GENOTYPE AND GROWTH REGULATOR EFFECTS ON SHOOT REGENERATION FROM PRIMARY AND SERIALLY-SUBCULTURED HORMONE-AUTONOMOUS CALLUS OF SUGARBEET (*BETA VULGARIS* L.).

By

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ABSTRACT

GENOTYPE AND GROWTH REGULATOR EFFECTS ON SHOOT REGENERATION FROM PRIMARY AND SERIALLY-SUBCULTURED HORMONE-AUTONOMOUS CALLUS OF SUGARBEET (*BETA VULGARIS* L.).

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Many in vitro genetic manipulations require a reliable shoot regeneration system. Some sugarbeet genotypes are capable of one-step shoot regeneration from hormone-autonomous callus (i.e. without subculture) when leaf disks are incubated on a Murashige and Skoog (MS) medium containing 1 mg/L N^6-benzyladenine (BA)(B1 medium). Leaf disks from plants from 16 populations were incubated on B1 at 31°C in darkness for 10 wk. Among and within populations differences were significant for frequencies of callus production (CALLUS) and shoot regeneration (REGEN), time to callus (CTIME), and lag period (LAG) between CALLUS and REGEN. Of 3018 leaf disk explants, 52.6% initiated callus in an average time of 43.0 d, and 30.1% of the calli regenerated shoots after a mean LAG of 14.7 d. All 16 populations produced callus, but 5 populations failed to regenerate shoots. The 8 monogerm populations had REGEN three times that of the 8 multigerm populations (41.9% vs. 13.6%). Clustering of the 16 populations by CALLUS and REGEN resulted in 7 response types, each of which may require a different medium to optimize REGEN.

To optimize REGEN within the response types, genotype x growth regulator interactions were investigated in 21 sugarbeet genotypes. B1 medium was used as the control in all experiments. Interactions of genotype with BA, naphthaleneacetic acid (NAA) and gibberellic acid (GA_3) suggest that a single medium is inadequate when screening germplasm for REGEN. Although CALLUS was somewhat independent of [BA], the [BA] needed for optimal REGEN varied with genotype. Optimal [BA] for REGEN was above 1.0 mg/L for 4 of 8 genotypes tested. Some genotypes had enhanced REGEN when NAA (0.01-0.1 mg/L) was included in B1, but for most genotypes, NAA (0.1 mg/L) inhibited and delayed callus

formation and reduced REGEN. Inclusion of GA_3 (1.0 to 3.0 mg/L) in B1 increased shoot number (SHOOTS) per leaf disk, and simultaneously inhibited root regeneration from callus.

REGEN declined when calli were subcultured every 3 wk on B1. To investigate this decline in REGEN, calli of 3 genotypes were initiated on B1 and subcultured to various media after 3 wk growth. Competence was assessed by REGEN and SHOOTS on maintenance (M) media and challenge (C) media. After 15 wk on B1, more than half of EL 45/2-108 calli were still regenerating shoots, while regeneration by calli of REL-1 and FC 607-O-20 was approaching zero. REGEN from calli maintained on B1 was increased after subculture to C medium B3 (MS + 3 mg/L BA). REGEN and SHOOTS were both enhanced by repeatedly doubling the [BA] at each subculture or by maintenance on B1 + 1 mg/L 2,3,5-triiodobenzoic acid (TIBA). Increases in REGEN were greater when both [BA] and [TIBA] were higher in the C medium relative to the M medium. Calli maintained in a non-regenerating state on hormone-free medium were induced to regenerate by transfer to B3. Manipulation of shoot regeneration with BA and TIBA is compatible with a model involving auxin/cytokinin ratio.

To the love of life,

and to all the good things which make life worth living.

To the study of life,

for each bit of understanding sheds a little light on our place on earth and in the universe.

To the possibility,

that through the study of life,

we will achieve a level of understanding that allows us to live in harmony with all the diverse life forms on earth, and to sustain forever the live-giving properties of the earth.

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INTRODUCTION

Plant breeding is the genetic manipulation of crop species by humans to fulfill human needs. It's successful application is truly an art passed down over the centuries from the time the first cereals and legumes were domesticated. With the discoveries of Mendel and the theories of quantitative genetics, plant breeding developed a solid scientific foundation. Modern plant breeders now have at their disposal a new array of molecular and biotechnological tools with which to enhance the efficiency of gene transfer. Some of these new tools, such as genetic transformation and protoplast techniques, allow for the movement of germplasm across natural biological barriers. In many cases, the application of these new tools to crop improvement is dependent on the ability to regenerate whole plants from in vitro cell cultures. Thus, research efforts to develop plant regeneration systems are necessary components in the progression of plant breeding into the biotechnological age.

Sugarbeet breeders are currently seeking methodologies to utilize the tools of biotechnology in genetic improvement programs. A reliable sugarbeet shoot regeneration system is needed for the application of some in vitro genetic manipulations such as somatic cell selection and protoplast techniques. A system of shoot regeneration utilizing hormone-autonomous callus of sugarbeet has been developed at Michigan State University (Saunders and Daub, 1984). The callus forms when leaf disks are incubated on Murashige and Skoog (MS) medium containing 1 mg/L N^6-benzyladenine (BA) as the sole growth regulator (B1 medium). For some genotypes, this callus subsequently regenerates shoots without subculture (i.e. the system is one-step).

The following research on shoot regeneration from hormone-autonomous callus of sugarbeet was divided into three manuscript style chapters, each of which addressed a distinct

aspect of the system: (1) genetic variation, (2) genotype x growth regulator interaction, and (3) long-term regeneration. The first two chapters involved the use of the one-step regeneration system developed by Saunders and Doley (1986). In Chapter 1, a wide range of germplasm was evaluated for in vitro behavior using the standard B1 system. Chapter 2 examined the effects of BA concentration and supplementation of B1 with naphthaleneacetic acid and/or gibberellic acid on shoot regeneration in a number of sugarbeet genotypes. In this chapter the essential features, i.e. one-step and hormone-autonomy, of the shoot regeneration system were maintained. The final chapter examined the effects of genotype, subculture interval, BA concentration and 2,3,5-triiodobenzoic acid on shoot regeneration from serially subcultured callus of three sugarbeet genotypes. The callus in Chapter 3 was hormone-autonomous, but the regeneration was no longer one-step since subculturing was involved.

In addition to potential applications by sugarbeet breeders and geneticists, this research also touched upon several concepts that are of general interest to plant developmental biologists and physiologists. These included genotype and growth regulator effects on the initiation of the habituated (hormone-autonomous) state, and the regulation of morphogenesis from undifferentiated tissue.

LITERATURE REVIEW

Sugarbeet (*Beta vulgaris* L.), a root crop grown mainly in temperate regions, supplies about 40% of the world supply of sucrose (Smith, 1987). Sucrose, or table sugar, is extracted from the roots at large processing plants near the growing areas. In 1987, sugarbeets were harvested from 8.6 million ha around the world, with an average yield of 31.2 Mg/ha (USDA, 1988). The leading sugarbeet producing countries include the Soviet Union, France, West Germany, the United States and Poland. In the USA, sugarbeets are mainly grown in North Dakota, Minnesota, Idaho, California and Michigan. Total USA acreage in 1987 was 3.9 million (1.6 million ha), with an average yield of 22.3 T/A (50.0 Mg/ha)(USDA, 1988).

A member of the Chenopodiaceae, sugarbeet is an allogamous species with a typically biennial life cycle. In the first season, the plant grows a foliar rosette and sucrose is stored in a large fleshy taproot. Following an overwintering experience and exposure to long-day conditions, a large bushy flower stalk emerges on which as many as 5000 seed often ripen under favorable conditions. The fruit is a nutlet, and in the case of multiple flowers at a node, an aggregate fruit containing several seed is produced. The terms monogerm and multigerm refer to fruits containing either one or several seed, respectively. Precocious production of a flower stalk during the first season is referred to as bolting, and is highly undesirable in sugarbeet production.

SUGARBEET BREEDING

Having been domesticated only in the past 200 yr, the sugarbeet is a relatively modern crop. Following the discovery by Marggraf in 1747 that beets contained sucrose, Achard developed a process for sucrose extraction and recovery from beets. Mass selection carried out in fodder beet from 1786 to 1830 by Achard and the von Koppy family improved the sugar

content of beets from 6% to 9% (Smith, 1987). This resulted in the cultivar White Silesian, thought by many to be the progenitor of all sugarbeets. The hypothesis that White Silesian arose from intercrosses between fodder beet and chard (both *B. vulgaris* L.) is supported by the efforts of Fischer (1989) to resynthesize the progenitor type.

Today's modern sugarbeet hybrids can approach 20% sucrose under favorable growing conditions. The genetic improvements facilitated by sugarbeet breeders within this short time frame are thus a remarkable achievement. Although these improvements are partially due to advancements in cultural practices, plant breeding has undoubtedly played a key role in enhancing sugarbeet productivity.

Before the 1950's, all commercial sugarbeet production involved open-pollinated multigerm populations. Due to the nature of the multigerm seed, thinning (stand reduction) was a routine aspect of sugarbeet cultivation. Two key genetic discoveries, i.e. cytoplasmic male sterility (CMS) and monogermness, led to the evolution of today's monogerm hybrid sugarbeet cultivars. After the discovery of CMS and its maintainer (O-type) by Owen (1945), and the monogerm trait by Savitsky (1950), monogerm hybrid sugarbeet cultivars first became available in 1958 (Hecker and Helmerick, 1985). Monogerm sugarbeet hybrids with high rates of germination allow precision planting, eliminating the need for the thinning operation. A typical hybrid results from a three-way cross involving: (a) a monogerm CMS inbred, (b) a monogerm O-type inbred, and (c) a broad-based multigerm pollinator. The pollinator may be diploid or tetraploid, giving rise to diploid and triploid hybrids, respectively. For detailed descriptions of sugarbeet breeding procedures, the reader is referred to reviews by Hecker and Helmerick (1985) and Smith (1987).

Breeding objectives in USA sugarbeet improvement programs include: sugar yield and quality, disease resistance (e.g. Cercospora leaf spot incited by *Cercospora beticola* Sacc., Rhizoctonia root rot incited by *Rhizoctonia solani* Kühn, black root incited by *Aphanomyces cochlioides* Drechsl., powdery mildew incited by *Erysiphe polygoni* DC., bacterial rot incited by *Erwinia carotovora* (Jones) Bergey et al. ssp. *betavasculorum* Thomson et al., curly top

virus, the yellows viruses and rhizomania), resistance to sugarbeet cyst nematode (*Heterodera schachtii* Schmidt), and bolting resistance. Secondary or minor breeding objectives include herbicide tolerance, cold tolerance and resistance to insects (sugarbeet root maggot (*Tetanops myopaeformis* Röder) and sugarbeet root aphid (*Pemphigus populivenae* Fitch). Additionally, use of a single source of CMS has resulted in worldwide cytoplasmic uniformity in sugarbeet hybrids rendering the crop potentially vulnerable to genetic disaster. Thus, sugarbeet breeders are searching for new sources of CMS to alleviate this concern. Using traditional approaches, plant breeders have made significant progress in most of these areas, but in the future many of these objectives will be addressed through a combination of traditional and biotechnological strategies. In vitro genetic manipulations, such as genetic transformation and somatic cell selection, require that an efficient and reliable method is available for the regeneration of whole plants from cell cultures.

SUGARBEET PLANT REGENERATION SYSTEMS

Systems of in vitro plant regeneration can broadly be divided into somaclonal, gametoclonal or protoclonal, relative to the starting material being somatic cells, gametic cells or protoplasts, respectively. Two recent reviews by Atanassov (1986a; 1986b) summarize the various in vitro manipulations which have been applied to sugarbeet. The following discussion of sugarbeet regeneration systems will be restricted to somaclonal systems, but the alternative systems deserve brief mention. Largely unsuccessful efforts to regenerate sugarbeet plants from anther culture (Rogozinska et al., 1977: Welander, 1974) have led to the development of gynogenic systems for the production of gametoclonal haploids. Following reports of low frequency plant regeneration from cultured ovules and ovaries (Bossotrout and Hosemans, 1985; Hosemans and Bossotrout, 1983), reliable protocols for the production of gynogenic sugarbeet haploids have been developed (Doctrinal et al., 1989; Goska, 1985; Van Geyt et al., 1987). Sugarbeet protoplasts have been successfully isolated and cultured (Bhat et al., 1985; Bhat et al., 1986; Szabados and Gaggero, 1985). Although no protocol for regeneration from protoplasts has been published, a report of variation among sugarbeet protoclones implies that

the technology has been developed (Steen et al., 1986).

Prior to 1984, shoot formation from somatic sugarbeet explants cultured in vitro was unpredictable and obtained only sporadically (De Greef and Jacobs, 1979; Hooker and Nabors, 1977). Systems for reliable regeneration of whole sugarbeet plants are now available and these can be grouped into four distinct approaches: regeneration from conventional callus (Tetu et al., 1987); regeneration from hormone-autonomous callus (Saunders and Daub, 1984; Saunders and Doley, 1986; Saunders and Shin, 1986); production of self-regenerating lines (Van Geyt and Jacobs, 1985); and direct regeneration from explants (Detrez et al., 1988; Freytag et al., 1988; Ritchie et al., 1989). There have been no reports of regeneration of sugarbeet plants from cell suspension cultures.

Regeneration from Conventional Callus

Conventional callus, as defined here, requires an exogenous source of both auxin and cytokinin for induction and continued growth, while hormone-autonomous callus is capable of sustained growth on medium devoid of growth regulators. The infrequent regeneration obtained by Hooker and Nabors (1977) occurred after compact, green callus induced on auxin and cytokinin was transferred to media with much lower levels of auxin. Regeneration did not occur on the compact callus, but on a soft callus derived from it after transfer.

Tetu et al. (1987) have reported regeneration from sugarbeet callus by three distinct protocols, and in each case an auxin was used to initiate the callus. Compact, green callus initiated on various levels of cytokinin and auxin became friable and formed buds at low frequency after transfer to a regeneration medium also containing auxin and cytokinin. High intensity shoot organogenesis was obtained when friable, green callus initiated conventionally and transferred to media containing cytokinin and the anti-auxin 2,3,5-triiodobenzoic acid (TIBA). They also reported a procedure for obtaining somatic embryogenesis, but it included several media and subcultures and appeared somewhat impractical.

Regeneration from Hormone-Autonomous Callus

Hormone-autonomous sugarbeet callus is initiated on medium containing only cytokinin, but is capable of sustained growth on hormone-free medium (Saunders and Daub, 1984). Initiation can also occur on hormone-free medium (Doley and Saunders, 1989), but the time required to initiate callus is considerably longer in the absence of the cytokinin stimulus. The white, friable callus is morphologically distinct from the compact, conventional callus described above. High-frequency regeneration can now be obtained using shoot culture leaf explants (Saunders and Shin, 1986) or whole plant leaf explants (Saunders and Doley, 1986) on MS medium containing 1 mg/L N^6-benzyladenine (BA). Shoot regeneration is one-step (i.e. subculture is not required). When explants are incubated at 28 to 31°C, callus initiation typically occurs 4 to 6 wk post-inoculation, and subsequent shoot regeneration from callus occurs after a lag period of 1 to 3 wk (Saunders and Doley, 1986). Using leaf blade and petiole explants from in vitro shoot cultures, Saunders and Shin (1986) demonstrated that the regeneration system involving hormone-autonomous callus is applicable to a wide range of sugarbeet germplasm.

Freytag et al. (1988) and Ritchie et al. (1989) described procedures for two-step regeneration from callus morphologically similar to the hormone-autonomous callus described above. Both procedures utilized shoot culture explants plated on medium containing BA, but the procedure of Freytag et al. (1988) also included 0.1 mg/L indole-3-acetic acid (IAA).

Regeneration from Self-Regenerating Lines

De Greef and Jacobs (1979) fortuitously obtained what they refer to as a self-regenerating line. The cell line was capable of continued growth on hormone-free medium, but required a cytokinin for successful shoot production. Attempts to repeat the procedure were not successful. Plants have been regenerated from this cell line after more than eight years in culture (Jacobs, personal communication).

A reproducible protocol for the initiation of self-regenerating lines from a number of genotypes has been reported by Van Geyt and Jacobs (1985). The lines were produced when

callus at the base of primary shoots was used for habituation. These authors obtained habituation by successively halving the hormone concentrations in the medium. The self regenerating lines were not true callus, but were composed of a mixture of undifferentiated cells, promeristems, meristems and leaf-like structures. Total dedifferentiation of these lines resulted in loss of the regenerative capacity.

Direct Regeneration from Explants

Early reports of direct adventitious shoot regeneration in sugarbeet from in vitro shoot cultures (Hussey and Hepher, 1978), from shoot culture derived leaf (Rogozinska and Goska, 1978) or petiole explants (Rogozinska and Goska, 1978; Saunders and Shin, 1986) or using flower buds from greenhouse plants (Miedema, 1982) stimulated interest in these procedures for clonal micropropagation as well as their potential use in genetic transformation. Since then, three systems of high frequency direct regeneration have been developed (Detrez et al., 1988; Freytag et al., 1988; Ritchie et al., 1989). These reports all utilized petiole explants from in vitro shoot cultures, but differed considerably in the growth regulator regimes employed. The medium used by Ritchie et al. (1989) contained 10 mg/L BA, that of Detrez et al. (1988) contained 3 mg/L BA and 3 mg/L NAA, while that of Freytag contained 0.4 mg/L BA and 0.1 mg/L IAA. All three reports also stressed the importance of donor culture environment to obtain optimum response. For example, the procedure of Detrez et al. (1988) involved the use of TIBA in the shoot culture medium.

Although the production of large numbers of regenerants has been demonstrated using these protocols, the likelihood that many of the shoot initials are preformed makes them unlikely components of in vitro gene transfer systems (Krens and Jamar, 1989). However, the genetic fidelity of plants regenerated directly from explants (Detrez et al., 1989) suggests that these procedures may find application in sugarbeet breeding programs as a cloning vehicle.

GENETIC VARIATION FOR IN VITRO RESPONSE

Considering the quantitative nature of plant growth regulator interactions which result in organ formation (Skoog and Miller, 1957), the numerous reports of genetic variation for in

vitro behavior should come as no surprise. Genetic differences for in vitro response have apparently been reported in every species where they have been sought. Many studies, ranging from analyses of progeny of a few crosses to full diallel mating designs, have attempted to elucidate the genetic control of the various in vitro responses. Interestingly, the results suggest that the genetic control of in vitro response ranges from as few as two major genes to a fully polygenic system.

Examples of crops where major gene activity has been reported include alfalfa (Reisch and Bingham, 1980), cucumber (Nadolska-Orczyk and Malepszy, 1989), petunia (Izhar and Power, 1977) and tomato (Koornneef et al., 1987). In both alfalfa and tomato, two dominant major genes were postulated to control shoot regeneration ability, while three dominant loci were postulated in cucumber. In petunia, 'a few genes' were thought to control regeneration from protoplasts.

On the quantitative side several fixed effects diallels have been performed. Significant general and specific combining abilities have been reported for regeneration from anther culture in rice (Miah et al., 1985) and wheat (Lazar et al., 1984), and for regeneration from callus in maize (Beckert and Qing, 1984), pigeonpea (Suresh Kumar et al., 1985) and tomato (Frankenberger et al., 1981). In most reports, additive genetic variance was more important than nonadditive genetic variance in controlling in vitro response. Heterosis has been reported for callus growth in alfalfa (Keyes and Bingham, 1979), maize (Tomes and Smith, 1985), pigeonpea (Suresh Kumar et al., 1985) and tobacco (Keyes et al., 1981). Heritability estimates for shoot regeneration include: 0.09 in cauliflower (Buiatti et al., 1974), 0.43 to 0.77 in cucumber (Nadolska-Orczyk and Malepszy, 1989), 0 to 0.62 in maize (Beckert and Qing, 1984), 0 to 0.19 in sweet potato (Templeton-Somers and Collins (1986), 0.98 in tomato (Frankenberger et al., 1981), and 0.60 to 0.72 in wheat (Lazar et al., 1984). Ranges of estimates usually involve different genetic material, but in some cases, detection of significant heritability was dependent on the medium that was used (Beckert and Qing, 1984)

The large number and variety of cytogenetic stocks available in wheat have facilitated

detailed analysis of in vitro genetics in that crop. Studies involving substitution analysis, monosomic analysis, aneuploids and trisomics suggest that loci effecting in vitro behavior can be found on several chromosomes. Group 2 chromosomes (Felsenburg et al., 1987; Kaleikau et al., 1989a; Kaleikau et al., 1989b; Szakacs et al., 1988), chromosome 4B (Felsenburg et al., 1987; Mathias and Fukui, 1986) and chromosome 5B (Agache et al., 1989; Felsenburg et al., 1987) have all been reported to significantly effect regeneration from callus.

In addition to nuclear control, cytoplasmic effects on in vitro behavior have been reported in barley (Powell and Caligari, 1987), rapeseed (Narasimhulu et al., 1989) and wheat (Felsenburg et al., 1987; Mathias et al., 1986).

From the many reports of genetic control of in vitro behavior, a few generalizations may be drawn. In wheat, where breeding behavior and cytogenetic stocks have facilitated the most thorough examination, it seems clear that major and minor nuclear genes, as well as cytoplasmic factors, are involved in the in vitro response. One might speculate that major genes affecting growth regulator metabolism interact with the overall quantitative nature of growth itself, and it would be expected that a similar duality of control is involved in the in vitro response of most species.

Further evidence for the genetic control of in vitro behavior comes from reports of progress made through selection. In alfalfa, Bingham et al. (1975) increased regeneration response from 12% to 67% in two cycles of recurrent selection using vigorous regenerated plants as parents. This selected population was released as 'Regen-S'. Petolino et al. (1988) were able to increase anther culture response in wheat from 3.5% to 23.4% in a single cycle of selection involving a cross between two anther-derived plants. In tomato, Koornneef et al. (1986) successfully introgressed the shoot regeneration capacity of *Lycopersicon peruvianum* into *L. esculentum*.

In sugarbeet, information on the genetics of in vitro response is limited. Many studies have involved explants derived from seedlings of hybrid cultivars germinated in vitro (Hooker and Nabors, 1977; Krens and Jamar, 1989; Tetu et al., 1987), and thus by definition are

analyses of population phenomena. Interspecific variation within the genus *Beta* has recently been described for ability to regenerate shoots from callus (Yu, 1989) and for direct shoot regeneration (Majewska-Sawka and Jassem, 1988; Mikami et al., 1989).

Saunders and Shin (1986) evaluated the in vitro response of a broad array of sugarbeet germplasm, as well as some fodder beet, leaf beet and red beet material. Using leaf blade and petiole explants from in vitro shoot cultures, they observed extensive genetic variation for the ability to initiate hormone-autonomous callus and subsequently regenerate shoots from that callus. The response of the sugarbeet material was superior to the small sample of related subspecies, and within the sugarbeet germplasm, callus of the monogerm material sampled had a greater ability to regenerate shoots.

Development of the regenerator clone REL-1 by conventional breeding procedures (Saunders, unpublished) demonstrated that in vitro behavior in sugarbeet is heritable. Isolation of a somaclonal variant for in vitro behavior in sugarbeet provides further evidence of genetic control (Saunders and Doley, 1986).

The availability of a sugarbeet monosomic addition series carrying chromosomes of *B. procumbens* (Lange et al., 1988; Van Geyt et al., 1988), as well as a full series of trisomic lines (Romagosa et al., 1986), should facilitate future efforts to elucidate the genetic control of in vitro behavior in sugarbeet. Additionally, advances in isozyme (Nagamine et al., 1989a; Smed et al., 1989; Van Geyt and Smed, 1984) and RFLP (Nagamine et al., 1989b) techniques as molecular markers in sugarbeet may lead to localization and characterization of the genes involved in response in culture.

GENOTYPE x MEDIUM INTERACTION FOR IN VITRO RESPONSE

Although reports of genetic effects in culture are common, reports of genotype x medium (G x M) interaction are somewhat rare. Many reports of genetic variation in vitro involve evaluation on two or more media, and thus contain information pertaining to G x M interaction (Behki and Lesley, 1976; Izhar and Power, 1977; Oelck and Schieder, 1983; Ozawa and Komamine, 1989; Seitz et al., 1987; Zelcer et al., 1984). Although it is difficult to

estimate the frequency of this type of interaction, it might safely be assumed that any crop which displays genetic variation for in vitro behavior also will display G x M interaction. G x M interaction for regeneration has been reported in alfalfa (Nagarajan et al., 1986; Saunders and Bingham, 1975), barley (Dunwell, 1981; Hanzel et al., 1985; Powell and Dunwell, 1987), petunia (Skvirsky et al., 1984), rice (Quimio and Zapata, 1990) and wheat (Mathias and Simpson, 1986). The practical effect of G x M interaction is to eliminate the possibility that a single in vitro culture system will be applicable to the range of germplasm within the species.

Mathias and Simpson (1986) reported G x M interaction in wheat tissue culture. When they initiated callus from immature embryos with and without coconut milk in the medium, they found that a single genotype had enhanced callus initiation with the coconut milk. All other genotypes tested were inhibited by this addition to the medium.

Powell and Dunwell (1987) utilized a quantitative approach for the analysis of G x M interaction in barley. The potential impact of G x M interaction in tissue culture was exemplified by the character dry weight, where more than 50% of the variation was explained by the G x M component of variance. A joint regression approach similar to that used by plant breeders for stability analysis was used to characterize the interactions. Regression of genotype means across media means was used to determine whether G x M interactions were additive for each response parameter, and therefore of predictive value in a linear model. For response parameters where the interaction was nonadditive, an analysis of variance of the square root of the variance was used to estimate in vitro sensitivity. Thus the in vitro performance of individual genotypes was assayed by both the mean response and the square root of the variance component over four 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations.

There have been few reports of G x M interaction in sugarbeet. Some studies have involved a single genotype or hybrid (DeGreef and Jacobs, 1979; Hooker and Nabors, 1977; Krens and Jamar, 1989). In a study involving 15 sugarbeet genotypes, Jarl and Bomman (1986) found significant G x M interaction for callus growth. Evidence for G x M interaction

for shoot regeneration in sugarbeet comes from a recent report by Doley and Saunders (1989). Three plants of 'L53', which had failed to regenerate shoots from callus in previous studies (see Chapter 1) utilizing B1 medium, regenerated shoots from callus on hormone-free medium.

LONG-TERM REGENERATION FROM CELL CULTURES

The ability of serially subcultured callus or cell suspensions to maintain their regenerative capacity is referred to as long-term regeneration. The cultures may be maintained in a continuous state of regeneration, or they may be induced to regenerate by transfer to a regeneration medium. Alternatively, the ability to regenerate under standard regeneration conditions may be lost and then restored by some manipulation of the culture environment that was previously not required to obtain regeneration.

The successful application to plants of new genetic technologies such as DNA transfer and somatic cell selection is dependent on the availability of reliable plant regeneration systems. Regeneration of whole plants from protoplasts, suspensions or callus must be maintained over a period of several weeks to months, most likely involving several subcultures.

