Plant Cell, Tissue and Organ Culture **33**: 211–217, 1993.

A simple protocol for micropropagating diploid and tetraploid watermelon using shoot-tip explants

Michael E. Compton, D.J. Gray & Gary W. Elmstrom
University of Florida, Institute of Food and Agricultural Sciences, Central Florida Research and Education Center, 5336 University Ave., Leesburg, Florida 34748

Received 23 July 1992; accepted in revised form 17 December 1992

Key words: Citrullus, seedless watermelon, triploid watermelon, tissue culture

Abstract

Shoot-tip explants from 21-day-old aseptically-germinated watermelon seedlings were incubated on solidified MS medium containing test concentrations of benzyladenine (BA) and kinetin (each at 0, 1, 5 or 10 μM), and thidiazuron (TDZ; 0, 0.1, 1 or 5 μM) for 8 weeks. Approximately 1.5x–2.8x more axillary shoots formed at the optimum BA level (1 μM) compared to the best TDZ (0.1 μM) or kinetin (10 μM) concentration. The ability of various diploid and tetraploid genotypes to undergo prolonged axillary shoot proliferation on medium with 1 μM BA was examined. Among the genotypes tested, the number of axillary shoots per explant was greater for 'Bush Jubilee' and 'Jubilee II' than for 'Minilee', 'Dixielee', and the tetraploid genotypes. For a majority of the genotypes tested, the number of shoots per explant was low (2.7–4.0) during the first month of culture, peaked (5.3–12.5) at 2 to 3 months, and then declined (3.7–7.7) at 6 months. In contrast, the number of shoots per explant was greatest (11.7) for 'Bush Jubilee' during the first month of culture and declined to 7.7 by the sixth subculture. The percentage of rooted shoots varied from 60% to 100% and the percentage of acclimatized plants ranged from 21% to 96% depending on the genotype and the length of time in culture. Using this procedure, 13,200 finished plants could be produced in 3 months from 250 seedlings.

Abbreviations: PGR – plant growth regulator, BA – benzyladenine, TDZ – thidiazuron, IBA – 1H-indole-3-butyric acid

Introduction

Seedless watermelons are the most desirable watermelon cultivars presently available to consumers and command a high price. A consumer survey comparing 'King of Hearts' (seedless) to 'Mirage' (seeded) revealed that consumers rated seedless melons of equal or greater quality than seeded watermelon and were willing to pay 50% more per lb. for seedless watermelons (Marr & Gast 1991). However, seedless watermelon production has been hampered by high seed cost ($150–$200/1000 seed; Marr & Gast 1991) and poor seed germination. High seed cost has generally been attributed to difficulties in obtaining a sufficient number of tetraploid individuals and the low number of seeds in tetraploid fruit. Seedless watermelons are triploid (3n = 3x = 33) and result from crossing a tetraploid (4n = 4x = 44) seed parent with a diploid (2n = 2x = 22) pollen parent (Andrus et al. 1971; Kihara 1951). New tetraploid plants possess low fertility and

This process is protected by United States patent No. 5,007,198. Those interested in using this technology must secure a license from the University of Florida.

may require 10 or more generations of self-pollination to raise fertility to an acceptable level. Gray & Elmstrom (1991) by-passed the obstacle of low fertility by developing and patenting a breeding system that features micropropagation as a means of increasing the number of tetraploid individuals through axillary shoot proliferation. The tetraploid regenerants would then be used for triploid seed production. Among other advantages, this breeding system could reduce the amount of time required for triploid seed production from the current 10 years to approximately 1 to 2 years and allow male-sterile tetraploids to be used as parents.

Previously published watermelon micropropagation protocols have utilized explants from immature zygotic embryo axes (Adelberg & Rhodes 1989), 1–3 mm seedling shoot tips (Barnes et al. 1978; Barnes, 1979) and apical meristems (Anghel & Rosu 1985; Gray & Elmstrom 1991). However, the procedures employed high levels (>5 μM) of BA or kinetin, and numerous transfers to different media that resulted in low numbers of shoots per explant, low rooting and poor plant acclimatization. Our goal was to develop a simple, commercially feasible protocol to micropropagate tetraploid watermelon lines for use in triploid seed production.

