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### RESEARCH ARTICLE

## Indirect regeneration from cotyledonary explants of watermelon (*Citrullus lanatus*) at in vitro culture

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### Abstract

Genetic improvement by using of tissue culture and biotechnology gives improvement products by introducing recombinant genes or generating somaclonal variants with better resistance to biotic or abiotic stresses. *In vitro* organogenesis of different cotyledon segments of watermelon was done in medium supplemented with 0.1, 0.5, 1.5, 2.5, 3.5 and 5 mg/L BA and 0.1 and 0.5 mg/L IAA. The best regeneration was obtained for the central region of cotyledon under light condition and for hypocotyl in darkness. Regeneration with increasing BA concentration (3.5-5 mg/L) increased in the central segments but decreased in hypocotyl segments. IAA did not efficiently affect the regeneration process. The data showed that *in vitro* organogenesis of watermelon occurred with higher efficiency, when cotyledon segments from the proximal region were cultured in MS medium including 3.5 mg/L BA and 0.5 mg/L IAA under a 16/8h photoperiod.

**Abbreviations:** Mourashige and Skoog (MS), Benzyl Adenine (BA), Indole Acetic Acid (IAA), Naphthalene Acetic Acid (NAA), Indole-3-Butyric Acid (IBA).

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### Introduction:-

Watermelon is an important cucurbitaceous members. It is grown worldwide and ranked sixth in world production of fruit crops (Shalaby et al., 2008). The cultivated area with watermelon is ranked second behind tomato and world production ranks third in metric tons among vegetables. The global production of watermelon is over 81 million metric tons. China produces most of the world's watermelon crop, accounting for about 71% (57.65 metric tons) of global production followed by Turkey (3.9 million metric tons), Iran (1.9 million metric tons), the US (1.78 million metric tons) and Egypt (1.45 million metric tons) (FAO, 2008).

Watermelon (*Citrullus lanatus* Thumb.) belonging to the family Cucurbitaceae, is widely known because it is a valuable source of water, A and C vitamins as well as important minerals like calcium, potassium and iron. Watermelon cultivars are different in size, shape, rind color and pattern, seed color, and maturity date. Most cultivars of watermelon are diploid and produce fruit that have a striped rind with small black seeds (Compton et al., 2004).

Destructive viral diseases in watermelon are critical to control. Plant biotechnology could be used as a means of

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shoot regeneration from cotyledon pieces and somatic embryogenesis. Regeneration of adventitious shoots has been reported from a wide range of diploid and tetraploids cultivars of watermelon (Compton et al., 1993). In all reports, cotyledons of *in vitro* germinated seedlings were the best source of explants. Cotyledon explants have been used severally for regeneration of watermelon (Blackmon and Reynolds, 1982; Adelberg and Rhodes, 1989; Compton and Gray, 1993b). These showed differences in genotype (Shalaby et al., 2008), plant growth regulator type, its concentrations and combinations of auxins and cytokinin (Khatun et al., 2010). Besides growth regulators, explant age, dissection method (Compton, 2000), explant type (Ibrahim et al., 2009) and culture conditions (Choi et al., 1994) also influence adventitious shoot development.

Therefore, the present study was done to establish a protocol for large scale *in vitro* multiplication of plantlets from different cotyledon explants of watermelon and light conditions.

## Materials and Methods:-

Seeds of Watermelon (cv. Crimson Sweet) were used as explant sources to investigate indirect regeneration condition. Seeds were surface sterilized for 15 min in commercial bleach solution (1.5% NaClO and 0.1% Tween 20), rinsed 3 times in distilled water, and soaked 2 days in distilled water in the dark. After removing the seed coat, the cotyledons were excised and 1-2 mm removed from the borders, and cut transversally into two halves, proximal and apical region (hypocotyl) and used as explants. To examine suitable concentrations of plant growth regulators on shoot formation, various concentrations and combinations of IAA and BA were tested. The regeneration medium was MS basal medium containing various combinations of IAA (0.1 and 0.5 mg/L) and BA (0.1, 0.5, 1.5, 2.5, 3.5 and 5 mg/L), supplemented with 3% sucrose and 0.8% agar. After cultivation, explants were incubated either in darkness or under a 16-h photoperiod at 25°C for a month and then subcultured to fresh medium with the same composition. After 1 month, the percentage of callus induction in explants and frequency of plantlet and leaf formation was recorded. After 2 months from culture, the regeneration/callus induction percentages were calculated as the number of explants differentiating adventitious shoots/ total cultured explants.

