Chapter 17

Optimisation of growing conditions for the apple rootstock M26 grown in RITA containers using temporary immersion principle

Li-Hua Zhu*, Xue-Yuan Li & Margareta Welander
Swedish University of Agricultural Sciences, Department of Crop Science, P.O. Box 44, SE-230 53 Alnarp, Sweden
**Corresponding author: tel: +46 40 415373, fax: +46 40 460442;*
e-mail: Li-Hua.Zhu@vv.slu.se

Abstract: The use of bioreactors may provide an efficient and economic tool for mass clonal propagation of plants if technical problems can be solved. In this paper, we report the results of experiments aimed at optimising conditions for apple rootstock M26 growth in RITA containers using the temporary immersion principle. We tested different types and sizes of explants, different concentrations of plant growth regulators (BAP, kinetin and IBA) in the multiplication and elongation phases, and medium exchange during the shoot elongation period. The results show that the higher concentrations of cytokinins were required during the shoot multiplication phase, while the lower concentrations were better during the shoot elongation phase. Hyperhydricity was increased with increasing concentrations of cytokinins during both shoot multiplication and shoot elongation phases. The best shoot production in terms of shoot number and shoot quality was obtained using 4.4 µmol BAP and 0.5 µmol IBA during the shoot multiplication phase and 1.1 µmol BAP and 0.25 µmol IBA during the shoot elongation phase. Medium exchange twice during the shoot elongation phase resulted in higher shoot production compared with no exchange of the medium. However, it also resulted in increased hyperhydricity. Immersion frequency of 16 times per day gave a higher multiplication rate and longer shoots than 8 times per day. The explant size of 0.5 cm or 1 cm resulted in a significantly higher shoot production rate compared with that of 1.5 cm, but shoot length and hyperhydricity were not affected by the explant size. Shoot cultures from the liquid media rooted normally in the RITA containers with more than 90 % rooting and the rooted plantlets acclimatised well in the greenhouse.

Key words: apple**,** bioreactor, micropropagation, RITA, temporary immersion

Abbreviations: BAP – benzylaminopurine; IBA – indole butyric acid; MS – Murashige and Skoog medium (1962)

A.K. Hvoslef-Eide and W. Preil (eds.), Liquid Culture Systems for in vitro Plant Propagation, 253–261.

1. Introduction

Clonal propagation by conventional tissue culture techniques is limited in commercial production due to high input of manual labour and a low degree of automation (Chu, 1995; Maene and Debergh, 1985; Simonton et al., 1991, Sluis and Walker, 1985). In contrast to conventional tissue culture techniques, the use of bioreactors may provide a promising tool for mass clonal propagation. Due to the use of liquid media, bioreactors have the following advantages compared with agar media: 1) a large number of plantlets can be more easily produced due to more uniform culture conditions and the ease with which, the explants can take up the nutrients; 2) time- and labour-saving in the handling of cultures because of the semi-automatic operation; 3) better growth and biomass production because of good aeration by forced oxygen supply; 4) the decrease of apical dominance and the stimulation of lateral bud growth, which is probably due to the loss of culture orientation. However, bioreactors developed in the past were mainly for bacterial cultures and not suitable for plant micropropagation because of the mechanical damage and hyperhydricity (Teisson et al., 1999). Recent years, researchers have developed different semi-automated systems using temporary immersion principle and without the impeller (Aitken-Christie and Jones, 1987; Simonton et al., 1991; Tisserat and Vandercook, 1985) to eliminate hyperhydricity and mechanical damage, respectively. The RITA temporary immersion system is one of those developed lately (Teisson and Alvard, 1995). The system provides a temporary contact between explants and liquid media to avoid culture hyperhydricity. Studies on micropropagation using this system have been reported in *Citrus* (Cabasson et al., 1997), pineapple (Escalona et al., 1999), potato (Jiménez et al., 1999), and sugarcane (Lorenzo et al., 2001). The aim of this study was to optimise micropropagating conditions for the apple (*Malus domestica*) rootstock M26 grown in RITA containers using the temporary immersion principle.

