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# Adventitious Shoot Induction and Plant Regeneration from Cotyledons of Mature Seed in Watermelon (*Citrullus lanatus* L.)

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Culture conditions of watermelon (*Citrullus lanatus* L.) were investigated for the purpose of establishing an efficient plant regeneration system. Adventitious shoots were induced efficiently (50%) on MS medium containing 10 mg/*l* IAA and 10 mg/*l* BA. Cotyledon explants from young seedlings precultured for 3 days on MS medium containing 10 mg/*l* IAA and 10 mg/*l* BA or precultured for 1 day on MS medium without any phytohormone gave a high frequency of shoot formation (46% and 42%, respectively). The basal region of cotyledon showed higher frequency of shoot formation (55. 3%) than the apical region (23. 0%). Varietal differences were observed in the efficiency of shoot regeneration. Cultivar 'Kikodama' showed the highest frequency of shoot formation (68%).

## Introduction

Watermelon (*Citrullus lanatus* L.) is an economically important crop. The genetic varieties were less than those of the closely related genus *Cucumis*. Genus *Citrullus* consists of only four species[1], therefore introduction of foreign genes has a great potential for the improvement of watermelon. Recently, success of transformation has been reported in many crops using *Agrobacterium* or micro particle bombardment. The success of genetic manipulation using those methods strongly depends on the presence of an efficient plant regeneration system. Although there were three reports on shoot formation of watermelon[2-4], the number of reports indicating efficient plant regeneration systems for watermelon were fewer than those of cucumber[5-7] and melon[8-10].

In three reports on shoot formation of watermelon[2-4], cotyledon[2-4] or hypocotyl[3] segments from 5-day-old to 8-day-old seedlings were used as the best explants. Some research has already reported that cotyledons of mature seeds of young seedlings have high ability for regeneration in melon[8-11] and cucumber[7]. Therefore we investigated the effects of combined treatment with IAA and BA using cotyledons of young seedlings (3-day-old seedlings) as explants. Moreover regional differences of the same cotyledon explants were investigated. The effects of seedling age and varietal differences, including seedless watermelon (triploid), on shoot formation were also tested.

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## Materials and Methods

Seeds of Watermelon (*Citrullus lanatus* L. cv. Shimaou-Max, YAMATO NOUEN Co. Ltd., Japan) were used to investigate the culture condition. Seventeen varieties described in **Table 3** were used for examining the varietal differences of the ability to form shoots. Peeled seeds were sterilized with sodium hypochlorite solution containing 1% active chlorine for 15 min. and then rinsed three times with sterile distilled water.

To examine suitable phytohormonal conditions for shoot formation, combinations of IAA and BA at various concentrations were tested. The regeneration medium was MS basal medium[12] containing various combinations of IAA and BA, 3% sucrose and 0.8% agar. Sterilized seeds were cultured on regeneration medium for 3 days. Then the hypocotyl was removed from the seed, and the cotylodons were cut crosswise and lengthwise into 4 pieces. Segments from basal region were placed back onto the regeneration media. All media, adjusted to pH 5.8, were autoclaved at 121°C for 15 min. The explants put on the regeneration medium in a plastic box (*ca.* 60×60×90 mm) were cultured in the dark at 25°C for a month and then cultured under 16h light (day fluorescent light *ca.* 2,000 lx)/day for a month. The frequency of shoot formation was examined after 2 months of culture, and the percentages were calculated as the number of explants differentiating adventitious shoots to total explants cultured. Each treatment consisted of 24 explants and was repeated twice.

Explants differentiating adventitious shoots were transferred to MS agar medium containing 2 mg/*l* IAA and 2 mg/*l* BA to elongate adventitious shoots. Elongated shoots were transferred to MS agar medium containing 2 mg/*l* IAA to induce adventitious roots. Plantlets with adventitious roots were potted in a mixture of sterilized soil and Perlite (1 : 1, v/v) and were grown in a greenhouse.

The preculture effect of donor seedlings was investigated to improve the frequency of shoot formation. Sterilized seeds were sown on regeneration medium (W-1 ; MS medium containing 10 mg/*l* IAA and 10 mg/*l* BA) or MS medium without any phytohormone. They were cultured at 25°C in dark. Basal region of cotyledons was used as explants. As for control sample, sterilized seeds were immediately cut and cultured on regeneration medium. In this experiment each treatment consisted of 12 explants and was repeated three times.

To determine the regional effects of cotyledon for shoot formation, sterilized seeds were cultured on W-1 medium for 3 days in the dark. Preparation of explants was stated above. The basal region and apical region of cotyledons were placed back onto W-1 medium. In this experiment each treatment consisted of 36 explants and was repeated twice. The total number of explants cultured and the number of explants regenerating adventitious shoots were shown in **Table 2**.