Each treatment consisted of 6 replicates and each replicate include 6 explants for central and 3 explants for hypocotyl explants.

Developed shoots were isolated and transferred to MS basal medium. After 3 weeks on shoot elongation medium, shoots longer than 1 cm were excised and transferred to Magenta GA7 vessels that contained 15 ml of rooting medium) supplemented with 0.1 mg/L IBA for a month. Plantlets with adventitious roots were acclimated in hydroponic culture. All media adjusted to pH 5.8 were autoclaved at 121°C for 15 min.

To detect significant differences, data were subjected to analysis of variance. Duncan's multiple range test was adjusted to separate mean differences.

## Results:-

### Callus induction and regeneration of hypocotyl segments of seed:-

Results showed that there is a significant difference ( $p \leq 0.01$ ) at callus induction between different levels of BA. By increasing BA concentration, the amount and intensity of callus induction accelerated (Fig 1). Formed callus in dark condition were white to yellow in color and soft texture, although, those of light condition were green in color with soft and consistent tissue (Fig 3). The lowest percentage of callus induction (68%) were observed at low concentrations of BA and the highest (100%) of callus induction were observed at high concentrations of BA (3.5-5 mg/L). In addition, the percentage of callus induction and its intensity increased with increasing BA concentration.

Light conditions and IAA did not cause significant differences in the percentage of callus induction in hypocotyl explants and their interactions on callus induction were not significant ( $p \leq 0.05$ ).

No significant difference was observed ( $p \leq 0.01$ ) between the percentage of regeneration at levels of BA. However, IAA did not effect on percentage of regeneration. The interaction between these two hormones on regeneration was not significant, but in contrast with dark condition (29%), the light conditions could have a significant effect on these traits so that the regeneration during light conditions was induced (5.49%). The highest percentage of regeneration in dark conditions was recorded in 1.5 mg/L BA but significantly decreased in the light condition; the regeneration was increased by increasing BA concentration (Fig 2 and 3).

Hypocotyl explants did not require high concentrations of BA for initiation of organogenesis and were regenerated at low concentrations of BA. In dark conditions, higher concentrations of BA led to an accelerated callus induction, and inhibition of regeneration. The highest percentage of regeneration in conditions of darkness and light was observed, 53 and 75% respectively.

Light condition and different concentrations of BA could significantly affect the number of leaves and plant regeneration from hypocotyl explants ( $p \leq 0.01$ ) but light condition with IAA could not significantly affect the number of leaves. As the regeneration rate in light condition increased, number of leaves and regenerated plants also followed this trend. The number of regenerated leaves increased in response to concentration of BA, but the leaves were very small and extremely dense; this trend was also seen in the number of regenerated plantlets. Maximum number of leaves and plantlet (4.01 and 1.64 respectively) was observed at 3.5 mg/L BA.

#### **Callus induction and plant regeneration of central part of cotyledon explants:-**

The effect of different concentrations of BA on callus induction ( $p \leq 0.01$ ) was significantly different in various cotyledon explants. Increasing BA concentration to 2.5 mg/L caused acceleration in callus induction but higher concentrations decreased the percentage and intensity of callus induction and expansion of the cell (Fig 2 and 6). Light conditions can also induce a significant difference ( $p \leq 0.01$ ). Callus induction and intensity in cotyledons placed in lighting conditions was poorer (67.85%) than in the dark (42.93%). All treatments had 0.1-0.5 mg/L IAA, but there seems to be no difference in callus induction significantly in response to this hormone. The interaction of BA and light conditions on callus induction was significant. At a concentration of 1.5 and 2.5 mg/L BA and dark condition, 100 % of callus induction was achieved while the lowest percentage of callus induction in initial and final concentrations of BA in both light conditions was observed, respectively.

As the percentage of callus induction was observed, the interaction effect of BA and light conditions on regeneration percentage was observed to be significant ( $p \leq 0.01$ ). Regeneration induction was much better in light condition in contrast to dark and on the other hand, regeneration induced in dark conditions with increasing BA concentration remained relatively constant while the regeneration in light situations by increasing hormone concentrations increased (Fig 5 and 6). It is evident that the rate of regeneration in terms of the extent of light-induced regeneration was quite detectable after a month.

