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DEVELOPMENT OF SUGARBEET TISSUE CULTURE SYSTEMS: SOMATIC EMBRYOGENESIS, AND EVALUATION OF ALTERNATIVE NITROGEN SOURCES

presented by

Chia-Jung Tsai

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J W Saunders
Major professor

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DEVELOPMENT OF SUGARBEET TISSUE CULTURE SYSTEMS : SOMATIC EMBRYOGENESIS, AND EVALUATION OF ALTERNATIVE NITROGEN SOURCES

By

Chia-Jung Tsai

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ABSTRACT

DEVELOPMENT OF SUGARBEET TISSUE CULTURE SYSTEMS : SOMATIC EMBRYOGENESIS, AND EVALUATION OF ALTERNATIVE NITROGEN SOURCES

By

Chia-Jung Tsai

Somatic embryogenesis as well as sole nitrogen (N) sources for shoot cultures, leaf disc cultures, and suspension culture platings were examined for the purpose of further developing sugarbeet (*Beta vulgaris* L.) tissue culture systems. Somatic embryos could be used for propagation, for artificial seeds, or for gene transfer procedures if adequate methods for initiation and development could be devised. Two genotypes, REL-1 and REL-2, were used to measure the level of embryo production from suspensions plated on MS (Murashige and Skoog) medium containing combinations of abscisic acid (ABA) with different concentrations of other growth regulators, sucrose, or different sole N sources. Abscisic acid at 0.1-0.3 mg/L consistently improved embryo production in all experiments, and stimulated shoot production as well. A combination of NAA (0.3 mg/L) and ABA (0.1 mg/L) gave the highest embryo yield (77 embryos per ml of suspension plated out). REL-2 was a much more prolific embryo and shoot producer than REL-1. The range of embryo production was more than thirty-five fold between the two genotypes, whereas the range of growth regulator effects was no greater than ten-fold. There was no difference in the conversion rate of embryos into complete plantlets according to different length categories (0.5-1.9; 2.0-2.9; 3.0-3.9 mm) in either REL-1 (78, 81, and 85%, respectively) or REL-2 (87, 89, and 87%, respectively).

In order to initiate development of a system for making artificial seeds, isolated somatic embryos were encapsulated. With REL-2, the encapsulation of embryos with 2% sodium alginate in MS medium or in H_2O resulted in 81%, and 64% conversion, respectively, when placed on nutrient

agar plates. After 64 days of storage at 4°C, the conversion rate of embryos encapsulated with alginate in MS medium or in H_2O fell to 45% and 20%, respectively.

In order to identify media for cell selection of biochemical mutants or of transgenics that might have reduced levels of nitrogenous processing impurities or to identify superior media for developmental responses, the ability of eight endogenous N-containing compounds to serve as sole N source (15, 30, 60, and/or 90 mM) for suspension plating callus growth, shoot culture, as well as leaf disc culture of clone REL-1 was evaluated. Nitrate (NO_3), ammonium (NH_4), glutamine (GLN), glutamate (GLU), and urea as sole N sources supported plating callus growth. NO_3, NH_4, urea, and GLN supported shoot multiplication and fresh weight increase of shoot cultures. NO_3, NH_4, urea, GLN, and proline (PRO) supported callus initiation from leaf discs; NO_3, urea, GLN, or PRO further supported shoot regeneration from the disc callus, compared to the MS N mix of NO_3 (20mM) and NH_4 (40mM). Glycine betaine (BET) and choline (CHO) did not support growth in any system. Platings on either NO_3 or NH_4 as sole N source did not differ in sensitivity to the nitrate uptake inhibitor phenylglyoxal (PGO), suggesting that PGO lacks the specificity for use in selection for mutants of nitrate uptake. The ability of GLN or GLU to serve as the N source may preclude their use for cell selection of genetic variants accumulating less of these processing impurities. Moreover, mutants or transgenics able to utilize BET might be selectable on media containing BET as a N source.

Dedicated
to my parents

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LITERATURE REVIEW

Sugarbeet, a naturally cross-pollinating biennial and herbaceous dicotyledon of the family *Chenopodiaceae*, is the most economically important sucrose-producing crop in the temperate and subtropical regions of the world. It supplies about 40 % of the world supply of sucrose, an extremely important commodity on the international market. Sugarbeets are cultivated, as a warm and cool season crop, between 30° and 60°N in Europe, Asia, North America, North Africa and in small areas in South America. Following the discovery by Marggraf in 1747 that beets contain sucrose, Achard developed a process for sucrose extraction and recovery from beets (Winner, 1993). Mass selection carried out in fodder beet from 1786 to 1830 by Achard and the von Koppy family improved the sugar content of sugarbeet from 6% to 9% (Smith, 1987). Presently, sugarbeet hybrids can approach 20% sucrose, or 19.1 tons of sucrose per hectare (in California) (Kaffka and Lemaux, 1996) under favorable growing conditions. Although advancements in cultural practices are partially responsible for these improvements, plant breeding has also played an important role in enhancing sugarbeet productivity.

Traditional methods for breeding sugarbeet are slow. New genetic manipulation techniques are being developed in order to isolate superior genotypes with favorable production characteristics and combining ability more rapidly and specifically. The in vitro techniques of shoot culture cloning, somatic cell selection, somaclonal variation, and gynogenesis have been developed for more efficient sugarbeet improvement (reviewed by Saunders et al., 1990). Currently, genetic transformation, and to some extent, somatic cell

selection, are the biotechnological vehicles for genetic improvement, especially for "stop loss" purposes, such as genetic resistance to pests and to herbicides.

Since progress using traditional plant breeding can be relatively slow, tissue culture has played an increasingly important part for plant breeding. Initially, different methods of in vitro multiplication were used in breeding programs. During subsequent years, progress was made in developing protocols for sugarbeet shoot regeneration with various systems, including organogenesis and somatic embryogenesis for use in somatic cell selection, protoplast manipulations, ovule culture, and genetic transformation. Current interest in sugarbeet somatic embryogenesis is related to proprietary procedures for gene transfer as well as to prospects for inexpensive field-oriented vegetative propagation systems for seed production or final crop establishment. The use of protoplasts as a tool in sugarbeet improvement has been refined in a novel way and applied to gene transfer in what may be the most efficient transformation system presently available. The quest to achieve reliable and efficient shoot regeneration that is genotype independent, if not long-term, continues to be critical for routine genetic transformation and somatic cell selection.

Adventitious shoot regeneration

Sugarbeet is somewhat unusual in that adventitious buds and shoots often form directly on in vitro petioles of in vitro treated with origin N^6-benzyladenine (BA). Early reports documented direct adventitious shoot regeneration on petioles or midribs of old leaves of in vitro shoots multiplied on 0.25 mg/L BA supplemented medium (Hussey and Hepher, 1978), from leaves of shoot cultures on media containing cytokinin and auxin (Rogozinska

and Goska, 1978), from vegetative apexes and cotyledons of young seedlings on medium with BA or BA & GA (Atanassov, 1980), from flower buds of greenhouse plants on ½ MS (Murashige and Skoog, 1962) medium supplemented with 10 umol/L BA (Miedema, 1982), from shoot cultures from floral stalks on MS medium containing 0.25 mg/L BA (Saunders, 1982), and from inflorescence tips of field grown sugarbeet and wild beet (*Beta maritima*) plants on MS medium with 1 mg/L BA (Zhong et al., 1993). High frequencies of direct bud regeneration were observed from petioles of in vitro shoot cultures on several media containing mixtures of hormones, including 3 mg/L BA, 3 mg/L 1-naphthalene acetic acid (NAA) and 1 mg/L 2,3,5-triiodobenzoic acid (TIBA) for induction, and 3 mg/L BA and 3 mg/L NAA for regeneration (Detrez, et al., 1988), 0.4 mg/L BA and 0.1 mg/L indole-3-acetic acid (IAA) (Freytag et al., 1988), and 10 mg/L BA (Ritchie et al., 1989). In red beet (*Beta vulgaris*), multiple adventitious shoot formation was observed from single cells of the upper epidermis of petioles of cultured plantlets on MS medium containing several combinations of cytokinins with other growth regulators (Harms et al., 1983). The common component in all these media is BA, a strong synthetic cytokinin.

Adventitious buds arising from explants of other species have been suggested as a means of clonal propagation (Miedema, 1982; Rogozinska and Goska, 1978). Nevertheless, Van Harten et al. (1981) found that adventitious buds were a source of somaclonal variation in some species, for example, in potato. Therefore, somaclonal variation might also occur in sugarbeet via adventitious buds. Low levels of triploids and tetraploids in sugarbeet regenerated directly from blade and petiole explants of diploid

donor plants were found by Slavova et al. (1982). Abdel-Latif and Saunders (1985) detected no somaclonal variation in sugarbeet plants from adventitious buds. Nevertheless, diploid plants have been regenerated directly from explants of tetraploid plants (Zagorska and Atanassov, 1985).

Multiplication of axillary shoots

In general, axillary shoots have been multiplied on media with BA. Axillary shoots are rooted on a medium containing an auxin. Margara (1970) first reported that sugarbeet was grown in vitro and shoots have been induced from the flower buds. Hussey and Hepher (1978) indicated that axillary shoots were induced from the axils of the older leaves of in vitro seedlings on media with 0.12 or 0.25 mg/L BAP. Miedema et al. (1980) reported that axillary shoots grew out very rapidly when petiole explants from greenhouse grown plants with axillary buds attached were placed on MS media containing sucrose. Coumans-Gilles et al. (1981) observed multiplication of axillary shoots from in vitro culture of inflorescence pieces on a medium containing IBA and BA. Miedema (1982) also reported that shoots were multiplied by axillary bud proliferation on a medium with 1.0 umol/L BA, after the adventitious shoots were initiated from flower buds. In fact, the technique of multiplication of existing shoot meristems by stimulating axillary shoot development was not successful with all genotypes (Miedema, 1982), since in some genotypes, their shoots were stunted or had thick and large leaves. Saunders (1982) observed the formation of generative shoots during plant regeneration from axillary buds on MS medium containing 1.1 uM BA. Majewska-Sawka and Jassem (1988) observed

that 2 mg/L BA in the medium was better for wild genotypes of the Section *Corollinae* and caused fast shoot culture initiation from flower-stalk tips.

Shoot regeneration from callus or suspension cultures

In general, callus requires an exogenous source of auxin and/or cytokinin for induction, growth and subsequent organ development. Hormone-autonomous cells or callus are capable of synthesizing part or all of the hormones to support growth, without needing exogenous auxin and/or cytokinin. De Greef and Jacobs (1979), and Kevers et al. (1981) obtained nonorganogenic habituated callus lines from leaf pieces which had been initiated on auxin plus cytokinin media, then subcultured on a basal medium without hormone. Saunders and Daub (1984) reported that hormone-autonomous callus, which was initiated in high frequency from shoot cultures on MS medium with 0.25 mg/L BA, sustained growth on hormone-free medium with no reduction in growth rate for at least 6 months, and it regenerated shoots as well. Callus was initiated from leaf discs of several germplasm sources on hormone-free MS medium (Doley and Saunders, 1989), with mean callus initiation time elevated to 96.7 days, which was considerably longer than was the case when 1.0 mg/L BA was included in the initial medium (4-6 weeks). Moreover, shoots were regenerated on the hormone-free medium without subculture of the callus (Doley and Saunders, 1989). Masuda et al. (1988) also indicated that hormone-autonomous callus was initiated from young leaf explants in liquid MS medium with 0.25 mg/L BA. Then, following transfer to hormone-free liquid medium, cells could further divide and grow autonomously in suspension cultures. Moreover, Van Geyt and Jacobs (1985) reported

that habituated (hormone-autonomous) cells were obtained following a gradual reduction in exogenous auxin and cytokinin levels, but subsequently the cells dedifferentiated and lost regeneration capacity. Hagege et al. (1991) indicated that habituated sugarbeet callus and cell suspensions exhibited a shorter linear growth phase, although they divided actively. The habituated cell lines were defective in cell expansion, cell differentiation and cell wall development under microscopic observations. Such cultures exhibit all the characteristics of vitrified tissue with much more watery. The peroxidase activities of those cell lines were very low (Hagege et al., 1990). Moreover, they produced much less ethylene than normal (auxin and cytokinin-dependent) callus (Gaspar et al., 1988, Hagege et al., 1990). Gaspar et al. (1992) also reported that habituated callus lines exhibited many characteristics of vitrified tissues. The vitrified shoots became habituated under micropropagation, then progressively lost the organogenic capacities.

Enomoto and Ohyama (1985) reported callus induction and shoot formation from in vitro petioles on various combinations of BA (0.1-5.0 mg/L) and NAA (0 or 0.1 mg/L). Saunders and Shin (1986), and Saunders and Doley (1986) developed a very simple system for obtaining callus and subsequent high-frequency shoot regeneration of several unrelated genotypes, using leaf explants on MS medium containing 1 mg/L BA. This high frequency but unconventional system was based on hormone-autonomous callus. Further, Owens and Eberts (1992) modified the original protocol of Saunders and Doley (1986) for regenerating shoots from leaf explant callus by plating multiple leaf discs (ten 4-mm discs/plate) on RV medium (an enriched modification of MS medium) containing 1 mg/L BA and solidified with 0.3% Gelrite, and by transferring the discs to fresh medium every

two weeks during the first month. Thereby, the modified protocol produced more callus per plate and higher rates of morphogenesis per unit dry weight of callus than did the one-step, and one disc/plate methods of Saunders and Doley (1986).

Yu (1989) examined callus induction and differentiation from leaf explants of 16 germplasm sources of the Genus *Beta* on MS medium containing BA. The leaf explants from most *Beta* species exhibited optimum callus formation and shoot differentiation at 31°C. A large amount of callus was induced in *B. vulgaris* L., *B. maritima* L., *B. intermedia* Bung., and *B. macrocarpa* Guss, but shoots were only regenerated in *B. vulgaris* and *B. macrocarpa*.

Hooker and Nabors (1977) reported that the anti-auxin TIBA in combination with exogenous BA promoted shoot initiation from cotyledon and hypocotyl callus, but the regeneration rate was low. However, Tetu et al. (1987) found that high percentages (52%) of shoot formation occurred with combinations of TIBA and BA. Doley (1990) also reported that TIBA stimulated shoot regeneration from subcultured callus of REL-1. Abe et al. (1991) indicated that 2 uM TIBA promoted shoot morphogenesis from callus culture (from the shoot region of the germinated seed) in the presence of 5 uM BAP. The combination of TIBA and BAP was also found to be the most favorable for callus induction from hypocotyl explants derived from 21 day-old-seedlings (Jacq et al., 1992), although the combination gave only a low callus formation from leaf explants from 6-month-old plants (Roussy et al., 1996).

Recently, Roussy et al. (1996) reported that the type of cytokinin is important factor for callus induction in sugarbeet, since callus induction frequency was significantly higher on a

medium containing 2.2 mg/L thidiazuron (TDZ) medium than on one containing 1 mg/L BAP.

Callus production for shoot regeneration was affected by explants and/or genotypes (Saunders and Doley, 1986; Ritchie et al., 1989; Catlin, 1990). Saunders and Doley (1986) mentioned that smaller less expanded leaves were more responsive than larger leaves from the same plant, and blade explants were superior to petiole explants for callus induction with the more expanded leaves. Therefore, they proposed that the critical leaf size may differ with various genotypes and might be associated with the incompleteness of leaf expansion. Genotype and medium interaction may also affect in vitro responses. Jarl and Bornman (1986) observed significant genotype and medium interaction for callus growth from 15 sugarbeet genotypes. Shimamoto et al. (1993) achieved shoot regeneration from friable calli initiated from the shoot region of the germinated seed for eight of the 21 sugarbeet germplasms.

Actually, genotype plays a key role in organogenesis and embryogenesis, for callus initiation, shoot regeneration and, to a lesser extent, root formation are affected by genotype. REL-1, an especially useful and tissue-culture friendly genotype, was released to the public by J.W. Saunders in 1987. REL-1 has a heterozygous dominant annualism nature, which allows it or its direct tissue culture genetic manipulation products to flower much more rapidly than the ordinary biennial beet the farmer grows. Another clone, REL-2, was developed specifically for superior embryogenesis and shoot regeneration for application in biotechnology, REL-1 being one of its parents.

Miscellaneous use of callus or suspension cultures

Welander (1976) mentioned that callus growth initiated from hypocotyl explants could be affected by varying nitrogen and sucrose concentration in the media but the influence could not clearly be related to any particular IAA level. The nitrogen content of the callus tissue was higher when nitrogen concentration in the media was increased. Nevertheless, the nitrogen content of the tissue decreased when the sucrose level was risen.

Zelmer and Gunther (1988a) examined the activities of glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamate synthase (GOGAT) from the suspension cultures of *Beta vulgaris* and *Chenopodium album*. The level of the enzymes was not change during the exponential growth of the cell culture. GDH and GS activities reached a high level, the value of GOGAT was found to be approximately 1/12 the amount of GS. Adding twice the amount of nitrate to the suspension medium, depressed growth, stimulated GS activity, and reduced GDH activity; adding twice the amount of ammonia, stopped cell growth, increased GDH activity, and eliminated GS activity. In a follow up study, Zelmer and Gunther (1988b) further reported that the inclusion of 13 herbicides in the suspension medium caused an inhibition of the enzymatic activities of GDH and a stimulation of GOGAT within 7 days after herbicide application.

Coutts et al. (1994) detected elicitation of stress-induced proteins in suspension-cultured sugarbeet cells. Phenylalanine ammonia lyase (PAL) activity increased after treated of the suspension-cultured cells with elicitors (including cell wall extracts of *Botrytis cinerea* or chitosan oligomers). The suspension cells responded to these elicitors by inducing the synthesis of more than 10 polypeptides.

Eckermann and Baumann (1995) demonstrated enzymatic changes in callus cultures of sugarbeet during the transition from photoheterotrophic to photoautotrophic growth. A decrease in the sucrose level of the medium caused an increase in Calvin cycle enzyme activity, whereas the activity of dissimilation related enzymes either decreased or was not significantly altered.

Somatic embryogenesis

One of the most dramatic achievements in plant tissue culture has been the discovery of the phenomenon of somatic embryogenesis. It demonstrated the totipotency of cells of higher plants. Somatic embryos, derived from somatic cells, are capable of developing into complete plantlets via a series of developmental stages and morphological changes, i.e., proembryo, globular, heart- and torpedo-shaped, as well as mature embryo. Somatic embryogenesis is strikingly similar to morphogenesis occurring after the fertilization of the egg cell. Somatic embryos have been induced from callus, suspension culture cells, zygotic embryos, or leaf and stem explants of many species. Multiple embryos form in many cases, under appropriate culture conditions, with genotypes and growth regulator dependency being common. The large-scale production of somatic embryos for many high-value crops is now becoming possible. However, only a few reports are available on somatic embryogenesis in sugarbeet, mostly anecdotal. Atanassov (1976) first mentioned somatic embryo occurrence from suspension cultures in sugarbeet. Tetu et al. (1987) concluded that multiple hormonal sequences were necessary for the induction and development of somatic embryos from calli derived from petiole explants. Freytag et al. (1988) noticed

somatic embryogenesis at a low frequency from callus (initiated from petioles of cultured shoots) in all six sugarbeet germplasm sources tested, using a highly supplemented medium containing 10 vitamins, 6 amino acids, and a combination of indole-3-butyric acid (IBA) and BA. Only 2 % of the callus pieces formed somatic embryos, although most embryos developed into plantlets. Pedersen and Andersen (1988) reported that embryogenic callus was induced on PG_o medium (De Greef and Jacobs, 1979) containing BAP, and the somatic embryos proliferated on 1/3 MS medium with BAP, IAA, and GA_3. Whether all these hormones were necessary is unclear. Doley and Saunders (1989) showed the simple production and partial germination of somatic embryos from leaf disc callus of a fodder beet cultivar without the use of growth regulators, although embryos were not seen for at least three months on the initial medium. Kubalakova (1990) obtained friable embryogenic callus at the base of shoot tip explants transferred onto MS or PG_o media without hormones, or on media containing BAP and kinetin. The embryogenic callus maintained its embryogenic ability for three years on hormone-free medium. Somatic embryos were induced from immature zygotic embryos by Tenning et al. (1992), who observed some directly secondary embryogenesis from the primary somatic embryos on MS medium containing 1 mg/L 2,4-D. D'Halluin et al. (1992) used embryogenic callus (which was initiated from seedlings on PG_o medium containing BA, IAA and GA_3) in an *Agrobacterium*-mediated genetic transformation system for sugarbeet.

Ovule culture

Gynogenesis has been the most effective technique for obtaining haploids in sugarbeet.

Haploid plants were obtained by Hosemans and Bossoutrot (1983) through gynogenesis by culturing mature excised ovules from male-sterile plants, with a haploid recovery frequency of only 0.23 %, based on initial number of ovules. In that case, 2.1 % ovules gave rise to calli or embryos, then 10.9 % of the structures developed into haploid plantlets after one or two months. Some of the roots on these haploid plantlets were uniformly diploid. In subsequent work, plants were recovered from 0.17 % of the unpollinated ovules (Bossoutrot and Hosemans, 1985). In that study, shoot meristems were haploid, but root tips were polyploid. Van Geyt et al. (1987) reported that up to 2.2 % of cultured ovules from male-fertile and male-sterile plants yielded haploid plantlets. The frequency of haploids was increased to 6.1%, when callus initiated from ovary culture was transferred to a medium containing 0.5% activated charcoal without 2,4-D. Thus, ovary culture may lead to haploid sugarbeet plants more efficiently than excised ovule culture. D'Halluin and Keimer (1986) showed that the variability in haploid recovery rate was affected by genotype, and the maximum response frequency achieved was 26%. Doctrinal et al. (1989) obtained haploid plants by in vitro culture and induction of embryogenesis of unpollinated ovules. In such gynogenesis, the rate of embryo induction and haploid plant regeneration was affected by both genotype and medium. Galatowitsch and Smith (1990) observed that haploid, diploid and tetraploid plants were regenerated from unfertilized ovule callus of diploid sugarbeet on MS medium. In addition, the vitrification of shoots was reduced when Gamborg's B5 (Gamborg et al., 1968) salts were used instead of MS salts in the regeneration medium. Ferrant and Bouharmont (1994) reported that haploid plants were obtained by gynogenesis directly via embryogenesis from

ovules on N6 medium containing IAA and kinetin, or BAP only. They also indicated that maturity of the female gametophyte was reached a few days prior to anthesis and therefore ovules from unopened flowers were already suitable for plating. A further study of the haploid cells of sugarbeet embryo sac during the first week of in vitro culture showed that the viable gynogenetic embryo originated only from the egg cell.

Anther culture

Regeneration of haploid plants has an important application in sugarbeet breeding. Various methods have been tried in order to obtain androgenic haploids in sugarbeet. Banbe and Tanabe (1972) induced only one single, non-rooted diploid shoot. Rogozinska et al. (1977) observed some regenerated shoots from one callus that was initiated from anther culture. Unfortunately, all of the regenerants were diploids, quite likely from somatic tissue, and no regeneration of haploid plants was obtained from anther culture. Van Geyt et al. (1985) indicated that macrocalli and nuclear division could be induced from anther cultures and free microspore cultures, but no plant regeneration was reported. Thus, so far, the recovery of haploid plants through androgenesis has not proven successful.

Protoplasts and somatic hybridization

Protoplast hybridization could be considered to study somatic cell genetic manipulations in sugarbeet. The major disadvantage of somatic hybridization for application in crop improvement has been the difficulty in achieving plant regeneration from protoplasts of

many plant species. In some early studies, no colony was formed from protoplasts which were isolated from leaves of greenhouse-grown sugarbeet plants (Nam et al., 1976; Binding et al., 1981; Bornman et al., 1982). Subsequently, callus colonies were induced from protoplasts of a sugarbeet cell suspension culture, but no plant was regenerated from protoplast derived cultures (Szabados and Gaggero,1985; Bhat et al., 1985). Bhat et al. (1986) only succeeded in regenerating roots (no shoots) from protoplast calli derived from shoot culture leaves in three diverse beet genotypes. None of these reports mentioned use of genotypes with demonstrated shoot or embryo regeneration capacity. Steen et al. (1986) only mentioned that the variability was found in regenerated plants from the protoplasts of the tetraploid line 68-3, but no detailed procedure for regenerating plants from protoplasts was provided. Krens et al. (1990) found that the inclusion of n-propyl gallate (a lipoxygenase inhibitor) in the isolation and culture media was crucial for cell division, colony formation and regeneration, although mesophyll protoplasts of sugarbeet showed a significant decrease in protoplast viability just one day after isolation. Nevertheless, plating efficiencies were still very low (from 0.005% to 1%). Subsequently, embedding protoplasts in alginate was found to increase the plating efficiency (Schlangstedt et al., 1992; Hall et al., 1993; Pedersen et al., 1993). In fact, the plating efficiencies of protoplasts from leaf mesophyll tissue was low and showed substantial variance (overview by Schlangstedt et al., 1994). Ritchie et al. (1989) proposed that sub-epidermal petiole parenchyma protoplasts might preferentially permit shoot regeneration. Schlangstedt et al. (1994) demonstrated that much higher plating efficiencies were obtained from petiole protoplasts (average 4.6%) than from mesophyll protoplasts

(average 1%). In addition, a higher proportion of protoplasts with doubled DNA content (i.e., nuclei in the G2/M stage of the cell cycle) was found in petiole protoplast populations via DNA histograms. However, no plantlets were obtained. Later, Lenzner et al. (1995) succeeded in regenerating plants from mesophyll protoplasts isolated from 2- to 3-week-old subcultured shoot cultures (instead of the methods described by Krens et al. (1990) using mesophyll protoplasts from seedlings). The mesophyll protoplasts from in vitro shoots showed high plating efficiencies up to 4%, then developed into callus. Two of the lines with friable callus containing starch grain cells frequently formed adventitious shoots in the light after cytokinin (BAP or TDZ) treatment (Lenzner et al., 1995). Recently, a high frequency of regeneration of plants was obtained via protoplast culture, and the technique was further applied to gene transformation (Hall et al., 1996). The protoplasts were isolated specifically from stomatal guard cells. The cells are the only progenitors of totipotent protoplasts in sugarbeet leaves, it was claimed, and were separated by using computer-assisted microscopy (Hall et al., 1995). Overall, many factors may influence shoot regeneration from protoplasts, such as genotype, source of explants for isolating protoplasts, isolating procedures, cultural conditions, media, plating density, and environment.

Somaclonal variation

Somaclonal variation in sugarbeet has been reviewed by Saunders et al. (1990). Somaclonal variation includes genetic or epigenetic change via in vitro procedures. Somaclonal variation arises during the tissue culture phase or from pre-existing genetic

variation within the explants. Reducing the length of the cell culture phase could reduce the occurrence of somaclonal variation. In sugarbeet, polyploidy and aneuploidy have been detected soon after the initiation of callus (Atanassov et al., 1978). Saunders and Doley (1986) found prolific regeneration from leaf disc callus on MS medium with 1 mg/L BA in a somaclonal variant. Steen et al. (1986) reported that within genotypes the frequency of variation was higher in plants regenerated from protoplasts than in plants regenerated from suspension cultures. In addition, the degree of variation was higher from suspension cultures than from callus. Yu (1987) observed that some regenerant plants from leaf disc callus displayed differences in leaf morphological and pigmentation. Plants of 36 and 38 chromosome number were obtained from 18 and 19 chromosome plants, respectively. Mixoploid plants containing roots with either doubled or undoubled chromosome number were also detected. Yu et al. (1991) further reported that polyploid regenerants usually developed wider leaf blades, while diploid regenerants developed smaller leaf length to width ratios. In one experiment, 73.5% of the experimental plants contained the 19-chromosome number of the donors, 23.1% of the plants had roots with doubled chromosomes, and 2.6% had mixoploids with sectors of diploid and tetraploid roots in the same plant (Yu et al., 1991).

Somatic cell selection

Somatic cell selection has contributed to crop improvement in sugarbeet. Somatic cell selection involves the application of some chemical or physical agents or environment to cultured cells or tissues with the intention of favoring the survival or multiplication of

certain variant cells in the cell population. For instance, stable resistance could be obtained by applying a herbicide concentration lethal to normal cells. Saunders et al., (1992) successfully recovered monogenic dominant sulfonylurea-resistant sugarbeet plants from somatic cell selection. They reported that acute somatic cell selection for sulfonylurea herbicide resistance involved unmutagenized cell clusters of clone REL-1 plated on MS agar medium with 2.8 nM chlorsulfuron and 1 mg/L BA. Shoots regenerated from the only surviving colony without subculture. Those shoots were further resistant to 28 nM chlorsulfuron incorporated in the rooting medium, a concentration that killed similar shoots of REL-1. Shoot resistance to chlorsulfuron in this single gene mutant, designated CR1-B, was 300 to 1000- fold greater than in REL-1. In greenhouse studies, CR1-B was cross-resistant to some other sulfonylurea herbicides tested (Hart et al., 1993). Two imidazolinone herbicide-resistant sugarbeet mutants, 93R30B and Sir-13, have been independently generated via somatic cell selection against the imidazolinone herbicide Pursuit (according to the system of Saunders et al., 1992) and prevent imidazolinone carryover injury to sugarbeets (Wright, 1997). Importantly, the mutants recovered from somatic cell selection do further express the resistance in whole plants, as well as transmit the resistance sexually for use in development of resistant hybrid cultivars of sugarbeet. The 93R30B mutant was recovered in a genotype bred for somatic cell selection as well as homozygous dominant for the earlier sulfonylurea resistance allele, and proved to be a second somaclonal mutation on the same allele (Wright, 1997). The imidazolinone-resistant mutants are currently being incorporated into commercial breeding lines.

Genetic transformation

Genetic engineering techniques have been used to overcome some factors limiting traditional plant breeding including barriers among species. Nevertheless, sugarbeet has been considered one of the most recalcitrant plant species to molecular methodologies. Part of the apparent recalcitrance has been due to the proprietary nature of much of the sugarbeet transformation research. Fortunately, these limitations involving low transformation rates and poor regeneration efficiencies appear to have been overcome by Hall et al. (1996), who have established a striking breakthrough system in the molecular manipulation of sugarbeet.

Lindsey and Jones (1987, 1989) first reported that gene expression and stable transformation could be obtained by electroporation-mediated protoplast transformation in sugarbeet, but the regeneration of transgenic plants via this system was limited due to technical difficulties in regeneration from protoplasts.

Wang et al. (1985) and Paul et al. (1987) demonstrated that sugarbeet is susceptible to infection by bacteria, such as *Agrobacterium tumefaciens* wild type nopaline strains and *A. rhizogenes*. Harpster et al. (1988) produced stably transformed callus by using *A. tumefaciens*, but no regeneration of plants was obtained. Krens et al. (1988) recognized that optimal transformation frequencies were dependent on sugarbeet genotype and *Agrobacterium* strain. They found that using *Agrobacterium* shooter mutants, or strains carrying an isolated cytokinin gene, in order to affect the endogenous hormone ratios prevented shoot regeneration from the transformed calli. Successful regeneration of transformed sugarbeet plants by using *A. tumefaciens* was reported by Lindsey and Gallois

(1990). Binary vectors were used to carry selectable (NPTII) and screenable genes (CAT and GUS). The use of cultured shoot-base tissues allowed rapid and prolific generation of putatively transgenic plants while avoiding a callus phase. This transformation rate was dependent on the explant sources, genotypes, and selection conditions. D'Halluin et al. (1992) developed *Agrobacterium*-mediated transformation from seedling embryogenic callus to recover transgenic plants with herbicide resistance by introducing either the bialaphos resistance gene (*bar*) or a gene encoding mutant acetolactate synthases (ALS). The transformants expressing the ALS gene were resistant to field levels of sulfonylurea compounds applied in the greenhouse, whereas the progeny of transformants expressing the *bar* gene were resistant to commercial levels of glufosinate-ammonium applied in the field.

In order to increase the level of gene expression in sugarbeet, Ingersoll et al. (1996) demonstrated that various types of promoters (osmotin, PR-S, *pin2*, and CaMV 35S) that were fused to the *gus* coding region and biolistically transferred into sugarbeet suspension cells did affect the level of expression. The expression of osmotin promoter-leader construct was the highest, about 2.5-fold higher than for 35S-*gus*. Expression of PR-S-*gus* and *pin2-gus* were intermediate but somewhat higher than 35S-*gus*.

Hall et al. (1996) achieved high transformation frequencies by using a polyethylene glycol (PEG)-mediated protoplast transformation technique. The protoplasts were derived from stomatal guard cells, which Hall et al. (1996) assert are the only progenitors of totipotent protoplasts in sugarbeet leaves. Transgenic plants resistant to glufosinate-ammonium-based herbicides were obtained within only 8 to 9 weeks. This system seems

to offer highly efficient regeneration and transformation as well as rapid development, although the genotype independence claimed was not rigorously tested. This optimized protocol can be applied to integrate useful genes in order to attain efficient transformation for genetic modification with herbicide resistance, disease resistance, and enhanced yield traits of sugarbeet in the near future.

So far, researchers have established good systems for superior somatic embryogenesis and shoot regeneration abilities in a few sugarbeet genotypes. These systems should be further developed to attain (1) efficient gene transformation for genetic modification and improvement, (2) mass propagation of male-sterile lines or of elite F_1 plants with field delivery capability for use as artificial seeds, and (3) novel schemes for somatic cell selection.

The general objectives of this research are to:

(1) determine the level of embryo production from suspensions of two genotypes, REL-1 and REL-2, plated onto MS media with combinations of ABA with different concentrations of growth regulators, sucrose, or different sole nitrogen sources, (2) detect the germination and conversion rates of different length categories of somatic embryos, (3) examine the germination and conversion rates of somatic embryos encapsulated with alginate in MS medium or in H_2O after different periods of cold storage at 4 °C, (4) determine if nitrate, ammonium, glutamine, glutamate, urea, proline, glycine betaine, and choline can be utilized as sole nitrogen sources for growth of suspension plate-out, shoot

culture, and induction of leaf disc callus and subsequent shoots of sugarbeet clone REL-1, (5) test glutamine, glutamate and glycine betaine for use as either sole carbon or carbon & nitrogen source, in order to ascertain their potential role in somatic cell selection schemes, and (6) examine the specificity of inhibition by nitrate uptake inhibitor phenylglyoxal on callus growth with either nitrate or ammonium alone and in combination.

REFERENCES

Abdel-Latif, T.H. and J.W. Saunders. 1985. Genetic fidelity of *Beta vulgaris* L. ramets derived from adventitious buds on shoot culture petioles. Agron. Abstr. Am. Soc. Agron. Madison. WI. p.130.

Abe, J., H. Nakashima, K. Mitsui, T. Mikami, and Y. Shimamoto. 1991. Tissue culture response of *Beta* germplasm: callus induction and plant regeneration. Plant Cell. Tissue Org. Cult. 27: 123-127.

Atanassov, A.I. 1976. Analysis of organogenetic abilities of continuously cultivated somatic tissues of sugar beet on the basis of obtaining single-celled clones and suspension cultures. *In* F.J. Novak (ed.). Use of tissue cultures in plant breeding. Institute of Experimental Botany, Olomouc. pp. 69-80.

Atanassov, A.I. 1980. Method for continuous bud formation in tissue cultures of sugar beet (*Beta vulgaris* L.). Z. Pflanzenzucht 84: 23-29.

Atanassov, A.I., T. Kikindonov, and G. Antonova. 1978. Cytological changes in permanent sugar beet tissue cultures cultivated in vitro. *In* M. Stoilov (ed.). Proc Int Symp on experimental mutation of plants. Bulg. Acad. Sci. Sofia. pp. 309-317.

