

## Morphogenetic Response of Cotyledon and Leaf Explants of Melon (*Cucumis melo* L.) cv. Amarillo Oro

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### ABSTRACT

Callus cultures from cotyledon and leaf explants of a Spanish cultivar of melon (Amarillo Oro) were tested for growth and morphogenic capacity on several culture media with different concentrations of IAA (indole-3-acetic acid) in combination with 1.0 mg.L<sup>-1</sup> BA (6-benzylaminopurine) or 6.0 mg.L<sup>-1</sup> KIN (kinetin). The best results were achieved with cotyledon explants. The leaf explants presented low bud formation capacity. Variability of organogenic response on cotyledons of different age (7, 5, 3 and 1-day-old) was evaluated. The age of explant had a significant influence on bud induction. Cotyledon explants from 7-day-old seedlings showed higher organogenic index and development of shoots when cultured onto MS medium supplemented with 1.5 mg.L<sup>-1</sup> of IAA and 1.0 mg.L<sup>-1</sup> of BA. The effect of cut type of cotyledonary explants on organogenic response was also investigated. Explants cut transversally showed the best results. The addition of copper sulfate in the culture medium promoted a qualitative improvement of the regenerated shoots.

**Key words:** Organogenesis, plant regeneration, leaf explant, cotyledon explant, cucurbitaceae

### INTRODUCTION

The melon (*Cucumis melo* L.) which belongs to the Cucurbitaceae family has an important social and economic role, mainly in the tropical regions of both hemispheres. Biotechnology offers tools which are capable of surpassing some limitations found by traditional plant breeding methods, by developing new material, through genetic engineering, molecular biology and tissue culture techniques. However, for the use of these possibilities it is necessary to develop of *in vitro* plant regeneration protocols, allowing the recovery of improved material for further propagation.

In earlier studies on morphogenesis of melon, methods for plant regeneration from primary explants of cotyledons and leaves were established (Moreno et al., 1985; Ortiz et al., 1987; García-Sogo, 1990; Bordas et al., 1991). Bordas (1994) carried out an additional study on the effect of type and concentration of gelling agents, besides confirming the effect of copper ion on the morphogenetic response in melon, as previously described by García-Sogo (1990). It has been demonstrated that morphogenetic response in melon is genotype dependent (Molina and Nuez, 1995; Souza, 1999). The establishment of a efficient plant regeneration protocol is also an essential step in the generation of new cultivars

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through plant transgenic technology. Considering these information, the objective of this work was to establish protocol of plant regeneration of the melon cultivar Amarillo Oro suitable for studies on plant genetic transformation.

## MATERIAL AND METHODS

### Plant Material

Cotyledon and two different types of leaves were used as explant source, namely, L1 - the first leaf that emerged from *in vitro* cultivated seedling and L2 - leaves from *in vitro* micropropagated plants. Approximately 1 cm<sup>2</sup> explants of all tissues were used in the experiments.

### Morphogenetic response of calli from cotyledon and leaf explants

Three sequential experiments were carried out. The culture media used for organogenesis

induction are described in Table 1. The variables studied in the three experiments were: **1) organogenic index:** (mean  $\pm$  SE), measured by the mean value obtained from arbitrary values (from 0 to 3) for each explant according to the intensity of the organogenic response. The arbitrary value considers the extension of the areas with the buds as well as their development, whereas: 0 = explant without bud formation; 1 = bud formation on less than half the explant extension; 2 = bud formation on approximately half the explant; 3 = bud formation covering almost the full extension of the explant; and **2) the number of shoots per callus** (mean  $\pm$  SE).

In all experiments, the explants were incubated under 16 h photoperiod at  $24 \pm 2$  °C, 70% relative humidity and fluorescent light providing 30  $\mu\text{E m}^{-2} \text{s}^{-1}$ .

