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A liquid-medium-based protocol for rapid regeneration from embryogenic soybean cultures

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Abstract Soybean [*Glycine max* (L.) Merrill] somatic embryos of the cultivar Jack underwent histodifferentiation in liquid Murashige and Skoog (MS) medium with 3% maltose, or according to the standard published procedure employing solidified MS media, permitting the recovery of an average of 8.1 and 3.9 embryos/mg of embryogenic tissue, respectively. Cotyledon-stage embryos that developed in liquid medium were ready for desiccation within 4 weeks, while the embryos from the standard procedure required a maturation step for an additional 4 weeks. Comparison of embryo development in MS medium with maltose or FN Lite-based medium without growth regulators and supplemented with maltose or an equimolar amount of sucrose revealed that sucrose promotes faster embryo histodifferentiation and maturation, and allows the recovery of up to 50% more mature, cotyledon-stage embryos within 3 weeks. The use of this liquid-medium-based protocol relative to the standard procedure led to a fourfold increase in the number of cotyledon-stage embryos recovered from other genotypes tested. In many cases, however, the percent germination was lower. Application of this new procedure also made it possible to harvest transgenic seed 9 months following biolistic bombardment, as compared to the 13 months required when the standard solid-medium-based protocol was used.

Key words Somatic embryogenesis · Embryo conversion · Microprojectile bombardment · Transformation

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Abbreviations *AC* Activated charcoal · *2,4-D* 2,4-Dichlorophenoxyacetic acid · *GUS* β-Glucuronidase · *HygR* Hygromycin resistant

Introduction

After their induction, somatic embryos can be maintained in a continuous, self-replicating state, whereby they can be proliferated on a large scale. When the amount of exogenous auxin falls below a threshold level, both primary somatic embryos and repetitive embryos undergo histodifferentiation to reach a cotyledon stage, then undergo a period of maturation. The conversion of somatic embryos into plants is the final stage in the process of plant regeneration via somatic embryogenesis, and is characterized by activation of the root and apical meristems, such that root and shoot growth occur (Merkle et al. 1995). Germination and conversion of soybean somatic embryos usually do not require exogenous growth regulators (Parrott et al. 1988), but somatic embryos must undergo physiological maturation (Ranch et al. 1985; Buchheim et al. 1989) prior to germination. Consequently, a series of different media has been used to accommodate the different stages of embryogenesis, making the regeneration process time and labor consuming (Bailey et al. 1993; Trick et al. 1997). The availability of a simple and more rapid protocol for soybean somatic embryogenesis could accelerate efficient recovery of fertile soybean plants for in vitro propagation and transformation. Therefore, the objective of this study was to develop a simple and quick procedure for histodifferentiation and maturation of soybean somatic embryos.

Materials and methods

The cultivar Jack was used as a model genotype to test new histodifferentiation and maturation protocols, and the new procedure was tested with Chapman, Williams 82, and F138. Of these, Chapman and F138 were known to have a high embryogenic capacity, while

Table 1 Composition of the media used for soybean somatic embryogenesis

Medium	Composition
FN Lite basal	FN Lite macro salts (Samoylov et al. 1998), MS micro salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 6.7 mM L-asparagine, pH 5.8
FN Lite	FN Lite basal medium, 22.6 μM (5 mg/l), 2,4-D, 29.2 mM (1% wt/vol) sucrose (Samoylov et al. 1998)
FNL0M3	FN Lite basal medium, 87.6 mM (3% wt/vol) maltose
FNL0S3	FN Lite basal medium, 87.6 mM (3% wt/vol) sucrose
MS0	MS basal salt, B5 vitamins, 87.6 mM (3% wt/vol) sucrose, pH 5.8, 0.2% Gelrite
MS0M3	MS basal salts, B5 vitamins, 87.6 mM (3% wt/vol) maltose, pH 5.8
MS0M6	MS basal salts, B5 vitamins, 175.3 mM (6% wt/vol) maltose, pH 5.8, 0.2% Gelrite (Finer and McMullen 1991)
MS0M6AC	MS basal salts, B5 vitamins, 175.3 mM (6% wt/vol) maltose, pH 5.8, 0.5% AC, 0.2% Gelrite (Bailey et al. 1993)
MSD20	MS basal salts, B5 vitamins, 90.4 μM (20 mg/l), 2,4-D, 87.6 mM (3% wt/vol) sucrose, pH 5.8 (Wright et al. 1991)