Loss of regenerative ability after serial subculture could be the result of altered genetic constitution of the culture or could result from the development of some new stable physiological state in which the culture no longer responds to conditions that previously induced morphogenesis (Smith and Street, 1974). The critical difference between these two explanations for loss of competence is that the genetically altered cell line will remain incompetent while the physiologically altered cell line should be capable of having competency restored through manipulation of the culture environment.

Changes in the genetic constitution of plant cells in culture have been reported in many species (Ahloowalia, 1983; Balzan, 1978; Bayliss, 1975; Lee and Phillips, 1988; McCoy et al., 1982; Meins, 1983; Singh, 1975; Skirvin, 1978; Vapper and Kallak, 1986) and their frequency of occurrence is apparently high. Variation resulting from these changes is referred to as somaclonal variation (Larkin and Scowcroft, 1981), and the frequency of this variation

has been reported to increase with time in culture (Armstrong and Phillips, 1988; Kasperbauer et al., 1979; Sutter and Langhans, 1981). Loss of organ-forming capacity in long-term cultures has been directly related to increased frequency of aneuploidy and polyploidy (Torrey, 1967), to increased nuclear DNA content (Chandler and Dodds, 1983), and to number of cell divisions (Meyer-Teuter and Reinert, 1973). It should be mentioned that although somaclonal variation is quite common, there are also examples of long-term cell cultures with relatively stable chromosomal constitutions (Evans and Gamborg, 1982; Franklin et al., 1989; Mo et al., 1989; Nagl and Pfeifer, 1988).

The regeneration of somaclonal variant plants implies that the genetic constitution has maintained some minimum level of organization relative to regenerative ability. In other cases, the genetic changes occurring in vitro are sufficient to preclude any further morphogenesis. These typically involve gross cytogenetic alterations such as aneuploidy and polyploidy. Habituation, the ability of a callus or suspension to continue growth without the addition of exogenous growth regulators, is an example of the development of a new stable physiology (for review, see Meins, 1982). Habituation is an epigenetic phenomenon that is inducible and often reversible. It seems likely that many other stable physiologies develop during cell culture that are not as easily detectable as habituation but might interfere with the regenerative ability of the culture. The genetic totipotency of the cultured cells can be masked by epigenetic events induced by the culture conditions and these events should be reversible by modifying those conditions.

Continuous regeneration in long-term culture has been reported in several species, including barley (Weigel and Hughes, 1985), birdsfoot trefoil (Orshinsky and Tomes, 1985), maize (Armstrong and Green, 1985), *Microcitrus* spp. (Vardi et al., 1986), oats (Heyser and Nabors, 1982), pea (Hussey and Gunn, 1984), rice (Heyser et al., 1983; Ozawa and Komamine, 1989), wheat (Heyser et al., 1985) and white clover (White, 1984). Culture age in these species does not seem to affect the ability to regenerate. In some cases, little effort was necessary to maintain the regenerative state (Orshinsky and Tomes, 1985; Vardi et al., 1986;

White, 1984), but in most reports competence was maintained by preferential subculture of a particular callus type or was dependent on some specific aspect of the culture system.

Competence is frequently maintained by preferential subculturing of regenerative callus. In oats, embryogenic callus is morphologically distinct from non-embryogenic callus (Heyser and Nabors, 1982). Embryogenic callus is also easily identified in wheat, proso millet and pearl millet (Nabors et al., 1983). A similar situation exists in maize where Type I and Type II callus, organogenic and embryogenic respectively, are also distinguished by callus morphology (Armstrong and Green, 1985), and selection for callus type has prolonged longevity (Lowe et al., 1985). Such morphological distinctions among callus types, while undoubtedly quite practical, are somewhat fortuitous and seem unlikely to be of general application.

Frequently, variations in the culture system are found to affect the longevity of the culture. Choice of explant can have a pronounced effect, as in the case of *Stylosanthes guyanensis* (Aubl.) Sw., for which leaf-derived callus had lost regenerative capacity after two years in culture, but hypocotyl-derived callus was still highly morphogenic (Meijer, 1984). In Afghan pine, regeneration from callus has been maintained for up to three years, and the regeneration capacity is enhanced by cycling the culture between the bud induction and the bud maturation media (Gladfelter and Phillips, 1987). Subculture frequency is another cultural consideration which has been reported to affect long-term regeneration in begonia (Cassells and Morrish, 1987), carrot (Bayliss, 1975), tobacco (Evans and Gamborg, 1982) and rice (Ozawa and Komamine, 1989).

The high frequency of genotype-dependent regeneration systems in many species suggests that genotypic effects may play a role in successful long-term regeneration systems. Although genotypic effects are common in cell cultures, there have been few reports of genotypic effects on long-term regeneration. Callus of two pea genotypes continually regenerated for three years, while three other genotypes produced little or no callus (Hussey and Gunn, 1984). White (1984) identified a single white clover genotype, out of 200 tested,

that had a high regeneration capacity. This cell line maintained its morphogenic capacity after two years in culture. These two examples of genetic differences in long term regeneration both reflect genetic differences in ability to initiate callus and regenerate shoots in primary cultures, rather than genetic variation for regeneration from long term cultures.

There have been at least three reports of genotypic effects on long-term regeneration. Of 16 pea genotypes tested, Malmberg (1979) found that six regenerated shoots after two months in culture. After four and six months in culture, only four and two of these genotypes, respectively, were still capable of shoot regeneration. Locy (1983) evaluated six *Lycopersicon* spp. and found that only two species formed shoots after subculture, and one of those retained its regenerative capacity for more than one year. In maize, 6-month-old type 2 callus of B73 x A188 regenerated more plants than A188 or A188 x B73 (Kamo and Hodges, 1986).

The most interesting reports of long-term regeneration are those in which the regenerative ability had been lost and then restored by some manipulation of the culture environment. In alfalfa, buds were induced on 32-month-old callus by a high cytokinin/low auxin ratio (Stavarek et al., 1980). The callus had continually been challenged to regenerate without success on a medium developed for alfalfa regeneration. Lowering the sucrose concentration from 3% to 1% promoted further development of the buds. The authors suggest that the higher sucrose was necessary as an energy source for bud induction, but this same level inhibited shoot elongation when the sucrose was thought to play an osmoregulatory role.

Competence has been restored in long-term rice callus by osmotic manipulation of the medium (Kavi Kishor and Reddy, 1986a; Kavi Kishor and Reddy, 1986b). When the rice callus was maintained on medium with 2% sucrose and 3% sorbitol or mannitol, regeneration did not decline after 1400 days (56 subcultures). Callus growing on 2% sucrose alone lost its shoot producing ability after 75 to 300 days, but was revived after 50 days on 2% sucrose plus 3% sorbitol or mannitol.

Callus cultures of haploid rape showed a decline in shoot production after the first subculture (Sacristan, 1981). After nearly three years in culture, regeneration was induced by

transfer of the callus to a new medium under high light intensity, followed by transfer back to the original medium while maintaining the high light intensity. Since the maintenance medium included 2,4-D and the induction medium did not, restoration of competence in this case may fit the 2,4-D withdrawal model of regeneration.

In the several reports of revival of regenerative ability in long-term callus, no clear pattern emerges. In all cases, some form of stable metabolism was developed and then overcome by an environmental modification, but in each report the effective modification was different.

Little information is available regarding regeneration from subcultured sugarbeet callus. Research on regeneration in sugarbeet has focused on regeneration from primary callus (Krens and Jamar, 1989; Saunders and Doley, 1986; Saunders and Shin, 1986) and from secondary callus (Freytag et al., 1989; Hooker and Nabors, 1977; Tetu et al., 1987). Plants have been regenerated from the original self-regenerating line of De Greef and Jacobs (1979) after more than 8 yr in culture. However, because the self-regenerating lines are not true callus but a leafy callus consisting of various levels of differentiation, it seems unlikely that they will be useful for such procedures as genetic transformation or somatic cell selection.

There have been no reports of regeneration from conventional sugarbeet callus beyond the first subculture. Callus derived from excised ovules lost regeneration capacity after the second subculture (Van Geyt et al., 1987). Saunders and Daub (1984) reported that hormone-autonomous sugarbeet callus regenerated occasional shoots when callus maintained for three monthly subcultures on hormone-free medium was transferred to medium containing 1 mg/L BA and 0.3 mg/L IAA. Hormone-autonomous callus maintained on hormone-free medium was capable of sustained growth for three 3 wk subcultures, but no shoot regeneration was observed (Saunders and Doley, 1986).

CHAPTER 1

Genetic Variability for Frequency and Chronology of Hormone-Autonomous Callus Initiation and Subsequent Shoot Regeneration in Sugarbeet (*Beta vulgaris* L.).

SUMMARY

A reliable shoot regeneration system is a prerequisite for the application of many in vitro genetic manipulations. Some genotypes of sugarbeet (*Beta vulgaris* L.) are capable of one-step shoot regeneration from hormone-autonomous callus (i.e. without subculture) when leaf disks from whole plants are incubated on Murashige and Skoog medium containing 1 mg/L N^6-benzyladenine (B1 medium). The frequency of this ability within the sugarbeet germplasm pool was investigated by testing leaf disks from 4 to 5 plants each from 16 sugarbeet populations, 8 multigerm and 8 monogerm. Leaf disks were incubated on B1 medium at 31°C in darkness for 10 wk. Significant differences among populations were found for frequencies of callus production and shoot regeneration, time to callus, and lag period between callus initiation and shoot regeneration. Significant differences among plants within populations were also found for all four parameters, suggesting that selection for in vitro behavior should be effective both within and between populations. Of 3018 leaf disk explants, 52.6% initiated callus in an average time of 43.0 d, and 30.1% of the calli regenerated shoots after a mean lag period of 14.7 d. All 16 populations produced at least some callus, but five populations failed to regenerate shoots. Overall, 76% of the plants produced some callus and 59% of these plants regenerated at least one shoot. The monogerm material had a shoot regeneration frequency three times that of the multigerm material (41.9% vs 13.6%). The concept of in vitro response types is proposed here as a mechanism to efficiently optimize shoot regeneration from all members of a germplasm pool. Clustering of the 16 populations

by frequencies of callus initiation and shoot regeneration resulted in 7 response types. Different media may be required to optimize regeneration within each response type.

INTRODUCTION

Sugarbeet (*Beta vulgaris* L.) supplies about 40% of the world's supply of sucrose (Smith, 1987). In 1987, sugarbeets were harvested from 8.6 million ha worldwide and yielded a total of 296 million Mg (USDA, 1988). The continued production of superior sugarbeet cultivars through genetic improvement will partly depend on the ability to efficiently apply the techniques of tissue culture and molecular biology in breeding programs.

In the past decade, sugarbeet tissue culture has progressed from a recalcitrant system with minimal shoot regeneration to the availability of at least three distinct protocols for high frequency shoot regeneration from callus. These include: (1) the production of self-regenerating lines (De Greef and Jacobs, 1979; Van Geyt and Jacobs, 1985), (2) regeneration from conventional auxin-dependent callus (Tetu et al., 1987) and (3) regeneration from hormone-autonomous callus (Saunders and Doley, 1986; Saunders and Shin, 1986). It is not clear whether somatic embryogenesis as reported by Tetu et al. (1987) was from conventional or hormone-autonomous callus. The regeneration system which utilizes hormone-autonomous callus has two major advantages. First, regeneration can be one-step, i.e. callus initiation and shoot regeneration can occur without subculture (Saunders and Doley, 1986). Secondly, the technique seems to be applicable to a wide range of sugarbeet germplasm (Saunders and Shin, 1986).

Saunders and Shin (1986) evaluated the in vitro response of a wide range of sugarbeet germplasm using petiole and blade explants derived from shoot cultures. Since then, methodology has evolved to obtain one-step regeneration using leaf disks from whole plants (Saunders and Doley, 1986). A regeneration system utilizing whole plants as explant donors has potential advantages relative to a breeding program. Application of gene transfer or somatic cell selection within elite germplasm may be more efficient if leaf disk explants could be obtained directly from plants growing in the greenhouse or in the field. Furthermore, if a

regeneration system could be developed which is applicable to the range of germplasm in the breeding pool, it could also be used for vegetative propagation. Regenerant plants thus obtained could provide information on qualitative traits, such as some forms of disease resistance, by essentially allowing a plant to be in two places at one time. The procedure would be restricted to the evaluation of qualitative traits since somaclonal variation at specific loci should be infrequent. This type of early screening would be particularly useful in sugarbeet breeding where initial seed supplies are frequently limited, and where evaluation of floral characteristics requires a minimum 10 wk vernalization treatment.

The one-step regeneration procedure has the unusual characteristic of a 4 to 6 wk delay between explant inoculation and callus initiation, and a 1 to 4 wk lag period between callus initiation and shoot regeneration (Saunders and Doley, 1986). If genetic variability exists for this in vitro chronology, it may be possible to select for rapid, as well as high-frequency, regeneration.

The objective of this research was to evaluate the frequency and chronology of initiation of hormone-autonomous callus, and subsequent shoot regeneration, from leaf disks taken from greenhouse grown plants of a wide range of sugarbeet germplasm.

MATERIALS AND METHODS

Plant Material

Sugarbeet plants used as donors of leaf explants were grown in soil in 15 cm plastic pots in a greenhouse with supplemental lighting (provided by high pressure sodium lamps) only during the first 4 months. The soil used was a 2:2:1:1 mixture of Baccto professional planting mix (Michigan Peat Co, Houston, TX, USA), a local greenhouse mix, vermiculite and perlite, respectively. The local greenhouse mix consisted of a 5:3:2 mixture of field soil of variable origin, peat and sand, respectively. Plants were fertilized every 2 wk with 200 mL Peters 20-20-20 water soluble commercial nutrient mix and monthly with Snyder's (1974) nutrient formulation. At approximately 6 months of age, the plants were transferred to 25 cm plastic pots and moved to a greenhouse without supplemental lighting.

All plants were grown from seed planted in December 1985 and represent a diverse sample of USA monogerm and multigerm sugarbeet germplasm, including several parental lines which have been used in commercial seed production. Five plants were sampled from each of eight monogerm and eight multigerm populations. Seed sources and characteristics for these populations are listed in Table 1. With the exceptions of Owen's Annual (OA) and 84M5-20, each of these lines is a genetically heterogeneous population.

Culture Procedures

Explants were taken in March 1986 and December 1986 from approximately three-month-old and one-yr-old donor plants, respectively. Two small partially expanded leaves, varying in length from 5 to 15 cm, per plant were used as the source of explants. Detached leaves were surface sterilized with two 20 min soakings in 15% commercial hypochlorite bleach solution with 0.01% sodium laurylsulfate, followed by six rinses with sterile distilled water. Square explants varying in size from 4 to 5 mm^2 were cut from leaf blade tissue with a scalpel. The same plants were sampled for the two experiments, and the two samples were considered distinct environments, since the first sample was in the Spring with plants maintained under supplemental lighting, while the second was in the Winter without supplemental lighting.

The culture medium was MS (Murashige and Skoog, 1962) salts, 3% (w/v) sucrose, 100 mg/L *myo*-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, 1.0 mg/L thiamine·HCl, 0.9% (w/v) Difco Bacto agar (Difco Laboratories, Detroit, MI, USA) and 1.0 mg/L N^6-benzyladenine (BA)(Sigma Chemical Co, St Louis, MO, USA), herein referred to as B1. The pH was adjusted to 5.95 with KOH prior to autoclaving for 20 min at 121°C. Thirty-five mL of medium was dispensed into each 15 x 100 mm Falcon Optilux disposable plastic Petri dish (Becton Dickinson & Co, Lincoln Park, NJ, USA) after autoclaving. Ten explants were taken per leaf and a single explant was placed in each dish. The dishes were sealed with two strips of Parafilm (American National Can, Greenwich, CT, USA). Cultures were maintained at 31°C in the dark. A total of 1560 and 1500 dishes were inoculated in the

Table 1. Seed sources and characteristics of populations studied.

Population	Genotypes sampled Exp 1	Exp 2	Germness[1]	Comments
EL 36[2]	4	4	mm	Breeding line; O-type
EL 40[2]	5	5	MM	Pollinator of USH 23
EL 44[2]	5	5	mm	Parent of cultivar; O-type
EL 45/2[2]	5	5	mm	Breeding line; O-type
EL 48[2]	5	5	mm	Breeding line
F1003[3]	5	5	MM	Breeding line
FC 506[4]	5	5	mm	Breeding line; O-type
FC 607[4]	5	5	mm	Breeding line; O-type
FC 701/5[4]	5	5	MM	Breeding line
FC 708[4]	4	5	mm	Breeding line
GWK[5]	5	4	MM	Commercial mangel beet
L53[6]	5	4	MM	High sugar breeding line
OA[7,2]	5	4	MM	Annual CMS tester
SP 6822[2]	5	5	MM	Pollinator of USH 20
SP 6926[8]	5	4	mm	Parent of cultivar; O-type
84M5-20[6]	5	5	MM	Trout pigmented genetic stock
Total	78	75		

1 MM denotes multigerm, mm denotes monogerm.

2 from JW Saunders, East Lansing, MI, USA

3 from DF Cole, Fargo, ND, USA

4 from GA Smith and RJ Hecker, Fort Collins, CO, USA

5 Garton's White Knight from I Linde-Laursen, Roskilde, Denmark

6 from JC Theurer, East Lansing, MI, USA

7 Owen's Annual

8 from G Coe, Beltsville, MD, USA

two experiments, respectively.

Experimental Design and Data Analysis

The experimental design was completely randomized. Sampling was hierarchical and completely nested, i.e. plants within populations, leaves within plants and samples (leaf pieces) within leaves. For all four variables analyzed, the experimental unit was the leaf and the response was calculated as the mean of the samples within the leaf. Data on callus initiation and shoot regeneration were recorded every other day for 10 wk after the experiments were initiated. Only the moist, friable callus described in previous reports (Saunders and Doley, 1986; Saunders and Shin, 1986) was scored as callus; compact callus, which arose from vascular tissue of some explants and did not continue to grow, was ignored. Time to callus was defined as the number of days from explant inoculation to first visual observation of callus. Lag time was defined as the number of days between callus initiation and first visual observation of shoot regeneration. Due to explant or plant death, data are presented from a total of 78 and 75 plants for the two experiments, respectively. Only plants that were sampled in both experiments were included in the combined analyses.

Statistical Analysis System Release 5.18 (SAS Institute, 1985) was used for most analyses. Data were generally unbalanced and handled by the General Linear Models (GLM) procedure. Populations were treated as fixed effects, while plants and leaves were treated as random effects. The two frequency variables displayed variance heterogeneity and were transformed using the arcsine function (Steel and Torrie, 1980). All means presented are actual values, but mean separation was accomplished using least squares marginal means computed by the LSMEANS option of GLM. Least squares means are the values expected had the design been balanced. The PDIFF option of LSMEANS performed all possible t-tests in a manner analogous to using an LSD. To avoid deflation of error terms, populations which gave no response for a variable were not included in the analysis for that variable. Approximate F tests involving synthesized mean squares were necessary in some cases (Satterthwaite, 1946).

Populations were clustered by frequencies of callus production and shoot regeneration from callus in the two experiments, i.e. by four variables. Time to callus and lag time were not included. Clustering was accomplished using the average linkage method of PROC CLUSTER of SAS. The smallest number of clusters which accounted for at least 90% of the variation among populations was chosen.

RESULTS

Leaf disk explants placed on B1 medium began to expand within 24 h. Final explant size, up to 35 times the original area, was usually reached within 1 wk. Callus initiation was first observed after 21 d, but the average time to callus was about 6 wk. The moist, white friable callus appeared to be the same as that reported by Saunders and Doley (1986) and Saunders and Shin (1986). The callus was hormone-autonomous; calli from all 16 populations was capable of sustained growth on hormone-free medium. When shoot regeneration occurred, it was after an average lag time of 2 wk. Blackening of leaf disks did not preclude callus initiation or shoot regeneration. In some cases, rapid explant death occurred; these explants failed to expand and lost all green pigmentation within 2 to 3 d. Rapid explant death is thought to involve moisture and/or heat stress of donor plants.

Since the cultures were incubated in darkness, etiolation of regenerant shoots was common. Shoots were frequently vitreous, but the proportion varied with genotype. Some non-vitreous shoots were readily converted into whole plants in all eleven populations which produced shoots. Since rooting of shoot cultures is routine (Saunders, 1982), the critical step for conversion to plants is the establishment of healthy, non-vitreous shoot cultures from the regenerant shoots. Healthy shoot cultures were sometimes obtained from semi-vitreous shoots, but rarely so from vitreous regenerants.

Reports of extensive bacterial contamination of sugarbeet explants (Hooker and Nabors, 1977; Miedema et al., 1980) have resulted in a general preference for the use of aseptically cultured seedlings as explant sources (Ritchie et al., 1989; Tetu et al., 1987). In our research, contamination of leaf disk explants taken from vegetative one-yr-old greenhouse

grown plants was not a significant problem. Overall contamination rates were less than 1.3% and no cases of systemic bacterial infection were observed. The use of young expanding tissue may have helped to minimize contamination. These results demonstrate that intact vegetative sugarbeet plants are a viable alternative as a source of high quality, uniform explants.

Germplasm Screening

All 16 populations produced at least some callus in the first experiment, and only OA failed to initiate any callus in the second. The overall frequency of explants producing callus was 55.5% and 49.5% for the first and second experiments, respectively, with more than 75% of the plants producing some callus in both (Table 2). Most populations responded similarly in both experiments. Some exceptions included a marked increase in callus initiation by L53 and notable decreases by EL 45/2 and EL 48, both of which had high rankings in the first experiment.

More than half of the genotypes which initiated hormone-autonomous callus regenerated shoots from the callus. Regeneration frequencies ranged from zero for five of the populations to 92.5% for EL 45/2 (Table 3). Overall, 30% of the explants which produced callus regenerated at least one shoot. Regeneration response was very consistent across the two experiments, with the notable exception of SP 6926 where regeneration increased from 24.5% to 53.2%.

Data on shoot number were not recorded, but some populations were noticeably more prolific than others. Some populations such as EL 48 typically produced only a few shoots per Petri dish, while others such as EL 45/2 were capable of producing dozens of shoots. There was an inverse relationship between callus mass and shoot mass. Explants which produced a large callus mass tended to regenerate few shoots, while those producing many shoots had a much smaller callus mass. This partitioning of in vitro biomass was highly dependent on genotype. The incidence of vitreous shoots seemed to increase with prolificacy.

Of the four variables, time to callus was the least consistent across experiments. The

Table 2. Frequency of callus formation from leaf disks on B1, and number of genotypes which produced callus from leaf disks.

Population	Callus frequency(%)			Number of genotypes		
	Exp 1	Exp 2	Mean	N[1]	Exp 1	Exp 2
SP 6926-O	95.9	98.8	97.2 a[2]	178	5/5	4/4
GWK	81.0	98.7	88.8 ab	178	5/5	4/4
EL 36	90.0	74.7	82.4 abc	159	4/4	4/4
EL 45/2	92.9	70.1	81.6 ab	196	5/5	5/5
FC 506	81.6	69.0	75.3 abc	198	5/5	5/5
EL 48	86.0	56.6	71.4 abc	199	5/5	5/5
FC 701/5	64.0	70.1	67.0 bc	197	5/5	5/5
FC 607	69.7	62.0	65.8 bc	199	4/5	4/5
SP 6822	59.8	54.1	56.9 cd	195	5/5	5/5
84M5-20	60.6	45.0	52.8 cde	199	5/5	5/5
EL 40	39.4	40.4	39.9 cde	198	2/5	3/5
FC 708	26.6	16.0	20.7 def	179	2/4	4/5
F1003	26.0	11.0	18.6 ef	199	2/5	2/5
L53	2.0	37.9	15.6 ef	179	1/5	4/4
OA	7.2	0	4.0 f	177	2/5	0/4
EL 44	6.1	1.1	3.7 f	188	2/5	1/5
Mean	55.5	49.5	52.6			
Total				3018	59/78	60/75

[1] Total number of explants sampled over both experiments.

[2] Means in the same column followed by the same letter are not significantly different based on a t-test at $P \leq 0.05$.

Table 3. Frequency of shoot regeneration from callus on B1, and number of genotypes which regenerated shoots from callus.

Population	Regeneration frequency(%)[1]			Number of genotypes		
	Exp 1	Exp 2	Mean	N[2]	Exp 1	Exp 2
EL 45/2	97.8	85.3	92.5 a[3]	160	5/5	5/5
FC 506	67.5	59.4	63.8 b	149	5/5	5/5
FC 607	60.9	62.9	61.8 b	131	4/4	4/4
EL 44	50.0	0	42.9[4]	7	2/2	0/1
SP 6926-0	24.5	53.2	37.6 bc	173	5/5	4/4
SP 6822	24.1	22.6	23.4 c	111	4/5	3/5
FC 701/5	23.4	14.7	18.9 c	132	2/5	2/5
EL 48	14.0	12.5	13.4 c	142	4/5	3/5
FC 708	9.5	6.3	8.1	37	1/2	1/4
GWK	6.2	3.9	5.1 c	158	2/5	2/4
EL 36	6.9	0	3.8 c	131	1/4	0/4
84M5-20	0	0	0	105	0/5	0/5
EL 40	0	0	0	79	0/2	0/3
F1003	0	0	0	37	0/2	0/2
L53	0	0	0	28	0/1	0/4
OA	0	0	0	7	0/2	0/0
Mean	30.9	29.2	30.1			
Total				1587	35/59	29/60

[1] Frequency of shoot regeneration from callus from those explants which produced callus.

[2] Total number of explants which produced callus over both experiments.

[3] Means in the same column followed by the same letter are not significantly different based on a t-test at $P \leq 0.05$.

[4] Means not followed by a letter had non-estimable least squares means and could not be statistically compared to other means.

overall mean differed by 5 d and there was some rearrangement in the rank order of the populations (Table 4). Mean time to callus by FC 506, EL 45/2 and EL 48 was greater than 10 d longer in the second experiment. Time to callus is viewed as the time required by the cells in the leaf disk to develop auxin autonomy. The significantly longer time to callus in the second experiment may be partially due to the increased age of the plants and/or environmentally induced physiological variation.

Population means for lag time were highly consistent across the two experiments (r = 0.90**). Lag time varied from just more than a week for EL 45/2 to one month for SP 6822 (Table 5). Although the callus initiation frequency for EL 44 was quite low, EL 44 and EL 45/2 exhibited a rapid regeneration response distinct from the other populations. The three populations which had the longest lag times all produced very large masses of callus and had only poor to moderate regeneration frequencies.

Although the population responses for the four variables were highly correlated in the two experiments (r = 0.79-0.90), the overall means for three variables changed significantly. In the second experiment, callus initiation was 6% lower, time to callus was 5 d longer and lag time was 1.3 d shorter (Tables 2, 4 and 5, respectively). In addition to differences in plant age, the two environments differed in temperature, light intensity and day length, any one of which may have influenced the altered response.

Interrelationships among the four variables were examined by correlation analysis (Table 6). Populations with high callus initiation frequencies tended to initiate callus more rapidly and have higher shoot regeneration frequencies. Populations with higher shoot regeneration frequencies tended to have shorter lag times. Although genetic parameters were not estimated, the consistent response of the populations in the two experiments suggests that genotype exerts a major influence on in vitro behavior in sugarbeet.

