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Full Length Research Paper

Efficient plant regeneration from cotyledonary node explants of *Cucumis melo* L.

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An efficient regeneration system was developed using cotyledonary node of two inbred line of melon, *Cucumis melo* 'CM-15' and 'CM-23'. The induction of shoots was achieved on Murashige and Skoog (MS) solid medium supplemented with different combinations of 6-benzylaminopurine (BA) and indole-3-acetic acid (IAA). The best shoot induction was observed when the explants were cultured on MS medium supplemented with BA (1.5 mg/L) and IAA (0.1 mg/L), and on MS medium supplemented with BA (1.5 mg/L) and IAA (0.5 mg/L) for 'CM-15' and 'CM-23', and inbred line CM-15 had the best regeneration rate. The shoot clumps were transferred to MS with BA (0.05 mg/L), resulting to the differentiation of most of the shoots initially into well developed shoots. Regenerated shoots were rooted on half-strength MS medium without plant growth regulators (PGRs). The rooted plants were established in soil with 100% success rate. This high frequency plant regeneration system provides improved technology to assist in genetic transformation of melon.

Key words: *Cucumis Melo* L., cotyledonary node, shoots regeneration, plant regeneration.

INTRODUCTION

Melon (*Cucumis melo* L.) is one of the oldest cultivated crops and an important horticultural crop grown in temperate, subtropical and tropical regions worldwide. According to the FAO, world production of melon in 2007 was about 26 million tons. Being a significant component of fresh fruit traded internationally, melon has a great potential for becoming a model plant for studying important traits in fruiting crops. Increasing demand for these crops will necessitate improving their agronomic characteristics, such as their resistance to disease and pest, in the past and present. Using traditional breeding methods, a large number of fine inbred, hybrid and elite lines of melon were cultivated by plant breeders. But under the pressures of limited land, expanding population, plant diseases and insect pests stresses, traditional breeding methods alone have not incorporate the great demand for melon in both quality and quantity. Consequently, several biotechnology approaches have received more emphasis, and genetically transformed melon plants have been obtained by various approaches, such as particle *Agrobacterium*-mediated (Awatef, 2007; Nuray et al., 2009). However, success or failure of melon genetic transformation largely depends on the ability of the transformed tissues.

Considerable work has been done on tissue culture and plant regeneration in melon. In earlier studies on cucurbits via somatic embryogenesis, methods of plant regeneration from primary explants of cotyledons and leaves were established (Valentine, 2010; Muthu et al., 2010; Curuk et al., 2003; Moreno et al., 1985; Ortz et al., 1987; Chaturvedi and Bhatnagar, 2001; Lee et al., 2003; Thomas and Sreejesh, 2004; Akasaka-Kennedy et al., 2004; Nuray et al., 2009).

However, only certain genotypes could be regenerated from leaf of low frequency, consequently, there is need to develop better procedures for melon plant regeneration from cotyledonary node, thus, the objective of this study was to develop a more efficient regeneration system through excised cotyledonary nodes to assist in more

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Abbreviations: BA, 6-Benzylaminopurine; **MS,** Murashige and Skoog; **IAA,** indole-3-acetic acid; **PGRs,** plant growth regulators.

