Carbon sources and polyethylene glycol on soybean somatic embryo conversion

Ana Paula Körbes[1] and Annette Droste[1]

[1]Universidade do Vale do Rio dos Sinos, Laboratório de Cultura de Tecidos Vegetais, Caixa Postal 275, CEP 93022-000 São Leopoldo, RS, Brazil. E-mail: korbes@netu.unisinos.br, adroste@bios.unisinos.br

Abstract – Somatic embryogenesis is an efficient method for the production of target cells for soybean genetic transformation. However, this method still offers low percentages of plant regeneration, and perhaps is related to the maturation process and high morphological abnormalities of the matured embryos. This study aimed to identify a maturation medium that could contribute to the outcome of more efficient plant regeneration results. Embryogenic clusters, derived from cotyledons of immature seeds of the soybean cultivars Bragg and IAS5, were used as starting material for embryos development. Different maturation media were tested by using 6% maltose, 3% sucrose or 6% sucrose, combined with or without 25 g L^{-1} of the osmotic regulator polyethylene glycol (PEG-8000). The histodifferentiated embryos were quantified and classified in morphological types. Percentages of converted embryos were analyzed. Cultivar Bragg resulted in higher matured embryo quantities, but lower percentages were obtained for the conversion in comparison to cultivar IAS5. While the addition of PEG did not affect the number of embryos converted into plants, 6% sucrose enhanced the conversion percent significantly.

Index terms: *Glycine max*, PEG-8000, sucrose, osmotic regulation.

Fontes de carbono e polietilenoglicol na conversão de embriões somáticos de soja

Resumo – A embriogênese somática é um eficiente método na produção de células-alvo para a transformação genética de soja. Entretanto, este método ainda apresenta baixas porcentagens de regeneração de plantas, o que pode estar relacionado ao processo de maturação e às altas anormalidades morfológicas dos embriões maturos. Este estudo visou a identificar um meio de maturação que pudesse contribuir com resultados mais eficientes de regeneração de plantas. Foram utilizados conjuntos embriogênicos derivados de cotilédones imaturos de sementes de soja das cultivares Bragg e IAS5 para o desenvolvimento de embriões. Testaram-se diferentes meios de maturação, com a utilização de 6% de maltose, 3% de sacarose ou 6% de sacarose, combinadas ou não com 25 g L^{-1} do regulador osmótico polietilenoglicol (PEG-8000). Os embriões histodiferenciados foram quantificados e classificados em tipos morfológicos. A porcentagem de embriões convertidos em plantas foi analisada. A cultivar Bragg apresentou maior quantidade de embriões maturos, mas foram obtidas menores porcentagens de conversão em comparação à cultivar IAS5. A adição de PEG não afetou o número de embriões convertidos em plantas. Entretanto, a concentração de 6% de sacarose aumentou significativamente a porcentagem de conversão.

Termos para indexação: *Glycine max*, PEG-8000, sacarose, regulação osmótica.

Introduction

Soybean crops are outstanding in the Brazilian agronomical scenery, and for this reason there is a special interest in genetic improvement programs by genetic transformation. An important prerequisite is the establishment of an in vitro culture capable of producing highly responsive tissues. Somatic embryogenesis, first reported in soybean by Christianson et al. (1983), is one of the most efficient methods to obtain regenerated plants, either on semi-solid media (Droste et al., 2002) or in liquid suspension cultures (Finer & Nagasawa, 1988; Droste et al., 2001). However, while a considerable number of protocols describe successfully the induction and proliferation of somatic embryos, the maturation and germination stages remain a goal, due to the low frequency of embryos converted into plants (Droste et al., 2001).

Difficulty in inducing embryo maturation is one of the main barriers for the success of somatic embryogenesis, not only for soybean (Walker & Parrott, 2001), but for other species such as *Pinus taeda* (Li et al., 1998) and various species of the genus *Picea* (Misra et al., 1993; Tremblay & Tremblay, 1995; Bozhkov & Arnold, 1998). Factors that determine the ability of embryos to convert into plants are the accumulation of enough storage materials (Bozhkov & Arnold, 1998) and desiccation tolerance (Blackman et al., 1992).

The quality of somatic embryos can be positively influenced by a low osmotic potential in maturation medium (McKersie & Brown, 1996; Walker & Parrott, 2001). Carbohydrates are commonly used as carbon sources for the development of the tissues on in vitro cultures (Iraqi & Tremblay, 2001). These compounds can be considered important for somatic embryo maturation of many species, not only being a nutrition source, but playing a role as osmotica (Li et al., 1998). The main studies in conifers show that an increased sugar concentration improves the somatic embryo maturation (Tremblay & Tremblay, 1995; Li et al., 1998; Iraqi & Tremblay, 2001). In alfafa, embryo maturation and conversion into plants, and the fresh weight of seedlings were increased by raising sucrose concentration in maturation medium (Lai & McKersie, 1994).