The in vitro performance of the monogerm populations was generally superior to that of the multigerm group. The monogerm material had higher frequencies of callus initiation and shoot regeneration, and when regeneration occurred it was after a shorter

Table 4. Mean time to callus initiation from leaf disks cultured on B1.

Population	Exp 1		Exp 2		Mean	
	N[1]	Time(days)	N	Time(days)	N	Time(days)
SP 6926-O	94	35.4	79	38.2	173	36.7 a[2]
GWK	81	34.0	77	40.0	158	36.9 ab
EL 48	86	34.7	56	45.2	142	38.8 abc
FC 506	80	33.4	69	45.0	149	38.8 abc
SP 6822	58	40.1	53	39.6	111	39.9 bd
EL 45/2	92	37.5	68	49.0	160	42.4 ab
84M5-20	60	40.9	45	47.4	105	43.7 cd
FC 607	69	42.0	62	46.7	131	44.2 ab
FC 701/5	64	43.5	68	45.6	132	44.6 b
EL 40	39	47.0	40	50.9	79	49.0[3]
OA	7	49.0	0	--	7	49.0
EL 36	72	53.4	59	53.6	131	53.5 d
F1003	26	53.8	11	52.8	37	53.5 d
L53	2	44.0	26	54.7	28	53.9
EL 44	6	52.7	1	65.0	7	54.5
FC 708	21	59.0	16	56.7	37	58.0
Mean		40.5		45.9		43.0
Total	857		730		1587	

[1] Number of explants which produced callus

[2] Means in the same column followed by the same letter are not significantly different based on a t-test at $P \leq 0.05$.

[3] Means not followed by a letter had non-estimable least squares means and could not be statistically compared to other means.

Table 5. Mean lag period between callus initiation and shoot production from leaf disks cultured on B1.

Population	Exp 1		Exp 2		Mean	
	N[1]	Time(days)	N	Time(days)	N	Time(days)
EL 44	3	7.3	0	--	3	7.3[2]
EL 45/2	90	8.0	58	6.9	148	7.6 a[3]
FC 506	54	16.8	41	15.2	95	15.4 b
EL 36	5	15.6	0	--	5	15.6
FC 607	42	17.1	39	11.7	81	16.2 b
FC 701/5	15	19.6	10	14.2	25	17.4
SP 6926-O	23	17.9	42	17.6	65	17.7 b
FC 708	2	21.0	1	12.0	3	18.0 ab
GWK	5	25.2	3	21.3	8	23.7
EL 48	12	26.4	7	27.4	19	26.8
SP 6822	14	30.2	12	30.2	26	30.2
Mean		15.3		14.0		14.7
Total	265		213		478	

[1] Number of explants which regenerated shoots from callus.

[2] Means not followed by a letter had non-estimable least squares means and could not be statistically compared to other means.

[3] Means in the same column followed by the same letter are not significantly different based on a t-test at $P \leq 0.05$.

Table 6. Correlations among population means for four parameters of in vitro behavior.

	Regeneration (%)	Time to callus (d)	Lag time (d)
Callus (%)	0.56**	-0.69**	0.12
Regeneration (%)		-0.46**	-0.48*
Time to callus (d)			-0.39

*, ** Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

lag period (Table 7). Genetic factors conditioning in vitro response may be localized in the chromosomal region controlling the monogerm trait, or the germness locus could exert a pleiotropic effect on in vitro behavior.

Variability Within and Between Populations

Significant differences were found both among and within populations for all four variables in both experiments (Tables 8, 9, 10 and 11). In addition to the observed quantitative variation, qualitative effects on callus initiation and shoot regeneration were also observed within populations. In EL 40, two plants produced callus at high frequency (95 to 100%) in both experiments, while the other three plants failed to produce callus. The same pattern occurred in F1003. In FC 701/5, one of the five plants accounted for 92% of the regenerating calli, and in FC 607, four plants regenerated shoots in both experiments, while the fifth plant never initiated callus. The same patterns of callus initiation were observed when explants from these same plants were tested on hormone-free medium (Doley and Saunders, 1989). Such bimodal responses indicate that genes with major effects on callus initiation and shoot regeneration are segregating in some sugarbeet populations. This extensive genetic variation should allow selection for in vitro behavior both within and between populations.

In Vitro Response Types

The concept of in vitro response types is proposed here as a tool for the classification of the members of a germplasm pool into discrete groups, each of which may require a different protocol for optimum shoot regeneration. It was obvious from a cursory examination of the data that similarities existed between some of the populations with regard to their in vitro profiles. When the relationships among the 16 populations analyzed for in vitro behavior were evaluated using cluster analysis, the populations fell into seven semi-discrete clusters, hereafter referred to as response types 1-7 (Figure 1; Table 12). Response types 1 and 2 respond well to the B1 regeneration system which was utilized in this research, while the regeneration responses of types 3, 4 and 5 populations may not be optimal under these conditions. Types 3 and 4 tended to rapidly initiate large calli and produce only a few shoots

Table 7. Comparison of in vitro behavior of monogerm populations vs multigerm populations.

	Multigerm Populations	t-test	Monogerm Populations
Callus (%)			
Experiment 1	43.5	**	68.3
Experiment 2	44.2	NS	56.9
Combined	43.8	NS	62.6
Regeneration (%)			
Experiment 1	15.1	**	43.0
Experiment 2	12.1	*	40.9
Combined	13.6	NS	41.9
Time to callus (d)			
Experiment 1	43.4	NS	41.5
Experiment 2	49.0	--[1]	48.6
Combined	46.3	NS	45.0
Lag Time (d)			
Experiment 1	24.7	*	16.7
Experiment 2	19.6	--	14.6
Combined	22.4	NS	15.7

NS, *, ** Not significant or significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

[1] Indicates comparison involved non-estimable least squares mean.

Table 8. Analysis of variance of frequency of callus production from leaf disks cultured on B1.

	Exp 1		Exp 2	
Source	df	MS(arcsine)	df	MS(arcsine)
Populations	15	2.263**	15	1.802**
Plants/Population	62	0.341**	59	0.344**
Leaves/Plant	78	0.059	74	0.039

** Significant at $P \leq 0.01$.

Table 9. Analysis of variance of frequency of shoot regeneration from callus on B1.

Source	Exp 1 df	Exp 1 MS(arcsine)	Exp 2 df	Exp 2 MS(arcsine)
Populations	10	1.858**	8	1.353**
Plants/Population	36	0.232**	32	0.243**
Leaves/Plant	44	0.031	37	0.041

** Significant at $P \leq 0.01$.

Table 10. Analysis of variance of time to callus initiation from leaf disks cultured on B1.

Source	Exp 1 df	Exp 1 MS	Exp 2 df	Exp 2 MS
Populations	15	390.7**	14	283.8*
Plants/Population	43	86.0**	45	128.6**
Leaves/Plant	53	17.8	51	20.8

*, ** Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table 11. Analysis of variance of lag period between callus initiation and shoot regeneration from leaf disks cultured on B1.

Source	Exp 1 df	Exp 1 MS	Exp 2 df	Exp 2 MS
Populations	10	240.3**	8	260.4*
Plants/Population	24	75.7**	20	81.6**
Leaves/Plant	23	18.6	19	14.7

*, ** Significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

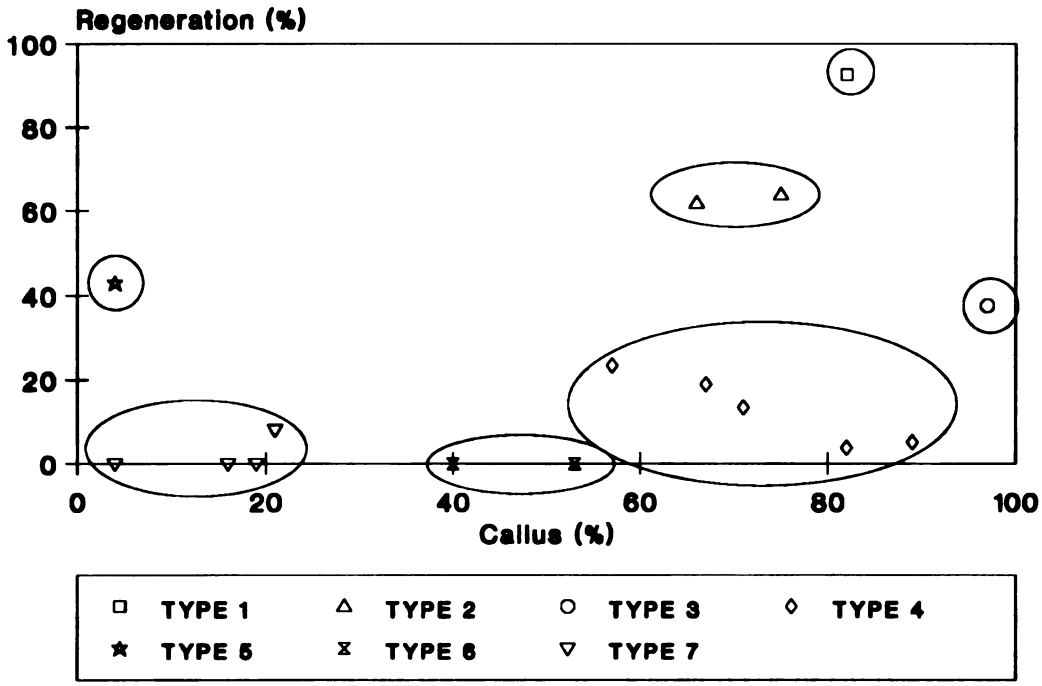


Figure 1. Graph of population means by in vitro response type for frequencies of callus initiation and shoot regeneration.

Table 12. In vitro response types, including member populations and their characteristics, generated by clustering populations by frequency of callus production and shoot regeneration.

Response type	Populations	Callus (%)	Regeneration (%)
1	EL 45/2	Good[1]	Excellent
2	FC 506, FC 607	Good	Good
3	SP 6926-O	Excellent	Moderate
4	EL 36, EL 48, FC 701/5, GWK, SP 6822	Good	Poor
5	EL 44	Poor	Moderate
6	EL 40, 84M5-20	Moderate	Poor
7	F1003, FC 708, OA, L53	Poor	Poor

[1] Excellent = > 90%, Good = 55-90%, Moderate = 25-55%, Poor = < 25%.

after a long lag period. The five populations which did not regenerate shoots in either of the samplings make up the type 6 and type 7 clusters. For these populations, incubation of leaf disks on B1 is certainly sub-optimal, but they may be capable of one-step shoot regeneration if the appropriate medium were employed.

DISCUSSION

Reproducible, high-frequency shoot regeneration from hormone-autonomous callus was observed in several sugarbeet populations, most noticeably EL 45/2, FC 506 and FC 607 (Table 3). Plants from these same populations were also found to be good regenerators when explants were derived from in vitro shoot cultures (Saunders and Shin, 1986). One-step shoot regeneration as described here was applicable to a wide range of germplasm; plants representing 11 of the 16 populations tested regenerated shoots from callus. Although plants of EL 45/2 were the most prolific regenerators, the high incidence of vitreousness detracts from their potential for in vitro genetic manipulations.

Overall favorable response rates coupled with minimal levels of contamination demonstrated that greenhouse grown vegetative sugarbeet plants could be sources of high quality uniform explants. Use of whole plants as explant donors has distinct advantages in breeding programs. In vitro behavior of breeding clones could be tested using available plant material without the need to initiate shoot cultures. Breeding efficiency could be enhanced by using regenerant plants as propagules to evaluate qualitative traits, such as some forms of disease resistance, where bias due to somaclonal variation would be minimal.

Extensive genotypic variation was found for in vitro behavior in the populations evaluated. The presence of significant variation both within and between populations, and the consistent response of the populations in the two experiments, suggested that genetic gain from selection for in vitro traits in sugarbeet should be possible. Effective selection for in vitro performance has been practiced in alfalfa (Bingham et al., 1975), maize (Beckert and Qing, 1984), tomato (Koornneef et al., 1986) and wheat (Petolino et al., 1988). In sugarbeet, the largely quantitative variation may be conditioned by qualitative factors as well. Populations

with significant within population variability could provide good starting material to evaluate the inheritance of in vitro behavior in sugarbeet, and might eventually allow isolation of genes which condition callus initiation and shoot regeneration.

The superior in vitro response of the monogerm populations over the multigerm populations (Table 7) is consistent with the results of Saunders and Shin (1986). All five populations which failed to regenerate shoots were multigerm, but some multigerm individuals had regeneration rates comparable to the best monogerm genotypes. This may be a reflection of a narrower genetic base in the monogerm material, but could also be due to linkage or pleiotropy.

In vitro response types are proposed as a mechanism to efficiently optimize shoot regeneration from all members of a germplasm pool. Each response type might require a specific growth regulator regime to promote optimal levels of shoot regeneration. In theory, once the specific medium required for each response type is identified, it should be possible to screen germplasm on B1, assign each to one of the response groups and to effectively predict the medium to be employed to optimize regeneration. Future research endeavors are necessary to determine the validity of the proposed concept.

We hypothesize that shoot regeneration from hormone-autonomous callus of sugarbeet involves complementation between endogenous auxin physiology and exogenous cytokinin, and is therefore compatible with the classic auxin/cytokinin ratio model (Skoog and Miller, 1957). Thus, genotypes from response types 1 and 2 are postulated to have an endogenous auxin physiology which when combined with 1 mg/L BA produces an auxin/cytokinin ratio in the range favorable for shoot regeneration. Genotypes in response types 3 to 7 may require higher concentrations of BA and/or additional growth regulators for optimal shoot regeneration.

The response of EL 44 provides some evidence that callus initiation and shoot regeneration may be partially independent events. Explants from EL 44 initiated callus at a low frequency (3.7%), but these rare calli had good regeneration potential (42.9%). In contrast

to some of the non-regenerating populations, the problem with EL 44 seems to lie with callus initiation rather than shoot regeneration. Independent inheritance of callus initiation and plant regeneration has been reported for wheat anther culture (Deaton et al., 1987; Lazar et al., 1984).

Some of the unexplained variability encountered in this research may have been due to gradients of endogenous growth regulators within the sampled leaves, as well as temporal variability due to circadian rhythms. Within leaf gradients of indole-3-acetic acid have been reported in orchardgrass (Wenck et al., 1988), and circadian rhythms have been shown to influence endogenous levels of cytokinin in carrot (Stiebeling and Neumann, 1987). In *Lolium multiflorum*, Joarder et al. (1986) found that leaf segment position influenced the frequency of callus initiation. In our research, no attempt was made to standardize the time of day when leaves were harvested or to examine the response of explants from various positions on the leaf.

Interest in regeneration systems involving leaf disks has been enhanced by successful *Agrobacterium*-mediated transformation of leaf disk explants (Horsch et al., 1985). Sugarbeet is susceptible to infection by *A. tumefaciens* (Huizing et al., 1988; Krens et al., 1988), and the sugarbeet leaf disk regeneration system appears to be a suitable candidate for incorporation into a transformation protocol. Because of its applicability to a wide range of germplasm, this system of regenerating shoots from callus is also appealing for somatic cell selection within elite breeding material.

CHAPTER 2

Genotype x Growth Regulator Interaction for Callus Initiation and Shoot Regeneration in Sugarbeet (*Beta vulgaris* L).

SUMMARY

Leaf disk explants of many sugarbeet (*Beta vulgaris* L.) genotypes are capable of one-step shoot regeneration from callus (i.e. without subculture) when placed on Murashige and Skoog (MS) medium containing cytokinin. Genotype x medium (G x M) interaction was investigated in 21 sugarbeet genotypes, representing 12 germplasm sources, as part of efforts to expand the range of sugarbeet germplasm which can be regenerated from callus. We investigated the effects of N^6-benzyladenine (BA) concentration and inclusion of naphthaleneacetic acid (NAA) and/or gibberellic acid (GA_3) on callus initiation and shoot regeneration. An MS medium with 1.0 mg/L BA (B1) was used as the control in all experiments. Significant G x M interactions were found in all seven experiments, including interactions with BA, NAA and GA_3. Although frequency of callus initiation was somewhat independent of BA concentration, genotypes varied in the BA concentration needed for optimal shoot regeneration. Optimal levels of BA for shoot regeneration were above 1.0 mg/L for four of eight genotypes tested. Some genotypes had enhanced rates of shoot regeneration from callus when B1 medium was supplemented with low concentrations of NAA (0.01-0.1 mg/L), but for most genotypes, NAA (0.1 mg/L) both inhibited and delayed callus formation and reduced regeneration frequency. Addition of GA_3 (1.0 to 3.0 mg/L) to B1 medium significantly increased shoot number per leaf disk, up to 3-fold in some genotypes, and significantly inhibited root regeneration from callus. The frequency of significant G x M interactions detected in this research suggests that a single medium is inadequate when

screening sugarbeet germplasm for the ability to regenerate shoots from callus. Manipulation of G x M interaction should lead to protocols for optimizing regeneration within in vitro response types.

Abbreviations

BA, N^6-benzyladenine
GA_3, gibberellic acid A_3
MS, Murashige and Skoog medium
NAA, 1-naphthaleneacetic acid

INTRODUCTION

In a breeding program, efficient application of molecular and cellular technologies may require that the entire germplasm pool be amenable to in vitro manipulation. However, in many crop species, only a subset of the breeding pool is generally capable of plant regeneration from cell cultures (Hanzel et al., 1985; Malmberg, 1979; Ozawa and Komamine, 1989; Saunders and Bingham, 1972; White, 1984). Genetic transformation and somatic cell selection would be more efficient if accomplished within elite breeding lines, but this strategy requires that plants can be regenerated from cells of such germplasm.

Genotype x medium (G x M) interaction describes a nonadditive relationship between the in vitro response of germplasm to different tissue culture media. If variability in shoot regeneration frequency is due to G x M interaction, different genotypes may require different media for optimum regeneration. G x M interaction for regeneration has been reported in alfalfa (Nagarajan et al., 1986; Saunders and Bingham, 1975), barley (Dunwell, 1981; Hanzel et al., 1985; Powell and Dunwell, 1987), petunia (Skvirsky et al., 1984), rice (Quimio and Zapata, 1990) and wheat (Mathias and Simpson, 1986), and might be an important tool for broadening the range of genotypes within a species that can be regenerated.

Application of cell culture and recombinant DNA techniques to genetic improvement of sugarbeet (*Beta vulgaris* L.) has been limited by low regeneration frequencies. Several recent reports demonstrated that shoot regeneration from callus can be achieved over a wide range of sugarbeet genotypes (Saunders and Shin, 1986; Chapter 1). In Chapter 1, plants from

11 of 16 sugarbeet populations sampled regenerated shoots when leaf disks were placed on MS medium with 1 mg/L BA (B1 medium). Significant variation was reported for four parameters that characterize sugarbeet regeneration from callus: callus initiation frequency, time to callus, shoot regeneration frequency, and lag time between callus initiation and shoot regeneration. Although many sugarbeet genotypes were regenerated from callus, shoot regeneration frequencies varied by genotype from poor to excellent, and a portion of those tested failed to regenerate.

Jarl and Bornman (1986) reported G x M interaction for callus growth in sugarbeet, and suggested that this may partially explain the apparent recalcitrance of the crop in vitro. Further evidence of G x M interaction in sugarbeet was reported by Doley and Saunders (1989). They observed shoot regeneration from callus of three genotypes of the population L53 when leaf disk explants were incubated on hormone-free medium. These same plants failed to regenerate shoots in two experiments utilizing B1 medium (Chapter 1). Thus, different sugarbeet genotypes may require different media for optimal shoot regeneration.

Two unusual features characterize the sugarbeet regeneration system: (1) One-step (i.e. without subculture) callus production and subsequent shoot regeneration, and (2) hormone autonomy. Throughout this research, various media were explored with the intent of maintaining these two attributes of the system. The objectives of this research were: (1) to assess the magnitude of G x M interaction for in vitro performance in several sugarbeet genotypes on a variety of media, and (2) to elucidate the medium components that might enhance the in vitro performance of specific sugarbeet genotypes.

MATERIALS AND METHODS

Plant Material

Sugarbeet plants used as donors of leaf explants were grown in soil in 15 cm plastic pots in a greenhouse without supplemental lighting or in 17 cm plastic pots in a controlled environment chamber (CEC) at 25°C under a 12 h photoperiod from cool white fluorescent bulbs (100 to 200 $\mu Em^{-2}s^{-1}$). The soil used was a 2:2:1:1 mixture of Baccto professional

planting mix (Michigan Peat Co, Houston, TX, USA), a local greenhouse mix, vermiculite and perlite, respectively. The local greenhouse mix consisted of a 5:3:2 mixture of field soil of variable origin, peat and sand, respectively. Plants were fertilized weekly with 200 mL Peters 20-20-20 water soluble commercial nutrient mix and monthly with Snyder's (1974) nutrient formulation.

During the course of these investigations, the experimental procedure evolved from the use of greenhouse grown plants to the use of shoot culture derived ramets (Saunders, 1982) grown in the CEC. Three types of plant material were evaluated: plants derived from seed and grown in the greenhouse, plants micropropagated by shoot culture and grown in the greenhouse, and micropropagated plants grown in the CEC.

The genotypes evaluated were chosen on the basis of diversity for in vitro performance. With the exceptions of BV-0 and REL-1, the genotypes were individuals isolated from the following breeding populations: EL 36, EL 44, EL 45/2, EL 48 and SP 6822 (JW Saunders, East Lansing, MI, USA); FC 607-O, FC 701/5 and FC 708 (GA Smith and RJ Hecker, Fort Collins, CO, USA); 'Garton's White Knight' (GWK)(I Linde-Laursen, Roskilde, Denmark); and SP 6926-O (G Coe, Beltsville, MD, USA). BV-0 (TA Thorpe, Calgary, Alberta, Canada) is an individual isolated from 'Primahill', and REL-1 (JW Saunders, East Lansing, MI, USA) is a clone bred and selected for superior regeneration ability from callus and suspension cultures.

Culture Procedures

The seven experiments were initiated over a period of 1.5 yr with plants of variable age and size between experiments, but uniform within experiments. Partially expanded leaves, varying in length from 3.5 to 11 cm, per plant were used as the source of explants. Detached leaves were surface sterilized with two 20 min soakings in 15% commercial hypochlorite bleach solution with 0.01% sodium laurylsulfate, followed by six rinses with sterile distilled water. Leaf disk explants were cut from leaf blade tissue with a No. 3 cork borer (7 mm i.d.), except in Exp 1 where square explants varying in size from 4 to 5 mm^2 were cut with a

scalpel.

The culture medium consisted of MS (Murashige and Skoog, 1962) salts, 3% (w/v) sucrose, 100 mg/L *myo*-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, 1.0 mg/L thiamine·HCl, 0.9% (w/v) Difco Bacto agar (Difco Laboratories, Detroit, MI, USA) and various concentrations of BA, NAA and GA_3 (all from Sigma Chemical Co, St Louis, MO, USA). The pH was adjusted to 5.95 with KOH prior to autoclaving for 20 min at 121°C. All growth regulators were added prior to autoclaving, except in Exp 6 where GA_3 for 2 treatments was filter sterilized using a 0.22 µ Acrodisc disposable 25 mm syringe filter (Gelman Sciences, Ann Arbor, MI, USA). B1 medium (1 mg/L BA) was used as the control. Thirty-five mL of medium was dispensed into each 15 x 100 mL Falcon Optilux disposable plastic Petri dish (Becton Dickinson & Co, Lincoln Park, NJ, USA) after autoclaving. The number of explants taken per leaf varied (see Table 1) and a single explant was placed in each dish. The dishes were sealed with two strips of Parafilm (American National Can, Greenwich, CT, USA). Cultures were maintained at 28°C under dim light provided by cool-white fluorescent bulbs (20-40 $\mu Em^{-2}s^{-1}$) in a controlled environment room.

Experimental Designs

The experimental designs were completely randomized genotype x medium factorials, with hierarchical and completely nested sampling of plants within genotypes, leaves within plants and samples within leaves. Table 1 summarizes some aspects of the experimental designs, and the genotypes sampled are listed in Table 2. The details of the seven experiments were as follows:

Experiment 1. Eight genotypes (EL 36-93, EL 45/2-96, EL 48-99, FC 607-O-92, FC 701/5-94, FC 708-91, GWK-97 and SP 6822-91) were evaluated over a concentration gradient of BA (0, 0.1, 0.3, 1.0, 3.0 or 10 mg/L). Plants were grown from seed and maintained in the greenhouse, and all were part of germplasm previously evaluated on B1 medium (Chapter 1). Explants were taken in August 1987. Because of variability in leaf size, two to six partially expanded leaves were sampled from each genotype. Samples per leaf varied from six (one set

Table 1. Summary of some components of experimental design for the seven experiments to evaluate genotype x growth regulator interaction in sugarbeet.

Experiment	Variables	Media[1]	Genotypes(G)[2]	Plants(P)/G	Leaves/(P)	Environment[3]	Source[4]
1	[BA]	6	8	1	2-6	GH	seed
2	[NAA]	2	4	1	2	GH	seed
3	[NAA]	6	3	1	4	GH	SC
4	[NAA]	6	4	1	3	GH	SC
5	[GA_3]	3	3	1	3	GH	seed
6	[GA_3]	5	3	2	2	CEC	SC
7	[BA],[NAA],[GA_3]	8	6	2	3	CEC	SC

[1] Total number of different media used in each experiment.

[2] See Table 2 for list of specific genotypes used in each experiment.

[3] Environment where explant donor plants were maintained; GH = greenhouse; CEC = controlled environment chamber.

[4] Source of explant donor plants; SC = micropropagated by shoot culture.

Table 2. Genotypes sampled in the seven experiments to evaluate genotype x growth regulator interaction in sugarbeet.

Genotype	Experiment 1	2	3	4	5	6	7
BV-0			X				
EL 36-93	X						
EL 36-98*				X			
EL 44-90							X
EL 44-90*			X				
EL 45/2-96	X	X			X		
EL 48-97*				X			
EL 48-99	X	X					
FC 607-O-20						X	X
FC 607-O-92	X	X			X		
FC 701/5-94	X				X		
FC 701/5-94*				X			
FC 701/5-116						X	X
FC 708-91	X						
GWK-93*							X
GWK-97	X						
REL-1			X			X	X
SP 6822-90*				X			
SP 6822-91	X						
SP 6926-O-3							X
SP 6926-O-94		X					

* indicates genotype was a regenerant from callus.

of treatments) to 66 (eleven sets), and a total of 20 samples per genotype was initiated on each treatment. The experiment was terminated after 14 wk.

Experiment 2. Four genotypes (EL 45/2-96, EL 48-99, FC 607-O-92 and SP 6926-O-94) were evaluated on B1 with NAA at 0 or 0.1 mg/L. Plants were derived from seed and maintained in the greenhouse, and all were previously evaluated on B1 medium (Chapter 1). Explants were taken in July 1987. Two leaves per plant and 12 explants per leaf were sampled, resulting in six treatment sets per leaf. The experiment was terminated after 14 wk.

