Influence of Medium Parameters on Somatic Embryogenesis from Hypocotyl Explants of Flax (*Linum usitatissimum* L.)

Effect of carbon source, total inorganic nitrogen and balance between ionic forms and interaction between calcium and zeatin.

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Summary

The effects of different carbon sources, total inorganic nitrogen concentration, nitrate to ammonium ratio and the interaction between calcium and zeatin (ZEA) levels on somatic embryogenesis from flax hypocotyl explants were studied in three independent factorial experiments. MS medium supplemented with the monosaccharides glucose or fructose at high concentrations (4%) gave consistently highly embryogenic cultures, with higher somatic embryos frequencies and higher growth rates when compared with media supplemented with sucrose or maltose. Although media with maltose had performed well in a 1 to 4% concentration range, media supplemented with sucrose at 4% appeared to inhibit the induction and development of somatic embryos. Independently of the effect of the nitrogen content, the balance between both ionic forms (NO_3^- and NH_4^+) played a dramatic role on the induction of somatic embryogenesis and somatic embryos growth. Nitrate is important to calli differentiation and growth and a high ammonium content increased somatic embryo frequency. The embryogenic vs. undifferentiated cell growth commitment of flax explants was determined by an interaction between calcium and ZEA levels, a high calcium/low ZEA affording very low embryogenic potencial and

high calli biomass. A high ZEA concentration was essential for the normal development of somatic embryos.

Key Words: *Linum usitatissimum L., flax, tissue culture, somatic embryogenesis, carbohydrates, nitrogen, calcium.*

Abbreviations: 2,4-D, 2,4 - dichlorophenoxyacetic acid; N.C., non-embryogenic calli; E.C., embryogenic calli; S.E., somatic embryos; ZEA, zeatin.

Introduction

Somatic embryogenesis is a suitable process for the regeneration of plants from valuable genotypes obtained either by in vitro manipulation or by breeding techniques. A one-step somatic embryogenesis protocol, that avoids sequential transference to different media, and maximizes somatic embryo yield and growth, should be achieved to obtain high efficiencies of micropropagation.

The knowledge of the interplay between some fundamental medium parameters allows not only the optimization of the micropropagation system but also gives models to investigate and rationalize the process of induction and development of somatic embryogenesis itself. Although there is a lot of work in the literature dealing with the effects of growth regulators and nutrient factors on somatic embryogenesis of several species, there is generally a great variability and some discrepancy in the reported results. The great variability in culture conditions (namely the use of complex media) and biological systems (cells, tissue explants, zygotic embryos, age, physiological state) used, along with the high species and genotype-dependent response may be responsible for part of this discrepancy.

Regeneration of flax (*Linum usitassimum* L.) through somatic embryogenesis has already been obtained using immature zygotic embryos (Pretova and Williams 1986) and hypocotyl segments from young in vitro germinated seedlings (Cunha and Fernandes-Ferreira 1996). However little is known about the effects of alternative carbon sources, nitrogen and calcium levels on flax somatic embryogenesis. In this study we report the effects of a) different types and concentrations of carbohydrates as carbon and energy sources, b) total inorganic nitrogen content, c) balance between the oxidized (NO_3^-) and the reduced (NH_4^+) ionic forms and d) interaction between calcium and zeatin in MS media on somatic embryogenesis from hypocotyl segments of flax seedlings.

Materials and Methods

Plant material and culturing conditions

Three experiments were performed to evaluate the effects of 1) the type and concentration of the carbon source; 2) total inorganic nitrogen content and relation between the oxidized and the reduced ionic forms and 3) the interaction between calcium and zeatin on somatic embryogenesis and on calli growth of flax. The somatic embryo yield, defined as the product of the percentage of induction, number of somatic embryos per explant and their mean fresh weight in a monthly basis, was taken here as a measure of medium efficiency for plantlet regeneration. Flax dry seeds were sterilized and allowed to germinate in vitro on MS medium (Murashige and Skoog 1962) without hormonal supplementation as described previously (Cunha and Fernandes-Ferreira 1996). Hypocotyl segments were excised from one week old seedlings and used as primary explants. To control eventual physiological and tissue age effects, only one segment per seedling was used, the sub-cotyledonary portion of about 1.5 cm long. The hypocotyl segments were placed in culture vessels on MS medium supplemented with

