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SOME GENOTYPIC AND PHYSIOLOGICAL ASPECTS OF SHOOT REGENERATION FROM HORMONE AUTONOMOUS CALLUS OF SUGARBEET (BETA VULGARIS L.)

presented by

KYUNGWON SHIN

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SOME GENOTYPIC AND PHYSIOLOGICAL ASPECTS OF SHOOT REGENERATION FROM HORMONE AUTONOMOUS CALLUS OF SUGARBEET (*BETA VULGARIS* L.)

By

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ABSTRACT

SOME GENOTYPIC AND PHYSIOLOGICAL ASPECTS OF SHOOT REGENERATION FROM HORMONE AUTONOMOUS CALLUS OF SUGARBEET (BETA VULGARIS L.)

by

Kyungwon Shin

The most effective shoot regeneration in sugarbeet involves the production of high frequency hormone autonomous callus. A wide range of germplasm in the species Beta vulgaris L. was screened for induction of hormone autonomous callus as well as bud or shoot formation from the petioles and callus using shoot culture leaf parts. Callus was induced on some genotypes from samples of each of seventeen germplasm sources tested. Buds or shoots were regenerated from callus of four forms of the species Beta vulgaris from thirteen of the seventeen germplasm sources.

Several genotypes capable of callus formation were chosen for further characterization. The characteristics tested were the sensitivity to 6-benzyladenine (BA) as well as other cultural factors affecting optimum regeneration rates such as size of callus piece, light intensity for donor shoot cultures, and hormone or nutrient supplements.

frequency of bud regeneration. Generally, monogerm genotypes showed a high frequency of ability to regenerate while most multigerm genotypes had poor ability.

Several genotypes capable of callus formation were chosen for further characterization. The characteristics tested were the sensitivity to 6-benzyladenine (BA) as well as other cultural factors affecting optimum regeneration rates such as size of callus piece, light intensity for donor shoot cultures, and hormone or nutrient supplements.

Of the four genotypes tested, BA by itself was found effective in inducing buds from callus in genotypes FC 607-0-20 and 6926-0-3. BA concentrations of 0.3-1.0 mg/l were optimal for bud induction in the permissive genotypes, whereas 10 mg/l BA was completely inhibitory for growth for all genotypes.

Of the medium changes screened, proline at 200 and 600 mg/l showed the most effect for the improvement of bud regeneration. Variations in concentrations of α-naphthalene acetic acid (NAA), 3-indoleacetic acid (IAA), 2,3,5-triiodobenzoic acid (TIBA), sucrose or inorganic salts had no significant effect on bud regeneration from callus.

Light intensity under which donor shoot cultures were grown was determined not to have a major influence on subsequent callusing or shoot regeneration from petiole

explants. However, age and size of callus did have a significant effect on regeneration in callus following subdivision, and such an effect might well explain the significant replication effects seen in other experiments.

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INTRODUCTION

Sugarbeet (Beta vulgaris L.) which is cultivated in about 40 countries, is an economically important agricultural plant resulting in the production of nearly 40% of the world's sugar (Martens, 1984). Despite the successes of producing superior varieties by conventional plant breeding, there are still a lot of valuable genetic combinations or additions that cannot be achieved by conventional methods (Butenko and Atanassov, 1971). Plant cell and tissue culture methods are also considered as important tools for basic studies of higher plant genomes and for agricultural improvement of crop plants (Green, 1978). At the present time, cell and tissue culture methods can be expected to lead to improvement of sugarbeet (Butenko and Atanassov, 1971; Ingram, 1971; Nickell and Torrey, 1969).

The primary purpose of plant tissue culture research is crop improvement. In order to apply cellular genetic techniques for this, efficient procedures for plant regeneration must be achieved. Most proposed schemes involve a tissue culture cycle including a phase of callus proliferation and subsequent regeneration of plants from

culture. In many species, a tissue culture cycle is a source of phenotypic and genotypic variation (Larkin and Scowcroft, 1981; Meins, 1983). This would provide a direct link between tissue culture research and conventional genetic and breeding procedures. Although regeneration of shoots from callus or cell suspensions remains a key, it is often the hardest step to overcome for many species (Evans, et al., 1981). In sugarbeet, desired applications for genetic manipulation could be the introduction of microbial betaine catabolic genes, selection of resistance to disease toxins, male sterile/fertile cytoplasm swapping, and rapid conversion to male fertility in specific cytoplasmic male sterile genotypes. From a functional viewpoint, one of the important characteristics of callus is that this unorganized growth has the potential to develop normal shoots and roots, or else embryoids which form plantlets. Therefore, regeneration of shoots from callus is needed to transfer genetic variation present at the cell level, whether induced, introduced or spontaneous, to the whole plant level for breeding application.

In sugarbeet, callus has been initiated from various types of explants such as shoot axes (Atanassov, 1980), flower buds (Margara, 1970), seedling explants (Butenko and Atanassov, 1971; Mohammad and Collin, 1979; Hooker and Nabors, 1977; Welander, 1976), anthers (Rogozinska et al., 1977; De Greef, 1978), leaf pieces from <u>in vitro</u> shoot

(Rogozinska and Goska, 1978), and embryos (Hooker and Nabors, 1977). Subsequent studies on calli generally proved that regeneration is possible even though difficult (Butenko and Atanassov, 1971).

The general characteristics of a callus involve a complex relationship among the plant material used to initiate the callus, the composition of the medium, and the environmental conditions during the incubation period. Regardless of the presence of apparently normal chloroplasts, chemical and physical conditions encountered during culture sharply limit the photosynthetic potential of the cultured cells. By developing an optimal culture medium such as the required amount of growth hormones, sucrose and other chemical factors under the proper environmental conditions, one can succeed in getting the shoots and roots (Murashige, 1978; Vasil and Vasil, 1980; Vasil et al., 1979).

This research was aimed to improve shoot regeneration by a combination of genetic and environmental approaches. A wide range of sugarbeet germplasm was screened to identify sources of the ability to callus and regenerate buds from callus. And, with several genotypes already known to be capable of callus formation, some characteristics were studied: optimal concentration of 6-benzyladenine (BA) as well as other cultural factors affecting regeneration rates such as size of callus piece, light intensity and chemical

supplements like proline or 2,3,5-triiodobenzonic acid (TIBA).

LITERATURE REVIEW

Tissue culture has been considered as a potentially important technique to solve practical problems for the improvement of sugarbeet (Butenko and Atanassov, 1971; Nickell and Torrey, 1969; Hooker and Nabors, 1977). Progress in the production of improved crop varieties can be achieved by developing protocols for high frequency regeneration of plants for a broad range of crop species for use in any of a wide array of genetic applications.

Identification of genotypes with high frequency shoot regeneration could facilitate long term selection procedure at the cellular level, so that the desired cell lines can be recovered as whole plants after long periods of cell culture. One weakness of the cell culture approach sometimes has been that morphogenetic potential has been lost by the cells during lengthy culture periods. Therefore, even though many valuable cell culture lines might have arisen, their ultimate benefits cannot be realized because of the inability to obtain whole plants. Salt and temperature tolerance in cell cultures of Nicotiana and Capsicum (Bopp, 1978), Phoma lingam toxin

resistance in Brassica napus (Sacristan, 1982), and fused protoplasts of male sterile and fertile cytoplasm in Beta vulgaris are a few examples (personal communication, Linda Schnabelrauch).

Callus cultures of tobacco pith tissues are normally found to require supplies of exogenous indoleacetic acid (IAA) and cytokinin in the culture medium for growth. Some cultures gradually lose the requirement for exogenous auxin. Although some tissue explants may initially have high endogenous auxin levels, the cultured tissues apparently develop auxin biosynthetic abilities. Other cultures have been found that require the addition of auxin but not cytokinin (Gautheret, 1955a). Callus that has lost the requirement for auxin, cytokinin or both is called habituated, also known as hormone autonomous.

Tissue of wild carrot in culture gradually loses its requirement for exogenous auxin (Gautheret, 1955a). There are reports that cultured tissues from various plant species may habituate for auxins, certain vitamins, and cytokinins (Gautheret, 1955b; Fox, 1963; Street, 1966). Gautheret (1955a) first proposed that habituation had an epigenetic basis, and involved "enzymic adaptation" rather than mutation since conversion of tissues to the autotrophic state was gradual and at least partially reversible.

Binns and Meins (1973) obtained 62 completely fertile tobacco plants from 13 different clones of habituated tissue. The important point was that cells derived from habituated clones were totipotent. These cells have also lost their habituated character. The experiments provide strong evidence that cytokinin habituation has an epigenetic basis because it is a progressive, gradual process involving epigenetic changes rather than classical genetic mutations. Epigenetic changes are defined as directed, heritable changes that are regularly reversible and limited in their expression by the genetic potential of the cell. Meins also showed that whole tobacco plants were regenerated from habituated callus. Different somatic cells in the same organism are thought to have the same complement of genes (Davidson, 1968; Waddington, 1956; Weiss, 1939). Because cells have not lost genes during development, their determination is still potentially reversible. This poses the fundamental problem of how cells with the same genotype can inherit different characters.

Saunders and Daub (1984) reported that a high frequency callus of sugarbeet could be induced in several genotypes of Michigan breeding origin, with habituation for auxin and cytokinin. In other words, callus growth proceeded on a simple basal medium without any hormones. With the presence of cytokinin 6-benzyladenine and the

auxin 3-indoleacetic acid, however, shoots were regenerated and grown into whole plants.

Since plant propagation through tissue culture was suggested by Haberlandt at the beginning of the 20th century, many studies about physiology of callus growth either in solid medium or in suspension cultures have been accomplished (Thorpe, 1978). However, few researchers have pursued similar studies on organogenesis, so that the knowledge of the organ forming process is not fully understood yet (Thorpe, 1978).

