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Plant regeneration from suspension culture and mesophyll protoplasts of *Medicago sativa* L.

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Abstract. A system was established for achieving plant regeneration from mesophyll protoplasts and cotyledon-derived cell suspension cultures of alfalfa, *Medicago sativa* L. Peeled leaflets or cells from 6-day-old cell suspensions were incubated in an enzyme mixture containing 1% Driselase, 1% Rhozyme, 0.1% Cellulase and 72 gl^{-1} mannitol at pH 5.8 for 2–16 h to liberate protoplasts. A complex Kao medium supported cell division and colony formation, whereas a high auxin/low cytokinin treatment on Schenk and Hildebrandt medium followed by culture on growth regulator-free Blaydes or Linsmaier and Skoog medium resulted in somatic embryo formation. Of the three varieties tested, Citation, Answer and Regen S, the latter two produced embryos from which plants could be regenerated.

Introduction

The application of tissue culture and genetic engineering techniques to crop improvement is dependent on our ability to regenerate plants from cultured tissues, cells and protoplasts. Current *in vitro* techniques being investigated to genetically alter alfalfa (*Medicago sativa* L.) whether via cell fusion, selection of genetic variants or recombinant DNA manipulation rely on the use of cultured cells and protoplasts. Previous reports have outlined somatic embryogenesis in alfalfa calli derived from a variety of tissues including immature embryos [21, 22, 25, 26, 27], petioles [7, 20, 26, 27], stems [20, 26, 27], cotyledons [20, 24], hypocotyls [20–22] and leaves [4, 16–18, 20]. In several of these studies [6, 16, 20, 21, 24, 25] whole plants have been recovered. Embryogenesis has been reported in cell suspension cultures [10, 17, 19–21] and plants recovered [16, 17, 19, 20] in some cases. Plants have also been regenerated from protoplasts [4, 7, 15–17, 28] and a variety of tissues including cotyledons [13, 14], leaves [4, 7, 9, 16], roots [13, 28], cell suspensions [16, 17] and leaf calli [15–17] have been used as donor tissue. Protoplasts have been isolated from mesophyll cells of other *Medicago* species [1, 6] and plants have been recovered in the case of *M. glutinosa* [1]. Work in North America on alfalfa has been restricted almost exclusively to only one line, Regen S, a recurrent selection of the cultivated varieties Saranac and Dupuis [2] which shows a superior in vitro embryogenic response.

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Because of the widespread use of Regen S as a model system in North America, we have based our technique development on this laboratory variety. During the course of this study we have also continually attempted to extend the results obtained with Regen S to other varieties. In the present work we report the successful regeneration of plants from cell suspension-derived protoplasts, outline the parameters which critically affect the success of the technique and compare the response of Regen S to two commercial varieties, Answer and Citation.

Materials and methods

Plant material. Seeds of *Medicago sativa* L. cv. Answer, Armor, Banner, Iroquois, Multileaf, Peak, Rambler, Saranac, 520, Thor and Citation were kindly provided by M. Faris, Agriculture Canada, Ottawa Research Station, Ottawa, Canada; cv. Rangelander by G. Lees, Agriculture Canada, Saskatoon Research Station, Saskatoon, Saskatchewan; cv. Regen S. by E. T. Bingham, University of Wisconsin, Madison, WI, U.S.A.

Seeds were surface-sterilized by immersion in 0.2% (w/v) $HgCl_2$, containing one drop of Tween 80 per 200 ml for 10 min followed by 3 washes of sterile distilled water and germinated on B_5 medium [5] lacking 2,4-dichlorophenoxyacetic acid and solidified with 9.0 gl^{-1} Bacto agar. Media were autoclaved at 121 °C for 17 min and all growth conditions were 25 °C under a 16 h photoperiod of fluorescent light (25 $\mu Em^{-2} s^{-1}$) unless indicated otherwise.

Shoot tips, isolated from 2- to 3-week-old seedlings, were maintained in 125 ml Erlenmeyer flasks on cotton pads soaked with B_5 medium lacking 2,4-D and modified to contain 80 mgl^{-1} Sequestrene Fe330 (Ciba-Geigy).

Plants maintained in growth chambers and the greenhouse were grown in flats of sterilized vermiculite, watered twice daily and fertilized once weekly with 20:20:20 CIL Plant Feeder (Canadian Industries Ltd., Kempville, Ontario), at a concentration of 2 gl^{-1}.

