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DIFFERENCE IN COMPETENCE FOR *IN VITRO* PROLIFERATION AND *EX VITRO* GROWTH OF GENETICALLY IDENTICAL MATURE AND JUVENILE CLONES OF APOMICTIC *MALUS* SPECIES

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Abstract

The apomictic system in *Malus* was used as a model to examine rejuvenation by generating genetically identical tissue culture lines that had two entirely different developmental origins: either embryo-derived tissues (juvenile clones) or somatic tissue from the adult/mature tree (mature clones). These two lines were then subsequently used to examine *in vitro* difference between mature (M) and juvenile (J) tissues in potential for shoot, root proliferation and *ex vitro* (glasshouse) growth. The M clones of *M. hupehensis* and *M. toringoides in vitro* had significantly fewer total shoots and shoot more than 2 cm in length per proliferating explant than the J clones and also rooted less efficiently. *Ex vitro* (glasshouse) juvenile clones had shorter internodes, a greater number of leaves and more dry weight compared to their mature counterparts.

Introduction

Plant tissue culture is an integral part of molecular approaches to plant improvement. The practical utilization of molecular biology approaches is greatly dependent on the development of reliable and efficient regeneration systems from somatic tissues. There are various factors affecting regeneration *in vitro*. Ontogenetic aging and physiological maturation, particularly in tree species, leading to the flowering state (known as "phase change") has several major consequences. One of these is the decrease and even loss of ability to produce adventitious roots coupled with the loss of vigour in shoot-tips and meristems isolated for *In vitro* culturing (Mitchell *et al*., 2004). No matter what the mechanism for phase change, mature characteristics accumulate during development. They accumulate because the characteristics are quite stable and transmitted developmentally through cell division from one somatic cell generation to the next, even into branches of the main axis. However, such stability does not mean that maturation-related characteristics are permanent and non-reversible. They can be wholly or partly reversed during sexual reproduction, apomictic reproduction or by chemical or cultural treatments (Hackett & Murray, 1997). Mitchell *et al*., (2004) stated that root system quality and stem size and form all decline with increasing hedge age. A decline in root system quality was particularly apparent and was observed prior to a decline in rooting efficiency. Williams *et al*., (2004) stated that vegetative and reproductive maturity are genetically and physiologically uncoupled in Eucalyptus. Nas *et al*., (2003) reported that micro propagated plants of mature origin may retain their physiologic maturity during long-term *In vitro* culture or regain it soon after they are taken out of the culture.

The process of apomixis provides a good tool to study the differences (*In vitro, In vivo*) between individuals having a different physiological state but of identical genetic make-up. The scientific value of such a system lies in the ability to obtain identical

genetic backgrounds that are expressed in two distinct morphological forms. Apomictic embryos can be germinated *In vitro* and used for basic studies on rejuvenation. By collecting vegetative apices from the same adult tree it is possible to introduce into culture identical germplasm in two totally different physiological conditions. Presumably the genetically identical germplasm has in one case been entirely re-programmed but in the other it has not. This is a situation rarely met in developmental biology and has considerable potential to throw light on basic developmental processes at the cell, tissue and organ level. This material can also be used for reducing experimental variability in the physiological response of tissue and enhance the prospects of obtaining reproducible results. The studies reported here were carried out to investigate the effects of the physiological state of the explants on *In vitro* organogenesis in apomictic *Malus* species (*Malus hupehensis* and *M. toringoides*) and thereafter on *ex vitro* (glasshouse) growth of transplanted shoots.

Materials and Methods

Plant materials: The *In vitro* establishment, multiplication and culture conditions of mature (M) and juvenile (J) clones of *M. hupehensis* and *M. toringoides* are described by (Ur-Rahman (1997). Genetic similarity between the M and the J clones was confirmed using RAPD molecular markers (Ur-Rahman *et al*., 1997).

