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Improved Induction of Somatic Embryo in Watermelon [*Citrullus lanatus* (Thunb.) Matsum. et Nakai]

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Abstract

In order to optimize somatic embryo induction of watermelon, three different explants from three different varieties were used to induce somatic embryo in medium contained BA in combination with either NAA or 2,4-D. 'Kangbingjingxin' cotyledon explant with all of PGR combination treatments has a high callus inductivity (75.6%-98.9%), but inductivity of somatic embryo was varied among different PGR combination, the highest inductivity (54.7%) was obtained in medium with BA 3.0 mg/L and NAA 0.05 mg/L. Somatic embryogenesis was different among 'Kangbingjingxin', 'Zaojia 8424' and 'Heimeiren', the inductivity was 46.2%, 34.1% and 15.7% respectively. Callus inductivity of three explants was 88.9%-98.9%, in which callus induced from cotyledon and cotyledon node was mostly embryonic callus while callus from hypocotyl was non-embryonic callus. Somatic embryo inductivity of cotyledon node (62.0%) was significantly higher than cotyledon (46.1%), there is no somatic embryo induced from hypocotyl. However, somatic embryos number per explant of cotyledon node (2.3) was significantly lower than cotyledon (6.0).

Keywords: somatic embryo, watermelon, BA, NAA, 2,4-D, explant, genotype

Abbreviations: PGR (plant growth regulator), BA (6-Benzyladenine), NAA (Naphthaleneacetic acid), 2,4-D (2,4-Dichlorophenoxyacetic acid)

1. Introduction

Watermelon [*Citrullus lanatus* (Thunb.) Matsum. et Nakai] is an important fruit crop in Cucurbitaceae family and it is widely planted as an economically important crop (Yang, 1991; Zhang et al., 1996). Conventional breeding of watermelon has the shortage such as long breeding cycle, great difficulty and genetic instability, while plant regeneration via organogenesis also has the defects of low frequency in regeneration and huge difference in gene expression. Somatic embryo could be mass-produced in a short time, the reproduction rate of somatic embryo is high, and it's easy to develop into plantlet (Song et al., 2004). Somatic embryo provides perfect material for artificial seed, haploid breeding and asexual propagation. Moreover, somatic embryo has little somaclonal variation while has high capability to accept exogenous DNA, so it is used as receptor system of genetic modification in many plant species (Yan, 2003).

Somatic embryogenesis in watermelon was first reported by Compton and Gray (1993), cotyledon from immature embryos was used as explants, and the highest induction rate was 30% while the PGR combination was 10 μM 2,4-D and 0.5 μM TDZ. After that Zhang (2004), Niu (2006) and Song et al. (2007) tried to induce somatic embryo using mature cotyledon and hypocotyl et al as explants. However, both the induction rate of somatic embryo and seedling regeneration rate were low, the quality of somatic embryo and the amount of somatic embryos per explant were as well.

In this study, three different explants from three different varieties were used to induce somatic embryo, while the PGR combination was researched.

2. Materials and Methods

2.1 Plant Materials and Culture Conditions

Three commercial watermelon varieties were used in this study. Seeds of 'Zaojia 8424' were bought from Ningbo Seed Corporation, China. Seeds of 'Heimeiren' were bought from Known-You Seed (China) Co. Ltd., China. Seeds of 'Kangbingjingxin' were bought from Hefei Fengle Seed Co. Ltd., China.

Shelled watermelon seeds were agitated in 75% ethanol for 30 s and washed three times with sterile distilled water. Then surface sterilized using 0.1% mercuric chloride solution for 8 min and rinsed five times with sterile distilled water. The seeds were sowed on 1/2 MS (Murashige & Skoog, 1962) medium with 3.0% sucrose and 0.7% agar. The cultures were first incubated in dark, three days later transfer them under a 16 h light/8 h dark photoperiod at 25°C under illumination at 2000 lx.

The medium pH used in experiments were adjusted to 5.8 with 1.0 M NaOH and then sterilized by autoclaving at 121°C for 20 min. Cultures were performed in 150 ml Erlenmeyer flasks with 30 ml medium.

