Journal of Tropical Crop Science Vol. 5 No. 3, October 2018
www.j-tropical-crops.com

Somatic Embryogenesis of Melon (*Cucumis melo* L.) As Affected by Culture Media and Composition of Plant Growth Regulators

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Abstract

Conventional production of melon hybrid seeds requires a long time. Propagation by tissue culture can be an alternative method to produce hybrid melon seedlings in order to fulfill the high demand for uniform seedlings. Our current study was aimed to determine the type of propagation media and the best concentration of picloram for the induction of somatic embryogenesis in melon young seed explants. The study was expanded to examine the effective concentrations of two of auxins, 2-4 D and NAA, combined with BAP to induce somatic embryogenesis in melon using hypocotyl explants. The experiment was conducted at Plant Tissue Culture Laboratory, Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University. The experiment was arranged in a randomized complete block design with three replications. The first experiment tested three types of planting media (MS, B5, and WPM) and four levels of picloram concentration (0,0.5, 1.0, 1.5 mg.L^{-1}). The second experiment tested auxin (2,4-D and NAA) concentrations of 0, 1, 2, 3, 4, 5 mg.L^{-1} and two BAP concentrations, 0 and 1 mg.L^{-1}. The first study showed that no somatic embryos were formed with the media types and picloram concentration tested; the seeds, however, germinated and formed callus. In the second study, there was an interaction between concentration levels of auxin (2,4-D and NAA) and BAP on induction of somatic embryos using hypocotyl explants. Somatic embryo formation can be induced with treatment of 1 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP and 2 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP. The highest yield of embryos formation was with the treatment of 1 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP at 0.53 embryos per explant.

Keywords: embryogenesis, tissue culture media, melon, Picloram, 2,4-D, BAP

Introduction

Melon is usually propagated from seeds; the seeds were collected from the mature fruits for the following planting season. Production of hybrid melon seeds can be done by crossing the parent's line that has desirable characteristics. However, this method will take time to produce hybrid seeds. One alternative to plant propagation is by tissue culture. Tissue culture provides many advantages including plants that can be propagated at any time without depending on the season, high multiplication rate, requires small size of planting materials, plants produced uniformly, and free of diseases, mainly bacteria and fungi (Wattimena et al., 1992).

In vitro propagation can also be done on melon, which potentially save time, more efficient and economical compared to conventional culture. Propagation can be done at any time in large quantities and will reduce production costs for the supplier of melon seeds. Multiplication can be done through organogenesis and embryogenesis pathways. The method of somatic embryogenesis have received much attention because the number of propagules produced was not limited and could be obtained in a shorter time (Purnamaningsih, 2002).

In vitro propagation studies on melon have been widely reported. In the mid-1980s research on somatic embryogenesis from callus suspension cultures was reported by Oridate and Oosawa (1986). The study used MS media and a combination of concentrations of 2,4-D and BA. Callus planting on MS media added 1 mg.L^{-1} 2,4-D + 0.1 mg.L^{-1} BA showed the best response for embryogenesis. Kageyama et al. (1991) reported the planting of old seed cotyledons on MS media added with 1 mg.L^{-1} 2,4-D + 1 mg.L^{-1} NAA + 0.1 mg.L^{-1} BA also affected the formation of somatic embryo characteristics.

Tabei et al. (1991) examined the role of auxin regulation for organogenesis and somatic embryogenesis in melons (*Cucumis melo* L.). The results of the study reported that adventitious shoot formation occurred at a low level of auxin concentration (0 to 0.01 mg.L^{-1} 2,4-D; 0 to 0.1 mg.L^{-1} NAA; 0 to 1.0 mg.L^{-1} IAA), and embryos formed at the high level of auxin concentration (1.0 to 2.0 mg.L^{-1} 2,4-D; 3.0 to 10.0 mg.L^{-1} NAA; 20 to 100 mg.L^{-1} IAA). The addition of IAA was more efficient in shoot and embryogenesis induction than other auxins tested.

