TETRAPLOID INDUCTION OF *ACACIA CRASSICARPA* USING COLCHICINE AND ORYZALIN

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Received February 2013

LAM HK, HARBARD JL & KOUTOULIS A. 2014. Tetraploid induction of *Acacia crassicarpa* using colchicine and oryzalin. Australian *Acacia*, including *Acacia crassicarpa*, are important plantation species but prolific seed production may lead to weediness concerns. Sterility via production of triploid *Acacia* is potentially a solution to combat weediness. The aim of this study was to describe tetraploid induction using colchicine and oryzalin on seeds of *A. crassicarpa* as the first step towards development of triploids. Seeds were nicked and exposed to different concentrations of colchicine and oryzalin for 24 hours, germinated and grown under natural light at 26 °C. Germination, subsequent growth and survival were assessed and efficiency of induced tetraploidy determined using flow cytometry when seedlings were 2 to 7 months old. The highest conversion rate achieved was 21% with 61% survival when seeds were exposed to 0.02% colchicine. The most effective oryzalin treatment was exposure to 0.005% oryzalin with 5% tetraploidy and 62% survival. There were no survivors in the highest concentration of colchicine tested (0.5%). Germination and survival were not severely impeded by the highest concentration of oryzalin (0.01%). Treatment in oryzalin also produced more mixoploids relative to the number of tetraploids than colchicine treatment. The 2C DNA amounts for *A. crassicarpa* of differing ploidy were estimated as 1.75 (diploid), 2.74 (triploid) and 3.47 pg (tetraploid).

Keywords: Polyploid, triploid, DNA doubling, flow cytometry

INTRODUCTION

Acacia crassicarpa (2n = 2x = 26) is one of the major tropical Australian *Acacia* species in South-East Asia with 3.3 m ha planted predominately in Indonesia and Vietnam (Griffin et al. 2011). Its utility is also evident in China with increased plantings in southern provinces for reforestation, reclamation of wastelands and industrial plantations (Yang et al. 2006). *Acacia crassicarpa* is planted in exotic monocultural stands and, in common with other species in this genus, they are prolific producers of seed with long-term viability, a combination leading to potential weediness (Richardson et al. 2011) and possible threats to local biodiversity. One approach to managing this problem could be to plant triploid clones produced from crosses of diploid and tetraploid parents. Such triploids are expected to show much reduced fertility as the uneven number of chromosomes can lead to defective gamete fusion resulting in seedlessness or endosperm failure with resultant infertile seed (Ramsey & Schemske 1998). An additional benefit might be that reproductive resources could be redirected to vegetative growth with a corresponding increase in harvestable biomass. Some triploid hops have been reported to be superior to their diploid counterparts with higher yield, more vigorous growth and much lower seed production (Roy et al. 2001). The reproductive biology of polyploid *A. crassicarpa* has not yet been studied but flowering of autotetraploid and diploid cytotypes of another species within the same taxonomic Section, *Acacia mangium*, has been compared by Nghiem et al. (2011). Pollen–pistil interactions in controlled pollinations between the cytotypes have also been investigated (Nghiem et al. 2013). Open-pollinated breeding systems of autotetraploid *A. mangium* trees have been compared with their diploid counterparts using molecular markers (Griffin et al. 2012).

The availability of genetically improved diploid germplasm, but low frequencies of both spontaneous tetraploids and triploids (Blakesley et al. 2002), makes tetraploid induction a logical first step for generating a polyploid breeding population. Artificial tetraploid induction

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has been reported in *Acacia mearnsii* (Moffett & Nixon 1960, Beck et al. 2003), *A. mangium* (Harbard et al. 2012, Blakesley et al. 2002) and *Acacia dealbata* (Blakesley et al. 2002) using mitotic inhibitors, colchicine or oryzalin.

The popular choice of colchicine over other mitotic inhibitors such as oryzalin (Barandalla et al. 2006), trifluralin (Rey et al. 2002), nitrous oxide (Kitamura et al. 2009) and amiprophos methyl (Khosravi et al. 2008) may be due to the ease of replicating a developed protocol. Colchicine, with its low affinity to tubulin binding sites in plant cells (Morejohn et al. 1987) has been shown to be less effective at lower dosages than oryzalin (Jones et al. 2008). For instance, chromosome doubling has been achieved by alternative mitotic inhibitors at about 100–1000 times lower concentrations than colchicine (Hansen et al. 2000). From a safety perspective, tetraploid induction using an alternative herbicide mitotic inhibitor such as oryzalin, should be explored as colchicine has been clinically proven to be highly toxic and carcinogenic to humans (Van Tuyl et al. 1992, Eeckhaut et al. 2004) due to its specific binding to animal tubulin dimers. Thus, the aim of this study was to compare tetraploid induction using colchicine and the less toxic oryzalin in *A. crassicarpa*.