2.2 Induction of Callus and Somatic Embryo

About five days after sowing 'Kangbingjingxin', the seeds germinated and the leaves turned green. Excise cotyledon from seedling and then cut the cotyledon in half longitudinally. Cotyledon segments were placed on MS medium with 3.0% sucrose and 0.7% agar. The medium contained BA (1.0, 2.0, 3.0, 4.0, mg/L) in combination with either NAA (0.05, 0.1, 0.5, 1.0, mg/L) or 2,4-D (0.1, 0.5, 1.0, 2.0, mg/L). There were six explants per Erlenmeyer flask. Each experimental unit consisted of five Erlenmeyer flasks and three replicates were tested per treatment. Set a treatment without PGR as control. The dark culture time was seven days. The cultures were subcultured by transferring the explants to fresh medium every 2 weeks. To study the effect of different genotypes on somatic embryo induction, Cotyledon explant from 'Zaojia 8424', 'Heimeiren' and 'Kangbingjingxin' were placed on MS medium with BA 3.0 mg/L and NAA 0.1 mg/L. To research the effect of explants, Cotyledon, cotyledon node and hypocotyl (Figure 1) explants from 'Kangbingjingxin' seedlings were excised and cultured on MS medium with BA 3.0 mg/L and NAA 0.1 mg/L.

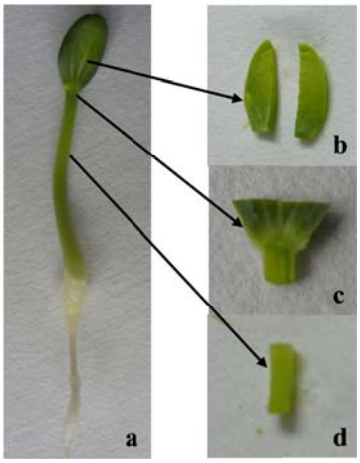

Figure 1. The type of explants

(a) Seedling of watermelon. (b) Cotyledon. (c) Cotyledon node. (d) Hypocotyl.

2.3 Histological Observation

For histological observation of somatic embryogenesis, samples were collected and fixed in FAA (formalin: glacial acetic acid: 70% ethanol at 5 : 5 : 90 by volume) at room temperature for 48 h. The fixed material was then stained with 1% safranine solution for 3 days. After dehydration through an alcohol-xylol series, the samples were embedded in paraffin wax (58-60°C). 8 μm slices were sectioned using a microtome and fixed on glass slides. The slices were de-waxed in xylene for 5-10 min and observed under a light microscope.

2.4 Statistical Analysis

The percentage of callus induction was recorded after 3 weeks, while somatic embryo was recorded after 6 weeks. All experiments were arranged as a completely randomized design. Percentage data were arcsine transformed prior

to analysis. All data were subjected to analysis of variance (ANOVA) using DPS v. 6.55. Tests for normality and homogeneity of variance were performed prior to ANOVA. The significant difference among the mean ± standard error was carried out using Least Significant Differences test ($P \leq 0.05$).

3. Results and Discussion

3.1 Effect of PGR Combination on Induction of Callus and Somatic Embryo

The initial size of cotyledon explant was about 2×5 mm, and the color was light green. Three days after culturing in dark, the cotyledon twisted and enlarged four times the previous size, and the color turned into yellowish-white, while a few callus appeared on the incision. The explant turned green when transferring it to light after one-week dark culture. Obvious callus could be observed after culturing 2-3 weeks. A great amount of callus was gained when cultured 4 weeks. The callus could be grouped in three types: The first is embryonic callus that was close-textured and vigorous, which color is dark green or green or light green and the shape is nodular or nodular or globular or granular; The second is embryonic callus that was loose structure and slow-growing, which color is yellowish or pale yellow-green and the shape is granular; The third is non-embryonic callus that was hard brittle yellow-green callus or hygrophanous loose white callus, the former callus become harder and hadn't somatic embryogenesis after culturing long time, while the latter growing weakly and browning and degenerating. Histological observation indicated that non-embryonic callus were seen to consist of sparse cells with small nucleus and was lightly stained (Figure 2 a). Embryonic callus were seen to consist of small, dense cells with large nucleus and was deeply stained (Figure 2 b). It was a long time from callus to the appearance of globular embryo, and globular embryo was soon started to develop once its appearance. Somatic embryo could originated from the surface of explant, or the near surface of callus (Figure 2 c), but mostly were originated from the inside of callus (Figure 2 d).

