Somatic embryogenesis, organogenesis and callus growth kinetics of flax (*Linum usitatissimum* L.)

Ana Cristina Gomes da Cunha & Manuel Fernandes Ferreira*

Departamento de Biologia, Universidade do Minho, Campus de Gualtar 4719 Braga Codex, Portugal

(* to whom correspondence should be addressed)

Key words: Auxins, cytokinins, *in vitro* culture, *Linum usitatissimum*, somatic embryos.

Abstract

The effects of plant growth regulators (PGR) on calli induction, morphogenesis and somatic embryogenesis of flax were studied. The organogenic and callus formation capacity were assessed for different types of source explants. Root and shoot explants were equally good material for calli production but the former produced calli without shoot regeneration capacity. Under the experimental conditions tested, 2,4-dichlorophenoxyacetic acid (2,4-D) + zeatin (ZEA) was the most efficient PGR combination on calli induction and biomass production. The calli were green but with no rhizogenic capacity. On the contrary, and at similar concentrations, indole-3-butyric acid (IBA) + kinetin (KIN) induced white or pale green friable calli with a good root regeneration capacity (60%). A factorial experiment with different combinations of 2,4-D + ZEA + gibberellic acid (GA_3) levels revealed that the direction of explant differentiation was determined by specific PGR interactions and concentrations. The results from these experiments revealed that the morphogenetic pathway (shoot versus root differentiation) can be manipulated on flax explants by raising the 2,4-D level from 0.05 to 3.2 mg l^{-1} in the induction medium. The induction and development of somatic embryos from flax explants was possible in a range of 2,4-D+ZEA concentrations surrounding 0.4 mg l^{-1} 2,4-D and 1.6 mg l^{-1} ZEA, the most efficient phytohormonal combination.

Abbreviations: C - Shoot segments with the cotyledonary leaves; 2,4-D - 2,4 - Dichlorophenoxyacetic acid, GA_3 - Gibberellic acid; H - Hypocotyl segments; IBA - Indole-3-butyric acid; KIN - Kinetin; NAA - α-naphthaleneacetic acid; PGR - plant growth regulators; R - Root segments; ZEA - Zeatin.

Introduction

Linum usitatissimum L. (flax) is a crop species widely adapted to warm and cool temperate climates (Green 1986). It has a long history of cultivation at the North of Portugal for textile workmanship industries. This species has also been used, for a long time as a source of industrial oil, for use in the production of paints, varnishes, inks and linoleum (Green & Marshall 1984). Genetic studies on flax are opening the possibility of introducing linseed oil into the edible oil industry for utilization as an edible vegetable oil (Green & Marshall 1984; Green 1986). Although tissue culture of *L. usitatissimum* has been carried out for 20 years (Rybczynsky 1975; Gamborg & Shyluk 1976; Mathews & Narrayanaswamy 1976; Murray *et al.* 1977; Lane 1979; McHughen & Swartz 1984), the knowledge of factors that control organogenesis and induction of somatic embryogenesis in this species is still scarce. Plant regeneration from isolated protoplasts has been reported (Barakat & Cocking 1983; Ling & Binding 1987), but the frequency was very low and genotype dependent (Ling & Binding 1987). These investigators also observed the occurrence of somatic embyos in *L. alpinum* protoplasts-derived calli but there are no reports on somatic embryogenesis in *L. usitatissimum*. In order to establish the basic operational conditions for the micropropagation of flax, we have started *in vitro* cultures of this species. In this paper we report the effects of PGR supplementation of the MS medium on the growth of calli, organogenesis and somatic embryogenesis induced from explants of *in vitro* grown flax seedlings.

Materials and methods

Plant materials and culturing conditions

Flax seeds were immersed in 70% ethanol for 2 min., surface sterilized in a 20% calcium hypochlorite filtered solution for 10 min. and then rinsed 5 times in sterile deionized water. Seeds were plated onto Murashige & Skoog's (1962) medium containing 2% sucrose and solidified with 0.8% agar (agar agar, J. M. Vaz Pereira, Lisboa Portugal) after pH adjustment to 5.7. The seeds were then cultured in a growth chamber under a 16 h light/8 h dark and 22°C regime for four weeks. The *in vitro* grown seedlings were used as a source of primary explants.

