Adventitious regeneration from haploid melon (*Cucumis melo* L.) leaves as an approach to increase the frequency of diploid plants

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Running head: Efficient haploid melon micropropagation

Abstract

An efficient process of adventitious regeneration from haploid plant leaves to increase the proportion of diploid plants of six elite melon lines 'Védrantais' French cultivar, 'Piel de Sapo' Spanish line 'T111' and four Near Isogenic Lines (NILs : SC 6-6, SC 7-2, SC 7-4, SC 8-4) has been developed. Several hormone combinations, *in vitro* culture conditions and leaf position on the plant stem, were analyzed to improve the regeneration frequency and the percentage of diploid plants in these lines. Flow cytometry showed that diploid plant production was optimized by regeneration from haploid leaves as the percentage of diploid plants increased from 11% with regeneration of diploid cotyledons to 60% in this work. Evaluating the four NILs, only the lines SC 6-6 and SC 8-4 showed a significantly higher percentage of diploidy with 80% and 72.7%, respectively. The leaf position factor had no effect on regeneration and ploidy level while darkness negatively influenced regeneration but had no effect on ploidy.

Furthermore, it was concluded that the hormonal combination (cytokinin plus auxin) induces low endoreduplication activity, which is the phenomenon responsible for the polyploidy. Furthermore, this study provided evidence that in melon polyploidy occurs during the process of organogenesis, and, therefore, one should focus on this phase to avoid polyploidization of cells and, thus, avoid polyploid plants.

Keywords: Melon; Haploid leaves; Diploid plants; Ploidy level; Endoreduplication

Introduction

Melon (*Cucumis melo* L.) is a diploid plant (2n = 24) belonging to the Cucurbitaceous family that is grown in the tropical, subtropical, and temperate regions where it is one of the most important crops in the fresh products market. Over the past decades, the production and marketing of melon has rapidly increased due to, among others, the development of *in vitro* culture techniques, such as the production of double haploid lines *via* parthenogenesis (Gonzalo *et al.* 2011), which provide pure lines for the production of commercial hybrids.

Genetic transformation in melon remains a difficult task, as it is a recalcitrant species (Yalcin-Mendi *et al.* 2004; Çürük *et al.* 2005; Chovelon *et al.* 2011) with a lower transformation efficiency than other horticultural plants (Nuñez-Palenius *et al.* 2008) ranging from 0 and 12.5% (Ren *et al.* 2012). However, a satisfactory increase in the transformation rate (23.3%) was reached after selecting some Near Isogenic Lines (NILs) with both a good regenerative capacity and transformation rate (Castelblanque *et al.* 2008; Menéndez *et al.* 2012). Nonetheless, the determination of ploidy level showed that 89% of the transgenic plants were tetraploid, which was considered a negative result since tetraploid plants in melon are mostly sterile and, therefore, failed to be perpetuated by seed. In comparison with diploid plants, tetraploids show important phenotypic alterations, such as short internodes, thicker leaves, and

a reduced productivity due to its low fertility (Nugent and Ray 1992). Furthermore, the fruits are not marketable as they are characterized by their smaller size than those from diploid plants, flattened or rounded shape, and their propensity to crack (Nugent and Ray 1992; our own unpublished results).

The spontaneous generation of tetraploid plants from diploid genotypes has been frequently reported in melon tissue cultures and is considered a limiting factor for its genetic manipulation (Ezura *et al.* 1992). While most regenerated melon plants were polyploid when regeneration started from cotyledons, regeneration from leaves of diploid plants gave lower percentages of polyploids (Guis *et al.* 2000; Souza *et al.* 2006; Chovelon *et al.* 2008).

In order to avoid this phenomenon, the present work focused on developing a protocol for the adventitious regeneration of plants from haploid plant leaves. The effects of genotype, hormonal combination, and leaf position on the plant stem and darkness during shoot induction were studied on the regeneration rates and ploidy level of the regenerated plants. Setting up this protocol would have the additional advantage of producing homozygous transgenic diploid plants after genetic transformation of haploid leaf tissue with the consequent reduction of time dedicated to self-pollination and selection of homozygous plants.

Materials and methods

Plant material

Six melon haploid lines were generated, following the protocol described in Gonzalo *et al.* (2011), from the French cultivar "Védrantais", the Spanish line "Piel de Sapo" 'T111', and four Near Isogenic Lines (NILs), namely ‘SC 6-6’, ‘SC 7-2’, ‘SC 7-4’, and ‘SC 8-4’, were used. These NILs, developed from a population of Doubled Haploid Lines (DHLs) and perpetuated by seed, were selected for their higher regeneration capacity within a population of 32 NILs (Castelblanque *et al.* 2008). This population was derived from a hybrid between the Korean cultivar "Songwhan Charmi" accession 'PI 161375' and the elite line 'T111' (Eduardo *et al.*

2005; Gonzalo *et al.* 2011). Each NIL contained a single genotype introgression from the Korean accession into the 'Piel de Sapo' genetic background. This Korean accession has alleles of beneficial effects for some traits of fruit quality, weight, and the sugar content (Monforte *et al.* 2004). Following the regeneration and genetic transformation protocol with cotyledon explants, the 'PI 161375' accession had a higher adventitious regeneration capacity and *Agrobacterium*-mediated genetic transformation efficiency than 'T111' (Castelblanque *et al.* 2008), which improved the response of some NILs with the genetic background of 'T111' (Menéndez *et al.* 2012).