Seventeen varieties containing pure-bred lines, $F_1$ commercial varieties (diploid) and seedless watermelons (triploid) were used for examining the varietal differences in shoot forming ability. Culture medium and culture method were the same as described above. In this experiment each treatment consisted of 24 explants and was repeated twice.

## Results and Discussion

### 1. *Effects of phytohormones*

The combination of 7~12 mg/*l* BA and 10~30 mg/*l* IAA induced adventitious shoots (**Table 1**). The adventitious shoots were directly regenerated from the surface of the cotyledon (**Fig. 1-A**). The lowest concentration of BA (5 mg/*l*) or IAA (5 mg/*l*) did not promote shoot formation. The highest concentration of 15 mg/*l* BA also inhibited it and the frequency of shoot formation was reduced at the highest concentration of IAA (30 mg/*l*) except in combination with 7 mg/*l* BA. The

**Table 1.** Effects of IAA and BA for shoot regeneration from cotyledon explants in watermelon (*Citrullus lanatus* L.).

| | | IAA (mg/ *l*) | | | | |
|---|---|---|---|---|---|---|
| | | 5 | 10 | 15 | 20 | 30 |
| BA (mg/*l*) | 5 | 0*[1] | 0 | 0 | 0 | 0 |
| | 7 | 0 | 0 | 0 | 13 | 13 |
| | 10 | 0 | 50 | 0 | 25 | 0 |
| | 12 | 0 | 0 | 31 | 19 | 0 |
| | 15 | 0 | 0 | 0 | 0 | 0 |

Each treatment consisted of 24 explants and all experiments were repeated twice.

*[1] The frequency indicates the percentage of explants regenerating shoots to total explants cultured.

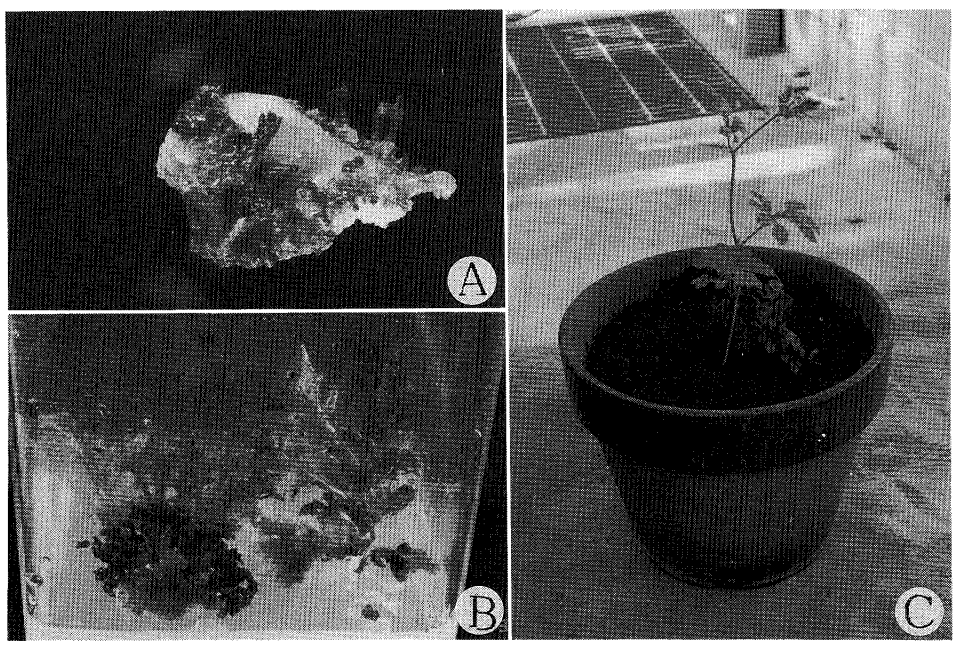


**Fig. 1** Plant regeneration from cotyledon explants of watermelon (*Citrullus lanatus* L.). **A** : Shoot regeneration from surface of cotyledon explants. **B** : Multiple shoots elongated on the shoot elongation medium. **C** : Plantlet growing in greenhouse.

combination of 10 mg/*l* BA and 10 mg/*l* IAA gave the highest frequency of shoot regeneration (50%) in this experiment. Therefore this phytohormonal combination was selected as a best condition for later experiments.