To study the effects of explant type and different auxin and cytokinin combinations on the callus induction and organogenesis, root segments (R), cotyledons containing shoot segments (C) and hypocotyl segments (H) were plated onto MS medium supplemented with three PGR combinations: 5.37 μM (1.0 mg l^{-1}) NAA + 2.32 μM (0.5 mg l^{-1}) KIN (medium 1); 2.95 μM (0.6 mg l^{-1}) IBA + 2.32 μM (0.5 mg l^{-1}) KIN (medium 2) and 2.26 μM (0.5 mg l^{-1}) 2,4-D + 2.28 μM (0.5 mg l^{-1}) ZEA (medium 3). The percentage of replicates (n=28) exhibiting development of callus, adventitious roots or shoots was recorded after a five week period. To determine the influence of auxins and cytokinins on calli growth and root regeneration, well-developed calli from medium 2 and 3 were transferred to MS medium supplemented with 0.6 mg l^{-1} IBA + 0.5 mg l^{-1} ZEA keeping also both controls (medium 2 and 3). The growth curves were determined on a dry weight basis from random samples of 6-8 calli measured once a week over a period of 8 weeks.

To evaluate the interplay of the main growth regulators on the differentiation of flax H explants, an experiment was conducted in which four different concentrations of 2,4-D, ZEA and GA_3 (0.05, 0.2, 0.8 and 3.2 mg l^{-1}) (for GA_3 1 mg l^{-1} <=> 2.887 μM) were combined according to a coordinate triangle (Fig. 3). H explants were inoculated with a total of 21 replicates per treatment and results recorded after 6 weeks. Taking into account the occurrence of a low frequency of somatic embryogenesis in one treatment, a second smaller scale experiment was conducted to estimate the hormonal optimum for the induction of somatic embryos. In this experiment three levels of 2,4-D (0.1, 0.2 and 0.4 mg l^{-1}) were tested in combination with three levels of ZEA

(0.4, 0.8 and 1.6 mg l^{-1}) in a complete 3x3 factorial design with 23-30 replicates per treatment.

Proportional data were statistically analyzed using the $\chi 2$ test and continuous variables with the Analysis of Variance test (ANOVA). Some specific *post hoc* comparisons between treatments were analyzed with the HSD Tuckey test (all the statistical analysis were performed with Statistica@ 4.1 from StatSoft).

Scanning electron microscopy

To observe earlier ontogenic steps of somatic embryogenesis, samples were freeze dried at 4 μbar during 48h (Alpha 2-4 LDC-1m) and subjected to gold metallization before observation with a scanning electron microscope (Leica S360).

Results and discussion

Effect of growth regulators and explant type on calli induction and organ regeneration

Within the experimental conditions tested, flax calli were induced independently from the type of PGR supplementation or type of primary explant used. However, the most efficient calli induction was obtained in the presence of 2,4-D+ZEA (Table 1). Flax calli maintained with this type of supplementation were green, grew vigorously but no roots were developed (Table 1). The highest percentage of shoot regeneration (53%) was induced on MS medium supplemented with IBA+KIN. Root regeneration was also high under this hormonal supplementation, but the calli induction was the lowest of the conditions tested (Table 1). Higher rates of calli induction were obtained when NAA was used instead of IBA. However the rate of shoot regeneration obtained in the presence of NAA+KIN was four times lower than that obtained with IBA+KIN (Table 1).

Table 1 - Effects of plant growth regulator hormonal supplementations on the induction of calli and adventitious organs (roots and shoots) from different flax primary explants types. For each variable and in each column, numbers followed by the same letters are not statistically different. C = Cotyledon, H = Hypocotyl, R = Root.

Experimental variables and variations tested		Calli induction (%)	Induced roots (%)	Induced shoots (%)
Primary explant	Type C	78 (a)	33 (b)	38 (a)
	Type H	87 (a)	53 (a)	23 (b)
	Type R	76 (a)	26 (b)	0 (c)
Plant Growth Regulator supplementation	NAA+Kinetin (medium 1)	82 (b)	68 (a)	13 (b)
	IBA+Kinetin (medium 2)	65 (c)	60 (a)	53 (a)
	2,4-D+Zeatin (medium 3)	97 (a)	0 (b)	25 (b)

