

Effect of colchicine and amiprofosh-methyl on the production of *in vitro* doubled haploid onion plants and correlation assessment between ploidy level and stomatal size

Efecto de la colchicina y del amiprofosh-metil en la producción *in vitro* de plantas dihaploides de cebolla y determinación de la correlación entre el nivel de ploidía y tamaño de los estomas

María Laura Foschi ¹, Liliana Estela Martínez ², María Teresa Ponce ²,
Claudio Rómulo Galmarini ^{1,3}, Borut Bohanec ⁴

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ABSTRACT

Doubled haploid onion (*Allium cepa L.*) plants allow the production of completely homozygous lines for a later production of hybrids. The haploid plants are normally produced using *in vitro* gynogenesis. The obtained haploid plantlets must be treated with different agents for doubling chromosomes. It is necessary to adjust the concentration and the length of treatment of the doubling agent. In this case, the effect of 250 and 500 mg.L⁻¹ colchicine and 15.2; 30 and 60 mg.L⁻¹ amiprofosh-methyl during 24 and 48 h was assessed over the rate of onion haploid plantlets chromosome doubling. The best duplication treatment was 250 mg.L⁻¹ colchicine for 48 h, which yielded 100% of doubled haploid plants. On the other hand, a positive correlation resulted from the ploidy level and stomatal size, and a negative correlation between the level of ploidy and stomatal density. Significant differences between the stomatal length, width and density in haploid

RESUMEN

La producción de plantas dihaploides de cebolla (*Allium cepa L.*), permite obtener líneas completamente homocigotas para luego producir híbridos. A través de la ginogénesis *in vitro* se ha logrado producir plantas haploides, las cuales deben ser sometidas a tratamientos de duplicación de sus cromosomas, siendo necesario ajustar la concentración del agente duplicador y la duración del tratamiento. En este trabajo se evaluó el efecto de la aplicación de 250 y 500 mg.L⁻¹ de colchicina y 15,2; 30 y 60 mg.L⁻¹ de amiprofosh-metil durante 24 y 48 h, sobre la tasa de duplicación de cromosomas en plántulas haploides de cebolla. El mejor tratamiento de duplicación fue 250 mg.L⁻¹ de colchicina, durante 48 h, logrando un 100% de plantas dihaploides. Por otra parte, se obtuvo una correlación positiva entre el nivel de ploidía y el tamaño de estomas y una correlación negativa entre el nivel de ploidía y la densidad estomática, registrándose diferencias

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- 1 Chair of Horticulture and Floriculture. Agricultural Faculty. National University of Cuyo. Alte. Brown 500. Chacras de Coria, Mendoza. Argentina. M5528AHB. mfoschi@fca.unco.edu.ar
- 2 Chair of Plant Physiology. Agricultural Faculty. National University of Cuyo. Instituto de Biología Agrícola de Mendoza-Consejo Nacional de Investigaciones Científicas y Técnicas (IBAM-CONICET). Alte. Brown 500, Chacras de Coria, Mendoza. Argentina. M5528AHB.
- 3 Estación Experimental Agropecuaria INTA La Consulta, Mendoza, Argentina - Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).
- 4 Biotechnical Faculty. University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia.

and doubled haploid plantlets were observed. An economical and quick method to test ploidy level in onion plantlets is proposed through the measurement of stomatal size and density.

Keywords

Allium cepa L. • antimitotic agents • duplication • gynogenesis • hybrids

Palabras clave

Allium cepa L. • agentes antimitóticos • duplicación • ginogénesis • híbridos

significativas entre los valores promedio de largo, ancho y densidad de estomas para plantas haploides y dihaploides. Se propone un método económico y rápido para verificar el nivel de ploidía de las plántulas de cebolla mediante la medición del tamaño y densidad estomática.

Abbreviations

APM: amiprofosh-methyl
DAPI: 4',6-diamidino-2- phenylindole
DH: doubled haploid
DMSO: dimethyl sulfoxide
MS: Murashige and Skoog

INTRODUCTION

The development of homozygous lines is a fundamental step in the production of onion hybrids. However, one of the drawbacks of conventional improvement methods for this species is the prolonged time required for obtaining pure lines through self-pollination. In onion, more than three inbreeding generations cannot be done without high depression levels due to consanguinity. As a result, only partial homozygous lines can be obtained (1). The production of doubled haploid (DH) plants is a strategy used in a large number of species (17, 18, 26). It allows the production of complete homozygosity plants in a short period of time.

