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Induction of somatic embryogenesis in soybean: physicochemical factors influencing the development of somatic embryos

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Abstract

The embryogenic capability of five soybean cultivars (Renascença, IAS-5, IAC-17, BR-16 and FT-Cometa) was studied at different auxin concentrations (8, 10 and 12 mg/l naphthalene acetic acid, NAA), at different pHs (5.8 and 7.0) and at low (8-12 $\mu Em^{-2} s^{-1}$) and high (27-33 $\mu Em^{-2} s^{-1}$) light intensities. The experimental design was completely randomized with four replications. Immature cotyledons 4-6 mm in length were placed in the six induction mediums evaluated and submitted to two light intensities. Twenty immature cotyledons per cultivar were placed on each Petri dish, which was considered to be one replication. The number of somatic embryos per treatment per replication was counted. The results showed genotype influence on somatic embryogenic capability of each cultivar, with the most embryogenic cultivars being BR-16, FT-Cometa and IAS-5. Auxin concentration and pH value also influenced somatic embryo production, with 10 mg/l NAA being the best auxin concentration and 7.0 the best pH value. The interactions cultivar x auxin, auxin x pH and pH x light were significant, while other double interactions were not. All triple and quadruple interactions were significant, except cultivar x pH x light. No significant differences in somatic embryo production were observed in medium with different pHs or when the Petri dishes containing immature cotyledons were exposed to the two light intensities evaluated. However, a higher number of somatic embryos was produced when the medium pH was adjusted to 7.0.

INTRODUCTION

Soybean [*Glycine max* (L.) Merril], one of the most important cultivated species, is outstanding as a source of vegetable oil and protein. Most by-products of soybean oil extraction are used in animal nutrition. Furthermore, the chemical composition of soybean seeds recommends it as one of the main solutions for global food deficits. Thus, the soybean has been the object of intense genetic improvement programs that have resulted in the development of new cultivars adapted to Brazilian climatic conditions.

As a consequence of the continuously growing global demand for food and protein, new commercial cultivars, with greater capacity to withstand environmental stresses and genetically improved to increase yield quantity and quality are desirable. Tissue culture and plant transformation techniques open new possibilities for improving soybeans (Hildebrand *et al*., 1991), and among them methods of *in vitro* regeneration and somatic embryogenesis stand out.

Considering the importance of both the identification of cultivars suitable for embryogenic propagation and the knowledge of the interaction between cultivars and inherent physicochemical factors involved in the induction of somatic embryogenesis, the aim of the present research was to evaluate the behavior of soybean cultivars in relation to embryogenic capacity at different auxin concentrations (naphthalene acetic acid, NAA), pH values, and light intensities, as well as to determine interactions between these factors.

MATERIAL AND METHODS

Soybean cultivars and treatments

The soybean cultivars IAS-5, IAC-17, Renascença, BR-16 and FT-Cometa were used as source of explants, and maintained in the greenhouse at a 14-h photoperiod and a temperature of approximately 30°C. Pods containing immature 4-6-mm long cotyledons were collected, transported to the laboratory and submitted to asepsis (Mauro *et al*., 1994). In a laminar flow hood, pods were excized and immature cotyledons extracted and placed on solid MS media (Murashige and Skoog, 1962) containing different auxin (NAA) concentrations (8, 10 and 12 mg/l). Two pH values of the media were also evaluated (5.8 and 7.0). Twenty cotyledons of each cultivar were placed on each Petri dish (100 mm in diameter), sealed with Parafilm, and incubated in a growth chamber at a 23-h photoperiod and approximately 26°C. Petri dishes containing the immature cotyledons were exposed to different light intensities (8-12 $\mu Em^{-2} s^{-1}$ (low) and 27-33 $\mu Em^{-2} s^{-1}$ (high)), since great attention has recently been given to light intensity in the growth chamber.

Experimental design and statistical analysis

The experimental design was an entirely randomized factorial model (5 x 3 x 2 x 2 x 4). The five soybean cultivars were combined with three auxin concentrations, two

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pH values, two light intensity levels, and four replications. The induction period was 90 days and cotyledons were transferred to fresh media at 15-day intervals. As somatic embryos appeared they were removed and counted. The suggestions of Snedecor and Cochran (1989) were followed for statistical analysis. The number of somatic embryos per treatment was transformed to the scale $\sqrt{x + 0.5}$ for data normalization (Banzato and Kronka, 1992).