2. Materials and methods

The experiments were conducted in RITA (VITROPIC, Saint-Mathieu-de-Tréviers, France) containers using temporary immersion principle. *In vitro* propagated shoot cultures of the apple rootstock M26 were used. The cultures were originally grown on the solid MS (Murashige and Skoog, 1962) medium supplemented with 4.4 μmol BAP (benzylaminopurine), 0.5 μmol IBA (indole butyric acid), 30 g l^{-1} sucrose and 0.7 % agar at pH 5.5.

In the RITA containers, 150 ml of liquid MS medium supplemented with 30 g l^{-1} sucrose at pH 5.5 was used for all treatments. In total, five

experiments were included in this study. The first experiment aimed to improve shoot production by using different combinations of growth regulators and different types of explants (for details see Table 1). Based on experiment 1, the second and third experiments were conducted aimed at optimising concentrations of growth regulators for shoot elongation. In the forth experiment, the influence of medium exchange on shoot production was studied, and the influence of explant size in the fifth experiment. The detailed treatments and conditions used in experiments 2-5 are presented in table 3. In all experiments, two phases were included, namely, the shoot multiplication phase with higher cytokinin concentrations (15 days for experiment 1 and 10 days for experiments 2-5) and the shoot elongation phase with lower cytokinin concentrations for 3-4 weeks. The multiplication media contained 4.4 μmol BAP and 0.5 μmol IBA except for experiment 1. The frequency of medium immersion was 8 times per day for experiments 1-3 and 16 times per day for experiments 4-5. The duration of medium immersion was 5 minutes per time for experiments 1-2 and 2 minutes per time for experiments 3-5. The media were not exchanged during the shoot elongation phase except for experiment 4.

Shoots of 1-1.5 cm in length from four treatments in experiment 1 were rooted in RITA containers using the rooting medium consisting of Lepoivre (Quoirin et al., 1977) macro and microelements, Walkey (1972) vitamins, 1.2 μmol IBA and 30 g l^{-1} sucrose at pH 5.5. The shoots were kept in the dark for 4 days, and then transferred to the identical rooting medium without IBA in light for 3 weeks.

Shoot number (≥ 0.5 cm) and shoot quality, measured as shoot length and hyperhydricity were recorded at the end of the experiments for experiments 1-5, and the root number and root length for experiment 1. All data were subjected to ANOVA analysis with Duncan's multiple range test using the Statgraphics program.

Rooted plantlets from the RITA containers were planted in pots in a mixture of peat and perlite, and acclimatised in the greenhouse conditions.

3. Results and discussion

3.1 Experiment 1

The results from experiment 1 are presented in table 1 and figure 1. Using 1.1 μmol BAP and 1.2 μmol IBA in the shoot multiplication medium resulted in a significantly higher shoot number compared with 4.4 μmol BAP

A B C D E

Figure 1: Shoot cultures of the apple rootstock M26 grown in the RITA containers after 5 weeks from experiment 1. A and B: Shoot tip and stem segment explants, respectively, 4.4 µmol BAP and 0.5 µmol IBA during the shoot multiplication phase and 0.5 µmol kinetin and 0.05 µmol IBA during the shoot elongation phase for both. C and D: Shoot tip and stem segment explants, respectively, 8.8 µmol BAP and 1.0 µmol IBA for the shoot multiplication phase and no growth regulators during the shoot elongation phase for both. E: Shoot tip explants, 1.1 µmol BAP and 1.2 µmol IBA during the shoot multiplication phase and no growth regulators during the shoot elongation phase.

and 0.5 µmol IBA, and 8.8 µmol BAP and 1.0 µmol IBA when shoot tips were used as explants. Stem segments gave a higher shoot number than shoot tips at low BAP and IBA concentrations. The shoot length decreased at the concentrations of 8.8 µmol BAP and 1.0 µmol IBA for both explant types. The percentage of hyperhydricity increased with increasing the concentration of the growth regulators. Stem segments resulted in higher hyperhydricity than shoot tip explants at the same concentration of the growth regulators.