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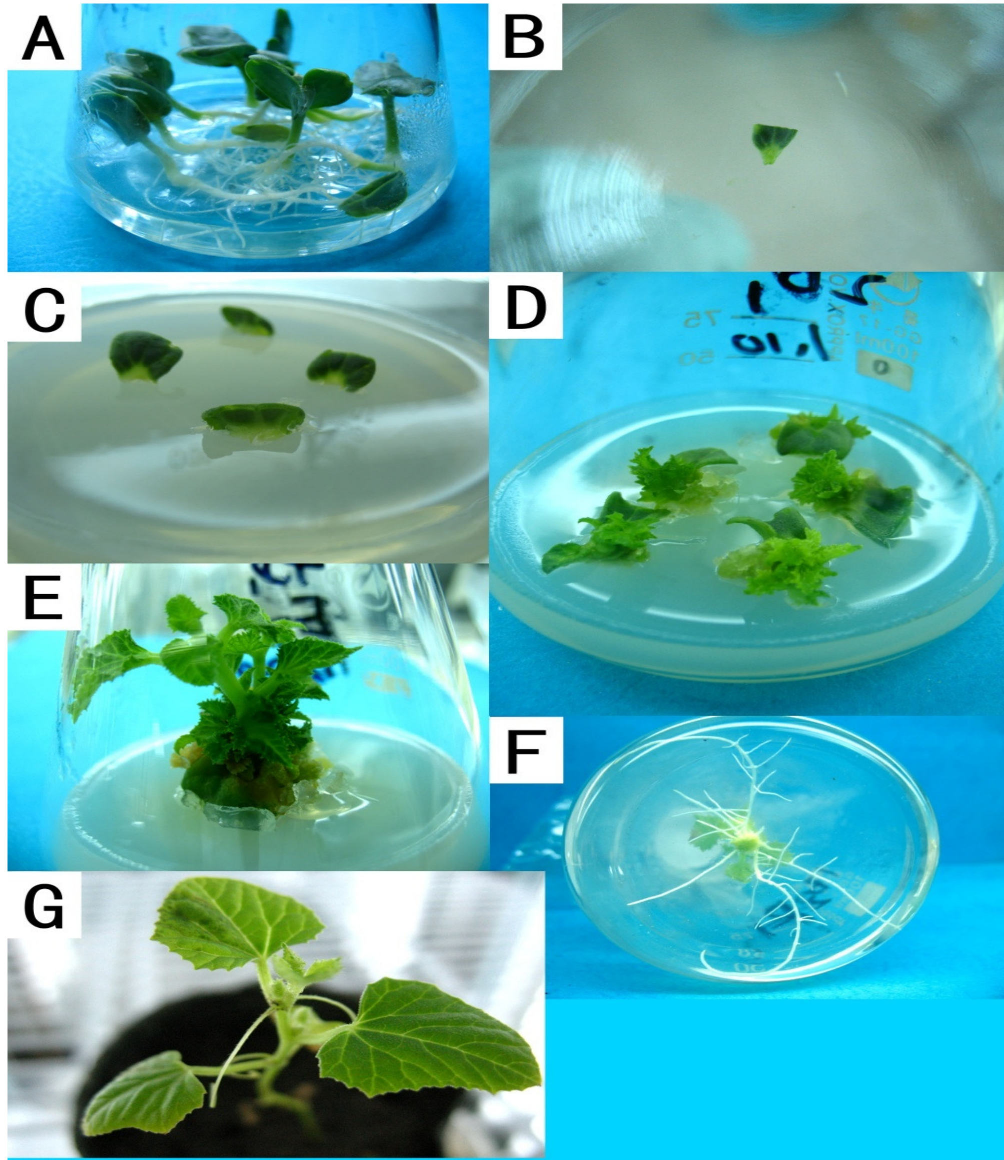

Figure 1. Several steps of explants in plant regeneration from cotyledon node explants of *C. melo* L A, Embryogenesis of melon CM-15, plants propagated by inbred line after 5 days in propagation medium; B and C, cotyledonary node explants prepared for regeneration; D, regenerated shoots after 3 to 4 weeks in regeneration medium; E, elongated shoots after 1 week in elongate medium; F, rooted shoots after 2 weeks in rooting medium; G, regenerated plants ready to be transferred to soil.

efficient genetic improvement method of melon.

MATERIALS AND METHODS

Matured embryo seeds were derived from viable seeds of melon inbred lines (CM-15). Seeds were supplied by the Watermelon and Melon Genetic Breeding Laboratory of Horticulture College at Northeast Agricultural University. Seeds were manually peeled and surface-sterilized with 70% (v/v) ethanol for 30 s followed by 10 min sterilization with 0.1% mercuric chloride ($HgCl_2$) and then rinsed with sterile distilled (3 min) water. Ten seeds were placed in a 100 ml triangle-bottle containing 25 ml of basal medium MS, supplemented with 30 g/L sucrose and solidified with 8 g/L agar (pH 5.8). All cultures were incubated in a growth chamber at 26 ± 2°C with a 16 h light/8 h dark photoperiod (cool-white fluorescent lights at 60 $mol \cdot m^{-2} \cdot s^{-1}$ photon flux) for 5 days.

Cotyledonary nodes were taken from 5 days old seedlings. Whole cotyledonary nodes were excised from aseptic seedling. Primary leaves and the epicotyls were detached from the seedling using sterile surgical blades. Then, the seedling was excised leaving it to be approximately 0.3 to 0.5 cm long (Figure 1A and B).

