

Full Length Research Paper

Optimized system for plant regeneration of watermelon (*Citrullus lanatus* Thumb.)

J. Li, X. M. Li, Y.G. Qin, Y. Tang, L. Wang, C. Ma and H. X. Li*

College of Horticulture, Sichuan Agricultural University, Ya'an 625014, Sichuan, People's Republic of China.

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The objective of this study was to establish an efficient and reproducible *in vitro* plant regeneration for *Citrullus lanatus* cv. Zaojia. To achieve optimal conditions for adventitious shoot induction, five explants (entire cotyledons, distal cotyledons, proximal cotyledons, cotyledonary node A and cotyledonary node B) were tested on MS medium supplemented with different concentrations and combinations of growth regulators (0 to 0.2 mg/L IAA and 1.0 to 5.0 mg/L BA), the results showed that entire cotyledons cultured in MS + BA (2.0mg/L) + IAA(0.2mg/L) achieved the highest regenerated rate (89.67%) and the optimal protocol screened in this experiment had 7.69 ± 0.10 shoots per explants. Adventitious shoots were able to elongate both on MS medium with 0.2 mg/L KT and 0.2 mg/L NAA; IBA 0.3mg/L was found to be effective in the production of root. Acclimatized plantlets transferred to pot resumed growth, and their stems and leaves elongated and expanded in one month.

Key words: Watermelon (*Citrullus lanatus* Thumb.), optimized system, regeneration, cotyledon explants, cotyledonary node.

INTRODUCTION

Watermelon (*Citrullus lanatus* Thumb.) is an important cucurbit crop species and it is a popular fruit which is native to Central Africa. It was first grown by ancient Egyptians and is believed to also have been cultured in Asia minor, Russia and the near and Middle East, thousands of years ago. Watermelon is an economically important crop and a valuable alternative source of water in desert areas, and its fruits are rich in carbohydrates, vitamins and minerals (Anonymous, 1992). The soluble fiber in watermelon may help to reduce cholesterol and risk of heart disease. It is a good source of fiber, which is important for keeping digestive track operating properly by preventing constipation, hemorrhoids and diverticular diseases. The nutritive value of the seeds is due to their high oil and protein content.

Watermelon is susceptible to a number of fungal,

bacterial and viral diseases, requiring annual field rotation, frequent chemical sprays and disease-resistant cultivars (Kim et al., 1998; Compton and Gary, 1999). In propagation, the introduction of new characters into watermelon by means of genetic manipulation is of great potential value, especially of the traits that would confer resistance to diseases and pests. Therefore, a major objective of watermelon breeding had been to develop cultivars with disease resistance by the application of recombinant DNA technology (Xiao et al., 1999). The success of genetic manipulation using these methods strongly depends on the presence of an efficient plant regeneration system.

However, to date, *in vitro* plant regeneration of watermelon has been successfully developed via organogenesis (Dong and Jia, 1991; Choi et al., 1994; Chen et al., 1998; Compton, 1999, 2000) and have been achieved using different sources of explants such as shoot tips (Compton et al., 1993a; Alper et al., 1994), immature embryos (Ahad et al., 1994), cotyledons (Blackmon and Reynolds, 1982; Adelberg et al., 1993; Compton, 1997), hypocotyls (Srivastava et al., 1989) and leaf (Sultana et al., 2004).

Regeneration of watermelon is largely dependent on

*Corresponding author. E-mail: hxli62@163.com. Tel: 0835-2882563.

Abbreviations: MS, Murashige and Skoog (1962); BA, benzyladenine; IAA, indoleacetic acid; NAA, naphthalene-acetic acid; IBA, indole-3-butyric acid; KT, kinetin.

