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Improvement of somatic embryogenesis and plant recovery in cassava

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Abstract
Methods for improving the efficiency of plant recovery from somatic embryos of cassava (*Manihot esculenta* Crantz) were investigated by optimizing the maturation regime and incorporating a desiccation stage prior to inducing germination. Somatic embryos were induced from young leaf lobes of *in vitro* grown shoots of cassava on Murashige and Skoog medium with 2,4-dichlorophenoxy acetic acid. After 15 to 20 days of culture on induction medium, the somatic embryos were transferred to a hormone free medium supplemented with activated charcoal. In another 18 days mature somatic embryos became distinctly bipolar and easily separable as individual units and were cultured on half MS medium for further development. Subsequent desiccation of bipolar somatic embryos resulted in 92% germination and 83% complete plant regeneration. The plants were characterized by synchronized development of shoot and root axes. Of the non-desiccated somatic embryos, only 10% germinated and 2% regenerated plants. Starting from leaf lobes, transplantable plantlets were derived from primary somatic embryos within 70 to 80 days.

Key words: *Manihot esculenta* - somatic embryogenesis - maturation - germination - desiccation

Abbreviations: 2,4-D, 2,4 dichlorophenoxyacetic acid; BA, Benzyl aminopurine; GA, Giberellic acid; MS, Murashige and Skoog; NAA, Naphthalene acetic acid.

Introduction

Cassava is a perennial woody shrub cultivated mainly in the tropics for its starchy tuberous roots. It belongs to the family Euphorbiaceae which also includes rubber (*Hevea brasiliensis*) and castor bean (*Ricinus communis*). Among tropical crops, rice, sugarcane, maize and cassava are the most important sources of calories for human consumption. Among these, cassava is the more cheaply cultivated. It also has important applications as animal food and in industry. Because of its broad adaptabilty to a variety of soil and climatic conditions, drought tolerance and ability to grow on depleted and marginal soils, cassava is very important to the agroeconomy of several tropical countries. The average agricultural yield varies widely, for example: from 7.7 metric tons/hectare in Zaire to 18.8 metric tons/hectare in India (FAO 1990). Some of the factors that contribute to this range of yield are varietal differences, losses due to diseases such as cassava bacterial blight, African cassava mosaic virus, tuber rot, and to insect pests, such as spider mites and mealybugs.

Several international groups are co-operating in the development of new cassava cultivars and improving existing cultivars for better yield, protein quality and resistance to diseases and pests. *In vitro* methods coupled with recombinant DNA technology offer attractive routes for improvement of plant cultivars. Efficient and reproducible plant regeneration methods linked with viable transgenic systems are essential pre-requisites for the success of these approaches. The purpose of this study was to establish an efficient system for plant recovery from somatic embryos in cassava.

There have been several reports of cassava plant regeneration via somatic embryogenesis (Stamp and Henshaw 1982, Stamp 1987, Stamp and Henshaw 1986, 1987 a, b, Szabados et al. 1987, Cabral et al. 1992). The efficiency of plant regeneration, however, has remained a limiting factor (Schopke et al. 1992a). Generally, a two step procedure was followed for plant regeneration through somatic embryogenesis: stage 1 - induction of somatic embryos on medium containing 2,4-D; stage 2 - transfer to medium with 2,4-D + BA with or without GA to promote shoot development, followed by adventitious rooting (Stamp and Henshaw 1987 b, Szabados et al. 1987). Somatic

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CLaSP. Since the expression of CLaSP was directed by a patatin promoter and message levels would be low in the leaves, the mRNA isolated from leaves was probed with the *npt* II coding region (Figure 3A). The northern using the LSP cDNA as a probe (Figure 3B) shows that the LSP gene is transcribed in the tubers of transgenic plants. These clones as well the the GUS expressing plants are currently being evaluated in field trials for bruise resistance, somoclonal variation, agronomic traits and tuber quality at various locations across the United States.