Unlike other species (ex. *Helianthus* sp., Punia & Bohorova 1992), the induction of flax calli seems to be independent from the type of primary explant. The percentage of induced calli was always above 75% (Table 1). Contrary to what happens with some other species (e.g. *Dianthus caryophyllus* L., Nakano *et al.* 1994), shoot and root regeneration of *L. usitatissimum* seems to be possible from any green part of the seedling, namely from shoot segments containing cotyledons (C) or not (H) (Table 1). However, while primary explants of C type induced a higher percentage of shoot regeneration, those of type H induced a higher root regeneration. Root regeneration was also induced when root segments were used as primary explants. However, no adventitious shoots were induced from this explant type. The substitution of KIN by ZEA in medium 2 gave rise to green calli and larger than those obtained in medium 2, and to a slight decrease in the rate of root regeneration (from 61 to 50%) so that no discernible role can be attributed to these cytokinins on this later process. On the other hand while no root regeneration occurred from calli grown on medium 3, 33% of the calli regenerated adventitious roots when 2,4-D from medium 3 was substituted by IBA. It is clear that from the growth regulators tested IBA is the most important in the induction of roots, and ZEA seems to be more efficient than KIN on biomass and chlorophyll accumulation by flax calli.

IBA and 2,4-D had opposite effects on shoot regeneration from H primary explants when their exogenous concentrations were increased from 0.25 mg l^{-1} to 1.0 mg l^{-1} in the presence of ZEA at 0.5 mg l^{-1}. After 40 days, 2,4-D at 0.25 mg l^{-1} was more favourable for shoot regeneration than IBA at the same concentration. However, the increase of the IBA concentration stimulated the induction and development of

adventitious shoots, whereas the increase of the 2,4-D concentration had a strong inhibitory effect (Fig. 1). These results suggest that 2,4-D, at least at concentrations above 0.25 mg l^{-1}, inhibits shoot regeneration from flax explants. In fact, as Figure 1 shows, the presence of 2,4-D in the seedlings growth medium (series A) had an inhibitory effect on the development of shoots. This effect was most likely due to an increase in the endogenous concentration of auxin in the primary explant tissues determined by the addition of 2,4-D to the germination medium. IBA seems to be a more suitable auxin for the induction of shoots from flax explants because it is effective in a wider range of concentrations and stimulates a higher shoot frequency from flax explants (data not shown).

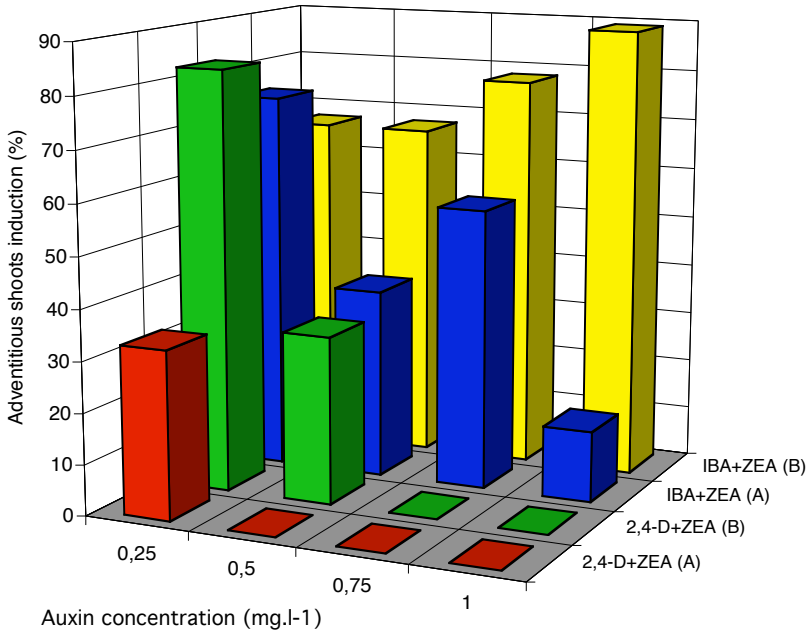

Figure 1 - Effect of increasing concentrations of auxin, added with 0.5 mg l^{-1} Zeatin, on the induction of adventitious shoots from H explants. The auxins tested were 2,4-D (first and second series in zz axis) and IBA (third and forth series). The explants derived from seedlings grown on MS medium supplemented with 0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} ZEA (letter A) or from seedlings grown on medium devoided from growth regulators (letter B).