Experiment 3. Three genotypes (BV-0, EL 44-90* and REL-1) were evaluated on B1 with a concentration gradient of NAA (0, 0.01, 0.03, 0.1, 0.3 or 1.0 mg/L). Plants were derived from shoot cultures and maintained in the greenhouse. Explants were taken in June 1988. Four leaves per plant and 12 explants per leaf were sampled, resulting in two treatment sets per leaf. The experiment was terminated after 10 wk.

Experiment 4. Four genotypes (EL 36-98*, EL 48-97*, FC 701/5-94* and SP 6822-90*) were evaluated on B1 with a concentration gradient of NAA (0, 0.01, 0.03, 0.1, 0.3 or 1.0 mg/L). Plants were derived from shoot cultures and maintained in the greenhouse, and all were regenerants from a previous evaluation (Chapter 1). Three leaves per plant and 12 explants per leaf were sampled, resulting in two treatment sets per leaf. The experiment was terminated after 10 wk.

Experiment 5. Three genotypes (EL 45/2-96, FC 607-O-92 and FC 701/5-94) were evaluated on B1 with 0, 1.0 or 3.0 mg/L GA_3. Plants were derived from seed and maintained in the greenhouse, and all were previously evaluated on B1 medium (Chapter 1). Explants were taken in September 1987. Three leaves per plant and six explants per leaf were sampled resulting in two treatment sets per leaf. The experiment was terminated after 14 wk.

Experiment 6. Three genotypes (FC 607-O-20, FC 701/5-116 and REL-1) were evaluated on B1 with GA_3 at concentrations of 0, 1.0 or 3.0 mg/L autoclaved or 0.1 or 1.0 mg/L filter sterilized. Plants were derived from shoot cultures and maintained in the CEC. Two plants per genotype, two leaves per plant and ten explants per leaf were sampled, resulting in two

treatment sets per leaf. The experiment was terminated after 10 wk.

Experiment 7. Six genotypes (EL 44-90, FC 607-O-20, FC 701/5-116, GWK-93*, REL-1 and SP 6926-O-3) were evaluated with a 2^3 factorial involving 1.0 or 3.0 mg/L BA, 0 or 0.1 mg/L NAA and 0 or 1.0 mg/L GA_3. Plants were derived from shoot cultures and maintained in the CEC. Two plants per genotype, three leaves per plant and eight explants per leaf were sampled. A single set of treatments was derived from each leaf, and each leaf was considered a replication. Individual dishes in the experiment were terminated 3 wk after shoot regeneration was observed. Dishes without callus were terminated after 10 wk, and those which initiated callus but did not regenerate shoots were terminated 8 wk after callus initiation.

Data Analyses

Data on callus initiation and shoot regeneration were recorded weekly until the experiments were terminated. The term callus will be used to describe friable callus which is typically hormone-autonomous, in contrast to compact callus which is typically hormone-dependent. Time to callus was defined as the number of days from initiation of the experiment to visual observation of friable callus. Lag time was defined as the number of days between initiation of friable callus and shoot regeneration. Shoot regeneration from compact callus did not occur. Number of shoots regenerated per explant was determined at the termination of most experiments, except in Exp 7 where they were counted 3 wk after shoot regeneration was first observed to provide a more valid comparison of factors effecting shoot number. Each shoot was judged for quality, with quality shoots defined as having relatively normal appearance, i.e. not developmentally deformed or vitreous, with a high probability of resulting in a healthy plantlet.

In some experiments data on the following variables were also recorded: frequency of initiation of compact callus, frequency of root regeneration from friable callus, and lag time between initiation of friable callus and root regeneration.

Statistical Analysis System (SAS Institute, 1985) was used for most analyses. Data were generally unbalanced and handled by the General Linear Models (GLM) procedure.

Genotypes and media were treated as fixed effects, while plants, leaves and samples were treated as random effects. The experimental unit for all variables was the leaf and response frequencies were calculated as the mean of the samples within the leaf. All frequency variables displayed variance heterogeneity and were transformed using the arcsine function (Steel and Torrie, 1980). All means presented are actual values, but mean separation was accomplished using least squares marginal means computed by the LSMEANS option of GLM. Least squares means are the values expected had the design been balanced. Mean separation was accomplished using the PDIFF option of LSMEANS which performs all possible t-tests in a manner analogous to using an LSD. Approximate F tests involving synthesized mean squares were necessary in some cases (Satterthwaite, 1946).

RESULTS

Significant G x M interactions were detected for some of the response parameters in each of the seven experiments. Time to callus and frequencies of callus initiation and shoot and root regeneration were each influenced by G x M interactions in three experiments. Lag time from callus initiation to shoot regeneration was the only variable not affected by G x M interactions.

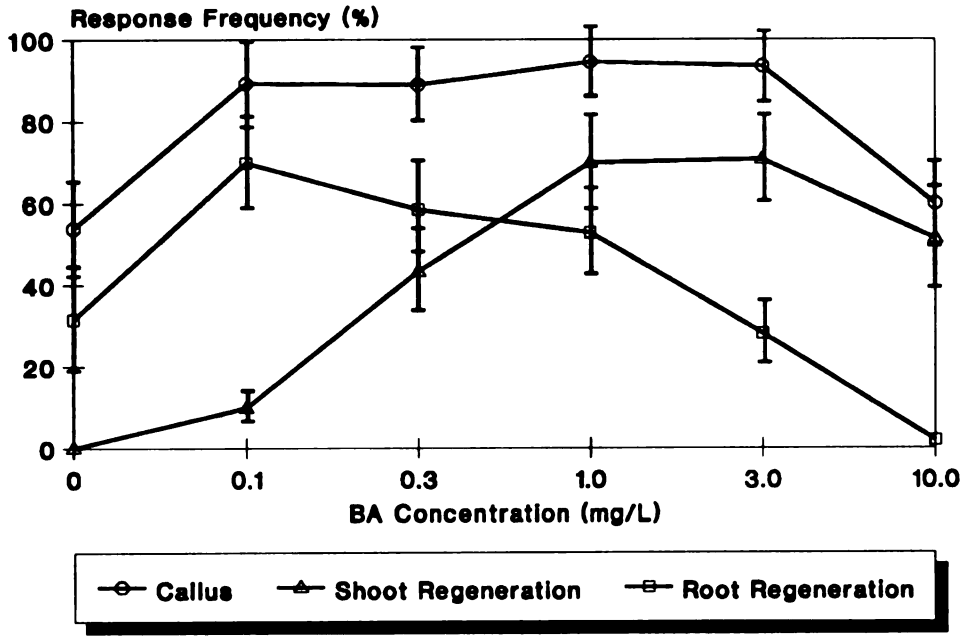
Effects of BA

Previous studies (Chapter 1) demonstrated that there was a diversity of in vitro response types among sugarbeet populations when leaf disk explants were placed on MS medium containing 1 mg/L BA (B1). In Exp 1, significant differences among the eight genotypes and the six BA concentrations were found for callus frequency, shoot regeneration frequency, time to callus and lag time. All eight genotypes initiated callus and subsequently regenerated shoots and roots.

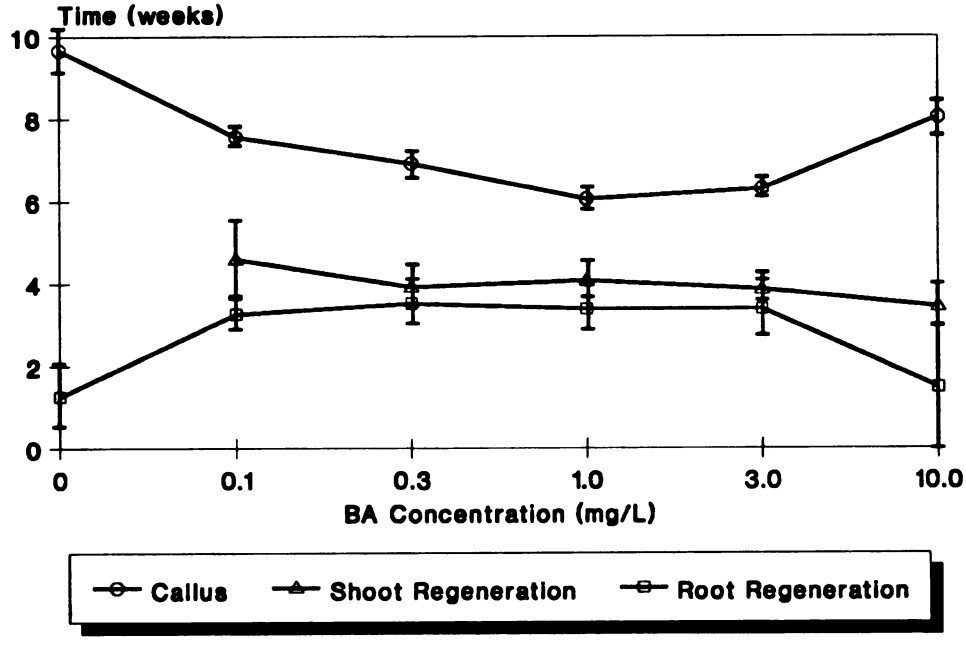
The intermediate four BA concentrations (0.1, 0.3, 1.0 and 3.0 mg/L) resulted in the induction of similar mean levels of callus frequency averaged over the eight genotypes (Figure 1a). The frequency of callus production on hormone-free medium (53.8%) demonstrated that high levels of callus initiation were possible without exogenous cytokinin. In contrast, the

Figure 1. The effect of BA concentration on (a) Mean callus initiation, shoot regeneration and root regeneration, and (b) time to callus, lag time to shoot regeneration and lag time to root regeneration summed over eight genotypes; and on (c) callus initiation frequencies, (d) time to callus, (e) shoot regeneration frequencies, and (f) root regeneration frequencies for five genotypes (EL 45/2-96, EL 49-99 and SP 6822-91 are not shown)(Exp 1). Vertical bars represent ± SE when larger than symbol.

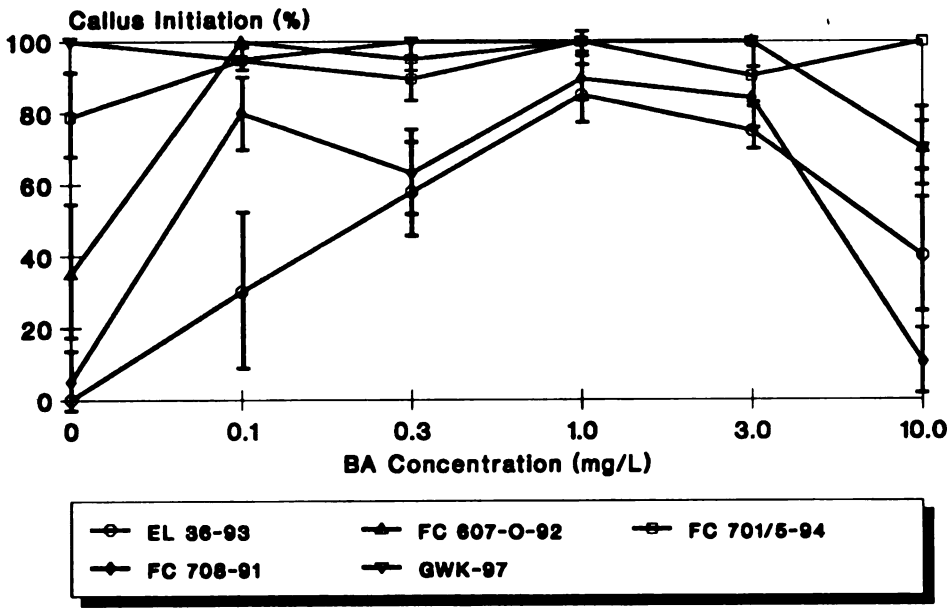
A


Response Frequency (%)
100
80
60
40
20
0
0
0.1
0.3
1.0
3.0
10.0
BA Concentration (mg/L)
Callus
Shoot Regeneration
Root Regeneration

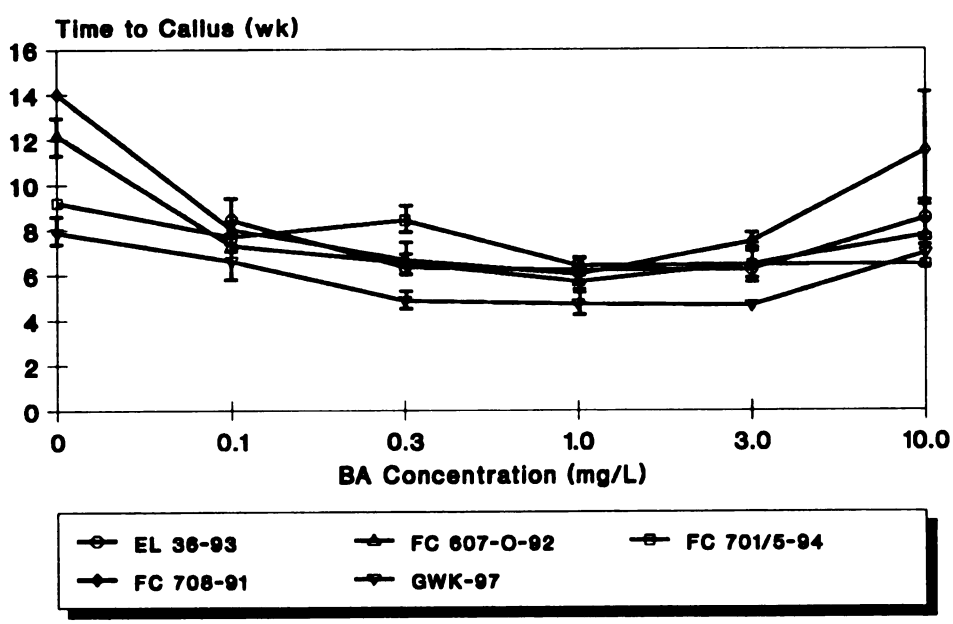
B


Time (weeks)
10
8
6
4
2
0
0
0.1
0.3
1.0
3.0
10.0
BA Concentration (mg/L)
Callus
Shoot Regeneration
Root Regeneration

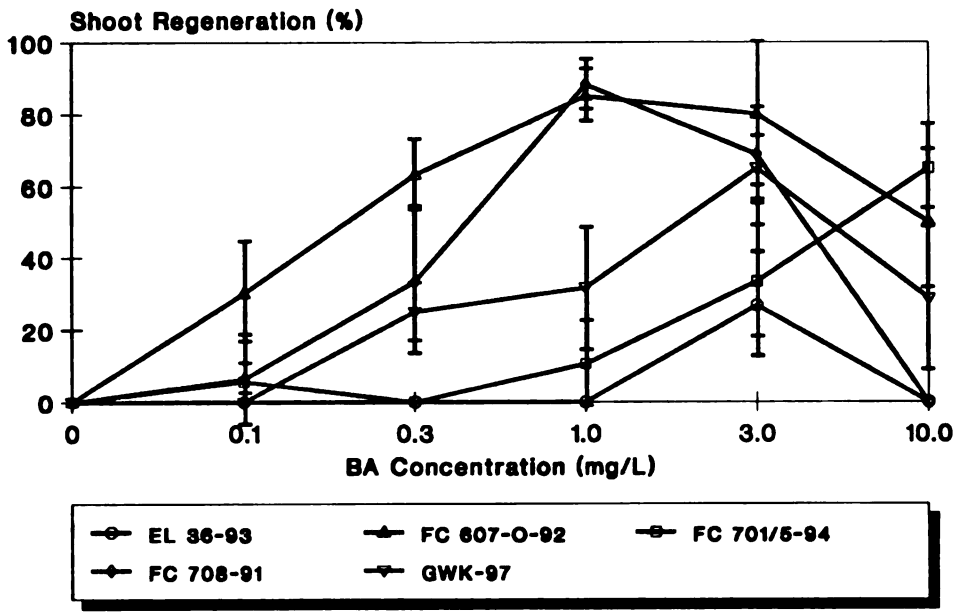
C


Callus Initiation (%)
100
80
60
40
20
0
0
0.1
0.3
1.0
3.0
10.0
BA Concentration (mg/L)
EL 36-93
FC 607-O-92
FC 701/5-94
FC 708-91
GWK-97

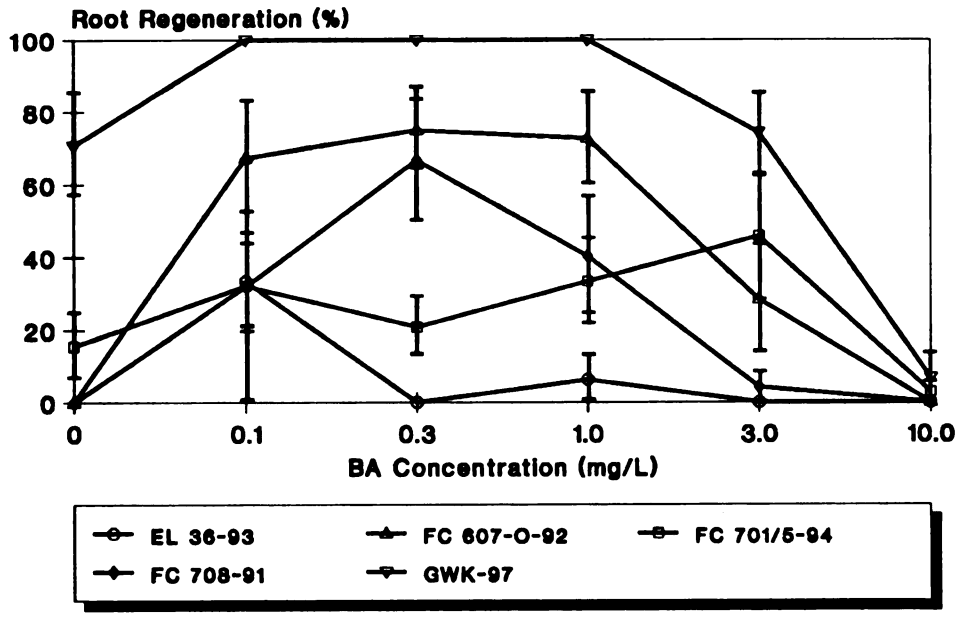
D


Time to Callus (wk)
16
14
12
10
8
6
4
2
0
0
0.1
0.3
1.0
3.0
10.0
BA Concentration (mg/L)
EL 36-93
FC 607-O-92
FC 701/5-94
FC 708-91
GWK-97

E


Shoot Regeneration (%)
100
80
60
40
20
0
0
0.1
0.3
1.0
3.0
10.0
BA Concentration (mg/L)
EL 36-93
FC 607-O-92
FC 701/5-94
FC 708-91
GWK-97

F


Root Regeneration (%)
100
80
60
40
20
0
0
0.1
0.3
1.0
3.0
10.0
BA Concentration (mg/L)
EL 36-93
FC 607-O-92
FC 701/5-94
FC 708-91
GWK-97

level of BA in the medium had a significant effect on the frequency of calli which regenerated shoots (Figure 1a). The overall optimum concentration was between 1.0 and 10.0 mg/L, with the greatest response at 1.0 and 3.0 mg/L. No shoot regeneration was observed on hormone-free medium. In contrast to shoot regeneration, root regeneration from callus (Figure 1a) was enhanced at lower levels of BA (0.1 to 1.0 mg/L). Direct root regeneration from leaf disk explants was occasionally observed, particularly on media with ≤ 1.0 mg/L BA (data not shown).

The chronology of the in vitro events discussed above is depicted in Figure 1b. Callus initiation was most rapid at 1.0 and 3.0 mg/L BA, and was slower as BA concentrations approached either 0 or 10.0 mg/L. The lag time between callus initiation and shoot regeneration was slightly longer at higher levels of BA, while the lag time to root initiation was significantly shorter on either 0 or 10.0 mg/L BA, the same two concentrations which delayed callus initiation.

G x M interaction was significant in Exp 1 for callus initiation and shoot regeneration frequencies and time to callus, but not for lag time. Since some genotypes had similar response curves, only five of the eight genotypes are shown in Figure 1c-f. The four genotypes with the lowest rates of callus initiation (i.e. EL 36-93, EL 45/2-94, FC 708-91 and SP 6822-91) on hormone-free medium also had the lowest responses on 10.0 mg/L BA (Figure 1c). The three genotypes (i.e EL 48-99, FC 701/5-94 and GWK-97) with high rates of callus initiation on hormone-free medium also had high rates of callus initiation on 10.0 mg/L BA. The ability to initiate callus on hormone-free medium may be related to the ability to tolerate high levels of BA.

G x M interaction for time to callus mainly involved differences in the delay of callus initiation on 0 and 10.0 mg/L BA (Figure 1d). The two genotypes with the slowest callus initiation on hormone-free medium (FC 607-O-92 and FC 708-91) also had low frequencies of callus initiation on that medium (Figure 1c). FC 708-91 and SP 6822-91 both had delayed callus initiation on 10.0 mg/L BA, while FC 701/5-94 was the only genotype whose time to

callus was not reduced on that medium.

The BA concentration which optimized shoot regeneration frequency varied with genotype (Figure 1e). Two genotypes, FC 607-O-92 and FC 708-91 had their highest level of shoot regeneration on 1.0 mg/L BA, while three others, EL 36-93, SP 6822-21 and GWK-97, had optimum shoot regeneration on 3.0 mg/L BA. If these genotypes had been evaluated on B1 medium, EL 36-93 may have been classified as a non-regenerator. Thus, B1 medium is not the optimal level for all genotypes. The response of FC 701/5-94 suggests that some genotypes may respond optimally to levels of BA greater than 3.0 mg/L.

Although root regeneration from callus was greatest on 0.1 mg/L BA, distinct response patterns were observed (Figure 1f). FC 607-O-92 and GWK-97 both had high rates of rhizogenesis within the 0.1 to 1.0 mg/L BA range. Inhibition of rhizogenesis on BA $\geq$ 1.0 mg/L was common for most genotypes, but FC 701/5-94 had its highest frequency of rhizogenesis on 3.0 mg/L BA.

BA effects (1.0 vs 3.0 mg/L) were also evaluated in Exp 7. BA at 3.0 mg/L significantly reduced callus initiation, delayed lag time and reduced shoot number. Although the main effect of BA was not significant for time to callus, REL-1 callused 1.5 wk sooner on 3.0 mg/L BA (Table 3). The reduced callus production on 3.0 mg/L BA was mainly due to a BA x GA_3 interaction. Medium containing 3.0 mg/L BA and 1.0 mg/L GA_3 were toxic regardless of genotype or NAA concentration (Figure 2). In the absence of GA_3 in the medium, the two BA concentrations gave almost identical levels of callus initiation. In contrast, the reduction in shoot number by 3.0 mg/L BA vs 1.0 mg/L BA can be directly attributed to the BA since GA_3 had a net stimulatory effect on shoot number (Table 4).

Effects of NAA

We thought that addition of small quantities of NAA to B1 might enhance shoot regeneration in some genotypes, without disturbing the hormone-autonomous nature of the callus. Exp 2 evaluated the response of four genotypes on B1 with and without 0.1 mg/L NAA. Supplementation of B1 with NAA significantly increased frequencies of callus

Table 3. Effect of concentrations of BA, NAA and GA_3 on time to callus (Exp 7).

	Time to Callus (wk)								
	BA			NAA			GA_3		
Genotype	1.0 mg/L	t-test	3.0 mg/L	0 mg/L	t-test	0.1 mg/L	0 mg/L	t-test	1.0 mg/L
GWK-93*	3.75	**	5.45	4.87	NS	4.14	3.25	**	6.05
FC 607-O-20	6.15	NS	6.60	5.73	*	6.87	5.81	**	7.44
REL-1	6.30	**	4.83	5.41	NS	6.17	4.75	**	8.09
SP 6926-O-3	6.35	NS	5.64	6.18	NS	6.00	5.20	**	7.73
FC 701/5-116	6.94	NS	6.54	6.20	*	7.43	6.50	*	7.71
EL 44-90	9.00		----[1]	9.00		----[1]	----[1]		9.00
Mean	5.84	NS	5.73	5.65	NS	5.96	5.05	**	7.20

[1] Explants of this genotype failed to initiate callus on media including this treatment.

NS,*,** Difference between means not significant or significant at $P \leq 0.05$ or $P \leq 0.01$, respectively.

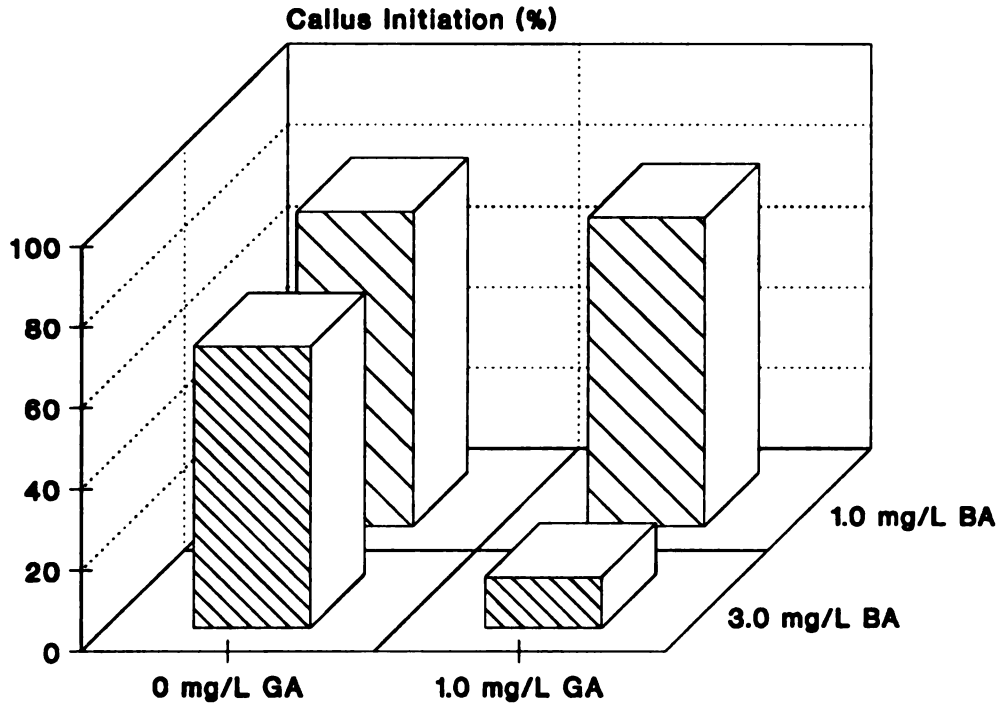


Figure 2. Effect of concentrations of BA and GA_3 on mean frequency of callus initiation summed over six genotypes (Exp 7).

Table 4. Effect of concentrations of BA and GA_3 on shoot number (Exp 7).

	Shoot Number					
	BA			GA_3		
Genotype	1.0 mg/L	t-test	3.0 mg/L	0 mg/L	t-test	1.0 mg/L
REL-1	22.17	**	14.50	16.62	**	25.90
SP 6926-O-3	19.10	**	5.33	13.58	NS	16.36
GWK 93*	11.69	*	6.85	9.52	NS	9.35
FC 701/5-116	8.12	NS	5.22	4.38	**	14.00
FC 607-O-20	7.44	NS	5.22	4.25	**	12.33
EL 44-90	1.00		----[1]	----[1]		1.00
Mean	13.94	**	7.67	9.93	**	14.61

[1] Explants of this genotype failed to initiate callus on media including this treatment.

