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A HIGHLY EFFICIENT REGENERATION SYSTEM FOR WATERMELON (*CITRULLUS LANATUS* THUNB.)

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Abstract

We developed a high frequency watermelon regeneration system using two inbred lines of watermelon (*Citrullus lanatus*), 'W1-4' and 'W1-12'. Shoots were induced from cotyledonary nodes cultured on Murashige and Skoog (MS) basal medium solidified with agar (7.0 g/L) and containing various concentrations of cytokinin (6-benzyladenine; 6-BA) and auxin (indoleacetic acid; IAA). The highest rate of bud organogenesis was on MS medium containing 1.5 mg/L 6-BA + 0.2 mg/L IAA for 'W1-4' and on MS medium containing 1.0 mg/L 6-BA + 0.1 mg/L IAA for 'W1-12'. The regeneration rate was higher in 'W1-12' than in 'W1-4'. The best medium for shoot elongation in both inbred lines was MS containing 0.05 mg/L 6-BA. Regenerated plants showed the best rates of root formation on 1/2 MS containing 0.1 mg/L IAA. The rooted plants were carefully washed to remove all medium from the roots, and then transferred to soil in a greenhouse. The plants showed a 100% survival rate when transferred to soil. This highly efficient regeneration system will be useful for regenerating plants in genetic engineering applications, and is a useful tool for further genetic transformation studies on watermelon.

Introduction

Watermelon [*Citrullus lanatus* (Thunb.) Mantsum. & Nakai] is an important horticultural crop and has been cultivated for more than 4000 years (Huang *et al*., 2006; Mustafa *et al*., 2010). *Citrullus* species originate from tropical Africa (Decoteau, 2000). Watermelon is a significant component of fresh fruit traded internationally, and it is popular with consumers because of its sweet taste and pleasant aroma (Marr & Gast, 1991; Jaskani *et al*., 2005). China produces the majority of the global watermelon crop. At present, there are several soil-borne pathogens that affect watermelon production, resulting in fruit losses of millions of metric tons (Martyn & Miller, 1996). Several methods have been used to address the problem of soil-borne pathogens. However, traditional methods have not solved this problem effectively, and have led to other problems such as environmental pollution and destruction of soil microorganisms.

With developments in biotechnology, the problem of soil-borne pathogens may be addressed using transgenic technology. Genetic engineering is an efficient and rapid method to introduce desirable agronomic characteristics, and it has already been used to produce transgenic watermelons. For example, *Agrobacterium*-mediated transformation has been used to improve fruit quality and to introduce resistance to disease and some environmental stresses (Ellul *et al*., 2003; Sang Mi Park *et al*., 2005; Akashi *et al*., 2005). However, for watermelon, *Agrobacterium* mediated genetic transformation is restricted to a few varieties because of a lack of efficient and reliable methods for *In vitro* plant regeneration (Gui *et al*., 2000). Genotype is a very important factor in the regeneration ability of watermelon; therefore, the rate of regeneration can differ markedly among different cultivars.

Consequently, an efficient plant regeneration system is necessary for transformation and propagation of watermelon. The aim of this study was to establish a high frequency, reliable, tissue culture-based regeneration system for watermelon, for applications in biotechnology and genetic engineering.

Materials and Methods

Plant materials and media: Seeds of *C. lanatus* 'W1-4' and 'W1-12' were provided by the Watermelon and Melon Genetic Breeding Laboratory of Horticulture College of Northeast Agricultural University, Harbin, China. In these experiments, the basal medium was MS (Murashige & Skoog, 1962; Asma *et al*., 2010) supplemented with 30 g/L sucrose, solidified with 7.0 g/L agar, and pH 5.8 adjusted with 1 mol/L NaOH or 1 mol/L HCl before autoclaving at 121°C for 14 min. Medium was dispensed into triangular jars (v=100 ml, 35 ml medium per bottle) for experiments.