In cotyledons explant, initiation of regeneration requires a minimum concentration of 1.5 mg/L BA in both light conditions and then with increasing concentrations of BA, an increase of regeneration with expanded cotyledons was observed so that 3.5 and 5 mg/L BA had no significant increase (58.84%). Increase BA concentration to 5 mg/L in some cases caused slightly expanded cotyledons, which resulted in deformity the original explant (Fig 4).

Effect of BA and IAA with light conditions on plant regeneration and leaf number was significant ( $p \leq 0.01$ ). Regeneration in darkness was hard and plantlets had low numbers of leaves. In contrast, in light conditions, number of leaves per regenerated plantlets was quite sensitive to increase in hormone concentration and enhancement of BA concentration led to an increasing in leaf number of plantlets, but at 5 mg/L BA, an increase in the number of regenerated leaves did not follow the enhancement in number of regenerated plantlet (Tables 1 and 2) because the leaves are smaller and denser, as a result, isolation of regenerated plantlets in 3.5 mg/L was more difficult. Increasing the concentration of IAA in the light conditions, especially in the hormonal range of 2.5-3.5 mg/L BA was able to increase the number of leaves of regenerated plantlets (Tables 1 and 2).

#### **Discussion:-**

The results showed that hypocotyls regeneration at low concentrations of hormone was performed under dark or light conditions. Callus induction increased in dark and regeneration induced in light condition. The central part of

the cotyledon explants required minimum concentrations of BA (1.5 mg/L) for regeneration. Light conditions in contrast to the dark, can induce optimum regeneration. The central part of cotyledon explants in light conditions caused most regeneration (85%). In this explant, the density of plantlets is less; hence, isolation was easier than those regenerated from the hypocotyl.

Regeneration of watermelon and other members of the cucurbit family with explants of cotyledons, hypocotyls, leaves, branches, tops, anther and protoplast have been reported (Bajaj, 1997). In all reports, *in vitro* cotyledons of germinated seeds were the best explants. Even more precisely, the selected part of explants affects the regeneration rate in the cucurbit family so that cells capable of forming side branches are surrounded with a specific area in the cotyledon near the end of each segment of the cotyledons (Ananthakrishnan et al., 2003). In watermelon, susceptible cells of regeneration are closely related to cotyledons because most sprouts accessories in the basal part of explants have evolved (Compton, 2000, Ameri et al., 2015). Li et al. (2013) used 5 different explants and 100% of node explants had regeneration, while the highest number of shoots per explant was in complete cotyledons. It seems that, watermelon cotyledons include high level of growth regulators (Maheshwari and Prakash, 1967). Cytokinin can cause cell developments in some tissues and organs. This effect in dicotyledons or plants with cotyledon leaves such as mustard, sunflower and cucumber are clearly seen (Taiz and Zeiger, 2006). Cytokinins are responsible for activating undifferentiated cells of callus or operate in direct regeneration of somatic cells for organogenesis (Ibrahim et al., 2009).

Some reports suggest that the effect of BA is dependent on its concentration (Khalekuzzaman et al., 2012). Concentrations of 1-2 mg/L BA alone or in combination with auxin to initiate organogenesis in melon explants have been reported (Li et al., 2013) and increasing concentrations of BA led to a reduction in the extent of regeneration, vitrification, somaclonal variation and inhibition of plant growth (Shahin, 2012).

Germination and seedling emergence of watermelon will rise in dark (Li et al., 2013). It was observed that seed germination in dark can increase the capacity of cotyledon in organogenesis (Bajaj, 1997). Compton (1999) also stated that pretreatment of cotyledon explants in dark, also regenerated shoots increased. Perhaps, the effect of dark is to maintain growth regulators and other endogenous plant chemicals. Microscopic studies have shown that etiolated tissues have more undifferentiated parenchyma cells. Since the dedifferentiation is a key part of the regeneration process, a higher proportion of undifferentiated cells improved regeneration. Etiolated tissues of plant have oxidative polyphenol compounds, and less compounds in the cell wall which is thinner with less vascular tissue (Shahin, 2012). Reduced wall thickness and composition may regulate and facilitate the movement of materials to the site of explants regeneration. Most reports discovered light is more effective (a photoperiod of 16/8 h) in comparison with dark condition (Suratman et al., 2010).