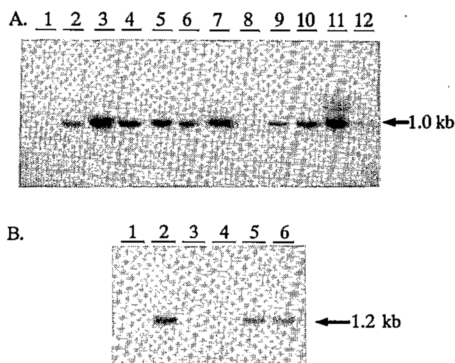

Fig. 3. Northern blot of total RNA extracted from: A. Tissue culture leaves of control Lemhi (Lane 1), transgenic Lemhi (Lanes 2-9), and Russet Burbank (Lanes 10-12); B. Russet Burbank control (Lane 1) and putative transgenic tubers (lanes 2-5). The mRNA was separated on an 0.8% agarose gel transferred to a nylon membrane. The blot in A was probed with a one kb ^{32}P-labelled DNA fragment encoding NPT II isolated from pCGN1547, and B was probed with a 1.2 kb ^{32}P-labelled DNA fragment encoding LSP. Note: Lane 3 contains RNA from a plant which escaped transformation and does not produce the LSP message.

The advantages of the method described here include the elimination of surface sterilization of the explant because microtubers are grown *in vitro*. Eyes or buds on the microtubers produce shoots which may serve the same function as a nurse culture. Also, if necrotic or unresponsive discs are removed, addition of an ethylene inhibitor such as $AgNO_3$ (De Block 1988) appears to be unnecessary. Based on these results we now use Stage I medium with a 10.0:0.3 μM ZR:IAA-AA ratio for routine *Agrobacterium*-mediated transformation and shoot regeneration from Lemhi, Russet Burbank, and Lenape (data not shown). The length of time on Stage I medium has been reduced to 1 month and transgenic shoots can appear on Lemhi within 14 days on Stage II medium, while culture time on Stage II medium should be extended to about 2 months or more for Russet Burbank. Multiple shoots usually form on the discs, although we did not attempt to determine if they arose from a single or multiple transformation event. We have found this microtuber transformation protocol to be more efficient and reliable than our previous method (Ishida *et al.* 1989), and as shown here the method works well with a variety of genotypes and might be useful for most potato cultivars.

Acknowledgements. The authors wish to acknowledge Betty Ishida and David Twell for their helpful suggestions on transformation and regeneration procedures, and Jody Steffens for her work with the CLaSP plants. References to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others that may also be suitable.

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embryos could also be recultured on 2,4-D medium for the production of secondary embryos (Stamp and Henshaw 1987b). Such cultures can be maintained as a continuous source for plant regeneration and transformation studies (Raemakers et al. 1992, and our observations). The development of somatic embryos in stage 2 was often limited to apical shoot elongation with no growth of the root axis (Henshaw 1991). Our studies with the previously described protocols showed: a) a low frequency of somatic embryo development, and b) those embryos exhibiting shoot elongation had to be excised and subcultured on shoot propagation medium for induction of adventitious roots and subsequent plant development.

In the present study, we describe a modified protocol for efficient plant recovery via somatic embryogenesis that is characterized by well defined stages of embryo development, maturation, germination and plant regeneration.

Materials and Methods

Explant source. In vitro clonal cuttings of cassava, *Manihot esculenta* Crantz of cultivar MCol 1505, obtained from CIAT, Colombia, were grown on MS medium (Murashige and Skoog, 1962) at 25° ± 2° C with a photoperiod of 16 h (flourescent tubes, Sylvania cool white, 90-110 $\mu Mm^{-2}s^{-1}$ PAR). Nodal explants, 2 to 3 cm long, were cultured on MS medium under the same conditions. Within 10 to 20 days, axillary buds sprouted and gave rise to young leaf lobes. These leaf lobes were used as explants for induction of somatic embryos.