MATERIALS AND METHODS

Plant material

Open-pollinated seeds of *A. crassicarpa* were obtained from single tree collections from five 8-year-old elite parent trees growing at a seed orchard managed by the Research Centre for Forest Tree Improvement at Hàm Thuận Nam District, Bihn Thuan Province, Vietnam. Seeds were stored in the dark at 4 °C before use. Equal numbers of seeds from each of the five parent trees were mixed and randomly chosen for all experiments.

Tetraploid induction via colchicine and oryzalin treatment on seeds

Induction and flow cytometry protocols were based on those developed for *A. mangium* (Harbard et al. 2012). Ungerminated seeds were nicked at the opposite end to the funicle/aril attachment and placed on filter paper in 9-cm Petri dishes (35 seeds/petri dish) and saturated with 4 mL aqueous solution of the following four colchicine concentrations: 0.005, 0.02, 0.05 and 0.5% w/v. A total of 140 seeds were treated for each concentration treatment. A control treatment of 140 seeds treated with sterile distilled water only was also included. Petri dishes were sealed with parafilm to retain moisture, followed by a 24-hour incubation at 25 ± 2 °C with a 12-hour photoperiod under a photon flux of 57–72 µmol m^{-2} s^{-1}. After 24 hours, the treated seeds were washed with sterile distilled water and transferred to fresh filter paper, soaked with sterile distilled water. The treated seeds were incubated under the previously mentioned conditions for germination and kept moist with sterile distilled water, as required. Germination rate was recorded after 3 days. Seeds were planted in composted pine bark-based potting mix and grown under natural light at 26 ± 2 °C. When using oryzalin, ungerminated seeds were treated identically as the colchicine treatment, except substituting colchicine with the following oryzalin concentrations: 0.0005, 0.005 and 0.01% w/v with controls as above. As oryzalin did not readily dissolve in water, a 100 mM oryzalin stock solution was prepared by dropping 98% ethanol until oryzalin dissolved and then made to volume at the required concentrations with sterile distilled water.

Nuclei isolating buffer and internal standard preparation for flow cytometry

Modified Galbraith et al. (1983) buffer with addition of 3.0% w/v polyvinylpolypryrolidone (Beatson et al. 2003) was used as the nuclei isolating buffer. Buffer was prepared in bulk and stored as frozen 45 mL aliquots at -20 °C to prevent oxidation. *Pisum sativum* (pea) cv. 'Torstag' (2C = 9.10 pg) (Bennett & Leitch 1995) was used as internal standard for flow cytometry. The pea plants were grown at 18–24 °C in an 18-hour photoperiod consisting of natural light extended morning and evening by high pressure sodium 400 W lamps in pendant mount fittings with acrylic diffusing covers delivering approximately 50 µmol m^{-2} s^{-1} at the pot surface. Young, healthy pea leaves (0.04 g pea leaf sample) were collected on the day of flow analysis and kept on ice. After the removal of mid veins and addition of a small amount of ice-cold nuclei isolating buffer, the leaves were

homogenised into fine pulp using a sharp scalpel blade in a straight up and down chopping motion for minimal damage to nuclei. The homogenate was filtered through a 75-µm stainless steel mesh filter and the collected filtrates were made up to desired volume, of which 1 g pea leaf can make up 20 mL standard, with addition of ice-cold nuclei isolating buffer.