Explants and *in vitro* culture conditions

Shoot tip multiplication cultures of haploid plants were maintained in basal medium without plant growth regulators and were transferred to fresh medium every four wk. Two wk after subculture to fresh medium, the youngest four unfolded leaves were collected from each shoot. Five transversal and parallel cuts were made on each leaf and across the midrib every 5 mm (± 1 mm). Leaves were placed on the induction medium with its abaxial side in contact with the culture medium and transferred to fresh medium after three wk of culture (d 21). Six wk after culture in the induction medium (d 42), shoots observed on the leaf explants were isolated and transferred to elongation medium (Fig. 1). Three wk later (d 63), a subculture was made to the same medium of elongation by placing each plant in a tube to favor shoot and leaf development and facilitate the determination of the ploidy level by flow cytometry.

The basal medium (BM) consisted of Murashige and Skoog (MS) salts (Murashige and Skoog 1962) supplemented with B5 vitamins (Gamborg *et al.* 1968), 30.0 g L^{-1} sucrose, 0.6 g L^{-1} MES, 1.0 mg L^{-1} $CuSO_4 \cdot 5H2O$ (all chemicals provided by Duchefa Biochemie B.V., Haarlem, The Netherlands), and 8.0 g L^{-1} Bacto agar (Difco Laboratories, Becton, Dickinson and Company, Le Pont de Claix, France). The pH was adjusted to 5.8 before autoclaving. The induction regeneration medium (RM) consisted of BM supplemented with 2.21 µM BAP (6-

Benzylaminopurine) and 0.47 μM IAA (Indole-3-acetic acid) and sterilized by filtration. The elongation medium was composed of the same BM plus 6.0 μM Silver Thiosulfate (STS), an inhibitor of ethylene action that promotes stem elongation (Nichols and Kofranek 1982; Altman and Solomos 1995; Sharp *et al.* 2000; Sharp 2002), (chemicals provided by Duchefa Biochemie B.V., Haarlem, The Netherlands). All cultures were maintained at 28ºC, a 12hr photoperiod, and a light intensity of 140 μmol m^{-2} s^{-1}.

Effect of leaf position on the stem

Two wk after subculture on fresh medium, the four youngest unfolded leaves were collected from each shoot, then starting at the apex their relative position from 1 to 4 was recorded. For each position, leaf size was as homogeneous as possible, as it is expected to influence regeneration (Kathal *et al.* 1988; Yadav *et al.* 1996). For each genotype and leaf position, a total of 151 leaves were cultured.

Effect of darkness during the induction phase

To determine the effect of darkness on regeneration, half of the cultured leaves from each genotype were placed in the dark and at 28°C during the first five-d of culture while the other half were cultured under 28ºC, a 12hr photoperiod, and a light intensity of 140 μmol m^{-2} s^{-1}. For each genotype and culture condition a total of 211 leaves were used.

Effect of hormones on inducing adventitious regeneration

The effect of three hormonal combinations were tested on the regeneration medium, (1) 2.21 μM BAP (6-Benzylaminopurine) (Castelblanque *et al.* 2008); (2) 2.21 μM BAP plus 0.47 μM IAA (Indole-3-acetic acid) (Menéndez *et al.* 2012); and (3) 1.0 μM BAP plus 1.0 μM 2iP (6-(γ,γ-dimethylallylamino)-purine (Guis *et al.* 2000). All cultures were maintained at 28ºC, a 12hr photoperiod, and a light intensity of 140 μmol m^{-2} s^{-1}. For each genotype, two leaves were placed in each medium, and the assay was repeated 5 times.

Determination of ploidy level by flow cytometry

Young leaves from actively growing plants were finely chopped and macerated into 2.0 mL LB01 lysis buffer (Dolcet- Sanjuan *et al.* 1997) with 2.0% (*v/v*) Triton X-100 (Sigma-Aldrich, Madrid, Spain) to release intact nuclei. The solution obtained was filtered through an 80 µm nylon mesh to remove cell debris. An aliquot of 500 µL was taken and 5.0 µL of propidium iodide (1.0 mg mL^{-1}) added, which is capable of binding to DNA and of emitting a proportional fluorescence to the amount of DNA in each nucleus once stimulated by an ultraviolet light. The computer system converts each fluorescent signal situated in different positions according to their intensity to a point on the screen. The resulting graphics (Fig. 2) sorted data based on nuclear DNA content on the horizontal axis and counted the number of nuclei with the same DNA content in the vertical axis. The cytometer was previously calibrated with a haploid and a diploid melon plant. The peak corresponding to the haploid cells was positioned to 200 units of fluorescence (FL3) while the peaks of diploid and tetraploid cells were positioned at 400 and 800 fluorescence units, respectively. The ploidy level of the analyzed plants was determined based on the position of the majority peak obtained for each plant.

Statistical analyses

Significance analysis of the effect of leaf position and darkness on the regeneration frequency and ploidy level was performed by χ^2-test ($P < 0.05$) using R statistical software. The significance of the effect of hormonal combinations on regeneration was analyzed by the ANOVA and Duncan's Multiple Range Test ($P < 0.05$) using the GenStat Discovery Edition 3 statistical software while the effect on ploidy level was carried out with the χ^2-test ($P < 0.05$) using R statistical software.