Germination experiments: Mature seeds were soaked for 12 h in water before removing the seed coat and sterilizing the seed with sodium hypochlorite (5%) for 10 min. The seeds were rinsed four times with sterile distilled water and then dried on sterile filter paper. To determine the optimum conditions for germination of watermelon seeds, the seeds were sown aseptically on various concentrations of MS media (Murashige & Skoog, 1962): full-strength MS, 1/2 MS, 1/4 MS, 1/8 MS, with corresponding reductions in sugar content. Fifty seeds were sown on each medium, and each treatment consisted of three replicates. The cultures were kept in the dark at 26±2°C for 2 days until the epicotyl emerged and then kept under a 16-h light/8-h dark photoperiod (light intensity, 2500 lux). Appearance of the radicle from seeds indicated successful germination. Seeds that produced strong, green cotyledons were considered acceptable (Li *et al*., 2011).

Shoot induction: Cotyledon explants were derived from 5-day-old seedlings with newly formed cotyledons that were yellow to pale green, rather than dark green (Fig. 1A). Cotyledonary node explants (approx. 0.5 cm long) were excised along with some epicotyl tissue (Fig. 1B)

using sterile surgical blades. The explants were cultured on MS basal medium containing 30 g /L sucrose, 7.0 g /L agar, and various concentrations of 6-benzyladenine (6-BA) and indoleacetic acid (IAA) (Table 1). To determine whether the orientation of the tissue affected growth responses, the explants were placed with either the petiole end (A) or the blade end (B) embedded in the medium (Fig. 2). We used 30 explants per treatment, and each treatment was replicated three times. All cultures were incubated at 26±2°C under a 16-h light/8-h dark photoperiod with a light intensity of 2500 lx during the light period. The rate of regeneration of adventitious buds was determined after 4 weeks. The regeneration frequency was calculated as follows:

$$\frac{\text{Number of differentiated explants}}{\text{Total number of explants}} \times 100\%$$

The number of shoots per explant was calculated as follows:

$$\frac{\text{Total number of shoots}}{\text{Total number of explants}} \times 100\%$$

Table 1. Murashige and Skoog (1962) basal medium containing 16 combinations of 6-BA and IAA.

Medium code	Basic medium	Concentrations of phytohormones ($mg \cdot L^{-1}$)	
		6-BA	IAA
MS11	MS	0.5	0.0
MS12	MS	0.5	0.1
MS13	MS	0.5	0.2
MS14	MS	0.5	0.5
MS21	MS	1.0	0.0
MS22	MS	1.0	0.1
MS23	MS	1.0	0.2
MS24	MS	1.0	0.5
MS31	MS	1.5	0.0
MS32	MS	1.5	0.1
MS33	MS	1.5	0.2
MS34	MS	1.5	0.5
MS41	MS	2.0	0.0
MS42	MS	2.0	0.1
MS43	MS	2.0	0.2
MS44	MS	2.0	0.5

Fig. 1. Several steps of the high efficiency regeneration system of watermelon using cotyledonary nodes as explants
A, Five-day-old seedlings on propagation medium; B and C, cotyledonary node explants prepared for regeneration; D, regenerated shoots; E, elongated shoots after 1 week in elongation medium; F, rooted shoots after 1 week in rooting medium; G, rooted shoots after 3 weeks in rooting medium; H, regenerated plants ready for transfer to soil; I, regenerated plants after transfer to soil.