***In vitro* shoot proliferation and rooting of mature (M) and juvenile (J) clones:** *Malus hupehensis* and *M. toringoides* explants from mature (M) and juvenile (J) clones were used. These two explant materials were systematically paired during all culture procedures. They were propagated for one year with sub-culture periods of 4-5 weeks, on multiplication medium (MS at full-strength plus BA 1.0 mg l^{-1}, IBA 0.1 mg l^{-1}, GA_3 1.0 mg l^{-1}, Oxoid Agar No.3 at 7.5 g l^{-1}, sucrose 30.0 g l^{-1}, pH 5.2 adjusted before autoclaving). After one year, shoot proliferation of the M and the J clones was compared on the same multiplication medium. To test rooting ability, micro cuttings of 2.0-2.5 cm length with an apical bud were used from these clones. Micro cuttings were first placed on a root induction medium (MS at full-strength plus IBA 3.0 mg l^{-1}, Oxoid Agar No.3 at 7.5 g l^{-1}, sucrose 30.0 g l^{-1}, pH 5.2 before autoclaving) for four days. After that, the micro cuttings were then sub-cultured onto a root development medium (MS at half-strength plus Oxoid Agar No.3 at 7.5 g l^{-1}, sucrose 30.0 g l^{-1}, pH 5.2 pre-autoclave).

For the shoot proliferation study, explants were cultured in 25 ml coulter pots containing about 8 ml of culture medium and one explant per pot. For comparing rooting ability the same size coulter pots were used, containing two explants per pot. The shoot proliferation experiment involved 4 replications with 6 explants per replication whereas the rooting experiment involved 4 replications with 12 explants per replication. Cultures were maintained under a 16-h (μmol m^{-2} s^{-1}) photoperiod at 23 ± 1°C.

Shoot proliferation traits such as the number of proliferating explants, total number of shoots per proliferating explant, and number of shoots of >2.0 cm length per proliferating explant, were recorded after 4 weeks. Rooting ability was recorded as the number of rooted explants, and the length and number of roots per rooted explant after 4 weeks on root development medium. Analysis of variance was used to analyse the data.

***Ex vitro* (glasshouse) performance of micro propagated mature (M) and juvenile (J) clones:** The glasshouse performance of these propagules was assessed after rooting *in vitro* and weaning the explants from both clones (M and J). Six weeks after weaning the plantlets were transferred to 6″ diameter pots filled with uniformly homogenised potting mixture. The plants in pairs (M and J) exhibiting similar initial height, and number of leaves were placed on the bench top in the glasshouse (25 ± 5°C day/night from April to September). Every 30-d until harvest (just before the cessation of growth) measurements were recorded for the following characteristics: height, stem diameter (2cm from the base), number of fully expanded leaves, internodal length (10 internodes, 10th to 20th from the base), number of axillary shoots, and at harvest the fresh weight of the whole plant excluding roots. The plants were dried in an oven at 80°C for a week and after cooling for about 20 min, they were weighed individually for dry weight and % dry weight was calculated as:

$$\% \text{ Dry weight} = \frac{\text{Dry weight (g)}}{\text{Fresh weight (g)}} \ 100\%$$

Eighteen pairs of the M and the J clone of *M. hupehensis* and 15 pairs of the M and the J clone of *M. toringoides* were compared in randomised block designs. Analysis of variance was used to analyse the data.

Results

In vitro comparisons of mature (M) and juvenile (J) clones

a: *M. hupehensis*

Shoot proliferation: There was no difference for shoot proliferation rate between the M and the J clone (100% explants proliferated for both the clones), however the physiological state of the explant affected significantly ($p<0.001$) the mean number of shoots per proliferating explant (6.67 for the M clone and 13.24 for the J clone). The mean number of shoots per proliferating explant over 2.0 cm in length was also significantly higher ($p<0.001$) in the J clone than for the M clone 2.83 and 9.86 respectively (Table 1).

