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A Simple Chromosome Doubling Technique Is Effective for Three Species of Cupressaceae

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***Abstract. Platycladus orientalis* (L.) Franco (syn. *Thuja orientalis* L.), *Thuja occidentalis* L., and *T. plicata* D. Don. are conifers often used in the landscape. Most of the available cultivars of these species share the character of having foliage that turns an off-color during winter as a result of photoinhibition. Tetraploids of the related japanese-cedar [*Cryptomeria japonica* (L. f.) D. Don.] have exhibited greener color retention than diploids during winter and a recent report described a simple technique to double its chromosomes. The technique used to double the chromosome number of *C. japonica* was applied to the three species mentioned to determine if it would be effective for inducing polyploidy and, if so, optimal duration of treatment. Seedlings were treated at the cotyledon stage for 0 (control), 10, 20, or 30 days with an aqueous solution containing 150 µM oryzalin + 0.1% Tween® 20 using a standard household spray bottle that created a fine mist. No tetraploids were observed for any species in control treatments, indicating all recovered tetraploids resulted from applying oryzalin. Tetraploids were observed for all other treatments except *T. plicata* at 30 days. Efficacy ranged from 0% to 27.1% of transplanted seedlings being tetraploid. There was a quadratic relationship between duration of treatment and percent tetraploids in *T. occidentalis* and *T. plicata* and a linear relationship for *P. orientalis*. Based on regression analysis, the optimal duration of treatment was 20.5 days for *T. occidentalis* and 13.9 days for *T. plicata*. The highest percent tetraploids recovered for *P. orientalis* was at 30 days and it is unclear if increasing duration beyond this would continue increasing percent tetraploids recovered. Morphology was not useful in early identification of tetraploids for any species.**

Platycladus orientalis, *Thuja occidentalis*, and *T. plicata* are evergreen conifers and from each species, cultivars have been selected to fill many niches in modern landscapes. *Platycladus orientalis*, oriental arborvitae, is native to China and Korea and is adaptable to a range of soil types and pH and is moderately resistant to deer browsing (Dirr, 2009). *Thuja occidentalis*, eastern or american arborvitae, is native to eastern North America and is tolerant of limestone soils, heat, and drought (Dirr, 2009). *Thuja plicata*, western red cedar or western arborvitae, is native to western North America from Alaska to northern California. Western red cedar forms a statuesque tree in the landscape, tolerates varying soil moisture, and is pH-adaptable (Dirr, 2009). Selections of these three species include varying growth forms, foliage morphology, and foliage color. Winter foliage color of available cultivars includes green, yellow, yellow–orange, bronze, brown, plum, and the range thereof; however, cultivars exhibiting green foliage during winter are the fewest in number (Dirr, 2009).

Foliage of most selections of the related japanese-cedar is yellow, brown, or bronze during winter. However, tetraploid forms of japanese-cedar have been reported to remain green during the photoinhibitory conditions of winter compared with diploid forms (Niwa and Sasaki, 2003). Previous research identified a technique to develop tetraploids ($2n = 4x = 44$) of japanese-cedar with the goal of developing new cultivars that remain green during winter (Contreras et al., 2010). The objectives of the current research were to evaluate the effectiveness of this technique and determine optimal treatment duration for doubling chromosome numbers in oriental arborvitae, american arborvitae, and western red cedar.

Materials and Methods

Plant material. Seeds labeled as *Platycladus orientalis* 'Compactus', *Thuja occidentalis*, and *Thuja plicata* were received from Lawyer Nurseries, Inc. (Plains, MT). The seeds of oriental arborvitae labeled as 'Compactus' likely were collected from the cultivar Sieboldii, which has the synonym 'Compacta' (Krüssman, 1985). There is no evidence for a cultivar named 'Compactus'. In this report I refer to the cultivar as Compacta as a result of its prevalence in the trade and its similarity to the labeled cultivar name. With the exception of controls, ≈1000 seeds of each species were sown in germination trays containing 6 Douglas fir bark [*Pseudotsuga menziesii* (Mirbel) Franco]:3 peat:1 pumice (v/v) and germinated under laboratory conditions in humidity chambers (100% relative humidity) with constant light (32 $\mu mol \cdot m^{-2} \cdot s^{-1}$) supplied by cool-white fluorescent lamps at 20 °C. Controls of the three species were grown under the same conditions; however, only 100 seeds were sown.

Inducing polyploidy. Beginning at germination (cotyledon stage), seedlings were sprayed to runoff daily for 0 (control), 10, 20, or 30 d with an aqueous solution containing 150 µM oryzalin (supplied as Surflan® AS; United Phosphorus, Trenton, NJ) + 0.1% Tween® 20 (Acros Organics, Geel, Belgium) using a standard household spray bottle that created a fine mist. Each species was replicated once per treatment duration for a total of 12 trays. After each treatment the seedlings were moved to a glasshouse with day/night set temperatures of 27/20 °C. When seedlings were 4 to 5 cm, they were transplanted into 32-cell trays (T.O. Plastics, Clearwater, MN) containing 1 bark mix above:1 Sunshine® SB40 patio mix (Sun Gro Horticulture, Bellevue, WA) and fertilized weekly with 100 ppm nitrogen with Jack's Professional® 20-8.7-16.6 (J.R. Peters, Inc., Allentown, PA).

