In vitro Ploidy Manipulation for Crop Improvement

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In vitro regeneration systems provide a powerful tool for manipulating ploidy to facilitate breeding and development of new crops. Polyploid induction can expand breeding opportunities, assist with the development of seedless triploid cultivars, enhance ornamental characteristics and environmental tolerances, increase biomass and restore fertility in wide hybrids. *In vitro* ploidy manipulation is commonly induced using antimitotic agents such as colchicine, oryzalin and trifluralin, while many other antimitotic agents have been relatively unexplored. Successful induction requires a synergistic pairing of efficient penetration of the antimitotic agent and may be dependent the length of exposure and concentrations of antimitotic agents, tissue types, and interactions with basal media and plant growth regulators. *In vitro* conditions vary among taxa and individual genera, species, and cultivars, often requiring unique treatments to maximize polyploid induction. In some taxa, the induction of polyploidy influences *in vitro* growth, development, and root formation. Here we provide an overview of mitotic inhibitors and their application for *in vitro* ploidy manipulation for plant breeding and crop improvement.

Keywords: chromosome doubling, *in vitro* regeneration, mitotic inhibitor, plant breeding, polyploidy, whole genome duplication

INTRODUCTION

Polyploidy, the condition of having more than two sets of chromosomes, has long been recognized as a major driver of plant evolution and speciation (Soltis et al., 2009). Naturally occurring polyploids have been identified in a wide range of taxa and recent estimates suggest that almost all extant angiosperms have experienced polyploid events in their evolutionary history (Soltis and Burleigh, 2009). Potential evolutionary/adaptive advantages of being polyploid include increased heterosis, gene redundancy and mutational robustness, and phenotypic plasticity (Comai, 2005; Sattler et al., 2015).

The artificial induction of polyploidy can provide a valuable tool to assist with understanding evolutionary processes and to facilitate plant breeding and improvement programs. Polyploids often possess improved traits, such as thicker, darker-colored leaves; larger, longer-lasting flowers and thicker petals; enhanced vigor; improved tolerances to environmental stresses, pests and pathogens; increased metabolite production and may restore fertility in sterile wide hybrids (Kehr, 1996; Comai, 2005; Ranney, 2006; Banyai et al., 2009). However, incorporating polyploids into plant breeding programs often necessitates the induction of new polyploids.

In vitro chromosome doubling has predominantly been induced using the antimitotic agent colchicine. However, the herbicides oryzalin and trifluralin, are often preferred due to their reduced toxicity, higher affinity to plant tubulins, and effectiveness at lower concentrations. The success of *in vitro* chromosome doubling protocols is dependent upon the effectiveness of antimitotic

agents to temporarily arrest cell division (cytokinesis) in actively growing tissue. While the length of exposure and concentrations of antimitotic agents is critical for chromosome doubling, several other factors such as tissue types, methods of application, culture conditions and species differences may influence the efficacy of *in vitro* chromosome doubling. Species-specific *in vitro* chromosome doubling protocols for diverse and valuable taxa have been widely reported (**Table 1**). In this review we will explore mitotic inhibitors and examine factors that impact *in vitro* polyploid manipulation and provide possible areas for further research.

HISTORY

Artificial induction of polyploids in plants was first reported in the late 1930s with Blakeslee and Avery (1937) demonstrating the use of colchicine for chromosome doubling of several species. Numerous studies investigating colchicine for ploidy manipulation soon followed this initial report (see Dunham and Banta, 1940). The interest in polyploidy grew rapidly, and in 1941, The American Naturalist published the 'Symposium on theoretical and practical aspects of polyploidy in crop plants' (The American Naturalist, 1941). In that issue, Emsweller and Ruttle (1941) first discussed the value of induced polyploidy for the improvement of ornamental plants. Since these early studies chromosome doubling has become an integral component of breeding programs for many economically important crops.

Advancements in plant tissue culture in the 1960s provided new opportunities for developing polyploids. Murashige and Nakano (1966) isolated tetraploid cells from the pith of diploid tobacco plants and used *in vitro* culture to stabilize and produce tetraploid plants. Soon after, Heinz and Mee (1970) reported the use of colchicine to induce polyploid sugarcane cell suspensions. Hussey and Hypher (1978) recovered tetraploid sugar beets by treating *in vitro* grown plantlets with colchicine. The past two decades have seen a significant increase in the use of *in vitro* polyploid induction. This increase may be attributed, in part, to the development and proliferation of tissue culture protocols for a diverse range of taxa.

ANTIMITOTIC AGENTS

In vitro polyploid manipulation is dependent upon disrupting the cell cycle to prevent polar migration of chromosomes during anaphase. Chemicals ranging from caffeine (Thomas et al., 1997) and nitrous oxide (Taylor et al., 1976) to antimicrotubule herbicides have all been shown to induce polyploidy. However, several antimicrotuble compounds, such as colchicine and oryzalin, have been predominantly used for successful *in vitro* polyploid induction (**Table 2**).

Colchicine [*N*-5,6,7,9-tetrahydro-1,2,3,10-tetra-methoxy-9-oxobenzo(a)heptalen-7-yl] acetamide is perhaps the most commonly used mitotic inhibitor and has been used to recover polyploids in a wide range of species (**Table 1**). Colchicine is extracted from the bulbs of autumn crocus (*Colchicum autumnale*) and is widely used as a medication to treat gout and other inflammatory diseases. As an antimitotic agent, colchicine disrupts the cell cycle beginning at metaphase where it destabilizes microtubules by binding to the β–tubulin subunit to form a colchicine-tubulin complex. As such, colchicine prevents microtubule polymerization, without influencing depolymerization, resulting in degradation of microtubules (Leung et al., 2015). For *in vitro* chromosome doubling, colchicine has advantages that it is soluble in aqueous solutions, heat-stable, and can be autoclaved and easily applied to plant tissues. However, colchicine has high binding affinity for animal microtubules and is potentially toxic to humans (Morejohn et al., 1984). In contrast, colchicine has relatively low binding affinity to plant microtubules which requires it to be used in high concentrations to maintain effectiveness.

Collectively, certain herbicides provide viable alternatives to colchicine for *in vitro* ploidy manipulation. It is estimated that approximately 25% of herbicides act by affecting mitosis (Vaughn and Lehnen, 1991). Herbicides consist of several different chemical classes with antimitotic activity, including dinitroanilines (oryzalin, trifluralin, pendamethalin) (Morejohn et al., 1987; Hugdahl and Morejohn, 1993), phosphorothioamidates (amipro-phos-methyl) (Murthy et al., 1994), benzamides (propyzamide) (Bartels and Hilton, 1973), cyanoacrylates (ethyl (2Z)-3-amino-2-cyano-4-ethylhex-2-enoate) (Tresch et al., 2005), and carbonates (chlorpropham, propham).

Dinitroanilines are the most common class of herbicides used for *in vitro* ploidy manipulation. Dinitroanilines have shown to have high binding affinity to plant tubulins at low concentrations while showing little binding affinity with animal tubulins (Morejohn et al., 1987; Hugdahl and Morejohn, 1993). This group of compounds works similarly to colchicine to disrupt mitosis in metaphase. Dinitroanilines bind to α-tubulin to form a tubulin-dinitroaniline complex to prevent microtubule polymerization.

The dinitroanilines contain numerous compounds that can be divided into symmetric (e.g., oryzalin, trifluralin) and non-symmetric (e.g., pendimethalin) compounds that differentially interact with α-tubulin (see Ma et al., 2010). In a comprehensive study evaluating the effect of 12 different dinitroanilines on the unicellular parasite, *Toxoplasma gondii*, expressing oryzalin sensitive wild-type and α-tubulin mutants conferring oryzalin resistance, Ma et al. (2010) found that non-symmetric compounds dinitramine and pendimethalin demonstrated increased inhibition. Similarly, several trifluralin analogs showed increased binding efficiencies to α-tubulin of the unicellular organism *Trypanosoma brucei* (Giles et al., 2009). These studies have suggested that small species-specific differences in the properties of α-tubulin binding sites may influence interaction with functional groups of different dinitroanilines.

For plant species, studies have shown that mutations to α-tubulin binding sites may alter binding affinities and confer resistance to the dinitroanilines, specifically oryzalin and trfluralin (Anthony and Hussey, 1999; Chu et al., 2018). This may have significance for *in vitro* ploidy manipulation, as studies have been primarily isolated to oryzalin and trifluralin (**Table 1**),

TABLE 1 | Reported *in vitro* polyploid induction of diverse crops utilizing varied tissues, antimitotic agents and concentrations, and exposure times.