The uses of growth retardant for shoot development *in vitro* was reported by Gaba et al. (1996). The combination of ancymidol and BA showed the best shoot development compared to ancymidol or BA separately. Kintzios and Taravira (1997) reported that somatic embryo induction was influenced by genotype and light intensity. Both of these factors have significant effects on the number of somatic embryos, embryo maturation, and plantlet regeneration so the response can be variety specific.

Further research by Nakagawa et al. (2001) examined the effect of sugar and ABA on the induction of melon somatic embryogenesis from cotyledons. Sucrose can induce somatic embryos while mannitol can not induce the formation of somatic embryos. The highest somatic embryo formation was obtained from 0.5 µM ABA treatment plus 200 mM sucrose. Rhimi et al. (2006) reported the use of 2,4-D (0.25 to 1.0 mg.L^{-1}) and BA (0.10 to 0.50 mg.L^{-1}) was successful to induce somatic embryogenesis of two Tunisian melon cultivars, “Maazoun” and “Beji”.

Factors that influence somatic embryogenesis include types of explants, sources of nitrogen and sugar, and plant growth regulators (Purnamaningsih, 2002). The composition of these three factors determines the success in somatic embryogenesis induction. The current study consists of two experiments; the first experiment aimed to determine the type of media and the best concentration of picloram for the induction of somatic embryogenesis using young seed explants; the second experiment was aimed at determining the best combination of auxins (2,4-D and NAA) and BAP to induce somatic embryogenesis using hypocotyl explants.

Materials and Methods

Induction of Somatic Embryogenesis in Different Types of Media and Picloram Concentrations

This experiment was carried out using a factorial design arranged in a randomized block design (RBD). The experiment consisted of two factors; the first factor was three types of planting media (MS, B5, and WPM) and four levels of picloram concentration (0, 0.5, 1.0, 1.5 mg.L^{-1}) as the second factor. The planting was carried out for three days with three replications, and each replication contained five bottles. So there were 12 treatments and 180 experimental units.

The planting materials used in this study was the young seeds of hybrid melon H-7 collection of IPB. H-7 melon has white flesh color with smooth texture, flesh thickness of 4 cm, and total dissolved solid of 10.5^{o} brix. Fifteen days after pollination, young fruits were collected and washed with a brush under running water then soaked with a solution of Dhitane (fungicide) and Agrept (bactericide) with a concentration of 2 g.L^{-1} for 12 hours. The fruit was rinsed with sterile water then soaked with a bleach solution 25% for 20 minutes. After that, the fruit was rinsed with sterile water and then cut and seeds were separated from the flesh. The seeds were sterilized with bleach 10% for 15 minutes. The seeds were rinsed twice with sterile water mixed with five drops of betadine. After that, the seeds were sown into the culture media. The planted seeds were stored in a dark room with a temperature of 20 ^{o}C. Observation was carried out every four days to examine the development of embryo.

Induction of Somatic Embryogenesis with Different Concentrations of Auxins and BAP

This experiment was carried out in a factorial design arranged in a randomized block design (RBD). The experiment consisted of two factors; the first factor was auxin concentration (2,4-D and NAA) which consist of six levels i.e. 0, 1, 2, 3, 4, 5 mg.L^{-1}. The second factor was BAP concentrations of 0 and 1 mg.L^{-1}. The experiment was carried out with three replications, and each replication consists of three bottles. Each bottle contained five explants.

The planting materials were mature seeds of hybrid H-7 from IPB collection. The explants were hypocotyls derived from *in vitro* germinated seeds. Melon seeds were first soaked in sterile water for 12 hours. Soaked seeds were put into bleach solution of 10% for 10 minutes then rinsed twice using sterilized water. Seeds were soaked with a bleach solution of 5% for 5 minutes and rinsed twice with sterilized water prior to planting on MS media. Seeds were left for for 15 days in a dark room. Hypocotyls from germinated seeds were cut at ± 1 cm above the radicle and then planted in the treatment media. The planted hypocotyls were stored in a dark room at a temperature of 20 ^{o}C and examined every three days.

Results and Discussions

Induction of Somatic Embryogenesis in Different Types of Media and Picloram Concentrations

The percentage of seeds that formed callus

Callus formation was affected by picloram concentration from 32 DAP (day after planting) to the end of the observation (Table 1) while callus formation was not affected by type of media, or by interaction between media types with picloram concentrations.