Ploidy analysis. Flow cytometry was used to screen all seedlings that survived treatments. Approximately 0.5 cm of leaf tissue was finely chopped in an extraction buffer (CyStain® Ultraviolet Precise P Nuclei Extraction Buffer; Partec, Münster, Germany) with a double-sided razor blade to extract nuclei. The nuclei suspension was passed through a 30-µm filter (Partec), nuclei were stained with 4',6-diamidino-2-phenylindole (CyStain® ultraviolet Precise P Staining Buffer; Partec), and nuclei were analyzed using a CyFlow® Ploidy Analyzer (Partec). All samples were analyzed with an internal standard (*Pisum sativum* L. 'Ctirad'; 2C = 8.76 pg) (Greilhuber et al., 2007) to correct for peak shifting and ensure correct interpretation of peak location.

Data analysis. Statistical analysis was not possible as a result of single replicates of each treatment. Percent tetraploids for each species and duration were calculated based on the number of observed tetraploids/number of surviving plants that were transplanted. Scatterplot, best fit curves, regression equations, and multiple correlation coefficients (R^2) were prepared in Excel® (Microsoft Corporation, Redmond, WA).

Results and Discussion

The number of tetraploids induced in *Platycladus orientalis*, *Thuja occidentalis*, and *T. plicata* after treatment with oryzalin

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for 10, 20, or 30 d ranged from 0 to 107 (Table 1). No tetraploids were recovered in the control (0 d) treatment for any species, indicating that tetraploids observed in the study were the result of treatments and not unreduced gametes or spontaneous chromosome doubling in embryonal initials (Khoshoo, 1959). Only diploids and tetraploids were recovered in the current study. In contrast, Contreras et al. (2010) observed 9.3% mixoploids after 30 d treatment of japanese-cedar. It is unclear why no mixoploids were recovered in the current study.

Tetraploids were observed for all three species in each of the treatment durations except the 30-d treatment of western red cedar. There was a quadratic relationship between percent tetraploids recovered and treatment duration for *T. occidentalis* ($y = –0.060x^2 + 2.47x – 0.86$; $R^2 = 0.96$) and *T. plicata* ($–0.032x^2 + 0.89x + 1.08$; $R^2 = 0.65$) and a linear relationship for *P. orientalis* 'Compacta' ($y = 0.22x – 0.3$; $R^2 = 0.99$) (Fig. 1). Observed values of percent tetraploids for american arborvitae increased from 15.3% at 10 d, to 27.1% at 20 d, and declined to 18.3% at 30 d (Table 1). By solving for the derivative of the quadratic formula, the optimal treatment duration for american arborvitae was determined to be 20.5 d. Observed values for percent tetraploids in oriental arborvitae increased from 1.5% at 10 d, to 3.8% at 20 d, and reached 6.4% at 30 d (Table 1). It remains to be seen if increasing treatment duration beyond 30 d will continue to increase the percent tetraploids recovered. Observed values for percent tetraploids of western red cedar were 10% at 10 d, 2.8% at 20 d, and 0% at 30 d (Table 1). By solving for the derivative of the quadratic formula, the optimal treatment duration for inducing tetraploidy in western red cedar was 13.9 d.

It was not possible to select tetraploids based on phenotype as can be done in pines, larix, japanese-cedar, and japanese cypress. A great deal of morphological variation among all seedlings including controls was observed, which likely contributed to the inability to select tetraploids based on phenotype. In japanese-cedar, Contreras et al. (2010) reported over 92% accuracy when selecting for tetraploids based on plants with thick and twisted needles at an early stage of growth, which reduced the number of seedlings that were screened by flow cytometry. Tetraploids of *Pinus ponderosa* Douglas ex Lawson and *P.* ×*attenuradiata* Stockw. & Righter polyploids exhibited thickened and shortened needles with a bluish tint but growth rate was similar to that of diploids (Hyun, 1953). Jensen and Levan (1941) described *Sequoia gigantea* (Lindl.) Decne. [*Sequoiadendron giganteum* (Lindl.) Buckholz] polyploids as having coarser and more erect branches and needles that were broader and thicker than normal. Johnsson (1975) reported altered morphology in tetraploids of *Pinus sylvestris* L., *P. contorta* Douglas ex Loud, *Picea abies* (L.) Karst., and *Larix sibirica* Ledeb. [*L. russica*. (Endl.) Sab. ex Trautv.]. Morphology of slash pine (*Pinus elliottii* Engelm.) polyploids was reported to be abnormal including darker needles, shorter and thicker cotyledons and primary needles, enlarged buds, fusion of needles, and reduced growth (Mergen, 1959). Most of the species described in previous studies have needle- or awl-like leaves that perhaps display the altered character of tetraploids more effectively than in species with scale-like leaves such as arborvitae. However, Kanezawa (1951) reported tetraploids of japanese cypress [*Chamecyparis obtusa* (Sieb. & Zucc.) Endl.], a species with scale-like leaves, to have coarser branches that were more erect, broader leaves that were squatter than diploids, and leaves roughly twice as thick as diploids. An important distinction is that Kanezawa (1951) was reporting on trees at least 3 years old, whereas the current study focused on examining seedling morphology for altered phenotypes to reduce the number of seedlings to be screened using flow cytometry.

