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In vitro Shoot Regeneration of *Citrullus vulgaris* Schrad (Watermelon)

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Abstract: In this study, shoot regeneration using cotyledons derived from seedlings of diploid and triploid yellow watermelon (cultivars Hwang Fong Yellow Queen, Round Dragon and Chin San Seedless) was investigated. Multiple shoots from auxiliary meristems were obtained without adventitious shoot. Shoot regeneration system for watermelon was successfully established from cotyledon sections of 4 to 5 day-old *in vitro* seedling. The explants were collected from the proximal cotyledon with hypocotyls segment. Highest mean number of multiple shoots were obtained (9.83±0.54) for cultivar Hwang Fong Yellow Queen on MS medium supplemented with 20 μM BAP as compared to (8.87±0.81) for Chin San Seedless on 5 μM of BAP and (6.00±0.32) for Round Dragon on 10 μM of BAP. From these results, cultivar Hwang Fong Yellow Queen was used for further experiment due to its highest percentage of germination and mean number of shoots. Subsequently, the cultivar successfully rooted (80%) on half strength MS medium supplemented with 0.5 μM IAA. Finally, 50% of these rooting plantlets were acclimatized on soil.

Key words: Plant tissue culture, cotyledons explants, watermelon, *Citrullus vulgaris*

INTRODUCTION

In vitro plant regeneration of watermelon (*Citrullus lanatus*) was previously studied using clonal micropropagation of shoot tips and nodes as well as adventitious shoot regeneration from cotyledon segment and somatic embryogenesis. Shoot regeneration of watermelon has been previously reported (Krug *et al*., 2005; Jaskani *et al*., 2004; Yalcin-Mendi *et al*., 2003; Pirinc *et al*., 2003; Compton, 1999; Choi *et al*., 1994; Tabei *et al*., 1993; Compton and Gray, 1993; Dong and Jia, 1991; Srivastava *et al*., 1989) in which cotyledons derived from *in vitro* germinated seedlings were found to be the best source of explants.

The greatest organogenic competency was reported to be demonstrated by cotyledon explants from 3 to 5-day-old seedlings (Krug *et al*., 2005; Compton *et al*., 2004; Jaskani *et al*., 2004; Pirinc *et al*., 2003; Yalcin-Mendi *et al*., 2003; Compton, 1999; Compton *et al*., 1996; Choi *et al*., 1994; Compton and Gray, 1993; Tabei *et al*., 1993; Dong and Jia, 1991). The proximal end of cotyledons generally has higher regeneration rate as compared to distal end of cotyledons explant (Krug *et al*., 2005; Tabei *et al*., 1993), for example, the number of explants producing shoots at the proximal end was 3.25±2.37 and distal end only produce 1.00±1.0 shoots (Krug *et al*., 2005). Tabei *et al*. (1993) also reported the proximal end of cotyledon showed higher frequency of shoot formation (55.3%) than distal end (23.0%). Meanwhile whole cotyledon bases or basal halves explant demonstrated 60-92% of shoot induction (Dong and Jia, 1991). Shoot regeneration from proximal end of cotyledons (cut lengthwise into 4 pieces) and the margins (1 mm) removed have also been reported (Compton, 1999; Compton *et al*., 1996; Tabei *et al*., 1993).

Previous reports on shoot organogenesis from watermelon demonstrated that the use of MS medium showed the highest frequency of shoot formation at 78, 72 and 55.3%, respectively (Yalcin-Mendi *et al*., 2003; Compton, 1999; Tabei *et al*., 1993). Maximum percentage of shoot regeneration has been achieved by supplementing the MS medium with 4.4-20 μM benzylaminopurine (BAP) as the only plant growth regulator (Akashi *et al*., 2005; Krug *et al*., 2005; Yalcin-Mendi *et al*., 2003; Pirinc *et al*., 2003; Compton, 1999; Compton *et al*., 1996; Srivastava *et al*., 1989). Dong and Jia (1991) further reported that adding another regulator (2.85 or 5.7 μM indole-3-acetic acids) in MS

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medium supplemented with 22 or 35.8 µM BAP improved the percentage of shoots regeneration per explant (96.7 or 93.9%, respectively) for some genotypes.

Shoot elongation has been a problem when cotyledon pieces are continuously cultured on medium supplemented with BAP (Compton *et al*., 2004). Improvement of shoot elongation was observed when shoots and buds were transferred to medium without plant growth regulator (Pirinc *et al*., 2003; Compton, 1999; Compton *et al*., 1996) or when supplemented with 0.92 µM kinetin (Krug *et al*., 2005; Dong and Jia, 1991). Shoots greater than 15 mm can be easily rooted in MS medium supplemented with 0.54-10.8 µM NAA (Sultana *et al*., 2004; Pirinc *et al*., 2003; Dong and Jia, 1991) or 11.4 µM IAA (Tabei *et al*., 1993) or 4.9 µM IBA (Krug *et al*., 2005). In addition, adventitious shoot regeneration systems have been demonstrated to be the most effective system for producing genetically transformed plants (Horsch *et al*., 1985). Based on the above observation and reports, the watermelon shoot regeneration using cotyledon with meristem region was chosen as a target material in this study. The establishment of a regeneration system for watermelon was studied as a step in producing a reproducible plant transformation system. The objective of this study is to develop an efficient shoot regeneration system for diploid (Round Dragon and Hwang Fong Yellow Queen) and triploid (Chin San Seedless) watermelon cultivars.

MATERIALS AND METHODS

Plant material: Seeds of three watermelon cultivars (Round Dragon, Hwang Fong Yellow Queen and Chin San Seedless) were purchased from a local supplier (ACE Seed Trading (M) Sdn Bhd, Malaysia).

Media preparation: In this study MS basal medium (Murashige and Skoog, 1962) was used as the basic medium in addition of standard salts and vitamins, 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose and 8 g L^{-1} agar powder. The pH was adjusted to 5.8±0.1 prior to the addition of agar and then autoclaved for 20 min at 121°C under 1.1 kg cm^{-2} pressure and media was dispensed into sterile containers under a laminar flow hood.

Plant growth regulator stocks solutions were prepared by dissolving initially in appropriate solvent and making up to 1 mM stock solutions in sterile distilled water. Plant growth regulators were added to the culture medium before autoclaving in all experiments, unless otherwise stated. Antibiotics were prepared by dissolving in nano-pure water to a stock solution of 50 mg mL^{-1} stock solution. Antibiotics were filter sterilized using 0.2 µm filters (Whatman, USA) and added to the media after autoclaving.

Seed sterilization and germination: In the preliminary study, germination of watermelon seeds with coat was tested. Seeds of three watermelon cultivar were surface disinfected for 20 min in 5% (v/v) Clorox, rinsed five times with sterile distilled water and soaked for 0, 16, 40 and 64 h in sterile distilled water. The seeds were dried on a filter paper. The embryos were later placed in culture tubes containing 10 mL MS basal medium.

In another method, the seeds were soaked for 30 min under running water to ease seed coat removal. Each ten peeled seeds were surface sterilized for 15 min in 100 mL (1.5 or 5% (v/v)) Clorox followed by five times rinses with 100 mL sterile distilled water. The seeds were germinated on MS basal medium in culture tubes containing 10 mL medium.