**Table 1.** The effect of light and plant growth regulators on plant regeneration from cotyledon explants

BA (mg/l)	0.1 and 0.5		1.5		2.5		3.5		5.0	
IAA (mg/l)	0.1	0.5	0.1	0.5	0.1	0.5	0.1	0.5	0.1	0.5
darkness	0.0h	0.0h	0.06e	0.06e	0.06e	0.08e	0.11 e	0.14 e	0.14 e	0.50 e
light	0.0h	0.0h	0.36 d	0.36 d	0.45d	1.08c	2.08b	2.58a	2.36ab	2.22ab

Means with similar letters at the 1% level according to Duncan's multiple range tests were not significantly different.

**Table 2.** The effect of light and plant growth regulators on leaf regeneration from cotyledon explants

BA (mg/l)	0.1 and 0.5		1.5		2.5		3.5		5.0	
IAA (mg/l)	0.1	0.5	0.1	0.5	0.1	0.5	0.1	0.5	0.1	0.5
Darkness	0.00 h	0.00 h	0.06gh	0.08gh	0.11gh	0.14gh	0.39fgh	0.50fg	0.45fgh	0.45fgh
light	0.00 h	0.00 h	0.61 f	0.72 f	1.17 e	3.64 d	4.50 c	6.11 b	7.20 a	6.92 a

Means with similar letters at the 1% level according to Duncan's multiple range tests were not significantly different.

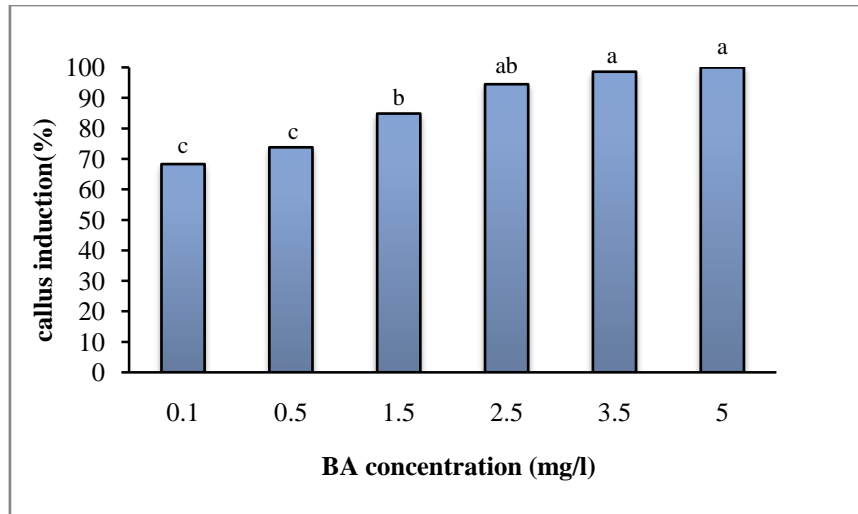


Figure 1:-The effect of BA concentration on callus induction in hypocotyl explants of watermelon.

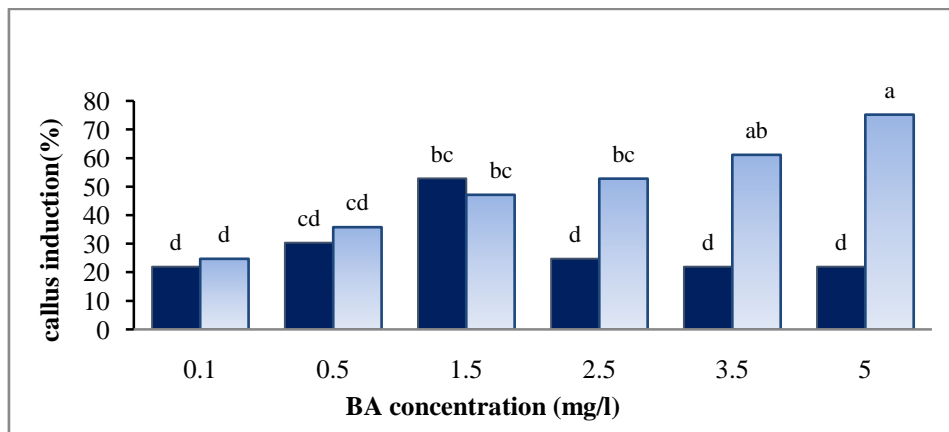


Figure 2:- The effect of BA concentration and light conditions on regeneration of watermelon hypocotyls explants (Black column: dark condition and white column: light condition).

