Improvement of *in vitro* induction of androgenesis in Phaseolus beans (*P. vulgaris* L. and *P. coccineus* L.)

L. C. Muñoz* & J.-P. Baudoin

COMPENDIO

Mejoramiento de la inducción* in vitro *de la androgénesis en los fríjoles* Phaseolus vulgaris *L. y* P. coccineus *L.

Se evaluaron diferentes factores que pueden mejorar la inducción de androgénesis en los fríjoles *Phaseolus vulgaris* y *P. coccineus*. La frecuencia más alta de inducción de callos se obtuvo para los genotipos de *P. vulgaris* con el medio de Murashige & Skoog, suplementado con kinetina (2 mg/l), 2,4-D (2 mg/l), las vitaminas del medio B5, caseína hidrolizada, agarosa y una fuente de carbono (12.5 a 25.0 g/l de sacarosa o maltosa), en las condiciones siguientes: 40 anteras en cajas de Petri con un diámetro de 55 mm que contienen 10 ml de medio en la oscuridad y a 26°C, con un pretratamiento al frío de los botones florales durante 1 ó 2 días. El análisis de los callos por microfluorometría reveló una alta frecuencia de células haploides.

Palabras claves: anteras, haploidía, Phaseolus, microfluorometría.

ABSTRACT

Different factors likely to improve androgenesis induction in *Phaseolus vulgaris* L. and *P. coccineus* L. were tested. The highest frequencies of callus induction were obtained for the P. vulgaris genotypes, with Murashige & Skoog based-medium, supplemented with kinetin (2 mg/l), 2,4-D (2 mg/l), the vitamins of B5 medium, casein hydrolysate, agarose and a carbon source (12.5 to 25 g/l of either sucrose or maltose), in the following conditions: 40 anthers in Petri dishes with a diameter of 55 mm, containing 10 ml of medium, in the dark and at 26°C, with a cold pretreatment of the flower buds of 1 or 2 days. The analysis of the calli by microfluorometry revealed a high frequency of haploid cells.

Keywords: anther culture, haploidy, Phaseolus bean, microfluorometry

INTRODUCTION

The induction of haploids from anthers cultured in vitro has been reported in many species. However, information concerning grain legumes are scarce, even if some data have been published for *Glycine max* L., *Arachis hypogea* L., *Vigna radiata* L. Wilezek, *Cajanus cajan* L. and *Cicer arietinum* L. (Kaltchuk-Santos *et al.*, 1997; Willeox *et al.*, 1991; Haddon and Northcote, 1976; Peters *et al.*, 1977; Bajaj *et al.*, 1980; Khan and Gosh, 1983).

Although calli have been induced from anthers, theses studies do not indicate the key factors in the *in vitro* response. No plants have been regenerated from callus.

In vitro androgenesis is a useful tool for geneticists, for breeding high performance genotypes, hastening character fixation, enlarging genetic variability and undertaking genetic studies. Because of the potential significance of haploids for food legume improvement, research works were conducted in our laboratory to devise an *in vitro* culture technique for Phaseolus beans and to study several factors likely to influence the rate of success.

Preliminary experiments were conducted to select wild and cultivated genotypes from three Phaseolus species (*P. vulgaris, P. coccineus and P. acutifolius*), presenting a significant response to anther culture (Muñoz and Baudoin, 1992). From the same studies, we also established that a better response was obtained when the plants were cultivated in controlled conditions (growth chambers), compared to cultivation in the glass greenhouse or in the field, to give anthers containing

Faculté Universitaire des Sciences Agronomiques de Gembloux, Unité de Phytotechnie des Régions intertropicales *2, passage des Déportés B-5030 Gembloux (Bélgica)*

* present address: Centro Internacional de Agricultura Tropical (CIAT), Unidad de Biotecnología Apartado aéreo 6713, Cali, Colombia

Email: lcmfo2@hotmail.com

microspores at the uninucleated stage (Muñoz, 1996). The experiments presented here aimed to determine the optimal culture medium composition (mineral salts and carbon source) and to study he effects of a cold pretreatment on flower buds and/or on anthers.