Rooting ability: The physiological state of the explant had a significant effect ($p=0.459$) on rooting rate: the J clone exhibited 98.81% rooting compared with 93.75% for the M clone, however there was no significant difference in the mean number of roots per rooted explant, nor the mean number of roots over 1.5 cm length (Table 2).

b: M. toringoides

Shoot proliferation: There was no difference in the rate of shoot proliferation between the M and the J clone and both clones exhibited 100% proliferation. However the J clone produced more shoots ($p=0.042$) per proliferated explant than the M clone (6.96 and 8.75 respectively). The mean number of shoots more than 2.0 cm in length per proliferated explant was also higher ($p=0.039$) in the J clone (5.60) than the M clone (3.83) (Table 3).

Table 1. Effect of the physiological state of the shoot explants on *in vitro* shoot proliferation in *Malus. hupehensis*.

Physiological state	Shoot proliferation rate (%)	Mean number of shoots/proliferated explants	Mean number of shoots >2.0 cm length/*P.Expl.
Mature (M)	100.00 (24)	6.67	2.83
Juvenile (J)	[1]100.00 (168)	[1]13.24	[1]9.86
Statistical significance:			
Mature × Juvenile		p < 0.001 $LSD_{5\%}$ (21 d.f.) 2.95	p < 0.001 $LSD_{5\%}$ (21 d.f.) 2.97

*P. Expl. = Proliferated explants.
[1]Mean of 7 clones.
Figures in parenthesis are the number of explants cultured.

Table 2. Effect of the physiological state of the shoot explants on *in vitro* rooting ability in *M. hupehensis*.

Physiological state	Rooting rate (%)	Mean number of roots/rooted explants	Mean number of roots >1.5 cm length/*R.Expl.
Mature (M)	93.75 (48)	7.84	6.52
Juvenile (J)	[1]98.81 (336)	[1]7.96	[1]6.37
Statistical significance:			
Mature × Juvenile	p = 0.459 $LSD_{5\%}$ (21 d.f.) 6.07	ns $LSD_{5\%}$ (21 d.f.) 1.52	ns $LSD_{5\%}$ (21 d.f.) 1.26

*R.Expl. = Rooted explants.
[1]Mean of 7 clones.
Figures in parenthesis are the number of explants cultured.

Table 3. Effect of the physiological state of the shoot explants on *in vitro* shoot proliferation in *Malus toringoides*.

Physiological state	Shoot proliferation rate (%)	Mean number of shoots/proliferated explants	Mean number of shoots >2.0 cm length/*P.Expl.
Mature	100.00 (24)	6.96	3.83
Juvenile (J_1)	[1]100.00 (48)	[1]8.75	[1]5.60
Statistical significance:			
Mature × Juvenile		p = 0.042 $LSD_{5\%}$ (6 d.f.) 1.48	p = 0.039 $LSD_{5\%}$ (6 d.f.) 1.46

*P.Expl. = Proliferated explants.
[1]Mean of 2 clones.
Figures in parenthesis are the number of explants cultured.

Rooting ability: Rooting ability was observed to be influenced by the physiological state of the explant (p=0.330): the J clone exhibited 94.80% rooting compared with 85.40% for the M clone, however the differences in mean number of roots and mean number of roots more than 1.5 cm in length per rooted explant were not significant (Table 4).

***Ex vitro* (glasshouse) growth of mature (M) and juvenile (J) clones:** After weaning, the survival and establishment in the glasshouse of both species for each clone was 100%.

a: *M. hupehensis*

Plant height: The M clone was significantly (p=0.006) taller compared to the J clone until the 30th day in the glasshouse. There was no significant difference at 5% LSD for height between the M and the J clone from the 60th to the 90th day, however the M clone again exhibited significantly (p=0.017) more height than the J clone until harvest on the 120th day (Fig. 1a).

Stem diameter: The J clone had significantly (p=0.02) greater stem diameter than the M clone at the 30th day, whereas the differences were not significant at the 60th, 90th and 120th days (Fig. 1b).

Number of leaves: The J clone bore a significantly (p=0.002, 0.003, <0.001 and <0.001 respectively for the 30, 60, 90 and 120th day) greater number of leaves compared to the M clone (Fig. 1c).

Internodes length: The M clone had significantly (p=0.001) longer internodes (average 2.2 cm) compared to the J clone (average 2.02cm) (Table 5).