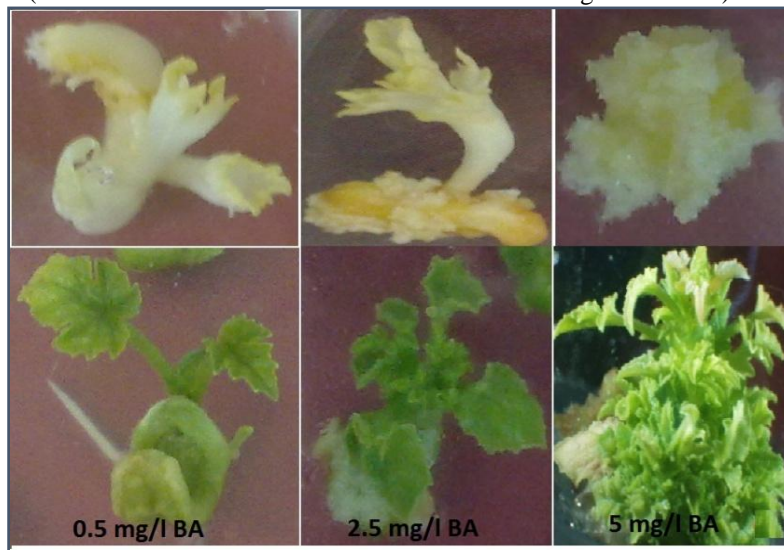


Figure 3:- Regeneration of watermelon hypocotyls in differenet hormonal treatments and light conditions (darkness (top) and light (bottom))