0.4 mg.L^{-1} 2,4-D + 1.6 mg.L^{-1} ZEA, which is known to be an embryogenic medium for flax (Cunha and Fernandes-Ferreira 1996). The MS nutrients and zeatin concentrations were modified according to each treatment as described below. Cultures were maintained in a growth cabinet under a 16 h photoperiod (ca. 50 μmol.m^{-2}.s^{-1}) at a constant temperature (25°C ± 2°C) for five weeks, or six in the case of the experiment changing calcium and ZEA levels. At the end of the experimental period, the material was harvested and the different types of tissues were separated: non-embryogenic calli (N.C.), embryogenic calli (E.C.) and the respective somatic embryos (S.E.).

Experimental treatments

Carbon sources and concentration

Sucrose, maltose, glucose and fructose at 1, 2 and 4% (w/v) were added to the induction medium before autoclaving. A 4x3 factorial design was performed with four randomized blocks and 14 replicates per treatment.

Total inorganic nitrogen content and balance between the oxidized (NO_3^-) and the reduced (NH_4^+) ionic forms

Two total inorganic nitrogen contents (30 and 60 mM) and three relative molar proportions (1:2, 1:1 and 2:1) of the two ionic forms (NO_3^-:NH_4^+) were tested. The nitrogen was added as MS salts (NH_4NO_3 and KNO_3). In the case of the 1:2 ratio the extra NH_4^+ was added in the form of $(NH_4)_2SO_4$. A 2x3 factorial design with three randomized blocks and 21 replicates per treatment was run.

Calcium levels and interaction with ZEA

Two $CaCl_2$ concentrations (1.5 and 3.0 mM) were tested in combination with two ZEA concentrations (0.4 and 1.6 mg.L^{-1}) in a 2x2 experiment with 21 replications. Earlier experiments revealed that the concentration of 0.4 mg.L^{-1} ZEA compared to the concentration of 1.6 mg.L^{-1} in MS medium containing 0.4 mg.L^{-1} of 2,4-D, was much less efficient on somatic

embryogenesis induction, stimulating preferentially the growth of undifferentiated callus (Cunha and Fernandes-Ferreira 1996).

In all experiments the percentage of somatic embryogenesis induction, mean number of somatic embryos produced per embryogenic explant, somatic embryos fresh weight and mean calli fresh weight were estimated.

Statistical analysis

Frequency data or counts (percentage of induction) presented as contingency multidimensional tables were analysed by Log-linear models. The results obtained with parametric variables were statistically analysed running the one way analysis of variance test (ANOVA). Before analysis, the variable "number of somatic embryos per embryogenic explant" was subjected to a square root transformation to meet test assumptions. However, data presented in the results are the true values. All the statistics were performed with the software Statistica@ 4.1 from StatSoft.

Results and Discussion

I - Effects of different carbohydrates, as carbon sources, on somatic embryogenesis from flax explants

Carbohydrate type and concentration have been found to play important roles in different stages of the somatic embryogenesis process. Although the majority of media used in plant tissue culture contain sucrose as the standard carbon and energy source, several reports indicate that the beneficial effects of sugar on that plant regeneration process appear to be species specific.

Two important features that came out from the bulk of results here reported were that the monosaccharides and the disaccharides performed differently in terms of somatic embryogenesis efficiency and calli growth, and that there was a very different overall response to sucrose when compared to maltose (Tab. 1).

Table 1 - Effects of the type and concentration of carbohydrates on somatic embryogenesis from flax hypocotyl explants after 5 weeks in culture. The variables analysed were the percentage of embryogenic explants, mean number of somatic embryos per embryogenic explant and their mean fresh weight and calli growth. Total somatic embryo fresh weight produced per explant was also estimated as a measure of total yield. In each column, means followed by the same letter were not statistically different.