In onion, the production of DH is achieved through haploids induction via gynogenesis (*in vitro* cultivation of flowers, ovaries or non-pollinated ovaries) and followed by doubling the chromosomes number resulting in highly homogenous individuals with a high degree of genetic stability (2, 3, 12, 13). The production of complete homozygous plants in a shorter period constitutes a critical stage and is affected by: explant type, doubling substance, treatment length and treatment conditions (34). The most widely used doubling agent either in onions or other species is colchicine (3, 4, 14, 20), with an average doubling efficiency of 70% (21, 24, 25, 32). Less frequently, and with variable efficiency levels, trifluralin, oryzalin and amiprofosh-methyl (APM) have been also used (4, 16, 22, 29).

Several studies about genome doubling in various species have been carried out. A protocol needs to be developed for 'Cobriza INTA' cultivar, there are several methods described in the literature for onion, but not for this cultivar. Another critical and laborious step in obtaining DH is the determination of ploidy level (8). The most frequently used methods have been chromosome counts in meristem root tips and the use of flow cytometry. The direct measurement of ploidy level has the disadvantage of being very laborious and time-consuming (10, 11, 30). Flow cytometry, allows the direct measurement of DNA quantity in nuclei from fresh leaves. Although it is a quick and hardly destructive technique, it measures nuclei in phases of high and low ploidy levels in a single plant; it also requires extremely costly equipment (9).

Measuring stomatal length, stomatal density and the number of chloroplasts in guard cells are indirect ways of determining ploidy levels. Such techniques are a quick, successful and harmless form of measurement techniques (6, 7) based on a highly positive correlation between the ploidy level and the stomatal length, and a highly negative correlation between the ploidy level and the stomatal density (23, 28, 37, 38, 39). These correlations have not been yet assessed in onions. In this work, an adjustment of an efficient method for chromosome number doubling in onion plantlets obtained via *in vitro* gynogenesis is proposed by using colchicine and APM. Also, it is proposed the use of measuring stomatal length and density for determining ploidy levels.

MATERIALS AND METHODS

Doubling conditions determination

Plant material

A total of 480 explants of 'Cobriza INTA' onion (19) from *in vitro* multiplication of a gynogenetic haploid embryo, according to the technique described by Kahane *et al.* (27), were used.

Doubling treatments

The effect of colchicine (Sigma) in two concentrations: 250 and 500 mg L⁻¹ (625 and 1250 µM), and APM (Fluka) in three concentrations 15.2 mg L⁻¹ 30 mg L⁻¹ and 60 mg L⁻¹ (50, 100, and 200 µM) were tested in 2 exposition periods: 24 and 48 h. Nine replications were used for colchicine treatments, and 5 replications for APM treatments. An experimental unit of 6 plants per replication was tested.

The culture media was constituted by half strength macro and micronutrients of Murashige and Skoog (31) (MS), without growth regulators, adding 40 g L⁻¹ sucrose, jelled with 7.5 g L⁻¹ Bacto-Difco-Agar™ (Becton Dickinson S. R. L., Argentina) and adjusted to pH 5.8 (30, 34, 36). Colchicine was dissolved in water, whereas APM was dissolved in dimethyl sulfoxide (DMSO). Both substances were added to the sterile culture medium before solidification, by previously filtering with 0.22 µM Millipore filters.

Plantlets were cut alongside in half to be exposed to antimitotic agents (figures 1A, 1B, pág. 159). During and after treatment, the plantlets were exposed to a photoperiod of 16 h of 70 µmol m⁻² h⁻¹ light and to a temperature of 26 ± 2°C. Once the treatments finished, the explants were placed into the same culture media, without growth regulators.

Ploidy analysis

The ploidy level was assessed two months after the doubling treatments were done, in order to assure that all of the budding leaves were originated from the cells treated with the doubling substances. All the samples were analyzed by flow cytometry (Model Partec PA, software Partec GMBH, Germany), belonging to the Institute of Floriculture - INTA, Castelar, Buenos Aires, Argentina, following Otto's methodology (33).