Results and Discussion

Effect of leaf position on regeneration and ploidy level

The results of the effect of the leaves' position on regeneration and ploidy level are shown in Table 1. The phenomenon of the regeneration of plants from explants depends on several factors. Some are obvious in terms of their effects on regeneration, for example, genotype, explant type, and growth medium. The number of publications in this sense is significant (Neidz *et al.* 1989; Tang *et al.* 2003; Petri and Scorza 2010). Nevertheless, there are some factors that are rarely studied, such as leaves' position on the axis of the stem, which is the reason of interest in this present study. Statistical analysis of the results showed a non-significant effect of the leaves' position on the regeneration with a $\chi 2$ calculated = 2.52 < $\chi 2$ tabular = 7.81, the *p-value* was 0.47 > to 0.05. Kintzios *et al.* (2000) demonstrated that leaves' position in chili pepper has an effect on the induction of somatic embryogenesis. However, as in this present work, Fuentes *et al.* (1998) were able to show in tomato that the factor of leaves' position does not affect regeneration. Therefore, it appears that the effect of the position of the leaves on regeneration depends on the species.

In the present study the age of the leaf was clearly associated with its position, as it decreases from the bottom to the top of the plant. Regarding the ploidy level, statistical analysis showed no significant effect among the four leaf positions on the ploidy, where $\chi 2 = 4.87$, with *p-value* = 0.18. . It was demonstrated in many species that polysomaty (ploidy level in tissues before regeneration) increases with the age (elongation and cell expansion) of the plant organ (Galbraith *et al.* 1991; Colijn-Hooymans *et al.* 1994; Smulders *et al.* 1994; Mishiba and Mii 2000; Fukai *et al.* 2002; Lukaszewska and Sliwinska 2007; Ogawa *et al.* 2012), but it appears that this phenomenon has exceptions and that it is dependent on the species and tissue type. Gilissen *et al.* (1993) and Castro *et al.* (2007) demonstrated that in cucumber and *Polygala vayredae* the pattern of the polysomaty (coexistence of different ploidy cells in the same tissue) of the leaves during expansion does not change in comparison to the other organs. These

observations may explain why in the present study there has not been a significant difference in ploidy between different leaf positions.

Many studies relate polyploidization plants originating from *in vitro* culture to the phenomenon of endoreduplication, which consists of repeated cycles of DNA synthesis without mitosis (Joubes and Chevalier 2000). Bulk *et al.* (1990) observed in tomato that there is a correlation between polysomaty and the frequency of polyploid plants. Otherwise, the study conducted by Bubner *et al.* (2006) showed that this correlation was significant among one genotype "Arizona" of *Nicotina attenuata* but that it was absent in another genotype "Utah" of the same species. This clearly demonstrates the existence of genotype effect on the determination of ploidy level on one hand. On the other hand, it seems that the difference between the two genotypes could be due to the failure of polyploid callus of genotype "Utah" to produce polyploid plants as if there was a genetic selectivity when regenerating plants from cells with different ploidy. This selective regeneration has been observed in melon by Ezura and Oosawa (1994a, b).

The question of whether there is or is not a relationship between the polysomaty and ploidy level in melon can be formulated differently: Is the ploidy level determined by endoreduplication before or during organogenesis process? Ezura and Oosawa (1994a) were able to demonstrate that frequency of tetraploid callus cells increases with culture time while the diploid callus cells decrease. Also, Kathal *et al.* (1992) revealed that the percentage of polyploid plants regenerated *via* direct organogenesis from melon leaf tissue increases with the evolution in time of the *in vitro* culture. Indeed, polyploidy varied from 50% after 1 mo of cultivation to 90% after 3 mo in culture. These two studies demonstrate that in melon the polyploidy occurs during organogenesis and that reducing the culture time would reduce the percentage of polyploidy.

Given the importance of knowing when endoreduplication occurs, the present study decided to make a pattern of polysomaty on the study's material and compare it with the ploidy level of regenerated plants in order to confirm whether the endoreduplication really occurs during the organogenesis process. The results were unequivocal since there was no relationship between the patterns of polysomaty (percentage of nuclei contained DNA with various "C-value") and the percentage of ploidy level of the plants obtained after regeneration (Fig. 3). According to the results of the present study's work and those of Ezura and Oosawa (1994a) and Kathal *et al.* (1992), the present study considers that in melon the endoreduplication (responsible phenomenon of the polyploidy) occurs during the organogenesis process, therefore there is probably no need to focus on the morphological and physiological characteristics (such as size and age) of the explant. One should rather focus on the process that occurs during the induction of the cells to avoid the tetraploid plants. Ezura *et al.* (1997) concluded by means of long-term work that in melon the endoreduplication can be avoided during the process of regeneration and therefore maintain stable ploidy along the culture time. The work was done from aggregates of meristematic apices induced from the culture of the apical bud in a liquid medium with agitation at low speed. Also, Adelberg (1998) demonstrated in African melon (*Cucumis metuliferus*) that the induction of the regeneration in a liquid medium may help reduce the frequency of the tetraploid plants insofar as the transfer in Agar medium is done at the appropriate time.

Effect of darkness on regeneration and ploidy level

The results show that only 23.66% of the leaves subjected to darkness regenerated while it was observed that half of the leaves regenerated in absolute light conditions. Darkness had a negative effect on regeneration since the statistical test was highly significant while there was no recorded difference regarding ploidy level between both conditions (Table 2).