All cultures were incubated in growth room for five days at 25°C with 16 h photoperiod and 20.50 µmols^{-1} m^{-2} of light intensity (light meter-LI-COR® Biosciences, USA). Fifty seeds from each treatment were cultured and repeated twice. After 5 days of incubation, the number of seedlings was counted. Contamination was determined by visual inspection for fungal and/or bacterial growth.

Effect of size, cotyledon age and different concentration of growth regulators (BAP) on shoot regeneration: The cotyledon explants from 3 and 5-day-old seedlings were excised 1-2 mm beyond the point of attachment to the hypocotyls. The cotyledon margins (1 mm) were removed and the cotyledons were cut transversely into two halves where the distal portions were discarded and the proximal tissues were used as explants. The two types of explants (Fig. 1a, b) were cultured abaxial side down on MS

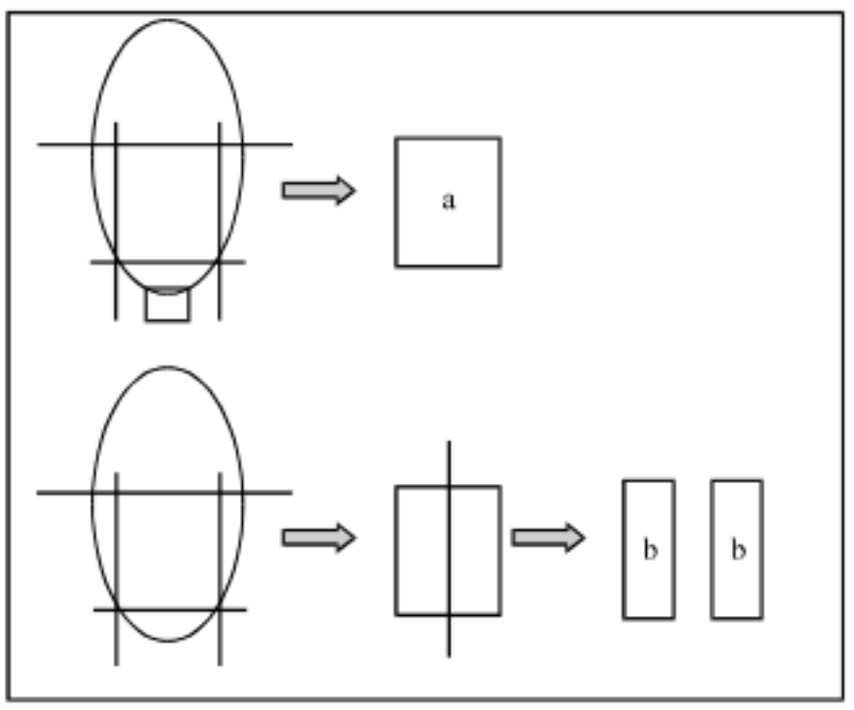

Fig. 1: Dissection of cotyledon explant from a 3 and 5-day-old seedling of yellow watermelon. Explant type a is the cotyledon proximal with 1mm margin removed, explant type b is the half cotyledon proximal

Table 1: The effects of age and size of the explants with different concentrations of BAP on shoot induction from cotyledon sections of cultivar Hwang Fong Yellow Queen

Explant age			
3 day-old		5-day-old	
Type	Concentration of BAP (μM)	Type	Concentration of BAP (μM)
a	0	a	0
	2		2
	4		4
	8		8
	16		16
b	0	b	0
	2		2
	4		4
	8		8
	16		16

medium containing different levels of BAP (0, 2, 4, 8 and 16 μM). The growth regulators were added as in Table 1. The number of shoot buds was counted using stereo microscope (Leica, German) after four weeks in culture.

Explants preparation for shoot regeneration from cotyledon and hypocotyl of watermelon: Explants of cotyledon from 5-day-old seedling were excised 1-2 mm including the part of hypocotyls (Fig. 2A-C). The hypocotyls were prepared by cutting 0.5-1.0 cm long section and culturing it horizontally on regeneration medium (MS medium containing different concentrations of BAP). The distal portions of cotyledons were discarded and the proximal tissues were cultured with abaxial side down. Explants were cultured in 100×15 mm plastic Petri dishes with 25 mL medium containing different concentration of growth regulators for 3 weeks at a 16 h photoperiod. The explants were subcultured to fresh medium in Magenta jar containing 50 mL of regeneration medium after 3 weeks and subculture for another 3 weeks. After 6 weeks in culture, explants with shoot and buds were transferred to MS medium without hormone (MSO) for shoot elongation for another 4 weeks to obtain *in vitro* plantlet.

The effect of growth regulators on shoot regeneration: To examine suitable growth regulators conditions for shoot formation, combination of various concentrations of IAA and BAP were tested. The medium tested were MS basal containing different concentrations of BAP (0, 1, 5, 10, 20 μM) and IAA (0, 0.5, 5 μM). BAP hormones were added before autoclaving and IAA were then added after autoclaving. In this experiment, each treatment consisted of 3 replicates with 10 explants per Petri dish. All experiments were repeated twice. The experiments were designed as shown in Table 2. The data were collected after 3 weeks in culture. The number of shoot buds was

