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In vitro polyploid induction of orchids using oryzalin

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ABSTRACT

A protocol for in vitro polyploid induction using oryzalin was developed for *Dendrobium*, *Epidendrum*, *Odontioda*, and *Phalaenopsis* orchids (Orchidaceae). Protocorms and protocorm-like bodies (PLBs) in liquid nutrient media were subjected to oryzalin treatments of 14.4, 28.9, and 57.7 μM (w/v) concentrations for 3 and 6 days. Higher concentrations and longer treatment durations lowered the survival rates of the explants, but increased the number of polyploids produced. Stomatal guard cell lengths as measured with digital image analysis software, helped to identify several polypoids from digital images of leaf imprints. The optimal treatments were: 14.4 μM for 6 days in *Dendrobium* and *Odontioda*; 57.7 μM for 6 days in *Epidendrum*; and, 14.4 μM for 3 days in *Phalaenopsis*.

1. Introduction

Polyploid induction plays a significant role in the hybridization and improvement of orchids (Orchidaceae). Show competition and consumers require growers to continually introduce new orchid varieties produced from interspecific and intergeneric hybridizations. High infertility is often experienced when breeding intergeneric and interspecific primary and advanced hybrids (Nakasone, 1962; Sanguthai et al., 1973; Wimber et al., 1987; Watrous and Wimber, 1988). Fertility is reduced in such hybrids by the improper synapsis and the variability of chromosome pairings during metaphase I of meiosis. Polyploid induction can help to restore fertility by doubling the number of chromosomes and creating allotetraploids. This permits the disomic pairing of homologous pairs of chromosomes during meiosis while creating balanced gametes (Ranney, 2006).

Polyploids can assist breeders in developing improved hybrids and novelty types by contributing floral or growth characteristics unobtainable from diploid forms. Plants which have been converted to polyploids expand the genetic potential of parental material allowing the expression of variants that could result in novel and improved plants for breeders to use (Griesbach, 1985; Wimber et al., 1987; Ramanna and Jacobsen, 2003; Thao et al., 2003). Polyploid orchids also have the desirable characteristics of larger flowers with greater substance, rounder conformation and intensified coloration. Thicker stems and leaves are also associated with polyploidy (MacLeod, 1947; Kamemoto and Kam, 1980; Griesbach, 1985; Wimber et al., 1987; Watrous and Wimber, 1988).

Antimitotic agents such as colchicine and oryzalin have been used to induce polyploids in several plant species. Several authors have reported the successful use of colchicine to convert diploid to tetraploid forms in: *Dendrobium* (Nakasone and Kamemoto, 1961; Nakasone, 1962; Sanguthai et al., 1973); *Cymbidium* (Menninger, 1963; Wimber and Van Cott, 1966); *Vanda* (Nakasone and Kamemoto, 1961; Nakasone, 1962; Sanguthai and Sagawa, 1973); *Phalaenopsis* (Griesbach, 1981, 1985); *Paphiopedilum* (Wimber et al., 1987; Watrous and Wimber, 1988); *Habenaria* and *Calanthe* (Tahara and Kato, 1987); and, *Cattleya* (De Mello e Silva et al., 2000). Though efficient in yielding polyploids, colchicine is highly toxic to humans. This is due to colchicine's stronger affinity in binding with animal tubulins (more so than with plant tubulins) which is necessary for correct polar migration of chromosomes during anaphase in mitosis (Morejohn et al., 1984).

In recent studies, oryzalin (3,5-dinitro-N4,N-dipropylsulphate), a dinitroaniline herbicide, has been utilized as an alternative to colchicine (Dhooghe et al., 2010). Polyploids in a number of flowering plants including: *Lilium* (Van Tuyl et al., 1992; Takamura et al., 2002); *Nerine* (Van Tuyl et al., 1992); *Gerbera* (Tosca et al., 1995); *Rhododendron* (Vainola and Repo, 2001); *Rosa* (Kermani et al., 2003); and *Spathiphyllum* (Eeckhaut et al., 2004) have been produced using oryzalin. It has been reported to be more effective in producing polyploids and less toxic to animals because of its greater affinity to binding with plant tubulins (Morejohn et al., 1987).