Assessed variables

In each treatment, the following variables were assessed: number of vitrescent plants (hyperhydric plants), survival percentage, the ploidy level of the plantlets and doubling efficiency (product between survival percentage and duplicate proportion of plantlets).

Experimental design and statistical analysis

The jars containing the plants were randomly distributed in culture chambers. The percentage of doubled plants without phytotoxicity symptoms was compared among treatments through the Chi-square test and proportions comparison was carried out for 95 % significance level (Statgraphics 4.0).

Correlations between ploidy level and stomatal size

Plant material

A total of 5 haploid genotypes and 5 diploid genotypes from 'Cobriza INTA' cultivar were used. The haploid plantlets were obtained through *in vitro* culture of unpollinated onion flowers, following the technique suggested by Ponce *et al.* (35). The diploid plantlets came from nectaries, placed at the bottom of the flowers, cultured *in vitro* and from dihaploid plantlets obtained through genome doubling treatments with antimitotic substances. The culture media was constituted by half strength macro and micronutrients of Murashige and Skoog (31), without growth regulators, adding 40 g·L⁻¹ sucrose, jelled with 7.5 g·L⁻¹ Bacto-Difco-Agar™ (Becton Dickinson S. R. L., Argentina) and pH 5.8. The plantlets were exposed to a photoperiod of 16 hours (70 µmol·m⁻²·h⁻¹) and 26 ± 2°C.

Ploidy level verification

The ploidy level of each plantlet was verified through chromosome counts in root meristems and also by flow cytometry. For direct chromosome countings, the root tips were stained following Feulgen's technique; which consists in immersing root tips in a 0.1% colchicine solution, then the material was fixed in a 3:1 ethanol: glacial acetic acid solution, followed by the disaggregation of proteins in HCl 1 N and rinse with 45% acetic acid. Finally, the material was stained with 1% hematoxylin in order to make microscope observations.

For flow cytometry, nuclear DNA content was stained with DAPI according to procedure modified by Otto (33). Nuclei of sample and standard species were released in 0.1 M citric acid containing 0.5% Tween 20. The suspension was filtered through a 30 µm nylon-mesh filter. A 3-4 volume of staining buffer containing 4 µg/ml DAPI in 0.4 M disodium hydrogen phosphate was added. Measurements were done on a Partec PAS flow cytometer using a linear scale. Samples were analyzed using an UV spectrum excited with a HBO lamp and emissions were measured through a GG 435 long-pass filter.

Stomatal size and density measurement

The length and width of 100 closed occlusive cells were measured on the epidermis of three completely developed leaves on *in vitro* plantlets. The stomatal density was calculated from the number of stomata per field. Fifteen fields were measured per plant. The number of stomata per visual field was then converted to stomata per mm². In order to remove foliar epidermis, the 'peeling' technique described by D'Ambrogio de Argüeso (15) was used.

The images were captured using a ZEISS (Axioplan, West Germany) video camera connected to a microscope and a computer. The observations were made with a 20 x 1.6 zoom. The measurements of stomatal length, width and number per field were carried out with AxioVision 3.0 image software (figures 2A, 2B).

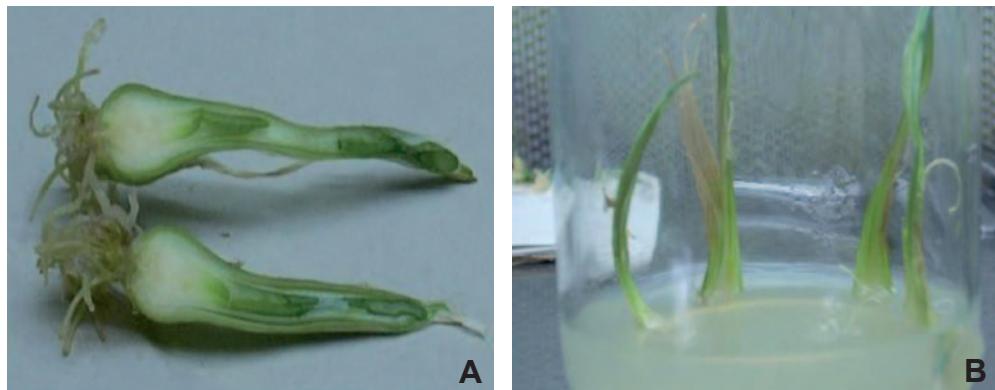


Figure 1. Conditioning of haploid plantlets in order to be exposed to antimitotic substances. Plantlet divided in half (A). Haploid plantlets on the solid culture medium during doubling treatments (B).