**Table 1** - Organogenesis induction media used in the experiments.

	Organogenesis Induction Media				
	IB 05/10	IB 075/10	IB 10/10	IB 15/10	IK 15/60
Basal Solution	MS*	MS	MS	MS	MS
Sucrose (g.L <sup>-1</sup> )	30.0	30.0	30.0	30.0	30.0
Inositol (g.L <sup>-1</sup> )	0.1	0.1	0.1	0.1	0.1
Thiamine-HCl (mg.L <sup>-1</sup> )	1.0	1.0	1.0	1.0	1.0
IAA (mg.L <sup>-1</sup> )	0.5	0.75	1.0	1.5	1.5
BA (mg.L <sup>-1</sup> )	1.0	1.0	1.0	1.0	1.0
KIN (mg.L <sup>-1</sup> )	-	-	-	-	6.0
CuSO <sub>4</sub> .5H <sub>2</sub> O (mg.L <sup>-1</sup> )	1.0	1.0	1.0	1.0	1.0

\*Murashigue and Skoog (1962).

### Experiment 1. Effect of explants source and the medium culture in growth and organogenic response

The experimental design was two-way factorial arrangement 3 x 4 on a CRD (three types of primary explants and four culture media) with 18 culture flasks with two explants per flask. repetition by treatment, each repetition represented by one explant. The explants used were leaves L1, leaves L2 and cotyledons from 7-day-old seedlings. The culture media IB 05/10; IB 075/10; IB 10/10; IB 15/10 were used. The experiment was evaluated after 30 days.

### Experiment 2. Effect of age, culture media and copper sulphate addition in the growth and morphogenetic response of cotyledon explants

Four cotyledons ages - 1, 3, 5 and 7-day-old seedlings, two culture media (IB 15/10 and IK 15/60) and two concentrations of copper sulphate (0.025 mg.L<sup>-1</sup> - the standard concentration of MS - and supplemented with 1.0 mg.L<sup>-1</sup>) were investigated. The experimental design was three-way factorial arrangement 4 x 2 x 2 on a CRD with 48 repetitions by treatment, each repetition represented by one explant. Two evaluations were carried out at 30 and 60 days of cultivation.

### Experiment 3. Effect of cut type, culture medium and copper sulphate on morphogenetic response of cotyledons explants

The experimental design was three way factorial arrangement 2 x 2 x 2 on a CRD with 20 culture flasks with two explants per flask. Each repetition was represented by one explant. Cotyledons from 7-day-old-seedlings were used as explant and divided transversal and longitudinal into two equals parts, constituting two types of primary explants: transversal segments (0.5 cm x 0.5 cm) and longitudinal segment (1.0 cm x 0.3 cm). Two culture media (IK 15/60 and IB 15/10) and the addition of copper sulphate were also investigated. The experiment was evaluated after 30 days.

#### Statistical Analysis

The transformation  $\sqrt{x+5}$  was applied to the data of the variables organogenic index and number of buds per callus. The effects of treatments were tested by Analysis of Variance; differences among means were tested by LSD range test ( $p < 0.05$ ).

## RESULTS AND DISCUSSION

### Effect of explant type and culture medium on growth and organogenic response

The choice of an appropriated explant constitutes a main step in the establishment of plant regeneration protocols. Several protocols for melon have been described using different explants such as hypocotyls, cotyledons, leaves and roots (Moreno et al., 1985; Trulson and Shahin, 1986; Niedz et al., 1989; Bordas et al., 1991; Tabei et al., 1991; Souza, 1999; Souza, 2001; Lotfi et al., 2003). The results have shown that protocols using leaf explants were less efficient. However, studies with cucumber

demonstrated that the frequency of polyploid cells was much lower in leaf explants, when compared with cotyledons and hypocotyl, which rise the interest for the working with more stable starting materials (Colijn-Hooymans et al., 1988). In cotyledon explants, callus formation on the cut surface was observed approximately at 15-20 days of cultivation, depending on the medium. The results of this study showed that the capacity of plant regeneration from leaf explant was much lower than cotyledons. Table 2 showed the effect of the interaction of explant with culture medium on the organogenic index, where cotyledons were superior in all studied culture media.

The most common regeneration protocols for melon use cotyledons as explant source, while the use of leaves is not frequent due to their lower morphogenetic response. One of the factors that contributed for this negative result was the occurrence of callus hyperhydricity. Yadav et al. (1996) reported different factors affecting melon regeneration from leaf explants including leaf age, the addition of silver nitrate to the medium and the type of gelling agent. Therefore, further studies on such subjects could offer an alternative for solving the problem.