Family	Species	Tissue	Agent	Concentration	Exposure	References
Actinidiaceae	*Actinidia chinensis*	Organogenesis from petioles	Colchicine	1.25–2.5 mM	4 h	Wu et al., 2011
Amaryllidaceae	*Clivia miniate*	Immature embryos	Colchicine	10–50 mM	10–30 days	Wang and Lei, 2012
	Allium cepa	Shoot apices	Colchicine	2.5 mM	24 h	Geoffriau et al., 1997
			Oryzalin	50 μM	24 h	
Apiaceae	*Centella asiatica*	Shoot apices	Colchicine	1.25–5 mM	12–24 h	Kaensaksiri et al., 2011
	Trachyspermum ammi	Seeds	Colchicine	0.06–1.25 mM	6–48 h	Noori et al., 2017
Araceae	*Spathiphyllum wallsii*	Somatic embryos	Oryzalin	10 μM	16 h	Eeckhaut et al., 2004
			Trifluralin	10 μM		
			Colchicine	100 μM		
	Zantedeschia sp.	Shoot apices	Colchicine	1.25 mM	1–4 days	Cohen and Yao, 1996
Asparagaceae	*Ophiopogon planiscapus*	Embryogenic callus	Oryzalin	7.5 μM	3–9 days	Gillooly et al., 2015
Asteraceae	*Echinacea purpurea*	Organogenesis from petioles	Colchicine	300 mM	28 days	Nilanthi et al., 2009
	Rudbeckia hirta	Shoot apices	Oryzalin	15 μM	3–5 days	Touchell personal communication
	Rudbeckia subtomentosa	Shoot apices	Oryzalin	15–60 μM	3–5 days	Palmer et al., 2008
	Rudbeckia maxima	Shoot apices	Oryzalin	60 μM	3 days	Palmer et al., 2008
	Gerbera jamesonii	Shoots	Colchicine	2.5–12.5 mM	2–8 h	Gantait et al., 2011
	Smallanthus sonchifolius	Nodal segments	Colchicine	3 mM	24 h 24–48 h	Viehmannová et al., 2009
			Oryzalin	20–25 μM		
	Artemisia annua	Organogenesis from leaves	Colchicine	25 mM	24 h	Banyai et al., 2009
Balsaminaceae	*Impatiens walleriana*	Shoot apices	Oryzalin	15–60 μM	12–48 h	Ghanbari et al., 2019
Bixaxeae	*Bixa orellana*	Hypocotyl segments	Oryzalin	15 μM	15 days	de Carvalho et al., 2005
Brassicaceae	*Raphanus sativus* x *Brassica oleracea*	Shoots	Amiprophos-methyl	10–30 μM	24 h	Niimi et al., 2015
	Brassica oleraceae var. *capitate*	Root cultures	Colchicine	5–10 mM	3–12 h	Yuan et al., 2015
	Brassica oleraceae var. *italica*	Root cultures	Colchicine	1.25 mM	6–12 h	Yuan et al., 2015
Cannabaceae	*Humulus lupulus*	Shoot apices	Colchicine	0.25–2.5 mM	24–72 h	Trojal-Golush and Skomra, 2013
	Cannabis sativa	Shoot apices	Oryzalin	20–60 μM	24 h	Parsons et al., 2019
Caryophyllaceae	*Lychnis senno*	Nodal segments	Colchicine	0.25–1.25 μM	3 days	Chen et al., 2006
	Dianthus caryophyllus	Nodal segments	APM	32.9 μM	24 h	Nimura et al., 2006
Cucurbitaceae	*Citrullus lanatus*	Hypocotyl segments	Colchicine	0.25 mM	4 days	Raza et al., 2003
	Citrullus lanatus	Shoot apices	Colchicine	1.5–2.5 mM	3–9 days	Nasr et al., 2004
			Oryzalin	25–100 μM	3–9 days	
			Ethalfluralin	25–100 μM	3–9 days	
			Butralin	25–100 μM	3–9 days	
			Dinitramine	25–100 μM	3–9 days	
	Cucumis sativus	Nodal segments/shoot apices	Colchicine	0.6–3.75 mM	18–36 h	Ebrahimzadeh et al., 2018
			Oryzalin	15–433 μM	18–36 h	
			Trifluralin	15–450 μM	18–36 h	
Ericaceae	*Rhododendron* 'Frangrantissimum Improved'	Organogenic callus	Oryzalin	7.5	14 days	Hebert et al., 2010
	Rhododendron	Seedlings	Oryzalin	0.3 mM	3 days	Eeckhaut et al., 2002
			Trifluralin	0.3 mM	3 days	
	Rhododendron	Shoots	Oryzalin	30 μM	24 h	Väinölä, 2000
Fabaceae	*Cercis glabra*	Shoot apices	Oryzalin	150 μM	12–96 h	Nadler et al., 2012
Haemodoraceae	*Anigozanthos* sp.	Axillary buds	Colchicine	2.5 mM	7 days	Griesbach, 1990
Hydrangea	*Hydrangea macrophylla*	Apical shoot	Oryzalin	15–30 μM	3–5 days	Touchell personal communication
	Hydrangea arborescence	Apical shoot	Oryzalin	15–30 μM	3–5 days	Touchell personal communication
Hypericaceae	*Hypericum* sp.	Organogenic callus	Oryzalin	30 μM	6 days	Meyer et al., 2009
Iridaceae	*Crocosmia aurea*	Seed	Colchicine	0.25–25 μM	12 h–3 days	Hannweg et al., 2013

(Continued)

TABLE 1 | Continued

Family	Species	Tissue	Agent	Concentration	Exposure	References
	Watsonia lepida	Hypocotyl segments	Oryzalin	25–250 µM	1–3 days	Ascough et al., 2008
Lamiaceae	*Thymus persicus*	Shoot apices	Colchicine	0.75–1.25 mM	12–48 h	Tavan et al., 2015
	Tetradenia riparia	Seed	Colchicine	0.025–0.25 mM	12–72 h	Hannweg et al., 2016b
	Plectranthus esculentus	Nodal segments	Colchicine	0.025250 mM	12–72 h	Hannweg et al., 2016a
Liliaceae	*Tulipa gesneriana*	Flower stems	Oryzalin	1.44–28.8 µM	1–14 days	Chauvin et al., 2005
	Lilium hybrid	Bulb segments	Oryzalin	30–200 µM	2–6 h	Chandanie et al., 2011
	Linum album	Nodal segments	Colchicine	1.25–5 mM	24–96 h	Javadian et al., 2017
ιLythraceae	*Lagerstroemia indica*	Nodal segments	Colchicine	0.25–0.75 mM	10 days	Zhang et al., 2010
Oleaceae	*Syringa* sp.	Nodal segments	Colchicine	0.05–0.25 mM	1–2 days	Rose et al., 2000
Orchidaceae	*Bletilla striata*	Protocorms	Colchicine	1.25–5 mM	12–48 h	Pan-pan et al., 2018
	Dendrobium chrysotoxum	Protocorms	Colchicine	1.0 mM	24 h	Artichart, 2013
Passifloroideae	*Passiflora edulis*	Hypocotyl segments	Colchicine Oryzalin	0.025–1.25 mM 5–30 µM	15 days 15 days	Rêgo et al., 2011
Plantaginaceae	*Hebe* 'Oratia Beauty'	Nodal segments	Colchicine Oryzalin	0.5–1.0 mM 11.5–289 µM	48 h 48 h	Gallone et al., 2014
Plumbaginoidaceae	*Plumbago auriculata*	Shoot apices	Pendimethalin	800 µM	7 days	Jiang et al., 2020
Poaceae	*Miscanthus sinensis*	Shoots	Colchicine Oryzalin	313 µM 5–15 µM	18 h 4–7 days	Petersen et al., 2002, 2003
Poaceae	*Miscanthus* x *giganteus*	Shoots	Oryzalin	15 µM	3–5 days	Touchell and Ranney, 2012
	Panicum virgatum	Embryogenic callus	Colchicine	1 mM	13 days	Yang et al., 2014
	Triticum aestivum	Microspore culture	Colchicine	3 mM	24–48 h	Hansen and Andersen, 1998
Polemoniaceae	*Phlox subulata*	Shoot apices	Colchicine	0.125–1.0 mM	10–30 days	Zhang et al., 2008
Ranunculaceae	*Ranunculus asiaticus*	Shoots	Colchicine Oryzalin Trifluralin	100–200 µM 0.5–3.0 µM 2.0 µM	16–24 h 6–10 weeks 6–10 weeks	Dhooghe et al., 2009a
	Helleborus niger	Shoots	Oryzalin Trifluralin	3 µM 3–10 µM		Dhooghe et al., 2009b
	Helleborus x nigercors	Shoots	Oryzalin Trifluralin	3 µM 3–10 µM		Dhooghe et al., 2009b
Rhamnaceae	*Ziziphus jijuba*	Shoot apices	Colchicine	1.25–2.5	24–72 h	Gu et al., 2005
Rosaceae	*Rosa* 'Therese Bugnet'	Shoot apices/nodal segments	Oryzalin	5–15 µM	14–28 days	Kermani et al., 2003
	Rosa rugosa	Nodal segments	Oryzalin	5 µM	12 h	Allum et al., 2007
	Rosa hybrida	Nodal segments	Oryzalin Trifluralin APM	6–24 µM 6–24 µM 6–24 µM	12–48 h	Khosravi et al., 2008
	Rosa persica	Nodal segments	Trifluralin APM	6–24 µM 6–24 µM	12–48 h	Khosravi et al., 2008
	Chaenomeles japonica	Nodal segments	Oryzalin Colchicine	10–50 µM 0.25–38 mM	1–2 days	Stanys et al., 2006
	Prunus laurocerasus	Shoots	Oryzalin	150 µM	1–2 days	Contreras and Meneghelli, 2016
	Malus x *domestica*	Axillary buds	Colchicine	10 mM	2 days	Hias et al., 2017
	Pyrus pyriflora	Shoots	Colchicine	0.25 mM	1–8 days	Kadota and Niimi, 2002
	Pyrus communis	Organogenesis from leaf	Colchicine	1 mM	24–72 h	Sun et al., 2009
Salicaceae	*Populus* sp.	Organogenesis from leaves	Colchicine	50–100 µM	2–4 days	Xu et al., 2016
	Populus hopeiensis	Organogensis from leaves	Colchicine	100 µM	96 h	Wu et al., 2020
Sapindaceae	*Acer platanoides*	Nodal segments	Oryzalin	15 µM	3 days	Lattier et al., 2013
Scrophulariaceae	*Buddleja* sp.	Nodal segments	Oryzalin	3–7 µM	1–3 days	Dunn and Lindstrom, 2007
Solanaceae	*Petunia axillaris*	Leaves, organogenesis	Colchicine	0.2 mg	15 days	Regalado et al., 2017
Vitaceae	*Vitis* sp.	Shoots	Colchicine	1.25 mM	24–48 h	Notsuka et al., 2000
Zingiberaceae	*Hedychium muluense*	Embryogenic callus	Colchicine Oryzalin	2.5 mM 20–120 µM	1–3 days 1–3 days	Sakhanokho et al., 2009