Materials and methods

Explant preparation and culture conditions

Watermelon [*Citrullus lanatus* (Thunb.) Matsum. & Nakai] seeds were surface-disinfested for 30 min in 2.5% NaOCl plus one drop (100 ml^{-1}) Triton X-100, rinsed five times with sterile-distilled water and soaked overnight in distilled water in the dark. The seed coat was removed and the embryos surface-disinfested for 10 min (20 min for 'Dixielee' and 'Jubilee II') in 0.75% NaOCl plus one drop (100 ml^{-1}) Triton X-100 prior to six sterile-distilled water rinses. Nine embryos were germinated in each Magenta GA_7 vessel (Magenta Corp., Chicago) containing 50 ml of medium [MS salts (Murashige & Skoog 1962), 20 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol, 2 mg l^{-1} glycine, 0.5 mg l^{-1} of both pyridoxine HCl and nicotinic acid, 0.1 mg l^{-1} thiamine HCl and 1 μM BA (Sigma Chemical Co., St. Louis, Mo.)]. The pH of all media was adjusted to 5.7 prior to the addition of 7 g l^{-1} TC agar (JRH Biosciences, Lenexa, KS) and autoclaving at 121°C and 98 kPa for 15 min. All cultures were maintained under a 16-h photoperiod (30–50 μmol m^{-2} s^{-1} from cool-white fluorescent lamps) at 25°C.

Shoot tips (1 cm) from 21-day-old seedlings with at least one axillary node were inserted vertically into MS medium as above but with 30 g l^{-1} sucrose and test concentrations of PGR's. Culture vessels were Magenta GA_7 vessels that contained 50 ml of medium. Similar explants were subcultured to fresh medium of the same composition every 4 weeks.

Effect of cytokinin type and concentration

To identify the best cytokinin for axillary shoot proliferation, MS medium (as above) was supplemented with BA, kinetin (Sigma Chemical Co., St. Louis, Mo.) or TDZ (Nor-Am Chemical Co., Wilmington, DE) each at four concentrations (0, 1, 5 or 10 μM for BA and kinetin, and 0, 0.1, 1, or 5 μM for TDZ). The diploid cultivar Minilee was used for medium optimization experiments. There were four vessels per treatment with five explants each. The number of axillary shoots per explant and shoot length were recorded monthly for 2 months.

Performance of diploid and tetraploid genotypes on medium with 1 μM BA

Shoot-tip cultures of 'Bush Jubilee' (2n), 'Dixielee' (2n), 'Jubilee II' (2n), 'Minilee' (2n), SP90-1 (4n), SP90-2 (4n) and SP90-4 (4n) were initiated as above and subcultured to fresh medium with 1 μM BA every 4 weeks. The number of axillary shoots per explant was recorded 1, 2, 3 and 6 months after culture initiation. There were 10 vessels per treatment with five explants each.

The ability of shoot tips to form roots was evaluated at 1, 3 and 6 months. Shoots (5–30 mm) were excised from the proliferating mass and all of the fully expanded leaves removed before transfer to MS medium as above but with 20 g l^{-1} sucrose and 5 μM IBA (Sigma Chemical

Co., St. Louis, Mo). After 2 weeks, plantlets were transplanted to plug trays (3.3 × 5.1 cm; 72 plugs/tray) filled with autoclaved medium (1 ProMix: 1 coarse vermiculite), covered with a clear plastic lid, and incubated in a 16-h photoperiod (30–50 μmol m^{-2} s^{-1}) at 24° ± 3°C. Seven days later, the plants were acclimatized to ambient humidity levels by gradually removing the lid over a 2-day period. Acclimatized plants were moved to the greenhouse 21 days after transfer to soil. The number of plants acclimatized to the ambient environment was recorded after 1 week in the greenhouse.