Tissue and cell culture. Leaf, hypocotyl and cotyledon explants from 2- to 3-week-old sterilized seedlings were induced to form callus on B_5 h medium which consisted of B_5 medium [5] modified to contain 1 mgl^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid), 0.2 mgl^{-1} kinetin, 30 000 mgl^{-1} sucrose, 3000 mgl^{-1} KNO_3, 895 mgl^{-1} $CaCl_2 \cdot 0$, 800 mgl^{-1} L-glutamine, 500 mgl^{-1} $MgSO_4 \cdot 7H_2O$, 100 mgl^{-1} serine, 10 mgl^{-1} L-glutathione, 1.0 mgl^{-1} adenine and 9 gl^{-1} Bacto agar. The medium was autoclaved at 121 °C for 17 min. Three week-old primary callus was used to establish cell suspension cultures in 125 ml Erlenmeyer flasks containing 45 ml of B_5 h medium (lacking Bacto agar) agitated at 125 rpm on a gyrotory shaker. The cells were maintained in a rapidly growing state by subculturing 2–5 ml of the topmost layer of settled cell clusters (Figure 1A) into 45 ml of fresh medium every 7 days.

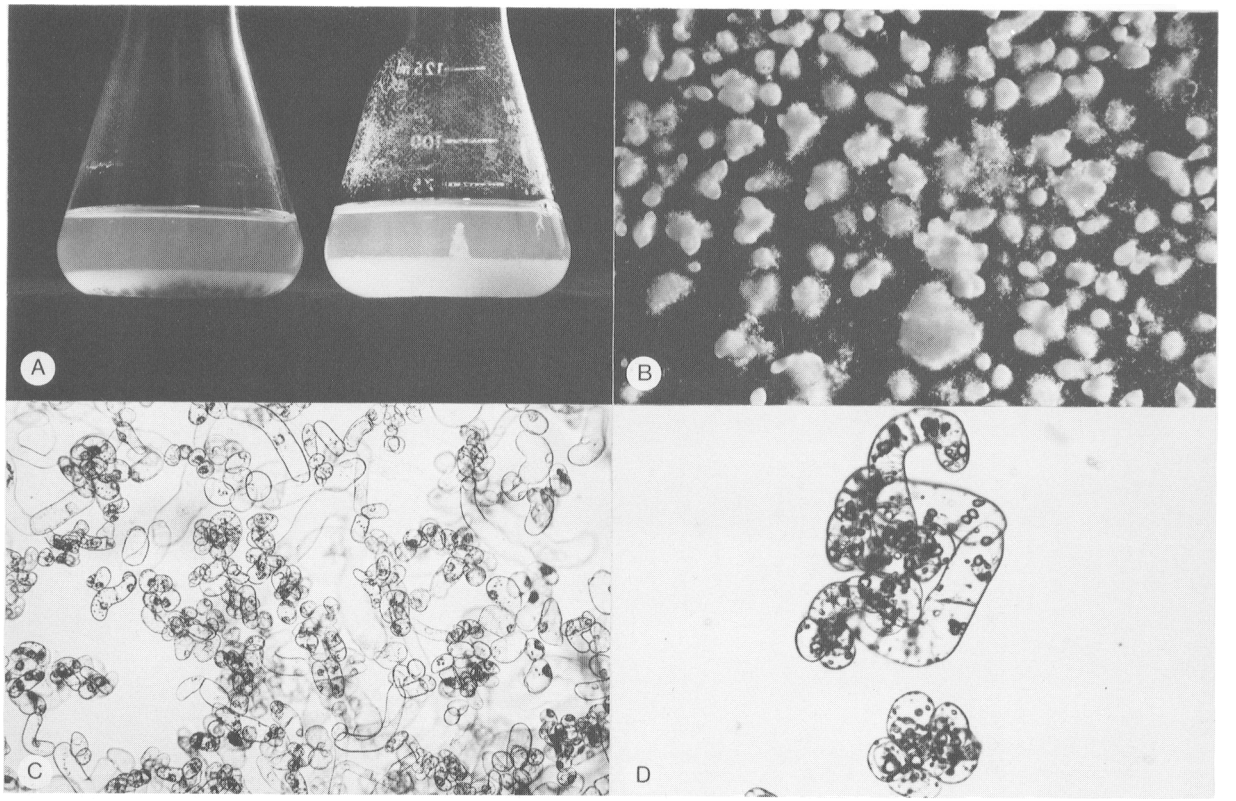

Figure 1. (A) Settled cell suspension of newly established heterogenous cell cultures of alfalfa cv. Rangelander (left) and selectively subcultured fine cell cultures of alfalfa cv. Regen S (right) in 125 ml Erlenmeyer flasks. (B) Thirty-eight-day-old hetergenous cell suspension of alfalfa cv. Answer (× 4). (C) Six-day-old fine cell suspension of alfalfa cv. Rambler used for protoplast isolation (× 160). (D) The two morphological cell cluster types of alfalfa cv. Regen S found in cell suspension cultures used for protoplasts isolation (× 500).