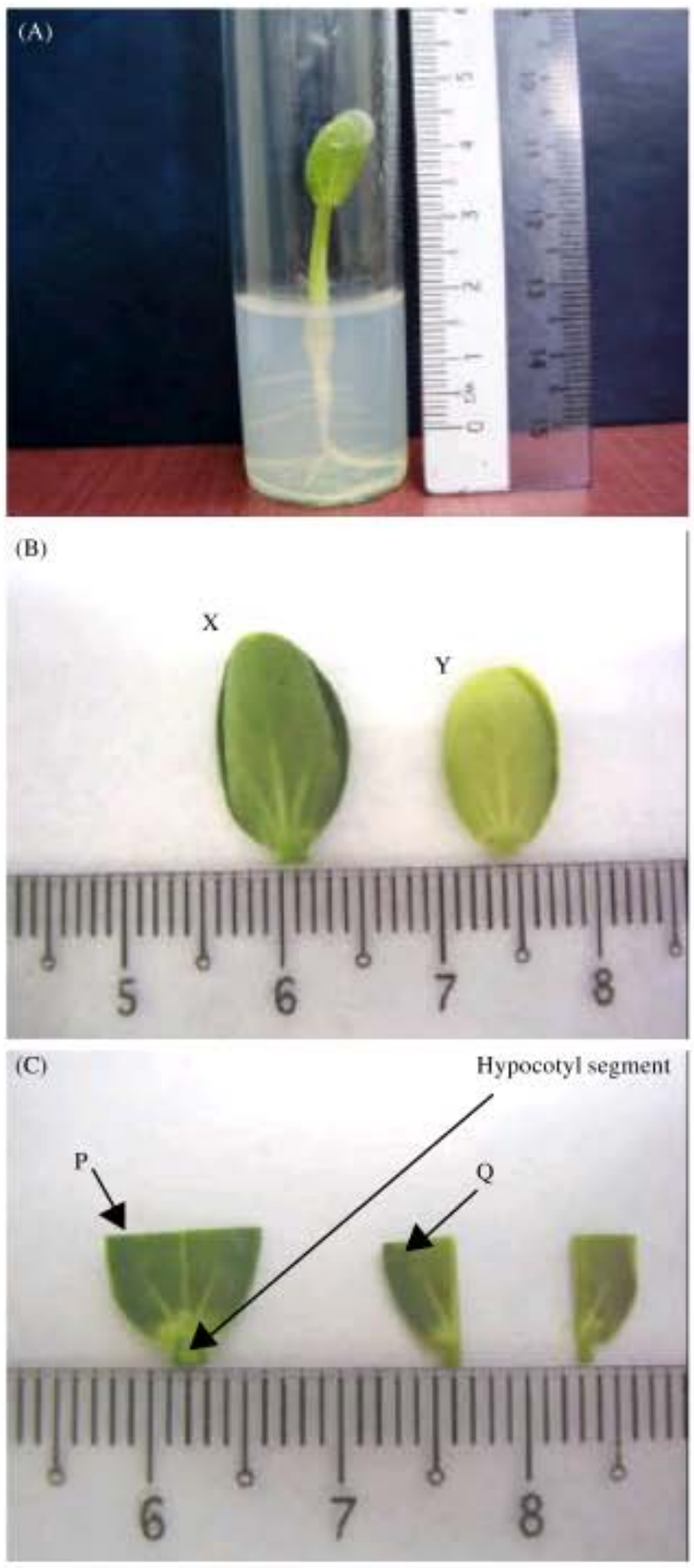

Fig. 2: (A) Five-day-old seedling used as plant material. Cotyledon of cultivar Hwang Fong Yellow Queen, (B) the whole size of cotyledon X=5-day-old, Y= 4-day-old and (C) the size of 5-day-old cotyledon after cutting (indicated by arrow). P: Full hypocotyl segment and Q: Half cotyledon segment

counted using stereo microscope. The explants with shoot buds were sub-cultured to fresh medium in Magenta jar containing 50 mL medium for another 3 weeks and remained on regeneration medium for a total of 6 weeks. Shoot explants were transferred to MSO medium for shoot elongation for another 4 weeks. All cultures

Table 2: The concentrations BAP and IAA used for shoot induction using cotyledon and hypocotyl explants of cultivar Hwang Fong Yellow Queen, Round Dragon and Chin San Seedless

	Cultivar											
	Hwang Fong Yellow Queen				Round Dragon				Chin San Seedless			
	Cotyledon		Hypocotyl		Cotyledon		Hypocotyl		Cotyledon		Hypocotyl	
Treatment	BAP	IAA	BAP	IAA	BAP	IAA	BAP	IAA	BAP	IAA	BAP	IAA
1	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
2	1	0.0	1	0.0	1	0.0	1	0.0	1	0.0	1	0.0
3	5	0.0	5	0.0	5	0.0	5	0.0	5	0.0	5	0.0
4	10	0.0	10	0.0	10	0.0	10	0.0	10	0.0	10	0.0
5	20	0.0	20	0.0	20	0.0	20	0.0	20	0.0	20	0.0
6	0	0.5	0	0.5	0	0.5	0	0.5	0	0.5	0	0.5
7	1	0.5	1	0.5	1	0.5	1	0.5	1	0.5	1	0.5
8	5	0.5	5	0.5	5	0.5	5	0.5	5	0.5	5	0.5
9	10	0.5	10	0.5	10	0.5	10	0.5	10	0.5	10	0.5
10	20	0.5	20	0.5	20	0.5	20	0.5	20	0.5	20	0.5
11	0	5.0	0	5.0	0	5.0	0	5.0	0	5.0	0	5.0
12	1	5.0	1	5.0	1	5.0	1	5.0	1	5.0	1	5.0
13	5	5.0	5	5.0	5	5.0	5	5.0	5	5.0	5	5.0
14	10	5.0	10	5.0	10	5.0	10	5.0	10	5.0	10	5.0
15	20	5.0	20	5.0	20	5.0	20	5.0	20	5.0	20	5.0

were maintained under a 16 h photoperiod at 25°C and 20.50 μmol sec^{-1} m^{-2} of light intensity (light meter-LI-COR® Biosciences, USA) during shoot regeneration.

The effect of growth regulators on root induction of cultivar Hwang Fong Yellow Queen: In rooting experiment, cotyledons were excised from 5-day-old seedlings of cultivar Hwang Fong Yellow Queen and cultured in rooting medium consisting of full or half strength MS media in 100×15 mm plastic Petri dishes. Determination of growth regulators condition for root formation were carried out by testing different concentrations of IAA (0.5, 1.0, 3.0 μM), IBA (0.5, 1.0, 2.5 μM) and NAA (0.5, 1.0, 3.0 μM). Each treatment consisted of 3 replicates with 10 explants per Petri dish and was repeated twice. Root induction was determined after 3 weeks in culture.

Effect of size and age on shoot regeneration of cultivar Hwang Fong Yellow Queen: This experiment was carried out to study the effect of size and age of explants on shoot regeneration using 4 and 5-day-old seedlings of cultivar Hwang Fong Yellow Queen. The effect of the size of explants were tested by cutting the cotyledon into half and cutting it crosswise and lengthwise into 4 pieces as half 5-day-old cotyledon (Fig. 2). The distal portions of cotyledons were discarded and the proximal tissues were cultured with abaxial side down. Excised explants were cultured on the shoot regeneration medium (MS medium containing 20 μM BAP), each experiment consisted of 5 replicates with 10 explants per plate. All experiments were repeated twice.

Statistical analysis: All data were analyzed statistically using SPSS for Windows software (SPSS Windows Version 12). One-Sample t-test was carried out for all data obtained. Analysis of variance (One-Way ANOVA) was used to compare means for more than one treatment on number of shoots per explant. Tukeys Honestly Significant Difference (HSD value) test was used to compare the treatments at $p<0.05$.

RESULTS AND DISCUSSION

Experiments using different concentration of BAP and IAA were conducted to determine the optimized condition for shoot regeneration from cotyledon and hypocotyl of all three watermelon cultivars. Number of shoot, root and callus were observed within three to four weeks in culture. Regeneration of watermelon cultivars was studied involving the following 4 parameters: 1) seed sterilization and germination, 2) shoot regeneration, 3) growth regulators effect on root formation and 4) the effect of explants size and age on shoot regeneration.

Seed sterilization and germination: In the preliminary study on germination of watermelon, the results show that the percentage of germination of watermelon seeds with coat was lower compared to the peeled seeds. Only yellow watermelon seeds with coat were germinated after 5 days in culture (10-40%). The percentage of contamination was higher as a result of water imbibitions on seeds germinated with coat than without coat on all cultivar after 64 h (30-80%). The percentage of mortality was the highest in Chin San Seedless and Round Dragon (100%) as compared to yellow watermelon (60-90%). Thus, the *in vitro* germination of watermelon is more effective by removing the seed coats and was used for further studies.

In the sterilization and germination using seeds without coat shows that contamination on Chin San Seedless was higher in the presence of 1.5% Clorox (35%), (Fig. 3A). Hwang Fong Yellow Queen produced the highest percentage of germination (96%) than Chin San Seedless (80%) and Round Dragon (60%). When the concentration of Clorox increased to 5%, the germination of Hwang Fong Yellow Queens still the highest (96%) followed by Chin San Seedless (64%) and Round Dragon (62%), (Fig. 3B). Contamination was only observed in Chin San Seedless (5%). Thus, the sterilization method using 5% (v/v) Clorox was used for further studies.

