Universidade Federal do Rio Grande do Sul
Instituto de Biociências

Effects of abscisic acid on soybean somatic embryo maturation and conversion to plants

Ricardo Luís Mayer Weber
Orientadora: Maria Helena Bodanese Zanettini

Trabalho apresentado como um dos requisitos para a obtenção do grau de Bacharel em Ciências Biológicas – Ênfase em Biologia Molecular, Celular e Funcional.

Porto Alegre
Janeiro, 2004

Agradecimentos

À minha orientadora, **Maria Helena Bodanese Zanettini**. Maria Helena, obrigado por ter-me acolhido no teu grupo de pesquisa, ensinado-me muito e colaborado a tornar-me mais otimista e prático. "Quando crescer quero ser como você!"

Às **meninas do laboratório (as verdinhas!)**, por terem-me recebido tão bem e colaborado muito para a realização deste trabalho (o auxílio de vocês foi fundamental, tanto na descontração, quanto nos experimentos e afins), vocês são muito especiais. Valeu por tudo!

À **Sílvia**, sem comentários, tu é + que d+! Brigadão por tudo.

À **Sidia**, pela colaboração nas análises estatísticas.

Lala, Mary, Mini e Martin (os "personagens"!), Taty e Alininha (plagiando a Mary – **My best friends!**) – sem vocês a graduação seria muito chata. Obrigado por terem-me proporcionado momentos felizes e terem-me dado apoio no que precisei, é bom ter pessoas assim ao redor. Vocês fazem parte da minha vida.

Ao **Marcos**, tu és "fora de série", obrigado por tudo mesmo, TÁ!

À toda minha família, em especial aos **meus pais** (Ricardo e Jane) e a **minha irmã** (Maria Eduarda – a Duda), por acreditarem nos meus projetos, darem todo o apoio que precisei e compreenderem minhas variações de humor e ausências. Obrigado por me tornarem a pessoa que sou. É um sonho que se realizou. Este é para vocês!

Vô Zé (*in memorian*): Eu consegui!

Aos **órgãos financiadores** deste estudo: PIBIC - UFRGS/CNPq, CNPq e FAPERGS.

A **todos** que, de algum modo, contribuíram para a realização deste projeto, MUITO OBRIGADO.

Effects of abscisic acid on soybean somatic embryo maturation and conversion to plants

Authors:

Ricardo Luís Mayer Weber[a]

Maria Helena Bodanese Zanettini[a] *

Sidia Maria Callegari-Jacques[a,b]

[a] Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul.

[b] Departamento de Estatística, Instituto de Matemática, Universidade Federal do Rio Grande do Sul.

Partial results of the present study were presented in 49° Congresso Brasileiro de Genética, Águas de Lindóia, São Paulo, September 16 – 19, 2003.

* Author for correspondence, e-mail: maria.zanettini@ufrgs.br, Caixa Postal 15053, 91501-970, Porto Alegre, RS, Brazil; ph: 55-51-33166725; fax: 55-51-3316-7311.

Effects of abscisic acid on soybean somatic embryo maturation and conversion to plants

Abstract

The effect of abscisic acid (ABA) on soybean somatic embryogenesis, embryo development, histodifferentiation, germination and conversion was investigated. Two soybean cultivars (IAS-5 and Conquista) were included in the study. ABA at 50μM was applied at two different stages of culture; namely at embryo globular stage in proliferation medium and at histodifferentiation stage in maturation medium. Cultivar IAS-5 showed a higher embryogenic potential, producing high number of histodifferentiated embryos and high germination and conversion rates. For cultivar IAS-5 the presence of ABA in proliferation medium improved embryo germination and conversion. The beneficial effect of ABA on embryo development was not observed for cultivar Conquista. Thus, the effects of ABA treatments were genotype-specific.

Key words – soybean, abscisic acid, somatic embryogenesis, germination, conversion.

Introduction

Soybean [*Glycine max* (L.) Merrill], one of the most important cultivated species, is an important source of oil and protein for which *in vitro* technology has considerable potential. However, this species has remained particularly recalcitrant to highly-efficient transformation due to the low regeneration rates of plants (Trick et al., 1997).