Protoplast isolation and culture. The enzyme solution used to generate protoplasts contained 10 gl^{-1} Driselase (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan), 10 gl^{-1} Rhozyme HP 150 (Rohm and Haas, Philadelphia, PA), 1 gl^{-1} Cellulase 'Onozuka' R-10 (Yakult Pharmaceutical Industry Co., Ltd., Nishinomiya, Japan) and 72.6 gl^{-1} mannitol with pH adjusted to 5.8 with KOH. The enzyme mixture was centrifuged at 1500 g for 5 min to remove the particulate matter and stored frozen in 50 ml lots at −20 °C. Just prior to use, the solution was thawed and filter-sterilized by successive passages through 1.2, 0.45 and 0.2 μm cellulose acetate/nitrate membrane filters.

For production of leaf mesophyll-derived protoplasts, donor plants whether maintained in the greenhouse or as a shoot tip culture were pretreated at 10 °C in the dark for 48 h. For greenhouse material, individual leaflets were sterilized by immersion in 70% ethanol for 15 s, and immersion in a 0.7% (w/v) calcium hypochlorite solution containing 1 drop of Tween 80 per 200 ml for 10 min followed by 3 rinses in sterile distilled water. The abaxial epidermal layer of the leaflets was then carefully peeled in a filter-sterilized solution of 72 gl^{-1} glucose and 200 mg^{-1} $CaCl_2 \cdot H_2O$ at pH 6.0 (EMI medium). The peeled leaflets were placed abaxial side down on 3 ml of enzyme solution in a 60 x 15 mm polystyrene petri dish, wrapped with parafilm and incubated in the dark at 25 °C for 5–7 h with gentle agitation once every hour.

For production of cell suspension derived protoplasts, 1.5 ml of settled cells (Figure 1A) from 6-day-old cultures was mixed with 8–10 ml of enzyme solution in 100 x 15 mm petri dishs, wrapped with parafilm 'M' laboratory film and incubated on a reciprocating shaker (40 strokes min^{-1}) at 25 °C under continuous fluorescent light (25 μEm^{-1} s^{-1}) for 16–18 h.

Following incubation, both types of protoplasts were separated from undigested cells and debris by successive passages through 125 and 44 μm nylon mesh filters, collected by centrifugation at 100 g for 3 min and washed 3× with EMI media. Yields were determined by haemocytometer count. Protoplasts were finally cultured in filter-sterilized Kao medium which consisted of Kao's [8] medium modified to contain 81 gl^{-1} glucose, 75 mg^{-1} penicillin G and 5 mgl^{-1} streptomycin sulfate. Leaf mesophyll were cultured in 50 μl droplets and cell suspension protoplasts were cultured in thin layers (3–5 ml) in 60 x 15 mm polystyrene petri dishes sealed with parafilm, under conditions of high humidity and low diffused fluorescent light (7 μEm^{-2} s^{-1}). After initiation of sell division (24 h), light intensity was raised to 25 μEm^{-2} s^{-1}. Plating density was approximately 5 x 10^3 ml^{-1} for leaf mesophyll and 1 x 10^5 ml^{-1} for cell suspension protoplasts.

Osmotic potential was reduced in the culture medium between day 17 and 21 by adding filter-sterilized-Kao_{12} medium which consisted of Kao's medium [8] modified to contain 36 gl^{-1} glucose and 5 gl^{-1} sucrose at a volume of 300–500 μl per petri dish. At 40–45 days, the cell colonies were diluted further with Kao_{12} medium and transferred to solid medium by