Statistical analysis on the number of axillary shoots produced per explant was conducted using the GLM procedure of the Statistical Analysis System and percentage data were analyzed using the catmod procedure for categorical data (SAS Institute, Inc. 1988). Mean comparisons were made using the standard error of the mean.

Results and discussion

Effect of cytokinin type and concentration

Axillary shoot proliferation of 'Minilee' shoot tips was dramatically influenced by the cytokinin type and concentration in the medium. The number of axillary shoots per explant was greatest for BA compared to TDZ or kinetin (Table 1). Approximately 1.5x–2.8x more shoots were formed at the optimum BA level (1 μM) compared to the best TDZ (0.1 μM) or kinetin (10 μM) concentration. Shoot tips incubated on medium without PGRs produced few axillary shoots.

Shoot length was also influenced by cytokinin. Shoots elongated the most (54.5 mm) when incubated in medium without cytokinin (Table 1). However, among the cytokinin treatments, shoot elongation was greatest (>31.3 mm) for explants incubated in medium supplemented with kinetin. Shoots obtained from explants incubated in medium with 1 or 5 μM BA were significantly shorter (6.7–6.9 mm, respectively) but were of sufficient size for subculture and rooting. Shoots derived from explants incubated in medium with more than 0.1 μM TDZ were stunted (≤4.3 mm) making them difficult to subculture and root. Hyperhydricity was common among shoot-tip cultures that were maintained in medium containing greater than 0.1 μM TDZ.

Table 1. Effect of cytokinin type and concentration on the number of 'Minilee' shoots per explant and shoot length.

PGR	Conc. (μM)	Number of shoots per explant[a]	Shoot length[b] (mm)
None		1.0 ± 0.1	54.5 ± 4.6
BA	1	6.2 ± 0.5	6.9 ± 0.3
	5	5.2 ± 0.3	6.7 ± 0.2
	10	4.4 ± 0.2	5.8 ± 0.2
Thidiazuron	0.1	4.2 ± 0.3	5.7 ± 0.2
	1	2.4 ± 0.3	4.3 ± 0.3
	5	2.1 ± 0.3	3.9 ± 0.3
Kinetin	1	1.1 ± 0.1	37.5 ± 3.6
	5	1.4 ± 0.1	44.2 ± 4.3
	10	2.2 ± 0.2	31.3 ± 2.6

[a]The number of explants per treatment ranged from 30 to 40.
[b]The number of shoots examined ranged from 37 to 187.
±values represent the standard error of the mean.

Our results differ from previous reports for watermelon that employed high levels of BA (10 μM) either alone (Adelberg & Rhodes 1989) or combined with 0.5–1 μM of either IAA or NAA (Anghel & Rosu 1985). We observed that high levels (>1 μM) of BA reduced both the number of axillary shoots per explant and shoot length, and increased the occurrence of hyperhydricity. Pasqualetto et al. (1986) observed that the occurrence of hyperhydric apple (*Malus domestica* 'Gala') was increased when the BA concentration in the medium was raised from 2.2 μM to 4.4 μM. Others have stated that kinetin was the best cytokinin for micropropagating triploid watermelon (Barnes 1979; Barnes et al. 1978). Our results with diploid watermelon shoot tips demonstrated that shoot elongation was promoted by kinetin, but at the expense of axillary shoot proliferation.

Performance of diploid and tetraploid genotypes on medium with 1 μM BA

The ability of various diploid and tetraploid genotypes to form axillary shoots was monitored over a period of 6 months. A significant interaction was observed between the genotype and the length of time (months) in culture. Among

the genotypes tested, the number of shoots per explant per month was greatest for 'Bush Jubilee' and 'Jubilee II' compared to 'Minilee', 'Dixielee', and the tetraploid genotypes (Table 2). The number of axillary shoots per explant was generally low during the first month of culture but nearly doubled during the two subsequent subcultures. In contrast, the number of 'Bush Jubilee' shoots per explant averaged 11.7 during the first month of culture and declined to 7.7 at 6 months. A 6-month-old 'Bush Jubilee' culture is shown in Fig. 1A. All the genotypes tested, with the exception of 'Jubilee II', exhibited a decrease in the number of shoots per explant between the third and sixth subculture. Shoot length for 'Jubilee II' ranged from approximately 5 mm to 26 mm (Fig. 1B). Similar results were obtained for the remaining genotypes except for 'Dixielee' and 'Minilee', which experienced a reduction in shoot length by the sixth subculture (data not shown).