Growth analysis of calli in media with different PGR compositions

The growth profiles of calli maintained on MS medium supplemented with different PGR

supplementations are represented in Figure 2. At the end of the experiment, calli mean dry weight was statistically different between most treatments. The difference between mean calli biomass with 2,4-D+ZEA and IBA+ZEA was significant only at the 6% level. The kinetic parameters specific growth rate (μ) and doubling time (dt) estimated for calli maintained in the presence of 2,4-D+ZEA (μ=0.049 d^{-1}, dt=14 d) and IBA+ZEA (μ=0.038 d^{-1}, dt=18 d) revealed that the rate of biomass increment was also higher with 2,4-D+ZEA. These results confirm previous qualitative observations that showed that 2,4-D+ZEA was the most efficient PGR combination for flax calli biomass production. Under similar culturing conditions, Fernandes-Ferreira *et al.* (1992) also concluded that among several auxins tested along with ZEA, 2,4-D promoted the best growth rates of *Euphorbia characias* calli. No references were found on growth kinetics of flax calli, but comparatived to other species, the specific growth rate of flax calli was low (0.085 d^{-1} for *Euphorbia characias*, Fernandes-Ferreira *et al.* 1989; 0.08 to 0.095 d^{-1} for *Sylibum marianum*, Fevereiro *et al.* 1986; 0.158 to 0.165 d^{-1} for *Cynara cardunculus*, Figueiredo *et al.* 1987)

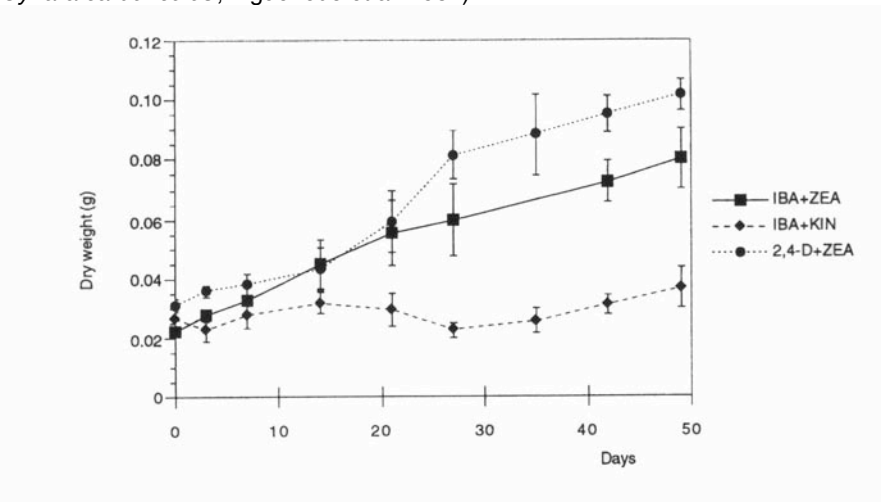

Figure 2 - Variation of mean dry weight of calli grown on MS medium supplemented with: ■ - 0.6 mg l^{-1} IBA + 0.25 mg l^{-1} ZEA, ◆ - 0.6 mg l^{-1} IBA + 0.5 mg l^{-1} KIN or ● - 0.5 mg l^{-1} 2,4-D + 0.25 mg l^{-1} ZEA. I - ± Standard error.

Effect of serial combinations of plant growth regulators on organogenesis and somatic embryogenesis

The action of 2,4-D, ZEA and GA_3 tested simultaneously on *in vitro* root differentiation from flax

explants was in agreement with what is described elsewhere (eg. *Digitalis purpurea*, Rucker 1982 in Scott 1984; Pierik 1987; Fowler 1992).