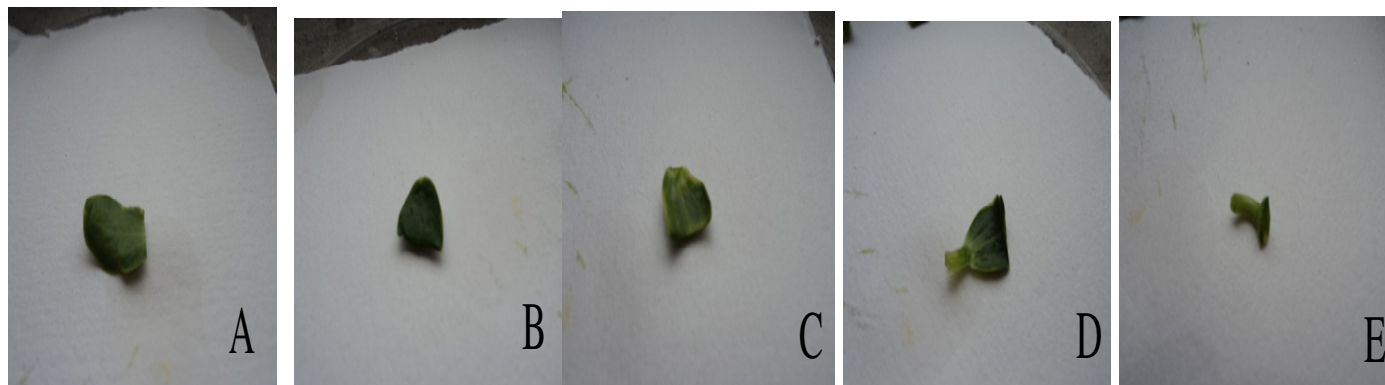


Figure 1. Types of different explants. (A) entire cotyledons; (B) distal cotyledons; (C) proximal cotyledons; (D) cotyledonary node A; (E) cotyledonary node B.

Table 1. MS basal medium containing ten combinations of IAA and BA.

Hormone	Culture medium number									
	MS1	MS2	MS3	MS4	MS5	MS6	MS7	MS8	MS9	MS10
IAA/mg/L	0	0	0	0	0	0.2	0.2	0.2	0.2	0.2
BA/mg/L	1	2	3	4	5	1	2	3	4	5

various factors such as genotype, explant types, explant ages and plant growth regulator. The objective of this study was to implement an efficient organogenesis protocol, followed by plant regeneration for watermelon cultivar Zaojia, for further studies of watermelon genetic transformation via *Agrobacterium*.

MATERIALS AND METHODS

C. lanatus cv. Zaojia mature seeds were used as explants sources. The seeds were rinsed thoroughly for 30 min under running tap water. The seed coat was dehusked and the embryos were surface sterilized for 30 min by immersing in 75% ethanol (v/v). Surface sterilization was carried out with 0.1% mercury chloride (HgCl₂) (w/v) for 10 min, followed by shaking gently. This is followed by successive three washings with sterile distilled water to remove the trace of HgCl₂. The seeds were then germinated on germination medium.

To investigate the influence of dark cultivation time on the rate of germination of watermelon, the seeds *in vitro* were divided into two sets (A and B) with 50 seeds per treatment, set A was maintained under 16 h photoperiod and set B was cultured in continuous darkness for 3 days, and then put under 16 h photoperiod. The statistics of the number of germination and seedlings were carried out after a week. Seeds that had normal elongated radicle were considered to have germinated. Seeds that had normal elongated epicotyl and cotyledons were considered as fine seedlings.

The basal medium used for all the experiments was MS (Murashige and Skoog, 1962) mineral formulation containing standard salts and vitamins, 30 g/l sucrose and 8 g/L agar. The pH was adjusted to 5.7 ± 0.1 before adding agar and the media were autoclaved for 20 min at 121°C under 1.1 kg/cm² pressure. Cultures were incubated at $25 \pm 1^\circ\text{C}$ at 1500 TO 2000 lux cool white fluorescent light. Each experiment was repeated three times with 24 cotyledons per treatment in each experiment.