NS,*,** Difference between means not significant or significant at $P \leq 0.05$ or $P \leq 0.01$, respectively.

initiation and shoot regeneration, and reduced time to callus (Table 5). Time to callus was sharply reduced by more than 2 wk. G x M interaction was also significant for callus initiation frequency and shoot number. The overall increase in callus initiation from 0.1 mg/L NAA was mainly due to the 91.7% response of SP 6926-O-94, vs no response on B1. The other three genotypes had similar levels of callus initiation on both media, but all had non-significant increases in shoot regeneration frequency on 0.1 mg/L NAA (Table 5).

Exp 3 and 4 were designed to determine if there is an optimal level of NAA in B1, and if this level varies with genotype. Both experiments involved B1 supplemented with either of six NAA concentrations from 0 to 1.0 mg/L. In Exp 3, there was a linear decline in callus initiation and shoot regeneration with increase in NAA concentration (Figure 3a). In contrast to the friable callus, initiation of compact callus was enhanced by higher levels of NAA. The initiation of both types of callus was strongly affected by genotype. REL-1 initiated friable callus at high frequency on NAA $\leq$ 0.3 mg/L and only formed compact callus on NAA $\geq$ 0.1 mg/L, with explants on 0.3 mg/L NAA commonly initiating both types of callus. In contrast, every explant of EL-40* initiated compact callus, but friable callus was only initiated on NAA $\leq$ 0.01 mg/L. In vitro chronology was not affected by NAA, except time to callus was longer on 1.0 mg/l NAA (Figure 3b). Although the G x M interaction for shoot regeneration was not significant, Figure 3c suggests that 0.01 mg/L NAA stimulated regeneration in BV-0. Shoot regeneration from compact callus did not occur.

Exp 4 was identical to Exp 3 except that a different set of genotypes was evaluated. Again, friable callus initiation and shoot regeneration declined with increasing NAA (Figure 4a), but the overall levels were higher than in Exp 3. Higher levels of NAA enhanced production of compact callus (Figure 4a), and slowed the initiation of friable callus by explants (Figure 4b). As in Exp 3, the effects on regeneration were mainly genotypic, but FC 701/5-94* had increased regeneration on 0.03 mg/L NAA (Figure 4c).

NAA had few significant effects in the factorial experiment (Exp 7). The only significant main effects were a reduction in shoot number and enhanced production of compact

Table 5. Effect of 0.1 mg/L NAA in B1 medium on frequency of callus initiation, frequency of shoot regeneration and time to callus (Exp 2).

Genotype	Callus Initiation (%)			Shoot Regeneration (%)[1]			Time to Callus (wk)		
	B1[2]	t-test	B1N.1[2]	B1	t-test	B1N.1	B1	t-test	B1N.1
EL 45/2-96	84.6	NS	100	76.9	NS	100	7.73	**	3.30
EL 48-99	84.6	NS	100	30.8	NS	38.5	7.18	NS	5.23
FC 607-92	100	NS	92.3	53.9	NS	92.3	8.54	*	5.75
SP 6926-94	0	*	91.7	----[3]		16.7	----[3]		8.91
Mean	68.6	**	96.1	41.2	*	62.8	7.86	**	5.67

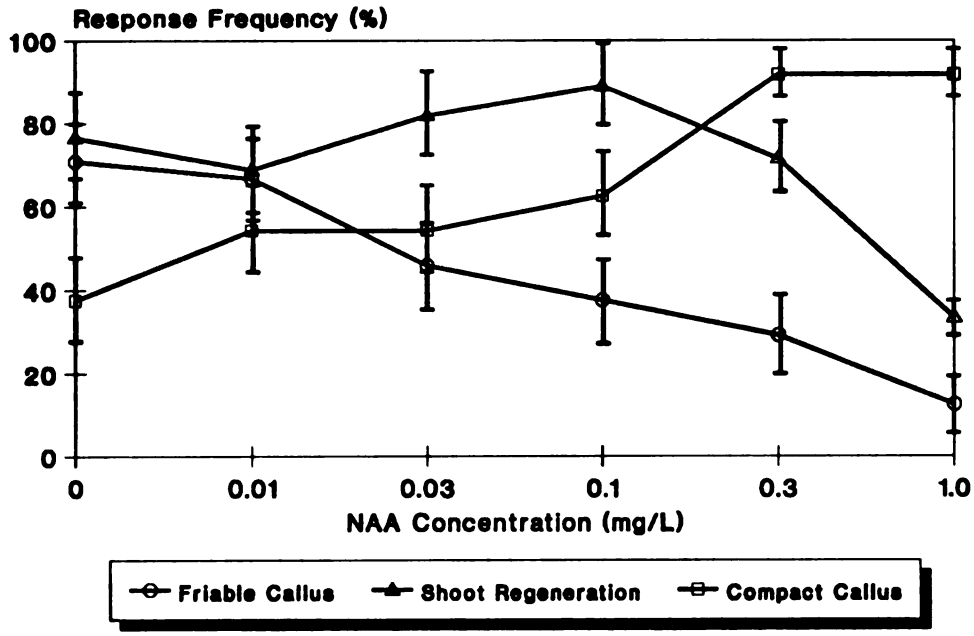
[1] Frequency of shoot regeneration from callus from those explants which produced callus.

[2] B1 = MS + 1.0 mg/L BA; B1N.1 = B1 + 0.1 mg/L NAA.

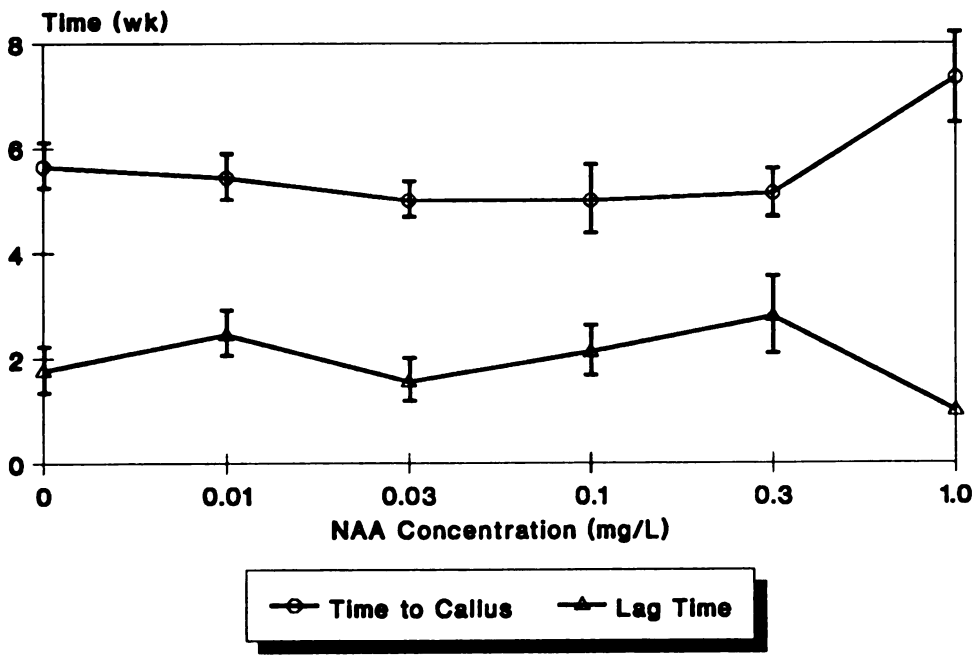
[3] No callus initiated on this medium.

NS,*,** Difference between maintenance and challenge means not significant or significant at $P \leq 0.05$ or $P \leq 0.01$, respectively.

A


Response Frequency (%)
100
80
60
40
20
0
0
0.01
0.03
0.1
0.3
1.0
NAA Concentration (mg/L)
Friable Callus
Shoot Regeneration
Compact Callus

B


Time (wk)
8
6
4
2
0
0
0.01
0.03
0.1
0.3
1.0
NAA Concentration (mg/L)
Time to Callus
Lag Time

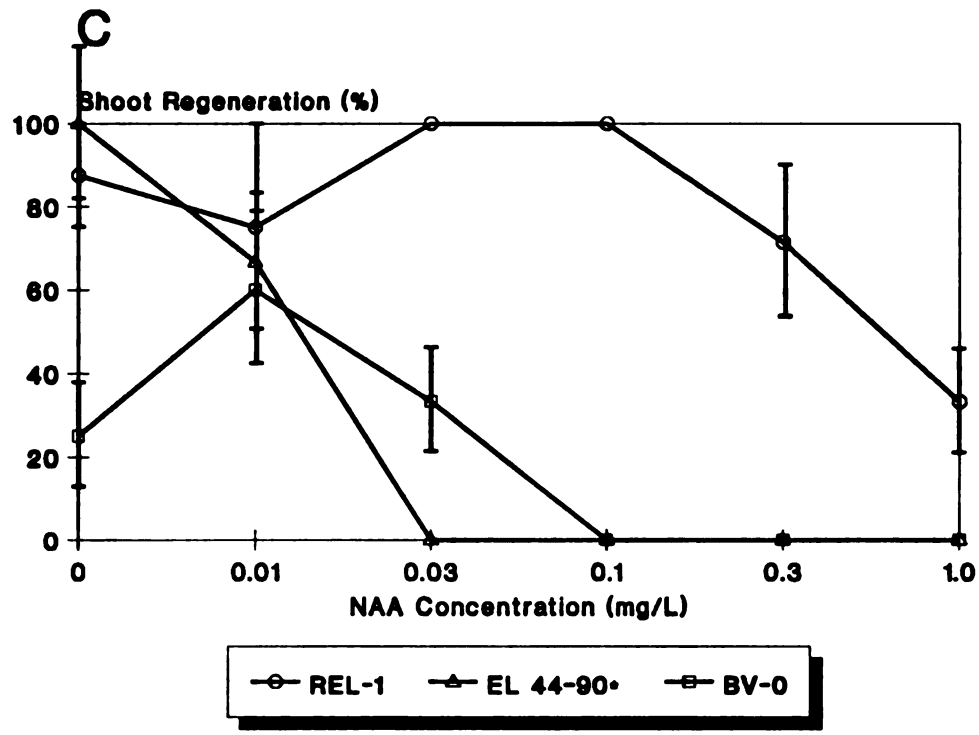
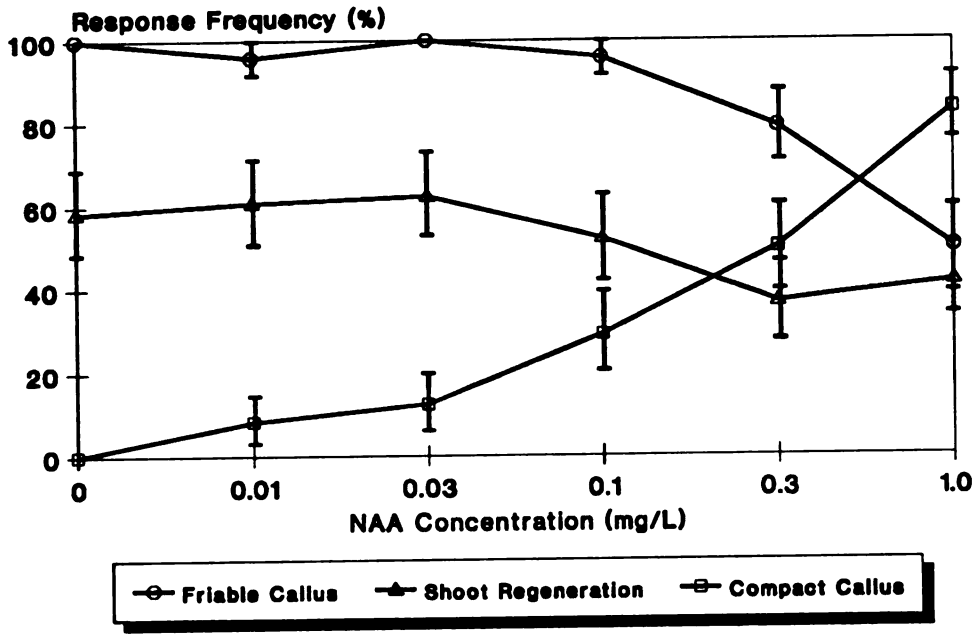
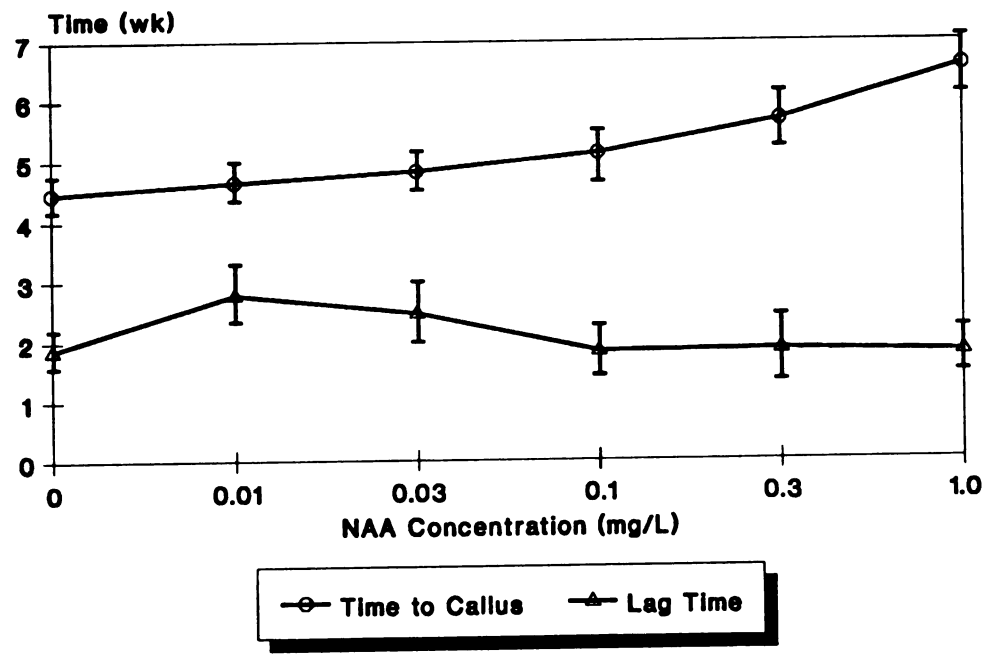


Figure 3. The effect of NAA on (a) mean callus initiation and shoot regeneration, and (b) time to callus and lag time to shoot regeneration summed over three genotypes; and on (c) shoot regeneration for three genotypes (Exp 3). Vertical bars represent ± SE when larger than symbol.

A


Response Frequency (%)
100
80
60
40
20
0
0
0.01
0.03
0.1
0.3
1.0
NAA Concentration (mg/L)
Friable Callus
Shoot Regeneration
Compact Callus

B


Time (wk)
7
6
5
4
3
2
1
0
0
0.01
0.03
0.1
0.3
1.0
NAA Concentration (mg/L)
Time to Callus
Lag Time

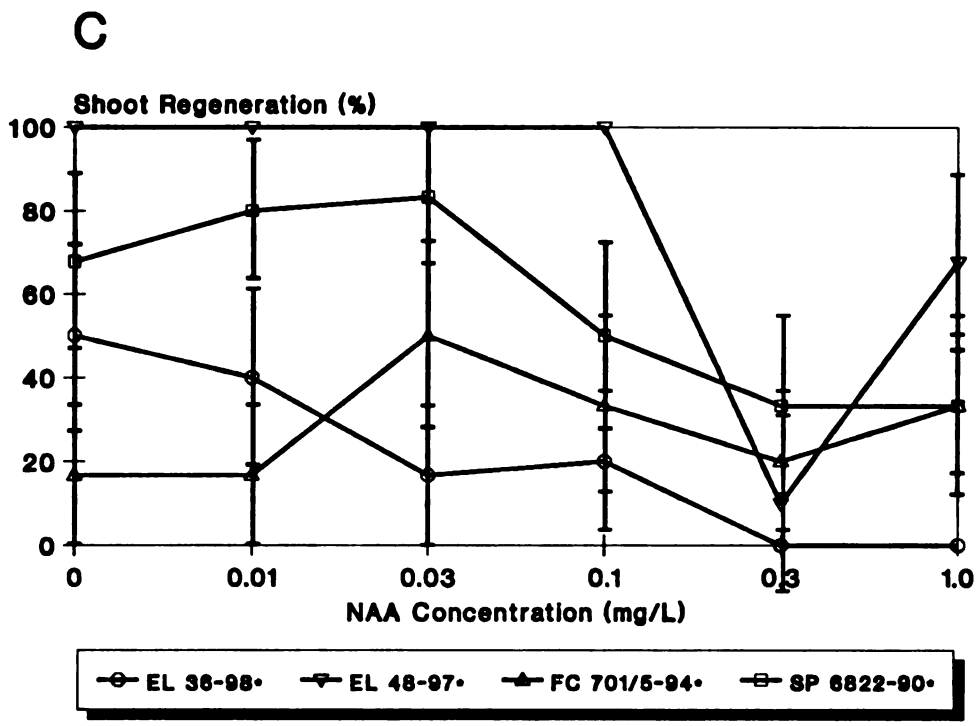


Figure 4. The effect of NAA on (a) mean callus initiation and shoot regeneration, and (b) time to callus, lag time to shoot regeneration summed over four genotypes; and on (c) frequencies of shoot regeneration for four genotypes (Exp 4). Vertical bars represent ± SE when larger than symbol.

callus. G x M interaction due to NAA involved significantly delayed callus initiation for FC 607-O-20 and FC 701/5-116, but not for the other genotypes tested (Table 3).

Effects of GA_3

Sugarbeet shoots regenerated from friable, hormone-autonomous callus are frequently vitreous. GA_3 was explored as a possible remedy to this problem (Miedema, 1984). Although GA_3 did not seem to affect vitreousness, other interesting effects on in vitro behavior were observed. In Exp 5, B1 supplemented with 1.0 or 3.0 mg/L GA_3 significantly enhanced shoot regeneration while inhibiting root regeneration (Table 6). For both of these variables, G x M interaction was also significant. Two genotypes had high levels of regeneration on all three media, while FC 701/5-94 failed to regenerate without GA_3.

In Exp 6, treatments with filter-sterilized GA_3 were included and the genotypes were different. G x M interaction was again significant for shoot regeneration. Shoot regeneration by callus of FC 701/5-116 was increased from 50% to 100% by all four GA_3 treatments (Table 7). This genotype is a member of the same population as the genotype whose shoot regeneration was enhanced by GA_3 in Exp 5.

Shoot number per leaf disk was significantly enhanced by all four media which contained GA_3 (Table 8). Regardless of whether it was filter-sterilized or autoclaved, GA_3 at 1.0 mg/L had similar results. The number of quality shoots per leaf disk was also enhanced by GA_3 (Table 8). Since the ratio of quality to total shoots was not greater with GA_3, the increase in quality shoots reflects an increase in total shoot number rather than a reduction in vitreousness.

In the factorial experiment (Exp 7), GA_3 significantly inhibited and delayed callus initiation, and inhibited root regeneration from callus. Overall, shoot regeneration frequency was excellent (94.7%) and none of the variables affected rate of shoot regeneration. GA_3 significantly enhanced the number of shoots (Table 4) and reduced the lag time to shoot regeneration.

GA_3 was involved in several interactions in Exp 7, including the toxic interaction with

Table 6. Effect of GA_3 concentration on frequencies of shoot and root regeneration from callus of three genotypes (Exp 5).

Genotype	Shoot Regeneration (%)[1]			Root Regeneration (%)[1]		
	B1[2]	B1G1[2]	B1G3[2]	B1	B1G1	B1G3
EL 45/2-96	100 a[3]	100 a	100 a	0 a	0 a	0 a
FC 607-O-92	87.5 a	100 a	87.5 a	87.5 a	12.5 b	50.0 ab
FC 701/5-94	0 a	75.0 b	87.5 b	75.0 a	50.0 ab	0 b
Mean	62.5 a	91.7 b	91.7 b	54.2 a	20.8 b	16.7 b

[1] Frequency of shoot or root regeneration from callus from those explants which produced callus.

[2] B1 = MS + 1.0 mg/L BA; B1G1 = B1 + 1.0 mg/L GA_3; B1G3 = B1 + 3.0 mg/L GA_3.

[3] For either shoot or root regeneration, means in the same row followed by the same letter are not significantly different based on a t-test at $P \leq 0.05$.

Table 7. Effect of GA_3 concentration on frequency of shoot regeneration from callus of three genotypes (Exp 6).

GA_3 (mg/L)	Shoot Regeneration[1]			
	Genotype			
	REL-1	FC 607-O-20	FC 701/5-116	Mean
0	100 a[2]	100 a	50.0 b	81.8 a
0.1 FS[3]	100 a	100 a	100 a	100 b
1.0 FS	100 a	100 a	100 a	100 b
1.0 A[3]	100 a	100 a	100 a	100 b
3.0 A	100 a	85.7 a	100 a	95.7 b
Mean	100 a	97.2 ab	90.0 b	95.7

[1] Frequency of shoot regeneration from callus from those explants which produced callus.

[2] Means in the same row or column followed by the same letter are not significantly different based on a t-test at $P \leq 0.05$.

[3] FS=filter sterilized; A=autoclaved

Table 8. Effect of GA_3 concentration on shoot number variables (Exp 6).

GA_3 (mg/L)	Shoot Number		
	Total[1]	Quality[2]	Q-Ratio (%)[3]
0	14.0 c[4]	1.58 b	11.29
0.1 FS[5]	21.1 bc	1.81 b	8.58
1.0 FS	29.5 a	2.78 a	9.42
1.0 A[5]	26.7 ab	2.83 a	10.60
3.0 A	22.6 ab	1.71 b	7.57
Mean	22.8	2.14	

[1] Total number of shoots per leaf disk.

[2] Number of quality shoots per leaf disk.

[3] Ratio of number of quality shoots to total shoot number.

[4] Means in the same column followed by the same letter are not significantly different based on a t-test at $P \leq 0.05$.

[5] FS=filter sterilized; A=autoclaved

BA mentioned above (Figure 2). Genotypes interacted with GA_3 for time to callus, shoot number and root regeneration from callus. The interaction for root regeneration was primarily due to GWK-93*, where rhizogenesis was reduced from 100% to 4.2% by 1.0 mg/L GA_3.

DISCUSSION

Concentrations of BA as low as 0.1 mg/L were enough to substantially increase callus production over hormone-free medium, however BA at 1.0 to 3.0 mg/L was needed to optimize shoot production from the callus. The BA in the medium may serve to speed up the initiation of an inevitable event, i.e. habituation. The explant requires only a minimal level of cytokinin to stimulate callus initiation, but the highly organogenic hormone-autonomous callus that is produced requires a 10-fold greater level of cytokinin in order to shift the organogenic response in favor of shoot production. In general, the BA concentrations which stimulate organogenesis are the same as those which stimulate callus production. Shoot production is

optimized on higher levels of BA, while root production is optimized on lower levels of BA. Although somatic embryos were not encountered in this study, we might speculate that somatic embryogenesis would occur near the BA concentration where root and shoot production are equally likely (Figure 1a).

Two distinct callus types, friable or compact, were initiated by sugarbeet leaf explants, but only the friable callus type regenerated shoots. Although some genotypes (e.g. EL 44-90) initiated compact callus on B1 medium, supplementation with NAA greatly enhanced its production, while inhibiting production of friable callus. In our hands, the friable callus has always proven hormone-autonomous (Doley and Saunders, 1989; Saunders and Daub, 1984; Saunders and Doley, 1986; Saunders and Shin, 1986; Chapter 1; Chapter 3). Three other studies have reported the initiation of both friable and compact callus by sugarbeet explants (Hooker and Nabors, 1977; Ritchie et al., 1989; Tetu et al., 1987), and, as in our research, shoot regeneration from compact callus did not occur. In two of these (Hooker and Nabors, 1977; Tetu et al., 1987), compact callus was initiated on medium containing auxin and cytokinin, but the shoot regeneration obtained was from a friable callus derived from the compact callus. Ritchie et al. (1989) obtained a white friable callus when leaf disks were incubated on MS + 0.5 mg/L BA. This callus developed green areas which developed into shoots after transfer to MS + 1.0 mg/L BA. The possibility that these regenerating friable calli were hormone-autonomous was not tested in these three studies.

Sugarbeet genotypes appear to differ in the BA concentration required for optimal shoot regeneration from friable callus. Cytokinin x genotype interactions affecting direct shoot regeneration from explant have been reported in petunia (Skvirsky et al., 1984) and cottonwood (Coleman and Ernst, 1989). Skvirsky et al. (1984) found that two inbred petunia cultivars differed in the concentration of BA needed to optimize shoot regeneration, but the response curves were fully reversed when zeatin was used. We have speculated that variation in endogenous hormone levels and/or sensitivities conditions in vitro response in sugarbeet

(Doley and Saunders, 1989; Chapter 1; Chapter 3), but we have not tested genotype-cytokinin combinations. Cytokinin structure-activity relationships, such as reported in *Phaseolus* spp. (Mok et al., 1978) and possibly operative in petunia (Skvirsky et al., 1984), may be involved in G x M interaction in sugarbeet.

Interactions between genotype and BA concentration may be useful in designing media to optimize regeneration within the response types proposed in Chapter 1. The three genotypes whose shoot regeneration was highest on 3.0 mg/L BA are all members of response type 4. Thus, the poor regeneration frequencies observed for members of response type 4 in Chapter 1 may have been enhanced had they been tested on higher levels of BA. A single medium is therefore inadequate for evaluating sugarbeet germplasm for the ability to regenerate shoots from callus.

Some genotypes had enhanced response when low levels of NAA (0.01 to 0.1 mg/L) were included in B1 medium (Table 2; Figure 3c), but the overall effect seemed to be neutral or possibly inhibitory. Although the effects of NAA on callus initiation and time to callus observed in Exp 2 were not realized in the other experiments involving NAA, two genotypes in Exp 7 had faster callus initiation with NAA. Thus, rather than routinely supplementing B1 with NAA, we recommend NAA use only for those genotypes or response types where a stimulatory effect has been demonstrated.

The inconsistent results obtained with GA_3 suggest that this hormone plays diverse roles in sugarbeet regeneration from callus. Supplementation of B1 with 1.0 mg/L GA_3 significantly enhanced shoot regeneration in Exp 5 and 6 mainly by boosting the regeneration frequency of plants of FC 701/5. Since GA_3 inhibited shoot formation from tobacco callus (Murashige, 1961; Murashige, 1964; Murashige, 1974), these results were unexpected. Jarret et al. (1981) found that GA_3 inhibited shoot meristem initiation from potato tuber disk explants, but was required for shoot development. A similar phenomenon could explain the response of sugarbeet callus to GA_3. GA_3 may be stimulating shoot development, rather than

shoot initiation. Consistent increases in shoot number by GA_3 in Exp 6 and 7, as well as reduction in lag time in Exp 7, support this hypothesis.