Banbe, H., and H. Tanabe. 1972. A study of anther culture in sugarbeet. Bulletin of Sugar Beet Research, Supplement 14: 9-16.

Bhat, S.R., B.V. Ford-Lloyd, and J.A. Callow. 1985. Isolation of protoplasts and regeneration of callus from suspension cultures of cultivated beets. Plant Cell Rep. 4: 348-350.

Bhat, S.R., B.V. Ford-Lloyd, and J.A. Callow. 1986. Isolation and culture of mesophyll protoplasts of garden, fodder and sugar beets using a nurse culture system : callus formation and organogenesis. J. Plant Physiol. 124: 419-423.

Binding, H., R. Nehls, R. Kock, J. Finger, and G. Mordhorst. 1981. Comparative studies on protoplast regeneration in herbaceous species of the dicotyledoneae class. Z. Pflanzenphysiol. 101: 119-130.

Bornman, J.F., C.H. Bornman, and L.O. Bjorn. 1982. Effects of ultraviolet radiation on variability of isolated *Beta vulgaris* and *Hordeum vulgare* protoplasts. Z. Pflanzenphysiol. 105: 297-306.

Bossoutrot, D. and D. Hosemans. 1985. Gynogenesis in *Beta vulgaris* L.: From in vitro culture of unpollinated ovules to the production of doubled haploid plants in soil. Plant Cell Rep. 4: 300-303.

Catlin, D.W. 1990. The effect of antibiotics on the inhibition of callus induction and plant regeneration from cotyledons of sugarbeet (*Beta vulgaris* L.). Plant Cell Rep. 9: 285-288.

Coumans-Gilles, M.F., C. Kevers, M. Coumans, E. Ceulemans, and T.H. Gaspar. 1981. Vegetative multiplication of sugarbeet through in vitro culture of inflorescence pieces. Plant Cell Tissue Org. Cult. 1: 93-101.

Coutts, R.H.A., D.J. Linton, and G.P. Bolwell. 1994. Patterns of protein synthesis in infected and stressed sugar beet (*Beta vulgaris* L.). J. Phytopathology 142: 274-282.

De Greef, W. and M. Jacobs. 1979. In vitro culture of the sugarbeet: description of a cell line with high regeneration capacity. Plant Sci. Lett. 17: 55-61.

Detrez, C., T. Tetu, R.S. Sangwan, and B.S. Sangwan-Norreel. 1988. Direct organogenesis from petiole and thin layer explants in sugar beet cultured in vitro. J. Exp. Bot. 39: 917-926.

D'Halluin, K., M. Bossut, E. Bonne, B. Mazur, J. leemans and J. Botterman. 1992. Transformation of sugarbeet (*Beta vulgaris* L.) and evaluation of herbicide resistance in transgenic plants. Bio/technology 10:309-314.

D'Halluin, K. and B. Keimer. 1986. Production of haploid sugarbeet (*Beta vulgaris*) by ovule culture. *In* Horn W., Jensen C.J., Odenbach W., Schieder O. (eds) Genetic manipulation in plant breeding. De. Gruyter. Berlin. pp. 307-309.

Doctrinal, M., R.S. Sangwan, and B. Sangwan-Norreel. 1989. In vitro gynogenesis in *Beta vulgaris* L.: effects of plant growth regulators, temperature, genotypes and season. Plant Cell Tissue Org. Cult. 17: 1-12.

Doley, W.P. 1990. Genotype and growth regulator effects on shoot regeneration from primary and serially-subcultured hormone- autonomous callus of sugarbeet (*Beta vulgaris* L.). Ph.D Dissertation, Michigan State University pp. 34-47.

Doley, W.P. and J.W. Saunders. 1989. Hormone-free medium will support callus production and subsequent shoot regeneration from whole plant leaf explants in some sugarbeet. (*Beta vulgaris* L.) populations. Plant Cell Rep. 8: 222-225.

Eckermann, N. and G. Baumann. 1995. Enzymatic changes in callus cultures of sugar beet during the transition from photoheterotrophic to photoautotrophic growth.

Photosynthetica 31: 163-175.

Enomoto, S. and K. Ohyama. 1985. Callus induction and shoot formation from explant of sugar beet. Japan. Jour. Crop Sci. 54: 281-282.

Ferrant, V., and J. Bouharmont. 1994. Origin of gynogenetic embryos of *Beta vulgaris* L.. Sex Plant Reprod. 7: 12-16.

Freytag, A.H., S.C. Anand, A.P. Rao-Arelli, and L.D. Owens. 1988. An improved medium for adventitious shoot formation and callus induction in *Beta vulgaris* L. in vitro. Plant Cell Rep. 7: 30-34.

Galatowitsch, M.W. and G.A. Smith. 1990. Regeneration from unfertilized ovule callus of sugarbeet (*Beta vulgaris* L.). Can. J. Plant Sci. 70: 83-89.

Gamborg, O.L., R.A. Miller, and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158.

Gaspar, T., C. Kevers, C. Penel, M. Crevecoeur, and H. Greppin. 1988. Biochemical characterization of normal and habituated sugarbeet calli. Relationship with anatomy, habituation and organogenesis. Potsdamer Forsch. B57: 20-30.

Gaspar, T., C. Kevers, M. Crevecoeur, C. Penel, J.M. Foidart, and H. Greppin. 1992. Habituation and vitrification of plants cultured in vitro: a reciprocal relationship. Wiss. Z. der Humboldt-Univ. zu Berlin, R. Mathematik/Naturwiss. 41: 35-40.

Hagege, D., C. Kevers, and F. Le Dily. 1990. NaCl dependent growth rate of normal and habituated calli, ethylene production and peroxidase activity. C.R. Acad. Sci, Ser. III, (Paris) 310: 259-264.

Hagege, D., C. Kevers, T. Gaspar, and T.A. Thorpe. 1991. Abnormal growth of habituated sugarbeet callus and cell suspensions. In Vitro Cell. Dev. Biol. 27: 112-116.

Hall, R.D., C. Pedersen, and F.A. Krens. 1993. Improvement of protoplast culture protocols for *Beta vulgaris* L. (sugarbeet). Plant Cell Rep. 12: 339-342.

Hall, R.D., T. Riksen-Bruinsma, G.J. Weyens, I.J. Rosquin, P.N. Denys, I.J. Evans, J.E. Lathouwers, M.P. Lefebvre, J.M. Dunwell, A. van Tunen, and F.A. Krens. 1996. A high efficiency technique for the generation of transgenic sugar beets from stomatal guard cells. Nature Biotechnology 14: 1133-1138.

Hall, R.D., H.A. Verhoeven, and F.A. Krens. 1995. Computer-assisted identification of protoplasts responsible for rare division events reveals guard cell totipotency. Plant Physiol. 107: 1379-1386.

Harms, C.T., I. Baktir, and J.J. Qertli. 1983. Clonal propagation *in vitro* of red beet (*Beta vulgaris* ssp.) by multiple adventitious shoot formation. Plant Cell Tissue Org. Cult. 2: 93-102.

Harpster, M.H., J.A. Townsend, J.D.G. Jones, J. Bedbrook, and P. Dunsmuir. 1988. Relative strengths of the 35S cauliflower mosaic virus, 1', 2' and nopaline synthase promoters in transformed tobacco, sugar beet and oilseed rape callus tissue. Mol. Gen. Gen. 212: 182-190.

Hart, S.E., J.W. Saunders, and D. Penner. 1993. Chlorsulfuron resistant sugarbeet : cross resistance and physiological basis of resistance. Weed Sci. 40: 378-383.

Hooker, M.P. and M.W. Nabors. 1977. Callus initiation, growth, and organogenesis in sugarbeet (*Beta vulgaris* L.). Z. Pflanzenphysiol. 84: 237-246.

Hosemans, D., and D. Bossoutrot. 1983. Induction of haploid plants from *in vitro* culture of unpollinated beet ovules (*Beta vulgaris* L.). Z. Pflanzenzuchtg. 91: 74-77.

Hussey, G. and A. Hepher. 1978. Clonal propagation of sugar beet plants and the formation of polyploids by tissue culture. Ann. Bot. (Lond) 42: 477-479.

Ingersoll, J.C., T.M. Heutte, and L.D. Owens. 1996. Effect of promoter-leader sequences on transient expression of reporter gene chimeras biolistically transferred into sugarbeet (*Beta vulgaris*) suspension cells. Plant Cell Rep. 15: 836-840.

Jacq, B., T. Tetu, R.S. Sangwan, A.D. Laat, and B.S. Sangwan-Norreel. 1992. Plant regeneration from sugarbeet (*Beta vulgaris* L.) hypocotyls cultured *in vitro* and flow cytometric nuclear DNA analysis of regenerants. Plant Cell Rep. 11: 329-333.

Jarl, C. and C.H. Bornman. 1986. Observations on genotypic variation in *Beta vulgaris* (sugar beet) tissues cultured in vitro. Hereditas 105: 55-59.

Kaffka, S. and P.G. Lemaux. 1996. Sweeter times ahead for sugarbeet growers. Nature Biotechnology 14: 1088.

Kevers, C., M. Coumans, W. De Greef, M. Hofinger, and T. Gaspar. 1981. Habituation in sugarbeet callus: auxin content, auxin protectors, peroxidase pattern and inhibitors. Physiol. Plant. 51: 281-286.

Krens, F.A., C. Zijlstra, W.v.d. Molen, D. Jamar, and H.J. Huizing. 1988. Transformation and regeneration in sugar beet (*Beta vulgaris* L.) induced by 'shooter' mutants of *Agrobacterium tumefaciens*. Euphytica S: 185-194.

Krens, F.A., D. Jamar, G.J.A. Rouwendal, and R.D. Hall. 1990. Transfer of cytoplasm from new *Beta* CMS sources to sugar beet by asymmetric fusion.1. Shoot regeneration from mesophyll protoplasts and characterization of regenerated plants. Theor. App. Gen. 79: 390-396.

Kubalakova, M. 1990. Somatic embryogenesis and cytoplasmic sterility in *Beta vulgaris* L. var. *Saccharifera*. Biol. Plant. 32: 414-419.

Lenzner, S., K. Zoglauer, and O. Schieder. 1995. Plant regeneration from protoplasts of sugarbeet (*Beta vulgaris* L.). Physiol. Plant. 94: 342-350.

Lindsey, K. and P. Gallois. 1990. Transformation of sugarbeet (*Beta vulgaris*) by *Agrobacterium tumefaciens*. J. Exp. Bot. 41: 529-536.

Lindsey, K. and M.G.K. Jones. 1987. Transient gene expression in electroporated protoplasts and intact cells of sugarbeet. Plant Mol. Biol. 10: 43-52.

Lindsey, K. and M.G.K. Jones. 1989. Stable transformation of sugarbeet protoplasts by electroporation. Plant Cell Rep. 8: 71-74.

Majewska-Sawka, A. and B. Jassem. 1988. The comparison of in vitro morphogenetic abilities in the *Corollinae* section of the Genus *Beta*. Arch. Zuchtungsforsch. Berlin 18: 285-293.

Margara, J. 1970. Neoformation de bourgeons in vitro chez la betterave sucriere, *Beta vulgaris* L. C.R. Acad. Sci. Ser. D. (Paris) 270: 698-701.

Masuda, H., R. Nakagawa and S. Sugawara. 1988. Hormone-autonomous suspension culture from leaf explants of sugar beets in liquid medium. Plant Cell Physiol. 29: 75-78.

Miedema, P. 1982. A tissue culture technique for vegetative propagation and low temperature preservation of *Beta vulgaris*. Euphytica 31: 635-643.

Miedema, P., P.J. Groot, and J.H.M. Zuidgeest. 1980. Vegetative propagation of *Beta vulgaris* by leaf cuttings. Euphytica 29: 425-432.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Nam, L.S., B. Landova, and Z. Landa. 1976. Isolation of protoplasts from sugar beet leaves. Biol. Plant. 18: 389-392.

Owens, L.D. and D.R. Eberts. 1992. Sugarbeet leaf disc culture: an improved procedure

for inducing morphogenesis. Plant Cell Tissue Organ Cult. 31: 195-201.

Paul, H., C. Zijlstra, J.E. Leeuwangh, F.A. Krens, and H.J. Huizing. 1987. Reproduction of the beet cyst nematode *Heterodera schachtii* schm. on transformed root cultures of *Beta vulgaris* L.. Plant Cell Rep. 6: 379-381.

Pedersen, C., R.D. Hall, and F.A. Krens. 1993. Petioles as the source for isolation and culture of *Beta vulgaris* and *Beta maritima* protoplasts. Plant Sci. 95: 89-97.

Pedersen, M.G., and J.M. Andersen. 1988. Callus morphology and endogenous cytokinins in regenerating sugar beet callus cultures. Eucarpia Meeting Beta, Helsinger.

Ritchie, G.A., K.C. short, and M.R. Davey. 1989. *In vitro* shoot regeneration from callus, leaf axils and petioles of sugar beet (*Beta vulgaris* L.). J. Exp. Bot. 40: 277-283.

Rogozinska, J. and M. Goska. 1978. Induction of differentiation and plant formation in isolated sugar beet leaves. Bull. Pol. Acad. Sci. XXVI: 343-345.

Rogozinska, J.H., M. Goska, and A. Kuzdowicz. 1977. Induction of plants from anthers of *Beta vulgaris* cultured *in vitro*. Acta. Soc. Bot. Poloniae. Vol.XLVI: 471-479.

Roussy, I., F. Dubois, R.S. Sangwan, and B.S. Sangwan-Norreel. 1996. *In planta* 2,3,5-triiodobenzoic acid treatment promotes high frequency and routine in vitro regeneration of sugarbeet (*Beta vulgaris* L.) plants. Plant Cell Rep. 16: 142-146.

Saunders, J.W. 1982. A flexible in vitro shoot culture propagation system for sugarbeet that includes rapid floral induction of ramets. Crop Sci. 22: 1102-1105.

Saunders, J.W., G. Acquaah, K.A. Renner, and W.P. Doley. 1992. Monogenic dominant sulfonylurea resistance in sugarbeet from somatic cell selection. Crop Sci. 32: 1357-1360.

Saunders, J.W. and M.E. Daub. 1984. Shoot regeneration from hormone-autonomous callus from shoot cultures of several sugarbeet (*Beta vulgaris* L.) genotypes. Plant Sci. Lett. 34: 219-223.

Saunders, J.W. and W.P. Doley. 1986. One step shoot regeneration from callus of whole plant leaf explants of sugarbeet lines and a somaclonal variant for *in vitro* behavior. J. Plant Physiol. 124: 473-479.

Saunders, J.W., W.P. Doley, J.C. Theurer, and M.H. Yu. 1990. Somaclonal variation in sugarbeet. *In* Y.P.S. Bajaj (ed.). Biotechnology in agriculture and forestry. II. Somaclonal variation in crop improvementI., Springer-Verlag, Berlin. pp. 465-490.

Saunders, J.W. and K. Shin. 1986. Germplasm and physiologic effects on induction of high frequency hormone autonomous callus and subsequent shoot regeneration in sugarbeet. Crop Sci. 26: 1240-1245.

Schlangstedt, M., B. Hermans, K. Zoglauer, and O. Schieder. 1992. Culture of sugar beet (*Beta vulgaris* L.) protoplasts in alginate- callus formation and root organogenesis. J. Plant Physiol. 140: 339-344.

Schlangstedt, M., K. Zoglauer, S. Lenzner, B. Hermans, and M. Jacobs. 1994. Improvement of sugar beet (*Beta vulgaris* L.) protoplast culture: Leaf petioles as a protoplast source. J. Plant Physiol. 143: 227-233.

Shimamoto, Y., H. Hayakawa, J. Abe, H. Nakashima, and T. Mikami. 1993. Callus induction and plant regeneration of *Beta* germplasm. J. Sugar Beet Res. 30: 317-319.

Slavova, Y., A. Zahariev, and G. Antonova. 1982. Improving and applying the method of sugar beet vegetative propagation in vitro. Plant Physiol. (Sofia) 8: 95-99.

Smith, G.A. 1987. Sugar beet. *In* W.R. Fehr (ed.). Principles of cultivar development. MacMillan. New York. pp. 577-625.

Steen, P, B. Keimer, K. D'Halluin, and H.C. Pedersen. 1986. Variability in plants of sugar beet (*Beta vulgaris* L.) regenerated from callus, cell-suspension and protoplasts. *In* W. Horn, C.J. Jensen, W. Odenbach, O. Schieder (eds.). Genetic manipulation in plant breeding. De Gruyter. Berlin. pp. 633-634.

Szabados, L, and C. Gaggero. 1985. Callus formation from protoplasts of a sugarbeet cell suspension culture. Plant Cell Rep. 4: 195-198.

Tenning, P., E.W. Weich, U-B. Kjarsgaard, M-A. Lelu and M. Nihlgard. 1992. Somatic embryogenesis from zygotic embryos of sugar beet (*Beta vulgaris* L.). Plant Sci. 81: 103-109.

Tetu, T., R.S. Sangwan, and B.S. Sangwan-Norreel. 1987. Hormonal control of organogenesis and somatic embryogenesis in *Beta vulgaris* callus. J. Exp. Bot. 38: 506-517.

Van Geyt, J., K. D'Halluin and M. Jacobs. 1985. Induction of nuclear and cell divisions in microspores of sugarbeet (*Beta vulgaris* L.). Z. Pflanzenzucht. 95: 325-335.

Van Geyt, J.P.C. and M. Jacobs. 1985. Suspension culture of sugarbeet (*Beta vulgaris* L.) Induction and habituation of dedifferentiated and self-regenerating cell lines. Plant Cell Rep. 4: 66-69.

Van Geyt, J., Speckmann Jr. G.J., K. D'Halluin, and M. Jacobs. 1987. In vitro induction

of haploid plants from unpollinated ovules and ovaries of the sugarbeet (*Beta vulgaris* L.). Theor. Appl. Genet. 73: 920-925.

Van Harten, A.M., H. Bouter, and C. Broertjes. 1981. In vitro adventitious bud techniques for vegetative propagation and mutation breeding of potato (*Solanum tuberosum* L.). II. Significance for mutation breeding. Euphytica 30: 1-8.

Wang, Y.Q., C.E. Lo, Y.Y. Lian, Q.Q. Shao, X.C. Jiang, Z.Q. Zhou, A.S. Lee, and Y.Q. Chen. 1985. Teratome induction and gene transfer in *Beta*. Acta. Agron. Sinica 11: 159-162.

Welander, T. 1976. Effects of nitrogen, sucrose, IAA and kinetin on explants of *Beta vulgaris* grown *in vitro*. Physiol. Plant. 36: 7-10.

Winner, C. 1993. History of the crop. *In* D.A. Cooke and R.K. Scott (eds.). The sugar beet crop. Chapman & Hall. Cambridge. pp. 1-36.

Wright, T.R. 1997. Development and characterization of imidazolinone-resistant sugar-beet somaclonal cell selections. Ph.D Dissertation. Michigan State University.

Yu, M.H. 1987. Observations on callus induction and somaclonal variation in beet species. Genetics 116: s17.

Yu, M.H. 1989. Callus induction and differentiation from leaf explants of different species of the Genus *Beta*. Crop Sci. 29: 205-209.

Yu, M.H., L.M. Pakish, and J.W. Saunders. 1991. Association of a nematode resistance bearing addition chromosome with a recurring leaf intumescence somaclonal variation in sugar beet. Genome 34: 477-485.

Zagorska, N. and A. Atanassov. 1985. Somaclonal variation in tobacco and sugar beet breeding. *In* R.R. Henke, K.W. Hughes, M.J. Constantin, and A. Hollaender. (eds.). Tissue culture in forestry and agriculture. Plenum. New York. pp. 371.

Zelmer I. And G. Gunther. 1988a. Activities of glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamate synthase (GOGAT) in suspension cultures of *Beta vulgaris* (sugar beet) and *Chenopodium album* (goosefoot). Biochem. Physiol. Pflanzen 183: 397-405.

Zelmer, I. And G. Gunther. 1988b. Influence of various herbicides on the activity dynamic of glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamate synthase (GOGAT) obtained from suspension cultures of *Beta vulgaris* (sugar beet) and *Chenopodium album* (goosefoot). Biochem. Physiol. Pflanzen 183: 407-415.

Zhong, Z., H.G. Smith, and T.H, Thomas. 1993. Micropropagation of wild beet (*Beta maritima*) from inflorescence pieces. Plant growth regulation 12: 53-57.

CHAPTER 1

SOMATIC EMBRYOS FROM CALLUS OF SUGARBEET BIOTECHNOLOGY CLONE REL-1

ABSTRACT

Somatic embryos could be used for proliferative propagation or for gene transfer procedures in sugarbeets (*Beta vulgaris* L.) if adequate methods for initiation and development could be devised. With sugarbeet model clone REL-1, plating of fresh suspension culture cells grown with hormone-free Murashige and Skoog (MS) medium onto further hormone-free MS medium elicited a low frequency of somatic embryogenesis, about one embryo per ml of suspension used. The inclusion of 0.1 or 0.3 mg/L abscisic acid (ABA) in the plate-out medium increased the number of somatic embryos in this system. A combination of naphthaleneacetic acid (NAA) (1 mg/L) and ABA (0.1 mg/L) gave the highest somatic embryo yield, 15 embryos per ml of suspension. After 22 to 40 days, embryos at various stages, ranging from globular, heart, and torpedo-shaped embryos to mature opaque white embryos with cotyledons and radicles, were present at the callus surface. The external morphology of several somatic embryos was examined by scanning electron microscopy. The somatic embryos developed into normal plantlets, exhibiting betalain pigmentation on hypocotyls after being transferred onto hormone-free MS medium. The conversion rate of somatic embryos of different lengths (1, 2, 3 mm) into complete plantlets was similar (78, 81, and 85 % respectively). The conversion rate

of encapsulated somatic embryos was 75 % with 1.5 % alginate in MS medium and 72 % with 1.5% alginate in H_2O.

INTRODUCTION

Somatic embryogenesis is the process of embryo initiation and development from somatic, i.e., non-germ, cells. In plant tissue culture, somatic embryos have most often been produced from callus or suspension culture cells under appropriate conditions, with genotype dependency being common. Somatic embryos are under investigation in species such as alfalfa and celery for use in production of artificial seeds (Gray and Purohit, 1991). Elite highly productive individual genotypes from genetically heterogeneous cultivars, or superior-combining male sterile clones, can be vegetatively reproduced on a large scale as somatic embryos and delivered to the field in a seedlike fashion following conditioning and coating, or alternatively, by fluid drilling. Somatic embryos also could be used in genetic transformation applications as exemplified in gene transfer with *Agrobacterium* in walnut (McGranahan et al.,1990), grape (Le Gall et al., 1994) and *Datura innoxia* (Ducrocq et al., 1994). Synthetic seed production, as well as gene transfer, would be more efficient if somatic embryogenesis also occurred via immediate subsequent cycles wherein multiple new embryos arose from existing ones. This self-replicating production is termed secondary somatic embryogenesis.

Somatic embryos in sugarbeet were first reported by Atanassov (1976) in suspension cultures. Tetu et al. (1987) concluded that multiple hormonal sequences were necessary for the induction and development of somatic embryos from callus. Somatic embryogenesis at a low frequency in callus was reported by Freytag et al. (1988) in all six germplasm sources tested.

Kubalakova (1990) described callus from at least one genotype that had maintained its embryogenic ability for three years on media without growth regulators. Somatic embryos were directly produced on sugarbeet zygotic embryos by Tenning et al. (1992), who also observed some direct secondary embryogenesis. Doley and Saunders (1989) reported the simple production and partial germination of somatic embryos from leaf disc callus of a fodder beet cultivar without the use of growth regulators. D'Halluin et al. (1992) used embryogenic callus from seedlings in an *Agrobacterium*-mediated genetic transformation system for sugarbeet.

REL-1 is a self-fertile diploid sugarbeet clone bred for ease of tissue culture manipulations. It exhibits a high frequency of leaf disc callusing, shoot regeneration from hormone autonomous callus, dispersed suspension cultures and resistance to shoot vitrification. REL-1 is also heterozygous for monogermness (*Mm*), annualism (*Bb*), and red hypocotyl (*Rr*). REL-1 has been used for the recovery of monogenic dominant sulfonylurea herbicide resistance by somatic cell selection (Saunders et al., 1992) and of other mutant traits (Saunders et al., 1990).

We report here (1) the initial success in obtaining somatic embryos from biotech clone REL-1, (2) converting somatic embryos into plantlets, (3) examination of later stages of somatic embryos under scanning electron microscopy, and (4) encapsulation of somatic embryos in initial efforts to produce artificial seeds.

MATERIALS AND METHODS

Plant materials

All experiments were performed with the diploid sugarbeet (*Beta vulgaris L.)* clone REL-1, released to the public in 1987. REL-1 has been maintained in shoot culture (Saunders, 1982) and is available upon request, either as in vitro shoots, whole plants, or S_1 seed.

Culture media

The culture media contained MS mineral salts (Murashige and Skoog, 1962), 100 mg/L myo-inositol, 1.0 mg/L thiamine·HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, and 30 g/L sucrose. Media used in plating out were gelled with 3.5 g/L phytagel. The growth regulators used were : 6-benzyladenine (BA) (1.0 mg/L), 2,3,5-triiodobenzoic acid (TIBA) (1.0 mg/L), 1-naphthaleneacetic acid (NAA) (0.25-1.0 mg/L), and/or (±)cis,trans-abscisic acid (ABA) (0.1-1.0 mg/L). The pH was adjusted to 5.95 prior to autoclaving. ABA was filter-sterilized and added into previously autoclaved and partially cooled media. Culture vessels were 125 mL Erlenmeyer flasks or 20 x 100 mm Falcon disposable plastic Petri plates. Medium volume per vessel was 35 mL. Flasks were closed with foam caps and aluminum foil. Petri plates were sealed with one layer of Parafilm.

Initiation of callus and maintenance of cell suspensions

Callus was initiated from leaf discs (8 mm diameter) from partially expanded leaves of greenhouse grown REL-1 on MS medium with 1 mg/L BA (Saunders et al., 1992) and 0.9 % Difco Bacto agar in Petri plates at 30 C in the dark, one disc per plate of 35 mL medium. Callus was first seen after one month, and after another month, 2 to 3 g of leaf-disc callus was transferred to hormone free liquid MS medium in 125 mL Erlenmeyer flasks for growth at

21±2 °C in the dark. The suspension cultures were shaken on rotary shakers at 125 rpm to aerate the cultures and to reduce cell cluster size.

Induction of somatic embryos

After 5-7 days, suspension cultures were subcultured with hormone-free MS medium for another 5-7 days (poured the fresh medium into the old suspension flask and mixed, then divided into two flasks). Suspensions used as inoculum for platings were pushed through a stainless steel sieve with 830 um openings. Sieved suspension cells were washed with hormone free liquid medium and plated on MS media with no growth regulators or with combinations of BA and NAA, or NAA, ABA, and TIBA. Each Petri plate received 1 mL of suspension preparation containing about 0.1g fresh weight, and was incubated in dim light (less than 5 umol m^{-2} s^{-1}) from fluorescent lamps (Philips cool white econowatt) at 25°C. Minimum size for a somatic embryo to be counted was 0.5 mm.

Germination and conversion of somatic embryos

Somatic embryos were transferred onto hormone-free MS medium under light (20-50 umol $m^{-2}s^{-1}$ from fluorescent lamps) at 25°C. The proportion that developed into normal plantlets with roots constituted the conversion rate of somatic embryos into complete plantlets.

Scanning electron microscopy

According to the procedures of Flegler et al. (1993) somatic embryos were fixed in 2 % glutaraldehyde in 0.1 M cacodylate buffer for 1 hour, then washed with two changes of buffer for 10 minutes each. Dehydration was accomplished by immersion for 10 minutes each in 25, 50, 75, 95 and 100 % ethanol with two additional changes of 100 % ethanol at the end. Fixed somatic embryos were dried in a Balzers critical point dryer, mounted and sputter-coated with gold, and examined with a JSM-6400 scanning electron microscope.

Encapsulation of somatic embryos

Somatic embryos (approximately 2-3 mm) were encapsulated with hormone-free MS medium including 1.5 % sodium alginate, or H_2O including 1.5 % sodium alginate. A pipette was used to suck up single embryos with the alginate, which were then dropped into 50 mM $CaCl_2 \cdot H_2O$ and stirred for 20 minutes, thus formed sodium alginate beads of a size large enough to cover the entire somatic embryo. Then, the germination and conversion rates of embryos in each encapsulation solution were determined, after the beads were placed on hormone-free MS agar plates.

Data analysis

Each experiment with 10 replications was performed 3 times with similar results. Analysis of variance was based on a randomized complete block design. The average number of somatic embryos per plate for each medium was subjected to ANOVA, and the least significant difference (LSD) test (P=0.05) was performed to permit individual treatment comparisons.

RESULTS AND DISCUSSION

Induction of somatic embryos

A low frequency of somatic embryogenesis, almost one embryo per Petri plate, was obtained following plating-out of suspension culture cells on hormone-free MS medium. NAA and BA tested either individually or in combination did not induce production of significantly more somatic embryos than did the hormone-free medium. Rather, the presence of BA at 0.5 or 1.0 mg/l markedly reduced the occurrence of somatic embryos (Table 1-1). On the other hand, ABA at 0.1 or 0.3 mg/l in the media increased the number of somatic embryos per plate up to eight-fold (Figure 1-1). The highest somatic embryo yield of 15 per plate was attained

with the combination 1 mg/l NAA and 0.1 mg/l ABA (Figure 1-1). ABA is best known for its positive effects in somatic embryogenesis where it normalizes development and inhibits precocious germination (Ammirato, 1974; 1983; Wilen et al., 1990). ABA also play a key part on conversion in somatic embryo systems of alfalfa and carrot (Fujii et al., 1990; Nickle and Yeung, 1993). TIBA did not stimulate somatic embryogenesis in REL-1 using the described procedure, even though Doley (1990) found that TIBA promoted bud formation in callus of REL-1. Tetu et al. (1987) reported a stimulatory effect of TIBA on bud formation in sugarbeet callus of another genetic background.

Development of somatic embryos

During the first two weeks following plating-out, white or light yellow callus proliferated. From the third to the sixth week, various late stages of somatic embryos from torpedo to mature opaque white embryos with cotyledons were clearly distinguishable at the callus surface (Figure 1-2 and 1-3). The simultaneous occurrence of embryos of different lengths (0.5 to 4 mm) indicated that somatic embryogenesis was not uniform and synchronous. After 40 days, most of the somatic embryos with cotyledons were around 2-3 mm long. Each somatic embryo (Figure 1-4) could be easily separated from the surrounding callus.

Conversion of somatic embryos into plantlets

After being transferred onto hormone-free MS medium, the cotyledons and radicles of the bipolar embryos gradually developed into shoots and roots simultaneously to form normal plantlets (Figure 1-5). Plantlets usually displayed betalain pigmentation on the hypocotyls, the colors including yellow, light red or deep red (Figure 1-6). All somatic embryos longer than 2 mm germinated. However, the proportion of conversion into complete plantlets was somewhat less because of subsequent callusing of somatic embryos or the formation of abnormal plants.

The conversion rate of somatic embryos of different sizes into complete plantlets was 85 % (Table 1-2).

Embryo morphology could have affected subsequent germination and conversion of somatic embryos into plantlets. Abnormal somatic embryos are common in other species, for example, caraway (Ammirato, 1974) and soybeans (Buchheim et al, 1989). Achieving a high degree of normal morphology usually involves optimizing the culture medium components and environmental conditions.

Scanning electron microscopy

The development of somatic embryos from REL-1 callus resulted in torpedo stage (Figure 1-7 and 1-8) and mature embryo stage with cotyledons (Figure 1-9) and radicles (Figure 1-10). Although somatic embryogeny is reported to mimic zygotic embryogeny in many respects (Crouch, 1982; Sharp et al, 1980), this has yet to be demonstrated completely in sugarbeet. Artschwager (1927) and Artschwager and Starrett (1933) have described the anatomy of embryo and seed development in sugarbeet. A more detailed comparison of somatic with zygotic embryogenesis would require detection of earlier stages of somatic embryogeny in callus or liquid cultures. Fowke et al. (1994) reported that the general morphology of the mature embryos and zygotic embryos was very similar at the same stage of development in white spruce, except for some differences in embryo size, orientation of cotyledons and surface wrinkling.

Some abnormal somatic embryos with possible vascular connection to the callus (Figure 1-11), or with swollen hypocotyls (Figure 1-12 and 1-13) were observed on the plate-out medium. These abnormal morphologies might reduce subsequent conversion of somatic embryos into normal plantlets.

Trichomes could be seen on the cotyledonary surface of some somatic embryos with SEM (Figure 1-14, 1-15 and 1-16). Goldberg et al. (1994) mentioned that trichomes were not present on wild-type cotyledons of *Arabidopsis* seedlings. Nevertheless, trichomes, which were markers for postembryonic development, occurred on leaf, stem and sepal surfaces in wild-type *Arabidopsis* plants (Hulskamp, et al., 1994). The mature leaf trichomes were regularly distributed in the epidermal layer. On the other hand, Gray et al. (1993) reported trichome development on precociously germinating cotyledonary stage embryos from *Cucumis melo.*

Encapsulation of somatic embryos

In some crops, such as alfalfa, celery, and carrot, somatic embryos have been used in efforts to make artificial seeds (Fujii et al, 1993; Kitto and Janick, 1985). Isolated somatic embryos (Figure 1-4) of REL-1 were utilized for encapsulation to study the possibility of making artificial seeds in sugarbeet (Figure 1-17). The somatic embryos encapsulated with 1.5 % alginate in MS medium resulted in 100 % germination and 75 % conversion; those encapsulated with 1.5 % alginate in H_2O resulted in 100 % germination and 72 % conversion, in each case after being transferred on agar plates for 20 days (Table 1-3).

This experiment indicated that encapsulation of somatic embryos reduced the conversion rate (about 10 %) as compared to nonencapsulated somatic embryos (according to Table 1-2 and Table 1-3). The same result was observed by Rao and Singh (1991), who reported that the conversion rate for encapsulated embryos was significantly lower (49.7 %) as compared to that of nonencapsulated somatic embryos of hybrid *Solanum melongena* L..

Based on the research reported here, the model biotech clone REL-1 is capable of at least moderate intensities of production of somatic embryos, which then are easily collected and

converted into plantlets. This is the first report of sugarbeet somatic embryos that describes the use and promotive effect of ABA on embryo number. In addition, its the first report of the encapsulation of somatic embryos in sugarbeet. In order for REL-1 somatic embryos to be useful in gene transfer research, conditions for secondary somatic embryo production must be developed. If somatic embryos are to find a direct application in the field, conditions for their massive proliferation in other genotypes must be found. It is encouraging to note that both Tenning et al. (1992) and Kubalakova (1990) reported some secondary embryogenesis in sugarbeet cultures.

REFERENCES

Ammirato, P.V. 1974. The effects of abscisic acid on the development of somatic embryos from cells of caraway (*Carum carvi L.*). Bot. Gaz. 135: 328-337.

Ammirato, P.V. 1983. The regulation of somatic embryo development in plant cell cultures: Suspension culture techniques and hormone requirements. Bio/Technology 1: 68-74.

Artschwager, E. 1927. Development of flowers and seed in the sugar beet. J. Agri. Res. 34: 1-25.

Artschwager, E. and R.C. Starrett. 1933. The time factor in fertilization and embryo development in the sugar beet. J. Agri. Res. 47: 823-843.

