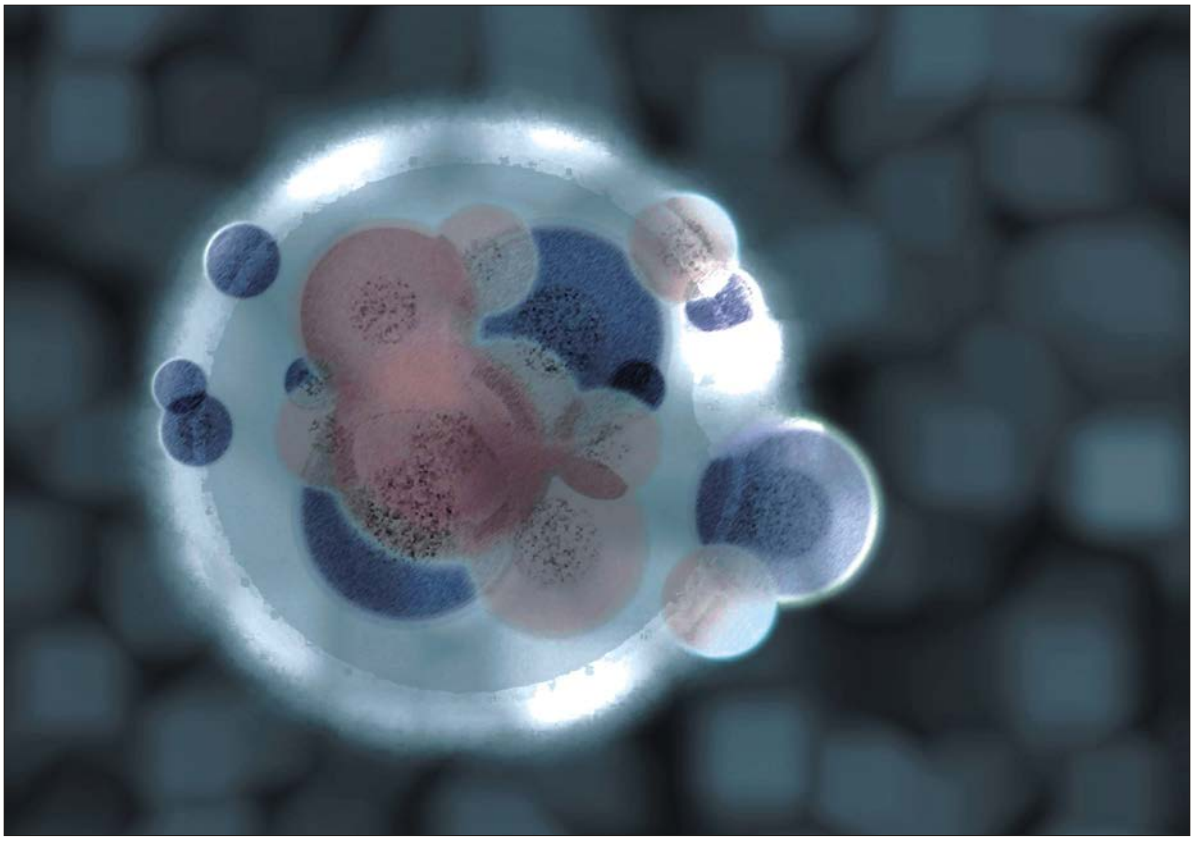

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In vitro Regeneration of *Citrullus lanatus* cv. Round Dragon

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Abstract: The objective of the current study was to establish an efficient and reproducible *in vitro* plant regeneration protocol using cotyledonary explant for *Citrullus lanatus* cv. Round Dragon. To achieve optimal conditions for adventitious shoot induction, cotyledon explants of 5-day-old seedlings, 7-day-old seedlings and 9-day-old seedlings were tested for regeneration potential on Murashige and Skoog (MS) media supplemented with 2.3 mg L^{-1} BAP. Results showed that high frequency of *in vitro* adventitious shoot regeneration was induced from the proximal region of 5-day-old seedlings (93%) with 19.80±0.99 shoots per responding explant after 6 weeks. Adventitious shoots induced from 5-day-old seedlings after 6 weeks were transferred to MS shoot regeneration medium without plant growth regulator for shoot elongation for 4 weeks. The influence of various concentrations of IBA, IAA and NAA on root initiation was examined on half-strength and full-strength of MS rooting medium. The best response for root initiation was obtained from the microshoots grown in full-strength MS rooting medium compared to the half-strength MS rooting medium. Furthermore, IBA was more efficient in promoting root induction than IAA and NAA, resulting in a higher rate of root initiation (100%) at the concentration of 0.1 mg L^{-1} IBA. Therefore, elongated shoots were rooted in MS medium supplemented with 0.1 mg L^{-1} IBA for 3 weeks. Rooted plantlets were acclimatized successfully under *ex vitro* conditions.

