12-1986

Embryo induction and plant regeneration from cultured anthers and pistils of orchardgrass (Dactylis glomerata)

David D. Songstad

To the Graduate Council:

I am submitting herewith a dissertation written by David D. Songstad entitled "Embryo induction and plant regeneration from cultured anthers and pistils of orchardgrass (Dactylis glomerata)." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plant, Soil and Environmental Sciences.

B. V. Conger, Major Professor

We have read this dissertation and recommend its acceptance:

Karen W. Hughes, Robert A. McLean, John H. Reynolds, Otto J. Schwarz

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Accepted for the Council:

Lewis Winkel

Vice Provost
and Dean of The Graduate School

EMBRYO INDUCTION AND PLANT REGENERATION FROM CULTURED ANTHERS AND PISTILS OF ORCHARDGRASS (*DACTYLIS GLOMERATA*)

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

David Dean Songstad

December 1986

ACKNOWLEDGMENTS

I am thankful for all the individuals who provided guidance, assistance and encouragement during my Ph.D. program. Special gratitude goes to the following:

Dr. Bob V. Conger for providing the opportunity for me to develop as a scientist.

Dr. Karen W. Hughes, Dr. Robert A. McLean, Dr. John H. Reynolds and Dr. Otto J. Schwarz for serving on my committee.

Plamen Denchev, Dr. Dennis J. Gray, Dr. Gary E. Hanning and Dr. Bob N. Trigiano for their friendship, encouragement and exchange of ideas.

Dr. Chen H. Chen, my former advisor at South Dakota State University, for his friendship and scientific techniques which he has taught me.

Most of all to my wife, Aseema, for her unending patience and support and to my Lord Jesus Christ for giving me the ability to complete this dissertation.

ABSTRACT

Direct embryogenesis was observed from Dactylis glomerata L. [orchardgrass (2N=4X=28)] anthers incubated at 25 C for 6 weeks on Schenk and Hildebrandt (SH) medium containing 3% sucrose and 30 μM dicamba [SH-30 (3,6 dichloro-o-anisic acid)]. Subsequent experiments showed that SH-30 supplemented with up to 5.0 g/l casein hydrolysate did not affect and 0.1 to 5.0 mg/l benzylaminopurine or kinetin hindered the embryogenic response. Furthermore, anther orientation had no significant effect on the embryogenic response and no embryos were obtained from culture of isolated microspores. However, SH-30 containing 9% sucrose promoted embryogenesis and an anther density of 10/ml initiated more embryos per anther than the 100 anthers/ml treatment.

Anthers initiated embryo-like structures from within microspores after culture on SH-30 for 6 weeks at 4 C. In another experiment, anthers were exposed to 0, 3, or 6 week cold pretreatments (4 C) and then cultured at 25 C on SH medium containing 0, 10, 20, or 30 μM dicamba and 9% sucrose. Embryo initiation was affected by an interaction of cold pretreatment and dicamba concentration. Stereomicroscopy showed that the 6 week cold pretreated anthers initiated indirect embryos from callus masses derived from microspores. Five of six plants regenerated from the 6 week cold pretreated anthers consisted of cells with 28 chromosomes. However, 1 plant had 14, 28, and up to 112 chromosomes and 1 to 8 nucleoli per nucleus.

Direct embryogenesis was observed from ovary and style regions of unpollinated pistils cultured on SH-30 for 3 to 4 weeks at 25 C. After 5 and 6 weeks culture, however, embryos disorganized and produced calli which proliferated into numerous secondary embryos. An upright pistil orientation initiated a significantly greater embryogenic response compared to those pistils cultured flat on the medium. All examined plants regenerated from pistil culture possessed the somatic chromosome number of 28 except for 1 mixoploid with 14, 28, and 56 chromosomes. Culture of excised unpollinated ovules failed to initiate any embryos when cultured on SH-30 with or without kinetin concentrations up to 5.0 mg/1. However, 1 of 60 ovules excised from pistils cultured for 3 weeks on SH-30 and then recultured on SH-30 initiated callus and embryos. Histology of pistils cultured for 3 weeks showed possible cell divisions within the embryo sac.

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I. INTRODUCTION

Orchardgrass (Dactylis glomerata L.), a cool season self incompatible autotetraploid forage grass, has a European origin but also has been grown in North and South America, Asia and higher elevations of Africa (Jung and Baker, 1985). It has been estimated that approximately 6.7 million acres of land in the United States were used to produce orchardgrass for livestock consumption either as pasture or provender (Martin, 1976). Furthermore, the nutrition provided by orchardgrass has been reported to be similar to that of alfalfa (Medicago sativa L.) (Jung and Baker, 1985).

Production of superior new orchardgrass varieties by conventional breeding techniques has been difficult (Poehlman, 1979). Self incompatibility prevents the use of inbreeding techniques to isolate homozygous breeding lines useful as source plants in breeding research. Therefore, current breeding strategies involve production of multivariety and multiclone synthetics through open pollination and selection (Poehlman, 1979).

The advent of anther and ovary culture to plant breeding and genetics has provided a potential means to obtain doubled haploid or homozygous plants from self incompatible cross pollinating species. Recently, anther culture derived plants have found utility in plant breeding applications. For example, doubled haploid plants of rye (Secale cereale L.) (Friedt et al., 1983) and wheat (Triticum aestivum

L.) (DeBuyser et al., 1985) have shown similar value to their inbred counterparts in agronomic breeding potential. Furthermore, haploid plant cell cultures have produced gametoclonal variants which may prove useful in conventional breeding programs (Evans et al., 1984).

The objective of this study was to obtain, observe and identify haploid and/or polyhaploid embryos/plants from orchardgrass anther-microspore and ovary-ovule cultures. Four approaches were undertaken to fulfill this objective. First, anther density and cold pretreatment experiments were conducted to determine the effect of these physical factors on anther embryogenesis. Second, various nutritional and hormonal supplements were added to Schenk and Hildebrandt (Schenk and Hildebrandt, 1972) agar or liquid medium after which anther/microscore and ovary/ovule cultures were examined for embryogenesis. Third, morphological and histological assessment of cultured anthers and ovaries was conducted at various time intervals. Finally, regenerated plants from anther and ovary cultures were screened for a possible haploid origin by chromosome and nucleoli counts and isozyme banding patterns.

II. LITERATURE REVIEW

Terminology

A haploid plant is one that consists of cells containing the gametic chromosome number. Additional nomenclature has been adopted to distinguish haploids derived from diploids vs. polyploids. A haploid plant derived from a diploid and consisting of a single basic genome has been called a monoploid (McClintock, 1933). The term polyhaploid was reserved for haploid plants, originating from polyploid sources, that have more than one basic genome (Myers, 1947). Furthermore, polyhaploids have also been categorized as autopolyhaploid or allopolyhaploid depending on the origin of the plant genomes (Kimber and Riley, 1963a).

Origin

In Vivo

A review by Kimber and Riley (1963a) described *in vivo* haploid induction by spontaneous origin, through polyembryonic seeds and various chemical or physical treatments. Haploids derived through spontaneous origin included all those species by which the mechanism of haploid induction was not known. Species such as corn (*Zea mays* L.) (Randolph, 1932), wheat (*Triticum aestivum* L.) (Riley and Chapman, 1958), and rice (*Oryza sativa* L.) (Yasui, 1941) have produced haploid plants by spontaneous origin.

Ramiaha et al. (1933) first reported haploid plants from polyembryonic seeds of rice through isolation of haploid-diploid twin seedlings. Possible origins of haploid-diploid twin seedlings have included development of twin embryos from two embryo sacs within the same ovule with only one being fertilized and, also, embryo formation from synergid or antipodal cells in association with the normal diploid zygote (Lacadena, 1974). Although the frequency of haploid-diploid twin seedlings has been low, Morgan and Rappleye (1951) reported that pollination of corn or Easter lily (Lilium longiflorum Thunb.) ovaries with the respective irradiated pollen resulted in increases of haploid-diploid twins.

Haploidy can be induced by the use of various chemical or physical treatments. The first chemical reported to induce haploid plants was colchicine by Levan (1945) with sugar beets (Beta vulgaris L.). In addition, physical treatments such as ionizing and nonionizing radiation have also induced haploidy in several plant species (Kimber and Riley, 1963a; Chase, 1969; Lacadena, 1974). In general, pollen was irradiated and used to pollinate the female counterpart with hopes of stimulating haploid plant initiation (Lacadena, 1974). Ionizing irradiation has been shown to induce haploidy by disturbing the fertility of pollen nuclei without affecting pollen tube stimulation of egg cell division (Montezuma de Carvalho, 1967).

Various plant hybridizations have led to the production of maternal, paternal, or zygotic haploids. Embryo sac gynogenesis, mediated by interspecific hybridization, has resulted in production

of maternal haploids (Kimber and Riley, 1963a; Rowe, 1974). Likewise, paternal haploids have also developed in the female parent but originate from the pollen of either interspecific or intraspecific crosses (Kostoff, 1934; Rhoades, 1948; Goodsell, 1961). Zygotic haploids of barley (Hordeum vulgare L.), first reported by Kasha and Kao (1970), arose through chromosome elimination of the Hordeum bulbosum genome after interspecific hybridization with H. vulgare. Unfortunately, the chromosome elimination technique has had only limited success in other plant species.

In Vitro

In vitro production of haploids involve the culture and regeneration of plants from anther/microspore or ovary/ovule explants. Guha and Maheshwari (1964, 1966) were the first to report embryo production and haploid plant regeneration from cultured anthers of Datura innoxia Mill. The initial objective of that research was to study the gametophytic development of Datura innoxia pollen, which illustrates the serendipity of this discovery (Maheshwari et al., 1982). Since this first report, haploid plants from over 171 different species have been regenerated through anther/microspore culture (Maheshwari et al., 1982).

Several possible origins have been proposed for androgenetic haploid plants. Repeated division of vegetative and/or generative nuclei along with that of undifferentiated uninucleate microspores have resulted in haploid plant initiation in several species (Sunderland and Evans, 1980; Vasil, 1980). In addition, plants with various

ploidy levels have originated from fusion and/or endoreduplication of vegetative and generative nuclei (Sunderland, 1974).

Successful haploid recovery from barley ovary culture was first reported by San Noecum (1976). Furthermore, a review by Yang and Zhou (1982) lists several species which ovary and ovule-derived haploid plants have been recovered. The number of plant species initiating haploids via gynogenesis is less than through androgenesis. Nevertheless, ovary culture may provide an alternative to species recalcitrant to anther/microspore culture (Yang and Zhou, 1982).

The possible origins of gynogenesis have been investigated in only a few species. In rice ovary culture, Zhou and Yang (1981) reported that haploid callus/embryoids developed from either the egg or synergid cells and usually not from the antipodals. A similar origin was also reported for barley ovary culture (Yang and Zhou, 1982). In either case, however, it was not clear if one or more than one callus/embryoid initiated per embryo sac.

Advantage of In Vitro vs. In Vivo Origin

Although haploid plants have been recovered by both *in vivo* and *in vitro* methods, some species appear to initiate haploids more efficiently through one method or the other. For example, haploids from barley were obtained more efficiently by chromosome elimination via interspecific crosses with *Hordeum bulbosum* than with *in vitro* culture of anthers (Huang et al., 1984). However, *in vitro* culture of anthers from various *Nicotiana* species initiated more haploid

plants than *in vivo* methods. For example, Nitsch and Nitsch (1969) regenerated hundreds of anther culture derived tobacco (*Nicotiana tabacum* L.) haploid plants whereas the twin seedling method initiated one haploid from 104,000 tobacco seeds (Nettancourt and Stokes, 1960). Maheshwari et al. (1980) concluded that anther and ovary culture allows for large-scale regeneration of haploid plants which was only a theoretical possibility by other methods.