Strong inhibition of rhizogenesis suggested that the role of GA_3 was more complex than simply organ elongation. If GA_3 interferes with the initiation of root meristems, their development may be more sensitive than that of shoot meristems. Regulation of phytomorphology by the interaction of GA_3 and cytokinin has been reported for leaf shape in tobacco (Engelke et al., 1973) and for normal shoot development in beans with restricted root systems (Carmi and Heuer, 1981). The simultaneous inhibition of roots and stimulation of shoots by GA_3 resembles a cytokinin effect and may partially explain the toxic interaction between BA and GA_3 observed in Exp 7.

Donor plant effects could have been involved in some of the inconsistent results with NAA and GA_3. Moderate shoot regeneration rates on B1, such as in Exp 2, might reflect less than optimal condition of the donor plants, and might leave room for a stimulatory effect from NAA or GA_3. In Exp 7, the overall shoot regeneration rate was excellent and supplementation with NAA or GA_3 could therefore not result in any improvement.

Further studies of G x M interaction in sugarbeet might involve inclusion of abscisic acid (ABA), triiodobenzoic acid (TIBA) or ethylene antagonists. ABA has been reported to enhance shoot production by cotyledonary explants of muskmelon (Neidz et al., 1989) and loblolly pine (Sen et al., 1989), and TIBA appears to enhance regeneration from sugarbeet callus (Tetu et al., 1987). Use of ethylene antagonists have been reported to increase shoot production from explants of Chinese cabbage (Chi and Pua, 1989), somatic embryogenesis by carrot suspension cultures (Roustan et al., 1989) and shoot regeneration from maize callus (Songstad et al., 1988). Sugarbeet leaf disks rapidly produce high levels of ethylene (Aharoni and Yang, 1983). This ethylene production may vary with genotype and inhibit in vitro response by the leaf disks.

Powell and Dunwell (1987) utilized a joint regression analysis to analyze G x M

interaction in barley. Regression of genotype means across medium means allowed characterization of specific interactions. A similar approach might facilitate analysis of G x M interaction in sugarbeet, and ultimately lead to physiological genetic interpretations of the G x M phenomenon.

In conclusion, G x M interaction was found to be a major component of in vitro response in sugarbeet. Information on G x M interaction should allow identification of media which will optimize regeneration of specific genotypes or of in vitro response types (Chapter 1). For example, it appears that shoot regeneration of response type 4 germplasm can be enhanced by incubating leaf disks on 3.0 mg/L BA rather than the standard 1.0 mg/L BA. Although only genotypes with a history of regeneration were evaluated in this research, manipulation of G x M interaction may lead to regeneration of previously recalcitrant sugarbeet germplasm.

CHAPTER 3

Effects of Genotype, Subculture Interval and Growth Regulators on Shoot Regeneration from Serially-Subcultured Hormone-Autonomous Sugarbeet (*Beta vulgaris* L) Callus.

SUMMARY

Many genotypes of sugarbeet (*Beta vulgaris* L.) initiate hormone-autonomous callus when leaf disks are incubated on Murashige and Skoog (MS) medium with 1.0 mg/L N^6-benzyladenine (BA) as the sole growth regulator (B1 medium). When this callus is serially subcultured on B1 medium, shoot regeneration frequency rapidly declines. We investigated the effects of genotype, subculture interval, BA concentration and 2,3,5-triiodobenzoic acid (TIBA) on shoot regeneration from serially-subcultured calli up to 18 wk old. Calli of three genotypes were initiated from leaf disks on B1 medium and subcultured to various MS based media after 3 wk growth. Competence was assessed by shoot regeneration frequency and shoot number per callus on maintenance media as well as after subculture to challenge media. When calli were subcultured every 3 wk on B1, genotypes differed significantly in rate of decline in shoot regeneration. After 15 wk on B1, more than half of EL 45/2-108 calli were still regenerating shoots, while regeneration by calli of REL-1 and FC 607-O-20 was approaching zero. Although EL 45/2-108 had excellent long term regeneration, the extreme vitrification of the regenerant shoots coupled with a very high shoot to callus ratio make this genotype a poor choice for applications involving serially-subcultured callus. Subculture interval did not effect subsequent shoot regeneration frequency, but calli subcultured more frequently were lighter in color and appeared less senescent. Regeneration frequency from calli maintained on B1 was increased after subculture to MS + 3 mg/L BA. The frequency of calli regenerating shoots and the number of shoots per callus were both significantly enhanced

by repeatedly doubling the BA concentration at each subculture or by maintenance on B1 + 1 mg/L TIBA. Calli of REL-1 were generally more responsive than calli of FC 607-O-20 to maintenance on TIBA. Increases in regeneration frequency were greater when concentrations of both BA and TIBA were higher in the challenge medium relative to the maintenance medium. Calli maintained in a non-regenerating state on hormone-free medium were induced to regenerate by transfer to challenge medium containing 3 mg/L BA. Manipulation of shoot regeneration with BA and TIBA appears to be compatible with a regeneration model involving auxin/cytokinin ratio.

Abbreviations

BA, N^6-benzyladenine
MS, Murashige and Skoog medium
SC, subculture interval
TIBA, 2,3,5-triiodobenzoic acid

INTRODUCTION

In less than 200 yr, sugarbeet (*Beta vulgaris* L.) breeders have increased the sucrose content of sugarbeets from 6% to more than 15%. Future increases in productivity will partially depend on the ability of plant scientists to apply the new array of biotechnologies to sugarbeet. Many of these new procedures are dependent on the ability to regenerate whole plants from cell cultures, and some, such as somatic cell selection and protoplast manipulations, require that this regenerative ability be maintained over a period of several subcultures in vitro.

Serially subcultured cell lines frequently lose their ability to regenerate whole plants (Lustinec and Horak, 1970; Malmberg, 1979; Smith and Street, 1974; Torrey, 1967). Loss of regenerative ability after serial subculture could be the result of altered genetic constitution of the culture or could result from the development of some new stable physiological state in which the culture no longer responds to conditions that previously induced morphogenesis (Smith and Street, 1974). The critical difference between these two explanations for loss of competence is that the genetically altered cell line will remain incompetent while the

physiologically altered cell line should be capable of having competency restored through manipulation of the culture environment.

The most interesting reports of long term regeneration are those in which the regenerative ability had been lost and then restored by some manipulation of the culture environment. In alfalfa, buds were induced on 32-month-old callus by a high cytokinin/low auxin ratio (Stavarek et al., 1980). The callus had continually been challenged to regenerate without success on a medium developed for alfalfa regeneration. Competence was restored in long term rice callus by osmotic manipulation of the medium (Kavi Kishor and Reddy, 1986a; Kavi Kishor and Reddy, 1986b). Callus growing on 2% sucrose alone lost its shoot producing ability after 75 to 300 days, but was revived after 50 days on 2% sucrose plus 3% sorbitol or 3% mannitol. Callus cultures of haploid rape showed a decline in shoot production after the first subculture (Sacristan, 1981). After nearly three years in culture, regeneration was induced by transfer of the callus to a new medium under high light intensity, followed by transfer back to the original medium while maintaining the high light intensity.

In the several reports of restoration of regenerative ability in long term callus, no clear pattern emerges. In all cases, some form of physiological adaptation was overcome by an environmental modification, but in each report the effective modification was different. Alternatively, when genetic instability is involved in loss of competence, it may be possible to minimize these changes through cultural procedures. Somaclonal variation has been shown to increase with time in culture (Armstrong and Phillips, 1988; McCoy et al., 1982), and shortening the subculture interval may be one means of reducing the variation (Cassells and Morrish, 1987; Evans and Gamborg, 1982). Chandler and Vasil (1984) found that shorter subculture intervals greatly enhanced the frequency of embryogenic callus formation in Napier grass cultures.

Leaf disk explants from whole plants of some sugarbeet genotypes are capable of one-step shoot regeneration, i.e. without subculture, from hormone-autonomous callus (Saunders and Doley, 1986). Callus forms after a minimum of 3 wk on B1 medium (MS + 1.0 mg/L

BA), and subsequent shoot regeneration occurs after a lag period of 1 to 4 wk. The response of sugarbeet germplasm to the B1 regeneration system varies extensively (Saunders and Shin, 1986; Chapter 1). One-step shoot regeneration of some genotypes can be enhanced by inoculating leaf disks onto medium containing 3 mg/L BA (Chapter 2). This suggests that the explants, and possibly the callus initiated from them, differ in endogenous auxin physiology and/or sensitivity to cytokinin.

The only report of shoot regeneration from serially subcultured hormone-autonomous sugarbeet callus was by Saunders and Daub (1984). They observed occasional shoots when callus maintained for three monthly subcultures was transferred to MS + 1 mg/L BA + 0.3 mg/L indole-3-acetic acid (IAA). Saunders and Doley (1986) reported increases in fresh weight of calli subcultured to hormone-free medium, but no shoot regeneration was described. Tetu et al. (1987) obtained regeneration from sugarbeet callus through three distinct protocols, all of which utilized an auxin for callus initiation and at least one subculture before regeneration was observed. One procedure, referred to as intense morphogenesis, consisted of subculturing green friable callus initiated with naphthaleneacetic acid and BA to media containing a cytokinin (BA or zeatin) and the anti-auxin TIBA. Since no treatments without TIBA were reported, the effect of the TIBA was not clear. Hooker and Nabors (1977) also reported a beneficial effect of TIBA on sugarbeet shoot regeneration, but shoot regeneration frequencies were quite low.

One plausible explanation for the decline in regenerative ability in hormone-autonomous sugarbeet callus is a buildup of tolerance or decreased sensitivity to BA. Alternatively, the same decline might be expected if the endogenous auxin level in the callus increases with time in culture, possibly as a direct result of habituation. It should be possible to remedy either situation by transfer of the calli to media with higher levels of BA. Addition of TIBA to the medium might also be capable of shifting the effective auxin/cytokinin ratio into the range required for shoot regeneration.

The objectives of this research were: (1) to characterize the decline in shoot

regeneration over time of serially-subcultured, hormone-autonomous callus of three sugarbeet genotypes, and (2) to evaluate the effects of subculture interval, BA concentration and TIBA on maintenance of the shoot regeneration ability of these calli.

MATERIALS AND METHODS

Plant Material

Sugarbeet plants used as donors of leaf explants were grown in soil in 18-cm pots in a controlled environment chamber at 25°C under a 12 h photoperiod from cool white fluorescent bulbs (100-200 $\mu Em^{-2}s^{-1}$). The soil used was a 2:2:1:1 mixture of Baccto professional planting mix (Michigan Peat Co, Houston, TX, USA), a local greenhouse mix, vermiculite and perlite, respectively. The local greenhouse mix consisted of a 5:3:2 mixture of field soil of variable origin, peat and sand, respectively. Plants were fertilized weekly with 200 mL Peters 20-20-20 water soluble commercial nutrient mix and monthly with Snyder's (1974) nutrient formulation.

The three genotypes evaluated are all known to have good regeneration capacity (Saunders and Shin, 1986; Chapter 2). FC 607-O-20 and EL 45/2-108 are individual plants isolated from the breeding populations FC 607-O (GA Smith, Fort Collins, CO, USA) and EL 45/2 (JW Saunders, East Lansing, MI, USA), respectively. REL-1 (JW Saunders, East Lansing, MI, USA) is a clone bred and selected for superior regeneration ability. Three plants of each genotype were produced from shoot cultures by the procedure developed by Saunders (1982), and the same plants were used in the four experiments. Data for EL 45/2-108 is presented for Exp 1 and 2 only. Use of EL 45/2-108, an unusually prolific regenerator, was discontinued midway through Exp 3 because the time required to subculture callus and count shoots (up to 200 per dish) of this genotype became prohibitive.

Culture Procedures

Explants for Exp 1, 2, 3 and 4 were taken in March, June, August and October 1988, respectively, from 4-, 7-, 9- and 11-month-old donor plants, respectively. Two small partially expanded leaves (three in Exp 4), varying in length from 3.5 to 10 cm, per plant were used as the source of explants. Detached leaves were surface sterilized with two 20 min soakings in

15% commercial hypochlorite bleach solution with 0.01% sodium laurylsulfate, followed by six rinses with sterile distilled water. Leaf disk explants were cut from leaf blade tissue with a No. 3 cork borer (7 mm i.d.).

The culture medium consisted of MS (Murashige and Skoog, 1962) salts, 3% (w/v) sucrose, 100 mg/L *myo*-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, 1.0 mg/L thiamine·HCl, 0.9% (w/v) Difco Bacto agar (Difco Laboratories, Detroit, MI, USA) and various concentrations of BA and TIBA (both Sigma Chemical Co, St Louis, MO, USA). The pH was adjusted to 5.95 with KOH prior to autoclaving for 20 min at 121°C. Thirty-five mL of medium was dispensed into each 15 x 100 mm Falcon Optilux disposable plastic Petri dish (Becton Dickinson & Co, Lincoln Park, NJ, USA) after autoclaving. Eight explants (ten in Exp 4) were taken per leaf and a single explant was placed on each dish. The dishes were sealed with two strips of Parafilm (American National Can, Greenwich, CT, USA). Cultures were maintained at 28°C under dim light provided by cool-white fluorescent bulbs (20-40 $\mu Em^{-2}s^{-1}$) in a controlled environment room.

Individual leaf disk explants initiated callus at from one to many sites 3 to 6 wk after inoculation. For subculturing, all callus on a leaf disk was treated as a single unit. Callus formed on individual explants was subcultured to fresh media when it was 21 d old. Each initial callus was removed from the explant and subdivided into three calli of 1 to 1.5 cm diameter which were placed equidistantly around the perimeter of the Petri dish. Calli from two explants per leaf were subcultured to each of the three treatments, except in Exp 4 where callus from one explant per leaf was subcultured to each of the eight treatments. Calli were subcultured to fresh media every 21 d unless otherwise noted. Callus age refers to the total length of time since callus was first observed on the leaf disk, regardless of the number of subcultures performed. All references to time (e.g. regeneration after 15 wk) are synonymous with callus age.

Media on which calli were maintained were referred to as maintenance media, while media used to assess regenerative ability were referred to as challenge media. B1 medium was

the maintenance control medium in all experiments. In many cases, portions of calli were simultaneously subcultured to both maintenance and challenge media. Regenerative ability on challenge media was assessed after 3 wk. Through the course of the four experiments, the challenge medium evolved from B1 to MS + 3 mg/L BA (B3) and finally to B3 supplemented with TIBA.

Experimental Designs

The experimental design for callus initiation was completely randomized, with hierarchical and completely nested sampling, i.e. plants within genotypes, leaves within plants and samples within leaves. In each of the four experiments, the calli were randomly subcultured to the various genotype x medium treatments, which were arranged factorially with the same nested sampling structure used for callus initiation. Table 1 summarizes the design of the four experiments, while Table 2 provides the BA and TIBA contents of the various media which were used. See Figure 1 for a flow chart of the callus maintenance and challenge regimes. The details of the four experiments were:

Experiment 1. Shoot regeneration of three genotypes was evaluated after maintenance on MS + 1.0 mg/L BA (B1), with calli subcultured at 7, 14 or 21 d. Shoot regeneration ability was assessed at 9 wk by challenge on B1 and at 15 wk by challenge on MS + 3.0 mg/L BA (B3).

Experiment 2. Shoot regeneration of three genotypes was evaluated after maintenance on hormone-free MS (MS0), B1 or a regime in which BA concentration in the medium was doubled at each subculture. The doubling regime began with 2.0 mg/L BA (B2) at the first subculture and ended with calli transferred to 32.0 mg/L BA (B32) at 15 wk. Calli maintained on MS or B1 were challenged to regenerate at 9, 12 and 15 wk by transfer to B3.

Experiment 3. Shoot regeneration of three genotypes was evaluated after maintenance on B1 supplemented with 0, 0.1 or 1.0 mg/L TIBA (B1, B1T.1 and B1T1, respectively). Evaluation of EL 45/2-108 was terminated after 9 wk of maintenance. All calli were challenged at 9 and 12 wk by transfer to B3, and at 15 wk by transfer to B3 + 1.0 mg/L TIBA (B3T1).

Experiment 4. Shoot regeneration was evaluated in a 2^4 factorial experiment involving two

Table 1. Summary of treatments and sampling structure of four experiments to evaluate shoot regeneration from serially-subcultured sugarbeet callus.

Experiment	Genotypes (G)	Plants (P)/G	Leaves/P	Variables	Treatments
1	3	3	2	SC[1]	7, 14, 21 d
2	3	3	2	[BA]	0, 1.0, 2X[2] mg/L
3	3[3]	3	2	[TIBA]	0, 0.1, 1.0 mg/L
4	2	3	3	SC [BA] [TIBA]	10, 20 d 0, 1.0 mg/L 0, 1.0 mg/L

[1] SC = subculture interval; the number of days between subcultures.

[2] 2X refers to a regime of doubling the BA concentration at each subculture, i.e. 1 mg/L, 2 mg/L, 4 mg/L, 8 mg/L, 16 mg/L, 32 mg/L

[3] EL 45/2-108 was discontinued from Exp 3 after 9 wk.

Table 2. BA and TIBA contents of the various media used throughout the four experiments.

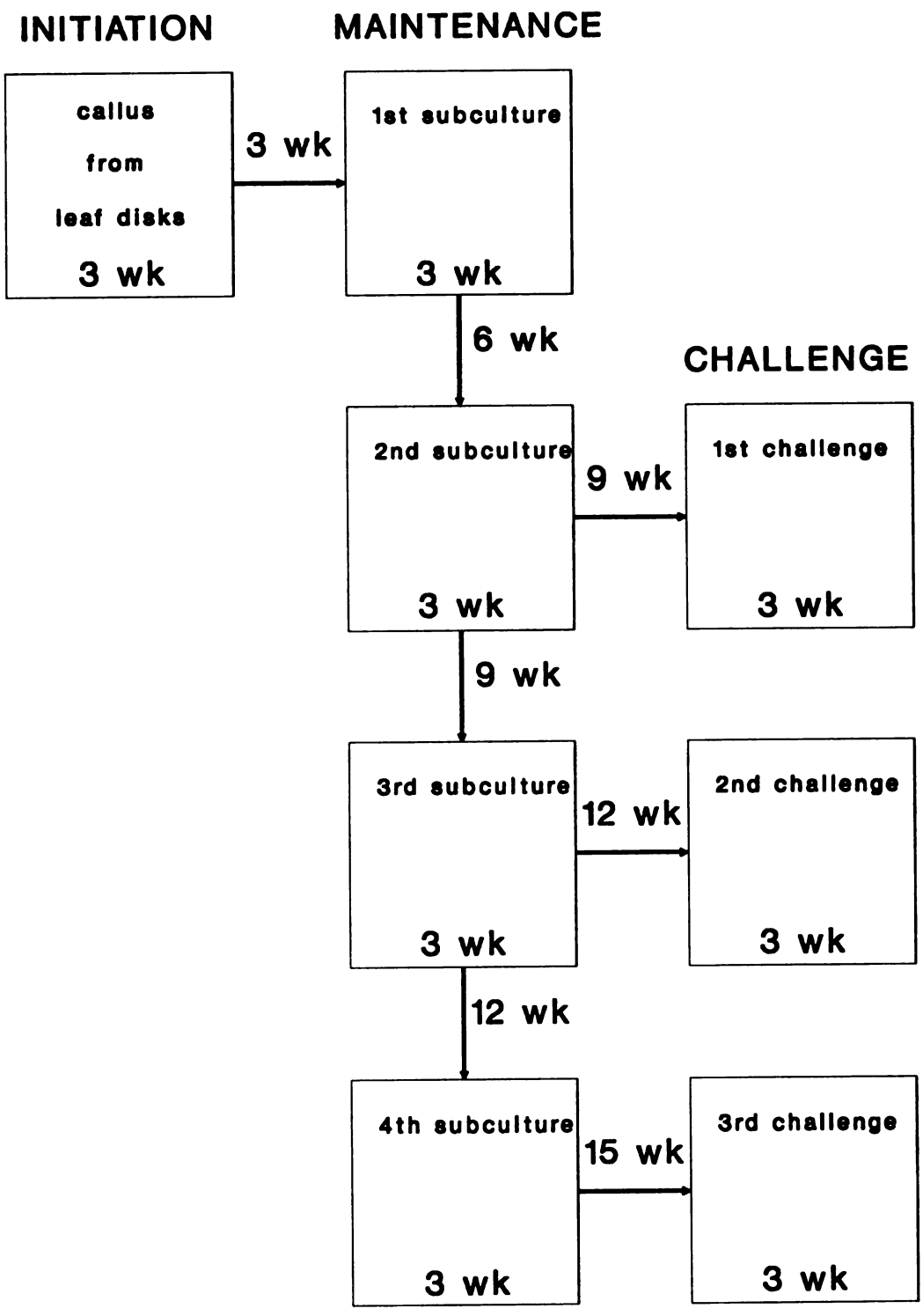
Medium	BA (mg/L)	TIBA (mg/L)
MS0	0	0
B1	1.0	0
B2	2.0	0
B3	3.0	0
B4	4.0	0
B8	8.0	0
B16	16.0	0
B32	32.0	0
B1T.1	1.0	0.1
B1T1	1.0	1.0
B3T1	3.0	1.0
B3T2	3.0	2.0
T1	0	1.0

genotypes (FC 607-O-20 or REL-1), two subculture intervals (10 or 20 d), two BA concentrations (0 or 1.0 mg/L)and two TIBA concentrations (0 or 1.0 mg/L). Subculture intervals of 10 and 20 d were chosen so that regeneration ability of the calli could be assessed every 20 d. Calli were challenged at 61 and 81 d by transfer to B3T1 and at 101 d by transfer to B3 with 2.0 mg/L TIBA (B3T2).

Data Analysis

Data on callus initiation and shoot regeneration were recorded every other day until the callus was first subcultured. Time to callus was defined as the number of days from explant inoculation to visual observation of callus. Lag time was defined as the number of days between callus initiation and visual observation of shoot regeneration. Number of shoots regenerated on individual calli was recorded at each subculture. Each shoot was judged for quality, with quality shoots defined as having relatively normal appearance, i.e. not developmentally deformed or vitreous, with a high probability of resulting in a healthy plantlet. The ratio of quality shoot number to total shoot number was referred to as the quality ratio. Quality shoot number and quality ratio were both calculated only from those calli which regenerated shoots.

Figure 1. Flow chart depicting maintenance and challenge subculture regimes utilized in the four experiments. The value adjacent each arrow represents the total age of the callus at the time of subculture. Exp 1 had no second challenge due to asynchrony of treatments, and callus age values in Exp 4 were slightly less due to 20 d subculture intervals.


INITIATION
MAINTENANCE
callus
from
leaf disks
3 wk
3 wk
1st subculture
3 wk
6 wk
CHALLENGE
2nd subculture
3 wk
9 wk
1st challenge
3 wk
9 wk
3rd subculture
3 wk
12 wk
2nd challenge
3 wk
12 wk
4th subculture
3 wk
15 wk
3rd challenge
3 wk

Statistical Analysis System (SAS Institute, 1985) was used for most analyses. Data were generally unbalanced and were handled by the General Linear Models (GLM) procedure. Genotypes and treatments were treated as fixed effects, while plants, leaves and samples were treated as random effects. The experimental unit for callus initiation and shoot regeneration frequencies was the leaf and response frequencies were calculated as the mean of the explants within a leaf. All frequency variables displayed variance heterogeneity and were transformed using the arcsine function (Steel and Torrie, 1980). For time to callus, lag time and shoot number, the experimental unit was the Petri dish and explants within a leaf were an additional source of variation. After the calli were subcultured, the experimental unit was the Petri dish for shoot regeneration frequency and the individual callus for shoot number. Shoot regeneration frequency was calculated as the mean of the three calli within the dish, i.e. 0, 33.3, 66.7 or 100%. Approximate F tests requiring synthesized mean squares were used when necessary (Satterthwaite, 1946).

When data were balanced, mean separation was accomplished using a standard LSD test. Data on shoot regeneration frequency and shoot number per callus were frequently balanced, but data for quality shoot number and quality ratio were by definition unbalanced. For unbalanced data, mean separation was accomplished using least squares marginal means computed by the LSMEANS option of GLM. Least squares means are the values expected had the design been balanced. Mean separation was accomplished using the PDIFF option of LSMEANS which performs all possible t-tests in a manner analogous to using an LSD.

RESULTS

The three genotypes studied were chosen on the basis of their in vitro profiles. Table 3 summarizes the mean leaf disk responses in Exp 1, 2 and 3. Since only those leaf disks whose callus was subcultured are included, callus frequency was 100% in all cases and the values for time to callus were biased downward. Leaf disk explants of all three genotypes initiated callus and regenerated shoots at high frequency, but the genotypes differed in time to callus, lag time, shoot number, quality shoot number and quality ratio. Explants of EL 45/2-

Table 3. Pooled mean values of in vitro response of leaf disks of three sugarbeet genotypes from cultures used to initiate Exp 1, 2 and 3 (N=108).

	Frequency (%)		Chronology (d)			Shoot Number		
Genotype	Callus Initiation	Shoot Regeneration	Time to Callus	Lag Time	Time to Regeneration	Total	Quality	Q-Ratio(%)
REL-1	100	90.8	26.7	11.9	38.6	5.84	1.40	32.04
FC 607-O-20	100	96.2	32.4	14.4	46.8	5.56	0.78	19.38
EL 45/2-108	100	99.8	33.3	8.5	41.8	43.79	2.18	7.47

108 were slow to callus and regenerated a large number of shoots after a short lag period, but the shoots were mostly of poor quality resulting in a very low quality ratio. Although lag time was longer, rapid callus initiation by REL-1 led to shoot regeneration 3 d earlier than EL 45/2-108. Slow callus initiation coupled with a long lag period by explants of FC 607-O-20 led to shoot regeneration 8 d later than REL-1. Calli of REL-1 and FC 607-O-20 produced similar numbers of shoots, but shoots of REL-1 were generally of better quality as indicated by the quality ratio.

The genotypes also differed in their callus morphology on the initiation medium. REL-1 callus tended to be bright white to yellowish and friable, but sometimes had green sectors which were more moist. The callus of FC 607-O-20 was off-white to tan with dark specks, friable and rarely green. EL 45/2-108 callus was tan to brown, crumbly and nodular and very difficult to extract from the large mass of shoots typically present.

