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Transmission of somatic embryogenesis via sexual hybridization in Dactylis glomerata L.

Anita M. Lujan

To the Graduate Council:

I am submitting herewith a thesis written by Anita M. Lujan entitled "Transmission of somatic embryogenesis via sexual hybridization in Dactylis glomerata L.." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant, Soil and Environmental Sciences.

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To the Graduate Council:

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We have read this thesis
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TRANSMISSION OF SOMATIC EMBRYOGENESIS VIA SEXUAL HYBRIDIZATION IN *DACTYLIS GLOMERATA* L.

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Anita M. Lujan

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ABSTRACT

The primary objective of this study was to determine if the ability of an orchardgrass (Dactylis glomerata L.) genotype to produce somatic embryos in in vitro culture could be sexually transmitted to genotypes not possessing this ability. Reciprocal crosses were performed between embryogenic and nonembryogenic genotypes. Seeds from crosses were germinated and plants maintained in a greenhouse. Leaf sections of F_1 plants (27 crosses and 4 selfs) were cultured on Schenk and Hildebrandt (SH) medium amended with 30 μM 3,6-dichloro-o-anisic acid (dicamba) and evaluated for embryogenic response. There were no significant differences between F_1 plants in which females were the embryogenic parents and those in which males were the embryogenic parents for the production (mean number) of somatic embryos and the ability of these somatic embryos to produce viable shoots. Five crosses individually compared with their reciprocals were not significantly different for the production (mean number) of somatic embryos and their ability to produce shoots. Two embryogenic parents compared to their F_1 progeny were not significantly different for the production of somatic embryos. However, one parent was significantly different from its F_1 progeny for the total shoots produced. The ratios of embryogenic to nonembryogenic F_1 progeny and of embryogenic females to embryogenic males were 1:1.

Secondary goals were to determine the influence of sucrose on the germination of somatic embryos and the development of meristematic

tissue in somatic embryos. Seventy percent of the somatic embryos that were cultured on SH with 3% sucrose produced shoots whereas, only 27% of those cultured on SH without sucrose produced shoots. Shoot and/or root apices from somatic embryos of F_1 derived leaf cultures were observed from histological sections of somatic embryos, but there was insufficient data to make any conclusions.

Transmission of somatic embryogenesis was demonstrated to be under control of nuclear gene(s) due to lack of significant effects for reciprocal crosses. The embryogenic trait was effectively incorporated into nonembryogenic genotypes via sexual hybridizations. This research provides preliminary information that will be useful for further understanding somatic embryogenesis in orchardgrass.

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CHAPTER I

INTRODUCTION

Orchardgrass is a perennial, cool season bunch grass producing an open sod. It is native to Europe and is commonly grown in the temperate regions of the United States as a forage grass (Jung and Baker, 1985). It is an autotetraploid having 2n=4x=28 chromosomes (Müntzing, 1937). The mode of reproduction is sexual, and the crop is commercially propagated from seed. Vegetative propagation from tillers is used during certain stages of breeding programs. Development of new cultivars of orchardgrass takes between 15 and 20 years. For example, the popular cultivar 'Potomac' took 19 years to develop through a mass selection breeding method. It was released in 1954 (cf. Wagner et al., 1972).

In recent years there has been an increasing interest in the application of plant cell and tissue culture research to agriculture. However, a necessary requirement for the application of this technology is the ability to regenerate whole plants from cultured cells and/or tissues. The two main processes of plant regeneration are organogenesis and somatic embryogenesis. Organogenesis, the development of unipolar structures (shoots and/or roots), is generally considered to be a multicellular event (Broertjes and Keen, 1980; Haccius, 1978), although unicellular derivation of meristems has also been suggested (Ammirato, 1985; Broertjes and Keen,

1980). Somatic embryogenesis is a process in which bipolar structures that resemble zygotic embryos are formed. The somatic embryos may be formed directly from cultured tissue, from intermediary callus, or from suspension cultures (Vasil, 1985). Embryos are thought to have a single cell origin either from individual cells or from proembryonal complexes that originate from single cells (Haccius, 1978; Halperin, 1969).

Plant regeneration by somatic embryogenesis has been reported in numerous monocot and dicot species (Tissert et al., 1979; Williams and Maheswaran, 1986) including various species of cereals and grasses (Vasil, 1985). A plant regeneration system has been developed for somatic embryos originating from leaf tissue, anthers, pistils and suspension cultures of Dactylis glomerata L. (orchardgrass) (Conger et al., 1983; Gray and Conger, 1985a,b; Hanning and Conger, 1986; Hanning and Conger, 1982; Songstad and Conger, 1986). Transfer of the somatic embryogenic trait into nonembryogenic genotypes possessing elite agronomic characteristics, and cloning these selected genotypes is important in the implementation of in vitro techniques in orchardgrass improvement (Conger et al., 1986).

The primary objective of this study was to determine if somatic embryogenesis, as expressed under certain cultural conditions (Hanning and Conger, 1986), could be transmitted sexually to progeny from crosses between embryogenic and nonembryogenic genotypes. Reciprocal crosses were made between embryogenic and nonembryogenic genotypes and offspring were cultured to observe the transmission of somatic

embryogenesis through both male and female parents. Secondary objectives were to determine the development of meristematic tissue of somatic embryos produced from some F_1 explants and their parents, and to determine the influence of sucrose on the development of somatic embryos into plantlets.

CHAPTER II

LITERATURE REVIEW

Successful regeneration from cell and tissue cultures depends on the genotype selected for study in conjunction with *in vitro* cultural conditions (Buiatti et al., 1974; Guha-Mukherjee, 1973; Lazar et al., 1983; Marsolais et al., 1984; Raquin, 1982; Templeton-Somers and Collins, 1986) and the physiological development of the initial explant (Lu et al., 1983; Ozias-Akins and Vasil, 1983; Vasil, 1985). These variables influence the initiation of cell and tissue cultures and their subsequent regeneration into whole plants. Legumes and grasses seem to be especially responsive to culture when genotypic selection is practiced (Oelck and Schieder, 1983). Therefore, the literature review on the effect of genotype on cell and tissue culture response will be focused on these species.

Genotypic Influence

Legumes

Bingham et al. (1975) reported that the regeneration potential of *Medicago sativa* L. (alfalfa) callus could be increased by using recurrent selection and *in vitro* screening procedures. Hypocotyls of F_1 plants were cultured and after 3 generations of screening calli for *in vitro* regeneration and 2 generations of *in vivo* recurrent selection, the frequency of plant regeneration increased from 12%

to 67%. In additional experiments involving regeneration from diploid alfalfa callus, the segregation ratios observed for high regenerator x low regenerator crosses suggested that bud differentiation was controlled by two dominant genes (Reisch and Bingham, 1980). The authors noted that transferring genes into diverse genetic backgrounds should be relatively easy since the regeneration trait was qualitatively inherited.

The genetic variability in callus cultures of Trifolium pratense L. (red clover) was assessed for growth, development and somatic embryogenesis using a complete diallel crossing design (Keyes et al., 1980). Red clover calli were propagated on PCL medium (Phillips and Collins, 1979) and on B5 medium (Gamborg et al., 1968), but no significant differences in somatic embryogenesis were found. Additive genetic variance was significant for callus initiation, growth and redifferentiation based on F_1 progeny observations. Therefore improvements in regeneration response could be achieved by breeding and selection for embryogenic genotypes.

The in vitro regeneration response for several other legume species was also shown to be affected by genotype (Oelck and Schieder, 1983). In their study seed of 19 cultivars representing 7 species (alfalfa, red clover, peanut--Arachis hypogaea L., soybean--Glycine max L., yellow sweet clover--Melilotus officinalis L., persian clover--Trifolium resupinatum L., and broad bean--Vicia faba L.) were germinated in petri dishes and evaluated for seedling vigor. Vigorous and nonvigorous shoots were cultured, and the shoots selected for high

vigor exhibited superior redifferentiation ability from callus as compared to nonselected materials.