Williams 82 was known to have a relatively poor embryogenic capacity under these protocols. Embryogenic cultures were initiated as described in Samoylov et al. (1998) and maintained in liquid FN Lite medium (Table 1) in 125-ml Erlenmeyer flasks on a gyratory shaker (130 rpm) at 26°C, a light intensity of 10 μmol m^{-2} s^{-1}, and a 24-h photoperiod, with a 3-week subculture period. For each experiment, morphologically uniform embryogenic clusters measuring 3 mm in diameter were individually picked out using forceps, 2 weeks after the last subculture. Embryogenic clusters were grouped into sets of ten, representing a replication, and the weight of each set was recorded before their transfer to auxin-free media.

The first experiment compared the use of the standard regeneration protocol (Bailey et al. 1993) with protocols in which liquid media were used to replace one or more steps with solidified media. Each set of ten embryogenic clusters was transferred to a 100×15 mm petri dish containing 20–25 ml of MS0M6AC medium (Table 1). Cultures were maintained under the conditions described above. After 4 weeks, the resulting cotyledon-stage embryos were counted, and five embryos from each cluster were transferred to 100×15 mm petri dishes containing 20–25 ml of MS0M6 medium (Table 1) for maturation, at a density of 25 embryos per dish. After 4 weeks on MS0M6, mature embryos were desiccated by placing 25 embryos into a 100×15 mm petri dish and sealed with Nescofilm (Karlan, Santa Rosa, Calif.). A small piece (approximately 1 cm^3) of MS0M6 medium was placed in each dish off to the side and away from the embryos, to permit gradual desiccation of embryos over a period of 5–7 days. Germination was induced by culturing desiccated embryos on MS0 medium (Table 1) in 100×15 mm petri dishes at 26°C, a light intensity of 60–80 μmol m^{-2} s^{-1}, and a 23-h photoperiod. Embryos which produced roots and a shoot with leaves within 3 weeks were scored as germinated.

To incorporate liquid medium into the protocol, a set of embryogenic clusters per replication was transferred from FN Lite into 125-ml Erlenmeyer flasks (one cluster per flask) containing 35 ml of liquid MS0M3 (Table 1) medium. Prior to transfer into a flask, each embryogenic cluster was gently mashed to partially separate the individual globular-stage embryos. The flasks were sealed with Bellco (Vineland, N. J.) silicon closures and placed on a gyratory shaker. Environmental conditions were the same as those described for the cultures in liquid medium. At four weeks, the resulting cotyledon-stage embryos were counted, and five embryos from each flask were transferred to solid MS0M6 medium for a 4-week maturation period, then desiccated as described for the standard treatment. For the third treatment, a set of embryogenic clusters was subcultured into liquid MS0M3 medium as described above. At four weeks, the number of cotyledon-stage embryos was counted, and five embryos from each flask were taken through the maturation, desiccation, and germination procedures as described before.

The next experiment compared two auxin-free liquid media for their ability to promote embryo histodifferentiation and maturation. The first medium, MS0M3 (Table 1), is a liquid version of the medium used in the standard protocol as defined by Bailey et al. (1993). The second medium, FNL0S3 (Table 1), was based on a formulation previously shown to be effective for promoting growth of embryogenic clusters (Samoylov et al. 1998). These two media were compared using the methodology described above, except that the transfer to MS0M6 medium prior to desiccation was omitted. Next, the effect of carbohydrate source on embryo histodifferentiation and maturation was determined by comparing FNL0M3 and FNL0S3 media (Table 1) to each other using the methodology described before.

Finally, the results obtained with best liquid medium, FNL0S3, as identified in the previous steps, were verified with different genotypes. Embryogenic cultures of Chapman, Williams 82, and F138 were subjected to histodifferentiation and maturation according to both the standard (Bailey et al. 1993) and new procedures, i.e., using FNL0S3 medium, as described above. The osmotic pressure of the liquid media prior to and after embryo development in each of three flasks from a treatment was determined using a μOsmette 5004 (Natick, USA).