Figure 4:- Induced deformation of watermelon cotyledons at 5 mg/l BA

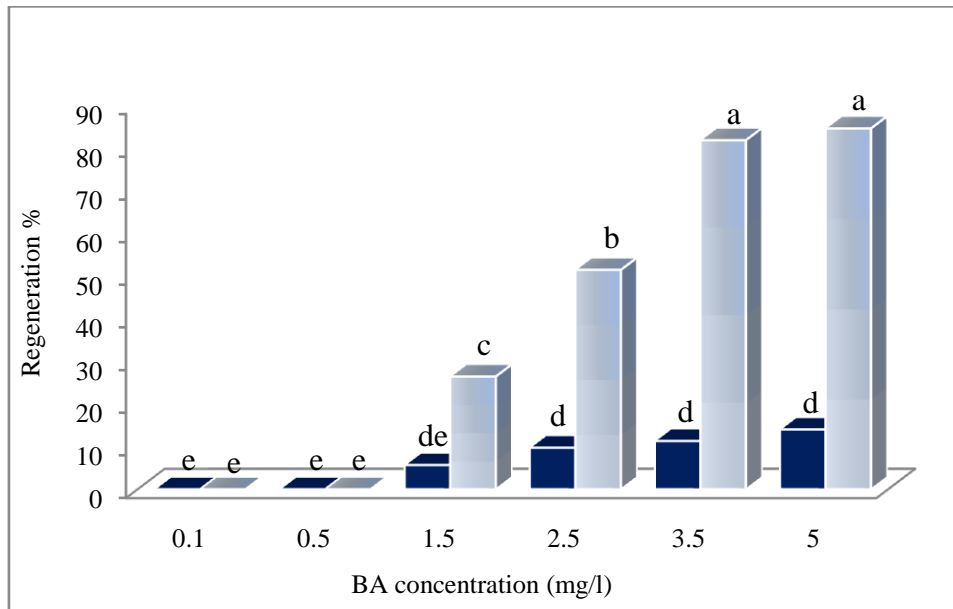
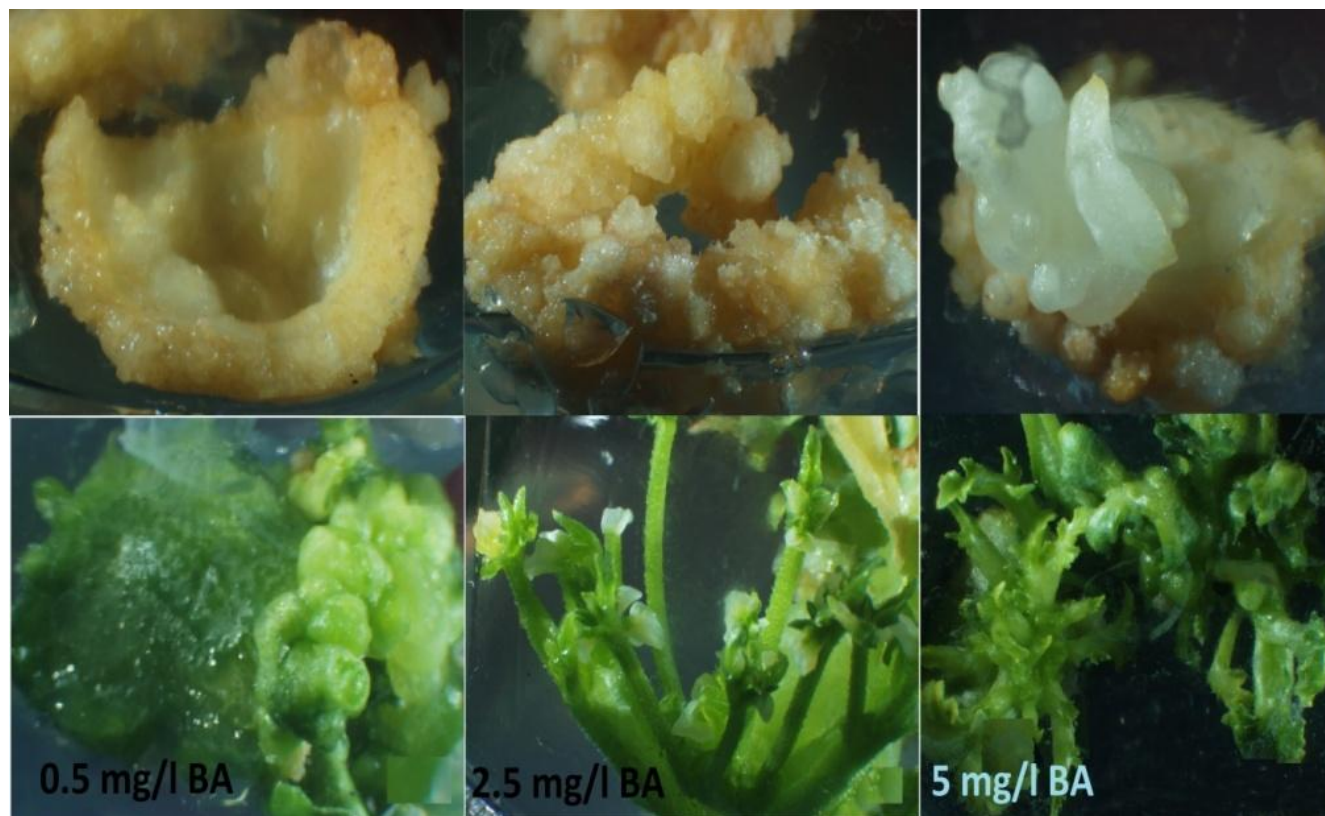


Figure 5:- Percentage of regeneration from cotyledons of watermelon in different BA concentrations and light conditions (Black column: dark condition and white column: light condition).





**Figure 6:-** Watermelon callus induction and regeneration from the central part of cotyledon in different BA treatments and light conditions (darkness (top) and light (bottom)).

### Conclusions:-

Light conditions with regeneration rate greater than 63% had more effect in compare with dark condition (25%). However, better regeneration was observed in hypocotyl explants in the dark and central cotyledon explants under light conditions. Enhancement of BA concentration increased regeneration so that maximum regeneration for hypocotyl explants (75%) and the central part of cotyledons (85%) in the range 3.5-5 mg/L BA was obtained. The concentration of IAA from 0.1-0.5 mg/L had no significant effect on regeneration, number of leaves and regenerated plants from hypocotyl, but high concentration of auxin increased the number of leaves and plant regeneration from cotyledon explant. In general, the results showed that central part of cotyledon as explant and 3.5 mg/l BA in combination with 0.5 mg/l IAA are the best condition for indirect regeneration of watermelon.

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