Panama Red' when compared with the tetraploid. Morphology of the induced polyploid compared with the standard cytotype is similar to that described by Menzel and Wilson (1963b). A spontaneous allododecaploid ($2n = 12x = 216$) arose from a hybrid between *H. radiatus* × *Hibiscus diversifolius* Jacq. that Menzel and Wilson (1963b) reported as having reduced vigor and smaller leaves than the standard F_1. The agreement between the morphology of the study by Menzel and Wilson (1963b) and the current study suggests that a similar response in form may be expected throughout section *Furcaria*. Reduced overall plant size has been reported frequently among other induced polyploids. In his review of the use of induced polyploidy in breeding of agronomic crops, Randolph (1941) reported that dodecaploid (12*x*) *Triticum aestivum* L., *Solanum tuberosum* L., and *Solanum andigenum* Juz. & Bukasov were all less vigorous than standard cytotypes. Conversely, tetraploids of *Solanum jamesii* Torr., *Solanum chacoense* Bitter, and *Solanum bulbocastanum* Dunal were as vigorous, or more so, than diploids. Tetraploid strains of maize (*Zea mays* L.) are also often taller than their diploid progenitors; however, octoploid forms are much less vigorous than tetraploids and are sterile.

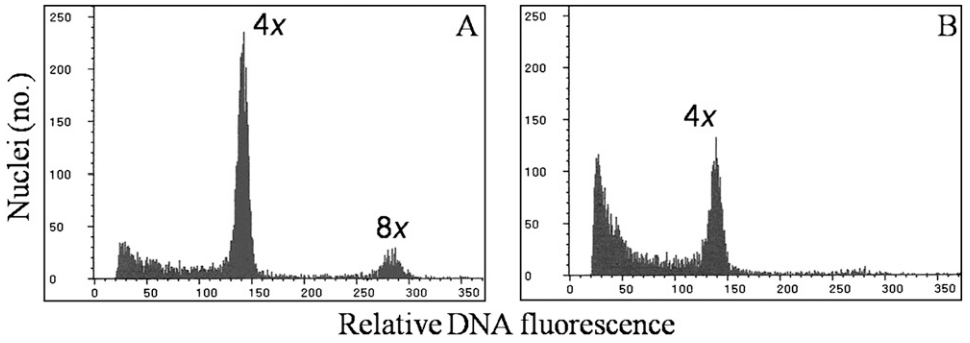

Fig. 4. Histograms generated using flow cytometry on root tissue of cytochimeras (HIB-33 and HIB-41) of *Hibiscus acetosella* 'Panama Red' PP21,120. (**A**) HIB-33 is L-III 4*x* + 8*x*; 4*x* mean relative fluorescence (MRF) = 141 and 8*x* MRF = 282 and (**B**) HIB-41 is L-III 4*x*; 4*x* MRF = 138.

Rigorous and formal investigation of fertility of the induced

Table 2. Morphological comparison of tetraploid (4*x*) *Hibiscus acetosella* 'Panama Red' PP20,121 to induced octoploid (8*x*). All material was propagated at the same time and was transplanted to the field on 14 May 2009 and grown under the same conditions at the University of Georgia, Tifton (USDA Zone 8a). For plant height and canopy volume, n = 2 for 4*x* and n = 3 for 8*x*, n = 3 (five subsamples) for internode length, leaf length, and leaf width. All data are presented as means; pairwise comparisons conducted using a *t* test.

	8*x*	'Panama Red'	*P*
Plant height 1 (cm)[z]	95.7	117.5	0.0370
Plant height 2 (cm)[z]	146.2	196.1	0.0022
Canopy volume (m^3)[y]	1.16	4.14	0.0002
Internode length (cm)[x]	2.25	4.09	<0.0001
Leaf length (cm)[w]	7.03	9.66	<0.0001
Leaf width (cm)	6.35	10.87	<0.0001

[z]The tallest branch on each plant was measured. Plant height 1 was recorded 15 July 2009, plant height 2 was recorded 3 Sept. 2009.
[y]Canopy volume was calculated by multiplying height × width 1 × width 2. Width 1 and width 2 were measured perpendicular to each other.
[x]Five internodes (subsamples) were measured between the sixth and eleventh nodes basal from the shoot tip on three stems.
[w]The sixth to eleventh leaves (five subsamples) basal from the shoot tip were measured on three stems.

Fig. 5. Container-grown octoploid (left) and -tetraploid (right) *Hibiscus acetosella* 'Panama Red' photographed 13 Aug. 2009.

polyploids was not conducted; however, much anecdotal evidence has been observed. *H. acetosella* 'Panama Red' produces large amounts of autonomously self-pollinated seeds when grown in a glasshouse. The induced octoploid produced no autonomously self-pollinated seeds to date, and has performed poorly as a staminate and pistillate parent in a limited number of controlled crosses. Eleven flowers on the octoploid were pollinated using pollen from *H. acetosella* 'Panama Red' in 2008 and only a single, abnormal seed was obtained, which did not germinate. Further evaluation of the octoploid using a greater number of controlled crosses with fertile and compatible mates is warranted. *H. acetosella* is an allotetraploid with the genome composition AABB and, on average, produces 34 bivalents (II) per pollen mother cell [(PMC) (Menzel and Wilson, 1961)]. We propose that the resulting induced polyploid is an autoalloctoploid with the genome composition AAAABBBB.