MATERIAL AND METHODS

Seeds were provided by the International Centre for Tropical Agriculture (CIAT, Cali, Colombia) and the National Botanic Garden of Belgium (Meise, Belgium). In *P. vulgaris*, the two selected wild forms originated from Colombia (NI922) and Mexico (X1862) and the cultivated form originated from Guatemala (X1730). Both cultivated forms of *P. coccineus* (NI15, NI16) originated from Rwanda.

The seeds were planted in jiffy-pots (8 cm wide), containing a soil mixture of compost, sand, soil in the proportion: 1.5, 1.0, 4.0 kg, respectively, with 140 g dry manure. The pots were placed in a seed tray (28-30 °C; 100 % relative humidity and light, provided by 2.400 W lamps). After development of primary leaves, the seedlings were transplanted to 2.0-l pots, containing the same mixture as that of the jiffy-pots.

The genotypes were grown in a growth chamber with the following environmental parameters as defined by Muñoz and Baudoin (1992): 24/20 °C for day/night temperature, 60-70% relative humidity, a day-length of 11.5 h and 513 ìmol/m^2.s light intensity.

In vitro culture

Flower buds (4.0-5.5 mm long) of *P. coccineus* and of the wild forms of *P. vulgaris* and 5.5-6.8 mm long buds of the cultivated forms of *P. vulgaris* were picked to isolate microspores at the uninucleated stage. Buds were sterilized in ethanol (70% v/v; 1 min) then transferred to a solution of Ca hypochlorite (1.25 g/l) for 4 min, before washing several times with sterilized distilled water. The pedicel tissues were removed and the anthers extracted. Forty anthers, collected from 4 flower buds, were then aseptically cultured in Petri dishes (55 mm diameter) containing 10 ml of culture medium. The cultures were kept in growth chambers at 26°C and in darkness, until callus induction.

Media

A first experiment was conducted in order to study the influence of medium composition. Two basal media, tested from previous experiments (Muñoz and Baudoin, 1993) were used: 67-V (Veliky and Martin, 1970) and MS (Murashige and Skoog, 1962). The main characteristics of the media concerned their nitrogen content, namely the balance between NO_3^- and NH_4^+ ions. The 67-V medium was an improved version of Gamborg *et al.* (1966) and was poor in NO_3^- (7.9 mM) and NH_4^+ (1.5 mM). On the contrary, MS medium is relatively rich in both NO_3^- (40 mM) and NH_4^+ (20 mM). Both media were supplemented with the vitamins of Gamborg *et al.* (1968), 2,4-D (2 mg/l), kinetin (2 mg/l) and casein hydrolysate (2 g/l) and solidified with agarose (5 g/l). pH was adjusted to 5.7. For both media, 50 g/l of sucrose or maltose as carbon sources was also tested.

In a second experiment, the same two basal media and the above-mentioned variations were used to study the influence of Petri dish size: dishes of 35 and 55 mm containing, respectively 4 and 10 ml medium with 20 or 40 anthers, i.e. 4 and 5 ml, were used.

Choice of carbon sources (sucrose or maltose), at three concentrations (12.5, 25 and 50 g/l) was evaluated in the MS-based medium, considered as the best medium on basis of preliminary experiments.

Cold pretreatment of buds and anthers

Cold pretreatments (4°C) were applied (2 or 4 days), either directly to flower buds or to anthers placed on MS media. The two wild *P. vulgaris* genotypes (X1862 and NI922) were used. The MS media again contained sucrose or maltose and at two concentrations (25 or 50 g/l).

Cytologic observations were also made on anthers cultured on all media, after 5, 10, 15 or 20 d of cultivation. The microspores, when available, were stained with acetic-orcein and observed microscopically.

Microfluorometry

The ploidy level of calli was determined by microfluorometry from the three *P. vulgaris* genotypes, using the Hoechst 33258 fluorochrome. Calli at different developing stages were examined. Time of incubation on the callus induction medium ranged from 30 to 60 days. At least 3 calli were collected randomly, at each stage and for each medium. The cells of root tips were used as diploid controls.