The above results show that higher concentrations of plant growth regulators resulted in a higher multiplication rate, but this was often accompanied by increased hyperhydricity. This means that the apple cultures in liquid media are more sensitive to plant growth regulators compared with those grown on solid medium where 4.4 µmol BAP and 0.5 µmol IBA are routinely used during the whole shoot culture period without hyperhydricity. The reason for the higher sensitivity of the cultures to the growth regulators is possibly due to an easy access of the cultures to growth regulators in liquid media. The experiment has also shown that stem segments showed

more hyperhydricity than shoot tips at the same concentration of the growth regulators. This might be because lateral buds are already differentiated in stem segments when they are cultured in the medium and there is no need for a high concentration of growth regulators. On the other hand, higher concentrations of growth regulators are required for meristems from shoot tips to induce new buds. It is well known that higher concentrations of cytokinins are necessary for the differentiation of new shoots, but that shoot elongation is often inhibited by a high concentration of cytokinins.

Table 2 shows that the rooting percentage was generally high, ranging from 91 to 100 %. Among the four different treatments, the lowest rooting percentage was obtained from the medium containing the highest BAP and IBA concentrations in the shoot multiplication medium. The root number and root length were also lower when the previous medium contained higher concentrations of BAP and IBA. Addition of kinetin in combination with IBA in the shoot elongation phase had no negative effects on rooting percentage and root number. These results suggest that high concentrations of cytokinins during the shoot multiplication phase inhibit rooting, which is also common in micropropagation of plants. The survival of the rooted plantlets in the greenhouse was 100% (data not shown).

3.2 Experiment 2 and 3

Based on experiment 1, experiments 2 and 3 were carried out to further optimise growth conditions and the results are presented in table 3. The highest kinetin concentration resulted in the highest shoot number and shoot length among the three kinetin concentrations, but it also caused the highest percentage of hyperhydricity. The increase of BAP concentration from 1.1 to 2.2 μmol did not give a better multiplication rate, but resulted in a higher percentage of hyperhydricity. These results further confirm that the high percentage of hyperhydricity is closely related to high BAP or kinetin concentrations in culture medium. In order to obtain more shoots with better quality from the RITA system, the concentration of cytokinin needs to be below a threshold level. The results also revealed that, at a similar concentration, BAP and kinetin gave a similar result for shoot production and shoot quality. Based on this, only BAP was used in the later experiments.

Table 1: Results of shoot multiplication and elongation of the apple rootstock M26 grown in RITA containers from experiment 1. Explant size was 0.5 cm for tips and 0.6-0.8 cm for stem segments, and no medium exchange during the shoot elongation period for all treatments. The shoot cultures were in the multiplication media for 15 days, and then transferred to the shoot elongation media

Exp.	Explant type	Treatment		Shoot No.	Shoot length (mm)	H (%)
		Multiplication	Elongation			
		BAP/IBA (µmol)	Kinetin/IBA (µmol)			
1	Shoot tip	4.4/0.5	0.5/0.05	2.5 a	13.4 b	0
	Stem seg.	4.4/0.5	0.5/0.05	3.5 b	13.3 b	33
	Shoot tip	8.8/1.0	0/0	3.2 ab	9.7 a	25
	Stem seg.	8.8/1.0	0/0	3.8 bc	9.6 a	50
	Shoot tip	1.1/1.2	0/0	4.3 c	13.3 b	50

Figures followed by different letters in each column differ significantly at P=0.05 (n=40). Exp.=experiment. seg.=segment. H.=Hyperhydricity.

Table 2: Rooting results of the apple rootstock M26 grown in the RITA containers where the shoots were derived from experiment 1. The shoots were rooted in the rooting medium consisting of Lepoivre macro- and micro nutrients, Walkey vitamins and 1.2 µmol IBA for 4 days in the dark, and then in the identical IBA-free rooting medium in light for 3 weeks

Explant type	Treatment prior to rooting		Root No. per shoot	Root length (cm)	Rooting %
	Multiplication	Elongation			
	BAP/IBA (µmol)	Kinetin/IBA (µmol)			
Shoot tip	4.4/0.5	0.5/0.05	6,48 b	2,5 b	96
Stem seg.	4.4/0.5	0.5/0.05	6,58 b	2,0 b	100
Shoot tip	8.8/1.0	0/0	4,95 a	1,2 a	100
Shoot tip	1.1/1.2	0/0	4,65 a	1,5 a	91

Figures followed by different letters in each column differ significantly at P=0.05 (n=20). seg.=segment.