TABLE 2 | Mitotic inhibitors that are used or have potential to interfere with the cell cycle to induce polyploids.

Mitotic inhibitor	Mode of action	Application
Miscellaneous		
Colchicine	Destabilizes β-tubulin	Seeds, shoots, see **Table 1**
Taxol	Stabilizes β-tubulin	Not reported for plant polyploidy
Nitrous oxide	Possible interacts with α-tubulin	Seeds Taylor et al. (1976)
Dinitroanilines		
Oryzalin	Destabilizes α-tubulin	Seeds, nodal segments, shoots, callus, see **Table 1** for examples
Trifluralin	Destabilizes α-tubulin	Nodal segments, shoots, callus, see **Table 1** for examples
Pendimethalin	Destabilizes α-tubulin	Limited use, Micro-shoots of *Nepta* (Mitrofanova et al., 2003)
Ethalfluralin	Destabilizes α-tubulin	Limited use, Micro-shoots of *Nepta* (Mitrofanova et al., 2003)
Benzamides		
Propyzamide	Destabilizes α-tubulin	Nodal segments of *Vitis davidii* (Cai et al., 2016)
Phosphorothioates		
Amiprophos-methyl (APM)	Destabilizes α-tubulin, same binding site as oryzalin	Nodal segments (Nimura et al., 2006)
Cyanoacrylates		
Ethyl (2Z)-3-amino-2-cyano-4-ethylhex-2-enoate (CA1)	Destabilizes α-tubulin, same binding site as oryzalin	No reports for plant polyploid induction
Carbamates		
Propham	Disrupt and fragment spindle poles	No reports for plant polyploid induction
Proteasome inhibitors		
Lactacystin	Interfere with regulatory proteins that govern metaphase, anaphase and cytokinesis transitions	No reports for plant polyploid induction
MG132	Same as lactacystin	No reports for plant polyploid induction
Cancer drugs		
Reversine	Inhibits anaphase in human breast tissue to form polyploid cells	No reports for plant polyploid induction

with only limited reports of alternative dinitroanilines such as pendamethalin (Ren et al., 2018), dinitramine (Nasr et al., 2004), ethylfluralin (Mitrofanova et al., 2003), and butralin (Nasr et al., 2004). With the structural diversity in dinitroanilines, different compounds may provide higher efficacy for recovering polyploids in recalcitrant species.

The phosphoric amides are another group of herbicides with antimitotic activity, of which amiprophos-methyl (APM) has been used for *in vitro* chromosome doubling (Khosravi et al., 2008). Amiprophos-methyl has shown high affinity for tobacco α-tubulin and may target the same binding sites as oryzalin

(Murthy et al., 1994). An advantage of APM is that it has increased solubility in water compared to dinitroanilines, thus reducing the use of additional solvents. Similarly, the benzamides, particularly propyzamide have shown potential for *in vitro* chromosome doubling (Cai et al., 2016). Propyzamide also targets the same binding sites as oryzalin (Bartels and Hilton, 1973). Cyanoacrylates are another class of antimitotic agents that have the same mechanisms as dinitroanilines (Tresch et al., 2005). Similar to APM and propyzamide, the cyanoacrylates, ethyl (2Z)-3-amino-2-cyano-4-ethylhex-2-enoate (CA1) and CA2 bind to α-tubulin at the same sites as oryzalin (Tresch et al., 2005). However, they have yet to be used for *in vitro* polyploid induction.

Nitrous oxide has also been reported to induce polyploids (Taylor et al., 1976). The mode of action has remained unclear. However, Kitamura et al. (2009) suggested that nitrous oxide may induce polyploidy by inhibiting microtubule polymerization. It is likely that nitrous oxide interacts with tyrosine to form nitrotyrosine (Neill et al., 2003). Nitrotyrosine may replace tyrosine in α-tubulin and influence polymerization (Blume et al., 2013; Lipka and Müller, 2014). Lipka and Müller (2014) found that in *Arabidopsis thaliana* nitrotyrosine alone inhibited microtubule polymerization, but reduced sensitivity to oryzalin due to changes in α-tubulin binding sites. However, Jovanović et al. (2010) demonstrated that nitrotyrosine increased sensitivity of *Nicotiana tabacum* L. cell cultures to oryzalin, suggesting nitrotyrosine could provide addition antimicrotubule qualities.

In contrast to antimicrotubule agents, the carbamate herbicides, such as propham or chlorpropham, act to disrupt mitosis without influencing the polymerization or destabilization of microtubules. Rather, carbamates act to disrupt and fragment spindle poles throughout the cell resulting in a multipolar, rather than bipolar, migration of chromosomes (Vaughn and Lehnen, 1991). As such, it is unlikely that carbamates will be effective in the development of polyploids.

There are no reports of the use of proteasome inhibitors being utilized for vitro ploidy manipulation. However, proteasome inhibitors such as lactacystin and MG132, interfere with key regulatory proteins that govern the metaphase, anaphase and cytokinesis transitions (Planchais et al., 2000). A drawback of proteasome inhibitors is that treatments are not fully reversible (Planchais et al., 2000).

Another group of cell cycle inhibitors, including hydroxyurea and aphididcolin, act to arrest cell cycle at the beginning of S-phase. Following the removal of the inhibitor, cells progress through S, G_2, and M phase in a synchronized manner (Darzynkiewicz et al., 2011). While these compounds do not directly affect polyploidy, they may have utility as treatments to facilitate synchronizing the cell cycle and maximize the number of cells affected by the antimicrotubule agent thereby reducing cytochimeras.

IN VITRO POLYPLOID INDUCTION – AN OVERVIEW

The success of *in vitro* polyploid induction is highly integrated with the development of efficient *in vitro* culture protocols. Plant

tissue culture systems have often proven difficult for many taxa, especially woody plants and only a limited number of species have successfully been grown in tissue culture. Protocol development often needs to be conducted for each species and often for each clone to optimize regeneration protocols that can be applied for *in vitro* polyploid induction. Nonetheless, successful chromosome doubling has been achieved for a significant number of species representing a diverse range of families and genera (**Table 1**).

KEY VARIABLES INFLUENCING *IN VITRO* POLYPLOID INDUCTION

Tissue Type

In vitro ploidy manipulation is highly dependent on the availability of successful *in vitro* regeneration systems. Although apical meristems can be treated *in vitro*, regeneration via somatic embryogenesis or shoot organogenesis is highly desirable for polyploid induction treatments. The ability to regenerate an entire plant from a single or only a few cells can improve the development of homogenous polyploid plants and minimizes the possibility of cytochimeras. Organogenic or embryogenic regeneration systems have been used for chromosome doubling for several species (**Table 1**). For *Echinacea purpurea*, polyloids were regenerated from petioles treated with colchicine (Nilanthi et al., 2009). Similarly, organogenesis from *Populus* sp. leaves treated with oryzalin resulted in polyploids. Sakhanokho et al. (2009) treated embryogenic callus of *Hedychium muluense* with colchicine or oryzalin to develop homogeneous polyploids. Further, *in vitro* regeneration systems are essential for developing dihaploids. Hansen and Andersen (1998) regenerated dihaploids from microspores of *Triticum aestivum* treated with colchicine. In an alternative approach, Yuan et al. (2015) regenerated dihaploids from *in vitro* roots treated with colchicine of haploid *Brassica* sp.