Number of axillary shoots: The J clone produced significantly (p<0.001) more axillary shoots per plant (on average 29.39) than the M clone (on average 16.17), (Table 5).

Fresh and dry weight: The M clone gained significantly (p=0.02) more fresh weight (on average 75.3g) than the J clone (on average 68.5g) per plant. There were no significant differences in dry weight, however average % dry weight was significantly (p<0.001) more in the J clone (65.84%) than the M clone (62.81%), (Table 5).

b: *M. toringoides*

Plant height: The physiological state of the clones affected significantly (p<0.001 for the 30, 60, 90th days) plant height in glasshouse. The M clone was significantly taller on the 30th day, but the J clone was taller than the mature clone on the 60th and 90th days (Fig. 2a).

Stem diameter: The J clone had significantly (p<0.001) greater stem diameter than the M clone on the 30th, 60th and 90th days and the difference in stem diameter increased over this period (Fig. 2b).

Number of leaves: The physiological state of the plants affected significantly (p<0.001) the number of leaves. Over the period of measurement, the rate of leaf production was greater for the J clone than the M clone (Fig 2c).

Internodes length: The J clone had significantly (p<0.001) shorter internodes (2.42 cm) than the M clone (3.49cm) (Table 6).

Table 4. Effect of the physiological state of the shoot explants on *in vitro* rooting ability in *M. toringoides*.

Physiological state	Rooting rate (%)	Mean number of roots/rooted explants	Mean number of roots >1.5 cm length/*R.Expl.
Mature	85.40 (48)	4.16	3.69
Juvenile (J_1)	[1]94.80 (96)	[1]4.63	[1]3.94
Statistical significance:			
Mature × Juvenile	p = 0.330	ns	ns
	$LSD_{5\%}$ (7 d.f.) 12.86	$LSD_{5\%}$ (6 d.f.) 0.90	$LSD_{5\%}$ (6 d.f.) 1.11

*R.Expl. = Rooted explants.
[1]Mean of 2 clones.
Figures in parenthesis are the number of explants cultured.

Table 5. Internode length, number of axillary shoots, fresh weight (whole plant except roots), dry weight, and percentage dry weight of glasshouse grown mature and juvenile clones of *M. hupehensis* at harvest (120th day after weaning).

Physiological state	*Internode (cm)	No. of axillary shoots	Fresh weight (g)	Dry weight (g)	Dry weight (%)
Mature (M)	2.2	16.17	75.3	47.19	62.81
Juvenile (J)	2.02	29.39	68.5	44.96	65.84
Statistical significance:					
M × J F. prob.	= 0.001	< 0.001	= 0.02	ns	< 0.001
$LSD_{5\%}$ (17 d.f.)	= 0.11	= 2.63	= 5.32		= 1.43

*Mean length of 10 internodes (10th to 20th internode from the base).
Fresh weight of the plant immediately after harvest (except roots).
Dry weight after drying at 80°C for one week.

Table 6. Internode length, fresh weight (whole plant except roots), dry weight, and percentage dry weight of glasshouse grown mature and juvenile clones of *M. toringoides* at harvest (90th day after weaning).

Physiological state	*Internode (cm)	Fresh weight (g)	Dry weight (g)	Dry weight (%)
Mature (M)	3.49	68.2	43.1	67.2
Juvenile (J)	2.42	112.9	63.3	56.3
Statistical significance:				
M × J F. prob.	< 0.001	< 0.001	< 0.001	= 0.002
$LSD_{5\%}$ (14 d.f.)	= 0.28	= 16.30	= 6.63	= 5.98

*Mean length of 10 internodes (10th to 20th internode from the base).
Fresh weight of the plant immediately after harvest (except roots).
Dry weight after drying at 80°C for one week.

Fresh and dry weight: The physiological state of the plantlets affected significantly ($p<0.001$) their fresh and dry weight. The J clone had greater fresh weight and dry weight (112.9g and 63.38g respectively) than the M clone (68.2g and 43.1g respectively). Percentage dry weight was significantly ($p=0.002$) greater for the M clone than for the J clone (67.2% and 56.3% respectively) (Table 6).