Haploid Induction of Forage Grasses

Haploid plant production from many forage grass species has been hindered by either a low haploid induction rate or limited recovery of haploid plants. For example, only one haploid plant of *in vivo* origin has been recovered each from bromegrass (*Bromus inermis* Leyss) (Elliott and Wilsie, 1948), Kentucky bluegrass (*Poa pratensis* L.) (Nielsen, 1946), and nodding stipa (*Stipa cernua* Stebbins and Love) (Love, 1944). Two haploid plants, one fertile and one sterile, were recovered "with a good deal of difficulty" from orchardgrass (Muntzing, 1943) and 7 haploids of desert wheatgrass (*Agropyron desertorum* (Fisch) Schult), 4 of which were fertile, have been identified (Dewey, 1961). Furthermore, Muntzing (1938) identified 11 haploids among 2201 plants (1 per 200) in a survey of 16 forage or cereal species from 11 different genera. *In vitro* anther culture of forage grasses, although currently recalcitrant in many species, has the potential for the efficient production of haploid plants (Clapham, 1977). In fact, haploid plants have been recovered in

some forage grasses. Clapham (1971), with Italian ryegrass (Lolium multiflorum Lam.), was the first to report haploid plant regeneration from any forage grass species. Furthermore, Kasperbauer et al. (1980) regenerated 22 haploid plants from tall fescue (Festuca arundinacea Schreb.) anther-panicle culture and Bui and Pernes (1982) regenerated 4 haploids and possibly 11 dihaploid plants from pearl millet (Pennisetum typhoides L.) anther culture. Unlike anther culture, there are no published reports of haploid initiation from ovary/ovule culture of any forage grass species. However, Bovo and Quarin (1983) reported plant regeneration from somatic tissues of combs paspalum (Paspalum almum Chase) ovary explants.

Factors Affecting In Vitro Anther/Ovary Culture

Genotype

One of the most important factors governing the in vitro androgenetic/gynogenetic response has been the genotype of the source plant (Maheshwari et al., 1980; Yang and Zhou, 1982). Genotypic differences in callus induction and/or plant regeneration have been reported for anther cultures of rice (Siva-Reddy, 1985), wheat (Lazar et al., 1984; Marsolais et al., 1984), corn (Genovesi and Collins, 1982), rye (Wenzel et al., 1977), and oats (Avena sativa L.) (Rines, 1983). Rice (Zhou and Yang, 1981) and corn (Troung-Andre and Demarly, 1984) ovaries cultured in vitro also showed genotype differences in the callus induction rate. In corn anther culture, Genovesi and Collins (1982) reported that genotypes possessing heterozygous genomes

were more responsive in culture than various homozygous genotypes. Furthermore, Jacobsen and Sopory (1978) reported that intercrossing of embryogenic anther-derived potato (Solanum tuberosum L.) plants resulted in progeny with greater embryogenic potential. In either case, however, the number and identity of the genes responsible for androgenesis were not reported.

Cold Pretreatment

Exposure of spikelets to various cold temperature pretreatments has improved callus/embryo initiation from anther and microspore culture of several species (Maheshwari et al., 1980, 1982). Pretreatment of corn tassels (Genovesi and Collins, 1982) and rice panicles (Genovesi and Magill, 1979) for 14 days at 4 C to 8 C and 10 C to 13 C, respectively, gave the maximum induction rate of calli and/or embryos from anthers cultured at 25 C. Furthermore, 21 day exposure of rye spikes at 6 C (Wenzel et al., 1977) and sugarcane (Saccharum spontaneum L.) panicles at 10 C (Fitch and Moore, 1983) resulted in a greater proportion of microspores producing calli and/or embryos than controls. In some cases, however, various cold pretreatments have not promoted in vitro androgenesis or gynogenesis. Rines (1983) reported that callus initiation from oat anthers previously exposed to a 4 C cold pretreatment were not significantly different from untreated controls. In wheat anther culture, Marsolais et al. (1984) reported a significant interaction of cold pretreatment and genotype which suggested that response to cold pretreatment was dependent upon the donor plant genotype. Rose et al. (1986) reported that

sorghum (Sorghum bicolor L.) anthers exposed to prolonged cold pretreatments did not promote callus or embryo initiation. Similarly, a review by Yang and Zhou (1982) reported that cold pretreatments were considered ineffective in ovary and ovule culture of most species.

Developmental Stage of Microspore/Embryo Sac

Haploid plant production from in vitro anther culture appears to be related to the stage of microspore development. In a review by Bajaj (1983), he stated that uninucleate microspores of many species initiated the most calli or embryos compared to tetrads or bi and trinucleate microspores. However, it has not been practical to examine microspores from each anther prior to culturing; therefore, anther length has been used to estimate microspore development prior to culture (Sopory and Maheshwari, 1976; He and Ouyang, 1984).

In contrast to anther culture, either bi, tetra, or octanucleate embryo sacs appear to be the best stages for haploid induction from ovary or ovule culture (Yang and Zhou, 1982). Microspore development and date of anthesis have been used as an indirect measure of embryo sac stage. Furthermore, Rangan (1984a) reviewed the literature and reported that ovary or ovule explants collected 24-48 hours prior to anthesis were of a proper developmental stage for haploid induction.

Basal Medium

A variety of basal media have been used to successfully initiate calli/embryos and regenerate haploid plants from anther/microspore and ovary/ovule explants of several plant species (Maheshwari et al., 1980; Yang and Zhou, 1982). Wenzel and Foroughi-Wehr (1984)

reported that it is difficult to draw conclusions concerning medium preference in anther culture because species and genotypic differences among donor plants may require specific nutritional modifications for haploid induction. Nevertheless, basal media such as MS (Murashige and Skoog, 1962), LS (Linsmaier and Skoog, 1965), H (Nitsch and Nitsch, 1969), B5 (Gamborg, 1970), SH (Schenk and Hildebrandt, 1972), N6 (Chu et al., 1975), and Potato II (Chuang et al., 1978) have been used to initiate haploids from anther-microspore and/or ovary-ovule cultures.

Sucrose Concentration

The concentration of sucrose in anther-microspore and ovary-ovule culture media was reported to be critical for optimum haploid induction (Vasil, 1980; Yang and Zhou, 1982; Wenzel and Foroughi-Wehr, 1984). Anthers of many angiosperm species initiated haploids when cultured on medium containing 3% sucrose (Maheshwari et al., 1980); however, anthers from several cereal and forage grass species showed optimal haploid induction when 6 to 12% sucrose was present in the medium (Wenzel and Foroughi-Wehr, 1984). Sucrose concentrations from 2 to 14% have been used in ovary and ovule culture of various species (Yang and Zhou, 1982). The mechanism by which sucrose promotes microspores or embryo sacs to form haploid plants is not known; however, the role may be either metabolic or osmotic.

Auxins and Cytokinins

Auxins and cytokinins used as media supplements appeared to induce callus/embryo production or promote regeneration of plants

from anther-microspore and ovary-ovule cultures of several angiosperm species (Maheshwari et al., 1980; Yang and Zhou, 1982). Nevertheless, haploids from some cereal and forage grass species can be induced from anthers cultured on basal medium containing no hormones (Wenzel and Foroughi-Wehr, 1984) and addition of the appropriate hormone(s) functioned only in the enhancement of embryogenesis. The exact biochemical role of auxins and cytokinins in anther-microspore and ovary-ovule culture is not known; however, optimal microspore and embryo sac embryogenesis appear to be related to relative auxin and/or cytokinin concentrations (Maheshwari et al., 1980, 1982; Rangan, 1984a).

Anther/Ovary Wall

The anther wall may have a beneficial effect on *in vitro* androgenesis (Maheshwari et al., 1980). The exact mechanism governing anther wall enhancement of androgenesis has not been reported; however, increased production of amino acids in the anther wall may be responsible for promotion of embryogenesis from microspores (Maheshwari et al., 1982). Horner and Pratt (1979) reported a 4- to 5-fold increase in the glutamine level of *in vitro* cultured tobacco anthers compared to newly collected anthers. Low rates of embryogenesis from pollen cultures compared to anther culture further indicate the beneficial aspects of the anther wall (Maheshwari et al., 1980; Vasil, 1980).

Anther/Ovary Orientation

The orientation of anther or ovary explants on culture media has an influence on the embryogenic response. Sopory and Maheshwari

(1976) first reported that Datura innoxia anthers cultured on edge (one lodicule in contact with the medium) initiated fewer embryos than those cultured flat (both lodicules in contact with the medium). Anther orientation experiments have also been reported by Hunter (1985) and Shannon et al. (1985) with barley. However, these reports showed that anthers cultured on edge initiated more microspore-derived embryos than those flat on the medium. Therefore, in light of the report by Sopory and Maheshwari (1976), Shannon et al. (1985) suggested that anther culture response to anther orientation may be species dependent. Ovary orientation also has an effect on the embryo sac embryogenic response. Yang and Zhou (1982) reported that ovaries explanted with the placenta side facing the medium responded more favorably than those randomly explanted.

Other Media Supplements

There are several media supplements that have also been tested for their effect on anther embryogenesis. Genovesi and Collins (1982) reported that casein hydrolysate promoted androgenesis in cultured corn anthers. However, casein hydrolysate may both benefit and hinder microspore embryogenesis. The amino acid glutamine, a constituent in casein hydrolysate, was shown to stimulate wheat microspore embryogenesis (Henry and DeBuyser, 1981) whereas Horner and Pratt (1979) reported that serine, another component of casein hydrolysate, was detrimental to tobacco anther culture.

Inositol was reported to both hinder and promote microspore callus/embryo induction (Wenzel and Foroughi-Wehr, 1984). For

example, Sunderland and Xu (1982) reported that 1000 mg/L m-inositol promoted barley callus initiation. However, inositol had either no effect (Genovesi and Collins, 1982) or inhibited (Brettel et al., 1981) corn microspore sporophytic development.

Activated charcoal and polyvinylpyrrolidone incorporated into culture media enhanced pollen embryo production in some species (Anagnostakis, 1974; Wenzel et al., 1977; Tyagi et al., 1981; Johansson, 1983). The exact mechanism by which these compounds act is not known. However, activated charcoal and polyvinylpyrrolidone may absorb substances originating from anther explants and/or culture medium that may inhibit microspore embryogenesis (Maheshwari et al., 1982).

The role of the previously stated media supplements discussed in this section is not known for ovary or ovule culture.

Identification of Androgenetic/Gynogenetic Plants

Chromosome counts have been the standard means for identification of haploid plants (Sadasivaiah, 1974). However, nucleoli counts per nucleus were reported to be a valid means of estimating ploidy level for haploid identification in the Gramineae (Reitberger, 1977; Wenzel and Foroughi-Wehr, 1984). A problem associated with haploid plant identification has been the separation of gametophytic dihaploids from somatic diploid regenerants (Keller and Armstrong, 1977). Chromosome and nucleoli counts from these plants have a potential to produce identical results and thus fail to indicate a gametophytic

or sporophytic origin. Isozyme banding patterns, however, have been used to differentiate dihaploid and diploid regenerated plants since each band acts as a genetic marker (Orton and Browers, 1985).

Uses of Haploids in Plant Research

Haploid plants have been used in various aspects of basic and applied research. The study of species origin has involved haploids to investigate pairing affinities of nonhomologous monohaploid (McClintock, 1933) and allopolyhaploid (Kimber and Riley, 1963b) chromosomes. Partial pairing of nonhomologous chromosomes of haploid plants may indicate chromosome duplication in monohaploids or similar ancestry in allopolyhaploid genomes (Kimber and Riley, 1963a; Sadasivaiah, 1974).

Haploid plants have also been used to study uniparental cytoplasmic inheritance. Mechanisms of uniparental inheritance that have been proposed include dominance, exclusion, compatibility, inactivation, and alteration of organelles (Birky, 1976). Vaughn et al. (1980) regenerated haploid albino Oryza and Hosta plants through anther culture and ultrastructural examination revealed organelle alteration as the probable cause of albinism. Pollen organelle alteration, therefore, appears to be the mechanism which promoted maternal uniparental inheritance in these species (Vaughn et al., 1980).

Genetic anomalies have been isolated or derived from haploid plants. Such genetic anomalies include expression of recessive mutant

traits (Zenk, 1974) and production of monosomics (Sears, 1954). Phenotypic expression of mutant alleles would be possible in monohaploid plants since the genes exist in a hemizygous state (Riley, 1974). Furthermore, crossing haploid with diploid plants of the same species has produced a variety of monosomics with potential uses in intraspecies chromosome substitution (Kimber and Riley, 1963a).

Perhaps the most valuable potential use of haploids involves the production of homozygous plants by doubling the chromosomes of haploid individuals with chemicals such as colchicine (Blakeslee and Avery, 1937). Doubled haploid (dihaploid) plants can be used in place of inbreds in many plant breeding applications. Dihaploid plants have the advantage of a 2 to 3 year period for their isolation and identification compared to 5 to 7 years for the inbred counterpart (Bajaj, 1983).