Regarding the mean number of shoots per callus, cotyledons presented a good development of the formed buds into shoots. The buds from leaf explants, however, did not show the capacity to develop into shoots. The best result was observed with cotyledons cultivated in IB 15/10 medium (Table 3).

The evaluated culture media did not show significant difference on organogenic index. However, for the number of shoots per callus, higher auxin concentration, combined with 1.0 mg.L<sup>-1</sup> of BA, favored the development of buds, resulting in a higher number of shoots (Table 3).

**Table 2** - Effect of culture medium and explant source on organogenic index.

Explant	Culture Media			
	IB 05/10	IB 075/10	IB 10/10	IB 15/10
Cotyledon	1.40±0.13 <sup>ab</sup>	1.60±0.14 <sup>aAB</sup>	1.90±0.12 <sup>aA</sup>	1.80±0.14 <sup>aAB</sup>
Leaf L1	0.78±0.13 <sup>bb</sup>	1.10±0.15 <sup>bb</sup>	1.00±0.15 <sup>bB</sup>	1.40±0.12 <sup>aA</sup>
Leaf L2	0.77±0.16 <sup>bBC</sup>	0.90±0.15 <sup>bAB</sup>	1.10±0.17 <sup>bA</sup>	0.47±0.11 <sup>bC</sup>

The numbers indicate mean ± SE. Values followed by different lower case letter within the column are significantly different at 5% level. Values followed by different upper case letter within the row are significantly different at 5% level.

**Table 3** - Effect of culture medium and explant source on number of shoots per callus.

Explant	Culture Media			
	IB 05/10	IB 075/10	IB 10/10	IB 15/10
Cotyledon	0.80±0.14 <sup>ab</sup>	1.10±0.15 <sup>ab</sup>	1.30±0.15 <sup>aA</sup>	1.50±0.14 <sup>aA</sup>
Leaf L1	0.20±0.09 <sup>ba</sup>	0.08±0.09 <sup>ca</sup>	0.05±0.07 <sup>ba</sup>	0.04±0.06 <sup>ba</sup>
Leaf L2	0.14±0.11 <sup>bb</sup>	0.60±0.12 <sup>ba</sup>	0.22±0.11 <sup>baB</sup>	0.08±0.07 <sup>bb</sup>

The numbers indicate mean ± SE. Values followed by different lower case letter within the column are significantly different at 5% level. Values followed by different upper case letter within the row are significantly different at 5% level.

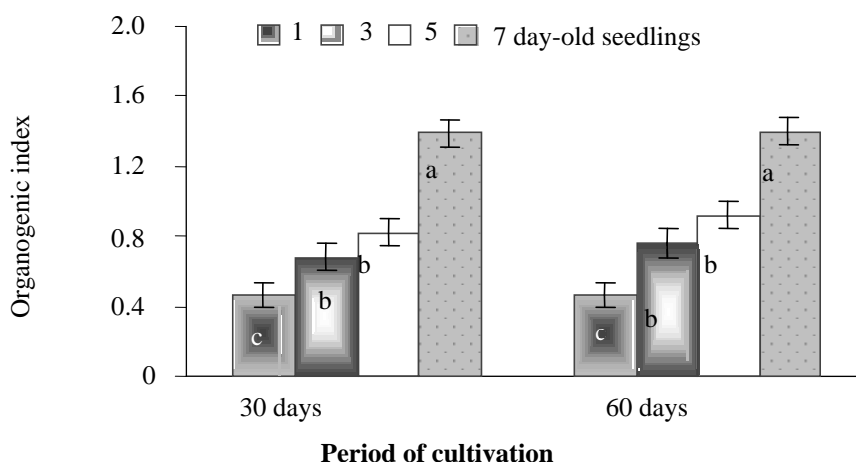
### Effect of age, culture medium and addition of copper sulfate on growth and organogenic response in cotyledon explants

According to the Analysis of Variance, the age of cotyledons was highly significant for both studied variables. The highest organogenic index and number of shoots per callus were observed in seven-day-old cotyledons after 30 and 60 days of cultivation (Figs. 1 and 2).