At the present time, plant regeneration has been reported in primary and subsequent callus, cell suspension and protoplast derived callus of many species (Vasil et al., 1979). After the classical study of Skoog and Miller (1957) who described hormonal control of shoot and root induction in tobacco tissue culture, that plant has been used as a model system for in vitro studies on regeneration. Somatic cells of many plant species has now been regenerated to whole plants by the application of appropriate aseptic procedures. This marvelous capacity has attracted a great deal of attention because of its potential importance in agricultural genetic manipulation as well as basic cell biology (Wareing and Phillips, 1981).

There is progressive differentiation of organs and tissues, giving rise to a wide range of different types of cells. However, not all of the genes of the total gene complement are expressed all the time and in all parts of the plant. Therefore, development must require that the right genes are expressed in the right cells at the appropriate time (Wareing and Phillips, 1981). In nature, development is a process involving selective gene expression and involves the activity of specific groups of genes which in turn control the synthesis of enzymes and other proteins characteristic of specialized cells. Thus the understanding of how specific culture procedures affect the tissue types obtained will facilitate production and recovery of the most desirable forms (Wareing and Phillips, 1981). Since somatic embryogenesis was discovered from carrot, Daucus carota (Steward et al., 1958), development of reliable methods for recovery of plants from additional species and culture systems has become a major objective in many laboratories. Shoot development in its simplest forms is now possible in many but not all species (Tisserat et al., 1979).

In sugarbeet, bud or shoot regeneration in vitro has been reported in callus derived from seedling explants (Butenko et al., 1972; Mohammad and Collin, 1979), anthers (Rogozinska et al., 1977), flower buds (Margara, 1977), and in habituated cell lines (De Greef and Jacobs, 1979;

Kevers et al., 1981; Saunders and Daub, 1984). Sub-cultured calli indicated that bud regeneration is possible (Hooker and Nabors, 1977). In all cases, Murashige - Skoog medium (Murashige and Skoog, 1962) was used for inorganic salts.

Given the reports of a range of genetic variability for in vitro callus response within many other species (e.g., Keys and Bingham, 1979; Marsolais et al., 1984) as well as within sugarbeet (Saunders and Daub, 1984), it is advisable to screen for genetic variability such as for habituation, shoot regeneration, hormone toxicity and pigmentation. One or more of these traits showing genetic variability could be the parameters for the development of an efficient system to identify cell fusion hybrids. In other words, progress in the application of plantlet regeneration from tissue culture to agriculture is closely related with the understanding of its genetic basis (Green, 1978; Sharp et al., 1982). Genetic lines of alfalfa with 60% regeneration were produced from inter-pollination of plants regenerated from hypocotyl callus with an initial plant regeneration frequency of only 12% (Bingham et al., 1975). Tomato shoot morphogenesis originating from cultured leaf discs with different regeneration potentials has been also examined (Frankenberger and Tigchelaar, 1980).

The most effective shoot regeneration in sugarbeet involoves the production of high frequency habituated callus from shoot cultures or isolated shoot culture petioles or blades, although after an incubation time of 4-10 weeks (Saunders and Daub, 1984). Many genotypes have the capability to make habituated callus at proper conditions (Saunders and Daub, 1984) such as high temperature, 32 C. In this case, callus will arise and maintain long term growth on basal medium (i.e. without any hormone), even though cytokinins can enhance both induction and rate of regeneration. While shoot regeneration from callus induced and maintained with auxins and cytokinins has been reported, and appeared to be of low reliability, induction of habituated callus in beets is highly reproducible (Saunders and Daub, 1984).

Cultures of plant tissue have been used to study factors involved in organogenesis in vitro. Most studies have shown that organ formation is strongly dependent on the growth regulator balance in the medium (for example, in alfalfa, Saunders and Bingham, 1975). In the classical study of Skoog and Miller (1957), it was demonstrated that the auxin to cytokinin ratio strongly influenced the pattern of organized development: a relatively high auxin to cytokinin ratio promoting root formation, and the reverse favoring shoot formation. But unfortunately, this mechanism cannot be applied as a general rule, even though

many species respond to the balance of auxin and cytokinin concentrations. The various growth regulating substances may need to be applied to cells, not only in the right amounts but also in the right sequence under the right culture conditions (Steward et al., 1967). These types of studies which are dealing with the manipulation of organogenesis by determining the optimum level of media additives, time of treatment, and/or the proper culture condition, still indicate little about the regulation of organ initiation. In order to increase the knowledge about how organogenesis is regulated at the tissue level, there are several aspects to be discussed (Thorpe, 1978): experimental system, physiological requirements, structural aspects and phytohormonal studies. If we accept the concept of cell totipotency, it should be possible to get shoot regeneration from all species under the right culture conditions and/or the proper additives to the medium (Thorpe, 1978), even though the failure of organogenesis in callus of many legumes over the last 30 years continues.

In sugarbeet, fresh habituated callus was stimulated to regenerate shoots by treatment with several combinations of 3-indoleacetic acid (IAA) and 6-benzyladenine (BA) (Saunders and Daub, 1984). The ability of callus to regenerate shoots can be affected by the relative amount of callus per milliliter of medium. High ratios of this can depress regeneration,

possibly because of premature depletion of benzyladenine or nutrient components.

There are three factors that appear to affect the shoot regeneration frequency from habituated callus in sugarbeet. First is the age of callus: more than three monthly subcultures on low benzyladenine eliminated shoot regeneration when the callus was placed on 1 mg/l BA (Saunders, personal communication). Second is the kind and amount of cytokinin (Saunders, 1982). This is because morphogenetic differentiation in vitro as well as in vivo is commonly thought to be dependent on supplies of auxin and cytokinin (Skoog and Miller, 1957) even though one cannot induce organ formation in vitro in all cases by only varying the supply of exogenous growth substances especially in monocots. The third one is the effect of genotypes (Saunders and Daub, 1984). Not all genotypes are capable of callus induction and of those that are, not all will regenerate shoots (Saunders and Daub, 1981).

MATERIALS AND METHODS

Shoot culture establishment and maintenance

Shoot cultures were established from seedlings or lateral buds of flower stalks. From two week old seedlings grown in the green house, a one cm stem piece with cotyledonary nodes attached was excised. Each one cm section was surface sterilized by soaking it with agitation for two 20 minutes periods in 15% chlorox and 0.01% sodium laurylsulfate. Then, they were rinsed at least five times with sterile distilled water. Each section was planted on 10 ml of M-20 medium in 25 ml glass screwtop vials and the vials were placed at room temperature under up to 50-60 $\mu Em^{-2}s^{-1}$ continuous fluorescent lighting. In the second method, 3 to 8 mm long lateral buds were taken from the axils of floral stalks in the green house during summer. The buds were surface sterlized as described before for seedling origin. After sterilization, each bud was placed on the M-20 in 25 ml vials containing 10 ml medium at room temperature under 50-60 $\mu Em^{-2}s^{-1}$ in continuous fluorescent light.

Shoot cultures from both origins grew large enough to be divided and be transferred to petri dishes for further maintenance as well as to provide petiole or blade explants for experimental use.

Shoot culture medium was contained in 100X20 mm Falcon plastic disposable petri dishes with 35 to 40 ml per plate. Shoots were subcultured at 4 to 6 week intervals, with three shoots transferred to each dish.

Each plate was sealed firmly twice with parafin film strips after callus or explant was placed on the medium. It allowed retention of moisture during the culture period.

These shoot stock cultures were maintained in a walk-in, temperature-controlled culture room at 26 ± 1 C with 24 hour continuous light supply. The light source was cool white fluorescent bulbs providing up to 50 $\mu Em^{-2}s^{-1}$ at culture level, depending on how plates were stacked. The characteristics of source populations for all experiments used here are listed (Table A).

Table A. Characteristics of source populations

Forms of B. vulgaris	source population	germ	characteristics	origin
Sugarbeet	FC 701/5	MM*	Rhizoctonia crown rot tolerant	Colorado
	EL 40	MM	Parental line for USH 23	Michigan
	6822	MM	Parental line for USH 20	Michigan Beltsville
	F 1003	MM	Low respiration	Soviet Union, N.Dakota
	EL 36	mm**	Type O	Michigan
	EL 44C3	mm	Cytoplasmic male sterile	Utah, Michigan
	EL 45	mm	Type O	Utah Michigan
	EL 45/2	mm	Type O	Utah Michigan
	6926-O	mm	Type O	Beltsville
	C 566cms	mm	Cytoplasmic male sterile	California
	FC 506	mm	Type O	Colorado
	FC 607cms	mm	Cytoplasmic male sterile	Colorado
	S1	mm	Breeding line	Michigan
Table beet	Detroit Dark Red	MM	Commercial	U.S.
Fodder beet	Gorton's White Knight	MM	Commercial	England
Leaf beet	Palak	MM	Annual P.I. 271438	India
	Fordhook Giant (chard)	MM	Commercial	U.S.

* MM multigerm
** mm monogerm

MS media preparation

1. Materials

1)Water: glass distilled water

2)Inorganic nutrients and organic compounds (Table B)

3)Agar: Bacto from Difco (Table C)

2. Procedures

Two stock solutions (ten times concentrated for the five major salts and one hundred times concentrated for the minor salts) were prepared in advance. The stock solutions were diluted to normal strength and inositol, sucrose, thiamine·HCl, pyridoxine·HCl and nicotinic acid were added to final concentrations of 100, 30000, 1.0, 0.5, and 0.5 mg/l respectively (Linsmaier and Skoog, 1965). Hormones were autoclaved in the medium, at concentrations depending on the experiment. The pH was adjusted to 5.95 by using KOH and sometimes HCl.

