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SOYBEAN [*GLYCINE MAX* (L.) MERRILL] EMBRYOGENIC CULTURES: THE ROLE OF SUCROSE AND TOTAL NITROGEN CONTENT ON PROLIFERATION

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SUMMARY

To improve proliferation of soybean cultures in liquid medium, the effects of sucrose; total inorganic nitrogen; content of NO_3^-, NH_4^+, Ca^{2+}, PO_4^{3-}, K^+; NH_4^+/NO_3^- ratio; and medium osmotic pressure were studied using cv. Jack. Sucrose concentration, osmotic pressure, total nitrogen content, and ammonium to nitrate ratio were found to be the major factors controlling proliferation of soybean embryogenic cultures. Growth decreased linearly as sucrose concentration increased from 29.7 m*M* to 175.3 m*M*. A sucrose concentration of 29.2 m*M*, a nitrogen content of 34.9 m*M* at 1 to 4 ammonium to nitrate ratio were found to be optimal for the fastest proliferation of soybean embryogenic cultures. There was no significant effect on proliferation of cultures when concentrations of NH_4^+, Ca^{2+}, PO_4^{3-}, and K^+ were tested in the range of 3.50 to 10.50, 1.02 to 3.06, 0.68 to 2.04, and 22.30 to 36.70 m*M*, respectively. The relative proliferation of embryogenic cultures of four soybean genotypes was evaluated in Finer and Nagasawa medium and in the new medium formulation. Despite genotype-specific differences in growth, the genotypes tested showed a biomass increase in the new formulation equal to 278, 269, 170, and 251% for Chapman, F138, Jack, and Williams 82, respectively, relative to their growth on standard FN medium. Due to its lowered sucrose and nitrogen content, we are referring to the new medium as FN Lite.

Key words: embryogenic cultures; carbohydrate content; osmotic pressure; total nitrogen content; ammonium to nitrate ratio; somatic embryogenesis.

INTRODUCTION

Biotechnologies for plant improvement are of interest because they can help overcome some of the traditional limitations of crop breeding. Somatic embryo cultures are one of the most important and convenient systems for plant genetic engineering and *in vitro* propagation. Therefore, somatic embryogenesis has been reported in many plant species and extensive studies have been performed to establish and evaluate factors that may affect the induction and proliferation of embryogenic cultures for a number of crops. The importance of carbohydrate source, total nitrogen, ammonium to nitrate ratio, and auxin type and level have been identified as major factors affecting the proliferation of embryogenic cultures (for review *see* Merkle et al., 1995).

Soybean [*Glycine max* (L.) Merrill] is the world's most important oil and protein crop, and efforts have been made to develop efficient techniques for its *in vitro* culture and genetic engineering. Such cultures can be a convenient system for genetic transformation of soybean via particle bombardment and recovery of transgenic plants (Finer and McMullen, 1991; Sato et al., 1993; Parrott et al., 1994; Stewart et al., 1996). Although maintenance of soybean embryogenic cultures in liquid medium was facilitated by the development of FN medium by Finer and Nagasawa (1988) the efficiency of soybean tissue culture manipulations *in vitro* still remains low relative to that of other crops. Hence, the availability of a suitable tissue culture technology for soybean embryo proliferation and regeneration may be a limiting step for efficient soybean genetic transformation.

Therefore, the objective of this study was to identify the roles that individual medium components have on somatic embryogenesis in order to improve proliferation of soybean suspension cultures in liquid medium. Towards that end, the effect of a number of factors, such as carbohydrate type and concentration, total nitrogen, ammonium, nitrate, and other macronutrients on proliferation of suspension embryogenic cultures were evaluated, resulting in the development of an optimized medium we refer to as FN Lite.

MATERIALS AND METHODS

Initiation and maintenance of cultures. Embryogenic cultures were initiated from immature cotyledons as described by Bailey et al. (1993) except that the sucrose concentration in the D40 induction medium was lowered to 3%. Cultures were maintained in liquid FN medium (Finer and Nagasawa, 1988) or in various medium formulations described below, in 125-ml Erlenmeyer flasks on a gyratory shaker (130 rpm) at 26° C under 10 $\mu Em^{-2}s^{-1}$ constant light, with a 3-wk subculture period. Cultivar Jack was used as a model genotype to test all medium modifications, and results were confirmed with Chapman, Jack, Williams 82, and F138, a breeding line derived from Fayette × PI 417138 (Parrott et al., 1994). Conversion of embryos into plants was conducted according to Bailey et al. (1993).