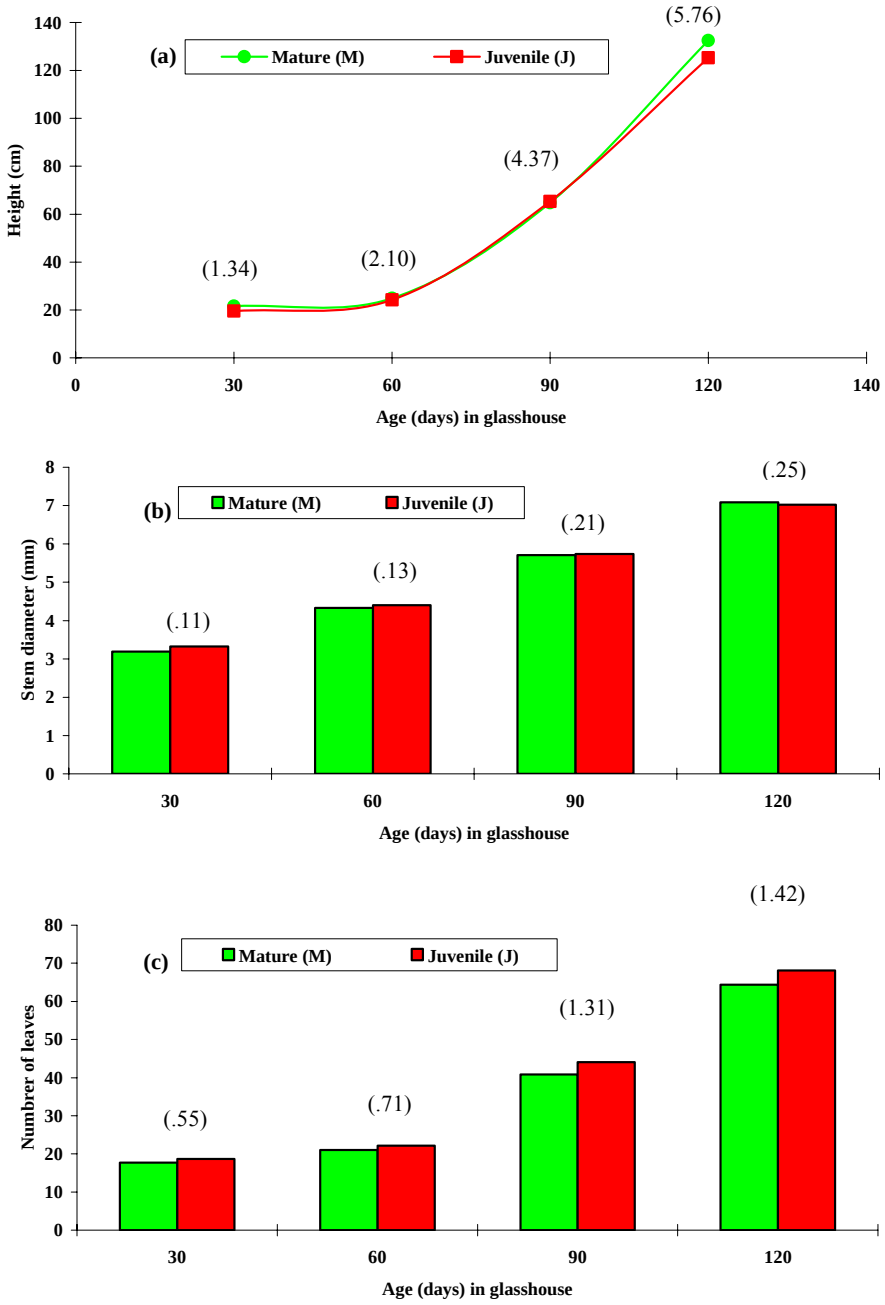

Fig. 1. Mean height (a), stem diameter (b), and number of leaves (c) of glasshouse grown mature and juvenile clones of *M. hupehensis*, 30, 60, 90, and 120 days after weaning. Values in parentheses indicate LSD at 5% (17d.f.).