Atanassov, A.I. 1976. Analysis of organogenetic abilities of continuously cultivated somatic tissues of sugar beet on the basis of obtaining single-celled clones and suspension cultures. *In* F.J. Novak (ed.). Use of tissue cultures in plant breeding. Institute of Experimental Botany, Olomouc. pp. 69-80.

Buchheim, J.A., S.M. Colburn, and J.P. Ranch. 1989. Maturation of soybean somatic embryos and the transition to plantlet growth. Plant Physiol. 89:768-775.

Crouch, M.L. 1982. Non-zygotic embryos of *Brassica napus* L. contain embryo -specific storage proteins. Planta 156: 520-524.

D'Halluin, K., M. Bossut, E. Bonne, B. Mazur, J. Leemans, and J. Botterman. 1992. Transformation of sugarbeet (*Beta vulgaris* L.) and evaluation of herbicide resistance in transgenic plants. Bio/Technology 10: 309-314.

Doley, W.P. and J.W. Saunders. 1989. Hormone-free medium will support callus production and subsequent shoot regeneration from whole plant leaf explants in some sugarbeet (*Beta vulgaris* L.) populations. Plant Cell Rep. 8: 222-225.

Doley, W.P. 1990. Genotype and growth regulator effects on shoot regeneration from primary and serially-subcultured hormone-autonomous callus of sugarbeet (*Beta vulgaris* L.). Ph.D Dissertation, Michigan State University.

Ducrocq C., R.S. Sangwan, and B.S. Sangwan-Norreel. 1994. Production of *Agrobacterium*-mediated transgenic fertile plants by direct somatic embryo-genesis from immature zygotic embryos of *Datura innoxia*. Plant. Mol. Biol. 25: 995-1009.

Flegler, S.L., J.W. Heckman Jr., and K.L. Klomparens. 1993. Scanning and transmission electron microscopy. W.H. Freeman, New York, pp. 151-167.

Fowke, L.C., S.M. Attree, and P.J. Rennie. 1994. Scanning electron microscopy of hydrated and desiccated mature somatic embryos and zygotic embryos of white spruce (*Picea glauca* Voss.) Plant Cell Rep. 13: 612-618.

Freytag, A.H., S.C. Anand, A.P. Rao-Arelli, and L.D. Owens. 1988. An improved medium for adventitious shoot formation and callus induction in *Beta vulgaris* L. in vitro. Plant Cell Rep. 7: 30-34.

Fujii, J.A.A., D. Slade, and R. Olsen. 1990. Alfalfa somatic embryo maturation and conversion to plants. Plant Sci. 72: 93-100.

Fujii, J.A.A., D. Slade, and K. Redenbaugh. 1993. Planting artificial seeds and somatic embryos. *In* K. Redenbaugh.(ed.), Synseeds. CRC Press. Davis, California. pp. 183-202.

Goldberg, R.B., G. Paiva, and R. Yadegari. 1994. Plant embryogenesis: zygote to seed. Science 266: 605-614.

Gray, D.J. and A. Purohit. 1991. Somatic embryogenesis in development of synthetic seed technology. Crit. Rev. Plant Sci. 10: 33-61.

Gray, D.J., D.W. McColley, and M.E. Compton. 1993. High-frequency somatic

embryogenesis from quiescent seed cotyledons of *Cucumis melo* cultivars. J. Amer. Soc. Hort. Sci. 118: 425-432.

Hulskamp, M., S. Misera, and G. Jurgens. 1994. Genetic dissection of trichome cell development in *Arabidopsis*. Cell 76: 555-566.

Kitto, S. and J. Janick. 1985. Production of synthetic seeds by encapsulating asexual embryos of carrot. J. Am. Soc. Hort. Sci. 110: 227-232.

Kubalakova, M. 1990. Somatic embryogenesis and cytoplasmic sterility in *Beta vulgaris* L. var. *saccharifera*. Biol. Plant. 32: 414-419.

Le Gall, O., L. Torregrosa, Y. Danglot, T. Candresse, and A. Bouquet. 1994. *Agrobacterium*-mediated genetic transformation of grapevine somatic embryos and regeneration of transgenic plants expressing the coat protein of grapevine chrome mosaic nepovirus (GCMV). Plant Sci. 102: 161-170.

McGranahan, G.H., C.A. Leslie, S.L. Uratsu, and A.M. Dandekar. 1990. Improved efficiency of the walnut somatic embryo gene transfer system. Plant Cell Rep. 8: 512-516.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Nickle, T.C. and E.C. Yeung. 1994. Further evidence of a role for abscisic acid in conversion of somatic embryos of *Daucus carota*. In Vitro Cell. Dev. Biol. 30: 96-103.

Rao, P.V.L. and B. Singh. 1991. Plantlet regeneration from encapsulated somatic embryos of hybrid *Solanum melongena* L.. Plant Cell Rep. 10: 7-11.

Saunders, J.W. 1982. A flexible *in vitro* shoot culture propagation system for sugarbeet that includes rapid floral induction of ramets. Crop Sci. 22: 1102 1105.

Saunders, J.W., W.P. Doley, J.C. Theurer, and M.H. Yu. 1990. Somaclonal variation in sugarbeet. *In* Y.P.S. Bajaj (ed.): Biotechnology in agriculture and Forestry. Vol II. Somaclonal variation in crop improvement. I. Springer-Verlag, Berlin Heidelberg. pp. 465-490.

Saunders, J.W., G. Acquaah, K.A. Renner, and W.P. Doley. 1992. Monogenic dominant sulfonylurea resistance in sugarbeet from somatic cell selection. Crop Sci. 32: 1357-1360.

Sharp, W.R., M.R. Sondahl, L.S. Caldas, and S.B. Maraffa. 1980. The physiology of in vitro asexual embryogenesis. *In* J. Janick, (ed.). Horticultural Reviews, vol 1, AVI , Westport, CT. pp. 268-310.

Tenning, P., E.W. Weich, U.B. Kjarsgaard, M.A. Lelu, and M. Nihlgard. 1992. Somatic embryogenesis from zygotic embryos of sugar beet (*Beta vulgaris* L.) Plant Sci. 81: 103-109.

Tetu, T., R.S. Sangwan, and B.S. Sangwan-Norreel. 1987. Hormonal control of organogenesis and somatic embryogenesis in *Beta vulgaris* callus. J. Exp. Bot. 38: 506-517.

Wilen, R.W., R.M. Mandel, and R.P. Pharis. 1990. Effect of abscisic acid and high osmoticum on storage protein gene expression in microspore embryos of *Brassica napus*. Plant Physiol. 94: 875-881.

Table 1-1: The effect of BA and NAA concentrations on somatic embryogenesis. One mL of suspension cells was plated onto media with various combinations of BA and NAA and scored 68 days after plating. Means marked with same letter are not significantly different according to LSD with $P<0.05$.

		Number of somatic embryos per plate			
		BA	**BA**	**BA**	**BA**
	mg/L	**0**	**0.25**	**0.5**	**1.0**
NAA	**0**	0.75 ab	0.25 bc	0 c	0 c
NAA	**0.25**	1.0 a	0.25 bc	0.08 c	0 c
NAA	**0.5**	0.67 ab	0 c	0 c	0 c
NAA	**1.0**	1.08 a	0.08 c	0 c	0.08 c

Table 1-2 : Effect of embryo length on the proportions of somatic embryos germinating and converting into complete plantlets.

Length of somatic embryos mm	Germination rate %	Conversion rate %
(0.5 - 1.9)	88 (61/69)	78 (54/69)
(2.0 - 2.9)	100 (96/96)	81 (78/96)
(3.0 - 3.9)	100 (28/28)	86 (24/28)

Table 1-3: Comparison of encapsulated somatic embryos with MS medium and H_2O, and effects on the germination and conversion rate on MS medium (genotype REL-1).

	Encapsulated with MS medium & 1.5% alginate	Encapsulated with H_2O & 1.5% alginate
Germination rate	100 % (16/16)	100 % (18/18)
Conversion rate	75 % (12/16)	72 % (13/18)

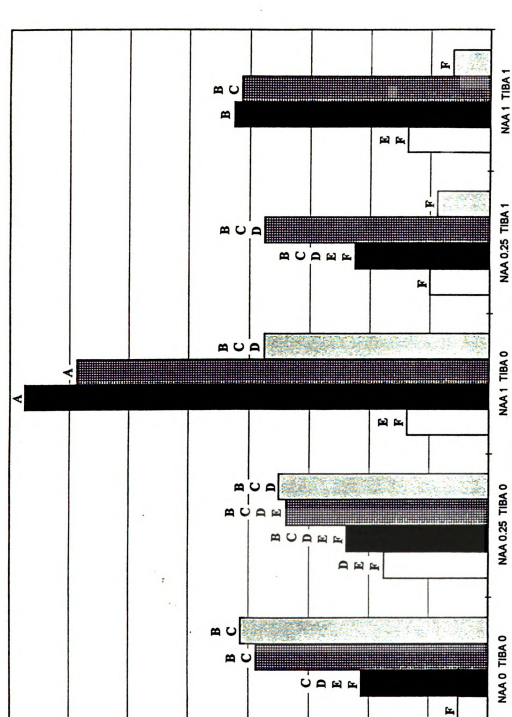

Figure 1-1: The number of somatic embryos per ml of suspension plated out onto media with combinations of ABA, NAA, and TIBA 33 days after plate out. Means marked with same letter are not significantly different according to LSD with $P < 0.05$.

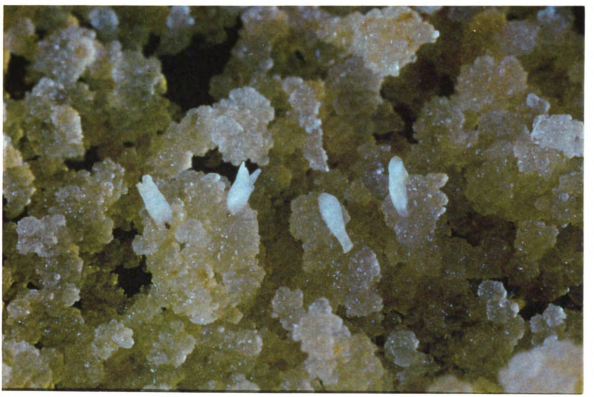

Figure 1-2: Opaque white somatic embryos (1.5 mm long) on the surface of callus tissue 22 days after suspension plating.

Figure 1-3: A 3 mm long tricotyledonary somatic embryo 30 days after suspension plateout.

Figure 1-4: Isolated somatic embryos (length: 0.5 - 4 mm).

Figure 1-5: Somatic embryo-derived complete plantlet, 10 days after being transferred to hormone-free MS medium (length: 20 mm).

Figure 1-6: Somatic embryo development into plantlet, and betalain pigment on hypocotyl after transfer onto hormone-free MS medium.

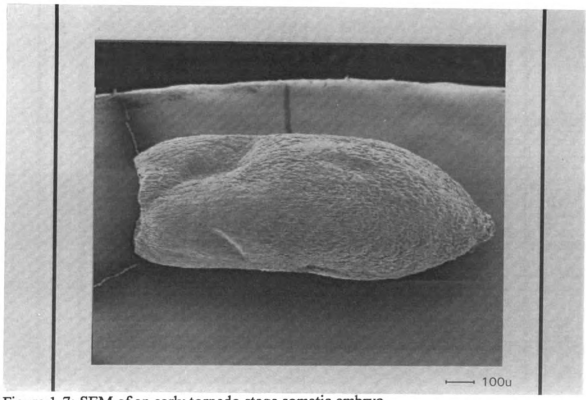

Figure 1-7: SEM of an early torpedo stage somatic embryo.

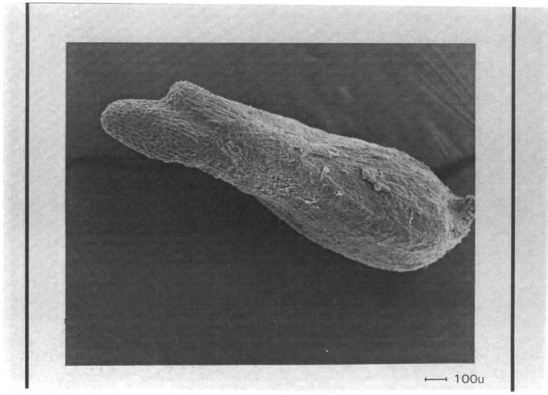

Figure 1-8: SEM showing a torpedo stage somatic embryo.

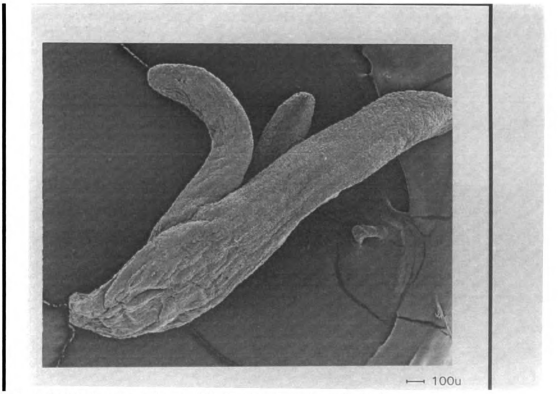

Figure 1-9: SEM showing a late cotyledonary stage somatic embryo.

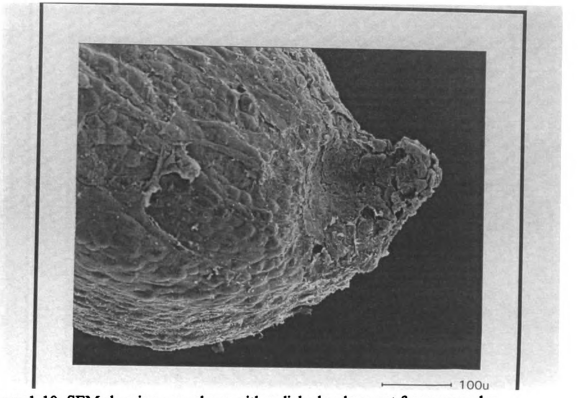

Figure 1-10: SEM showing an embryo with radicle development from one polar.

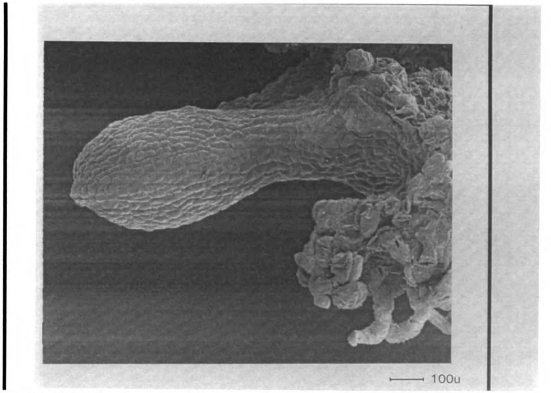

Figure 1-11: SEM showing a torpedo stage of somatic embryo attached to the callus.

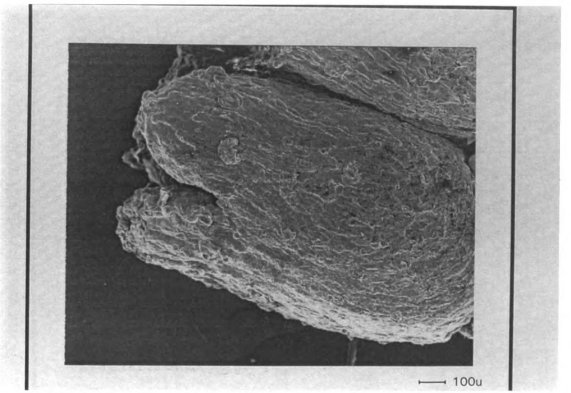

Figure 1-12: SEM showing a somatic embryo with cotyledons and a little swollen hypocotyl.

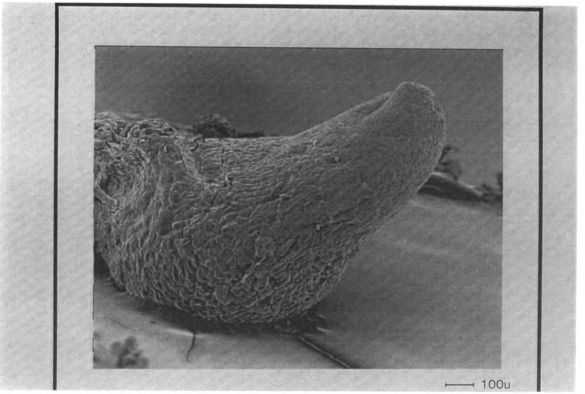

Figure 1-13: SEM showing an abnormal embryo with swollen a swollen hypocotyl.

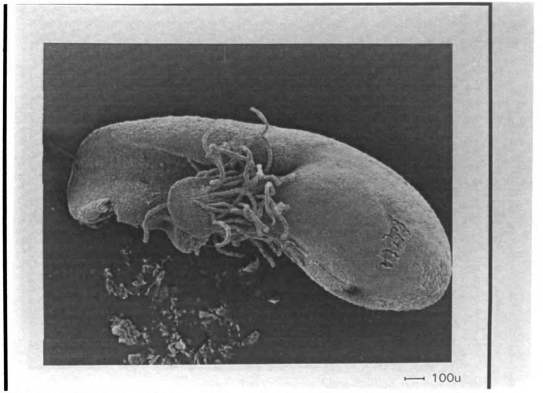

Figure 1-14: SEM showing trichomes only occurred on the cotyledonary surface of embryo.

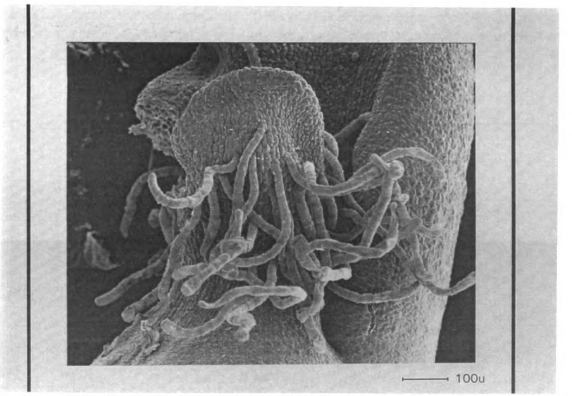

Figure 1-15: SEM showing the magnification of partially area from Figure 1-14. The trichomes appeared on the cotyledons of the somatic embryo.

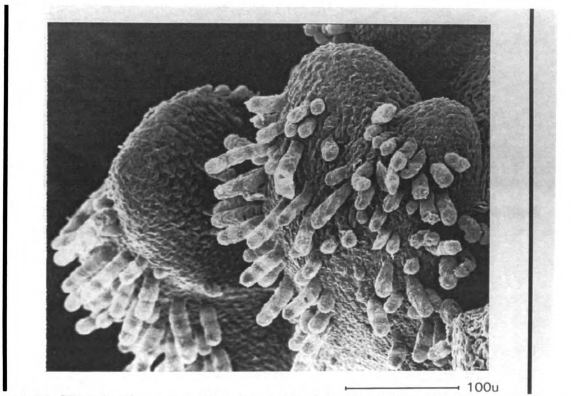

Figure 1-16: SEM showing a somatic embryo with trichomes on the cotyledonary surface.

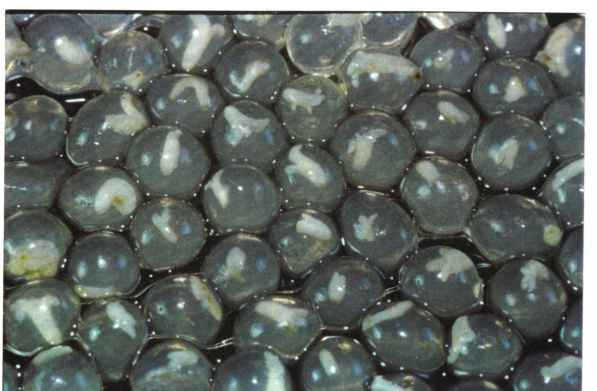

Figure 1-17: Encapsulation of somatic embryos. The diameter of each gel bead is about 4-5 mm.

CHAPTER 2

MEDIA AND GENOTYPE EFFECTS ON SOMATIC EMBRYOGENESIS FROM SUGARBEET CALLUS.

ABSTRACT

Suspension platings of two sugarbeet genotypes, REL-1 and REL-2, were used to determine the effects on somatic embryo production under varied combinations of abscisic acid (ABA) with the growth regulators 1-naphthalene acetic acid (NAA), 6-benzyladenine (BA), triiodobenzoic acid (TIBA), 2,4-dichloroacetic acid (2,4-D), different sucrose concentrations, or different sole nitrogen sources with the goal of improving somatic embryo production. Higher BA concentration was reduced embryo numbers but increased shoot regeneration for both clones. At some BA levels, the presence of ABA improved somatic embryo and/or shoot numbers up to twenty-fold. TIBA in the range of 0.1-3.0 mg/L had a promotive effect on somatic embryo production only in combination with ABA. 2,4-D was ineffective in improving somatic embryo numbers. Three and five percent sucrose were superior to one, seven, and nine percent for promoting somatic embryo production. At some concentrations, urea and glutamine as sole nitrogen sources, as well as NAA, stimulated greater embryo production over the control, but only in REL-1, which in general was a much less prolific somatic embryo and shoot producer than REL-2, one of its progeny. Moreover, ABA at certain concentrations consistently improved embryo production in all experiments, and was observed to stimulate shoot production. The range of embryo production was more than thirty-five

fold between genotypes (REL-2 and REL-1), whereas the range of growth regulator effects was no greater than ten-fold. Use of these clones for transgenic efforts, somatic cell selection or synthetic seeds should be facilitated by this research.

INTRODUCTION

Plant tissue culture is playing an important part in plant biotechnology directly for mass clonal propagation and indirectly as a central tool for somatic cell selection or genetic engineering, in plant modification and improvement. In plant tissue culture, somatic embryos have most often been produced from callus or suspension culture cells under appropriate culture conditions, with growth regulators or genotype dependency being common. Somatic embryogenesis was first reported in 1958 with carrot (Steward et al., 1958). Since then, the number of species producing somatic embryos has rapidly increased. The list is well diversified and includes field crops, vegetables, ornamentals and trees. This success is attributed to a number of factors, such as the search for amenable genotypes, rapid communication of successful media, common patterns of media induction of embryogenesis, and a better understanding of the embryo developmental process. Despite the lengthy period of development of these procedures, somatic embryogenesis is still a poorly understood phenomenon for most crops. Further research is required to fully understand and control its use.

Plant growth regulators play a very important role in eliciting organogenesis or embryogenesis from callus or suspensions. An auxin is usually required in the medium for induction of somatic embryogenesis and maintenance of proliferative growth (Ammirato and Steward, 1971; Vasil et al., 1982). Although 2,4-D was more effective in promoting

embryogenesis compared to other auxin sources in some species, such as barley or white clover (Ziauddin and Kasha, 1990; Parrott, 1991), NAA was a more useful auxin for production of somatic embryos that converted into normal plantlets in asparagus (Levi and Sink, 1991) and pecan (Rodriguez and Wetzstein, 1994).

Cytokinin is often required for induction of shoot organogenesis, but is not required for initiation of somatic embryogenesis (Ammirato, 1983). Sometimes, cytokinin in combination with auxin were essential for growth and induction of somatic embryogenesis as in carrot cell suspensions (Fujimura and Komanine, 1980). Levi and Sink (1991) reported that the combination of auxin (NAA or 2,4-D) and cytokinin (kinetin) dramatically affected the frequency of somatic embryos.

ABA has distinct effects on morphogenesis (Ammirato, 1974 ; 1983). ABA, at the appropriate concentrations, can inhibit precocious germination and abnormal development in alfalfa (Fujii et al., 1990), as well as foster normal maturation and conversion in rapeseed (Finkelstein et al., 1985) and *Liriodendron tulipifera* (Merkle et al., 1990). ABA and osmoticum (such as sucrose) can promote maturation of embryos in pea (Barratt and Clark, 1989; Misra et al., 1993). Xu et al. (1990) reported that precocious germination of developing alfalfa embryos could be suppressed by ABA and high osmotic potential in culture. However, an osmoticum did raise the endogenous ABA level in maize (Rivin and Grudt, 1991), and then the increased ABA may further promote the induction of embryogenesis. Besides, ABA also enhanced the conversion of somatic embryos into plantlets in asparagus (Li and Wolyn, 1995; Li and Wolyn, 1996).

Response in tissue culture inducing somatic embryogenesis varies greatly with genotype, e.g., soybean (Bailey et al., 1993), watermelon (Compton and Gary, 1993), and

asparagus (Delbreil and Jullien, 1994). Thus, somatic embryogenesis and regeneration ability could probably be increased in vitro by selecting highly responsive genotypes and subsequent breeding. In alfalfa, Brown and Atanassov (1985) found that 50 of 76 cultivars failed to produce somatic embryos. Li and Wolyn (1996) reported that only three of six asparagus genotypes produced somatic embryos.

Somatic embryos in sugarbeet callus or suspension cultures have been reported anecdotally by a number of authors (Atanassov, 1976; Tetu et al., 1987; Freytag et al., 1988; Doley and Saunders, 1989; Kubalikova, 1990; Tenning et al., 1992; D'Halluin et al., 1992). Tetu et al. (1987) concluded that multiple hormonal sequences in the medium were necessary for the induction and development of somatic embryos from callus. In contrast, Doley and Saunders (1989) reported the simple production and partial germination of somatic embryos from leaf-disc hormone-autonomous callus of a fodder beet cultivar without the use of growth regulators. Tsai and Saunders (1995) extended this work to sugarbeet biotech clone REL-1 by demonstrating that somatic embryos, albeit in low frequency (one per ml of plated suspension), could be recovered from callus after plating suspensions grown with Murashige-Skoog (MS) (1962) medium plus 1 mg/L BA onto MS hormone-free medium. This same work determined that certain concentrations of ABA in the plating medium increased the somatic embryo yield of REL-1 as much as fifteen-fold.

In order to increase somatic embryo number, two sugarbeet genotypes (REL-1 and REL-2) were used to measure the level of somatic embryo production from suspensions plated onto MS medium with combinations of ABA with different concentrations of growth regulators, sucrose, or different sole nitrogen sources in this studies.

MATERIALS AND METHODS

Plant materials

The experiments were performed with several genotypes of sugarbeet (*Beta vulgaris* L.). REL-1 (J.W. Saunders, East Lansing, MI.) is a self-fertile diploid sugarbeet clone bred for ease of tissue culture manipulations. It exhibits a high frequency of leaf disc callusing, shoot regeneration from hormone autonomous callus, dispersed suspension cultures and resistance to shoot vitreousness. REL-1 has been used for the recovery of monogenic dominant sulfonylurea herbicide resistance by somatic cell selection (Saunders et al., 1992). REL-2 (also known as LTR-41) is an F_1 hybrid clone between EL 45/2-108 (Doley, 1990) and REL-1. Three additional clones were tested in one experiment: Clone 6926cms12 (from SP6926-01) (G. Coe, Beltsville, MD), Clone GWK-R, an individual plant from the mating of two somatic-embryo-derived plants from the fodder beet cultivar Garton's White Knight, and Clone 6822-08 from SP6822 (J.W. Saunders, East Lansing, MI).

Both REL-1 and REL-2 have been maintained in shoot culture (Saunders,1982). LTR-41 has been released as REL-2 so that sugarbeet researchers can use its superior embryogenic and shoot regeneration abilities for application in biotechnology.

Culture media

The culture media contained MS mineral salts (Murashige and Skoog, 1962), supplemented with 100 mg/L myo-inositol, 1.0 mg/L thiamine·HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine· HCl, and 30 g/L sucrose. Media used for leaf disc callusing

were gelled with 9 g/L Difco Bacto agar, whereas media used in plating out were gelled with 3.5 g/L phytagel. The growth regulators used were NAA (0.3-10 mg/L), BA (0.1-1.0 mg/L), TIBA (0.1-10 mg/L), 2,4-D (0.1-1.0 mg/L), and/or ABA (0.02-3.0 mg/L). The pH was adjusted to 5.95 with KOH or HCl before autoclaved (15 psi, 121 °C, 20 min). ABA was filter-sterilized and added into previously autoclaved and partially cooled media, as were sole nitrogen sources. Nitrate (NO_3), ammonium (NH_4) urea, glutamine (GLN), glutamate (GLU), proline (PRO), glycine betaine (BET) and choline (CHO) were tested as sole nitrogen source at 60 mM atomic nitrogen. KNO_3 (20 mM) and NH_4NO_3 (40 mM) were used as the standard MS nitrogen. Nitrogen-free (MS-N) served as the control. NH4 was provided as the chloride salt and was accompanied by half that concentration of succinic acid.

Culture vessels were 125 mL Erlenmeyer flasks for suspension culture or 20 x 100 mm Falcon disposable plastic Petri plates for callus initiation and suspension plate-out. Medium volume per vessel was 35 ml. Flasks were closed with foam caps and aluminum foil. Petri plates were sealed with one layer of Parafilm.

Callus was initiated from leaf discs (8 mm diameter) cut from partially expanded leaves of greenhouse grown plants. Leaves were sterilized for twenty minutes with a dilute (1:5 v/v) commercial (5.25 % w/v) sodium hypochlorite (NaOCl) bleach containing 100 mg/L sodium lauryl sulfate detergent followed by five rinses in sterile distilled water. Leaf discs were placed on MS medium with 1 mg/L BA (Saunders et al., 1992) in Petri plates incubated at 30 °C in the dark, one disc per plate of 35 mL medium. Callus was first observed after one month, and after another month (from REL-1); or after 2-3 weeks (from REL-2), 2 to 3 g of leaf-disc callus was transferred to liquid hormone-free MS

medium in flasks for growth at 21±2 °C in the dark. However, REL-2 required only half a month before callus was taken for suspension culture because it grew faster. The suspension cultures were shaken on rotary shakers at 125 rpm to aerate the cultures and to reduce cell cluster size.

Suspension cultures were subcultured with hormone-free MS medium every 5-7 days. After 10 to 14 days, suspensions used as inoculum for platings were pushed through a stainless steel sieve with 830 um openings. For experiments with different nitrogen sources or sucrose levels, sieved suspensions were washed with nitrogen-free or sucrose-free liquid medium, respectively. Sieved suspension cell clusters were plated on MS media with the indicated combinations of growth regulators. Plates were inoculated with 1 ml (approximately 0.1 g fresh weight) of suspension by plating out with disposable plastic Pasteur pipettes. After inoculation, plates were sealed with Parafilm strips and incubated in dim light (less than 5 umol $m^{-2}s^{-1}$) from fluorescent lamps (Philips cool white econowatt) at 25 °C. Experiments were terminated after 4-5 weeks. Minimum size for an embryo to be counted was 0.5 mm.

The number of somatic embryos and shoots, as well as fresh weight of somatic embryos and callus were statistically analyzed. The two factor randomized complete block design with 6-10 replications per treatment was used, and experiments were performed with similar results 2 or 3 times each. The analysis of variance was performed using MSTAT. The least significant difference (LSD, P=0.05) was used to separate treatment mean.

RESULTS AND DISCUSSION

Initiation of leaf disc callus and suspension cultures

In preliminary experiments, clone REL-2 was developed primarily as an embryogenic genotype, using somatic embryo production on primary leaf disc callus initiating on hormone-free medium as evaluation criterion (Figure 2-1). Callus initiation time was 2 months (twice as long compared to the response on MS medium with 1 mg/L BA), and after 3 months, about a third of such calli had a rough fuzzy white area of what appeared to be somatic embryos or white embryogenic tissue (Figure 2-2).

When leaf disc callus was initiated on MS medium with 1 mg/L BA in darkness at 30 °C, shoot regeneration was ultimately seen with genotypes REL-1 (Figure 2-3) and REL-2 (Figure 2-4). REL-1 leaf disc callus first appeared after about one month, and after another month, the friable callus was transferred to liquid hormone-free MS medium for suspension culture (Figure 2-5). Most REL-2 leaf disc callus underwent shoot regeneration quickly after callus was observed. Therefore, REL-2 only required two weeks before callus was transferred to hormone-free MS liquid medium for suspension culture since its callus quickly changed color to brown after a short time. These suspension culture establishment procedures were applied to the following experiments.

Induction of somatic embryos with ABA and NAA

With genotype REL-1, a low frequency of somatic embryogenesis, less than one embryo per petri plate, was seen following plating-out of subsequent suspension cultures on hormone-free MS medium. Suspensions of REL-1 plated onto media with

combinations of NAA and ABA produced low numbers of embryos (Figure 2-6), but ABA at 0.1 or 0.3 mg/L in the medium increased the number of somatic embryos per plate up to nine fold (Figure 2-7). Nearly one embryo per plate was found on the basal medium without containing NAA and ABA. Maximal production, more than six embryos per plate, occurred on the medium with 1.0 mg/L NAA and 0.1 mg/L ABA. ABA is well known for its positive effects in somatic embryogenesis, where it normalizes development and inhibits precocious germination (Ammirato, 1974, 1983).

For genotype 6926cms12, a very low number of somatic embryos was produced when similar combinations of NAA and ABA were used (Figure 2-8). This genotype produced embryos only with 1 mg/L NAA + 0.1 mg/L ABA (one embryo per plate) or 3 mg/L NAA + 0.1 mg/L ABA (0.3 embryo per plate).

For genotype GWK-R and genotype 6822-08, no somatic embryos were obtained in this system when similar combinations of NAA and ABA were used.

With genotype REL-2, much higher frequency of somatic embryogenesis (Figure 2-9) was seen, with nearly 40 embryos per plate on basal medium (Figure 2-10). The inclusion of 0.1 or 0.3 mg/L ABA alone in the plate out medium significantly increased the production of somatic embryos (Figure 2-11, 2-12). Maximum average somatic embryo yield observed was 77 per ml of suspension plated out on the MS medium containing 0.3 mg/L NAA and 0.1 mg/L ABA. On the other hand, high levels of ABA (3 mg/L) or NAA (3 and 10 mg/L) decreased the combined fresh weight of somatic embryos and callus per plate (Figure 2-13).

Actually, different genotypes strongly affected somatic embryogenesis and the number of somatic embryos, as least as much as any growth regulator combination did. Similarly,

other reports showed how different genotypes affect somatic embryogenesis such as with grape (Emershad and Ramming, 1994), and radish (Takahata et al., 1996). Genotype along with auxin had a significant effect on the frequency of somatic embryogenesis in soybean (Parrot et al., 1989) and in chickpea (Eapen and George, 1994).

Induction of somatic embryos with ABA and 2,4-D

Concentrations of 2,4-D from 0.1 to 1.0 mg/L with or without ABA did not strongly affect the frequencies of somatic embryogenesis with REL-2 (Figure 2-14). Media containing ABA at 0.1 mg/L had significantly higher numbers of somatic embryos than did those with 0, 0.3 or 1.0 mg/L. Thus, ABA was far more effective for promoting somatic embryos than was 2,4-D. In some species, 2,4-D was more effective in promoting somatic embryogenesis than were other auxins (NAA or IAA). For instance, 2,4-D was superior to NAA or IAA for embryogenic callus initiation or maintenance in barley (Ziauddin and Kasha, 1990). Nevertheless, Rodriguez and Wetzstein (1994) reported that more pecan embryos were induced by 2.4-D, but NAA was more effective in inducing somatic embryos morphologically resembling zygotic embryos and their subsequent conversion into plantlets.