In vitro regeneration systems via organogenesis and somatic embryogenesis, however, have only been developed for relatively few species, and this approach may result in greater somaclonal variation due to mutations and epigenetic changes (Bairu et al., 2007). For many crops, the development of *in vitro* regeneration systems provides unique challenges and alternative tissues may need to be considered. As such, nodal segments and shoot apices have been the most widely used tissues for *in vitro* chromosome doubling (**Table 1**).

To obtain homogenous polyploids using nodal segments and shoot apices, all initial cells within the three histogenic layers of the meristems need to be affected by the antimitotic agent (Dermen, 1953; Klekowski, 2003). If all the initial cells are not affected, mixoploids or cytochimeras may form. Mixoploids have been widely observed in *in vitro* chromosome doubling of a wide range of species, including; *Acer platanoides* (Lattier et al., 2013), *Helleborus* sp. (Dhooghe et al., 2009b), *Hypericum* sp. (Meyer et al., 2009), *Lagerstroemia indica* (Zhang et al., 2010), *Rhododendron* hybrids (Väinölä, 2000; Hebert et al., 2010), *Rosa rugosa* (Allum et al., 2007), *Ranunculus asiaticus*

(Dhooghe et al., 2009a), *Tulipa gesneriana* (Chauvin et al., 2005), and *Vitis* sp. (Notsuka et al., 2000; see **Table 1**).

Mixoploid tissue are often unstable and have a high tendency for diplontic selection and may revert to their original ploidy. Diplontic selection may occur when diploid (or lower ploidy) cells (having less DNA) can replicate and divide faster than neighboring higher ploidy cells (Dermen, 1953; Klekowski, 2003). Over time, the proportion of lower ploidy cells increases resulting in the loss of converted cells. For example, Hussey and Hypher (1978) documented cytochimeral sugar beets after treatment with colchicine and observed that polyploid cells disappeared over subsequent subcultures. Similarly, Lattier et al. (2013) found that for *Acer platanoides*, mixoploid tissues reverted to diploids over a 6-month period.

Another approach is to treat seeds with antimitotic inhibitors prior to establishing *in vitro* cultures. Hannweg et al. (2013) treated *Crocosmia aurea* seed with 0.25 µM colchicine overnight or 25 µM colchicine for 3 days before using introducing them into tissue culture. The highest induction of homogenous tetraploids (29.82%) was achieved with 0.25 µM overnight.

Selection and Exposure to Antimitotic Agents

Polyploid induction is highly variable between species and cultivars and is dependent upon antimitotic agent, tissue type, and culture conditions (**Table 1**). While the dinitroaniline herbicides have a high affinity for plant tubulins and, more recently, have been increasingly used for *in vitro* chromosome doubling, colchicine remains a highly effective mitotic agent for many species. Morejohn et al. (1984) showed that binding efficiencies of colchicine to plant tubulins varied substantially between species, and colchicine still remains the most efficient and preferred antimitotic agents for species such as *Populus hopeiensis* (Wu et al., 2020).

Cholchicine is clearly the most used antimitotic agent (**Table 1**), regardless of the well-documented negative drawbacks (Dhooghe et al., 2009a), and has been used for a diverse species ranging from herbaceous (e.g., *Echinacea purpurea;* Nilanthi et al., 2009) to woody crops (e.g., *Chaenomeles japonica;* Stanys et al., 2006). The wide success of colchicine as an antimitotic inhibitor has led to its continued and regular use in more recent studies, for example *Bletilla striata* (Pan-pan et al., 2018), *Petuna axillaris* (Regalado et al., 2017), and *Populus hopeiensis* (Wu et al., 2020). In comparison, oryzalin has been used successfully to create polyploid lines of woody and semi-woody plants including *Rosa* (Kermani et al., 2003), *Rhododendron* (Hebert et al., 2010), *Chaenomeles* (Stanys et al., 2006), *Hypericum* (Meyer et al., 2009), and *Acer platanoides* (Lattier et al., 2013; **Table 1**). Further, oryzalin has been shown to be more efficient than colchicine for *in vitro* chromosome doubling for *Chaenomeles japonica* (Stanys et al., 2006), *Rhododendron* sp. (Väinölä, 2000), *Watsonia lepida* (Ascough et al., 2008), and *Ranunculus asiaticus* (Dhooghe et al., 2009a).

The concentration and length of exposure to antimitotic compounds are factors that are consistently investigated. While low levels of exposure are non-effective and high levels are

lethal, the interaction between exposure time and concentration is not fully understood. For oryzalin, Meyer et al. (2009) found that the concentration significantly affected survival and ploidy induction of *Hypericum* sp. callus; however, length of exposure had no effect. Similarly, concentration and exposure duration of oryzalin were not significant factors in inducing polyploidy in *Rhododendron* hybrids (Väinölä, 2000). For *Populus hopeiensis* both concentration and exposure time to colchicine were highly significant factors influencing polyploidy. For *Rosa* sp. the relationship between concentration and exposure time to oryzalin seemed to be dependent upon tissue type (Kermani et al., 2003; Allum et al., 2007). Kermani et al. (2003) found reduced duration to oryzalin was required when using 1 mm nodal segments compared to shoot apices. Similarly, Allum et al. (2007) found nodal segment size influenced exposure duration to oryzalin. Allum et al. (2007) suggested that to ensure chromosome doubling, exposure time should be long enough to maximize the number of cells in mitosis and exposure to antimitotic compounds.

Solubility in relation to binding affinity to tubulin is another factor to consider when selecting an antimitotic agent. For example, colchicine is highly soluble in water (>1.5M) and can be readily added to standard culture media, but has a relatively low binding affinity to plant tubulins (Morejohn et al., 1987). In contrast, dinitroanilines are relatively insoluble in aqueous solutions, with oryzalin reaching saturation at $< 7.5\ \mu M$ in water, but often bind to plant tubulins at concentrations below 500 nM (Morejohn et al., 1987). Interestingly, working concentrations of oryzalin commonly used for polyploid induction regularly exceed both binding affinity and solubility concentrations (**Table 1**). While species differences in oryzalin specificity to tubulins may account for some variability (Ma et al., 2010), the interaction between exogenous oryzalin concentrations and intracellular tubulin remain unclear.

The most common method for the *in vitro* application of antimitotic inhibitors is via treatment in a liquid solution or media, followed by recovering tissues on a regeneration or propagation medium, though, in some cases, the addition of antimitotic agents to solidified media has been successful for developing polyploids. For *Hypericum* sp., polyploids were developed after exposing regenerative callus to oryzalin in a liquid media for 3 to 9 days (Meyer et al., 2009). In contrast, Dhooghe et al. (2009a) used solid media containing oryzalin to induce polyploidy in *Ranunculus asiaticus*.

Commonly, the antimitotic agents are added to media with similar composition to the *in vitro* growth media. It is possible that media components may also interact with antimitotic agents to influence chromosome doubling. For example, the efficiency of dinitroanilines binding to α-tubulin is significantly influenced by pH and sucrose (Morejohn et al., 1987; Hugdahl and Morejohn, 1993). In tubulin binding assays, Hugdahl and Morejohn (1993), showed a threefold increase in oryzalin binding affinity when pH was increased from 6.0 to 7.0. Further, these authors noted that sucrose may alter the interaction between oryzalin and tubulin (Hugdahl and Morejohn, 1993). Sucrose and pH are two key components in plant tissue culture media and need to be considered when developing *in vitro* polyploidy protocols.

Plant growth regulators may also interact with antimitotic agents. Lattier et al. (2013) showed the addition of the cytokinin 6-benzyl amino purine (BA) to medium containing oryzalin increased shoot mortality at higher oryzalin concentrations. They suggested that BA may act to increase cell cycling, producing cells that more quickly transition from metaphase to anaphase where they may be susceptible to oryzalin (Lattier et al., 2013). Considering media components when developing *in vitro* ploidy manipulation protocols may maximize chromosome doubling efficiencies.

INFLUENCE OF PRE AND POST-TREATMENTS

In some cases, pre-treatments have been utilized to facilitate synchronizing the cell cycle to maximize the effect of antimitotic agents. Lattier et al. (2013) found for nodal segments of *Acer platanoides*, a 7-day culture on media supplemented with a combination of 4 μM BAP and 1 μM IAA prior to oryzalin treatments, significantly increased the number of stable tetraploids recovered. Similarly, Wu et al. (2020) found leaf blades of *Populus hopeiensis* cultured on 1.78 μM BAP, 0.07 μM TDZ and 0.53 μM IAA for 7 days prior to colchicine treatment significantly increased polyploid induction. In contrast, for *Tulipa gesneriana*, incubating stem disks for 2 weeks on media supplemented with 4 μM BAP, 15 μM 2-iP, and 4 μM NAA did not influence polyploid induction (Chauvin et al., 2005).