Embryogenic tissue was first identified a target for genetic transformation of soybean by Parrott et al. (1989) using *Agrobacterium tumefaciens* – mediated transformation. Further studies showed embryogenic tissue to be amenable to transformation via particle bombardment (Finer & McMullen, 1991; Sato et al., 1993; Simmonds & Donaldson, 2000; Droste et al., 2002). The development of a system with high efficiency on conversion of plants from soybean somatic embryos could increase the potential for production of large numbers of independent transgenic lines.

The phytohormone abscisic acid (ABA) is a sesquiterpenoid synthesized from xanthophylls. ABA regulates several important aspects of plant growth and development (Gaspar et al., 1996). It accumulates at high levels when a plant is subjected to certain abiotic stresses, such as hydric stress, and during seed development. ABA provided by the mother plant and synthesized in the seed itself contributes to the regulation of embryo development and maturation. Furthermore, crucial physiological processes, such as germination, which is inhibited by ABA, are regulated by ABA catabolism (Nambara & Marion-Poll, 2003). Seed maturation not only includes growth and development of the

embryo (embryogenesis); but also involves accumulation of storage reserves and preparation for desiccation - which occurs in the last stages of seed maturation. ABA induces storage protein synthesis and affects the induction and maintenance of some aspects of dormancy in seeds (Rock & Quatrano, 1995).

In tissue culture, manipulation of cultural conditions to prolong and improve embryo maturation, and to prevent precocious germination, will probably increase the similarity observed between zygotic and somatic embryos. That is to say that somatic embryos produced will come to have the vigor and germination associated with their zygotic counterparts (Merkle et al., 1995). Studies have reported beneficial effects of ABA on somatic embryo development. ABA, exogenously supplied, has effects on storage protein expression. In embryos of hybrid larch (Gutmann et al., 1996), sugarcane (Nieves et al., 2001) and geranium (Madakadze & Senaratna, 2000) cultured *in vitro*, exogenous ABA induced an increased of storage proteins.

Exogenously supplied ABA could increase the frequency of somatic embryo reaching maturity. This has been described for conifers that, in the absence of ABA, maturation resulted in poorly developed somatic embryos which often exhibited abnormal morphology asynchronous development and precocious germination. Exogenously supplied ABA in the maturation medium promoted the development of high quality somatic embryos in large quantities. Under appropriate conditions, these embryos germinated and developed into plants at a high frequency (Lelu et al., 1994; Gutmann et al., 1996).

In soybean, initial reports on somatic embryogenesis have reported that ABA may affect normal embryo induction and maturation (Ranch et al., 1985; Lazzeri et al., 1987). These studies, however, contained no substantial data and little analysis about the effects of ABA. Further study showed that ABA promoted embryo growth, development, maturation, and improved embryo germination when applied at the globular stage (Tian & Brown, 2000).

In order to further analyze the effect of ABA on soybean somatic embryogenesis, this plant growth regulator was added to proliferation and maturation medium and the embryos were monitored for their ability to continue development and to convert into plants.

Material and Methods

Two soybean cultivars (IAS-5 and Conquista) were used in this study. Young pods with immature seeds were harvested from field-grown plants and surface sterilizes during 1 min in 70% ethanol and 10 min in diluted commercial bleach (4% sodium hypoclorite) containing a trace amount of Tween-20. Following four rinses in sterile distilled water, immature seeds (3-6 mm) were aseptically excised; the cotyledons were removed and used as explants for culture. Cotyledon halves were placed with the abaxial side facing the D40 induction medium, which contains MS salts (Murashige & Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 40 mg/l 2,4-D, 6% sucrose, 0,3% Phytagel™, pH 7.0 (Bailey et al., 1993). Twenty cotyledon halves (explants) were cultured in 10cm petri dishes containing 30 ml of medium and incubated at 25°±1°C under fluorescent light at an intensity of 22.5$\mu Em^{-2}s^{-1}$ and a 16h light photoperiod.

After 4 weeks on induction medium, explants were transferred to D20 proliferation medium (D40 medium containing 20 mg/l 2,4-D, 3% sucrose, pH 5.8) (Wright et al., 1991). The proliferative tissue was subcultured every 15 days on the same medium and incubation conditions described above during 150 days.