Picloram is a type of auxin that stimulate cell elongation (Evans et al., 2003). In addition, auxins also play important roles in inducing callus formation, apical dominance and root initiation (Wattimena et al., 1992). Picloram in low doses has been reported to induce callus formation. Purba (2009) reported that picloram at 0.5 and 1.0 mg.L^{-1} was able to induce the formation of embryos in mangosteen. Media containing high concentrations of auxin can induce callus growth (Purnamaningsih, 2002). However, no embryo was formed in this study, it only formed callus and did not experience further development.

Table 1. Recapitulation of variance in percentage of seeds that formed callus

Days after planting	Media	Picloram Concentration	Interaction	CV (%)
16 ^{t}	ns	ns	ns	50.70
24 ^{t}	ns	ns	ns	85.21
32 ^{t}	*	*	ns	53.42
40	ns	**	ns	44.74
48	ns	**	ns	30.98
56	ns	**	ns	26.96
64	ns	**	ns	23.92
72	ns	**	ns	23.45
80	ns	**	ns	22.46
88	ns	**	ns	23.15
96	ns	**	ns	22.88

Note: ns not significantly different at p<0.05
* significant different at p<0.05
** significant different at p<0.01
^{t} transformed data ($\sqrt{x+0.5}$)

The concentration of picloram significantly affected the percentage of callus formation. Based on the observations presented in Table 2, when the explants were at 96 DAP, the average percentage of seeds that formed callus was the highest with the treatment of 0.5 mg.L^{-1} picloram (97.78%). However, it was not significantly different from the treatment of 1.0 and 1.5 mg.L^{-1}, i.e. 94.44% and 92.41%, respectively (Table 2).

The percentage of germinated seeds

The percentage of seed germination was influenced by the growing media, but it was not affected by the concentration level of picloram (Table 3). There was no interaction between media types and picloram concentrations in affecting the percentage of germination seeds.

Tabel 2. Effect of picloram concentrations on the percentage of callus formation

Picloram level (mg.L^{-1})	Days after planting				
	32 ^{t}	48	64	80	96
0	11.11 ab	25.00 c	30.83 b	33.75 b	35.56 b
0.5	17.78 a	68.89 a	90.56 a	96.11 a	97.78 a
1.0	10.56 ab	67.22 a	86.67 a	91.67 a	94.44 a
1.5	3.89 b	47.41 b	74.63 a	89.07 a	92.41 a

Note: Means followed by the same letters within the same columns are not significantly different at p<0.05 according to Duncan's multiple range test
^{t} transformed data ($\sqrt{x+0.5}$)

B5 media gave the best response to the average percentage of germination seeds, i.e. 89.17% (Table 4). Germination rate in B5 media was significantly higher than the WPM and MS media which only gave an average of 70.14 and 57.50% seed germination, respectively.

Nitrogen is a major factor in driving morphogenesis of *in vitro* culture (Purnamaningsih, 2002). Nitrogen in the tissue culture media is supplied in the form of NO^{3-} and NH^{4+} ions (Beyl, 2005), and nitrogen in the form of KNO_3 tends to promote explant growth. In B5 media which contains high nitrogen possibly had influenced the percentage of germinating seeds. Seed germination was not expected in somatic embryo induction. Germinating seeds will inhibit the formation of the embryo. Therefore, MS media was the best media used for embryo induction.

Induction of Somatic Embryogenesis with Different Concentrations of Auxins and BAP

Number of embryos and explants that produced embryo

Treatment of auxin (2,4-D and NAA) combined with BAP concentration interacted in affecting embryo formation per explant and number of explants that produced embryos. The recapitulation of variance is presented in Table 5.