It is likely that the induced autoallooctoploid produces an increased number of multivalents, which contributes to its sterility. Increased multivalent production in induced polyploids has been observed by Menzel and Wilson (1961), who reported bivalent pairing (17.9 II per cell) in diploid *H. cannabinus* (2*n* = 2*x* = 36), but in the induced tetraploid (2*n* = 4*x* = 72), there were nearly 10 quadrivalents (IV) per cell. Unfortunately, they did not report on the fertility of the autotetraploid. Tetraploid *H. acetosella* has been reported to produce an average of 34II, 0.1III, and 0.6IV per cell (Menzel and Wilson, 1961). Based on these findings, it is probable that an increase in the production of multivalents has occurred in the induced autoallooctoploid *H. acetosella*, which has led to the reduction in fertility. Support for this idea has been observed in autotetraploid forms of maize (Gilles and Randolph, 1951), pearl millet [*Pennisetum glaucum* (L.) R. Br.] (Gill et al., 1969; Jauhar, 1970), and safflower (*Carthamus tinctorius* L.) (Schank and Knowles, 1961) where it was observed that after selection for increased fertility, there was a corresponding decrease in multivalent formation, thus showing an inverse relationship between multivalent formation and fertility. However, analysis of meiotic pairing in the autoallooctoploid is necessary to draw final conclusions as to the mechanism of sterility at work. Regardless of the mode of sterility, it is of utility to have reduced fertility in *H. acetosella*. In many areas where it will be grown, the flowers would experience freezing before seed set; however, in extreme southern climates, it has the potential to become a prolific seed producer. With reduced fertility, there is reduced potential for invasion of landscapes or native ecosystems. Because 'Panama Red' and the resulting autoallooctoploid are propagated by stem cuttings, reduced fertility does not affect commercial production.

This study is the first time oryzalin has been successfully used to induce polyploidy in *Hibiscus* section *Furcaria*. The resulting autoallooctoploid was more compact, had shorter internodes, had smaller leaves, and exhibited a reduction in fertility. Of these characters, the more compact habit, shorter internodes, and reduction in fertility represent marked improvements over commercially available cultivars of *H. acetosella*. Oryzalin may be regarded as a preferred chromosome-doubling agent because it is safer for researchers to use than colchicine. Additionally, colchicine requires a 1000-fold higher concentration to produce quantitatively similar effects (Upadhyaya and Noodén, 1977). Optimization of the protocol, including reducing the concentration of agar and increasing the number or duration of applications, could lead to higher incidence of chromosome doubling.

Literature Cited

Akpan, G.A. 2000. Cytogenetic characteristics and the breeding system in six *Hibiscus* species. Theor. Appl. Genet. 100:315–318.

Bates, D.M. 1965. Notes on the cultivated Malvaceae 1. Hibiscus. Baileya. 13:56–96.

Bennett, M.D. and I.J. Leitch. 2005. Plant DNA C-values database, release 4.0. 15 July 2009. <http://data.kew.org/cvalues/>.

Bennett, M.D., P. Bhandol, and I.J. Leitch. 2000. Nuclear DNA amounts in angiosperms and their modern uses: 807 new estimates. Ann. Bot. (Lond.) 86:859–909.

Contreras, R.N. and J.M. Ruter. 2009. An oryzalin induced polyploid from a hybrid of *Hibiscus acetosella* × *H. radiatus* (Malvaceae) exhibits reduced fertility and altered morphology. HortScience 44:1177 (Abstr.).

Dermen, H. 1960. Nature of plant sports. Amer. Hort. Mag. 39:123–173.

Fryxell, 1988. Malvaceae of Mexico. Systematic Bot. Monogr. 25:1–522.