Data were analyzed by analysis of variance (ANOVA) using the SAS system for Microsoft Windows, release 6.10. Significant differences between means was determined by F-LSD at $P \leq 0.05$. All experiments were replicated three times.

Regeneration of transgenic plants

The transformation procedures of Stewart et al. (1996) were used to obtain transgenic cell lines for use in the medium comparison work. Three plates (0.3–0.5 g of tissue per plate) of 9-month old Jack embryogenic cultures growing on MSD20 medium were subjected to particle bombardment with a DuPont PDS 1000/He system using pTRA140 (Zheng et al. 1991). This plasmid contains the *hph* and *gusA* genes, each driven by the CaMV 35 S promoter. After bombardment, embryogenic clusters were cultured in FN Lite medium for 2 weeks, after which, 5 mg/l hygromycin was added as a selection agent. The hygromycin concentration was increased by 5 mg/l every 2 weeks over the subsequent 8-week period, to a final concentration of 25 mg/l. Transgenic clones were selected within 3 months following bombardment.

To compare the efficiency of regeneration of transgenic plants using the standard and the FNL0S3 liquid-medium-based protocol, one 3-mm embryogenic cluster from each transgenic clone was randomly selected, and the globular-stage embryos were separated as described before. Tissue was divided into two even halves, and each half was subcultured in FN Lite medium for a period of 8 or 3 weeks. An 8-week propagation period was necessary to obtain enough tissue (i.e., 25 3-mm embryogenic clusters) to take through the standard protocol. However, since the FNL0S3 protocol required much less tissue to obtain an equivalent number of cotyledon-stage embryos, only a 3-week propagation period was required. Embryogenic tissue from each half was allowed to histodifferentiate into cotyledon-stage embryos and mature according to either the standard procedure based on a solid media sequence, or the new procedure using FNL0S3 liquid medium, followed by desiccation as described before. Germinated embryos were treated as described by Parrott et al. (1994), and 11 plants from each treatment were transferred to a greenhouse to assess survival and fertility. Seeds were harvested from mature T_0 plants.

β-Glucuronidase (GUS) activity in selected hygromycin resistant (HygR) embryogenic clones and leaves of T_0 and T_1 plants was determined histochemically according to Jefferson et al. (1987). PCR amplification of a 933-bp *hph* gene fragment in T_0 embryogenic cul-

tures and T_1 plants was carried out with the following primers: 5′-GTC TCC GAC CTG ATG CAG CTC TC-3′ forward and 5′-GGC GTC GGT TTC CAC TAT CG-3′ reverse primers. Amplifications were done on a Stratagene RoboCycler Gradient 96 Temperature Cycler with the following program: 95°C for 5 min; followed by 35 cycles at 95°C for 1 min, 53°C for 1 min, 71°C for 1 min; 71°C for 7 min; soak 6°C. Amplified products were loaded into 1.1% agarose gels and separated by electrophoresis in TBE buffer for 4 h at 50 V. DNA was visualized with ethidium bromide. Images were scanned using an Eagle Eye II Still Video System (Stratagene) with a Mitsubishi Video Copy Processor P68U.

For Southern blot analysis, leaf material was harvested from young leaves of T_1 plants and DNA extracted as described in Keim et al. (1988), except that 25:24:1 phenol:chloroform:isoamyl alcohol was used instead of 24:1 chloroform:isoamyl alcohol, and the centrifugation time was increased to 20 min. After that, the aqueous phase was decanted and 20 ml of 24:1 chloroform:isoamyl alcohol was added. The phases were once again separated by centrifugation at 3,500 *g*. For each plant, 10 μg of DNA were digested with *Eco*RV (NEB) according to the manufacturer's instructions. Electrophoresis and blotting was as described by Lee et al. (1996). The probe was prepared by using PCR to amplify part of the *gus*A coding region from pTRA140. In this case, the primers were 5′-GTG GCA GTG AAG GGC GAA CAG-3′ forward and 5′-GCA GCC CGG CTA ACG TAT CC-3′ reverse primers, and the 100 μM of dCTP in the reaction was replaced with 0.825 μM of ^{32}P-labeled dCTP. The initial cycle consisted of 95°C for 5 min; followed by 30 cycles at 94°C for 30 s, 60°C for 75 s, and 72°C for 90 s. Probing was also as described by Lee et al. (1996), except that the prehybridization and hybridization temperatures were lowered to 60 °C. The resulting filters were visualized using a Molecular Dynamics Storm 860 phosphoimager with ImageQuaNT software.