To induce maturation clumps (~3mm) of globular embryos were placed on MSM6 maturation medium (Finer & McMullen, 1991), containing MS salts, B5 vitamins, 6% maltose, 0,3% Phytagel™, pH 5.8. After 4 weeks, the embryos were separated and subcultured on fresh MSM6 medium for further 4 weeks. Histodifferentiated embryos were counted and classified in 7 morphological

types, adapted from Buchheim (1989) and Santos (1997). A sample of 100 histodifferentiated embryos per treatment per cultivar were placed on empty sterile dishes without medium for 48 h to promote partial desiccation. Subsequently, the embryos were placed on MSO conversion medium, containing MS salts, B5 vitamins, 3% sucrose, 0,3% Phytagel™, pH 5.8. Germinated embryos were transferred individually to 110ml vessels with the same medium. After 60 days, embryos were evaluated for conversion. Germination refers to root and/or shoot development, while conversion was recorded as the development of the primary root and formation of at least one a trifoliolate leaf.

ABA was applied at two different stages of embryo development: in the proliferation stage and maturation stage. ABA was added to the D20 and/or MSM6 media at concentration of 50µM before autoclaving. ABA (Sigma Chemical Co., 99+% purity) was dissolved in diluted NaOH solution, filter-sterilized and added to the autoclaved medium. Duration of treatments was 30 days. In proliferation stage, ABA was applied at the last month before transfer of embryo clumps to MSM6 medium. In maturation stage, ABA was applied in the first month after transfer of embryo clumps onto MSM6 medium. The experiment was delineated as shown in Figure 1.

A 4x2 factorial analysis of variance was used to evaluate the effect of the four treatments on the number of histodifferentiated embryos obtained in the two cultivars, as well as on the percentage of embryo germination and of embryo conversion. In order to compare the treatments in relation to the frequency of different embryo morphologies, a classical χ^2 test of association

was used. The residuals (observed value minus expected value) for each cell of the table were individually analyzed, to identify punctual associations between treatment and morphological class.

Results and Discussion

After 2 months on maturation medium, somatic embryos were counted and classified in to 7 morphological types following Buchheim's classification (1989), with minor modifications. The number of histodifferentiated embryos as well as the frequencies of each class in each treatment were recorded and are presented in TABLES 1 and 2.

The total numbers of histodifferentiated embryos obtained for cultivars IAS-5 and Conquista were 2275 and 1187, respectively. The analysis of variance showed significant differences between cultivars ($F = 49.2$; $df = 1, 24$; $p < 0.001$) and among treatments ($F = 14.4$; $df = 3, 24$; $p < 0.001$). No interaction was observed between these two factors ($F = 1.09$; df: 3, 24; $p < 0.374$). The number of histodifferentiated embryos per Petry dish observed in the IAS-5 cultivar (average±SE: 142 ± 44) was significantly higher than that obtained for Conquista (74 ± 33). On the other hand, treatments that used ABA in the proliferation medium yielded significantly less embryos (average±SE: T4: 74 ± 34; T3: 79 ± 43) than those without ABA in this medium (T2: 140 ± 54; T1: 140 ± 47) independently of the presence/absence ABA in the maturation medium.

The relative frequencies of morphological classes varied among treatments for cultivar IAS-5 (χ^2= 236,933; df = 18; $p < 0,001$) and Conquista (χ^2= 77,836; df = 18; $p < 0.001$). A subsequent analysis of adjusted residuals showed essentially the same results for both cultivars (TABLE 2). Monocotyledonous were more frequent that expected in treatments that used

ABA in the proliferation medium (T3 and T4). Dicotyledonous were more frequent in treatments with ABA in both media, proliferation and maturation (T4). In contrast, dicotyledonous were less frequent than expected in treatment without ABA (T1).