Culture conditions following treatment with antimitotic agents have also been shown to influence chromosome doubling. Niimi et al. (2015) found that culturing *Raphanobrassica* hybrids on media containing either silver nitrate ($AgNO_3$) or aminoethoxyvinylglycine (AVG) after treatment with the mitotic inhibitor APM increased in tissue survival and polyploids. These compounds inhibit ethylene production and were shown to reduce chlorosis resulting in higher recovery rates of tissues affected by APM.

CROP IMPROVEMENT

The effects of whole genome duplication may cause significant genetic changes in gene expression and gene function and may have significant effects for crop breeding and development. The specific effects of polyploidy may vary greatly between species and polyploid induction events.

Morphological changes are regularly reported in response to chromosome doubling. Increased stomatal size has been commonly used to identify chromosome doubling (Stanys et al., 2006; Dhooghe et al., 2010). Traits such as leaf size and thickness (Väinölä, 2000; Dunn and Lindstrom, 2007), inflorescence size and number (Kermani et al., 2003; Allum et al., 2007; Oates et al., 2012) internode length and plant height (Liu et al., 2007; Oates et al., 2012) have all been reported to be influenced by chromosome doubling. Increases in polyploidy have also been linked to an increase in size and biomass. For example,

many grasses used as bioenergy feedstocks are polyploids (Lambertini, 2019).

In vitro polyploid induction may also facilitate the development of improved, non-invasive, seedless nursery crops. One of the most effective means for developing seedless plants is to create triploids (plants with three sets of chromosomes) by crossing a diploid with artificially induced tetraploid (Ranney, 2006). This approach has been successful for several species including _Miscanthus sinensis_ (Rounsaville et al., 2011).

Hybrid sterility, also referred to as chromosomal sterility, often occurs due to improper chromosome pairing during meiosis as a result of structural differences in parental chromosomes (Ranney, 2006; Contreras et al., 2007). In many cases, doubling chromosomes of sterile hybrids, thereby developing allotetraploids, provides a homolog for chromosomes to pair with during meiosis and restores fertility. Doubling chromosomes has been successful in restoring fertility in the wide hybrids _Rhododendron_ 'Fragrant Affinity' (Contreras et al., 2007), × _Chitalpa tashkentensis_ (Olsen et al., 2006), and _Rudbeckia_ sp. (Oates et al., 2012). Further, chromsosome doubling restored fertility to the interspecific triploid bioenergy grass, _Miscanthus × giganteus_ (Touchell and Ranney, 2012).

OPPORTUNITIES FOR THE FUTURE

The future development and improvement of efficient, reliable and repeatable _in vitro_ ploidy manipulation protocols may consider multiple variables:

(1) The development of effective and efficient regeneration systems. Regeneration through organogenesis and somatic embryogenesis can facilitate reducing cytochimeras and increase the production of homogeneous polyploids. However, regeneration systems have been developed for a small number of crops and in most cases polyploid induction studies have focused on using shoot apices or nodal segments.

(2) Exploring the diversity of mitotic inhibitors. _In vitro_ ploidy manipulation studies have focused primarily on a small number of antimitotic agents. Considering the diversity in the structural range of antimicrotubule agents, especially herbicides, there is significant opportunity to further explore their efficacy in _in vitro_ ploidy manipulation.

(3) Considering interaction with media components. _In vitro_ procedures are often species-specific and each taxa requires unique media compositions. The effect of the interactions of media components on polyploid induction has received little attention. Considering pH, sucrose, and growth regulators may interact with mitotic inhibitors it may be important to integrate _in vitro_ protocols with ploidy manipulation.

(4) Refining cultures conditions before and after treatments. Some studies have found pre- and post-treatments beneficial in maximizing survival and homogeneous polyploids. These treatments may act to synchronize the cell cycle and moderate physiological responses to stresses imposed by mitotic inhibitors.

AUTHOR CONTRIBUTIONS

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REFERENCES

Allum, J. F., Bringloe, D. H., and Roberts, A. V. (2007). Chromosome doubling in a _Rosa rugosa_ Thunb. hybrid by exposure of in vitro nodes to oryzalin: the effects of node length, oryzalin concentration and exposure time. _Plant Cell Rep._ 26, 1977–1984. doi: 10.1007/s00299-007-0411-y

Anthony, R. G., and Hussey, P. J. (1999). Double mutation in _Eleusine indica_α-tubulin increases the resistance of transgenic maize calli to dinitroaniline and phophorothiomidate herbicides. _Plant J._ 18, 669–674. doi: 10.1046/j.1365-313x.1999.00484.x

Artichart, P. (2013). Polyploid induction by colchicine treatments and plant regeneration of _Dendrobium chrysotoxum_. _Thai J. Agric. Sci._ 46, 59–63.

Ascough, G. D., van Staden, J., and Erwin, J. E. (2008). Effectiveness of colchicine and oryzalin at inducing polyploidy in _Watsonia lepida_ N.E. Brown. _Hortscience_ 43, 2248–2251. doi: 10.21273/hortsci.43.7.2248

Bairu, M. W., Stirk, W. A., Dolezal, K., and Staden, J. V. (2007). Optimizing the micropropagation protocol for the endangered _Aloe polyphylla_: Can meta-topolin and its derivatives serve as replacement for benzyladenine and zeatin? _Plant Cell Tissue Organ. Cult._ 90, 15–23. doi: 10.1007/s11240-007-9233-4

Banyai, W., Sangthong, R., Karaket, N., Inthima, P., Mii, M., and Supaibulwatana, K. (2009). Overproduction of artemisinin in tetraploid _Artemisia annua_ L. _Plant Biotechnol._ 27, 427–433. doi: 10.5511/plantbiotechnology.10.0726a

Bartels, P., and Hilton, J. (1973). Comparison of trifluralin, oryzalin, pronamide, propham, and colchicine treatments on microtubules. _Pest. Biochem. Physiol._ 3, 462–472. doi: 10.1016/0048-3575(73)90072-2

Blakeslee, A., and Avery, A. (1937). Methods of inducing doubling of chromosomes in plants by treatment with colchicine. _J. Hered._ 28, 393–411. doi: 10.1093/oxfordjournals.jhered.a104294

Blume, Y. B., Krasylenko, Y. A., Demchuk, O. M., and Yemets, A. I. (2013). Tubulin tyrosine nitration regulates microtubule organization in plant cells. _Front. Plant Sci._ 4:530. doi: 10.3389/fpls.2013.00530

Cai, D.-Y., Lin, J.-H., and Deng, J.-P. (2016). Studies of propyzamide on polyploidy mutation of _Vitas davidii_. _J. Cent. S. Univ. For. Technol._ 7, 103–108.

Chandanie, M. A., Singh, S. K., Sindhu, S. S., Singh, A., Tomar, S. M. S., and Prasad, K. V. (2011). Efficacy of oryzalin as a potent chemical for _in vitro_ induction of

polyploids in Asiatic lily (*Lilium hybrid* L.) var. Polyanna. *Indian J. Genet.* 71, 262–268.

Chauvin, J. E., Label, A., and Kermarrec, M. P. (2005). In vitro chromosome-doubling in tulip (*Tulipa gesneriana* L.). *J. Hortic. Sci. Biotechnol.* 80, 693–698. doi: 10.1080/14620316.2005.11512000

Chen, L.-P., Wang, Y.-J., and Zhao, M. (2006). *In vitro* induction and characterization of tetraploid *Lychnis senno* Siebold et Zucc. *Hortscience* 41, 759–761. doi: 10.21273/hortsci.41.3.759

Chu, Z., Chen, J., Nyporko, A., Han, H., Yu, Q., and Powles, S. (2018). Novel α-tubulin mutations conferring resistance to dinitroaniline herbicides in *Lolium rgidum. Front. Sci.* 9:97. doi: 10.3389/fpls.2018.00097

Cohen, D., and Yao, J.-L. (1996). *In vitro* chromosome doubling of nine *Zantedeschia* cultivars. *Plant Cell Tissue Organ. Cult.* 47, 43–49. doi: 10.1007/BF00224065

Comai, L. (2005). The advantages and disadvantages of being polyploid. *Nature* 6, 836–846. doi: 10.1038/nrg1711

Contreras, R. N., and Meneghelli, L. (2016). In vitro chromosome doubling of *Prunus laurocerasus* 'Otto Luyken' and 'Schipkaensis'. *Hortscience* 51, 1463–1466. doi: 10.21273/hortsci11329-16

Contreras, R. N., Ranney, T. G., and Tallury, S. P. (2007). Reproductive behavior of diploid and allotetraploid *Rhododendron* L. '*Fragrant Affinity*'. *Hortscience* 42, 31–34. doi: 10.21273/hortsci.42.1.31

Darzynkiewicz, Z., Halicka, H. D., Zhao, H., and Podhorecka, M. (2011). Cell synchronization by inhibitors of DNA replication induces replication stress and DNA damage response: analysis by flow cytometry. *Methods Mol. Biol.* 761, 85–96. doi: 10.1007/978-1-61779-182-6_6

de Carvalho, J. F. R. P., de Carvahlo, C. R. P., and Otoni, W. C. (2005). *In vitro* induction of polyploidy in annatto (*Bixa orellana*). *Plant Cell Tissue Organ. Cult.* 80, 69–75. doi: 10.1007/s11240-004-8833-5

Dermen, H. (1953). Periclinal cytochimeras and origin of tissues in stem and leaf of peach. *Am. J. Bot.* 40, 154–158.