Grasses

Research advances in cell and tissue culture of grasses have been limited due to difficulties in regenerating plants across a wide range of genotypes (Beckert and Qing, 1984; Hodges et al., 1986). It has been suggested that genotype may be more critical than manipulations of environmental conditions (Bullock et al., 1982; Lazar et al., 1983, 1984a,b; Maddock et al., 1983; Sears and Deckard, 1982). Several experiments have been conducted in attempt to sexually transmit the regeneration response. Hodges et al. (1986) confirmed Armstrong and Green's (1985) report that genotype A188 of Zea mays L. (corn) maintained a high frequency of regeneration via somatic embryogenesis when immature embryos were used as the initial explant. Although genotype A188 was not agronomically desirable, regenerative capabilities made it useful to study the heritability of regeneration potential. A188 was used as the pollen parent in crosses with 25 agronomically important inbred lines. Immature embryos from hybrid seeds were cultured and varied greatly in their responses, i.e., some produced only embryogenic callus, and others developed further to produce plants. Results clearly indicated that embryogenesis and subsequent plant formation were under control of dominant nuclear genes. Furthermore, expression of genes for regeneration appeared to be dependent on specific media components, i.e., the type and concentration of nitrogen. Hodges et al. (1986)

found that MS medium (Murashige and Skoog, 1962), modified with 20.6 mM NH_4^+ and 39.4 mM NO_3^- was generally superior to N6 medium (Chu et al., 1975), which contains 7 mM NH_4^+, 28 mM NO_3^-, and 20 mM proline, for the production of embryogenic callus in corn. Beckert and Qing (1984) used a diallel breeding design to test for genetic transmission of high regeneration ability in corn after 5 months of callus growth. They reported that regeneration was heritable in the F_1 generation; however, the percentage of embryos producing plants varied from 0-92%. Bruneau (1985) also conducted a detailed study to determine plant regeneration rate in corn. Observations were made on percentage of immature embryos that produced at least one plant per total number of embryos cultured, and number of plants regenerated per individual embryo cultured. Reciprocal crosses were made among low, medium and high regenerators. Genotype had the greatest influence on the number of somatic embryos produced. The different concentrations of growth regulators 2,4-D (2,4-dichlorophenoxyacetic acid) and pCA (para-chlorophenoxyacetic acid) seemed to have the same effect on the production of somatic embryos.

Genetic background has also been demonstrated to be very important in regeneration of hexaploid Triticum aestivum L. em. Thell. (wheat) from tissue culture. Inflorescences and immature embryos from 25 wheat cultivars of spring and winter wheat were cultured on MS medium and examined for variation in initiation and development of shoots from callus (Maddock et al., 1983). Plants from all 25 cultivars produced shoots, but there were significant

differences in morphogenic capacity among the different cultivar explants, i.e., the plants produced from cultured embryos of glasshouse grown material ranged from 14-82% and from 12-96% in field grown material. Another study utilizing a different set of 25 wheat cultivars and 3 culture media confirmed that callus proliferation rate and differentiation of wheat cultures varied significantly among plants of different cultivars (Lazar et al., 1983). Additional studies showed that transmission of genes for androgenesis (development of a haploid embryo from a male nucleus) to F_1 progeny was accomplished when androgenic genotypes were crossed to nonandrogenic genotypes (Lazar et al., 1984b; Bullock et al., 1982). Most of the variability was attributed to nuclear genes; however, reciprocal effects for androgenesis were also found to be significant, indicating that cytoplasmic genes were also involved (Lazar et al., 1984b). Conversely, in a similar study Bullock et al. (1982) did not demonstrate significant differences between reciprocal crosses. This indicated that the androgenic trait was nuclear in origin; however, the possibility of interaction between nuclear and cytoplasmic genes was not excluded. Both studies suggested that anther culture response may be improved by increasing the androgenetic potential of currently nonandrogenic genotypes coupled with alteration of environmental conditions that enhance this response.

Sixty rice varieties were cultured on plant growth medium amended with 2,4-D (Abe and Futsuhara, 1986). Genotypic variability for callus formation and plant regeneration was observed among and

within three Oryza sativa L. (rice) subspecies and one hybrid. Most of the variation was attributed to genotype, although concentrations of endogenous phytohormones and 2,4-D were considered to affect the regeneration response.

Dunwell (1981) demonstrated that immature embryos from three Hordeum vulgare L. (barley) genotypes differed in water content of callus, dry weight of callus and in their ability to regenerate plants on MS media supplemented with 3, 6, 9 or 12% sucrose, and 0, 2, 4 or 8 mg/l 2,4-D. Results demonstrated that specific differences in genotypes were difficult to confirm because of the complexity of interactions in this system. Several barley cultivars were tested for high androgenic and nonandrogenic responses (Foroughi-Wehr et al., 1982). Anthers from the F_1 progeny were cultured on Clapham medium (1973). Callus formation ranged from 3.3 to 73.2 per 1000 anthers cultured, and plant regeneration varied from 0 to 12.7 green plants formed per 1000 anthers cultured. These results clearly indicated that regeneration was controlled by genetic factors and suggested that it was more reasonable to transfer the androgenic response to nonandrogenic wheat breeding material rather than testing many culture conditions (Foroughi-Wehr et al., 1982).

Orchardgrass

Somatic embryogenesis and plantlet regeneration from leaf segments of orchardgrass were first reported by Hanning and Conger (1982). Embryos were produced either directly from the leaf tissue or from embryogenic callus. The basal section of the innermost

leaf segment produced the greatest amount of callus and/or somatic embryos. The external appearance and histological studies of the embryos indicated that the plants were formed by somatic embryogenesis, although evidence for the unicellular origin of embryogenic callus and embryos was not conclusive. A later histological study of somatic embryogenesis using paraffin sections identified direct embryos arising from mesophyll cells (Conger et al., 1983).

Embryogenesis in cell suspension cultures of orchardgrass was initially reported by Gray et al. (1984). Somatic embryogenesis in suspension cultures was demonstrated to be dependent on the addition of 3,6-dichloro-o-anisic acid (dicamba, a synthetic auxin) and casein hydrolysate (Gray and Conger, 1985a). Hanning and Conger (1986) determined that the optimum response for somatic embryogenesis in cultured leaf segments occurred in medium amended with 30 μM dicamba or 10 μM 2,4-D acid. They reported that plants regenerated from somatic embryos showed an increase in regeneration potential relative to the original donor plant. The embryogenic and regeneration responses were maintained through four cycles of *in vitro* culture. A cycle was completed when a somatic embryo, formed from a cultured leaf section, produced a plant (cycle I) which in turn produced embryos in culture that regenerated plants (cycle II), etc.

Genotype was suggested to have a predominant influence on the embryogenic response (Hanning and Conger, 1982, 1986; Hanning, 1984). Differential genotypic responses for somatic embryogenesis were observed among plants grown from seed in which 5% produced

embryogenic callus (Hanning and Conger, 1986). Twenty-five percent of the F_1 progeny tested from an open-pollinated population of embryogenic and nonembryogenic genotypes produced embryogenic callus.

CHAPTER III

MATERIALS AND METHODS

The orchardgrass clones used in this study were from the cultivar 'Potomac'. Embryogenic genotypes were specifically derived from a single plant designated number 10. Initially leaves from plants were cultured *in vitro* according to the methods of Hanning and Conger (1982, 1986). Plant number 10 produced several somatic embryos (Conger et al., 1983). A somatic embryo was germinated, the resulting plant established in a jiffy-7 peat moss pellet (A. H. Hummert Seed Co., St. Louis, MO), transplanted to a 20-cm-diameter pot, and maintained in the greenhouse. This plant, designated cycle I, was vegetatively propagated in the greenhouse and used for further *in vitro* culture experiments to obtain cycle II plants. This procedure of obtaining somatic embryos and regenerating plants was repeated twice to obtain cycle III and cycle IV plants. Nomenclature for the regenerated plants was as follows: A Roman numeral was designated for each cycle, followed by two Arabic numerals, e.g., II-10-10. The first Arabic numeral was assigned to genotype, and the second identified plants from that genotype. For simplicity of discussion, the plants were redesignated letters shown in Table 1. Plants from the first three cycles (Table 1) were transplanted in the field and arranged within a Latin square crossing block with plants that had been previously screened and displayed no embryogenic

Table 1. Plant material derived from the cultivar 'Potomac' used in crosses of embryogenic x nonembryogenic genotypes.[a]

Embryogenic Genotypes						Nonembryogenic Genotypes	
Cycle I	ID	Cycle III	ID	Cycle III	ID		ID
I-10-3	A	II-10-1	I	III-10-8	O	106	S
I-10-8	B	II-10-2	J	III-10-12	P	133	T
I-10-25	C	II-10-3	K	III-10-22	Q		
I-10-30	D	II-10-5	L	III-10-34	R		
I-10-39	E	II-10-7	M				
I-10-68	F	II-10-10	N				
I-10-80	G						
I-10-84	H						

[a] The 3 category nomenclature is shown under each cycle, and ID stands for the letter identification for genotypes. A-H were vegetatively propagated clones from one cycle I plant (10). Plants I-T were obtained from individual somatic embryos.

capabilities. Crosses were made among cycle I, II and III embryogenic plants and plants of two nonembryogenic genotypes (Table 2). Crosses were also made between cycle I and cycle II plants (Table 3).

Pollinations

In late April and early May of 1985, panicles of designated female parents were isolated by placing clear shoot tector bags (Carpenter Paper Co., Des Moines, IA) on them before anthesis. The bags were closed with paperclips and attached to bamboo stakes by twist ties. After anthesis (May 10-27), tillers with bagged panicles from plants designated as male parents were clipped near the soil line and the cut ends placed in water filled lab specimen jars to insure pollen viability. The clear shoot tector bags were removed from the female panicles and the jars containing tillers of the male parents were placed at the base of the designated female parents so that the panicles of the male were positioned slightly above those of the female parents. Panicles of both parents were placed inside Kraft water resistant pollen tector bags (Carpenter Paper Co., Des Moines, IA) which were closed with paperclips and attached to bamboo stakes with twist ties for support. The bags were shaken twice weekly to disperse the pollen. A total of 162 crosses were made. Self-pollinations were attempted by leaving the panicles isolated in shoot tector bags.