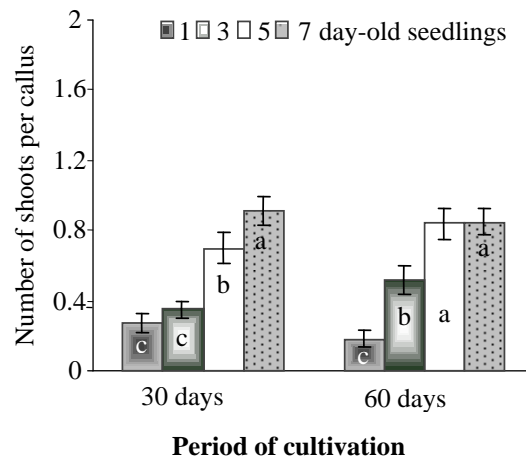
Moreno et al. (1985) tested several culture media with different concentrations of IAA and KIN in Amarillo Oro cultivar. The most effective combination for organogenic response was 1.5 mg.L<sup>-1</sup> of IAA and 6.0 mg.L<sup>-1</sup> of KIN (IK 15/60). Since then, such medium has been the most commonly used in melon regeneration studies with different melon cultivars (Bordas, 1994). However, preliminary experiments conducted in our laboratory has shown that IB 15/10 medium was more efficient than IK 15/60 in plant regeneration of some melon cultivars (data not shown). Therefore, this experiment was also

carried out to investigate the effect of IB 15/10 medium in the cultivar Amarillo Oro. The analysis of number of shoots per callus, at 30 days of cultivation confirmed that IB 15/10 was more efficient than IK 15/60 (Fig. 4).

Organogenic response in Cucurbitaceae is highly genotype dependent. Ficcidenti and Rotini (1995) reported shoot production in 11 melon cultivars using BA as growth regulator. In addition, Dabaúza (1995) obtained an expressive organogenic response in cotyledon explants from *Citrullus colocynthis*, using only BA as growth regulator. Recently, Souza (1999) reported the organogenic response of explants from several cultivars in two culture media, where in one of them only 1.0 mg.L<sup>-1</sup> of BA was added, while in the other IAA and KIN were supplemented. The number of shoots per callus was higher in the cultivars Shipper and Melón de Onteniente when BA was used in the culture medium, while for the cultivars Eldorado 300, Amarelo and Cantaloup Charentais, IK 15/60 medium was more efficient.



**Figure 1** - Effect of cotyledon age on organogenic index at 30 and 60 days of culture the two culture media. Columns with different letter in the same period of cultivation are statistically different at 5% level; the bars represent the stand error.

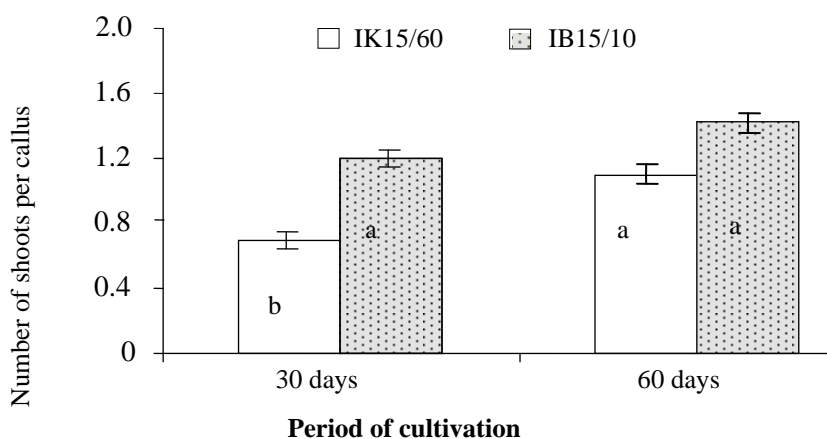


**Figure 2** - Effect of cotyledon physiological stage on number of shoots per callus at 30 and 60 days of culture in the two culture media. Columns with different letter in the same period of cultivation are statistically different at 5% level; the bars represent the stand error.