Polyethylene glycol (PEG) has proven to be an important osmoticum in maturation stage of different species of the genus *Picea* (Misra et al., 1993; Tremblay & Tremblay, 1995; Svobodová et al., 1999). Nevertheless, there are few studies for dicotyledonous species (Linossier et al., 1997), including *Glycine max* (Walker & Parrott, 2001). PEG is a non-plasmolysing osmoticum. Its large molecules are not able to pass through the cell wall, leading to a restriction of water uptake, a reduced turgor pressure and a more negative intracellular osmotic potential (Misra et al., 1993).

Bailey et al. (1993) described a semi-solid maturation medium for soybean, containing maltose as carbon source. Most laboratories use this protocol. Unfortunately, the frequencies of embryo maturation and, specially, of embryo conversion are low.

The objective of this work was to investigate whether supplementation of the maturation medium with sucrose and PEG would improve maturation and conversion frequencies of soybean somatic embryos.

Material and Methods

Two soybean cultivars were used in this study – Bragg and IAS5 –, both North American-adapted cultivars, commonly used in genetic improvement programs and for commercial growing in the Brazilian State of Rio Grande do Sul (Costa, 2000). Pods containing immature seeds, 3–5 mm in length, were harvested from field-grown plants approximately 25 days postfertilization. Pods were surface desinfested for 30 sec in 70% ethanol and 15 min in 4% sodium hypochlorite containing Tween-20. After desinfestation the pods were rinsed four times in sterile distilled water. The immature seeds were excised, the cotyledons were removed and used as explants for culture. Cotyledon halves were placed with the abaxial side facing the modified D40 induction medium (Bailey et al., 1993), which contains MS salts (Murashige & Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 40 mg L^{-1} 2,4-dichlorophenoxiacetic acid (2,4-D), 3% sucrose, 0.3% Phytagel™, pH 7.0. Cultures were illuminated 16 hours per day with 22.5 µmol m^{-2} s^{-1} light intensity and exposed to 25±1ºC.

After four weeks on D40 medium, proliferative embryogenic tissues were transferred to D20 proliferation medium (modified D40 medium, containing 20 mg L^{-1} 2,4-D, 3% sucrose, pH 5.8) (Wright et al., 1991). The proliferating tissues were subcultured every 14 days.

Clusters of globular-stage embryos, 4 mm in diameter, were selected for the treatments. All media were based on MSM6 maturation medium (Finer & McMullen, 1991) containing MS salts, B5 vitamins, 0.3% Phytagel™, pH 5.8. These media were supplemented with: (1) 6% maltose; (1P) 6% maltose + 2.5% PEG-8000 (Sigma, St. Louis, USA); (2) 3% sucrose; (2P) 3% sucrose + 2.5% PEG); (3) 6% sucrose; (3P) 6% sucrose + 2.5% PEG. Four dishes, each containing four clusters, were used for each treatment. After four weeks, the histodifferentiated embryos were individualized and subcultured on the same maturation medium for additional four weeks. Mature embryos from each treatment were counted and classified in eight morphological types, according to Buchheim et al. (1989). For each treatment, 120 embryos were randomly chosen and placed in dry, sterile dishes with up to twenty embryos per dish for two days to allow partial desiccation. The embryos were transferred to MSO conversion medium, containing MS salts, B5 vitamins, 3% sucrose, 0.3% Phytagel™, pH 5.8. The frequency of conversion was taken after eight weeks on MSO.

The effect of PEG and cultivars on percentages of conversion were independently analyzed based on Fisher's Exact test. Comparison among carbon sources was made using the chi-square test. Interactions among cultivars, carbon sources and media with or without PEG were analyzed by a logistic regression. To compare the percentages of morphologic types of histodifferentiated embryos obtained in the treatments, cultivars and carbon sources were analyzed as isolated variables by chi-square test.

Results and Discussion

In maturation stage, cultivars Bragg and IAS5 produced a number of 2,094 and 1,345 histodifferentiated somatic embryos, respectively. For each cultivar, 120 randomly chosen histodifferentiated embryos per treatment were submitted to partial desiccation and transferred to conversion medium. Obtained conversion percentages refer to the development of the primary root and at least a trifoliolate leaf. Many embryos germinated precociously during the second month of the maturation step and converted into plants. These plants were counted and the percentages of precocious conversion were calculated over the number of maturated embryos for each treatment/cultivar.

Initially, the data obtained for the three parameters studied, PEG, carbon sources and cultivars, were analyzed independently. The concentration of PEG used did not enhance the conversion percent of embryos. Supplementation with 2.5% PEG resulted in 18.7% of conversion, compared with 15.8% without PEG ($p = 0.157$), and in 7.6% of precocious conversion, compared with 9.3% without PEG ($p = 0.073$) (Figure 1A).