Carbohydrates tested	Concentration		Somatic embryogenesis induction (%)	No. somatic embryos per embryogenic explant	Mean calli fresh weight (g)	Mean somatic embryos fresh weight (mg)	Somatic embryo yield (mg/month/ explant)
	(%, w/v)	(mM)					
	1	28	64,3	3.1 a	0.4 a	nm	-
Sucrose	2	59	64,3	6.1 ab	0.6 ac	14	45
	4	117	35,7	3.8 a	1.28 b	5	5
	1	28	64,3	3.5 a	0.4 a	0,9	2
Maltose	2	59	78,6	5.0 ab	0.6 ac	6	19
	4	117	71,4	6.3 ab	0.85 bc	24	92
	1	56	0	-	0.64 ac	-	-
Glucose	2	111	42,9	5.0 ab	0.98 b	45	81
	4	222	71,4	11.4 b	1.18 b	58	423
	1	56	35,7	2.8 a	0.55 ac	2	7
Fructose	2	111	35,7	4.4 ab	1.05 b	27	37
	4	222	71,4	8.3 ab	1.04 b	72	312

Post-hoc comparisons were performed running Duncan test (critical range 0.05) and Tukey (HSD) test to analyse mean number of somatic embryos and calli fresh weight results. nm - not measurable, below weighing limit.

At lower concentrations (1 and 2%), disaccharides were much more effective than monosaccharides on the induction of somatic embryogenesis. The high levels of somatic embryogenesis induction obtained by disaccharides at 1 and 2% were attained by the monosaccharides only at 4%. The rise of maltose concentration to 4% didn't result in significant variations in the percentage of somatic embryogenesis induction. In the case of sucrose, however, there was a decrease of about 50% when its concentration was doubled to 4% (Tab. 1). This decrease was paralleled by a reduction in the somatic embryo frequency and growth rate (Tab. 1). The reduction in the embryogenic capacity of flax explants determined by the increase of sucrose concentration to 4%, could not be attributed to an osmotic effect since similar or higher media osmotic strengths were used

with maltose or with the monosaccharides respectively and here no such results were observed. Using another medium formulation (Monnier's medium, supplemented with 5% of sucrose), Pretova and Williams (1986) also failed to induce somatic embryos from immature zygotic embryos of flax unless glutamine and yeast extract were added to the medium. Similarly, King and Kasha (1994) obtained a maximum number of somatic embryos from barley immature embryo-derived calli with maltose at 6%, and a very low one with sucrose at concentrations of 3 and 6%. The same authors considered that this was not an osmotic effect. Although at concentrations somewhat higher than those observed for flax, inhibitory effects of high sucrose concentrations were also recorded for somatic embryogenesis of other plant species namely, from petiole of *Medicago sativa* (Meijer and Brown 1987); from immature cotyledons of cassava (Konan *et al.* 1994) and feijoa (Canhoto and Cruz 1994). On the contrary, other authors found very different results. Sucrose concentrations higher than 5% and 150 mM and sucrose concentrations up to 252 mM; 350 mM and 12% were needed to maximize induction levels and frequencies of somatic embryogenesis from immature cotyledonary tissue of *Azadirachta indica* (Shrikhande *et al.* 1993); cotyledonary explants of cucumber (Lou and Kako 1995); seedling's shoot apices of *Pisum sativum* (Loiseau *et al.* 1995); immature zygotic embryos of sunflower (Jeannin *et al.* 1995) and immature zygotic embryos of *Zea mays* (Lu *et al.* 1982) respectively. Inhibitory effects of maltose on somatic embryogenesis from cotyledonary explants of cucumber (Ladyman and Girard 1992) and from immature cotyledonary or embryonal axes of peanut (Eapen and George 1993) have been reported. Inefficiency of fructose on the induction of somatic embryogenesis from sunflower immature zygotic embryos (Jeannin *et al.* 1995) and from asparagus calli (Levi and Sink 1990) have been also reported. At low concentrations, the monosaccharides tested revealed to be very inefficient for embryogenesis induction as well as for somatic embryos