III. MATERIALS AND METHODS

Panicle Induction, Sterilization and Anther Staging

An embryogenic genotype of orchardgrass (Dactylis glomerata L.) (Hanning and Conger, 1982; Conger et al., 1983) was reared in a growth chamber with an 8 hour light/16 hour dark photoperiod at 13 C/4 C for 4 weeks to simulate short-cool day conditions. These plants were then exposed to a 16 hour light/8 hour dark photoperiod at 25 C/13 C to induce panicle development and maturation. Panicles were collected prior to anthesis, separated into spikelets and surface sterilized in a 50% aqueous bleach solution containing 1 ml/l Triton-X (surfactant) for 3 minutes. Spikelets were then rinsed in 3 changes of sterile deionized-distilled water. Anthers were excised from these panicles and microspore development assessed using a microscope. Uninucleate microspores were observed in anthers 2.5 mm to 3.5 mm long; anthers within this size range were considered suitable for culturing.

Embryo Induction and Plant Regeneration

Embryos from anther and pistil explants were, unless otherwise stated, initiated while cultured on Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) containing 30 μM dicamba, 3% sucrose, 0.8% agar and a volume of 1N KOH to adjust the pH to 5.5 prior to autoclaving (this medium is hereafter referred to as SH-30).

The embryogenic response was quantified in all anther culture experiments after 6 or 8 weeks culture by embryo counts per 10 anthers and by embryo and/or seedling counts as directed in the pistil and ovule culture experiments. Embryo germination and seedling development were obtained by transfer of cultures to SH medium without dicamba (SH-0) and incubation at 25 C in a lighted growth chamber (16 hour light/8 hour dark photoperiod) for 3 weeks. Embryogenesis was quantified and plants were regenerated by these methods in all anther, pistil, and ovule culture experiments in this research.

Cold Pretreatment

Sterile spikelets were explained in 100 mm X 15 mm petri dishes containing 25 ml SH-30 per dish and placed in a refrigerator at 4 C for up to 6 weeks. After 0, 1, 2, and 6 weeks, panicles were fixed in a 3 parts absolute ethanol:1 part glacial propionic acid containing 60 mM $FeCl_3$ for 24 to 48 hours (this solution is hereafter referred to as 3:1 fixative). Anthers were then excised and stained in 1% propiocarmine and microspores examined for nuclear divisions with a microscope.

Anther Density

Anthers were collected from 3 week cold pretreated spikelets and cultured in Falcon #3047 multiwell plates containing 1 ml SH-30 per well to determine the effect of anther density on embryogenesis. In a total of 1600 anther explants, one set of 800 anthers was cultured

at a density of 100 anthers per ml on 8 wells of media and the other set was cultured at 10 anthers per ml on 80 wells of media. The embryogenic response was quantified by embryo number per 10 anthers in both density treatments after 6 weeks culture at 25 C. Anthers with embryos were either fixed in 2.5% glutaraldehyde for histology and scanning electron microscopy (Gray et al., 1984) or transferred to SH-0 for embryo germination.

Sucrose Concentration

Anthers from spikelets exposed to a 6 week cold pretreatment at 4 C were explanted on SH-30 containing 3, 6, 9, or 12% sucrose. Sixteen replications of the 4 sucrose treatments were dispensed as 1 ml aliquots into 64 wells of multiwell plates. Ten anthers were explanted in each of the 64 wells. This experiment was duplicated using anthers from 6 week cold pretreated panicles. Regardless of the experiment, the embryogenic response was assessed by embryo counts after 6 weeks culture in the dark at 25 C.

Anther Cold Pretreatment X Dicamba Concentration

Anthers collected from spikelets exposed to 0, 3, or 6 week cold pretreatments (4 C) were explanted on SH medium supplemented with 0, 10, 20 or 30 μM dicamba, 9% sucrose and 0.8% agar to determine the effects of cold pretreatment and dicamba concentration on the anther embryogenic response. A total of 2640 anthers were cultured in two separate experiments on media dispensed as 1 ml aliquots in

264 wells of multiwell plates. In the first experiment, 840 anthers were randomly explanted on 84 wells of media. Ten anthers were cultured per well and there were 7 replications of the 12 treatments representing all possible combinations of cold pretreatments and dicamba concentrations. In the second experiment, 1880 anthers were explanted "on edge" in 180 wells of media at a density of 10 anthers per ml. There were 15 replications of the 12 above stated treatments. The embryogenic response was quantified in both experiments by embryo counts after 8 weeks culture at 25 C.

Anther Orientation

Anthers from spikelets exposed to a 3 week cold pretreatment were explanted "flat" (both lodicules contacting medium) or "on edge" (one lodicule contacting medium) on SH-30 dispensed in 1 ml volumes into 160 wells of multiwell plates to determine the effect of anther orientation on anther embryogenesis. A total of 1600 anthers were cultured in two sets of 800 representing the flat and on-edge orientations. Ten anthers were explanted per well and embryogenesis assessed by embryo counts 6 weeks after culture initiation.

Casein Hydrolysate, Kinetin, and Benzylaminopurine

Three week cold pretreated anthers were cultured on SH-30 supplemented with 9% sucrose and 0, 1, 5, or 10 gm/L casein hydrolysate. There were 30 replications of the 4 treatments dispensed as 1 ml aliquots into 120 wells of multiwell plates. A total of 1200 anthers

were explanted at a density of 10 anthers per well. Embryogenesis was quantified by embryo number after 6 weeks culture at 25 C.

Anthers exposed to 3 weeks cold at 4 C were cultured in two separate experiments investigating the effect of kinetin and benzylaminopurine (BAP) on embryogenesis. Kinetin at concentrations of 0, 0.47, 4.7, or 23.3 μM was added to SH-30 containing 9% sucrose and dispensed as 1 ml volumes into 120 wells of multiwell plates so that each treatment was replicated 30 times. Ten anthers were explanted per well of medium. The treatment regime of 0, 0.1, 1.0, or 5.0 mg/l was used in a subsequent experiment investigating the effect of BAP on anther embryogenesis. There were 30 replications of the 4 BAP treatments dispensed as 1 ml units into 120 wells of multiwell plates. Ten anthers were cultured per well (total of 1200). Both kinetin and BAP experiments were repeated using anthers exposed to a 6 week cold pretreatment. The embryogenic response from anthers cultured in both kinetin and BAP experiments was assessed by embryo counts after 6 weeks culture at 25 C.

Microspore Culture

A total of 1800 orchardgrass anthers, 900 from each of 0 and 6 week cold pretreated anthers, were collected and sets of 300 ground with a sterile mortar and pestle in an isolation medium containing 3% sucrose and 50 mM $Ca(NO_3)_2$ to release microspores (Hinchee and Fitch, 1984). Microspores were isolated from most anther wall debri by washing through a 80 μm mesh screen and concentrated by centrifugation at 59 g for 5 minutes and resuspending in 1.0 ml of isolation

medium. This volume was then layered on top of 10 ml SH-30 containing 9% sucrose and 20% ficoll and centrifuged at 1475 g for 10 minutes. Microspores were collected at the ficoll-isolation medium interface and also from the pellet (after resuspending in isolation medium) and microspore density quantified using a haemocytometer. Microspores were inoculated onto 0.9 ml of the final culture medium (SH-30 containing 9% sucrose and 20% ficoll) dispensed in multiwell plates by layering 0.1 ml of the microspore suspension. Approximately 1,000,000 microspores were cultured from each cold pretreatment in the dark at 25 C for 8 weeks.

Embryogenic Response from Cultured Pistils

Unpollinated orchardgrass pistils were randomly explanted on SH-30 dispensed as 25 ml volumes into each of 18 100 mm x 15 mm petri plates. A total of 270 pistils were cultured so that each petri dish contained 15 pistils. The embryogenic response was followed at weekly intervals with stereoscopic and scanning electron microscopy (Gray et al., 1984). Embryogenesis was quantified on a weekly basis by counting seedlings resulting from transferring 45 pistils per week (for 6 weeks) from SH-30 to SH-0. Pistils were cultured on SH-0 for 3 weeks at 25 C in a lighted incubator (16 hours light per 24-hour cycle) prior to seedling enumeration.

In a second experiment, the effect of pistil orientation on embryogenesis was investigated by explanting pistils either flat so that the ovary, styles, and stigmas contacted the medium (SH-30) or upright with just the placenta contacting the medium. A total

of 150 pistils were cultured with two sets of 75 representing each orientation. A sampling of representative pistils from both orientations were collected after 0, 1, 2, and 3 weeks culture for paraffin histology (Sass, 1958). The embryogenic response of the remaining pistils was assessed after 3 weeks on SH-30 through seedling counts after transfer to SH-0 for 3 weeks.

Ovule Culture

Unpollinated orchardgrass ovules were excised from ovaries and cultured on SH medium supplemented with various levels of dicamba and/or kinetin. A total of 120 ovules were explanted on SH medium containing 0, 10, 20, or 30 μM dicamba dispensed as 1 ml volumes into 24 wells of multiwell plates. Five ovules were cultured per well and there were six replications of each treatment. In a separate experiment, 120 ovules were explanted on SH-30 containing 0, 0.1, 1.0, or 5.0 mg/l kinetin distributed as 1 ml quantities into 24 wells of multiwell plates. Five ovules were cultured per well and each treatment was replicated six times. In both dicamba and kinetin experiments the embryogenic response was assessed by embryo counts per ovule after 8 weeks culture.

In a second experiment 60 pistils were cultured for 1, 2, or 3 weeks on SH-30 after which ovules were excised and recultured on SH-30. Twenty ovules were cultured in each treatment. Ovules were assessed for embryogenesis by embryo counts after 8 weeks culture at 25 C.

Identification of Polyhaploid Plants

Regenerated plants were screened as to possible gametic or somatic origin by chromosome counts, nucleoli counts and isozyme banding patterns. Basal leaf meristems from approximately 150 orchardgrass plants regenerated from anther and pistil cultures were fixed in 3:1 fixative for 24 to 48 hours and transferred to a 5% aqueous pectinase solution for 4 hours before staining with 1% propiocarmine for 24 to 48 hours. The leaf tissue was macerated and prepared for examination with a microscope according to the methods of Sapre and Barve (1982). Approximately 10 to 20 cells were examined per plant.

Nucleoli from two plants regenerated from 6 week cold pretreated anthers and a control plant were examined in nuclei of cells from basal leaf meristems. The procedure utilized in preparing these cells was identical to that described above for chromosome counts except for a 48 to 96 hour staining period in 1% propiocarmine. In each plant 100 cells with visible nucleoli were assessed for nucleoli number per nucleus. Length of nucleoli were also measured with an ocular and stage micrometer.

Isozyme banding patterns of peroxidase enzymes of leaf tissue from orchardgrass plants regenerated from 6 week cold pretreated anthers were assessed for polyhaploidy using polyacrylamide gel electrophoresis (PAGE). Crude protein extracts from the lower 5 cm portion of orchardgrass leaf tissue were isolated in a buffer containing 8.3% sodium ascorbate and 16.7% sucrose (Shields et al., 1983).

Twenty µl of protein extract was loaded per well on to 10% acrylamide 0.8% bisacrylamide gels and electrophoresed at 100 volts for approximately 8 hours. The gel and running buffers for peroxidase isozyme electrophoresis were from that used in corn (Vallejos, 1983). Peroxidase isozymes were stained by agitating the gel in 20 mM Tris-500 mM NaCl solution (TBS), adjusted to pH 7.5, containing 60 mg 4 fluoronapthol/100 ml TBS for 30 minutes in the dark before addition of 60 µl 30% hydrogen peroxide.

IV. RESULTS

Cold Pretreatment

Anthers exposed to no cold pretreatment contained microspores in a mid to late uninucleate stage (Plate I, Figure 1). No sign of embryogenesis was observed in microspores exposed to a 1 week cold pretreatment; however, some of these microspores contained three nuclei of which two were enclosed by what appeared to be a common cell wall (Figure 2). Microspores exposed to a 2 week cold pretreatment contained tetranucleate structures where the 4 nuclei appeared to be associated as 2 pairs; both nuclei pair were contained within separate cell walls (Figure 3). No microspore contained more than 1 tetranucleate structure. Anthers exposed to a 6 week cold pretreatment contained microspores with multinucleate embryo-like structures (Figure 4).