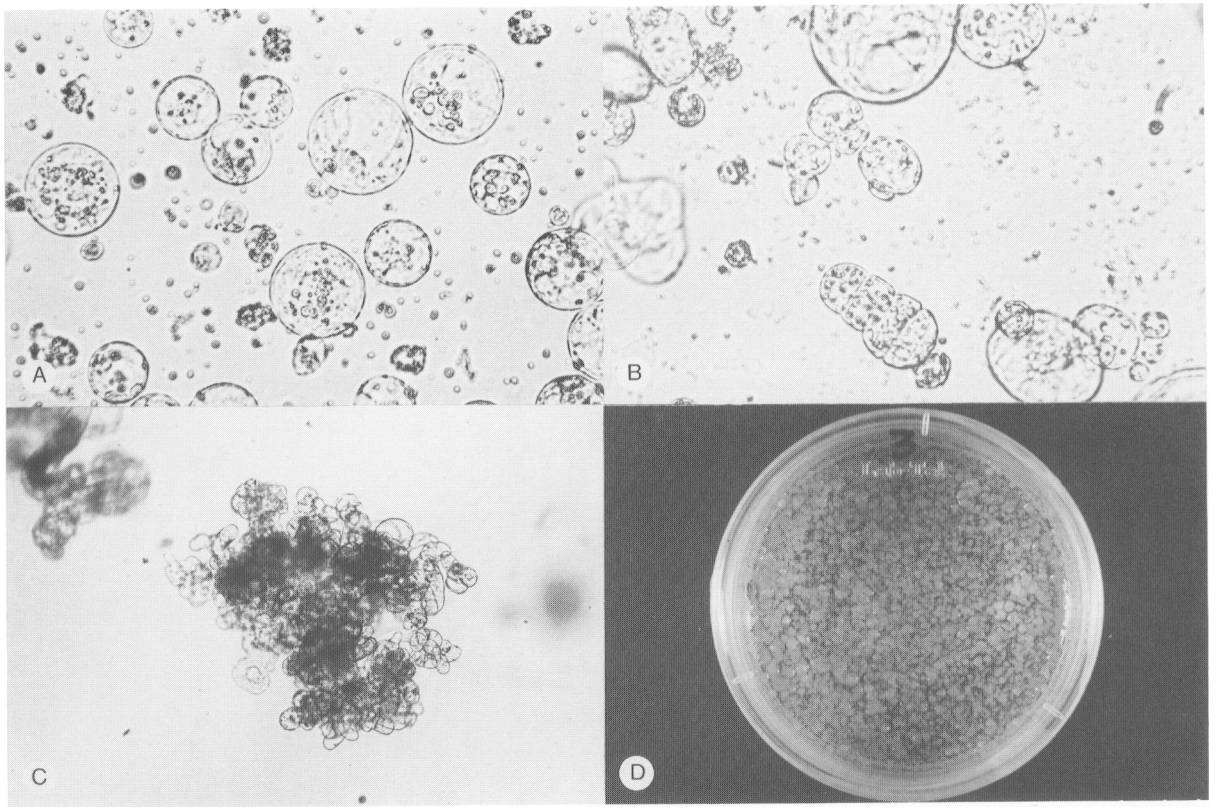

Figure 2. (A) Protoplasts of alfalfa cv. Regen S isolated, washed and plated in Kao_2 medium (X 640). (B) First and second cell divisions in seven-day-old protoplasts of alfalfa cv. Regen S (X 640). (C) Twenty-one-day-old colony of alfalfa cv. Regen S in Kao_2 liquid medium (X 100). (D) Forty-two-day-old colonies of alfalfa cv. Regen S in Kao_2/B_5h solid medium in a 60 X 15 mm petri dish.

mixing the Kao's medium containing the cell colonies with an equal volume of warm B_5h medium modified to contain 12 gl^{-1} Bacto agar (Kao_{12}/B_5h medium). When individual colonies were large enough to be handled with a small spatula (approximately 70 days from protoplast isolation), they were transferred to B_5h medium. For maintenance, colonies were subcultured onto B_5h medium every 28 days.

Plant regeneration. Cell colonies growing on either Kao_{12}/B_5h or B_5h could be induced to form embryos by transferring individual cell colonies (Figure 2D) onto SHb medium [26] which consisted of Schenk and Hildebrandt [23] inorganic and organic constituents plus 1 mgl^{-1} 2,4-D, 1 mgl^{-1} kinetin and 8 gl^{-1} Bacto agar. After 21 days on SHb, cell colonies were transferred to BOi2Y media [2] which consisted of modified Blaydes [2] inorganic and organic constituents plus 100 mgl^{-1} meso-inositol, 2 gl^{-1} yeast extract, and 10 gl^{-1} Bacto agar. Similar results could be obtained by substituting Linsmaier and Skoog [12] inorganic and organic components for those of Blaydes (SHb and BOi2Y media were autoclaved at 121 °C for 17 min). After 21–42 days on BOi2Y, large green embryos which had large fused cotyledons (Figure 3B) could be transferred to modified Schenk and Hildebrandt [23] or B_5 [5] medium lacking growth regulators with 10 gl^{-1} sucrose and 9 gl^{-1} Bacto agar. Individual somatic embryos continued development, were well rooted after approximately 14 days (Figure 3C) and could be transferred to Jiffy-7 peat moss containers in a mist chamber at this time. After 7–12 days in the mist chamber (Figure 3D), plants could successfully be potted and moved to the greenhouse.