Many researchers noted that the incubation of cell cultures under a reduced density of photosynthetic photon flux or in the dark would be preferable for the induction of somatic embryos in many species, such as melon (Gray *et al.* 1993; Guis *et al.* 1997; Kintzios and Taravira 1997), cucumber (Cade *et al.* 1988; Colijn-Hooymans *et al.* 1988), and chili pepper (Kintzios *et al.* 2000); but as far as induction of shoots (organogenesis), it was demonstrated that in melon light is necessary for the regeneration of cotyledons explants (Leshem *et al.* 1995). Yet, it seems that the effect of light depends on the type of explants. Curuk *et al.* (2003) demonstrated that in melon the direct regeneration from hypocotyl occurs at a high level both under the light and darkness while they confirmed the requirement of light for regeneration of the cotyledons. In this study, it has been shown that the leaves of melon also require light to regenerate plants.

Effect of genotype on regeneration and ploidy level

The effect of genotype on regeneration in this work has been well established (Table 3) confirming what was also obtained in many studies on melon (Valdez and Gatica 2009; Kiss-Baba *et al.* 2010; Chovelon *et al.* 2011; Ren *et al.* 2012). In terms of its effect on ploidy level, this study has demonstrated from the rate of diploid plants obtained (varying between 37.5% and 80%) and its statistical analysis that the polyploidization frequency depends on the genotypes (Table 3). Many studies had the same conclusion (Guis *et al.* 1997; Ellul *et al.* 2003; Bubner *et al.* 2006). Among the four NILs, only SC 6-6 and SC 8-4 lines have shown a highest percentage of diploidy with 80% and 72.7%, respectively. This study has noted through many studies in melon that the frequency of regeneration *via* organogenesis was more efficient in cotyledons than leaves; however, the percentage of diploid regenerated plants was higher when using leaves than cotyledons as original explants (Guis *et al.* 2000; Nora *et al.* 2001; Souza *et al.* 2006; Nuñez-Palenius *et al.* 2007; Chovelon *et al.* 2011).

Effect of hormonal induction on regeneration and ploidy level

In the present study, it was demonstrated that the hormones used in different culture media have an effect on the frequency of regeneration (Fig. 4). Analyses of variance for the effect of culture media, genotypes, and their interaction on the number of regenerated plants were significant, with *p-values* of 0.004, < 0.001 and 0.030, respectively, at significance level $\alpha = 0.05$.

The beneficial effect of BAP in combination with IAA on shoot induction has been observed in melon by Kathal *et al.* (1986), Neidz *et al.* (1989), and Kiss-Baba *et al.* (2010). It was demonstrated in melon that auxin IAA offers greater efficiency in shoot formation and embryogenesis than other auxins (Tabei *et al.* 1991). It is well known that cytokinins stimulate cell division and induce shoot formation and that within this type of hormones, the BAP is by far the most widely used for its efficiency in the process of organogenesis in melon (Nuñez-Palenius *et al.* 2008) while auxins exert a strong influence on the initiation of cell division and induce the formation of roots.

The choice of using BAP alone in the second medium was based on the observation of significantly higher callus growth on explants cultured in the medium containing auxin (BAP plus IAA). Indeed, the second test came to confirm the role of IAA, namely, that its addition to a medium carrying BAP increased callus production and relatively inhibited shoot formation, which explained the reduction in the number of plants grown in the BAP plus IAA medium with respect to the medium that carried only BAP (Fig. 4). The same phenomenon was observed in other genotypes of melon by Dirks and Buggenum (1989) and also in watermelon by Compton and Gray (1993). As to the low rate of regeneration (0 to 41.66%) observed in the carrying medium BAP plus 2IP, it is explained by the fact that many shoots were vitrified, a typical effect of an excessive concentration of cytokinins in melon (Leshem *et al.* 1988a, b).

In this paper, the effect of hormones on the ploidy level has been demonstrated (Table 4; Fig. 5). This study has already noted that the polyploidization of plants is related to the phenomenon of endoreduplication, which is intensified, among some species for some kinds of

explants, by the increasing size (elongation and expansion) of the cells. In fact, Valente *et al.* (1998) demonstrated in tobacco that during cell elongation the nuclei increased in size and showed that a clear pattern of polyploidy development correlated with the age and size of the cells. Also, it was possible to demonstrate that the signal of the presence of auxin (NAA) alone in the medium induced elongation (80% of the cells) and DNA endoreduplication while the signal of an auxin (NAA) plus cytokinin (BAP) combination induced cell division starting with an amitosis led to the deduplication of DNA, then served a normal mitosis cycle and thereby decreased the polyploidy of cells. The same hormonal effect (auxin, cytokinin / auxin) on the ploidy level was observed from different tissues of legumes (Joubes and Chevalier 2000). In another study done from haploid plants of *Petunia*, a genetic stability was observed when BAP was used alone in the induction medium. While with this medium 80% of the regenerated plants were haploid and 90% of the regenerated plants were diploid with a medium containing only IAA (Liscum and Hangarter 1991). Another type of auxin (2.4.5-trichlorophenoxyacetic acid) induced the same phenomenon of cell expansion and DNA endopoliploidization in mesocarpic cells of parenchymal of the apricot fruit (Bradley and Crane 1955). It has been proven at the molecular level that the cytokinins induced rapid activation of the cell cycle regulator via dephosphorylation of tyrosine, which is required to initiate the mitosis process (Zhang *et al.* 1996). It appears that cytokinins played an important role in the induction of the mitosis process and therefore in the stabilization of the ploidy level while auxins allowed the endoreduplication genetic material, thus giving rise to the phenomenon of polyploidization.