In July bags were removed from panicles of the designated female parents. Panicles were threshed individually in a single

Table 2. Crosses made with parental material shown in Table 1.[a]

Embryogenic Female	CID	Embryogenic Male	CID
	Cycle I		
I-10-25 x 133	CT	*	
I-10-39 x 133	ET	*	
I-10-68 x 106	FS	133 x I-10-68	TF
*		133 x I-10-84	TH
*		106 x I-10-3	SA
*		106 x I-10-8	SB
	Cycle II		
II-10-2 x 106	JS	106 x II-10-2	SJ
II-10-3 x 106	KS	106 x II-10-3	SK
II-10-3 x 133	KT	133 x II-10-3	TK
II-10-7 x 133	MT	133 x II-10-7	TM
II-10-10 x 133	NT	*	
*		133 x II-10-5	TL
	Cycle III		
III-10-12 x 106	PS	*	
III-10-22 x 106	QS	*	
III-10-34 x 133	RT	133 x III-10-34	TR
III-10-8 x 133	OT	*	
*		106 x III-10-8	SO

[a] CID represents the letters used to identify crosses made between plants, and an asterisk indicates that a reciprocal cross was not made.

Table 3. Crosses made between Cycle I and Cycle II embryogenic parents from material shown in Table 1.[a]

Cycle I x Cycle II			
Cycle II x Cycle I	CID	Cycle I x Cycle II	CID
II-10-1 x I-10-84	IH	*	
II-10-3 x I-10-30	KD	*	
II-10-7 x I-10-84	MH	*	
*		I-10-80 x II-10-2	GJ
*		I-10-25 x II-10-10	CN
*		I-10-30 x II-10-1	DI

[a] CID represents the letters used to identify crosses made between plants, and an asterisk indicates that a reciprocal cross was not made.

head thresher which was thoroughly cleaned between samples. One hundred florets from 20 of the selfed plants were checked for caryopsis development to determine percent seed set. Caryopses utilized resulted from 27 crosses and 4 selfs. Three seeds from each cross and one seed from each self were germinated and plants established in the greenhouse in 20-cm-diameter pots using Pro-Mix (Premier Co., Canada), a nonsoil commercial mix.

Leaf Culture

Growth of plants in the greenhouse was adequate for leaf culture by November. The two innermost leaves from a tiller were separated and split lengthwise along the midvein as reported by Hanning and Conger (1982, 1986). The basal 30 mm of the leaves were surface sterilized in a 2.6% NaOHCl solution containing 0.1% w/v Triton X for 1.5 minutes and rinsed in 3 changes of sterile distilled water for 1 minute each (Conger et al., 1983). The basal 18 mm of the four leaf-halves were cut into six, equal sections (24 total) and plated serially from the basal to the most distal segment on modified Schenk and Hildebrandt (1972) basal medium, amended with 3% sucrose, 5 mg thiamine, 8 g/l agar and 30 µM 3-6-dichloro-o-anisic acid (dicamba), in 9-cm-diameter petri dishes (Conger et al., 1983; Hanning and Conger, 1982, 1986). The pH of the final medium was adjusted to 5.4-5.5 before autoclaving for 15 minutes at 121 °C. Hereafter this medium will be referred to as SH-30. Three replications from each F_1 plant and self were incubated in the dark at 25 °C.

Leaf sections were evaluated for embryogenesis and/or callus production after 5 weeks. The number of embryos per leaf section was estimated, and ranked from 1-6 as follows: 1=1-19, 2=20-39, 3=40-59, 4=60-79, 5=80-99, and 6≥100. Figure 1 illustrates the scale (1-6) that was used to estimate the number of embryos/leaf section. The leaf section shown in Figure 1A was assigned a rating of 1; the production of embryos was sparse and most of the leaf section was visible. A rating of 2 was assigned to the leaf section shown in Figure 1B; the embryos were more numerous and covered a larger portion of the leaf surface. Figure 1C shows a leaf section that was designated 3; the embryos, which are individually discernible, covered approximately 60% of the leaf surface. The leaf section shown in Figure 1D was rated 4; the embryos were densely packed and only a small percentage of the leaf section was visible. A rating of 5 was assigned to the leaf section shown in Figure 1E; the leaf section was completely covered with embryos. Figure 1F represents a leaf section that has been designated a rating of 6; the embryos were so numerous that the leaf section could not be seen.

The number of leaf segments producing callus per leaf segments cultured was also recorded. After evaluation, leaf sections with embryos and/or callus were transferred *in toto* to basal medium without dicamba (SH-0) for embryo germination and seedling development. Cultures were incubated in the light at 25 °C as described by Hanning and Conger (1986). Shoots were counted after 3 weeks. Enumeration

Figure 1. Leaf sections ranked from 1-6 for embryo production.

A. Leaf section assigned a 1 (1-19 embryos/leaf section).

B. Leaf section rated a 2 (20-39 embryos/leaf section).

C. Leaf section designated a 3 (40-59 embryos/leaf section).

D. Leaf section assigned a 4 (60-79 embryos/leaf section).

E. Leaf section rated a 5 (80-99 embryos/leaf section).

F. Leaf section designated a 6 (100 embryos/leaf section and more).

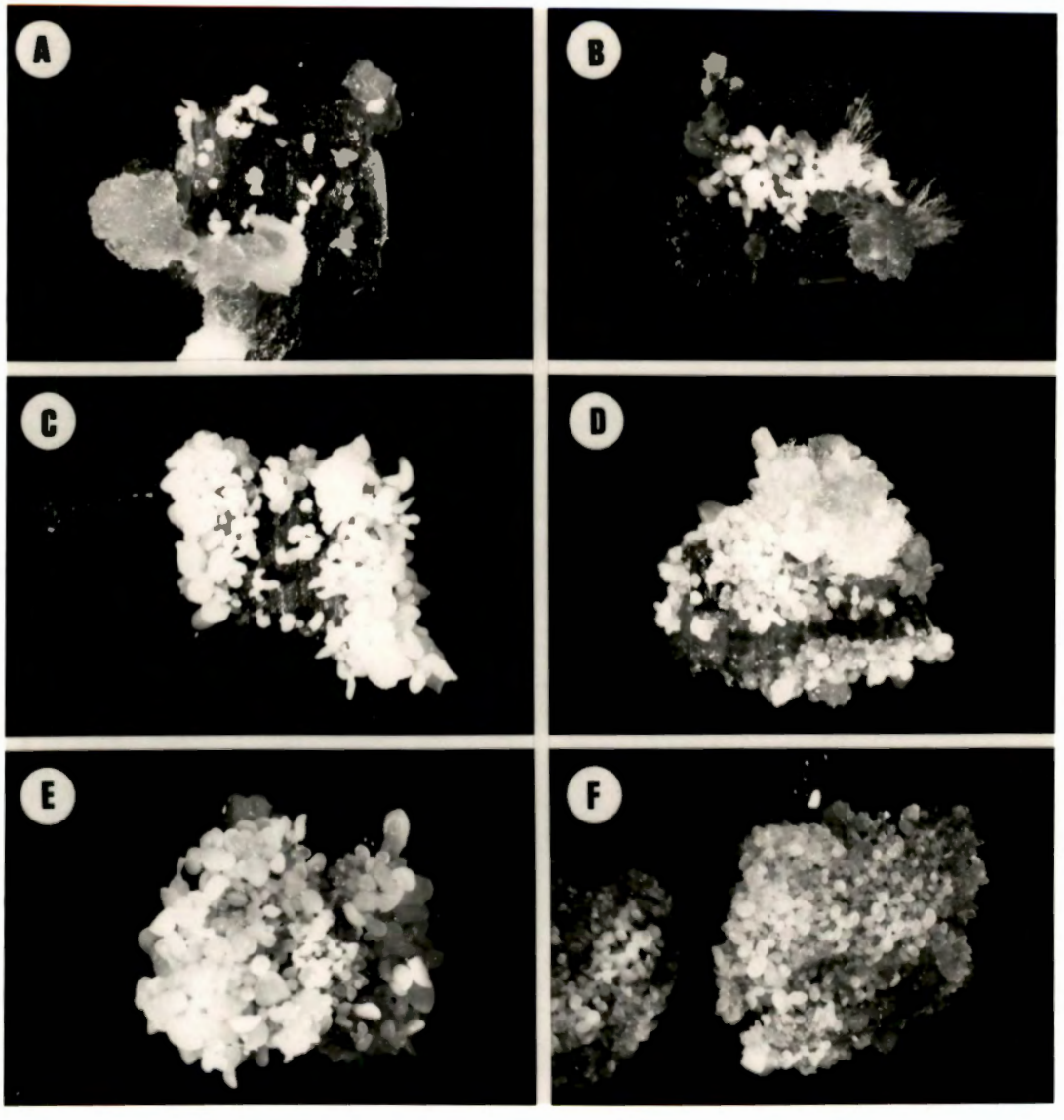

A
B
C
D
E
F

Figure 1, Page 20, Anita M. Lujan, M.S. March 1987

of shoots produced *in toto* was a second method for estimating embryos.