The medium was distributed to petri dishes after autoclaving. When vials were used, autoclaving of the medium occured after it was put into the vials. The medium was autoclaved at 121 C for 20 minutes the autoclave has reached this temperature. At the end of the 20 minute

Table B. Inorganic salt compositions of the plant tissue culture media used

constituents	MS	B5	NT
	mg/l		
NH_4NO_3	1650	---	825
KNO_3	1900	2500	950
$CaCl_2 \cdot 2H_2O$	440	150	220
$MgSO_4 \cdot 7H_2O$	370	250	1233
KH_2PO_4	170	---	680
$(NH_4)_2SO_4$	---	134	---
$NaH_2PO_2 \cdot H_2O$	---	150	---
KI	0.83	0.75	0.83
H_3BO_3	6.2	3	6.2
$MnSO_4 \cdot 4H_2O$	22.3	---	22.3
$MnSO_4 \cdot H_2O$	---	10	---
$ZnSO_4 \cdot 7H_2O$	8.6	2	---
$ZnSO_4 \cdot 4H_2O$	---	---	8.6
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.025	---
$CoSO_4 \cdot 7H_2O$	---	---	0.03
Sequestrene 330Fe	---	28	---
$FeSO_4 \cdot 7H_2O$	27.8	---	27.8
$Na_2EDTA \cdot 2H_2O$	37.3	---	37.3

Key: MS= Murashige and Skoog (1962)
B5= Gamborg et al. (1968)
NT= Nagata and Takebe (1971)

Table C. Chemical composition of Difco agar*

Constituents	Bacto-agar
Ash	4.50 %
Calcium	0.13 %
Barium	0.01 %
Silica	0.19 %
Chloride	0.43 %
Sulphate	2.54 %
Nitrogen	0.17 %
Iron	11 mg/l
Magnesium	285 mg/l
Copper	5 mg/l

* from Pierik (1971)

period, the steam was turned off and the pressure was allowed to return slowly to the atmospheric level. Agar solution was autoclaved separately from the other nutrient solutions. Agar was used at a concentration of 0.9% (w/w). The code of MS media used here was listed (Table 1) according to the concentration.

Table 1. MS medium code

	M-0	M-10	MA-17	M-20	MA-25
BA	0	1.0 mg/l	1.0 mg/l	0.25 mg/l	1.0 mg/l
IAA	0	0	0.3 mg/l	0	0.1 mg/l

Experiment 1. Effect of benzyladenine on bud regeneration and callus growth

Four to six weeks old shoots of one fodder beet (GWK-3) and three sugarbeet clones (6926-0-3, FC 607-0-20 and FC 701/5 -116) multiplied on M-20 served as sources of petiole explants.

For callus induction, one cm long petiole segments were placed onto M-20 with eight in each Falcon 20X100 mm plastic petri dish containing 40 ml medium. These plates were kept at 32 C in 5-10 $\mu Em^{-2}s^{-1}$ continuous fluorescent light for 8 to 10 weeks.

In order to examine the effect of BA on bud regeneration and callus growth, primary calli, generally from 60 to 120 mg in weight, were divided into small pieces approximately 10 mg each and five calli were inoculated onto each 40 ml plate of test medium containing one of the BA concentrations (0 to 10 mg/l). These plates were cultured under the same conditions as the petioles for four weeks, at which time the fresh weight and the proportions of calli with buds were recorded. At least 5 sets (replications) were made for each genotype.

Expriment 2. The capability of callus induction and bud regeneration according to genotypes

Two different methods were used for the screening of Beta vulgaris germplasm. This was because other work done with beet callus indicated that either quick or higher frequency bud regeneration resulted from modifications in the procedure.

1) Two step screening

Shoot cultures of forty-one genotypes from 12 germplasm sources were established by the procedure described earlier for seedling origin.

For callus induction, eight one cm long petiole segments of each genotype's shoot culture were placed on Falcon 20X100 plastic petri dishes containing 35 to 40 ml M-20. The plates were cultured at 32 C in 5 to 10 $\mu Em^{-2}s^{-1}$ continuous fluorescent light. Starting four weeks later, callus or adventitious bud appearance on the petioles was recorded as the proportion of responding tissue pieces per plate at two weeks intervals until senescence, at most 12 weeks.

For the test of bud regeneration ability, several callusing petiole plates of each genotype were selected and the callus divided into about 10 mg pieces. Each five callus pieces were inoculated onto MA-17 plates. These plates were incubated under the same conditions as callus induction.

After four weeks, the proportion of bud regeneration was recorded as proportion of responding callus pieces per dish. Where bud regeneration occured some of these developed further into shoots, but it was felt that bud regeneration was a better measure of the ability of unorganized callus to differentiate, especially considering the limited time given to the experiment.

ii) One step screening

There was no distinct stage between callus induction and bud regeneration in one step screening. Therefore, only one kind of medium (M-10) was used. Shoot cultures for this were established from either seedling or lateral buds by the same procedure described earlier. For callus induction and bud regeneration, eight petiole segments (one cm long) were placed on M-10. This was for genotypes established in shoot cultures from seedlings. On the other hand, for genotypes established by lateral bud, one cm long petiole section or one cm blade section was placed on each petri dish contain-

ing 25 ml M-10. The plates were kept at 32 C in 5-10 $\mu Em^{-2}s^{-1}$ continuous fluorescent light.

Starting four weeks from the initiation of this culture, callusing or adventitious bud appearance as well as shoot regeneration were recorded until senescence, at most 12 weeks, with two week intervals.

Experiment 3. The effect of hormone and nutritional variables on bud regeneration and callus growth

Four to six week old shoot cultures of three marginally regenerating genotypes (6822-15, GWK-3 and FC 701/5-116) were cultured on M-20.

For callus induction, petioles of stock shoot cultures were cut into about one cm long segments and placed on M-20. Each plate had eight petiole sections. Falcon 20x100 mm plastic petri dishes were used containing 35-40 ml media. Callus induction occured at 32 C in a growth chamber under 5 to 10 $\mu Em^{-2}s^{-1}$ continuous fluorescent light for 4 to 6 weeks. 6926-0-3 protoplast callus was used for the source callus in a limited quantity. This callus was obtained on M-10 after protoplasts were isolated from suspension culture.

For the comparison of shoot regeneration according to different medium compositions, calli were divided into small pieces (10 mg). Five calli were inoculated onto each petri dish containing 40 ml of the test media. Compositions of test media are shown in the results. MS inorganic salts were used in all cases unless the effects of other salt formulations were being examined.

These plates were cultured four to five weeks under the same conditions as callus induction. The experiments were terminated by measurement of the fresh weight of each callus piece after determining how many had formed buds.

EXPERIMENT 4. The effect of primary callus age and size on bud regeneration

Four to six week old shoot cultures of one standard regeneration genotype, EL 36-18, on M-20 medium served as sources of one cm long petiole segments for the callus induction.

In order to induce the callus, one cm petiole sections were placed on each petri dish containing 25 ml M-20. These plates were incubated at 32 C growth chamber under 5 to 10 $\mu Em^{-2}s^{-1}$ continuous fluorescent light. The calli were collected according to the planned callus size between 7 and 12 weeks and numbered. In addition to callus size, callus age and culture age were also calculated. Callus age was obtained by calculating the difference between the dates from when callus was first observed and when it was collected. Culture age was the sum of callus age plus time prior to the first observation time for any individual callus.

After collection, all calli were weighed aseptically and were divided into about 10 mg standard sized pieces. Each five of these calli were placed on MA-17 for the bud regeneration test. These plates were cultured under the same conditions as callus induction.

After four weeks, plates were examined. Regeneration frequency was expressed by calculating the number of calli with regenerated shoots as a percentage of the total number of calli for each treatment.

EXPERIMENT 5. Effect of light intensities during shoot culture on subsequent callus initiation and bud regeneration

In separate experiments, shoot cultures of two sugarbeet genotypes (EL 36-18 and 6926-0-3) were grown on M-20 medium under four different light intensities (0, 5-10 $\mu Em^{-2}s^{-1}$, 40-50 $\mu Em^{-2}s^{-1}$, and 80-90 $\mu Em^{-2}s^{-1}$) for 5 weeks with EL 36-18 and for 7 weeks with 6926-0-3. For this experiment, three different growth chambers equipped for continuous light and constant temperature (22-23 C) control were used. For the dark treatment, plates were wrapped completely with aluminium foil and put into one of the growth chambers. The continuous light source was cool white fluorescent bulbs.

Explants from shoot cultures grown under these four treatments were then challenged to callus in a single uniform procedure. A single one cm long petiole segment per plate was used for the callus induction test. Each petiole was inoculated onto M-20 medium, 35 ml per petri dish. These plates were incubated at 31 C with continuous light supply from fluorescent lamps with an intensity of 10 to 20 $\mu Em^{-2}s^{-1}$ until bud regeneration occured. If bud regeneration was not found, plates were cultured until senescence, at most 8 to 12 weeks.

In order to estimate the time needed for callus induction, the plates were checked twice every week to note the first day of callus appearance or bud regeneration.

RESULTS

Experiment 1. Effect of benzyladenine on bud regeneration and callus growth

1. Induction of callus

The petioles of shoot cultures of four genotypes (FC 701/5-116, 6926-0-3, FC 607-0-20 and GWK-3) were used as explants on M-20 medium for callus induction. It took about 4 to 6 weeks for the callus to appear on them depending on each genotype. Not all petiole sections produced callus. The callus arose at random locations around the petioles explant, usually in only a few (1-3) clumps. Calli from all genotypes were white or yellow and friable. Calli were removed from the plates when they reached around 60 to 120 mg.

2. 6-benzyladenine effects on bud regeneration.

There was no bud regeneration at any concentration of BA for FC 701/5-116 and very little regeneration on GWK-3 (only a single event in the absence of BA) (Fig 1-a and Fig 1-d).

In 6926-0-3, bud regeneration was observed in the BA range between 0.1 and 3 mg/l, with 0.3 mg/l showing the highest proportion (80%) (Fig 1-b). There was no bud regeneration at either lower (0 to 0.03 mg/l) or higher(10 mg/l) concentrations (Fig 1-b). The bud regeneration of FC 607-0-20 displayed a similar pattern to 6926-0-3 even though the frequency is very low (Fig 1-c). BA at 0.3 mg/l made the highest frequency regeneration (25%) (Fig 1-c).