Anther Density

An explant density of 10 anthers per ml of medium initiated a significantly greater embryogenic response compared to the 100 anther per ml treatment (Table 1). An average of nearly 14 embryos per 10 anthers were obtained from the 10 anther per ml treatment whereas 6 embryos per 10 anthers were obtained from the 100 anther per ml treatment. Embryo initiation from anthers cultured at both densities appeared to originate from somatic tissue (Plate II,

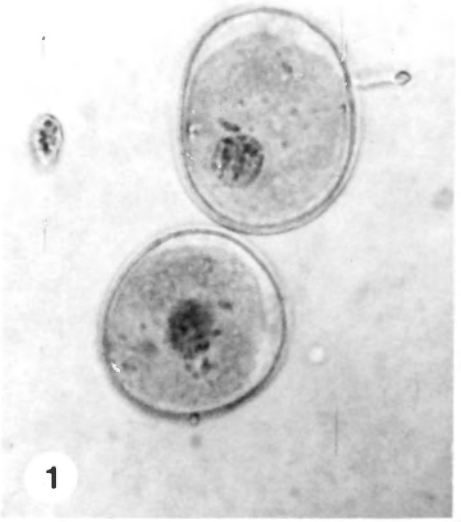
Plate I. Orchardgrass microspores after various cold pretreatments at 4 C.

Figure 1. Uninucleate microspores after no cold pretreatment (1400 x).

Figure 2. Three nuclei of which two (small arrows) are enclosed with a common cell wall (larger arrow) within a microspore after a 1 week cold pretreatment (3000 x).

Figure 3. Five nuclei in which four (small arrows) are separated into pairs by a common cell wall (large arrow) in a 2 week cold pretreated microspore (2800 x).

Figure 4. Embryo-like structure (arrow) contained within a 6 week cold pretreated microspore (2900 x).



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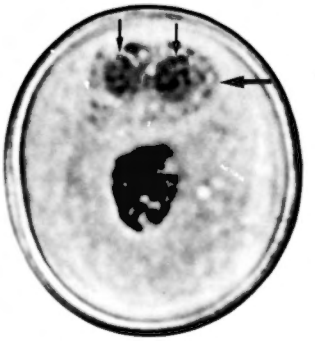

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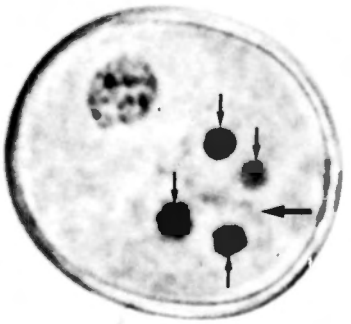

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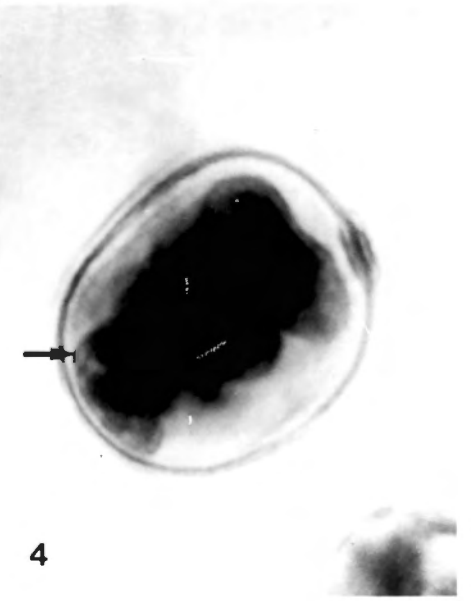

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Plate I

Table 1. Effect of anther density on embryo production from cultured anthers of orchardgrass.

Anther Density	Embryo Mean Per Ten Anthers
10	13.8a*
100	6.0b

*Different letters indicate significant differences according to the Student's t-test at 0.05 probability level.

Plate II. Orchardgrass embryo/callus development and chromosomes from plants regenerated from anther cultures.

Figure 5. Somatic embryo with a coleoptile (C), suspensor (S), and anther wall (AW) origin (80 x).

Figure 6. Histology of orchardgrass somatic embryo emerging from an anther wall (AW) (350 x).

Figure 7. Callus (C) with possible microspore origin developing within a 6 week cold pretreated anther (86 x).

Figure 8. Fourteen chromosomes in a cell of a plant regenerated from a 6 week cold pretreated anther (4200 x).

Figure 9. Chromosomes (28) from a regenerated plant obtained from a 6 week cold pretreated anther (3000 x).

Figure 10. Numerous chromosomes (possibly 112) from a cell of a regenerant from a 6 week cold pretreated anther (1200 x).

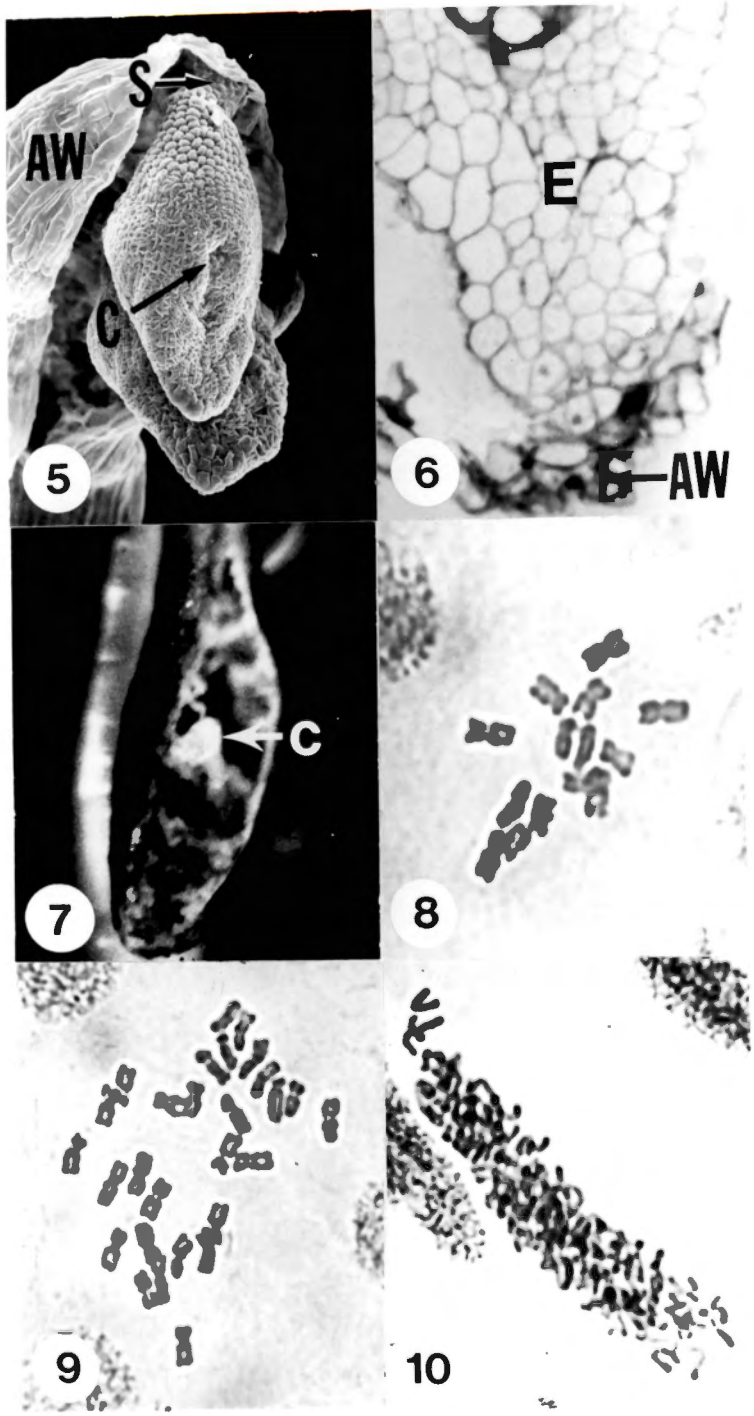
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Plate II

Figure 5) after 2 to 4 weeks culture. Morphological features of these embryos included a suspensor, scutellum and coleoptile (Figure 6). Chromosome counts from cells of basal leaf meristems of 51 plants showed the normal somatic number (2n=4x=28).

Sucrose Concentration

Anthers exposed to a 3 week cold pretreatment initiated an average of 26.3 embryos per 10 anthers when cultured on medium containing 9% sucrose (Table 2). This was not significantly different from the average of 25.8 embryos per 10 anthers initiated from medium containing 12% sucrose. However, embryo production rates were 6.5 and 7.5 per 10 anthers for the 3 and 6% sucrose treatments, respectively. Although these were not significantly different from each other, they were significantly different from that of the 9 and 12% sucrose treatments.

Anthers exposed to a 6 week cold pretreatment initiated an average of 4.8 embryos per 10 anthers when cultured on medium containing 9% sucrose (Table 2). This average rate of embryogenesis was significantly greater than that of 0.5, 1.8, and 0.2 embryos per 10 anthers from the 3, 6, and 12% sucrose treatments. A total of 559 plants regenerated from 3 and 6 week cold pretreated anthers had a normal phenotype. Chromosome counts from 37 of these plants showed the normal somatic number of 28.

Table 2. Effect of sucrose concentration on orchardgrass embryo production from anthers exposed to 3 and 6 week cold pretreatments at 4 C.

Sucrose (%)	Embryo Mean Per Ten Anthers	
	3 Week at 4 C	6 Week at 4 C
3	6.5a	0.5b*
6	7.5a	1.8b
9	26.3b	4.8a
12	25.8b	0.2b

*Means in the same column with identical letters are not significantly different according to Duncan's New Multiple Range Test with a 0.05 probability level.

Cold Pretreatment X Dicamba Concentration

Experiment I

Embryo production. A significant cold pretreatment X dicamba concentration interaction ($P < 0.05$) indicated that the anther embryogenic response was affected by a combination of these two factors (Table 3). Analysis of the 12 treatment means indicated that 3 week cold pretreated anthers cultured on medium containing 30 μM dicamba initiated a significantly greater average number of embryos (20 per 10 anthers) than to the 11 other treatments (Table 4). Those embryos initiated from anthers exposed to 0 or 3 week cold pretreatments at all dicamba levels appeared to initiate directly from the anther wall and filament, i.e., there was no evidence of a microspore origin.

Six week cold pretreated anthers initiated what appeared to be calli from microspores after 6 weeks culture (Figure 7). In general, one microspore derived callus was obtained per approximately 40 cultured anthers. Furthermore, these callus masses initiated embryos after another 2 to 3 weeks culture on medium regardless of dicamba concentration (Table 4).

Chromosome and nucleoli counts. Chromosome counts from 5 of 6 plants regenerated from 6 week cold pretreated anthers showed cells with 28 chromosomes (named regenerants 1 through 5). The remaining plant (regenerant 6) was a mixoploid and contained chromosome numbers of 14, 28, or 56 (Figures 8 and 9) and occasionally up to approximately 112 (Figure 10).

Table 3. Analysis of variance for the effect of cold pretreatment and dicamba level on orchardgrass embryo production from anther culture (Experiment I).

Source of Variation	df	Mean Square	F-ratio
Cold Pretreat (Cold)	2	357.0	
Cold X Rep (Error A)	10	22.8	
Dicamba (Dic)	3	134.0	
Cold X Dic	6	151.0	12.8*
Error B	45	11.8	

*Significant at a 0.05 probability level.

Table 4. Mean number of embryos per ten anthers from various combinations of cold pretreatments and dicamba levels (Experiment I).

	Weeks of Cold Pretreatment at 4 C		
Dicamba (μM)	0	3	6
0	0.3a*	3.0a	0.5a
10	1.6a	2.0a	0.2a
20	1.0a	4.3a	0.8a
30	0.7a	20.0b	0.2a

*Mean value and letter indicating significant difference according to Duncan's New Multiple Range Test with a 0.05 probability level.

Nucleoli counts from 100 nuclei of cells from regenerants 3 and 6 and a control plant are listed in Table 5. The control plant and regenerant 3 contained cells with 1, 2, 3, or 4 nucleoli per nucleus. Two nucleoli per nucleus (Plate III, Figure 11) were observed in 58 and 49% of the cells from the control and regenerant 3. However, 35% of the nuclei examined in regenerant 3 contained 4 nucleoli per nucleus (Figure 12) as compared to 3% in the control plant. No cells from these plants contained more than 4 nucleoli per nucleus.