Key words: *Citrullus lanatus*, round dragon, cotyledon explants, regeneration, watermelon

INTRODUCTION

Citrullus lanatus (watermelon) is an important cucurbit crop species and one of the major fruit grown in the warmer region of the world. The development of an efficient *in vitro* regeneration system for *Citrullus lanatus* is essential for further genetic transformation studies and application of gene transfer technology. Dong and Jia (1991) had reported earlier that less attention has been given to *in vitro* regeneration of watermelon via tissue culture, where the only *in vitro* system available was propagation by shoot tip culture (Barnes, 1979). In addition, Pech *et al*. (2007) noted that regeneration of watermelon is largely dependence on various factors such as genotype, explant types, explant ages and plant growth regulator inclusion for the capacity to regenerate. Krug *et al*. (2005) suggested that the choice of explant and seedling age at the time of explant preparation could influence the efficiency of *in vitro* shoot regeneration of watermelon.

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

Shoot regeneration for watermelon from cotyledon explants has received attention in the recent past and had promising applications in the areas of genetic transformation (Pirinc *et al*., 2003). In addition, Debeaujon and Branchard (1993) claimed that adventitious shoots of Cucurbits had a higher regeneration potential via somatic embryogenesis when explants derives from seedlings especially cotyledons were used. Several protocols of *in vitro* regeneration of *Citrullus lanatus* have been established via somatic embryogenesis (Compton and Gray, 1993; Compton *et al*., 1996; Chaturvedi and Bhatnagar, 2001).

Thus, the purpose of this study is to develop an efficient and reproducible *in vitro* plant regeneration protocol for *Citrullus lanatus* cv. Round Dragon. In the present study, cotyledon were used as explants to

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establish shoot regeneration in *Citrullus lanatus* via somatic embryogenesis and a study was undertaken to develop *in vitro* rooting with suitable plant growth regulator supplemented on MS solid medium. As an initial step to achieving this goal, the ability of different cotyledon explant ages to induce shoots and the effectiveness of type as well the concentration of various plant growth regulators to initiate roots were evaluated in order to establish significant regeneration system of *Citrullus lanatus*.

MATERIALS AND METHODS

Plant tissue culture media: Germination medium used was Murashige and Skoog (MS) salt supplied with 20 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol and 3.2 g L^{-1} phytagel. Shoot-regeneration medium (MS medium) supplied with 2.3 mg L^{-1} BAP, 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol and 3.2 g L^{-1} Phytagel. Root-inducing medium (MS medium) contained 0.1 mg L^{-1} IBA, 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol and 3.2 g L^{-1} Phytagel.

Plant material and shoot regeneration: *Citrullus lanatus* cv. Round Dragon was used as experimental material in this study. *Citrullus lanatus* seeds imported from Taiwan were purchased from local supplier ACE Seed Trading (Malaysia) Sdn Bhd. The seeds were rinsed thoroughly for 30 min under running tap water. The seed coat was removed and the embryos were surface sterilized for 15 min by immersing in 5% (v/v) sodium hypochlorite (commercial bleach) followed by successive five times rinses with sterile distilled water to remove the trace of sterilent. The seeds were then germinated on germination medium.

Cotyledons were excised from 5-day-old seedlings and cut crosswise into distal and proximal halves with 2 mm of hypocotyls segments using scalpel blade. The distal half was discarded and the proximal tissue was cultured horizontally in 90×15 mm plastic Petri dishes that contained 25 mL of shoot regeneration medium. All cultures were incubated for 3 weeks at 25±1°C with 16 h photoperiod under 12.16 μmol/m/sec from cool white fluorescent lamps in a tissue culture chamber. After 3 weeks, explants with adventitious shoots were subcultured to fresh shoot-regeneration medium in Magenta jar for another 3 weeks. Explants with shoots were transferred to Magenta jar that contained 50 mL of shoot regeneration medium without hormone for 4 weeks for shoot elongation.