Results and discussion

Preliminary studies with cell suspensions and callus cultures derived from cotyledon explants indicated that the embryogenesis response, especially in callus cultures, was strongly genotype dependent. Three of 13 varieties tested, Rangelander, Regen S and Rambler responded with 100, 67 and 53 percent, respectively, of individuals of those varieties forming embryos on the B_5h → SHb → BOi2Y media sequence. The varieties Answer, Armor, Banner, Citation, Iroquois, Multileaf, Peak, Saranac, Thor and 520 responded with between 20 and 0 percent of individuals forming embryos under the same media treatments. Various stages of differentiation were observed in the newly established cell suspensions of all above varieties but as observed in callus cultures not all individuals from each variety were capable of embryogenesis under the above conditions. These observations are consistent with those of Bingham et al. [2] who found similar variation in nine cultivars screened for regeneration from hypocotyl-derived callus. Kao and Michayluk [9, 10] have also reported this genotypic variation for embryogenesis in callus-derived from leaf mesophyll protoplasts and cell suspensions

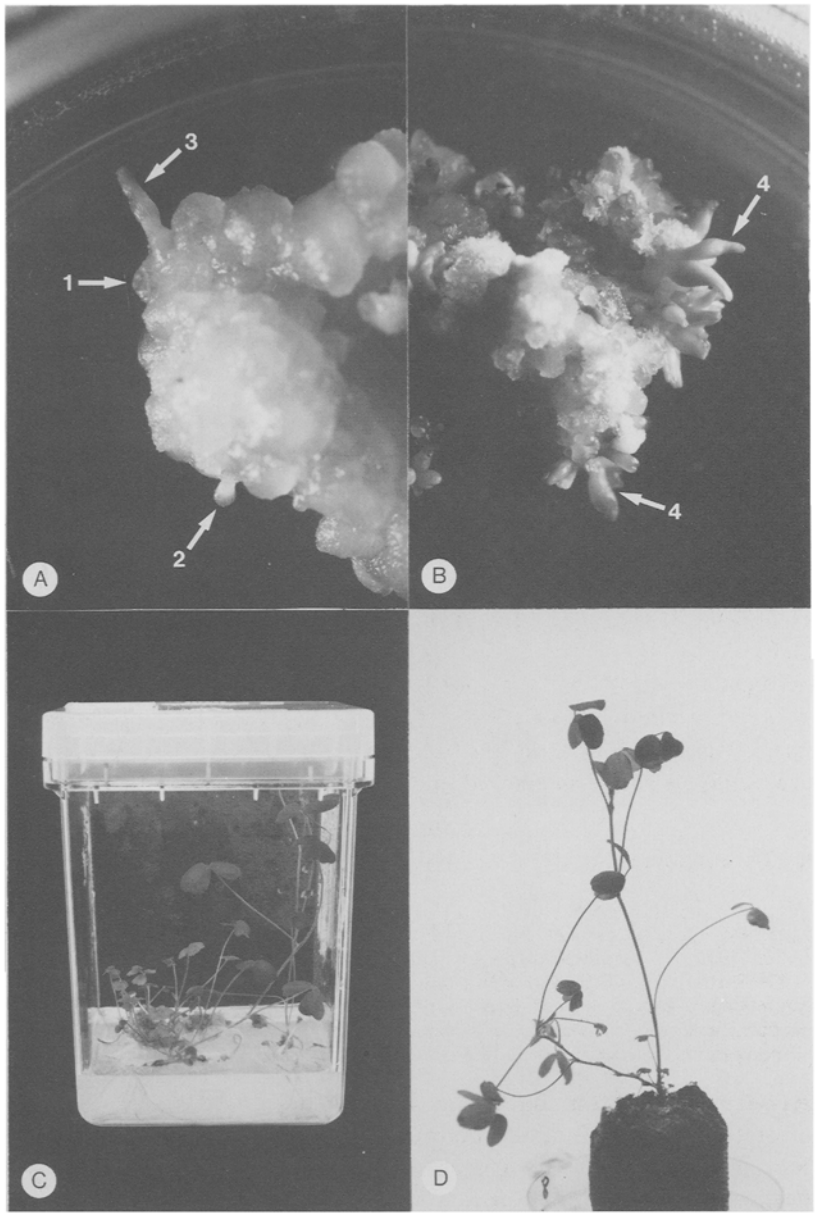

Figure 3. (A) Early embryo production on callus from cell suspension-derived protoplasts of alfalfa cv. Regen S on BOi2Y medium after about 100 days. The numbered arrows indicate progressively stages of embryo formation (4 ×). (B) Mature somatic embryos of alfalfa cv. Regen S on BOi2Y medium after about 120 days. The numbered arrows indicate clusters of mature embryos prior to germination (× 2.4). (C) Germinating isolated embryos of alfalfa cv. Rambler on B_5 medium lacking growth regulators (× 0.75). (D) Rooted seedling of alfalfa cv. Regen S during removal from mist bed (× 0.5).