Shoot induction

Cotyledonary node explants of 5 days from seeding germination

Table 1. The shoot regeneration on 12 different media of the whole cotyledonary node of melon C-15 and C-23.

z	IAA (mg/L)	C-15		C-23	
		Percentage of shoot induction	Average number of shoots/explant (%)	Percentage of shoot induction	Average number of shoots/explant (%)
0.5	0.0	2.50±2.50^{h}	3.33±2.89^{h}	2.50±2.50g^{h}	5.00±1.00^{fg}
0.5	0.1	10.83±1.44^{fg}	13.33±1.44^{g}	11.67±3.82^{ef}	14±4.16e^{fg}
0.5	0.5	10.00±0.00^{fg}	15.00±2.50^{g}	15.00±4.33^{e}	21.67±7.64^{def}
1.0	0.0	64.16±5.20^{c}	70.00±433^{c}	50.83±5.20^{b}	36.67±8.78^{cd}
1.0	0.1	78.33±3.82^{b}	89.17±7.64^{b}	40.83±3.82^{c}	55.83±28.43^{ab}
1.0	0.5	36.66±3.82^{e}	42.50±2.50^{e}	25.83±6.29^{d}	30.83±8.78^{cde}
1.5	0.0	35.83±1.44^{e}	40.00±2.50^{e}	30.00±2.50^{d}	36.67±6.29^{cd}
1.5	0.1	97.50±2.50^{a}	105.83±7.64^{a}	54.17±2.89^{ab}	70.83±16.65^{a}
1.5	0.5	45.00±5.00^{d}	52.50±6.61^{d}	57.55±6.66^{a}	45.83±10.10^{bc}
2.0	0.0	13.33±1.44^{f}	23.33±3.82^{f}	15.83±3.82^{e}	21.00±1.32^{def}
2.0	0.1	9.16±1.44^{g}	17.50±2.50^{fg}	7.50±5.00^{fg}	13.33±3.21^{efg}
2.0	0.5	10.83±1.44^{fg}	18.33±3.82^{fg}	5.00±2.50^{gh}	7.33±5.84^{fg}

The data reported correspond to the mean values which were determined from triplicate experiments. The differences within each column were obtained using Duncan new multiple range text (DNMRT). Means (±standard deviation) with different small letters are significantly different at p≤0.05 level.

(Figure C) contains 25 ml of basal MS medium solidified with 8 g/L agar (pH 5.8, imported from Japanese agar), supplemented with 30 g/L sucrose and different combinations of PGR: BA (1.0, 1.5, 2.0 mg/L) and IAA (0.0, 0.1, 0.5 mg/L). All cultures were incubated in a growth chamber at 25 ± 2°C with 16 h light/8 h dark photoperiod (cool-white fluorescent lights at 60 mol·m^{-2}·s^{-1}photon flux).

Shoot elongation

After 30 days in shoot induction, explants with shoots from CM-15 were transferred to triangle-bottle containing MS medium solidified with 8 g/L agar and BA 0.05 mg/L, pH 5.8, supplemented with 30 mg/L sucrose without other growth regulators.

Rooting

One hundred (100) plantlets from CM-15 were excised and transferred to rooting medium. Regenerated shoots with over 3 cm height were placed onto rooting medium for evaluation of root development. Rooting medium was half-strength MS medium (devoid of casein hydrolysate, L proline and thiamine hydrochloride) supplemented with 30 g/L sucrose and was solidified with 8 g/L agar. The pH of the medium was adjusted to 5.8 before autoclaving. All cultures were incubated in a growth chamber at 26 ± 2°C with a 16 h light/8 h dark photoperiod (cool-white fluorescent lights at 60mol·m^{-2}·s^{-1}photon flux). After 3 weeks, well rooted plants were washed with tap water and transferred to plastic cups containing soil (obtained from a regional supplier), irrigated with water, under glasshouse conditions. Each cup was covered with a plastic bag and the plants grew up for 10 days by gradually reducing the humidity by making holes in the bag and transferred to the glasshouse following the same procedure used as control.

Experimental design and statistical analysis

All experiments were repeated a minimum of three times (except the soil establishment experiment) following a completely randomized block design. Data were analyzed by means of analysis of variance (ANOVA) on the statistical package of SAS (Version 9.0), the means were analyzed by Duncan new multiple range test (DNMRT) (p = 0.05) to evaluate the effects of different concentrations of BA and IAA on shoot induction.