growth. The production of translucid leafy structures (foliose) was observed in flax cultures maintained in the presence of 1% glucose or 1% fructose and 2% of glucose (data not shown). However, at 4%, the higher number of somatic embryos induced together with higher growth rates determined the highest somatic embryo yields from flax hypocotyl explants (Tab. 1). Apparently, independent from the effect on somatic embryogenesis determined by both the type (Pearson χ^2= 22,16; p = 0,14) and concentration (Pearson χ^2= 26,4; p = 0,09) of carbohydrate, there was a strong effect of carbohydrate concentration on calli growth. Concentrations from 2 to 4% of monosaccharides and of 4% of disaccharides, resulted in the development of large and compact calli (Tab. 1). Nuutila *et al.* (1991), in a statistical approach, also found an optimum sucrose concentration value for somatic embryogenesis induction from betula calli lower than the optimum value estimated for calli growth (3,4%). Similar results were obtained for calli derived from several citrus cultivars (Kochba *et al.* 1982).

The estimation of the somatic embryo fresh weight productivity on a monthly basis provided a comparative index to evaluate media performance (last column of Tab. 1). From the results obtained it seems reasonable to conclude that glucose or fructose, at high concentrations, in MS medium, are adequate carbohydrate choices, for the establishment of a somatic embryo micropropagation system from flax seedling's hypocotyl segments. Sucrose, at high levels, could be an adequate carbohydrate for calli biomass production purposes.

II - Effects of the total inorganic nitrogen content and of the balance between the nitrate and ammonium ions

Nitrogen (N) is also a fundamental element for plant cell and tissue cultures, being essential for the synthesis of DNA, RNA and proteins (Oksman-Caldentey *et al.* 1994). Of all the nutrients, the form of nitrogen (oxidized or reduced, organic or inorganic) has probably the most pronounced effects on the growth and differentiation of cultured plant tissues (Gamborg *et al.*

1968). In the modified MS media used in our experiments, nitrogen was included in the form of the basal inorganic salts, comprising the two ionic forms (nitrate and ammonium) available for uptake by the plant cells and tissues and 2 $mg.L^{-1}$ glycine. There is a lot of work done trying to study the effect of both N inorganic ions on the process of somatic embryogenesis but the results are somewhat contradictory. Although it is believed that nitrate has a main role in supporting the growth of plant tissues with essentially low embryogenic capacity, the role of reduced nitrogen is not well understood. According to Rangaswamy (1986) reduced N is essential, specially at the induction phase. However, Kamada and Harada (1979) concluded that the induction phase is independent of nitrogenous compounds while the somatic embryo developmental phase requires reduced N.

Table 2 resumes the results obtained in our experiments. Superimposed to the N level effect (Pearson χ^2, p = 0,07), a highly significant effect (Pearson χ^2; p = 0,50) of NO_3^-:NH_4^+ ratio was observed on somatic embryogenesis induction. The maximum percentage of induction (90,5%) was obtained with a well-defined ratio of 1:1, being higher than that obtained with normal MS medium (total nitrogen level of 60 mM and a ratio of 2:1). However, the drop was steeper for the lower N content (30 mM). Likewise, the growth of somatic embryos showed roughly the same trend, with a maximum at 1:1 ratio, but in this case, the extreme nitrate contents (10 and 40 mM) determined the lower growth rates (Tab. 2). Probably, the low viability of the plant tissues maintained with 10 mM nitrate (pale yellow primary explants with incipient callus and direct somatic embryogenesis) and the lower embryogenic capacity of calli induced on the 40 mM nitrate treatment when compared to the other at the same N level, could explain this result. The number of somatic embryos produced was not significantly affected by nitrogen level or NO_3^-:NH_4^+ ratio *per se*. Only the treatment with the highest ammonium content (40 mM) together with a NO_3^-:NH_4^+ ratio (1:2)