Induction of somatic embryogenesis. Young leaf lobes 4 to 6 mm long were isolated and cultured on medium with MS (Murashige and Skoog 1962) minerals and vitamins, 2% sucrose and 4 mg/l 2,4- D. The medium was supplemented with additional copper in the form of 2 µM $CuSO_4$ to improve frequency of somatic embryogenesis as reported by Schopke et. al. (1992b). After adjusting the pH to 5.8, the medium was solidified with 0.2% Phytagel (Sigma) before autoclaving. Leaf lobe cultures for somatic embryo induction were kept in dark at 25° ± 2° C and were evaluated after 14 to 20 days.

Maturation of somatic embryos. The term "maturation" as used in this study denotes the development of globular stage somatic embryos into distinct bipolar structures with defined shoot and root axes and expanded green cotyledons.

Primary somatic embryos with adjoining callus were transferred to medium with MS minerals and vitamins consisting of 10 mg/l thiamine HCl, 1.5 mg/l pyridoxine HCl, 1.5 mg/l nicotinic acid, 2mg/l glycine, 100 mg/l inositol (referred to as "cassava vitamins" in this paper), 3% sucrose and 0.5% charcoal solidified with 0.3% Phytagel. After 18 to 20 days of culture on maturation medium individual somatic embryos were isolated for further development.

Secondary somatic embryos from a two year old culture (repetitive embryos) were also used as explants for studies on embryo maturation and development. These were derived from leaf lobes of cultivar MCol 1505 and had been maintained as somatic embryo clumps by subculture at monthly intervals on MS medium with 2 mg/l 2,4-D at a photoperiod of 16 h (flourescent tubes, Sylvania cool white, 20-30 $\mu Mm^{-2}s^{-1}$ PAR).

Secondary somatic embryo clumps, about 2 to 3 mm size and comprising roughly 50 to 60 globular stage embryos, were cultured on the following media for maturation: (1) MS basal medium, (2) MS medium supplemented with 0.01 mg/l 2,4-D and 0.1 mg/l BA (after Stamp and Henshaw 1987 a, b), (3) MS medium supplemented with 0.1 mg/l BA, 0.01 mg/l NAA and 1 mg/l GA (after Szabados et al. 1987) and (4) modified MS medium with charcoal (same as the maturation medium for primary somatic embryos).

Desiccation. Some of the somatic embryos were subjected to desiccation treatment at various stages of development to study the effects of desiccation on plant regeneration. Somatic embryos were sealed in empty petri plates for a period of 6 to 8 days so as to lose approximately 17% of their initial fresh weight. They were then cultured on germination medium.

Germination and plant regeneration. In this study, the term "germination" denotes the elongation of the primary root; "conversion" or "plant regeneration" refers to the development of plantlets with a well established root system and shoot with at least the first pair of leaves.

After maturation, individual somatic embryos with or without desiccation were cultured on medium with half MS major salts, MS minor salts, cassava vitamins and 2% sucrose gelled with 0.2% Phytagel for germination and plant regeneration. Frequencies of germination and plant regeneration were scored at the end of 30 and 40 days for primary and secondary somatic embryos, respectively.

All cultures at maturation, desiccation, germination and plant regeneration stages were kept at 25°C with a photoperiod of 16 h (flourescent tubes, Sylvania cool white, 90-110 $\mu Mm^{-2}s^{-1}$ PAR) and 60% relative humidity.

Results

Induction of somatic embryos. Within 7 to 10 days of culture, leaf lobes on somatic embryo induction medium showed dedifferentiation and globular projections at cut ends and on the adaxial surface of the leaf. At the end of 2 weeks, globular embryos became visible in 85% of 336 leaf lobe explants with 61.6% showing more than 10 such embryos per leaf lobe (Fig. 1).