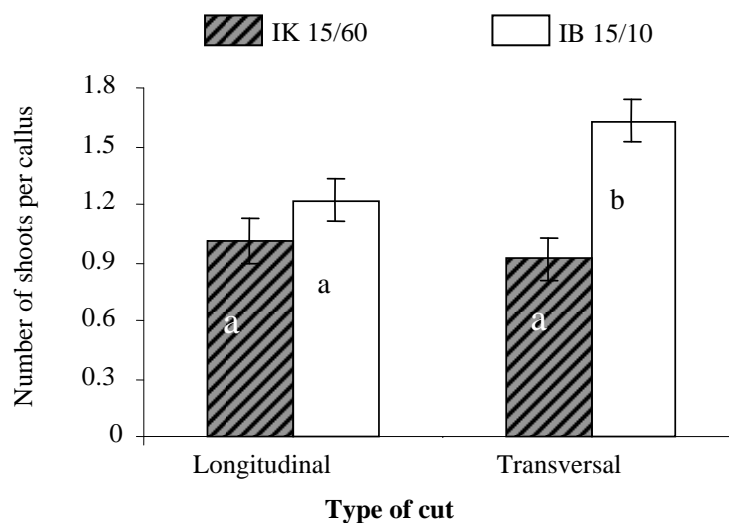
Although significant improvement by the addition of copper sulfate on culture media was observed in many melon cultivars (García-Sogo, 1990; Bordas, 1994; Souza, 1999), in our experiment, there was no significant change on the organogenic index and number of shoots per callus. Our results indicated that the age of cotyledon had a great influence on organogenic response. Therefore, further experiments were conducted using 7-day-old cotyledon explants.

#### Effect of cut type, culture medium and copper sulfate addition on growth and organogenic response in primary cotyledons explants

Even though the isolated effect of cut type was not significant, the interaction between with the culture medium was significant for both organogenic index and number of shoots per callus. The highest values were registered when cotyledons were cut transversally and cultivated in IB 15/10 medium (Fig. 4).



**Figure 3** - Effect of culture medium on number of shoots per callus at 30 and 60 days of culture. Columns with different letter in the same period of cultivation are statistically different at 5% level; bars represent the stand error.



**Figure 4** - The effect of cut and culture medium on numbers of shoots per callus. Columns with different letter in the same type of cut are statistically different at 5% level; the bars represent the stand error.

The culture medium IB 15/10 promoted a significant increase on both organogenic index and number of shoots per callus. As the concentration of IAA was the same in both culture media, it suggested that, for this cultivar,  $1.0 \text{ mg.L}^{-1}$  of BA was more effective than  $6.0 \text{ mg.L}^{-1}$  of KIN on organogenic induction.

There are few reports on morphogenesis in cucurbits that describe the effect of cut type of explants. In cucumber, Gambley and Dodd (1991) evaluated many cut types on cotyledons and found that when the basal region was not present in the explant, there was no shoot production, independently of the culture medium used. In this work, although the results of copper sulfate addition in the culture medium were not quantitatively significant, it promotes a qualitative improvement of the regenerated plants (data not shown). The plant regeneration protocol established in this work was suitable and currently in use at our laboratory for plant genetic transformation studies.

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#### RESUMO

Explantes de cotilédones e folhas do cultivar de melão Amarillo Oro foram cultivados para avaliação do potencial morfogênico em diferentes meios de cultura com diversas concentrações de ácido indolacético (AIA), em combinação com  $1,0 \text{ mg.L}^{-1}$  benziladenina (BA) e  $6,0 \text{ mg.L}^{-1}$  de cinetina (CIN). Os melhores resultados foram obtidos com explantes de cotilédones, sendo que explantes de folhas mostraram uma capacidade baixa na indução de gemas. A variação na resposta organogênica em cotilédones de diferentes idades fisiológicas (1, 3, 5 e 7 dias após a germinação) mostraram que os melhores resultados foram obtidos com cotilédones de sete dias cultivados no meio MS suplementado com  $1,5 \text{ mg.L}^{-1}$  de AIA e  $1,0 \text{ mg.L}^{-1}$  de BA. O efeito do tipo de corte na resposta organogênica de explantes cotiledonares foi também avaliado. Os melhores resultados foram obtidos com explantes cortados transversalmente. Adição suplementar de sulfato de cobre ao meio de cultura resultou em uma melhora na qualidade da dos brotos regenerados.

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