# COLCHICINE-MEDIATED POLYPLOID INDUCTION IN *Acacia* FOR THE BREEDING OF SEEDLESS TRIPLOID TREES

**J. W. Yap[1*], M. M. Clyde[2], K. I. Kandasamy[1], M. Madon[3] & A. Koutoulis[4]**

[1]Tissue Culture Laboratory, Forest Research Institute Malaysia (FRIM)
[2]Faculty of Science and Technology, National University of Malaysia (UKM)
[3]Cytogenetics Laboratory, Malaysian Palm Oil Board (MPOB)
[4]School of Plant Science, University of Tasmania (UTAS), Australia

**(yapjingwei@yahoo.com)[*]**

## INTRODUCTION

Introduced *Acacia* such as *A. mangium* and *A. auriculiformis* are an important group of forestry crops throughout tropical Asia. However, there is concern that *Acacia* may potentially behave as weeds under certain conditions. The development of seedless triploid planting material for clonal forestry represents a possible strategy for overcoming this problem. In addition, triploids may have superior traits than diploids, as has been observed in other commercial crops. Moreover, the near sterility of triploids makes them a good candidate for transformation studies.

The first step in developing triploids is to develop tetraploids through the use of mitotic inhibitors, such as colchicine. These tetraploids can later be crossed with diploids to produce seedless triploid progeny. In this study we describe the effects of varying colchicine concentrations and exposure times on the frequency of polyploid induction on a selected $F_1$ *Acacia* hybrid clone and report the nuclear genome sizes for *A. mangium*, *A. auriculiformis* and the $F_1$ hybrid.

## OBJECTIVE

To develop methodologies for inducing polyploidy in *Acacia* under *in vitro* conditions toward the development of seedless planting material.

## MATERIALS & METHODS

### Plant material

The *Acacia* material consisted of *in vitro* micro-shoot cultures growing at the Tissue Culture Laboratory, FRIM. *Glycine max* 'Polanka' were provided as seed by MPOB and raised in the open. Sample tissues were stored at 4°C on moistened filter paper until ready for use.

### Colchicine immersion treatments

The *in vitro Acacia* explants consisted of 1.0 cm length shoot-tips from healthy cultures of a selected $F_1$ hybrid clone 'AH:M4' that had been carefully excised and divested of leaves. Explants were aseptically removed and transferred into 50 mL conical flasks (10 to 15 explants per flask) containing 10 mL of colchicine solution at varying concentrations (0.00, 1.25, 2.50, 5.00 and 10.00 mM). Flasks were secured in a rotary platform incubator shaker and incubated for a range of exposure times (0, 6, 12, 24, 48 and 96 h) at 28°C in the dark at 10 rpm.

After incubation period had elapsed, treated explants were aseptically transferred into 50 mL conical flasks and gently washed three times with approx. 20 mL of sterile ultra pure water to remove excess colchicine. Finally, the treated explants were transferred into test tubes containing 10 mL of *Acacia* multiplication media and incubated at 23 ± 5°C under a 16 h photoperiod regime. The cultures were inspected periodically for new apical growth and non-responsive or necrotic explants discarded after 16 weeks.

**Preparation of nuclei suspensions**

For DNA ploidy analysis, nuclei suspensions of the first emerging phyllodes from the surviving regenerating plantlets were prepared according to Galbraith *et al*. (1983) by rapidly chopping the phyllodes in 900 µl of ice-cold General Plant Buffer (Loureiro *et al*. 2007). For genome size estimation, nuclei suspensions from phyllodes of *A. mangium*, *A. auriculiformis* and the $F_1$ hybrid were each co-processed with approx. equal amounts of *G. max* (soybean) ($2C$ = 2.50 pg) leaves as an internal reference standard.

The resulting homogenates were removed with disposable syringes and filtered through 50 µm CellTrics® (Partec) nylon disposable filters to remove large debris into 3.5 mL sample tubes (Sarstedt) containing 50 µL of 1.0 mg/mL PI, as a fluorochrome, and 50 µL of 1.0 mg/mL RNAse, as an additive to prevent staining of double-stranded RNA.