A third method for estimating embryos was also utilized. Twenty embryos were individually removed from leaf sections of each F_1 hybrid and self-pollinated plant that produced embryos. Ten embryos were placed on each of two plates of SH-0 medium at the same time leaf sections with remaining embryos were transferred to SH-0. Individual embryos were incubated as described above, and after 3 weeks shoots were counted. A fraction was obtained from the number of shoots per 10 embryos cultured. This will be referred to as shoot forming rate. Shoot forming rate was used as a correction factor to estimate the number of embryos formed per experimental unit (petri dish). The final formula was [number of estimated embryos = shoots counted *in toto*/shoot forming rate]. For convenience this will be referred to as corrected estimate of embryos.

In spring of 1986, embryogenic and nonembryogenic plants were collected and established in the greenhouse. The materials were subsequently cultured to confirm the embryogenic ability of the parents.

Statistical Analysis

A nested analysis using the General Linear Models Procedure of Statistical Analysis System (SAS Inst. Inc., 1985) was performed to determine significant differences in visual estimate of embryos,

total shoots, shoot forming rate, corrected estimate of embryos and percent of leaf sections producing callus for the F_1's of embryogenic x nonembryogenic genotypes and their reciprocals. The nested analysis consisted of the following classes: cycles (I, II, III), crosses within cycles (7 in cycle I, 10 in cycle II and 6 in cycle III), plants within crosses within cycles (3 for each cross), and reps (3 for each plant) within plants within crosses within cycles (Table 4). T-tests were conducted to determine differences among reciprocals in the F_1 generation, and differences between parents and their F_1 progeny. Two chi square analyses for goodness of fit were performed to determine if the ratios of embryogenic to nonembryogenic F_1 progeny and of embryogenic females to embryogenic males were 1:1. In order to perform these analyses two assumptions were made, i.e., embryogenesis is controlled by a single gene, and the limited numbers of this study can be considered a representative sample from the population. The error level used for all tests was $\alpha = .05$.

Histology

Histological procedures were performed on embryos from 10 crosses, two parents and one self. Four embryos from one inner and one outer leaf were removed and fixed in 3% gluteraldehyde in 0.067M phosphate buffer pH 7.0 (McDaniel et al., 1982). Samples were placed in a vacuum desiccator (-1 atm) for approximately 15 minutes, and stored at 4 °C for a minimum of 24 hours. Embryos were oriented for longitudinal sectioning in a molten but cooled

Table 4. Sources of variation and degrees of freedom for nested analysis of controlled crosses.

Sources of Variation	Degrees of Freedom
Cycles	2
Crosses within Cycles	20
Cycle I	6
Cycle II	9
Cycle III	5
Plants within Crosses within Cycles	43
Leaves within Plants within Crosses within Cycles	128

1.2% gel of Sea Plaque Agarose (FMC Rockland, ME). Preparations were dehydrated for an hour on each of 11 solutions of a graded ethanol-tertiary butyl alcohol (TBA) series (see Appendix A) and then subjected to 3 changes of TBA for 20 minutes each. After the third change of TBA, 1 ml of TBA and 3-4 chips of para-plast plus (Sherwood Medical Supply Co., St. Louis, MO) paraffin were added to the sample vials and placed in a forced air oven at 60 °C. Paraffin was added periodically until the volume was tripled. Samples remained in the oven for approximately 16 hours. Preparations were removed from the oven and embedded in paraffin contained in 10 ml plastic microbeakers. After the paraffin hardened, the embryos were oriented for sectioning by trimming to the appropriate dimensions and mounted on supporting blocks. The blocks were refrigerated at 4 °C for 24 hours, and then secured into position on a rotary microtome for sectioning. Ten µm longitudinal sections of embryos were affixed to glass slides with Haupt's adhesive medium (Johansen, 1940), and 4% formaldehyde solution. The formaldehyde was evaporated on a slide warmer over 24 hours at 37 °C. Sections were stained with a modified Fleming triple stain series (Johansen, 1940), utilizing safranin (CI No. 50240), 15 minutes, crystal violet (CI No. 42555), 45 seconds and fast green FCF (CI No. 42053), 45 seconds (see Appendix B). Permanent slides were prepared using Permount medium and 24 x 50 mm cover slips. Slides were dried on a slide warmer at 47 °C for 24 hours before microscopic examination.

Influence of Sucrose on Somatic Embryo Germination

Twenty leaf bases from several plants of the genotype II-10-10 were cultured as previously described. After 5 weeks of culture on SH-30. sections from one half of each of the two innermost leaves were transferred to SH-0 with 3% sucrose, and sections from the sister halves were transferred to SH-0 without sucrose. Additionally, individual embryos were removed from SH-30 and plated onto SH-0 with 3% sucrose or SH-0 without sucrose. The shoots and the roots were counted after 21 days in culture at 25 °C. The data were analyzed using a t-test for paired variates.

CHAPTER IV

RESULTS

A sample of 20 panicles was randomly selected from the 67 self-pollinations attempted. Five self-pollinations (25%) of the 20 sampled produced one or more seeds. The seed set from selfed plants ranged from 1-6% (Table 5). Compared to the seed from F_1 hybrids, the seeds were reduced in size and had shrunken endosperms.

One hundred forty-four controlled crosses were successful in setting seed from the 162 attempted (89%). No data were obtained for percent seed set from controlled crosses; however, visual observations indicated that seed set was greater than in self-pollinations.

The inner leaf sections proximal to the base produced the most callus and/or somatic embryos. Most embryos were formed from callus; a few were formed directly without an intervening callus. Figure 2 illustrates the response of leaf sections from parent material and F_1 generation crosses after they were cultured on SH-0 basal medium. Response of nonembryogenic parents is shown in Figure 2A, few leaf sections responded to culture, but only callus and/or roots were produced. Two cycle II embryogenic parents (Figure 2B) illustrate the prolific growth of somatic embryos into shoots. In one cycle I plant the leaf sections of reciprocal crosses varied for embryo and shoot production as shown in Figure 2C. All of the leaf sections from reciprocal crosses of cycle II did not appear to be

Table 5. Seed set from 100 florets examined from each selfed nonembryogenic and embryogenic plant.

Nonembryogenic Plants	Percent Seed Set	Embryogenic Plant	Percent Seed Set
8	0		0
11	0	I-10-30	6
33	0	I-10-21	1
34	0	I-10-25	1
39	0	II-10-1	3
42	0	II-10-2	0
49	0	II-10-3	0
106	0	II-10-5	0
133	1	II-10-7	0
		II-10-10	0
		II-10-16	0

Figure 2. Response of leaf sections from parents and F_1 plants cultured on SH-30 for 5 weeks and then transferred to SH-0 for 3 weeks to allow embryo germination and seedling development.

A. Root and callus formation on nonembryogenic parents.

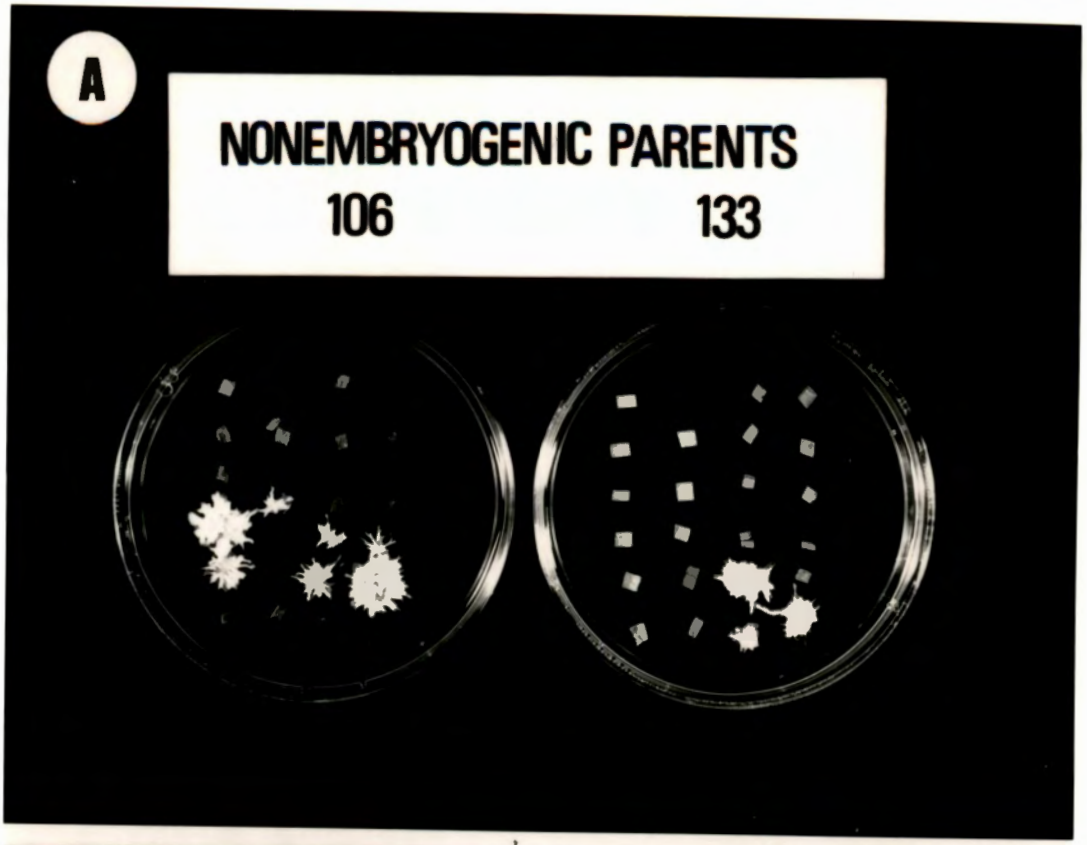
B. Prolific shoot production from two cycle II embryogenic parents.