After shoot regeneration for a total of 10 weeks, shoots longer than 2 cm were excised and transferred to 50 mL of root induction medium in Magenta jar for 3 weeks. After 3 weeks on rooting medium, the plantlets were transplanted under *ex vitro* condition into plastic pots containing autoclaved soil and sand in the proportion of 1:1. All the pots were covered with clear plastic covers for 7 days to maintain high humidity and incubated at 25±1°C under 16 h photoperiod in a tissue culture chamber. Plantlets in the pot were watered every 2 days with nutrient solution. After 7 days, the humidity was reduced by progressively removing the lids over 3 days. During this time, plants are watered 2 to 3 times alternatively with nutrient solution and water.

Optimizing shoot regeneration: To optimize shoot regeneration experiments, the effect of ages of *in vitro* germinated seedlings were tested for regeneration potential. The influence of cotyledonary explants age was evaluated using cotyledon excised from 5 days, 7 days and 9 days old seedlings after placement of the seeds onto germination medium. Cotyledons were excised 1-2 mm beyond the point of attachment to the stem and the distal halves were discarded. The proximal tissue was cultured abaxial side down in 90×15 mm plastic Petri dishes (Brandon) that contained 25 mL of regeneration medium for 3 weeks.

The explants were subcultured to fresh medium in sterile wide mouth conical flask (GQ) containing 100 mL of regeneration medium after 3 weeks and remained in conical flask for a total of 6 weeks. Shoot induction efficiency based on cotyledonary explant age was assessed using two criteria: the percentage of explants with shoots and the number of shoots per responding explant were counted by stereomicroscope after 6 weeks on shoot induction medium.

Rooting of micropropagated shoots: Prior to rooting studies, an adequate number of microshoots for rooting experiment were established from 5-day-old seedlings germinated *in vitro*. Regenerated adventitious shoots (with length >2 cm) were excised aseptically from the cotyledon explants for rooting after 3 weeks. The shoots were then cultured individually in Magenta jar (MAGENTA) with 50 mL of rooting medium having different strength and combination of auxins. Rooting *in vitro* were tested on half-strength MS salts (1/2 MS) and full strength MS salts supplemented with 30 g L^{-1}sucrose, 100 mg L^{-1} myo-inositol, 3.2 g L^{-1} Phytagel and different concentrations of IBA (0, 0.1, 0.5, 1.0 and 10.0 mg L^{-1}) , IAA (0, 0.1, 0.5, 1.0 and 3.0 mg L^{-1}) and NAA (0, 0.1, 0.5, 1.0 and 10.0 mg L^{-1}). Auxins were filter sterilized prior adding into the autoclaved medium. Cultures were incubated under a 16 h photoperiod of

12.16 µmol/m/sec from cool white fluorescent lamps at 25±1°C in a tissue culture chamber for 3 weeks. At week 3, the frequency of shoots that developed roots and the number of roots per shoot were counted.

Statistical analysis: All statistical analysis was performed using SPSS for Windows software (SPSS Windows Version 15). The experimental design were completely randomized with each experiment were performed with three replicates per treatment and each set within the replicate consisted of 10-20 explants. Data were analyzed using one-way ANOVA (analysis of variance) and Tukey's Honestly Significant Difference Test (HSD value) and the significance were determined at the $p<0.05$ level. Data are presented as means and standard errors.

RESULTS

Effect of explant age on shoot regeneration: Cotyledon explants from 5, 7 and 9 days old seedlings were tested for the efficiency of shoot regeneration on MS medium supplemented with 2.3 mg L^{-1} BAP alone for 6 weeks. Six weeks exposure of explants on MS medium containing 2.3 mg L^{-1} BAP led to high frequency of shoot formation on 5-day-old seedlings. The shoots on 5-day-old seedlings were longer and appeared earlier and grew vigorously than those on 7 and 9-day-old seedlings (Fig. 1a-f). Apart from that, 5-day-old seedlings produced significantly higher number of shoots than 7 and 9-day-old seedlings (Table 1). On the other hand, explants from 7-day-old seedlings produced fewer adventitious shoots while shoots from 9-day-old seedlings remained short and smaller in number even after 6 weeks in culture. In this experiment, cotyledon explants from 5-day-old seedlings of *Citrullus lanatus* found to be more potential to regenerate rather than 7 and 9-day-old seedlings.