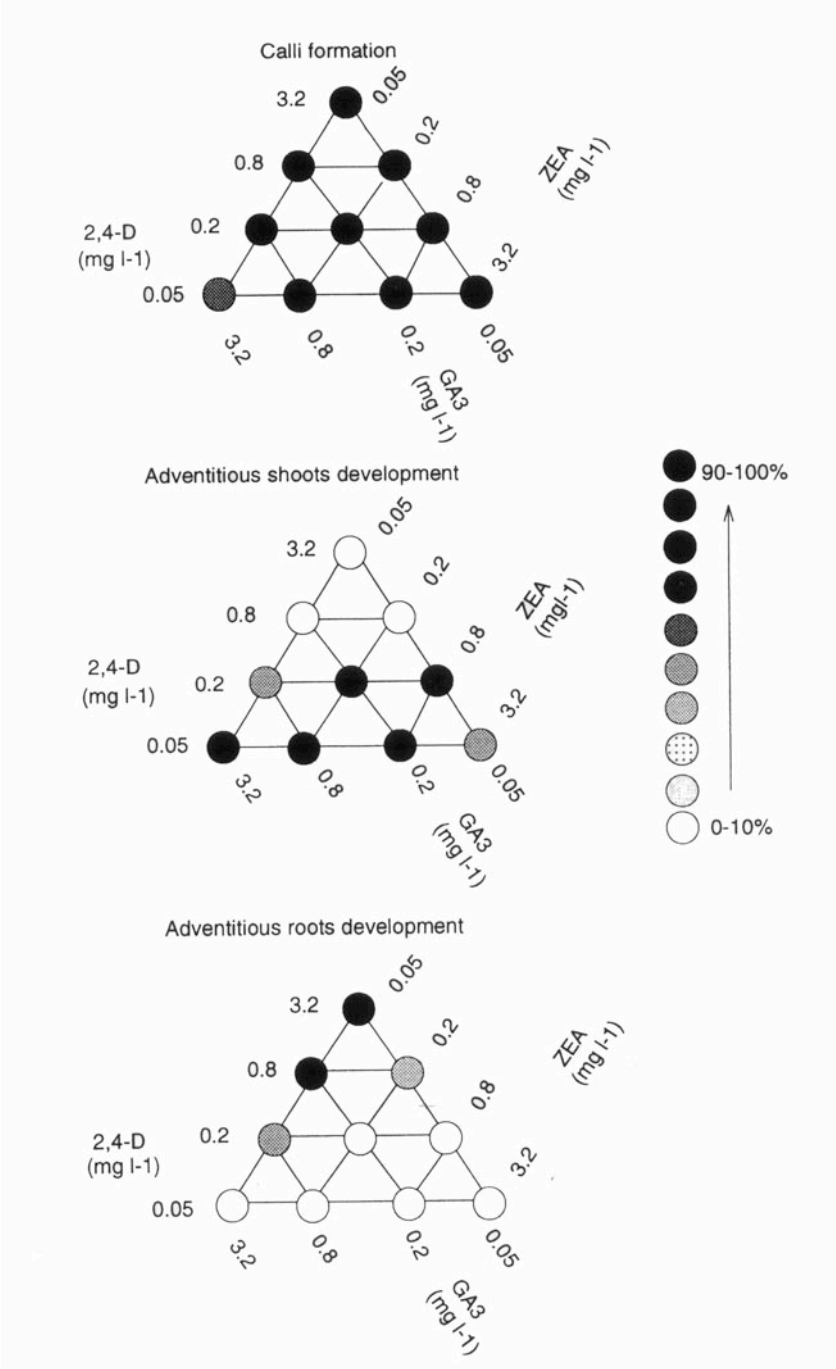

Figure 3 - Percentage of calli induction and shoot and root regeneration from flax explants inoculated on MS medium supplemented with different level combinations of 2,4-D + Zeatin + GA_3.

Root formation was sensitively stimulated by increasing concentrations of 2,4-D and was inhibited under the influence of ZEA (Fig.3). However, in the presence of 0.5 mg l^{-1} ZEA, IBA is more efficient on root regeneration than 2,4-D used at the same concentration. This result explains also the absence of root regeneration from flax calli obtained in the presence of 0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} ZEA in the first experiment (Table 1). The promotive effect of 2,4-D was probably overruled by the high ZEA concentration. Although the use of very low auxin:cytokinin ratios is common for *in vitro* shoot induction of flax (Cullis & Clearly 1986; Marshall & Courduries 1992), the development of adventitious shoots seems to be determined by a low 2,4-D concentration and not only by a low auxin:cytokinin ratio. From these observations and considering the results expressed in Figure 1, it seems reasonable to conclude that independent of the concentration of ZEA used, increasing concentrations of 2,4-D suppress shoot development from flax explants (0.5 mg l^{-1} being completely inhibitory). Similarly, Yuan *et al.* (1994) found that when the concentration of 2,4-D increased from 0 to 0.5 mg l^{-1}, the formation of shoots from *Catharanthus roseus* explants was inhibited, independent of the absence or presence of BA (0 to 7 mg l^{-1}). On the contrary and as we had obtained with IBA, increasing concentrations of NAA (up to 1.0 mg l^{-1}) resulted in a stimulation of shoot formation. The induction of calli from flax H explants occurred with almost all the PGR combinations tested. The exception was the inhibition of calli formation in the presence of low 2,4-D concentrations with increasing GA_3 concentrations. Although at a very low rate, somatic embryos started to develop from calli on the 0.2 mg l^{-1} 2,4-D + 0.8 mg l^{-1} ZEA + 0.05 mg l^{-1} GA_3 treatment (Fig.4).