In the present study, seeds were soaked for 30 min under running water for ease in removing seed coat. The peeled seeds were disinfected for 15 min in 1.5% (v/v) or 5% (v/v) Clorox followed by rinsing 5 times with sterile distilled water (Krug *et al*., 2005; Jaskani *et al*., 2004; Yalcin-Mendi *et al*., 2003; Dong and Jia, 1991).

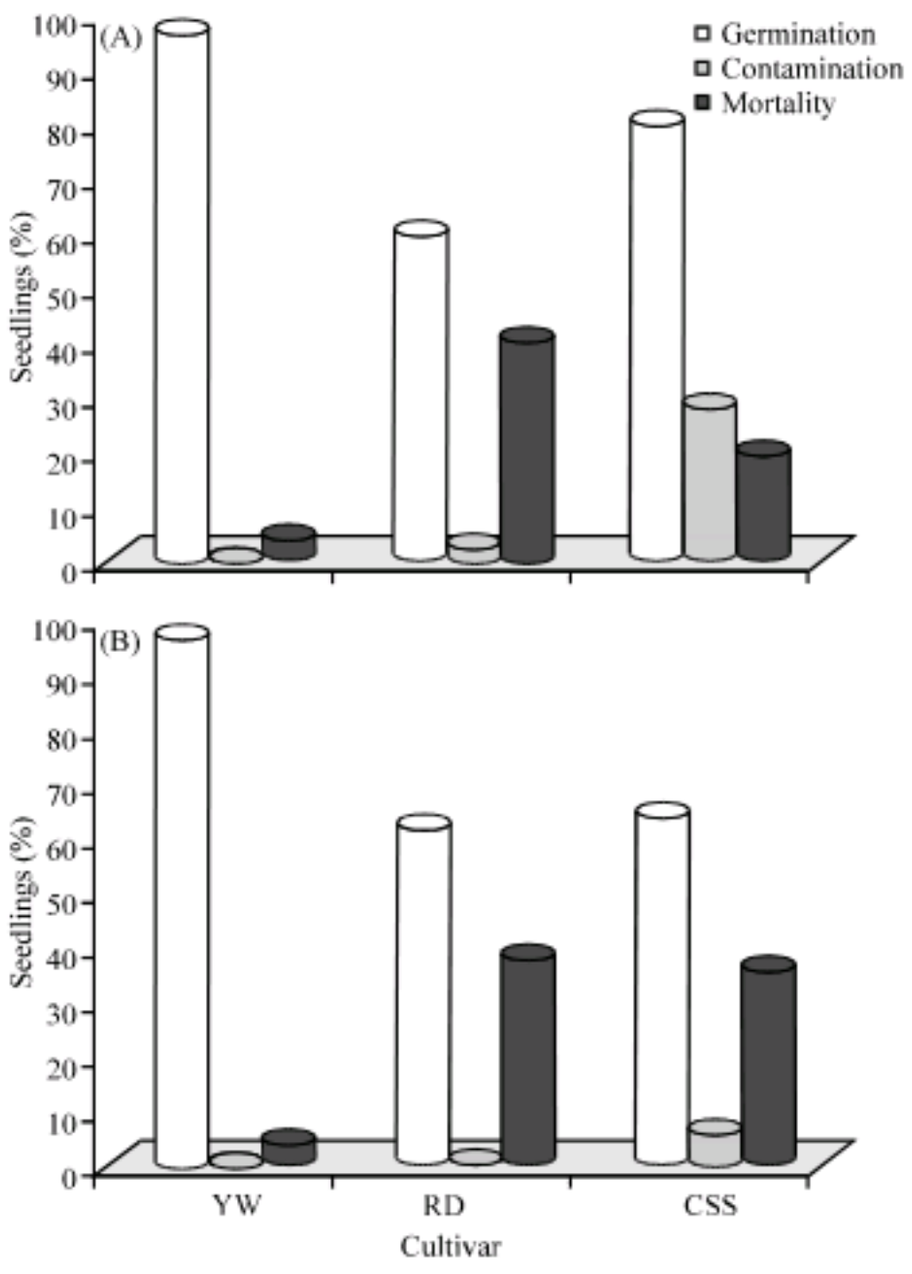

Fig. 3: Percentage of germination, contamination and mortality of watermelon using, (A) 1.5% (v/v) Clorox or (B) 5% (v/v) Clorox after 5 days in culture YW = Hwang Fong Yellow Queen, RD = Round Dragon, CSS = Chin San Seedless. Each treatment consisted of 50 explants and all experiments were repeated twice

The thickness of seed coat could cause *in vitro* germination difficulty. Removal of seed coat could be carried out soaking seed in concentrated acid, chemical or hot water, or removing the seed coats manually (Halimathul, 1995). Other studies on regeneration of watermelon also suggested extracting the seed from the seed coats for the *in vitro* germination of seedling (Srivastava *et al*., 1989; Dong and Jia, 1991; Compton and Gray, 1993; Tabei *et al*., 1993; Compton *et al*., 1996; Compton, 1999; Yalcin-Mendi *et al*., 2003; Pirinc *et al*., 2003; Jaskani *et al*., 2004; Krug *et al*., 2005).

To establish seedlings *in vitro*, seeds were disinfected in sodium hypochlorite (NaOCl) followed by rinsing three to five times with sterile distilled water and soaked for 2 h or overnight in sterile distilled water to ease seed coat removal (Krug *et al*., 2005; Pirinc *et al*., 2003; Compton *et al*., 1996). Others reported the used of running water to soak seeds to ease seed coat removal (Jaskani *et al*., 2004). Peeled watermelon seeds were surface sterilized in commercial bleach (Clorox) solution or NaOCl for 15-30 min followed by rinsing with sterile distilled water three to five times (Krug *et al*., 2005; Jaskani *et al*., 2004; Yalcin-Mendi *et al*., 2003; Dong and Jia, 1991).

Preliminary study on shoot regeneration using cotyledon sections: Adventitious shoot regeneration of cotyledon sections was studied by manipulating parameters such as size of explants (type a and b), age of explants (3-day-old seedling and 5-day-old seedling) and different concentration of BAP (0, 2, 4, 8 and 16 μM). Cotyledon explants were shown to form adventitious shoots and calli after four weeks on MS medium supplemented with BAP at concentration of 2, 4, 8 and 16 μM. The adventitious shoots were formed at the cut edge of cotyledon sections. An accurate determination of the number of shoot buds developed per explant is very difficult, because organogenesis occurred in clusters and not as individual buds (Fig. 4). The explants with multiple shoots were transferred to elongation media, however single shoots were found difficult to be separated for development of complete plants.

The use of half proximal tissue (type b) from 3-day-old seedling explants resulted in higher percentage (92%) of explants that induced adventitious shoots and the mean number of shoots per explant (7.63±0.96) as compared to explants from 5 day-old seedling (63% and 1.96±0.39) when cultured on MS medium supplemented with 16 μM of BAP (Fig. 5A, B). However, callus was observed at the cut edges of the explants before it turned necrosis and died during continuous subcultures and resulted in inhibition of shoot formation and the size of shoot.