***In vitro* rooting experiment:** For the initiation of roots, microshoots (>2 cm) derived from 5-day-old seedlings were individually transferred to half strength and full strength MS medium each supplemented with various concentrations of IBA, IAA and NAA for 3 weeks. Among different concentrations of auxins tested in half strength media, IBA was found to be comparatively better to response than IAA and NAA. It was observed that the microshoots induced high frequency of roots in half strength MS medium supplemented with 0.1 mg L^{-1} IBA followed by 0.5 and 1.0 mg L^{-1} IBA, however no significance differences were observed for these concentrations (Table 2). On the other hand, microshoots

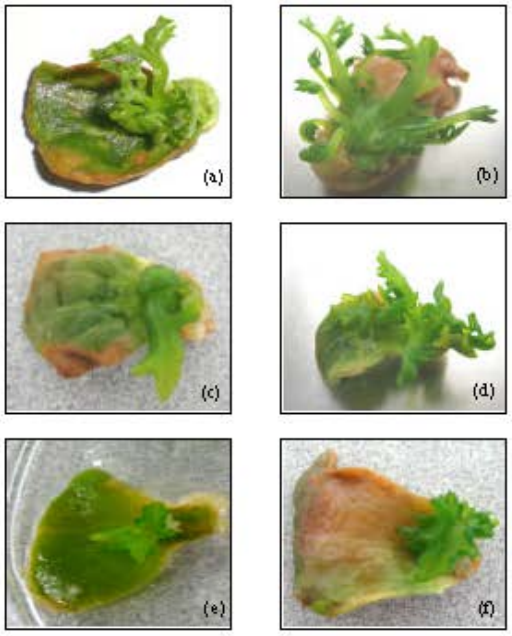

Fig. 1: Adventitious shoot development (a) based on explant ages. Shoot initiation on 5-day-old seedlings after 3 weeks and after (b) 6 weeks. Shoot growth on (c) 7-day-old seedlings after 3 weeks and (d) 6 weeks. Shoot development on (e) 9-day-old seedlings after 3 weeks and (f) 6 weeks

Table 1: Shoot regeneration from different explant ages after six weeks in culture on MS medium supplemented with 2.3 mg L^{-1} BAP

Seedling age (days)	Frequency of explants with shoots (%)	No. of shoots per responding explant
5	93	19.80±0.99[a]
7	73	7.27±0.90[b]
9	47	1.97±0.45[c]

Values within a column followed by different superscripted letters are significantly different at the $p<0.05$ level

on half strength MS medium supplemented with IAA gave a slightly higher level of response compared to NAA. However, no significance differences on root formation were observed between IAA and NAA.

Root initiation on full-strength MS media showed that IBA as an auxin source was significantly better than IAA and NAA (Table 3). Optimal root development was observed on full strength MS medium supplemented with 0.1 mg L^{-1} IBA yielded the best regeneration rate of 100% followed by 0.5 and 1.0 mg L^{-1} IBA. The IBA promoted root development more efficiently than IAA and NAA. The IBA induced several long primary roots which grew more rapidly and branched more frequently. However, IAA induced normal lateral shoots and abundant callus at the higher concentration nevertheless NAA led to thicker

Table 2: Effect of different concentrations of auxins in half strength MS medium on root formation from microshoots in *citrullus lanatus*

Auxin	Auxin concentration (mg L^{-1})	Frequency of rooted microshoots (%)	No. of root per microshoot (Mean±SE)
Control	0.0	43	5.90±1.29^{ab}
IBA	0.1	87	38.70±2.85^{cd}
	0.5	70	53.17±6.47^{d}
	1.0	60	56.43±8.56^{d}
	10.0	40	10.00±2.29^{ab}
IAA	0.1	63	13.90±1.98^{ab}
	0.5	47	18.93±3.80^{abc}
	1.0	43	27.13±5.82^{bc}
	3.0	30	26.87±7.65^{bc}
NAA	0.1	47	16.97±3.39^{abc}
	0.5	27	14.83±4.57^{ab}
	1.0	23	15.67±5.27^{ab}
	10.0	13	1.80±0.86^{a}

Values within a column followed by different superscripted letters are significantly different at the p<0.05 level

Table 3: Effect of different concentrations of auxins in full strength MS medium on root formation from microshoots in *Citrullus lanatus*

Auxin	Auxin concentration (mg L^{-1})	Frequency of rooted microshoots (%)	No. of root per microshoot (Mean±SE)
Control	0.0	60	12.43±1.93^{ab}
IBA	0.1	100	58.77±0.55^{de}
	0.5	80	84.20±7.87^{e}
	1.0	67	84.17±11.05^{e}
	10.0	53	18.53±3.23^{abc}
IAA	0.1	73	21.47±2.52^{abc}
	0.5	57	32.73±5.42^{abcd}
	1.0	60	45.43±7.00^{cd}
	3.0	43	48.10±10.25^{cd}
NAA	0.1	67	38.37±5.09^{bcd}
	0.5	53	45.87±7.97^{cd}
	1.0	43	40.00±8.50^{cd}
	10.0	27	6.10±1.89^{a}