The percentage of shoots that rooted ranged from 60% to 100% depending on the genotype and the amount of time in culture (Table 2). Shoots of SP90-1 and SP90-2 rooted best during the first month of culture; less rooting occurred for subsequent subcultures. Shoots of 'Bush Jubilee', SP90-4 and 'Minilee' rooted best after 6

Table 2. Performance of diploid and tetraploid shoot tips maintained on MS medium with 1 μM BA for 6 months.

Genotype	Number of shoots per explant[a]	Rooted plants[b] (%)	Plants to soil[c] (%)
	1 month		
Bush Jubilee	11.7 ± 1.0	–[d]	–
Minilee	4.0 ± 0.2	86.1 ± 5.8	67.7 ± 8.4
Jubilee II	3.4 ± 0.2	60.0 ± 21.9	66.7 ± 27.2
Dixielee	3.4 ± 0.3	66.7 ± 12.2	90.0 ± 9.5
SP90-2	3.0 ± 0.3	95.0 ± 4.9	77.8 ± 9.8
SP90-4	2.9 ± 0.4	–	–
SP90-1	2.7 ± 0.2	100	72.7 ± 9.8
	2 months[e]		
Bush Jubilee	9.5 ± 0.6	–	–
Minilee	7.6 ± 0.4	–	–
Jubilee II	12.5 ± 0.7	–	–
Dixielee	6.4 ± 0.4	–	–
SP90-2	6.9 ± 0.5	–	–
SP90-4	6.5 ± 1.1	–	–
SP90-1	6.0 ± 0.4	–	–
	3 months		
Bush Jubilee	9.2 ± 0.6	66.7 ± 9.1	72.2 ± 10.6
Minilee	6.3 ± 0.4	62.5 ± 0.8	65.0 ± 10.7
Jubilee II	8.3 ± 0.5	88.9 ± 7.4	93.8 ± 6.1
Dixielee	6.6 ± 0.4	76.7 ± 7.7	75.0 ± 9.7
SP90-2	6.3 ± 0.5	75.9 ± 5.8	63.2 ± 7.8
SP90-4	5.3 ± 0.4	74.1 ± 8.4	62.9 ± 8.2
SP90-1	5.3 ± 0.3	74.1 ± 5.9	65.0 ± 10.7
	6 months		
Bush Jubilee	7.7 ± 0.5	92.6 ± 3.6	96.0 ± 2.8
Minilee	2.4 ± 0.2	96.3 ± 3.6	21.7 ± 8.6
Jubilee II	7.7 ± 0.5	75.0 ± 5.1	85.1 ± 5.2
Dixielee	4.3 ± 0.3	86.8 ± 4.7	36.3 ± 7.3
SP90-2	4.1 ± 0.2	66.7 ± 6.4	70.6 ± 7.8
SP90-4	3.7 ± 0.2	90.3 ± 5.3	69.2 ± 9.1
SP90-1	NA[f]	–	–

[a]The number of explants per treatment ranged from 15 to 50.
[b]The number of shoots placed on rooting medium ranged from 5 to 72.
[c]The number of plantlets ranged from 3 to 50.
[d]There were insufficient numbers of shoots for rooting.
[e]Rooting was not evaluated at the second subculture.
[f]All cultures became contaminated.
±values represent the standard error of the mean.