Considering only the carbon sources, embryos maturated in MSM6 medium with 6% sucrose had the highest conversion and precocious conversion frequencies (25.6% and 14.2%, respectively), a 2-fold increase in comparison with MSM6 supplemented with 3% sucrose or 6% maltose (Figure 1B). There was no significant difference between 3% sucrose and 6% maltose.

Embryos of cultivar IAS5 showed the highest conversion and precocious conversion percentages independently of carbon sources and PEG. While cultivar Bragg showed 7.8% of conversion, IAS5 showed a 3-fold higher percentage (27.6%) (Figure 1C). The same relation can be made with precocious conversion frequencies (4.2% for Bragg and 14.9% for IAS5).

Analysis of combined data, PEG and carbon sources, for both cultivars, by a logistic regression, corroborate the results obtained when variables were analyzed independently. Cultivar IAS5 had a significantly higher conversion percentage, independently of all other variables and all interactions. Likewise, 6% sucrose led to significantly higher conversion rates, independently of the other variables and all interactions.

Walker & Parrott (2001) evaluated the effect of osmotica on somatic embryogenesis of other soybean cultivars. Nevertheless, the authors used a liquid maturation medium (Samoylov et al., 1998), so that direct comparisons with data obtained are difficult. They reported that, for cultivar "Jack", supplementation with 5% PEG-4000 resulted in 30% conversion, which was higher than conversion frequency from maturation medium without PEG. The osmoticum failed to enhance conversion frequency of cultivar "Fayette", demonstrating the influence of genotype.

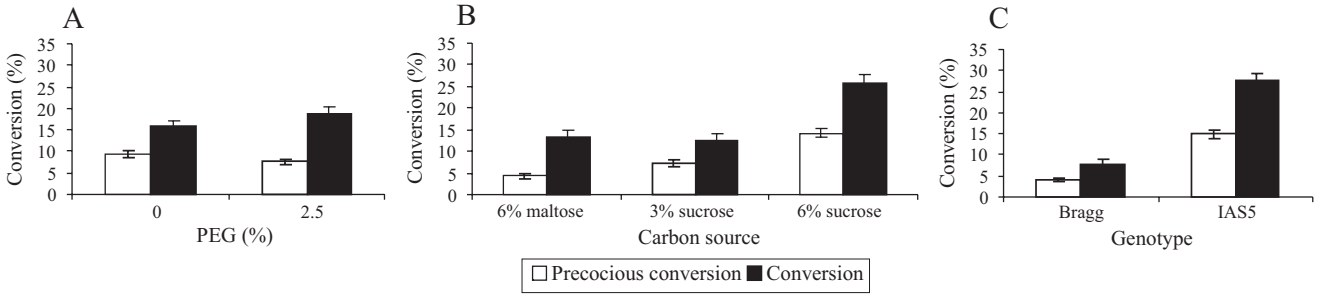

Figure 1. Conversion frequencies of somatic embryos from cultivars Bragg and IAS5 (mean±SD), obtained through maturation medium supplementation with 0% and 2.5% PEG and with maltose or sucrose.

Although PEG can improve maturation of somatic embryos, previous studies demonstrated an opposite effect on some species. For *Pinus taeda*, different concentrations of PEG combined with sucrose produced a large number of precotyledonary embryos. Nevertheless, these embryos had a low conversion frequency to the cotyledonary stage (Li et al., 1998). Bozhkov & Arnold (1998) showed that somatic embryos of *Picea abies* treated for ten weeks with 7.5% PEG-4000 had a low capacity of regeneration. The osmotic stress induced by PEG seems to be responsible for a small embryo size, due to a decreasing of the ratio of cellulose:hemicellulose in the cell wall. It results in decreased elasticity of the cell wall and in reduced ability of cellular expansion (Iraki et al., 1989). Another relevant aspect is the rapid influx of water when placing embryos on germination medium (Bozhkov & Arnold, 1998). The low germination frequency seems to be more important than the positive effect of osmotic stress treatment. Otherwise, a lower concentration of PEG-4000 (3.75%) and a shorter treatment (six weeks) permitted an increased number of maturated embryos and converted plants for the same species (Svobodová et al., 1999). According to the authors, the treatment accelerated maturation of embryos in two weeks and led to an enhanced synthesis of proteins and lipids.

Carbon sources proved to be osmotic regulators for other soybean cultivars (Walker & Parrott, 2001). The authors showed that supplementation of maturation medium with 3% sorbitol enhanced conversion frequencies to 50%–60% for both cultivars "Jack" and "Fayette". The influence of sucrose over conversion capacity was studied for other species. Lai & McKersie (1994) observed that raising the concentration of sucrose from 3% to 5% on maturation medium promoted a higher fresh weight and increased maturation and conversion frequencies of alfafa embryos. For black spruce, the osmotic effect of sucrose was studied by Tremblay & Tremblay (1995). The concentration of this carbon source in medium decreased from 6% initially to 0.3% at the end of a 6-week maturation period. This decrease in sucrose was correlated with an increase in fructose and glucose leading to an increased osmotic pressure of the medium.