One hundred randomly chosen histodifferentiated embryo per treatment per cultivar were submitted to desiccation treatment and then transferred to conversion medium. Germinated embryos were transferred individually to vessels with the same medium. The effects of treatments on embryo germination and conversion are presented in TABLE 3. Both cultivar and treatment showed highly significant effect ($p < 0.001$), but the most conspicuous feature of the data is an interaction between cultivar and treatment. This interaction was significant for percentage of germinated embryos ($F = 4.31$; $df = 3, 24$; $p = 0.014$) and for converted embryos ($F = 3.56$; $df = 3, 24$; $p = 0.029$). Cultivar IAS-5 showed a higher percentage of embryo germination (54.3 ± 6.2) and conversion (25.0 ± 3.9) when compared with Conquista (27.5 ± 4.9; 6.7 ± 3.2, respectively). However, the percentage of germinated and converted embryos of IAS-5 were significantly higher when ABA was added to proliferation medium, (T3 and T4) independently of the presence/absence ABA in the maturation medium. At the same way, for cultivar Conquista, differences on germination and conversion frequencies were detected among treatments. The percentage of germinated embryos was significantly higher in T3 which used ABA only in proliferation medium. Although the results suggest a higher frequency of conversion in the same treatment (T3), the statistical analysis did not show significant differences among treatments.

In soybean, differences among cultivars as to ability to produce somatic embryos from immature cotyledons have been documented in several reports (Parrott et al., 1989; Bailey et al. 1993; Santos et al., 1997; Droste et al., 2001; Meurer et al., 2001). In the present study, cultivar IAS-5 presented a higher number of histodifferentiated embryos as well as higher frequencies of embryo germination and conversion. This finding indicated a higher potential of IAS-5 for embryogenic response when compared with Conquista.

Histodifferentiated embryos were of diverse morphologies and included the types described by Buchheim et al. (1989) and Santos et al. (1997). The high percentage of abnormal somatic embryos found in the present study is in agreement with previous reports and appears to be the rule, rather than an exception (Buchheim et al., 1989; Bailey et al., 1993; Santos et al., 1997; Droste et al., 2001).

We observed that, for cultivar IAS-5 in all treatments, the frequencies of germination and conversion were higher than the frequency of morphological normal embryos (compared the frequencies of normal dicotyledonous embryos in TABLE 2 with the frequencies of germination and conversion in TABLE 3). Thus, as with Buchheim et al. (1989), Bailey et al. (1993) and Santos et al. (1997), our data indicated that a large number of IAS-5 abnormal embryos were capable of germinate and convert into plants.

This study clearly demonstrated that exogenously supplied ABA can effect histodifferentiation, germination and conversion stages of the soybean somatic embryos. Only embryos at the globular stage (proliferation stage)

198 showed a response to ABA. The same observation was also reported by Tian &
199 Brown (2000).

200 The presence of ABA in proliferation medium resulted in decreased
201 histodifferentiated embryo number in both cultivars (TABLE 1). On the other
202 hand, ABA treated-globular-embryos (T3 and T4) of IAS-5 exhibited an
203 increased germination and conversion capability (TABLE 3). For Conquista, the
204 results are not consistent. Although the presence of ABA in proliferation
205 medium (T3) increased germination rate, the effect was not confirmed in T4.

206 These results could be explained by genotype variation on response to
207 ABA. The cultivar IAS-5 could be more responsive while Conquista would be
208 less sensitive to this growth regulator. Genotype-specific responses to ABA
209 have been reported in previous studies. Sloger & Caldwell (1970) showed that
210 only 14 of 34 soybean cultivars responded to ABA application as indicated by
211 leaf senescence, abscission and reduced stem growth. In tissue culture, ABA
212 treatments at concentrations of 50 and 500μM resulted in increased embryo
213 sizes for cultivar X52, while for cultivar Jack, a high level of ABA (500μM)
214 completely stopped embryo development (Tian & Brown, 2000). The authors
215 assumed that high level of ABA was detrimental for embryogenesis and
216 proliferation of embryogenic cultures of cultivar Jack.

217 Increased germination and conversion frequencies suggested that IAS-5
218 embryos treated with ABA are physiologically more mature. Maturation of
219 somatic embryos is a process during which a large amount of nutrients
220 accumulate (Buchheim et al., 1989; Merkle et al., 1995). ABA has been

221 reported to stimulate protein accumulation of soybean zygotic embryos cultured
222 *in vitro* during the early phase of embryogenesis (Ackerson, 1984).
223
224 **Acknowledgments**
225 Research support was provided by Conselho Nacional de
226 Desenvolvimento Científico e Tecnológico (CNPq, Brazil) and Fundação de
227 Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS, Brazil).