Medium reformulation. The initial reformulation of FN medium began by replacing the FN macro salts with modified macro salts of N6 medium (Chu et al., 1975), modified by addition of $CaCl_2$ for a final concentration of 2.0 m*M*, and 27.9 m*M* KNO_3, 3.5 m*M* $(NH_4)_2SO_4$, 1.4 m*M* KH_2PO_4, and 1.5 m*M* $MgSO_4$ (Table 1). The MS micro salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), L-asparagine, and 2,4-D (2,4-dichlorophenoxyacetic acid) remained as in FN medium. The sucrose content of FN medium was lowered to 87.6 m*M*, which corresponds to 3% (wt/vol). The working

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TABLE 1

MACRO SALTS (mM) COMPOSITION IN MEDIA FOR SOYBEAN EMBRYOGENIC CULTURES

Macro salts	Media[a]			
	MS	FN	FNN6	N6
KNO_3	18.9	29.9	27.9	27.9
NH_4NO_3	20.6	9.9	—	—
$(NH_4)_2SO_4$	—	—	3.5	3.5
$CaCl_2$	2.9	2.9	2.0	1.1
$MgSO_4$	1.5	1.5	1.5	0.8
KH_2PO_4	1.2	1.2	1.4	2.9
$(NH_4):NO_3$	1:2	1:4	1:4	1:4
Total nitrogen	60.0	49.9	34.9	34.9

[a]MS = Murashige and Skoog (1962) medium; FN = Finer and Nagasawa (1988) medium; FNN6 = defined in Materials and Methods.

designation for this medium was FNN6. Then, individual components were modified to determine the optimal concentration of each. The pH was adjusted to 5.8 with KOH prior to autoclaving. All media were autoclaved at 121° C and 103 kPa for 20 min.

Experimental design. To evaluate proliferation, embryogenic cultures of cultivar Jack first were conditioned in the reformulated media for at least 3 wk before growth rates were measured. To measure growth rates, 90 to 130 mg of embryogenic tissue were placed into each of five flasks containing 35 ml of the respective media. After 3 wk, tissue from each set of five flasks was combined and its weight was measured. Total biomass increase was calculated according to the following formula: $(W_f - W_i)/W_i$, where W_i and W_f are initial and final fresh weight of tissue, respectively (Conner and Meredith, 1985). Each experiment was conducted using a completely randomized design and was performed in triplicate. Data were analyzed by analysis of variance (ANOVA) or regression analysis, using the SAS System for Microsoft Windows, Release 6.10. Significance between means was determined by F-LSD at $P = 0.05$.

Growth of cultures in FN medium was initially set at 100%, and the biomass increase of different soybean genotypes in FN medium with 87.6 mM sucrose [3% (wt/vol)] and FNN6 medium was measured relative to that in FN. Thereafter, the value of soybean proliferation in FNN6 medium, formulated as above, was set as a standard at 100% to study the effect of variables in modified media. A sucrose content of 87.6 mM (3%) in FNN6 medium was constant in all experiments unless specified otherwise.

Effect of 2,4-D content. Evaluation of the 2,4-D effect on proliferation of cultures was conducted in FN and FNN6 basal media supplemented with 22.6 and 45.2 μM 2,4-D. Proliferation of cultures in FN medium with 22.6 μM 2,4-D was used as a standard at 100%.

Effect of sucrose content. To evaluate the effect of sucrose content on soybean proliferation, FNN6 basal medium was supplemented with 8.75, 17.5, 29.2, 58.4, 87.6, 116.9, 146.1, and 175.3 mM sucrose, which correspond to sucrose content of 0.3, 0.6, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0% (wt/vol), respectively.

Evaluation of the effect of medium osmotic pressure on proliferation of cultures was conducted in FNN6 basal medium with 29.2, 87.6, or 175.3 mM sucrose, 29.2 mM sucrose + 160.1 mM mannitol, or 29.2 sucrose + 175.4 mM mannitol. In a second experiment, an equivalent amount of sorbitol was used instead of mannitol as a source of an osmoticum (Osm). The osmotic pressure of the media was measured using a μOsmette 5004 (Advanced Instruments, Inc., Norwood, MA, USA) according to the manufacturer's recommendations.

Effect of total inorganic nitrogen. To evaluate the effect of total nitrogen, amounts of $(NH_4)_2SO_4$ and KNO_3 in FNN6 basal medium were changed proportionally to maintain a 1:4 ammonium to nitrate ratio. This is the ammonium to nitrate ratio found in both FN and N6 macro salts. Media with 17.5, 26.2, 34.9, 39.4, 43.7, 48.1, and 52.5 mM of the total nitrogen were formulated, which correspond to 50, 75, 100, 112.5, 125.0, 137.5, and 150.0% of the total nitrogen content in FNN6 basal medium, respectively.