Sample preparation and ploidy analysis by flow cytometry

Young healthy phyllodes were harvested on the day before flow cytometry was conducted and stored immediately at 4 °C. Nuclei were extracted by chopping a 1-cm^2 piece of phyllode sample in a straight up and down motion in a Petri dish lid sitting on ice using a single-edged razor blade and 2 drops of ice-cold nuclei isolating buffer. The Petri dish lid was held at an angle while the fine pulp was scraped to the bottom edge of the Petri dish lid and washed down with 800 µL of internal standard. The preparation was gently swirled and filtered through a 40-µm nylon cell strainer into the cold Petri dish base. The filtrate was transferred into a 1.5 mL eppendorf tube and kept on ice. For each sample, 300 µL aliquot was transferred into a 1.2 mL RNase- and DNase-free microtiter tube, which included 100 µL propidium iodide (50 µg mL^{-1}). Nuclei were analysed using a Coulter Elite ESP Cell Sorter equipped with 100-W high pressure mercury arc lamp. Air-cooled argon laser emitting light at 488 nm was used with fluorescence measured using a 575-nm band pass filter. The propidium iodide induced fluorescence of 5000 nuclei counts was measured and analysed using WinMDI software (version 2.9). Samples were recorded as diploid (2x), tetraploid (4x), mixoploid (i.e. having both diploid and tetraploid peaks) or triploid (3x) (Figure 1). The 2C DNA amounts of 2x, 3x and 4x were quantified based on mean of 10 samples for each ploidy level (except 3x due to only one plant detected in this study) using the estimates of the ratio of the DNA relative fluorescent intensity of the internal standard, *P. sativum* and *A. crassicarpa,* using the formula as described in Dolezalova et al. (2002).

Statistical analysis

All statistical analyses were performed using the SAS 9.1 (2003) statistical software package. Raw data of germination, survival and tetraploid induction were analysed by χ^2 test based on Pearson's error distribution using FREQ procedures. If pair-wise χ^2 tests were conducted to compare performances between treatments. Results of such tests were adjusted using the Dunn-Sidak Multiple Comparison Procedure (Sokal & Rohlf 1995). Heights of seedlings were analysed by analysis of variance using general linear model procedures. Proper transformation of data was conducted where necessary conforming to normality and homoscedasticity. When significant effect of treatment was detected with probability $\alpha \leq 0.05$, a post-hoc Tukey's test was conducted to further differentiate differences between treatment groups.

RESULTS

Tetraploid induction, germination and survival

Tetraploids were successfully induced by three out of four colchicine treatments of 24-hour duration with concentrations 0.005, 0.02 and 0.05%. There were significant differences between colchicine treatments in the percentage of tetraploids induced ($p < 0.0001$), with 0.02% colchicine being most effective to achieve 21% tetraploid induction with germination of 93% and survival of 61% or equivalent to 12.81% induction efficiency (Table 1). Survival decreased with increasing colchicine concentration and no plants survived the highest concentration of colchicine treatment despite 79% germination (Table 1). Mixoploid plants were produced in all treatments where there were surviving plants.

Two out of three oryzalin treatments produced tetraploids under the same exposure of 24 hours with concentrations of 0.005 and 0.01%. The percentage of tetraploids induced varied significantly between treatments ($p = 0.0093$). Treatment with 0.01% oryzalin induced 6% tetraploids with 86% germination and 39% survival, whereas the 0.005% oryzalin treatment had slightly lower level of tetraploid induction (5%) with 80% germination and 62% survival (Table 1). Tetraploid induction efficiencies of 0.005 and 0.01% oryzalin treatments were 3.1 and 2.34% respectively (Table 1). More mixoploid plants were produced relative to the number of tetraploids using oryzalin than in the colchicine treatments. A triploid plant was found among the

