Abstract. Embryogenic callus growth of sweetpotato Ipomoea batatas (L.) Lam.] was selectively enhanced by subculture on basal callus proliferation medium modified to contain 15 μM NH₄NO₃. Embryogenic callus production was doubled on basal callus proliferation medium modified to contain 60 mM K+, while nonembryogenic callus production was reduced 40%. Additions of up to 40 mM NaCl to basal callus proliferation medium did not affect callus proliferation. The development of embryos from callus subculture to embryo production basal medium was unaffected by the KCl or NaCl treatments of the callus proliferation phase. However, embryo production was increased by subculturing callus from callus proliferation medium containing 20 mM NH₄⁺ to embryo production medium containing 10 mM NH₄⁺. Our results demonstrate that changes in mineral nutrition, in addition to growth regulator differences between callus proliferation and embryo production media, are important factors in sweetpotato somatic embryogenesis.

Materials and Methods

Embryogenic callus of sweetpotato ‘White Star’ was obtained from shoot apices and proliferated as reported by Chée and Cantliffe (1988b). The basal medium contained the inorganic salts of Murashige and Skoog (1962), 500 μM myo-inositol, 5 μM thiamine·HCl, 10 μM nicotinic acid, 5 μM pyridoxine·HCl, 87.6 mM sucrose, and 0.6% w/v Phytagar (GIBCO Laboratories, Grand Island, N.Y.). Callus proliferation media (CP) contained 10 μM 2,4-D and 1 μM BA (Chée and Cantliffe, 1988b); embryoid production media (EP) were hormone free (Chée and Cantliffe, 1988a). The basal CP and EP media were modified, as indicated for each experiment, by using combinations of NH₄NO₃, NH₄Cl, (NH₄)₂citrate, NaNO₃, KNO₃, KCl, and NaCl to obtain the desired balance of ions. The pH was adjusted to 5.8 with 1 N NaOH before autoclaving at 121°C and 110 kPa for 15 min.

Embryogenic callus fragments (calli), 1 mm in diameter (1 mg average fresh weight), of 8-week-old cultures were used in the experiments. Calli were placed individually on 12 ml of medium contained in 60 x 15-mm plastic petri dishes. There were 10 dishes per treatment. Incubation was at 27°C in the dark with unmonitored light interruptions during daily observations. Embryogenic and nonembryogenic callus production on CP media was determined using fresh weights at 8 weeks. Sweetpotato embryogenic callus is firm, yellow, and opaque; nonembryogenic callus is friable, white, and translucent (Chée and Cantliffe, 1988b; Liu and Cantliffe, 1984). Embryo production on
EP media was determined by counting embryos at the heart, torpedo, and cotyledonary stages of development at 21 days (Chée and Cantliffe, 1988a).

In Expt. I, we investigated N form on callus growth. Basal CP medium was modified to contain NH₄NO₃ at 2.5, 5.0, 7.5, 10, 15, or 20 mM. The other N source of basal CP medium was 20 mM KNO₃. Calloused in the first passage were obtained on basal CP medium, which contained 20 mM NH₄NO₃. After 8 weeks, calli were subculture to the same respective NH₄NO₃ treatments for the second passage.

In Expt. II, we compared K concentration on callus growth. Basal CP medium was modified to contain K⁺ at 6.25, 11.25, 16.25, 21.25, 31.25, 41.25, 51.25, or 61.25 mM. Each in each case 1.25 mM of K⁺ came from KH₂PO₄ of the basal CP medium. The four lower K⁺ concentrations were obtained using KNO₃. The four higher K⁺ concentrations were obtained using KNO₃ at 20 mM and KCl. The N levels were complemented with NaNO₃ to a total of 60 mM for each treatment. Calli used in the first passage originated on basal CP medium that contained 20 mM K⁺. After 8 weeks, calli were subculture to the same respective K⁺ treatments for the second passage. Calli of each passage were subculture onto basal EP medium to test for residual effects of the K⁺ treatments on subsequent embryo production.

In Expt. III, we compared the effect of NaCl with that of KCl on callus growth. Basal CP medium was supplemented with NaCl or KCl at 10, 20, 30, or 40 mM. The basal CP medium contained 20 mM K⁺, 6 mM Cl⁻ and no Na⁺. The calli used were subculture from basal CP medium. The residual effects of NaCl and KCl on subsequent embryo production were tested by subculturing calli onto EP basal medium.