The effect of NO_3^- content was evaluated by changing the amount of KNO_3 to formulate media with 13.9, 20.9, 27.9, 34.9, and 41.9 mM of NO_3^-, which correspond to 50, 75, 100, 125, and 150% of the nitrate concentration in basal FNN6, respectively. Ammonium content was held constant at 7 mM.

Likewise, the amount of $(NH_4)_2SO_4$, was changed accordingly to formulate media with 3.5, 4.75, 7.0, 8.25, and 9.0 mM of NH_4^+, which correspond to 50, 75, 100, 125, and 150% of the ammonium content in FNN6 basal medium, while holding the nitrate level at 27.9 mM to evaluate the effect of NH_4^+ content on proliferation of cultures.

In order to evaluate the effect of ammonium to nitrate ratio in FNN6 basal medium, the $(NH_4)_2SO_4$ and KNO_3 were changed to 35.0, 28.0, 23.3, 17.5, 11.7, 7.0, or 0 mM of NH_4^+, and 0, 7.0, 11.7, 17.5, 23.3, 28.0, or 35 mM of NO_3^-, to create media with NH_4^+/NO_3^- ratios equal to 1:0, 4:1, 2:1, 1:1, 1:2, 1:4, and 0:1, respectively. However, no attempts were made to compensate the amount of K^+ in the media with lower KNO_3 content, because such adjustment would have required the addition of a large amount of salts not normally present in tissue culture media. The effect of K^+ content on proliferation was evaluated separately.

Effect of other macro salts. To evaluate the effect of Ca^{2+}, the amount of $CaCl_2$ was altered to obtain media with 1.0, 1.5, 2.0, 2.5, and 3.0 mM of Ca^{2+}, which correspond to 50, 75, 100, 125, and 150%, respectively, of the calcium content in FNN6 basal medium.

The effect of PO_4^{3-} content was evaluated by altering the amount of KH_2PO_4 in FNN6 basal medium to obtain media with 0.7, 1.0, 1.7, 1.7, and 2.0 mM of PO_4^{3-}, which correspond to 50, 75, 100, 125, and 150% of the phosphate level in FNN6 basal medium, respectively.

Finally, to evaluate the effect of K^+ content on proliferation of cultures, the amount of KNO_3 was lowered from 27.9 mM to 20.7 mM, and 3.5 mM $(NH_4)_2SO_4$ were replaced with 7.0 mM NH_4NO_3. Therefore, this formulation contained the same amount of total nitrogen, ammonium, and nitrate as FNN6 basal medium, although the amount of K^+ was lowered from 29.4 to 22.3 mM, which corresponds to 76.1% of K^+ in FNN6. Thereafter, 3.3, 7.0, 10.7, and 14.3 mM of KCl were added to obtain media with 25.7, 29.4, 33.0, and 36.7 mM of K^+, which correspond to 87.5, 100, 112.5, and 125.0% of the K^+ level in FNN6 basal medium, respectively.

The final medium formulation was evaluated for precipitation-dissolution of salts using the Metal Speciation Equilibrium Model for Surface and Ground Water (MINTEQA2), v. 3.11 (Center for Exposure Assessment Modeling, Athens, GA), and Problem Definition Program (PRODEFA2) for MINTEQA2 v. 3.11.

Results and Discussion

A recent review on somatic embryogenesis of legumes (Parrott et al., 1995) revealed that MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrand, 1972), or B5 (Gamborg et al., 1968) basal media were used almost exclusively. In the case of soybean, MS basal medium is used exclusively for induction and conversion, while a modified version, FN medium, is used to proliferate embryogenic cultures. Yet MS medium was optimized for the growth of tobacco callus, and SH medium was optimized for growth of monocot callus. Only B5 medium comes close to meeting requirements for legumes, having been optimized for growth of soybean root cells.

There are examples in the legume literature to demonstrate that basal media optimized for individual species yield superior results over the standard, commonly used basal media. For example, the L2 basal medium was optimized for red clover (Collins and Phillips, 1982), the L6 basal medium was optimized for moth bean (Kumar et al., 1988a), and EC6 medium was developed for somatic embryogenesis of white clover (Maheswaran and Williams, 1984). L6 medium also proved optimal for tepary bean (Kumar et al., 1988b).