Results and discussion

Comparison of different media

Embryogenic clusters placed in liquid MS0M3 yielded an average of 8.1±0.4 cotyledon-stage embryos/mg tissue, while those placed on solid MS0M6AC yielded an average of 3.0±0.3 embryos/mg tissue. However, embryos differentiated in liquid MS0M3 started to germinate after transfer to solid MS0M6 medium, and the germination rate of the embryos from this treatment was lower than that of the other two treatments. Germination of embryos on solid MS0M6 medium was an indication that embryos differentiated for 4 weeks in liquid MS0M3 medium had already reached physiological maturity and were competent for germination, and that consequently an additional maturation step on solid MS0M6 medium was not required. Overall, the percent germination was 33.0±2.0 for embryos from the standard treatment consisting of 4 weeks on MS0M6AC followed by 4 weeks on MS0M6. The rate from the treatment using 4 weeks on MS0M3 followed by 4 weeks on MS0M6 was essentially the same (29.0±2.6%), while that for embryos from 4 weeks in MS0M3 was 38.3±0.7%.

When embryo development was compared in MS0M3 and FNL0S3 liquid media, embryos in MS0M3 required 4 weeks for histodifferentiation and remained green (Fig. 1A). In contrast, embryos cultured in FNL0S3 medium differentiated into yellow-green cotyledon-stage embryos within 3 weeks. According to their shape, size

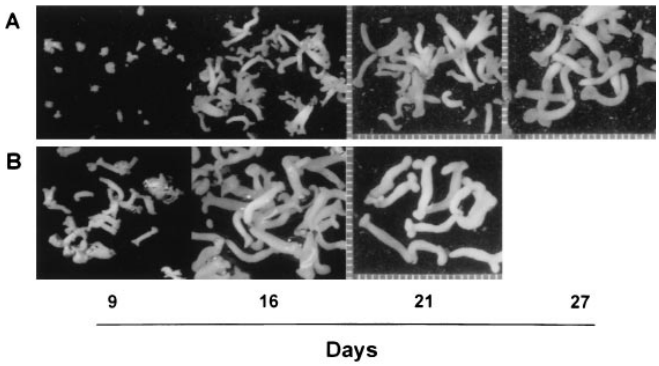

Fig. 1 Histodifferentiation of embryos in MS0M3 (**A**) or FNL0S3 medium (**B**) at 9, 16, 21, and 27 days in culture. Scale is in millimeters. Embryos are shown under the same magnification

(Fig. 1B) and color, these were ready for desiccation. In contrast, and besides the difference in growth rate, 10.8±2.1 cotyledonary-stage embryos/mg initial tissue were recovered in FNL0S3 medium, compared to 15.7±0.5 which were obtained in MS0M3. The germination of embryos into plants was 47.3±3.2% for the embryos derived from FNL0S3, and 70.7±2.4% for those from MS0M3 medium. Overall, gains in embryo recovery were offset by a lower germination frequency when FNL0S3 medium was used. Nevertheless, the use of FNL0S3 medium for histodifferentiation yielded desiccation-tolerant embryos a full week earlier than MS0M3 medium. In the future, adjustment of the osmotic potential of the liquid medium might circumvent the low germination of embryos obtained with this protocol.