In Expt. IV, we evaluated N source and level on embryo production. Basal EP medium was modified to contain NH₄NO₃ at 2.5, 5.0, 7.5, 10, 15, and 20 mM. The other N source of basal EP medium was 20 mM KNO₃. The calli used were subculture from basal CP medium that contained 20 mM NH₄NO₃. In a replicate experiment, the calli used in each treatment were subculture from CP media with the same NH₄NO₃ concentrations.

In Expt. V, embryo production was compared in response to NH₄⁺ and total N in a factorial experiment. Ammonium nitrate was withheld from the basal EP medium and (NH₄)₂ citrate was added at 0, 2.5, 5.0, and 10 mM. Total N levels of 40 and 60 mM were obtained by complementing the media containing 20 mM KNO₃ with an appropriate amount of NaNO₃. The calli used in the experiment were subculture from basal CP medium.

**Results and Discussion**

In the first passage of Expt. I, both embryogenic and nonembryogenic callus fresh weights were similar among NH₄NO₃ treatments (Table 1). However, upon subculture to the same NH₄NO₃ treatments in the second passage, embryogenic callus production increased to a maximum at 15 mM NH₄NO₃, and nonembryogenic callus production decreased as NH₄NO₃ concentration increased. Calli used in the first passage originated on CP medium containing 20 mM NH₄NO₃. Consequently, subculturing embryogenic callus on CP medium containing 15 mM instead of the 20 mM NH₄NO₃ of the MS salts selectively enhanced embryogenic callus proliferation. The enhanced embryogenic callus growth by increased NH₄NO₃ could be a response to increased total N level, NH₄⁺ concentration, or NH₄⁺/NO₃⁻ and NH₄⁺/K⁺ concentration ratios.

In Expt. II, where total N level and NH₄⁺/NO₃⁻ ratio were held constant, embryogenic callus growth was promoted by increased K⁺ concentrations and decreased NH₄⁺/K⁺ ratios (Table 2). Decreasing the NH₄⁺/K⁺ ratio promoted in vitro meristem initiation and growth in Vitis (Galzy, 1972). However, the combined results of Expt. I, where K⁺ was held constant and N varied (Table 1), and Expt. II, where N was held constant and K⁺ varied (Table 2), show an ambiguous relationship between embryogenic callus growth and NH₄⁺/K⁺ ratios, possibly because the varying NH₄⁺/NO₃⁻ ratio may complicate the K⁺-NH₄ relationship in Expt. I. In Expt. I, increased embryogenic callus growth corresponded to increased NH₄⁺/K⁺ ratios with highest yields at NH₄⁺/K⁺ = 0.75, while in Expt. II, increased embryogenic callus growth corresponded to decreasing NH₄⁺/K⁺ ratios with highest yields at NH₄⁺/K⁺ = 0.3. Our results suggest that K⁺ at 20 mM became the limiting factor for embryogenic callus growth when NH₄⁺ exceeded 15 mM (Table 1). Cell metabolism is disrupted when NH₄⁺ relatively high levels competes successfully with K⁺ for negative charges within the cells (Marschner, 1986). Appropriately, when K⁺ concentration was increased from 20 to 60 mM in the presence of 20 mM NH₄⁺, embryogenic callus growth was promoted (Table 2), probably because the readily exchangeable K⁺ was then reestablished in its role in neutralizing organic and inorganic anions.

High K⁺ concentrations promoted embryogenic callus growth whether calli were subculture from basal CP medium, containing 21.25 mM K⁺, or from CP media of passage 1 containing respectively the same K⁺ levels (Table 2). Doubling the K⁺ concentration of the MS salts doubled embryogenic callus yields, while nonembryogenic callus growth decreased 40%. Thus, subculturing calli on high-K CP medium selectively promoted embryogenic callus growth. The K⁺ concentration in the callus proliferation stage did not affect subsequent embryo production on basal EP medium (Table 2). The basal EP medium contained 20 mM K⁺, a concentration found optimum in embryogenesis of Daucus carota L. (Brown et al., 1976).