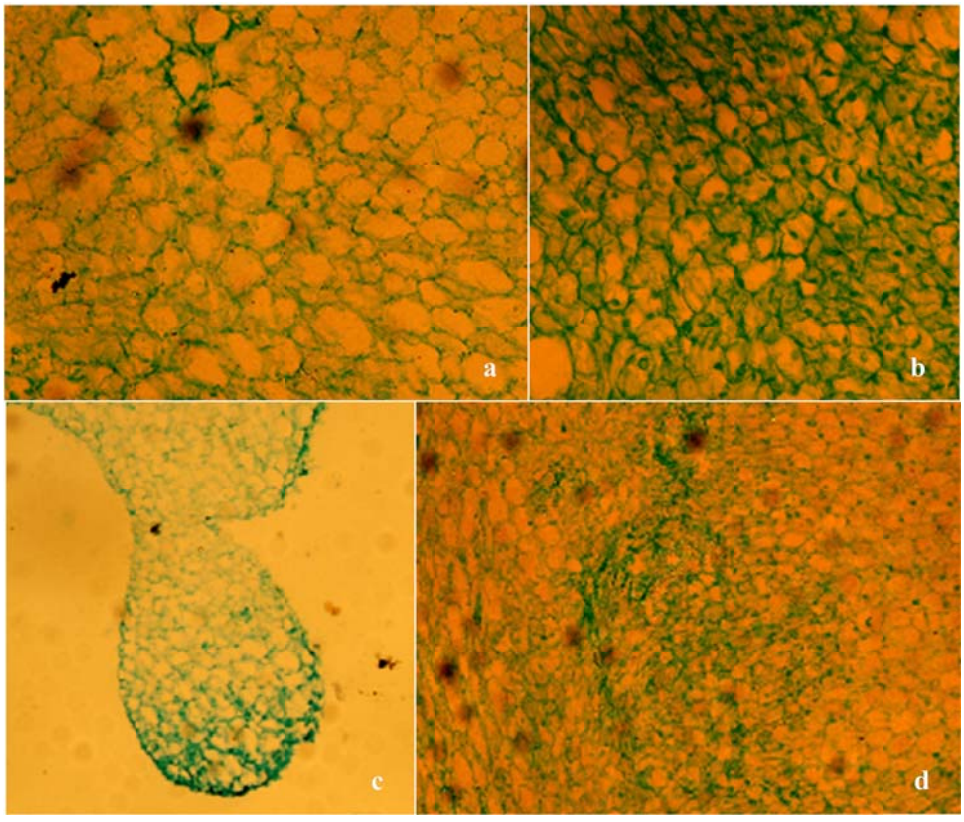

Figure 2. Histological observation for callus and somatic embryogenesis

(a) Non-embryonic callus. (b) Embryonic callus. (c) Globular embryo. (d) Heart-shape embryo.

Most of callus induced by BA and NAA is the first and second type embryonic callus (Figure 3 a), these two types of callus emerged later than the third, somatic embryos in different stages could be observed on these embryonic callus after 7-10-week culture (Figure 3 g-k). Ten weeks later normally developed and prematurely

germinated somatic embryos appeared on some explants. The third non-embryonic callus was mostly induced by BA and 2,4-D (Figure 3 b). The quality of callus and inductivity of somatic embryo decreased as the increase of 2,4-D concentration. All of the callus induced is non-embryonic callus when 2,4-D concentration is higher than 1.0 mg/L. The cotyledon explant without PGR just slightly rolled and enlarged, there isn't callus on them while there is adventitious root on some explants. This reflects PGR is necessary for induction of callus and somatic embryo, which was the same as the report on melon (Yuan, 2007).

The inductivity of callus was high in all PGR combinations, the lowest inductivity is 75.6% when BA 4.0 mg/L with NAA 0.05 mg/L (Table 1). The highest inductivity of somatic embryo (54.7%) was gained in medium supplemented with BA 3.0 mg/L and NAA 0.05 mg/L, the second (45.6%) with BA 3.0 mg/L and NAA 0.1 mg/L, these two treatments were significantly higher than others. The inductivity of somatic embryo and number of somatic embryos per explant were first increased and then decreased as the increase of BA concentration, the inductivity of somatic embryo gained the highest when the concentration of BA is 3.0 mg/L, while the number of somatic embryos per explant gained the highest (7.8) in the same concentration. These show that 3.0 mg/L was the best BA concentration for induction of somatic embryo. The inductivity of somatic embryo and number of somatic embryos per explant were decreased as the increase of NAA concentration, 0.05 mg/L was the best NAA concentration for induction of somatic embryo. When the concentration of NAA added to 1.0 mg/L, callus induced was non-embryonic callus which couldn't produce somatic embryo, and a large number of adventitious roots occurred on it (Figure 3 c).