References

ACKERSON, R.C. Regulation of soybean embryogenesis by abscisic acid. **Journal of Experimental Botany**, v.35, p.403-413, 1984.

BAILEY, M.A.; BOERMA, H.R.; PARROTT, W.A. Genotype effects on proliferative embryogenesis and plant regeneration of soybean. **In Vitro Cellular Development Biology**, v.29, p.102-108, 1993.

BUCHHEIM, J.A.; COLBURN, S.M.; RANCH, J.P. Maturation of soybean somatic embryos and the transition to plantlet growth. **Plant Physiology**, v.89, p.768-775, 1989.

DROSTE, A.; LEITE, P.C.P.; PASQUALI, G.; MUDSTOCK, E.C.; BODANESE-ZANETTINI, M.H. Regeneration of soybean via embryogenic suspension culture. **Scientia Agricola**, v. 58, n.4, p.753-758, 2001.

DROSTE, A.; PASQUALI, G.; BODANESE-ZANETTINI, M.H. Transgenic fertile plants of soybean [*Glycine max* (L.) Merrill] obtained from bombarded embryogenic tissue. **Euphytica**, v.127, p.367-376, 2002.

FINER, J.J.; McMULLEN, M.D. Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. **In Vitro Cellular Development Biology**, v.27, p.175-182, 1991.

GAMBORG, O.L.; MILLER, R.A.; OJIMA, K. Nutrient requirements of suspension cultures of suspension cultures of soybean root cells. **Experimental Cell Research**, v.50, p.151-158, 1968.

GASPAR, T.; KEVERS, C.; PENEL, C.; GREPPIN, H.; REID, D.M.; THORPE, T.A. Plant hormones and plant growth regulators in plant tissue culture. **In Vitro Cellular Development Biology**, v.32, p.272-289, 1996.

GUTMANN, M.; von ADERKAS, P.; LABEL, P.; LELU, M-A. Effects of abscisic acid on somatic embryo of hybrid larch. **Journal of Experimental Botany**, v.47, n.305, p.1905-1917, 1996.

LAZZERI, P.; HILDEBRAND, D.F.; COLLINS, G.P. Soybean somatic embryogenesis: Effects of hormones and culture manipulations. **Plant Cell, Tissue and Organ Culture**, v.10, p.197-208, 1987.

LELU, M-A.; BASTIEN, C.; KLIMASZEWSKA, K.; CHAREST, P.J. An improved method for somatic plantlet production in hybrid larch (*Larix* x *leptoeuropaea*). 2. Control of germination and plantlet development. **Plant Cell, Tissue and Organ Culture**, v.36, p.117-127, 1994.

MADAKADZE, R.M.; SENARATNA, T. Effect of growth regulators on maturation of geranium (*Pelargonium* x *hortorum*) somatic embryos. **Plant Growth Regulation**, v.30, p.55-60, 2000.

MERKLE, S.A.; PARROTT, W.A.; FLINN, B.S. Morphogenic aspects of somatic embryogenesis. In: THORPE, T..A. (Ed.) ***In vitro* embryogenesis in plants.** Dordrecht: Kluwer Academic Publishers, 1995. cap.5, p.155-203.

MEURER, C.A.; DINKINS, R.D.; REDMOND, C.T., McALLISTER, K.P.; TUCKER, D.T.; WLAKER, D.R.; PARROTT, W.A., TRICK, H.N.; ESSIG, J.S.; FRANTZ, H.M.; FINER, J.J.; COLLINS, G.B. Embryogenic response of multiple soybean [*Glycine max* (L.) Merr.] cultivars across three locations. **In Vitro Cellular Development Biology - Plant**, v.37, p.62-67, 2001.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue culture. **Physiologia Plantarum**, v.15, p.473-497, 1962.

NAMBARA, E.; MARION-POLL, A. ABA action and interactions in seeds. **Trends in Plant Science**. v.8, n.5, p.213-217, 2003.

NIEVES, N.; MARTÍNEZ, M.E.; CASTILLO, R.; BLANCO, M.A.; GONZÁLEZ-OLMEDO, J.L. Effect of abscisic acid and jasmonic acid on partial desiccation of encapsulated somatic embryos of sugarcane. **Plant Cell, Tissue and Organ Culture**, v.65, p.15-21, 2001.