RESULTS AND DISCUSSION

Table I shows significant differences (P<0.01) in somatic embryo production between cultivars and also that auxin level had an important influence. The influence of cultivar genotype on somatic embryo formation was also reported by Parrot *et al.* (1989) and Komatsuda and Ko (1990). Significant differences were also observed in auxin levels (P<0.01) and pH (P<0.05), indicating that the production of somatic embryos was independently influenced by these two factors. The two light intensities had no effect on somatic embryo production. The double interactions were significant for cultivar x auxin (P<0.01), auxin x pH and pH x light (P<0.05). Triple and quadruple interactions were all significant (P<0.01), except for cultivar x pH x light. These results show that each factor is influenced by the other. Coefficient of variation was 18.04%, suggesting reliable results.

Analysis of variance applied to axin levels x cultivars and cultivar x auxin levels (Table II) showed that different auxin concentrations had important effects (P<0.01) on the somatic embryo formation of each cultivar. Similarly, the same Table shows that each cultivar produced a different number of somatic embryos at each auxin concentration (P<0.01). Considering auxin levels within pH values, the results showed that auxin had significant effects (P<0.01) on somatic embryo production at each medium pH. On the other hand, the same Table also shows that the evaluated medium pHs only had significant effects (P<0.01) when 12 mg/l NAA was added to the induction medium. Regarding light intensities within pH values and pH values within light intensities, although Tables I and II showed some interaction between these factors, no differences among means of somatic embryo production at the two light intensities and pH values were observed by the Tukey test (Table III). This may indicate that further studies with an increased range and number of light intensities are necessary.

Table IV shows the mean number of somatic embryos obtained from cotyledons of the five soybean cultivars induced at three different auxin concentrations. Each cultivar produced a significantly higher number (P<0.05) of somatic embryos when the MS media were supplemented with 10 mg/l NAA. Interestingly, in most research on somatic embryogenesis in the soybean this was the concentration used and the reason why modified MS medium was termed N10 (Lazzeri *et al.*, 1985; Hartweck *et al.*, 1988;

Table I - Variance analysis results of number of somatic embryos in an entirely randomized factorial model.

Source of variation	d.f.	Mean square
Cultivar	4	12.02**
Auxin	2	21.89**
pH	1	0.93*
Light	1	0.48^{NS}
Cultivar x auxin	8	0.96**
Cultivar x pH	4	0.31^{NS}
Cultivar x light	4	0.35^{NS}
Auxin x pH	2	0.78*
Auxin x light	2	0.17^{NS}
pH x light	1	1.14*
Cultivar x auxin x pH	8	1.49**
Cultivar x auxin x light	8	1.85**
Cultivar x pH x light	4	0.34^{NS}
Auxin x pH x light	2	2.07**
Cultivar x auxin x pH x light	8	1.29**
Error	180	0.20

CV% = 18.04
Mean = 2.52

**Significant at 1% level of probability (P<0.01). *Significant at 5% level of probability (P<0.05). ^{NS} Nonsignificant. d.f., Degrees of freedom.

Table II - Analysis of variance for the partition of degrees of freedom (d.f.) in auxin levels x cultivars, cultivars x auxin levels, pH values x auxin levels, auxin levels x pH value, light intensities x pH values and pH values x light intensities.

Source of variation	d.f.	Mean square
Auxin x Renascença	2	5.72**
Auxin x IAS-5	2	2.14**
Auxin x IAC-17	2	6.30**
Auxin x BR-16	2	1.48**
Auxin x FT-Cometa	2	10.10**
Cultivar x 8 mg/l NAA	4	6.34**
Cultivar x 10 mg/l NAA	4	3.10**
Cultivar x 12 mg/l NAA	4	4.50**
Auxin x pH 5.8	2	8.00**
Auxin x pH 7.0	2	14.67**
pH x 8 mg/l auxin	2	0.17^{NS}
pH x 10 mg/l auxin	2	0.06^{NS}
pH x 12 mg/l auxin	2	2.26**
Light x pH 5.8	2	0.07^{NS}
Light x pH 7.0	2	1.55**
pH x 8-12 $\mu Em^{-2} s^{-1}$ light	2	2.06**
pH x 27-33 $\mu Em^{-2} s^{-1}$ light	2	0.01^{NS}