Nutritionally important ions are usually added to culture media in the form of sodium salts or chlorides, since Na⁺ and Cl⁻ do not influence growth in many plant tissue culture systems at concentrations up to 40 mM (Brown et al., 1976; Heller, 1953). In Expt. III, additions of 10 to 40 mM NaCl to CP media did not affect callus growth, while similar KCl additions promoted a linear increase in embryogenic callus production (Table 3). Thus, increased embryogenic callus growth can be attributed to increased K⁺ rather than increased Cl⁻ concentrations. Neither NaCl nor KCl additions to CP media were detrimental to subsequent embryo production on EP media (Table 3).

In Expt. IV, embryo production was stimulated by decreasing NH₄NO₃ from 20 mM in the CP medium to 10 mM in the EP medium (Table 4). A further decrease in NH₄NO₃ was not beneficial. Embryo production was similar for all NH₄NO₃ concentrations when callus was subculture from CP media containing 2.5 to 20 mM NH₄NO₃ to EP media with the same respective NH₄NO₃ concentration. Increased embryo production in response to decreased NH₄NO₃ between CP and EP media could result from decreased total N level NH₄⁺ or NH₄⁺/N ratio. This was investigated in Expt. V where NH₄⁺ was varied in the EP medium from 0 to 20 mM at two total N levels (Table 5). Total N did not affect embryo production within each NH₄⁺ treatment. However, overall embryo production was increased by subculturing calli from basal CP medium containing 20 mM NH₄⁺ to EP media containing 10 mM NH₄⁺.

Auxin removal has been the classical permissive trigger for
embryo development (Ammirato, 1983). However, requirements for the reduction form of N for embryo initiation and maturation seem well supported (Halperin, 1966; Halperin and Wetherell, 1965; Walker and Sato, 1981; Wetherell and Dougall, 1976). In carrot cultures, ammonium additions of 10 mM \( \text{NH}_4\text{Cl} \) to media containing 12 to 40 mM \( \text{KNO}_3 \) were found optimal for embryogenesis (Wetherell and Dougall, 1976). In \textit{Medicago sativa} (L.), optimal somatic embryo development required a minimum of 12.5 mM \( \text{KNO}_3 \); for passage 2, calli were subcultured from the respective treatments of passage 1.

In alfalfa, embryo development followed 2,4-D withdrawal combined with increase from a 2.6 to 5 mM range to a 10 to 12.5 mM range (Seitz Kris and Bingham, 1988; Walker and Sato, 1981). In \textit{Glycine max}, embryo development required removal of 2,4-D and a coordinated decrease of 40 to 20 mM \( \text{KNO}_3 \) and increase \( \text{NO}_3^- \) from 0 to 40 mM (Christianson, 1985).

Here we enhanced embryo production in sweetpotato by increasing both embryogenic callus growth and embryo development (Ammirato, 1983). However, requirements for the reduction form of N for embryo initiation and maturation seem well supported (Halperin, 1966; Halperin and Wetherell, 1965; Walker and Sato, 1981; Wetherell and Dougall, 1976). In carrot cultures, ammonium additions of 10 mM \( \text{NH}_4\text{Cl} \) to media containing 12 to 40 mM \( \text{KNO}_3 \) were found optimal for embryogenesis (Wetherell and Dougall, 1976). In \textit{Medicago sativa} (L.), optimal somatic embryo development required a minimum of 12.5 mM \( \text{KNO}_3 \); for passage 2, calli were subcultured from the respective treatments of passage 1.