These results indicated that bud regeneration was affected strongly by BA concentration, in the absence of other hormones. Therefore, choice of BA concentration would optimize the bud regeneration.

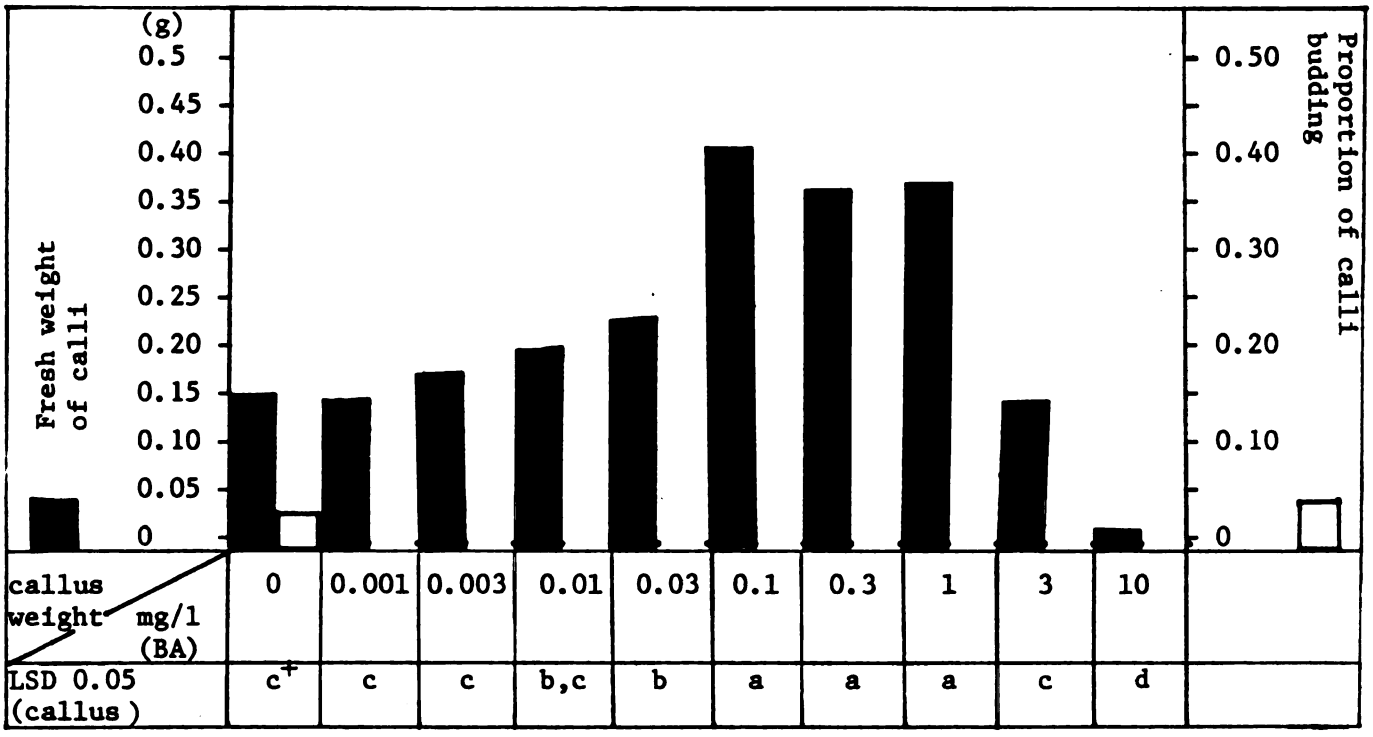
3. 6-benzyladenine effects on callus growth

The calli which were transferred from M-20 medium to tested media (0-10 mg/l BA) were weighed after 4 weeks. Although callus growth occured on basal medium (M-0) without BA, its growth was up to 2.5 times more in the presence of some BA concentrations, such as 0.1 mg/l for GWK-3. The BA concentration range from 0.1 to 1 mg/l most stimulated callus growth in GWK-3 while with 6926-0-3 a slight increase in callus growth rate was noticed from 0 to 0.1 mg/l. With FC 607/0-20, there was little difference among callus growth rate at 0-0.3 mg/l. FC 701/5-116 grew very poorly through the whole range of concentrations.

Even though the sensitivity of BA in terms of callus growth was found to differ according to the genotype, all four genotypes responded very poorly at high concentrations of BA such as 3 to 10 mg/l. Only GWK-3 showed a little growth at 3 mg/l.

F-test and LSD multiple range test were used for the comparison of callus fresh weight and proportion of calli budding depending on BA concentrations.

Figure 1-a. Effect of benzyladenine concentration on callus fresh weight growth and on bud regeneration from callus of the genotype GWK-3

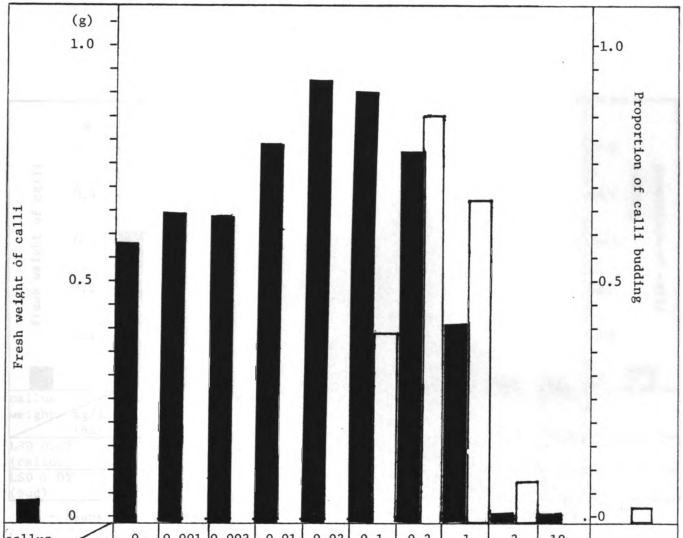


callus weight mg/l (BA)	0	0.001	0.003	0.01	0.03	0.1	0.3	1	3	10	
LSD 0.05 (callus)	c+	c	c	b,c	b	a	a	a	c	d	

+ Means from the same row followed by the same letter did not differ significantly at the 0.05 level according to LSD multiple range test.

Figu

Figure 1-b. Effect of benzyladenine concentration on callus fresh weight growth and on bud regeneration from callus of the genotype 6926-0-3

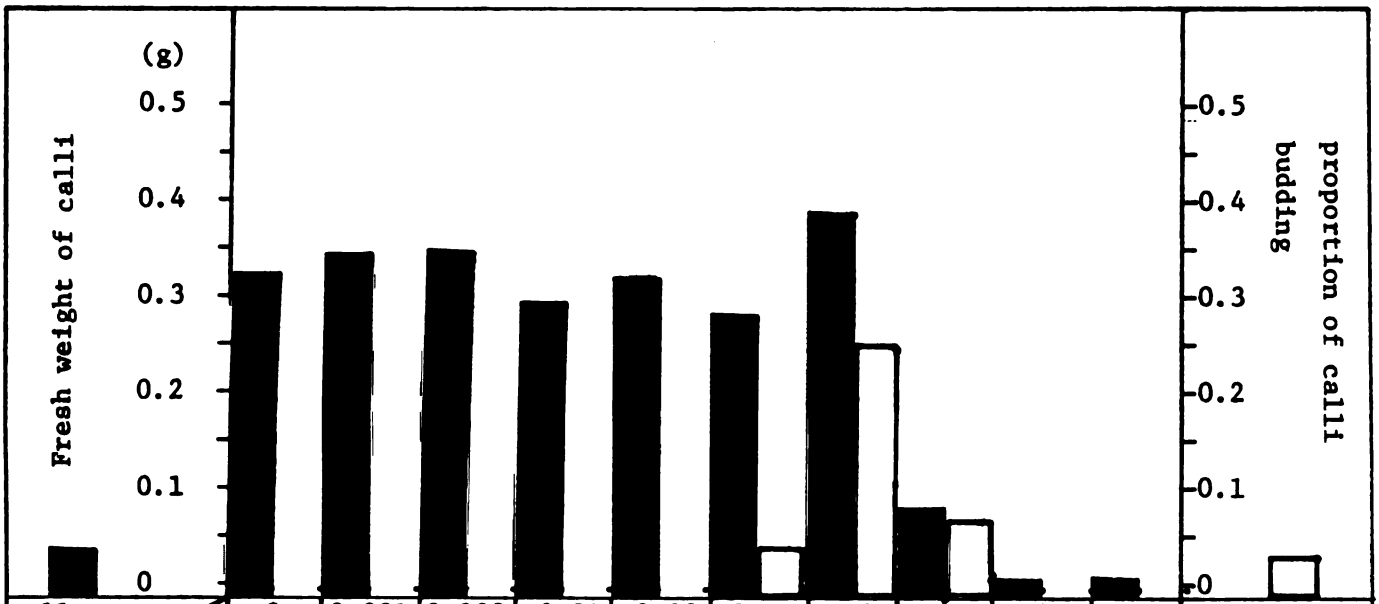


callus weight / mg/l (BA)	0	0.001	0.003	0.01	0.03	0.1	0.3	1	3	10
LSD 0.05 (callus)	c+,d	b,c	b,c	a,b	a	a	a,b,c	d	e	e
LSD 0.05 (bud)	d	d	d	d	d	a	b	c	d	d

+ Means from the same row followed by the same letter did not differ significantly at the 0.05 level according to LSD multiple range test.