Blackmon and Reynold[2] induced adventitious shoots from cotyledon of watermelon using a combination of 10 mg/*l* 2ip and 0.1 mg/*l* 2-naphthoxyacetic acid. Dong and Jia[4] also induced adventitious shoots from cotyledon of watermelon with the combination of 5~7 mg/*l* BA and 0~3 mg/*l* IAA. These reports showed that shoot formation in watermelon required high concentrations of cytokinin. These results agreed with ours. But Srivastava *et al.*[3] induced adventitious shoots from cotyledon or hypocotyl in watermelon by 4.5 $\mu$M BA. This result was far different from other reports[2,4] and ours. In the closely related genus *Cucumis*, adventitious shoots were induced by 1 to

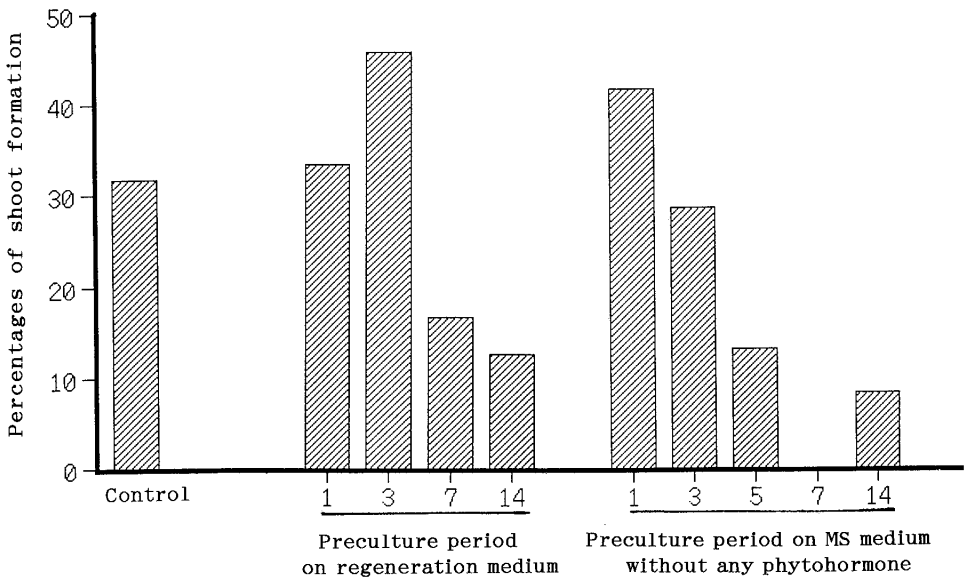


**Fig. 2** Effect of preculture period for shoot regeneration.
The frequency indicates the percentage of explants regenerating shoots to total explants cultured. Each treatment consisted of 12 explants and all experiments were repeated three times. In control experiment, sterilized seeds were immediately cut and cultured on regeneration medium.

2 mg/*l* BA[9,11,13,14]. Trulson and Shahin[15] failed to regenerate plants from cotyledon or roots by using the culture condition of cucumber. These facts suggested that watermelon required a higher concentration of cytokinin to regenerate adventitious shoots. On the other hand, our results showed that a high concentration of IAA (10~20 mg/*l*) was also essential for shoot formation in watermelon. While other reports showed that shoot formation in watermelon was enhanced by the addition of a lower concentration of auxin or no auxin in the regeneration medium[2-4]. Generally speaking, a higher concentration of auxin inhibits shoot formation and promotes callus proliferation[16-18]. Adequate auxin concentration for shoot regeneration in watermelon determined in this experiment was different from those of other reports[2-4]. The genotype or age of the donor seedlings in our experiment were different from those in other reports[2-4]. The reason for this remained to be clarified.

**2. *Effect of preculture***

The frequency of shoot formation varied remarkably depending on the preculture period. Cotyledon explants from seedlings precultured for 3 days on W-1 medium or 1 day on phytohormone-free MS medium showed the highest frequency of 46% and 42%, respectively (**Fig. 2**). Otherwise the frequency of shoot formation in the control experiment was 32%. On the other hand, when precultured for 14 days on W-1 medium or phytohormone-free MS medium, the frequencies decreased to 13% and 8%, respectively. By culturing cotyledon explants of mature seeds in melon, adventitious shoots[9,18] or somatic embryos[8,18] were formed efficiently. Yoshioka *et al.*[11] reported that cotyledons of 1-day-old seedlings were the best source for shoot formation in melon. Similarly, the shoot forming ability of the cotyledon explants dramatically decreased when the age of donor seedlings exceeded 7 days in watermelon[4]. Cotyledon explants from 7-day-old seedlings of melon gave lower frequency of shoot formation and could not regenerate somatic embryos[18]. Niedz *et al.*[19] reported that the frequency of shoot formation from cotyledon explants in melon was also

**Table 2.** Regional differences for shoot regeneration between basal and apical regions of cotyledon in watermelon (*Citrullus lanatus* L.).

| Region of cotyledon | No. of explants cultured (A) | No. of explants with adventitious shoots (B) | Percentage of regeneration (B/A×100) |
|---|---|---|---|
| Basal region | 87 | 47 | 55. 3 |
| Apical region | 85 | 18 | 20. 7 |

All experiments were repeated twice. Total number of explants cultured and number of explants regenerating adventitious shoots were shown.