C. Response from cycle I F_1 generation plants, in which embryogenic females x nonembryogenic males showed greater shoot production than embryogenic males x nonembryogenic females.

D. Shoot production on leaf sections from cycle II F_1 generation plants, showing similar responses of reciprocal crosses.

E. Regeneration of shoots from cycle I x cycle II cross.

F. Cycle II x cycle I cross showing similar results as the reciprocal cross in Figure 1E, page 19.


A
NONEMBRYOGENIC PARENTS
106
133

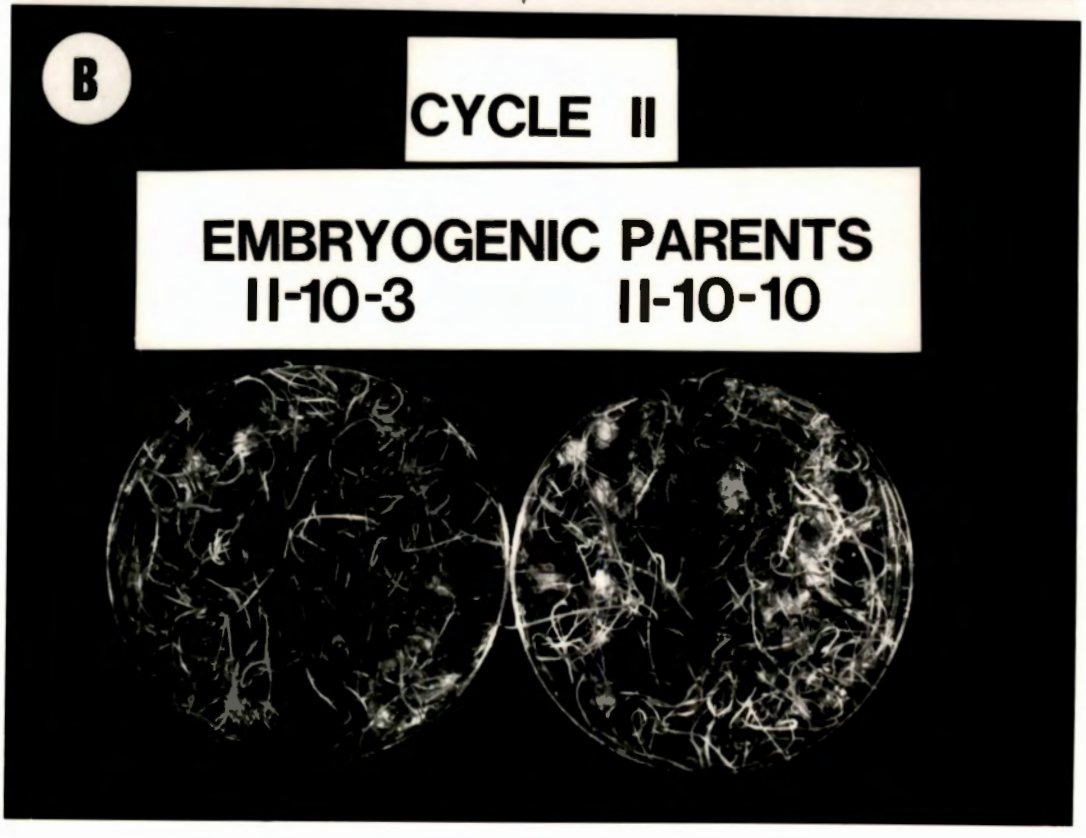

B
CYCLE II
EMBRYOGENIC PARENTS
II-10-3
II-10-10

Figure 2, Page 29, Anita M. Lujan, M.S. March 1987

C

CYCLE I F1 GENERATION

EMBRYOGENIC MALE EMBRYOGENIC FEMALE

D

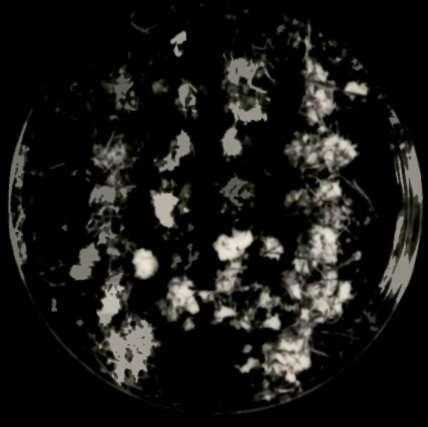
CYCLE II F1 GENERATION

EMBRYOGENIC MALE EMBRYOGENIC FEMALE

Figure 2 continued, Page 30, Anita M. Lujan, M.S. March 1987

E

CYCLE I X CYCLE II



F

CYCLE II X CYCLE I

different for embryo and subsequent shoot production as illustrated in Figure 2D. Response of leaf sections from F_1 plants in Figures 2E and 2F show that reciprocal crosses between cycles I and II were not different for embryo and shoot production.

The nested analysis of variance indicated that none of the traits evaluated were significantly different among crosses and among cycles (Table 6). There were significant differences among plants for the visual estimate of the number of embryos, total number of shoots, corrected estimate of embryos, and percent of leaf sections producing callus. No significant differences among plants were detected for shoot forming rate of individually plated embryos.

A t-test was used to compare reciprocal crosses as a whole that produced F_1 progeny (Table 7). There were no significant differences between F_1 plants in which females were the embryogenic parents and those in which males were the embryogenic parents for visual estimate of embryos, total shoots, shoot forming rate and corrected estimate of embryos.

An additional t-test was used to compare each cross individually with its reciprocal for visual estimate of embryos, total shoots, shoot forming rate and corrected estimate of embryos (Table 8). There were no significant differences between crosses SJ and JS for visual estimate of embryos and total shoots. When S was crossed to K it was not significantly different from its reciprocal (KS) for visual estimate of embryos and total shoots. Data were not collected for shoot forming rate and corrected estimate of embryos

Table 6. Mean squares for visual estimate of embryos, total shoots, shoot forming rate, corrected estimate of embryos and percent of leaf sections producing callus.

Source of Variation	df	Mean Squares: Visual Estimate of Embryos	Total Shoots	Shoot Form. Rate	Corrected Est. of Embryos	Callus Produc.
Among Cycles	2	0.05	47261.39	0.17	59411.50	0.35
Among Crosses	20	0.04	34119.20	0.07	44545.37	0.16
Among Plants	43	0.03*	24415.81*	0.06	3259.90*	0.11*

*Significant at $\alpha = 0.05$.

Table 7. Means of 5 embryogenic females x nonembryogenic males (SJ, SK, TK, TM, TR) versus 5 nonembryogenic females x embryogenic males (JS, KS, KT, MT, RT) compared for several characteristics of *in vitro* culture.

Characters	Mean Emb. Female x Nonemb. Male	Mean Emb. Male x Nonemb. Female	t-Value
Visual Est. of Embryos	235.0	222.0	-0.07 NS
Total Shoots	15.0	30.0	0.53 NS
Shoot Form. Rate	0.38	0.29	-0.38 NS
Corrected Est. of Embryos	66.0	94.0	0.31 NS

NS = Not significant at $\alpha = 0.05$.

Table 8. Comparison of 5 nonembryogenic x embryogenic F_1's and their reciprocals for several traits of *in vitro* culture.