gave higher frequencies when compared with the other treatments (interaction between variables $p < 0.001$). The differential effects of total nitrogen content and NO_3^-:NH_4^+ ratio on the different parameters of somatic embryogenesis resulted in high somatic embryo yield with 1:1 ratio treatments and high ammonium contents (last column of Tab. 2). With respect to calli growth, all N-factors tested gave significant effects (Tab. 2). At N=30 mM, calli growth was linearly related to increasing nitrate concentrations, revealing a nitrate limitation. At N=60 mM, calli fresh weight were lower than the maximum obtained for the low N level. Although it is possible to conceive an inhibitory effect of high N level over growth, the variation observed for non-embryogenic calli (N.C.) was not clear. Nuutila *et al.* (1991) also found an optimum N level for betula calli cultures of 28 mM, which was lower than that estimated for somatic embryogenesis induction (35 mM). Another interesting fact was the difference between N.C. and E.C. fresh weights obtained with a non-limiting N level. This difference could be explained by a carbon allocation from the embryogenic calli to the growing embryonary tissues or by the existence of different metabolic pathways more energy/carbon consuming.

That was visible also for the N=30 mM, 2:1 treatment (Tab. 2). Although the absence of either of the N ionic forms in the medium was not tested, we believe that our results support the idea of a minimal nitrate concentration to support growth of the tissues (explants, callus or somatic embryos) and that high ammonium concentrations (40 mM) is important for the production of somatic embryos (Tab. 2). The requirement for NH_4^+ (or of N in a reduced form) for embryogenic induction and differentiation was noticed by other authors in different species and culturing systems (Halperin and Wetherell 1965, Wetherell and Dougall 1976, Kamada and Harada 1979, Walker and Sato 1981, Meijer and Brown 1987, He *et al.* 1989). With respect to the effect of NO_3^-:NH_4^+ ratio on somatic embryogenesis induction, although some authors obtained similar results (Wetherell and Dougall 1976,

Tremblay and Tremblay 1991), there are few reports in the literature regarding this factor mainly due to the use of organic N in media formulations which can replace total or partially for NH_4^+.

Table 2 - Effect of two inorganic nitrogen levels tested in three different nitrate:ammonium proportions on somatic embryogenesis from flax hypocotyl explants after 5 weeks in culture. The variables analysed were the percentage of embryogenic explants, mean number of somatic embryos per embryogenic explant and their mean fresh weight. Calli growth was divided in N.C. and E.C. fresh weight. Total somatic embryo fresh weight produced per explant was also estimated as a measure of total yield. In each column, means followed by the same letter were not statistically different (calli fresh weight was analysed as one variable so, for comparison, the 2 columns can be regard as just one).

Total Nitrogen (mM)	Nitrate:ammonium		Somatic embryogenesis induction (%)	No. somatic embryos per embryogenic explant	Mean N.C fresh weight (g)	Mean E.C fresh weight (g)	Mean somatic embryos fresh weight (mg)	Somatic embryo yield (mg/month/explant)
	ratio	(mM)						
30	1:2	10:20	38,1	3.1 a	0.016 a	0.016 a	nm	nm
30	1:1	15:15	90,5	4.2 ac	0.333 b	0.349 b	12	39
30	2:1	20:10	57,1	3.3 a	0.817 e	0.694 c	7,4	13
60	1:2	20:40	71,4	6.6 bc	0.692 c	0.377 b	10,5	40
60	1:1	30:30	90,5	2.8 a	0.439 b	0.424 b	14,6	32
60	2:1	40:20	71,4	4.5 a	0.55 d	0.391 b	4,3	10

Post-hoc comparisons were performed running Duncan test (critical range p=0,05) to analyse mean number of somatic embryos and calli fresh weight results. nm - not measurable, below weighing limit.

Comparing the tables 1 and 2, it is possible to see the need for testing alternative carbon sources together with different nitrogen formulations, especially for the ionic balance, for the optimization of MS media for calli growth and somatic embryogenesis from flax hypocotyl explants.