Figur

Figure 1-c. Effect of benzyladenine concentration on callus fresh weight growth and on bud regeneration from callus of the genotype FC 607-0-20

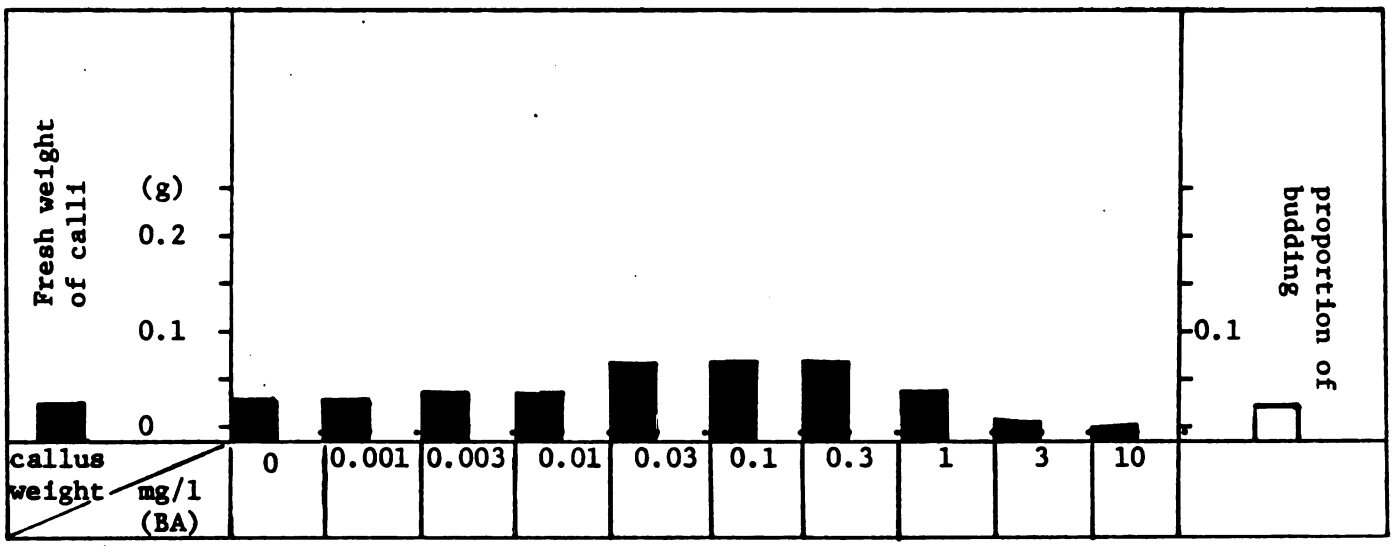


callus weight mg/l (BA)	0	0.001	0.003	0.01	0.03	0.1	0.3	1	3	10	
LSD 0.05 (callus)	a+,b	a,b	a,b	b	b	b	a	c	d	d	
LSD 0.05 (bud)	b	b	b	b	b	b	a	b	b	b	

+ Means from the same row followed by the same letter did not differ significantly at the 0.05 level according to LSD multiple range test.

Fig

Figure 1-d. Effect of benzyladenine concentration on callus fresh weight growth and on bud regeneration from callus of the genotype FC 701/5-116



EXPERIMENT 2. The capability of callus induction and bud regeneration according to genotype

The objective of this expertiment was the screening of a wide range of Beta vulgaris germplasm for the ability of leaves from shoot cultures to form habituated callus, and for their ability to regenerate buds from that callus. Two separate germplasm screenings took place, employing somewhat different procedures.

1. Induction of Callus

A. Leaves derived from shoot cultures of seedling origin

1) Two step screening

Callus formation varied widely among the beet genotypes examined. After about 4 to 6 weeks of culture without response, white or yellow translucent callus started to appear adjacent to the petiole section depending on the genotype. Forty-one genotypes from 12 germplasm sources were evaluated for the capability of callus induction in this two step procedure. Four genotypes (EL 44C3-301, EL 36-305, EL 40-301 and F 1003-1) out of forty-one did not induce callus at all (Table 2-a). On the other hand, all

genotypes from GWK and EL 45/2 showed 100% callusing. In most cases, the proportion of callusing was very different within the same germplasm source, such as S1 (41.6 to 100%), EL 40 (0 to 100%), 6822 (25 to 100%), C 566 cms (37.5 to 97.7%), FC 506 (50 to 100%), FC 607 cms (12.5 to 93.8%) and FC 701/5 (62.5 to 75%) while all the genotypes from EL 44C3 and EL 36 had low frequency of callusing.

ii) One step screening

This method was developed for faster screening of the ability for callusing and bud formation. Rather than using separate media for callus induction and for bud regeneration steps, the change from 0.25 to 1.0 mg/l BA allowed many genotypes to regenerate buds and shoots without subculture. Most genotypes tested in both ways gave similar results in both tests, except FC 607 cms-302 (Table 2-a).

B. Leaves derived from shoot cultures of lateral bud origin

Petiole and blade sections were used as the sources for callusing in this experiment. In most cases, blade sections were the better source for callusing. Especially, in FC 701/5 germplasm, the capability of callusing through blade section was considerably more effective.

Table 2-a. Effect of genotype on callus induction, adventitious budding on petiole segments and bud regeneration on callus (seedling origin)

Genotypes		Callusing on M-20	Regeneration on MA17 (%)	Adventitious bud on M-20	Callusing on M-10	Regeneration on M-10 (%)	Adventitious bud on M-10
S1+	-23	48/48	6.7	4/48	13/16	0	1/16
	-25	10/24	5.5	1/24	6/24	0	0/24
	-26	10/16	15	0/16	12/16	42	0/16
	-34	16/24	12	16/24	13/16	0	1/16
EL 44C3+	-301	0/16	---	1/16	0/16	---	4/16
	-302	9/24	100	0/24	2/16	50	0/16
EL 36+	-301	Not Tested			3/16	0	1/16
	-303	6/48	0	0/48	4/24	0	2/24
	-304	5/16	0	0/16	Not Tested		
	-305	0/24	---	2/24	0/16	---	0/16
EL 45/2+	-107	16/16	52	0/16	13/16	38.4	1/16
	-108	16/16	40	0/16	9/16	55.6	0/16
C 566 cms+	-301	9/24	70	0/24	Not Tested		
	-302	22/24	8.3	2/24	7/16	0	0/16
FC 506+	-20	24/24	92	3/24	9/16	56	0/16
	-21	40/40	92	6/40	21/24	90	0/16
	-22	4/8	68	0/8	Not Tested		
	-25	24/24	37.5	3/24	15/16	86.7	0/16
	-26	15/16	8	5/16	8/32	22.2	2/32
FC 607cms+	-301	15/16	8	1/16	11/16	45.5	3/16
	-302	22/24	80	4/24	0/16	---	0/16
	-303	22/24	52	0/24	16/16	87.5	2/16
	-304	24/24	68	1/24	13/16	69.2	0/16
	-307	5/24	Not Tested	5/24	10/16	80	0/16
	-308	2/16		4/16	24/32	87.5	4/32

Continue Table 2-a.

Genotypes		Callusing on M-20	Regeneration on MA17 (%)	Adventitious bud on M-20	Callusing on M-10	Regeneration on M-10 (%)	Adventitious bud on M-10
GMK++	-10	48/48	0	5/48	10/16	0	0/16
	-11	8/8	3.3	1/8	8/8	0	0/8
	-12	24/24	0	1/24	16/16	0	2/16
EL 40++	-20	4/24	0	4/24	5/16	0	4/16
	-301	0/16	---	2/16	5/16	80	1/16
	-302	16/16	0	0/16	15/16	0	1/16
	-303	17/32	0	4/32	20/24	0	0/24
	-305	15/24	3.3	12/24	32/32	6.3	2/32
6822++	-21	7/8	70	1/8	Not Tested		
	-22	Not Tested			5/16	20	1/16
	-23	6/24	0	0/24	4/16	0	3/16
	-25	4/8	0	2/8	6/16	0	1/16
	-28	24/24	0	1/24	13/16	0	0/16
	-29	7/8	16	0/8	13/24	0	3/24
	-32	8/8	0	1/8	16/16	0	2/16
F 1003++	-1	0/24	---	3/24	0/24	---	1/24
FC 701/5++	-302	Not Tested			0/8	---	0/8
	-304	12/16	0	1/16	16/16	6.2	0/16
	-305	5/8	3.8	1/8	Not Tested		

Key:

\+ Monogerm

++ Multigerm

--- bud regeneration could not be observed because of no callusing.

Table 2-b. Summary of callusing and bud regeneration from leaves of shoot cultures of seedling origin

germness	source	Number of genotypes	Number of callusing	Number with A.buds* on callus	Number with A.buds on petiole
Monogerm	FC 506	5	5	5	4
	FC 607 cms	6	6	6	6
	EL 36	4	3	0	3
	S1	4	4	4	3
	EL 45/2	2	2	2	1
	EL 44C3	2	1	1	1
	C 556 cms	2	2	2	1
Total		25	23	20	19
Multigerm	6822	7	7	3	7
	FC 701/5	3	2	2	2
	GWK	3	3	1	3
	EL 40	5	5	2	5
	F 1003	1	0	0	1
Total		19	17	8	18

Key: * Adventitious buds

Forty-two genotypes from nine germplasm sources were tested in this study (Table 2-c). In six genotypes, this work was done twice because the age of the donor shoot culture might affect the capability to callus. In four of these cases there was no difference in callusing response, but in the other two cases a much higher callusing resulted. Only FC 701/5 germplasm among multigerm sources showed high frequency of callusing while monogerm sources had a high frequency of callusing.

2. Bud Regeneration

A. Leaves derived from shoot cultures of seedling origin

There were large differences within as well as among germplasm sources for the capability of bud regeneration regardless of whether indirect bud regeneration through two steps or direct bud regeneration through one step (Table 2-a).