Cultivar Bragg presented a higher number of histodifferentiated embryos, while showing a lower frequency of conversion than cultivar IAS5, 7% and 28%, respectively (Figure 1C). The differences in conversion percentages among cultivars are in agreement with results obtained for soybean cultivars in previous studies (Bailey et al., 1993; Droste et al., 2001). The data suggest that a soybean genotype with high efficiency of somatic embryo maturation may have low conversion rates, indicating that embryo maturation and conversion must be under independent genetic control. So, both maturation and conversion capacities of each cultivar have to be considered.

Histodifferentiated somatic embryos were of different morphologies and include the types described by Buchheim et al. (1989) (Table 1). Although a high percentage of the resultant somatic embryos was of abnormal types, conversion data indicated that a part of them were capable to germinate. Similar results were obtained by Bailey et al. (1993) and Droste et al. (2001). Droste et al. (2001) obtained 77% and 79% of abnormal embryos for cultivars Bragg and IAS5, respectively. In the study, 78%–79% of the plants of both cultivars converted from abnormal morphologies of the somatic embryos. High abnormality

Table 1. Percentage of morphological classes of somatic embryos from cultivars Bragg and IAS5 after 60 days on MSM6 maturation medium(1).

Form	Bragg						IAS5					
	1	1P	2	2P	3	3P	1	1P	2	2P	3	3P
Dicotyledonous	9.2	14.2	5.8	9.2	15.0	17.5	30.8	15.8	22.9	27.5	23.3	30.8
Monocotyledonous	12.5	20.8	11.7	9.2	10.8	15.0	18.3	21.7	20.0	15.8	18.3	16.7
Polycotyledonous	6.7	6.7	4.2	5.0	1.7	2.5	2.5	10.0	10.0	13.3	5.0	11.7
Fused cotyledons	47.5	38.3	27.5	35.8	37.5	46.7	35.8	39.2	20.0	19.2	30.0	24.2
Trumpet	10.0	14.2	24.2	28.3	10.8	6.7	2.5	3.3	10.0	5.0	7.5	3.3
Proximal fusion	5.0	4.2	7.5	5.8	8.3	4.2	5.0	5.0	8.6	5.8	5.0	6.7
Fasciated embryos	7.5	0.8	7.5	3.3	6.7	6.7	0.8	2.5	1.4	7.5	5.8	4.2
Vestigial cotyledons	1.7	0.8	11.7	3.3	9.2	0.8	4.2	2.5	7.1	5.8	5.0	2.5

(1)Medium 1: 6% maltose; Medium 1P: 6% maltose + 2.5% PEG; Medium 2: 3% sucrose; Medium 2P: 3% sucrose + 2.5% PEG; Medium 3: 6% sucrose; Medium 3P: 6% sucrose + 2.5% PEG.

percentages were obtained as a rule, rather then exception, among soybean somatic embryos production studies (Buchheim et al., 1989; Finer, 1995).

According to chi-square test, both cultivars and carbon sources influenced the relative frequencies of morphological classes of somatic embryos obtained in the present study. Just comparing the morphologies obtained for each cultivar, embryos with fused cotyledons and trumpet-like embryos were found more frequently in cultivar Bragg, whereas dicotyledonous, monocotyledonous and polycotyledonous embryos were more frequent in cultivar IAS5. Independently of the cultivar, maturation media supplemented with 6% maltose gave rise to a higher percentage of fused cotyledons embryos, and, on the average, with 3% sucrose, higher percentages of embryos with vestigial cotyledons and trumpet-like embryos were obtained. Media supplemented with 6% sucrose permitted a higher percentage of dicotyledonous embryos. Embryos with fused or vestigial cotyledons and trumpet-like embryos are morphologies that rarely convert into plants due to the absence or abnormalities of their shoot apex. These findings can partially explain the lower conversion percentages for Bragg and for media supplemented with 6% maltose or 3% sucrose. On the other hand, cultivar IAS5 showed histodifferentiated embryos morphologies with higher capacity of conversion. Additionally, supplementation of maturation medium with 6% sucrose led to a higher frequency of dicotyledonous embryos, contributing to conversion.

Conclusions

1. Sucrose, at high concentration, can both act as carbon source and osmoticum, substituting maltose in soybean somatic embryogenesis standard protocols and, therefore, minimizing the costs of the culture medium.

2. The morphology of maturated somatic embryos is genotype-dependent and influences the conversion capacity of each soybean cultivar.

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