Optimizing Embryogenic Callus and Embryo Growth of a Synthetic Seed System for Sweetpotato by Varying Media Nutrient Concentrations

Raymond P. Chée¹, Daniel I. Leskovar², and Daniel J. Cantliffe

Vegetable Crops Department, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL 32611

Additional index words. *Ipomoea batatas*, nitrogen, potassium, somatic embryogenesis

Abstract. Embryogenic callus growth of sweetpotato [*Ipomoea batatas* (L.) Lam.] was selectively enhanced by subculture on basal callus proliferation medium modified to contain 15 mM NH_4NO_3 . 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 calli 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 NH_4^+ to embryo production medium containing 10 mM NH_4^+ . 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.

Clonal propagation via somatic embryogenesis has been proposed by Cantliffe et al. (1988) for synthetic seed production of sweetpotato. Sweetpotato somatic embryo production is accomplished in two stages (Chée and Cantliffe, 1988a). Embryogenic callus is continuously proliferated by subculture on media containing growth regulators; embryos are produced by transferring embryogenic callus to media without growth regulators. Formation of embryogenic callus was enhanced by addition of 10 μM (2,4-dichlorophenoxy)acetic acid (2,4-D) and 1 μM *N*-(phenylmethyl)-1 *H*-purine-6-amine (BA) (Chée and Cantliffe, 1988b). Further improvements in embryogenic callus growth and embryo production could be expected by optimizing inorganic salts in the basal medium. However, information on the mineral nutrition of embryogenic callus is insufficient (Ammirato, 1983; Vasil, 1984).

Nitrogen is quantitatively the major mineral nutrient taken up during growth of plant tissues (Marschner, 1986). Supplying N in its reduced rather than oxidized form to tissue culture likely would be more efficient for growth, since NO_3^- reduction is energetically expensive (Clarkson and Hanson, 1980). However, cultured cells were unable to grow on NH_4^+ as the sole N source when media did not include organic acids as buffers, or without continuous titration of the medium (Chaleff, 1983; Dougall and Verma, 1978; Fukunaga et al., 1978; Gamborg and Shyluk, 1970; Martinet et al., 1977). This result is to be expected since NH_4^+ uptake is physiologically acidic (Marschner, 1986). The relative and absolute amounts of NH_4^+ and NO_3^- also influence embryogenesis in cell cultures (Tazawa and Reinert, 1969). Generally, it is thought that the ionic form of N affects growth and nutrient uptake by altering cation-anion balance, changing organic anion accumulation and ultimately affecting medium and cytosol pH values (Marschner, 1986; Poole, 1978;

Spanswick, 1981). Ammonium directly competes for uptake with K, which is the major cation involved in maintaining cation-anion balance and the most common counterion for organic anions (Marschner, 1986). Potassium is also required in the cytoplasm for active conformation of numerous enzymes and maintenance of osmotic potential (Clarkson and Hanson, 1980). External N and K ions may also influence growth through their direct effects on the plasmalemma proton pump that is stimulated preferentially by K^+ , NH_4^+ , and Na^+ , in that order (O'Neill and Spanswick, 1984).

In this study we evaluated the effects of NH_4^+ , NO_3^- , and K^+ , and their companion ions Na^+ and Cl^- , on embryogenic callus and embryo growth in sweetpotato.

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); embryo 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 NH_4NO_3 , NH_4Cl , $(\text{NH}_4)_2\text{citrate}$, NaNO_3 , KNO_3 , 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 × 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

Received for publication 16 Aug. 1991. Accepted for publication 10 Feb. 1992. Florida Agricultural Experiment Station, Journal Series no. R-9657. This work was supported in part by an IFAS/Gas Research Inst. cooperative grant. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

¹SIGMA Chemical Co., Cell Culture, PO Box 14508, St. Louis, MO 63178.

²Texas Agr. Expt. Sta., Texas A&M Univ., 1619 Gamer Field Rd., Uvalde, TX 78801.

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 NH_4NO_3 at 2.5, 5.0, 7.5, 10, 15, or 20 mM. The other N source of basal CP medium was 20 mM KNO_3 . Calloused in the first passage were obtained on basal CP medium, which contained 20 mM NH_4NO_3 . After 8 weeks, calli were subculture to the same respective NH_4NO_3 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, 11.2, 16.2, 21.2, 31.2, 41.2, 51.2, or 61.2 mM. In each case 1.25 mM of K^+ came from KH_2PO_4 of the basal CP medium. The four lower K^+ concentrations were obtained using KNO_3 . The four higher K^+ concentrations were obtained using KNO_3 at 20 mM and KCl. The N levels were complemented with NaNO_3 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 NH_4NO_3 at 2.5, 5.0, 7.5, 10, 15, and 20 mM. The other N source of basal EP medium was 20 mM KNO_3 . The calli used were subculture from basal CP medium that contained 20 mM NH_4NO_3 . In a replicate experiment, the calli used in each treatment were subculture from CP media with the same NH_4NO_3 concentrations.