The present work has come to the same conclusions as it is the medium combining BAP plus IAA that provided more diploid plants (Table 4); therefore it is the one that induced higher mitotic activity and lower endoreduplicative activity. Furthermore, this study has observed that the combination of cytokinins and auxins not only induced better gene stability compared to

the effect of auxins as pointed out above but also in comparison to the effect of the presence of cytokinin alone in the medium or a combination of cytokinins (Table 4).

It should be noted that this study has not obtained haploid plants regenerated from explants unlike the work done in *Petunia* (Liscum and Hangarter 1991) and *Larix decidua* (Aderkas and Anderson 1993). The failure to obtain regenerated haploid plants may be due to the types of culture medium used in the study, which are unfavourable for haploid plant regeneration. Additionally, based on this study's previous experiences, with the production of doubled haploid lines in melon (Gonzalo *et al.* 2011) as well as in cucumber (Claveria *et al.* 2005) ploidy level of the plantlets determined, *in vitro* is the same as their derived mature plants acclimated and transferred to the greenhouse for self-pollination. Morphologically, *in vitro* doubled haploid plantlets compared with their *in vitro* haploid progenitors used as tissue donors have significantly wider, broader, and thicker leaves.

Conclusion

The present study demonstrated that the proportion of diploid adventitious plants was increased with the regeneration from haploid leaves. Also, it showed the importance of the induction phase in the control of the endoreduplication phenomenon, therefore in the control of the ploidy level of regenerated plants. This control should necessarily go through the study and understand the factors (such as hormones, physical culture medium, and culture time) that affect this phenomenon. Since the principal objective of this study is to obtain fertile homozygous diploid transgenic plants, future work will focus on the genetic transformation of haploid plant leaves from the SC 6-6 and SC 8-4 lines with the best percentage of diploid plants.

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Author contributions

YMM and RDS conceived, designed the study, and wrote the manuscript. YMM, EM, and EC carried out laboratory experiments. The authors have read and approved the final manuscript.

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Declarations

The authors declare that they have no conflict of interest.

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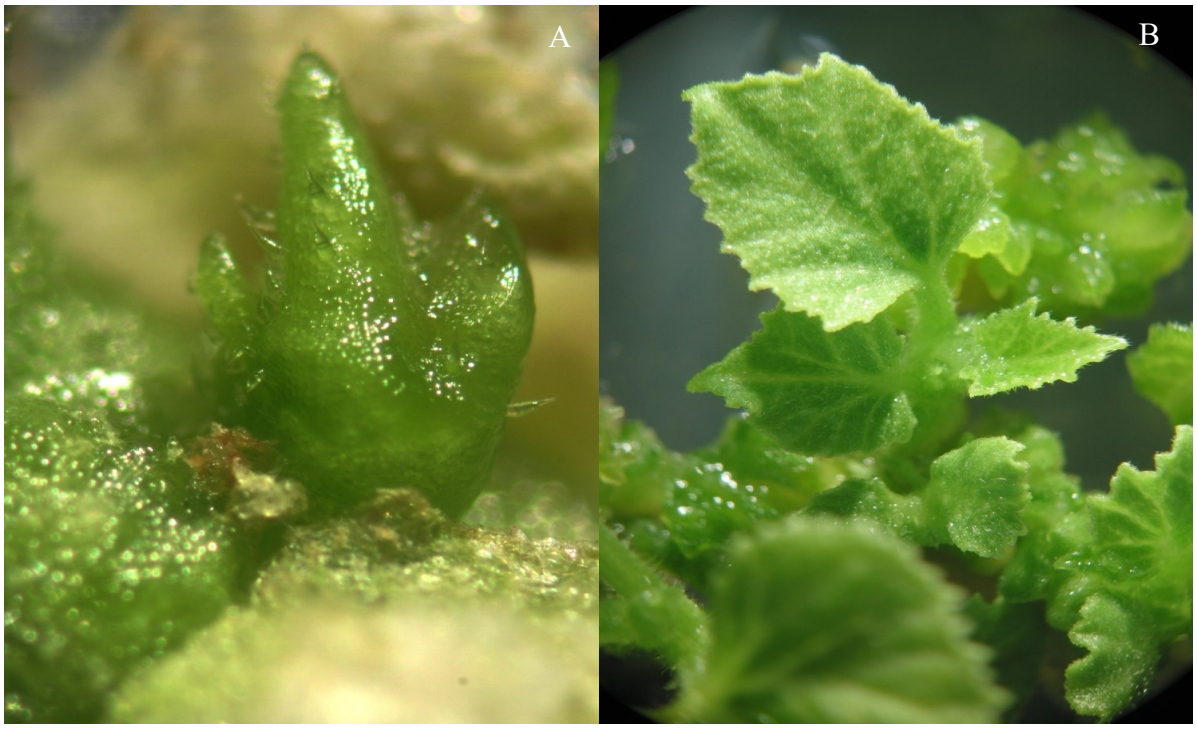

Figure 1. Adventitious regeneration from haploid *Cucumis melo* L. leaves, after six wk of culture in induction medium (A), and after three additional wk in elongation medium (B).