III - Effect of calcium and ZEA levels on calli growth and somatic embryogenesis from flax explants

The participation of intracellular calcium in a large number of physiological processes in plants and the modulation of its level and compartmentalization by many external stimuli have been largely documented (reviewed by Hepler and Wayne 1985). Calcium acts also as a second messenger in

extracellular signal transduction and is responsible for membrane stability and wall rigidity (Montoro *et al*. 1995). Although reports on the effect of calcium on this developmental process are scarce, there is evidence for an important role of this ion as a culture parameter, affecting cell differentiation and particularly somatic embryogenesis (Jansen *et al*. 1990, Montoro *et al*. 1995, Timmers *et al*. 1996). Cytokinins are known by their regulatory role of cell division in tissue culture and their ability to affect development of somatic embryos to plantlets is also described (Veisseire *et al*. 1994). A cytokinin-Ca^{2+} interaction has been noted in several systems (see Hepler and Wayne 1985 for references) although not associated with somatic embryogenesis. In this sense, the main goal of this experiment was to study the effects of calcium and of the calcium-cytokinin interaction on the process of somatic embryogenesis of flax.

The results obtained are represented in figures 1a) to 1d). Comparing figures 1a) and 1b), it is well evident that the induction of the embryogenic pathway was inversely related to the induction of disorganized callus growth. Only the strong interaction between factors explained the switch in the growth pattern observed at high Ca^{2+}/low ZEA concentrations. Unexpectedly in this treatment adventitious shoots were produced (data not shown). These results apparently contradict previous results where an increase in Ca^{2+} concentration positively affected somatic embryogenesis from suspension cultures of *Daucus carota* (Jansen *et al*. 1990), or didn't have a significant effect over a wide range of concentrations in other cultures such as immature zygotic embryos of wheat (He *et al*. 1989) and immature zygotic embryos derived calli of *Hevea brasiliensis* (Montoro *et al* 1995). Due to the strong interaction between calcium and cytokinins, calcium alone (Montoro *et al* 1995) or of both (Misra and Chaturvedi 1991) with inorganic nitrogen content, it was not possible to make direct comparisons with the referred reports. Albeit the strong effect on induction, these factors *per se* or their interaction didn't have a significant role on the mean number of

somatic embryos produced (fig. 1c)). It seems reasonable to picture that once the commitment to the embryogenic pathway was achieved, these factors, at least in the range of concentrations tested, didn't have a significant effect on somatic embryo frequency. High levels of ZEA (1.6 $mg.L^{-1}$) were nevertheless necessary for somatic embryo development (fig. 1d)) as expected. No statistics were possible when analysing somatic embryo mean fresh weight because somatic embryos were weighted together, but the eventual increment observed when doubling calcium level may be explained by its structural role more than by its regulatory one. ZEA had obviously a dramatic effect on somatic embryo yield (data not shown) the highest value having been obtained with the modified MS medium supplemented with 1.6 $mg.L^{-1}$ ZEA and 3 mM $CaCl_2$). Further increases in calcium and ZEA levels may eventually improve yield.