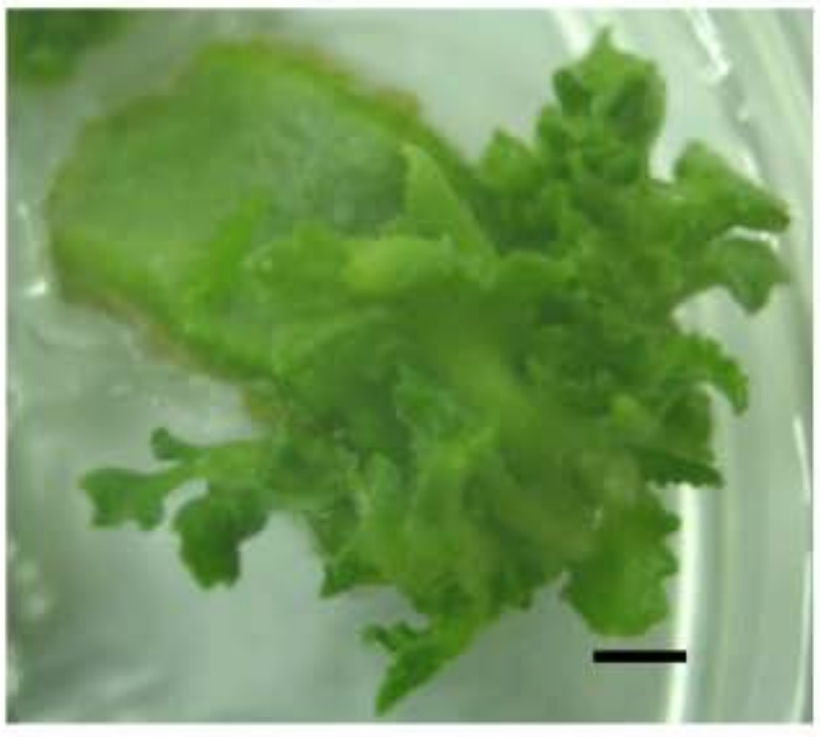

Fig. 4: *In vitro* organogenesis of cultivar Hwang Fong Yellow Queen from cotyledon sections of 3-day-old seedling Adventitious shoots on half proximal explant after 4 weeks on MS medium supplemented with 16 µM BAP. Bar scale = 5 mm

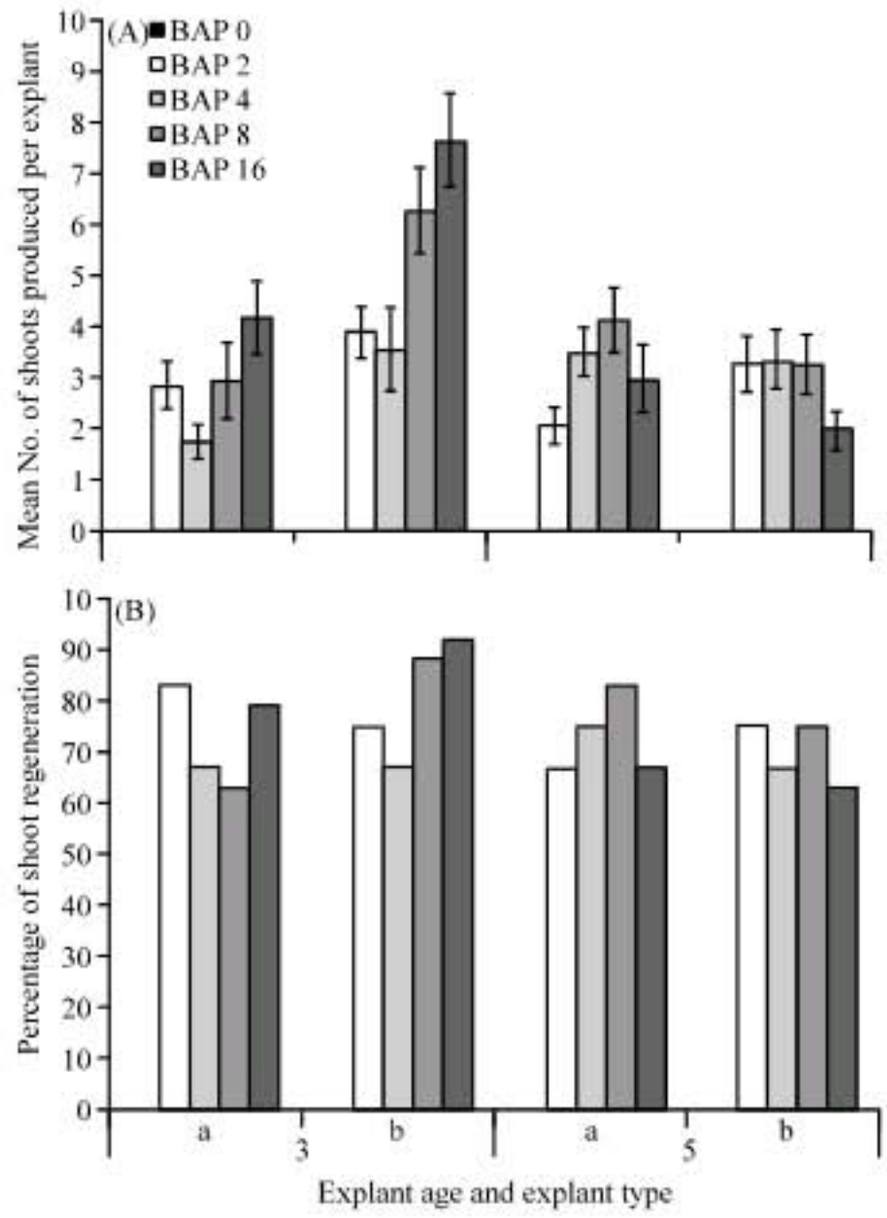

Fig. 5: (A) Effects of different BAP concentrations on different sizes and ages of explants and production of adventitious shoots. Data were subjected to ANOVA with 8 explants per treatment tissue. (B) Percentage of shoot regeneration from different sizes and ages of cultivar Hwang Fong Yellow Queen cotyledons sections. Data presented as means±SE Each treatment had three replicates and the experiment was conducted twice a = Proximal tissues b = Half proximal

From one way ANOVA analysis, the explants from 3-day-old and 5-day-old seedling (type b) were significantly different on mean number of shoots produced per explant. The results also shows that lower concentration of BAP (2 and 4 µM) produced shoots without callus. The high BAP concentration (8 and 16 µM) resulted in higher number of shoot buds with the presence of some callus.

Cotyledon segments obtained from 3-day-old seedlings responded with adventitious bud formation at a much higher percentage than explants collected from 5-day-old seedlings. The results from one way ANOVA analysis showed mean number of adventitious shoot regenerated from 3-day-old (size b) was significantly difference with 5-day-old seedling. Tabei *et al*. (1993) reported that cotyledon from 1-day-old and 3-day-old seedlings showed the highest frequency of shoot regeneration in watermelon. This was similar to Krug *et al*. (2005) who reported that cotyledon segments of watermelon collected from 3-day-old seedling responded with high adventitious bud percentage than explants collected from either 1 or 5-day-old seedlings. It was reported that highest shoot were obtained from cotyledon derived from 7 days old seedling of *Impatiens balsamina* as compared to 14 and 21 days old seedlings (Taha *et al*., 2009). A possible explanation for the above observation is that young cotyledons are physiologically very active and respond efficiently to exogenous hormones (Dong and Jia, 1991).