Liquid media are particularly adept at allowing the large-scale proliferation of somatic embryos, and their use has been incorporated into transformation systems for soybean (Finer and McMullen, 1991; Sato et al., 1993; Parrott et al., 1994; Stewart et al., 1996). Initial attempts at soybean somatic embryogenesis using a liquid medium were made by Christianson et al. (1983), in which the nitrogen-containing salts from MS medium were eliminated and replaced with 20

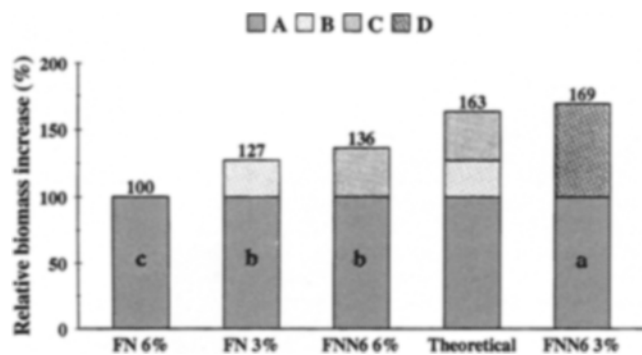

FIG. 1. Effect of sucrose and total nitrogen content on proliferation of soybean somatic embryo cultures in different medium compositions. *A*, general composition; *B*, increase attributable to sucrose; *C*, increase attributable to total nitrogen; *D*, observed. Means with the same letter are not significantly different at $P = 0.05$. FN = Finer and Nagasawa (1988) medium; FNN6 = defined in Materials and Methods.

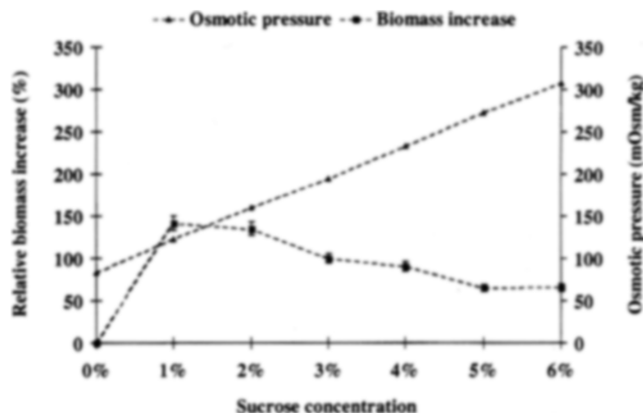

FIG. 2. Effect of sucrose content and osmotic pressure on proliferation of soybean embryogenic cultures in FNN6 medium. *Vertical bars* represent standard error (SE). FNN6 = defined in Materials and Methods.

m*M* ammonium citrate. This formulation was successful at allowing the limited proliferation of somatic embryos. Later, Ranch et al. (1985) obtained proliferation of somatic embryos in MS medium, but could not maintain the proliferative state if ammonium citrate was used to replace the inorganic nitrogen in MS medium. Finally, Finer and Nagasawa (1988) adjusted the ammonium:nitrate ratio to produce 10A40N medium, which greatly facilitated the use of embryogenic cultures in soybean. This medium eventually came to be known as Finer and Nagasawa medium, or simply FN medium.

Proliferation of cultures in FNN6 basal medium. In our initial study, a 27% increase in growth of proliferating embryos was obtained in FN medium with 3% sucrose as compared to standard FN with 6% sucrose (Fig. 1), although tissue harvested from FN (3% sucrose) had more necrotic spots than tissue from standard FN (6% sucrose). Growth in FNN6 6% sucrose revealed a 36% yield increase over growth in FN 6% sucrose, putatively attributable to the lower nitrogen content. Based on these two comparisons, a medium with 3% sucrose and the salt composition of FNN6 basal medium was expected to show a 63% (27 + 36%) increase in growth rate if no interactions are present between sucrose and nitrogen effects. In fact, a 69% growth increase in FNN6 (3% sucrose) medium was observed. Moreover, cultures derived in FNN6 medium lacked necrotic spots. Thus, sucrose concentration and total nitrogen content were initially identified as major factors controlling the growth rate of soybean embryogenic cultures. Based on these results, the medium formulation with the modified N6 macro salts and the 3% sucrose concentration was temporarily designated as FNN6, and used as the basis for further optimization work.

Effect of 2,4-D content. It was previously demonstrated that 2,4-D level could significantly affect embryo proliferation in liquid medium (Finer and Nagasawa, 1988). Therefore, 22.6 and 45.2 μ*M* 2,4-D were tested in FN and FNN6 media. Relative to the FN control (22.6 μ*M* 2,4-D), the greatest biomass increase (174.5%) was obtained in FNN6 containing 22.6 μ*M* 2,4-D. Doubling the 2,4-D concentration significantly ($P \leq 0.05$) decreased the growth rate in the FNN6 background to 129.3% of that of the control, but led to no significant changes in the FN background. Thus, FNN6 medium supplemented with 22.6 μ*M* 2,4-D was used in all further studies on media optimization.