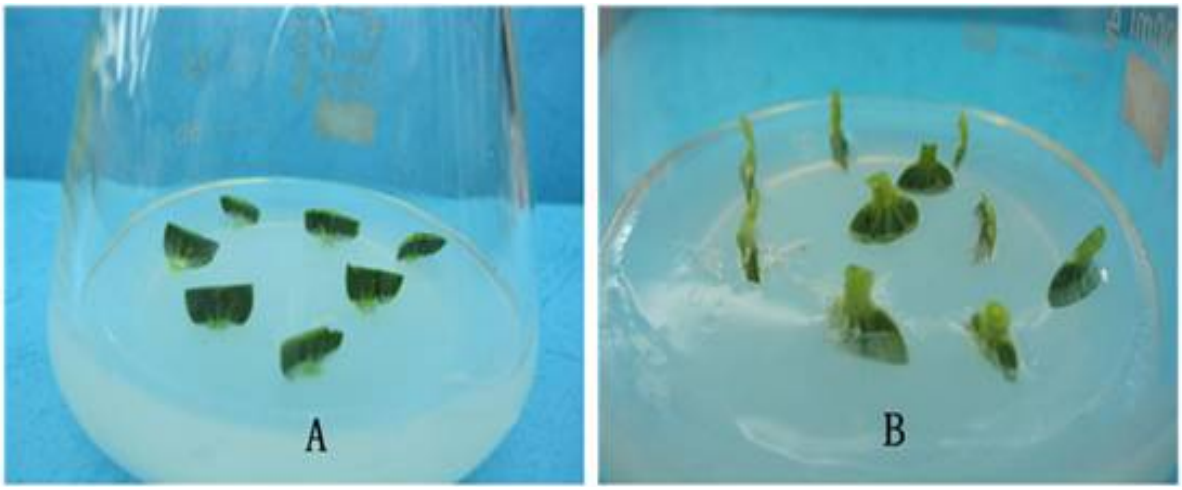

Fig. 2. Cotyledonary node explants with petiole end (A) or blade end (B) embedded in the medium.

Shoot elongation

Adventitious buds will only elongate if they are separated from each other, and therefore, clumps of adventitious buds must be separated and transferred to new medium for the elongation process. After 4 weeks of shoot induction, buds on explants were removed, separated into individual shoots, and transferred to elongation medium (MS supplemented with 0.05 mg/L 6-BA, pH 5.6). The shoots were cultured at the same temperature and photoperiod as those used for shoot induction.

Rooting: When the adventitious buds reached 2-4cm in height, they were excised and transferred to rooting medium for root induction. The rooting medium was 1/2 MS medium supplemented with 30 g/L sucrose, 7 g/L agar, and 0.1 mg/L IAA. After 3 weeks, the sealing membrane was removed from the jars and the rooted plantlets were allowed to adapt to the ambient conditions for 3 hours. Then, the plantlets were removed from the jars, washed with tap water to remove all medium, transferred to plastic containers filled with soil, and watered. The containers were covered with perforated transparent polyethylene sheets and kept at room temperature for 5 days. The covers were gradually removed to reduce the humidity, and after 3 weeks of acclimatization, the plants were transferred to a greenhouse.

Data analysis: We used a completely randomized design for these experiments. We used three replicates per treatment for experiments on the effects of different media on germination and shoot induction frequencies. The experimental data were subjected to analysis of variance using SPSS (17.0) software, and significant means were separated at p=0.05 using least significant difference tests.

Results and Discussion

Effect of different media on rate of emergence: Reducing the concentration of the MS medium enhanced the germination and growth rate to some extent (Table 2). Seeds cultured on full-strength MS medium showed a lower germination rate and grew more slowly than those cultured on 1/8 MS medium. The germination rate of the two inbred lines on 1/8MS medium was 98% (Table 2). The important factors for seed germination are moisture, air, and temperature. In this experiment, the only difference between 1/8 MS and MS medium was moisture availability, because sucrose regulates the osmotic pressure of the medium. In 1/8 MS medium, there were lower concentrations of sugar and other ingredients. Therefore, the osmotic pressure of the medium was lower than that of full-strength medium. The lower osmotic pressure meant that seeds imbibed water more easily, thus promoting the seed germination rate. Therefore, reducing the concentration of the MS salts and sucrose promoted germination of watermelon seeds.