Calli and root tips were fixed in a in a TRIS/EDTA buffer (10 mM TRIS; 10 mM N_a2EDTA; 100 mM NaCl; pH 7.2), complemented with 40 g/l formaldehyde. The cells were isolated by treatment of calli with cellulase 1 U/mg; Rio, Serva, Onozuka, Japan) and of the root tips with cellulase (1 U/mg) and pectinase (0.9 U/mg; 5S, Serva, Onozuka, Japan). The enzymes were diluted in the TRIS/EDTA buffer (2% for the cellulase solution, 1% for the cellulase/pectinase

solution). Callus suspensions were centrifuged (5 min; 150 g) and the supernatant removed. The appropriate enzymatic solution was added and the mixture was maintained at 20°C under constant agitation for 1 to 1.5 h until optimal separation. For root tip suspensions, separation was maximal after 45 min at 40°C for the wild-type genotypes and after 60 min, at the same temperature, for the cultivated genotypes. Hoechst 33258 (30 µg/ml) was then added for ploidy determination.

Fluorescence measurements of nuclei were made on a Leitz microscope (40 x 0.8 mm diaphragm) equipped with a photometer (Leitz MPV compact). The light source was a 100 W high intensity mercury lamp with the following filter combination: an excitation filter BP (340-380), a dichroic mirror RKP400 and a barrier filter LP430. Calibration was performed with 10 mm superbright fluorospheres (Coulter Electronic). The photomultiplier was calibrated to read 400 when a fluorescing filter was excited. Results were obtained from the measurement of 100 nuclei. Background cell fluorescence was substracted from nuclear fluorescence.

Statistical analyses

The rates of callus induction (%) were calculated from the following ratio:

Number of anthers giving at least one callus / number of anthers isolated

According to the tested combinations, two or three-way ANOVA were used to examine the effect of the treatments. The Student-Newman-Keuls test was applied, where necessary, for multiple comparison of means (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Cytological observations performed on calli of *P. vulgaris* genotype NI922 showed first division of microspores 15 days after culture. Microcalli were observed after 25-30 days and were verified as originating from the inside of the anther and not from the diploid anthers. Three different callus morphotypes could be distinguished: friable calli, compact nodular callus types, calli with roots. Globular structures were observed on the second callus type, after transfer on a regeneration medium (data not presented here).

Table 1 indicates the results obtained for the response of the anthers to the choice of mineral salts and carbon sources. With the exception of the NI16 genotype, best responses were obtained with MS salts but differences were only significant ($P < 0.01$) for the wild *P. vulgaris* genotype X1862. Media with sucrose gave a better response but the differences were significant ($P < 0.01$) only for a second wild-type genotype of *P. vulgaris* (NI922). The cultivated forms of *P. vulgaris* (X1730) and *P. coccineus* (NI15) gave a poor callus induction.

The results of the second experiment demonstrated the possibility of interactions between genotype and medium composition. A significantly higher ($P < 0.01$) response was observed for the X1862 genotype with MS salts, whereas the NI16 genotype gave a better response ($P < 0.05$) with the 67-V salts. The trends were already observed in the first experiment. The response of the NI15 genotype was not significantly affected by the mineral composition of the medium but significantly by the carbon source ($P < 0.01$). Its response was higher than that observed in the first experiment. The experiment also demonstrated the advantage of the use of large Petri dishes (55 mm diameter) for anther culture ($P < 0.01$). Our results did not confirm those of Peters et al (1977) who considered 67-V medium as the best source of mineral salts. A genotype effect can not be excluded to explain the discrepancy between both experiments.

Maltose and sucrose are often used as carbon sources in anther cultures but few comparative data are available for Leguminosae. In studies on somatic embryogenesis, Ranch *et al.* (1986) and Strikland *et al.* (1987) obtained a higher response with maltose for both soyabean and alfalfa, respectively. Our data indicated a better response with sucrose. This could be ascribed either to the faster hydrolysis of sucrose or to differences in nutrients provided by the two disaccharides. However, an interaction between carbon source and genotypeshas often been observed, like in *Glycine*, for example (Komatsuda *et al.*, 1991).

Use of large Petri dishes had a positive effect on the anther response than the small ones, although the ratio of concentration "anthers/ml medium" was nearly identical. The difference could come from either the small difference in concentration or by a difference in atmosphere volume (Muñoz, 1996). According to Dunwell (1979), ethylene produced during the culture inhibits callus induction. In the large Petri dishes, ethylene is more diluted in the atmosphere, reducing its negative effect. However, a specific experiment on that topic should be carried out to confirm this hypothesis.