Effect of sucrose content and/or medium osmotic pressure. Sucrose concentration was one of the major factors affecting proliferation. Therefore, to determine the minimum sucrose concentration required for embryo proliferation, FNN6 basal medium with 8.75 or 17.5 m*M* sucrose was tested, and biomass increase was found to be 53.7 and 100.7%, relative to medium supplemented with 87.6 m*M* sucrose. In additional tests using sucrose concentrations of 29.2, 58.4, 87.6, 116.9, 146.1, or 175.3 m*M*, which correspond to 1, 2, 3, 4, 5, or 6% sucrose, respectively, in FNN6 basal medium, the resulting biomass increases were 143.3, 136.9, 100.0, 89.8, 63.6, and 63.1%, respectively, relative to the growth obtained in FNN6 with 3% sucrose (Fig. 2). Thus, maximum growth occurs at 29.2 m*M* sucrose, and growth decreases as sucrose levels decrease or increase from 29.2 m*M*, though this decrease was not statistically significant at 58.4 m*M* sucrose.

In tissue culture media, each component serves as a nutrient or growth regulator and as a source of osmotic pressure. Below 29.2 m*M*, sucrose presumably becomes limiting as a nutrient, while sucrose levels above 29.2 m*M* may inhibit embryo proliferation due to osmotic pressure. The osmotic pressures of FNN6 basal medium with 0, 29.2, 58.4, 87.6, 116.9, 146.1, or 175.3 m*M* sucrose were measured and found to be 85.7 ± 1.45, 116.0 ± 0.58, 160.3 ± 1.288, 186.3 ± 0.33, 232.7 ± 0.89, 272.7 ± 1.45, and 294.3 ± 0.33 mOsm kg^{-1}, respectively.

To study the effect of osmotic pressure on proliferation, mannitol was used as an Osm. Mannitol is generally considered to be a nonplasmolyzing Osm, although plant cells have a limited capacity to metabolize it (Thompson et al., 1986). A medium with 29.2 m*M* sucrose content and an osmotic pressure of 290.3 ± 1.20 mOsm kg^{-1}, which corresponds to a medium with 175.3 m*M* sucrose, was obtained by addition of mannitol. The molarity of FNN6 basal medium supplemented with 29.2 m*M* sucrose + 146.1 m*M* mannitol is 175.3 m*M*, and, thus, theoretically it should have an osmotic pressure equal to 295 mOsm kg^{-1}. However, the actual osmotic pressure was found to be 274.0 ± 0.58 mOsm kg^{-1}. Consequently, the amount of mannitol was increased to 160.3 m*M* to obtain an osmotic pressure of 290.3 ± 1.20 mOsm kg^{-1}. The relative biomass increase of cultures in media with 29.2, 87.6, or 175.3 m*M* sucrose, which correspond to 1, 3, and 6% sucrose, respectively, and a medium with 1% sucrose + 160.1 m*M* mannitol is illustrated in Fig. 3. Osmotic pres-

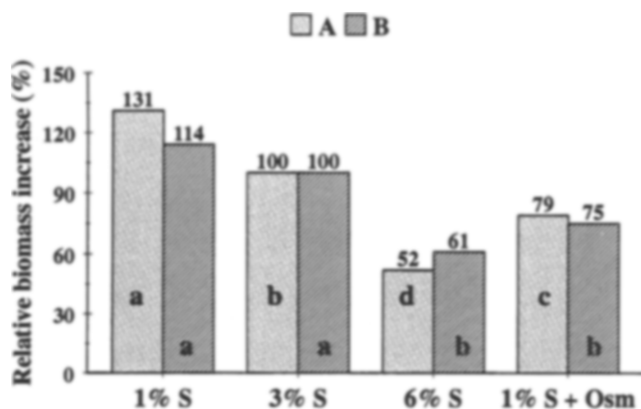

FIG. 3. Proliferation of suspension cultures in FNN6 media with different sucrose (S) concentrations and osmotic pressures in two different experiments using mannitol (A) or sorbitol (B) as the source of an osmoticum (Osm). For each experiment, growth in FNN6 with 3% sucrose was set as a standard at 100%, and growth in the other formulations was evaluated relative to the standard. Within a given experiment, means with the same letter are not significantly different at $P = 0.05$. FNN6 = defined in Materials and Methods.