Maturation. After 17 days of culture on induction medium, somatic embryos from 100 leaf lobes were transferred to maturation medium with the adjoining callus. After another 18 days, somatic embryos from 80 of the leaf lobes produced mature bipolar green embryos (Fig. 2) while the rest gave stumpy callus with spontaneous root formation. Mature somatic embryos above 5 mm in length (Fig. 3) were isolated for further development while the remaining tissue, with embryos at early stages of development, were re-cultured on fresh maturation medium. As shown

in Table 1, mature somatic embryos were periodically harvested from these cultures. In 52 days a total of 1178 mature somatic embryos were harvested. Studies with various media showed that MS medium with charcoal was superior to all the other media tested for maturation of somatic embryos from two year old cultures (Table 2, Fig. 4). The morphological quality of the somatic embryos, in terms of normality of shape, definition of cotyledons, presence of apical meristem and root axis, was much better than those isolated from other media. On MS medium with charcoal, bipolar units of somatic embryos were easily separable while in other media foliose structures developed which were not necessarily individual bipolar structures.

TABLE 1. PERIODIC HARVEST OF SOMATIC EMBRYOS FROM LEAF LOBE CULTURES

Period from last harvest of somatic embryos (days)	Culture period on maturation medium (days)	Number of Somatic Embryos (%)		
		Isolated Size 5-11 mm	Germinated with desiccation	Regenerated plants
First harv	18	168	157 (93.5%)	143 (85.1%)
7	25	182	150 (82.4%)	144 (79.1%)
15	40	214	139 (64.9%)	104 (48.6%)
4	44	304	233 (76.9%)	185 (60.8%)
8	52	310	175 (65.5%)	133 (42.9%)

Number of initial leaf lobes: 80; Culture period on induction medium: 17 days

Germination of somatic embryos and plant regeneration. Desiccation played a significant role in increasing the frequency of germination of somatic embryos and plant regeneration (Table 3, Figs 5-7). After desiccation treatment, primary somatic embryos germinated within 6 to 10 days followed by shoot development, while non-desiccated somatic embryos germinated in 20 to 30 days. At the end of one month plantlets from desiccated somatic embryos formed shoots 6 to 10 cm tall with 4 to 6 well developed leaves. In comparison, the shoots in plantlets from non-desiccated somatic embryos were only 1 to 3 cm tall with poor leaf development. Somatic embryos smaller than 5 mm in size were poor converters with only 3% plant regeneration despite the desiccation treatment (data not shown).

TABLE 2. EFFECT OF MEDIA CONSTITUENTS ON MATURATION OF SECONDARY EMBRYOS

Medium	No. of somatic embryos (size> 5mm.) isolated per clump Mean ± S. E.
MS	0.2 ± 0.3
MS + 2,4-D + BA	0.4 ± 0.4
MS + 2,4-D + BA + GA	0.7 ± 0.2
MS + Activ. Charcoal	3.4 ± 0.8

No. of replicates: 5; No. of clumps/replicate: 6

Desiccation of somatic embryos from cultures maintained in repetitive embryogenic cycle for two years gave 62% germination and 48% plant regeneration whereas non-dessicated somatic embryos gave only 15% and 3% germination and plant regeneration, respectively (Table 4). In secondary somatic embryos germination was observed after 10 to 20 days as compared to 5 to 10 days in primary somatic embryos.

Discussion

In the present study 85% of the leaf lobes islolated from cassava cv. MCol grown in vitro gave rise to somatic embryos. Szabados et al. (1987) reported an average frequency of somatic embryo formation in 47% of leaf lobe explants. The major differences between our protocol and those described earlier (Stamp and Henshaw 1982, 1987a, b, Szabados et al. 1987) are: (a) maturation of somatic embryos on charcoal supplemented medium which facilitated development of easily separable individual bipolar units, and (b) desiccation treatment of somatic embryos leading to synchronous development of shoot and root axes.

The improved development of somatic embryos with the addition of activated charcoal has been reported in several studies. Fridborg et al. (1978) showed that activated charcoal adsorbs a number of compounds including auxins and culture metabolites which often inhibit a specific developmental stage(s)

of somatic embryos.