Induction of somatic embryos and shoots with ABA and BA

Combinations of ABA with BA were evaluated for effects on production of somatic embryos and shoots in genotypes REL-1 and REL-2. A low frequency of somatic embryogenesis (not higher than six embryos per mL of plated suspension) was seen following plating onto media containing various combinations of BA and ABA from

genotype REL-1 (Figure 2-15). However, the yield of shoots obtained through organogenesis was progressively higher as concentrations of both BA and ABA increased (Figure 2-16). Approximately 68 shoots per plate were induced with 1.0 mg/L BA and 2 mg/L ABA (Figure 2-17).

With genotype REL-2, a similar pattern of lower embryo and higher shoot numbers with progressively higher BA concentration was observed following plating-out of suspension (Figure 2-18). Anyway, the yields of somatic embryos and shoots were considerably higher than with REL-1. REL-2 produced nine times more embryos in the absence of BA when averaged over ABA concentrations, and 3.5 and 2 times more shoots at 0.3 and 1.0 mg/L BA, respectively. Moderate ABA concentrations (0.02 or 0.2 mg/L) stimulated embryo production, and the effect was significant at 0 and 0.1 mg/L BA. BA inhibited embryogenesis at all concentrations, regardless of the presence or absence of ABA. ABA significantly stimulated shoot production in combination with 0.3 or 1.0 mg/L BA (Figure 2-19).

However, ABA had a strong positive influence on both somatic embryo and shoot production in an interactive manner: at least one of the three ABA concentrations enhanced either somatic embryo or shoot production at the two best BA levels, respectively, with both genotypes REL-1 and REL-2 (Figure 2-15, 2-18). With the less productive clone REL-1, progressive increases in shoot number were evident with increasing initial ABA concentrations at the two highest starting BA levels, to the point where at the combination of highest levels of both ABA and BA, shoot production reached an intensity matching that seen in the absence of ABA in the starting medium with REL-2.

Induction of somatic embryos and shoots with ABA and TIBA in the presence of 1.0 mg/L BA

Combinations of TIBA and ABA were tested at 1.0 mg/L BA, a level very conducive to shoot production but not very permissive of somatic embryo production. TIBA at 10 mg/L severely inhibited both somatic embryo and shoot formation in both REL-1 and REL-2 genotypes (Figure 2-20, 2-21). Overall, TIBA in the range of 0.1-3.0 mg/L had a promotive effect on somatic embryo production only in combination with ABA, to a minor degree, and more so for REL-1 than for REL-2. By itself, TIBA had no promotive effect on shoot formation with either genotype, but for REL-1 in the TIBA concentration range 0.1-3.0 mg/L (4 treatment levels), it interacted positively with ABA at the 2.0 mg/L treatment level to double or even triple shoot numbers per plate (Figure 2-20). From the ABA perspective, genotype REL-1 was much more responsive, and this was seen as an interactive effect on shoot production.

Induction of somatic embryos with ABA and sucrose

With genotype REL-2, the influence of sucrose concentration in the plating medium on somatic embryo production was examined with and without ABA at 0.2 mg/L. Increasing the sucrose concentration from 1 to 3 or 5% significantly increased embryo production, in the absence of ABA (Figure 2-22). ABA had no significant effect at 1, 7, and 9% sucrose, but approximately doubled embryo production with 3 and 5% sucrose. The positive effect of sucrose concentration on somatic embryo production has also been documented by Gray et al. (1993) with *Cucumis melo*. Komura et al. (1990) reported that 2-3 % sucrose

was the optimum concentration for embryogenic callus induction in asparagus, but somatic embryos only developed with 5 % sucrose.

Induction of somatic embryos with ABA and sole nitrogen sources

Combinations of sole nitrogen sources and ABA (0.02, 0.2, 2 mg/L) as the only growth regulator were examined in the plating medium for both genotypes, REL-1 and REL-2. With REL-1, only urea and GLN dramatically increased embryo production on the media containing 2.0 and 0.2 mg/L ABA, respectively, over the MS nitrogen mix control (Figure 2-23). Embryo production for most nitrogen sources was greater for at least one of the ABA concentrations than in its absence. Media containing no nitrogen, GLU, BET, or CHO formed almost no embryos. In general, shoot formation for each nitrogen source reflected embryo production. Very few embryos or shoots were produced on BET, CHO, or in the absence of nitrogen.

Maximum embryo production was four times as high with REL-2 than with REL-1, and no nitrogen source gave a significantly higher production than the MS nitrogen mix control (Figure 2-24). Only urea, GLN and the MS nitrogen mix produced considerable numbers of somatic embryos. In addition, with the more embryogenic genotype REL-2, shoot production did not reflect embryo production when nitrogen sources were compared to genotype REL-1. BET, CHO, and the nitrogen-free medium (MS-N) were unproductive of embryos or shoots.

In other crops, supplemental PRO stimulated embryogenic callus in carrot (Nuti-Ronchi et al., 1984), maize (Armstrong and Green, 1985) and *Agrostis alba* (Shetty and Asano, 1991). Supplemental GLN promoted somatic embryogenesis in suspension cultures of

Gossypium klotzschianum (Price and Smith, 1979). Moreover, supplementation with GLN also increased somatic embryo size and increase storage protein in alfalfa (Lai and McKersie, 1994).

Development of somatic embryos

During the first two weeks following plating-out, white or light yellow callus proliferated. From the third to the sixth week, various late stages of somatic embryos, from torpedo to mature opaque white embryos with cotyledons, were clearly distinguishable at the callus surface (Figure 2-25). The simultaneous occurrence of somatic embryos of different lengths (0.5 to 4 mm) indicated that somatic embryogenesis was not uniform and synchronous. After 40 days, most of the somatic embryos with cotyledons were around 2 - 3 mm long. Each somatic embryo could be easily separated from the surrounding callus.

The promotive effect of ABA on somatic embryo number as initially reported by Tsai and Saunders (1995) with REL-1 has been much more extensively confirmed with the second genotype REL-2. Different genotypes and media under varied combinations of ABA with other growth regulators (NAA, 2,4-D, BA, TIBA), different sucrose concentrations or different sole nitrogen sources strongly affected somatic embryo production. Bell et al. (1993) also reported that exogenous ABA promoted somatic embryo number in orchardgrass, and high internal levels of ABA have been implicated in permitting somatic embryogenesis from leaf explants of Napier grass (Rajasekaran et al., 1987). The involvement of ABA in regulating the continued normal development of

somatic embryos was demonstrated by Ammirato (1974; 1983). The application of ABA also prevented precocious germination and improved desiccation tolerance in somatic embryos of alfalfa (Senaratna et al., 1989; Anandarajah and McKersie, 1990). During the later stages of embryogeny, high osmoticum affected accumulation of storage proteins in rapeseed embryo development (Finkelstein and Crouch, 1986). ABA might suppress water uptake to attain the desiccation phase, since desiccation is required to complete embryo maturation. On the other hand, application of high concentrations of ABA to carrot somatic embryos caused dedifferentiation to embryogenic callus-like structures (Iida et al., 1992). Thus, the growth regulator ABA plays an important role from the initiation of somatic embryogenesis and further development as well as conversion into plantlets.

In this research, sugarbeet genotype REL-2 was first found to have superior somatic embryogenesis and shoot regeneration abilities. In addition, this research also showed that exogenous ABA significantly promoted the frequencies of somatic embryo and shoot production from both genotypes (REL-1 and REL-2). Therefore, we can utilize these clones in future studies of somatic cell selection, artificial seeds and genetic transformation.

REFERENCES

Ammirato, P.V. 1974. The effects of abscisic acid on the development of somatic embryos from cells of caraway (*Carum carvi* L.). Bot. Gaz. 135: 328-337.

Ammirato, P.V. 1983. The regulation of somatic embryo development in plant cell cultures: Suspension culture techniques and hormone requirements. Bio/Technology 1: 68-74.

Ammirato, P.V. and F.C. Steward. 1971. Some effects of the environment on the development of embryos from cultured free cells. Bot. Gaz. 132: 149-158.

Anandarajah, K. and B.D. McKersie. 1990. Manipulating the desiccation tolerance and vigour of dry somatic embryos of *Medicago sativa* L. with sucrose, heat shock and abscisic acid. Plant Cell Rep. 9: 451-455.

Armstrong, C.L. and C.E. Green. 1985. Establishment and maintenance of friable, embryo -genic maize callus and the involvement of $_{L}$-Proline. Planta 164: 207-214.

Atanassov, A.I. 1976. Analysis of organogenetic abilities of continuously cultivated somatic tissues of sugar beet on the basis of obtaining single-celled clones and suspension cultures. *In* F.J. Novak (ed.). Use of tissue cultures in plant breeding. Institute of Experimental Botany, Olomouc. pp. 69-80.

Bailey, M.A., H.R. Boerma, and W.A. Parrott. 1993. Genotype effects on proliferative embryogenesis and plant regeneration of soybean. In Vitro Cell Devel. Biol. 29: 102-108.

Barratt, D.H.P. and J.A. Clark. 1989. Proteins arising during the late stages of embryogenesis in *Pisum sativum* L. Planta 184: 14-23.

Bell, L.M., R.N. Trigiano, and B.V. Conger. 1993. Relationship of abscisic acid to somatic embryogenesis in *Dactylis glomerata*. Env. Exp. Bot. 33: 495-499.

Brown, D.C. and A.I. Atanassov. 1985. Role of genetic background in somatic embryogenesis in *Medicago*. Plant Cell Tissue Org. Cult. 4: 111-122.

Compton, M.E. and D.J. Gray. 1993. Somatic embryogenesis and plant regeneration from immature cotyledons of watermelon. Plant Cell Rep. 12: 61-65.

Delbreil, B. and M. Jullien. 1994. Evidence for in vitro induced mutation which improves somatic embryogenesis in *Asparagus officinalis* L. Plant Cell Rep. 13: 372-376.

D'Halluin, K., M. Bossut, E. Bonne, B. Mazur, J. Leemans, and J. Botterman. 1992. Transformation of sugarbeet (*Beta vulgaris* L.) and evaluation of herbicide resistance in transgenic plants. Bio/Technology 10: 309-314.

Doley, W.P. and J.W. Saunders. 1989. Hormone-free medium will support callus production and subsequent shoot regeneration from whole plant leaf explants in some sugarbeet (*Beta vulgaris* L.) populations. Plant Cell Rep. 8: 222-225.

Eapen, S. and L. George. 1994. Somatic embryogenesis in *Cicer arietinum* L.: Influence of genotype and auxins. Biologia Plantarum 36: 343-349.

Emershad, R.L. and D.W. Ramming. 1994. Somatic embryogenesis and plant development from immature zygotic embryos of seedless grapes (*Vitis vinifera* L.). Plant Cell Rep. 14: 6-12.

Finkelstein, R.R. and M.L. Crouch. 1986. Rapeseed embryo development in culture on high osmoticum is similar to that in seeds. Plant Physiol. 81:907-912.

Finkelstein, R.R., K.M. Tenbarge, J.E. Shumway, and M.L. Crouch. 1985. Role of ABA in maturation of rapeseed embryos. Plant Physiol. 78: 630-636.

Freytag, A.H., S.C. Anand, A.P. Rao-Arelli, and L.D. Owens. 1988. An improved medium for adventitious shoot formation and callus induction in *Beta vulgaris* L. in vitro. Plant Cell Rep. 7: 30-34.

Fujii, J.A.A., D. Slade, R. Olsen, S.E. Ruzin, and K. Redenbaugh. 1990. Alfalfa somatic embryo maturation and conversion to plants. Plant Sci. 72: 93-100.

Fujimura, T. and A. Komamine. 1980. Mode of action of 2,4-D and zeatin on somatic embryogenesis in a carrot cell suspension culture. Z. Pflanzenphysiol. 99: 1-8.

Gray, D.J., D.W. McColley, and M.E. Compton. 1993. High-frequency somatic embryogenesis from quiescent seed cotyledons of *Cucumis melo* cultivars. J. Amer. Soc. Hort. Sci. 118: 425-432.

Iida, Y., K. Watabe, H. Kamada, and H. Harada. 1992. Effects of abscisic acid on the induction of desiccation tolerance in carrot somatic embryos. J. Plant Physiol. 140: 356-360.

Komura, H., S. Chokyu, and Y. Ikeda. 1990. Micropropagation of asparagus through somatic embryogenesis, 1: Somatic embryogenesis and plant regeneration from seedling. Bulletin-of-the-Hiroshima-Prefectural-Agricultural-Experiment-Station (Japan) 53: 43-50.

Kubalakova, M. 1990. Somatic embryogenesis and cytoplasmic sterility in *Beta vulgaris* L. var. *saccharifera*. Biol. Plant. 32: 414-419.

Lai, F.M. and B.D. McKersie. 1994. Regulation of starch and protein accumulation in alfalfa (*Medicago sativa* L.) somatic embryos. Plant Sci. 100: 211-219.

Levi, A. and K.C. Sink. 1991. Somatic embryogenesis in asparagus: the role of explants and growth regulators. Plant Cell Rep. 10: 71-75.

Li, B. and D.J. Wolyn. 1995. The effects of ancymidol, abscisic acid, uniconazole and paclobutrazol on somatic embryogenesis of asparagus. Plant Cell Rep. 14: 529-533.

Li, B. and D.J. Wolyn. 1996. Temperature and genotype affect asparagus somatic embryogenesis. In Vitro Cell. Dev. Biol. 32: 136-139.

Li, B. and D.J. Wolyn. 1996. Abscisic acid and ancymidol promote conversion of somatic embryos to plantlets and secondary embryogenesis in *Asparagus officinalis* L. In Vitro Cell. Dev. Biol. 32: 223-226.

Merkle, S.A., A.T. Wiecko, R.J. Sotak, and H.E. Sommer. 1990. Maturation and conversion of *Liriodendron tulipifera* somatic embryos. In Vitro Cell. Dev. Biol. 26: 1086-1093.

Misra, S., S.M. Attree, I. Leal, and L.C. Fowke. 1993. Effect of abscisic acid, osmoticum, and desiccation on synthesis of storage proteins during the development of white spruce somatic embryos. Ann. Bot. 71: 11-22.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Nuti-Ronchi, V., M.A. Caligo, M. Nozzolini, and G. Luccarini. 1984. Stimulation of carrot somatic embryogenesis by proline and serine. Plant Cell Rep. 3: 210-214.

Parrott, W.A. 1991. Auxin-stimulated somatic embryogenesis from immature cotyledons of white clover. Plant Cell Rep. 10: 17-21.

Parrott, W.A., E.G. Williams, D.F. Hildebrandt, and G.B. Collins. 1989. Effects of genotype on somatic embryogenesis from immature cotyledons of soybean. Plant Cell Tissue Organ Cult. 16: 15-21.

Price, H.J. and R.H. Smith. 1979. Somatic embryogenesis in suspension cultures of *Gossypium klotzschianum*. Planta 145: 305-307.

Rajasekaran, K., M.B. Hein, and I.K. Vasil. 1987. Endogenous abscisic acid and indole-3-acetic acid and somatic embryogenesis in cultured leaf explants of *Pennisetum purpur-*

eum schum. Effects *in vitro* and *in vitro* of glyphosate, fluridone, and paclobutrazole. Plant Physiol. 84: 47-51.

Rivin, C.J. and T. Grudt. 1991. Abscisic acid and the development regulation of embryo storage proteins in maize. Plant Physiol. 95: 358-365.

Rodriguez, A.P.M. and H.Y. Wetzstein. 1994. The effect of auxin type and concentration on pecan (*Carya illinoinensis*) somatic embryo morphology and subsequent conversion into plants. Plant Cell Rep. 13: 607-611.

Saunders, J.W. 1982. A flexible in vitro shoot culture propagation system for sugarbeet that includes rapid floral induction of ramets. Crop Sci. 22: 1102-1105.

Saunders, J.W., G. Acquaah, K.A. Renner, and W.P. Doley. 1992. Monogenic dominant sulfonylurea resistance in sugarbeet from somatic cell selection. Crop Sci. 32: 1357-1360.

Senaratna, T., B.D. McKersie, and S.R. Bowley. 1989. Desiccation tolerance of alfalfa (*Medicago sativa* L.) somatic embryos. Influence of abscisic acid, stress pretreatments and drying rates. Plant Sci. 65: 253-259.

Shetty, K. and Y. Asano. 1991. The influence of organic nitrogen sources on the induction of embryogenic callus in *Agrostis alba* L. J. Plant Physiol. 139: 82-85.

Steward, F.C., M.O. Mapes, and K. Mears. 1958. Growth and organized development of cultured cells. II. Organization of cultures grown from freely suspended cells. Am. J. Bot. 45: 705-708.

Takahata Y., H. Komatsu, and N. Kaizuma. 1996. Microspore culture of radish : influence of genotype and culture conditions on embryogenesis. Plant Cell Rep. 16: 163-166.

Tenning, P., E.W. Weich, U.B. Kjarsgaard, M.A. Lelu, and M. Nihlgard. 1992. Somatic embryogenesis from zygotic embryos of sugar beet (*Beta vulgaris* L.) Plant Sci. 81: 103-109.

Tetu, T., R.S. Sangwan, and B.S. Sangwan-Norreel. 1987. Hormonal control of organogenesis and somatic embreyogenesis in *Beta vulgaris* callus. J. Exp. Bot. 38: 506-517.

Tsai, C.J. and J.W. Saunders. 1995. Somatic embryos from callus of sugarbeet biotechnology clone REL-1. J. Sugar Beet Res. 32: 215-226.

Vasil, I.K., V. Vasil, C. Lu, P. Ozias-Akins, Z. Haydu, and D. Wang. 1982. Somatic embryogenesis in cereals and grasses. *In* E. Earle and Y. Demarly (eds.), Variability in plants regenerated from tissue culture. Praeger Press, New York. pp. 3-21.

Xu, N., K. M. Coulter, and J.D. Bewley. 1990. Abscisic acid and osmoticum prevent germination of developing alfalfa embryos, but only osmoticum maintains the synthesis of developmental proteins. Planta 182: 382-390.

Ziauddin, A. and K.J. Kasha. 1990. Long-term callus cultures of diploid barley (*Hordeum vulgare*). I. Auxin effects on culture initiation and maintenance. Euphytica 48: 171-176.

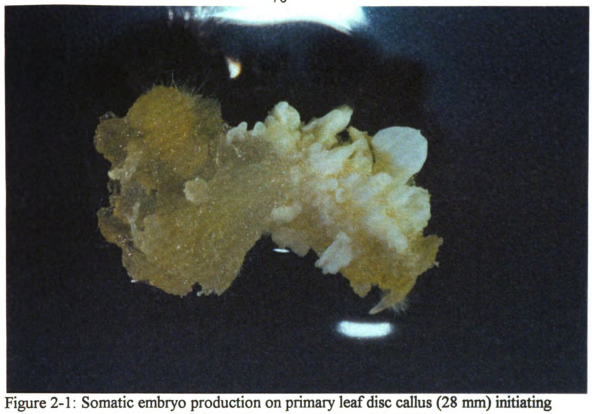

Figure 2-1: Somatic embryo production on primary leaf disc callus (28 mm) initiating on hormone-free MS medium after 58 days. (REL-2)

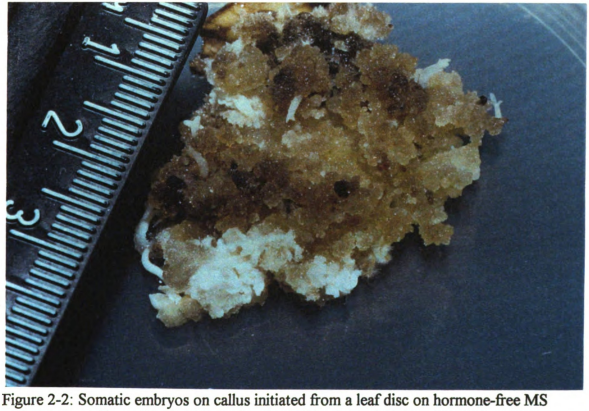

Figure 2-2: Somatic embryos on callus initiated from a leaf disc on hormone-free MS medium. About a third of such calli have rough fuzzy white embryogenic tissue. (REL-2)

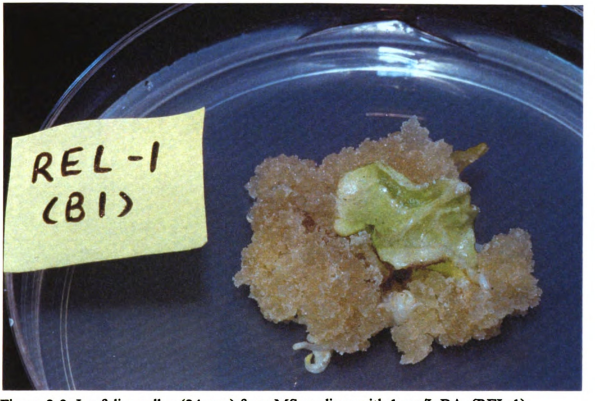

Figure 2-3: Leaf disc callus (24 mm) from MS medium with 1 mg/L BA. (REL-1)

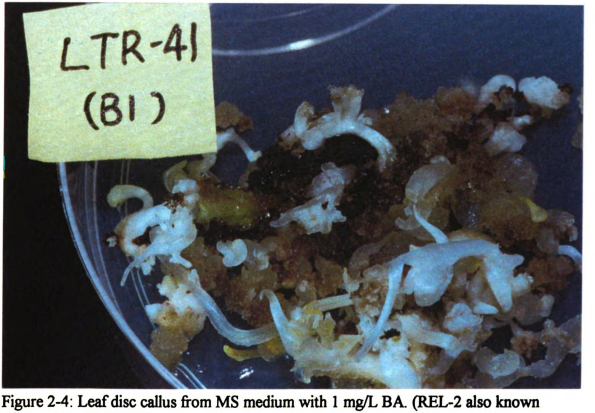

Figure 2-4: Leaf disc callus from MS medium with 1 mg/L BA. (REL-2 also known as LTR-41)

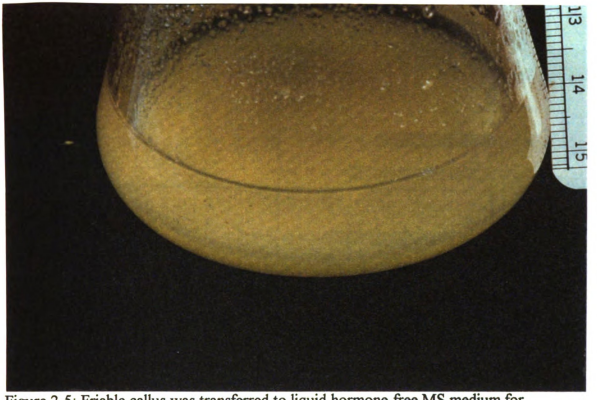

Figure 2-5: Friable callus was transferred to liquid hormone-free MS medium for suspension culture. (REL-1)

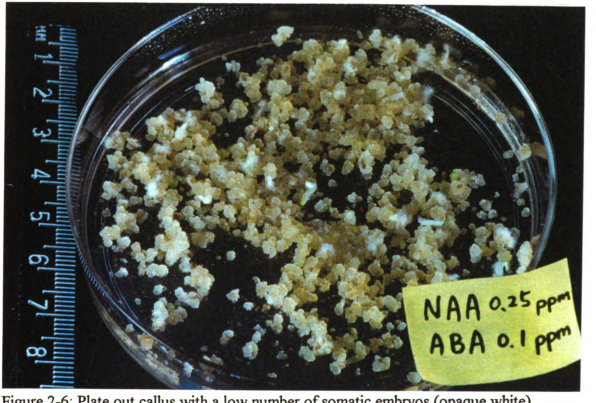

Figure 2-6: Plate out callus with a low number of somatic embryos (opaque white) on MS medium with 0.25 mg/L NAA and 0.1 mg/L ABA after 29 days. (REL-1)

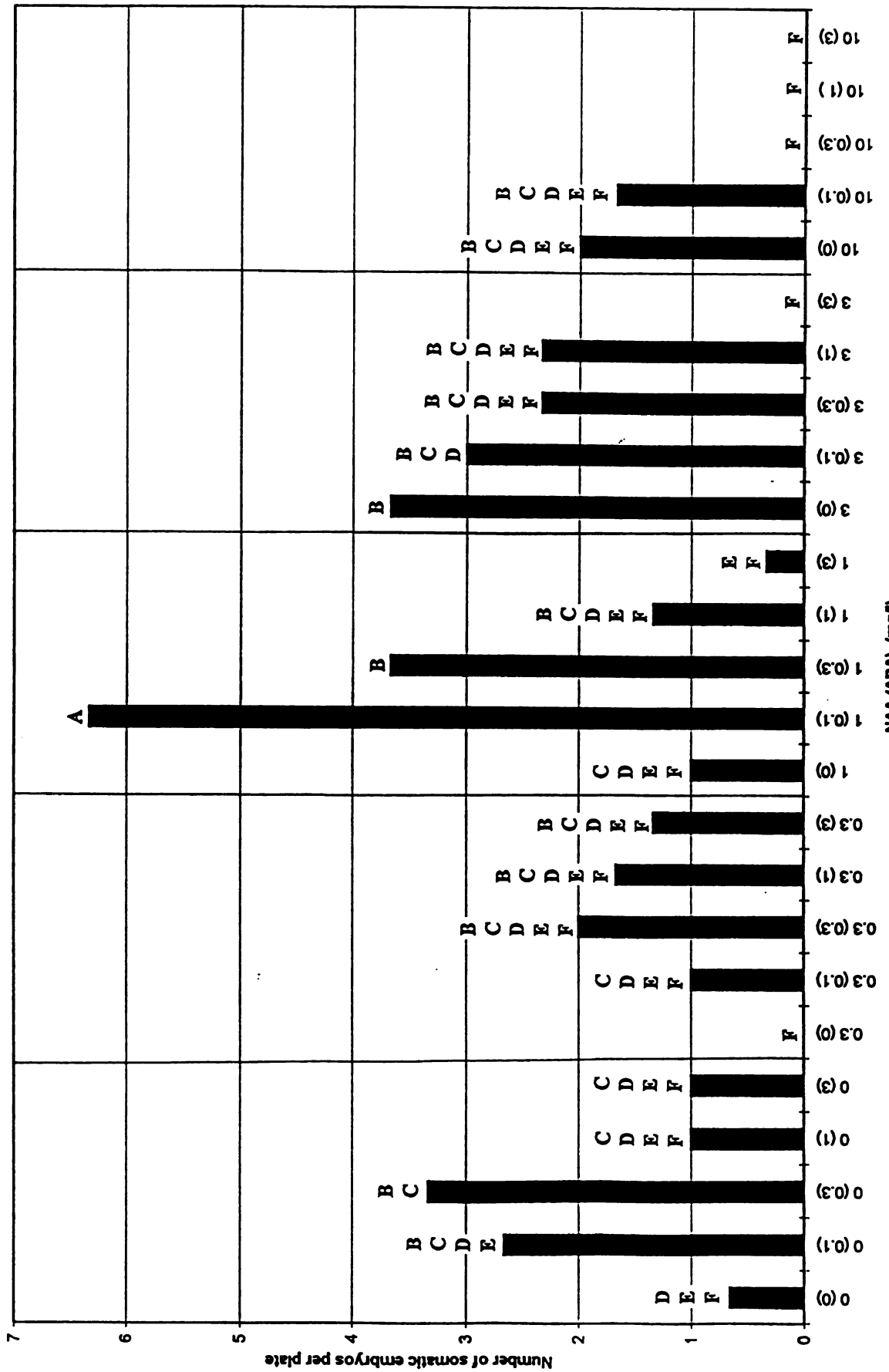

Figure 2-7: The influence of NAA and ABA on somatic embryo production with genotype REL-1 after 29 days. Means marked with same letter are not significantly different according to LSD ($P<0.05$).

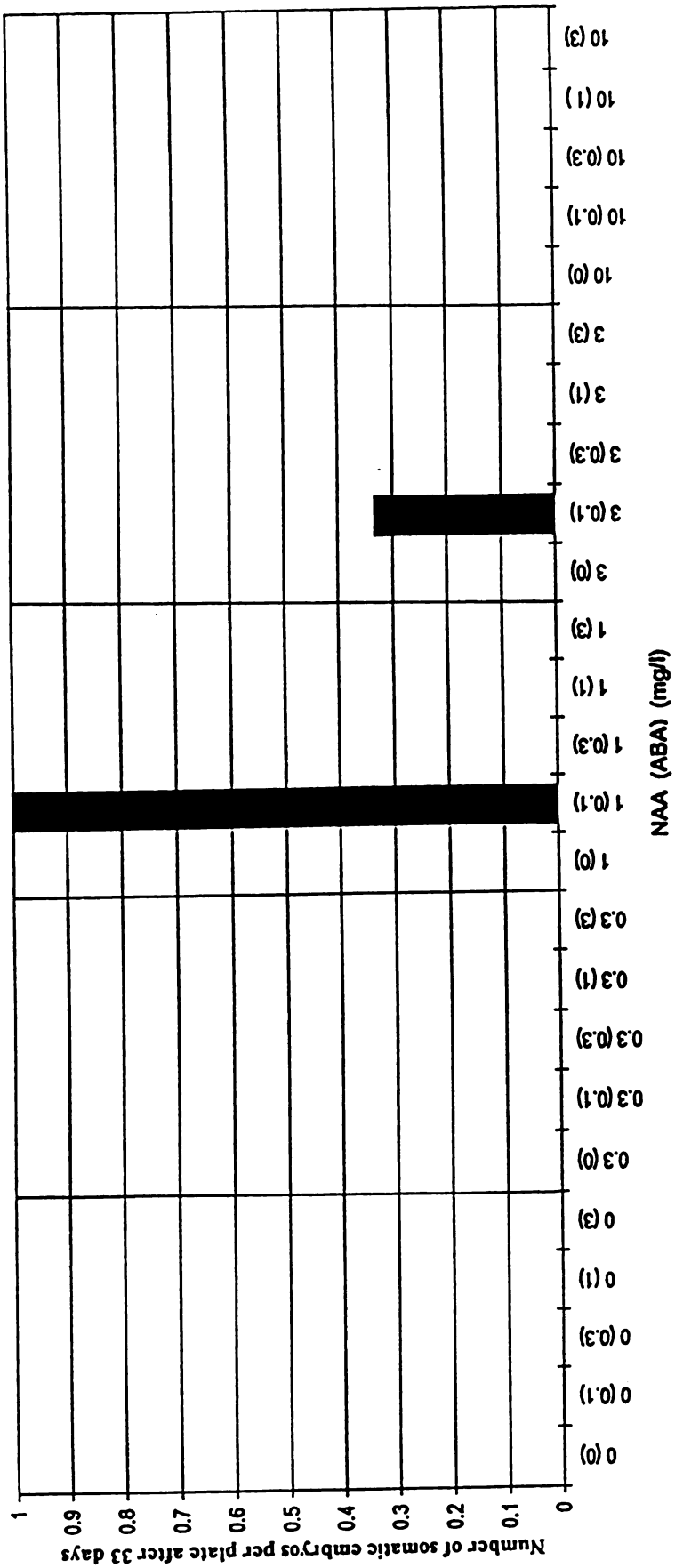

Figure 2-8: The influence of NAA and ABA on somatic embryo production with genotype 6926cms12 after 33 days.

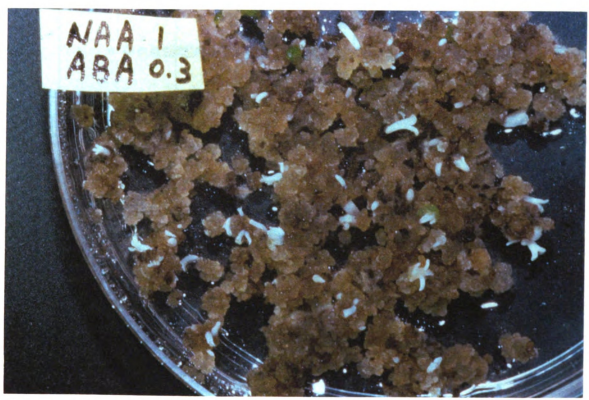

Figure 2-9: Plate out callus with somatic embryos on MS medium with 1 mg/L NAA and 0.3 mg/L ABA after 40 days. (REL-2)

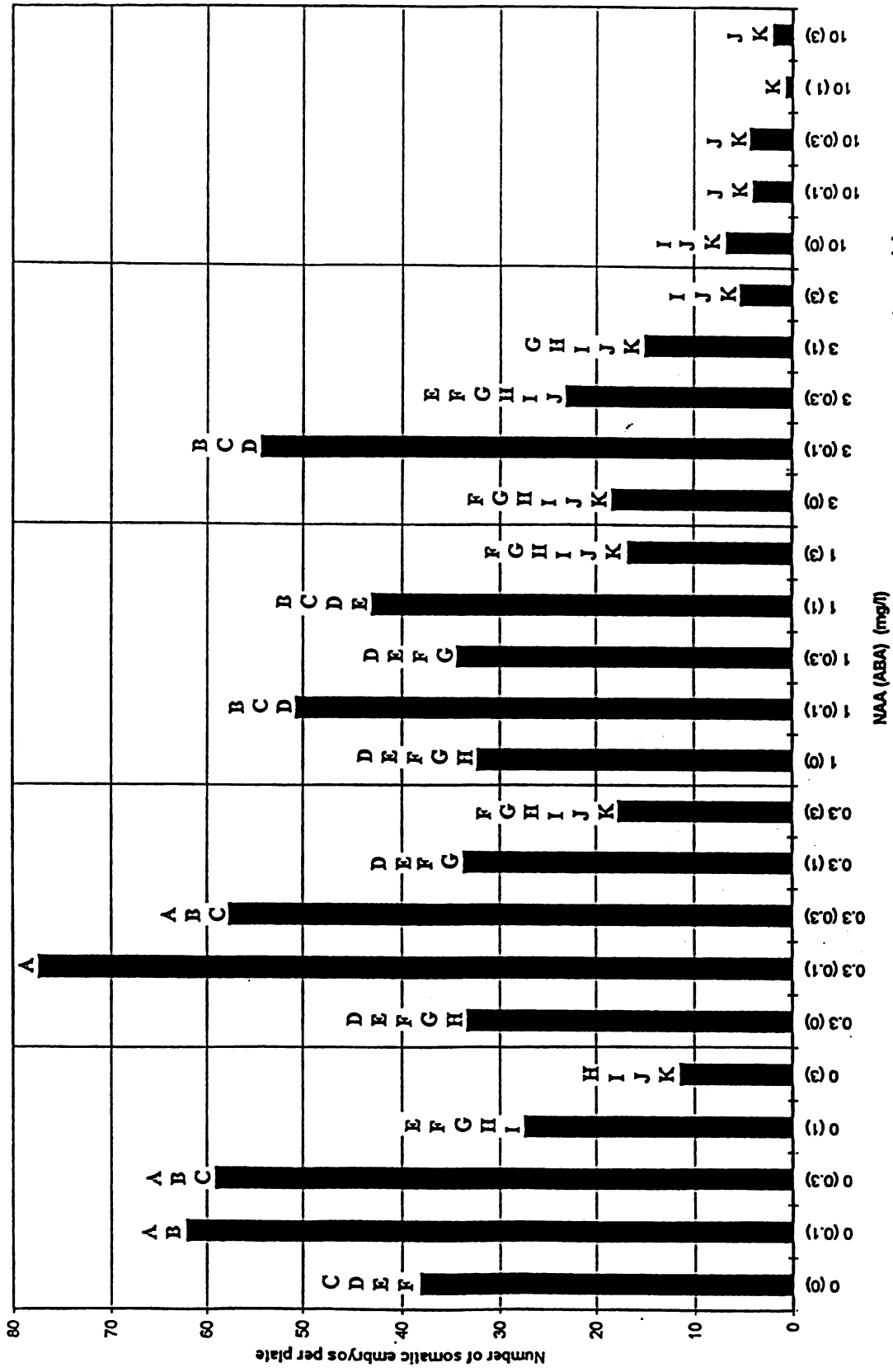

Figure 2-10: The influence of NAA and ABA on somatic embryo production with genotype REL-2 after 31 days. Means marked with the same letter are not significantly different according to LSD (P<0.05).