Besides seedling age, explants size has shown to be important for morphogenesis induction because shoot formation in cucurbit seems to be restricted to specific cotyledon regions (Choi *et al*., 1994; Compton, 2000; Ananthakrisnan *et al*., 2003). The proximal end of cotyledons has the greatest regeneration rate than distal end of cotyledon in term of number of explants with shoots (Krug *et al*., 2005). Compton (2000) reported the percentage of cotyledon explants that produced adventitious shoots about 52% of explants prepared from the proximal region of cotyledons formed shoots, whereas only 6% of distal explants did so. In the study, many shoots appeared from proximal end of basal region and cut edge at the vein of cotyledon. These facts agrees with the earlier finding that the proximal end of basal region and cut edge of vein has high potential for shoot formation (Compton, 2000; Compton and Gray, 1993).

The results from preliminary study on shoot regeneration of cotyledon sections showed the percentage of cotyledon explants induced shoots or the mean number of shoots per explant was the highest in half proximal tissue (size b) from 3-day-old seedling when cultured on MS medium supplemented with 16 µM of BAP (7.63±0.96). High BAP concentration also resulted in higher number of shoot buds but the shoots were small and calli were observed at the cut edge of the explants. Shoot regeneration observed in this study was not much different to Krug *et al*. (2005) who reported almost the same mean number of shoots per explant from 3-day-old seedling of watermelon when the MS medium was supplemented with 13.2 µM of BAP (4.60±2.70). They also reported higher number of explants with adventitious shoots from cotyledon of watermelon when using a combination of BAP (4.4 µM) with coconut water (10% v/v) but no significant differences were detected when compared with the treatment of 8.8 µM BAP. Srivastava *et al*. (1989) also reported the induction of adventitious shoots from cotyledon or hypocotyl of watermelon on 4.5 µM BAP. These reports showed that shoot formation in watermelon required lower concentrations of BAP. In *Robinia pseudoacacia* L. it was also reported that lower concentration of BAP is required to induce shoots from callus (Kanwar *et al*., 2009). Shoots were produced on MS medium supplemented with 0.5 µM NAA and 1 µM BAP and the shoots were multiplied on MS medium containing 1.5 µM BAP. Similarly in *Impatiens balsamina*, highest rate of shoot induction was obtained on MS medium containing 1 mg L^{-1} BAP (Taha *et al*., 2009). On the other hand, however, it was reported in high starch sweet potato (*Ipomoea batatas* L.) shoots were only produced on medium containing IAA or NAA and not on medium containing BAP (Santa-Maria *et al*., 2009). Only compact callus were obtained on medium containing BAP.

Although, a high number of adventitious shoots apparently formed on the explants in the preliminary study, determination of the number of buds developed per explant is very difficult, because organogenesis occurs in clusters and not as individual buds. Transferring the explants with multiple shoots to grow up in rooting media makes the explants difficult to be separated and results in very low number to develop. Similar observation with previous report of Krug *et al*., (2005) which observed that the apparently large number of buds during induction resulted in low number of plants after transfer to MSO medium for elongation. Therefore, for the subsequent study, cotyledon with hypocotyl segment was used as an explant to regenerate shoots direct from the meristem region.

The effect of growth regulators on shoot regeneration: To study the effects of growth regulators on shoot regeneration the cotyledon was exposed to MS basal medium containing different concentrations of BAP and IAA. Results show that multiple shoot buds differentiated directly from meristem region of cotyledon after 2 weeks in culture. Hwang Fong Yellow Queen showed the highest percentage of shoots production (100%) when cultured on 20 µM BAP at 9.83±0.54 mean number of shoots per explant and significantly different when compared to 0, 1 and 5 µM ($p = 0.00$, 0.00 and 0.01) (Table 3). However, it is not significantly different to 10 µM BAP and combinations of 10 µM BAP: 0.5 µM IAA, 20 µM BAP: 0.5 µM IAA and 20 µM BAP: 0.5 µM IAA.

Table 3: Effect of different combination and concentrations of BAP and IAA on shoot induction from cotyledon of cultivar Hwang Fong Yellow Queen, Round Dragon and Chin San Seedless, after 3 weeks in culture

Growth regulators (µM)		Cultivar: Hwang Fong Yellow Queen		Round Dragon		Chin San Seedless	
BAP	IAA	Mean No. of shoot per cotyledon±SE	Percentage of shooting	Mean No. of shoot per cotyledon±SE	Percentage of shooting	Mean No. of shoot per cotyledon±SE	Percentage of shooting
0	0.0	0.93±0.05^{d}	93	0.77±0.08^{z}	73	0.87±0.12^{s}	77
1	0.0	4.43±0.32^{c}	97	1.47±0.20^{y}	73	3.37±0.32^{r}	100
5	0.0	7.07±0.85^{b}	93	3.77±0.39^{x}	87	8.87±0.81^{p}	100
10	0.0	8.67±0.80^{a}	100	6.00±0.32^{w}	100	7.73±0.98^{p}	93
20	0.0	9.83±0.54^{a}	100	5.83±0.64^{w}	87	6.27±0.56^{q}	97
0	0.5	0.97±0.03	95	0.73±0.08	77	1.00±0.00	97
1	0.5	3.03±0.21	97	2.00±0.30	70	2.80±0.37	97
5	0.5	3.30±0.38	93	4.50±0.40	93	7.43±0.76	93
10	0.5	8.50±0.78	93	5.23±0.38	97	8.73±0.89	93
20	0.5	8.43±0.76	98	5.80±0.46	90	7.17±0.98	90
0	5.0	1.00±0.00	100	0.60±0.09	30	1.03±0.10	90
1	5.0	3.10±0.38	93	3.07±0.19	100	2.27±0.34	73
5	5.0	7.13±0.93	95	3.67±0.41	95	2.87±0.39	87
10	5.0	8.70±0.77	100	4.17±0.43	90	6.17±0.67	93
20	5.0	7.63±0.74	97	5.27±0.49	95	7.97±0.88	90

Each treatment consisted of 30 explants and all experiments were repeated twice. Data were subjected to ANOVA with 10 explants per treatment. Data presented as means±SE. Each treatment had three replicates and this experiment was conducted twice. a, b, c and d were significant differences at $p \leq 0.05$. The significance levels (Tukey HSD) between treatments

Chin San Seedless shows significantly highest percentage of shoots production (100%) when cultured on 5 μM of BAP at 8.87±0.81 mean number of shoots per explant as compared to 0, 1, 20 μM BAP (p-values = 0.00, 0.00 and 0.037) (Table 3). However, it is not significantly different to 10 μM BAP and combinations of 10 μM BAP:0.5 μM IAA and 20 μM BAP:5 μM IAA. Round Dragon shows significantly highest percentage of shoots production (100%) when cultured on 10 μM of BAP at 6.00±0.32 mean number of shoots per explant as compared to 0, 1, 5 μM BAP (p = 0.00) (Table 3). However, it is significantly different to 20 μM BAP and combination of 20 μM BAP: 0.5 μM IAA.