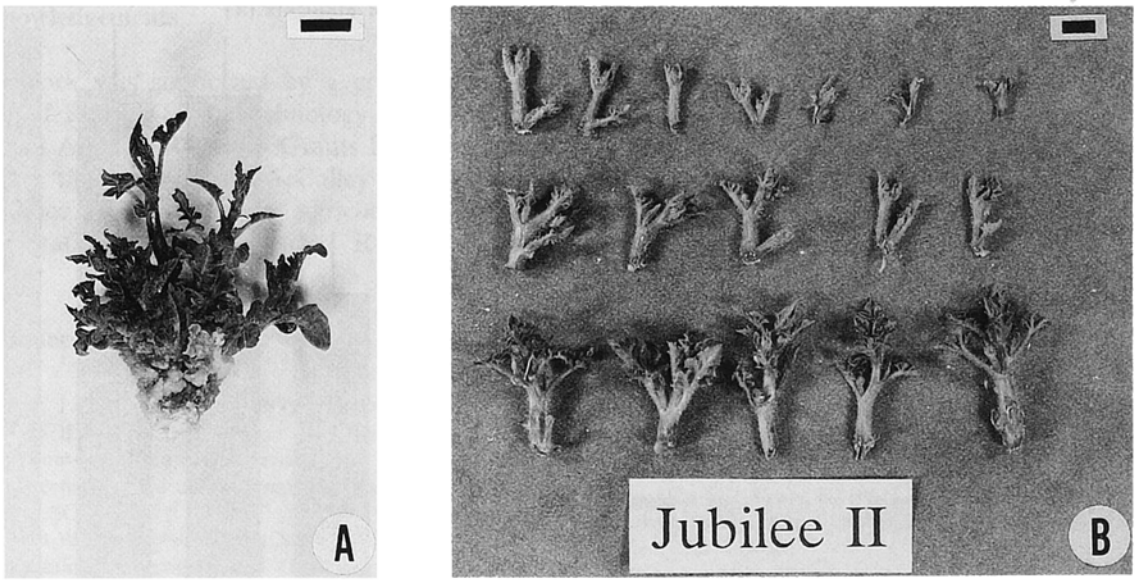

Fig. 1. (*A*) 'Bush Jubilee' shoot-tip culture after 6 months of recurrent subculture on medium with 1 μM BA. (*B*) Axillary shoots obtained from a 2-month-old 'Jubilee II' shoot-tip culture; Bar = 5 mm.

months. The trend in rooting was more complicated for 'Dixielee'. Shoots rooted better at 6 months than at 1 month after culture initiation; however, there was no difference between the first and third, and third and sixth subculture. Culture duration did not affect the ability of 'Jubilee II' shoots to root. Rooted diploid and tetraploid shoots are shown in Fig. 2A.

Plantlets were acclimatized in plastic flats filled with soilless medium. The genotype and the length of time in culture influenced the ability of some genotypes to become acclimatized. The percentage of 'Bush Jubilee' plants that survived in the greenhouse rose 23.8% between the third and sixth subculture, whereas the percentage of 'Minilee' and 'Dixielee' plants that acclimatized after the sixth subculture declined (Table 2). The percentage of 'Jubilee II', SP90-1, SP90-2 and SP90-4 plantlets acclimatized to the greenhouse was not affected by recurrent subculture. Acclimatized plants had well developed root systems 2 weeks after transfer to the greenhouse (Fig. 2B).

Poor acclimatization rates for 'Dixielee' and 'Minilee' may have been related to the size of the plantlets at the time of acclimatization. Many shoots from 6-month-old cultures failed to elongate on rooting medium and were less than 10 mm in length when transferred to soil. We have observed that a low percentage (≤56%) of plantlets shorter than 15 mm survived the transition from culture to the ambient environment. On the other hand, approximately 90%–100% of the plantlets greater than 15 mm survived acclimatization (Fig. 3). This occurred regardless of genotype or ploidy level, and indicates that plant height at the time of acclimatization is extremely important.