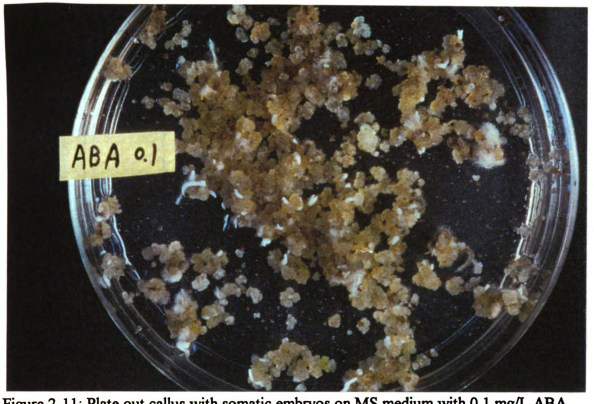

Figure 2-11: Plate out callus with somatic embryos on MS medium with 0.1 mg/L ABA after 31 days. (REL-2)

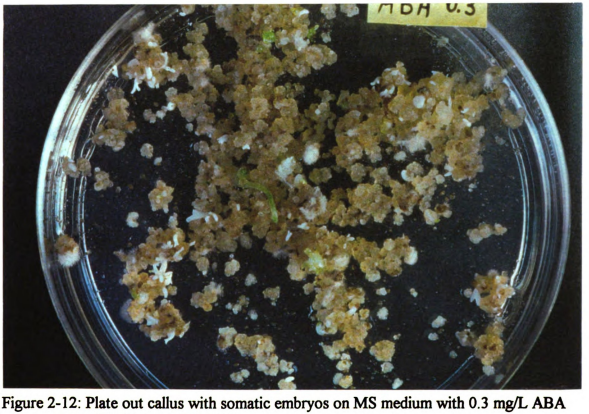

Figure 2-12: Plate out callus with somatic embryos on MS medium with 0.3 mg/L ABA after 31 days. (REL-2)

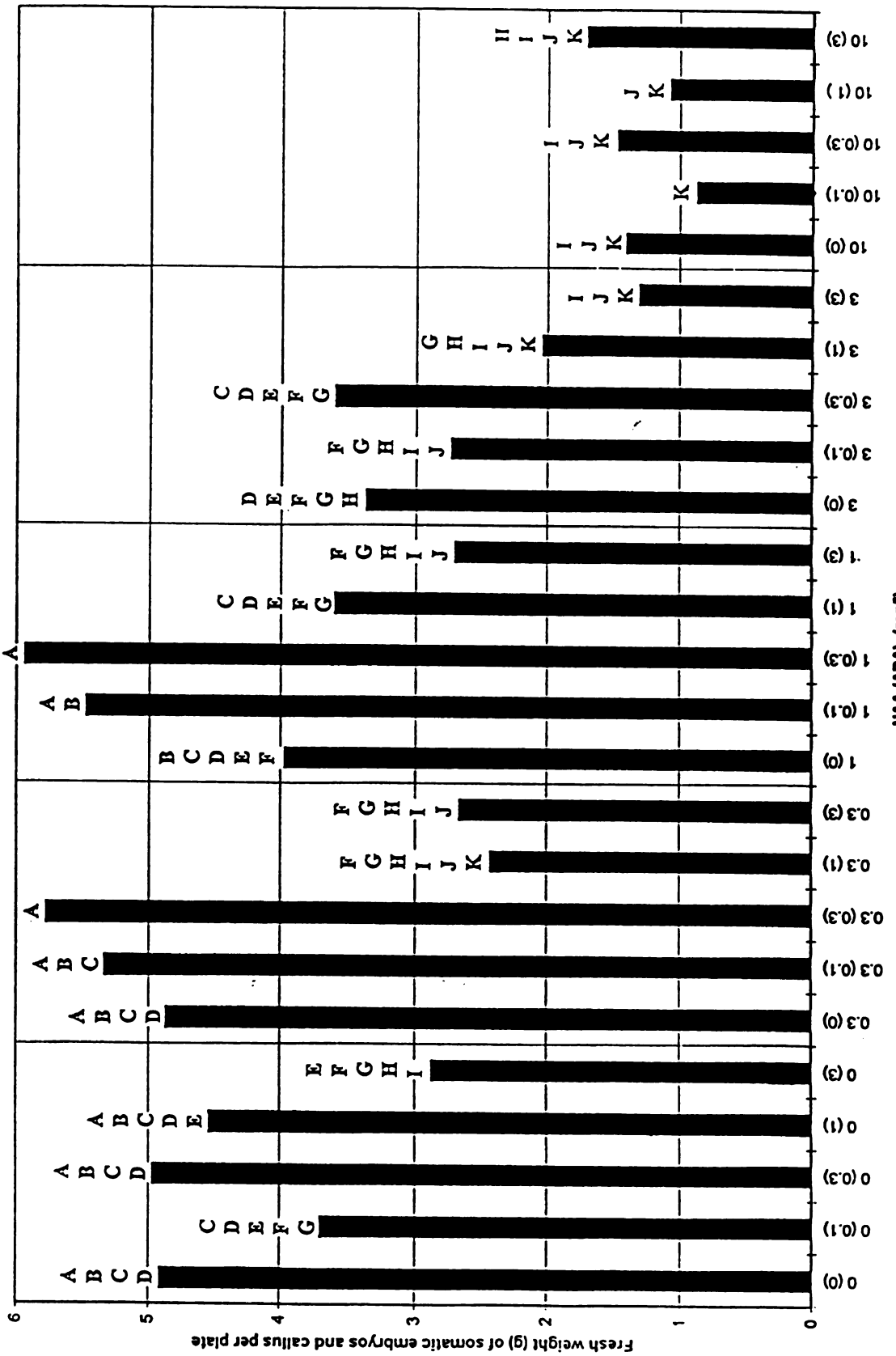

Figure 2-13: The influence of NAA and ABA on combined fresh weight of callus and somatic embryos with genotype REL-2 after 31 days. Means marked with the same letter are not significantly different according to LSD($P<0.05$).

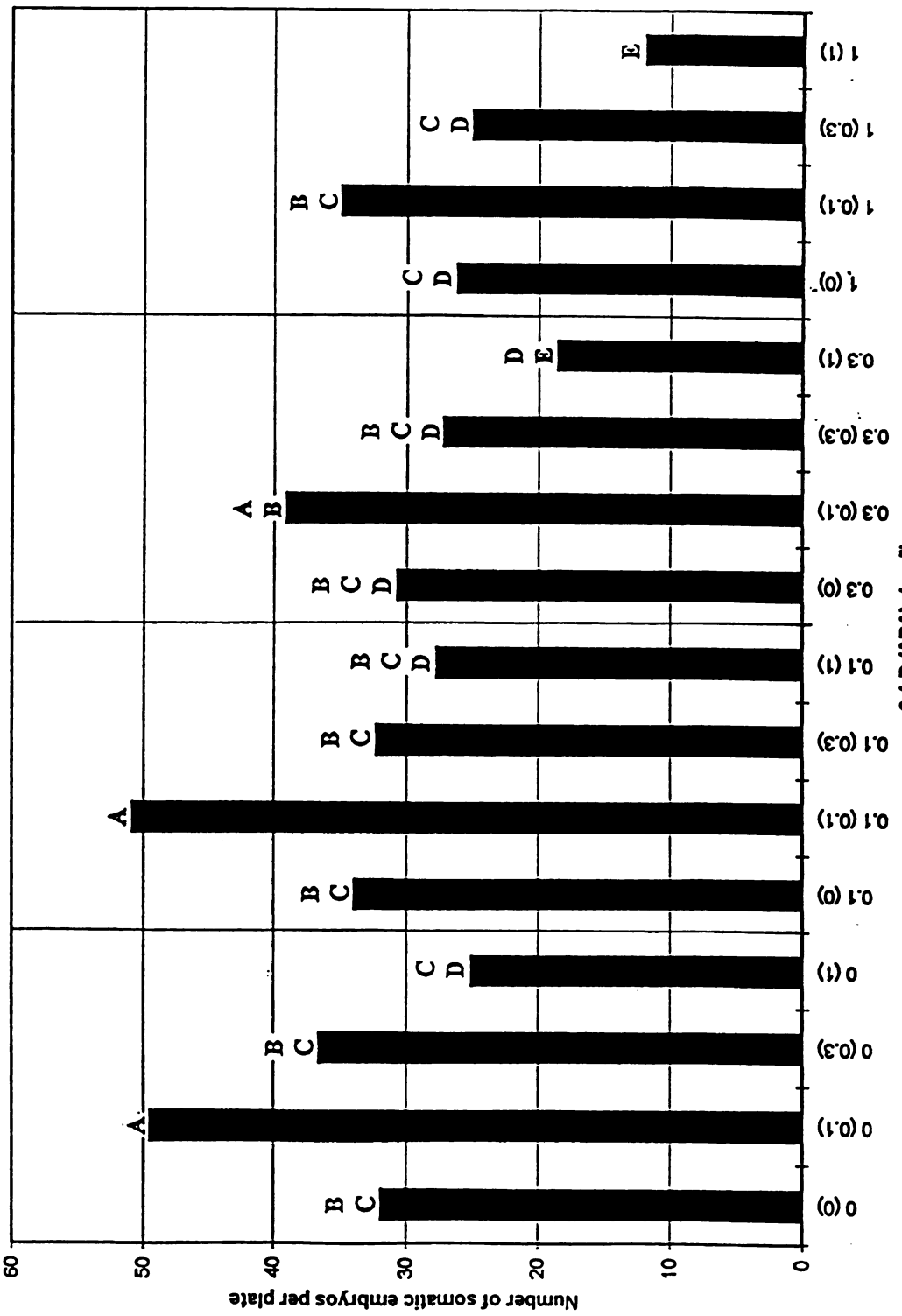

Figure 2-14: The influence of 2,4-D and ABA on somatic embryo production with genotype REL-2 after 32 days. Means marked with the same letter are not significantly different according to LSD ($P<0.05$).

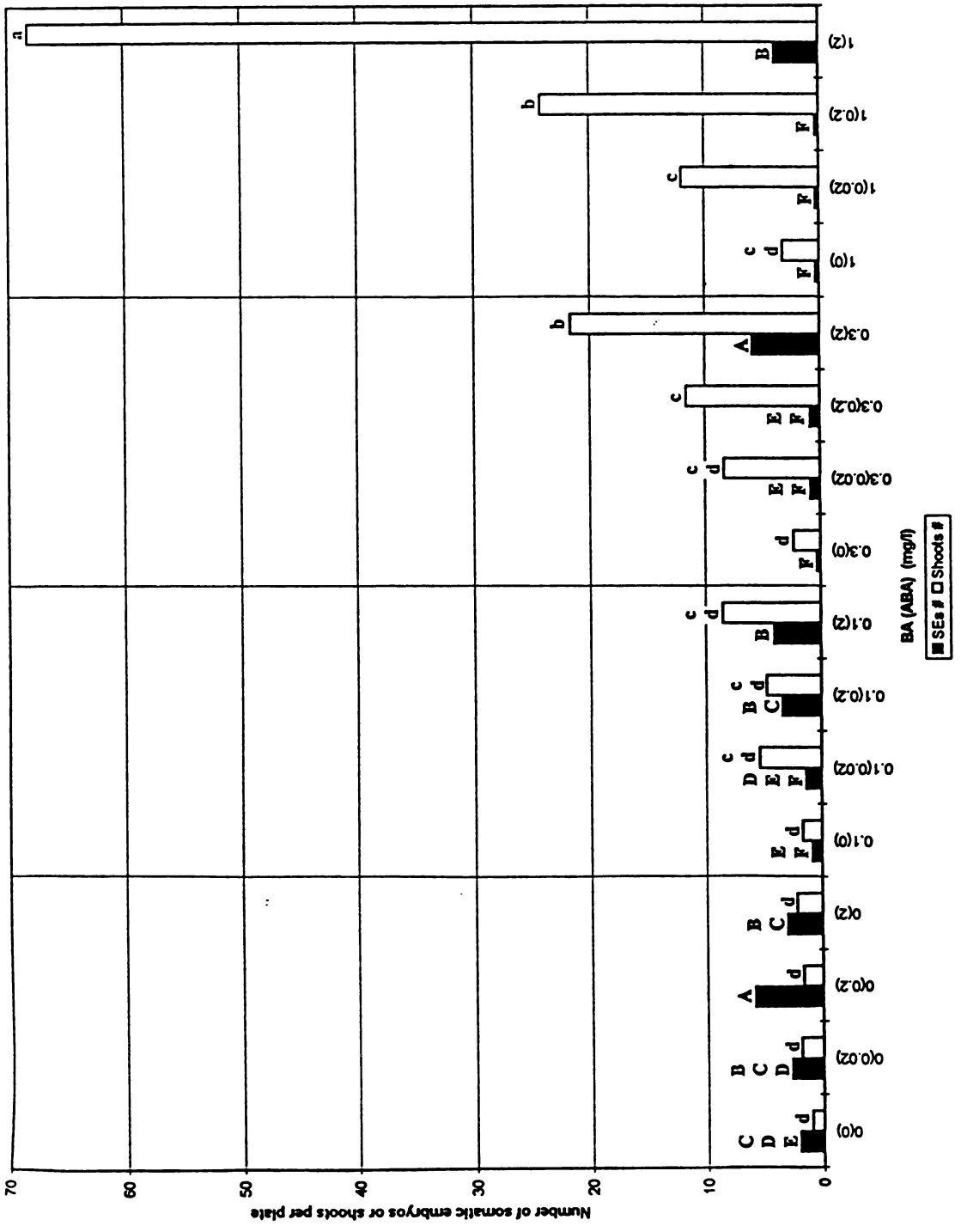

Figure 2-15: The influence of BA and ABA on somatic embryo and shoot production with genotype REL-1 after 33 days. Means marked with the same letter are not significantly different according to LSD ($P<0.05$).

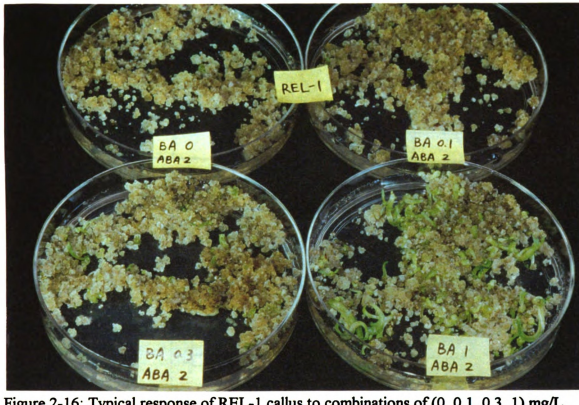

Figure 2-16: Typical response of REL-1 callus to combinations of (0, 0.1, 0.3, 1) mg/L BA and 2 mg/L ABA media after 35 days.

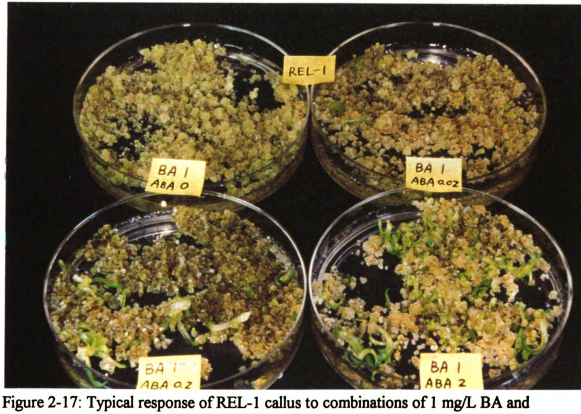

Figure 2-17: Typical response of REL-1 callus to combinations of 1 mg/L BA and (0, 0.02, 0.2, 2) mg/L ABA media after 35 days.

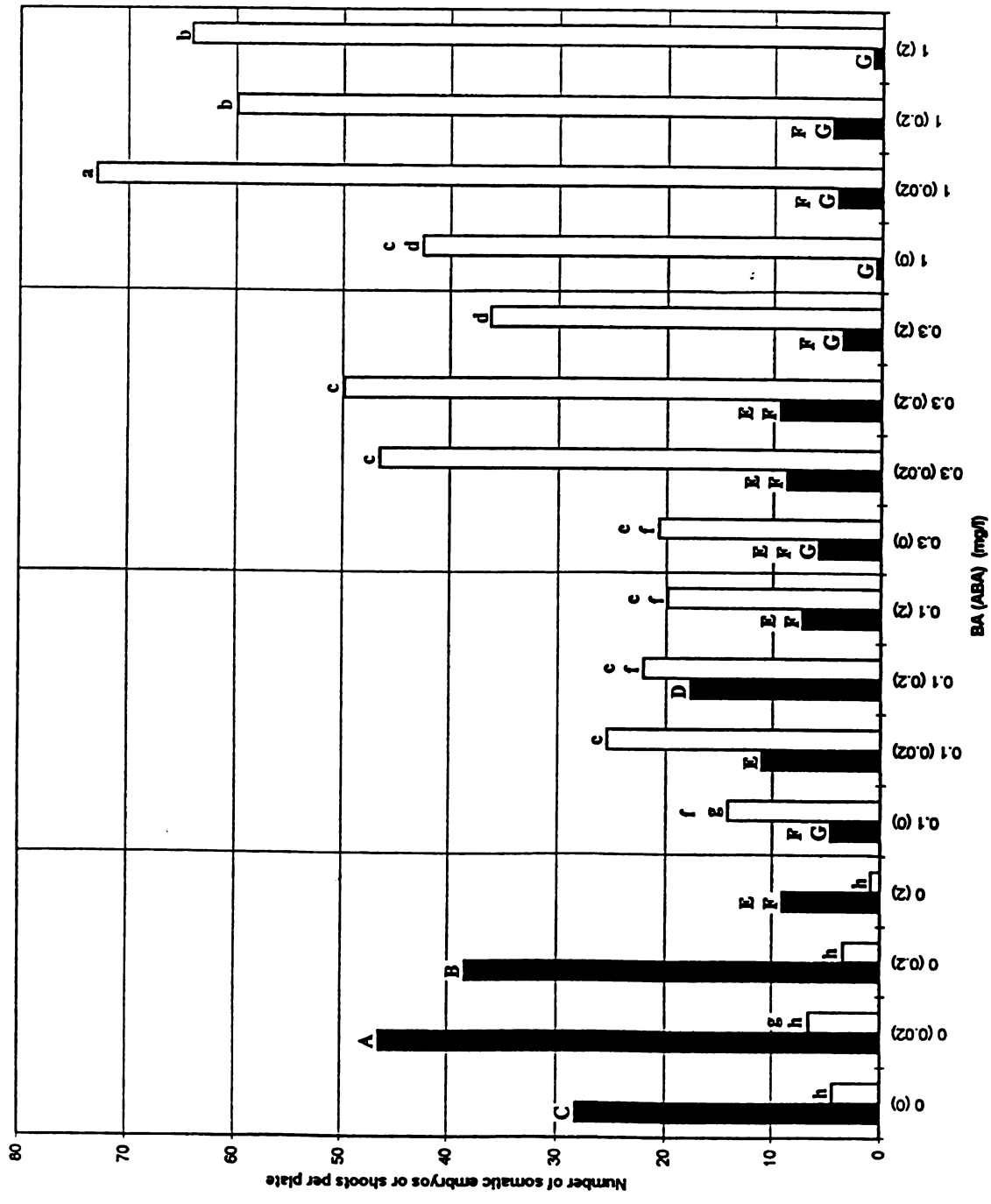

Figure 2-18: The influence of BA and ABA on somatic embryo and shoot production with genotype REL-2 after 34 days. Means marked with the same letter are not significantly different according to LSD ($P< 0.05$).

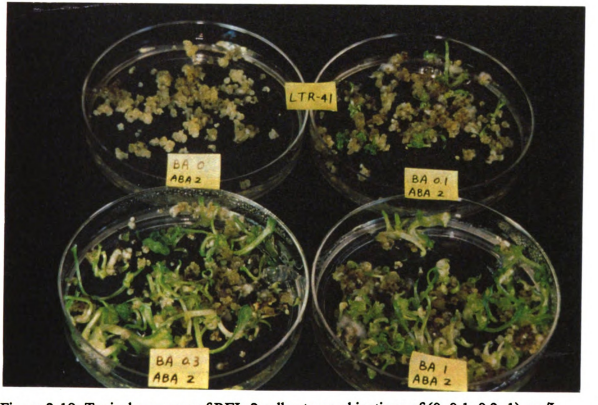

Figure 2-19: Typical response of REL-2 callus to combinations of (0, 0.1, 0.3, 1) mg/L BA and 2 mg/L ABA media after 35 days.

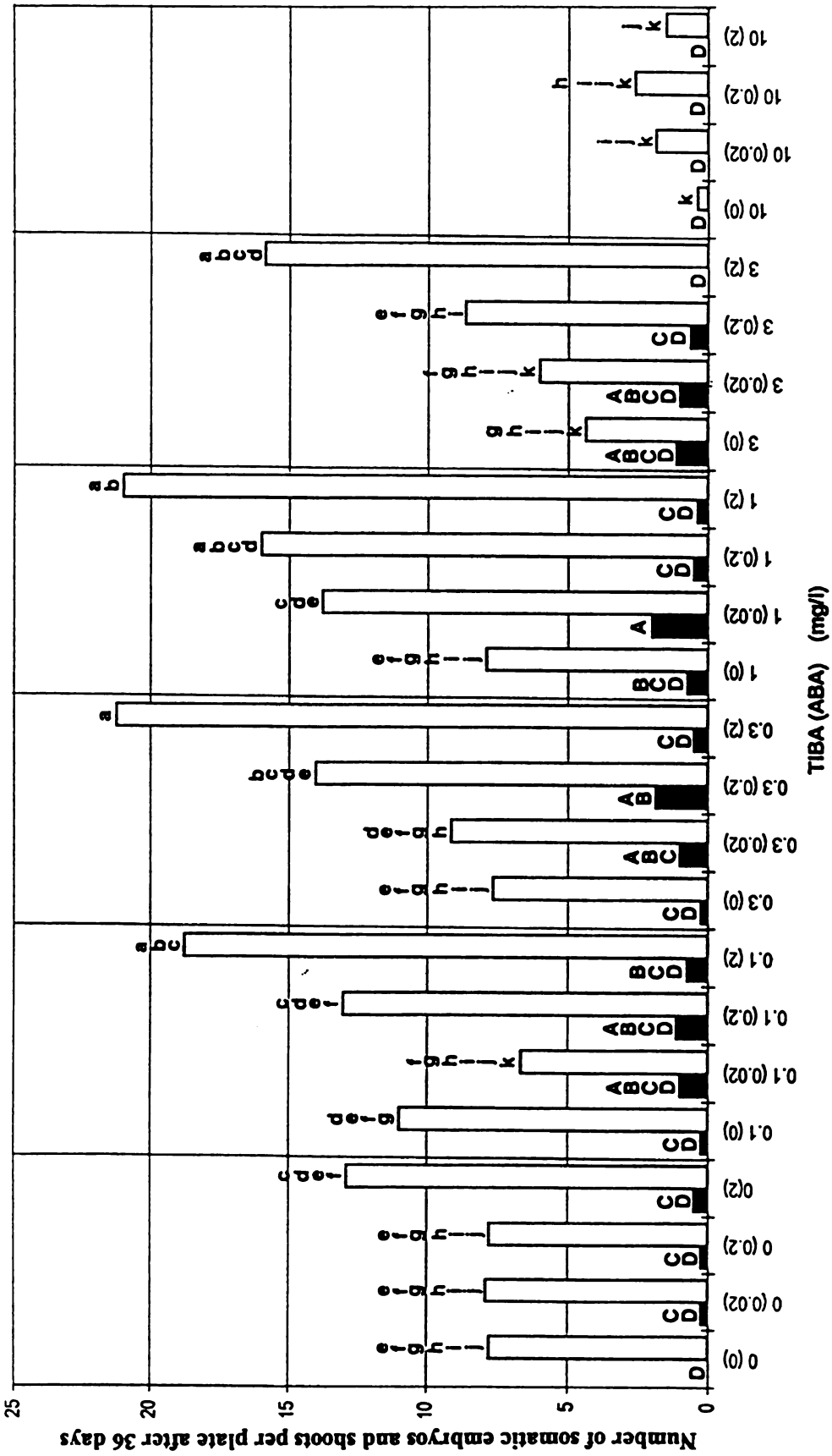

Figure 2-20: The influence of TIBA and ABA at 1 mg/L BA on somatic embryo and shoot production with genotype REL-1 after 36 days. Means marked with the same letter are not significantly different according to LSD ($P<0.05$).

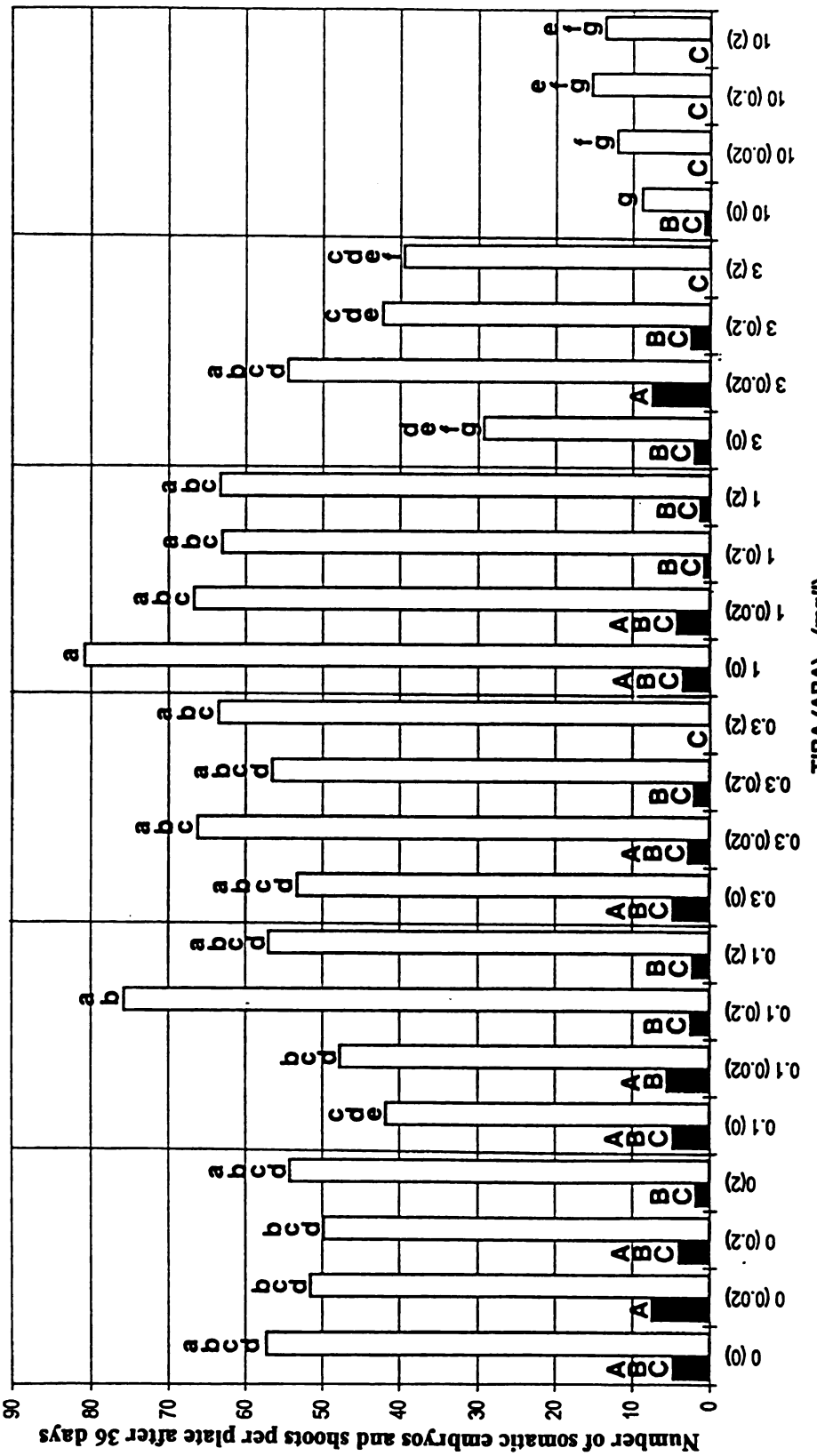

Figure 2-21: The influence of TIBA and ABA at 1 mg/L BA on somatic embryo and shoot production with genotype REL-2 after 36 days. Means marked with the same letter are not significantly different according to LSD ($P<0.05$).

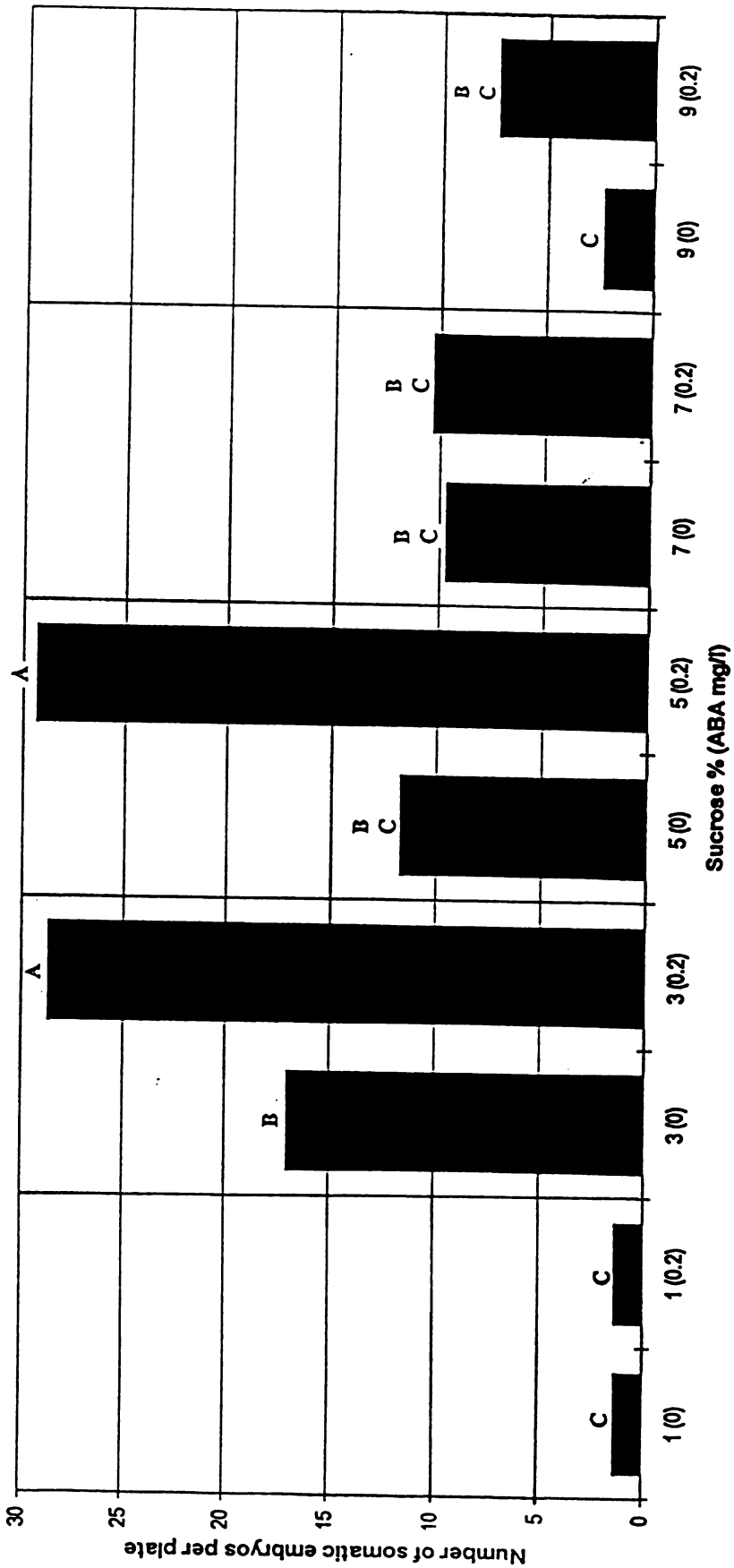

Figure 2-22: The influence of sucrose concentration and presence or absence of 0.2 mg/L ABA on somatic embryo production with genotype REL-2 after 35 days. Means marked with the same letter are not significantly different according to LSD ($P<0.05$).

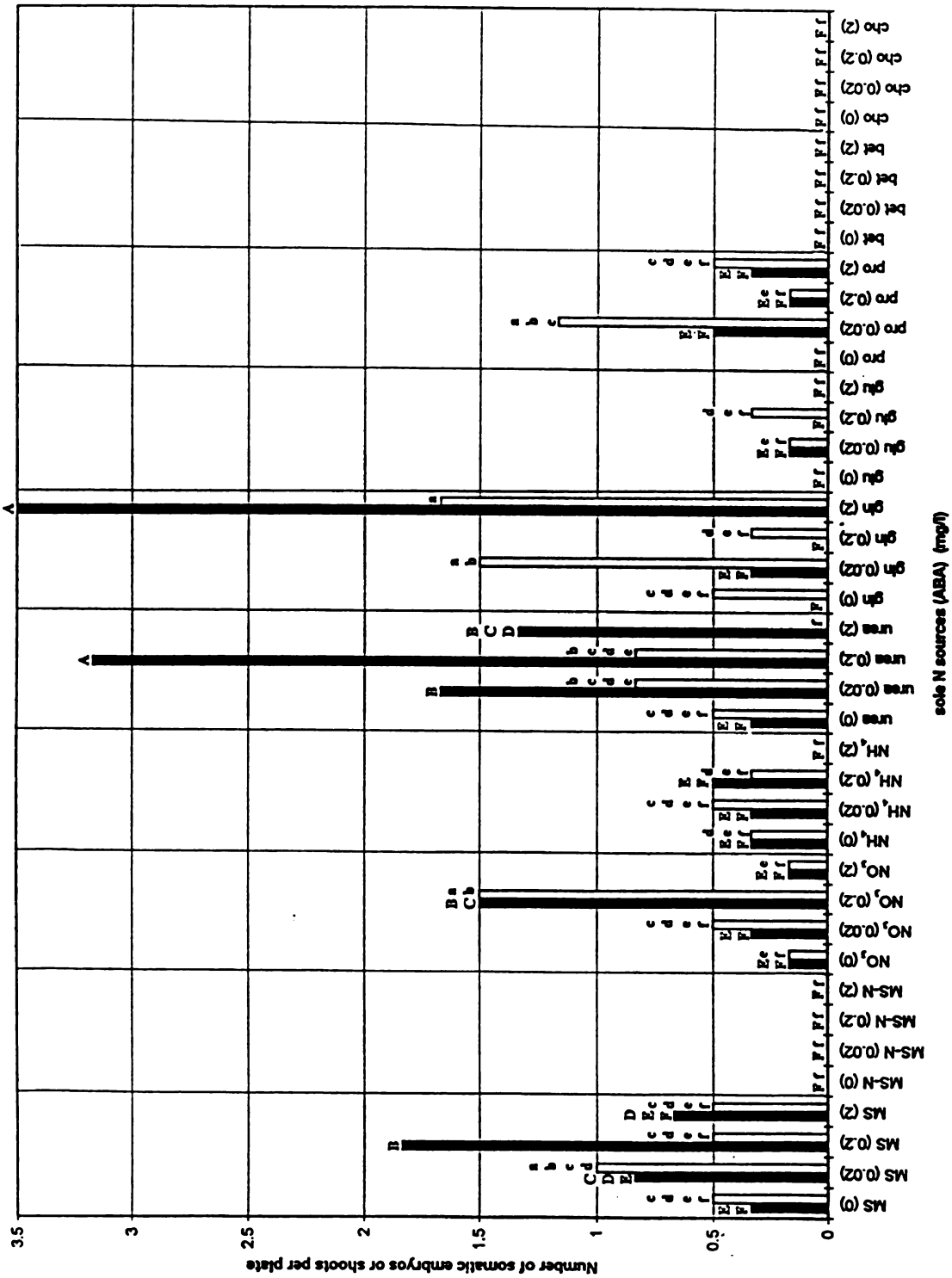

Figure 2-23: The influence of sole nitrogen source and ABA on somatic embryo and shoot production with genotype REL-1 after 34 days. Means marked with the same letter are not significantly different according to LSD ($P<0.05$).