To determine the regional effects of cotyledon for shoot formation, five explants were tested in this experiment as follows: entire cotyledons, distal cotyledons and proximal cotyledons (cotyledons were cut crosswise into distal and proximal halves), cotyledonary node A (the terminal buds were rejected and the distal halves of cotyledons were cut off, but 2 mm of hypocotyls were reserved and then the remaining were slivered into two parts along the hypocotyl) and cotyledonary node B (the terminal buds were rejected and the two entire cotyledons were excised, followed by cutting of the hypocotyl off from cotyledon base) (Figure 1). All explants in the test were excised from 6-day-old (when the cotyledons of the asepsis seedlings were not yet unfolded absolutely and their color was yellowish-green) seedlings processed with dark cultivation.

To examine suitable phytohormonal conditions for shoot formation, combinations of IAA and 6-BA at various concentrations were tested. The regeneration medium was MS basal medium containing ten combinations of IAA and BA (Table 1). The explants put on the culture tubes containing 30 ml of regeneration medium were cultured in the dark for 3 days and then cultured under 16 h photoperiod at 1500 to 2000 lux cool white fluorescent light. After 4 weeks, explants with adventitious shoots were subcultured to fresh shoot-regeneration medium for another 4 weeks. The frequency of shoot formation was then examined and the percentages were calculated as the number of explants differentiating adventitious shoots from total explants cultured.

In order to study the effect of different growth regulator on the elongation of adventitious buds, shoots longer than 0.8 cm were isolated and transferred to MS supplemented with 0.2 mg/L KT and 0.2 mg/L NAA, respectively for shoot elongation. Shoots longer than 2 cm were transferred to rooting medium supplemented with 0.3 mg/L indole-3-butyric acid (IBA) which consists of half strength micro- and macronutrients in culture tubes.

After 3 weeks on rooting medium, the plantlets were transplanted under *ex vitro* condition into plastic pots containing autoclaved soil. All the pots were covered with clear plastic covers for 7 days to maintain high humidity and incubated at $25 \pm 1^\circ\text{C}$ under 16 h photoperiod in a tissue culture chamber. Plantlets in the pots were

Table 2. Effect of dark cultivation time on rate of emergence.

Dark cultivation time (day)	Number of germination (pill)	Rate of germination (%)	Number of seedling (%)	Rate of seedling (%)
0	38	76	24	64
3	48	96	45	94

Rate of germination = number of germination/sum total of seeds inoculated × 100%; Rate of seedlings = number of seedlings/number of germination × 100%.

Table 3. Effects of IAA and BA on adventitious buds regeneration.

Culture medium number	Entire cotyledon		Proximal cotyledon		Cotyledonary node A		Cotyledonary node B	
	Inductivity (%)	Number of shoot per explant	Inductivity (%)	Number of shoot per explant	Inductivity (%)	Number of shoot per explant	Inductivity (%)	Number of shoot per explant
MS1	61.67	6.43±0.03	60.44	6.15 ± 0.10	90.37	3.85±0.20	89.17	4.05±0.22
MS2	89.57	7.63±0.05	87.42	7.31 ± 0.07	87.34	4.25 ± 0.23	88.08	4.39± 0.11
MS3	88.33	5.70±0.01	87.21	5.60±0.39	85.65	3.70±0.30	87.52	4.12±0.20
MS4	69.13	4.42±0.09	76.23	4.34±0.23	83.16	2.82 ± 0.17	85.48	3.68± 0.34
MS5	67.50	4.04±0.08	65.76	3.89 ± 0.37	80.33	2.82 ± 0.14	81.15	3.46 ± 0.18
MS6	64.44	6.56±0.07	63.12	6.30 ± 0.25	100	3.87± 0.17	100	3.86±0.10
MS7	89.67	7.69±0.10	88.56	7.43± 0.32	100	4.37 ± 0.35	100	4.56 ± 0.34
MS8	80.00	6.04±0.08	78.94	5.73±0.14	100	3.80± 0.30	100	4.32± 0.27
MS9	68.66	5.03±0.06	69.01	5.00±0.25	100	3.48±0.36	100	4.12±0.14
MS10	67.81	4.67±0.05	65.49	4.56±0.26	100	3.26± 0.48	100	3.95±0.20

watered every 3 days. After 9 days, the humidity was reduced progressively by removing the lids for 3 days. During this time, plants were watered 2 to 3 times alternatively with nutrient solution and water.