Early methods of inducing polyploidy in orchids were in vivo treatments of colchicine to the meristems of seedlings, inflorescences, and mature plants (MacLeod, 1947; Moore, 1947; Rotor, 1958). These experiments often resulted in the formation of

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chimerical or mixoploid shoots that included diploid and tetraploid tissues. Wimber and Van Cott reported in 1966 the successful production of tetraploid plantlets from protocorms and protocorm-like bodies (PLBs) of *Cymbidium* treated with colchicine in vitro in liquid nutrient solution.

Polyploid induction in orchids using oryzalin has not been reported. The objective of this experiment was to develop in vitro protocols for the polyploid induction of orchids using oryzalin. *Cymbidium*, *Dendrobium*, *Epidendrum*, *Odontioda,* and *Phalaenopsis* were chosen for this study because these genera represent a broad range of commonly cultivated orchids, and are economically important commodities in the floriculture market (NASS, 2010).

2. Materials and methods

2.1. Plant materials

PLBs of *Cymbidium* 'Promised Land' and *Dendrobium* 'Gatton Sun Ray' were obtained by the methods described by Wimber (1963) and Kim et al. (1970). For protocorms, seed capsules of *Odontioda* 'Emma Sander', *Phalaenopsis bellina* and *Epidendrum* 'Helen's Pride' were harvested at approximately four months after pollination and before dehiscence. Each capsule was surface sterilized by scrubbing with antibiotic soap and soaking in 0.975% sodium hypochlorite solution (15%, v/v Clorox Regular Bleach: The Clorox Company; Oakland, CA) for 15 min. Seed pods were aseptically dissected and seeds were removed and sown in 125 ml Erlenmeyer flasks containing 75 ml of liquid modified VW medium. Protocorms developed approximately six weeks after sowing.

2.2. Culture media

For seed germination and shoot tip proliferation, a modified Vacin & Went (1949) liquid medium was used. This medium was modified according to Sagawa and Kunisaki (1984) with 2% (w/v) sucrose, and 15% (v/v) coconut water (modified VW). The pH was adjusted to 5.0 with 1 N HCl or NaOH. Orchid Maintenance Medium (#P668, PhytoTechnology Laboratories; Shawnee Mission, KS) was used after oryzalin treatments for shoot differentiation and growing. This medium was solidified using 7 g of Phytagar gellan gum (Sigma–Aldrich; St. Louis, MO). Both media were autoclaved at 121 °C for 20 min prior to use.

2.3. In vitro culture conditions

Culture vessels were agitated continuously on a gyrotory shaker set at 100 rpm. Medium was aseptically drawn from the culture vessels and replaced with fresh medium weekly. All in vitro cultures, including oryzalin treatment cultures were continuously illuminated by cool white fluorescent lamps (30–40 μmol m^{-2} s^{-1}). Culture room temperatures were kept between 25 °C and 28 °C.

2.4. Oryzalin treatment

Oryzalin (PhytoTechnology Laboratories; Shawnee Mission, KS) was prepared by dissolving 500 mg in 2 ml of NaOH (1 N) then diluting with deionized water to make up a final stock volume of 100 ml. This solution was vacuum filter sterilized (0.22 μm pore) then aseptically added to previously autoclaved liquid modified VW medium to constitute final concentrations of 14.4, 28.9 and 57.7 μM (w/v) oryzalin, which are the equivalent to 5 mg/l, 10 mg/l and 20 mg/l, respectively. Under sterile conditions, 20 ml of these solutions were dispensed into sterilized 50 ml Erlenmeyer flasks which served as treatment vessels. Modified VW media with no oryzalin were used for controls. Using a sterile spatula, roughly 2.5 ml amount of protocorms or PLB masses were transferred from their mother flasks to their treatment flasks. These were kept in solution and agitated continuously on a gyrotory shaker set at 50 rpm. Protocorms and PLBs were removed from treatment after 3 and 6 days. Protocorms or PLBs from each treatment were rinsed three times in sterile deionized water then spread on solidified orchid maintenance media (25 ml) set in Petri dishes. A completely randomized (CRD) 2 × 4 factorial treatment design was set up with 10 protocorms or PLBs per treatment. Two treatment durations (days) and four treatment levels (control + oryzalin concentration treatments) were used in this experiment and replicated five times. Protocorm and PLB survival were recorded at 8 weeks after oryzalin treatment. The surviving protocorms and PLB masses (observed as live, solid, and light to dark green) were transferred to 250-ml Erlenmeyer flasks containing 50-ml of solid orchid maintenance medium for further development.