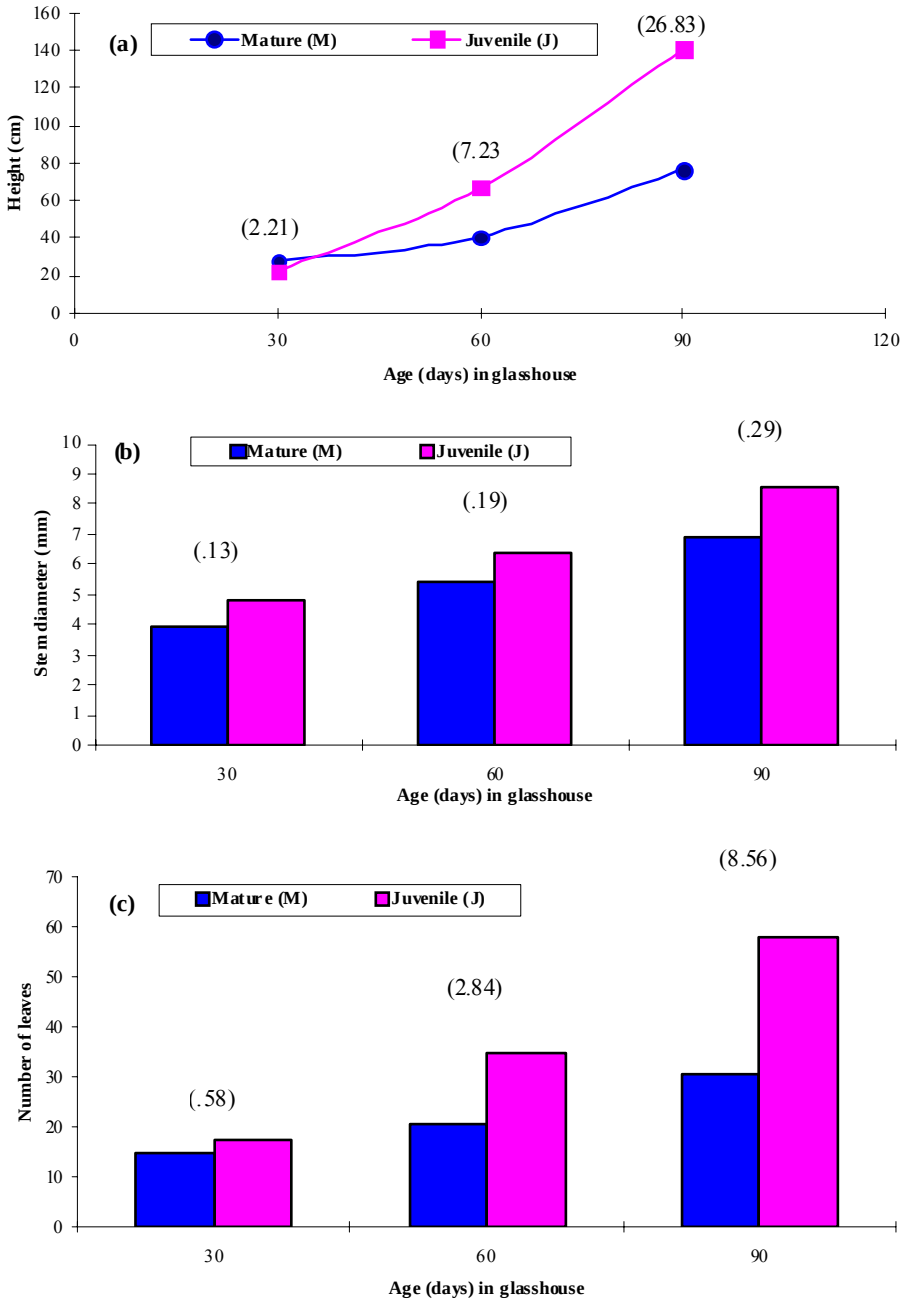

Fig. 2. Mean height (a), stem diameter (b), and number of leaves (c) of glasshouse grown mature and juvenile clones of *M. toringoides*, 30, 60, and 90 days after weaning. Values in parenthesis indicate LSD at 5% (14 d.f.).