Table 1. Effect of BA and NAA combination on induction of callus and somatic embryo

Concentration of PGR (mg/L)		Inductivity of callus (%)	Inductivity of somatic embryo (%)	Number of somatic embryos per explant induced somatic embryo
BA	NAA			
1.0	0.05	82.2±6.9ghi	17.4±4.9efg	4.4±0.8c
1.0	0.1	88.9±1.9cdefg	14.9±3.5efg	2.7±0.3g
1.0	0.5	95.6±2.0abc	11.6±5.2g	2.7±0.2g
1.0	1.0	96.7±3.4ab	0h	0h
2.0	0.05	80.0±5.8hi	26.2±5.0cd	3.5±0.6def
2.0	0.1	89.0±3.8cdefg	26.3±4.0cd	4.0±0.2cd
2.0	0.5	92.2±1.9abcd	19.4±8.8de	3.0±0.9fg
2.0	1.0	97.8±1.9a	0h	0h
3.0	0.05	83.3±8.8fgh	54.7±2.9a	7.8±0.7a
3.0	0.1	90.0±3.3bcdef	45.6±4.7b	6.0±0.2b
3.0	0.5	87.8±1.9defg	29.2±6.4c	6.3±0.4b
3.0	1.0	96.7±3.4ab	0h	0h
4.0	0.05	75.6±2.0i	19.0±6.4def	3.2±0.4efg
4.0	0.1	80.0±5.8hi	15.2±1.4efg	3.8±0.5cde
4.0	0.5	84.4±5.1efgh	11.8±3.5fg	4.3±0.7c
4.0	1.0	91.1±3.8abcde	0h	0h

Data represented mean ± SE of three replicates.

Means having the same letter in a column were not significantly different by Least Significant Differences test ($P = 0.05$).

