In Vitro Polyploid Induction of *Ophiopogon planiscapus*

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Index Words: chromosome doubling, flow cytometry, oryzalin, polyploidy, somatic embryogenesis, tissue culture, whole genome duplication

Significance to Industry: Mondo grass (*Ophiopogon* spp.) are versatile and valuable landscape plants with a variety of ornamental uses (2). The diversity of species and traits in this genus provide opportunities for the breeding and development of new cultivars. However, variations in ploidy levels complicate breeding systems. Embryogenic callus derived from embryos of the diploid cultivar *Ophiopogon planiscapus* 'Nigrescens' was subjected to *in vitro* ploidy manipulation to produce tetraploids that will be evaluated for ornamental character, size and vigor, and for use as breeding lines to hybridize with other naturally occurring tetraploid species.

Nature of Work: The genus *Ophiopogon* contains approximately 65 species, many of which are popular as ornamental landscape plants due to their versatility and adaptability (3). Of particular interest is the cultivar *Ophiopogon planiscapus* 'Nigrescens' which is characterized by dark, almost black, grass-like foliage and upright flowers. Although 'Nigrescens' is highly valued for its unique foliage color its propagation and production is typically hindered by its slow growth. Development of tetraploid 'Nigrescens' may enhance vigor and facilitate hybridization with existing polyploid species. Induced polyploids often show an increase in size and growth rate with larger flowers and longer bloom periods (5, 8). These induced tetraploids may also provide new opportunities for interspecific hybridization with naturally occurring tetraploids of *Ophiopogon japonicus* and may be useful in intergeneric crosses with the tetraploid *Liriope* species *L. gigantea, L. muscari,* and *L. platyphylla* (3, 8). The dinitroanaline herbicide oryzalin (Surflan®) has been effectively used as a mitotic inhibitor to induce chromosome doubling (6); however, the concentrations and exposure times can vary according to species (1). The objectives of this study were to produce tetraploid *Ophiopogon planiscapus* clones for future breeding work and to determine the optimal concentration and duration of exposure to oryzalin for chromosome doubling.

Somatic embryogenesis: Embryogenic callus was induced from mature embryos excised from open pollinated seeds of *Ophiopogon planiscapus* 'Nigrescens' collected in September 2012. Callus induction and proliferation medium consisted of Murashige and Skoog's (MS) basal salts and vitamins (7) supplemented with 2% sucrose, 5 µM

benzylamino purine (BAP), 5 µM naphthalene acetic acid (NAA), and 0.08 g/L adenine hemisulfate. Medium was adjusted to a pH of 5.75 ± 0.03 and solidified with agar at 0.65%. Callus was subcultured onto fresh medium every 6 to 8 weeks and incubated in the dark at 23 ºC (73.4 ºF) to allow callus proliferation.

Oryzalin Treatment: A liquid medium consisting of MS basal salts and vitamins and 2% sucrose was prepared for chromosome doubling. Medium was adjusted to a pH of 5.75 ± 0.03. A 3 mM stock solution of oryzalin was prepared in 95% ethanol. This stock solution was added to flasks containing cooled autoclaved media to obtain the concentrations of 7.5 µM, 15 µM, and 30 µM oryzalin. The control solution (0 µM oryzalin) received the addition of 5 ml of 95% ethanol. Fifteen jars per solution concentration were prepared.

The experimental design consisted of a completely randomized, four × three factorial, with four oryzalin concentrations (0, 7.5, 15 and 30 µM) and three exposure durations (3, 6 or 9 days). Each treatment combination consisted of five replications with each replication containing five calli (subsamples).

On day zero of the experiment, five callus pieces were placed in each jar. Jars were sealed with parafilm and placed on a rotary shaker in the dark. Five jars of each concentration (0, 7.5, 15 and 30 µM) were removed from the shaker after 3, 6, or 9 days. Upon removal, calli were transferred to a liquid MS medium and replaced on the rotary shaker for 24 hours to remove residual oryzalin. Calli were then transferred onto embryogenic maintenance MS medium and incubated in the dark. After 8 weeks, data was collected on the number of surviving calli.

Flow Cytometry: Calli were allowed to recover in the dark for a period of at least 28 days and thereafter resulting shoots that arose from the callus were analyzed using flow cytometry. Samples were prepared by placing leaf tissue in a petri dish with 400 µL of nuclei extraction buffer and chopping finely using a razor blade. The resulting solution was filtered and 1600 µL of a nucleotide staining buffer solution (CyStain UV Precise P Staining Buffer, Partec, Munster, Germany), containing 4', 6-diamidino-2-phenylindole (DAPI), was added to the solution. Stained nuclei were analyzed using a flow cytometer (Partec, PA-II).