2.5. Guard cell measurements and ploidy estimations

Between 16 and 24 weeks after oryzalin treatments, the surviving seedlings and plantlets from the treated populations were observed for gross morphological characteristics of higher ploidy. Seedlings or plantlets exhibiting shorter shoots and thicker leaves were used for stomatal assay. The fully expanded leaves from the top internode of treated seedlings or plantlets were used for making leaf imprints and viewing stomata. Leaves were harvested between 9:00 a.m. and 10:00 a.m. to avoid any variation from the natural opening or closing of stomata. Following procedures based on Stoddard (1965), clear nail polish (Scherer, Inc.; Rochester Hills, MI) was applied to the abaxial surfaces of the leaves. After drying (approximately 60 s), a piece of Scotch 3M Transparent Tape (Scotch 3M; St. Paul, MN) was set on top of the dried polish. The polish with imprint affixed to the tape was then peeled off the leaf samples and mounted onto microscope slides for observation. Stomata were viewed using a National DC3-163 Model Digital Compound Biological Microscope (National Optical & Scientific Instruments; San Antonio, TX) which displayed the images on a computer display monitor via the microscope's built-in camera. Digital images of the leaf and stomata imprints were viewed at 400× magnification, observed at 1024 × 768 pixel resolution, and saved as digital tagged image files (.tif) for later analysis. The images of the leaf imprints were analyzed and stomatal lengths measured using the image analyzing software, Motic Image Plus v.2 for Windows (Motic Group; China). Stomatal measurements made on the images were converted by the software into micrometer (μm) units. At least three plants from the screened treated population and controls with three leaves and ten to fifteen randomly selected stomata per leaf sample were measured. Stomatal lengths ≥1.25× the lengths of the controls were identified as polyploids (Russell, 2004).

2.6. Statistical analysis

Data were evaluated by SPSS Version 17.0 statistical software (IBM Corporation; Somers, NY). Analysis of variance (ANOVA) and post hoc comparison of means by Dunnett's Test comparing tested values against control measures were used at significance levels of $P \leq 0.05$ for survival rates and $P \geq 0.05$ for stomatal length measurements.

3. Results

Shorter durations and lower oryzalin concentrations yielded towards greater survival rates for each genus. At two weeks after oryzalin treatments, protocorms and PLBs began to vitrify and die off, marked by their watery appearances and yellow to tan coloration. Eight weeks after treatments, the number of surviving