All cultivars tested showed that the combination of BAP with 0.5 or 5 μM IAA did not improve the mean number of shoots per explant. The results also showed that the increment of BAPs concentration (10-20 μM) resulted in higher number of shoot buds. However, the shoots were smaller. Lower concentration of BAP (1-5 μM) resulted in bigger and longer shoots (Fig. 6A-C). Similar results were obtained when the combined concentration of BAP and 0.5 or 5 μM IAA were used. When culture proceeded, the shoot buds became elongated and generates distinct shoots after 6 weeks in culture. After sub-cultured on MS basal without hormone, shoots were elongated and some of the shoots induced roots (Fig. 7A, B).

Shoots regenerated from cotyledon with hypocotyl segment of the three watermelon cultivar were also assessed. The results showed that the mean number of shoots per explant was highest in Hwang Fong Yellow Queen (9.83±0.54) when cultured on MS medium supplemented with 20 μM BAP than, Chin San Seedless (8.87±0.81) with 5 μM of BAP and Round Dragon (6.00±0.32) with 10 μM of BAP. In this study, BAP was best in the range of 5-20 μM concentration for shoot formation of the three cultivars of watermelon cotyledons.

Shoot regeneration observed for yellow watermelon hybrid used in this study is similar to the study by Yalcin-Mendi *et al*. (2003) which demonstrated that the shoot regeneration of watermelon cotyledons was best obtained on 20 μM BAP. In contrast, Pirinc *et al*. (2003) found that the highest mean number of shoots per explant from 5-day-old seedlings cotyledon of diploid watermelon cultivar Surme was obtained when explants were cultured on 2 and 4 μM BAP. Furthermore, shoots obtained on 16 μM BAP or higher were abnormal (thick and stunted). The difference on the effect of BAP reported by the various studies may be due to the different in cultivar used.

Experiment for shoot regeneration using hypocotyl showed no response to the treatments where shoot buds was not observed. However, roots were only induced on

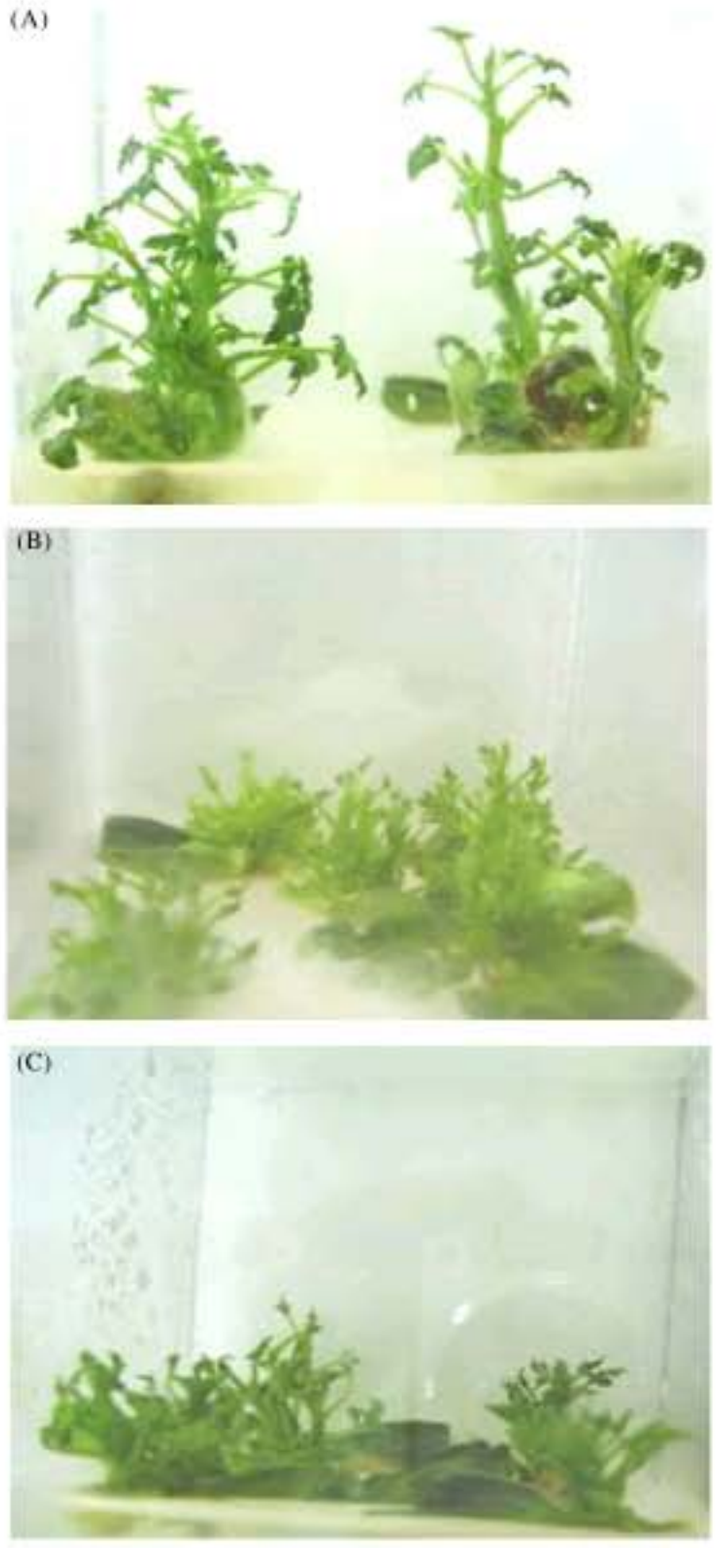

Fig. 6: Shoots regenerated from cotyledon explants of cultivar Hwang Fong Yellow Queen on MS medium supplemented with different concentration of BAP after 5 weeks in culture, (A) 1 μM BAP, (B) 5 μM BAP and (C) 10 μM BAP. Bar = 12 mm

MS medium supplemented with either 0.5 or 5 μM IAA after 3 weeks on culture. All of hypocotyl explants used only expanded in size. Therefore, only the cotyledon was used as explants in subsequent experiments.

The current study showed that cultivar Hwang Fong Yellow Queen proximal explants produced the highest percentage of shoots (100%) when cultured on MS medium containing 20 μM BAP. In contrast, other researchers reported that highest percentage of adventitious shoot had been achieved on 4.4-10 μM BAP (Compton, 1999). The results obtained in this study also indicated that the optimum shoot regeneration of watermelon was achieved when cotyledon of Hwang Fong Yellow Queen was used. Thus, the factor

Fig. 7: Shoot elongation (A) shoot regeneration on MS medium containing 20 µM BAP after 6 weeks in culture and (B) shoot elongation after 3 weeks on MS basal without hormone. Bar = 10 mm

influencing shoots regeneration that need to be considered includes the source of seed and genotype (Nasr *et al*., 2004).