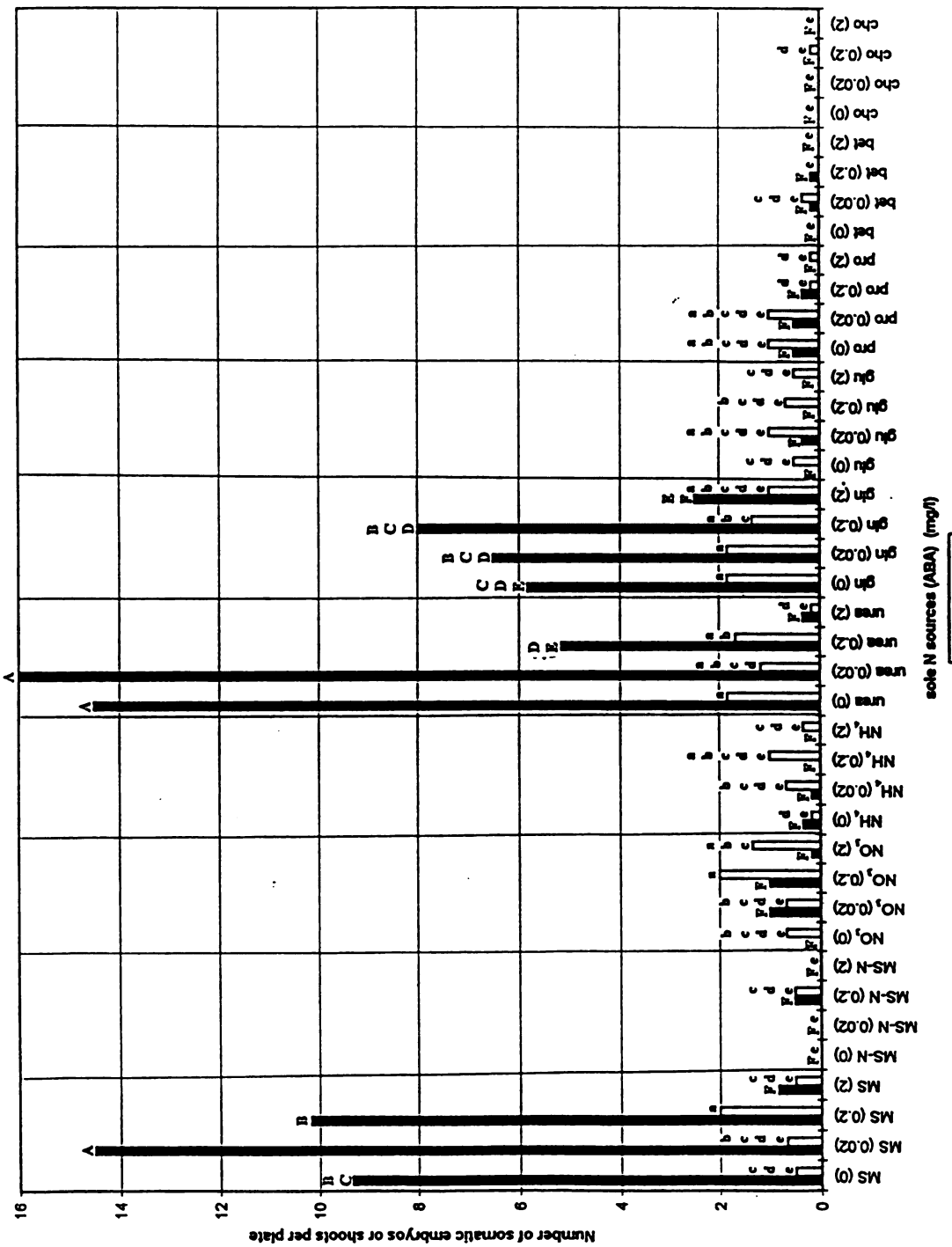

Figure 2-24: The influence of sole nitrogen source and ABA on somatic embryo and shoot production with genotype REL-2 after 34 days. Means marked with the same letter are not significantly different according to LSD (P<0.05).

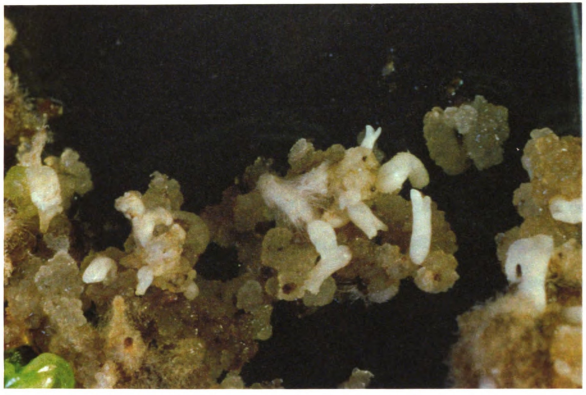

Figure 2-25: Different stages of somatic embryos were clearly distinguishable at the callus surface. (REL-2)

CHAPTER 3

ENCAPSULATION, GERMINATION, AND CONVERSION OF SOMATIC EMBRYOS IN SUGARBEET CLONE REL-2

ABSTRACT

Experiments with encapsulation and subsequent germination and conversion of sugarbeet somatic embryos to plantlets were performed with 2% sodium alginate, in initial efforts necessary for development of artificial seeds. Factors examined were encapsulated embryo size, alginate companion solution, cold storage duration, and germination substrate. Somatic embryo length category (0.5-1.9, 2.0-2.9, 3.0-3.9 mm) did not affect germination or conversion rates (87, 89, 87 %, respectively) into complete plantlets on hormone-free Murashige-Skoog (MS) medium in the absence of encapsulation. Alginate companion solutions (either MS medium or H_2O) had no differential effect on germination rate (100 %) but did differ in converting embryos to plantlets (81 vs. 64 %, respectively). No loss of conversion ability at 25 °C occurred after 21 days of cold storage at 4 °C of encapsulated embryos. After 64 days of storage at 4 °C, the germination and conversion at 25 °C of embryos encapsulated with alginate in MS medium had dropped to 70 % and 45 %, respectively; with alginate in H_2O respective rates were 60 % and 20 %. In addition, the germination rate in soil plates after 64 days cold storage for alginate capsules in MS medium or in H_2O was 38 % or 25 %, respectively. This initial research showed that somatic embryos, either nonencapsulated or encapsulated converted into plants at high frequencies (88% and 81%, respectively) without cold treatment. Cold storage did not

improve the conversion rate of encapsulated embryos, but did slow their development. However, this experiment indicated that the nonencapsulated and encapsulated embryos from clone REL-2 were capable of direct germination after planting on the agar plates and soil.

INTRODUCTION

The concept of using somatic embryos in synthetic seeds was the first proposed by Murashige (1977). The encapsulation of somatic embryos in a protective coating with alginate was initially reported by Redenbaugh et al. (1984), whereby alfalfa somatic embryos were mixed with sodium alginate and dropped into a calcium salt solution to form calcium alginate capsules. Practical synthetic seed technology would combine the efficient delivery aspects of seed propagation with elite germplasm conservation and plant propagule production via in vitro somatic embryogenesis. The advantage of such a synthetic seed system would be the use of conventional seed-handling techniques for embryo delivery and storage. Somatic embryos would be delivered from tissue culture through mechanical handling to automated planting. Research on encapsulated embryos has been reported for several species, and recovery of plants from encapsulated embryos has been obtained for some crops, e.g., carrot (Redenbaugh et al., 1987a; Li, 1993; Wake et al., 1995), orchardgrass (Gray et al., 1987), alfalfa (Redenbaugh et al., 1986, 1987b, 1988, 1990; Zhong and Wang, 1989), barley (Datta and Potrykus, 1989), eggplant (Rao and Singh, 1991), celery (Kim and Janick, 1987; Li et al., 1990; Nadel et al., 1995), wheat (Li et al., 1990), and horseradish (Shigeta and Sato, 1994).

The principal limiting problem for synthetic seeds is the difficulty in producing high quantities of quality somatic embryos, which is critical to the development of complete plantlets. In general, promoting embryo maturation with abscisic acid (ABA) or cold treatment did improve the quality of somatic embryos (Fujii et al., 1993). Additionally, the choice of hydrogel for encapsulation of somatic embryos is crucial. Sodium alginate, the most widely used hydrogel for encapsulation of embryos, can dissolve easily and remain

stable at room temperature (Redenbaugh et al., 1993). The problem after encapsulation is maintenance of embryo viability and capsule integrity, as the conversion rate may fall dramatically after short or long-term preservation. Thus, cold storage was tested in some systems (Datta and Potrykus, 1989; Li et al., 1990; Arrillaga et al. 1994). Cold treatments have improved the conversion rate from somatic embryos of *Vitis* (Rajasekaran et al., 1982). Kitto and Janick (1985) reported cold treatment hardened carrot embryos and promoted better survival after embryo desiccation. For alfalfa somatic embryos, cold exposure for up to 12 weeks did not decrease the conversion rate in soil in a growth chamber, with the conversion rate of the alfalfa embryos in soil still more than 40 % after 18 weeks of cold storage (Fujii et al., 1993). Nevertheless, cold storage could slow development, or biochemical and metabolic changes in the embryos.

A fairly prolific system for primary somatic embryogenesis has been established for sugarbeet clone REL-2 (Chapter 2). This system produces large enough numbers of somatic embryos for experiments on their utilization. The purposes of this research were to (1) examine the germination and conversion rates of different length categories of somatic embryos of sugarbeet clone REL-2, (2) and to examine the germination and conversion rates of somatic embryos encapsulated with alginate in MS medium or in H_2O (i.e., without nutrients) after different periods of cold storage at 4 °C.

MATERIALS AND METHODS

Experiments were performed with diploid sugarbeet (*Beta vulgaris* L.) clone REL-2, released to the public in 1996. REL-2 clone had been developed specifically for superior embryogenesis and shoot regeneration, annual biotech clone REL-1 (Tsai and Saunders, 1995) being one of its parents. Clone REL-2 was maintained in shoot culture (Saunders, 1982) and is available upon request, either as in vitro shoots, whole plants, or S_1 seeds.

The basic culture medium contained MS mineral salts (Murashige and Skoog, 1962), 100 mg/L myo-inositol, 1.0 mg/L thiamine·HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, and 30 g/L sucrose. Media used for leaf disc callusing were gelled with 9 g/L Difco Bacto agar, whereas media used in plating were gelled with 3.5 g/L phytagel. The pH was adjusted to 5.95 with KOH prior to autoclaving (15 psi, 121°C, 20 min). ABA was filter-sterilized and added into previously autoclaved and partially cooled media. Culture vessels were 125 mL Erlenmeyer flasks or 20 x 100 mm Falcon disposable plastic Petri plates. Medium volume per vessel was 35 mL. Flasks were closed with foam caps and aluminum foil. Petri plates were sealed with one layer of Parafilm.

Induction of somatic embryos

Callus was initiated from leaf discs (8 mm diameter) from partially expanded leaves of greenhouse-grown plants on MS medium with 1.0 mg/L BA (Saunders et al., 1992) in Petri plates at 30 °C in the dark. Callus was first seen after one month. Then after 15 days, 2 to 3 g of leaf-disc callus (Figure 3-1) was transferred to hormone-free liquid MS medium in 125 mL Erlenmeyer flasks for growth at 21±2 °C in the dark. Suspension cultures were shaken on rotary shakers at 125 rpm to aerate the cultures and to reduce cell cluster size.

After five to seven days, suspension cultures were subcultured with liquid hormone-free MS medium for another five to seven days by pouring fresh medium into the old suspension flask, mixing, then dividing the suspension evenly into two flasks. Suspensions used as inoculum for platings were pushed through a stainless steel sieve with 830 um openings. Sieved suspension cell clusters were plated on MS medium with 0.3 mg/L NAA and 0.1 mg/L ABA. Each Petri plate received 1 ml of suspension preparation containing about 0.1 g fresh weight of cells, and was incubated under dim light (less than 5 umol $m^{-2}s^{-1}$)from fluorescent lamps (Philips cool white econowatt) at 25 °C.

Germination and conversion of somatic embryos

Somatic embryos from three length categories (0.5-1.9 mm, 2.0-2.9 mm, 3.0-3.9 mm) were transferred onto hormone-free MS agar medium or soil, then placed under fluorescent lamps (20-50 umol $m^{-2}s^{-1}$) at 25 °C. Germination and conversion rates were determined for each length category. Germination was defined as development into cotyledons and/or radicles, conversion as germination and development into plantlets with shoots and roots.

Encapsulation of somatic embryos

Somatic embryos (2-3 mm) were immersed in 2 % sodium alginate with either hormone-free MS medium containing 3% sucrose or with H_2O. A pipette was used to suck up single embryos with the alginate, which were then dropped into 50 mM $CaCl_2 \cdot 2H_2O$ and stirred (80 rpm) for 20 min, thus forming sodium alginate beads of a size (4-5 mm) large enough to cover the entire somatic embryo. The beads were rinsed with water and then directly placed on the agar plates or in soil in plates in the light at 25 °C, or stored in darkness at 4 °C.

After storage for 0, 9, 21, 30, 41, 50, or 64 days, the somatic embryos encapsulated with alginate in MS medium or H_2O were transferred to agar plates with MS medium or to Petri

plates with moistened sterile soil (Baccto professional planting mix, Michigan Peat Co, Houston, TX, USA), then the germination and/or conversion rates were determined in the light during the next 14 days. Data analysis was based on a chi-square at $p \leq 0.05$.

RESULTS AND DISCUSSION

Induction of somatic embryos

According to previous work (chapter 2), a high frequency of somatic embryogenesis was seen following plating-out of suspension cultures of REL-2 on favorable media. The inclusion of 0.3 mg/L NAA and 0.1 mg/L ABA in the plate-out medium significantly increased the production of somatic embryos when compared to the plate-out medium without hormones. Maximum average yield was 77 somatic embryos per ml of suspension plated out after 31 days (Figure 3-2). Embryos of different lengths (0.5 mm to 4 mm) were clearly present simultaneously at the callus surface (Figure 3-3). This indicated that somatic embryogenesis was not synchronous in this system. Some somatic embryos (as in Figure 3-4) would directly develop into complete plantlets without transfer. Most often, after transfer of the somatic embryo onto hormone-free MS medium, its opposing ends gradually developed into shoots and roots simultaneously to form seedling-like plantlets (Figure 3-5, 3-6).

Sometimes, a very low proportion of somatic embryos experienced some secondary embryogenesis (Figure 3-7). In sugarbeet, Tenning et al. (1992) reported that such secondary somatic embryos were produced from the primary embryos.

In general, the different developmental stages of somatic embryos also correspond to different sizes of somatic embryos. The examination of the germination and conversion frequency for three relatively advanced length categories of somatic embryos which were placed on hormone-free MS agar plates (Table 3-1) indicated that there was no significant difference among germination rates (98 %, 99 %, 100 %) or conversion rates (87 %, 89 %, 87 %) when embryos from three length categories (total range = 0.5 to 3.9 mm) were placed on agar plates. The difference between germination rates and conversion rates into complete plantlets was 10-13% (Table 3-1) because of subsequent callusing or the development of abnormal advanced embryos and plantlets. Nevertheless, the germination or conversion rates might be differentially affected at different stages of embryonic development with other genotypes or species. For instance, the smallest length category (0.5-1.9 mm) had more difficulty germinating and converting into complete plantlets than did the other embryo sizes (2.0-2.9 mm; 3.0-3.9 mm) in earlier experiments with genotype REL-1 (Tsai and Saunders, 1995). Furthermore, with REL-2, respective germination and conversion rates were similar for nonencapsulated embryos placed on the MS medium agar plates or on the soil plates (Table 3-2).

Encapsulation of somatic embryos

We utilized isolated somatic embryos (Figure 3-8) for encapsulation to initiate study of the possibility of making artificial seeds (Figure 3-9). The diameter of each gel bead in diameter was approximately 4-5 mm (Figure 3-10). Somatic embryos encapsulated with 2% sodium alginate in MS medium demonstrated 100 % germination and 81 % conversion in the absence of any storage; 2 % sodium alginate in H_2O (i.e., without nutrients) resulted in 100 % germination and 64 % conversion when placed on agar plates with MS medium

without any storage (Table 3-3). Several reports have indicated that encapsulation of somatic embryos inhibited the conversion rates as compared to nonencapsulated somatic embryos. For instance, Rao and Singh (1991) reported a lower conversion rate for encapsulated (49.7%) in comparison with unencapsulated somatic embryos of *Solanum melongena* L.. A contrasting pattern was apparently observed by Shigeta et al. (1990) who noted a significantly greater conversion frequency in carrot with 54 % (nonencapsulated) and 74 % (encapsulated). Datta et al. (1989) reported that alginate-encapsulated somatic embryos of barley performed better than nonencapsulated embryos, perhaps by limiting oxygen access. In any case, the condition of the embryos entering encapsulation, as well as the actual encapsulation method or material, could account for such response differences.

At 25 °C encapsulated embryos started germinating quickly (Figure 3-11, 3-12, 3-13) after being placed on MS agar plates. Some encapsulated embryos developed into normal plantlets, and produced betalain pigment on hypocotyls (Figure 3-12, 3-13). In addition, some encapsulated embryos germinated out of the alginate, and then developed cotyledons and radicle nearly simultaneously (Figure 3-14). Storage durations of 0-21 days at 4 °C did not significantly affect response. In alfalfa, conversion of alginate-encapsulated somatic embryos was inhibited by exposure to 4 °C for only 1 week (Janick et al., 1989; Redenbaugh et al., 1991). In contrast, Arrillaga et al. (1994) reported that a 15-day cold treatment significantly increased the conversion rate for nonencapsulated black locust embryos (from 70 % increasing to 95 %) and for encapsulated embryos (from 41 % increasing to 80 %), but longer periods of cold storage drastically reduced the conversion rate. In this sugarbeet study, after 64 days of storage at 4 °C, the germination and conversion rate of encapsulated embryos with MS medium was 70 % and 45 %,

respectively; compared with 60% and 20% for beads without nutrients (alginate in water) (Table 3-3). The longer the encapsulated embryos were stored, the lower the conversion rate. The lowest conversion frequency was found in the encapsulated coats with alginate in water when compared to the encapsulated coats with alginate in hormone-free MS medium. A few somatic embryos germinated out of encapsulation in an unbalanced manner (Figure 3-15) after 41 days of cold storage. However, a very low percentage of the encapsulated embryos with alginate in MS medium still were viable after 191 days of storage at 4 °C, but most germinated directly out of the beads and became brown (data not shown). Datta and Potrykus (1989) reported that they succeeded in preserving encapsulated barley embryos for six months at cold temperatures (4°C), and at the end of this period achieved 37 % germination. In black spruce, the germination frequency showed a sharp reduction after one month of cold storage whether encapsulated or not (Lulsdorf et al., 1993). In addition, Gupta and Durzan (1987) found that encapsulated somatic embryos of loblolly pine did not convert into plantlets after storage at 4 °C for 4 months. In all such studies, the quality of the somatic embryos would be critical. Li et al. (1990) noted that with carrot, the vigor index, dehydrogenase activity, and respiration rate all decreased as storage duration increased. An ideal system would delay the further growth of somatic embryos, preserve the encapsulated embryos for high viability, and then further maintain or improve the conversion rate.

Without cold treatment, 53 % of encapsulated embryos from sugarbeet clone REL-2 germinated in soil (Table 3-4). Zhang et al. (1990) reported that the germination rate of encapsulated carrot embryos was as high as 87 % in nonsterile vermiculite when nutrient medium and a preservative were included in the alginate capsule. Fujii et al. (1987)

observed that the plant recovery rates were unacceptably low if the hydrated encapsulated embryos of some crop species (alfalfa, carrot, celery, and cauliflower) were planted in soil.

In another experiment, the cold treated encapsulated embryos were directly transferred onto the soil mix in Petri plates to examine only the germination rate (Table 3-4). There was no significant difference between germination rates with the beads containing MS medium and those without nutrients (i.e., H_2O) with 50 or fewer days of cold storage. After 64 days of storage, the germination rate was only 38 % and 25 %, respectively, when beads coated with 2% alginate in MS medium or in H_2O were placed in soil. The germination rates were dramatically lower when encapsulated embryos were planted on soil compared to MS medium agar plates (Table 3-3, 3-4).

In conclusion, aside from the quality of somatic embryos, the optimized capsule supportive complex, the capsule companion solution and the planting medium all appear to affect the germination and conversion rates, as well as storability. In order to establish a good system for encapsulating somatic embryos, we need to consider all of these factors in the future experiments.

REFERENCES

Arrillaga, I., J.J. Tobolski, and S.A. Merkle. 1994. Advances in somatic embryogenesis and plant production of black locust. Plant Cell Rep. 13: 171-175.

Datta, S.K. and I. Potrykus. 1989. Artificial seeds in barley: encapsulation of microspore derived embryos. Theor. Appl. Genet. 77: 820-824.

Fujii, J.A.A., D. Slade, and K. Redenbaugh. 1993. Planting artificial seeds and somatic embryos. *In* Synseeds. K. Redenbaugh. (ed.). CRC Press, California. pp 183-202.

Fujii, J.A.A., D.T. Slade, K. Redenbaugh, and K.A. Walker. 1987. Artificial seeds for plant propagation. Trends Biotechnol. 5: 335-338.

Gray, D.J., B.V. Conger, and D.D. Songstad. 1987. Desiccated quiescent somatic embryos of orchardgrass for use as synthetic seeds. In Vitro Cell. Dev. Biol. 23: 29-34.

Gupta, P.K. and D.J. Durzan. 1987. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Bio/technology 5: 147-151.

Janick, J., S.L. Kitto, and Y.-H. Kim. 1989. Production of synthetic seed by desiccation and encapsulation. In Vitro Cell Dev. Biol. 25: 1167-1172.

Kim, Y.-H. and J. Janick. 1987. Production of synthetic seeds of celery. HortSci. 22:89-91.

Kitto, S. and J. Janick. 1985. Hardening treatments increase survival of synthetically-coated asexual embryos of carrot. J. Amer. Soc. Hort. Sci. 110: 283-286.

Li, X.-Q. 1993. Somatic embryogenesis and synthetic seed technology using carrot as a model system. *In* Synseeds. K. Redenbaugh, (ed.). CRC Press. Davis, California. pp. 289-304.

Li, X.-Q., M.L. Deng, Z.Y. Wang, D.F. Chen, and Y. Wang. 1990. Germination and plant conversion of artificial seeds of carrot, celeriac and wheat under both sterile and nonsterile conditions, Acta Agr. Borali-Sin. 4:1-5.

Li, X.-Q., C. Zhu, M.J. Huang, M.L. Deng, X.M. Niu, B.Y. Chen, F. Liu, C. X., and T.H. Zhang. 1990. Production, storage and germination of artificial seed of high-nutrient carrot and mechanical screening of carrot somatic embryos. *In* Artificial seeds, Chen, Z.H. and Redenbaugh, K., (eds.). High Education Press of China. Chap. 11.

Lulsdorf, M.M., T.E. Tautorus, S.I. Kikcio, T.D. Bethune,and D.I. Dunstan 1993. Germination of encapsulated embryos of interior spruce and black spruce. Plant Cell Rep. 12: 385-389.

Murashige, T. 1977. Plant cell and organ cultures as horticultural practices. Acta Hort. 78: 17-30.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473-497.

Nadel, B.L., A. Altman, and M. Ziv. 1995. Somatic embryogenesis and synthetic seed in *Apium graveolens*. *In* Biotechnology in agriculture and forestry 31. Somatic embryogenesis and synthetic seed II. Y.P.S. Bajaj (ed.). Springer-Verlag, Berlin Heidelberg. pp. 306-322.

Rajasekaran, K., J. Vine, and M.G. Mullins. 1982. Dormancy in somatic embryos and seeds of *Vitis*: changes in endogenous abscisic acid during embryogeny and germination. Planta 154: 139-144.

Rao, P.V.L. and B. Singh. 1991. Plantlet regeneration from encapsulated somatic embryos of hybrid *Solanum melongena* L. Plant Cell Rep. 10: 7-11.

Redenbaugh, K. 1990. Application of artificial seeds to tropical crops. HortSci. 25: 251-255.

Redenbaugh, K., J. Fujii, and D. Slade. 1988. Encapsulated plant embryos, *In* Advances in biotechnological processes. A. Mizrahi, (ed.). Liss. New York, 9: 225-248.

Redenbaugh, K., J.A. Fujii, and D. Slade. 1991. Synthetic seed technology, *In* Cell culture and somatic cell genetics of plants: scale-up and automation in plant tissue culture. I. Vasil, (ed.). Academic Press, Orlando, pp. 35-74.

Redenbaugh, K., J.A.A. Fujii, and D. Slade. 1993. Hydrated coatings for synthetic seeds. *In* Synseeds. K. Redenbaugh, (ed.). CRC Press. Davis, California. pp. 35-46.

Redenbaugh, K., J. Nichol, M.E. Kossler, and B. Paasch. 1984. Encapsulation of somatic embryos for artificial seed production. In Vitro 20: 256-258.

Redenbaugh, K., B. Paasch, J. Nichol, M. Kossler, P. Viss, and K. Walker. 1986. Somatic seeds: encapsulation of asexual embryos. Bio/technol. 4: 797-801.

Redenbaugh, K., D. Slade, P. Viss, and J. Fujii. 1987a. Encapsulation of somatic embryos in synthetic seed coats. HortSci. 22:803-809.

Redenbaugh, K., P. Viss, D. Slade, and J. Fujii. 1987b. Scale-up: artificial seeds, *In* Plant tissue and cell culture. C. Green, D. Somers, W. Hackett, and D. Biesboer (eds.). Liss, New York. pp. 473-493.

Saunders, J.W. 1982. A flexible in vitro shoot culture propagation system for sugarbeet that includes rapid floral induction of ramets. Crop Sci. 22: 1102-1105.

Saunders, J.W., G. Acquaah, K.A. Renner, and W.P. Doley. 1992. Monogenic dominant sulfonylurea resistance in sugarbeet from somatic cell selection. Crop Sci. 32: 1357-1360.

Shigeta, J., T. Mori, K. Toda, and H. Ohtaka. 1990. Effect of capsule hardness on germination frequency of encapsulated somatic embryos of carrot. Biotech. Tech. 4: 21-24.

Shigeta, J.I. and K. Sato. 1994. Plant regeneration and encapsulation of somatic embryos of horseradish. Plant Sci. 102: 109-115.

Tenning, P., E.W. Weich, U-B. Kjarsgaard, M-A. Lelu, and M. Nihlgard. 1992. Somatic embryogenesis from zygotic embryos of sugar beet. (*Beta vulgaris* L.). Plant Sci. 81: 103-109.

Tsai, C.J. and J.W. Saunders. 1995. Somatic embryos from callus of sugarbeet biotechnology clone REL-1. J. Sugar Beet Res. 32:215-226.

Wake, H., H. Umetsu, and T. Matsunaga. 1995. Somatic embryogenesis and artificial seed in carrot. *In* Biotechnology in agriculture and forestry 31. Somatic embryogenesis and synthetic seed. II. Y.P.S. Bajaj (ed.). Springer-Verlag, Berlin Heidelberg. pp. 170-198.

Zhang, T.-H., C.-X. Lu, M.-L. Deng, and X.-Q. Li. 1990. Studies on coats and preservatives for carrot artificial seeds. *In* Studies on artificial seeds of plants. X.-Q. Li., (ed.). Peking University Press, Beijing. pp. 41-50.

Zhong, H. and F. Wang. 1989. Somatic embryogenesis in cell suspension culture of alfalfa roots and encapsulation of embryoids. Chin. J. Bot. 1: 35-44.

Table 3-1: Effect of length category on the proportions of somatic embryos germinating and converting into complete plantlets in **the absence of encapsulation.**

Length of somatic embryos(mm)	Germination rate	Conversion rate
0.5 - 1.9	98%(82/84) a	87%(73/84) a
2.0 - 2.9	99%(95/96) a	89%(85/96) a
3.0 - 3.9	100%(38/38) a	87%(33/38) a

Means within columns followed by the same letters were not significantly different based on a chi-square at $p \leq 0.05$.

Table 3-2: Germination and conversion rates of **nonencapsulated** somatic embryos on the MS medium or the soil without cold storage.

MS medium (agar 9 g/L)	germination rate	100% (16/16)	NS
	conversion rate	88% (14/16)	NS
Soil	germination rate	100% (10/10)	NS
	conversion rate	90% (9/10)	NS

NS indicated that germination and conversion rates on MS medium or soil were not significantly different based on a chi-square at $p \leq 0.05$.

Table 3-3: Comparison of germination and conversion rates on **MS medium** of somatic embryos **alginate-encapsulated** with either MS medium or H_2O after storage at 4 °C.

Days of storage at 4 °C	Germination response alginate encapsulated with		Conversion response alginate encapsulated with	
	MS medium	**H_2O**	**MS medium**	**H_2O**
0	100%(16/16)a	100%(14/14)a	81%(13/16)a	64%(9/14)aefh
9	95%(19/20)ac	85%(17/20)ad	75%(15/20)ac	65%(13/20)aefi
21	95%(19/20)ac	75%(15/20)bcde	60%(12/20)ad	55%(11/20)aefj
30	85%(17/20)ad	80%(16/20)ae	40%(8/20)bde	40%(8/20)bde
41	80%(16/20)ae	75%(15/20)bcde	45%(9/20)bcdf	45%(9/20)bcdf
50	80%(16/20)ae	80%(16/20)ae	50%(10/20)aefg	35%(7/20)bdghij
64	70%(14/20)bde	60%(12/20)bde	45%(9/20)bcdf	20%(4/20)b

Means for germination or conversion followed by one or more of the same letters were not significantly different based on a chi-square at $p \leq 0.05$.

Table 3-4: Comparison of germination rates in **soil** of **encapsulated** somatic embryos with either MS medium or H_2O and effects on the germination rates after storage at 4°C.

Days of storage at 4 °C	Germination response alginate encapsulation with	
	MS medium	**H_2O**
0	53% (16/30) NS	53% (16/30) NS
9	40% (8/20) NS	45% (9/20) NS
21	45% (9/20) NS	50% (10/20) NS
30	50% (5/10) NS	40% (4/10) NS
41	50% (5/10) NS	40% (4/10) NS
50	40% (4/10) NS	40% (4/10) NS
64	38% (3/8) NS	25% (2/8) NS

NS indicated that the germination rates of embryos encapsulated with alginate in either MS medium or H_2O were not significantly different based on a chi-square at $p \leq 0.05$.

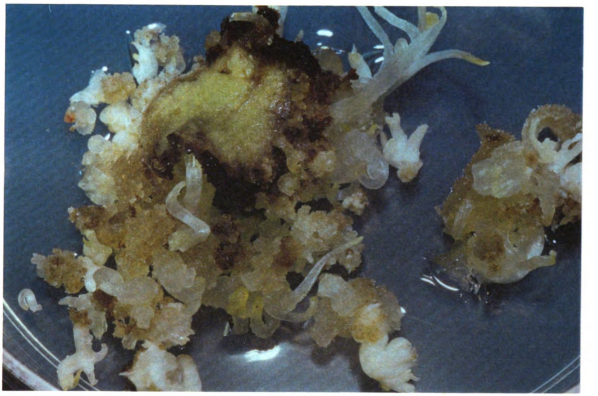

Figure 3-1: Leaf disc callus from MS medium containing 1 mg/L BA.

Figure 3-2: Somatic embryogenesis after suspension plate-out on MS medium with 0.3 mg/L NAA and 0.1 mg/L ABA after 31 days.

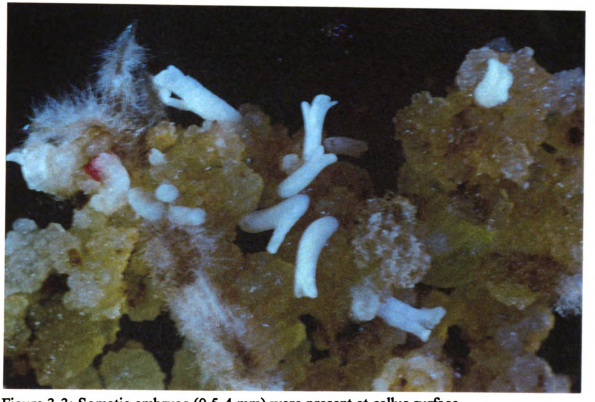

Figure 3-3: Somatic embryos (0.5-4 mm) were present at callus surface.

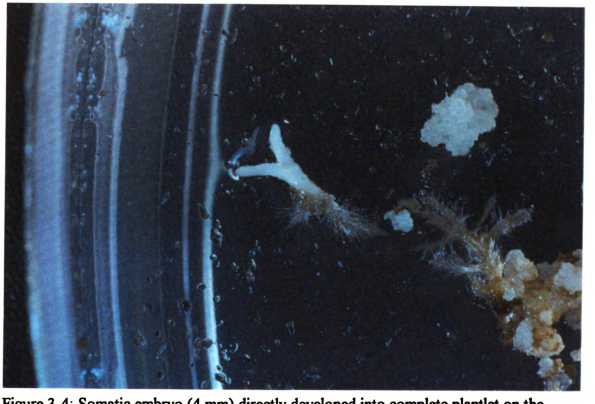

Figure 3-4: Somatic embryo (4 mm) directly developed into complete plantlet on the suspension plate-out medium.

Figure 3-5: Somatic embryo with bipolar converted into cotyledons and roots simultaneously, 6 days after being transferred to hormone-free MS medium (length: 14 mm).

Figure 3-6: Somatic embryos develop into complete plantlet, 9 days after being transferred to hormone-free MS medium (length: 19 mm).

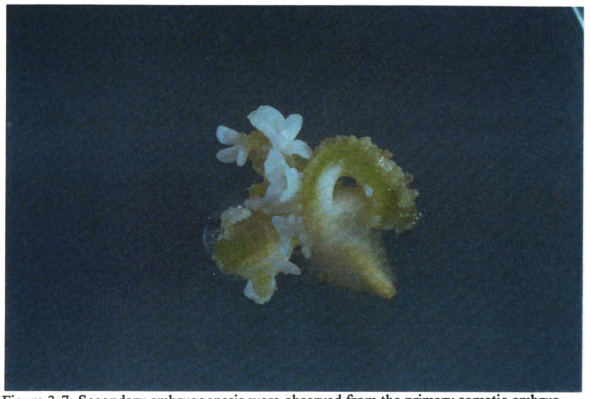

Figure 3-7: Secondary embryogenesis were observed from the primary somatic embryo (8 mm), 14 days after being transferred to hormone-free MS medium.