Cross	Mean Values			
	Visual Est. of Embryos	Total Shoots	Shoot Form. Rate	Corrected Est. of Embryos
SJ/JS	218.0/0.0 NS	22.0/0.30 NS	0.30/-	64.0/-
SK/KS	0.0/342.0 NS	0.30/36.0 NS	-/0.50	-/144.0
TK/KT	3.0/228.0 NS	23.0/32.0 NS	0.20/0.50 NS	217.0/148.0 NS
TM/MT	420.0/27.0 NS	27.0/11.0 NS	0.58/0.66 NS	54.0/81.0 NS
TR/RT	283.0/0.0 NS	76.0/0.0 NS	0.71/-	221.0/-

NS = Not significant at $\alpha = 0.05$.

- Not determined.

for both JS and SK to compare to values obtained for SJ and KS. The two F_1 plants from crosses of TK and KT were not significantly different for any of the characters measured. The F_1 plant TM was not significantly different from MT, for total shoots produced, shoot forming rate, corrected estimate of embryos, and visual estimate of embryos. Reciprocal crosses TR and RT were not significantly different for total shoots and visual estimate of embryos. There were no data collected from RT for shoot forming rate and corrected estimate of embryos to compare to values obtained for TR.

A third t-test demonstrated that genotype K was significantly different from KS, KT, SK and TK for total shoots produced (Table 9). All other traits measured for K versus KS, K versus KT, and K versus TK were not significantly different. Cross SK and parent K were not significantly different for visual estimate of embryos. No data were available to compare SK to K for corrected estimate of embryos, and shoot forming rate from individually plated embryos. Characteristics measured for NT were not significantly different from the same characteristics measured for the embryogenic parent N.

Fifty-six percent or 13 of the 23 F_1 progeny produced from embryogenic x nonembryogenic crosses and their reciprocals produced embryos at varying rates (Table 10). The other 10 produced callus or did not respond at all. The chi square value for goodness of fit for a 1:1 ratio was 0.174 and the probability of obtaining at least as large a deviation by chance was 0.75 (Table 11). Six of the 13 F_1 embryogenic progeny were produced from crosses of embryogenic

Table 9. Comparison between embryogenic parents to their F_1 progeny for visual estimate of embryos, total shoots, shoot forming rate and corrected estimate of embryos.

		Mean Value			
Parent	Cross	Visual Est. of Embryos	Total Shoots	Shoot Form. Rate	Corrected Est. of Embryos
K		933.0	451.0	0.69	656.0
N		915.0	280.0	0.70	381.0
	KS	342.0	36.0 *	0.50	144.0
	KT	228.0	32.0 *	0.50	148.0
	NT	590.0	141.0	0.80	285.0
	SK	0.0	0.30 *	-	-
	TK	3.0	23.0 *	0.20	217.0

*Significantly different from parent $\alpha = 0.05$.

Table 10. Mean number of embryos estimated from corrected and visual methods.[a]

Cross	Mean Number for Corrected Estimate of Embryos	Mean Number for Visual Estimate of Embryos
SB	103.0	258.0
FS	-	2.0
SJ	64.0	218.0
TK	217.0	3.0
TM	54.0	420.0
KS	144.0	342.0
KT	148.0	228.0
MT	81.0	27.0
NT	285.0	590.0
TL	500.0	446.0
SO	85.0	-
TR	221.0	283.0
PS	87.0	101.0
JS	-	0.0
SA	-	0.0
TF	-	0.0
TH	-	0.0
CT	-	0.0
ET	-	0.0
SK	-	0.0
QS	-	0.0
RT	-	0.0
OT	-	0.0

-Not calculated.

[a]Numbers were obtained from 3 replications of 3 F_1 embryogenic x nonembryogenic plants and reciprocals.

Table 11. Chi square analysis for goodness of fit to a 1:1 ratio for embryogenic versus nonembryogenic F_1 progeny.

	Classes			
	Embryogenic	Nonembryogenic	df	Chi Square
Observed	13.0	10.0	1	0.174 *
Expected	11.5	11.5		

*P > 0.75.

females by nonembryogenic males. The remaining 7 F_1 embryogenic progeny were produced by the reciprocal of the aforementioned cross. Chi square analysis for the two classes, embryogenic females and embryogenic males for a 1:1 ratio, yielded a value of 0.07 (Table 12), and the probability of obtaining at least as large a deviation by chance was 0.80. Thus, a 1:1 ratio for embryogenic females to embryogenic males was a good fit for these data.

When the leaf sections with more than 80 embryos were transferred from induction medium (SH-30) to regeneration medium (SH-0) most embryos either failed to develop roots and/or shoots or exhibited limited shoot development. One possible explanation is that the embryos were not anatomically mature. Histological preparations of 41 embryos were microscopically examined. Results are tabulated in Table 13. No meristematic tissue was observed in 22% of the embryos sampled. Thirty-seven percent of those embryos observed had both root and shoot meristematic tissue, and 41% had only root meristematic tissue. Two embryos sampled from parent K had root and shoot apices. Twenty percent of the embryos of cross KS had root and shoot apices, 40% had root apices and 40% had no meristematic tissue. The single embryo examined from cross KT had only a root apex. Cross NT produced embryos of which 66% had both root and shoot apices, and 33% had a root apex, as compared to the embryo observed from the parent N which had a root and shoot apex.

There were significant differences in shoot production from embryos that had been cultured on SH-0 with 3% sucrose as compared

Table 12. Chi square analysis for goodness of fit to a 1:1 ratio for embryogenic females to embryogenic males.

	Classes			
	Embryogenic Females	Embryogenic Males	df	Chi Square
Observed	6	7	1	0.07 *
Expected	6.5	6.5		

*P > 0.80.

Table 13. Histological observations on 10 crosses, two parents and one self.[a]

Cross or Parental	Sample Size	Root Apex	Root and Shoot Apex	No Meristematic Tissue
SB	1	1		
TM	1	1		
KS	10	4	2	4
KT	2	1		1
MT	2	1	1	
NT	6	2	4	
TL	10	4	4	2
PS	1	1		
IH	1			1
CN	1		1	
I ⊗**	3	2		1
K	2		2	
N	1		1	

[a]The number of embryos with root apex, shoot apex and no meristematic tissue are indicated.

**Selfed plant.

to those cultured on SH-0 with 0% sucrose for all the traits measured according to analysis of data with a t-test (Table 14). Figure 3 illustrates differences in the somatic embryogenic response of cultures that had 3% sucrose and those that had 0% sucrose. One or two embryos cultured on 0% sucrose produced shoots, as compared to numerous embryos producing shoots in cultures with 3% sucrose.

Table 14. Comparison of embryos cultured on regeneration media with 3% sucrose or 0% sucrose.

Characters Measured	Mean Value for 3% Sucrose	Mean Value for 0% Sucrose	t-Value
Total Shoots	110.0	7.0	4.55 *
Shoot Forming Rate	0.70	0.27	6.22 *
Corrected Est. of Emb.	195.0	34.0	3.97 *

*Significant at $\alpha = 0.05$.

Figure 3. Shoot production from somatic embryos after culturing on regeneration media with 3% or 0% sucrose.


SH-0
3% SUCROSE
SH-0
NO SUCROSE

Figure 3, Page 46, Anita M. Lujan, M.S. March 1987

CHAPTER V

DISCUSSION

In these experiments the results showed that seed set from self-pollinations was very low. In controlled crosses foreign pollen was excluded by isolating females before anthesis; therefore, seed produced from crosses of desired genotypes were considered to be genuine hybridizations. These findings are in agreement with previous reports on the self-incompatibility in orchardgrass which assures that the genetic nuclear complement of the hybrid seed is equally derived from two parents (Lentz et al., 1983; Lundqvist, 1969; Wolfe, 1925).

In a previous study the total number of shoots was used to estimate somatic embryo production (Hanning and Conger, 1986). This method considered only the embryos that were capable of producing shoots. The visual rating method approximated the embryo number on the leaf sections including some that did not germinate; however, it did not estimate the embryos formed on the leaf surface in contact with the medium. Moreover, the rating system for estimating embryos had a broad range of 20 embryos, i.e., 1=1-19 embryos, which minimized accuracy and may have produced erroneous results. The corrected method used here for estimating embryo number is probably more accurate since it considered the embryos that were on both surfaces. Shoot formation rate from individual embryos is a correction factor that

accounted for embryos that did not germinate (Trigiano et al., submitted). If no seedlings or a small number of seedlings were produced after the leaf sections were transferred to regeneration medium, the corrected estimate of embryos could not be utilized because somatic embryos were not individually plated to obtain a shoot forming rate which in turn was necessary to calculate the corrected estimate of embryos.

Lack of significant differences among cycles and crosses was due to a large amount of background variability in the experimental material and too few replications of cultured leaf sections. Previous reports have determined that embryo production on the inner leaf was significantly greater than the outer leaf, and embryo production decreases from the basal to the distal portion of the leaf (Conger et al., 1983; Hanning and Conger, 1986). The nested analysis also showed significant differences among plants which may be a result of segregation of gene(s) for the embryogenic trait.