Thirty-eight percent of the cells from regenerant 6 contained 5 to 8 nucleoli per nucleus (Table 5; Figure 13). Of these cells, nearly 50% of the nuclei contained nucleoli ranging from 5 to 14 μm in diameter. Furthermore, many cells contained nucleoli that appeared to show either nucleolar fusion or division (Figure 14). Nevertheless, 28% of the cells contained 4 nucleoli per nucleus; this was the most frequent nucleolar cell-type among the 8 nucleolar cell-types observed from regenerant 6.

Peroxidase isozyme electrophoresis. Peroxidase isozyme electrophoresis banding patterns from 2 control plants (lanes 1 and 10), 6 plants regenerated from 6 week cold pretreated anthers (lanes 2 through 7) and 2 plants from pistil culture (lanes 8 and 9) showed similar bands regardless of plant origin (Plate IV, Figure 15). Eight peroxidase isozyme bands were observed in each lane; furthermore, the peroxidase isozyme band migration distances observed in the controls were nearly identical for those in the other 8 lanes. However, the intensity of some isozyme bands varied within and among lanes.

Table 5. Percent of nuclei containing one to eight nucleoli in a control plant and plants regenerated from orchardgrass anthers cold pretreated at 4 C for 6 weeks.

Nucleoli Number	Percent		
	Control	Regenerant 3	Regenerant 6
1	28	8	4
2	58	49	25
3	11	8	6
4	3	35	28
5	0	0	13
6	0	0	7
7	0	0	8
8	0	0	9

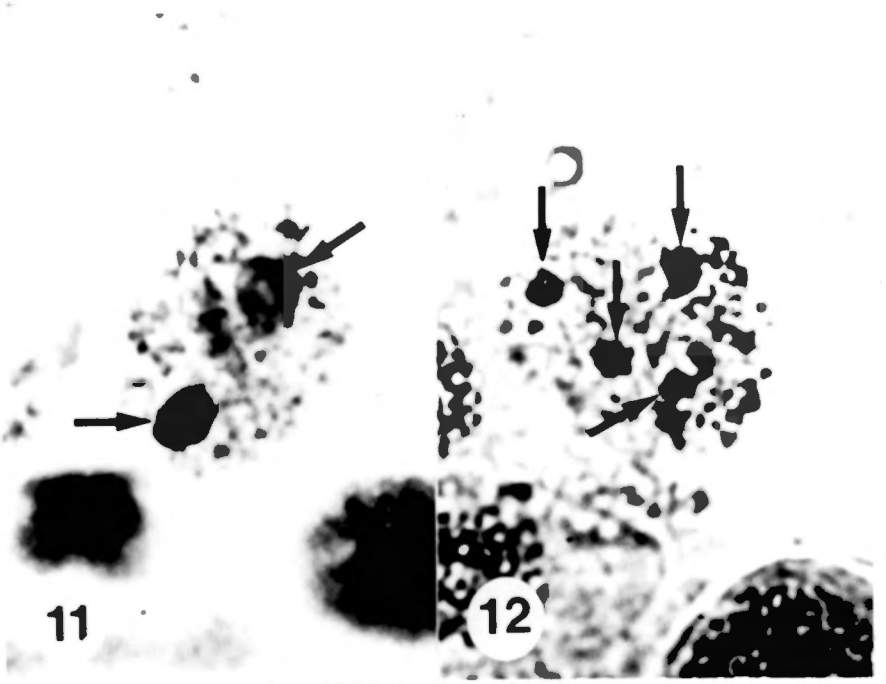
Plate III. Nucleoli from orchardgrass plants regenerated from 6 week cold pretreated (4 C) anthers.

Figure 11. Two nucleoli per nucleus (arrows) (4000 x).

Figure 12. Four nucleoli per nucleus (arrows) (3500 x).

Figure 13. Seven nucleoli of various sizes (arrows show large and small nucleoli) (2800 x).

Figure 14. Dividing or fusing nucleoli (arrow) (3800 x).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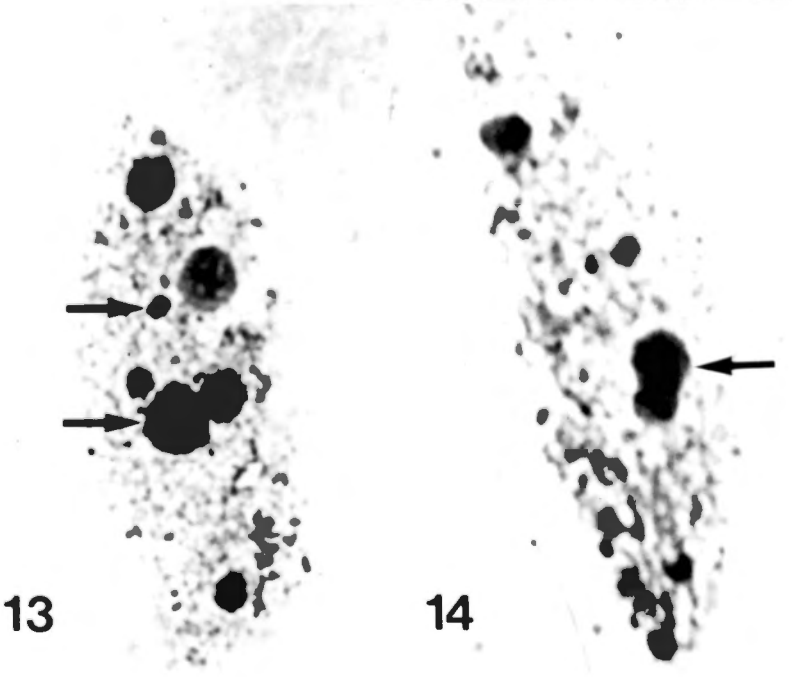
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Plate III

Plate IV. Peroxidase isozymes from orchardgrass leaf tissues.

Figure 15. Lanes 1 and 10 represent tissue culture-derived source plant controls. Lanes 2-7 represent 6 plants regenerated from 6 week cold pretreated (4 C) anthers. Lanes 8 and 9 represent plants from pistil culture.

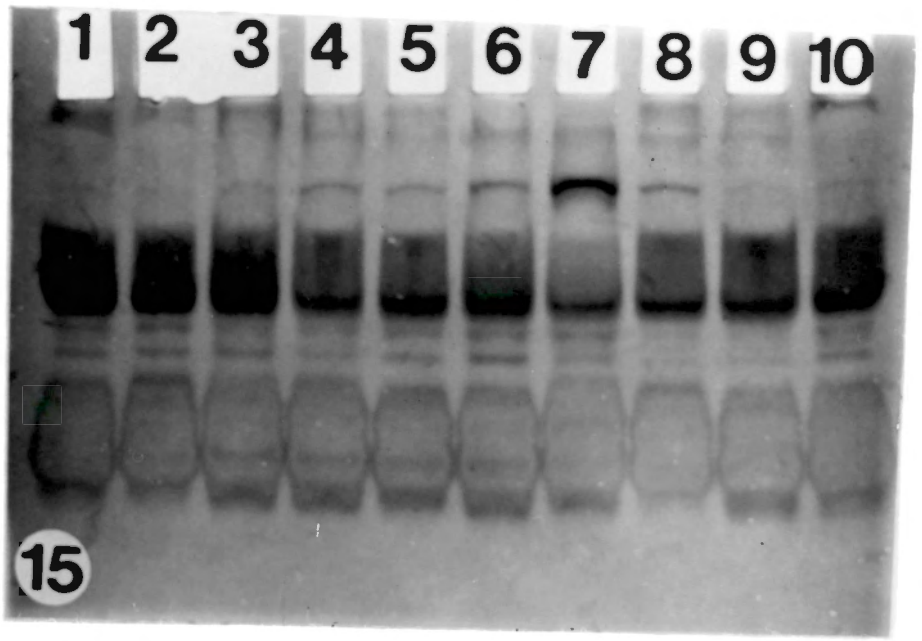
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Plate IV

Experiment II

The embryogenic responses from orchardgrass anthers cultured in the second experiment did not show a significant cold pretreatment X dicamba interaction; the effect of cold pretreatment was the only factor which showed significance (Table 6). Analysis of means showed that 22.8 embryos per 10 anthers from the 0 week cold pretreatment was significantly greater than that of 10.4 and 12.9 from the 3 and 6 week cold pretreatments (Table 7). Furthermore, means of 16.2, 14.3, 13.1, and 18.0 embryos per 10 anthers for the 0, 10, 20, and 30 μM dicamba treatments, respectively, showed no significant differences (Table 7). Most of the embryos initiated in this experiment appeared to have a somatic origin; however, calli with a possible microspore origin were observed in some cultures at a rate of 1 per 100 cultured anthers.

Anther Orientation

A statistically nonsignificant increase in mean embryo number was observed for the upright compared to the flat anther orientation (Table 8). An average of 21.6 embryos per 10 anthers was initiated from anthers cultured on-edge compared to 18.1 embryos per 10 anthers from those cultured flat. Furthermore, an average of 1.9 of the 10 anthers per experimental unit of the upright orientation responded whereas 1.8 embryos per 10 anthers responded in the flat orientation. About 15% of those anthers originally explanted in an upright orientation were later observed to be in a flat orientation. Microspores

Table 6. Analysis of variance for the effect of cold pretreatment (4 C) and dicamba level on orchardgrass embryo production from anther culture (Experiment II).

Source of Variation	df	Mean Square	F-ratio
Cold Pretreat (Cold)	2	2613.6	4.45*
Cold X Rep (Error A)	28	586.2	
Dicamba (Dic)	3	230.8	1.21 ns
Cold X Dic	6	19.0	0.10 ns
Error B	124	193.1	

*, ns Significant and not significant respectively at a 0.05 probability level.

Table 7. Mean number of embryos per ten anthers from a factorial arrangement of dicamba combinations and cold pretreatments (4 C) of Experiment II.

Dicamba (μM)	Embryo Mean	Cold Pretreatment	Embryo Mean
0	16.2a	0	22.8a*
10	14.3a	3	10.4b
20	13.1a	6	12.9b
30	18.0a		

*Means with different letter within each treatment indicates significant differences according to Duncan's New Multiple Range Test at a 0.05 probability level.

Table 8. Effect of "flat" vs. "on edge" anther orientation on embryo production in orchardgrass anther cultures.

Orientation	Embryo Mean Per Ten Anthers
Flat	18.1a*
On Edge	21.6a

*Means with same letter are not significantly different according to Duncan's New Multiple Range Test with a 0.05 probability level.

were shed from most of the anthers cultured in a flat orientation and remained inactive thereafter. However, microspores within the uppermost lodicule of anthers cultured in an upright orientation were never in direct contact with the medium. Occasionally, callus masses which had a possible gametic origin were observed from anthers cultured upright.

Casein Hydrolysate

Casein hydrolysate, at a level up to 5 grams per liter, appeared to have no positive effect on embryo initiation from orchardgrass anther cultures; however, a significant decrease in embryo production was observed from anthers cultured on medium containing 10 grams/liter casein hydrolysate (Table 9). An average of 2.2 embryos was produced per 10 anthers cultured on media containing 0 (control) or 1 gram/liter casein hydrolysate whereas means of 1.8 and 0.4 were obtained from the 5 and 10 gram/liter treatments, respectively. The rate of embryogenesis was about 8 to 10 fold less in this experiment as compared to controls of previous anther culture experiments. Nevertheless, in a preliminary experiment 20.4 and 18.6 embryos per 10 anthers were obtained from 0 and 0.5 gram per liter casein hydrolysate treatments (Table 9). These means were also not significantly different.

Kinetin

The presence of kinetin in the culture medium had a significant effect on embryo induction from orchardgrass anther explants (Table 10).

Table 9. Effect of casein hydrolysate on the mean embryo production from cultured anthers of orchardgrass.

Casein Hydrolysate (g/l)	Embryo Mean Per Ten Anthers
0	2.2a*
1	2.2a
5	1.8a
10	0.4b

*Means with same letter are not significantly different according to Duncan's New Multiple Range Test with a 0.05 probability level.

Table 10. Effect of 0 to 23.3 μM kinetin on embryo production in orchardgrass anther cultures.

Kinetin (μM)	Embryo Mean Per Ten Anthers
0	10.7a*
0.46	5.6b
4.6	1.3c
23.3	0.7c

*Means with same letter are not significantly different according to Duncan's New Multiple Range Test with a 0.05 probability level.