Table 2 shows the results of the effect of the type and concentration of the carbon source on the rate of callus induction in anther culture: the lower the concentration, the better the callus induction. The means

Table 1. Influence of the mineral salts and the carbon source (50 g/l) in the medium on the rate of callus induction in anther culture of *Phaseolus vulgaris* and *P. coccineus* genotypes.

Mineral salts		MS				67-V				Significance[a]	
		Sucrose		Maltose		Sucrose		Maltose		Salts	Csource
	Dish diameter (mm)	n	induction (%)	n	induction (%)	n	induction (%)	n	induction (%)		
Experiment 1											
Pv X1862	55	840	28.8a	880	31.5a	960	16.3b	840	8.8b	0.01	ns
Pv NI922	55	280	48.2a	360	15.6b	400	36.5a	360	2.8b	ns	0.01
Pv X1730	55	200	11.5	320	2.2	320	0.9	200	3.5	ns	ns
Pc NI15	55	320	1.6	240	0.8	320	0.9	280	0	ns	ns
Pc NI16	55	360	11.1	360	12.2	320	19.7	320	8.8	ns	ns
Experiment 2[b]											
Pv X1862	55	720	22.2a	720	28.3a	560	19.3a	600	5.7b	0.01	ns
	35	340	9.4a	300	14.0a	240	12.9a	260	2.7b		
Pc NI15	55	160	28.1a	120	17.5a	160	28.1a	0b	0b	ns	0.01
	35	60	5.0a	60	0b	60	11.3a	80	0b		
	55	280	3.6	280	7.1	400	10.3	400	11.0	0.05	ns
	35	140	0a	140	2.9b	180	5.0b	180	6.7b		

n: number of anthers in culture; induction: % of induced calli; Pv: *Phaseolus vulgaris*; Pc: Phaseolus coccineus.
a,b: the means with different superscripts differ signifiantly in a same row ($P < 0.05$).
a For the second experiment, the significance of the "salt" and of the "C source" concerns both the small and large Petri dishes. No significant "salt x C source" interaction was observed, with the exception of Pv X1862 in Expt 2 ($P < 0.01$).
b the higher response obtained with the large Petri dishes was significant in all cases ($P < 0.01$).

Table 2. Influence of the type and concentration of the C source in a MS-medium on the rate of callus induction in anther culture of *Phaseolus vulgaris* genotypes.

Genotype	Concentration	Sucrose		Maltose	
	(g/l)	n	induction (%)	n	induction (%)
X1862	12.5	240	45.8a	240	43.3a
	25.0	240	41.7a	240	25.4a
	50.0	240	18.3b	240	15.8b
NI922	12.5	200	46.0a	240	49.6a
	25.0	280	34.3[a]	320	27.5a
	50.0	320	10.3b	320	5.3b
X1730	12.5	200	35.0a	200	35.0a
	25.0	120	48.3a	120	28.0a
	50.0	160	5.0b	120	0b

n: number of anthers. Induction: % of induced calli
a,b: means with different superscripts differ significantly in a same column ($P < 0.05$)

were always higher for the lowest concentration tested (12.5 g/l).

In tissue culture, the response to carbon source depends on both the nature type and concentration but also on the potential interaction with the other parameters. For species such as potato or cauliflower, a high concentration (60-140 g/l) of sucrose favours the induction of androgenesis and plant regeneration (Tiainen, 1992; Yang *et al*, 1992). The present data suggested the necessity to limit the concentration for Phaseolus anther culture. Other examples in grain legumes support this assumption. Concentrations higher than 60 g/l inhibited callus induction in anther culture of *Arachis hypogea* (Martins and Rabechault, 1976). For somatic embryogenesis of *Glycine max*, using immature zygotic embryos, Lippmann and Lippmann (1984) and Lazzeri *et al.* (1987) also obtained better responses with very low carbon source concentrations (15 to 30 g/l). These authors also observed a positive response for sucrose, compared to glucose as carbon sources.