The lack of significant differences between reciprocal crosses for embryo production and shoot formation indicated that somatic embryogenesis in these particular F_1 plants was inherited through nuclear gene(s). These results were similar to those obtained for the transmission of the androgenic trait in wheat (Bullock et al., 1982; Lazar et al., 1984) and the somatic embryogenic trait in corn (Hodges et al. 1986). Furthermore, a t-test demonstrated that five of the F_1 crosses (SJ, SK, TK, TM, TR) and their reciprocals were not significantly different for embryo production and total shoots

produced, and therefore indicated nuclear inheritance of the embryogenic trait. There appear to be large differences in the means of SJ, SK, TK and TR and their reciprocals for visual estimate of embryos, although statistical analyses demonstrated that the differences are not significant. This discrepancy also occurred when the mean of parent K was compared to the means of SK and TK for visual estimate of embryos. Statistical differences were not detected because of the inherent variability present in the system. Apparent conflicting data also exists between K and its F_1 generation progeny, i.e., K was not significantly different from the F_1 for embryo production; however, the subsequent development of embryos into shoots was significantly different. The data illustrated a flaw in using total shoot production to estimate embryos. Discrepancies between embryo production and total shoots may be due to lack of development of shoot apices as observed in the histological sections. Shoot and/or root apices were observed from histological sections of somatic embryos from the self, both parents, and 9 of 10 crosses, but there was not sufficient data to make any conclusions. The differences may also be due to different gene(s) conditioning embryo production and seedling production as suggested for callus initiation, callus proliferation and shoot differentiation frequency in wheat cultures (Lazar et al., 1983).

Somatic embryogenesis in orchardgrass was also indicated to be under the control of nuclear gene(s) as confirmed by the chi square analysis. These data indicated that somatic embryogenesis

can be transmitted from an embryogenic female or male to its F_1 progeny when the other parent is nonembryogenic. A proposed model for the embryogenic trait of orchardgrass, an autotetraploid, may be the simplex Aaaa. When the simplex was crossed to a nonembryogenic nulliplex aaaa the resulting F_1 generation plants were: 1 Aaaa: 1 aaaa. The results from embryogenic plants crossed to other embryogenic plants, that is Aaaa x Aaaa yielded 3 A___:1 aaaa which also fits the proposed model. Data from selfed plants yielded a 1:3 ratio for embryogenic to nonembryogenic plants which did not agree with the proposed model. According to this model there should have been a 3:1 ratio for embryogenic to nonembryogenic genotypes. This apparent conflict may have resulted from a limited number of seed produced per self (1 to 6 seed). In addition the genetic load may have decreased the overall fitness of the selfed plants. Another possible explanation is that a key step in somatic embryogenesis is blocked or missing since somatic embryogenesis itself may be multigenic. Reports in other species suggested that regeneration in maize (Hodges et al., 1986) and bud differentiation in alfalfa (Reisch and Bingham, 1980) were controlled by at least two genes.

Results from this study agree with those previously published on orchardgrass which indicated the most important factor influencing the somatic embryogenic response was probably genotype (Hanning and Conger, 1986; Hanning, 1984). Studies of somatic embryogenesis in corn (Lu et al., 1982, 1983) and on 12 species of the Poaceae (Vasil, 1985) reported that somatic embryogenesis was dependent

on medium components and the developmental stage of the explant rather than genotype. These factors most likely had some effect on the embryogenic and regeneration responses of orchardgrass genotypes in this study; e.g., sucrose was necessary for embryos to germinate on SH-0 medium. The medium components and the optimum stage for culturing orchardgrass leaves for expression of embryogenesis were determined in previous studies (Conger et al., 1983; Hanning and Conger, 1986) and were held constant in this study. Assuming that the factors affecting embryogenesis of parental plants can be applied to the F_1 hybrids used in this study, it is reasonable to conclude that formation of somatic embryos of F_1 progeny is dependent on genotype. It is likely that a given orchardgrass genotype with nuclear genes for embryogenic potential may be enhanced by specific medium components, as was the case in maize regeneration and plant formation (Hodges et al., 1986).

Findings in these experiments are similar to other research involving transfer of regeneration capacity from embryogenic genotypes to F_1 progeny in the Fabaceae and Poaceae (Beckert and Qing, 1984; Bruneau, 1985; Hodges et al., 1986; Hanning and Conger, 1986; Keyes et al., 1980; Lazar et al., 1983). Interestingly, species of the genera Nicotiana, Datura, and Petunia, the so called "model plants" for in vitro culture systems, do not appear to be genotype specific for callus and/or embryo formation and regeneration. It has been suggested that "model plants" have nuclear genes coding directly for regeneration capacity that are not present in the Fabaceae and

the Poaceae (Wenzel and Schieder, 1980). Until the difference in regeneration capacity of legumes and cereals is better understood, genotype selection will continue to be a more effective method than testing many culture conditions for the ability to regenerate plants (Oelck and Schieder, 1983; Foroughi-Wehr et al., 1982).

This study demonstrated that somatic embryogenesis was inherited by F_1 progeny, and appeared to be controlled by nuclear gene(s). The results provide preliminary knowledge of the somatic embryogenic trait in orchardgrass. Further information on the genetics of embryogenic genotypes could be obtained by performing backcrosses and by testing more F_1 progeny for somatic embryogenesis. Transfer of the embryogenic trait to nonembryogenic genotypes may be advantageous in the future utilization of *in vitro* culture techniques for the improvement of this species (Hanning and Conger, 1986).

LITERATURE CITED

LITERATURE CITED

Abe, T., and Futsuhara, Y. 1986. Genotypic variability for callus formation and plant regeneration in rice (Oryza sativa L.). Theor. Appl. Genet. 72:3-10.

Ammirato, P.V. 1985. Patterns of development in culture. In: Tissue Culture in Forestry and Agriculture. Henke, R. R., Hughes, K. W., Constantin, M. J., and Hollaender, A. (eds.). Plenum Press, New York. pp. 9-29.

Armstrong, C. L., and Green, C. E. 1985. Establishment and maintenance of friable, embryogenic maize callus and the involvement of ℓ-proline. Planta 164:207-214.

Beckert, M., and Qing, C. M. 1984. Results of a diallel trial and a breeding experiment for in vitro aptitude in maize. Theor. Appl. Genet. 68:247-251.

Bingham, E. T., Hurley, L. V., Kaatz, D. M., and Saunders, J. W. 1975. Breeding alfalfa which regenerates from callus tissue in culture. Crop Sci. 15:719-721.

Broertjes, C., and Keen, A. 1980. Adventitious shoots: Do they develop from one cell? Euphytica 29:73-87.

Bruneau, R. 1985. Régénération in vitro á partir de cals d'embryons immatures de mais. Aspects quantitatifs et transmission héréditaire. Agronomie 5:591-596.

Buiatti, M., Baroncelli, S., Bennici, A., Pagliai, M., and Tesi, R. 1974. Genetics of growth and differentiation in vitro of Brassica oleracea var. botrytis. Z. Pflanzenzüchtg. 72: 269-274.

Bullock, W. P., Baenziger, P. S., Schaeffer, G. W., and Bottino, P. J. 1982. Anther culture of wheat (Triticum aestivum L.) F_1's and their reciprocal crosses. Theor. Appl. Genet. 62:155-159.

Chu, C. C., Wang, C. C., Sun, C. S., Hsu, C., Yin, K. C., Chu, C. Y., and Bi, F. Y. 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sin. 18:659-668.

Clapham, D. 1973. Haploid Hordeum plants from anthers in vitro. Z. Pflanzenzüchtg. 69:142-155.

Conger, B. V., Trigiano, R. N., and Gray, D. J. 1986. Dactylis glomerata: A potential model system for in vitro mutagenesis and mass propagation in cereals and grasses. Nuclear Techniques and In Vitro Culture for Plant Improvement. International atomic energy agency, Vienna. pp. 371-383.

Conger, B. V., Hanning, G. E., Gray, D. J., and McDaniel, J. K. 1983. Direct embryogenesis from mesophyll cells of orchardgrass. Science 221:850-851.

Dunwell, J. M. 1981. Influence of genotype and environment on growth of barley embryos in vitro. Ann. Bot. 48:535-542.

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

Foroughi-Wehr, B., Friedt, W., and Wenzel, G. 1982. On the genetic improvement of androgenetic haploid formation in Hordeum vulgare L. Theor. Appl. Genet. 62:233-239.

Gray, D. J., and Conger, B. V. 1985a. Influence of dicamba and casein hydrolysate on somatic embryo number and culture quality in cell suspensions of Dactylis glomerata (Gramineae). Plant Cell Tissue Organ Culture 4:123-133.

Gray, D. J., and Conger, B. V. 1985b. Somatic embryo ontogeny in tissue cultures of orchardgrass. In: Tissue Culture in Forestry and Agriculture. Henke, R. R., Hughes, K. W., Constantin, M. J., and Hollaender, A. (eds.). Plenum Press, New York, pp. 49-57.

Gray, D. J., Conger, B. V., and Hanning, G. E. 1984. Somatic embryogenesis in suspension and suspension-derived callus cultures of Datylis glomerata. Protoplasma 122:196-202.