Values within a column followed by different superscripted letters are significantly different at the p<0.05 level

and much shorter roots with a few lateral roots. In this experiment, both half strength MS medium supplemented with 0.1 mg L^{-1} IBA (87%) and full strength MS medium supplemented with 0.1 mg L^{-1} IBA (100%) improved the regeneration frequency of the roots. However, regeneration efficiency in full strength MS medium supplemented with 0.1 mg L^{-1} IBA was slightly increased and better compared to half strength MS medium supplemented with 0.1 mg L^{-1} IBA. Thus, the presence of 0.1 mg L^{-1} IBA in full strength MS medium was essential to initiate and to induce *in vitro* root induction in *Citrullus lanatus*.

Plant regeneration: Cotyledon explants of 5-day-old seedlings were excised and the proximal region (Fig. 2a) was cultivated on MS regeneration medium supplemented with 2.3 mg L^{-1} BAP. The BAP alone induced adventitious shoot bud differentiation from cotyledon explants in *Citrullus lanatus*. Cotyledonary explants on MS medium expanded considerably after 1 week and

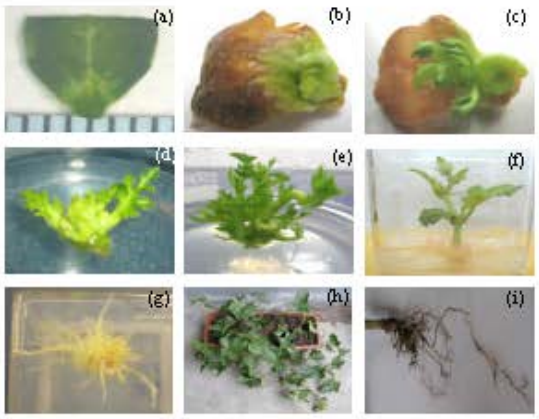

Fig. 2: Adventitious shoot development and plant establishment in *Citrullus lanatus*. (a) Proximal region from 5-day-old seedling used as cotyledon explants. (b) Initial development of callus at proximal end after 1 week. (c) Adventitious shoots on explants at week 3. (d) Adventitious shoot induction on MS medium with 2.3 mg L^{-1} BAP at week 6. (e) Shoot elongation on MS medium without hormone. (f) Rooted plantlet on MS medium with 0.1 mg L^{-1} IBA. (g) Induction of root on MS medium supplemented with 0.1 mg L^{-1} IBA. (h) Regenerated plantlets 1 month after transferred to pot. (i) *Ex vitro* rooting of *Citrullus lanatus*

gradually turned to yellowish-green as little green callus appeared directly at the proximal end of cotyledon segments (Fig. 2b). The cotyledon explants kept increasing in size and after 3 weeks on MS regeneration medium supplemented with 2.3 mg L^{-1} BAP, cotyledon explants produced adventitious shoots from the proximal end without intervening callus (Fig. 2c).

Moreover, the cotyledon explants transferred to fresh MS regeneration medium containing 2.3 mg L^{-1} BAP for another 3 weeks increase the size of the adventitious shoots and subsequently initiates a well-developed shoot tips (Fig. 2d). Further, MS medium lacking of hormone induced elongation of adventitious shoots with numerous shoot tips and leaves within 4 weeks (Fig. 2e). Independent fully developed shoots were transferred to MS medium containing 0.1 mg L^{-1} IBA to developed roots at their base resulting in complete plantlets with expanded leaves (Fig. 2f, g). The plantlets with roots (3-5 cm with >1 roots) which were transferred and acclimatized in pot resume growth and their stems and leaves elongated and expanded in 1 month (Fig. 2h, i).