Tables 3 and 4 summarize the effects of a cold pretreatment on flower buds and anthers and the interaction of this treatment with two carbon sources at

Table 3. Effect of a cold pretreatment (4°C) of the flower buds for 0, 2, 4 or 0, 1, 3 days on callus induction in anther cultures of two *Phaseolus vulgaris* wild forms.

	Concentration (g/l)	Sucrose			Maltose		
Duration Expt 1 (day)		0	2	4	0	2	4
Duration Expt 2 (day)		0	1	3	0	1	3
X1862	25	40.8a	55.0a	31.7b	59.2a	50.8a	24.2b
	50	13.3ab	35.0a	10.0b	39.1a	15.8b	4.2b
NI922	25	11.7a	34.2b	11.7a	9.2	12.5	5.0
	50	1.7a	29.2b	2.5a	0	0.8	0.7

a,b: means with different superscripts differ significantly ($P < 0.05$) in a same row, for each C source.

Table 4. Effect of a cold pretreatment (4°C) on anthers, for 0, 2, 4 days, on callus induction in cultured anthers of *Phaseolus vulgaris* wild forms.

C source concentration (g/l)	25			50		
Cold pretreatment (d)	0	2	4	0	2	4
Sucrose						
X1862	38.3a	2.5b	1.7b	17.5a	6.7b	0b
NI922	45.0a	19.2b	8.3ab	16.7a	8.3a	0b
Maltose						
X1862	65.8a	37.5b	0c	16.7a	7.5ab	0b
NI922	16.7"	1.7b	2.5b	5.8a	0b	0b

a,b: means with different superscripts differ significantly ($P < 0.05$) in a same row, for each C source.

different concentrations. There was a significant effect on callus induction for the two genotypes tested. The highest rate of induced calli was obtained with a 2-day pretreatment.

Genotype X1862 responded to the presence of a carbon source in the medium but not to the type of carbon, whereas the genotype NI922 was significantly influenced by both factors ($P < 0.01$). Both genotypes also presented a significant interaction between type of carbon source and duration of pretreatment. With sucrose, the optimal duration was 2 days whereas with maltose, the results differed according to genotypes.

On the contrary, a cold pretreatment of the anthers should be avoided because it significantly reduces callus induction, irrespective of the type or concentration of the carbon source (Table 4). Different hypotheses were proposed to explain the effect of cold. Cold can slow down anther senescence and thus maintain a maximal number of viable microspores (Maheswari *et al,* 1980). It could also modify the first haploid mitoses, thereby producing similar nuclei instead of a programmed generative and a vegetative nucleus (Sunderland and Roberts, 1977). Sangwan and Camefort (1978) explained this as an increase in the rate of free amino acids that would reduce or inhibit some protein synthesis that normally happens during the male gametophyte formation.

Ploidy level of the calli

The results of the ploidy level determinations are presented in Figure 1. For genotype X1862, the peak of relative fluorescence intensity of the root nuclei (diploid) was situated between 370 and 470 whereas those of the calli were between 70 and 270. Nuclei showing a low intensity (< 370) were considered to be haploid. They represented 66-69 % of the total, the others were considered as diploid, tetraploid or polyploid.

For genotype NI922, two peaks of relative intensity were observed: one situated at about 370 (diploid cells) and the other at 770 (tetraploid cells). The calli presented their peak of intensity between 150 and 250 and the assumed haploid nuclei (< 370) represented from 41 to 86 % of the total.

The relative fluorescence intensity of the root nuclei of the X1730 genotype situated between 470 and 570 and those of the calli, between 150 and 250. Th frequency of haploid cells (< 470) was estimated to range from 86 to 90 % of the total. In all cases, the highest frequencies were always obtained with the youngest calli (27 to 37 d).