**Flow cytometry analyses**

Samples were analysed with a CyFlow Space (Partec®) flow cytometer equipped with an air-cooled argon-ion laser tuned to 15 MW and operating at 488 nm at Faculty of Science and Technology, UKM. Fluorescence was collected through a 645 nm dichroic long-pass filter in reflecting mode and a 620 nm band pass filter. The resulting output was acquired using Partec® FloMax (version 2.52) FCM Data Acquisition and Analysis Software.

The nuclear genome size was estimated using to the following formula:

$$\text{Sample } 2C \text{ nuclear DNA content (pg)} = \frac{\text{Sample peak } G_0/G_1 \text{ peak mean}}{\textit{G. max} \text{ peak } G_0/G_1 \text{ peak mean}} \times 2.50$$

## RESULTS & DISCUSSION

| Species | No. of individuals | $2C$ DNA estimate (pg) | $1C$ (pg) | *$1C$ (Mbp) |
|---|---|---|---|---|
| *A. mangium* | 3 | $1.48^{a} \pm 0.01$ | 0.740 | 723.72 |
| *A. auriculiformis* | 3 | $1.49^{a} \pm 0.01$ | 0.745 | 728.61 |
| $F_1$ hybrid | 3 | $1.46^{b} \pm 0.01$ | 0.730 | 713.94 |

Means followed by the same superscript letter (a or b) are not significantly different according to a Tukey test at $P \leq 0.05$.
*DNA content (bp) = $(0.978 \times 10^9) \times$ DNA content (pg) (Doležel *et al*. 2003)

**Table 1.** FCM genome size estimates for *A. mangium*, *A. auriculiformis* & the $F_1$ hybrid

| Col (mM) | Ploidy | Mean frequency (%) ± S.D. 3 h | 6 h | 12 h | 24 h | 48 h | 96 h |
|---|---|---|---|---|---|---|---|
| 0.0 | Mortality | 0.00±0.00 | 2.78±4.81 | 9.09±9.09 | 5.56±9.62 | 6.67±5.77 | 8.33±14.43 |
| | Mixoploid | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1.25 | Mortality | 19.44±17.35 | 18.80±3.70 | 25.64±19.36 | 17.98±8.71 | 29.70±4.27 | 72.44±36.32 |
| | Mixoploid | 2.56±4.44 | 0.00±0.00 | 0.00±0.00 | 8.89±8.39 | 0.00±0.00 | 2.78±4.81 |
| 2.5 | Mortality | 3.03±5.25 | 13.75±12.16 | 18.65±5.63 | 34.60±10.29 | 38.89±39.38 | 68.08±32.69 |
| | Mixoploid | 10.26±11.75 | 12.82±22.21 | 2.78±4.81 | 16.92±7.96 | 16.67±16.67 | 5.13±8.88 |
| 5.0 | Mortality | 16.03±7.39 | 35.35±18.27 | 44.58±15.02 | 55.34±15.60 | 82.60±15.78 | 100.0±0.00 |
| | Mixoploid | 11.11±12.73 | 25.76±21.48 | 14.17±15.07 | 7.91±7.70 | 9.71±10.86 | 0.00±0.00 |
| 10.0 | Mortality | 26.92±21.41 | 79.83±12.31 | 76.92±20.35 | 80.05±4.44 | 86.67±17.64 | 94.66±4.64 |
| | Mixoploid | 0.00±0.00 | 10.26±11.75 | 7.69±13.32 | 2.78±4.81 | 8.33±14.43 | 0.00±0.00 |

**Table 2.** Effect of colchicine concentrations and exposure times on mixoploid frequencies and mortality of a selected $F_1$ *Acacia* hybrid clone 'AH:M4'

### Estimation of nuclear genome size

In this study, we obtained estimated the nuclear genome size of *A. mangium* ($2n$ = 26), *A. auriculiformis* (2n = 26) and the $F_1$ hybrid ($2n$ = 26) from the positions of the 2*C* DNA histograms of various diploid *Acacia* samples against *G. max* 'Polanka' as an internal reference standard (TABLE 1). We found the mean genome sizes of *A. mangium* and *A. auriculiformis* to be very close with a difference of only 0.01 pg, would be expected of two closely related species. Our estimates differ from previous published estimates of *A. mangium* by Blakesley *et al*. (2002) and Mukherjee and Sharma (1995) (1.30 pg and 2.30 pg respectively), and *A. auriculiformis* by Mukherjee and Sharma (1995) (2.22 pg), and probably reflect differences in estimation techniques and choice of reference standard species.