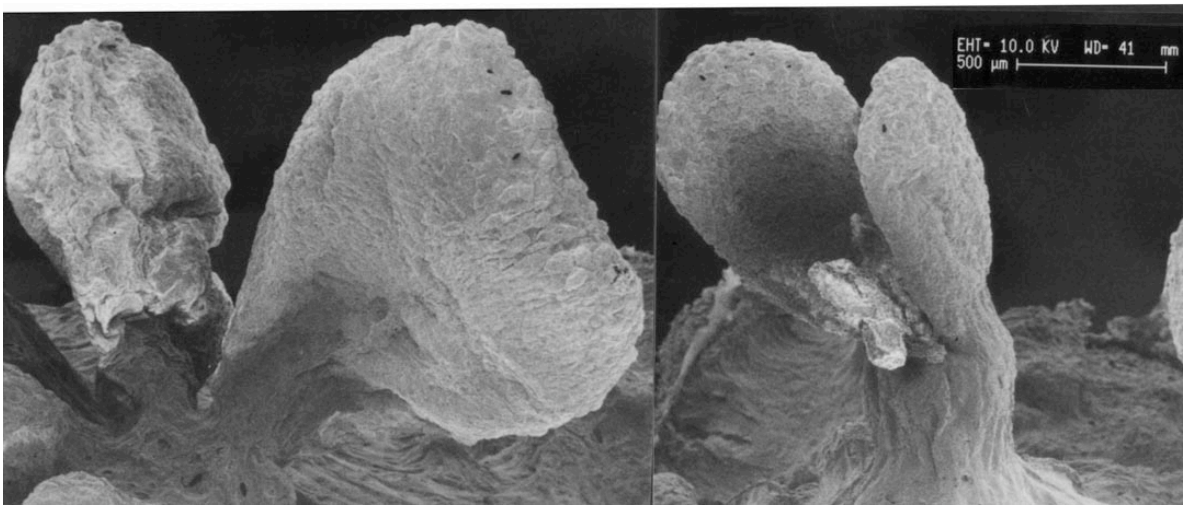

Figure 4 - Three different developmental phases during somatic embryogenesis on H explant-derived *calli*. From left to right, embryonic structures at the torpedo and heart shape stage and the true cotyledonary stage are observed.

In the 3x3 experiment (MS medium devoid of GA_3) we could observe a reduction in the time of embryo emergence from 4 weeks to 10 days. Simultaneously, with the exception of the 0.1 mg l^{-1} 2,4-D + 1.6 mg l^{-1} ZEA treatment, an increase of the frequency of embryogenic explants to values higher than 75% (Table 2).

Table 2 - Effect of the combined action of 2,4-D+Zeatin on the induction of embryogenic flax explants. Each plant growth regulator was tested in 3 different concentrations in a complete factorial design. The variables estimated were percentage of induction and percentage of embryo conversion after a period of 6 weeks in growth regulator-free medium. In each column numbers followed by the same letters are not statistically different.

2,4-D+ZEA (mg.l-1)	Embryogenic explants (%)	Number of embryos inocculated	Rooted embryos (%)
0.1+0.4	93.3 (a)	40	18.4 (a)
0.1+0.8	87.5 (a,b)	22	15.0 (a,b)
0.1+1.6	29.2 (c)	12	0.0
0.2+0.4	100 (a)	16	25.0 (a,b)
0.2+0.8	87 (a,b)	18	22.0 (a,b)
0.2+1.6	95.8 (a)	24	8.3 (a)
0.4+0.4	75.0 (b)	27	19.2 (a,b)
0.4+0.8	100 (a)	24	16.7 (a,b)
0.4+1.6	100 (a)	24	41.7 (b)