Table 3: Results of shoot production and shoot quality of the apple rootstock M26 grown in the RITA containers from experiments 2-5. The cultures were grown in the multiplication medium (basal MS plus 4.4 µmol BAP and 0.5 µmol IBA) for 10 days, and then in the shoot elongation media for 4 weeks as shown in the table. The frequency of medium immersion was 8 times per day for experiments 2-3 and 16 times per day for experiments 4-5. ANOVA analysis with Duncan's multiple range test was carried out within one experiment

Exp.	Treatment					Shoot No. per explant	Shoot length (mm)	H. (%)
	Kinetin/ BAP (µmol)		IBA (µmol)	Times of exchanging medium [a]	Explant size (cm)			
	K	0.5	0.05	0	0.5	2.2 a	11.3 a	5
2	K	2.3	0.25	0	0.5	3.3 b	18.8 b	43
	BAP	2.2	0.25	0	0.5	2.8 ab	16.5 b	50
	K	0.5	0.05	0	0.5	1.6 a	16.5 a	5
3	K	1.2	0.25	0	0.5	1.8 a	20.1 a	6
	BAP	1.1	0.25	0	0.5	2.1 a	17.1 a	0
	BAP	1.1	0.25	0	0.5	6.4 a	23.4 a	20
4	BAP	1.1	0.25	1	0.5	8.6 b	25.0 ab	25
	BAP	1.1	0.25	2	0.5	9.0 b	28.4 b	44
	BAP	1.1	0.25	0	0.5	7.2 b	25.9 a	0
5	BAP	1.1	0.25	0	1.0	8.1 b	25.8 a	0
	BAP	1.1	0.25	0	1.5	5.2 a	23.8 a	0

Figures followed by different letters in each column differ significantly at P=0.05 (n=40). Exp.=experiment. K=kinetin. H.=Hyperhydricity. [a] 0=no medium exchange; 1=one time of medium exchange with the interval of 15 days; 2=two times of medium exchange with the interval of ten days during one month of subculture.

3.3 Experiment 4

Experiment 4 was conducted to study the influence of medium exchange on shoot production and quality during the shoot elongation phase. The results are presented in table 3. Exchanging the medium once or twice during the elongation phase increased the shoot number significantly, and the shoot length non-significantly. However, this was accompanied by an increase in hyperhydricity when the medium was exchanged twice. This is clearly associated with the persistent presence of cytokinin in the medium due to the more frequent exchange of the fresh media. Therefore, we can conclude that exchanging the medium during the shoot elongation period has no obvious advantages for higher quality shoot production and from the economic point

of view. As shown in table 3, shoot number and shoot length were higher in this experiment than those in experiments 2 and 3. This is possibly due to the increased frequency of tissue immersion to the culture medium since the tissue was immersed 16 times per day in experiment 4, while only 8 times in experiments 2-3. The multiplication rate in this experiment was much higher than that on agar medium where 1-3 shoots can usually be produced from normal shoot cultures in our case.

3.4 Experiment 5

Experiment 5 was carried out to study the influence of explant size on shoot production and quality. Table 3 shows that the shoot number was not significantly affected when the explant size was within 1 cm. However, when the explant size was 1.5 cm, shoot number was significantly reduced. Hyperhydricity was not affected by the explant size. The decreased shoot number with larger explant size was likely due to poor transport of cytokinin from the cut surface to buds and tips.