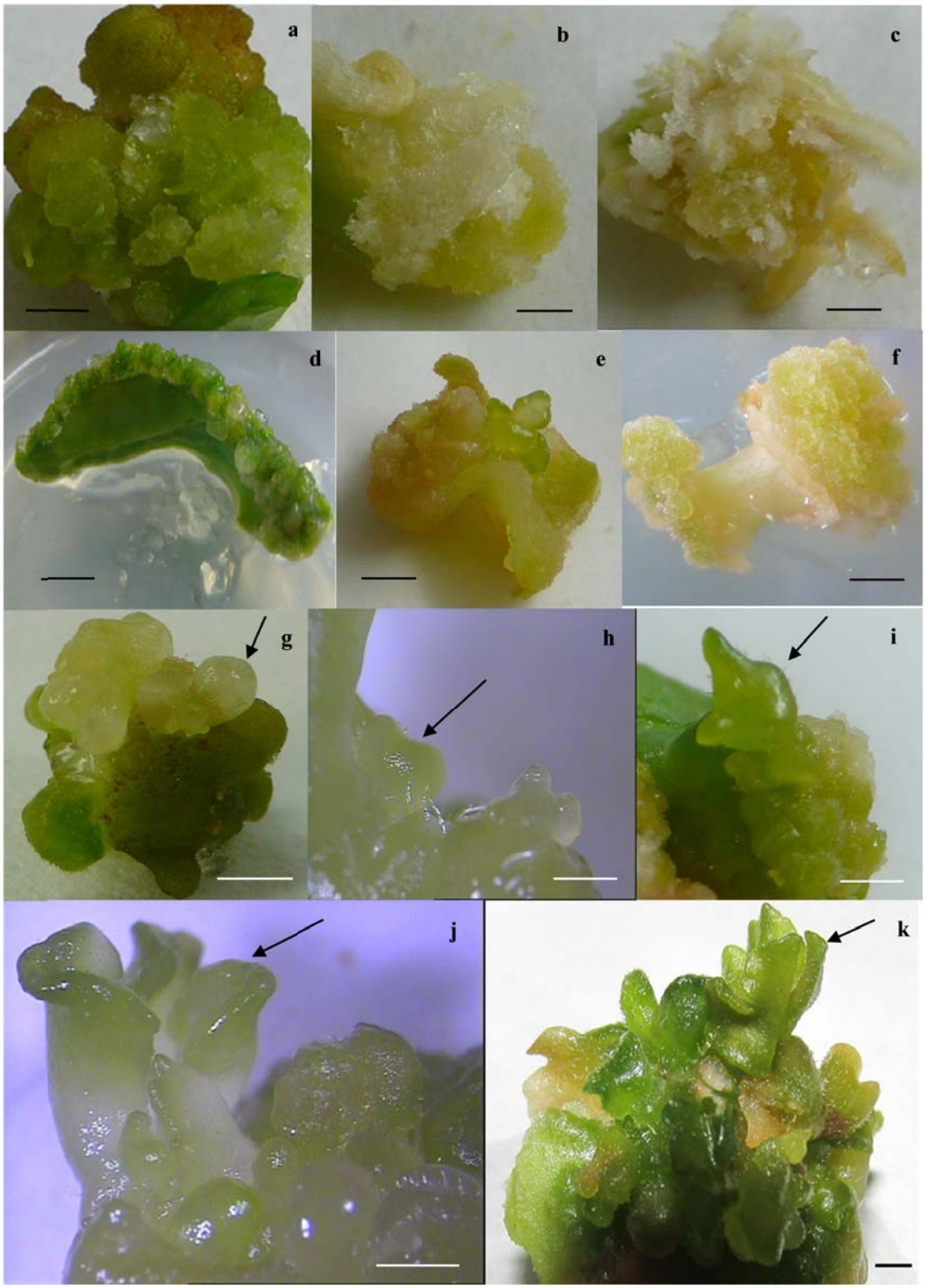

Figure 3. Induction of callus and somatic embryo

(a) Embryonic callus induced by BA 3.0 mg/L and NAA 0.1 mg/L. (b) Non-embryonic callus induced by BA 1.0 mg/L and 2,4-D 2.0 mg/L. (c) Adventitious roots induced by BA 1.0 mg/L and NAA 1.0 mg/L. (d) Callus on cotyledon. (e) Callus on cotyledon node. (f) Callus on Hypocotyl. (g) Globular embryo. (h) Heart-shape embryo. (i) Torpedo-shape embryo. (j) and (k) Cotyledonary embryo. (*bar* = 5 mm)

Inductivity of callus was slightly decreased as the increase of BA concentration and was increased as the increase of 2,4-D concentration (Table 2). It shows that low concentration BA in combination with high concentration 2,4-D was good for callus induction. When the concentration of BA increased, the inductivity of somatic embryo increased but there is no significant difference, while the number of somatic embryos per explant decreased and there is significant difference between low and high concentration. The inductivity of somatic embryo and number of somatic embryos per explant decreased as the increase of 2,4-D concentration. The inductivity and number gained maximum (12.6% and 6.9) when 2,4-D concentration is 0.1 mg/L. When 2,4-D concentration reach 1.0 mg/L, most of induced callus was non-embryonic callus. There is no somatic embryo induced when 2,4-D concentration is 2.0 mg/L. It proves that high 2,4-D concentration was not advantageous to induce somatic embryo from cotyledon explant.

Somatic embryogenesis is a complex procedure which was affected and regulated by multiple factors, and the composition of medium is the most important factor (Guis et al., 1998; Guan et al., 2009). The inductivity of somatic embryo obtained maximum in medium with BA 3.0 mg/L and NAA 0.1 mg/L. In the process of *in vitro* culture, some explants were browned, while there is abnormality and premature germination in somatic embryogenesis, these problems restricted the induction of high frequency and high quality somatic embryo.

Table 2. Effect of BA and 2,4-D combination on induction of callus and somatic embryo

Concentration of PGR (mg/L)		Inductivity of callus (%)	Inductivity of somatic embryo (%)	Number of somatic embryos per explant induced somatic embryo
BA	2,4-D			
1.0	0.1	92.2±1.9bcde	9.6±4.1abc	6.9±1.4a
1.0	0.5	93.3±3.4abcde	9.5±1.8abc	4.2±0.8bcd
1.0	1.0	93.3±3.4abcde	4.7±2.1d	2.2±1.0efg
1.0	2.0	97.8±3.9ab	0e	0h
2.0	0.1	93.3±3.4abcde	10.8±3.7ab	5.4±1.0b
2.0	0.5	91.1±1.9cde	9.7±2.0abc	3.9±1.4cd
2.0	1.0	94.4±2.0abcd	5.9±2.0cd	2.5±0.5ef
2.0	2.0	98.9±1.9a	0e	0h
3.0	0.1	91.1±1.9cde	11.0±3.9ab	4.6±0.8bc
3.0	0.5	93.3±6.7abcde	10.6±3.2ab	2.7±0.4ef
3.0	1.0	93.4±5.8abcde	7.0±3.3bcd	1.3±0.3g
3.0	2.0	95.6±5.1abc	0e	0h
4.0	0.1	87.8±1.9e	12.6±1.9a	3.1±0.3de
4.0	0.5	88.9±1.9de	10.0±2.0ab	2.6±0.4ef
4.0	1.0	90.0±0.0cde	4.9±2.1d	1.5±0.5fg
4.0	2.0	90.0±3.3cde	0e	0h

Data represented mean ± SE of three replicates.