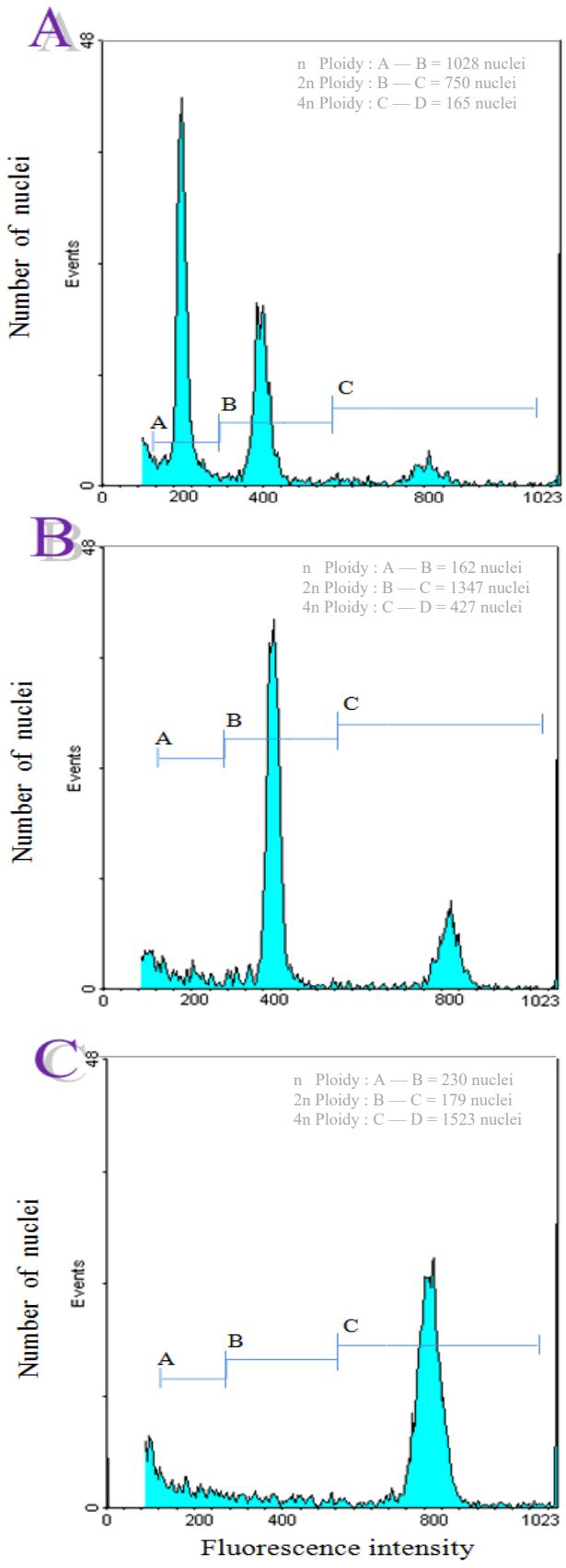

Figure 2. Ploidy level by flow cytometry of haploid (A), diploid (B), and tetraploid *Cucumis melo* L. plants (C), derived from the NIL SC 8-4.