The repeated harvest of mature somatic embryos on hormoneless medium is noteworthy. Secondary somatic embryos continuously budded from the hypocotyl base of the developing embryo. After 17 days exposure to 2,4-D, 80 leaf lobes gave a total of 1178 somatic embryos in a period of 52 days; 709 of these embryos gave rise to plantlets (see Table 1). Similar to these observations in cassava, somatic embryos of walnut and pecan underwent repetitive somatic embryogenesis and maturation on basal medium devoid of growth regulators after a single exposure to induction medium with hormone (Deng and Cornu 1992, Wetzstein et al. 1989). It should also be noted that although the number of somatic embryos increased at each harvest the percent germination and plant regeneration declined in successive cycles. Investigations are in progress to increase the frequency of plant recovery from secondary somatic embryos.

Starting with leaf lobe explants, transplantable plantlets from primary somatic embryos were obtained in 70-80 days in contrast to the 140-180 days required for field establishment of somatic embryo plantlets from cotyledon explants (Stamp and Henshaw 1987b). More than 9 plantlets per leaf lobe were obtained in the present study compared to 1.15 plantlets per primary explant obtained in earlier studies (Schopke et al. 1992a).

Globular somatic embryos of cassava could be maintained by repeated subculture on medium with 2 mg/l 2,4-D as reported by Stamp and Henshaw (1987b) and Szabados et al. (1987). We observed that as many as 100 to 200 somatic embryo clumps could be generated per leaf lobe in a period of 4 months.

Morphological and biochemical evidence suggest close homology between zygotic and somatic embryos (Ammirato 1987, Crouch 1982,). As with most zygotic embryos, to promote the development of morphologically normal plants it is important to maintain somatic embryos in a developmental mode until they are fully formed and have accumulated sufficient reserves to permit successful germination and seedling growth. The acquisition of desiccation tolerance could be viewed as a signal of the potential autonomy of the embryo. Desiccation or maturation drying terminates the developmental mode and "switches" the embryo into a germination mode (Kermode et al. 1989, Attree et al. 1991). It is possible that changes in turgor pressure caused by desiccation initiate a set of biochemical events affecting membrane permeability and levels of endogenous ABA which in turn induce expression of specific genes involved in plant development (Oishi and Bewley 1990, Skriver and Mundy, 1990). In

TABLE 3. EFFECT OF DESICCATION ON GERMINATION AND PLANT REGENERATION FROM PRIMARY SOMATIC EMBRYOS DERIVED FROM LEAF LOBE CULTURES

Treatment	Size of somatic embryos (mm)	Number of somatice embryos (%)		
		Initial	Germinated	Regenerated plants
Without desiccation	5 to 8	36	3 (8.3%)	1 (2.8%)
	8 to 11	48	5 (12.5%)	1 (2.1%)
With desiccation	5 to 8	36	32 (88.9%)	29 (80.1%)
	8 to 11	48	45 (93.8%)	41 (85.4%)

TABLE 4. EFFECT OF DESICCATION ON GERMINATION AND PLANT REGENRATION FROM SECONDARY SOMATIC EMBRYOS DERIVED FROM TWO YEAR OLD CULTURES

Treatment	Number of Somatic Embryos (%)		
	Initial	Germinated	Regenerated plants
Without Desiccation	69	10 (14.5%)	2 (2.9%)
With Desiccation	69	43 (62.3%)	33 (47.8%)

cassava somatic embryos smaller than 5mm in size were poor converters even after desiccation.

Besides providing a framework for genetic manipulations, a vigorous and efficient embryogenic system would facilitate development of compact storage, packaging and distribution methods for elite varieties of cassava.