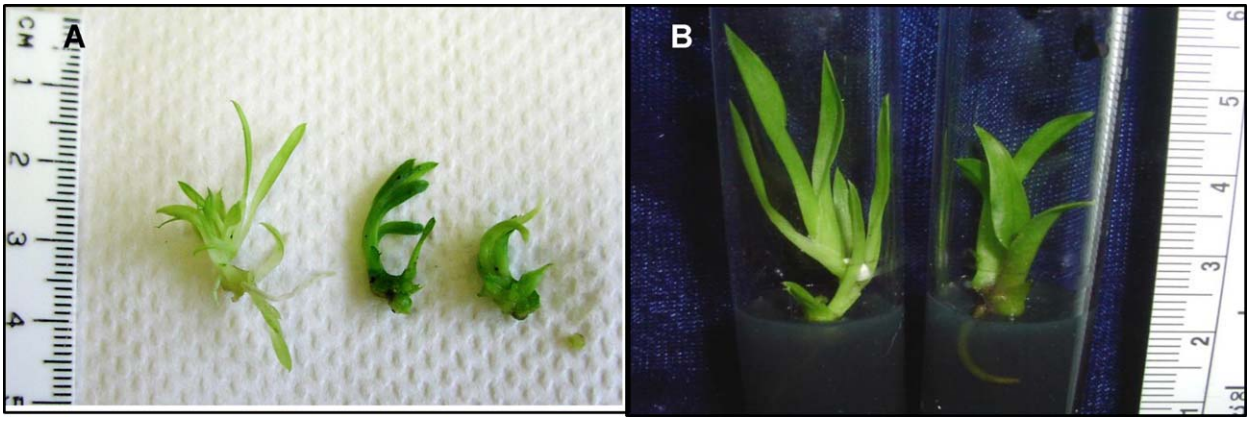

Fig. 1. Oryzalin's effect on growth of *Odontioda* Emma Sander'. (A) Shoot development at 14 weeks. From left to right: Control plant, 3 days 28.9 μM treatment, and 6 d 28.9 μM treatment. (B) Plant development at 22 weeks; control (left) and 6 d 28.9 μM treatment (right).

protocorms or PLBs were recorded. Surviving explants began differentiating and developing into shoot and leaf primordials. The highest number of surviving protocorms and PLBs were observed in *Cymbidium* (86%), *Dendrobium* (88%) and *Epidendrum* (82%). The least number of survival were recorded in *Odontioda* (50%) and *Phalaenopsis* (43%). In general, decreased rates of protocorm or PLB survival corresponded with the number of polyploids identified (Table 1).

The development of protocorms and PLBs from shoot primordial to the expansion of leaves were also affected by the exposure to oryzalin. Most of the surviving treated protocorms and PLBs produced healthy plants. However, variation in growth characteris-

Table 1
Effects of oryzalin concentrations and treatment durations on the survival of protocorms and PLBs, and the polyploidy conversion of *Cymbidium*, *Dendrobium*, *Epidendrum*, *Odontioda*, and *Phalaenopsis* orchids.

Genera	Plant material treated	Duration of treatment (days)	Concentration (μM)[a]	% Survival of PLBs or protocorms[b]	Mean stomatal lengths ± S.E. (μm)[c]	No. of polyploids identified[d]
Cymbidium	PLBs		0	100.0	38.17 ± 0.90	0
		3	14.4	82.9	40.23 ± 0.42	0
			28.9	84.3	37.63 ± 1.38	0
			57.7	84.3	41.04 ± 1.06	0
		6	14.4	81.4	40.34 ± 1.47	0
			28.9	80.0	41.00 ± 0.75	0
			57.7	78.6	40.41 ± 0.74	0
Dendrobium	PLBs		0	100.0	30.64 ± 0.30	0
		3	14.4	85.7	33.78 ± 0.63	0
			28.9	85.7	33.97 ± 0.73	0
			57.7	84.3	33.83 ± 0.97	0
		6	14.4	85.7	39.12 ± 0.92a	3
			28.9	84.3	42.81 ± 3.06a	3
			57.7	80.0	43.32 ± 1.10a	3
Etpidendrum	protocorms		0	100.0	33.42 ± 0.71	0
		3	14.4	82.9	40.17 ± 3.71	0
			28.9	81.4	36.24 ± 2.11	0
			57.7	77.1	39.76 ± 1.59	0
		6	14.4	77.1	38.95 ± 1.96	0
			28.9	77.1	40.70 ± 4.44	0
			57.7	58.6a	46.19 ± 5.32a	2
Odontioda	protocorms		0	100.0	20.57 ± 0.27	0
		3	14.4	48.6a	22.43 ± 1.09a	0
			28.9	41.4a	23.76 ± 0.53a	0
			57.7	34.3a	23.53 ± 0.26a	0
		6	14.4	27.1a	26.83 ± 0.41a	3
			28.9	25.7a	25.85 ± 0.95a	2
			57.7	20.0a	28.41 ± 1.50a	3
Phalaenopsis	protocorms		0	100.0	23.61 ± 0.75	0
		3	14.4	35.7a	32.17 ± 1.88a	3
			28.9	24.3a	33.29 ± 0.43a	3
			57.7	22.9a	35.56 ± 1.95a	3
		6	14.4	25.7a	38.73 ± 2.27a	3
			28.9	18.6a	39.55 ± 1.80a	2
			57.7	18.6a	35.97 ± 0.60a	3