The use of NAA combined with BAP showed a response to the formation of the embryo. Two treatments can induce the formation of embryos, namely a combination of 1 $mg.L^{-1}$ NAA + 0.1 $mg.L^{-1}$ BAP and a combination of 2 $mg.L^{-1}$ NAA + 0.1 $mg.L^{-1}$ BAP. Treatment of 1 $mg.L^{-1}$ NAA + 0.1 $mg.L^{-1}$ BAP gave the highest response compared to treatment of 2 $mg.L^{-1}$ NAA + 0.1 $mg.L^{-1}$ BAP and other treatments. Embryos formed in 1 $mg.L^{-1}$ NAA + 0.1 $mg.L^{-1}$ BAP treatment was 0.53 per explant, whereas the treatment of 2 $mg.L^{-1}$ NAA + 0.1 $mg.L^{-1}$ BAP only produced 0.07 embryos per explant (Table 6). Combination of 1 $mg.L^{-1}$ NAA + 0.1 $mg.L^{-1}$ BAP had the highest response in embryo formation (0.2 explants) whereas only 0.05 explants produced embryo in the treatment of 2 $mg.L^{-1}$ NAA + 0.1 $mg.L^{-1}$ BAP (Table 6).

Addition of auxin and cytokinins can induce the formation of embryo, with high ration of auxin to cytokinin (Wattimena et al., 1992). This is in line with the research of Kageyama et al. (1991) who reported

Table 3. Recapitulation of variance in the percentage of germinated seeds

Days after planting	Media	Picloram concentration	Interaction	CV (%)
16^t	ns	*	ns	65.69
24^t	ns	**	ns	47.29
32	ns	**	ns	55.82
40	**	**	ns	36.86
48	**	ns	ns	36.64
56	**	ns	ns	28.54
64	**	ns	ns	26.61
72	**	ns	ns	21.03
80	**	ns	ns	20.05
88	**	ns	ns	20.15
96	**	ns	ns	20.08

Note: ns not significantly different at p<0.05
* significant different at p<0.05
** significant different at p<0.01
^{t} transformed data ($\sqrt{x+0.5}$)

Table 4. The effect of media types on the percentage of germinated seeds

Media Type	Days after planting				
	32	48	64	80	96
MS	13.75	24.58 b	37.50 c	50.83 c	57.50 c
B5	22.92	50.42 a	67.92 a	84.17 a	89.17 a
WPM	14.48	33.61 b	49.69 b	64.86 b	70.14 b

Note: Means followed by the same letters within the same columns are not significantly different at p<0.05 according to Duncan's multiple range tests

that the use of 1 mg.L^{-1} 2,4-D + 1 mg.L^{-1} NAA + 0.1 mg.L^{-1} BA was able to induce somatic embryos from cotyledons of old seeds.

Embryogenic Callus Formation

Auxins (2,4-D and NAA) and BAP interacted in affecting embryogenic callus formation from 30 DAP until the end of the observation at 72 DAP (Table 7 and 8).

Combination of the type of auxins with BAP or auxin alone did not affect embryogenic callus formation. The use of NAA without BAP also did not affect embryogenic callus formation. Combination of NAA (1, 2, 3, 4, and 5 mg.L^{-1}) with BAP 0.1 mg.L^{-1} can produce embryogenic callus. Table 8 shows that treatments of 2 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP, 3 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP, and 5 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP produced the best response in embryogenic callus formation.

Table 5. Recapitulation of variance of the number of embryos per explant and number of explants that produced embryo at 72 days after planting

Parameter	Auxin	BAP	Interaction	CV (%)
Number of embryo per explant [t]	**	ns	**	8.41
Number of explants that produced embryo [t]	**	*	**	3.15

Note ns not significantly different at p<0.05
* significant different at p<0.05
** significant different at p<0.01
[t] transformed data ($\sqrt{x+0.5}$)

Table 6. The interaction between concentration of auxins (2,4-D and NAA) with BAP in affecting the number of embryo per explant and number of explants that produced embryo