Figura 1. Acondicionamiento de las plántulas haploides para ser expuestas a las sustancias antimitóticas. Plantas divididas a la mitad (A). Plántulas haploides en medio de cultivo sólido durante el tratamiento de duplicación (B).

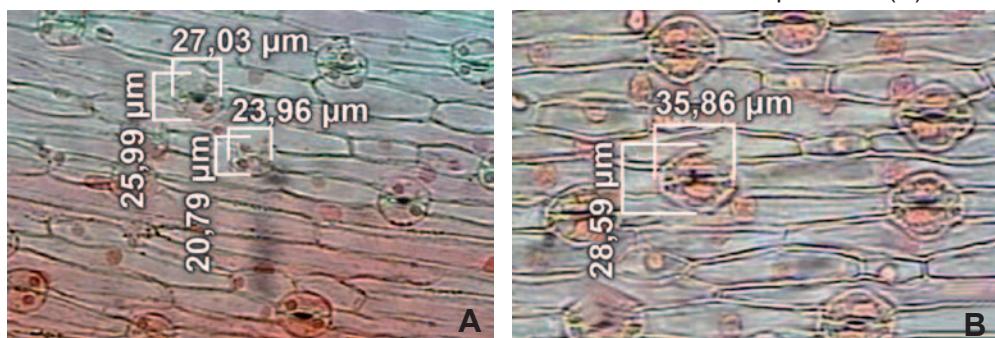


Figure 2. Measurement of stomatal length and width with AxioVision microscope in *in vitro* onion plantlets of 'Cobriza INTA' cultivar. Haploid (A) and diploid plantlets (B).

Figura 2. Medidas del largo y ancho de los estomas realizadas con microscopio AxioVision en plántulas *in vitro* del cultivar 'Cobriza INTA'. Plántulas haploides (A) y plántulas diploides (B).

Statistical analysis

Stomatal size, length, width and density were assessed. T-test was used to compare samples with different variances. Also, normal reliability intervals were used for ploidy levels (95% significance level). Correlation analysis between stomatal length, width and density and the ploidy level were carried out for 95% significance level (Statgraphics 4.0).

RESULTS AND DISCUSSION

Doubling experiment

Those plants treated with $250 \text{ mg} \cdot \text{L}^{-1}$ and $500 \text{ mg} \cdot \text{L}^{-1}$ colchicine for 48 h showed 42.6 and 44.4% vitrescence percentages, respectively (table 1).

Table 1. Percentage of vitrecence, survival and ploidy level in haploid plantlets of cv. 'Cobriza INTA' under the effect of different colchicine treatments.

Tabla 1. Porcentaje de vitrescencia, sobrevivencia y nivel de ploidía en plántulas haploides de cebolla cv. 'Cobriza INTA' con diferentes tratamientos de colchicina.

Antimitotic Products	Treatments		Nº of Treated Explants	Vitrescence (%) *	Survival (%) *	Ploidy Level (%)*				Doubling Efficiency
	Concentration mg L ⁻¹	Duration h				n	n + 2n	2n	4n	
Control	0	0	54	0.0 a	98.1 c	100 c	0.0 a	0.0 a	0.0 a	0.0
Colchicine	250	24	54	9.3 b	68.5 b	37.8 b	5.4 b	51.3 b	5.4 a	35.1
	250	48	54	42.6 c	70.4 b	0.0 a	0.0 a	100 c	0.0 a	70.4
	500	24	54	16.7 b	48.1 b	0.0 a	65.4 c	26.9 b	7.7 b	12.9
	500	48	54	44.4 c	24.1 a	0.0 a	0.0 a	100 c	0.0 a	24.1

* Different letters indicate significant differences in each column for $\alpha=0.05$ (χ^2 and comparison of proportions).