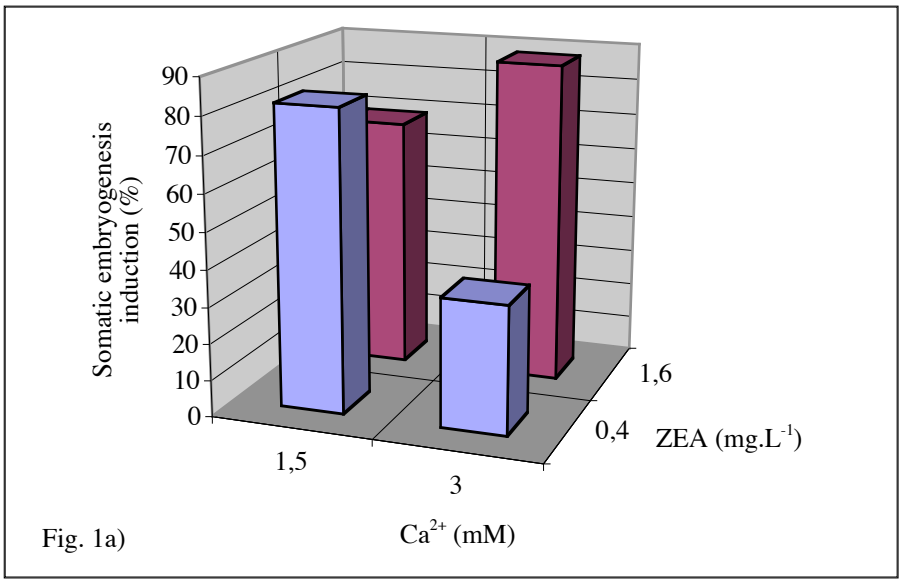

Fig. 1a)

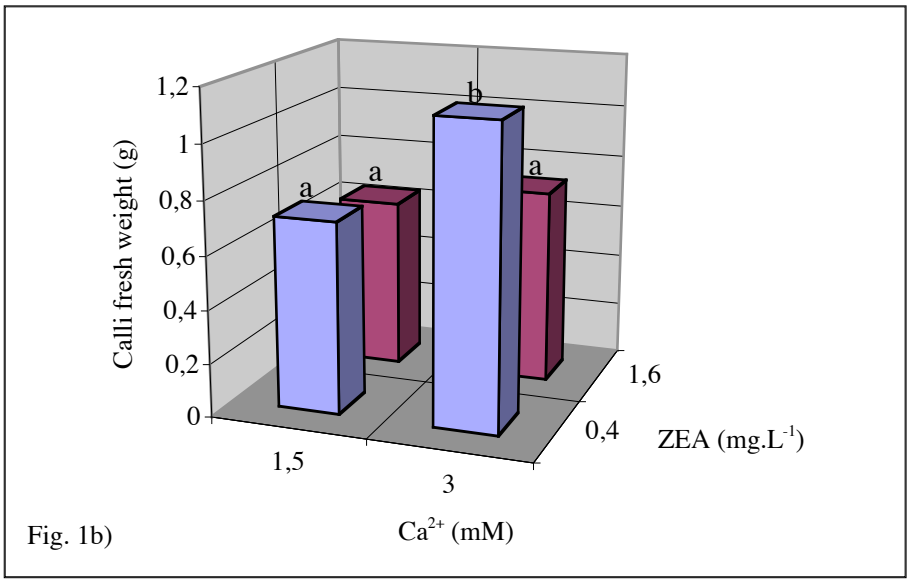

Fig. 1b)

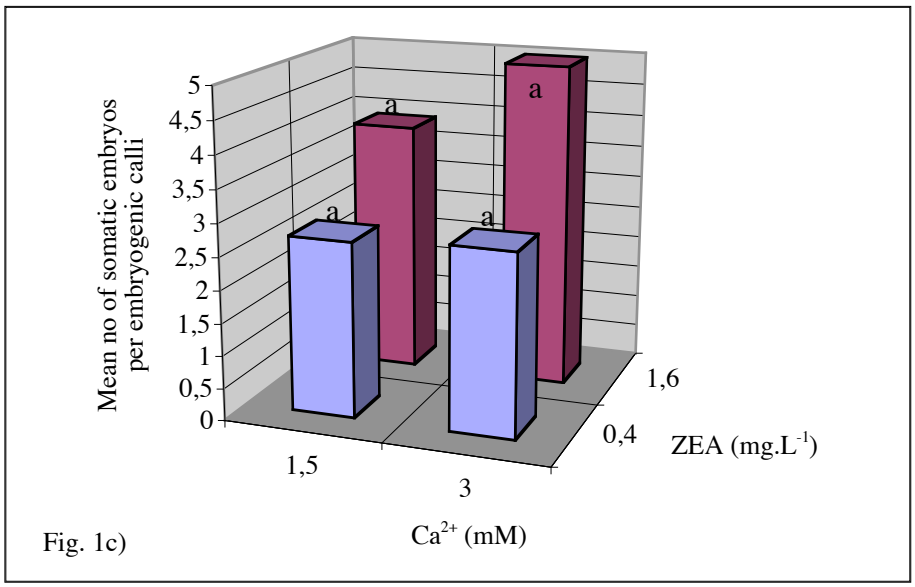

Fig. 1c)