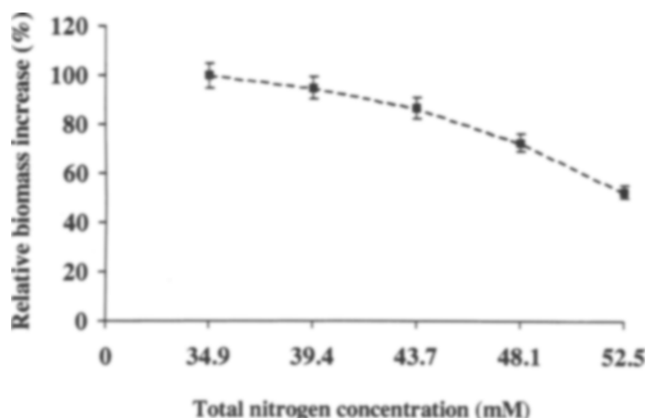

FIG. 4. Effect of total inorganic nitrogen content in FNN6 medium on proliferation of cultures. *Vertical bars* represent standard error (SE). FNN6 = defined in Materials and Methods.

sure explains approximately half of the decrease in tissue growth obtained at the higher sucrose concentration, with the remainder apparently attributable to sucrose itself. These results generally agree with those described by Gleddie et al. (1983), who observed inhibition of eggplant somatic embryogenesis on media supplemented with increased amounts of mannitol up to 0.6 *M*. To ensure that the decrease in proliferation of cultures was related to the medium osmotic pressure and not to the addition of mannitol, sorbitol was also used as a source of Osm and the relative biomass increase in media with 29.2, 87.6, or 175.3 m*M* sucrose, which correspond to 1, 3, or 6%, respectively, and a medium with 1% sucrose + 160.1 m*M* sorbitol was found to be 113.7, 100, 60.5, and 74.9%, respectively (Fig. 3).

Effect of total inorganic nitrogen. Growth of embryogenic cultures in media with lowered total nitrogen content (i.e., 50 and 75% of the amount in FNN6) revealed that repetitive somatic embryos lost re-

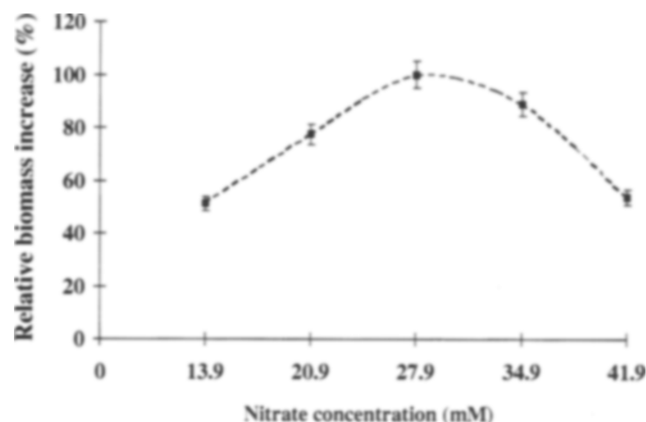

FIG. 5. Effect of NO_3^- content in FNN6 medium on proliferation of cultures. *Vertical bars* represent standard error (SE). FNN6 = defined in Materials and Methods.

TABLE 2

EFFECT OF NH_4^+ CONTENT IN FNN6 MEDIUM ON PROLIFERATION OF EMBRYOGENIC CULTURES[a]

NH_4^+ concentration (m*M*)	NH_4^+ content as a % of that in FNN6	Relative growth (%)
3.50	50	100.9a
5.25	75	84.9b
7.00	100	100.0a
8.75	125	100.9a
10.50	150	90.5ab

[a]Means followed by the same letter are not significantly different at $P = 0.05$. FNN6 = defined in Materials and Methods.

petitiveness and the tissue became nonembryogenic. Thus, the total nitrogen content in N6 macro salts appears to define the minimum concentration necessary to maintain repetitiveness of soybean somatic embryos. Therefore, these lowest two nitrogen levels were excluded from the experiment. Results at the end of the 3-wk period are in Fig. 4. The best embryo proliferation was obtained with 34.9 m*M* of total nitrogen, with a significant trend ($r^2 = 0.74$) towards less growth as nitrogen concentration increased, suggesting that the total nitrogen content of N6 macro salts is also the optimal concentration. A decrease in quality of embryogenic cultures was also apparent with the increase in total nitrogen. Cultures grown in FNN6 medium were represented by green compact embryogenic clusters consisting of multiple small embryos. As the amount of nitrogen increased, embryogenic clusters gradually changed from green to yellow-green or yellow-brown, and consisted of fewer, larger embryos. Thus, total nitrogen content is one of the major factors that controls proliferation of soybean cultures and embryogenesis, with only a very narrow range of concentrations being effective.