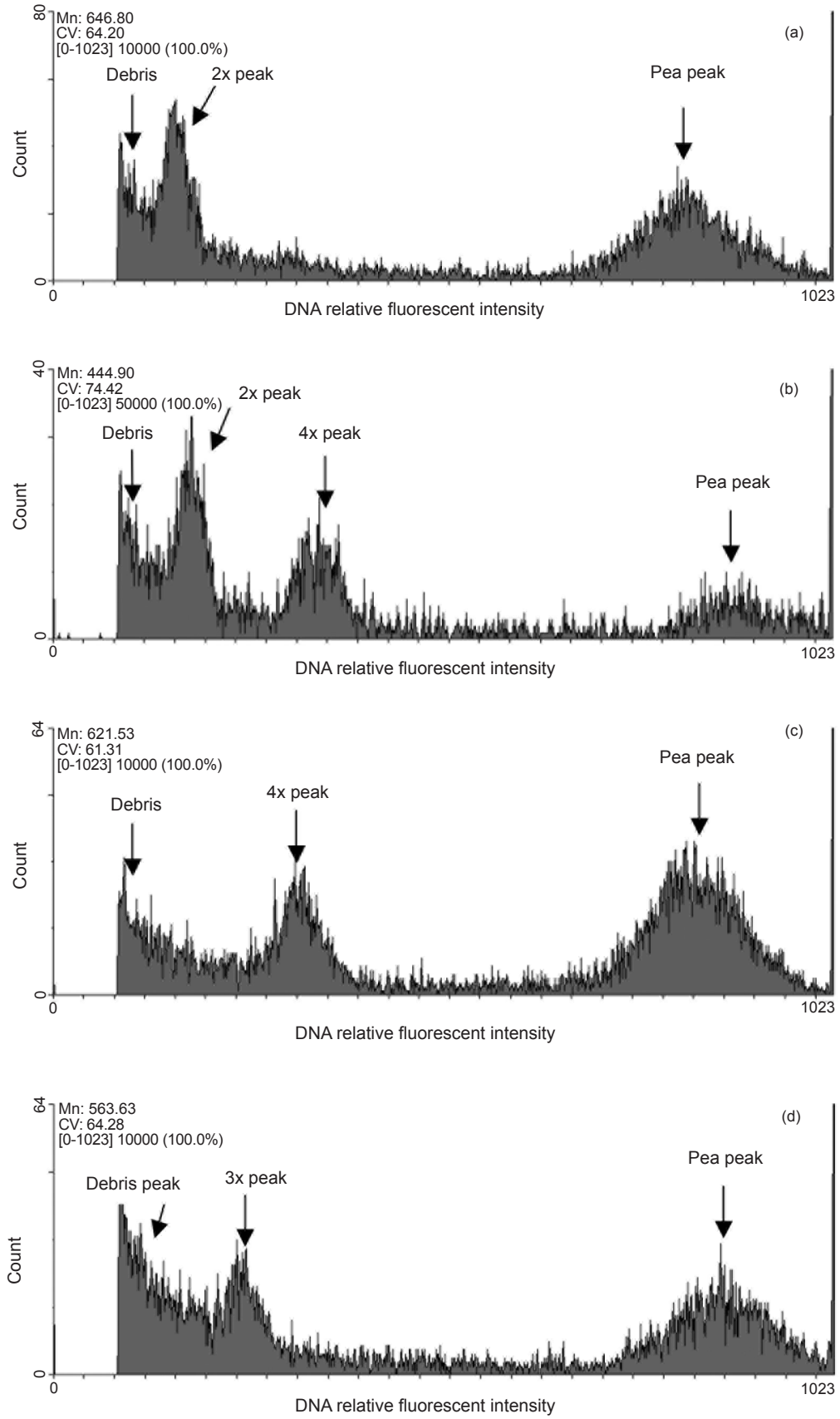

Figure 1 Representative flow cytometry histograms of nuclei isolated from phyllodes of (a) diploid, (b) mixoploid, (c) tetraploid and (d) triploid in *Acacia crassicarpa*, each with internal standard of *Pisum sativum*; 2x = diploid, 4x = tetraploid

Table 1 Effects of different concentrations of colchicine and oryzalin on tetraploid induction in *Acacia crassicarpa*

Mitotic inhibitor	Concentration (%)	Germination (%)	Survival (%)	Diploid seedling (%)	Mixo seedling (%)	Tetraploid seedling (%)	Tetraploid induction efficiency* (%)
Colchicine	0	86	95	95	0	0	0
	0.005	87	89	67	16	6	5.34
	0.02	93	61	9	31	21	12.81
	0.05	87	7	0	4	3	0.21
	0.5	79	0	0	0	0	0
Oryzalin	0	90	89	89	0	0	0
	0.0005	85	96	96	0	0	0
	0.005	80	62	36	21	5	3.10
	0.01	86	39	8	26	6	2.34

n = 140 for colchine and oryzalin treatments on seeds; *calculated as % survival × % tetraploid seedlings

280 plants in the mitotic inhibitor-free control treatments corresponding to a triploid frequency of 0.36%.

