In-vitro Polyploid Induction of *Rudbeckia spp.*

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Significance to the Industry: The genus *Rudbeckia* contains many diverse, adaptable, and desirable ornamental species. The development of new polyploids, through chromosome doubling, may increase ornamental characteristics, expand breeding opportunities, and restore fertility in sterile hybrids – ultimately leading to the development of improved cultivars. In-vitro treatments, ranging from 15 to 60 µM oryzalin over 3 to 5 days, were effective at inducing polyploidy, depending on taxa. New tetraploids of *R. maxima, R. subtomentosa*, and a novel interspecific hybrid were successfully developed and will be evaluated for ornamental characteristics and utilized in an ongoing breeding program to create improved hybrids.

Nature of the Work: The genus *Rudbeckia* contains approximately 30 species of annuals, biennials and perennials easily distinguished by their signature colorful ray corollas and disk shaped receptacles. *Rudbeckia maxima* is one of the tallest species in the genus, reaching over seven feet, and is noted for its blue-green foliage and large inflorescences with prominent cones. *Rudbeckia subtomentosa* has vibrant yellow florets and is a reliable perennial with broad adaptability and disease resistance (1). *Rudbeckia subtomentosa* 'Henri Eilers' is a unique cultivar with attractive quilled ray florets. The hybrid H062 is a cross between the durable perennial *R. subtomentosa* and showy annual *R. hirta*, developed at NC State University, but appears to be sterile. Polyploidy occurs naturally in *R. fulgida* and *R. hirta* species (3). The creation of tetraploids in *R. maxima*, *R. subtomentosa* 'Henri Eilers,' and H062 could enhance ornamental traits, facilitate hybridization with other tetraploids, and restore fertility in the interspecific hybrid H062. Oryzalin has been found to be an effective chromosome doubling agent, but optimal treatments (including dose and duration of exposure) vary for different species (2). The objectives of this study were to evaluate the efficacy of varied dosages and durations of oryzalin exposure as an in-vitro chromosome doubling treatment for *Rudbeckia spp*. and to develop new tetraploid clones for use in breeding projects.

Tissue culture: Material for this study was selected from *R. maxima*, *R.* H062, and *R. subtomentosa* 'Henri Eilers' tissue culture collections maintained at the Mountain Horticultural Crops Research and Extension Center, Fletcher, NC. Cultures were maintained on Murashige and Skooge (MS) basal media supplemented with 3% sucrose and 2 µM benzylamino purine (BAP). The pH was adjusted to 5.75 ± 0.03 and the media was solidified with 0.8% agar. Stock cultures were maintained at 23 ^{o}C under

cool white fluorescent lights (130 μmol·m^{-2}·s^{-1}) with a 16 h photoperiod and subcultured every four weeks to create a sufficient population sample size for each taxa. A minimum of 30 shoots (3-5 mm in length) were harvested for oryzalin treatments 20-24 days after subculturing.

Oryzalin treatment: Oryzalin (1 M stock solution, dissolved in 70 % ethanol) was filter sterilized and added to cooled liquid MS media after autoclaving. For *R. subtomentosa*, shoot apices were excised from in-vitro grown plantlets and treated with different concentrations of oryzalin (0, 15, 30, 60, or 90 μM) for 3, 5 or 7 d in a factorial combination. *Rudbeckia maxima* and *R.* H062 were only treated with 0, 60 or 90μm for 3 or 5 d due to lower amounts of available tissue. Shoot apices were placed in baby food jars containing 25 mL of oryzalin solution and placed on an orbital shaker. After treatment, the shoot apices were rinsed in distilled water for ten minutes, three separate times, and placed on shoot regeneration medium (MS media with 2μM BAP). Cultures were then placed under standard culture conditions in a completely randomized experimental design. Each taxa was treated as a separate experiment. Mortality was recorded four weeks after the completion of each treatment. Survival data were analyzed using analysis of variance and LSD means separation (SYSTAT 10, SYSTAT, Chicago, IL).

Flow Cytometry: Holoploid, 2C DNA contents (i.e., DNA content of the entire non-replicated, chromosome complement irrespective of ploidy level) were determined via flow cytometry for each surviving shoot eight weeks after initial treatment. Approximately 0.25 sq. in. (1.6 sq. cm.) of leaf, shoot or callus tissue was chopped with a razor blade in a petri dish containing 400 μL of cold extraction buffer (CyStain UV Precise P, Partec, Münster, Germany). The suspension was filtered through 50-μm nylon mesh and nuclei were stained using 1.6 mL staining buffer containing 4', 6-diamidino-2-phenylindole (DAPI) (CyStain UV Precise P, Partec). The suspension was analyzed using a flow cytometer with fluorescence excitation provided by a mercury arc lamp (PA-I Ploidy Analyzer, Partec). The mean fluorescence of each sample was compared with an internal standard of known ploidy and DNA content. Only samples with sufficient tissue were analyzed; remaining samples will be tested at a later date.