Table 1. Frequency of embroyogenesis in 38 day-old cell suspension cultures derived from cotyledon tissue used for protoplast production

Variety	Cell cluster stage (frequency)				
	Unorganized	Globular embryo	Topedo embryo	Cotyledon embryo	Roots
Answer	205 (0.24)	103 (0.12)	37 (0.04)	12 (0.02)	490 (0.58)
Citation	186 (0.22)	375 (0.44)	78 (0.09)	25 (0.03)	186 (0.22)
Regen S	352 (0.39)	372 (0.41)	116 (0.13)	26 (0.03)	38 (0.04)

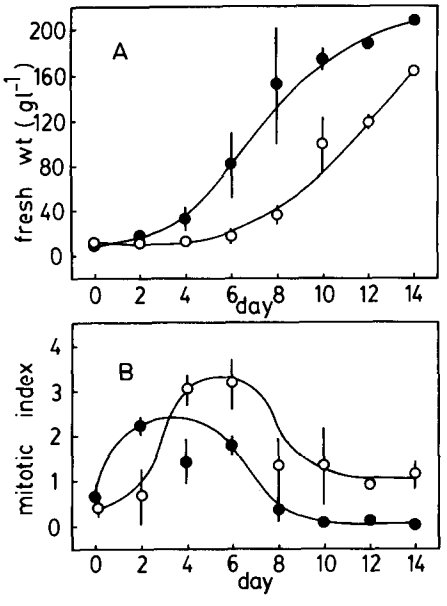

Figure 4. (A) Fresh weight (gl^{-1}) of a 10-month-old slow-growing culture (open symbols) and a 14-month-old fast-growing (closed symbols) culture of alfalfa cv. Regen S. (B) Mitotic index of slow-growing 10-month-old cell suspension (open symbols) and a fast-growing 14-month-old cell suspension (closed symbols) of alfalfa cv. Regen S. The mitotoic index was calculated as (# cells in cell division/# cells observed) X 100. Each point represents the mean ± standard error of three replicate experiments.

derived from isolated shoot tips. Coupled with our observations with cotyledon, hypocotyl and leaf-derived cell suspensions, and callus cultures, genotypic variation in embryogenesis would appear to be a widespread phenomenon with alfalfa. This may not be surprising as alfalfa is an open-pollinated species with a great deal of intervarietal heterogeneity.

Callus tissue formed readily on cotyledon, hypocotyl and leaf tissue cultured on B_5h solid medium and would disperse into a cell suspension when transferred to liquid B_5h medium. The newly established cell suspensions (Figure 1A, B) had a heterogenous cell population with respect to cellular morphology and contained not only single cells and small cell clusters but

also somatic embryos at various stages of formation (Table 1). The protoplast yield and seven day division efficiencies of this type of cell suspension was poor (i.e. 10^4 protoplast ml^{-1} yield and 2% division efficiency 7 days after isolation) and up to eight subcultures of selection was required to obtain a more homogenous culture as shown in Figure 1C, D.

A strict subculture/sampling protocol was found to be necessary to maintain the cell suspensions in a state which was most responsive to protoplast isolation. When grown at low density under a 7 day subculture cycle, cells were small, meristematic-like and grew in clusters of about 8–16 cells as shown in Figure 1C, D. Under these conditions, cells had a characteristic growth curve with about a 4 day lag phase (Figure 4A) and a peak of cell division at about day 6 (Figure 4B). After about 8 months, a more dense, faster growing population of cells arose. In contrast, this second, fast growing cell suspension exhibited little or no lag phase with cells beginning to divide immediately after subculturing and a peak in cell division at about day 3 (Figure 4A, B). This latter pattern of cell growth is similar to that reported for a diploid cell line HG2 [19]. The shift in cell growth pattern was correlated with an increase of elongate, vacuolated cells and a reduction in division efficiencies and regeneration ability in the isolated protoplasts. In HG2 cell suspensions [19] and HG2 and Saranac callus cultures [19, 22], a loss of regeneration capacity was also observed with repeated subcultures. The rapid loss of regeneration capactiy (within four months) in HG2 was correlated with an increase in the ploidy level of the cells. We observed a more gradual loss of regeneration capacity in protoplast-derived colonies from cell suspensions of Regen S with about 13% of the protoplast derived callus colonies still capable of regeneration after 16 months in culture. This compares to an original regeneration rate of 48% after 3 months in culture. About 50% of the calli retained the tetraploid level with about 40% undergoing polyploidization to the $8x$ level and 10% to the $16x$ level. Some chromosome loss was observed at each ploidy level. Older cell suspensions (>8 months) were particularly prone to this type of growth pattern shift.