An average of 10.6 embryos per 10 anthers was initiated in the treatment containing no kinetin whereas 5.6, 1.3, and 0.7 embryos per 10 anthers were obtained from the treatments containing 0.1, 1.0, and 5.0 mg/liter kinetin. Anther explants as well as associated embryos obtained from culture on media containing at least 0.1 mg/liter kinetin became brown and appeared less vigorous when compared to those cultured in the absence of kinetin. Furthermore, the initial response from some of the anthers cultured on media containing any of the kinetin levels tested was the production of brown callus masses; however, these calli then initiated embryos which appeared to have a normal phenotype.

Benzylaminopurine

Anthers cultured in the presence of 0.1 to 5.0 mg/liter benzylaminopurine (BAP) resulted in a significant decrease in the embryogenic response as compared to those anthers cultured in the absence of BAP (Table 11). An average of 11.6 embryos was observed per 10 anthers when cultured on medium containing no BAP. However, embryo means of 1.5, 0.8, and 0.3 were observed for anthers cultured in the presence of 0.1, 1.0 or 5.0 mg/liter BAP, respectively. Most anthers cultured on media containing 0.1 to 5.0 mg/liter BAP turned dark brown or black after 3 to 4 weeks incubation. However, the plants regenerated from anthers cultured on the control and BAP treatments were of a normal phenotype.

Table 11. Effect of 0 to 5 mg/l benzylaminopurine (BAP) on the mean embryo production from cultured orchardgrass anthers.

BAP (mg/l)	Embryo Mean Per Ten Anthers
0	11.6a*
0.1	1.5b
1.0	0.8b
5.0	0.3b

*Means with same letter are not significantly different according to Duncan's New Multiple Range Test with a 0.05 probability level.

Microspore Culture

Most (90%) of the microspores centrifuged in a ficoll/sucrose density gradient were collected at the bottom of the centrifuge tube; the remaining microspores were collected at the ficoll interface. A density of 10,000 to 50,000 microspores per ml medium was cultured in 48 wells of multiwell plates. However, no sign of embryogenesis was observed from approximately 1 to 2 million microspores regardless of anther cold pretreatment or microspore pellet/ficoll interface collection.

Pistil Culture

Stereo and Scanning Electron Microscopy

Orchardgrass pistils cultured for 1 week on SH-30 showed no sign of embryogenesis (Plate V, Figure 16); however, a root-like protrusion emerged basipetally from approximately 15% of these pistils (Figure 17). After 2 weeks culture pistils developed tumid regions which appeared to be young embryos (Figure 18). Pistils initiated direct embryos from these regions after 3 weeks culture (Figure 19 and Plate VI, Figure 22). Four week cultured pistils contained both direct and indirect embryos (Plate V, Figure 20) where the indirect embryos developed via callus originating through disorganization of preexisting direct embryos (Figure 21). Furthermore, embryos emerged from the style regions after 4 weeks culture (Plate VI, Figure 23). Direct and indirect embryos continued to develop from pistils after 5 and 6 weeks culture.

Plate V. Embryogenic and morphogenic responses from pistils cultured for up to 4 weeks on SH-30.

Figure 16. Pistil after 1 week culture with no sign of embryogenesis (45 x).

Figure 17. Pistil after 1 week culture with a root-like protrusion (arrow) emerging basipetally (30 x).

Figure 18. Tumid regions (arrows) on ovary and style of a pistil cultured for 2 weeks (50 x).

Figure 19. Embryos (arrows) emerging directly from tumid regions of a 3 week cultured pistil (54 x).

Figure 20. Embryos (small arrow) arising indirectly from callus (large arrow) formed by disorganization of direct pistil-derived embryos after 4 weeks culture (24 x).

Figure 21. Mature embryo (left) and disorganizing embryo (right) from pistil culture leading to callus production (90 x).

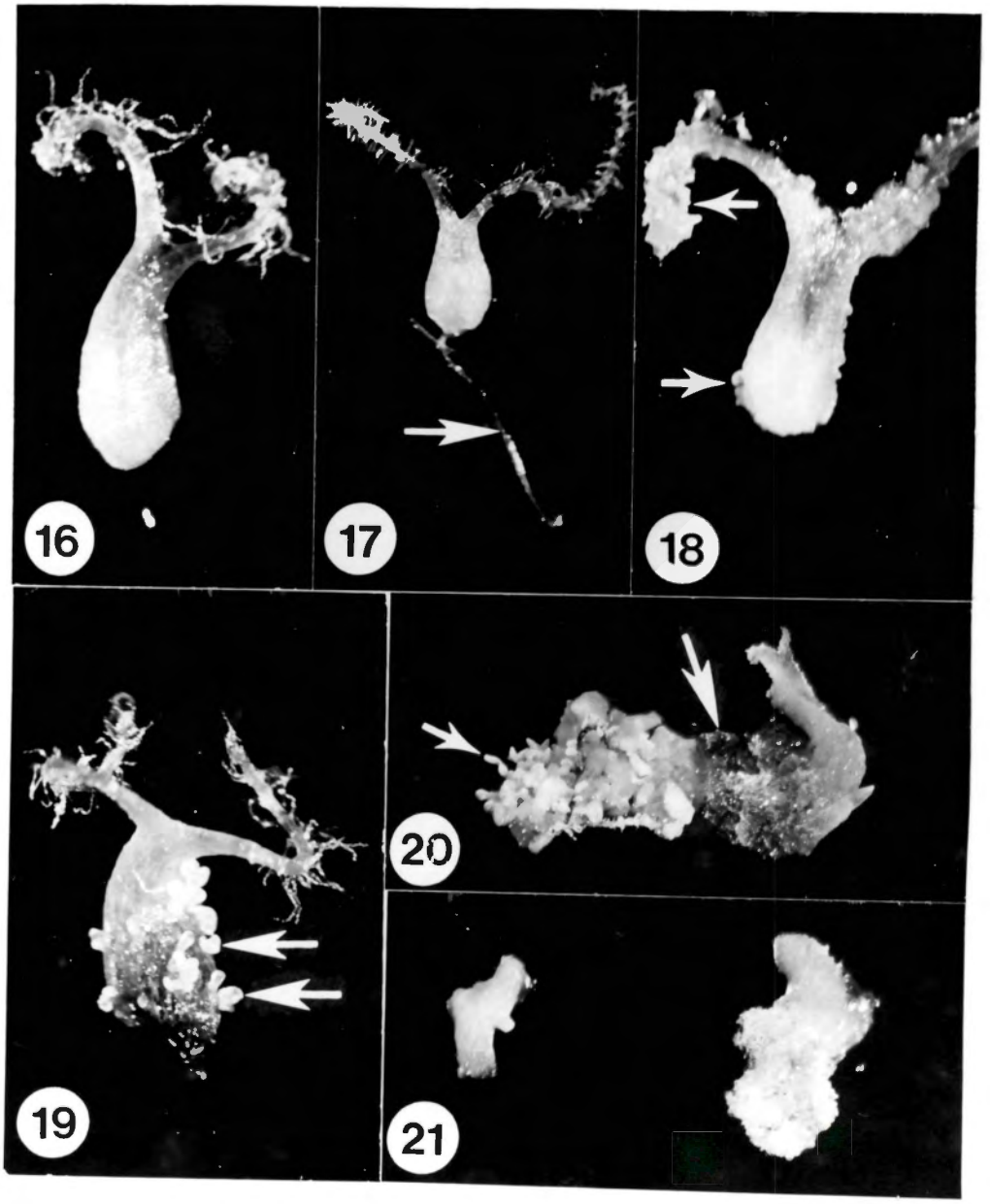
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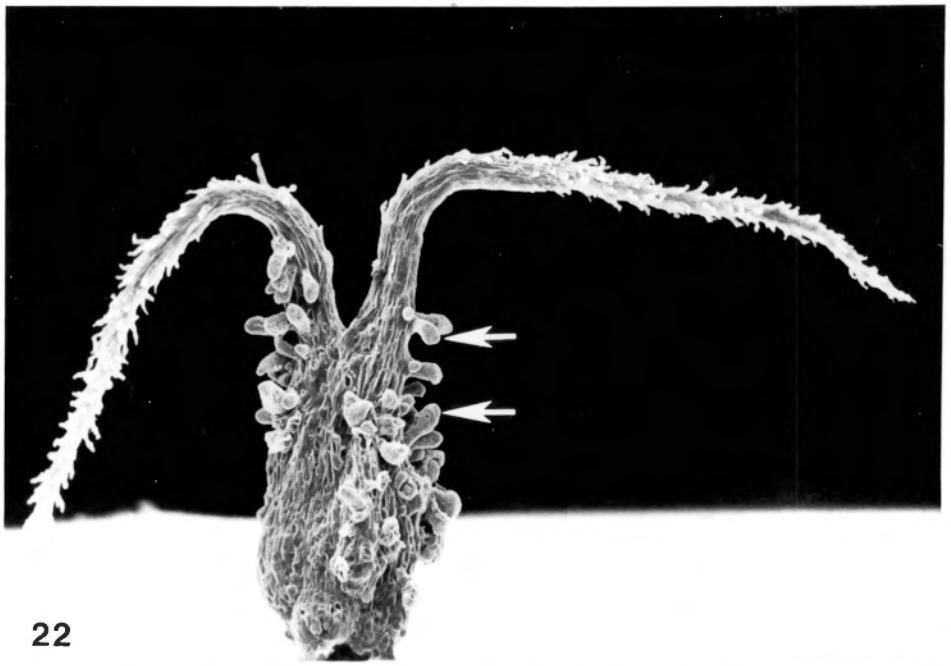
Plate V

Plate VI. Scanning electron micrographs of pistil derived embryos.

Figure 22. Embryos (arrows) arising directly from ovary regions of a pistil after 3 weeks culture (112 x).

Figure 23. Embryo (E) initiating directly from style (ST) of a pistil after 4 weeks culture. A stigma (SG) is also observed. (840 x).



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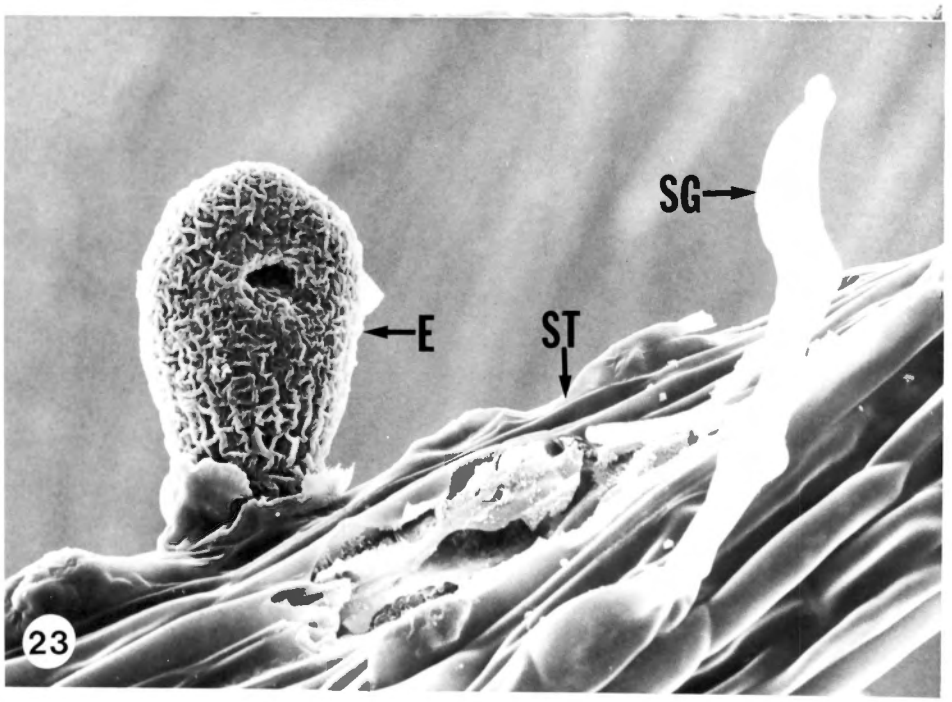

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Plate VI

Assessment of Embryogenesis/Chromosome Counts

The embryogenic response from 45 pistils cultured for 1 week on SH-30 was estimated by an average rate of 0.1 regenerants per pistil (Table 12). This regeneration rate increased to 0.3 and 1.2 regenerants per pistil after 2 and 3 weeks culture, respectively, but decreased to 0.8 after 4 weeks culture. This was followed by a significant increase in the average regenerant number per pistil of 2.1 and 5.1 for the 5 and 6 week cultures, respectively.