RESULTS

Effect of dark cultivation time on rate of emergence

Dark cultivation could enhance the rate of germination and seedlings in a certain degree (Table 2). Rate of germination and seedlings without dark cultivation were 76 and 64%, respectively lower than that cultured in the dark for three days, which were 96 and 94% respectively.

Effects of IAA and BA on adventitious buds regeneration

Explants were cultured on MS medium with various levels of 6-BA alone or in combination with 6-BA and IAA for adventitious bud induction. Morphogenic potentialities of the explants were found to differ which depend on the growth regulator supplements shown in Table 3.

The explant of distal cotyledons resulted in swellings, but did not form any adventitious buds. There was no

significant difference of inductivity between mediums with BA alone (MS1-MS5) and with 0.2 mg/L IAA (MS6-MS10) for the explants of entire cotyledons and proximal cotyledons. The best performance was shown in MS medium supplemented with 2.0 mg/L BA (Table 3). The inductivity and number of shoots per explant of entire cotyledons were 89.57% and 7.63 ± 0.05 on the medium with 2.0 mg/L BA alone (MS2), and were 89.67% and 7.69 ± 0.10 on the medium with 2.0 mg/L 6-BA + 0.2 mg/L IAA (MS7). Similar observation was found in proximal cotyledons, the highest inductivity and number of shoots per explant were 87.42% and 7.31 ± 0.07 (MS2), and 88.56% and 7.43 ± 0.32 (MS7). It was not conducive to the inductivity and regeneration of adventitious shoots when the concentration of BA was too high (MS4 and MS5) or too low (MS1).

The experiment showed that the inductivity of cotyledonary node A and cotyledonary node B were much higher than that of cotyledons. Moreover, the inductivity of the two explants cultured on the medium with 0.2 mg/L IAA (MS6-MS10) were up to 100%, obviously, higher than that on medium with BA alone (MS1-MS5). That is to say, 0.2 mg/L IAA contributed to the enhancement of shoots inductivity. However, there was no obvious action of 0.2 mg/L IAA to increase the number of shoots per explant. In addition, it was clear that as the concentration of BA was increased, the inductivity of adventitious shoots reduced

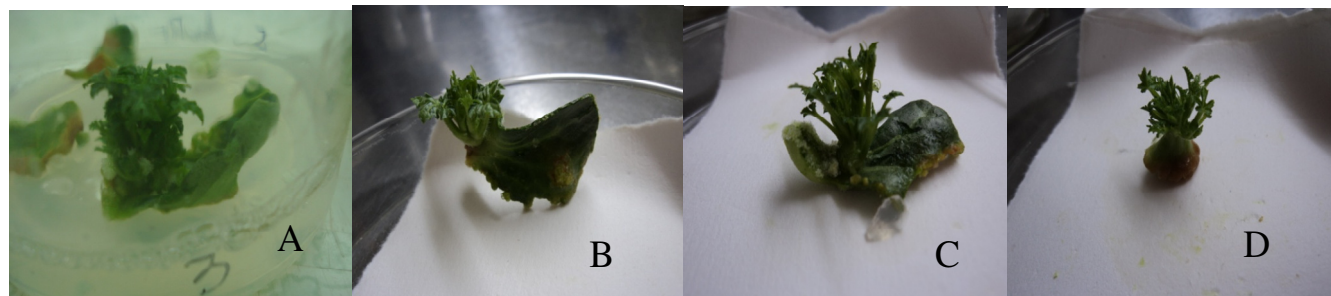


Figure 2. Effect of explant types on the induction of adventitious buds. (A) Adventitious buds in entire cotyledons; (B) adventitious buds in proximal cotyledons; (C) adventitious buds in cotyledonary node A; (D) adventitious buds in cotyledonary node B.

Table 4. Effect of explant types on the induction of adventitious buds.

Explant type	Entire cotyledon	Proximal cotyledon	Cotyledonary node A	Cotyledonary node B
Inductivity (%)	89.27	88.5	100	100
No. of shoots per explant	7.53 ± 0.16	7.39 ± 0.81	4.44 ± 0.4	4.66 ± 0.34



Figure 3. Adventitious shoots elongation.