Although there was some variation in the growth pattern of the cell suspensions as outlined above, a day 6 sample time in the slow growing cell suspensions consistently represented cells in the late lag to early logarithmic phase of growth. These cells yielded large quantities of protoplast which varied in size (Figure 2A) from 10–100 μ in diameter with about 95% of the protoplasts in the 10–20 μ range. Protoplast yields from 1×10^5 cell clusters/ml of cells suspensions were about 1×10^4 after 2 h, 1×10^5 after 4 h, 1×10^6 after 16 h. On a 7 day subculture cycle, protoplasts produced from cells sampled at day 6 showed the highest division efficiencies between 7 and 10 days after isolation. Although division efficiencies of 30–50% are typical, efficiencies of over 80% have been observed with Rambler. Immediately after subculture of the cell suspension a dramatic drop was observed in the division efficiency response of the protoplasts (Figure 5A). During this time a low

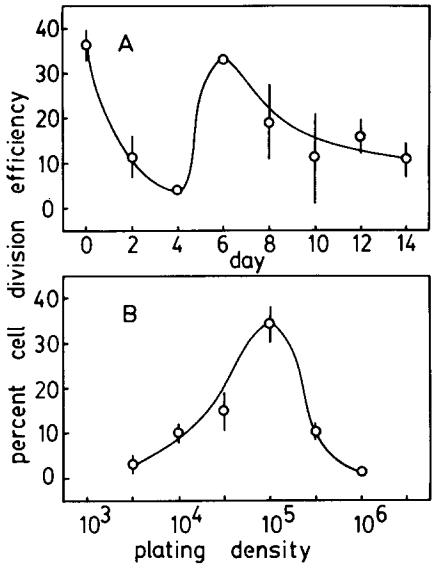

Figure 5. (A) Percent cell division efficiency at day 7 of protoplasts sampled from an alfalfa cv. Regen S cell suspension at different days after subculture and plated at 1×10^5 cells ml^{-1}. Each point represents the mean ± standard error of three replicate experiments. (B) Percent cell division efficiency at day 7 for protoplasts plated in a thin layer at different densities. Each point represents the mean ± standard error of four replicate experiments.

Table 2. Growth regulator combinations used for culture of protoplasts in modified Kao's [12] medium

	Media					
Compound (mgl^{-1})	Kao_1	Kao_2	Kao_3	Kao_4	Kao_5	Kao_6
2,4-dichlorophenoxyacetic acid	0.2	0.2	0.2	0.2	0.2	–
α-napthaleneacetic acid	1.0	1.0	1.0	1.0	1.0	–
Zeatin	0.5	–	–	0.25	0.2	–
Benzylamino purine	–	0.5	–	0.25	0.2	–
Kinetin	–	–	–	–	0.2	0.5
Benzothiazole-2-oxyacetic acid	–	–	–	–	–	10.0

mitotic index was also observed (Figure 4B) in the donor cell suspensions. Indeed, the rise and subsequent fall in the division efficiency response of the protoplasts correlates well with an accompanying rise and fall of the mitotic index of the cell suspension (Figures 4B, 5A). This is not surprising as donor tissue conditions and isolation conditions can dramatically affect both the yield and stability of isolated protoplasts as well as their subsequent metabolic activity and/or division efficiency [11]. As with the sample time, protoplast plating density also appears to have a relatively narrow optimum

of about 1×10^5 protoplasts ml^{-1} when plated as a thin layer (Figure 5B). Highest division efficiencies were consistently observed in dishes where protoplasts were plated as thin layer rather than as hanging drops or small (50 μl) droplets.

Protoplasts, when cultured in Kao_2 media, divided as early as 24 h after isolation with a peak in cell division activity between 7 and 10 days. The small, less vacuolated protoplasts appeared to divide at the highest frequency (Figure 2A, B) and small cell colonies (Figure 2C, D) could be isolated as early as 10 days after protoplast production. Of the six growth regulator combinations tested (Table 2), the combination of 0.2 mgl^{-1} 2,4-D, 1.0 mgl^{-1} NAA (α-napthaleneacetic acid) and 0.5 mgl^{-1} BAP (benzylamino purine) gave the best results (Table 3). In combination with 2,4-D and NAA, BAP was clearly a superior cytokinin to zeatin and kinetin for induction of cell division and cell colony formation. As well, the cytokinin supply appeared to be important during the time of embryo induction. In contrast to the stimulatory effect in protoplast culture, BAP was inhibitory to embryo formation and kinetin appeared to favor embryogenesis (Table 4). Furthermore, when 'induced colonies' were transferred to Linsmaier and Skoog media containing yeast extract or media containing 0.1 mgl^{-1} 2,4-D plus one of 1 mgl^{-1} kinetin, BAP, 2iPA (2-isopentyladenine) or zeatin, embryos developed only on media lacking growth regulators that had been induced by either a high 2,4-D media with kinetin or 2iPA. The presence of BAP or zeatin in the induction media or the presence of cytokinins in combination with a low level of 2,4-D during the time of embryo development was clearly inhibitory.