In Expt. V, embryo production was compared in response to NH_4^+ and total N in a factorial experiment. Ammonium nitrate was withheld from the basal EP medium and $(\text{NH}_4)_2$ 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 KNO_3 with an appropriate amount of NaNO_3 . 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 NH_4NO_3 treatments (Table 1). However, upon subculture to the same NH_4NO_3 treatments in the second passage, embryogenic callus production increased to a maximum at 15 mM NH_4NO_3 , and nonembryogenic callus production decreased as NH_4NO_3 concentration increased. Calli used in the first passage originated on CP medium containing 20 mM NH_4NO_3 . Consequently, subculturing embryogenic callus on CP medium containing 15 mM instead of the 20 mM NH_4NO_3 of the MS salts selectively enhanced embryogenic callus proliferation. The enhanced embryogenic callus growth by increased NH_4NO_3 could be a response to increased total N level, NH_4^+ concentration, or $\text{NH}_4^+/\text{NO}_3^-$ and NH_4^+/K^+ concentration ratios.

In Expt. II, where total N level and $\text{NH}_4^+/\text{NO}_3^-$ ratio were

held constant, embryogenic callus growth was promoted by increased K^+ concentrations and decreased NH_4^+/K^+ ratios (Table 2). Decreasing the NH_4^+/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 NH_4^+/K^+ ratios, possibly because the varying $\text{NH}_4^+/\text{NO}_3^-$ ratio may complicate the K^+/NH_4^+ relationship in Expt. I. In Expt. I, increased embryogenic callus growth corresponded to increased NH_4^+/K^+ ratios with highest yields at $\text{NH}_4^+/\text{K}^+ = 0.75$, while in Expt. II, increased embryogenic callus growth corresponded to decreasing NH_4^+/K^+ ratios with highest yields at $\text{NH}_4^+/\text{K}^+ = 0.3$. Our results suggest that K^+ at 20 mM became the limiting factor for embryogenic callus growth when NH_4^+ exceeded 15 mM (Table 1). Cell metabolism is disrupted when NH_4^+ at 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 NH_4^+ , 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 NH_4NO_3 from 20 mM in the CP medium to 10 mM in the EP medium (Table 4). A further decrease in NH_4NO_3 was not beneficial. Embryo production was similar for all NH_4NO_3 concentrations when callus was subculture from CP media containing 2.5 to 20 mM NH_4NO_3 to EP media with the same respective NH_4NO_3 concentration. Increased embryo production in response to decreased NH_4NO_3 between CP and EP media could result from decreased total N level, NH_4^+ , or NH_4^+/N ratio. This was investigated in Expt. V where NH_4^+ 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 NH_4^+ treatment. However, overall embryo production was increased by subculturing calli from basal CP medium containing 20 mM NH_4^+ to EP media containing 10 mM NH_4^+ .

Auxin removal has been the classical permissive trigger for

Table 1. Callus growth in response to NH_4NO_3 concentration in sweetpotato.^z

Treatment (mM)							Callus fresh wt (mg) ^y			
							Passage 1		Passage 2	
NH_4NO_3	KNO_3	KH_2PO_4	NO_3^-	N	NH_4^+/K^+	$\text{NH}_4^+/\text{NO}_3^-$	Embryogenic	Non-embryogenic	Embryogenic	Non-embryogenic
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
Significance							NS	NS	L**Q*	L**Q*
Mean (SE)							19 (1)	172 (21)	---	---

^zStatistical analysis used the component of variance method: Significance = F test, L** = linear at $P = 0.01$, Q* = quadratic at $P = 0.05$, NS = not significant.

^yInocula were 1-mg embryogenic callus fragments; for passage 1, calli were subcultured from callus proliferation media containing 20 mM NH_4NO_3 ; for passage 2, calli were subcultured from the respective treatments of passage 1.

Table 2. Effect of K concentration on callus growth in sweetpotato and residual effect on embryogenesis after callus transfer to media for embryo production.^z

Treatment (mM)									Passage 1 ^y			Passage 2 ^y		
									Embryogenic callus fresh wt (mg)	Non-embryogenic callus fresh wt (mg)	Embryos/inoculum (no.)	Embryogenic callus fresh wt (mg)	Non-embryogenic callus fresh wt (mg)	Embryos/inoculum (no.)
KNO_3	KCl	KH_2PO_4	NaNO_3	NH_4NO_3	K ⁺	N	$\text{NH}_4^+/\text{NO}_3^-$	NH_4^+/K^+						
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
Significance									L**	NS	NS	L**	L**	NS
Mean (SE)									---	116 (5)	7 (2)	---	---	8 (1)

^zStatistical analysis used the component of variance method: Significance = F test, L** = linear at $P = 0.01$, NS = not significant, NA = not available because of insufficient replications.