Gill, B.S., J.O. Minocha, D. Gupta, and D. Kumar. 1969. Chromosome behaviour and seed setting in autotetraploid pearl millet. Indian J. Genet. Plant Breed. 29:462–467.
Gilles, A. and L.F. Randolph. 1951. Reduction of quadrivalent frequency in autotetraploid maize during a period of ten years. Amer. J. Bot. 38:12–17.
Greilhuber, J., E.M. Temsch, and J.M. Loureiro. 2007. Nuclear DNA content measurement, p. 67–101. In: J. Doležel, J. Greilhuber, and J. Suda (eds.). Flow cytometry with plant cells: Analysis of genes, chromosomes and genomes. Wiley-VCH, Weinheim, Germany.
Hochreutiner, B.P.G. 1900. Revision du genre *Hibiscus*. Annuaire de Conservatoire du Jardin Botaniques de Geneve 4:23–191.
Jauhar, P.P. 1970. Chromosome behaviour and fertility of the raw and evolved synthetic tetraploids of pearl millet, *Pennisetum typhoides* Stapf et Hubb. Genetica 41:407–424.
Jones, J.R., T.G. Ranney, and T.A. Eaker. 2008. A novel method for inducing polyploidy in *Rhododendron* seedlings. J. Amer. Rhododendron Soc. 62:130–135.
Kao, K.N. 1975. A nuclear staining method for plant protoplasts, p. 60–62. In: Gamborg, O.L., and L.R. Wetter (eds.). Plant tissue culture methods. National Research Council of Canada, Ottawa, Ontario, Canada.
Kumar Sen, N. and H.R. Chheda. 1958. Colchicine induced tetraploids of five varieties of black gram. Indian J. Genet. Plant Breed. 18:238–248.
Kuwada, H. 1977. Interspecific hybridization between *H. acetosella* and *H. radiatus*: Studies on interspecific and intergeneric hybridization in the Malvaceae XVI. Jpn. J. Breeding 27:351–355.
Lee, S.K. and C.S. Kim. 1976. Studies on artificial polyploidy forest trees XIII: Some morphological and physiological characteristics of colchitetraploid *Hibiscus syriacus* L. J. Korean Forestry Soc. 32:73–86.
Madon, M., M.M. Clyde, H. Hashim, Y. Mohd Yusuf, H. Mat, and S. Saratha. 2005. Polyploidy induction of oil palm through colchicine and oryzalin treatments. J. Oil Palm Res. 17:110–123.
Meister, A. and M. Barow. 2007. DNA base composition of plant genomes, p. 177–215. In: J. Doležel, J. Greilhuber, and J. Suda (eds.). Flow cytometry with plant cells: Analysis of genes, chromosomes and genomes. Wiley-VCH, Weinheim, Germany.
Menzel, M.Y. and F.D. Wilson. 1961. Chromosomes and crossing behavior of *Hibiscus cannabinus*, *H. acetosella*, and *H. radiatus*. Amer. J. Bot. 48:651–657.
Menzel, M.Y. and F.D. Wilson. 1963a. Cytotaxonomy of twelve species of *Hibiscus* section *Furcaria*. Amer. J. Bot. 50:262–271.
Menzel, M.Y. and F.D. Wilson. 1963b. An allododecaploid hybrid of *Hibiscus diversifolius* and some related F1 hybrids. J. Hered. 54:55–60.
Menzel, M.Y., K.L. Richmond, C.S. Contolini, and P. Huang. 1986. New intergenomic hybrids among some African diploid species of *Hibiscus* sect. *Furcaria*. Amer. J. Bot. 73:304–309.
Olsen, R.T., T.G. Ranney, and Z. Viloria. 2006. Reproductive behavior of induced allotetraploid ×*Chitalpa* and in vitro embryo culture of polyploid progeny. J. Amer. Soc. Hort. Sci. 131:716–724.
Omran, A. and B. Mohammad. 2008. Polyploidization effect in two diploid cotton (*Gossypium herbaceum* L. and *G. arboreum* L.) species by colchicine treatments. Afr. J. Biotechnol. 7:102–108.
Randolph, L.F. 1941. An evaluation of induced polyploidy as a method of breeding crop plants. Am. Nat. 75:347–363.
Sanyal, P. and B.C. Kundu. 1959. Cytological study of *H. radiatus* × *H. cannabinus* hybrid and genomic relationship of the two species. Nucleus 2:99–108.
Schank, S.C. and P.F. Knowles. 1961. Colchicine induced polyploids of *Carthamus tinctorius* L. Crop Sci. 1:342–345.
Siemonsma, J.S. and K. Piluek (eds.). 1993. Plant resources of southeast Asia. No. 8. Pudoc Scientific Publishers, Wageningen, The Netherlands.
Singsit, C. and P. Ozias-Akins. 1992. Rapid estimation of ploidy levels in *in vitro*-regenerated *Arachis* hybrids and fertile triploids. Euphytica 64:183–188.
Skovsted, A. 1941. Chromosome numbers in the Malvaceae II. C. R. Traveaux du Laboratoire Carlsberg, Serie Physiologique 23:195–242.
Stebbins, G.L., Jr. 1950. Variation and evolution in plants. Columbia University Press, New York.
Upadhyaya, M.K. and L.D. Noodén. 1977. Mode of dinitroaniline herbicide action I. Analysis of the colchicine-like effects of dinitroaniline herbicides. Plant Cell Physiol. 18:1319–1330.
van Borssum Waalkes, J. 1966. Malesian Malvaceae revised. Blumea 14:1–213.
Van Laere, K., J. Van Huylenbroeck, and E. Van Bockstaele. 2006. Breeding strategies to increase genetic variability within *Hibiscus syriacus*. Acta Hort. 714:75–82.
Wilson, F.D. 1994. The genome biogeography of *Hibiscus* L. section *Furcaria* DC. Genet. Resources Crop Evol. 41:13–25.
Wilson, F.D. and M.Y. Menzel. 1964. Kenaf (*Hibiscus cannabinus*), roselle (*Hibiscus sabdariffa*). Econ. Bot. 18:80–91.
Zhuang, D. and J. Song. 2005. The characters of pollen grains and stomatal apparatus in *Hibiscus* L. in relation to the ploidy. J. Trop. Subtrop. Bot. 13:49–52.