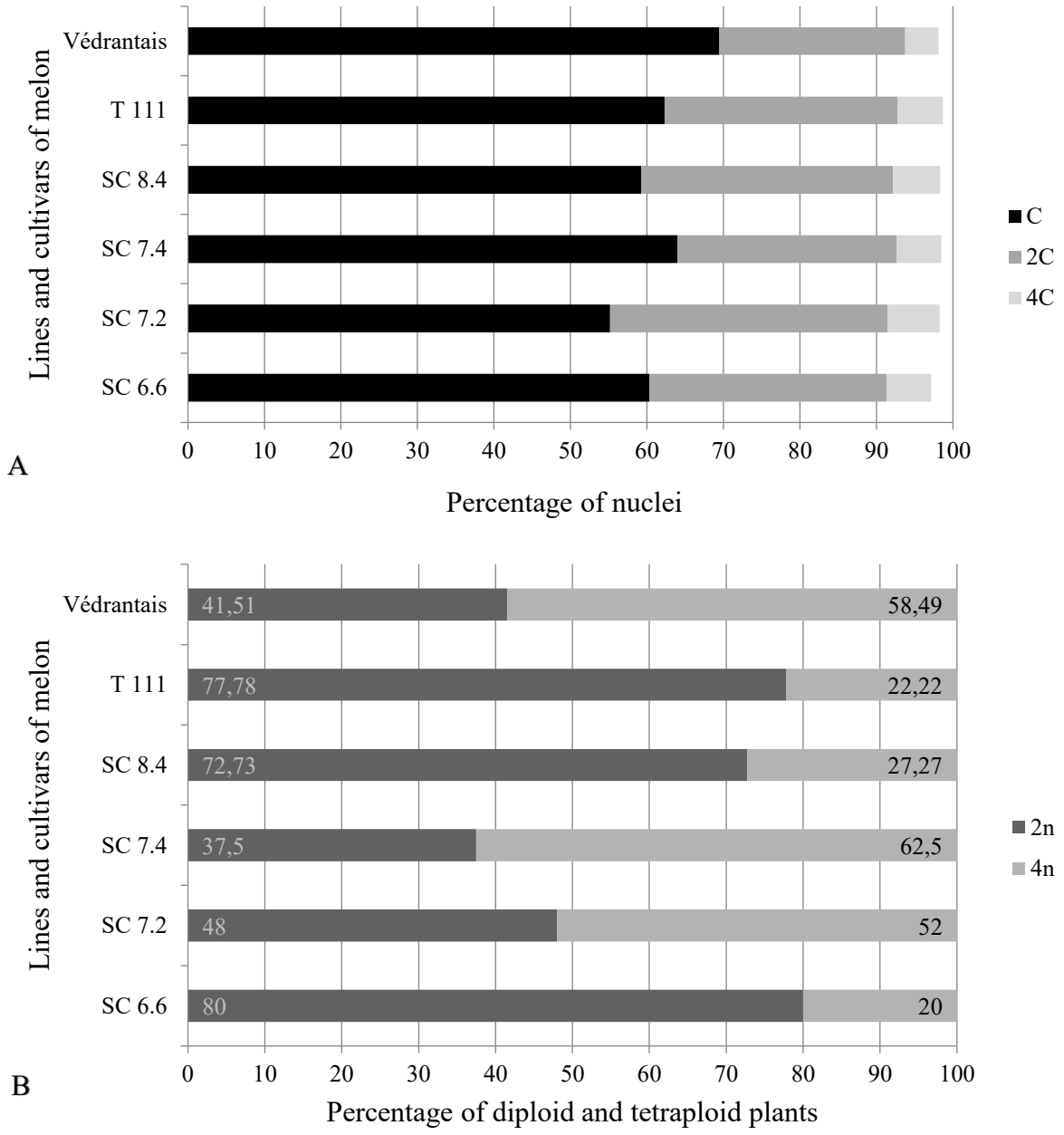

Figure 3. Polysomaty pattern in leaves of haploid *Cucumis melo* L. plants (A), without significant differences between genotypes (*p-value* = 0.867). Ploidy level of plants regenerated from haploid leaves (B), with significant differences between genotypes (*p-value* = 0.015). There is no relationship between the patterns of polysomaty and the percentage of ploidy level of the plants obtained after regeneration.

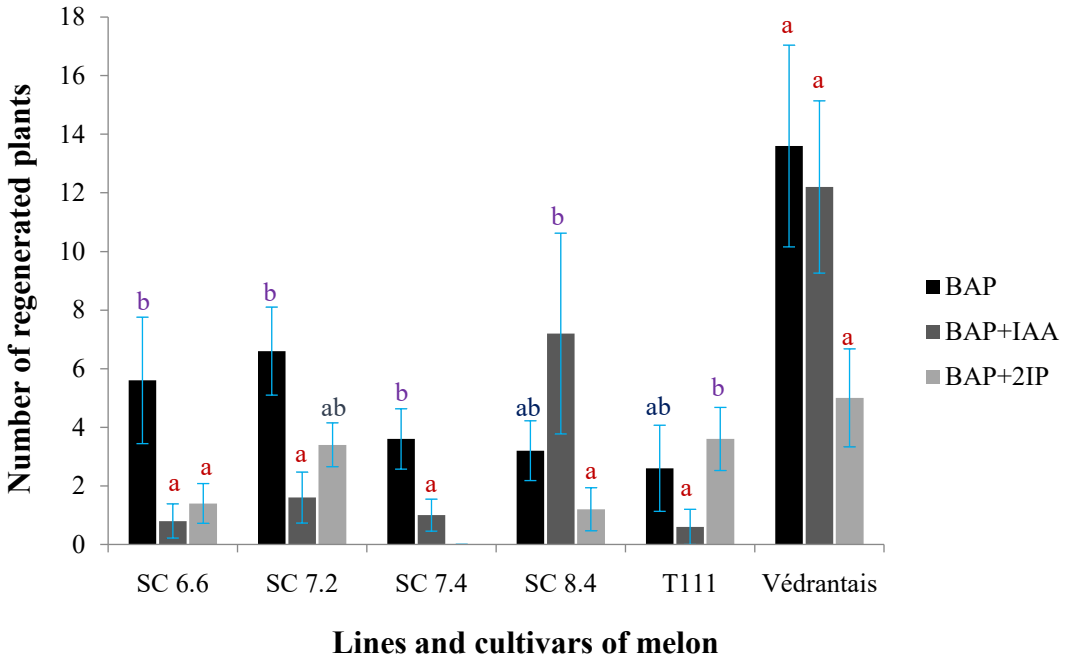

Figure 4. Effect of three hormonal combinations on the number of *Cucumis melo* L. regenerated plants from leaves of four haploid NILs (SC 6.6, SC 7.2, SC 7.4 and SC 8.4) and two haploid cultivars (T 111 and Védrantais). Vertical bars show standard errors. Bars with the same letters are not significantly different by Duncan's Multiple Range Test (p = 0.05).