Table 2 Germination and growth of watermelon seedlings on various concentrations of Murashige & Skoog (1962) medium.

Medium	Number of germinated seeds (W1-4/W1-12)	Germination rate (%) (W1-4/W1-12)	Number of seedlings W1-4/W1-12	Rate of seedling production (%) W1-4/W1-12
MS	36/32	72/64	31/24	86/75
1/2MS	41/35	82/70	36/30	88/ 86
1/4MS	44/43	88/86	44/40	100/ 93
1/8MS	49/49	98/98	49/49	100/100

Germination rate = Number of germinated seeds/number of seeds sown × 100%
Rate of seedling production = Number of seedlings/number of germinated seeds × 100%

Effects of 6-BA and IAA on shoot induction: Explants were cultured on MS medium with different concentrations of 6-BA and IAA to induce adventitious bud formation (Table 3). On MS medium containing 1.5 mg/L 6-BA, the bud induction rate of W1-4 was 55.62%, while on MS containing 1.5 mg/L 6-BA + 0.2 mg/L IAA, it was 92.37%. On MS medium containing 1.0 mg/L 6-BA, the bud induction rate of W1-12 was 51.27%. Addition of 0.1 mg/L IAA resulted in a bud induction rate of 98.48%. A few buds formed on MS medium containing only 6-BA, but addition of IAA as well as 6-BA resulted in a significant increase in the number of buds ($p<0.05$). Therefore, cytokinin is critical for shoot induction and auxin is important for shoot regeneration. These results differ from those in other studies. Li *et al*., (2011) found that 6-BA alone induced a high frequency of bud formation from cotyledon explants of watermelon. Michael *et al*., (1993) and Srivastava *et al*., (1989) reported that the combination of cytokinin and auxin inhibited shoot induction, possibly because the cytokinin suppressed the effects of auxin on shoot regeneration. Li *et al*., (2011) reported that 2.0 mg/L BA + 0.2 mg/L IAA induced the highest number of adventitious shoots from cotyledonary nodes in watermelon. In some studies on watermelon, high concentrations of cytokinin were required for a high frequency of shoot regeneration (Dong & Jia, 1991; Michael *et al*., 1993). In the present study, W1-4 showed the highest rate of shoot induction (92.37%) on MS containing 1.5 mg/L 6-BA + 0.2 mg/L IAA (MS33), and formed 10.27±0.40 shoots per explant. The best medium for shoot formation in W1-12 was MS containing 1.0 mg/L 6-BA + 0.1 mg/L IAA (shoot induction rate, 98.48%; shoots per explant, 8.80±0.85). The concentrations of plant growth regulators that were optimal for growth responses of both W1-4 and W1-12 were significantly lower than those reported by Tabei *et al*., (1993), who found that 10–20 mg/L IAA was essential for shoot formation (Muhammad *et al*., 2011). In the present study, however, 0.5 mg/L IAA resulted in browning of explants, increased callus formation, and fewer adventitious buds.

Table 3. Effects of 6-BA and IAA on shoot induction. Values are mean ± S.E.