4. Conclusion

In conclusion, the micropropagation of the apple rootstock M26 in the RITA system included two steps, i.e. shoot multiplication and shoot elongation. The optimised conditions include explant size of 0.5-1.0 cm, 4.4 μmol BAP and 0.5 μmol IBA in the shoot multiplication phase, 1.1 μmol BAP and 0.25 μmol IBA in the shoot elongation phase, no exchange of medium during the shoot elongation period and tissue immersion 16 times per day. No obvious differences in shoot production and shoot quality between BAP and kinetin when they were used at similar concentrations during shoot elongation phase. The multiplication rate in the RITA system was higher than than on agar media. The shoots produced from the RITA system could root normally and the rooted plantlets could easily acclimatise under greenhouse conditions.

Acknowledgements

We wish to thank Swedish Foundation for Innovation Centre (Stiftelsen för Innovationscentrum) for the financial support to this research.

References

Aitken-Christie J & Jones C (1987) Towards automation: radiata pine shoot hedges *in vitro*. Plant Cell Tiss. Org. Cult. 8: 185-196

Cabasson C, Alvard D, Dambier D, Ollitrault P & Teisson C (1997) Improvement of Citrus somatic embryo development by temporary immersion. Plant Cell, Tiss. Org. Cult. 50: 33-37

Chu I (1995) Economic analysis of automated micropropagation. In: Aitken-Christie J, Kozai T & Smith MAL (eds) Automation and Environmental Control in Plant Tissue Culture (pp.19-26). Kluwer Academic Publishers, Dordrecht

Escalona M, Lorenzo JC, González B, Daquinta M, González JL, Desjardins Y & Borroto CG (1999) Pineapple (*Ananas comosus* L. Merr) micropropagation in temporary immersion systems. Plant Cell Reports 18: 743-748

Jiménez E, Pérez N, Feria M de, Barbón R, Capote A, Chávez M, Quiala E & Pérez JC (1999) Improved production of potato microtubers using a temporary immersion system. Plant Cell, Tiss. Org. Cult. 59: 19-23

Lorenzo JC de los Angeles Blanco M, Peláez O, González A, Cid M, Iglesias A, González B, Escalona M, Espinosa P & Borroto C (2001) Sugarcane micropropagation and penolic excretion. Plant Cell, Tiss. Org. Cult. 65: 1-8

Maene L & Debergh P (1985) Liquid medium additions to established tissue cultures to improve elongation and rooting *in vitro*. Plant Cell, Tiss. Org. Cult. 5: 23-33

Murashige F & Skoog F (1962) A revised medium for rapid growth and bioassys with tobacco tissue cultures. Physiol. Plant. 15: 473-492

Quoirin M, Lepoivre P & Boxus P (1977) Un premier bilan de dix années de recherche sur les cultures de méristèmes et la multiplication *in vitro* de fruitiers ligneux (in French). Compte rendu des recherches, Station des Cultures Fruitières et Marrîchères de Gembloux 1976-1977, 93-117

Simonton W, Robacker C & Krueger S (1991) A programmable micropropagation apparatus using cycled medium. Plant Cell, Tiss. Org. Cult. 27: 211-218

Sluis CJ & Walker KA (1985) Commercialization of plant tissue culture propagation. International Association for Plant Tissue Culture. Newsletters 47: 2-12

Teisson C & Alvard D (1995) A new concept of plant *in vitro* cultivation liquid medium: temporary immersion. In: Terzi M et al. (eds) Current Issues in Plant Molecular and Cellular Biology (pp. 105-110). Kluwer Academic Publishers, Dordrecht

Teisson C, Alvard D, Lartaud M, Etienne H, Berthouly M, Escalona M & Lorenzo JC (1999) Temporary immersion for plant tissue culture. In: Plant Biotechnology and *In Vitro* Biology in the 21st Century, Proceedings of the IXth International Congress of Plant Tissue and Cell Culture, Section H: Novel micropropagation methods (pp. 629-632). Jerusalem

Tisserat B & Vandercook CE (1985) Development of an automated plant culture system. Plant Cell, Tiss. Org. Cult. 5: 107-117

Walkey DG (1972) Production of apple plantlets from axillary bud meristems. Canadian Journal of Plant Science 52: 1085-1087