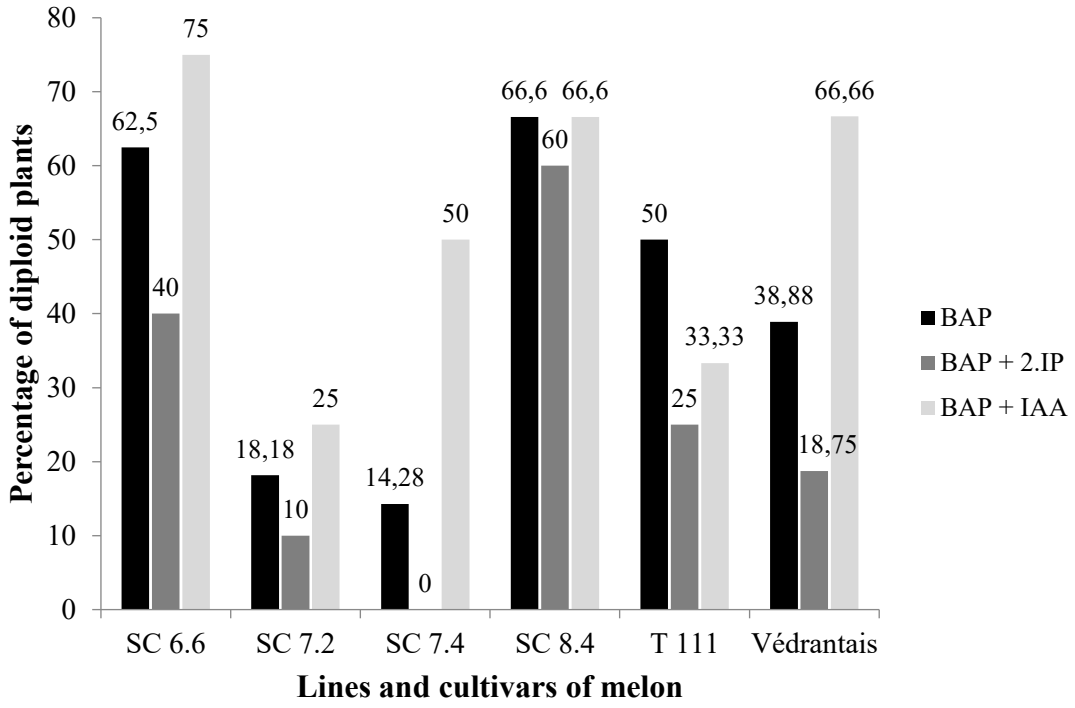

Figure 5. Effect of three hormonal combinations on the percentage of diploid *Cucumis melo* L. plants regenerated from leaves of four haploid NILs (SC 6.6, SC 7.2, SC 7.4 and SC 8.4) and two haploid cultivars (T 111 and Védrantais). Significant difference between the three hormonal combinations (*p-value* = 0.0015).

Table 1. Effect of leaves position on regeneration and ploidy level in *Cucumis melo* L.

LPS	Nº TL	Nº LWLOP [NS-1]	PLR	Nº OP	PL [NS-2]	
					% 2n	% 4n
1	60	18	30	59	53.7	46.3
2	53	14	26.41	32	62.5	37.5
3	25	11	44	26	31.58	68.42
4	13	4	30.77	8	37.5	62.5

LPS = Leaves position on the stem, Nº TL = Number of total leaves, Nº LWLOP = Number of leaves with at least one plant, PLR = Percentage of leaves that have regenerated, Nº OP = Number of obtained plants, PL = Ploidy level.
[NS-1] Non-significant statistical test (*p-value* = 0.47 > 0.05)
[NS-2] Non-significant statistical test (*p-value* = 0.18 > 0.05)

Table 2. Effect of darkness (5-d) on regeneration and ploidy level in *Cucumis melo* L.

EC	Nº TL	Nº LWLOP ***	PLR	Nº OP	PL [NS]	
					% 2n	% 4n
Darkness	93	22	23.66	66	50	50
Light	118	59	50	186	54.95	45.05

EC = Environmental conditions, Nº TL = Number of total leaves, Nº LWLOP = Number of leaves with at least one plant, PLR = Percentage of leaves that have regenerated, Nº OP = Number of obtained plants, PL = Ploidy level.
*** Significant statistical test (*p-value* = 9.36e-05 < 0.05)
[NS] Non-significant statistical test (*p-value* = 0.57 > 0.05)

Table 3. Effect of genotypes on regeneration and ploidy level in *Cucumis melo* L.

Genotypes	Nº TL	Nº LWLOP ***	PLR	Nº OP	PL *	
					% 2n	% 4n
SC 6-6	42	10	23.81	15	80	20
SC 7-2	31	17	54.84	61	48	52
SC 7-4	18	08	44.44	13	37.5	62.5
SC 8-4	48	10	20.83	40	72.73	27.27
T 111	26	07	26.92	09	77.78	22.22
Védrantais	46	29	63.04	105	41.51	58.49

Nº TL = Number of total leaves, Nº LWLOP = Number of leaves with at least one plant, PLR = Percentage of leaves that have regenerated, Nº OP = Number of obtained plants, PL = Ploidy level.
*** Significant statistical test (*p-value* = 5.4e-05 < 0.05)
* Significant statistical test (*p-value* = 0.015 < 0.05)

Table 4. Effect of three different media on percentage of ploidy level in *Cucumis melo* L.

Media of culture	N° of 2n plants	N° of 4n plants	Ploidy Level **	
			% 2n	% 4n
BAP	22	34	39.28	60.72
BAP plus IAA	31	21	59.61	41.39
BAP plus 2IP	13	39	25.00	75.00

** Significant statistical test (*p-value* = 0.0015 < 0.05)