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Legends to figures

1. Somatic embryo induction from leaf lobes on 2,4-D medium.
2. Maturation of somatic embryos on charcoal medium.
3. Mature somatic embryos isolated for desiccation.
4. Secondary somatic embryos for maturation
   at left : on MS medium with charcoal
   at right : on MS medium with 2,4-D and BA
5. Germination of desiccated somatic embryo after six days of culture on 1/2 MS medium.
6. Regenerated plants after 25 days of culture.
7. A regenerated plant ready for transplanting to soil.

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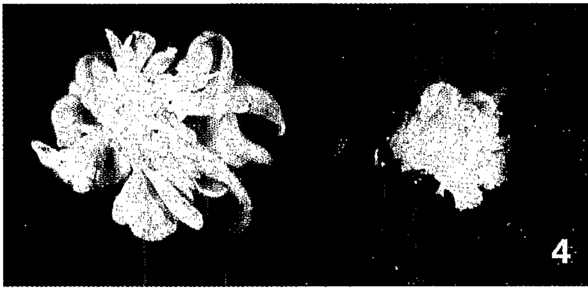
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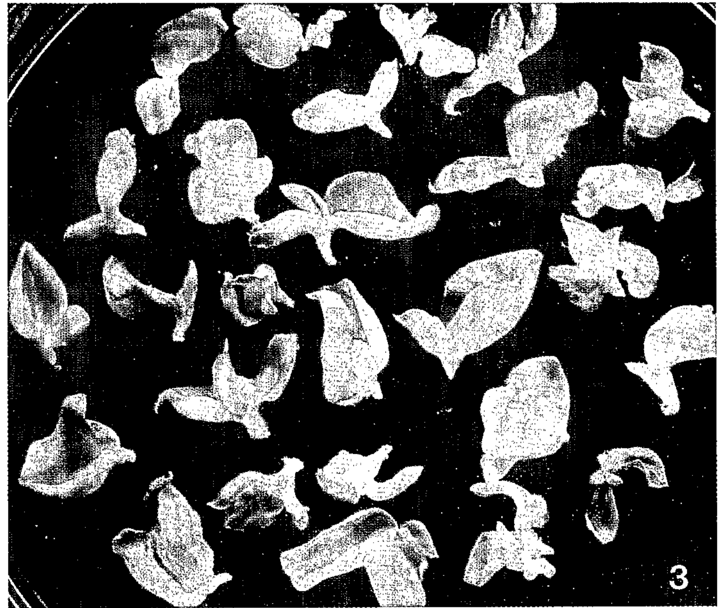
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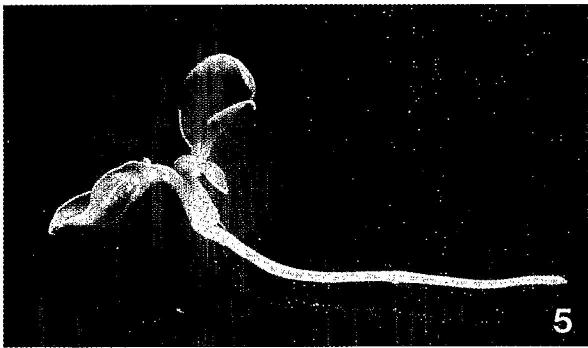
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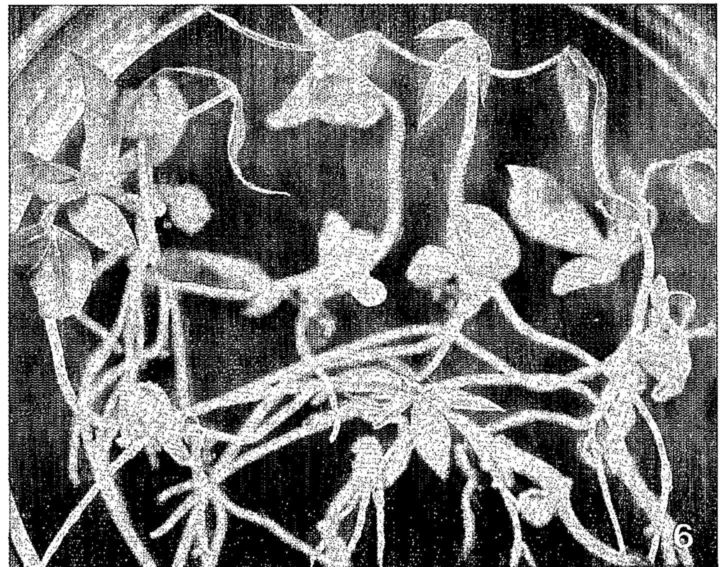
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Improvement of anther culture methods for doubled haploid production in barley breeding