Contreras et al. (2010) previously reported on effectively using oryzalin to double the

Table 1. Results of treating *Platycladus orientalis* 'Compacta', *Thuja occidentalis*, and *Thuja plicata* seedlings to develop tetraploids by spraying shoot tips of seedlings at the cotyledon stage with and aqueous solution of 150 µM oryzalin + 0.1% Tween® 20 for 0, 10, 20, or 30 d.

Taxon	Duration (d)	Seedlings transplanted (no.)[z]	No. 4*x* (%[y])
P. orientalis 'Compacta'	0 (control)	50	0 (0)
P. orientalis 'Compacta'	10	133	2 (1.5)
P. orientalis 'Compacta'	20	156	6 (3.8)
P. orientalis 'Compacta'	30	377	24 (6.4)
T. occidentalis	0 (control)	72	0 (0)
T. occidentalis	10	400	61 (15.3)
T. occidentalis	20	395	107 (27.1)
T. occidentalis	30	240	44 (18.3)
T. plicata	0 (control)	72	0 (0)
T. plicata	10	432	43 (10.0)
T. plicata	20	761	21 (2.8)
T. plicata	30	37	0 (0)

[z]For treatments except control, 1000 seeds were planted. Surviving seedlings were transplanted and tested. For controls, 100 seeds were planted and all surviving seedlings were transplanted and tested.
[y]Percentage of transplanted seedlings that were tetraploid.

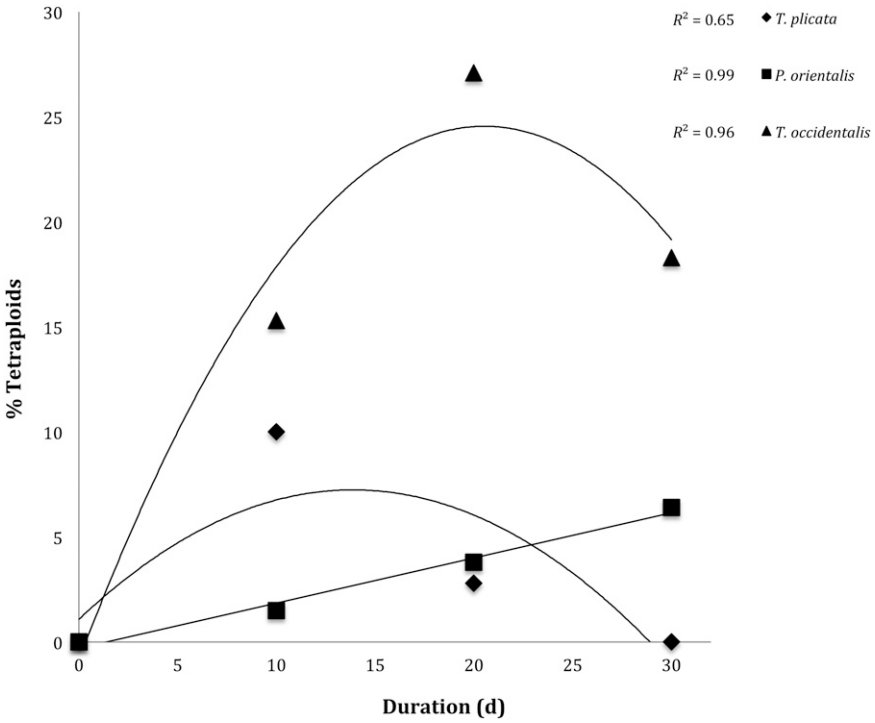

Fig. 1. Percent tetraploids resulting from treating seedlings of *Thuja plicata*, *Thuja occidentalis*, and *Platycladus orientalis* 'Compacta' at the cotyledon stage with a fine mist of aqueous 150 µM oryzalin + 0.1% Tween® 20 for 0, 10, 20, or 30 d. *Thuja plicata* ($y = –0.032x^2 + 0.89x + 1.08$) and *T. occidentalis* ($y = –0.060x^2 + 2.47x – 0.86$) exhibited a quadratic relationship and *P. orientalis* 'Compacta' displayed a linear relationship ($y = 0.22x – 0.30$).

chromosomes of japanese-cedar using the method described here. Applying oryzalin as a mist to seedlings of three conifer species for 10, 20, or 30 d resulted in the recovery a total 308 tetraploids, demonstrating the efficacy of oryzalin using this treatment to induce tetraploidy among three species in two genera of Cupressaceae.

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