Effect of NO_3^- and NH_4^+ content. To further explore the effect of total inorganic nitrogen content, the relative biomass increase of cultures in FNN6 basal medium altered to contain different NO_3^- levels is depicted in Fig. 5. In contrast, increasing or decreasing the NH_4^+ content by 50% from that in FNN6 basal medium resulted in very little if any effect on proliferation of cultures (Table 2). No differences in quality of cultures or morphology of embryogenic clusters were observed. Although it has long since been recognized that a minimal

TABLE 3

EFFECT OF CA^{2+} CONTENT IN FNN6 MEDIUM ON PROLIFERATION OF EMBRYOGENIC CULTURES[a]

Ca^{2+} concentration (m*M*)	Ca^{2+} content as a % of that in FNN6	Relative growth (%)
1.02	50	86.9a
1.53	75	100.7a
2.04	100	100.0a
2.55	125	102.3a
3.06	150	104.1a

[a]Means followed by the same letter are not significantly different at $P = 0.05$. FNN6 = defined in Materials and Methods.

TABLE 4

EFFECT OF PO_4^{3-} CONTENT IN FNN6 MEDIUM ON PROLIFERATION OF EMBRYOGENIC CULTURES[a]

PO_4^{3-} concentration (m*M*)	PO_4^{3-} content as a % of that in FNN6	Relative growth (%)
0.68	50	102.9a
1.02	75	101.7a
1.36	100	100.0a
1.69	125	103.9a
2.04	150	99.8a

[a]Means followed by the same letter are not significantly different at $P = 0.05$. FNN6 = defined in Materials and Methods.

amount of NH_4^+ must be present in the medium for somatic embryogenesis to occur (Walker and Sato, 1981; Meijer and Brown, 1987), it is evident for soybean that above such a minimal concentration, the effects of NH_4^+ concentration are negligible. However, overly high levels of NH_4^+ have been considered to be detrimental for somatic embryogenesis of red bud, another legume (Trigiano et al., 1988).

Effect of ammonium to nitrate ratio. The effect of nitrogen on proliferation of cultures may depend on four interrelated factors: total nitrogen, nitrate content, ammonium content, and ammonium to nitrate ratio. Therefore, the effect of each factor was evaluated separately, although altering the nitrogen composition in a culture medium unavoidably leads to changes in the ammonium to nitrate ratio, and this ratio has long since been recognized as an important regulatory factor in plant tissue culture (Smith and Krikorian, 1989).

As has been the case with other species, the ammonium to nitrate ratio was found to be another major factor that controls embryogenesis of soybean in media with 34.9 m*M* total inorganic nitrogen and 22.6 μ*M* 2,4-D. When ammonium was used as a sole source of nitrogen (1:0 ratio), embryos lost repetitiveness and cultures consisted of yellow-brown morphogenic tissue. However, quality of cultures derived in such a medium also could be partially affected by the K^+ content, which was lowered from 29.4 to 1.4 m*M*. In this experiment, the best embryogenic clusters were derived in the medium with the 1:4 ratio. These clusters consisted of numerous small, repetitive green embryos and had a compact morphology. When nitrate was used as the only source of nitrogen, embryogenic clusters consisted of fewer but larger embryos of yellowish color.

A 1:2 ammonium:nitrate ratio was found to be optimal for eggplant (Gleddie et al., 1983), while 1:3 was previously reported to be the best for soybean (Nadolska-Orczyk and Orczyk, 1994) and sweet orange (Niedz, 1994). By contrast, the ammonium:nitrate ratio in MS macro salts is 1:2, and that in FN and N6 is 1:4. Optimal tissue growth in this study was obtained with this 1:4 ratio. It is clear from these results that, while important, the ammonium to nitrate ratio is not independent of the total inorganic nitrogen content of the medium.