Three germplasm sources (GWK, EL 36 and EL 40) showed a low frequency of bud regeneration or no regeneration through all genotypes. In some cases, the capability of bud regeneration was very different within the same germplasm source. For example, 6822-21 was the only one genotype to show high frequency of shoot regeneration among 7 genotypes

of 6822. C 566 cms, FC 506 and FC 607cms also showed similar variability (Table 2-a). The summary of this result is shown in table 2-b.

B. Leaves derived from shoot cultures of lateral bud origin

EL 45 had a high frequency of bud regeneration with good callusing frequency while most genotypes from FC 701/5 and 6926-0 had low bud regeneration even under high frequency of callus induction. FC 607 cms showed very different ability of bud regeneration depending on the individual genotype. In FC 607 cms genotype 303 had a high frequency of callusing (100%) as well as high degree of bud regeneration (85.7%) while the other two genotypes tested did not induce callus at all. The data of this experiment are shown in table 2-c. The summary of this result is shown in table 2-d.

Table 2-c. Effect of genotypes on callus induction, adventitious budding on petiole or blade segments and bud regeneration on callus (lateral bud origin)

Genotype	Age of Shoot culture (days)		Adventitious bud on explant (M-10)		Callusing (M-10)		Regeneration on callus (M-10) (%)
			petiole	blade	petiole	blade	
FC 607cms+	-101	28	0	0	0	0	---
	-102	28	0	0	0	0	---
	-104	28	1/10	0	10/10	5/5	85.7
EL 36+	-8	70	0	0	2/10	4/5	0
EL 45+	-203	56	1/10	0	2/10	4/5	83.3
	-203	28	1/10	0	10/10	5/5	100
	-206	70	0	0	2/10	3/5	80
	-207	51	0	0	2/10	1/5	100
6926-0+	-102	28	0	0	4/10	4/5	25
	-107	28	1/10	0	7/10	4/5	0
	-109	28	0	0	4/10	4/5	0
DOR++	-101	60	3/10	2/5	0	0	---
	-103	58	3/10	2/5	0	0	---
	-104	35	5/10	1/5	0	2/5	0
	-105	79	4/10	0	0	0	---
	-106	63	2/10	1/5	1/10	0	0
	-109	28	0	0	0	0	---
	-111	28	1/10	0	0	0	---
	-112	77	4/9	1/5	0	0	---
F 1003++	-2	98	0	1/5	0	0	---
	-2	28	0	0	0	0	---
	-3	79	2/10	0	1/10	1/5	0
	-5	65	0	0	0	0	---
	-6	58	0	0	0	1/5	0

Continue Table 2-c

Genotype	Age of Shoot Culture (days)		Adventitious bud on explant (M-10)		Callusing (M-10)		Regeneration on callus (M-10) (%)
			petiole	blade	petiole	blade	
FC 701/5++	-101	64	5/10	2/5	0	0	---
	-102	28	0	0	0	4/5	0
	-103	74	0	3/5	2/10	3/5	0
	-104	28	0	0	1/10	5/5	0
	-201	108	0	0	0	4/5	100
	-202	98	0	0	2/10	1/5	0
	-202	28	1/10	2/5	1/10	2/5	0
	-203	82	0	0	1/10	5/5	0
	-203	28	0	0	7/10	5/5	0
Fordhook Giant++	-1	65	0	0	1/10	1/5	0
	-2	86	0	0	0	0	---
	-2	28	0	0	0	0	---
	-3	62	1/10	0	0	1/5	0
	-4	51	0	2/5	2/10	4/5	16.7
	-5	84	0	2/5	0	0	---
	-9	28	0	0	0	3/5	0
Palak++	-1	92	0	0	1/10	4/5	0
	-1	28	0	0	2/10	2/5	0
	-2	63	1/10	0	0	1/5	0
	-4	28	0	1/5	0	0	---
	-6	43	2/10	0	0	0	---
	-7	70	0	0	0	0	---

Key: + Monogerm
++ Multigerm

Table 2-d. Summary of callusing and bud regeneration from leaves of shoot cultures of lateral bud origin

germness	source	Number of genotypes	Number of callusing		Number with A.buds* on callus		Number with A.buds on petiole	
			petiole	blade	petiole	blade	petiole	blade
Monogerm	FC 607cms	3	1	1	1	0	1	1
	EL 36	1	1	1	0	0	0	0
	EL 45	3	3	3	3	3	0	0
	6926	3	3	3	1	1	1	0
Total		10	8	8	5	4	2	1
Multigerm	DDR	8	1	1	0	0	7	5
	F 1003	6	1	2	0	0	1	1
	FC 701/5	7	4	6	0	1	2	3
	Fordhook Giant	6	2	4	1	1	1	2
	Palak	5	1	2	0	0	2	1
Total		32	9	15	1	2	13	12

Key: * Adventitious buds

EXPERIMENT 3. The effect of hormone and nutritional variables on bud regeneration and callus growth

A. 3-indoleacetic acid

The effect of IAA on bud regeneration and callus growth with the two marginal bud regenerating genotypes (6822-15 and GWK-3) was tested (Table 3-a). Based on Saunders and Daub (1984), MA-17 was used as a control. Data from MA-17 in 6822-15 was lacking due to contamination.

Bud regeneration was found only at one replication (set B) of 6822-15, but for all IAA levels, so that it could not be considered an effect of IAA. This replication (set) effect is most likely explained by the use of particular calli or parts of some calli.

Callus fresh weight did not show any significant statistical differences among treatments of either genotype. 6822-15 produced greater amounts of callus fresh weight compared to GWK-3 over the concentration range of IAA. F-test was used for the analysis of variance at the 0.05 level of probability.

Table 3-a. The effect of IAA concentration on bud regeneration and callus fresh weight of two genotypes with BA at 1.0 mg/l

conc.(mg/l) / Geno-type		0		0.03		0.1		1.0		3.0		10.0		MA-17	
	set	F.W. (g)	Bud* (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)
6822-15+	A	1.13		1.29		0.93		0.90		0.69		0.63			
	B	0.78	80	1.23	40	0.75	20	1.10	40	0.66	40	0.88			
	C	0.43		0.29		0.22		0.27		0.14		0.01			
	D	0.53		0.43		0.38		0.39		0.4		0.29			
	$\bar{x}$	0.72		0.81		0.57		0.67		0.47		0.45			
GWK 3+	A	0.23		0.33		0.25		0.23		0.28		0.12		0.02	
	B	0.13		0.22		0.22		0.24		0.19		0.09		0.18	
	C	0.13		0.15		0.19		0.18		0.29		0.18		0.26	
	$\bar{x}$	0.16		0.23		0.22		0.22		0.25		0.13		0.15	

Key: + Callus fresh weight was not significantly different at the 0.05 level by F-test according to the concentration of IAA.

* No entry indicates no bud regeneration (= 0 %).

B. α-naphthaleneacetic acid

6822-15 was the major genotype used for this experiment while GWK-3 petiole callus and 6929-0-3 protoplast callus were tested in quite a limited degree (Table 3-b).

Bud regeneration was not observed at any NAA concentration for 6822-15 and GWK-3 while one set from 6926-0-3 protoplast callus showed it at all concentrations except the highest. This cannot be considered as an NAA effect but the effect of the callus sample itself. Even though callus growth was least at the highest concentration (10 mg/l) over all three genotypes, it did not show a statistically significant difference.

C. Triiodobenzoic acid

Despite lack of significance on the F-test TIBA appeared to have an inhibitory effect on callus growth at the high levels of 1.0 and 10.0 mg/l. Bud regeneration was observed in genotypes 6822-15 and FC 701/5-116 only. TIBA did not appear to stimulate bud regeneration in a significant way over the control medium, although the data suggest a small effect (Table 3-c).

Table 3-b. The effect of NAA concentration on bud regeneration and callus fresh weight of three genotypes with BA at 1.0 mg/l

conc.(mg/l) / Geno-type		0.01		0.03		0.1		1.0		3.0		10.0	
		F.W. (g)	Bud* (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)
	set												
6822-15+	A	0.18		0.17		0.08		0.07		0.11		0.01	
	B	0.33		0.31		0.35		0.28		0.18		0.06	
	C	1.00		1.02		0.85		0.54		0.27		0.10	
	D	0.23		0.18		0.18		0.15		0.09		0.05	
	E	0.26		0.48		0.39		0.26		0.21		0.15	
	F	0.52		0.53		0.88		0.32		0.36		0.42	
	$\bar{x}$	0.42		0.45		0.46		0.27		0.20		0.13	
GWK 3	A	0.17		0.12		0.13		0.09		0.11		0.06	
6926-0-3+	A	0.31		0.52		0.50		0.40		0.31		0.10	
	B	0.50	20	0.57	40	0.45	60	0.30	40	0.25	40	0.14	
	$\bar{x}$	0.40	10	0.55	20	0.48	30	0.35	20	0.28	20	0.12	

Key: + Callus fresh weight was not significantly different at the 0.05 level by F-test according to the concentration of NAA.

* No entry indicates no bud regeneration (= 0 %).

Table 3-c. The effect of TIBA concentration on bud regeneration and callus fresh weight of four genotypes with BA at 1.0 mg/l

conc.(mg/l) / Geno-type	set	0 F.W. (g)	0 Bud* (%)	0.001 F.W. (g)	0.001 Bud (%)	0.01 F.W. (g)	0.01 Bud (%)	0.1 F.W. (g)	0.1 Bud (%)	1.0 F.W. (g)	1.0 Bud (%)	10.0 F.W. (g)	10.0 Bud (%)
6822-15+	A	0.05		0.07		0.04		0.06		0.10		0.01	
	B	0.24	40	0.16		0.19		0.24	20	0.09	20	0.01	
	C	0.15		0.40		0.31	20	0.05	20	0.08	20	0.01	
	D	0.53		0.11		0.30	20	0.28	20	0.08	20	0.01	
	$\bar{x}$	0.24	10	0.19		0.21	10	0.16	15	0.09	15	0.01	
GWK-3	A	0.10		0.20		0.16		0.09		0.12		0.05	
	B	0.08		0.08		0.18		0.15		0.14		0.11	
	$\bar{x}$	0.09		0.14		0.17		0.12		0.13		0.08	
FC 701/5-116	A	0.88	60	--	--	0.30	80	0.30	100	0.16	100	0.01	
	B	0.50	60	0.40	60	0.52	100	0.24	100	0.55	60	0.01	
	$\bar{x}$	0.69	60	0.40	60	0.41	90	0.27	100	0.11	80	0.01	
6926-0-3		1.22		0.74		0.43		0.67		0.10		0.01	

Key: + Callus fresh weight was not significantly different at the 0.05 level by F-test according to the concentration of TIBA.