Dhooghe, E., Denis, S., Eeckhaut, T., Reheul, D., and Van Labeke, M.-C. (2009a). In vitro induction of tetraploids in ornamental *Ranunculus. Euphytica* 168, 33–40. doi: 10.1007/s10681-008-9876-1

Dhooghe, E., Grunewald, W., Leus, L., and Van Labeke, M.-C. (2009b). In vitro polyploidisation of *Helleborus* species. *Euphytica* 165, 89–95. doi: 10.1007/s10681-008-9763-9

Dhooghe, E., Laere, K. V., Eeckhaut, T., Leus, L., and Huylenbroeck, J. V. (2010). Mitotic chromosome doubling of plant tissues in vitro. *Plant Cell Tissue Organ. Cult.* 104, 359–373. doi: 10.1007/s11240-010-9786-5

Dunham, H. H., and Banta, A. M. (1940). Some effects of colchicine on heredity in *Daphnia longispina. Genetics* 25, 310–325.

Dunn, B. L., and Lindstrom, J. T. (2007). Oryzalin-induced chromosome doubling in *Buddleja* to facilitate interspecific hybridization. *Hortscience* 42, 1326–1328. doi: 10.21273/hortsci.42.6.1326

Ebrahimzadeh, H., Soltanloo, H., Shariatpanahi, M. E., Eskandari, A., and Ramezanpour, S. S. (2018). Improved chromosome doubling of parthenogenetic gaploid plants of cucumber (*Cucumis sativus* L.) using colchicine, trifluralin, and oryzalin. *Plant Cell Tissue Organ. Cult.* 135, 407–417. doi: 10.1007/s11240-018-1473-y

Eeckhaut, T., Samijn, G., and Van Bockstaele, E. (2002). In vitro polyploidy induction in *Rhododendron simsii* hybrids. *Acta Hortic.* 572, 43–49. doi: 10.17660/actahortic.2002.572.4

Eeckhaut, T., Werbrouck, S., Leus, L., Van Bockstaele, E., and Debergh, P. (2004). Chemically induced polyploidization in *Spathiphyllum wallisii* Regel through somatic embryogenesis. *Plant Cell Tissue Organ. Cult.* 78, 241–246. doi: 10.1023/b:ticu.0000025659.19232.04

Emsweller, S. L., and Ruttle, M. L. (1941). Induced polyploidy in floriculture. *Am. Nat.* 75, 310–328. doi: 10.1086/280967

Gallone, A., Hunter, A., and Douglas, G. C. (2014). Polyploid induction *in vitro* using colchicine and oryzalin on *Hebe* 'Oratia Beauty': production and characterization of vegetative traits. *Sci. Hortic.* 179, 59–66. doi: 10.1016/j.scienta.2014.09.014

Gantait, S., Mandal, N., Bhattacharyya, S., and Das, P. K. (2011). Induction and identification of tetraploids using in vitro colchicine treatment of *Gerbera jamesonii* Bolus cv. Sciella. *Plant Cell Tissue Organ. Cult.* 106, 485–493. doi: 10.1007/s11240-011-9947-1

Geoffriau, E., Kahane, R., Bellamy, C., and Rancillac, M. (1997). Ploidy Stability and in vitro chromosome doubling in gynogenic clones of onion (*Allium cepa* L.). *Plant Sci.* 28, 201–208. doi: 10.1016/s0168-9452(96)04556-6

Ghanbari, M. A., Jowkar, A., Salehi, H., and Zarei, M. (2019). Effects of polyploidization on petal characteristics and optical properties of *Impatiens walleriana* (Hook.). *Plant Cell Tissue Organ. Cult.* 138, 299–310. doi: 10.1007/s11240-019-01625-3

Giles, N. L., Armson, A., and Reid, S. A. (2009). Characterization of trifluralin binding with recombinant tubulin from *Trypanosoma brucei. Parasitol. Res.* 104, 893–903. doi: 10.1007/s00436-008-1271-2

Gillooly, D. A., Touchell, D. H., and Ranney, T. G. (2015). In vitro polyploid induction of *Ophiopogon planiscapus. SNA Res. Conf.* 60, 181–185.

Griesbach, R. J. (1990). A fertile tetraploid *Anigozanthis* hybrid produced by in vitro colchicine treatment. *Hortscience* 25, 802–803. doi: 10.21273/hortsci.25.7.802

Gu, X. F., Yang, A. F., Meng, H., and Zhang, J. R. (2005). In vitro induction of tetraploid plants from diploid *Ziziphus jujube* Mill. Cv.Zhanhua. *Plant Cell Rep.* 24, 671–676. doi: 10.1007/s00299-005-0017-1

Hannweg, K., Sippel, A., and Bertling, I. (2013). A simple and effective method for the micropropagation and *in vitro* induction of polyploidy and the effect on floral characteristics of the South African iris, *Crocosmia aurea. S. Afr. J. Bot.* 88, 357–372.

Hannweg, K., Steyn, W., and Bertling, I. (2016a). In-vitro induced tetraploids of *Plectranthus esculentus* are nematode-tolerant and have enhanced nutritional value. *Euphytica* 207, 343–351. doi: 10.1007/s10681-015-1547-4

Hannweg, K., Visser, G., de Jager, K., and Bertling, I. (2016b). In vitro-induced polyploidy and its effect on horticultural characteristics, essential oil composition and bioactivity of *Tetradenia riparia. S. Afr. J. Bot.* 106, 186–191. doi: 10.1016/j.sajb.2016.07.013

Hansen, N. G. P., and Andersen, S. B. (1998). In vitro chromosome doubling with colchicine during microspore culture in wheat (*Triticum aestivum* L.). *Euphytica* 102, 101–108.

Hebert, C. J., Touchell, D. H., Ranney, T. G., and LeBude, A. V. (2010). In vitro shoot regeneration and polyploidy induction of *Rhododendron* 'Frangrantissimum Improved'. *Hortscience* 45, 801–804. doi: 10.21273/hortsci.45.5.801

Heinz, D. J., and Mee, G. W. P. (1970). Colchicine-induced polyploids from cell suspension cultures of sugarcane. *Crop Sci.* 696–699. doi: 10.2135/cropsci1970.0011183x001000060030x

Hias, N., Leus, L., Davey, M. W., Vanderzande, S., Van Huylenbroeck, J., and Keulemans, J. (2017). Effect of polyploidization in two apple (*Malus x doemestica*) genotypes. *Hortic. Sci.* 44, 55–63. doi: 10.17221/7/2016-hortsci

Hugdahl, J. D., and Morejohn, L. C. (1993). Rapid and reversible high-affinity binding of the dinitroaniline herbicide oryzalin to tubulin from *Zea mays* L. *Plant Physiol.* 102, 725–740. doi: 10.1104/pp.102.3.725

Hussey, G., and Hypher, A. (1978). Clonal propagation of sugar beet plants and the formation of polyploids by tissue culture. *Ann. Bot.* 42, 477–479. doi: 10.1093/oxfordjournals.aob.a085483

Javadian, N., Karimzadeh, G., Sharifi, M., Moieni, A., and Behmanesh, M. (2017). In vitro polyploid induction: changes in morphology, podophyllotoxin biosynthesis, and the expression of the related genes in *Linum album* (Linaceae). *Planta* 245, 1165–1178. doi: 10.1007/s00425-017-2671-2

Jiang, Y., Liu, S., Hu, J., He, G., Liu, Y., Chen, X., et al. (2020). Polyploidization of *Plumbago auriculate* Lam. *In vitro* and its characterization including cold tolerance. *Plant Cell Tissue Organ. Cult.* 140, 315–325. doi: 10.1007/s11240-019-01729-w

Jovanović, A. M., Durst, S., and Nick, P. (2010). Plant cell division is specifically affected by nitrotyrosine. *J. Exp. Bot.* 61, 901–909. doi: 10.1093/jxb/erp369

Kadota, M., and Niimi, Y. (2002). In vitro induction of tetraploid plantsfrom a diploid Japanese pear cultivar (*Pyrus pyrifolia* N. cv. Hosui). *Plant Cell Rep.* 21, 282–286. doi: 10.1007/s00299-002-0509-1

Kaensaksiri, T., Soontornchainaksaeng, P., Soonthorncharennon, N., and Prathanturarug, S. (2011). In vitro induction of polyploidy in *Centella asiatica* (L.) Urban. *Plant Cell Tissue Organ. Cult.* 107, 187–194. doi: 10.1007/s11418-013-0761-4

Kehr, A. (1996). Woody plant polyploidy. *Am. Nurseryman* 183, 38–47.