To further determine the role carbohydrate type had on the faster growth of embryos, embryo histodifferentiation and maturation were compared in FNL0S3 medium and in FNL0M3 medium, in which sucrose was replaced with an equimolar concentration of maltose. An average of 9.9±1.1 and 6.8±0.2 cotyledon-stage embryos were recovered per milligram of initial tissue placed for histodifferentiation in FNL0S3 or FNL0M3 medium, respectively. After 3 weeks in culture, embryos in FNL0M3 medium resembled embryos developed in MS0M3 medium of the same age (Fig. 1A), and hence they also required an additional week to complete histodifferentiation and maturation, relative to those on FNL0S3. Moreover, cultures in FNL0S3 medium yielded 46% more cotyledon-stage embryos than cultures in FNL0M3 medium. These data indicate that relative to maltose, sucrose promotes embryo growth and significantly ($P \leq 0.05$) increases the number of cotyledon-stage embryos recovered during histodifferentiation and maturation in liquid medium.

Conversion of other genotypes

To test whether the new liquid-medium-based procedure was suitable for other soybean genotypes, histodifferentiation and germination of embryogenic cultures of three soybean genotypes were evaluated using the standard pro-

Table 2 Efficiency (mean±SE) of embryo histodifferentiation and germination according to the standard and new differentiation procedures for embryogenic cultures of three soybean genotypes

	Chapman		F138		Williams 82	
	Standard	FNL0S3	Standard	FNL0S3	Standard	FNL0S3
Amount of embryogenic tissue prior to transfer (mg)	86.6±2.6	85.6±3.2	119.2±6.2	119.1±8.2	102.6±4.5	99.0±6.5
Number of cotyledon-stage embryos recovered/1 mg initial tissue	2.8±0.2	10.8±1.2	3.2±0.1	9.9±1.0	2.6±0.1	8.4±0.6
Germination (%)	60.0±8.0	30.7±11.8	56.7±1.8	70.8±8.5	28.7±6.4	0