* Letras distintas en cada columna indican diferencias significativas para $\alpha=0.05$ (χ^2 y comparación de proporciones).

Vitrescence is a physiological disorder which, on certain occasions, appears on plants under *in vitro* conditions. This disorder has multiple causes, such as culture conditions, genotype, and presence of some growth regulators or other chemical substances like colchicine and APM (8). Similarly, all treatments with APM showed significantly superior vitrescence levels (23.3% to 30%) than that of the control (3.3%) (table 2). Nevertheless, the high vitrescence levels finally did not influence the efficiency of the treatment since many of these plants improved their condition and budded once they were transferred to a fresh media without antimitotics agents.

Table 2. Percentage of vitrecence, survival and ploidy level in haploid plantlets of cv. 'Cobriza INTA' under the effect of different APM treatments.

Tabla 2. Porcentaje de vitrescencia, sobrevivencia y nivel de ploidía en plántulas haploides de cebolla cv. 'Cobriza INTA' con diferentes tratamientos de APM.

Antimitotic Products	Treatments		Nº of Treated Explants	Vitrescence (%) * ^a	Survival (%) * ^a	Ploidy Level (%)*				Doubling Efficiency
	Concentration mg.L ⁻¹	Duration h				n	n + 2n	2n	4n	
Control	0	0	30	3.3 a	93.3 c	100 c	0.0 a	0.0 a	0.0 a	0.0
APM	15.2	24	30	23.3 b	73.3 b	68.8 b	31.3 b	0.0 a	0.0 a	0.0
	15.2	48	30	30.0 b	70.0 b	11.5 a	30.8 b	57.7 b	0.0 a	40.4
	30	24	30	33.3 b	56.7 b	45.5 b	36.4 b	18.2 b	0.0 a	10.3
	30	48	30	26.7 b	63.3 b	16.7 b	22.2 b	61.1 b	0.0 a	38.7
	60	24	30	23.3 b	53.3 b	36.0 b	0.0 a	64.0 b	0.0 a	34.1
	60	48	30	23.3 b	66.7 b	39.1 b	60.9 c	0.0 a	0.0 a	0

* Different letters indicate significant differences in each column for $\alpha=0.05$ (χ^2 and comparison of proportions).

* Letras distintas en cada columna indican diferencias significativas para $\alpha=0.05$ (χ^2 y comparación de proporciones).

The use of 500 mg·L⁻¹ colchicine for 24 h showed the lowest doubling efficiency (12.9). In the remaining treatments, such variable diminished between 24.1 and 70.4 respect to the control. Treatment with 500 mg·L⁻¹ colchicine for 24 h exposition, induced a high rate of mixoploids (65.4%), followed by treatment 250 mg·L⁻¹ - 24 h (5.4%). In the remaining treatments statistically significant differences were not observed (table 1, page 160). The rate of mixoploids obtained from the use of APM ranged from 22.2% to 60.9 %. These proportions may indicate that the concentration was not enough. Although an increase of such concentration could raise the doubling rate, it would also increase phytotoxicity, diminishing the total efficiency of the treatment.

The variability of responses shown in the bibliography, as that obtained in this work, highlight the great dependence of the doubling efficacy on the genotype, thus justifying the need to determine the most adequate treatment in each case.

An inverse relationship between the doubling percentage and survival was recorded. Therefore, by using 500 mg·L⁻¹ colchicine for 48 h, 100% doubling and the lowest survival rate were achieved (24.1%). By diminishing the concentration at 250 mg·L⁻¹ with the same exposition duration, the doubling percentage was maintained, while the survival rate increased up to 70.4% (table 1, page 160). Regarding those treatments in which APM was used, the best one was 15.2 mg·L⁻¹ for 48 h with 57.7% doubling and 70 % plantlet survival (table 2, page 160).

By comparing both antimitotic substances, the treatment with colchicine, 250 mg·L⁻¹ during 48 h, proved to be the most doubling efficiency (70.4), due to the achievement of the highest doubling rate (100%) at the same survival percentage as the treatment with APM (15.2 mg·L⁻¹ during 48 h), in which only 57.7% doubling was achieved and doubling efficiency was 40.4.