PARROTT, W.A.; HOFFMAN, L.M.; HILDEBRAND, D.F.; WILLIAMS, E.G.; COLLINS, G.B. Recovery of primary transformants of soybean. **Plant Cell Reports**, v.7, p.615-617, 1989.

RANCH, J.P.; OGLESBY, L.; ZIELINSKI, A.C. Plant regeneration from embryo-derived tissue culture of soybeans. **In Vitro Cellular Development Biology.,** v.21, p.653-658.

ROCK, C.D.; QUATRANO, R.S. The role of hormones during seed development. In: Davies, P.J. (Ed.) **Plant hormones: physiology, biochemistry and molecular biology**. Dordrecht: Kluwer Academic Publishers, 1995. cap.10, p. 671-697.

SANTOS, K.G.B.; MUNDSTOCK, E.; BODANESE-ZANETTINI, M.H. Genotype-specific normalization of soybean somatic embryogenesis through the use of an ethylene inhibitor. **Plant Cell Reports**, v.16, p.859-864, 1997.

SATO, S.; NEWELL, C.; KOLACZ, K.; TREDO, L.; FINER, J.J.; HINCHEE, M. Stable transformation via particle bombardment in two different soybean regeneration systems. **Plant Cell Reports**, v.12, p.408-413, 1993.

SIMMONDS, D.H., DONALDSON, P.A. Genotype screening for proliferative embryogenesis and biolistic transformation of short-season soybean genotypes. **Plant Cell Reports**, v.19, p.485-490, 2000.

SLOGER, C.; CALDWELL, B.E. Response of cultivars of soybean to synthetic abscisic acid. **Plant Physiology**, v.46, p.634-634, 1970.

TIAN, L.; BROWN, D.C.W. Improvement of soybean somatic embryo development and maturation by abscisic acid treatment. **Canadian Journal of Plant Science**. v.80, p.271-276, 2000.

TRICK, H.N.; DINKINS R.D.; SANTAREM E.R.; DI, R.; SAMOYLOV, V.; MEURER C.A.; NORRIS, B.L.; PARROTT, W.A.; FINER, J.J.; COLLINS, G.B. Recent advances in soybean transformation. **Plant Tissue Culture Biotechnology**, v.3, p.9-26, 1997.

WRIGHT, M.S.; LAUNIS, K.L.; NOVITZKY, R.; DUESILING, J.H.; HARMS, C.T. A simple method for the recovery of multiple plants from individual somatic embryos of soybean [*Glycine max* (L.) Merrill]. **In Vitro Cellular Development Biology**, v.27, 153-157, 1991.