* No entry indicates no bud regeneration (= 0 %).

D. Proline

Proline at 200 and 600 mg/l stimulated callus growth, most noticeably in 6822-15 and FC 701/5-116 which had more sets. However, these differences were not statistically significant. Bud regeneration was also stimulated by the 200 and 600 mg/l levels of proline even though the frequencies of regeneration were low (15 to 32%). No bud was found on the extreme concentrations (0 and 1800 mg/l). GWK-3 and 6926-0-3 protoplast callus did not make bud regeneration at any concentration (Table 3-d)

E. Sucrose concentration

Bud regeneration was found at all concentrations except 10%. Results obtained indicated that bud regeneration was not affected significantly by concentrations below 10%.

FC 701/5-116 was the only genotype investigated for effect of sucrose concentration (Table 3-e). Callus fresh weight was not noticeably affected by the concentration, except that the slowest growth was found at the highest sucrose concentration (10%).

Table 3-d. The effect of proline concentration on bud regeneration and callus fresh weight of four genotypes with BA at 1.0 mg/l

conc.(mg/l) / genotype		0		200		600		1800	
		F.W. (g)	Bud* (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)
	set								
6822-15+	A	--	--	0.88		0.90	40	0.24	
	B	--	--	0.79	20	0.93	60	0.13	
	C	0.65		0.95	20	0.52		0.82	
	D	0.51		0.32		0.65		0.34	
	E	0.10		0.28		0.15		0.08	
	F	0.20		0.47	60	0.40	40	0.39	
	$\bar{x}$	0.37		0.62	17	0.59	23	0.33	
	(1)	--	--	0.86		1.23		0.48	
	(2)	--	--	1.04	20	0.29		0.30	
	$\bar{x}$	--	--	0.95	20	0.76		0.39	
	grand mean	0.37		0.79	19	0.68	13	0.36	
GWK 3	A	0.11		0.32		0.13		0.04	
	B	0.06		0.06		0.02		0.01	
	$\bar{x}$	0.09		0.19		0.08		0.03	
FC 701/5-116+	(1)	0.34		0.62	80	0.78		0.69	
	(2)	0.32		0.46	40	0.43		0.48	
	(3)	0.35		0.83		0.51		0.74	
	(4)	0.30		0.92	20	1.03		0.64	
	(5)	0.31		0.43	20	0.48		0.62	
	$\bar{x}$	0.32		0.65	32	0.65		0.63	
6926-0-3		0.18		0.33		0.02		0.01	

Key: + Callus fresh weight was not significantly different at the 0.05 level by F-test according to the concentration of proline.

* No entry indicates no bud regeneration (= 0 %).

Table 3-e. The effect of sucrose concentration on bud regeneration and callus fresh weight

sucrose / geno-type	set	1% F.W. (g)	1% Bud* (%)	2% F.W. (g)	2% Bud (%)	3% F.W. (g)	3% Bud (%)	4% F.W. (g)	4% Bud (%)	5% F.W. (g)	5% Bud (%)	7% F.W. (g)	7% Bud (%)	10% F.W. (g)	10% Bud (%)
FC 781/5-116+	A	0.17		0.25	80	0.14		0.33	100	0.40	80	0.29	80	0.13	
	B	0.15	40	0.01		0.26	60	0.44	60	0.26	20	0.13		0.13	
	C	0.22	40	0.25	80	0.22		0.43	100	0.34	100	0.22	40	0.20	
	D	0.14		0.16	60	0.15		0.23	40	0.08	20	0.06		0.07	
	E	0.15		0.17	60	0.07		0.07		0.20	60	0.04		0.06	
	F	0.11		0.05		0.19		0.11	60	0.10		0.09		0.04	
	G	0.17		0.07		0.19		0.15	20	0.20		0.10	20	0.01	
	H	0.10	20	0.09	20	0.27	40	0.04	20	0.21	20	0.05	20	0.04	
	$\bar{x}$	0.15	13	0.14	38	0.19	13	0.23	50	0.24	38	0.12	20	0.09	

Key: + Callus fresh weight was not significantly different at the 0.05 level by F-test according to the sucrose concentration.

* No entry indicates no bud regeneration (= 0 %).

F. Mineral composition of medium

Most bud regeneration was found with FC 701/5-116 through all treatments except on MA-17. The capability of bud regeneration was large, from 76% (B5-17) to 44% (NT-17). GWK-3 did not form bud regeneration at all at any case (Table 3-f).

6822-15 and FC 701/5-116 were tested to compare the effect of different mineral compositions. The callus growth did not differ statistically according to the treatment.

Callus of FC 701/5-116 was able to regenerate on either Murashige-Skoog, Nakata-Takebe or Gamborg B5 inorganic salts. Compared over both hormone combinations (17 and 25), which are not very different, it can be said that no inorganic salt formula used here is much different from the others for regeneration.

Table 3-f. The effect of mineral composition on bud regeneration and callus fresh weight of two genotypes

geno-type		NT-17		B5-17		MA-25		NT-25		B5-25	
		F.W. (g)	Bud* (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)	F.W. (g)	Bud (%)
	set										
6822-15	A	0.59		0.37	40	0.91	20	0.50		0.34	
	B	0.05		0.34		0.45		0.56		0.54	
	$\bar{x}$	0.32		0.36	20	0.68	10	0.53		0.44	
FC	A	0.37	60	0.68	80	0.05		0.47	80	0.52	40
701/5-116+	B	0.39	80	0.24	60	0.24	80	0.29	80	0.30	40
	C	0.08	40	0.35	60	0.34	80	0.47	80	0.48	60
	D	0.05	20	0.20	100	0.29	60	0.42	80	0.27	80
	E	0.07	20	0.40	80	0.29	80	0.35	40	0.41	20
	$\bar{x}$	0.19	44	0.37	76	0.24	60	0.40	72	0.40	48

Key: + Callus fresh weight was not significantly different at the 0.05 level by F-test according to the mineral composition.

* No entry indicates no bud regeneration (= 0 %).

MA Murashige - Skoog medium (1962)

NT Nagata - Takebe medium (1971)

B5 Gamborgs B5 medium (1968)

EXPERIMENT 4. The effect of primary callus age and size on bud regeneration

EL 36-18 was used to test for bud regeneration with a standard regenerating genotype. Source callus weight of the initial callus obtained from petioles of shoot cultures had an effect only at larger weights (Table 4-a). In other words, callus pieces from the largest primary calli did not regenerate. Regeneration from subdivided callus over 520 mg was infrequent.

Depending on the growth rate of each individual callus, the size was more or less proportional to the age after first callus observation. When bud regeneration was calculated against source callus age, a similar result appeared (Table 4-b). Subdivided pieces of primary calli more than 26 days old rarely regenerated.

Source culture age showed no similar effect (Table 4-c). The oldest cultures were no less likely to regenerate. Culture age did not correspond much with callus age or weight.

Table 4-a. Effect of source callus weight on proportion of calli regenerating buds for genotype EL 36-18

callus * wt. / bud regeneration	mg													
	40	80	120	160	200	240	280	320	360	400	440	480	520	over
proportion %	36/65 55.4	65/191 34.0	60/121 49.6	48/75 64.0	33/83 39.8	41/105 39.0	29/45 64.4	44/83 53.0	38/61 62.3	59/119 49.6	18/49 36.7	16/49 32.7	37/110 26.4	8/175 4.6
number of primary calli sampled	18	31	13	6	5	5	2	3	2	3	1	1	2	3

* Each callus was categorized every 40 mg unit increment according to the callus weight.

Table 4-b. Effect of source callus age on proportion of calli regenerating buds for genotype EL 36-18

age / bud regeneration	9	12	14	16	18	20 (days)	22	24	26	28	30	over
proportion	24/51	72/132	98/178	85/137	89/220	36/65	43/93	31/43	42/162	0/20	5/115	7/115
%	47	54.5	55.1	62.0	40.5	55.4	46.2	72.1	25.9	0	4.3	6.1
number of primary calli sampled	13	22	16	8	14	5	3	2	6	1	3	2

Table 4-c. Effect of source culture age on proportion of calli regenerating buds for genotype EL 36-18

age / bud regeneration	(days)								
	50	55	60	65	70	75	80	85	Over
proportion	66/185	81/111	69/122	108/400	65/196	41/84	79/171	6/14	17/31
%	35.7	73.0	56.6	27	33.2	48.8	46.2	42.9	54.8
number of primary calli sampled	6	10	10	17	14	13	13	4	8

EXPERIMENT 5. Effect of light intensities during shoot culture on subsequent callus initiation and bud regeneration

EL 36-18 and 6926-0-3 genotypes were tested under the same conditions but in separate experiments. The shoot cultures which had been cultured under the different light intensities in the growth chamber had developed different appearances. The shoot culture leaves under 'light' or 'bright' treatments did not elongate much while those under 'dim' or 'dark' condition elongated noticeably and appeared to be very pale green.

The subsequent ability of explants after the four treatments to regenerate buds and callus did not differ much according to the light intensity in either genotype (Table 5-a and Table 5-b). Using a chi-square test used for fractional (enumerational) data, the frequency of bud regeneration in 6926-0-3 was found to be significantly lower (21.2%) under the light treatment (Table 5-b).