### Table 1. Callus growth in response to \( \text{NH}_4\text{NO}_3 \) concentration in sweetpotato.

| Treatment (mM) | Passage 1 | Passage 2 |
|---------------|-----------|-----------|
|               | Embryogenic | Non-embryogenic | Embryogenic | Non-embryogenic |
| \( \text{NH}_4\text{NO}_3 \) | \( \text{KNO}_3 \) | \( \text{K}_2\text{PO}_4 \) | \( \text{NO}_3^- \) | \( \text{N} \) | \( \text{NH}_4^+/\text{K}^+ \) | \( \text{NH}_4^+/\text{NO}_3^- \) | Fresh wt (mg) | Fresh wt (mg) |
| 2.5           | 18.8      | 1.2       | 21.3     | 23.8 | 0.13 | 0.12 | 18 | 139 | 14 | 346 |
| 5.0           | 18.8      | 1.2       | 23.8     | 28.8 | 0.25 | 0.21 | 16 | 126 | 21 | 307 |
| 7.5           | 18.8      | 1.2       | 26.3     | 33.8 | 0.37 | 0.29 | 19 | 171 | 37 | 146 |
| 10.0          | 18.8      | 1.2       | 28.8     | 38.8 | 0.50 | 0.35 | 17 | 228 | 47 | 80  |
| 15.0          | 18.8      | 1.2       | 33.8     | 48.8 | 0.75 | 0.44 | 21 | 140 | 65 | 84  |
| 20.0          | 18.8      | 1.2       | 38.8     | 58.8 | 1.00 | 0.52 | 22 | 227 | 55 | 110 |

### Table 2. Effect of \( \text{K} \) concentration on callus growth in sweetpotato and residual effect on embryogenesis after callus transfer to media for embryo production.

| Treatment (mM) | Passage 1 | Passage 2 |
|---------------|-----------|-----------|
|               | Embryogenic | Non-embryogenic | Embryogenic | Non-embryogenic |
| \( \text{KNO}_3 \) | \( \text{KCl} \) | \( \text{K}_2\text{PO}_4 \) | \( \text{NaNO}_3 \) | \( \text{NH}_4\text{NO}_3 \) | \( \text{K}^+ \) | \( \text{NH}_4^+/\text{NO}_3^- \) | Embryogenic | Fresh wt (mg) | Embryogenic | Fresh wt (mg) |
| 5             | 0         | 1.25      | 15        | 20    | 6.25 | 60 | 0.5 | 3.2 | 5 | 112 | 3 | 18 | 389 | 9 |
| 10            | 0         | 1.25      | 10        | 20    | 11.25| 60 | 0.5 | 1.8 | 6 | 142 | 3 | 18 | 400 | 9 |
| 15            | 0         | 1.25      | 5         | 20    | 16.25| 60 | 0.5 | 1.2 | 5 | 144 | 4 | 18 | 478 | 5 |
| 20            | 0         | 1.25      | 0         | 20    | 21.25| 60 | 0.5 | 0.9 | 9 | 134 | 13 | 19 | 251 | 8 |
| 20.10         | 1.25      | 0         | 20        | 31.25 | 60   | 0.5 | 0.6 | 10 | 166 | 9 | 41 | 274 | 8 |
| 20.20         | 1.25      | 0         | 20        | 41.25 | 60 | 0.5 | 0.5 | 15 | 45 | 6 | 23 | 271 | 13 |
| 20.30         | 1.25      | 0         | 20        | 51.25 | 60 | 0.5 | 0.4 | NA | NA | NA | 40 | 98 | 8 |
| 20.40         | 1.25      | 0         | 20        | 61.25 | 60 | 0.5 | 0.3 | NA | NA | NA | 44 | 160 | 6 |

### Table 3. Effect of \( \text{NaCl} \) and \( \text{KCl} \) on callus growth of sweetpotato and residual effect on embryogenesis after callus transfer to media for embryo production.

| \( \text{NaCl} \) or \( \text{KCl} \) (mM) | Total \( \text{K}^+ \) (mg) | Embryogenic callus fresh wt (mg) | Nonembryogenic callus fresh wt (mg) | Embryogenic callus inoculum (no.) |
|-----------------|-------------------|-------------------------------|------------------------------------|----------------------------------|
| 10              | 20                | 32                            | 102                               | 5                                |
| 20              | 20                | 29                            | 29                                | 6                                |
| 30              | 20                | 29                            | 12                                | 6                                |
| 40              | 20                | 38                            | 31                                | 7                                |

### Statistical analysis used the component of variance method: Significance = \( F \) test, \( L^{**} = \) linear at \( P = 0.01 \), \( \text{NS} = \) not significant, \( \text{NA} = \) not available because of insufficient replications.

**Inocula were 1-mg embryogenic callus fragments; for passage 1, calli were subcultured from callus proliferation media containing 20 mM \( \text{NH}_4\text{NO}_3 \); for passage 2, calli were subcultured from the respective treatments of passage 1.**

In alfalfa, embryo development followed 2,4-D withdrawal combined with increase from 0 to 40 mM K*; for passage 2, calli were subcultured from the respective treatments of passage 1.