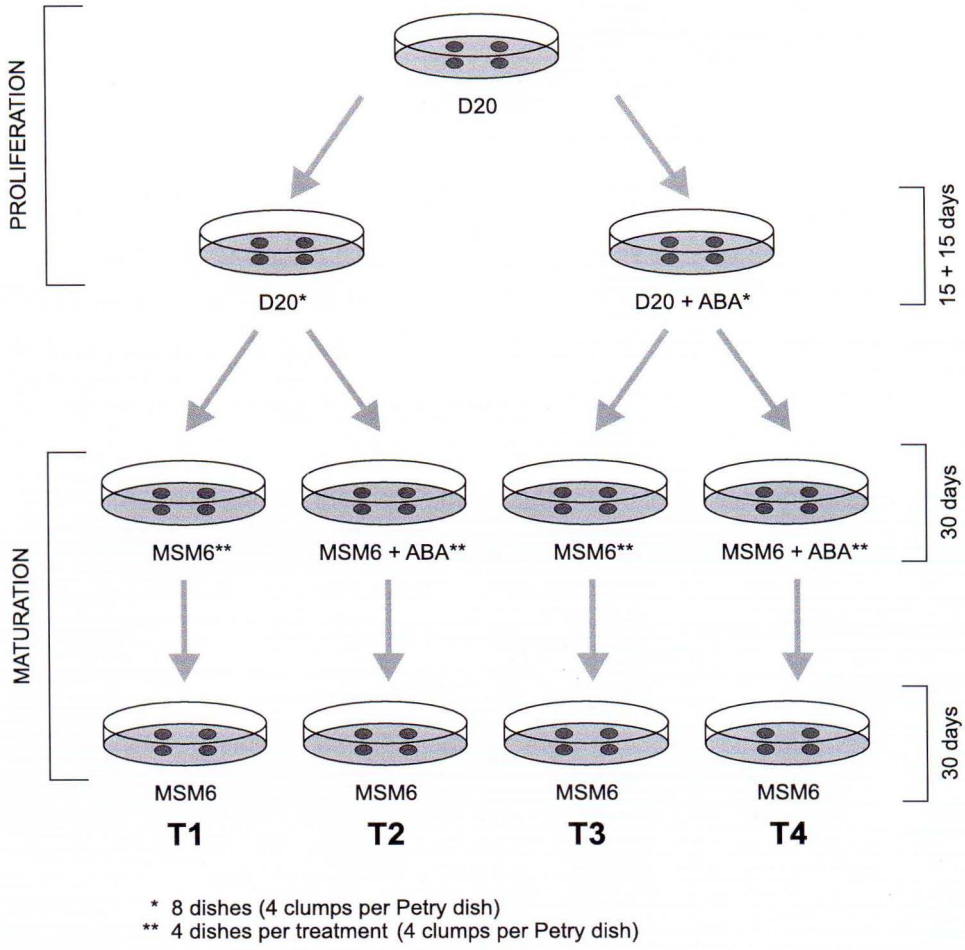

Figure 1 – Experimental design

TABLE 1 – Effect of treatments on the average number of histodifferentiated embryos per Petry dish obtained after maturation in two soybean cultivars

Treatment	ABA		Cultivars		
	Proliferation medium	Maturation medium	IAS-5	Conquista	Overall [1]
			Average ± SE		Average ± SE
T1	no	no	170 ± 24	109 ± 10	140 ± 24 a
T2	no	yes	186 ± 13	94 ± 12	140 ± 27 a
T3	yes	no	117 ± 10	42 ± 7	79 ± 21 b
T4	yes	yes	96 ± 19	52 ± 5	74 ± 17 b
Overall [2]			142 ± 22	74 ± 17	

[1] Comparison among treatments: F = 14.4; df = 3, 24; p < 0.001. Means indicated by the same letter do not differ significantly (Student-Newman-Keuls test; 0.05 level).
[2] Comparison between cultivars: F = 49.2; df =1, 24; p < 0.001.

TABLE 2 – Effect of treatments on the percentage of morphological classes after maturation, in two soybean cultivars

Form	Cultivars							
	IAS-5				Conquista			
	T1	T2	T3	T4	T1	T2	T3	T4
Monocotyledonous	5.0 (–)	9.0	15.0 (+)	18.5 (+)	5.0	4.2 (–)	10.8 (+)	10.2 (+)
Dicotyledonous	1.3 (–)	4.3	5.1	11.0 (+)	4.3 (–)	8.2	10.2	17.5 (+)
Polycotyledonous	0.4 (–)	1.7	2.7	3.9 (+)	0.9	1.8	3.0	1.9
Fused cotyledons	55.7 (+)	46.3	45.2	39.0 (–)	65.2 (+)	54.8	56.6	45.6 (–)
Trumpet	28.9 (+)	27.0 (+)	16.0 (–)	8.8 (–)	17.6	19.8 (+)	7.2 (–)	14.0
Fused embryos	6.5	7.5	8.0	7.8	3.9	4.5	3.6	3.4
Forms not-classified	2.2 (–)	4.0	8.3 (+)	9.4 (+)	3.0	6.6	8.4	7.3

+/– Indicate significant increased (+) or decreased (–) frequencies in relation to the expected under the no association hypothesis.