The mean relative fluorescence for each sample was compared with that of a confirmed diploid *Ophiopogon* (3, 4) to determine if ploidy levels had been affected by treatment. All tetraploid and any strongly mixoploid (>50% tetraploid nuclei) shoots were subcultured into jars containing shoot maintenance medium consisting of MS basal salts and vitamins supplemented with 2% sucrose and 10 µM BAP. Medium was adjusted to a pH of 5.75 ± 0.03 and solidified with 0.65% agar. Cultures were placed under cool white fluorescent lights (60 µmol $m^{-2}s^{-1}$) with a 16 h photoperiod at 23 ºC (73.4 ºF). After a period of regrowth these shoots were retested to confirm their ploidy. Diploid shoots were discarded and any strongly mioxploid or tetraploid shoots were subcultured. A population of diploid *O. planiscapus* was also maintained in culture on a solidified MS medium containing 10 µM BAP for use as a control for flow cytometry.

Results and Discussion: Regression analysis showed there was no influence of treatment duration or interaction between duration and concentration of oryzalin on callus survival or shoot ploidy (Proc GLM, SAS version 9.4; SAS Institute, Cary, NC). However, callus survival did decrease with increasing oryzalin concentration in a linear fashion ($y = -1.0108x + 90.933$, $R^2 = 0.9882$, $p < 0.0002$) (Fig. 1). This reduction in survival illustrates that the oryzalin was affecting the callus at these concentrations.

Oryzalin concentration also influenced the number of diploid and mixoploid shoots recovered (Fig. 2). The mean number of diploid shoots recovered after treatment followed a quadratic model ($y = 0.2306x^2 - 8.5134x + 98.373$, $R^2 = 0.9894$, $p < 0.0001$) where the percentage of diploid shoots decreased and then began to rise again as concentration increased (Fig. 2). This trend was seen in reverse in the mean number of mixoploid shoots recovered ($y = -0.2002x^2 + 7.7026x - 0.6843$, $R^2 = 0.9979$, $p < 0.0001$). As the concentration of oryzalin increased the mean number of mixoploids increased until it began to fall again at higher concentrations (Fig. 2). Although there was no significant effect of concentration on the number of tetraploid shoots recovered, a limited number of tetraploids were successfully recovered from treated callus (7.5 and 15 µM) evidencing that oryzalin is an effective agent for polyploid induction in *Ophiopogon planiscapus* (Fig. 2). A number of mixoploid shoots that were kept in tissue culture eventually stabilized as tetraploids at a later date. Due to variations in the rate of cell division and cell fitness, lineages of different cell cytotypes may eventually outcompete one another through this type of endocytotypic selection.

Clones of all tetraploids are currently being multiplied and will subsequently be rooted on a half-strength medium consisting of Murashige and Skoog's (MS) basal salts and vitamins supplemented with 2% sucrose and 5 µM (NAA). Clones will be removed as they root and placed in a greenhouse under intermittent mist to acclimate for use in future breeding projects.

Acknowledgements: This work was funded, in part, by the North Carolina Agricultural Research Service, Raleigh; the North Carolina Biotechnology Center, Research Triangle Park, NC; and the Kenan Institute, Raleigh, NC. Thanks are also expressed to Tom Eaker, Nathan Lynch, Joel Mowrey, Andra Nus and the staff at the Mountain Horticultural Crops Research and Extension Center for their technical assistance.

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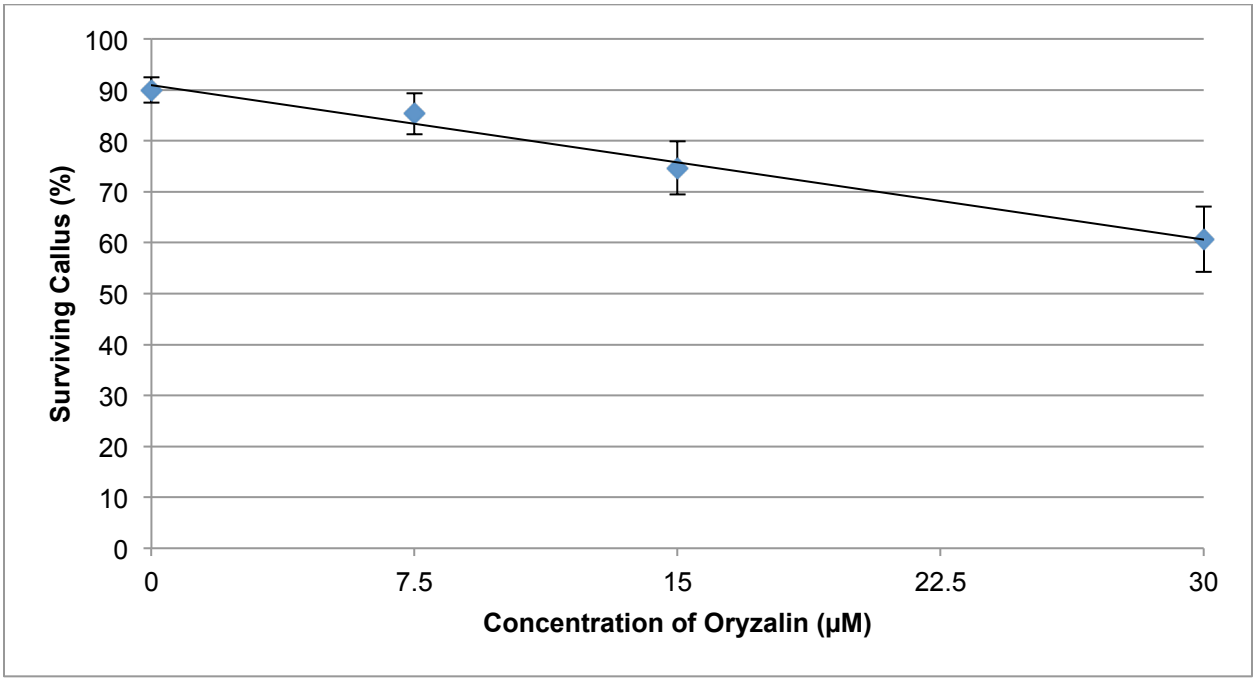