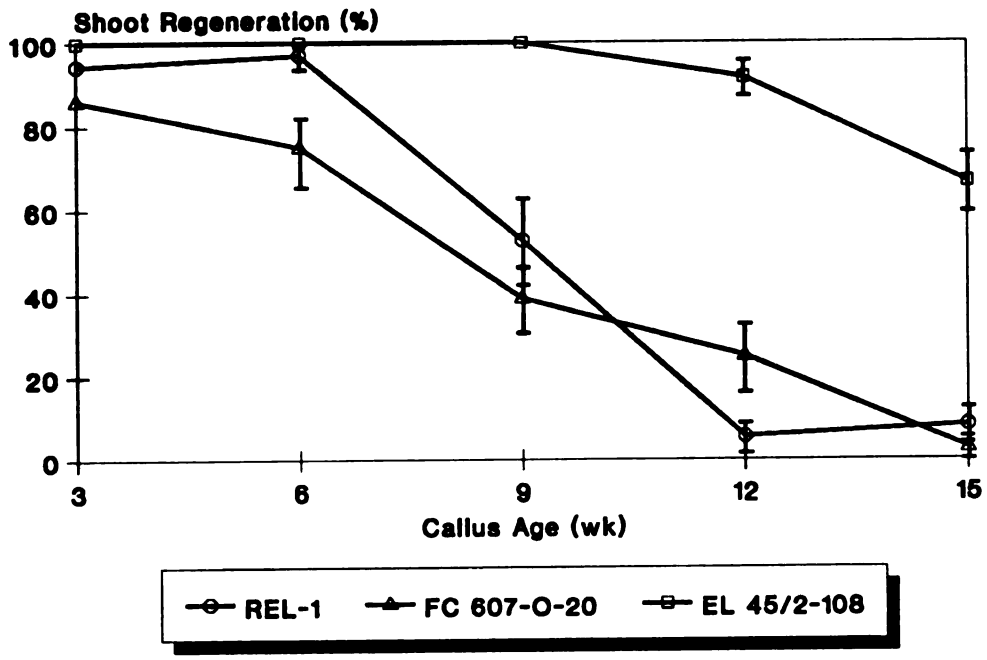
Effects of Genotype

All four experiments included genotypes as a variable. Genotypes differed significantly for frequency of shoot regeneration and number of shoots per callus at each subculture in the four experiments, except the final two subcultures in Exp 4. Figures 2a and 2b depict the decline in shoot regeneration frequency and shoot number per callus, respectively, of the three genotypes when subcultured every 3 wk on B1 in Exp 1. Similar data from Exp 2 are displayed in Figures 3a and 3b. Maintenance on B1 was the control in each experiment and the relative performance of the genotypes was always similar. After one subculture (6 wk), callus of all three genotypes still regenerated shoots at high frequency. By 9 wk, regeneration frequencies of REL-1 and FC 607-O-20 had sharply declined, but all calli of EL 45/2-108 continued to produce shoots. Shoot regeneration by calli of EL 45/2-108 remained above 50% after 15 wk on B1, while regeneration by REL-1 and FC 607-O-20 was near zero. The long term regeneration profiles of REL-1 and FC 607-O-20 were quite similar, but REL-1 typically had higher levels of shoot regeneration and shoot number per callus.

The high shoot number of EL 45/2-108 was the reason that this genotype was dropped

Figure 2. (a) Shoot regeneration frequency and (b) shoot number per callus over time for three genotypes in Exp 1. Vertical bars represent ± SE when larger than symbol.

A


Shoot Regeneration (%)
100
80
60
40
20
0
3
6
9
12
15
Callus Age (wk)
REL-1
FC 607-O-20
EL 45/2-108

B

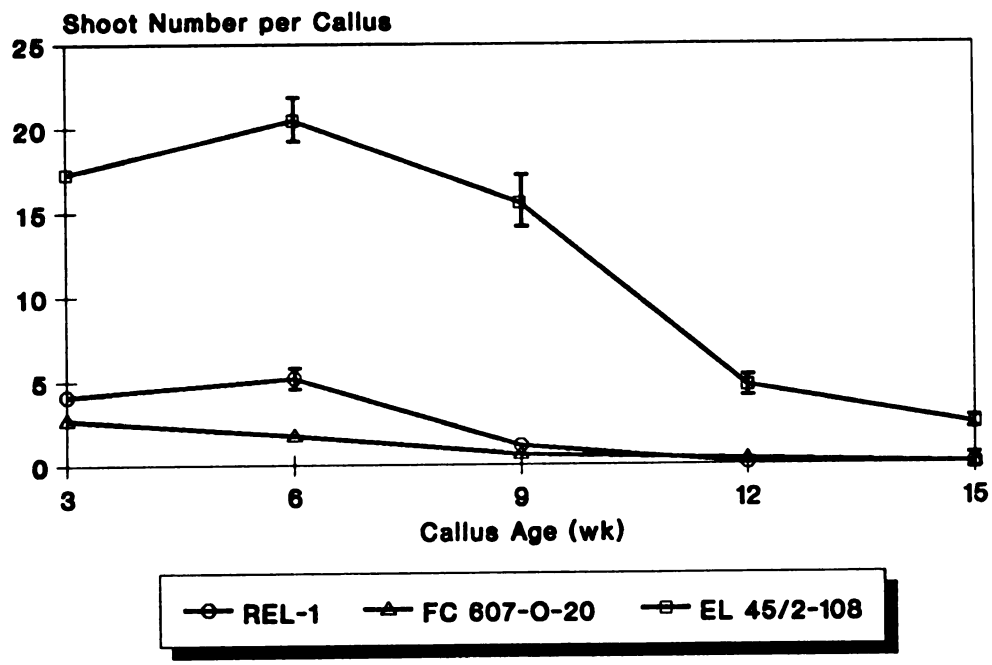
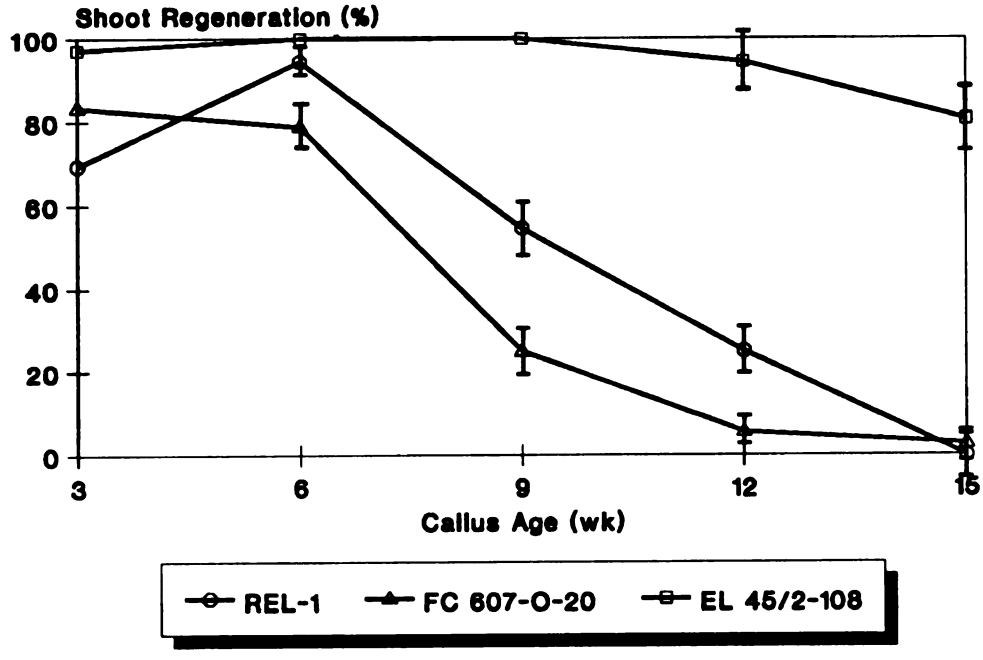
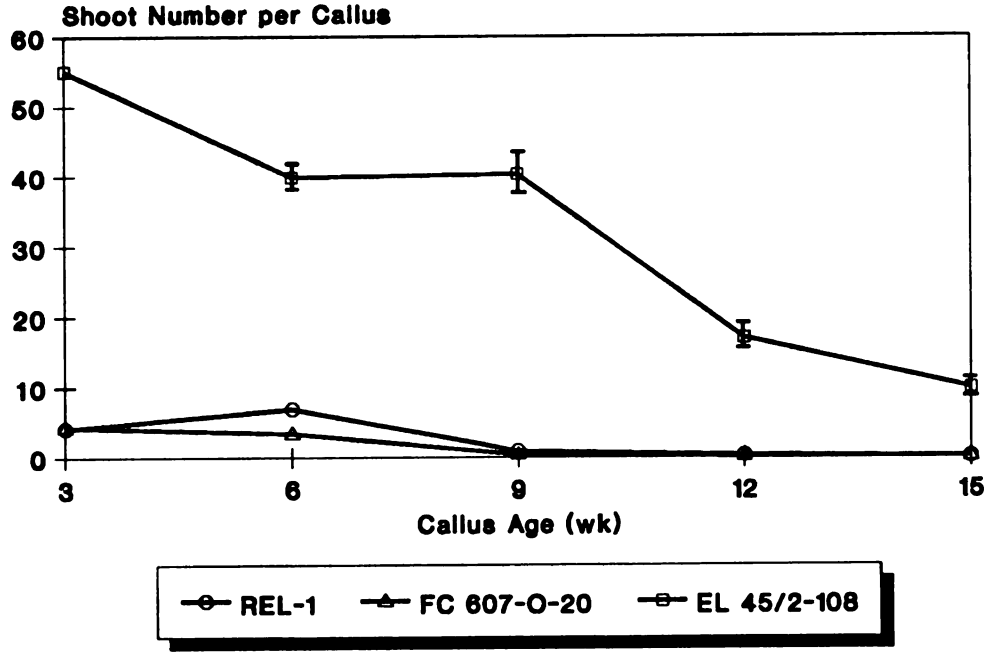

Shoot Number per Callus
25
20
15
10
5
0
3
6
9
12
15
Callus Age (wk)
REL-1
FC 607-O-20
EL 45/2-108

Figure 3. (a) Shoot regeneration frequency and (b) shoot number per callus over time for three genotypes in Exp 2. Vertical bars represent ± SE when larger than symbol.

A


Shoot Regeneration (%)
100
80
60
40
20
0
3
6
9
12
15
Callus Age (wk)
REL-1
FC 607-O-20
EL 45/2-108

B


Shoot Number per Callus
60
50
40
30
20
10
0
3
6
9
12
15
Callus Age (wk)
REL-1
FC 607-O-20
EL 45/2-108

from this research midway through Exp 3. When EL 45/2-108 was subcultured on media containing BA, the intense regeneration observed on initiation dishes continued for several subcultures, resulting in up to 200 shoots on many dishes. The amount of time required to count the shoots on the three calli on each dish and to subculture the small amount of callus proved to be prohibitive. Although shoots were removed at each subculture, subcultured callus of EL 45/2-108 may have contained undetected shoot primordia.

Subculture Interval

Routine callus maintenance was performed at 3 wk intervals, but at the end of the 3 wk period some calli were darker and approaching senescence. We thought that more frequently subcultured callus might be less senescent, and therefore more likely to be capable of shoot regeneration. Exp 1 investigated the effects of subculturing callus on B1 at 1, 2 or 3 wk intervals. The three treatments could only be compared at 9 and 15 wk since these were the only times the calli were all the same age since initiation. Regeneration frequency and shoot number data from 9 and 15 wk calli challenged to B1 and B3, respectively, for 3 wk are presented in Table 4. In both cases, genotypes (G) differed significantly for both traits, but subculture interval (SC) and G x SC interaction had no effect. Calli subcultured more frequently were lighter in color and appeared less senescent. When calli were challenged on B1 at 9 wk, the challenge medium was the same as the maintenance medium. In this case the longer SC resulted in more frequent regeneration and more shoots per callus. The calli subcultured more frequently had been exposed to more BA and may have developed a greater tolerance to BA. B3 medium was used for the challenge at 15 wk to see if the shorter SC treatments had a regeneration potential not realized with the B1 challenge. On the B3 challenge, the 1 wk SC gave the highest values for both traits. Although these were not significant differences, it suggested that shorter SC might have a beneficial effect on subsequent shoot regeneration. EL 45/2-108 was most notable in this regard; shoot number per callus was 5.0, 1.6 and 0.9 from the calli subcultured at 1, 2 and 3 wk intervals, respectively.

Table 4. Mean values of shoot regeneration frequency and shoot number per callus of three genotypes when challenged at 9 and 15 wk after maintenance with differing subculture interval in Exp 1.

		B1 Challenge at 9 wk		B3 Challenge at 15 wk	
Genotype	SC(wk)	Shoot Regeneration(%)	Shoot Number	Shoot Regeneration(%)	Shoot Number
REL-1	1	0	0	13.9	0.14
	2	0	0	5.6	0.08
	3	5.6	0.08	0	0
FC 607-O-20	1	8.3	0.11	2.8	0.03
	2	2.8	0.03	0	0
	3	25.0	0.33	0	0
EL 45/2-108	1	80.0	2.80	60.0	4.97
	2	88.9	3.36	55.6	1.64
	3	91.7	4.72	47.2	0.92
Means by SC	1	26.5	0.86	23.5	1.52
	2	30.6	1.13	20.4	0.57
	3	40.7	1.71	16.2	0.31

SC was also evaluated in Exp 4. In this case, SC of 10 and 20 d were used so that the calli could be compared every 20 d. No significant effects involving SC were found in the three challenges in Exp 4, but, as in Exp 1, there were some instances where shorter SC appeared beneficial. When first challenged to B3T1 at 81 d, the 10 d SC calli of REL-1 maintained without BA had a shoot regeneration rate more than double that of the 20 d SC calli. When challenged again to B3T1 at 101 d, the effect was not seen. Maintenance of calli with SC less than 3 wk, while clearly not of significant benefit, does not appear to be detrimental.

BA Concentration

Chronic exposure to BA by maintenance of calli on B1 could possibly intensify development of tolerance to BA. The effect of BA concentration was examined in Exp 2. Genotypes, media and their interaction had significant effects on regeneration frequency and shoot number at each subculture except at 6 wk. Doubling the BA concentration at each

subculture maintained the regeneration frequency (Figure 4a) and shoot number (Figure 4b) at levels significantly greater than maintenance on B1 or MS0. As expected, maintenance on MS0 resulted in a more rapid decline in both variables. The significant genotype x medium interactions were mainly due to EL 45/2-108 not regenerating more on the BA doubling regime vs. on B1. Since EL 45/2-108 maintained high-frequency regeneration on B1 (Figure 3a), any boost due to the BA doubling regime was more restricted than for the other two genotypes.

When calli maintained on MS0 or B1 were challenged to B3 at 9, 12 and 15 wk, significant increases in regeneration and shoot number were observed. In all cases the boost was greater for dishes maintained on MS0 vs. those on B1. The response of calli challenged on B3 at 9 and 12 wk was compared to calli of the same age on maintenance media. These comparisons for 12- and 15-wk-old calli are presented in Tables 5 and 6, respectively. Calli maintained on MS0 had regeneration rates near zero, but transfer to B3 produced regeneration rates similar to calli maintained on B1. Shoot number of MS0 calli was also greatly enhanced by B3, but it was still less than half the shoot number of B1 calli. The stimulation in regeneration from the B3 challenges did not bring these two treatments up to the regeneration level observed on dishes in the BA doubling regime (Figures 4a and 4b).

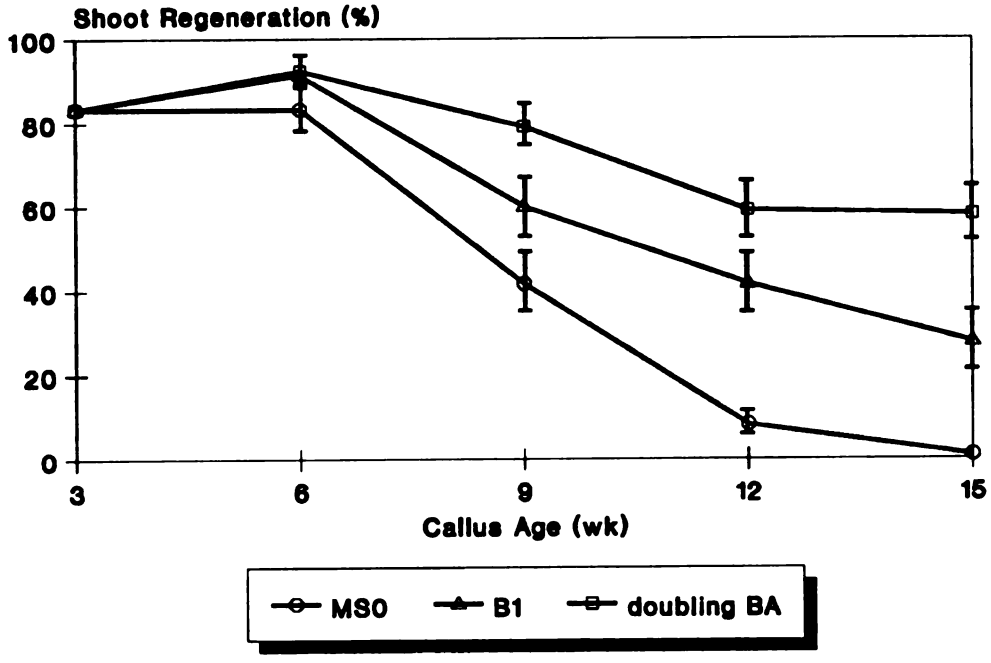
The effects of BA concentration were also examined in Exp 4, but a doubling regime was not included. As in Exp 1, regeneration by calli maintained on MS0 declined more rapidly than calli maintained on B1, but regeneration rates of MS0 calli approached those of B1 calli when challenged to B3T1. Challenge data from both experiments demonstrated that the lack of regeneration by calli maintained on MS0 or B1 was not indicative of loss of competence.

Effects of TIBA

If continuous exposure to a constant level of BA results in a decreased sensitivity to BA, we thought it might also be possible to counteract this with the anti-auxin TIBA. Calli were maintained on B1 with three levels of TIBA in Exp 3. When calli of two genotypes

Figure 4. (a) Shoot regeneration frequency and (b) shoot number per callus over time for three BA regimes in Exp 2. Vertical bars represent ± SE when larger than symbol.

A


Shoot Regeneration (%)
100
80
60
40
20
0
3
6
9
12
15
Callus Age (wk)
MS0
B1
doubling BA

B

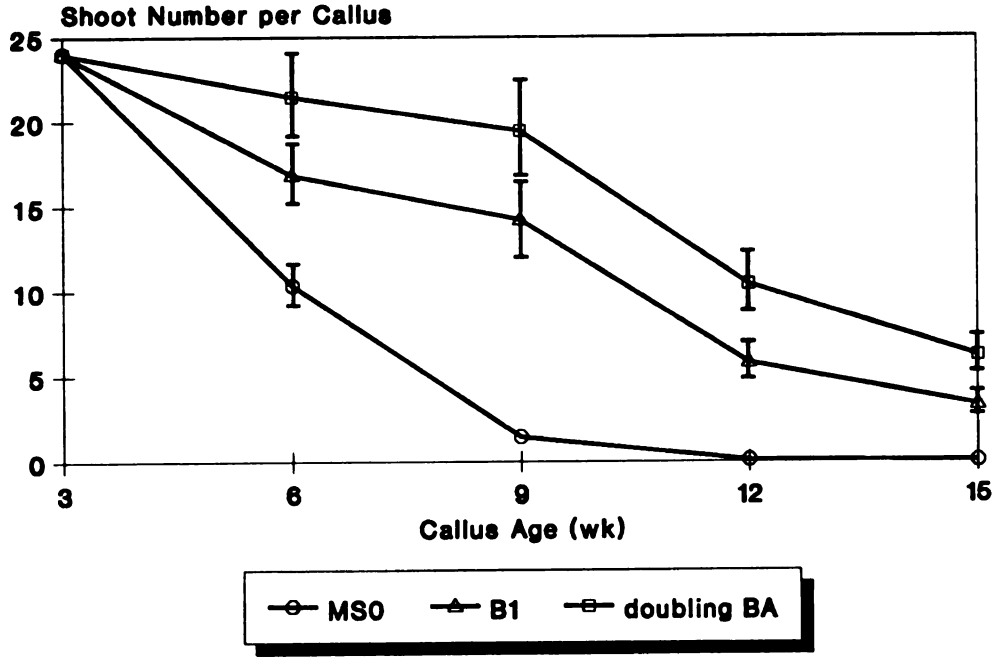

Shoot Number per Callus
25
20
15
10
5
0
3
6
9
12
15
Callus Age (wk)
MS0
B1
doubling BA

Table 5. Mean values of shoot regeneration frequency and shoot number per callus of 12-wk-old calli of three genotypes maintained with and without BA in Exp 2. At 9 wk, each callus was subcultured to both maintenance medium and challenge medium (B3).

Genotype	Maintenance Medium	Shoot Regeneration(%) Maintenance		Challenge	Shoot Number Maintenance		Challenge
REL-1	MS0	5.6	**	33.3	0.06	NS	0.58
	B1	25.0	NS	24.2	0.28	NS	0.30
FC 607-O-20	MS0	2.8	NS	16.7	0.03	NS	0.22
	B1	5.6	NS	22.2	0.06	NS	0.36
EL 45/2-108	MS0	16.7	**	88.9	0.19	**	7.67
	B1	94.4	NS	91.7	17.03	NS	16.58
Means by Medium	MS0	8.3	**	46.3	0.09	**	2.82
	B1	41.7	NS	46.7	5.79	NS	5.90
Grand Mean		25.0	*	46.5	2.94	*	4.34

NS,*,** Difference between maintenance and challenge means not significant or significant at $P \leq 0.05$ or $P \leq 0.01$, respectively.

Table 6. Mean values of shoot regeneration frequency and shoot number per callus of 15-wk-old calli of three genotypes maintained with and without BA in Exp 2. At 12 wk, each callus was subcultured to both maintenance medium and challenge medium (B3).

Genotype	Maintenance Medium	Shoot Regeneration(%) Maintenance		Challenge	Shoot Number Maintenance		Challenge
REL-1	MS0	0	**	38.9	0	*	0.81
	B1	0	NS	13.9	0	NS	0.14
FC 607-O-20	MS0	0	NS	2.8	0	NS	0.03
	B1	2.8	NS	5.6	0.03	NS	0.06
EL 45/2-108	MS0	3.0	**	61.1	0.03	*	1.64
	B1	80.6	NS	88.9	9.83	**	8.72
Means by Medium	MS0	1.0	**	34.3	0.01	**	0.82
	B1	27.8	NS	36.1	3.29	NS	2.97
Grand Mean		15.0	**	35.2	1.67	NS	1.90

NS,*,** Difference between maintenance and challenge means not significant or significant at $P \leq 0.05$ or $P \leq 0.01$, respectively.

(REL-1 and FC 607-O-20) were maintained on B1T1, regeneration at each subculture was significantly greater than from calli maintained on B1 (Figures 5a and 5b). Calli maintained on B1T.1 were generally intermediate in response, but did not regenerate significantly more than those on B1. Significant genotype x medium interactions were detected for shoot regeneration at 12 wk, for shoot number at 12 and 15 wk, and for both variables after the first B3 challenge (12 wk). In all cases, these were due to a greater response to TIBA by REL-1 vs FC 607-O-20.

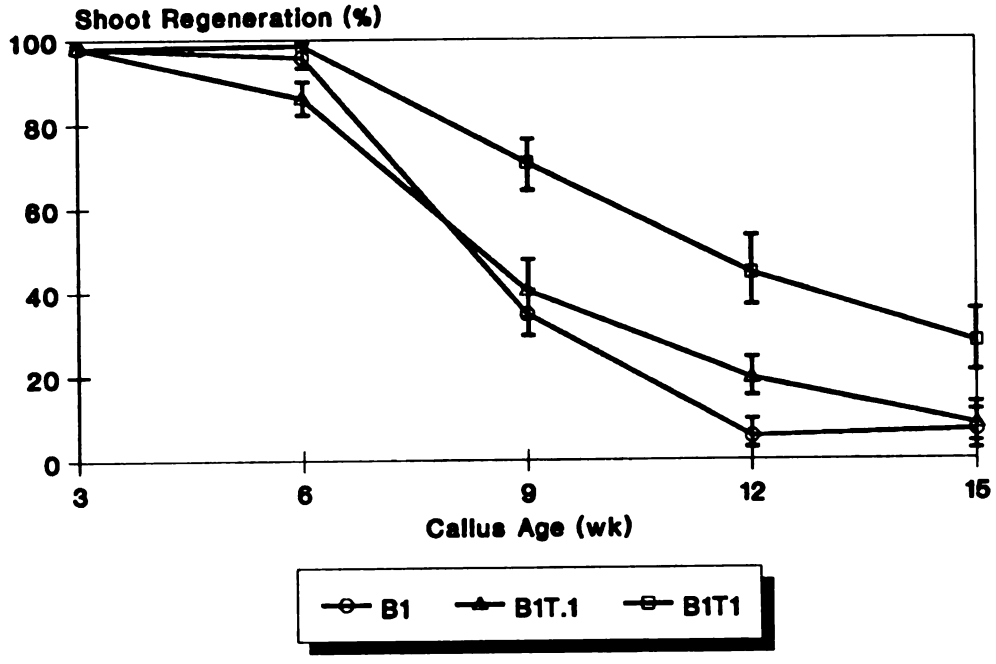
As in Exp 2, calli challenged at 9 and 12 wk could be compared to calli of the same age on maintenance media. With the exception of 12-wk-old calli of FC 607-O-20 on B1T.1, calli transferred to B3 had increased levels of shoot regeneration (Tables 7 and 8). In contrast, shoot number per callus was not enhanced by the B3 challenges, and calli maintained on B1T1 frequently had reduced shoot numbers after transfer to B3. The possibility that the removal of TIBA was responsible for the shoot number reduction in the challenged calli led to the inclusion of 1.0 mg/L TIBA in the medium used for the third challenge at 15 wk. Statistical comparison of the response of 15-wk-old calli before and after challenge to B3T1 was confounded by callus age, but maintenance of the 15 wk response levels in the 18-wk-old challenged calli could be viewed as a reversal or delay of the expected decline in response (Table 9). Increased regeneration of the calli maintained on B1 and B1T.1, but not of those maintained on B1T1, suggests that calli respond to increases in TIBA as well as increases in BA in the challenge medium.

In Exp 4, TIBA at levels of 0 and 1.0 mg/L were evaluated in combination with BA at the same concentrations. Calli maintained on B1T1 again had significantly higher regeneration rates than calli maintained on B1 (Figure 6a), and shoot number was enhanced for three subcultures (Figure 6b). The regeneration promoting effects of TIBA were only realized in the presence of BA, with calli on TIBA alone (T1) performing similar to those on hormone-free medium (MS0).

Neither of the B3T1 challenges significantly increased regeneration rates or shoot

Figure 5. (a) Shoot regeneration frequency and (b) shoot number per callus over time for three levels of TIBA in Exp 3. Vertical bars represent ± SE when larger than symbol.