^yInocula 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.

Table 3. Effect of NaCl and KCl on callus growth of sweetpotato and residual effect on embryogenesis after callus transfer to media for embryo production.^z

NaCl effect ^y					KCl effect ^y			
NaCl or KCl (mM)	Total K ⁺ (mM)	Embryogenic callus fresh wt (mg)	Nonembryogenic callus fresh wt (mg)	Embryos/inoculum (no.)	Total K ⁺ (mM)	Embryogenic callus fresh wt (mg)	Nonembryogenic callus fresh wt (mg)	Embryos/inoculum (no.)
10	20	32	102	5	30	36	32	7
20	20	29	29	6	40	37	17	9
30	20	38	12	6	50	46	14	7
40	20	38	31	7	60	55	21	9
Significance		NS	NS	NS	L**		NS	NS
Mean (SE)		34 (3)	44 (19)	6 (1)	---		21 (3)	8 (1)

^zStatistical analysis used the component of variance method: Significance = F test, L** = linear at $P = 0.01$, NS = not significant.

^yInocula were 1-mg embryogenic callus fragments subcultured from callus proliferation media containing 20 mM K⁺.

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 NH_4Cl to media containing 12 to 40 mM KNO_3 were found optimal for embryogenesis (Wetherell and Dougall, 1976). In *Medicago sativa* (L.), optimal somatic embryo development required a minimum of 12.5 mM NH_4^+ (Walker and Sato, 1981).

In alfalfa, embryo development followed 2,4-D withdrawal combined with NH_4^+ 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 *Glycine max*, embryo development required removal of 2,4-D and a coordinated decrease of NH_4^+ from 40 to 20 mM and increase NO_3^- from 0 to 40 mM (Christianson, 1985).

Here we enhanced embryo production in sweetpotato by increasing both embryogenic callus growth and embryo devel-

Table 4. Embryo production in response to NH_4NO_3 concentration in sweetpotato and residual effect on embryogenesis of the NH_4NO_3 concentrations used in the callus proliferation phase.^z

Treatment (mM)							Embryos/inoculum ^y (no.)	
NH_4NO_3	KNO_3	KH_2PO_4	NO_3^-	N	NH_4^+/K^+	$\text{NH}_4^+/\text{NO}_3^-$	Calli from CP with 20 mM NH_4NO_3	Calli from CP with 2.5–20 mM NH_4NO_3
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**	NS
Mean (SE)							---	11.2 (1.6)

^zStatistical analysis used the component of variance method: Significance = F test, Q** = quadratic at $P = 0.01$, NS = not significant.

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

Table 5. Embryo production in response to ammonium concentration and total N in sweetpotato.^{z,y}

		Total N = 40 mM			Total N = 60 mM		Main effect NH_4^+
NH_4^+ (mM)	K^+ (mM)	NH_4^+/K^+	NH_4^+/N	Embryos/inoculum (no.)	NH_4^+/N	Embryos/inoculum (no.)	Embryos/inoculum (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
				L*		L*	L*Q*

^zStatistical 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 $\times$ NH_4^+ were not significant at $P = 0.05$.

^yInocula were 1-mg embryogenic callus fragments subcultured from callus proliferation media containing 20 mM NH_4^+ and 60 mM total N.

opment separately. First, the best embryogenic callus growth obtained with high levels of 10 to 20 mM ammonium was further promoted by increasing K from 20 mM to a 40 to 60 mM range. Second, the development of embryos triggered by the removal of 2,4-D and BA was enhanced by decreasing ammonium from 20 to 10 mM. We also demonstrated that while decreasing ammonium levels affected embryo development, decreasing levels of the metabolically competing K ion had no effect. Our experiments also show that the companion ions Na^+ and Cl^- at concentrations up to 40 mM have no deleterious effects on somatic embryo development.

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 NH_4^+ 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 NH_4^+ and 20 mM K^+ with a total N of 40 mM.

Literature Cited

Ammirato, P.V. 1983. Embryogenesis, p. 82-123. In: D.A. Evans et al. (eds.) Handbook of plant cell culture Macmillan, New York.

Brown, S., D.F. Wetherell, and D.K. Dougall. 1976. The potassium

requirement for growth and embryogenesis in wild carrot suspension cultures. *Physiol. Plant.* 37:73-79.