Discussion

Plants undergo a series of profound developmental changes throughout their lifetimes in response to both external environmental factors and internal intrinsic ones. When these changes are abrupt and dramatic, the process is referred to as phase change. Examples of these transitions include changes from juvenile to adult leaf formation, vegetative to inflorescence meristem development and inflorescence to floral meristem initiation (Chuck & Hake, 2005). Maturation is closely related to changes in meristematic activity in the shoot apex, once the shoot apical meristem has reached maturity the meristem is competent to respond to a floral stimulus with floral evocation followed by differentiation and development of flower primordia (Waaseth, 2004). The higher shoot proliferation rate, number of shoots and shoot length in the juvenile clone (J) in the present study may be due to the physiological status of the explant. The physiological state of the explant affected significantly the rooting percentage, shoot proliferation rate, mean number of shoots and mean number of shoots over 2.0 cm in length, in both M and J clones of *M. hupehensis*. A similar effect of the physiological state of the explant was shown by *M. toringoides*. The fact that the J clone exhibited a greater potential for adventitious rooting than the M clone is consistent with many reports emphasizing the inhibitory effect of the maturation process on the capacity to form adventitious roots (Capuana & Giannini 1997; Nas *et al*., 2003; Mitchell *et al*., 2004). Various endogenous factors presumed to be involved in reduced rooting capacity during maturation have been reviewed by Waaseth, (2004). For example, following reception of a root inducing signal, mature explant material may show reduced potential for biosynthesis, especially protein synthesis, in comparison with juvenile explants. Several morphological and physiological differences are associated with the mature and juvenile phases of a plant. These vary from species to species and include leaf shape, shoot growth vigour, apical dominance, pigment production and thorniness. The M and J clones of *M. toringoides* and *M. hupehensis* exhibited several differences in the glasshouse despite being genetically identical. There were no differences in leaf shape between the M and J clones of either species, however the J clones throughout the growth period had a greater number of leaves than the M clones. Although the internode length of the J clones for both the species was shorter than in the M clones, the height of the J clones was on average greater than the M clone (*M. toringoides*) or not significantly different (*M. hupehensis*). Most of the plants of the M clone of *M. toringoides* ceased growth after first flush (40-d) and remained quiescent until harvest, whereas all of the J plants grew continuously after the first flush until harvest and had significantly greater stem diameter. Both the M and J clone of *M. hupehensis* continued growing after the first flush until harvest and exhibited no significant difference in stem diameter; however the J clone had a greater number of axillary shoots. Tetsumura *et al*., (2004) reported that micro propagation caused reinvigoration, but not true rejuvenation. Nas *et al*., (2003) reported that *In vitro* rejuvenated and micro propagated plants of mature origin may retain their physiologic maturity during long-term *in vitro* culture or regain it soon after they are taken out of the culture. They may set fruit earlier then seedling plants, similar to conventionally (asexually) propagated plants. They further concluded that some mature phase phenotypic characteristics were unstable during long-term *In vitro* culture of mature phase shoot-tips. However, complete reversal (rejuvenation) of maturation does not occur *In vitro* and individual maturation-related characteristics are not equally

affected. The M and J clones from both the species in this study also differed in fresh and dry weight and possible reasons might be the greater number of leaves, greater height and stem diameter and because of higher potential for biomass production in J than M clones. In the previous studies seedling material was compared with mature material and seedlings might be of a heterozygous nature. However, in these studies use of the J clones of apomictic nature made the comparisons far more sensitive. *In vitro* and *ex vitro* comparison of genetically identical (apomictic) the mature (M) and juvenile (J) clones of *M. hupehensis* and *M. toringoides* exhibited difference in growth which indicates the differential expression of genes into two physiologically different but genetically identical clones. This study has paved the path to clone phase related genes.

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