A


Shoot Regeneration (%)
100
80
60
40
20
0
3
6
9
12
15
Callus Age (wk)
B1
B1T.1
B1T1

B

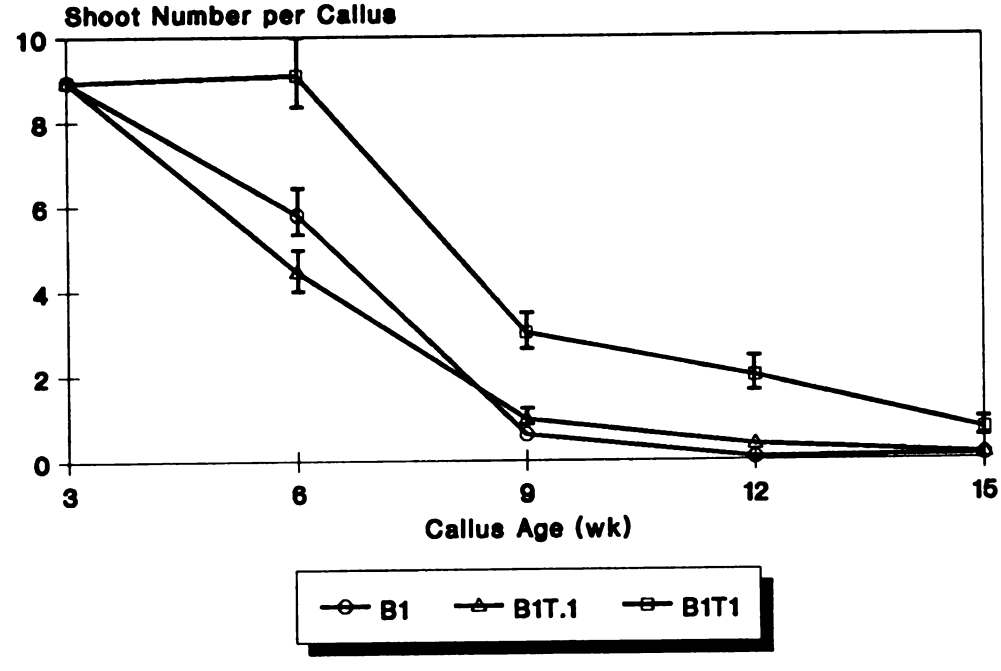

Shoot Number per Callus
10
8
6
4
2
0
3
6
9
12
15
Callus Age (wk)
B1
B1T.1
B1T1

Table 7. Mean values of shoot regeneration frequency and shoot number per callus of 12-wk-old calli of two genotypes maintained on B1 with and without TIBA in Exp 3. At 9 wk, each callus was subcultured to both maintenance medium and challenge medium (B3).

Genotype	Maintenance Medium	Shoot Regeneration(%) Maintenance		Challenge	Shoot Number Maintenance		Challenge
REL-1	B1	11.1	NS	19.4	0.14	NS	0.25
	B1T.1	19.4	NS	27.8	0.25	*	0.61
	B1T1	72.2	NS	77.8	3.39	**	2.50
FC 607-O-20	B1	0	NS	25.9	0	NS	0.30
	B1T.1	19.4	NS	16.7	0.50	NS	0.25
	B1T1	16.7	NS	24.2	0.58	NS	0.51
Means by Medium	B1	5.6	NS	22.2	0.07	NS	0.27
	B1T.1	19.4	NS	22.2	0.38	NS	0.43
	B1T1	44.4	NS	52.2	1.99	**	1.55
Grand Mean		23.2	**	32.4	0.81	NS	0.76

NS,*,** Difference between maintenance and challenge means not significant or significant at $P \leq 0.05$ or $P \leq 0.01$, respectively.

Table 8. Mean values of shoot regeneration frequency and shoot number per callus of 15-wk-old calli of two genotypes maintained with and without TIBA in Exp 3. At 12 wk, each callus was subcultured to both maintenance medium and challenge medium (B3).

Genotype	Maintenance Medium	Shoot Regeneration(%) Maintenance		Challenge	Shoot Number Maintenance		Challenge
REL-1	B1	11.1	NS	18.5	0.19	NS	0.22
	B1T.1	11.1	NS	26.7	0.19	NS	0.57
	B1T1	47.2	NS	55.5	1.33	NS	1.11
FC 607-O-20	B1	2.8	NS	5.6	0.03	NS	0.06
	B1T.1	5.6	NS	13.9	0.08	NS	0.25
	B1T1	8.3	NS	16.7	0.11	NS	0.42
Means by Medium	B1	6.9	NS	11.1	0.11	NS	0.13
	B1T.1	8.3	NS	19.7	0.14	NS	0.39
	B1T1	27.8	NS	33.3	0.72	NS	0.71
Grand Mean		14.4	NS	21.4	0.32	NS	0.41

NS indicates that maintenance and challenge means are not significantly different at $P \leq 0.05$.

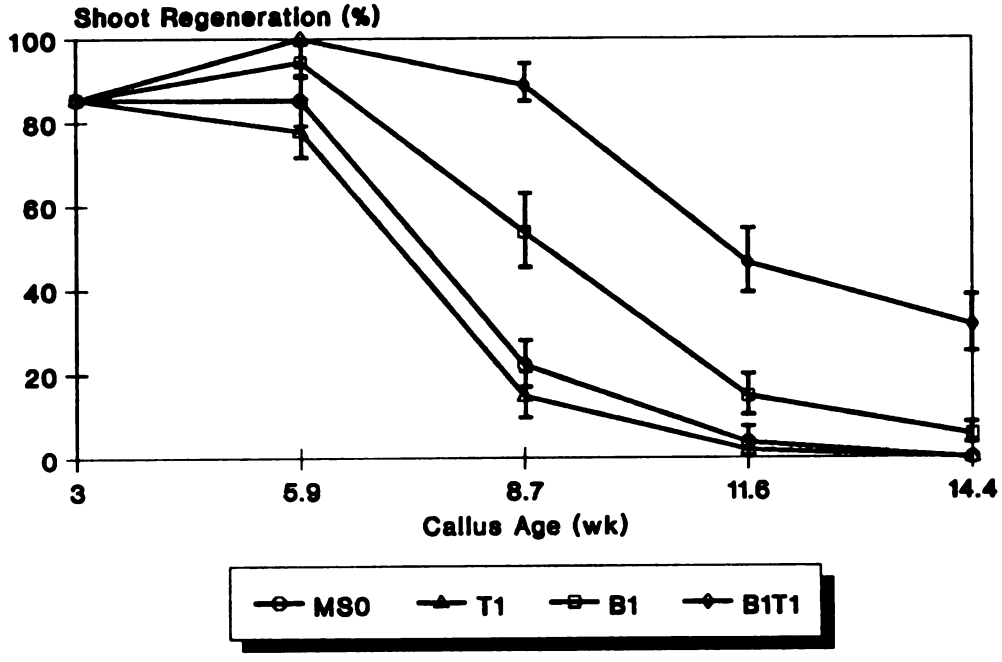
Table 9. Mean values of shoot regeneration frequency and shoot number per callus of two genotypes maintained on B1 with and without TIBA in Exp 3. The response of calli after 15 wk on maintenance media was compared with the same calli after 3 wk on challenge medium B3T1 (18-wk old). At 15 wk, all calli were subcultured from their respective maintenance media to the challenge medium.

	Maintenance	Shoot Regeneration(%)			Shoot Number		
Genotype	Medium	Maintenance		Challenge	Maintenance		Challenge
REL-1	B1	11.1	NS	22.2	0.19	NS	0.28
	B1T.1	11.1	*	33.3	0.19	NS	0.64
	B1T1	47.2	*	27.8	1.33	**	0.64
FC 607-O-20	B1	2.8	NS	0	0.03	NS	0
	B1T.1	5.6	NS	8.3	0.08	NS	0.08
	B1T1	8.3	NS	5.6	0.11	NS	0.06
Means by Medium	B1	6.9	NS	11.1	0.11	NS	0.06
	B1T.1	8.3	*	20.8	0.14	NS	0.36
	B1T1	27.8	NS	16.7	0.72	*	0.35
Grand Mean		14.4	NS	16.2	0.32	NS	0.28

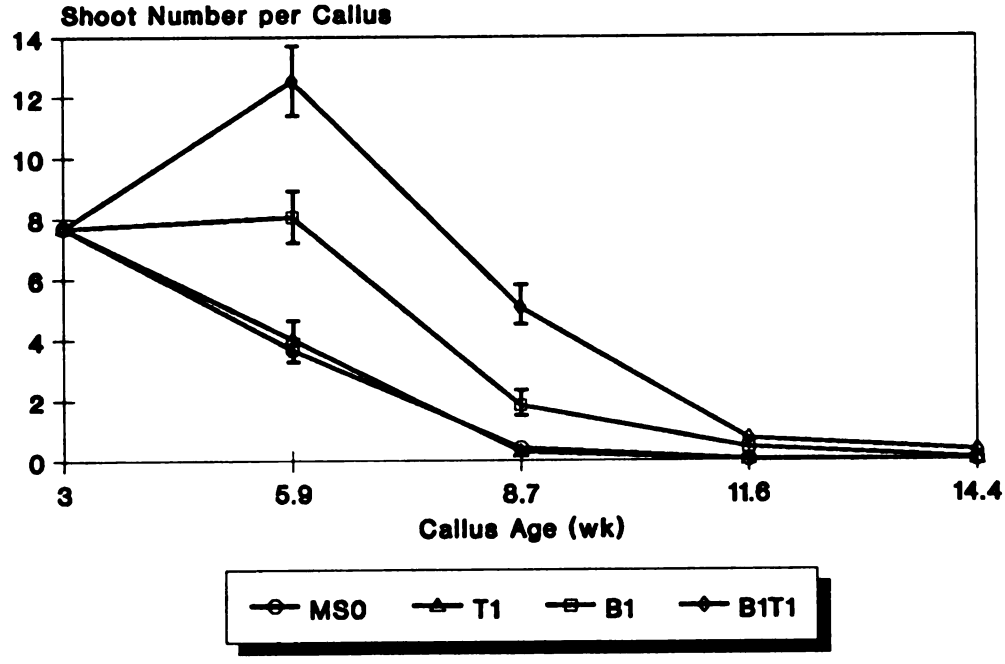
NS,*,** Difference between maintenance and challenge means not significant or significant at $P \leq 0.05$ or $P \leq 0.01$, respectively.

Figure 6. (a) Shoot regeneration frequency and (b) shoot number per callus over time for four BA x TIBA treatments in Exp 4. Vertical bars represent ± SE when larger than symbol.

A


Shoot Regeneration (%)
100
80
60
40
20
0
3
5.9
8.7
11.6
14.4
Callus Age (wk)
MS0
T1
B1
B1T1

B


Shoot Number per Callus
14
12
10
8
6
4
2
0
3
5.9
8.7
11.6
14.4
Callus Age (wk)
MS0
T1
B1
B1T1

numbers over the maintenance levels (Tables 10 and 11). These tables include only data from the long SC calli since valid maintenance regeneration data could not be obtained from the short SC calli. This reduction in sample size may partially explain why significant differences were not detected even though some large increases in regeneration frequency were observed. The poor response to B3T1 by calli maintained on B1T1 provides further evidence that calli respond to increases in TIBA. The final challenge in Exp 4 utilized B3T2, but regeneration levels were too low to draw any conclusions.

DISCUSSION

The model proposed for shoot regeneration from hormone-autonomous sugarbeet callus involves complementation of endogenous auxin physiology and exogenously supplied cytokinin (Chapter 1). We hypothesize that as the calli are subcultured on B1 there is a gradual decrease in sensitivity to exogenous cytokinin such that the effective auxin/cytokinin ratio exceeds the range in which shoot regeneration is possible. That is, the cells are still competent, but the stimulus required for determination is no longer perceived. B1 medium is clearly an effective stimulator of regeneration in primary callus, but its effectiveness declines with repeated subculture. The decreased sensitivity, or tolerance, to cytokinin could be due to an altered auxin physiology in the habituated callus. Kinetin was capable of eliminating the auxin requirement of tobacco callus by maintaining endogenous auxin levels (Syono and Furuya, 1972). BA might have a similar effect on hormone-autonomous sugarbeet callus. This shift in the effective auxin/cytokinin ratio can apparently be avoided by either steadily increasing the exogenous cytokinin level or by maintenance on medium containing the anti-auxin TIBA.

Information on endogenous hormone levels in habituated soybean callus (Wyndaele et al., 1988) provides some precedent for this hypothesis. Two fully-habituated soybean cell lines were found to contain higher levels of endogenous IAA than an auxin-habituated cell line, which in turn had higher levels of IAA than a non-habituated cell line. These authors also found that exogenously applied kinetin increased endogenous IAA in a fully-habituated

Table 10. Mean values of shoot regeneration frequency and shoot number per callus of 11.6-wk-old calli of two genotypes maintained with and without BA and/or TIBA in Exp 4. At 8.7 wk, each callus was subcultured to both maintenance medium and challenge medium (B3T1). Data presented is for long SC plates only.

Genotype	Maintenance Medium	Shoot Regeneration(%) Maintenance		Challenge	Shoot Number Maintenance		Challenge
REL-1	MS0	7.4	NS	11.1	0.07	NS	0.11
	T1	0	NS	7.4	0	NS	0.07
	B1	18.5	NS	40.7	0.70	NS	0.70
	B1T1	63.0	NS	63.0	1.00	NS	1.41
FC 607-O-20	MS0	0	NS	0	0	NS	0
	T1	3.7	NS	7.4	0.04	NS	0.11
	B1	11.1	NS	14.8	0.15	NS	0.22
	B1T1	29.6	NS	33.3	0.44	NS	0.52
Means by Medium	MS0	3.7	NS	5.6	0.04	NS	0.06
	T1	1.9	NS	7.4	0.02	NS	0.09
	B1	14.8	NS	27.8	0.43	NS	0.46
	B1T1	46.3	NS	48.2	0.72	NS	0.96
Grand Mean		16.7	NS	22.2	0.30	NS	0.39

NS indicates that maintenance and challenge means are not significantly different at $P \leq 0.05$.

Table 11. Mean values of shoot regeneration frequency and shoot number per callus of 14.4-wk-old calli of three genotypes maintained with and without BA and/or TIBA in Exp 4. At 11.6 wk, each callus was subcultured to both maintenance medium and challenge medium (B3T1). Data presented is for long SC plates only.

Genotype	Maintenance Medium	Shoot Regeneration(%) Maintenance		Challenge	Shoot Number Maintenance		Challenge
REL-1	MS0	0	NS	11.1	0	NS	0.11
	T1	0	*	25.9	0	**	0.70
	B1	3.7	NS	11.1	0.07	NS	0.11
	B1T1	22.2	NS	48.2	0.30	NS	0.59
FC 607-O-20	MS0	0	NS	0	0	NS	0
	T1	0	NS	7.4	0	NS	0.07
	B1	3.7	NS	18.5	0.04	NS	0.30
	B1T1	22.2	NS	7.4	0.33	NS	0.07
Means by Medium	MS0	0	NS	5.6	0	NS	0.06
	T1	0	NS	16.7	0	NS	0.39
	B1	3.7	NS	14.8	0.06	NS	0.20
	B1T1	22.2	NS	27.8	0.31	NS	0.33
Grand Mean		6.5	NS	16.2	0.09	NS	0.25

NS,*,** Difference between maintenance and challenge means not significant or significant at $P \leq 0.05$ or $P \leq 0.01$, respectively.

cell line derived from a crown gall tumor induced by wild-type *Agrobacterium tumefaciens*. If the habituation response in soybeans and sugarbeet is similar, hormone-autonomous sugarbeet callus may contain elevated levels of IAA, particularly when maintained on BA. Stimulation of regeneration with TIBA supports this possibility.

Endogenous IAA levels in nonorganogenic habituated sugarbeet callus have been compared to those of organogenic habituated and nonorganogenic auxin-dependent calli. Organogenic habituated callus derived from the original self-regenerating line of DeGreef and Jacobs (1979) had less than one-sixth the endogenous IAA as the auxin-dependent callus (Kevers et al., 1981b), while the two nonorganogenic cell lines contained similar levels of IAA (Kevers et al., 1981a). In these studies, each callus type was represented by a single long term cell line, and all three were derived from the same original cell line. Considering the magnitude of genetic variation for in vitro behavior in sugarbeet (Doley and Saunders, 1989; Saunders and Shin, 1986; Chapter 1), substantiation of the physiological differences between auxin-dependent and hormone-autonomous calli should include primary calli of a number of genotypes.

Genetic differences in long term regeneration have been reported in maize (Kamo and Hodges, 1986), pea (Malmberg, 1979) and tomato (Locy, 1983). In this research, sugarbeet genotypes differed in their long term regeneration profiles on maintenance media as well as in their response to the various challenge media. Genetic variation for shoot regeneration longevity highlights the need for careful choice of genotype for procedures involving hormone-autonomous sugarbeet callus.

The observed genotypic differences in long term regeneration could also be explained by the same tolerance hypothesis described above. The short lag time and prolific regeneration characteristic of explants of EL 45/2-108 is thought to be due to low levels of endogenous auxin and/or sensitivity to BA (Doley and Saunders, 1989). The variation in sensitivity observed in explants could also carry through to callus derived from these explants.

If EL 45/2-108 is more sensitive to cytokinin, it might take longer to develop the tolerance thought to result in the rapid decline in regeneration in other genotypes. In contrast, genotypes like REL-1 may contain higher levels of endogenous auxin, but not too high to interact favorably with the exogenous cytokinin to regenerate shoots from primary callus. Tolerance to BA may develop rapidly in these genotypes due to rapid increases in endogenous IAA, and genetic variation for long term regeneration may reflect genetic variation in IAA physiology in habituated calli.

The long term regeneration response of EL 45/2-108 was excellent, but it cannot be recommended for general use in procedures requiring callus subculture. The extremely high shoot to callus ratio made working with callus of this genotype very difficult and time consuming. Additionally, since the majority of the regenerant shoots were vitreous, the number of quality shoots was not much greater than other genotypes. Maintenance of EL 45/2-108 calli on hormone-free medium suppressed shoot regeneration to a more manageable level, representing one option for in vitro manipulation of this genotype. EL 45/2 germplasm might be useful in efforts to obtain regeneration from protoplasts.

Although shoot regeneration of REL-1 and FC 607-O-20 declined sharply after 6 wk in culture, shoot regeneration of both genotypes was enhanced by challenge with B3 medium. Both genotypes had enhanced rates of shoot regeneration and shoot number when maintained on B1T1, but REL-1 was generally more responsive to maintenance on TIBA. Results from Exp 4 demonstrated that the effect of TIBA on shoot regeneration was only realized in the presence of BA. Thus, TIBA acts synergistically with BA to produce an effect much greater than that of either one alone. Hormone-autonomous sugarbeet callus is capable of sustained growth on hormone-free medium, but shoot regeneration is generally dependent on cytokinin. If TIBA is acting as an anti-auxin in our system, its failure to stimulate regeneration without BA fits the proposed auxin/cytokinin regeneration model. TIBA has been shown to prevent a buildup in endogenous IAA in tobacco petiole explants (Cassells et al., 1982). Similarly, as

sugarbeet calli age, TIBA may reduce a buildup in endogenous auxin, resulting in an auxin level that can interact favorably with B1 medium.

Calli were also maintained in a non-regenerating state of competence on hormone-free medium (MS0), and then induced to regenerate by transfer to media containing BA. In this case, removal of the exogenous cytokinin supply increased the effective auxin/cytokinin ratio to a level that was more conducive to root formation than to shoot production. Although the calli were hormone-autonomous, their morphogenetic response was manipulated with exogenous hormone regimes. When calli maintained on MS0 in Exp 2 were challenged to regenerate on B3, highly significant increases in shoot regeneration and shoot number per callus were realized (Tables 5 and 6). In contrast, regeneration by calli maintained on B1 was enhanced, but not significantly, by the B3 challenges. This suggests that the maintenance medium may influence the physiological state of the calli, and thus influence the response to a given challenge medium. In this research, I have used the term 'challenge medium' in preference to 'regeneration medium' since it is clear that the medium required to optimize regeneration in subcultured calli depends on the genotype as well as the maintenance medium. Whereas B3 was capable of greatly enhancing regeneration in calli maintained on MS0, higher levels of BA may be needed to optimize regeneration in calli maintained on B1.

GENERAL DISCUSSION

The proposed model for shoot regeneration from hormone-autonomous sugarbeet callus involves complementation of endogenous auxin physiology with exogenously supplied cytokinin. Results from all three chapters supported this hypothesis which is compatible with the auxin/cytokinin ratio model of Skoog and Miller (1957). The specific underlying mechanisms, which were outside the scope of this research, remain unclear. In Chapter 1, a great range of variation was encountered when leaf disks of 78 genotypes were incubated on B1 medium. The leaf disks of the various genotypes tested were presumed to differ in either levels of, or sensitivity to, endogenous auxin and/or sensitivity to the exogenously supplied cytokinin. Differential response of genotypes to a range of BA concentrations in Chapter 2 provided evidence that the leaf disk response could be manipulated by varying the dose of exogenous cytokinin. Similarly, manipulation of shoot regeneration with BA and TIBA in Chapter 3 was explained by temporal as well as genetic variation in the endogenous auxin physiology and/or sensitivity to exogenous cytokinin of the subcultured callus.

An implicit assumption of the proposed model is that cell growth and division cannot occur in the complete absence of auxin and cytokinin. That is, both types of plant growth regulators must be present in at least minute quantities to stimulate callus initiation. Can plant cells divide in an environment devoid of auxin? I presume not, and therefore must assume that the hormone-autonomous sugarbeet callus initiated on B1 medium contains IAA, and must have the capacity to synthesize IAA. Meins (1982) has shown that habituated cell lines produce the substances for which they are habituated, but no data are available for hormone-autonomous sugarbeet callus.

Genetic variation for in vitro chronology was a curious aspect of this shoot

regeneration system. Genotypes varied not only for the frequency of callus initiation, but also for the time required to initiate the callus. I have hypothesized that this is the time required for one or more cells in the explant to attain auxin-autonomy, however, there is no direct evidence in support of this. If the hypothesis is correct, there is genetic variation in sugarbeet for the probability that a cell will become auxin habituated.

The BA gradient experiment in Chapter 2 demonstrated that levels of BA as low as 0.1 mg/L could significantly increase the frequency of callus initiation as well as reduce the time to callus. Kinetin has been shown to maintain endogenous IAA levels in tobacco callus, thereby replacing the auxin requirement of the callus (Syono and Furuya, 1972). Perhaps the BA in our medium stimulates IAA synthesis in the explant, increasing the probability and speed of callus initiation. Also working with sugarbeet, Van Geyt and Jacobs (1985) reported that cytokinin habituation occurs spontaneously, while auxin habituation requires induction. We know that initiation of hormone autonomous sugarbeet callus does not require exogenous growth regulators (Doley and Saunders, 1989), and BA appears to stimulate induction of auxin autonomy (Chapter 2). The BA-induced auxin-autonomous callus may spontaneously become cytokinin habituated. In soybean, Wyndaele et al. (1988) found that callus habituated for both auxin and cytokinin could be derived from an auxin-habituated cell line, but not from a cytokinin-habituated cell line.

Lag time, the other parameter of in vitro chronology, also was affected by genetic variation. This may be a reflection of variation for sensitivity to the exogenous cytokinin. The tendency for prolific regenerators, such as plants of EL 45/2, to have short lag times supported this possibility. Since the procedure is one-step, I cannot separate the physiology of the callus from that of the explant. The response of subcultured calli in Chapter 3 provided evidence that the physiology of the primary callus is at least partially maintained in the absence of the explant.

The results of Chapter 1 suggested that the ability to initiate callus and the ability to regenerate shoots from that callus may be independently inherited. If callus initiation is

dependent on auxin physiology and shoot regeneration is dependent on cytokinin physiology, there may be a hormonal basis for the postulated independence. Independence of these two in vitro parameters has been reported in other species, and could be a unifying aspect of plant tissue culture.

RECOMMENDATIONS

Genetic Variability

Even with an array of different media, one-step regeneration may not be achievable across the entire range of germplasm. Thus for some genotypes, a two-step procedure may be the only alternative. Subculture of fresh callus of recalcitrant genotypes to B3 or B3T1 may be helpful. Subculture of fresh callus to liquid MS0 or B1, followed by plating out on B1T1 or B3T1 might be a method of maximizing the number of regenerants obtainable in a limited period of time. Possibly, immersing the cells in liquid medium results in an increased probability of becoming determined for shoot regeneration.

Further testing of some of the hypotheses advanced in this research could include measuring endogenous levels of growth regulators in leaf disks of genotypes diverse for their in vitro behavior. These measurements should be done before culture, periodically after the explants have been plated, and after callus has been initiated. Methodology for detection and quantification of endogenous IAA have been published by Cassells et al. (1982) and Syono and Furuya (1972).

With regard to vitreousness, some possible remedies (GA_3, phloridzin, lower BA concentrations) were tested in the leaf disk system. The results were not encouraging, except possibly the use of B5 salts in place of MS. The many hypotheses as to the causes of vitreousness deal with shoot cultures and plantlets. Perhaps callus initiation and shoot regeneration are so far removed from normal growth and development that the remedies for vitreous shoot cultures are not applicable. It has been suggested that hormone-autonomous sugarbeet callus is vitreous by nature (Crevecoeur et al., 1987), but there is certainly genetic variation for the quality of the regenerant shoots.

Genotype x Medium Interaction

Some suggestions for further research on G x M interaction in sugarbeet were presented at the end of Chapter 2. These included use of ABA, GA_3, TIBA, and ethylene antagonists. Another possibility is 2,4-dinitrophenol which is mentioned by Atanassov (1986b). He cites work of Slavova (unpublished) showing that this compound enhanced regeneration in sugarbeet. The ultimate goal of the G x M interaction work is to develop protocols, if possible, to obtain shoot regeneration from all members of the sugarbeet germplasm pool. As mentioned above, attainment of this goal may involve utilization of a two-step procedure for some germplasm. Although they appear somewhat inhibitory in the callus initiation step (Doley, unpublished; Chapter 2), TIBA and GA_3 may be quite useful in two-step procedures.

Long-Term Regeneration

In my research, various maintenance schemes were employed to evaluate their effects on continuous regeneration, while only a few different media were explored as challenges. Maintenance of large numbers of calli on MS0 or B1 followed by subculture to media with various combinations of BA, TIBA, GA_3, etc may lead to formulation of a 'best' challenge medium, which might be worthy of the title 'regeneration medium'. Maintenance on low levels of BA (e.g. B.1) might have an advantage in prolonging responsiveness to BA.

Maintenance of calli on B1 with increasing levels of TIBA at each subculture might provide further support for the tolerance hypothesis. Maintenance of calli on B1T1 may only postpone an inevitable decline in shoot regeneration. Calli on B1T1 had enhanced regeneration for about two subcultures and then started to decline at a rate similar to the B1 calli. The B1T1 calli thus exhibited the response of younger calli.

Another unexplored area of long term regeneration is the effects of light. Preliminary studies suggested that transfer of calli from the dark to the light stimulated regeneration (Doley, unpublished; Galatowitsch and Smith, 1990). Experiments to evaluate the effect of light should involve transfer of dark-maintained calli to both dark and lighted environments.

The importance of long term regenerability is partly determined by the nature of the protocol to be used. For procedures such as somatic cell selection with acute exposure, sugarbeet genotypes with sufficient longevity of regeneration are available (e.g. REL-1), but cultural modifications might still be able to enhance the probability of obtaining a mutant regenerant. On the other hand, the available longevity, except in the case of EL 45/2, may be insufficient for the regeneration of sugarbeet plants from protoplasts.

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