A total of 135 plants were regenerated from pistil cultures transferred to SH-0. These plants appeared to have a normal phenotype except for one which was less vigorous. Chromosome counts from this plant revealed a mixoploid with 14, 28, or 56 chromosomes (Table 13).

Pistil Orientation

Pistil orientation was a significant factor affecting the embryogenic response. An upright orientation resulted in an average of 1.9 regenerants per pistil compared to 1.2 for the flat orientation (Table 14).

Histology

Histological examination of pistils not previously cultured showed an embryo sac enclosed by the nucellus which was within the ovule (Plate VII, Figure 24). Ovules appeared to be emerging through the placenta of some of the pistils cultured for 1 week on SH-30 (Figure 25). After 2 weeks culture, some pistils contained ovules with multicellular structures which may have resulted from repeated

Table 12. Average number of plants regenerated per cultured orchardgrass pistil after transfer from SH-30 to SH-0 for 3 weeks.

Weeks on SH-30	Average Number of Plants Regenerated
1	0.1a*
2	0.3ac
3	1.2b
4	0.8bc
5	2.1d
6	5.1e

*Means with same letter are not significantly different according to Duncan's New Multiple Range Test with a 0.05 probability level.

Table 13. Frequency of cells with 14, 28, or 56 chromosomes from a mixoploid plant regenerated from orchardgrass pistil culture.

Chromosome Number	Number of Cells	Percent
14	7	23.3
28	22	73.3
56	1	3.3

Table 14. Effect of "flat" vs. "upright" pistil orientation on the average number of plants regenerated (per pistil) from orchardgrass pistil cultures.

Orientation	Average Number of Plants Regenerated Per Pistil
Flat	1.2a*
Upright	1.9b

*Means with same letter are not significantly different according to Duncan's New Multiple Range Test with a 0.05 probability level.

Plate VII. Histology of cultured pistils and morphology of cultured ovules of orchardgrass.

Figure 24. Intact ovule within a pistil prior to culture. Nucellar tissue (N) is observed (620 x).

Figure 25. Ovule (O) emerging from the basal end of a pistil cultured for 1 week (430 x).

Figure 26. Multicellular structures within the ovule of 2 week cultured pistils containing 2 (small arrow) and 4 (large arrow) cells (900 x).

Figure 27. Embryos (E) emerging directly from the ovary wall of pistils cultured for 4 weeks (68 x).

Figure 28. Ovule after 1 week culture (arrow) (90 x).

Figure 29. Ovule with necrotic embryo sac (arrow) after 3 weeks culture (60 x).

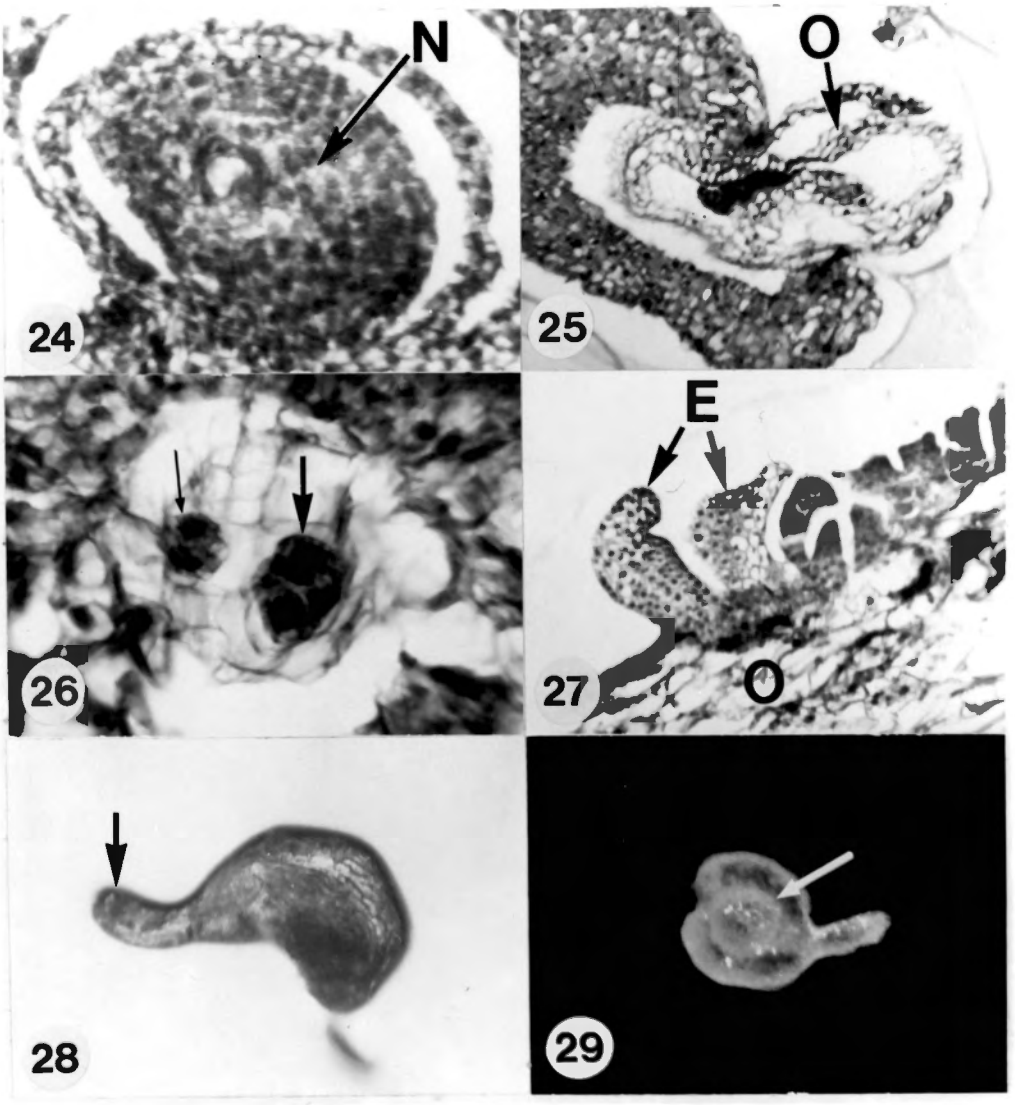
N
24
O
25
26
E
27
O
28
29

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Plate VII

division of a gametic cell within the embryo sac (Figure 26). However, ovules appeared to be degenerating within pistils cultured for 4 weeks. Nevertheless, several direct embryos were observed arising from the outer wall of the ovary and style regions of these pistils (Figure 27).

Ovule Culture

Ovules cultured on SH medium containing 0, 10, 20, or 30 μM dicamba maintained a translucent color after 1 to 2 weeks culture (Figure 28). However, some of these ovules developed necrotic regions within the ovule (Figure 29) and no sign of embryogenesis was observed. In subsequent experiments, ovules were cultured on SH-30 containing 0, 0.1, 1.0, or 5.0 mg/l kinetin. These explants enlarged during the first week of culture but also developed internal necrotic regions. No sign of embryogenesis was observed from these ovule explants.

Ovules removed from pistils cultured for up to 3 weeks and then recultured on SH-30 initially showed no sign of embryogenesis. Most ovules became brown and appeared lifeless. However, after continued culturing one ovule initiated callus which then developed somatic embryos (Plate VIII, Figure 30).

Plate VIII. Orchardgrass ovule from 3 week precultured pistil.

Figure 30. Ovule (O) initiating callus (C) and an embryo (E) after 6 weeks culture on SH-30 (85 x).

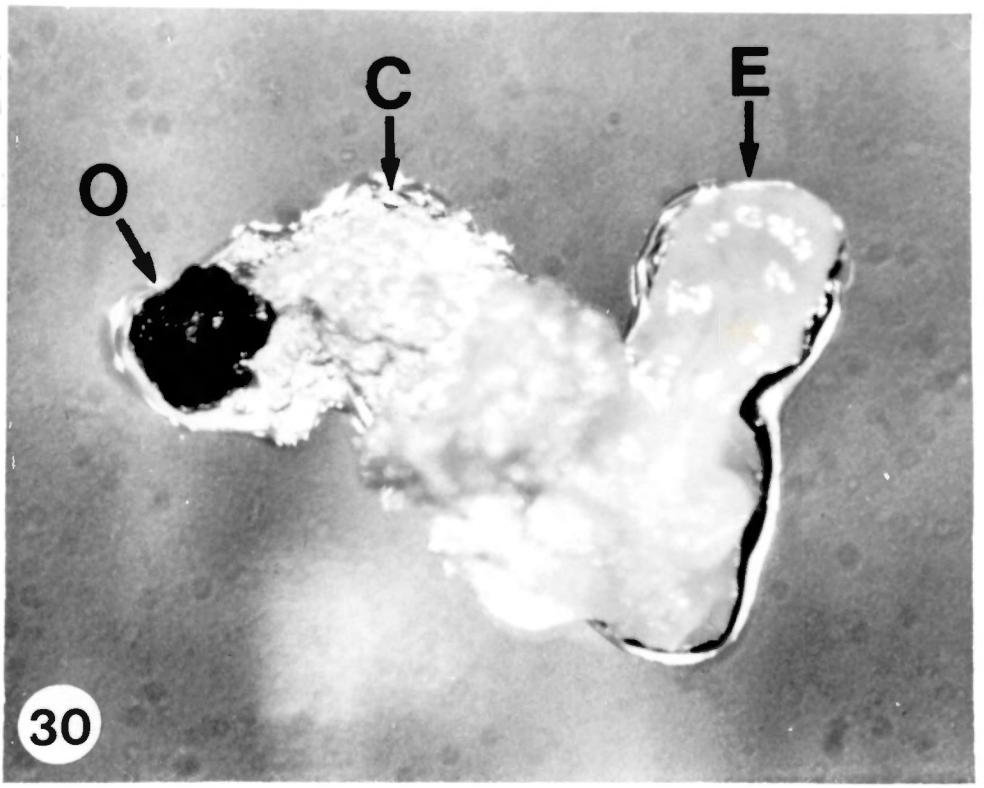
O
C
E
30

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Plate VIII

V. DISCUSSION

Embryo initiation and plant regeneration from anther and pistil cultures of orchardgrass show the utility of these tissues as new explant sources for future tissue culture research. Previously, calli have been initiated and/or plants regenerated from root and seedling (Atkin and Barton, 1973), caryopsis (Conger and Carabia, 1978), mature embryo (McDaniel et al., 1982), leaf (Hanning and Conger, 1982; Conger et al., 1985) and inflorescence (Chen et al., 1982, 1985; Conger and McDonnell, 1983) explants of orchardgrass. Anthers and pistils are more likely than some other explant sources to be free of bacterial and fungal contamination, especially in field grown material, and, therefore, more amenable to successful culture initiation.

Most of the nonzygotic embryos which were initiated from orchardgrass anther and pistil explants arose directly from somatic tissues. However, those microspores which responded in culture appeared to initiate callus masses which then developed into embryos. The reason for this dichotomy in the orchardgrass embryogenic response is not known. However, a review by Williams and Maheswaran (1986) suggested that such direct/indirect embryogenic responses can be attributed to a low/high density of totipotent cells and the physiological state of the explant. Therefore, differences within and among explants with regard to their physiology as well as the density

of totipotent cells may have resulted in expression of direct embryogenesis from orchardgrass anther somatic tissue compared with indirect embryogenesis from the microspores.

Embryo-like multicellular structures were observed within orchardgrass microspores exposed to a 6 week cold pretreatment. There are several explanations for the effect of cold pretreatments in microspore induction in several species. Fitch and Moore (1983) reported that a sugarcane anther cold pretreatment killed young microspores while mature microspores were converted from a gametic to a sporophytic development. In rye, Wenzel et al. (1977) suggested that a cold pretreatment killed weak microspores, arrested the remaining microspores at a uninucleate stage and provided a stimulus for initiation of endogenous conditions required for cellular development. It is not known if exposure of orchardgrass anthers to a cold pretreatment promoted or hindered callus/embryo induction from microspores. However, a 6 week cold pretreatment appeared to decrease the embryogenic response from somatic tissues of cultured anthers. Therefore, one possible role for a cold pretreatment in orchardgrass anther culture may be to limit embryogenesis from somatic tissues so that microspore embryogenesis can be observed.