Effects of treatments and ploidy levels on seedling height and morphology

In the colchicine treatment groups, the trend of decreasing mean height with increasing concentration was observed with each increase in colchicine level producing shorter plants ($p = 0.0005$, Table 2). The same trend occurred in oryzalin treatments ($p < 0.0001$). Height of seedlings was significantly different between ploidy levels in *A. crassicarpa* treated with colchicine ($p < 0.0001$) but not oryzalin ($p = 0.399$). Diploids that had been through the colchicine treament were the tallest, followed by tetraploids and mixoploids. However, no interaction was found between effects of concentration and ploidy level in either colchicine ($p = 0.58$) or oryzalin-mediated ($p = 0.23$) tetraploid induction. High frequencies of foliar abnomalites were observed in seedlings exposed to both mitotic inhibitors with 55% of plants assessed with abnormal phyllode morphology in colchicine-treated plants and 57% in oryzalin-treated plants.

DNA amounts of different ploidy levels

The 2C DNA amount of diploid *A. crassicarpa* was estimated as 1.75 pg (SD ± 0.09) (Table 3), whereas the DNA amount of tetraploid was estimated as 3.47 pg (SD ± 0.14). The 2C DNA amount of the single spontaneous triploid was estimated as 2.74 pg.

DISCUSSION

Healthy and actively-growing tetraploids were produced from both colchicine and oryzalin treatments over 24 hours. In this study, 0.02% colchicine induced 21% tetraploids with 93% germination and 61% survival as observed after 7 months post-treatment. Moffat and Nixon (1960) also found a reasonable balance between survival and tetraploidy at 0.02–0.03% colchicine on chipped seed for 24 hours or 0.01–0.02% for 48 hours. The 0.02% colchicine treatment was also optimal for *A. mangium* (Harbard et al. 2012), but that study evaluated treatments after only 16 hours exposure and found a 7% tetraploid conversion rate. An 18.9% tetraploid induction with 93% germination in *A. mangium* was achieved by imbibing unscarified seeds in 0.025% colchicine for a longer duration of 50 hours under aseptic conditions (Blakesley et al. 2002). It has also been reported that an 11% tetraploid induction was observed when 0.02% colchicine was applied to seeds of Japanese barberry (*Berberis thunbergii*) but with a tetraploid induction efficiency of 8% due to lower survival (Lehrer et al. 2008). Therefore, tetraploid induction efficiency is a good indicator of effective treatments because it considers both seedling survival and rate of conversion. The highest colchicine concentration of 0.5% had

Table 2 Effects of different concentrations of colchicine and oryzalin as well as different ploidy levels on seedling height of *Acacia crassicarpa*

Treatment concentration (%)		Height (cm)* (mean ± SE)
Mitotic inhibitor × concentration	Colchicine	
	0	15.35 ± 0.54 a
	0.005	14.97 ± 0.43 b
	0.02	14.50 ± 0.68 c
	0.05	14.07 ± 0.50 d
	0.5	00.00 ± 0.00 e
	p value	= 0.0005
	Oryzalin	
	0	5.48 ± 1.50 a
	0.0005	4.70 ± 2.34 b
	0.005	3.57 ± 2.58 c
	0.01	2.71 ± 1.17 d
	p value	< 0.0001
Mitotic inhibitor × ploidy	Colchicine	
	Diploid	15.23 ± 0.50 a
	Mixoploid	14.37 ± 0.63 b
	Tetraploid	14.61 ± 0.59 c
	p value	< 0.0001
	Oryzalin	
	Diploid	4.83 ± 2.28 a
	Mixoploid	2.80 ± 1.14 a
	Tetraploid	3.03 ± 1.46 a
	p value	= 0.399
Concentration × ploidy	Colchicine p = 0.5769	
	Oryzalin p = 0.2338	

SE = standard error; different letters indicate significant difference between mean values at $\alpha = 0.05$ level by post-hoc Tukey's test; *heights of colchicine- and oryzalin-treated seedlings were recorded at 8 months and 5 months respectively

Table 3 DNA amounts of *Acacia crassicarpa* of differing ploidy estimated by propidium iodide flow cytometry

Ploidy	2C DNA amount (pg ± SD)
Diploid (2x)	1.75 ± 0.09
Triploid (3x)	2.74 ± 0.00*
Tetraploid (4x)	3.47 ± 0.14

SD = standard deviation; *SD is 0.00 as only one triploid plant was identified

100% lethality indicating that the most effective treatment following 24 hours exposure could be optimised within the range chosen for this study.