The commercial production of triploid seed requires approximately 60 kg of seed annually for each triploid cultivar to meet the current market needs. Approximately 220 tetraploid plants are needed to produce 1 kg of triploid seed, hence about 13,200 tetraploid plants are required to meet current demands for triploid seed. Based on our tetraploid shoot proliferation rates (2.8, 6.5 and 5.8 for the first, second and third subculture, respectively), rooting (76%) and acclimatization (66%) percentages (Table 2), it would cost about $0.26 per acclimatized plant (13,237 plants) to micropropagate tetraploid watermelon lines for triploid seed production. This was based on the calculations by Anderson et al. (1977) that were modified using current cost for labor, laboratory and equipment, and rental of greenhouse space. The price

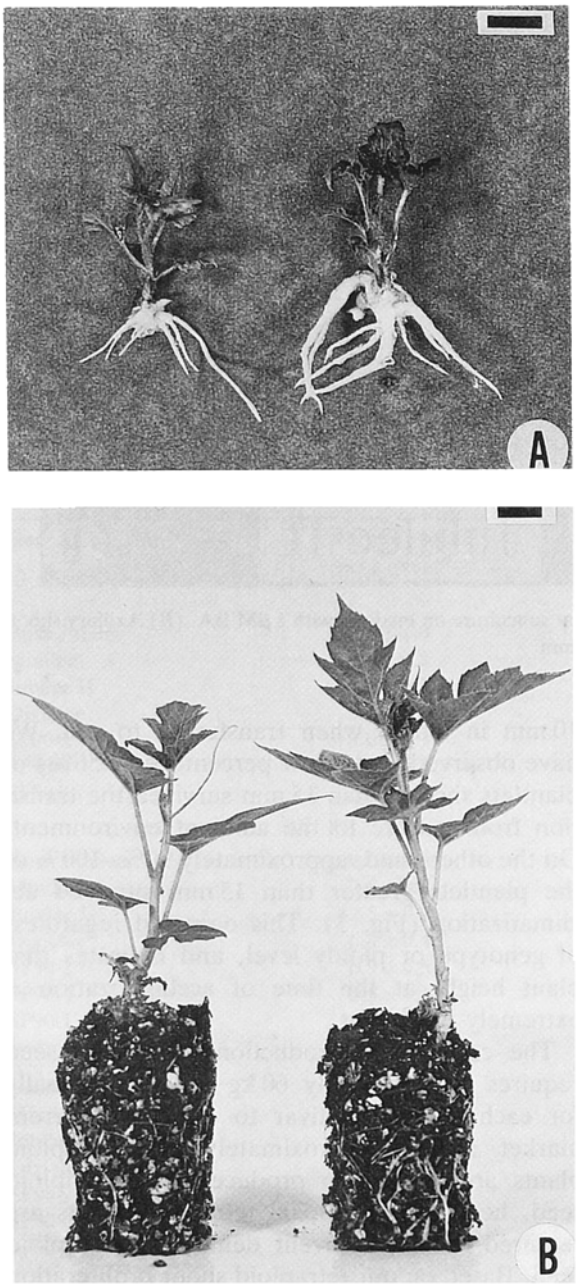

Fig. 2. (*A*) 'Jubilee II' (left) and SP90-2 (right) plantlets 2 weeks after transfer to root induction medium. (*B*) 'Bush Jubilee' (left) and SP90-2 (right) plants 2 weeks after transfer to the greenhouse; bar = 1 cm.