**Inocula were 1-mg embryogenic callus fragments; for passage 1, calli were subcultured from callus proliferation media containing 20 mM K*; for passage 2, calli were subcultured from the respective treatments of passage 1.**

**Inocula were 1-mg embryogenic callus fragments subcultured from callus proliferation media containing 20 mM K*.
Table 4. Embryo production in response to NH₄NO₃ concentration in sweetpotato and residual effect on embryogenesis of the NH₄NO₃ concentrations used in the callus proliferation phase.

| Treatment (mM) | Embryos/inoculum<sup>a</sup> (no.) | Calli from CP with 20 mM NH₄NO₃ | Calli from CP with 2.5-20 mM NH₄NO₃ |
|---------------|------------------------------------|-------------------------------|-----------------------------------|
| NH₄NO₃, KNO₃, KH₂PO₄, NO₃⁻ | NH₄⁺/K⁺ | NH₄⁺/NO₃⁻ | NH₄⁺/K⁺ | NH₄⁺/NO₃⁻ |
| 2.5 | 18.8 | 1.2 | 21.3 | 23.8 | 0.13 | 0.12 | 8.5 | 9.4 |
| 5.0 | 18.8 | 1.2 | 23.8 | 28.8 | 0.25 | 0.21 | 8.9 | 18.1 |
| 7.5 | 18.8 | 1.2 | 26.3 | 33.8 | 0.37 | 0.29 | 14.1 | 11.1 |
| 10.0 | 18.8 | 1.2 | 28.8 | 38.8 | 0.50 | 0.35 | 17.0 | 11.2 |
| 15.0 | 18.8 | 1.2 | 33.8 | 48.8 | 0.75 | 0.44 | 12.4 | 10.2 |
| 20.0 | 18.8 | 1.2 | 38.8 | 58.8 | 1.00 | 0.52 | 9.4 | 7.3 |

Significance: Q** = quadratic at P = 0.01, NS = not significant.

<sup>a</sup>Statistical analysis used the component of variance method: Significance = F test, Q** = quadratic at P = 0.01, NS = not significant.

Inocula were 1-mg embryogenic calli fragments; CP = callus proliferation media.

Table 5. Embryo production in response to ammonium concentration and total N in sweetpotato.

| NH₄⁺ (mM) | K⁺ (mM) | NH₄⁺/K⁺ | NH₄⁺/N | Embryos/ inoculum<sup>b</sup> (no.) | NH₄⁺/N | Embryos/ inoculum<sup>b</sup> (no.) | Embryos/ inoculum<sup>b</sup> (no.) |
|-----------|---------|---------|--------|------------------------------------|--------|------------------------------------|------------------------------------|
| 0         | 20      | 0.0     | 0.0    | 3.8                                | 0.0    | 2.4                               | 3.1                                |
| 5         | 20      | 0.25    | 0.13   | 5.9                                | 0.08   | 3.4                               | 4.8                                |
| 10        | 20      | 0.50    | 0.25   | 6.8                                | 0.17   | 6.1                               | 6.4                                |
| 20        | 20      | 1.00    | 0.50   | 5.8                                | 0.33   | 5.1                               | 5.5                                |

<sup>b</sup>Statistical analysis used the component of variance method: Significance = F test, L* = linear at P = 0.05, Q* = quadratic at P = 0.05; Main effect of total N and interaction total N × NH₄⁺ were not significant at P = 0.05.

Inocula were 1-mg embryogenic callus fragments subcultured from callus proliferation media containing 20 mM NH₄⁺ and 60 mM total N.

Our results demonstrate that embryo production of species such as sweetpotato can be greatly improved by optimizing the inorganic constituents of the culture media. Our recommendation for sweetpotato is to grow embryogenic callus by subculture every 8 weeks on a basal CP medium modified to contain 20 mM NH₄⁺ and 50 mM K⁺ with a total N of 60 mM. The medium for embryo production should be a basal EP medium modified to contain 10 mM NH₄⁺ and 20 mM K⁺ with a total N of 40 mM.

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