Previous studies on shoot regeneration resulted in adventitious shoot from diploid watermelon cotyledons excision by making a cut 1-2 mm beyond the point of attachment to the hypocotyl (Krug *et al*., 2005; Pirinc *et al*., 2003; Compton, 1999). The results showed that multiple shoot buds differentiated directly from meristem region of cotyledon. Failure to obtain adventitious shoot may be due to the inhibition of the adventitious shoots by meristemic protrusion. Tabei *et al*. (1993) reported that the proximal end of cotyledon segment regenerate shoots more effectively from the cut surface at the border of hypocotyl and cotyledon. These facts supported that the proximal end which was very close to the meristem region had the high potential for shoot formation.

Plant growth regulators effect on rooting of cultivar Hwang Fong Yellow Queen cotyledons: Root was induced from cotyledons when cultured on either full or-half strength MS medium. The IAA and IBA were found to be most effective at different concentration tested for inducing roots as compared to NAA. A total of 100% of explants induced roots on half-strength MS containing 0.5 and 3.0 µM IAA and 2.5 µM IBA (Table 4). The result shows that half-strength MS+3.0 µM IAA induced the formation of more adventitious or root hairs but the roots are thinner and longer. The adventitious or root hairs formed bigger and longer on half strength MS containing 2.5 µM IBA than on half-strength MS containing 3.0 µM IAA. Therefore, half-strength MS containing 2.5 µM IBA was chosen for subsequent experiment for root induction.

Table 4: The effect of different concentrations of auxins on root formation from the cotyledon of cultivar Hwang Fong Yellow Queen after 3 weeks in culture

		Rooting (%)	
Type of auxin	Concentration (µM)	Half strength MS	Full strength MS
Control	0.0	20	47
IAA	0.5	100	50
	1.0	85	80
	3.0	100	87
IBA	0.5	70	40
	1.0	87	97
	2.5	100	87
NAA	0.5	87	85
	1.0	93	77
	3.0	85	47

In the preliminary study on rooting, the percentage of root induction was the highest on half strength MS containing 2.5 µM IBA, however, many of the leaves were necrosis after 4 weeks culture. This may be due to the high auxin concentration inhibited the shoot development (Compton and Gray, 1993). When the shoots were successfully rooted on different concentration of IAA (0.5, 1.0 and 3.0 µM) and IBA (0.5, 1.0 and 3.0 µM) on half-strength MS medium, the result showed that 80% of the shoots induced root after four weeks on medium containing 0.5 µM IAA. Compton and Gray (1993) reported that shoots can be easily rooted in medium with 1 µM IBA. However, 0.5 µM IAA worked well in present studies and it may be due to variable genotypic response to auxins. In contrast, Sultana and Bari (2003) described that 100% shoots were rooted on half-strength MS medium with 0.54 µM NAA. Similar observation reported by Dong and Jia (1991), where roots were successfully induced on 0.54 µM NAA. In Mexican husk tomato (*Physalis ixocarpa* Brot.), roots were successfully regenerated on half-strength MS medium supplemented with 2.85 µM IAA (Escobar-Guzman *et al*., 2009). However, on the other hand, in *Impatiens balsamina* IBA produced highest percentage of rooting on half-strength MS medium containing IBA as compared to IAA (Taha *et al*., 2009). But, IAA produced more hairy roots

Fig. 8: Regeneration of cultivar Hwang Fong Yellow Queen plants, (A) plantlet on half strength MS + 2.5 μM IBA after 4 weeks, (B) plantlet on half strength MS + 0.5 IAA after 4 weeks and (C) regenerated plant on soil after 3 weeks. Bar = 15 mm

and longer roots. Similarly, in *Daphne* L. species, root development was best obtained on medium containing either IBA or NAA as compared to IAA (Noshad *et al*., 2009). Generally it was also reported that no roots were formed in medium absence of any auxins. It was also observed that better rooting was observed on half-strength MS medium. It was earlier reported that the favorable effects of a lower concentration (diluted) mineral solution on rooting can be explained by the reduction of nitrogen concentration. Fotopoulus and Sotiropoulus, (2005) has reported the increased in rooting percentage in *Prunus persica* on reduced mineral concentration of MS medium (half-strength MS) supplemented with IBA.

Root induction of regenerated shoots on half strength MS with auxins: The rooting experiment was carried out on regenerated shoots using different concentrations of IAA (0.5, 1.0 and 3.0 μM) and IBA (0.5, 1.0 and 2.5 μM). The result showed 80% of the shoots induced root after four weeks on half-strength MS containing 0.5 μM IAA (Table 5). The healthy plantlets were also obtained on this medium (Fig. 8A-C). After four weeks in rooting medium, the plantlets were transferred onto pots containing 1:1 (v/v) soil and vermiculate and incubated in conditions similar to *in vitro* cultures (16 h photoperiod at 25°C) for one week. Plants (F_0) were watered every day and then the plants were transferred onto soil (Fig. 8C). Using these conditions, 50% of the plants died after 3 weeks under mist because the plants became saturated and rapidly rot. The time frame required from seedling germination to acclimatization of the first plant was about 15 weeks.

Table 5: The effect of different concentrations of auxins on root induction from regenerated shoots

Type of auxin	Concentration (μM)	Percentage of rooting (%)
IAA	0.5	80
	1.0	60
	3.0	70
IBA	0.5	65
	1.0	70
	2.5	75

The ability of shoots to produce root or plants to survive acclimatization is dependent on the conditions provided. Plantlet should be well developed (minimum 16 mm high), possessing a minimum of one tap root at least 1 cm length and placed in small containers (3.3×5.1 cm plugs) containing medium amended with an equal volume of course grade vermiculite or perlite (Compton *et al*., 1993). In present study, the plantlets were transferred into pots containing 1:1 (v/v) soil and vermiculate and incubated in conditions similar to *in vitro* cultures (16 h photoperiod at 25°C) with high humidity for one week. Plantlets were watered every day and then the plantlets were transferred to the greenhouse. Using these conditions, only 50% of the plantlets were acclimatized into soil and plants were died after 3 weeks. This may be due to different place or condition of planting area. In a previous study, only watermelon shoots longer than 1.6 cm was capable of efficient rhizogenesis and acclimatization at 90-100% (Compton and Gray, 1993). Chaturvedi and Bhatnagar (2001) also reported that regenerated shoots and plantlet were established in pots with 55% success. Factors that affect percentage of plantlet surviving during acclimatization include the

cultivar, the genotype, the amount of time in culture and different techniques (Pirinc *et al*., 2003). Therefore, studies on these external factors such as containers, soilless medium amended with equal volume of vermiculite or perlite and time of containers covered with a plastic humidity dome are important and the use of the growth chamber before transplanting into soil was suggested.

CONCLUSION

In this study, we have successfully evaluated the shoot regeneration ability using cotyledons derived from seedlings of diploid and triploid yellow watermelon (cultivars Hwang Fong Yellow Queen, Round Dragon and Chin San Seedless). Shoot regeneration system for watermelon was successfully established using cotyledon sections of 4 to 5 day-old *in vitro* seedling. It was found that cultivar Hwang Fong Yellow Queen showed the highest percentage of germination and mean number of shoots as compared to the other two cultivars. Subsequently the cultivar also successfully rooted on half strength MS medium supplemented with 0.5 µM IAA. Fifty percent from these rooted plantlets were successfully acclimatized on soil.

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