Figure 3-8: Isolated somatic embryos (length: 0.5-4 mm).

Figure 3-9: Encapsulation of somatic embryos with sodium alginate. The gel bead is about 4-5 mm.

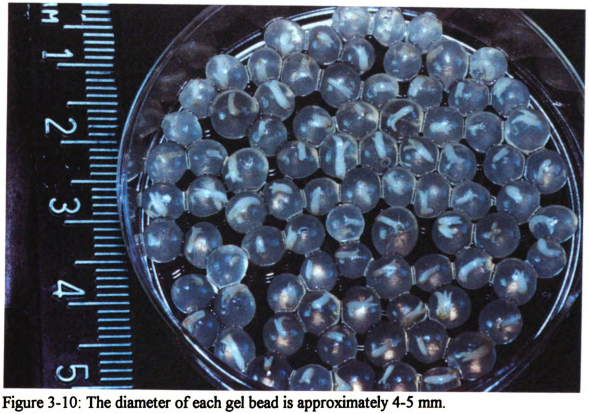

Figure 3-10: The diameter of each gel bead is approximately 4-5 mm.

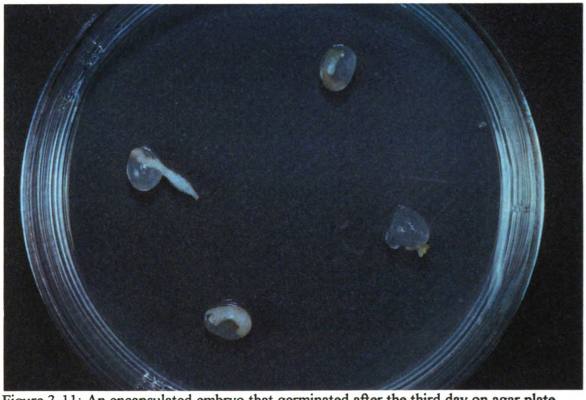

Figure 3-11: An encapsulated embryo that germinated after the third day on agar plate.

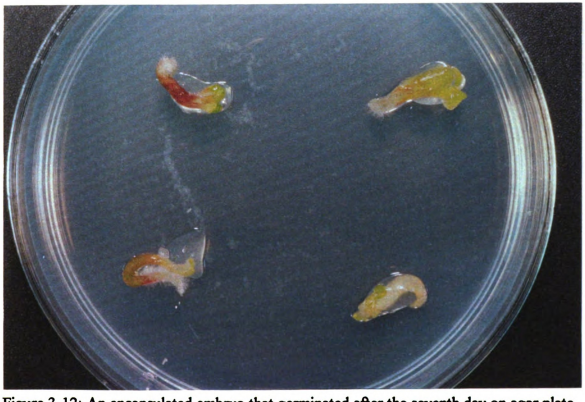

Figure 3-12: An encapsulated embryo that germinated after the seventh day on agar plate, with betalain pigment on the hypocotyls.

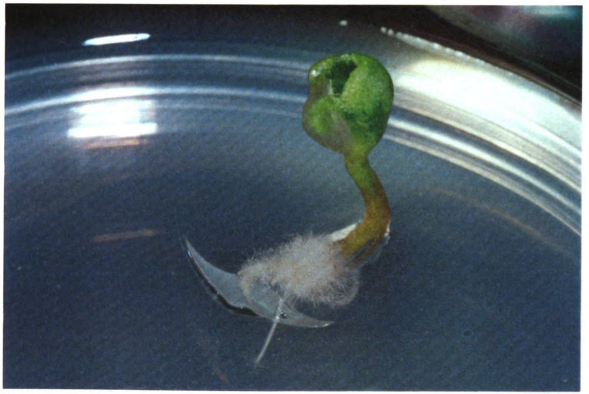

Figure 3-13: An encapsulated embryo that converted into a plantlet after fourteenth day on hormone-free MS agar plate, with betalain pigment on the hypocotyl (length: 15 mm).

Figure 3-14: An encapsulated embryo with cotyledons and radicle development simultaneously after 21 days of storage at 4 °C (length: 14 mm).

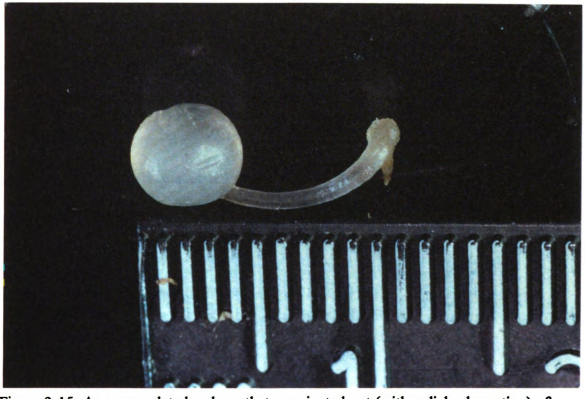

Figure 3-15: An encapsulated embryo that germinated out (with radicle elongation) of encapsulated coat after 64 days of cold storage without transfer. Capsule is about 4 mm in diameter.

CHAPTER 4

EVALUATION OF ALTERNATIVE NITROGEN SOURECES FOR SUGARBEET SUSPENSION CULTURE PLATINGS IN DEVELOPMENT OF CELL SELECTION SCHEMES

ABSTRACT

Nitrogenous impurities are negative quality factors in sugarbeet (*Beta vulgaris* L.) processing. In order to identify nutrient media for cell selection of biochemical mutants or transgenics that might have reduced levels of these impurities, the ability of eight endogenous compounds to serve as sole nitrogen source in suspension culture platings was evaluated with clone REL-1. Nitrate (NO_3), ammonium (NH_4), glutamine (GLN), glutamate (GLU), and urea as sole nitrogen sources were moderately supportive of plating callus growth based on fresh weight compared with the standard 60 mM mix of NO_3 and NH_4 in Murashige-Skoog (MS) medium. Proline (PRO) was poorly supportive, and glycine betaine (BET) and choline (CHO) were non-supportive. No callus growth occurred when GLN, GLU or BET was provided as the sole carbon or carbon plus nitrogen source. Platings on either NO_3 or NH_4 as sole nitrogen source did not differ in sensitivity to the nitrate uptake inhibitor phenylglyoxal (PGO), suggesting that PGO lacks the specificity for use in selection for mutants of nitrate uptake. The ability of GLN or GLU to be used as the nitrogen source may preclude their use for selection of genetic variants accumulating less of these processing impurities. However, mutants or transgenics able to utilize GLN, GLU or BET might be selectable on media containing any one of

these as carbon, nitrogen or carbon plus nitrogen source, respectively, that is incapable of supporting wild type cell growth.

INTRODUCTION

Somatic cell selection has potential for identifying novel single gene variations in crop species (Duncan and Widholm, 1986). The extent of the correlation between in vitro and whole plant responses, as well as the recognition of cell selective regimes targeting specific metabolic and regulatory areas, may limit improvement of the range of crop characteristics by the method.

The recovery of monogenic dominant sulfonylurea herbicide resistant plants by using somatic cell selection has been demonstrated in sugarbeet (Saunders et al., 1992). Their system involved a diploid clone (REL-1) bred for fine suspension culture texture and easy regeneration of nonvitreous shoots, as well as an acute (one time) exposure of plated suspension cell clusters to a lethal concentration of chlorsulfuron, a specific inhibitor of acetolactate synthase. This selection step was performed on a shoot regeneration medium to maximize chances of recovering shoots from any surviving colonies.

In order to increase the efficiency of industrial sucrose extraction, reducing the concentration of impurities in the harvested sugarbeet root has been a breeding objective (Smith, 1988; Smith and Martin, 1989). These impurities are the small molecules that are not removed by precipitation at the temperature and pH conditions employed in the sucrose purification process. The major nitrogenous impurities or their derivatives in sugarbeet molasses are GLN, GLU, and BET. One way to reduce the level of processing impurities would be to determine if the individual impurities would serve as sole nitrogen

sources in vitro. If any major nitrogen impurity would not serve as a sole nitrogen source, then a medium containing such an impurity could be used as a positive selective regime for cell variants that had acquired the ability to utilize that compound. A precedent for this approach in higher plants was the selection of a glycerol utilizing mutant in tobacco cells that was later recovered in sexual progeny (Chaleff and Parsons, 1981).

In contrast, if individual impurities or other endogenous metabolites could serve as sole nitrogen sources for wild-type cultured cells, then such media could be used in formulation of a different kind of positive cell selection scheme that relies on a specific inhibitor of an immediate assimilation step of that sole nitrogen source. Use of the single source of nitrogen would be important to make the cells susceptible to such an inhibitor. Expression of a mutation or epigenetic change conferring resistance to the inhibitor could permit cell survival. Heimer and Filner (1970) recovered a tobacco cell line resistant to growth inhibition by threonine when nitrate was the sole nitrogen source. Sugarbeet mutants with resistance mechanisms involving enzyme overproduction might have a reduced concentration of the respective impurity at the whole plant level. Furthermore, use of sole nitrogen sources could help characterize mutants in nitrogen metabolism, such as in soybean where different classes of urease mutants are defined according to tissue specificity (Polacco et al., 1989).

Thus, in order to determine their potential role in somatic cell selection schemes, glutamine, glutamate, and glycine betaine were evaluated for use as either a sole nitrogen, carbon, or a combination carbon and nitrogen source. Several other endogenous nitrogen compounds were evaluated as sole nitrogen sources because of interest in devising selection schemes using inhibitors for recovery of overproducing mutants for steps in

nitrogen assimilation, with the goal of ultimately improving nitrogen use efficiency. Lastly, phenylglyoxal (PGO), an inhibitor of nitrate assimilation (Dhugga et al., 1988), was tested with either nitrate or ammonium as sole nitrogen source to evaluate its suitability as a selective agent.

MATERIALS AND METHODS

All experiments were performed with suspension cultures of the diploid sugarbeet clone REL-1. Suspension cultures were initiated from leaf disc callus (Saunders et al., 1992). All experimental media contained Murashige and Skoog (MS) (1962) inorganics, 3 % sucrose, 100 mg/L myo-inositol, 10 mg/L thiamine·HCl, 1.0 mg/L nicotinic acid, 1.0 mg/L pyridoxine·HCl. 0.25 mg/L 6-benzyladenine (BA), and 0.9 % Difco Bacto agar (w/w). Nitrate (NO_3), ammonium (NH_4), glutamine (GLN), glutamate (GLU), proline (PRO), urea, glycine betaine (BET), and choline (CHO) were tested as sole nitrogen sources at 15, 30, 60, and/or 90 mM. KNO_3 (20 mM) and NH_4NO_3 (40 mM) together were used as the standard MS nitrogen source, except when alternative sole nitrogen sources were tested. In such cases the sources of potassium were from the 2.0 mM KH_2PO_4 in MS, any KOH used in pH adjustment, or stoichiometric quantities from KNO_3 used in providing the various levels of NO_3. NH_4 was provided as the chloride salt and was accompanied by half that concentration of succinic acid. Sucrose was omitted in tests of alternative carbon (C) or carbon & nitrogen (C+N) sources. GLN, GLU or BET were included at 33 and 66 mM and compared with 29 mM sucrose when these three compounds were tested as C or C+N sources. GLU was provided as the sodium salt, BET, CHO and PRO as the free bases. Individual K^+, Na^+ or Cl^- concentrations were not adjusted to maintain equivalency.

Suspension cultured cells used as inoculum were washed once through decanting in medium lacking either N, C, or C+N sources, then passed through a stainless steel sieve with 830 uM openings using additional wash medium. Solutions of all N, C, or C+N sources and of PGO (Sigma Chemical Co., St. Louis, MO) were adjusted to pH 5.95 with KOH or HCl prior to filter sterilization, and added during cooling of the medium, which had undergone pH adjustment with KOH before autoclaving (15 psi, 121°C, 20 min).

Thirty-five mL of medium was dispensed into 100 x 20 mm Falcon disposable plastic Petri plates. Plates were inoculated with 1.0 mL of log phase suspension using disposable plastic Pasteur pipettes. Each plate received, from experiment to experiment, about one hundred to three hundred viable cell clusters (20-35 mg total dry weight). After inoculation, plates were sealed with two Parafilm strips and incubated in dim light (less than 5 umol m^{-2} s^{-1} from fluorescent lamps) at 25°C. Final fresh weight was measured and each treatment was replicated 8 to 13 times, and experiments were performed with similar results 2 or 3 times each. Final callus fresh weight for each medium treatment were subjected to ANOVA for a randomized complete block design and the LSD (P=0.05) was used to compare individual treatments.

RESULTS

The N sources NO_3, NH_4, GLN, GLU, and urea supported callus growth, but not as well as the standard 60 mM NO_3/NH_4 nitrogen mixture in MS medium (Figure 4-1). Callus growth on BET, CHO, and PRO was not significantly different (P=0.05) from the nitrogen-free control (Figure 4-1).

The nitrogenous processing impurities GLN, GLU, and BET evaluated for use as sole C or C+N sources did not support growth (Figure 4-2).

Since either NO_3 or NH_4 as sole nitrogen source supported moderate growth, an experiment was conducted to test the specificity of inhibition of callus growth by PGO with each nitrogen source, alone and in combination. PGO at 2100 uM almost completely inhibited growth regardless of source of N. PGO at 700 uM also inhibited, but inhibition was greater with NO_3 than with NH_4 (Figure 4-3).

DISCUSSION

In order to identify opportunities for selecting biochemical mutants at the cell level, alternative nitrogen sources were investigated for plated callus growth of sugarbeet suspensions. Mutants of interest for use in sugarbeet improvement include those for modified (1) metabolism of processing impurities that might lead to reduced levels of those impurities at the plant level in harvested tap roots, and (2) nitrogen assimilation, given the recognized major influence of nitrogen on sugarbeet root yield, sucrose percentage, and processing efficiency.

A wide range (15, 30, 60, and/or 90 mM) of endogenous nitrogen compounds was tested as sole nitrogen sources. These included processing impurities (GLN, GLU, BET), a metabolite (PRO, from GLU), a precursor (CHO, to BET), inorganic forms (NO_3, NH_4) and an intermediate sometimes used in crop fertilization (urea). All of the tested compounds except BET, CHO and PRO were at least moderately supportive of growth in the presume of the MS mixture of NO_3 and NH_4. Sugarbeet callus could utilize NO_3 as sole nitrogen source, unlike sugarcane callus which could not rely on it (Veith and Komor,

1991). Sugarbeet was not capable of utilizing BET for the growth of callus from plating. This may be related to the apparent lack of BET metabolism in the whole plant (Hanson and Wyse, 1982).

When processing impurities GLN (largely converted to GLU and pyroglutamate in the heat of the extraction and purification process), GLU and BET were also evaluated as sole C and C+N sources for platings, none was effective. Hence, these could be used for positive selection to recover mutants that have acquired the ability to utilize these impurities. Such mutants might also possess altered metabolism and altered accumulation of the impurities at the whole plant level. Media with GLN or GLU as C or C+N source, or BET as N, C or C+N source might also be used to select for transgenic cells carrying genes permitting utilization of any of these processing impurities. For example, genes for catabolism of BET in *Rhizobium melliloti* (Le Rudulier et al., 1984) could be cloned and potentially used in transformation of sugarbeet.

An inhibitor of a specific step of nitrogen assimilation, in combination with a sole nitrogen source just upstream of the step sensitive to the inhibitor, should permit isolation of resistant mutants that produce excess enzyme or an altered enzyme insensitive to the inhibitor. Mutants overproducing the target enzyme by gene amplification or upregulation could be of greater value for crop improvement than amino acid sequence changes resulting in reduced inhibitor binding, provided agronomic performance or crop quality were improved in the former. Additionally, resistance to the inhibitor might be used as a pleiotropic marker for easy and early identification of the overproducing segregates in progeny. The possibility of such an indirect selection scheme, where selection for resistance to an inhibitor could lead to isolation of a mutant for altered nitrogen

assimilation, was investigated in this research. Dhugga et al. (1988) demonstrated that NO_3 and NH_4 as sole N sources, the specificity of growth inhibition by PGO, inhibited NO_3 uptake. Unfortunately, the inhibition of growth by PGO was not found to be specific to NO_3 as a nitrogen source (Figure 4-3), and thus selection for resistance to PGO may not be useful for recovering nitrate uptake mutants. A further possibility for this kind of indirect selection for enzyme overproducing mutants of nitrogen assimilation (and possibly of agronomic performance) would involve concomitant use of urease inhibitor N-(n-butyl) thiophosphoric triamide (Bremner and Krogmeier, 1988) with urea as sole N source.

Selection schemes to recover biochemical mutants with new characteristics or higher levels of endogenous enzyme activity are of interest because their occurrence in crop or higher plants is limited, where descriptions of hypomorphs or nullimorphs predominate. Reports on in vitro recovery of mutants in nitrogen metabolism that involve enzyme overproduction, acquisition of function, or beneficial effects on agronomic performance are even less common. Sabir et al. (1992) found that an apparent overproducer of glutamate dehydrogenase was recovered as a somaclonal variant in sugarbeet. Donn et al. (1984) recovered a cell line of alfalfa overproducing glutamine synthetase (GS) following somatic cell selection for resistance to the GS inhibitor phosphinothricin. The basis for resistance was amplification of the GS gene. The trait was interpreted to be dominant as it was expressed in hybrid protoplasts (Deak et al., 1988).

Some of the media with alternative sole N sources were completely or partially ineffective in supporting callus growth, with or without use of inhibitors, for example, PRO, BET and CHO. Such media could be used to isolate transgenic cells expressing heterologous genes for utilization of endogenous nitrogenous compounds, and/or to select

transgenic cells carrying "activation tagging" constructs generating dominant phenotypes by the overexpression of genes (Hayashi et al., 1992).

REFERENCES

Bremner, J.M. and M.J. Krogmeier. 1988. Elimination of the adverse effects of urea fertilizer on seed germination, seedling growth and early plant growth in soil. Proc. Natl. Acad. Sci. 85: 4601-4604.

Chaleff, R.S. and M.F. Parsons. 1978. Isolation of a glycerol-utilizing mutant of *Nicotiana tabacum*. Genetics 89: 723-728.

Deak, M., G. Donn, A. Feher, and D. Dudits. 1988. Dominant expression of a gene amplification related herbicide resistance in *Medicago* cell hybrids. Plant Cell Rep. 7: 158-161.

Dhugga, K.S., J.G. Waines, and R.T. Leonard. 1988. Nitrate absorption by corn roots. Inhibition by phenylglyoxal. Plant Physiol. 86: 759-763.

Donn, G., E. Tischer, J.A. Smith, and H.M. Goodman. 1984. Herbicide-resistant alfalfa cells: an example of gene amplification in plants. J. Mol. Appl. Genet. 2: 621-635.

Duncan, D.R. and J.M. Widholm. 1986. Cell selection for crop improvement. Plant Breeding Rev. 4: 153-173.

Hanson, A.D. and R. Wyse. 1982. Biosynthesis, translocation, and accumulation of betaine in sugarbeet and its progenitors in relation to salinity. Plant Physiol. 70: 1191-1198.

Hayashi, H., I. Czaja, H. Lubenow, J. Schell, and R. Walden. 1992. Activation of a plant gene by T-DNA tagging: auxin-independent growth in vitro. Science 258: 1350-1353.

Heimer, Y.M. and P. Filner. 1970. Regulation of the nitrate assimilation pathway of cultured tobacco cells II. Properties of a variant cell line. Biotech. Biophys. Acta 215: 152-165.

Le Rudulier, D., A.R. Strom, A.M. Dandekar, L.T. Smith, and R.C. Valentine. 1984. Molecular biology of osmoregulation. Science 224: 1064-1068.

Murashige, T. and F.A. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15: 476-497.

Polacco, J.C., A.K. Judd, J.K. Dybing, and S.R. Cianzio. 1989. A new mutant class of soybean lacks urease in leaves but not in leaf-derived callus or in roots. Mol. Gen. Genet. 217: 257-262.

Sabir, A., H.G. Newbury, G. Todd, J. Catty, and B.V. Ford-Lloyd. 1992. Determination of genetic stability using isozymes and RFLPs in beet plants regenerated in vitro. Theor. Appl. Genet. 84: 113-117.

Saunders, J.W., G. Acquaah, K.A. Renner, and W.P. Doley. 1992. Monogenic dominant sulfonylurea resistance in sugarbeet from somatic cell selection. Crop Sci. 32: 1357-1360.

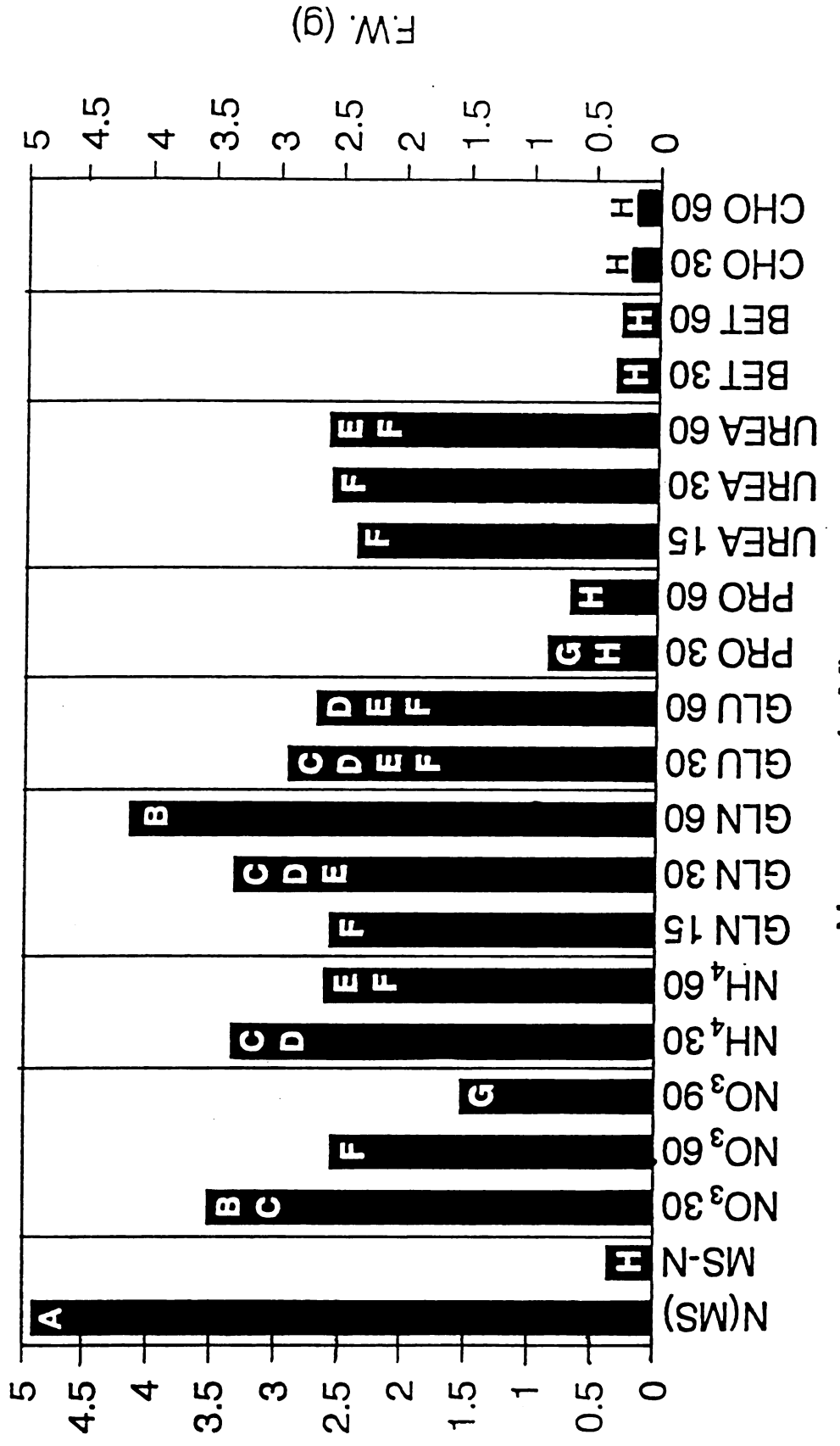

Figure 4-1: Fresh weight of plating callus after 30 days on MS medium, MS minus nitrogen (MS-N), or media with various sole nitrogen sources. The mM concentration of the sole nitrogen source is the number following the designation of nitrogen form. Means marked with same letter are not significantly different according to LSD with $P<0.05$.

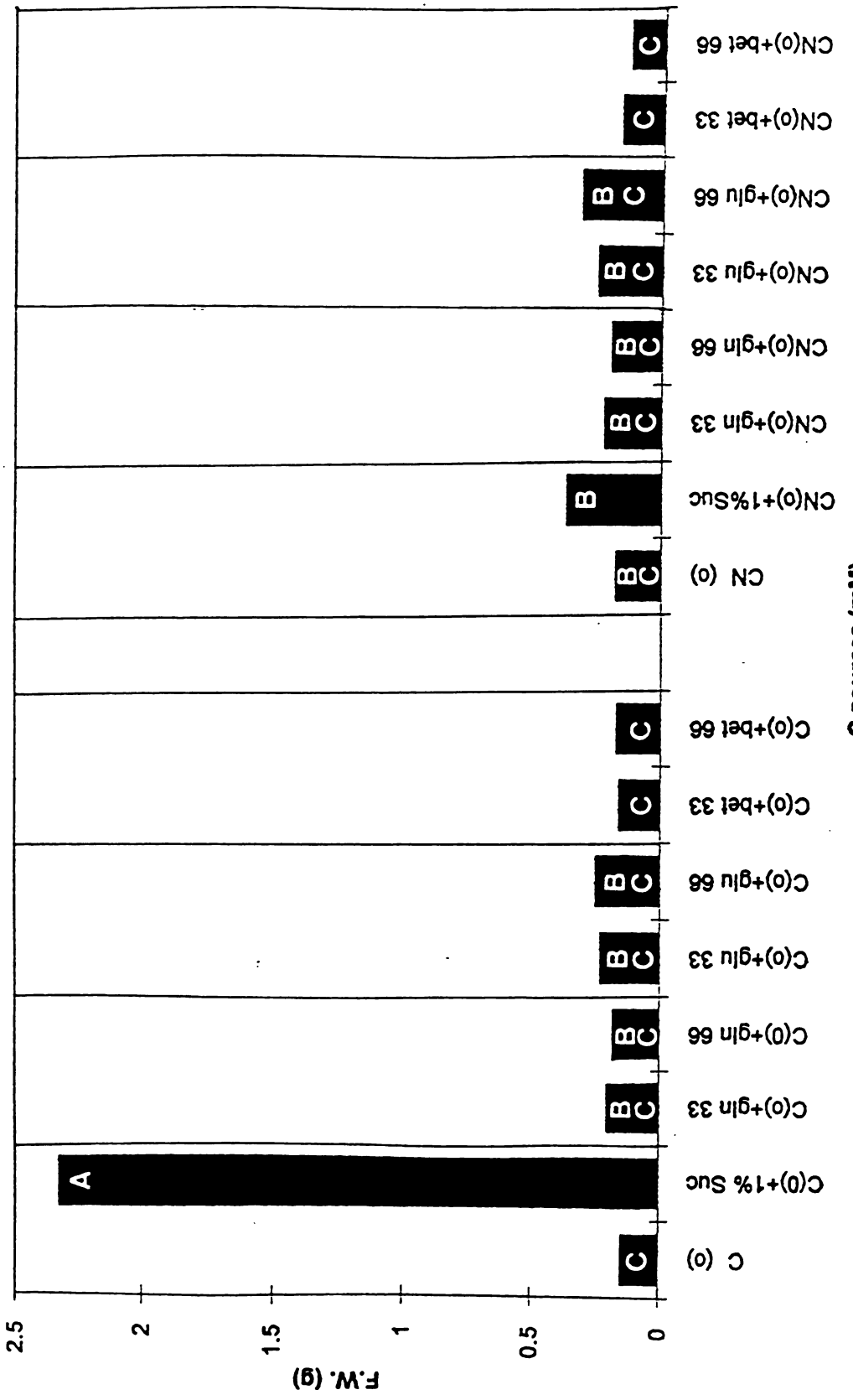

Figure 4-2: Ability of GLN, GLU and BET to serve as sole C or C+N sources on fresh weight of plating callus. Means marked with the same letter are not significantly different according to LSD with $P<0.05$.

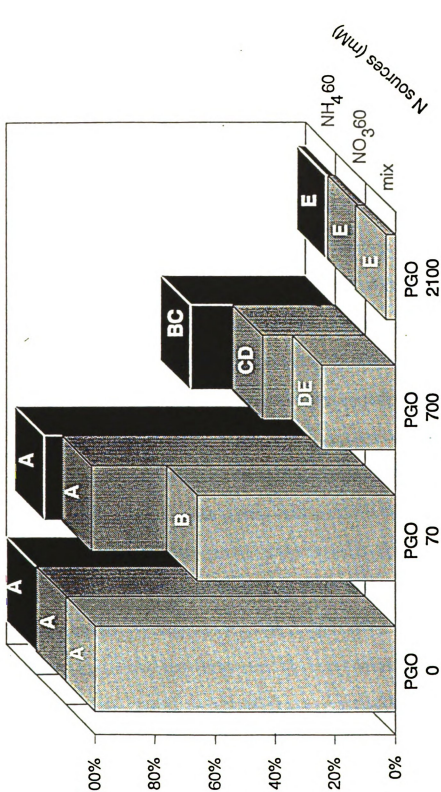

Figure 4-3: Effect of PGO on fresh weight of plating callus after 35 days on media with 60 mM NO_3, NH_4, or a mixture of these (total N=120 mM). Response to PGO is displayed as the percentage of the fresh weight in the absence of PGO for each N regime. Means marked with the same letter are not significantly different according to LSD with P<0.05.

CHAPTER 5

EVALUATION OF SOLE NITROGEN SOURCES FOR SHOOT CULTURES AND LEAF DISC CULTURES OF SUGARBEET

ABSTRACT

Eight endogenous nitrogen-containing sources (nitrate, ammonium, glutamine, glutamate, urea, proline, glycine betaine, and choline) were examined for their ability to serve as sole nitrogen source for shoot culture as well as leaf disc culture of sugarbeet (*Beta vulgaris* L.) clone REL-1. Nitrate (NO_3), ammonium (NH_4), urea, and glutamine (GLN) as sole nitrogen sources were moderately supportive of shoot multiplication and fresh weight increase of shoot culture growth compared to the Murashige-Skoog (MS) nitrogen mix of 40 mM NO_3 and 20 mM NH_4. Glutamate (GLU) and proline (PRO) were at best poorly supportive, and glycine betaine (BET) and choline (CHO) were non-supportive, of shoot culture growth. Callus initiation from leaf discs was moderately supported only by sole nitrogen sources NO_3, NH_4, urea, GLN, and PRO. Shoot regeneration from the disc callus was further supported on media with GLN, NO_3, urea or PRO, although at least three times more shoots per callusing disc were produced on the MS nitrogen mix. Overall, the MS nitrogen mix was superior or equal to any of the single nitrogen sources. However, single nitrogen source media with NO_3, NH_4, urea, GLN or PRO could be used for shoot cultures of mutants with impaired nitrogen assimilation or in comparative physiology studies.

INTRODUCTION

Commonly used basic media for plant tissue culture use various sources and quantities of nitrogen (Murashige and Skoog, 1962; Blaydes, 1966; Gamborg et al., 1968; Schenk and Hildebrandt, 1972; Lloyd and McCown, 1980). Most basic media have a mixture of nitrate and ammonium, and some have an additional form of organic nitrogen. Comparison of growth or developmental responses on these media permits few conclusions about quantitative or qualitative differences in nitrogen content because the effects of many other nutritional factors confound interpretations.

Nitrogen is a very important nutrient for sugarbeet crop yield and processing quality. Glutamine, glutamate and glycine betaine are nitrogenous impurities in the harvested beet that interfere with industrial sucrose extraction efficiency (Smith, 1988). Glutamine and glutamate, but not glycine betaine, were found to support sugarbeet plating callus growth in a test of eight sole nitrogen sources conducted in support of efforts to develop cell selection regimes for mutants in nitrogen assimilation (Chapter 4). In order to determine the feasibility of using shoot and leaf disc systems to study growth and metabolism in wild-type and prospective nitrogen metabolism mutants, we investigated the responses of cultured sugarbeet shoots and leaf discs.

MATERIALS AND METHODS

Plant materials: All experiments were performed with shoot cultures and leaf discs of sugarbeet clone REL-1, a diploid self-fertile annual clone, which is a superior regenerator of shoots from leaf callus, and easily maintained by in vitro shoot culture (Saunders et al., 1990).

Media: All media were formulated according to Murashige and Skoog (MS) (1962) inorganics, 10 mg/L thiamine·HCl, 1.0 mg/L nicotinic acid, 1.0 mg/L pyridoxine·HCl, 100 mg/L myo-inositol, 3 % sucrose, and 0.9 % agar w/v. Concentration of 6-benzyladenine was 1.0 mg/L for leaf disc culture; 0.25 mg/L for shoot culture. Nitrate (NO_3), ammonium (NH_4), glutamine (GLN), glutamate (GLU), proline (PRO), urea, glycine betaine (BET), and choline (CHO) were tested as sole nitrogen sources at 15, 30, 60, and/or 90 mM in the cases of both shoot cultures and leaf disc cultures. NO_3 (40 mM) and NH_4 (20 mM) together were used as the standard MS nitrogen, except when alternative sole nitrogen sources were tested. A nitrogen-free medium served as a negative control. In such cases the sources of potassium were from the 2.0 mM KH_2PO_4 in MS, any KOH used in pH adjustment, or stoichiometric quantities from KNO_3 used in providing the various levels of NO_3. NH_4 was provided as the chloride salt and was accompanied by half that concentration of succinic acid. GLU was provided as the sodium salt, BET, CHO and PRO as the free bases. Individual K^+, Na^+ or Cl^- concentrations were not adjusted to maintain equivalency.

Shoot culture: Cultures for shoot multiplication had been established from axillary shoots on a flower stalk in 1985 and subcultured by division every 2-3 months. Each 20- by 100-

mm Petri dish contained three shoots (initial mean fresh weight of a shoot was approximately 0.15 g) on 35 ml of MS medium with BA 0.25 mg/L, and were grown at 25 °C and under 10 to 20 umol $m^{-2}s^{-1}$ from fluorescent lamps. For the media comparison, total fresh weight of shoots per plate was measured after 35 days and the threesomes were subcultured to fresh respective media. After a further 26 days, each plate's shoots were measured to produce a 61 day cumulative fresh weight as well as shoot number increase. The minimum size for a shoot was 0.5 cm. Final medium pH was also measured on the 61st day.

Leaf disc culture: Leaf discs (8 mm diameter) were taken from incompletely expanded leaves of greenhouse-grown vegetative plants and disinfected with a dilute (1:5 v/v) commercial (5.25 % w/v) hypochlorite (NaOCl) bleach containing 100 mg/L sodium laurylsulfate. One 8 mm leaf disc was placed on 35 ml of MS agar medium with 1 mg/L BA in a 20- by 100- mm Falcon disposable Petri dish. Plates were kept at 30 °C in the dark (Saunders et al., 1992). Percent of discs callusing was observed after 42, 61, and 86 days of incubation. Mean number of shoots per callusing disc was counted after 106 days of culture. Expansion of leaf discs was measured after 15 days, and expressed as increase in diameter.