Cantliffe, D.J., J.R. Liu, and J.R. Schultheis. 1988. Development of artificial seed of sweet potato for clonal propagation through somatic embryogenesis, p. 183-195. In: W.H. Smith and J.R. Frank (eds.). Methane from biomass: a systems approach. Elsevier, New York.

Chaleff, R.S. 1983. Induction, maintenance, and differentiation of rice callus cultures on ammonium as sole nitrogen source. *Plant Cell Tissue Organ Cult.* 2:29-37.

Chée, R.P. and D.J. Cantliffe. 1988a. Somatic embryony patterns and plant regeneration in *Ipomoea batatas* Poir. In *Vitro Cell. Dev. Biol.* 24:955-958.

Chée, R.P. and D.J. Cantliffe. 1988b. Selective enhancement of *Ipomoea batatas* Poir. embryogenic and non-embryogenic callus growth and production of embryos in liquid culture. *Plant Cell Tissue Organ cult.* 15:149-159.

Christianson, M.L. 1985. An embryogenic culture of soybean: towards a general theory of somatic embryogenesis, p. 83-103. In: R.R. Henke et al. (eds.). Tissue culture in forestry and agriculture. Plenum, New York.

Clarkson, D.T. and J.B. Hanson. 1980. The mineral nutrition of higher plants. *Annu. Rev. Plant Physiol.* 31:239-298.

Dougall, D.K. and D.C. Verma. 1978. Growth and embryo formation in wild-carrot suspension cultures with ammonium as sole nitrogen source. In *Vitro Cell. Dev. Biol.* 14:180-182.

Fukunaga, Y., J. King, and J.J. Child. 1978. The different effects TCA-cycle acids on the growth of plant cells cultured in liquid media containing various nitrogen sources. *Planta* 139:199-202.

- Galzy, R. 1972. Remarques sur la nutrition minérale des apex de *Vitis rupestris*. C.R. Acad. Sc. Paris 275:561-564.
- Gamborg, O.L. and J.P. Shyluk. 1970. The culture of plant cells with ammonium salts as sole nitrogen source. Plant Physiol. 45:598-600.
- Halperin, W. 1966. Alternative morphogenetic events in cell suspensions. Amer. J. Bot. 53:443-453.
- Halperin, W. and D.F. Wetherell. 1965. Ammonium requirement for somatic embryogenesis *in vitro*. Nature (London) 205:519-520.
- Heller, R. 1953. Recherches sur la nutrition minérale des tissus végétaux cultivés *in vitro*. Ann. Sci. Natl. Bot. Biol. Veg. 14:1-223.
- Liu, J.R. and D.J. Cantliffe. 1984. Somatic embryogenesis and plant regeneration in time culture of sweet potato (*Ipomoea batatas* Poir.). Plant Cell Rpt. 3:112-115.
- Marschner, H. 1986. Mineral nutrition of higher plants. Academic, New York. p. 71-114.
- Martin, S.M., D. Rose, and V. Hui. 1977. Growth of plant cell suspension cultures with ammonium as sole source of nitrogen. Can. J. Bot. 55:2838-2843.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
- O'Neill, S.D. and R.M. Spanswick. 1984. Characterization of native and reconstitutive plasma membrane H⁺-ATPase from the plasma membrane of *Beta vulgaris*. J. Membr. Biol. 79:245-256.
- Poole, R.J. 1978. Energy coupling for membrane transport. Annu. Rev. Plant. Physiol. 29:437-460.
- Seitz Kris, M.H. and E.T. Bingham. 1988. Interactions of highly regenerative genotypes of alfalfa (*Medicago sativa*) and tissue culture protocols. In Vitro Cell. Dev. Biol. 24:1047-1052.
- Spanswick, R.M. 1981. Electronic ion pumps. Annu. Rev. Plant Physiol. 32:267-289.
- Tazawa, M. and J. Reinert. 1969. Extracellular and intracellular chemical environments in relation to embryogenesis *in vitro*. Protoplasma 68:157-173.
- Vasil, I.K. 1984. Cell culture and somatic cell genetics of plants. Plant regeneration and genetic variability. Academic, New York. p. 19-26.
- Walker, K.A. and S.J. Sato. 1981. Morphogenesis in callus tissue of *Medicago sativa*: the role of ammonium ion in somatic embryogenesis. Plant Cell Tissue Organ Cult. 1:109-121.
- Wetherell, D.F. and D.K. Dougall. 1976. Sources of nitrogen supporting growth and embryogenesis in cultured wild carrot tissue. Physiol. Plant 37:97-103.