Kermani, M. J., Sarasan, V., Roberts, A. V., Yokoya, K., Wentworth, J., and Sieber, V. K. (2003). Oryzalin-induced chromosome doubling in *Rosa* and its effect

on plant morphology and pollen viability. *Theor. Appl. Genet.* 107, 1195–1200. doi: 10.1007/s00122-003-1374-1

Khosravi, P., Kermani, M. J., Nematzadeh, G. A., Bihamta, M. R., and Yokoya, K. (2008). Role of mitotic inhibitors and genotype on chromosome doubling of Rosa. *Euphytica* 160, 267–275. doi: 10.1007/s10681-007-9571-7

Kitamura, S., Akutsu, M., and Okazaki, K. (2009). Mechanism of action of nitrous oxide gas applied as a polyploidizing agent during meiosis in lilies. *Sex. Plant Reprod.* 22, 9–14. doi: 10.1007/s00497-008-0084-x

Klekowski, E. J. (2003). Plant clonality, mutation, diplontic selection and mutational meltdown. *Biol. J. Linn. Soc.* 79, 61–67. doi: 10.1046/j.1095-8312.2003.00183.x

Lambertini, C. (2019). Why are tall-statured energy grasses of polyploid species complexes potentially invasive? A review of their genetic variation patterns and evolutionary plasticity. *Biol. Invasions* 21, 3019–3041. doi: 10.1007/s10530-019-02053-2

Lattier, J. D., Touchell, D. H., Ranney, T. G., and Smith, J. C. (2013). Micropropagation and Polyploid Induction of *Acer platanoides* 'Crimson Sentry'. *J. Environ. Hortic.* 31, 246–252.

Leung, Y. Y., Hui, L. L. Y., and Kroaus, V. B. (2015). Colchicine – update on mechanisms of action and therapeutic uses. *Semin. Arthritis Rheum.* 45, 341–350. doi: 10.1016/j.semarthrit.2015.06.013

Lipka, E., and Müller, S. (2014). Nitrosative stress triggers microtubule reorganization in *Arabidopsis thaliana*. *J. Exp. Bot.* 65, 4177–4189. doi: 10.1093/jxb/eru194

Liu, G., Li, Z., and Bao, M. (2007). Colchicine-induced chromosome doubling in Platanus acerifolia and its effect on plant morphology. *Euphytica* 157, 145–154. doi: 10.1007/s10681-007-9406-6

Ma, C., Tran, J., Gu, F., Ochoa, R., Li, C., Sept, D., et al. (2010). Dinitroaniline activity in *Toxoplasma gondii* expressing wild-type or mutant α-tubulin. *Antimicrob. Agents Chemother.* 54, 1453–1460. doi: 10.1128/AAC.01150-09

Meyer, E. M., Touchell, D. H., and Ranney, T. G. (2009). *In vitro* shoot regeneration and polyploid induction from leaves of *Hypericum* species. *Hortscience* 44, 1957–1961. doi: 10.21273/hortsci.44.7.1957

Mitrofanova, I. V., Zilbervarg, I. R., Yemets, A. I., Mitrofanova, O. V., and Blume, Y. B. (2003). The effect of dinitroaniline and phosphorothioamidate herbicides on polyploidisation *in vitro* of *Nepeta* plants. *Cell Biol. Int.* 27, 229–231. doi: 10.1016/S1065-6995(02)00317-7

Morejohn, L. C., Bureau, T. E., Molebajer, J., Bajer, A. S., and Fosket, D. E. (1987). Oryzalin, a dinitroaniline herbicide, binds to plant tubulin and inhibits microtubule polymerization *in vitro*. *Planta* 172, 252–264. doi: 10.1007/BF00394595

Morejohn, L. C., Bureau, T. E., Tocchi, L. P., and Fosket, D. E. (1984). Tubulins from different higher plant species are immunologically nonidentical and bind colchicine differentially. *Proc. Natl. Acad. Sci. U.S.A.* 81, 1440–1444. doi: 10.1073/pnas.81.5.1440

Murashige, T., and Nakano, R. (1966). Tissue culture as a potential tool in obtaining polyploid plants. *J. Hered.* 57, 114–118.

Murthy, J. V., Kim, H.-H., Hanesworth, V. R., Hugdahl, J. D., and Morejohn, L. C. (1994). Competitive inhibition of high-affinity oryzalin binding to plant tubulin by the phosphoric amide herbicide amiprophos-methyl. *Plant Physiol.* 105, 309–320. doi: 10.1104/pp.105.1.309

Nadler, J. D., Pooler, M., Olsen, R. T., and Coleman, G. D. (2012). *In vitro* induction of polyploidy in *Cercis glabra* Pamp. *Sci. Hortic.* 148, 126–130. doi: 10.1016/j.scienta.2012.09.024

Nasr, M. L., Habib, H. M., Ibrahim, I. A., and Kapiel, T. Y. (2004). "In vitro induction of autotetraploid watermelons using colchicine and four dinitroaniline compounds," in *Proceedings of the International Conference On Engineering & Application*, Sharm Elsheik. doi: 10.13140/2.1.3144.3840

Neill, S. J., Desikan, R., and Hancock, J. T. (2003). Nitric oxide signaling in plants. *New Phytol.* 159, 11–35.

Niimi, H., Wantanabe, M., Serizawa, H., Koba, T., Nkamura, I., and Mii, M. (2015). Amiprophosmethyl-inducedeffecient *in vitro* production of polyploids in rhapanobrassica with the aid of aminoethoxyvinylglycin (AVG) in the culture medium. *Breeed. Sci.* 65, 396–402. doi: 10.1270/jsbbs.65.396

Nilanthi, D., Chen, X.-L., Zhao, F.-C., Yang, Y.-S., and Wu, H. (2009). Induction of tetraploids from petiole explants through colchicine treatments in *Echinacea purpurea* L. *J. Biomed. Biotechnol.* 2009:343485. doi: 10.1155/2009/343485

Nimura, M., Kato, J., Horaguchi, H., Mii, M., Sakai, K., and Katoh, T. (2006). Induction of fertile amphidiploids by artificial chromosome-doubling in interspecific hybrid between *Dianthus caryophyllus* L. and *D. japonicas* Thunb. *Breed. Sci.* 56, 303–310. doi: 10.1270/jsbbs.56.303

Noori, S. A. S., Norouzi, M., Karimzadeh, G., Shirkool, K., and Niazian, M. (2017). Effect of colchicine-induced polyploidy on morphological characteristics and essential oil composition of ajowan (*Trachyspermum ammi* L.). *Plant Cell Tissue Organ Cult.* 130, 543–551. doi: 10.1007/s11240-017-1245-0

Notsuka, K., Tsurs, T., and Shiraishi, M. (2000). Induced polyploid graps via *in vitro* chromosome doubling. *J. Jpn. Soc. Hortic. Sci.* 69, 543–551. doi: 10.2503/jjshs.69.543

Oates, K. M., Ranney, T. G., and Touchell, D. H. (2012). Influence of Induced Polyploidy on Fertility and Morphology of *Rudbeckia* Species and Hybrids. *Hortscience* 47, 1217–1221. doi: 10.21273/hortsci.47.9.1217

Olsen, R. T., Ranney, T. G., and Viloria, Z. (2006). Reproductive behavior of induced allotetraploid x *Chitalpa* and in vitro embryo culture of polyploidy progeny. *J. Am. Soc. Horict. Sci.* 131, 716–724. doi: 10.21273/jashs.131.6.716

Palmer, I. E., Touchell, D. H., and Ranney, T. G. (2008). In vitro polyploid induction of *Rudbeckia* sp. *SNA Res. Conf.* 53, 186–189.

Pan-pan, H., Wei-xu, L., Hui-hui, L., and Zeng-xu, X. (2018). In vitro induction and identification of autotetraploid of *Bletilla striata* (Thunb.) Reichb.f. by colchicine treatment. *Plant Cell Tissue Organ. Cult.* 132, 425–432. doi: 10.1007/s11240-017-1339-8

Parsons, J. L., Martin, S. L., James, T., Golenia, G., Boudko, E. A., and Hepworth, S. R. (2019). Polyploidization for the genetic improvement of *Cannabis sativa*. *Front. Plant Sci.* 10:476. doi: 10.3389/fpls.2019.00476

Petersen, K. K., Hagberg, P., and Kristiansen, K. (2002). In vitro chromosome doubling of *Miscanthus sinensis*. *Plant Breed.* 121, 445–450. doi: 10.1046/j.1439-0523.2002.738314.x

Petersen, K. K., Hagberg, P., and Kristiansen, K. (2003). Colchicine and oryzalin mediated chromosome doubling in different genotypes of *Miscanthus sinensis*. *Plant Cell Tissue Organ. Cult.* 73, 137–146.