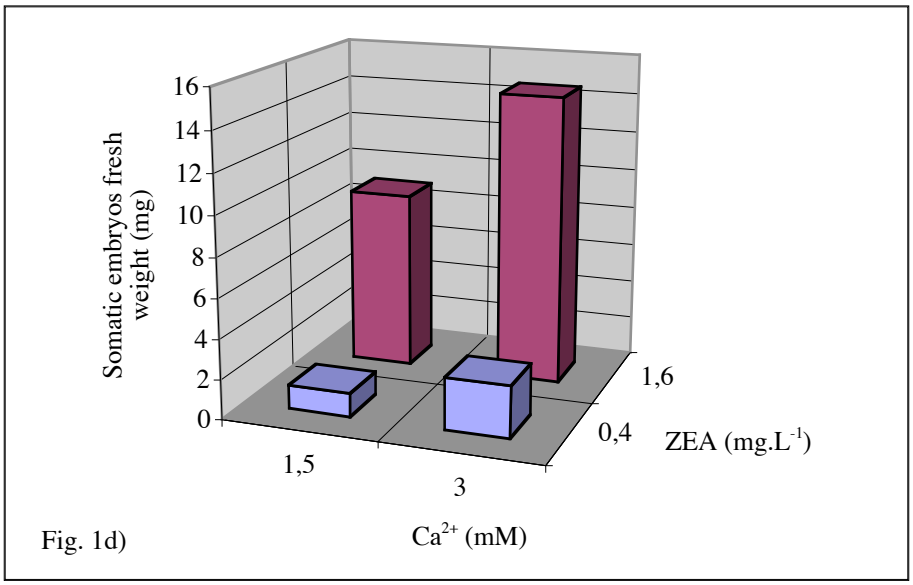

Fig. 1d)

Figure 1 – Effect of different $CaCl_2$ and zeatin levels on a) induction of somatic embryogenesis from hypocotyl flax explants; b) mean calli fresh weight; c) mean number of somatic embryos per embryogenic explant; d) mean somatic embryo

fresh weight. In each graph, columns with the same letters were not statistically different.

It was suggested that initiation of somatic embryogenesis involves a weakening of the cell-cell interaction gradient which coordinates normal bipolar development of the embryo (Pretova and Williams 1986). In the presence of a continuing stimulus for mitotic divisions, cells which are relatively undifferentiated and retain their internal pre-determination for embryo morphogenesis may escape the overall group control to re-initiate the embryogenic pathway independently as somatic embryoids (Pretova and Williams 1986). Our preliminary results seem to corroborate this explanation for the triggering of somatic embryogenesis. Considering that the concentration of cytokinin is sufficient to provide the mitotic stimulus, induction and expression takes place. At low ZEA concentrations only lowering the concentration of Ca^{2+}, and possibly weakening the cell-cell interaction, it was possible to revert the disorganized calli growth to embryogenic commitment (figs. 1a) and b)).

Many factors make the understanding of the role of specific compounds required for growth and differentiation, a very laborious task. Factors inherent to the species and to the type of explant could determine not only differences in the metabolism that is active (Kochba *et al*. 1982), but also differences in the endogenous levels of nutrients and growth regulators. In the case of zygotic embryos, an important factor is the presence of surrounding maternal tissue (Timmers *et al*. 1996). Other main factor, is the network of relationships between nutrients and growth regulators, that restricts the inference of mechanistic relations with the process of somatic embryogenesis. Most probably, even avoiding the variability due to the use of heterogeneous tissues, carryover problems and the use of complex additives in medium formulations, only with a vast number of experiments and comparing the independent results will be possible to draw more

accurate conclusions about the mechanisms of somatic embryogenesis induction. In this sense, the output results obtained in our experiments should be considered not only as a guideline for the establishment of somatic embryogenesis protocols of flax but also as an informative contribution for the understanding of the putative roles of the studied factors on such morphogenic developmental pathway.

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