Letter "a" indicates means significantly different within the column and genera group according to Dunnett's test at $P < 0.05$.

[a] Zero represents control, modified VW medium without oryzalin.

[b] Ten protocorms or PLBs per treatment with 5 replications

[c] Measured from screened seedlings or plantlets (ten to fifteen stomata per treatment with 3 replications).

[d] Identified by stomatal lengths of treated plants equal to or greater than 1.25× that of the controls.

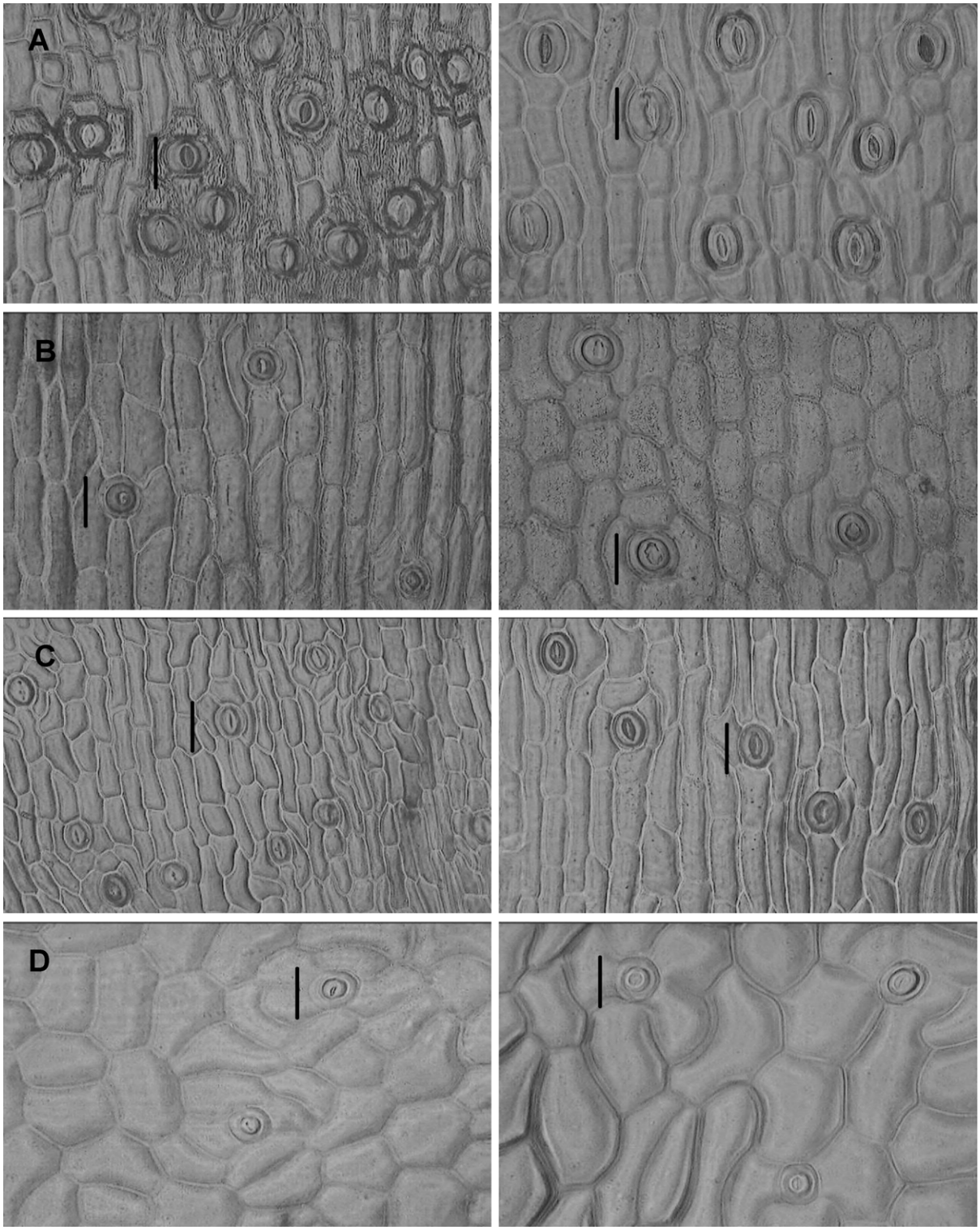

Fig. 2. Guard cells of Dendrobium (A), Epidendrum (B), Odontioda (C), and Phalaenopsis (D). Controls (left) and oryzalin induced polyploids (right). 400× magnification. Bars = 40 μm.

tics was observed between treatment durations (Fig. 1). *Cymbidium* were not affected by any of the treatment exposures. Rapid growth was observed, and the morphological characteristics of treated plants were indifferent to those of their controls. Data analysis of survival and for increases in stomatal lengths was also statistically insignificant in all treatments in *Cymbidium*.

The number of polyploids identified for each treatment are detailed in Table 1. Stomatal measurements of suspected polyploid plants were taken between 16 and 24 weeks depending on plant development and the availability of fully expanded leaves in culture. Stomatal lengths of treated plants evaluated as 1.25× the controls (stomatal lengths ≥ control stomatal lengths) (Russell, 2004) were identified as polyploids (Fig. 2). No polyploids were identified in *Cymbidium* (stomatal lengths ≤ 47.71 μm). Nine polyploids (stomatal lengths ≥ 38.30 μm) were identified in all 6 d concentrations in *Dendrobium*. *Epidendrum* had a significant

measure in the 6 d 57.7 μM concentration, and its stomatal length was significant at 46.19 ± 5.32 μm (≥41.78 μm). At this treatment level, two polyploids were identified. *Odontioda* had eight polyploids (stomatal lengths ≥25.71 μm) identified in the 6 d treatments. *Phalaenopsis* had significant differences between all treatment exposures and in the lengths of stomata (≥29.51 μm). This genus yielded 17 polyploids as the result of oryzalin treatments.