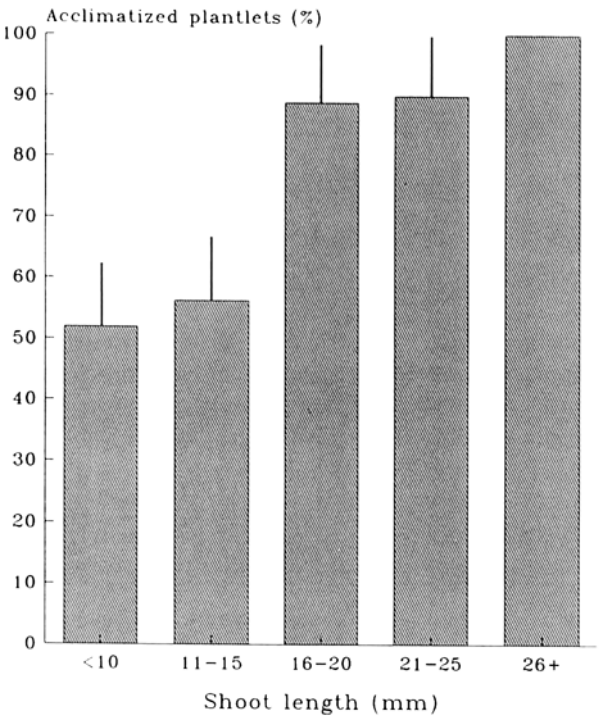

Fig. 3. Effect of shoot length on the ability of micropropagated plantlets to survive acclimatization to ambient conditions. Data for 'Bush Jubilee', 'Jubilee II', Minilee' and SP90-2 were combined as a result of a nonsignificant genotype x shoot length interaction. The number of plantlets per group (shoot length) ranged from 5 to 24. Vertical bars represent the standard error of the mean.

per finished plant could be reduced 16% if the rooting and acclimatization percentages were increased to 90% and 80%, respectively, and could be reduced further if rooting and acclimatization were accomplished simultaneously *in vivo*.

These results demonstrate that shoot tips of diploid and tetraploid watermelon genotypes can be repeatedly subcultured for up to 6 months without a significant decrease in the rooting percentage or the percentage of acclimatized plants. We have obtained similar results using shoot tips (1 cm) and single node explants from greenhouse-grown plants as well as small (≤3 mm) shoot tips from plants grown in the field (Compton & Gray 1992; Gray & Elmstrom 1991).

Plants obtained from this procedure were normal and true-to-type. Plants grown in the field produced normal male and female flowers that developed into normal fruit. Fruit yield, fruit quality, and number of seeds per plant were equivalent to plants grown from seed.

Acknowledgements

This work was supported by a grant from the State of Florida High Technology and Industry Council Applied Research Grants Program. The authors thank Mr. Dan McColley for technical assistance. This is Florida Agricultural Experiment Station Journal Series No. R-02551.

References

Adelberg J & Rhodes BB (1989) Micropropagation from zygotic tissues of watermelon. In: Thomas CE (Ed) Proceedings of the Cucurbitaceae 89: Evaluation and Enhancement of Cucurbit Germplasm (pp 110–112). Charleston, SC

Anderson WC, Meagher GW & Nelson AG (1977) Cost of propagating broccoli plants through tissue culture. HortScience 12: 543–544

Andrus CF, Seshadri VS & Grimball PC (1971) Production of seedless watermelons. Agricultural Research Service, United States Department of Agriculture Technical Bulletin No. 1425

Anghel I & Rosu A (1985) *In vitro* morphogenesis in diploid, triploid and tetraploid genotypes of watermelon – *Citrullus lanatus* (Thumb.) Mansf. Rev. Roum. Biol. – Biol. Végét. 30: 43–55

Barnes LR (1979) *In vitro* propagation of watermelon. Scientia Hort. 11: 223–227

Barnes LR, Cochran FD, Mott RL & Henderson WR (1978) Potential uses of micropropagation for cucurbits. Cucurbit Growers Coop. 1: 21–22

Compton ME & Gray DJ (1992) Micropropagation as a means of rapidly propagating triploid and tetraploid watermelon. Proc. Fla. State Hort. Soc. (in press)

Gray DJ & Elmstrom GW (1991) Process for the accelerated production of triploid seeds for seedless watermelon cultivars. United States Patent no. 5,007,198

Kihara H (1951) Triploid watermelons. Proc. Amer. Soc. Hort. Sci. 58: 217–230

Marr CW & Gast KLB (1991) Reactions by consumers in a 'farmers' market to prices for seedless watermelon and ratings of eating quality. HortTechnology 1: 105–106

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Pasqualetto P-L, Zimmerman RH & Fordham I (1986) Gelling agent and growth regulator effects on shoot vitrification of 'Gala' apple *in vitro*. J. Amer. Soc. Hort. Sci. 111: 976–980

SAS Institute, Inc. SAS/STAT user's guide (1988) Release 6.03. SAS Institute, Inc., Cary, N.C.