(MS1-MS5). The best performance occurred on MS medium with 2.0 mg/L BA + 0.2 mg/L IAA (MS7), the inductivity and number of shoots per explant were 100% and 4.37 ± 0.35 (cotyledonary node A) and 100% and 4.56 ± 0.34 (cotyledonary node B), respectively (Table 3).

Effect of explant types on the induction of adventitious buds

Explants tested in this experiment were excised from 6-

day-old plantlets, their entire and proximal cotyledons were cultured on MS medium with 2.0 mg/L BA alone, and cotyledonary node A and cotyledonary node B were cultured on MS medium with 2.0 mg/BA + 0.2 mg/L IAA .

There were some significant differences of adventitious buds inductivity among different explants. The four explants (entire cotyledons, proximal cotyledons, cotyledonary node A and cotyledonary node B) all had inductive adventitious buds in different extent (Figure 2). The explant of distal cotyledons just resulted in swellings, but did not form any adventitious buds. Adventitious buds were only found to develop from the petiole of proximal cotyledons as well as the entire cotyledons, and their adventitious bud inductivity were similar too, 88.56 and 89.27%, respectively (Table 4). For cotyledonary node A and B, although, the cutting method was different, there was no obvious difference between the two, both were 100% which were higher when compared to the cotyledons.

The number of adventitious buds per explant was as follows: 7.53 ± 0.16 shoots for entire cotyledons; 7.39 ± 0.81 shoots for proximal cotyledons; 4.44 ± 0.46 shoots for cotyledonary node A; 4.66 ± 0.34 shoots for cotyledonary node B (Table 4.). The experiment showed that entire cotyledons could induce a little more adventitious shoots than proximal cotyledons, but the number of adventitious buds per explant of the two cotyledons was higher than cotyledonary node explants. On the other hand, adventitious shoots that developed from cotyledonary explants were green and strong, and elongated faster. However, those from cotyledonary node A and B were the opposite, lightgreen, thin and elongated slower. To sum up, proximal cotyledons were suitable for *Agrobacterium* infection, because they had more wounds,

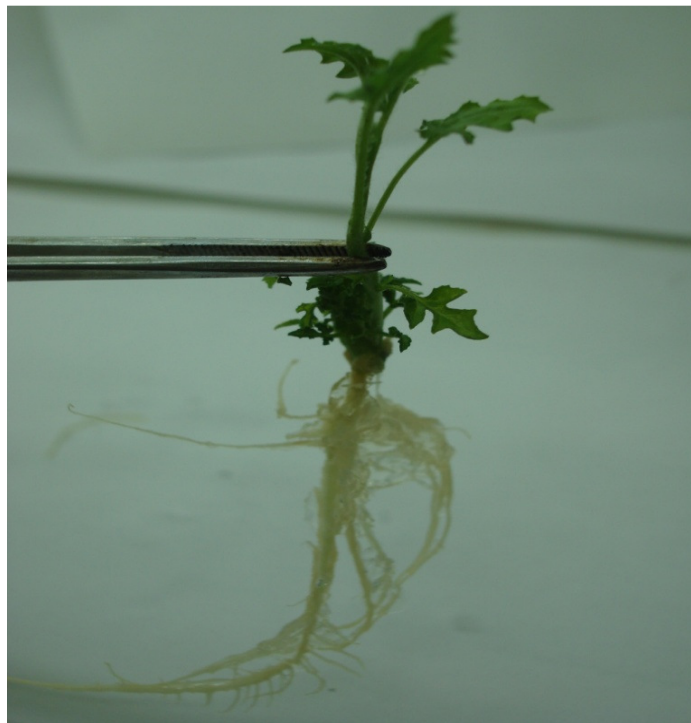


Figure 4. Induction of roots

Flattened and were beneficial for absorbing nutrient. Therefore, proximal cotyledons were the optimal explant screening in this experiment.