Means having the same letter in a column were not significantly different by Least Significant Differences test ($P = 0.05$).

3.2 Effect of Genotypes on Induction of Callus and Somatic Embryo

The callus inductivity of ‘Zaojia 8424’ and ‘Kangbingjingxin’ was 94.4% and 88.9% respectively, there is no significant difference between these two varieties (Table 3). However, the explant enlargement degree, callus initiation time and callus inductivity of ‘Heimeiren’ was weaker than the previous two varieties significantly. Inductivity of somatic embryo among different genotypes has significant difference, the highest inductivity was gained from ‘Kangbingjingxin’ (46.2%) cotyledon, then ‘Zaojia 8424’ (34.1%) and ‘Heimeiren’ (15.7%). However, the number of somatic embryos per responding explant was similar for all the genotypes tested.

The different ability of somatic embryogenesis induction among different genotypes was reported in other Cucurbitaceae plants. Nadolska-Orczyk and Malepszy (1989) suggested a genetic determinism in the ability to regenerate somatic embryo derived plants from cucumber (*Cucumis sativus* L.) leaf explants. Carol et al. (1995) studied the somatic embryogenesis of six squash cultivars, all cotyledons produced somatic embryos after 11 to 17 weeks on induction medium. However, the optimal culture time and the rate of plant regeneration were significantly different between the six cultivars. Significant differences of somatic embryogenesis were also observed among different cultivars in melon (Toshiro et al., 1992). It proved that genotype was a important factor to affect somatic embryo induction. Most plants have potential to induce somatic embryo, but the sensitivity and difficulty of inducing is different; and the frequency of somatic embryogenesis is widely different as the difference of genotype in the same species.

Table 3. Effect of genotypes on induction of callus and somatic embryo

Genotype	Inductivity of callus (%)	Inductivity of somatic embryo (%)	Number of somatic embryos per explant induced somatic embryo
Zaojia 8424	94.4±2.0a	34.1±4.9b	6.2±0.6a
Heimeiren	77.8±5.1b	15.7±1.9c	6.3±0.5a
Kangbingjingxin	88.9±3.8a	46.2±3.9a	6.0±0.2a

Data represented mean ± SE of three replicates.

Means having the same letter in a column were not significantly different by Least Significant Differences test ($P = 0.05$).

3.3 Effect of Explants on Induction of Callus and Somatic Embryo

Callus inductivity of cotyledon node and hypocotyl was higher than cotyledon in MS medium with BA 3.0 mg/L and NAA 0.1 mg/L (Table 4). Callus induced from cotyledon and cotyledon node was mostly embryonic callus while callus from hypocotyl was non-embryonic callus which cannot induce somatic embryo (Figure 3 d-f). Somatic embryo inductivity of cotyledon node (62.0%) was significantly higher than cotyledon (46.1%) and hypocotyl (0%). However, somatic embryos number per explant of cotyledon node (2.3) was significantly lower than cotyledon (6.0). It pointed that explant type was an extremely important factor in somatic embryo induction.

Somatic embryogenesis is reported using diverse types of explants in Curcurbitaceae, for example, petiole (Punja et al., 1990), root (Trulson & Shahin, 1986), protoplast (Oridate & Oosawa, 1986; Zhang & Liu, 1998), unfertilized ovaries (Wang et al., 2008), leaf disc (Usman et al., 2011), hypocotyl (Zheng et al., 2003), cotyledon (Song et al., 2007), hairy roots (Biljana et al., 2004), unfertilized ovules (Xie et al., 2006) and nucellus (Kwack & Fujieda, 1988). In our experiment, cotyledon and cotyledon node were successfully induced somatic embryo while hypocotyl cannot induce somatic embryo.

Table 4. Effect of explants on induction of callus and somatic embryo

Explant	Inductivity of callus (%)	Inductivity of somatic embryo (%)	Number of somatic embryos per explant induced somatic embryo
Cotyledon	88.9±3.8b	46.1±3.9b	6.0±0.2a
Cotyledon node	96.7±3.4a	62.0±7.8a	2.3±0.2b
Hypocotyl	98.9±1.9a	0c	0c

Data represented mean ± SE of three replicates.

Means having the same letter in a column were not significantly different by Least Significant Differences test ($P = 0.05$).

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