Each experiment with 10 replications was performed 3 times with similar results. Final fresh weight, shoot multiplication factor, percentage of discs callusing, mean shoots per callusing disc, and expansion of leaf discs for each medium treatment were subjected to ANOVA and the LSD (P=0.05) was performed to compare individual treatments.

RESULTS AND DISCUSSION

Shoot culture: Shoot culture response to the eight sole nitrogen sources as measured by cumulative fresh weight of initial culture (35 days) and subculture (26 days) (Figure 5-1), and shoot number increase (Figure 5-2) indicated that NO_3, NH_4, GLN and urea supported growth at best 81 % as well as the MS nitrogen mix. GLU, PRO, BET and CHO were ineffective. In general, the intensity of shoot multiplication on each medium corresponded to the respective fresh weight response.

The final medium pH in the shoot cultures (Figure 5-3) was not associated with the relative increase of shoots and fresh weight. The final pH values with NO_3 and GLU were above 6; MS, nitrogen-free (MS-N), NH_4 and urea were around 5; GLN, PRO, BET and CHO were below 5. Gamborg and Shyluk (1970) reported that there was no apparent correlation between final pH and growth of the soybean cells with the various ammonium salts, when the soybean cells grew on ammonium salts as the sole nitrogen source. Therefore, the abilities of sole nitrogen sources to support growth do not seem to be dependent on the value of the final medium pH.

Leaf disc culture: Callus was initiated from leaf disc only after at least 25 days of inoculation. Callus initiation response was not designed to involve amount of callus produced. GLU, BET, CHO and nitrogen-free media initiated no callus within 86 days of observation (Figure 5-4). All leaf discs had initiated callus from NH_4 (30 mM), GLN (15 mM) and MS media after an inoculation period of 42 days (Figure 5-4). Overall, callus initiation slowed after six weeks. Furthermore, shoots regenerated from callusing leaf

discs on MS, NO_3 (30, 60 mM), urea (15, 60 mM), GLN (15, 30, 60 mM) and PRO (30, 60 mM) (Figure 5-5) within 106 days of culture. GLN was the best sole nitrogen source for shoot production, but it was only about a third as productive as the MS nitrogen mix. Shoot production from callusing discs was lower at the higher GLN concentrations. All media that induced callus from leaf discs also induced some shoot regeneration from that callus, except for 30 mM urea and the NH_4 media (Figure 5-6, Table 5-1). The nonuniform number of discs that callused, and the low number of callusing discs that regenerated shoots on some media made statistical analysis of shoot regeneration impractical. Although the media with urea and NH_4 induced high percentages of disc callusing, the frequency of regenerated shoots was low and zero, respectively. Numerous studies have shown that most plants respond more favorably to nitrate than to ammonium as the sole nitrogen source. Filner (1966) found that ammonium did not support growth in cultured tobacco cells. Yatazawa and Furuhoshi (1968) reported that rice callus grew on agar media containing ammonium citrate but not on ammonium sulfate. Anyway, it really depends how the NH_4 is presented to the cultures, i.e., the companion anion. Some studies apparently not aware of this.

Leaf disc expansion (Figure 5-7) was not directly associated with callus initiation; note especially the response to NO_3 vs. PRO (Figure 5-4). All leaf discs attained their final expansion during the initial 15 days of inoculation. However, the results indicated that the NO_3, NH_4 and GLN sole nitrogen media were similar to the MS nitrogen mix medium for leaf disc expansion.

Unlike the shoot culture tests, PRO responded positively in the callus initiation of leaf disc. Moreover, shoot regeneration response on leaf disc callus did not correspond to

shoot culture response; only GLN and NO_3 induced a moderate response relative to the 100 % obtained with the standard MS nitrogen mix.

Hanson and Wyse (1982) reported that BET could not be utilized for cell culture growth, it might be associated with the apparent lack of BET utilization in the whole plant. Thus, ability of any sole nitrogen source to support growth in leaf disc and/or shoot culture indicates that the nitrogen source is being metabolized. This might also reflect in differential nitrogen response at the whole plant level. From this research, callus and shoots could be regenerated using GLN as a sole nitrogen source to produce independently-derived shoot regenerates. This could permit a degree of quantitative cell selection for ability to assimilate GLN, which might be reflected later in altered nitrogen response at the whole plant level.

REFERENCES

Atanassov, A.I. 1980. Method for continuous bud formation in tissue culture of sugar beet (*Beta vulgaris* L.). Z. Pflanzenzucht. 84: 23-29.

Blaydes, D.F. 1966. Interaction of kinetin and various inhibitors in the growth of soybean tissue. Physiol. Plant. 19: 748-753.

Coumans-Gilles, M.F., C. Kevers, M. Coumans, E. Ceulemans, T.H. Gaspar. 1981. Vegetative multiplication of sugarbeet through in vitro culture of inflorescence pieces. Plant Cell Tissue Org. Cult. 1: 93-101.

Filner, P. 1966. Regulation of nitrate reductase in cultured tobacco cells. Biochim. Biophys. Acta 118: 299-310.

Freytag, A.H., S.C. Anand, A.P. Rao-Arelli, and L.D. Owens. 1988. An improved medium for adventitious shoot formation and callus induction in *Beta vulgaris* L. in vitro. Plant Cell Rep. 7: 30-34.

Gamborg, O.L., R.A. Miller and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158.

Gamborg, O.L. and J.P. Shyluk. 1970. The culture of plant cells with ammonium salts as the sole nitrogen source. Plant Physiol. 45: 598-600.

Hanson, A.D. and R. Wyse. 1982. Biosynthesis, translocation, and accumulation of betaine in sugarbeet and its progenitors in relation to salinity. Plant Physiol. 70: 1191-1198.

Hooker, M.P. and M.W. Nabors. 1977. Callus initiation, growth, and organogenesis in sugarbeet. Z. Pflanzenphysiol. 84: 237-246.

Lloyd, G. and B. McCown. 1980. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Intl. Plant Prop. Soc. Proc. 30: 421-427.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-497.

Owens, L.D. and D.R. Eberts. 1992. Sugarbeet leaf disc culture: an improved procedure for inducing morphogenesis. Plant Cell Tissue Organ Cult. 31: 195-201.

Saunders, J.W., G. Acquaah, K.A. Renner and W.P. Doley. 1992. Monogenic dominant sulfonylurea resistance in sugarbeet from somatic cell selection. Crop Sci. 32: 1357-1360.

Saunders J.W. and W.P. Doley. 1986. One step shoot regeneration from callus of whole plant leaf explants of sugarbeet lines and a somaclonal variant for in vitro behavior. J. Plant Physiol. 124: 473-479.

Saunders, J.W., W.P. Doley, J.C. Theurer, et al. 1990. Somaclonal variation in sugarbeet. *In* Y.P.S., Bajaj. (ed.). Biotechnology in agriculture and forestry. Vol. II. Somaclonal variation in crop improvement I. Springer-Verlag, Berlin. Heidelberg. pp.465-490.

Saunders, J.W. and K. Shin. 1986. Germplasm and physiologic effects on induction of high-frequency hormone autonomous callus and subsequent shoot regeneration in sugarbeet. Crop Sci. 26: 1240-1245.

Saunders, J.W., C.J. Tsai and E. Samper. Evaluation of alternative nitrogen and carbon sources for sugarbeet suspension culture platings in development of cell selection schemes. In Vitro Cell. Dev. Biol. Submitted.

Schenk, R.V. and A.C. Hildebrandt. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50: 199-204.

Smith, G.A. 1988. Effects of plant breeding on sugarbeet composition. *In* Clarke, M.A. and M.A. Godshall (ed.) Chemistry and processing of sugarbeet and sugarcane. Elsevier, Amsterdam. pp 9-19.

Yatazawa, M., and K. Furuhoshi. 1968. Nitrogen sources for the growth of rice callus tissue. Soil Sci. Plant Nutr. 14: 73-79.

Table 5-1: Leaf disc culture with single nitrogen sources after 106 days of inoculation.

(106 days)	% of discs callusing	(LSD)	% of callusing discs that had at least 1 shoot	proportion of shoots/per callusing disc
MS	100	(A)	100	(10/10)
MS-N	0	(F)	0	(0/0)
NO_3 30	60	(CD)	33.33	(2/6)
NO_3 60	20	(EF)	50	(1/2)
NO_3 90	0	(F)	0	(0/0)
NH_4 30	100	(A)	0	(0/10)
NH_4 60	70	(BC)	0	(0/0)
urea 15	90	(AB)	11.11	(1/9)
urea 30	80	(ABC)	0	(0/8)
urea 60	100	(A)	10	(1/10)
gln 15	100	(A)	80	(8/10)
gln 30	80	(ABC)	62.5	(5/8)
gln 60	70	(BC)	14.29	(1/7)
glu 30	0	(F)	0	(0/0)
glu 60	0	(F)	0	(0/0)
pro 30	90	(AB)	11.11	(1/9)
pro 60	40	(DE)	25	(1/4)
bet 30	0	(F)	0	(0/0)
bet 60	0	(F)	0	(0/0)
cho 30	0	(F)	0	(0/0)
cho 60	0	(F)	0	(0/0)

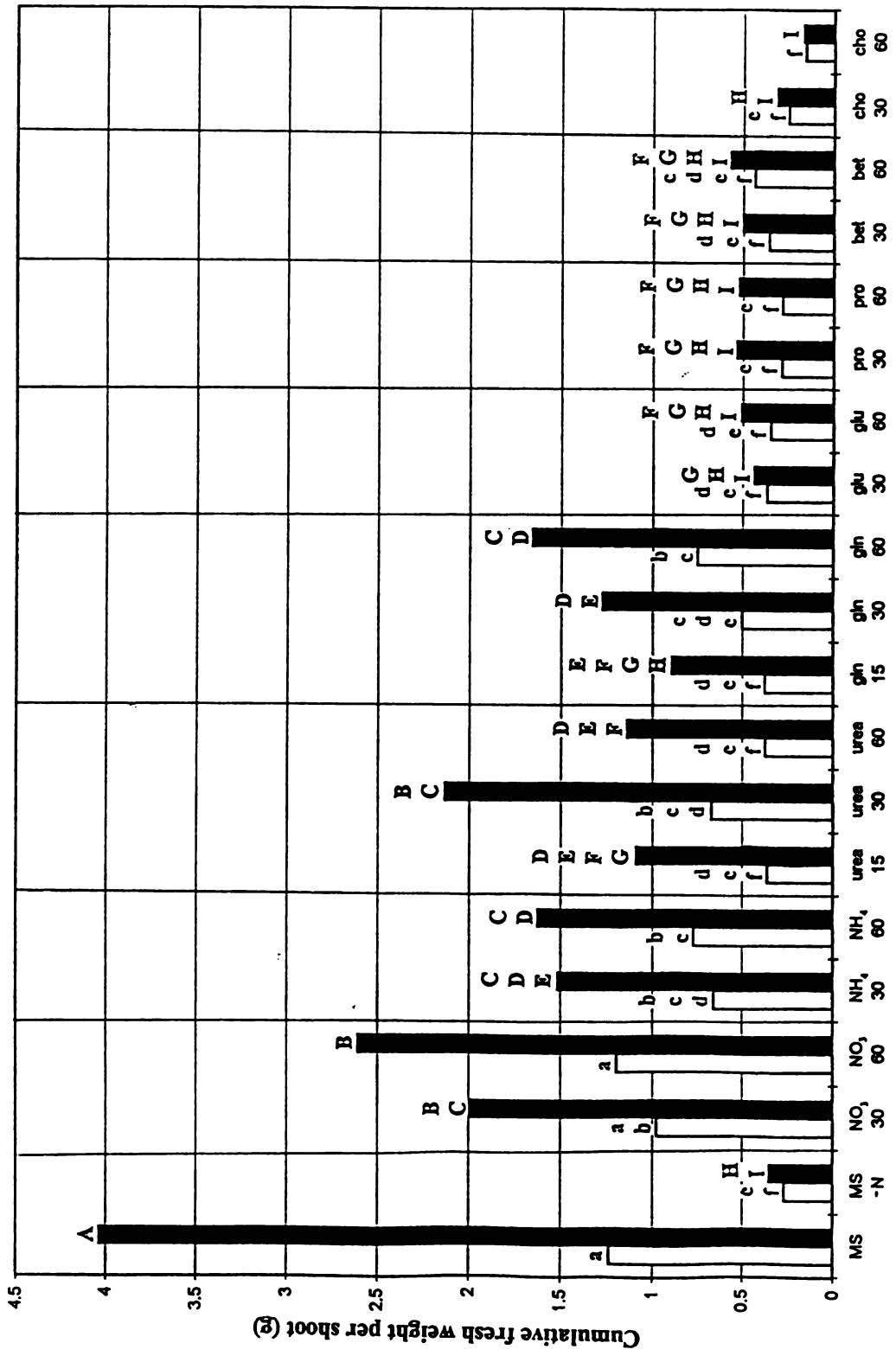

Figure 5-1: Cumulative fresh weights of shoot cultures growing with single nitrogen sources after initial culture (35 days) and subculture (subsequent 26 days), respectively. Means marked with same letter are not significantly different according to LSD ($P<0.05$).

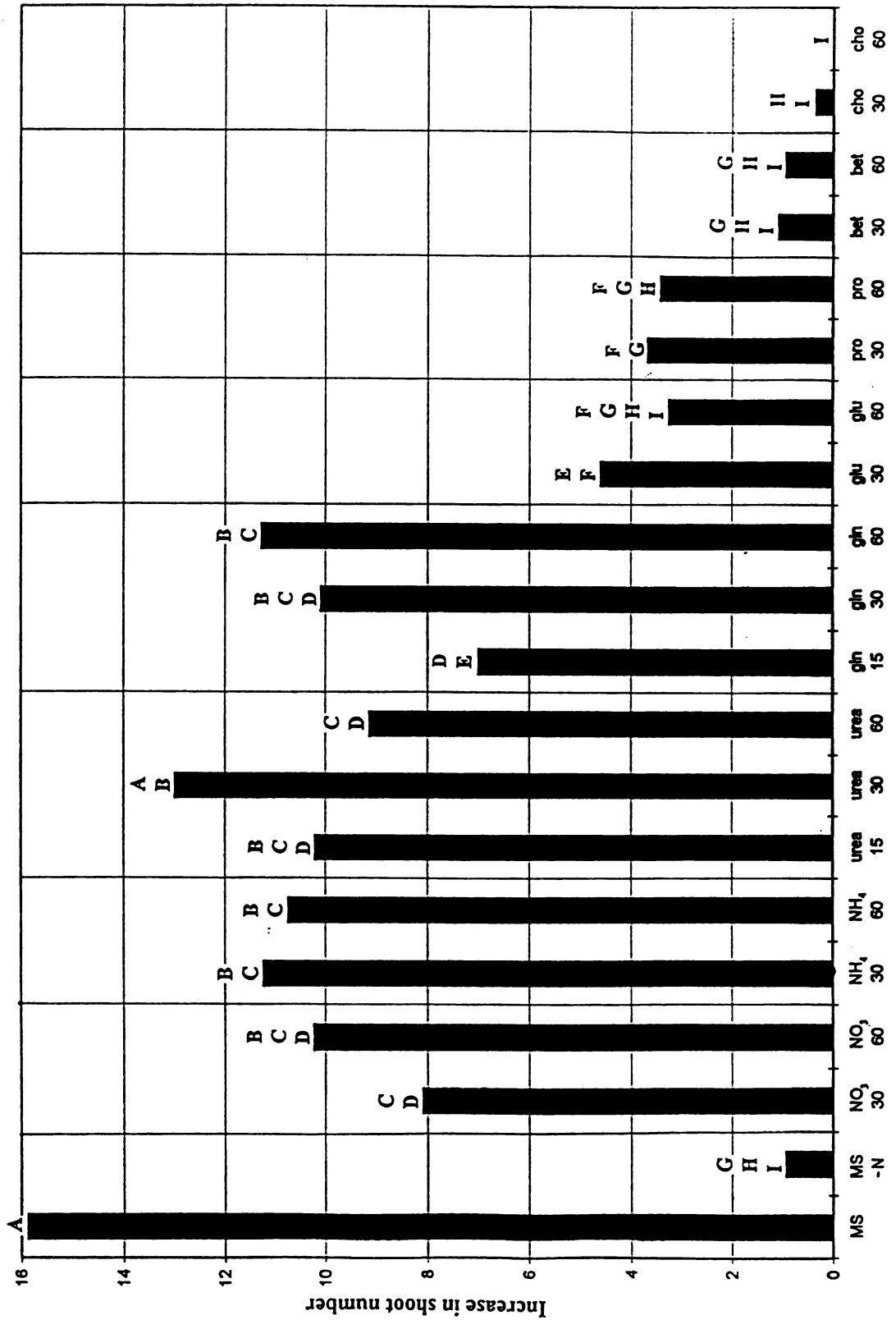

Figure 5-2: Increase in shoot number in shoot cultures with single nitrogen sources after 2 months. Means marked with same letter are not significantly different according to LSD ($P<0.05$).

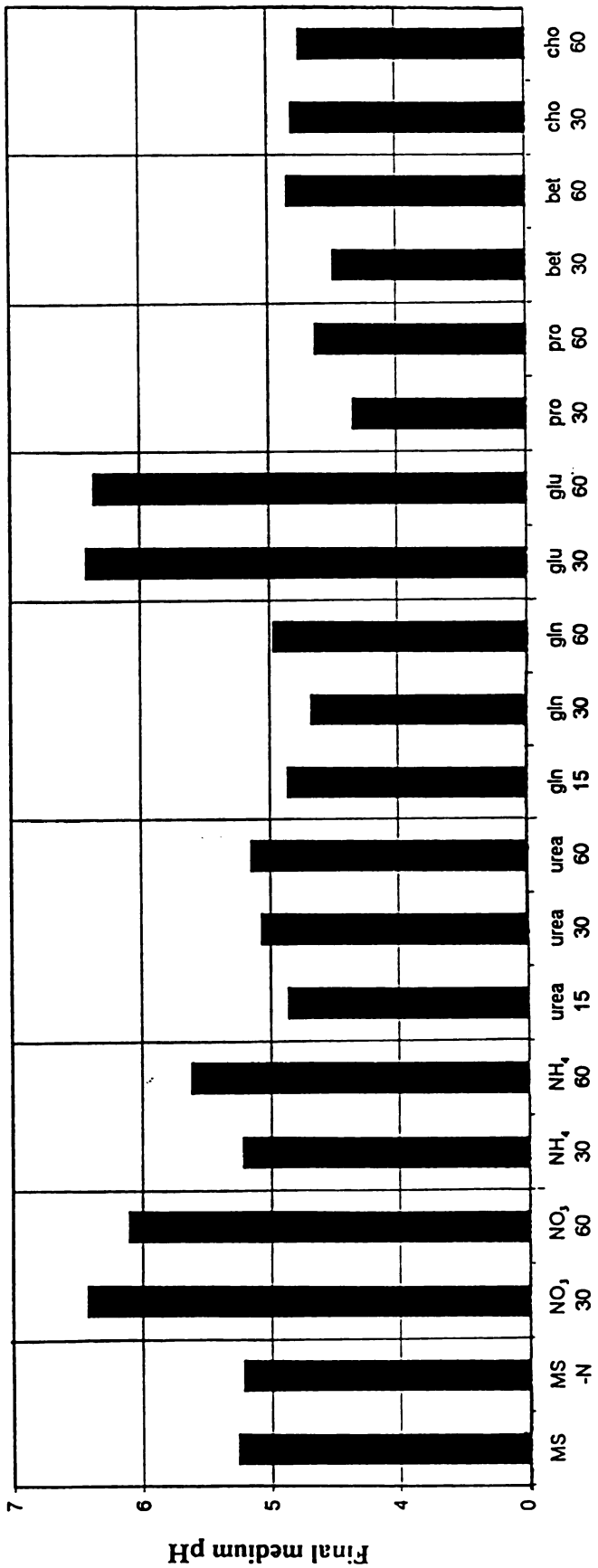

Figure 5-3: Final medium pH after shoot culture with single nitrogen sources after 2 months.

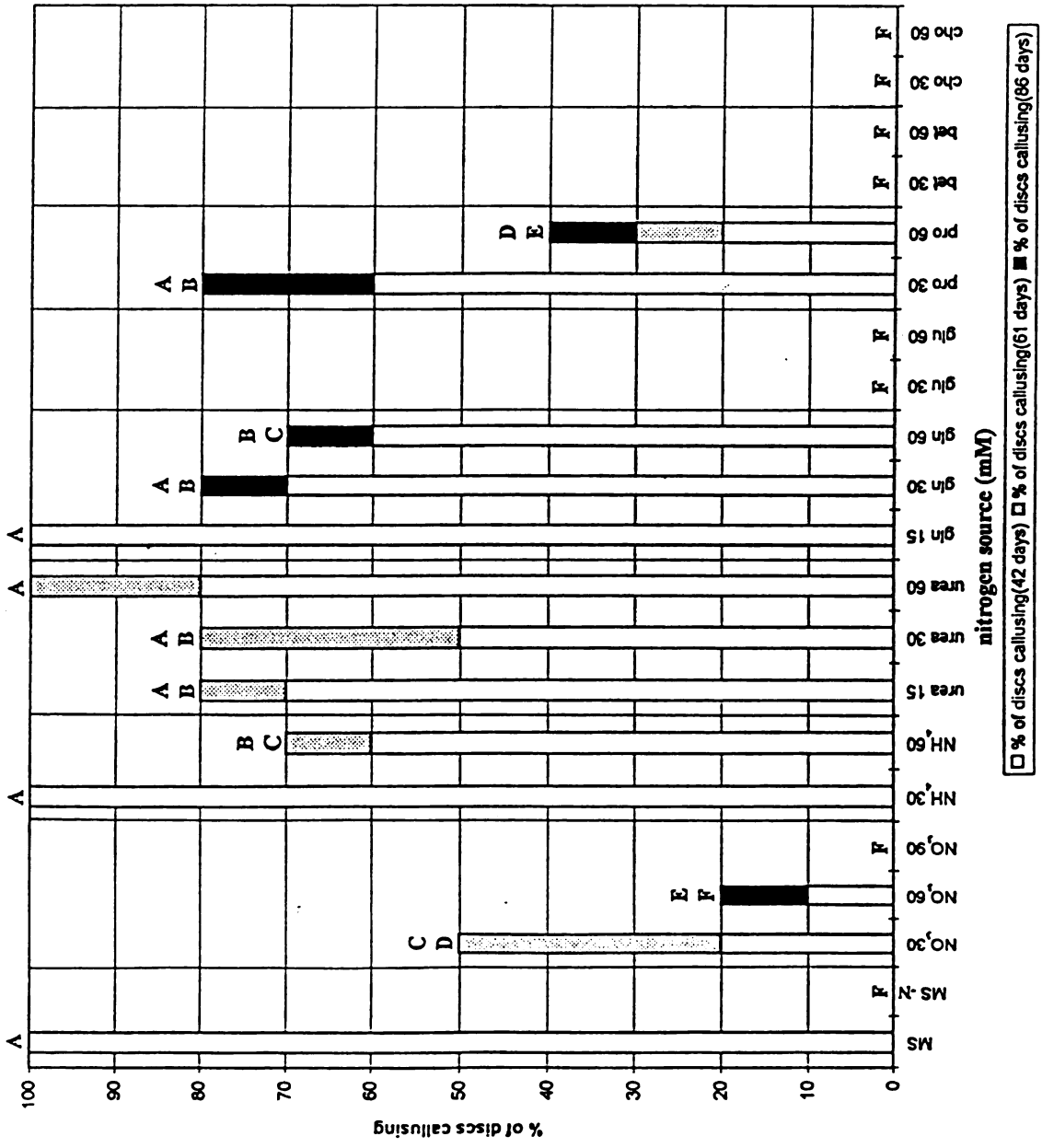

Figure 5-4: Callusing response of cultured leaf discs with single nitrogen sources. Means of 86 day values marked with same letter are not significantly different according to LSD ($P<0.05$).

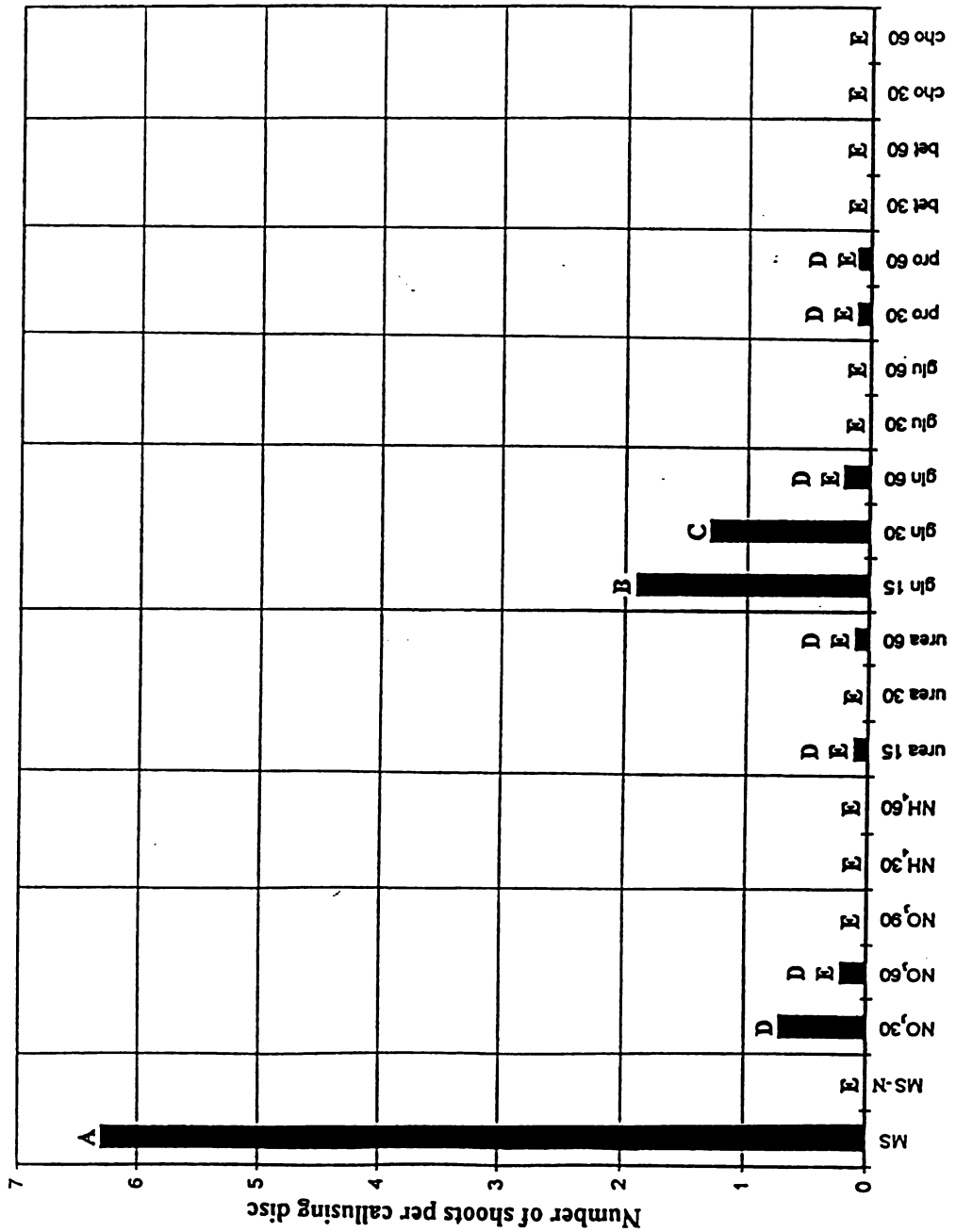

Figure 5-5: Number of shoots per callusing leaf disc on single nitrogen sources after 106 days. Means marked with same letter are not significantly different according to LSD ($P<0.05$).

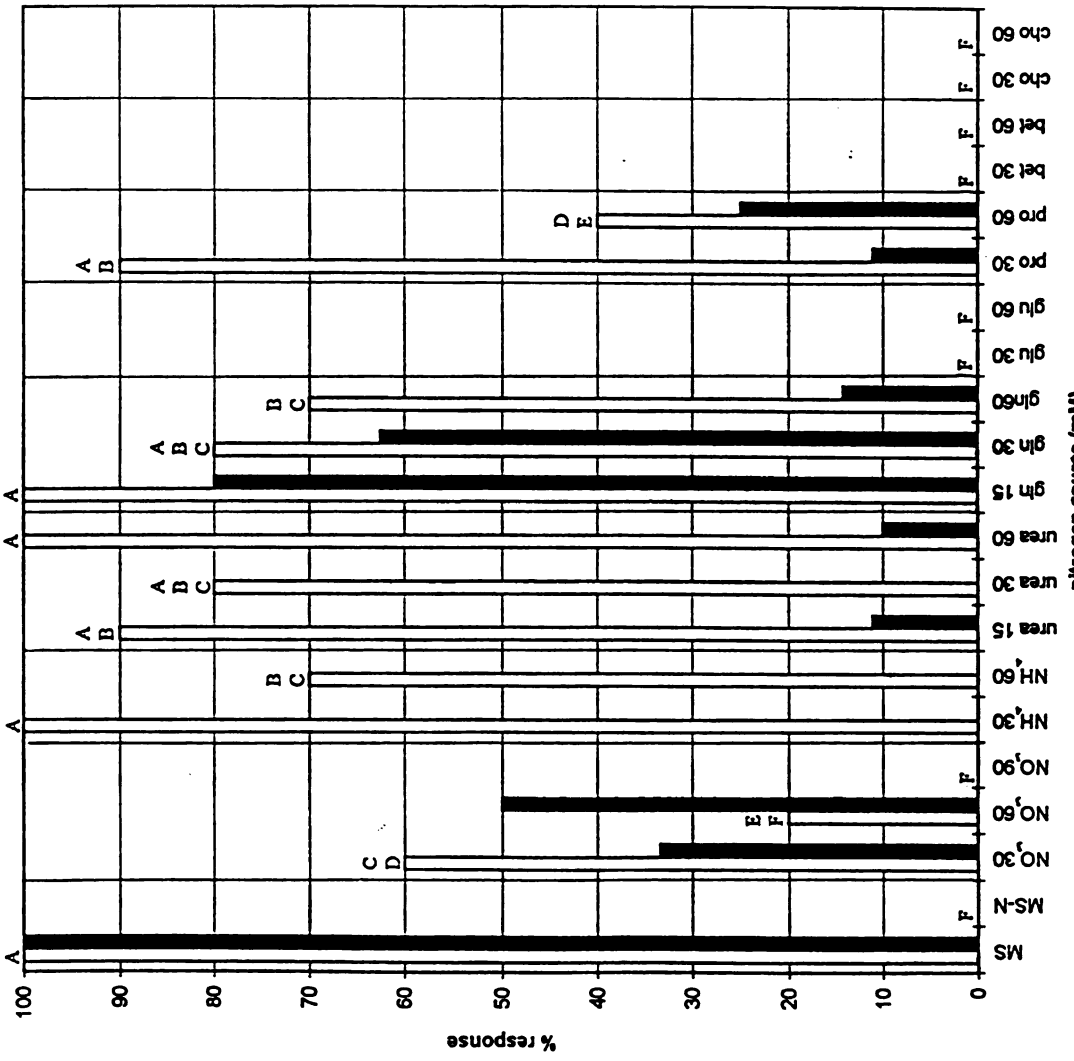

Figure 5-6: Leaf disc culture with single nitrogen sources after 106 days. For percentage of disc callusing only, means marked with same letter are not significantly different according to LSD (P<0.05).

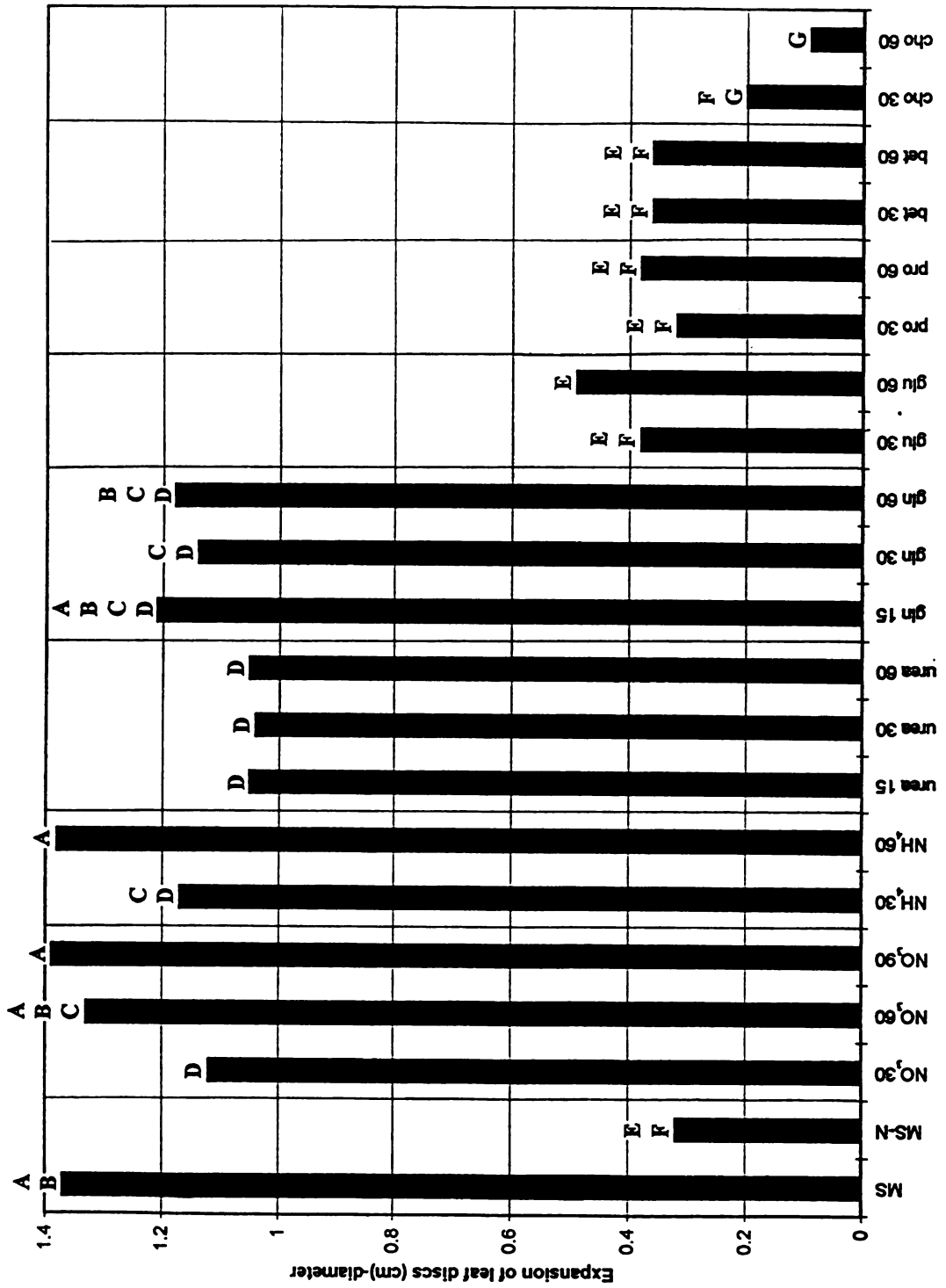

Figure 5-7: Leaf disc expansion with single nitrogen sources after 15 days. Means marked with same letter are not significantly different according to LSD ($P<0.05$).

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