TABLE 3 – Effectof treatments on average percentage of germination and conversion of somatic embryos of soybean cultivars IAS-5 and Conquista

Treatment	Number of dishes	Cultivars			
		IAS-5		Conquista	
		average ± SE[1]		average ± SE[1]	
		germinated	converted	germinated	converted
T1	4	36.0 ±13.0 b	11.0 ± 7.0 b	21.0 ± 5.7 b	2.0 ± 2.0 a
T2	4	34.0 ± 9.3 b	19.0 ± 5.3 b	22.0 ± 7.4 b	1.0 ± 1.0 a
T3	4	75.0 ± 3.0 a	29.0 ± 3.4 a,b	54.0 ± 7.4 a	21.0 ± 10.0 a
T4	4	72.0 ± 4.3 a	42.0 ± 6.0 a	13.0 ± 4.1 b	3.0 ± 1.9 a
Overall[2]		54.3 ± 6.2	25.0 ± 3.9	27.5 ± 4.9	6.7 ± 3.2

[1] Means indicated by the same letter do not differ significantly (Student-Newman-Keuls test; 0.05 level).
[2] Comparison between cultivars: F = 25.9; df = 1. 24; p< 0.001 for % of germinated embryos; F = 23.5; df = 1. 24; p< 0.001 for % of converted embryos.

SCIENTIA AGRICOLA

ISSN 0103-9016 *printed version*
ISSN 1678-992X *online version*

INSTRUCTIONS TO AUTHORS

- Scope and editorial policy
- General instructions
- Editorial fees

Scope and editorial policy

Scientia Agricola is a journal of the University of São Paulo, Campus "Luiz de Queiroz", Piracicaba, SP, Brazil that publishes original articles which contribute to the scientific development of Agricultural Sciences.

The Journal has a broad spectrum, including Crop and Animal Production, Agricultural Engineering, Economics and Sociology, Agroindustrial Technology, Forestry, Environmental and Soil Sciences, and Applications of Basic Sciences in Agriculture.

The articles submitted to the journal must be original with no simultaneous submission to other periodicals. Notes, View Points and Letters to the Editor may also be submitted. Reproductions are permitted only with indication of the source.

General instructions

Original: four copies and a 3,5" diskette, including text and illustrations.

Language: Portuguese or with preference in English.

Format: Word for Windows.

Space/Side margins: 3 cm, double-space

Paper: A4 paper with numbered lines

Pages: total of 30 pages, numbered consecutively, illustrations (figures, tables) included.

Title page

a. title of the paper (maximum of 15 words)
b. names of the author(s), corresponding author should be indicated using an asterisk
c. scientific affiliation of the authors / Institution / Department / Section
d. indication of sponsorship and eventual presentation of the paper at a scientific meeting
e. postal code, city, state, country, e-mail address, if available, and phone for contact

Structure of the manuscripts

Do not include authors name.

- title, abstract (a maximum of 250 words) and keywords (maximum of 5). A translation of these will be provided by the journal for non-Portuguese-speaking authors
- introduction (including literature review)
- material and methods
- results and discussion
- conclusions (optional)
- acknowledgements
- references

Citations

a. the citations of authors in the text must be in low case, followed by the year of publication
b. in the case of two authors, the last names of both must be spelled out, separated by "&"
c. for more than two authors, only the first author's last name will appear, followed by et al. (no italic)
d. personal notes, work in progress and unpublished papers must be mentioned as footnotes; they are not to be listed in the references

Examples: Cunha (1985); Silva & Mattos (1993); Reichardt et al. (1996)

References

References must be listed in alphabetical order by author's last name, as indicated below:

- 2 or more authors should be separated by (;)
- titles of journals should not be abbreviated

Articles of periodicals

MORTATTI, J.; MORAES, J.M.; RODRIGUES JR., J.C.; VICTORIA, R.L.; MARTINELLI, L.A. Hydrograph separation of the Amazon river using 18O as an isotopic tracer. **Scientia Agricola**, v.54, n.3, p.167-173, 1997.

Books

PINDYC, R.S.; RUBINFELD, D.L. **Econometric models and economic forecasts**. 3.ed. New York: McGraw-Hill, 1991. 596p.

Chapters in books

FRIED, W.M.; WARNER, J.R. Organization and expression of eukaryotic ribosomal protein genes. In: STEIN, G.S.; STEIN, J.L., (Ed.) **Recombinant DNA and cell proliferation**. Orlando: Academic Press, 1984. cap.1, p. 169-192.

Meetings

CHANDRA, S. Tropical crop statistics: a world perspective. In: SYMPOSIUM OF THE INTERNATIONAL SOCIETY FOR TROPICAL ROOT CROPS, 6., Lima, 1983. **Proceedings**. Lima: International Potato Center, 1984. p. 41-46.

Thesis and dissertations