Treatment	Number of embryo per explant	Number of explants that produced embryos
A0B0	0 b	0 b
A1B0	0 b	0 b
A2B0	0 b	0 b
A3B0	0 b	0 b
A4B0	0 b	0 b
A5B0	0 b	0 b
A6B0	0 b	0 b
A7B0	0 b	0 b
A8B0	0 b	0 b
A9B0	0 b	0 b
A10B0	0 b	0 b
A0B1	0 b	0 b
A1B1	0 b	0 b
A2B1	0 b	0 b
A3B1	0 b	0 b
A4B1	0 b	0 b
A5B1	0 b	0 b
A6B1	0.53 a	0.20 a
A7B1	0.07 b	0.05 b
A8B1	0 b	0 b
A9B1	0 b	0 b
A10B1	0 b	0 b

Note: Means followed by the same letters within the same columns are not significantly different at p<0.05 according to Duncan's multiple range tests (DMRT).
A0 : without auxin; A1 to A5 : 2,4-D at 1, 2, 3, 4, 5 mg.L^{-1}, respectively; A6 to A10: NAA at 1, 2, 3, 4, 5 mg.L^{-1}, respectively; B0: without BAP; B1: BAP at 0.1 mg.L^{-1} BAP.

Table 7. Recapitulation of variance of embryogenic callus formation

Days after planting (DAP)	Auxin	BAP	interaction	CV (%)
30	**	**	**	62.58
36	**	**	**	48.05
42	**	**	**	22.80
48	**	**	**	17.65
54	**	**	**	10.76
60	**	**	**	8.96
66	**	**	**	8.12
72	**	**	**	5.37

Note: ** Means are significantly different at p <0.01

Table 8. The interaction between concentration level of auxin (2,4-D and NAA) with BAP on the percentage of embryogenic callus formation

Treatment	Days after planting (DAP)			
	36	48	60	72
A0B0	0 c	0 b	0 b	0 c
A1B0	0 c	0 b	0 b	0 c
A2B0	0 c	0 b	0 b	0 c
A3B0	0 c	0 b	0 b	0 c
A4B0	0 c	0 b	0 b	0 c
A5B0	0 c	0 b	0 b	0 c
A6B0	0 c	0 b	0 b	0 c
A7B0	0 c	0 b	0 b	0 c
A8B0	0 c	0 b	0 b	0 c
A9B0	0 c	0 b	0 b	0 c
A10B0	0 c	0 b	0 b	0 c
A0B1	0 c	0 b	0 b	0 c
A1B1	0 c	0 b	0 b	0 c
A2B1	0 c	0 b	0 b	0 c
A3B1	0 c	0 b	0 b	0 c
A4B1	0 c	0 b	0 b	0 c
A5B1	0 c	0 b	0 b	0 c
A6B1	85.55 a	94.44 a	97.77 a	97.77 b
A7B1	84.44 a	97.77 a	97.77 a	100 a
A8B1	76.67 ab	97.77 a	96.67 a	100 a
A9B1	86.67 a	95.55 a	97.77 a	97.78 b
A10B1	66.66 b	91.11 a	100 a	100 a

Note: Values followed by the same letters within the same column are not significantly different at p<0.05 according to Duncan's multiple range test.
A0 : without auxin; A1 to A5 : 2,4-D at 1, 2, 3, 4, 5 mg.L^{-1}, respectively; A6 to A10: NAA at 1, 2, 3, 4, 5 mg.L^{-1}, respectively; B0: without BAP; B1: BAP at 0.1 mg.L^{-1} BAP.

In general, embryogenic callus induction requires high ratio of auxin to cytokinin (Wattimena et al., 1992). These results of this study are in line with Chee (1990) who reported that the use of 2 mg.L^{-1} 2,4-D + 0.5 mg.L^{-1} kinetin induced the highest percentage of embryogenic callus in cucumber.

Conclusion

Our study demonstrated that no somatic embryos were formed using the media and picloram concentrations tested. The seeds, however, germinated and formed callus. Auxin concentrations interacted with BAP in affecting somatic embryo formation using hypocotyl explants. Somatic embryos can be induced with 1 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP and 2 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP. The highest embryo formation was with the treatment of 1 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP to produce 0.53 embryo per explant. Moreover, the average number of explants that form the highest embryo in the treatment of 1 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP at 0.2 explants. Number of explants that produced embryo was the highest with the treatment of 1 mg.L^{-1} NAA + 0.1 mg.L^{-1} BAP.

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