4. Discussion

Determining an effective, non-lethal treatment is the key for the successful creation of polyploids. Durations of exposure and treatment concentrations affected survival rates of protocorms and PLBs for which polyploids were identified. An inverse relationship between survival and the identified polyploids was noted (Table 1). Similar results were reported in experiments conducted by Thao et al. (2003); Ascough et al. (2008); and, Karimiani et al. (2009). Since literature on the use of oryzalin in orchids was not found, trials on herbaceous perennials (Van Tuyl et al., 1992; Van Duren et al., 1996; Takamura et al., 2002; Thao et al., 2003) assisted in establishing the degree of oryzalin applications in our experiments.

Wimber and Van Cott (1966) and Griesbach (1981) reported that undifferentiated embryonic cells such as protocorms and PLBs are the best plant materials to use for polyploid induction in orchids. Antimitotic chemicals must come in direct contact or be in close proximity to meristematic tissues for mitotic inhibition to occur. Explants must be exposed to chemicals at levels high enough to saturate plant tissues and induce polyploidy without redoubling of epidermal tissues before reaching the meristem (Kermani et al., 2003; Allum et al., 2007). The lack of response in *Cymbidium* may be attributed to the advanced development at which the PLBs were at when exposed to oryzalin; and/or, treatment durations and concentrations were not strong enough for it to permeate tissues and meristems. *Cymbidium* had the highest survival rates, and was the earliest to differentiate into shoots within 4 weeks after treatment. In related studies, 0.005% (144.4 μM) oryzalin treatment was optimum for inducing ploidy in *Lilium* (Van Tuyl et al., 1992). The concentrations used for *Cymbidium* were considerably less than this. Further experiments are necessary to determine oryzalin's effectiveness to produce polyploids with oryzalin in *Cymbidium*.