Guha-Mukherjee, S. 1973. Genotypic differences in the in vitro formation of embryoids from rice pollen. J. Exp. Bot. 24:139-144.

Haccius, B. 1978. Question of unicellular origin of non-zygotic embryos in callus cultures. Phytomorphology 28:74-81.

Halperin, W. 1969. Morphogenesis in cell cultures. Annu. Rev. Plant Physiol. 20:395-418.

Hanning, G. E., and Conger, B. V. 1986. Factors influencing somatic embryogenesis from cultured leaf segments of Dactylis glomerata. J. Plant Physiol. 123:23-29.

Hanning, G. E. 1984. Callus formation and somatic embryogenesis from leaf cultures of orchardgrass (Dactylis glomerata L.) Ph.D. Dissertation, University of Tennessee, Knoxville. 69 p.

Hanning, G. E., and Conger, B. V. 1982. Embryoid and plantlet formation from leaf segments of Dactylis glomerata L. Theor. Appl. Genet. 63:155-159.

Hodges, T. K., Kamo, K. K., Imbrie, C. W., and Becwar, M. R. 1986. Genotype specificity of somatic embryogenesis and regeneration in maize. Purdue University Agric. Exp. Sta. Journal No. 10,236.

Johansen, D. A. 1940. Plant Microtechnique. McGraw Hill, New York, pp. 20, 84-89.

Jung, G. A., and Baker, B. S. 1985. Orchardgrass. In: Forages. Heath, M. E., Metcalfe, D. S., and Barnes, R. F. (eds.). Iowa State University Press, Ames, Iowa, pp. 224-232.

Keyes, G. J., Collins, G. B., and Taylor, N. L. 1980. Genetic variation in tissue cultures of red clover. Theor. Appl. Genet. 58:265-271.

Lazar, M. D., Baenziger, P. S., and Schaeffer, G. W. 1984a. Combining abilities and heritability of callus formation and plantlet regeneration in wheat (Triticum aestivum L.) anther cultures. Theor. Appl. Genet. 68:131-134.

Lazar, M. D., Schaeffer, G. W., and Baenziger, P. S. 1984b. Cultivar and cultivar x environment effects on the development of callus and polyhaploid plants from anther cultures of wheat. Theor. Appl. Genet. 67:273-277.

Lazar, M. D., Collins, G. B., and Vian, W. E. 1983. Genetic and environmental effects on the growth and differentiation of wheat somatic cell cultures. J. Hered. 74:353-357.

Lentz, E. M., Crane, C. F., Sleper, D. A., and Loegering, W. Q. 1983. An assessment of preferential chromosome pairing at meiosis in Dactylis glomerata. Can. J. Genet. Cytol. 25: 222-232.

Lu, C., Vasil, V., and Vasil, I. K. 1983. Improved efficiency of somatic embryogenesis and plant regeneration in tissue cultures of maize (Zea mays L.). Theor. Appl. Genet. 66:285-289.

Lu, C., Vasil, I. K., and Ozias-Akins, P. 1982. Somatic embryogenesis in Zea mays L. Theor. Appl. Genet. 62:109-112.

Lundqvist, A. 1969. Self-incompatibility in Dactylis glomerata L. Hereditas 61:353.

Maddock, S. E., Lancaster, V. A., Risiott, R., and Franklin, J. 1983. Plant regeneration from cultured immature embryos and inflorescences of 25 cultivars of wheat (Triticum aestivum). J. Exp. Bot. 34:915-926.

Marsolais, A. A., Séguin-Swartz, G., and Kasha, K. J. 1984. The influence of anther cold pretreatments and donor plant genotypes on in vitro androgenesis in wheat (Triticum aestivum L.). Plant Cell Tissue Organ Culture 3:69-79.

McDaniel, J. K., Conger, B. V., and Graham, E. T. 1982. A histological study of tissue proliferation, embryogenesis, and organogenesis from tissue cultures of Dactylis glomerata L. Protoplasma 110:121-128.

Müntzing, A. 1937. The effects of chromosomal variation in Dactylis. Hereditas 23:113-235.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant 15:473-479.

Oelck, M. M., and Schieder, O. 1983. Genotypic differences in some legume species affecting the redifferentiation ability from callus to plants. Z. Pflanzenzüchtg. 91:312-321.

Ozias-Akins, P., and Vasil, I. K. 1983. Improved efficiency and normalization of somatic embryogenesis in Triticum aestivum (wheat). Protoplasma 117:40-44.

Phillips, G. C., and Collins, G. B. 1979. In vitro tissue culture of selected legumes and plant regeneration from callus cultures of red clover. Crop Sci. 19:59-64.

Raquin, C. 1982. Genetic control of embryo production and embryo quality in anther culture of Petunia. Theor. Appl. Genet. 63:151-154.

Reisch, B., and Bingham, E. T. 1980. The genetic control of bud formation from callus cultures of diploid alfalfa. Plant Sci. Lett. 20:71-77.

SAS User's Guide: Statistics. 1985. Cary, North Carolina: SAS Institute, Inc., pp. 113-138, 569-574, 795-800, 817-824.

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

Sears, R. G., and Deckard, E. L. 1982. Tissue culture variability in wheat: Callus induction and plant regeneration. Crop Sci. 22:546-550.

Songstad, D. D., and Conger, B. V. 1986. Direct embryogenesis from cultured anthers and pistils of Dactylis glomerata. Amer. J. Bot. 73:989-992.

Templeton-Somers, K. M., and Collins, W. W. 1986. Heritability of regeneration in tissue cultures of sweet potato (Ipomoea batatas L.). Theor. Appl. Genet. 71:835-841.

Tisserat, B., Esan, E. B., and Murashige, T. 1979. Somatic embryogenesis in angiosperms. Hort. Rev. 1:1-78.

Trigiano, R. N., Conger, B. V., and Songstad, D. D. The effects of mefluidide and dicamba on in vitro growth and embryogenesis of Dactylis glomerata (orchardgrass). J. Plant Growth Regul. (submitted).

Vasil, I. K. 1985. Somatic embryogenesis and its consequences in the Graminae. In: Tissue Culture in Forestry and Agriculture. Henke, R. R., Hughes, K. W., Constantin, M. J., and Hollaender, A. (eds.). Plenum Press, New York, pp. 31-47.

Wagner, R. E., Hein, M. A., and Henson, P. R. 1972. Potomac (Reg. No. 1). Agriculture Handbook 170. U.S. Department of Agriculture, Washington, D.C., p. 52.

Wenzel, G., and Schieder, O. 1980. Zell-und Gewebekultur in der Kartoffelzüchtung. Der Kartoffelbau 31:225-227.

Williams, E. G., and Maheswaran, G. 1986. Somatic embryogenesis factors influencing coordinated behavior of cells as an embryogenic group. Ann. Bot. 57:443-462.

Wolfe, T. K. 1925. Observations on the blooming of orchardgrass flowers. J. Amer. Soc. Agron. 17:605-618.

APPENDICES

APPENDIX A

ALCOHOL DEHYDRATION SERIES

Table 15. Embryo preparations dehydrated for one hour in 11 solutions.

Total Alcohol	Water (ml)	95% ETOH (ml)	Tertiary Butyl Alcohol (ml)	Absolute ETOH (ml)
10	425	50	-	-
20	375	100	-	-
30	325	150	-	-
40	275	200	-	-
50	225	200	50	-
60	175	200	100	-
70	125	200	150	-
80	75	200	200	-
90	25	200	250	-
95*	-	175	300	-
100	-	-	350	125

*The 95% alcohol concentration was saturated with Erythrosin B dye, C.I. #45430.

APPENDIX B

MODIFIED FLEMING TRIPLE STAIN

Table 16. Embryo sections placed in each solution for time specified.

Solution	Time
100% Histoclear	5 min
100% Histoclear	5 min
Histoclear + 100% ETOH (50-50 v/v)	3-5 min
100% ETOH	↓
95% ETOH	
70% ETOH	
50% ETOH	
Safranin O in 50% ETOH	15 min
Water	rinse
Crystal Violet (aq.)	45 sec
Water	rinse
50% ETOH	3-5 min
70% ETOH	↓
95% ETOH	
100% ETOH	
Fast Green FCF	45 sec
100% ETOH	3-5 min
100% ETOH	↓
Histoclear + 100% ETOH (50-50 v/v)	
Histoclear	
Histoclear	

VITA

Anita M. Lujan was born on March 29, 1961, in Santa Fe, New Mexico. She is the daughter of Mr. and Mrs. Lawrence Lujan of Santa Fe, New Mexico. She was graduated from Santa Fe High School in 1979. She entered New Mexico State University in August of 1979 and received her Bachelor of Science degree in Agriculture in May of 1984. She began her Master of Science in Plant and Soil Science at The University of Tennessee, Knoxville, in September of 1984 and plans to receive said degree in March of 1987. The author is a member of Gamma Sigma Delta.