Contrary to expectation, we found the mean genome size of the $F_1$ hybrid, not to be intermediate, but lower than either parental species. While the genome sizes of most interspecific hybrids fall between that of their parental species (Buitendijk *et al*. 1997), the curious phenomenon of hybrids having lower than expected genome size is not unknown and has previously been documented in some $F_1$ hybrids of other genera such as *Cirsium* (thistle) (Bureš *et al*. 2004). While the cause of this discrepancy is still poorly understood, selective elimination of certain parts of genome following hybridization, as observed in *Helianthus* (sunflower) (Baack *et al*. 2005), has been proposed as a possible explanation.

### Colchicine-mediated in vitro polyploid induction

Colchicine is a phytotoxin that is known retard and kill plant tissue. The effects of exposure to varying colchicine concentrations for different periods of time on shoot tip survival and growth were evaluated (TABLE 2).

Compared to controls, explants in colchicine treatments all displayed slower early growth compared to controls. We observed a degree of mortality in all but one of the treatments (0.0

mM colchicine for 3 h), which suggests that the shoot tips were fragile and could be damaged even by the immersion treatment. Generally, the mortality increased with increasing colchicine concentration and exposure time which concurs with findings from other colchicine-mediated polyploid induction studies (Roy *et al*. 2001 and Zhang *et al*. 2010). For exposure periods of 12 h and below, low concentrations of colchicine (1.25 mM) was found to cause higher mortality for the equivalent exposure periods at a higher colchicine concentration (2.5 mM). This peculiar pattern agrees with findings from Caperta *et al*. (2006) who demonstrated in *Secale cereal* (rye) that short exposure to a high colchicine concentration induced the formation of viable reconstituted tetraploid nuclei, the equivalent exposure time in low colchicine concentration caused deleterious microtubulin disruptions.

We estimated the DNA ploidy of the surviving shoot tips indirectly using FCM analysis on the first emerging leaf of apical origin. Although our analysis did not detect any pure tetraploids, putative 'mixoploids' or cytochimeras composed of a mixture of 2*C* diploid and 4*C* tetraploid cells and, in one case, even 8*C* octaploid cells were revealed at various frequencies from the various treatments (TABLE 2).

In general, appreciable (>5%) frequencies of putative mixoploids were obtained only by using colchicine concentrations of 2.5 mM and greater with the highest mean frequency of mixoploids (25.76%) obtained using 5.0 mM colchicine at for 6h. Extending the exposure time for more than 6h generally did not increase putative mixoploid frequency in a meaningful manner. In fact, for colchicine concentrations of 2.5 to 10 mM and greater, exposure for 12h resulted in a drop in the mean putative mixoploid frequency. While the reason for this drop is not known, it is postulated that it may be related to the duration of the cell cycle in *Acacia*.

Mixoploidy is a common product of colchicine-mediated polyploid induction (Rose *et al*. 2000; Roy *et al*. 2001; Greplová *et al*. 2009). Only cells which are actively dividing are susceptible to colchicine (Satina *et al*. 1940). Thus, mixoploidy is likely a result of the shoot tip meristem being comprised of a mixture of cells at different phases of the cell cycle.

Mixoploids are considered to be valuable from a breeding perspective, as there is a possibility that they may behave reproductively as tetraploids (Koutoulis *et al*. 2005) in terms of producing diploid germ cells. Also, there is a chance of us using mixoploids to recovering full tetraploids through *in vitro* culture techniques as has previously been reported in *Humulus lupulus* (Roy *et al*. 2001), *Vitis vinifera* (Franks *et al*. 2002).

In conclusion, this study has successfully developed polyploid induction methodologies for *Acacia* which will be used to produce material for an *Acacia* triploid breeding program at FRIM.

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