DISCUSSION

Cotyledon from *in vitro* germinated seedlings used as an explant in this study was the most efficient system for plant regeneration in *Citrullus lanatus*. These finding support previous research that cotyledon tissues has been successfully regenerate into adventitious shoots in diverse type of cucurbits species like *Citrullus vulgaris* (Srivastava *et al*., 1989), *Citrullus colocynthis* (Dabauza *et al*., 1997) and *Cucumis melo* L. (Gaba *et al*., 1999). Furthermore, cotyledon regions in cucurbits consist of high competent cells which easily response to exogenous plant growth regulator and enable to regenerate adventitious shoots (Choi *et al*., 1994; Compton, 2000; Ananthakrisnan *et al*., 2003). In current experiment, cotyledon explants were excised and the proximal region of the cotyledon segment was effectively used to induce adventitious shoot on MS medium supplemented with 2.3 mg L^{-1} BAP. It has been reported that a higher percentage of explants forming adventitious buds was obtained from the cotyledon proximal region (Krug *et al*., 2005). Moreover, Compton and Gray (1993) have claimed that competent cells, which retained the capacity for a particular kind of cellular differentiation or regeneration located at the proximal region of the cotyledon segments in watermelon. In fact, proximal region of the cotyledon segments used in this study resulted in high adventitious shoot regeneration frequency.

Together with the explant type, seedling age is also important for adventitious shoot development. In this study, cotyledon explants were collected from 5-day-old seedlings was highly effective for the regeneration of shoots from the cotyledon explants. This phenomenon suggested that 5-day-old seedling is significantly different from 7 and 9-day-old seedlings in its efficiency level for shoot initiation. A possible explanation is that the young cotyledons are physiologically very active and response easily and efficiently to exogenous hormones (Dong and Jia, 1991). Another possible explanation for this is that young explants with many meristematic and undifferentiated cells are susceptible to tissue culture manipulation, thus regenerating shoots from their normal developmental pathway (Shin *et al*., 2000). Comparing the available information for *Citrullus vulgaris* with results obtained in *Citrullus lanatus*, it seems that 5-day-old cotyledons were the most sensitive to shoot induction compared to seedlings older than 7 days. Immature cotyledon of 5-day-old seedlings used as an explant showed to be an excellent source for plant regeneration on MS medium containing 2.3 mg L^{-1} BAP in this study.

The type and the concentration of phytohormone in the induction media were found to be another crucial factor in regeneration of *Citrullus lanatus*. The presence of BAP alone (without auxin) in the MS medium promoted the highest frequency of shoot regeneration from cotyledon explants. This is accordance with earlier studies of the same *Citrullus* species (Chaturvedi and Bhatnagar, 2001; Pirinc *et al*., 2003). In addition, shoots originated at proximal region of cotyledon explants on MS medium supplemented with 2.3 mg L^{-1}BAP have well-developed shoots tips and highly elongated with expanded leaves. These result are consistent with those of Pirinc *et al*. (2003), who reported that the shoot regeneration for watermelon cotyledons was best on MS medium with BAP alone (1 or 2 mg L^{-1}). Compton and Gray (1993) and Srivastava *et al*. (1989) detected an inhibition of shoot organogenesis when both cytokinin and auxin was added to shoot induction medium indicate that auxin counteracts the effect of cytokinin on shoot regeneration. However, the presence of auxin is absolute necessary for root regeneration to occur. Microshoots cultured in full strength MS medium supplemented with auxin comparatively better and improve root initiation than the microshoots cultured in half-strength MS medium. Furthermore, these results are consistent with those of the other studies which found full strength MS medium supplemented with auxin induced high frequency in root formation (Thakur *et al*., 2005).

Full strength MS medium supplemented with 0.1 mg L^{-1} IBA achieved high frequency of root initiation in *Citrullus lanatus*. It was significantly difference compared to various concentrations of NAA and IAA that has been tested for rooting efficiency. These findings agrees with Dabauza *et al*. (1997) and Ahn *et al*. (2007) that high frequency of shoots rooted and grew normally on MS medium supplemented with IBA. Moreover, IBA produced longer primary roots with numerous lateral roots on MS medium compared to IAA and NAA which promoted poor root development. For this reason, full strength MS medium supplemented with 0.1 mg L^{-1} IBA was chosen as the optimum condition for root initiation in *Citrullus lanatus* cv. Round Dragon.

CONCLUSIONS

In current study, we have established a reproducible *in vitro* plant regeneration protocol using cotyledonary explant for *Citrullus lanatus* cv. Round Dragon. The

adventitious shoot regeneration was successfully induced from the proximal region of 5-day-old seedlings on Murashige and Skoog (MS) media supplemented with 2.3 mg L^{-1} BAP. For rooting, MS media was added with 0.1 mg L^{-1} IBA and further incubated for another 3 weeks for the plantlets to grow under *ex-vitro* conditions. Establishments of some conditions would facilitate for future study in genetic transformation and seed production of transgenic *Citrullus lanatus* cv. Round Dragon.

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