When callusing response was tested, the speed of callus initiation, to first visible size, was also determined. With EL 36-18 callus induction appeared to be proportional to light intensity experienced by the shoot cultures (Table

5-a). This pattern was clearly not seen with 6926-0-3. No significant differences were seen for time to regenerate buds after callus initiation in either genotype (Table 5-a and Table 5-b). Uneven sample size was largely due to contamination.

There was some indication that lighting intensity on the shoot cultures might affect the frequency of adventitious buds on the petiole explants used for callusing. Petiole segments that made adventitious buds were not counted for callusing totals.

Table 5-a. Effect of light intensities during shoot culture on subsequent callus initiation and bud regeneration for EL 36-18

light intensity	callusing proportion i)	%	buds on those that callused proportion ii)	%	days to initiate the callus	days from 1st callus to regeneration	adventitious bud
Dark (no light)	8/16	50.0	5/8	62.5	33.75 (+12.17)	22.40 (+5.46)	---
Dim (5-10 µE)	24/43	55.8	15/24	62.5	45.96 (+16.19)	25.87 (+9.87)	4/47
Light (40-50 µE)	13/21	61.9	10/13	76.9	58.00 (+11.31)	27.40 (+4.33)	5/26
Bright (80-90 µE)	34/48	70.8	21/34	61.8	63.94 (+14.62)	23.30 (+8.51)	5/53

i) non significant by Chi-square at 0.05 level
ii) non significant by Chi-square at 0.05 level

Table 5-b. Effect of light intensities during shoot culture on subsequent callus initiation and bud regeneration for 6926-0-3

light intensity	callusing proportion i)	%	buds on those that callused proportion ii)	%	days to initiate the callus	days from 1st callus to regeneration	adventitious bud
Dark (no light)	9/22	40.90	6/9	66.6	39.89 (+7.56)	14.00 (+7.89)	---
Dim (5-10 µE)	31/53	58.55	15/31	48.4	38.12 (+6.46)	15.67 (+4.32)	---
Light (49-50 µE)	33/51	64.70	7/33	21.2	41.00 (+8.12)	12.14 (+6.74)	---
Bright (80-90 µE)	21/46	45.70	13/21	61.9	39.09 (+7.73)	16.92 (+6.93)	3/49

i) non significant by Chi-square at 0.05 level
ii) significant by Chi-square at 0.05 level

DISCUSSION

The goal of this research was to develop an understanding of the shoot regeneration process in sugarbeet so that it can be used with greatest efficiency in future applications.

Habituated sugarbeet callus can be induced at high frequency and stimulated to form buds or shoots when transferred to media containing several combinations of BA and IAA (Saunders and Daub, 1984). Cytokinins alone were found effective as the hormone to induce buds on primary callus (Saunders, 1982) and two of four genotypes tested here also showed this result. The range of benzyladenine for bud induction was between 0.1 to 3.0 mg/l. Only one bud was seen on GWK-3 callus without BA, but this was a single event and may represent carryover of some kind of organized body from the previous medium, which had an initial BA concentration of 0.25 mg/l. BA at 0.25 mg/l was sufficient to induce buds on callus of other genotypes in the first passage after induction, although no buds were noticed on callus attached to the petiole explant in the previous experiment.

It was not possible to determine with confidence whether there was genotype specific response patterns of the callus to different BA concentrations. Others such as GWK-3 showed optimal growth at intermediate levels. It is important to note that the main purpose of these initial experiments was to determine the range and optimum BA concentrations for bud regeneration. Callus growth rate at low BA concentrations and at 0 may be influenced by any residual BA carried over from the medium on which the callus was induced. Also, these experiments with a range of BA concentrations were carried out only once.

Using three somewhat different medium/explant procedures, a wide range of germplasm in the species Beta vulgaris L. was screened for formation of habituated callus as well as bud or shoot formation from the petioles and callus. Ability to respond in these ways was widespread in the species. Four morphological beet types were represented: sugarbeet (13 sources), table beet (1 source), fodder beet (1 source), leaf beet (2 sources). Callus formation was seen on some individual genotypes in samples of all seventeen germplasm sources. Buds or shoots regenerated from callus from some genotypes of thirteen of the seventeen sources.

In the work reported here (Exp. 2), variation was seen both within and among germplasm sources for both habituated

callusing potential and regeneration capability. Significant differences among genotypes in the capability of callusing have been reported (Cumming et al., 1976; Green et al., 1974; Hanzel et al., 1985) and among cultivars have been reported in tobacco for callus habituation (Bennici et al., 1972). Three monogerm genotypes (FC 506, FC 607 cms, and EL 45) were clearly the superior sources of high frequency regenerator germplasm while most multigerms are very poor sources regardless of the capability for callusing. Multiple sources of shoot regeneration would help broaden the scope of sugarbeet tissue culture research and potentially speed application to practical problems. There should be no need to breed specifically for improved regeneration frequency as has been done in alfalfa (Bingham et al., 1975). Thus not all genotypes are capable of callus induction and of those that are, not all will regenerate buds or shoots. Some genotypes produce callus that regenerates so fast and completely as to leave little residual callus while others initiate callus very rapidly but lack the ability to regenerate under the standard conditions employed.

Although each of the germplasm source populations are probably quite genetically heterogeneous, some of the variability in response within germplasm sources may be due to the fact that conditions have not been optimised for highest frequency callus and bud formation, or that sample

size from each genotype was limited. For example, there were a few genotypes where shoot culture age was variable. In some of these, the four week old shoot cultures gave a much higher callus induction frequency than eight or twelve week old shoot cultures. Another variable that seemed to make a difference was whether leaf blade or petiole was used for explant. Blade tissue formed callus at higher frequency than petiole tissue in genotypes where both were used. A sensible way to look at the data is that under optimum conditions probably a higher proportion of the genotypes examined would show callusing and bud or shoot regeneration. The proportions determined here probably were underestimated. It is interesting that the combination of characteristics of each genotype is somewhat variable in terms of the ability for callusing and bud or shoot regenerating. For example, GWK germplasm has almost no bud regeneration despite very fast callusing, while EL 44C3 and C 566 cms had high frequency of bud regeneration under very poor callusing capability. These are extreme cases.

The formation of adventitious buds directly on petiole explants was a common response for many genotypes. All seventeen germplasm sources gave at least one genotype each that showed adventitious buds. Over all, this response did not seem to be either positively or negatively associated with ability to form callus or regenerate buds or shoots from the callus, which is also a type of adventitious

response. A positive relationship between adventitious bud development on petiole explants and bud regeneration from callus or from intact plants (Saunders and Mahoney, 1982) might allow quick screening for good regenerators.

The germplasm screening project undertaken here has identified several sources of good bud regeneration capability among U.S. monogerm parental lines as well as in a population bred for Michigan conditions. The ideal germplasm source for tissue culture in sugarbeet is still unsolved. Although use of adapted material would permit more rapid utilization in potental line development, it probably would be wiser to use the best regenerating genotype and then rely on backcrossing to insert any desirable new character into adapted germplasm. Back-crossing would also eliminate any detrimental new genetic variation that arose somaclonally during the in vitro operations.

In efforts to screen several medium supplements or alternative components, callus of several genotypes with marginal shoot regenerating properties were used. The goal was to improve the shoot regeneration procedure in general. Because of the screening nature of these experiments, the callus available had to be spread among several treatments. Thus low numbers of sets (replications) resulted. Proline supplements of 200 and 600 mg/l appeared to be the most

promising change in the medium to be investigated for future use. TIBA should also be tested again as there was an increase in bud regeneration above control levels with no TIBA, although this was statistically nonsignificant. TIBA has been used to obtain shoots from nonhabituated sugarbeet callus (Hooker and Nabors, 1977).

Since the nutrient and hormone requirements for maximal growth of callus or shoot regeneration in tissue cultures differ from species to species, it is important to understand the optimal medium composition for each case. In general, the results of broad spectrum experiments such as these should not be taken as conclusive. The role is to point out promising aspects, which should then be pursued in more detail in the future.

There were often large differences in callus growth among the sets, especially in 6822-15 (Table 3-a). This indicates that the degree of callus growth might be effected by factors related to sampling of different calli, perhaps of different ages since habituated callus induction in beets is quite nonsynchronous, or different areas of callus.

The experiment on the age and size of source callus was done to see if callus quality would change with time. This might explain some variability between sets in other experiments. The results indicated that regeneration

frequency fell off sharply for the largest and oldest primary callus. Source culture age, in contrast, did not show similar effect. This might be explained by the fact that sampling of the calli was attempted so that large calli and small calli would not be taken from cultures of different ages. It was impossible even in an experiment of this size, with more than 1300 callus pieces, to evenly schedule sampling when date of appearance or growth rate of the primary callus was quite unpredictable. It is important to know that this callus induction system has callus that takes at least four weeks to be initiated, with large variation in its time of appearance.

Another result of unpredictable scheduling is that fewer larger and older source calli are used. The largest catagory of callus size provided an average of fifty eight test callus pieces, approximately 10 mg each, whereas the smallest category had an average of four test pieces per source callus. Thus the critical sizes and ages that produced low regeneration frequency are represented by only a few source calli.

The experiments with good regenerating genotypes (6926-0-3 and EL 36-18) and shoot culture light intensity were done in attempting to find sources of variability in callus induction and regeneration from calli. Shoot cultures routinely used as sources of explants for all

callus initiation experiments are kept in stacks of petri dishes in a growth chamber, thus light intensity differs from one plate to another. This light effect experiment indicated that such differences in light intensity from one shoot culture plate to another are probably not a major source of variability in responses of callus derived from petiole segments of those shoot cultures.

In summary, the parameters most influential for bud or shoot regeneration from callus studied here were genotype and benzyladenine concentration. In addition, proline supplements showed promising result in the change of medium. Light intensity under which donor shoot cultures were grown did not seem to have a major role, while age and size of source callus were found to have the effect on bud regeneration.

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