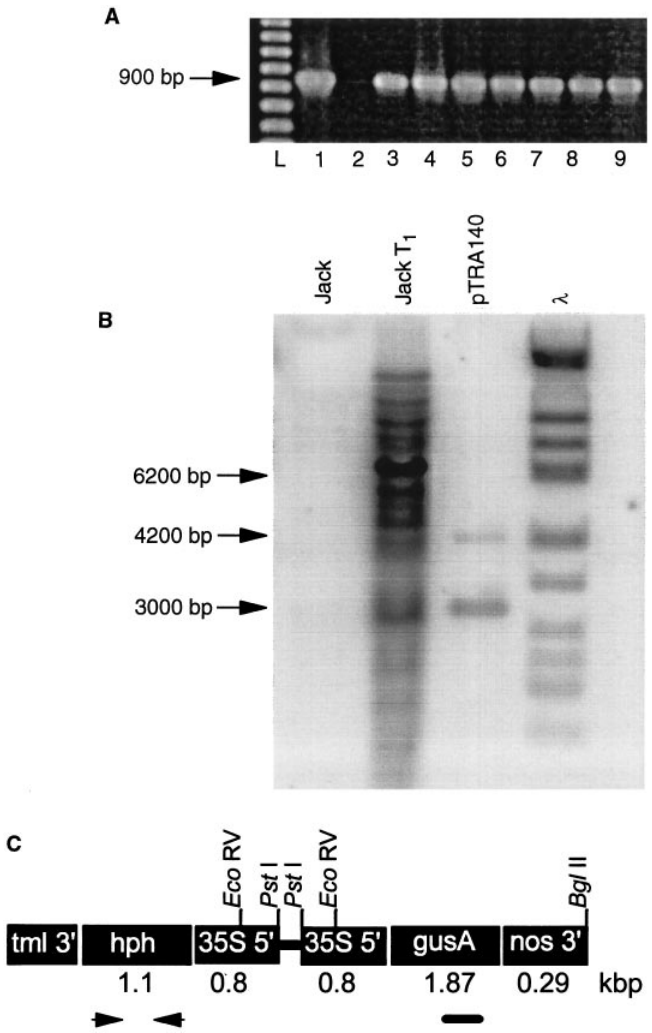

Fig. 2 **A** PCR amplification of the *hph* gene in HygR T_0 plants selected as a result of transformation with pTRA140 via microprojectile bombardment of soybean cv. Jack embryogenic cultures (*lane 1* pTRA140, *lane2* cv. Jack, *lanes 3–9* transgenic clones, *L* 100-bp ladder). **B** Southern blot analysis using the *gus*A coding sequence (*lane 1* non-transgenic Jack soybean control, *lane 2* a T_1 plant of Jack transformed with pTRA140 and digested with *Eco*RV, *lane 3* pTRA140 digested with *Pst*I/*Bgl*II to release the GUS cassette, *lane 4* phage λ DNA digested with *Hin*dIII and *Sty*I). **C** Map of the coding sequences of pTRA140. The restriction sites used to obtain the Southern blot are indicated. *Opposing arrowheads* indicate the primers used to obtain the PCR reaction in **A**. The *bar* under the *gus*A coding sequence indicates the probe used to obtain the Southern blot in **B**

cedure or FNL0S3 medium, which was the best liquid medium, as described above. Results are shown in Table 2. Increases in the number of cotyledon-stage embryos obtained in FNL0S3 were 400, 309, and 323% of the embryo yield from MS0M6AC medium for Chapman, F138, and Williams 82, respectively. However, genotypes were more variable in their germination response, with embryos from FNL0S3 medium having germination frequencies either higher or lower than those obtained using the standard procedure. In our experiments, the osmotic pressure of fresh FNL0S3 medium with 87.6 mM sucrose was 186.3±0.33 mOsm kg^{-1} at the time embryogenic tissue was added, and 5±1.5 mOsm kg^{-1} after 3 weeks, indicating a consumption of almost all the nutrients in the medium as the embryos reached the cotyledonary stage. The role of osmoticum in promoting proper embryo maturation has been well established (Kermode 1995), and it is possible that changes in osmotic conditions during the embryo histodifferentiation and maturation processes could be contributing to the lower germination and conversion frequency of these embryos.

Regeneration of transgenic plants

To test the new regeneration protocol with transgenic lines, we first obtained seven independent transgenic clones transformed with pTRA140. GUS expression was monitored through all stages of embryo, T_0 plant, and seed development, and was also confirmed in T_1 plants. Plants from all seven cell lines also yielded the expected 933-bp PCR amplification fragment (Fig. 2) for the *hph* gene. Southern blot analysis performed on a T_1 plant selected at random showed that the multiple copies of the transgene were transmitted to the progeny.

HygR embryogenic cultures were converted into plants with the standard and the FNL0S3-medium-based procedures in parallel. From any given independent transformation event, it was possible to harvest seeds 13 months after bombardment if the standard protocol was used, but only 9 months were required if liquid FNL0S3 medium was used for embryo histodifferentiation (Fig. 3). Two factors appear to account for this difference. First, since the standard protocol yielded about 20% of the embryo number obtained in liquid FNL0S3 medium, more tissue was necessary for the standard protocol, so a transgenic cell

Fig. 3 Soybean cv. Jack plants transformed with pTRA140 and recovered from the same transgenic cell line using the standard (*left*) and new liquid (*right*) regeneration protocols. A meter stick was used as a size standard. Photographs were taken on the same day

Fig. 4 Histodifferentiation of transgenic embryogenic clusters on solid MS0M6AC medium at 4 weeks in culture (*left*) as compared to embryos derived from one cluster in FNL0S3 medium at 3 weeks in culture (*right*). The left plate was initiated with 250 mg of tissue, while the right was initiated with just 10 mg. Embryos are pictured in 100×15 mm petri dishes

line had to be propagated for a longer period of time before enough tissue was available to begin the embryo histodifferentiation process. Secondly, as discussed previously, embryo development proceeds more rapidly in liquid FNL0S3 medium (Fig. 4).