A general trend observed here was that calli that had spent the minimum time on medium were those which represented the highest frequency of haploid nuclei. When the number of culture days was increased, that of haploid cells decreased and that of diploid cells

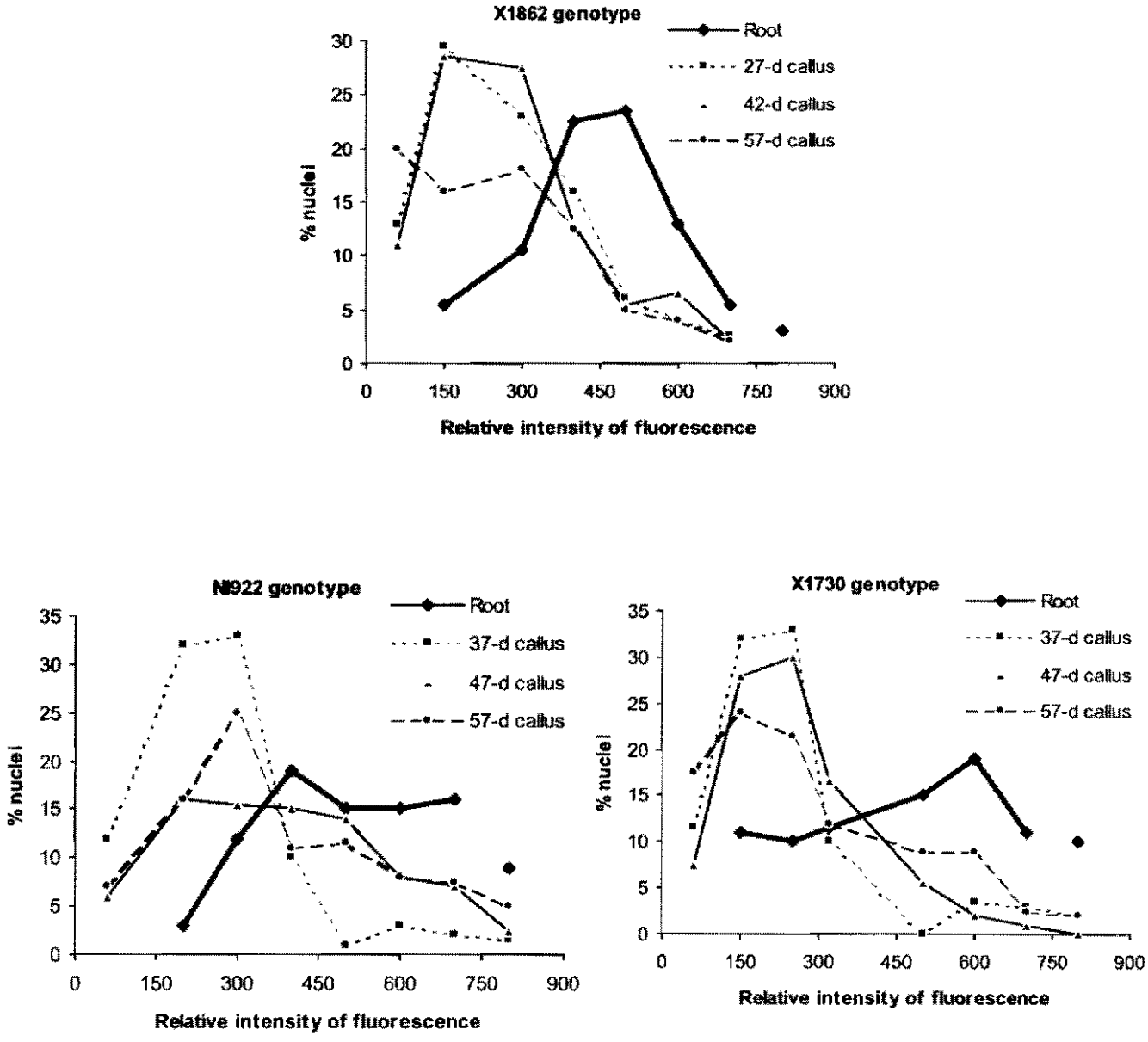

Figura 1. Frecuency of relative fluorescence intensity of the nuclei of tree *P. vulgaris* genotypes.

increased. The presence of the latter in calli could be due to a spontaneous doubling of the chromosomes in the original haploid cells.

CONCLUSIONS

Our data demonstrated that protocols, adapted for grain legumes, could be developed through systematic evaluation of the multiple parameters involved in the techniques. The present work led to the induction of calli, with a high frequency of haploid cells. However, a significant effect of genotype was highlighted, suggesting that the medium components and the treatments must be adapted according to the genotype.

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