**Significant at 1% level of probability (P<0.01). ^{NS} Nonsignificant.

Table III - Means of somatic embryos (NE) obtained from cotyledons induced at two pH values and at two ligtht intensities.

pHs	Light intensities/means*(NE)	
	8-12 $\mu Em^{-2} s^{-1}$	27-33 $\mu Em^{-2} s^{-1}$
5.8	2.56 a A	2.52 a A
7.0	2.31 a A	2.53 a A

*Means followed by the same lower case letters in the column and by the same upper case letters in the line did not differ at 5% level of probability by the Tukey test.

Parrot *et al.*, 1988, 1989; Komatsuda *et al.*, 1991; Dahmer *et al.*, 1991; Mauro *et al.*, 1994, 1995a; Nóbrega, 1996).

Considering the best auxin concentration (10 mg/l NAA) and the behavior of each cultivar within auxin concentrations (Table IV), cultivar influence on embryo formation can be seen, confirming the results obtained by Komatsuda and Okyama (1988), Parrot *et al.* (1989), Calvo (1989), Komatsuda *et al.* (1992), Mauro *et al.* (1995b) and Nóbrega (1996). Thus, cultivars IAS-5, FT-Cometa and BR-16 were the most embryogenic, as also observed by Ferreira *et al.* (1990), Bodanese-Zanetini *et al.* (1993) and Droste *et al.* (1993).

Comparisons among means for somatic embryo production at three auxin levels and two pH values (Table V) confirmed that the best auxin concentration for inducing somatic embryo formation is 10 mg/l NAA. No differences were observed in somatic embryo production between the two pH values when 8 or 10 mg/l NAA was added to the induction medium; however, a higher mean number of somatic embryos was obtained when the medium pH was adjusted to 7.0. This result disagrees with Pierik (1989), who emphasized that a pH in the 5.0 to 6.5 range is suitable for somatic embryo development, and may explain why most of the recent papers have used pH = 7.0 in the induction medium.

Regression in the number of somatic embryos produced by each cultivar as a function of the three auxin concentrations is shown in Figure 1. The results demonstrate an increasing response to auxin concentration up to 10 mg/l NAA. Greater concentrations decreased somatic embryo formation. The largest determination coefficient (R^2) was obtained for IAC-17 ($R^2 = 0.90$) and the smallest value for IAS-5 ($R^2 = 0.20$). The R^2 value obtained for cultivar IAS-5 may be due to a variation in embryo production related to auxin concentration, and may be possibly associated with some endogenous or physiological factor in the cultivar.

Table IV - Means of somatic embryos (NE) obtained from cotyledons of the five soybean cultivars induced at three different auxin concentrations.

Cultivars	Auxin concentrations (NAA)/means*(NE)		
	8 mg/l	10 mg/l	12 mg/l
Renascença	1.64b B	2.78bc A	1.90bc B
IAS-5	2.68a B	3.33a A	2.71a B
IAC-17	1.38b B	2.51c A	1.48c B
BR-16	2.61a B	3.17ab A	2.68a B
FT-Cometa	2.64a B	3.62a A	2.05b C
Mean	2.19b	3.09a	2.17b

*Means followed by the same lower case letters in the column and by the same upper case letters in the line did not differ at 5% level of probability by the Tukey test.

Table V - Means of somatic embryos (NE) obtained from cotyledons induced at two pH values and at three different auxin concentrations.

pHs	Auxin concentrations (NAA)/means*(NE)		
	8 mg/l	10 mg/l	12 mg/l
5.8	2.24 a B	3.05 a A	2.33 a B
7.0	2.15 a B	3.11 a A	2.00 b B

*Means followed by the same lower case letters in the column and by the same upper case letters in the line did not differ at 5% level of probability by the Tukey test.