The inductive role of 2,4-D in somatic embryo formation has been demonstrated [22, 25, 26], however, the induction phenomenon is not specific to 2,4-D [10] as NAA will replace 2,4-D in some cases. Also, the presence of a cytokinin appears not to be essential for embryo induction [22], however, Walker et al. [27] suggests that both 2,4-D and kinetin are required in the induction medium for precise control of organ formation. Our results (Table 4) suggest that the cytokinin requirement during embryo induction is not kinetin specific but kinetin appears to give a better response than BAP, 2iPA and zeatin. Furthermore, there is a nutrition/growth regulator interaction [25, 27] which has been attributed to the ammonium ion concentration [25]. There has been no systematic evaluation of nutrition/growth regulator interactions and only 2,4-D and kinetin interactions have been studied in any detail [22, 27].

In our studies, embryos developed in an asymchronous manner, often in small clusters, at or near the surface of the callus growing on BOi2Y medium (Figure 3A, B). Embryos do not germinate readily on BOi2Y medium especially when left in contact with the callus tissue or other smbryos (Figure 3B). A high proportion show abnormal development such as fused cotyledons

Table 3. Protoplast colony recovery in Kao [12] medium when planted at $\simeq 1 \times 10^5$/dish

Media	% division efficiency at 7 days				No. colonies/dish at 45 days			
	Answer	Citation	Regen S	Reagen S (mesophyll)	Answer	Citation	Regen S	Regen S (mesophyll)
Kao_1	6.5	13.4	6.3	2.1	296	264	336	0
Kao_2	10.1	21.1	34.2	13.3	750	1424	2712	336
Kao_3	7.3	8.2	6.8	6.6	330	296	384	204
Kao_4	9.1	11.5	15.7	11.6	560	288	1770	162
Kao_5	7.0	4.1	7.3	4.7	280	245	368	72
Kao_6	3.3	2.1	0.1	3.2	8	12	18	2

Table 4. Frequency of embroygenesis in protoplast-derived cell colonies as influenced by various cytokinins on Schenk and Hildebrandt [28] medium followed by Linsmaier and Skoog [16] medium as outlined in the Materials and methods

Media combinations	Regen S (leaf mesophyll)		Regen S (cell suspension)		Answer (cell suspension)		Citation (cell suspension)	
	% of colonies with embryos	Mean No. of mature embryo/colony	% of colonies with embryos	Mean No. of mature embryo/colony	% of colonies with embryos	Mean No. of mature embryo/colony	% of colonies with embryos	Mean No. of mature embryo/colony
SH_1 11 mgl^{-1} 2,4-D 1 mgl^{-1} kinetin	83	12	28	3	6	3	0	0
SH_2 11 mgl^{-1} 2,4-D 1mgl^{-1} BAP	72	13	0	0	0	0	0	0
SH_3 11 mgl^{-1} 2,4-D 1 mgl^{-1} 2iPA	72	18	17	10	0	0	0	0
SH_4 11 mgl^{-1} 2,4-D 1 mgl^{-1} zeatin	56	11	0	0	0	0	0	0

and recallusing with a recovery rate of about 26%. All of the recovered plants came from embryos which were isolated from both callus and adjacent embryo tissue (Figure 3C, D) or had the growing shoot excised and adventitious roots induced on B_5 medium lacking growth regulators. Similar observations have been noted for culture of European Lucerne [4].

Finally, our observations that regeneration is via embryogenesis rather than organogenesis is consistent with the majority of tissue culture studies [1, 4, 9, 10, 13, 15, 16, 17, 18, 20, 25, 28] on alfalfa. Reports of organogenesis come mainly from European labs which have not used the Regen S variety and where developing structures were referred to as 'buds' [17, 21, 22, 24]. In other cases [2, 25, 26, 27], it is apparent that the 'buds' that were first described to be a result of organogenesis may actually have been embryos. The only other reports of alfalfa regeneration via organogenesis [4, 14, 28] come from one laboratory and are cited with only one variety, cv. European Lucerne.

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