Figure 1: Percentage of callus surviving as a function of oryzalin concentration. ($y = -1.0108x + 90.933$, $R^2 = 0.9882$, $p < 0.0002$).

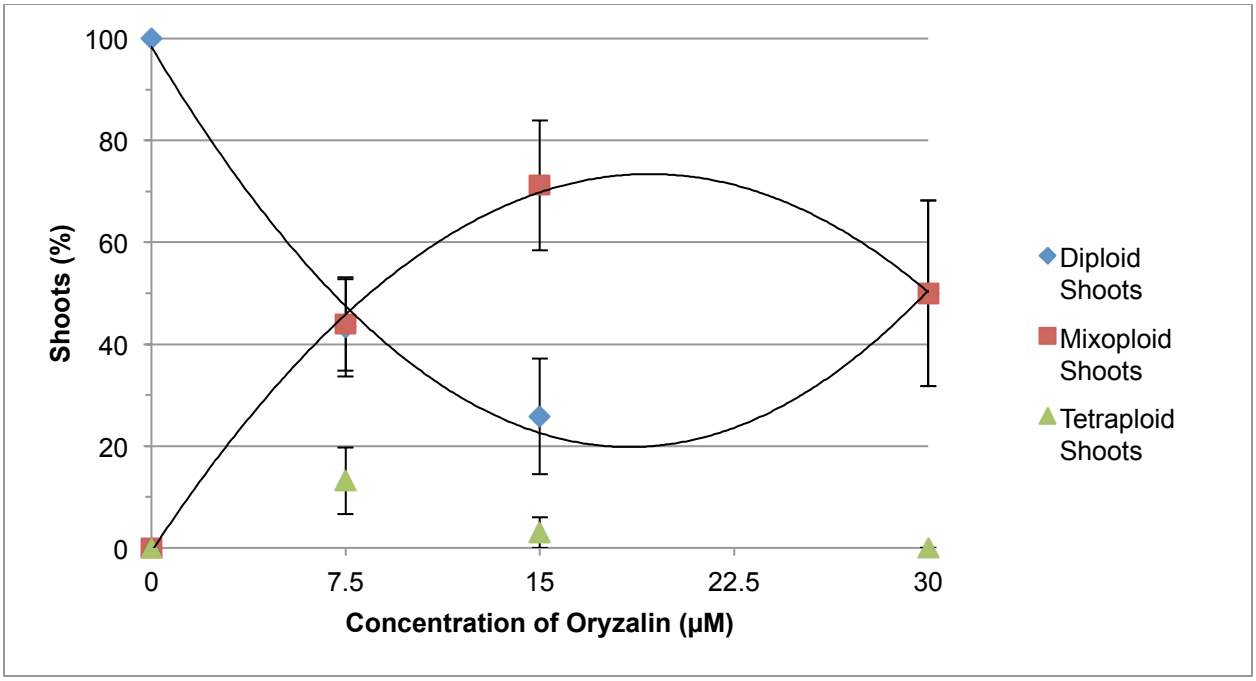

Figure 2: Percentage of diploid, mixoploid and tetraploid shoots recovered as a function of oryzalin concentration.
(Diploid: $y = 0.2306x^2 - 8.5134x + 98.373$, $R^2 = 0.9894$, $p < 0.0001$).
(Mixoploid: $y = -0.2002x^2 + 7.7026x - 0.6843$, $R^2 = 0.9979$, $p < 0.0001$).