Tetraploid conversion rates of 5 (0.005% treatment concentration) and 6% (0.01% treatment concentration) using oryzalin in this study was comparable with the 4% tetraploid conversion rate reported in *A. dealbata* by Blakesley et al. (2002). The tetraploid induction efficiency of the highest oryzalin concentration tested (0.01%) was 2.34%. The best performing oryzalin concentration tested was 0.005%, representing a tetraploid induction efficiency of 3.10%. It should be noted that 98% ethanol was used to dissolve oryzalin in this study instead of the more commonly used solvent, dimethyl sulfoxide (DMSO). DMSO was avoided for safety reasons

because this chemical was found to cause neurodegeneration (Hanslick et al. 2009). This may contribute to the lower availability of dissolved oryzalin to target cells because ethanol evaporates rapidly. Tetraploids induced by colchicine may also show higher reversion to diploids. A 4 and 2%-reversion rate to diploid was reported among tetraploids induced by colchicine and oryzalin respectively (Lehrer et al. 2008). For colchicine-induced *A. mangium,* reclassification of tetraploids to both diploids and mixoploids was observed when reassessed at 22 months (Harbard et al. 2012). Oryzalin has substantial advantage on safety grounds so further optimisation experiments are justified. Assessed across all concentrations tested both mitotic inhibitors produced mixoploids. However, more mixoploids were produced relative to the number of tetraploids in the oryzalin treatments compared with colchicine.

Observation of seedlings after exposure to both colchicine and oryzalin revealed a number of morphological abnormalities. These abnormalities included foliar irregularities such as curvy, wrinkled phyllodes and irregular surfaces, mixtures of different leaflet sizes on compound leaves and irregular phyllodes as well as stunted growth. Similar phenotypic differences have been reported by Harbard et al. (2012) and Lehrer et al. (2008). Exposure can lead to abnormalities without changing ploidy (Harbard et al. 2012). High incidence of abnormalities reported here relative to the number of confirmed mixoploid and tetraploid plants also indicated deleterious effect of the mitotis inhibitor on growth irrespective of a change in ploidy. The effect of concentration of oryzalin and colchicine on seedling height was also evident and expected. However, we expect that plants from both treatments would recover after this initial retardation of growth. Ploidy effect on plant morphology has been well documented and quantifiable variations in response to different ploidy levels can be exploited as a pre-screening method for putative polyploids. For example, leaf index, length, width of stomata, stomata index, stomatal count and chloroplast number per stomata guard cell have all been reported as useful parameters for screening putative tetraploids as well as reducing subsequent cytological workload for ploidy determination (Shao et al. 2003, Zhang et al. 2009).

Interestingly, one of the 280 seedlings in control treatments appeared to be triploid, as revealed by flow cytometry. This may indicate a possible low frequency of spontaneous triploids in the Vietnamese *A. crassicarpa* plantation where the plant material was sourced. A low percentage of triploids and tetraploids in natural populations of *A. dealbata* in Australia has also been detected (Blakesley et al. 2002).

The 2C DNA amount of tetraploid *A. crassicarpa* as estimated in our study (3.47 pg) was lower than that of 5.46 pg (SD ± 0.85) reported by Mukherjee and Sharma (1995). Similarly, differences were also observed when the 2C DNA amounts of *A. dealbata* and *A. mangium* reported by Blakesley et al. (2002) were compared with those estimates by Mukherjee and Sharma (1993, 1995). This might be explained by differences in the use of internal standards as the 2C DNA amount of *Allium cepa*, the standard used by Mukherjee and Sharma (1993, 1995), was 33.5 pg, which was three-fold larger than the pea standard used in this study.

In summary, tetraploid *A. crassicarpa* plants were successfully induced by colchicine and oryzalin treatments applied to scarified seeds at the time of germination. The most useful was 0.02% colchicine with 21% conversion to tetraploidy. The promising results using 0.005% oryzalin with 5% tetraploid conversion rate should be further investigated by using a solvent less detrimental to plant tissues, higher concentrations and adjustment of treatment duration. The discovery of a spontaneous triploid in this study was unexpected but encouraging as it showed that plants of this level of ploidy could be viable. Success in tetraploid induction has laid a foundation for breeding population from which triploids can be developed.

ACKNOWLEDGEMENTS

This research was funded by Australian Centre for International Agricultural Research (ACIAR) project FST/2008/007. We thank D Ratskowski, G Jordan and KY Yu for statistical advice and M Cozens for technical assistance with flow cytometry.

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