According to some authors (Santos *et al.* 1994), explant age may play a definite role in embryogenic competence of calli. In this experiment, explants from young seedlings (4 weeks) were used which may explain the success in the induction of somatic embryogenesis. Another interesting feature is the relatively low, effective 2,4-D concentrations for flax somatic embryogenesis (0.1 - 0.4 mg l^{-1}) when compared with other species (5 - 60 mg l^{-1} for peanut (Baker & Wetzstein 1994); 2 - 5 mg l^{-1} for barley (King & Kasha 1994); 38 - 132 mM for soybean (Schoemaker *et al.* 1991)). The results are recorded as the mean number of somatic embryos per embryogenic explant (Fig. 5). After a square root transformation of data for normalization of the variable distribution and stabilization of variances, significant differences in the number of embryos per explant between groups were detected using the ANOVA test. Although the overall effect of 2,4-D on somatic embryo induction was statistically significant, the test revealed that the most striking source of variability (very highly significant) was the interaction between auxin and cytokinin. Therefore, and according to Compton (1994), the influence of 2,4-D and ZEA on flax somatic embryogenesis should not be discussed separately. On Figure 5, we can observe a bimodal optimum PGR combination for embryo induction that corresponds to the 0.1 mg l^{-1} 2,4-D + 0.8 mg l^{-1} ZEA and 0.4 mg l^{-1} 2,4-D + 1.6 mg l^{-1} ZEA combinations, and three minima, one on the highest (16x) and two on the lower (1x and 2x) ZEA/2,4-D ratios. All other combinations are not statistically different. This indicated that unlike other species, ex. *Hevea brasiliensis* (Etienne *et al.* 1993), the expression of somatic embryogenesis is not just related to the establishment of a specific balance between different phytohormones but perhaps to the particular levels of the phytohormones in action. After transfer of the embryos to a phytohormone-free MS medium for root development, it was observed that the embryos induced in MS medium supplemented with 0.1 mg l^{-1} 2,4-D + 1.6 mg l^{-1} ZEA had no rooting capacity and the treatment with 0.4 mg l^{-1} 2,4-D + 1.6 mg l^{-1} ZEA induced the most conversion-competent embryos (Table 2). These results suggest that although we had a relatively wide range of 2,4-D+ZEA combinations which induce high embryo multiplication rates (>7/explant), supplementation with 0.4 mg l^{-1} 2,4-D + 1.6 mg l^{-1}ZEA is most likely to provide true bipolar embryos at high frequencies.

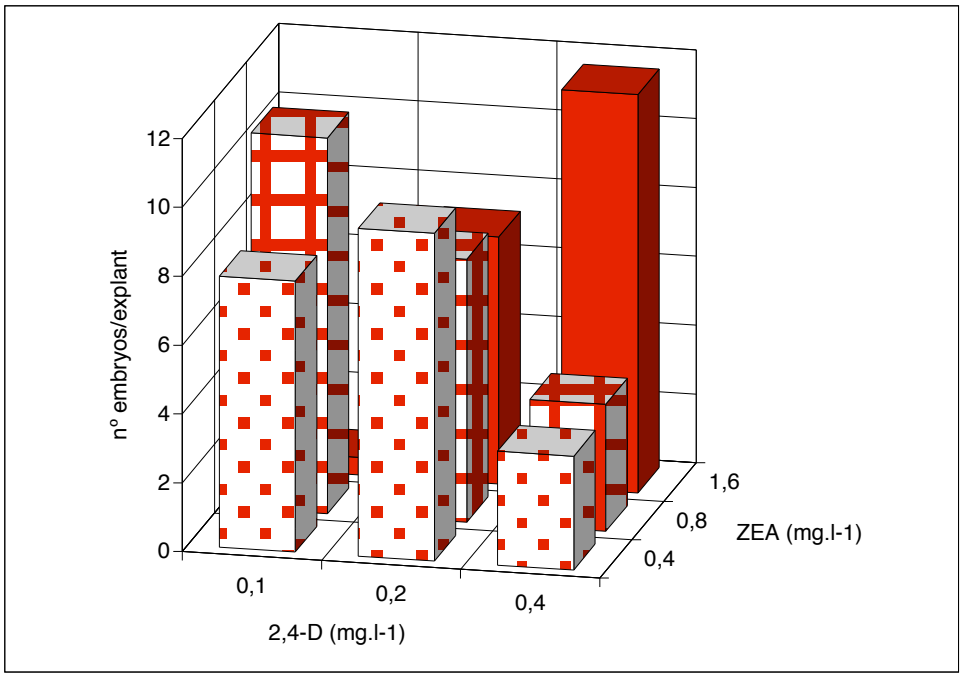

Figure 5 - Effect of different 2,4-D + Zeatin concentrations on the frequency of somatic embryos induced from H flax explants.

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