Adventitious shoots elongation and rooting experiment

“Adventitious shoots isolated from explants were able to elongate both in MS medium supplemented with 0.2mg/L KT and 0.2mg/L NAA respectively, and the percentage of elongation was almost the same, so whatever 0.2mg/L KT or 0.2mg/L NAA was suitable for shoots elongation (Fig. 3). It was observed that elongated shoots induce high frequency of roots cultured in half strength MS medium supplemented with 0.3mg/L IBA (Fig. 4). The plantlets with roots (with more than one 2-3cm roots) which were transferred and acclimatized in pot resume growth and their stems and leaves elongated and expanded in one month

DISCUSSION

Dark cultivation tested in the experiment promoted the rate of germination of watermelon. Rate of germination was enhanced and the seedlings came out evenly when seeds were cultured in the dark for the 3 days first.

Together with dark cultivation, explant type has shown to be important for morphogenesis, since competent cells

for adventitious shoots formation in cucurbits seem to be restricted to specific cotyledon regions (Choi et al., 1994; Compton, 2000; Ananthakrishnan et al., 2003). For watermelon, it has been shown that organogenic competent cells concentrated at the proximal region of the cotyledon (Compton and Gray, 1993b), since most of the adventitious buds developed at the explants basal region (Compton, 2000). It has been reported that a higher percentage of explants forming adventitious buds was obtained from the cotyledon proximal region (Krug et al., 2005). In fact, the entire cotyledons and proximal cotyledons used in this study resulted in high adventitious shoot regeneration frequency. However, we could not induce any adventitious shoots from distal cotyledons which was different from Chi et al. (1994)'s report of efficient response from the distal portions of the watermelon cotyledons, with a F1 hybrid watermelon (cvs. Sweet Gem and Gold Metal). So far, there is hardly any report about cotyledonary node as an explant in watermelon tissue culture, as a result, we tried in this study and had gotten a high inductivity. Our observation suggests that adventitious shoots came up principally in the petiole of cotyledons, which is the proximal end. The polarity appeared in tissue culture of watermelon; however, the polarity might be as a result of the meristem in the proximal end and also the transport of plant growth regulator which is polar translocation.

The type and the concentration of phytohormone in the induction media were found to be another crucial factor in regeneration of watermelon and the presence of cytokinin is critical for shoot induction. Compton and Gary (1993a) and Srivastava et al. (1989) detected an inhibition of shoot organogenesis when both cytokinin and auxin counteracts the effect of cytokinin on shoot regeneration. Dong and Jia (1991) observed adventitious shoot differentiation from cotyledons of watermelon on a medium containing 5 to 7mg/L BA + 0 to 3 mg/L IAA. Compton and Gary (1991, 1993) made similar observations on all the cultivars of watermelon tested. These reports showed that the high frequency shoot regeneration in watermelon required a high concentration of cytokinin. Hoque et al. (1995) found that a combination of 1.5 mg/L BA and 0.1 mg/LNAA was more suitable for adventitious multiple shoot formation, whereas in this experiment, 2.0 mg/L BA + 0.2 mg/L IAA was observed to be best for the production of multiple shoots from cotyledonary node A and B in watermelon. Moreover, we had found that the inductivity of using IAA or not was more or less the same, in other words, using BA alone also could induce high frequency adventitious buds for cotyledon explants in watermelon and 2 mg/L BA was the suitable concentration. Then, we could draw a conclusion that low concentration of cytokinin could induce high inductivity too. However, Tabei et al. (1993) reported that high concentrations of 10 to 20 mg/L IAA was also essential for shoot formation in watermelon.

Another important finding in this study is that multiple

shoots regenerated from explants must be transferred to elongation medium, as prolonged culture on shoot induction medium not only stimulates callus formation but also produce abnormal shoots. The same pattern of events is reported for muskmelon (Dirks and Van Bugenum, 1989).

Thakur et al. (2005) had found that full strength MS medium supplemented with auxin induced high frequency in root formation. Dabauza et al. (1997) and Ahn et al. (2007) reported that high frequency of shoots rooted and grew normally on MS medium supplemented with IBA. The plantlets with well developed roots were successfully transplanted in soil and the percentage of survivability was 63.73%.

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