Results and Discussion: Tetraploids were successfully induced in all three taxa, demonstrating that oryzalin is an effective mitotic inhibitor for inducing polyploidy in *Rudbeckia* species (Table 1). Oryzalin has also been used successfully to double chromosome numbers of in-vitro grown shoots of *Buddleja*, *Miscanthus*, *Syringa,* and *Rhododendron* (2, 4, 5, 6, and 7). Oryzalin-treated shoot apices were observed to grow slower than non-treated shoot apices. In some treatments, slow growth limited the amount of material sufficient for ploidy analysis at this time. It was also observed that an increase in exposure and concentration of oryzalin treatments resulted in greater callus and less organized shoot growth in the surviving shoot apices (data not presented).

There was a significant interaction between oryzalin concentration and duration of exposure on shoot apex survival for all three taxa ($P<0.05$). In general, tissues were more sensitive to increasing oryzalin concentrations as duration of exposure increased, resulting in reduced survival at the higher concentration/duration combinations (Table 1). For *R. subtomentosa*, all individuals subjected to the 7 d duration (with the exception of 1 shoot treated with a 30 µM concentration) and/or the 90 µM oryzalin concentration died, indicating that those treatments were excessive. For *R.* H062 and *R. maxima*, all plants died following 5 day treatments of either 60 or 90 µM oryzalin also indicating that those treatments were too extreme.

There were no significant effects of oryzalin dose or duration on the percent of homogeneous tetraploid formation for any of the taxa. However, for *R. subtomentosa*, tetraploids were induced in both the 3 d, 15 and 30µM treatments and in the 5 d, 30 and 60 µM treatments which produced 25, 38, 14, and 22% tetraploids respectively (Table 1). For *R.* H062 a mixaploid and 3 tetraploids (43%) were produced by the 3 d, 60 µM concentration treatment. *Rudbeckia* H062 receiving the 5 d, 60 µM treatment were not large enough to sample for ploidy analysis at this time. Mixaploids, a conglomeration of cells of varying ploidy levels, may result from oryzalin not penetrating or affecting all initial cells and histogenic layers or due to asynchronous cell cycling among initial cells. Only one *R .maxima* tetraploid (6%) was recovered from the 3 d, 60 µM treatment. The remaining three *R. maxima* survivors from the 3 d, 90 µM treatment were not of sufficient size for testing and will be tested at a later date. Lack of clear trends in dose and duration of oryzalin treatments on polyploid induction were also reported for *Rhododendron* hybrids (6) and may reflect the random variation in cell cycles, tissue sensitivity, and chemical penetration.

Results from this study have shown that oryzalin is effective for in-vitro induction of polyploids in *Rudbeckia spp*. In-vitro treatments, ranging from 15 to 60 µM oryzalin over 3 to 5 days, were taxa dependent but effective at inducing polyploidy, while minimizing mortality. Polyploids developed from these studies will be evaluated for ornamental merit and use in ongoing breeding efforts.

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Table 1: Effects of oryzalin treatments on survival and polyploid induction in selected *Rudbeckia* taxa.

Taxa	Concentration (µM)	Duration (Days)	Survival (%)[Z]	Ploidy Level (%) 2x	Mix[Y]	4x
R. subtomentosa 'Henry Eilers'	0	3	100 A	100	0	0
		5	100 A	100	0	0
		7	100 A	100	0	0
	15	3	70 B	75	0	25
		5	-[X]	-[X]	-[X]	-[X]
		7	0 E	-	-	-
	30	3	47 C	63	0	38
		5	20 D	85.71	0	14.29
		7	3 E	-[W]	-[W]	-[W]
	60	3	23 D	100	0	0
		5	23 D	78	0	22
		7	0 E	-	-	-
	90	3	0 E	-	-	-
		5	0 E	-	-	-
		7	0 E	-	-	-
R. H062	0	3	100 A	100	0	0
		5	100 A	100	0	0
	60	3	67 B	50	12.5	37.5
		5	0 D	-	-	-
	90	3	43 C	-[W]	-[W]	-[W]
		5	0 D	-	-	-
R. maxima	0	3	100 A	100	0	0
		5	100 A	100	0	0
	60	3	63 B	94	0	6
		5	0 D	-	-	-
	90	3	17 C	100	0	0
		5	0 D	-	-	-

[Z]Means followed by different letters within columns for a given taxa are significantly different, LSD $P < 0.05$.

[Y]Mixaploid (cytochimera) tissue.

[X]Missing treatment.

[W] Insufficient tissue for testing.