Planchais, S., Glab, N., Inzé, D., and Bergounioux, C. (2000). Chemical inhibitors: a tool for plant cell cycle studies. *FEBS Lett.* 476, 78–83. doi: 10.1016/s0014-5793(00)01675-6

Ranney, T. G. (2006). Polyploidy: From evolution to new plant development. *Combined Proc. Intl. Plant Propagators Soc.* 56, 137–142.

Raza, H., Jaskani, M. J., Khan, M. M., and Malik, T. A. (2003). *In vitro* induction of polyploids in watermelon and estimation based on DNA content. *Intern. J. Agric. Biol.* 5, 298–302.

Regalado, J. J., Carmona-Martin, E., Querol, V., Veléz, C. G., Encina, C. L., and Pitta-Alvarez, S. I. (2017). Production of compact petunias through polyplidization. *Plant Cell Tissue Organ. Cult.* 129, 61–71.

Rêgo, M. M., Rêgo, E. R., Bruckner, C. H., Finger, F. L., and Otoni, W. C. (2011). In vitro induction of autotetraploids from diploid yellow passion fruit mediated by colchicine and oryzalin. *Plant Cell Tissue Organ. Cult.* 107, 451–459. doi: 10.1007/s11240-011-9995-6

Ren, J., Wu, X., Song, C., Liang, Y., Gao, W., and Wang, Y. (2018). Induction of polyploid tillered onion using colchicine and pendamethalin. *Sains Malaysiana* 47, 2617–2624. doi: 10.17576/jsm-2018-4711-04

Rose, J. B., Kubba, J., and Tobutt, K. R. (2000). Chromosome doubling in sterile *Syringa vulgaris* x *S. pinnatifolia* hybrids by in vitro culture of nodal explants. *Plant Cell Tissue Organ. Cult.* 63, 127–132.

Rounsaville, T. J., Touchell, D. H., and Ranney, T. G. (2011). Fertility and reproductive pathways in diploid and triploid *Miscanthus sinensis*. *Hortscience* 46, 1353–1357. doi: 10.21273/hortsci.46.10.1353

Sakhanokho, H. F., Rajasekaran, K., Kelley, R. Y., and Islam-Faridi, N. (2009). Induced polyploidy in diploid ornamental ginger (*Hedychium muluense* R. M. Smith) using colchicine and oryzalin. *Hortscience* 44, 1809–1814. doi: 10.21273/hortsci.44.7.1809

Sattler, M. C., Carvalho, C. R., and Clarindo, W. R. (2015). The polyploidy and its key role in plant breeding. *Planta* 281–296. doi: 10.1007/s00425-015-2450-x

Soltis, D. E., and Burleigh, J. D. (2009). Surviving the K-T mass extinction: new perspectives of polyploidization in angiosperms. *PNAS* 106, 5455–5456. doi: 10.1007/s00425-015-2450-x

Soltis, D. E., Albert, V. A., Leebans-Mack, J., Bell, C. D., Paterson, A. H., Zheng, C., et al. (2009). Polyploidy and angiosperm diversification. *Am. J. Bot.* 96, 336–348. doi: 10.3732/ajb.0800079

Stanys, V., Weckman, A., Staniene, G., and Duchovkis, P. (2006). *In vitro* induction of polyploidy in Japanese quince (*Chaenomeles japonica*). *Plant Cell Tissue Organ. Cult.* 84, 263–268. doi: 10.1007/s11240-005-9029-3

Sun, Q., Sun, H., Li, L., and Bell, R. L. (2009). *In vitro* colchicine-induced polyploid plantlet production and regeneration from leaf explants of the diploid pear (*Pyrus communis* L.) cultivar, 'Fertility'. *J. Hortic. Sci. Biotechnol.* 84, 548–552. doi: 10.1080/14620316.2009.11512564

Tavan, M., Mirjalili, M. H., and Karimzadeh, G. (2015). In vitro polyploidy induction: changes in morphological, anatomical and phytochemical characteristics of *Thymus persicus* (Lamiaceae). *Plant Cell Tissue Organ. Cult.* 122, 573–583. doi: 10.1007/s11240-015-0789-0

Taylor, N. L., Anderson, M. K., Queenberry, K. H., and Watson, L. (1976). Doubling the chromosome number of *Trifolium* species using nitrous oxide. *Crop Sci.* 16, 516–518. doi: 10.2135/cropsci1976.0011183x001600040019x

The American Naturalist (1941). Symposium on theoretical and practical aspects of polyploidy in crop plants. *Am. Nat.* 75, 289–365. doi: 10.2135/cropsci1976.0011183x001600040019x

Thomas, J., Chen, Q., and Howes, N. (1997). Chromosome doubling of haploids of common wheat with caffeine. *Genome* 40, 552–558. doi: 10.1139/g97-072 doi: 10.1139/g97-072

Touchell, D. H., and Ranney, T. G. (2012). Chromosome doubling and fertility restoration in *Miscanthus × giganteus*. *HortScience* 47, S334. doi: 10.1139/g97-072

Tresch, S., Plath, P., and Grossman, K. (2005). Herbicidal cyanoacrylates with antimicrotubule mechanism of action. *Pest. Manag. Sci.* 61, 1052–1059. doi: 10.1002/ps.1093

Trojal-Golush, A., and Skomra, U. (2013). Artificially induced polyploidization in *Humulus lupulus* L. and its effect on morphological and chemical traits. *Breed. Sci.* 63, 393–399. doi: 10.1270/jsbbs.63.393

Väinölä, A. (2000). Polyploidization and early screening of *Rhododendron* hybrids. *Euphytica* 112, 239–244.

Vaughn, K. C., and Lehnen, L. P. Jr. (1991). Mitotic disrupter herbicides. *Weed Sci.* 39, 450–457. doi: 10.1017/s0043174500073215

Viehmannová, I., Fernández Cusimamani, E., Bechyne, M., Vyvadilová, M., and Greplova, M. (2009). In vitro induction of polyploidy in yacon (*Smallanthus sonchifolius*). *Plant Cell Tissue Organ Cult.* 97, 21–25. doi: 10.1007/s11240-008-9494-6

Wang, C., and Lei, J. (2012). *In vitro* induction of tetraploids from immature embryos through colchicine treatments in *Clivia miniata* Regal. *J. Agric. Res.* 7, 3712–3718.

Wu, J., Sang, Y., Zhou, Q., and Zhang, P. (2020). Colchicine in vitro tetraploid induction of *Populus hopeiensis* from leaf blades. *Plant Cell Tissue Organ. Cult.* 141, 339–349. doi: 10.1007/s11240-020-01790-w

Wu, J.-H., Ferguson, A. R., and Murray, B. G. (2011). Manipulation of ploidy for kiwifruit breeding: in vitro chromosome doubling in diploid *Actinidia chinensis* Planch. *Plant Cell Tissue Organ. Cult.* 106, 503–511. doi: 10.1007/s11240-011-9949-z

Xu, C., Huang, Z., Liao, T., Li, Y., and Kang, X. (2016). In vitro tetraploid plants regeneration from leaf explants of multiple genotypes in *Populus*. *Plant Cell Tissue Organ. Cult.* 125, 1–9. doi: 10.1007/s11240-015-0922-0

Yang, Z., Shen, Z., Tetreault, H., Johnson, L., Friebe, B., Frazier, T., et al. (2014). Production of autopolyploid lowland switchgrass lines through *in vitro* chromosome doubling. *Bioenergy Res.* 7, 232–242. doi: 10.1007/s12155-013-9364-x

Yuan, S., Su, Y., Liu, Y., Li, Z., Fang, Z., Yang, L., et al. (2015). Chromsome doubling of microspore-derived plants from cabbage (*Brassica oleraceae* var, *capitate* L.) and broccoli (*Brassica oleraceae* var. *italica* L.). *Front. Plant Sci.* 6:1118. doi: 10.3389/fpls.2015.01118

Zhang, Q. Y., Luo, F. X., Liu, L., and Guo, F. C. (2010). *In vitro* induction of tetraploids in crape myrtle (*Lagerstoemia indica* L.). *Plant Cell Tissue Organ. Cult.* 101, 41–47. doi: 10.1007/s11240-009-9660-5

Zhang, Z., Dai, H., Xiao, M., and Liu, X. (2008). In vitro induction of tetraploids in *Phlox subulata* L. *Euphytica* 159, 59–65. doi: 10.1007/s10681-007-9457-8