**Table 3.** Varietal differences for shoot regeneration of watermelon (*Citrullus lanatus* L.).

| | Variety | Percentage of shoot regeneration |
|---|---|---|
| Pure-bred line | Asahiyamato | 13 |
| | Kurobe | 33 |
| | Charleston Grey | 19 |
| | Kahou | 3 |
| | Otome | 8 |
| | Benikodama | 13 |
| | Kikodama | 68 |
| $F_1$ variety | Fujihikari | 25 |
| (diploid) | Simaou-Max | 29 |
| | Tahichi | 3 |
| | Piromasuta | 13 |
| | Akakodama | 31 |
| | New-kodama | 50 |
| Seedless | Tanenasi-Simaou | 11 |
| (triploid) | 3XSK | 19 |
| | Benizakura | 13 |
| | Seedless 202 | 23 |

Percentage indicates the number of explants regenerating shoots to total explants cultured. Each treatment consisted of 24 explants and all experiments were repeated twice.

reduced on 18-day-old seedlings when contrasted to explants from 4-day-old or 7-day-old seedlings. In a family of *Cucurbitaceae*, these facts suggest that cotyledons of mature seeds or young seedlings have high potential for shoot formation or somatic embryogenesis, and the potential of regeneration tends to disappear according to the aging of donor seedlings.

### 3. *Regional difference for the potential of shoot formation*

The frequency of shoot formation was different in the apical region and basal region of cotyledon (**Table 2**). The basal region gave higher frequency (55. 3%) than did the apical region (23. 0%). Shoot regeneration from explants of the basal region showed polarity, and many shoots were regenerated from the proximal end. Monacelli *et al.*[20] found that the proximal end of each

cotyledon segment regenerated adventitious shoots more effectively than the distal end. Homma *et al.*[10] showed that somatic embryos were induced most efficiently from the cut surface at the border of hypocotyl and cotyledon. This region was very close to the proximal end of basal region. In this experiment, many shoots appeared from the proximal end of the basal region. These facts support that the proximal end of basal region has high potential for shoot formation.

**4. *Varietal difference of potential for shoot formation***

Cotyledon explants of 17 varieties were used to investigate the varietal differences in shoot formation (**Table 3**). Regenerated shoots were obtained from all cultivars tested. However, varietal differences were observed in the efficiency of shoot formation. The shoot formation frequencies of pure-bred lines and $F_1$ commercial varieties (diploid) distributed from 3% to 68%, and those of seedless watermelons (triploid) distributed from 11% to 23%. Kikodama and New-kodama showed higher frequency of shoot formation than other cultivars. The fruit size of 'Kikodama' and 'New-kodama' were small. 'Akakodama' was also a small fruit type and it showed a relatively high frequency of shoot formation. Although 'Otome' and 'Benikodama' were small fruit watermelon, the shoot formation frequencies of these two cultivars were low. In the present condition, morphological characteristics related to the shoot formation ability was not observed.

We have established efficient and reproducible culture system in watermelon. We are sure that this system will be useful for obtaining transformants by using *Agrobacterium* or micro particle bombardment.

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《和文要約》

# スイカの種子子葉部からの不定芽誘導と植物体再生

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スイカの不定芽再分化条件と植物体再生条件について検討した．不定芽再分化のためのホルモン条件を検討するため，各種濃度のIAAとBAを組み合わせた再分化培地に，滅菌した種子を播種し，3日目の実生から子葉を切り出して外植片とした．外植片は再び再分化培地に置床して不定芽の再分化率を検討した結果，10 mg/*l* IAAと10 mg/*l* BAを組み合わせた培地（W-1）で最も高率（50%）に不定芽が誘導された．次に，種子の前培養が再分化に及ぼす影響について検討した．その結果，W-1で3日間またはホルモンを含まないMS培地で1日間前培養した実生由来の子葉を用いることで高頻度な再分化が認められた．5日以上前培養した実生は，前培養の日数の増加とともに再分化率は低下した．子葉の部位による再分化に及ぼす影響を検討した結果，基部の再分化率が55.3%と先端部の23.0%に比べ高率であった．さらに，再分化に及ぼす品種間差異を検討した結果，供試した全ての品種から再分化個体が得られたが，明らかな品種間差異が認められ，特に‘黄小玉’及び‘赤小玉’が高い再分化率を示した．