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Abstract. There is potential to accelerate cultivar development with a doubled haploid system for breeding line production. Anther culture methodology was evaluated for U.S.A. spring barley (*Hordeum vulgare* L.) breeding applications. Gelrite was found to be an acceptable replacement for ficoll in the induction medium to reduce costs while maintaining embryoid and plant production levels. Beneficial effects of 28 d cold pretreatment of donor spikes for anther culture were confirmed with Pacific Northwest USA barley genotypes. A 3 d mannitol solution pretreatment of fresh anthers was shown to be less effective for green plant production compared to 28 d cold pretreatment of donor spikes. Extended donor spike cold pretreatment from 28 to 42 d did not reduce anther culture productivity. Based on this research, anther culture techniques show promise for economical and convenient application in spring barley breeding.

Abbreviations: DH, doubled haploid - LS, Linsmaier and Skoog basal medium - BAP, benzylaminopurine - GLM, Generalized Linear Model - SAS, Statistical Analysis System

Introduction

The effective use of doubled haploid (DH) plants for accelerating cultivar development in barley breeding has been established. Although the *Hordeum bulbosum* method has been favored commercially for doubled haploid plant production in barley, the efficiency of androgenesis via anther culture has been improved markedly (Pickering and Devaux 1992; Luckett and Smithard 1992; Kuhlmann and Foroughi-Wehr 1989). In addition, anther culture has greater potential for DH production than the *H. bulbosum* method due to the large number of microspores compared to egg cells per spike.

Unfortunately, an efficient level of DH production has been achieved only with the cultivar Igri and a few other barley genotypes (Hunter 1985; Olsen 1987; Kuhlmann and Foroughi-Wehr 1989; Kao et al. 1991), and a large genotype effect has been noted in barley (Pickering and Devaux 1992). There is now a greater need to modify the available methods for economic reasons, as well as to minimize the genotype effect, rather than to maximize methods for the few most responsive genotypes (Kuhlmann and Foroughi-Wehr, 1989).

One of the high cost components for anther culture is the gelling/viscosity modifying agent in culture media. Ficoll 400 has been used by many researchers to improve embryoid production and green plant regeneration from cultured anthers (Olsen 1987; Kuhlmann and Foroughi-Wehr 1989; Kao et al. 1991). Sea plaque agarose has been used widely to solidify media and to avoid inhibitory effects of agar (Hunter 1985; Olsen 1987; Luckett and Smithhard 1992). However, both ficoll and sea plaque agarose are expensive, and ficoll is also difficult to mix into the medium. Recently, Gelrite, a gellan gum, has been investigated as a gelling agent and proved to be advantageous in rye (*Secale cereale* L.) anther culture (Flehinghaus et al. 1991). No information on the use of gelrite for barley anther culture has been available.

Cold pretreatment of donor spikes is a factor that adds to the complexity of barley anther culture technology. Beneficial effects of 28 d cold pretreatment have been reported (Huang and Sunderland 1982, Powell 1988), while conflicting results on the length of cold period have also been observed (Kuhlmann and Foroughi-Wehr 1989; Szarejko and Kasha 1991). These contradictions could be from different genotypes and/or donor plant environments used in the various investigations. Pretreatment of fresh anthers in 0.3 M mannitol solution for 3 d has been shown to be a potential substitute for 28 d cold pretreatment in barley microspore culture (Kasha

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