Representative data from one cell line are presented in Table 3. Overall, a greater proportion of the plants obtained from liquid FNL0S3 medium were phenotypicaly normal, and none were sterile. In contrast, sterility was present in 2 of the 11 plants obtained from the standard protocol. Furthermore, the plants obtained from the liquid FNL0S3 medium had a higher seed set. The reason for this is unknown,

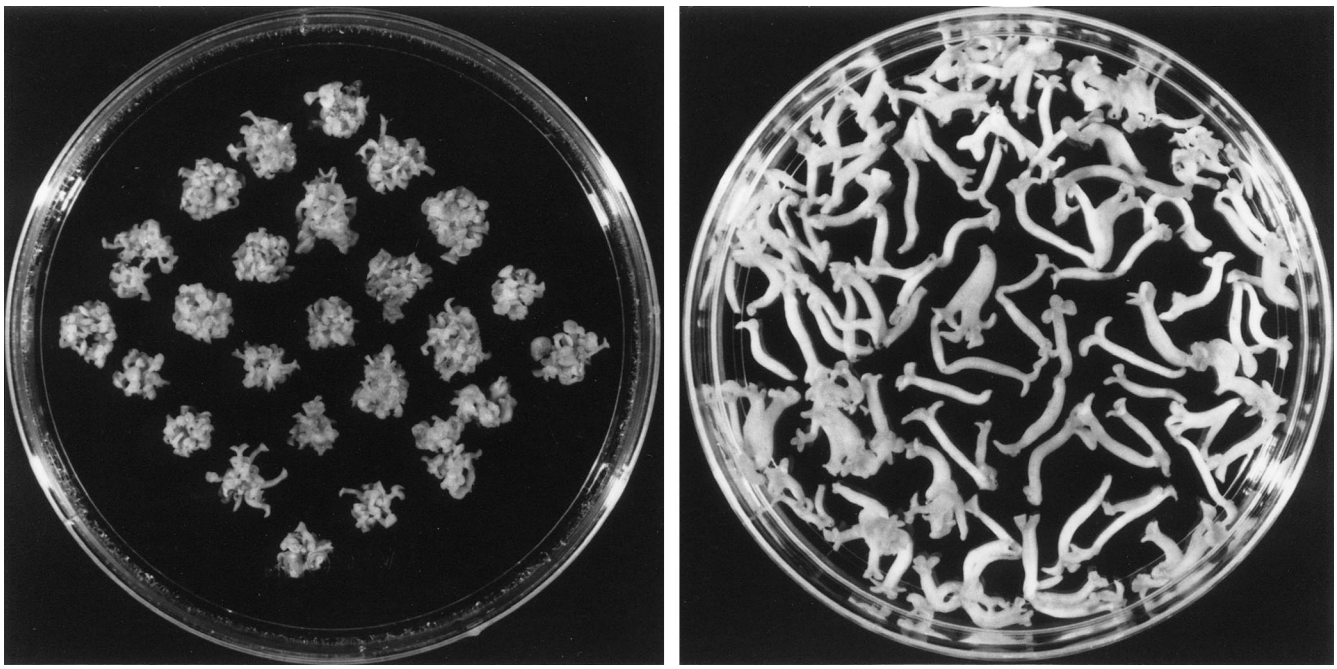

Table 3 Characterization of T_0 plants regenerated according to the standard and new protocols

	Regeneration protocol	
	Standard	FNL0S3
Number of phenotypically normal plants	3/11	8/11
Number of seeds per plant	309.7±26.3	433.5±24.5
Average seed weight (mg)	150.1±2.2	157.5±3.3
Time between microprojectile bombardment and seed harvest (months)	13	9

but does suggest that the general quality of somatic embryos influences the quality of the plants which are eventually obtained from them.

Although there has been one previous report on the use of liquid MS-based medium for soybean somatic embryo histodifferentiation and maturation (Rajasekaran and Pellow 1997), a direct comparison had never been made between the use of solid and liquid media for this purpose, nor had different liquid media been compared with each other. In this study, a basal medium, FN Lite, which was previously shown to facilitate the growth of soybean embryogenic cultures (Samoylov et al. 1998) also facilitated the recovery of mature, cotyledon-stage embryos. With this procedure, embryos develop faster, and far less tissue needed to be carried through the tissue culture process to obtain a given amount of somatic embryos, resulting in savings of time, labor, and supplies. Application of FNL0S3 medium could be especially useful when transgenic cell lines are involved, as the amount of time required to increase a transgenic cell line until enough tissue is available to start the plant recovery procedure, and for the plant recovery process itself, is greatly reduced.

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