Medium code	W1-4		W1-12	
	Regeneration frequency (%)	Number of shoots per explant (%)	Regeneration frequency (%)	Number of shoots per explant (%)
MS11	2.68 ± 0.49	3.10 ± 0.41	2.46 ± 0.32	2.09 ± 0.78
MS12	14.99 ± 1.66	6.21 ± 0.66	17.53 ± 0.81	5.68 ± 0.83
MS13	33.46 ± 1.00	8.45 ± 0.46	12.47 ± 0.84	6.82 ± 1.08
MS14	11.72 ± 1.06	5.75 ± 0.58	8.62 ± 0.92	3.94 ± 0.58
MS21	37.69 ± 1.79	6.72 ± 0.21	51.27 ± 2.32	4.07 ± 0.45
MS22	66.67 ± 1.75	7.46 ± 0.34	98.48 ± 1.28	8.80 ± 0.85
MS23	75.19 ± 1.54	8.36 ± 0.25	85.10 ± 1.47	6.11 ± 0.59
MS24	46.63 ± 1.01	6.96 ± 0.54	36.73 ± 1.17	4.76 ± 0.19
MS31	55.62 ± 0.83	7.78 ± 0.19	35.82 ± 0.89	4.98 ± 0.24
MS32	85.01 ± 1.92	8.91 ± 0.24	74.99 ± 1.64	7.84 ± 1.07
MS33	92.37 ± 1.46	10.27 ± 0.40	52.34 ± 1.45	6.09 ± 0.61
MS34	67.01 ± 1.73	6.55 ± 0.21	31.06 ± 1.36	4.16 ± 0.70
MS41	12.95 ± 0.66	4.73 ± 0.16	3.16 ± 0.46	4.11 ± 0.54
MS42	24.86 ± 2.60	5.95 ± 0.18	13.62 ± 0.90	6.35 ± 0.46
MS43	41.63 ± 1.33	7.18 ± 0.56	10.82 ± 0.44	5.80 ± 0.14
MS44	19.63 ± 1.07	4.99 ± 0.22	5.96 ± 0.37	7.8 ± 0.20

Effect of genotypes on induction of adventitious buds: The effects of 6-BA and IAA on shoot induction differed between W1-4 and W1-12, both in terms of adventitious shoot induction and the number of shoots per explant (Table 3). The highest rate of shoot induction in W1-4 was 92.37%, compared with 98.48% in W1-12. The highest number of shoots per explant in W1-4 was 10.27±0.40, compared with 8.80±0.85 in W1-12. We can conclude that induction of adventitious shoots is not only affected by phytohormones, but also by genotype. In other studies, the genotype played an important role in the shoot response of watermelon (Michael & Gray, 1994; Szalai, 1995; Sun *et al*., 2008) and also in muskmelon (Krug *et al*., 2005; Huijun *et al*., 2011).

Effect of cotyledon orientation on induction of adventitious buds: We placed explants with either the petiole end (A) or the blade end (B) embedded in the medium (Fig. 2). Those with the petiole end embedded in the medium formed adventitious buds from the cotyledon proximal region. Explants with the blade end embedded in the medium formed a few buds from the cotyledon proximal region and none from the abaxial region. Therefore, the induction of adventitious buds was restricted to specific cotyledon proximal regions. Michael & Gray (1993) and Krug *et al*., (2005) also reported that adventitious buds formed from the cotyledon proximal region. These differences may be because the transport of plant growth regulators is affected by the polarity of the tissues during culture.

Elongation and rooting: Adventitious buds must be transferred to elongation medium, otherwise the shoots rapidly yellow and/or die. However, it requires to some experience to judge the best time to segment and transfer shoot clumps, because only adventitious buds that are completely differentiated can grow on elongation medium. If the clumps are segmented prematurely, they will grow slowly or die. If the clumps are left too long before segmenting, they cannot absorb nutrients. Adventitious buds must be grown on elongation medium for approximately 20 days. Some produce abnormal shoots that cannot elongate, and subsequently die. Li *et al*., (2011) reported similar findings. Therefore, not all adventitious can regenerate successfully.

After 10 days' growth on MS containing 0.1 mg/L IAA, some short roots had formed from the tubercle (Fig. 1F) and then expanded rapidly (Fig. 1G). Thakur *et al*., (2005) reported a high frequency of root formation on shoots grown on MS medium containing auxin. Huijun *et al*., (2011) reported that 1/2 MS medium without plant growth regulators induced a high frequency of root formation.

Plant regeneration: It was important that well-rooted plantlets were washed thoroughly under tap water to remove excess agar, otherwise they rotted in the soil. High humidity was also essential in the first few days after transferring the rooted plantlets into soil.

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