Effect of other macro salts. The fact that cultures in media with Ca^{2+} contents increased or decreased by 50% relative to that in FNN6 revealed no significant effect on growth of cultures (Table 3). However, an increase in the number of necrotic spots was observed on embryogenic clusters derived from the medium with 1.0 m*M* Ca^{2+}. Likewise, no effect of PO_4^{3-} content on proliferation of cultures was observed within the range of PO_4^{3-} concentrations tested. The total

TABLE 5

EFFECT OF K^+ CONTENT IN FNN6 MEDIUM ON PROLIFERATION OF EMBRYOGENIC CULTURES[a]

K^+ concentration (m*M*)	K^+ content as a % of that in FNN6	Relative growth (%)
22.35	76.1	90.9a
25.68	87.5	87.9a
29.35	100.0	100.0a
33.02	112.5	91.4a
36.69	125.0	68.6b

[a]Means followed by the same letter are not significantly different at $P = 0.05$. FNN6 = defined in Materials and Methods.

biomass increase of cultures in media with the various phosphate contents is shown in Table 4. No differences in quality or morphology of embryogenic clusters were observed between treatments. Likewise, the effect of K^+ content on proliferation was negligible within the range tested (Table 5). No effect of Mg^{2+}, SO_4^{2-}, and Cl^- contents on proliferation of cultures was observed when NH_4Cl was used as a source of ammonium and, therefore, Mg^{2+}, SO_4^{2-}, and Cl^- contents in FNN6 medium were increased from 1.5 to 4.1 m*M*, 4.2 to 5.2 m*M*, and 4.1 to 11.1 m*M*, respectively (data not shown), relative to FNN6 basal medium, to facilitate the adjustments of other salts during the optimization process.

Proliferation of other genotypes in FNN6 medium. To ensure that FNN6 was suitable for other soybean genotypes, the proliferation of embryogenic cultures of three soybean genotypes was evaluated over a 3-wk period, and found to be 2.7 ± 0.15- and 7.4 ± 0.27-fold for Chapman in FN and FNN6 media, respectively; 12.4 ± 0.69- and 33.3 ± 2.13-fold for F138; 7.8 ± 1.52 and 19.5 ± 0.45-fold for Williams 82. This corresponds to biomass increases in FNN6 equal to 278, 269, and 251% for Chapman, F138, and Williams 82 relative to their growth in FN. Thus, while there were genotype-specific differences in growth, all genotypes experienced a similar magnitude of growth increase in FNN6 over FN medium.

Final medium composition. As a result of this study, we determined that four factors affect proliferation of soybean embryogenic cultures in liquid medium, and these factors are carbohydrate content, osmotic pressure, total nitrogen content, and ammonium to nitrate ratio. As compared to FN medium (Table 1), the new formulation has the amount of KNO_3 reduced from 29.9 to 27.9 m*M*, and the 9.9 m*M* NH_4NO_3 replaced with 3.5 m*M* $(NH_4)_2SO_4$. The final medium contains the macro salts as listed in Table 1, and supplemented with MS micro salts, B5 vitamins, 6.7 m*M* L-asparagine, and 22.6 μ*M* 2,4-D.

TABLE 6

PRECIPITATION OF ANIONS AND CATIONS AT EQUILIBRIUM IN MEDIA USED FOR SOYBEAN EMBRYOGENIC CULTURES

	Precipitated (%)		
Medium[a]	Ca^{2+}	PO_4^{3-}	Mn^{2+}
FN	39.6	64.9	100
FNN6	37.5	41.2	100
MS	39.7	65.1	100

[a]FN = Finer and Nagasawa, 1988; FNN6 = defined in Materials and Methods; MS = Murashige and Skoog, 1962.

Although the best growth was obtained at 29.2 to 58.4 m*M* sucrose, sufficient growth could be obtained with sucrose levels as high as 87.6 m*M*. Although the differences in media composition appear to be small, they have consistently yielded 170–250% greater growth than the original formulation, highlighting just how sensitive embryogenic cultures can be to certain factors in the culture medium.

Evaluation of the final FNN6 medium formulation using MINTEQA2 v. 3.11 revealed that at equilibrium at 25° C and pH 5.8, all medium components are dissolved, with the exception of 37.5, 41.2, and 100% of the Ca^{2+}, PO_4^{3-}, and Mn^{2+}, respectively. These cations and anions create insoluble compounds and, consequently, slight precipitation in the medium occurs. This precipitation also occurs in other commonly used media (Table 6), although it occurs to a lesser extent in FNN6.

Summary

It has been possible to optimize the existing medium for soybean somatic embryogenesis, resulting in an average growth rate increase for the genotypes tested up to 250% over that obtained in FN medium. The soybean embryogenic cultures grew equally well over a wide range of ammonium, calcium, potassium, and phosphate concentrations. However, the cultures have a very strict requirement for total inorganic nitrogen content, and are sensitive to the ammonium to nitrate ratio. The cultures are also sensitive to the sucrose concentration and osmotic pressure of the medium. Because the optimal medium composition differs from the original FN medium primarily by having lower concentrations of sucrose and total nitrogen, we have designated it as FN Lite medium.

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