The doubling efficiency obtained with colchicine in this work is the highest one recorded for onion up to the present (3, 4, 21, 32). Concerning the use of APM, the doubling percentages obtained were similar to, and in most cases, higher than those reported in the bibliography (4, 21, 24, 26), but lower than those obtained with colchicine.

Correlation determination between ploidy level and stomatal size

Differences in the stomatal length and density between haploid and diploid plants cultured *in vitro* were detected ($P < 0.05$). The haploid plants had lower stomatal length and width, compared to diploid plants. The stomatal length and width of haploid plants were, $27.2 \pm 0.3 \mu\text{m}$ and $25.5 \pm 0.2 \mu\text{m}$, respectively, compared to the diploid ones, which showed $39.1 \pm 0.4 \mu\text{m}$ and $31.0 \pm 0.3 \mu\text{m}$ respectively. In the same way, the number of stomata per mm² also showed significant differences between haploid and diploid plants, being higher in haploid ones (table 3, page 162).

These results agree with those obtained by several authors in other species with plants coming from *in vitro* culture as well as from field crops. Zhang *et al.* (40) assessed the stomatal length in *in vitro* cultured phlox plants and found a larger number of stomata in tetraploid plants than in diploid ones. Aryavand *et al.* (5) also

concluded that stomatal size and number can be used to distinguish tetraploid plants from hexaploid ones in *Aegilops neglecta*. In other species, such as *Vitis vinifera* L. and wheat, significant differences were obtained in polyploid and diploid plants (28, 39).

Table 3. Comparison of stomatal length, width (μm) and density (stomata.mm^{-2}) among haploid and diploid 'Cobriza INTA' plantlets.

Tabla 3. Comparación del largo y ancho de estomas estomas (μm) y densidad (estomas. mm^{-2}) entre plántulas haploides y diploides de cebolla cv. 'Cobriza INTA'.

Ploidy level	Length (μm)*	Width (μm)*	Density (stomata. mm^{-2})*
Haploids	27.2 ± 0.3 a	25.5 ± 0.2 a	157.6 ± 4.7 b
Diploids	39.1 ± 0.4 b	31.0 ± 0.3 b	129.0 ± 5.1 a

* Different letters in each column indicate significant differences (T test, $p<0.05$).

* Letras distintas en cada columna indican diferencias significativas (T test, $p<0.05$).

A positive correlation ($P<0.05$), between the stomatal size and ploidy level was observed, which indicate that stomatal length (correlation coefficient: 0.62) and width (correlation coefficient: 0.41) can be directly related to the increase of ploidy level. On the other hand, a negative correlation between stomatal density and ploidy level (correlation coefficient: -0.25) was recorded, indicating that a decrease in stomatal number per mm^2 resulted in an increase in ploidy level.

Based on these results, confidence intervals were carried out to determine ploidy level by stomatal density and size for a 95 % significance level. The ranges in which one *in vitro* onion plant can be considered haploid or diploid are the following: for haploid plant: length (26.6 - 27.8 μm), width (24.9 - 25.9 μm), density (148.3 - 166.9 stomata. mm^{-2}) and for diploid plant: length (38.3 - 39.8 μm), width (30.4 - 31.5 μm) and density (118.8 - 139.2 stomata. mm^{-2}). The stomatal measurement can be a useful and economic tool for ploidy level determination in onion.

CONCLUSIONS

The low percentage of spontaneous replication and stability of onion haploid plants confirms the need for an efficient protocol genome duplication using antimitotic substances. Generally, genome duplication of haploid plantlets increases with increased concentration and duration of treatment. In this paper the use of 250 mg L^{-1} of colchicine for 48 h, in solid medium, was the most efficient as it produced the highest rate of genome duplication onion plantlets, consistent with the reduced phytotoxicity and high doubling efficiency.

By the results obtained, it can say that it is possible to determine the ploidy level of haploid and diploid *in vitro* onion plantlets, through stomata size and density. This confirms that the measurement of the stomata can be very important to determine different ploidy levels in most species. Therefore, it is proposed to measure the length, width and / or density of stomata onion plants from *in vitro* cultivation, as an alternative to determine ploidy important since it is a rapid, simple, inexpensive and effective procedure.

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