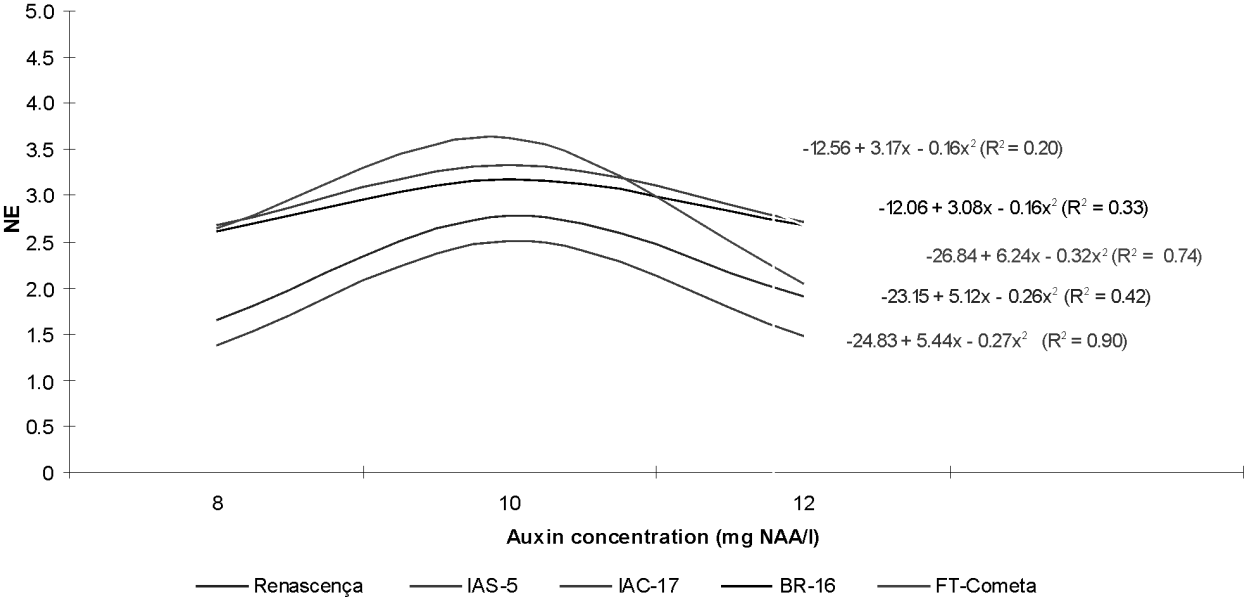

Figure 1 - Regression of the number of somatic embryos (NE) produced by each cultivar as a function of the three auxin concentrations.

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RESUMO

A capacidade embriogênica de cinco cultivares de soja (Renascença, IAS-5, IAC-17, BR-16 e FT-Cometa) foi estudada em diferentes concentrações de auxina (8, 10 e 12 mg/l de NAA), em diferentes valores de pHs (5,8 e 7,0) e em baixa (8-12 $\mu Em^{-2} s^{-1}$) e em alta (27-33 $\mu Em^{-2} s^{-1}$) intensidade luminosa. O delineamento experimental foi o inteiramente casualizado com quatro repetições. Cotilédones imaturos com 4-6 mm de tamanho foram depositados nos seis meios indutivos e submetidos às duas intensidades luminosas. Vinte cotilédones imaturos de cada cultivar foram depositados em cada placa de Petri, que foi considerada como uma repetição. O número de embriões somáticos por tratamento, por repetição foi contado e os resultados evidenciaram a influência do genótipo na capacidade embriogênica de cada cultivar, revelando-se mais embriogênicos os cultivares FT-Cometa, IAS-5 e BR-16. A concentração de auxina e o valor do pH tiveram influência no número de embriões somáticos produzidos, sendo 10 mg/l de NAA a melhor concentração de auxina e 7,0 o melhor valor de pH. As interações cultivar x auxina, auxina x pH e pH x luz revelaram-se significativas, e as demais duplas interações foram não significativas. Todas as interações triplas e quádruplas foram significativas, exceto cultivar x pH x luz. Não foram observadas diferenças significativas na produção de embriões somáticos nos meios com diferentes pHs e nem quando as placas de Petri contendo os cotilédones imaturos foram submetidas às duas intensidades luminosas avaliadas, porém maior número de embriões somáticos foi produzido quando o pH do meio foi ajustado para 7,0.

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