RESULTS AND DISCUSSION

Shoot induction and elongation using BA and IAA

The effects of different concentrations of 6 BA and IAA in MS medium were studied using melon inbred lines CM-15 and CM-23 after 3-weeks on shoot induction medium, when most explants had formed a large number of shoots (Table 1), but CM-23 had formed small number of shoots (Table 1). After 2-weeks on shoot induction medium, most explants formed a large number of callus, different combinations of MS medium with respect to their callus induction capacity in cotyledonary node base explants, including low concentrations of BA (0.1 to 0.5 mg/L). In the media without BA, there was no shoot formation (not listed in Table 1). BA in the callus induction medium strongly promoted the frequency of callus induction and the development of compact and embryogenesis callus in melon inbred line. The production of soft and watery callus was reduced on BA containing medium. Callus on medium containing BA tended to turn compact and brown more easily than that on medium high of BA. After 4 weeks, most explants from the CM-15 had formed a large number of shoots (Figure 1D) and the results were significant (p<0.05). The highest induction rate (97.5%) was obtained when 1.5 mg/L BA and 0.1 mg/L IAA were supplemented for shoots. Several dozens of shoots per explants were obtained. Table 1 shows the number of

well developed shoots. The medium that produced most shoots per explants contained 1.0 mg/L BA and 0.1 mg/L IAA. Nevertheless, shoots that arose on medium with 1.5 mg/L BA, and 0.1 mg/L IAA were produced in large numbers. Considering quality and quantity together, it was clear that the best culture medium for shoot production was BA (1.5 mg/L) and IAA (0.1 mg/L). Significant genotypic differences were recorded among the inbred line CM-15 which was the most responsive; CM-23 showed poor response. In terms of shoot quality, CM-15 gave the best response in BA (1.5 mg/L) and IAA (0.1 mg/L). Genotypes are reported to play an important role in shoot response in various crop plants including melon (Krug, 2005; Awatef, 2007). We have found that MS supplemented with BA (1.5 mg/L) and IAA (0.1 mg/L) was inbred line CM-15 which has the best quality and quantity of shoot.

Elongation and rooting

The inbred line CM-15 plantlets was from medium transferred to elongation medium (Figure 1E). After three weeks, the plantlets were transferred to rooting medium. After 2 weeks, all of them formed roots (Figure 1F). This was repeated twice with the same results.

Plant regeneration

After 3 weeks, regeneration of plants with strong root systems was achieved (Figure 1F). To examine normal growth and development status, well-rooted plants with a similar number of seed-initiated plants were removed from culture medium, rinsed in water to remove culture media and transplanted into a mixture of equal parts (v/v) of sterilized soil and vermiculite grown under humid conditions in a growth room for 2 weeks. Plants were subsequently transplanted into a green-house environment and grown to maturity (Figure 1G). No morphological differences were observed among regenerates, or between regenerates. All plants were found to be fertile and set viable seeds which germinated and produced morphologically normal plants. The number of regenerated plants per explant obtained in this study was compared with reports of other melon species, where protocols considered as very efficient were developed using other methodologies (Moreno et al., 1985; Fang and Grumet,1990 ; Rhimi et al., 2006, 2007; Shyamali et al., 2007), it is clear that the method developed in this study for shoot regeneration from cotyledonary nodes of melon inbred line produced a quite similar amount of shoots per explant, and therefore can be considered very efficient as well. Additionally, as the system developed in this study was planned to be used for further transformation experiments, only the elongated shoots that were clearly independent from each other were cut and considered for evaluation (to prevent having more than one plant coming from the same transformation event in the future). Nevertheless, it is worth highlighting that as can be seen in Figure 1, the number of shoots' primodia that arose in most induction medium was very much higher than that in well formed shoots reported in Table 1. Therefore, if the shoot clumps stayed longer than one week in elongation medium, many more elongated shoots could be recuperated. This observation may be useful if the protocol is intended for some other applications, like plant micropropagation or basic studies on plant regeneration.

Although, in most reports, cotyledonary nodes are not used, possibly it could increase the efficiency of regeneration and the quality of the regenerated shoots in some cases. On the basis of this study, it would be advisable to include that it is a growth regulator when trying to establish a regeneration system for a new melon species, because although it is more expensive, it can lead to much better results. In this study, it can be concluded that an efficient method for shoot regeneration from cotyledonary node explants of melon has been developed. It can be used to perform further experiments to obtain transgenic plants of this species as well as for other biotechnological or agricultural approaches.

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