Polyploids were identified only in the 6 d 14.4, 28.9, and 57.7 μM treatments of *Dendrobium*. It appears that this genus requires at least this amount of time for mitosis inhibition with oryzalin. No significant differences were seen between treatment concentrations. *Epidendrum* had only one significant treatment level at 6 d duration and 57.7 μM concentration. This treatment produced two identified polyploids. The survival rate of *Epidendrum* sharply declines at the 6 d 57.7 μM concentration. The 6 d 57.7 μM concentration treatment appears optimal for *Epidendrum*. Polyploids in *Odontioda* were identified only among the 6 d treatment concentrations, and unlike *Dendrobium*, survival rates for all treatment durations were significantly different from their controls. Comparing the 3 d 14.4 μM and 6 d 14.4 μM treatments, it appears that *Odontioda* requires at least 6 d at this concentration for induction to occur.

Of the genera treated, *Phalaenopsis* produced the most identified polyploids. It was also observed that this genus was the most sensitive to oryzalin treatments. Significant differences were noted in survival, and upon visual examination, it also had the greatest morphological differences between the treated plants and controls. Leaves of treated plants were noticeably thicker and shorter than the control plants. A 1.25× factorial of the control meant that stomatal lengths ≥29.51 μm (2× the control) were polyploids (Russell, 2004). Polyploids were identified in both the 3 d and 6 d treatments.

Stomatal assay by examination of leaf imprints is a functional and economical method for determining ploidy in the Orchidaceae. Investigators have used this method to study the genetic characteristics of *Stanhopea* (Ferry et al., 2000), and for evaluating the ploidy levels in the following orchids: *Cymbidium* (Wimber and Van Cott, 1966; Russell, 2004); *Calanthe* (Tahara and Kato, 1987); *Paphiopedilum* (Watrous and Wimber, 1988); and, *Phalaenopsis* (Chen et al., 2009). Earlier reports of stomatal measurements used arbitrary units based on a graticule scale built into a microscope's ocular eyepiece (Wimber and Van Cott, 1966; Watrous and Wimber, 1988; Russell, 2004). The software used in our experiment converted calibrated graphical lines drawn on digital images of stomata from digital pixel units into micrometers. We think this technology and software provided increased accuracy and improved the process of taking stomatal measurements.

Ploidy assessment by guard cell assay allows breeders the opportunity for early screening of potential polyploids without investing in the time and space for growing out large population of plants. This method is less time consuming which permits simple and rapid analysis of large groups of plants. For the purpose of our experiment, guard cell measurements were used to screen out potential polyploids from plants growing in vitro; plant materials to grow out, later evaluate, and use as stud plants for breeding. However, ploidy determination by morphological or anatomical assays alone have its limitations (Chen et al., 2009; Dhooghe et al., 2010). Guard cell assays estimate a change in ploidy at the L1 level, discounts the presence of mixoploids or periclinal chimeras, and do not verify ploidy levels or chromosome numbers. Therefore, other methods such as flow cytometry and chromosome counting of root mitotic cells should be employed in conjunction with stomatal measurements to further confirm ploidy levels (Zlesak et al., 2005; Vanstechelman et al., 2010; Winarto et al., 2010).

Using oryzalin, several factors can influence successful polyploid induction including: the type of explants used; the closeness in proximity of the chemical to the meristematic tissues; and, how great the concentrations of oryzalin are to induce doubling of chromosomes without causing the occurrence of chimeric plants and mixoploids (Allum et al., 2007).

The methods and treatments utilized in our study demonstrates that oryzalin is effective in inducing polyploids in Orchidaceae. These findings can be used to further develop protocols for the in vitro polyploid induction of orchids with oryzalin.

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