Orchardgrass embryos were initiated from anthers exposed to a 6 week cold pretreatment regardless of the presence or absence of dicamba in the medium. This may suggest either that the anthers produce endogenous auxins that will initiate embryogenesis or that exogenous auxins are not required for this response. In a like manner,

Hinchee and Fitch (1983) reported that sugarcane callus/embryo induction from anthers cultured on media devoid of auxins either promoted or was not significantly different from those cultured in the presence of auxins. Furthermore, Hanning and Conger (1986) found that leaf segments from an embryogenic orchardgrass genotype initiated somatic embryos in low frequency when cultured on medium without hormones. The orchardgrass genotype used by Hanning and Conger (1986) was the same as used in the research in this dissertation.

The presence of eight nucleoli as well as 14, 28, 56 or up to 112 chromosomes per nucleus of one plant regenerated from 6 week cold pretreated anthers indicates polyhaploidy and subsequent endoreduplication. Ordinarily a plant can possess up to one nucleolus per chromosome genomic set (Esau, 1965). Since orchardgrass is an autotetraploid no more than four nucleoli per cell in any normal plant should be expected. Therefore, eight nucleoli per nucleus would suggest an octaploid genome. Similar results of haploidy and endoreduplication have been reported from anther cultures of species such as rye (Wenzel et al., 1977), wheat (DeBuyser and Henry, 1980), corn (Genovesi and Collins, 1982), and sugarcane (Fitch and Moore, 1984).

An anther density of 10 anthers per ml medium initiated a greater embryogenic response than those cultured at a 100 per ml density. Xu et al. (1981) reported that a density of 20 anthers per ml initiated the greatest embryogenic response from barley anther cultures. However, in that study the highest anther density evaluated

was 20 anthers per ml. This suggests that an optimum anther density for orchardgrass anther culture may lie between 10 and 100 anthers per ml. The physiological explanation for the higher embryogenic response at the lower anther density was not investigated. However, it is likely that anthers cultured at a density of 100 per ml utilized the available nutrients more quickly than those cultured at 10 per ml.

A 9% sucrose concentration promoted embryogenesis from 3 and 6 week cold pretreated orchardgrass anthers cultured in vitro. Similar results were obtained in barley (Clapham, 1971; Kao, 1981), rice (Chen, 1978), and triticale (Ono and Larter, 1976) anther cultures. The role of sucrose in orchardgrass anther cultures is not completely understood. However, Maheshwari et al. (1980, 1982) suggested that sucrose may provide the most favorable osmotic and/or nutritive factors required for microspore embryogenesis.

Anther orientation was not a significant factor controlling orchardgrass embryogenesis; however, orientation did have a significant effect on pistil embryogenesis. Anther orientation was a significant factor in barley (Hunter, 1985; Shannon et al., 1985) and tobacco (Sopory and Maheshwari, 1976) anther cultures. However, Shannon et al. (1985) suggested that variation in response to anther orientation may be due to species differences. A different orchardgrass embryogenic genotype may also show a positive response due to anther orientation during in vitro culture. In contrast to anther culture, an upright pistil orientation may have allowed placental tissues to

facilitate nutrient movement throughout the explant. San Noecum (from a review by Yang and Zhou, 1982) obtained a better response when barley ovary explants were inoculated with the placenta contacting the medium.

Embryo initiation from orchardgrass anther cultures was not affected by up to a 5 g/l supplement of casein hydrolysate to the medium. This is contrary to results obtained with orchardgrass suspension cultures (Gray et al., 1984; Gray and Conger, 1985) where up to 4 g/l casein hydrolysate promoted embryogenesis. Since the same genotype and basal medium were used in orchardgrass anther and suspension cultures, the dichotomy which exists may be due to the effects of solid vs. liquid culture. For instance, callus cultures generally establish a "vertical nutrition gradient" (Gamborg and Shyluk, 1981) whereas suspension cultures are continuously agitated in liquid medium and no gradient develops. Therefore, in orchardgrass anther cultures, a similar nutrition gradient may have occurred which limited the uptake fo casein hydrolysate.

The presence of the cytokinins BAP and kinetin at 0.1 mg/l or greater inhibited the initiation of embryos from cultured orchardgrass anthers. Anther cultures of other species have also shown no apparent requirement for cytokinins either in callus/embryo induction (Reynolds, 1984; Monfort, 1985) or in plant regeneration (Henry and DeBuyser, 1981; Rines, 1983). The exact reason for this inhibitory response is unknown; however, the concentration of these cytokinins may have been too high to promote the embryogenic response. Furthermore,

the putative cytokinin receptor site (Horgan, 1984) of orchardgrass may bind with kinetin or BAP and elicit a detrimental response. Perhaps the use of a naturally occurring cytokinin, such as zeatin (Letham, 1973) or zeatin conjugates (Horgan, 1984), may be more applicable to orchardgrass anther culture.

The inability of isolated orchardgrass microspores to form embryos while cultured on media which were suitable for polyhaploid induction from microspores via anther culture suggests a need for the anther wall to promote androgenesis. Several reports suggest that the anther wall and/or panicle tissue may provide endogenous growth regulators or nutrients which promote microspore embryogenesis (Kasperbauer et al., 1980; Maheshwari et al., 1980, 1982; Heberle-Bors, 1985). Various amino acids and/or carbohydrates have been isolated from the anther wall of sugarcane (Hinchee et al., 1984) and tobacco (Horner and Pratt, 1979; Aruga and Nakajima, 1985) anther cultures. However, it has not been proven whether the somatic wall of orchardgrass anther cultures produce endogenous compounds.

Orchardgrass microspores were separated into two density bands with a ficoll gradient by centrifugation. This suggests that two developmentally and/or physiologically different types of microspores may exist within orchardgrass anthers. Previous reports with tobacco have also shown microspores of various densities separated by percoll gradients (Wernicke et al., 1978; Heberle-Bors and Reinert, 1980). In those reports the less dense microspores initiated embryos after 6 weeks culture and haploid plants were recovered. The proper culture

conditions to stimulate embryogenesis in orchardgrass microspore culture is not known. However, the nutritional and environmental conditions required for embryo induction may be different for the two types of microspores observed.

The rate of microspore-derived callus initiation was 1 per 40 for the 6 week cold pretreated anthers in the first experiment and 1 per 100 anthers in the second experiment. This reflects the inherent variability in orchardgrass anther cultures. Furthermore, different rates of embryogenesis observed between like treatments of the two cold pretreatment X dicamba concentration and casein hydrolysate experiments also suggests the innate variability of this system. Variation in embryo production has also been seen in orchardgrass leaf cultures (Hanning, 1984). This implies that a myriad of physiological and environmental factors may affect the response of cultured anthers.

One desirable attribute of anther/ovary culture of most diploids is the recovery of haploid phenotypes which, after chromosome doubling, exhibit homozygous dominant and recessive traits (Maheshwari et al., 1982). However, orchardgrass is a self incompatible autotetraploid which exhibits a high degree of genic heterozygosity. Furthermore, an orchardgrass source plant with a 1:4:6:4:1 genomic distribution of nulliplex (aaaa), simplex (Aaaa), duplex (AAaa), triplex (AAAa) and quadriplex (AAAA) loci should produce microspores with gametic genomic ratios of 2AA:4Aa:2aa (see Conger et al., 1986 for theoretical allelic frequencies). Therefore, approximately 50% of the loci of

regenerated plants from orchardgrass anther culture would be heterozygous. Reculture of anthers from polyhaploid (2X) orchardgrass regenerants might result in monoploid (1x) plant recovery with all loci in a homozygous state. However, orchardgrass microspore-derived regenerants may exhibit a high degree of spontaneous endoreduplication therefore rendering monoploid plant recovery by this method impossible.

In vitro recovery of nearly 100% of the loci in a homozygous state would be possible by the nested explanting [using regenerated plant as an explant source for subsequent tissue culture experiments] of anthers from several cycles of tissue culture derived polyhaploid plants. By this method, the first, second and third regeneration cycle polyhaploid plants would result in approximately 50%, 75% and 87.5% of the regenerated plant's loci in a homozygous state. Nested culturing of anther explants from subsequent regeneration cycles would follow the equation: $\%\ \text{Homozygosity}=[1-(0.5)^{n}] \times 100$ where n equals the number of tissue culture regeneration cycles employed. Therefore, after 6 tissue culture regeneration cycles nearly 98% of the loci should be in a homozygous form.

No significant differences between orchardgrass source plants or those regenerated from anther or pistil culture were noted for peroxidase isozyme banding patterns. Corduan (1976) noted no differences in fox glove (Digitalis purpurea L.) anther-derived plants of gametic vs. somatic origin regarding isozyme banding patterns of several enzymes, including peroxidase. However, isozymes of other enzymes showed differences between gametic and somatic derived plants.

Therefore, in orchardgrass, isozymes of other enzymes need to be assessed in order to possibly show a gametic origin of these plants.

Several hundred plants of somatic origin and one mixoploid of possible gametic origin were regenerated from pistil cultures of orchardgrass. Furthermore, the presence of cell clusters in the embryo sac of orchardgrass pistils also indicates a gametic origin. This is the first report of gynogenesis in orchardgrass as well as in any other forage grass species. However, mixoploids of embryo sac origin have also been regenerated from corn (Truong-Andre and DeMarly, 1984), petunia (Petunia axillaris (Lam.) B. S. P.) (DeVerna and Collins, 1984), rice (Zhou and Yang, 1981) and sugarbeet (Bossoutrot and Hosemans, 1985) ovary and/or ovule cultures.

The origin of the root-like structure which emerged basipetally from 1 week cultured orchardgrass pistils is not known. However, Mullins and Srinivasnan (1976) reported that a root-like structure which emerged from cultured grape ovules was due to elongation of the integuments. Whether orchardgrass pistils underwent elongation of the integuments remains unknown; however, histology of such pistils showed emergence of the ovule. Perhaps the root-like structure developed from the emerged ovule.

Most cultured orchardgrass ovules failed to initiate embryos whereas cultured pistils initiated numerous embryos. This suggests that the ovule lacks one or more endogenous compound(s) required for embryogenesis. However, one orchardgrass ovule initiated callus after reculture following excision from pistils precultured for

3 weeks on SH-30. This may suggest that the factor(s) responsible for embryogenesis in somatic tissue of these pistils was (were) also present in the ovules after pistil preculture. Furthermore, pollinated ovules of several species have been cultured successfully *in vitro* and developing embryos recovered or plants regenerated (Rangan, 1984b; Topfer and Steinbis, 1985). Therefore, orchardgrass unpollinated ovule culture might initiate polyhaploid plants if the correct medium constituents are present to simulate those conditions within fertilized ovules.

Callus which initiated from scutellar and coleorhizal regions of orchardgrass pistil-derived embryos was similar to that from zygotic embryo explants of several Gramineae species (Dale, 1980; Vasil and Vasil, 1981; Lu and Vasil, 1982; Lu et al., 1982; McDaniel et al., 1982; Ozias-Akins and Vasil, 1982). Although embryos developed from the callus of those zygotic embryo explants, it is not clear as to whether such somatic embryos could dedifferentiate and proliferate into an embryogenic callus if left on the callus induction medium. In orchardgrass pistil cultures, however, direct somatic embryos clearly dedifferentiated and proliferated into callus which reinitiated indirect embryos in a cyclic fashion. The dramatic increases in plant regeneration after 5 and 6 weeks of culture are considered to be through secondary (indirect) embryogenesis from direct embryo-derived callus. This system provides a potential for long-term toti-potency through dedifferentiation and redifferentiation of somatic embryos.

VI. SUMMARY AND CONCLUSIONS

Media supplements such as benzylaminopurine, kinetin (both at up to 5 mg/l) and casein hydrolysate (up to 10 g/l) hindered whereas anther orientation and microspore culture experiments did not promote orchardgrass androgenesis or embryogenesis . Furthermore, in one experiment anthers, regardless of cold pretreatment, cultured on media containing 0 to 30 μM dicamba did not show any significant differences in embryo production. The only factors which clearly promoted embryogenesis from cultured anthers were a low anther density (10 anthers/ml medium) and a 9% sucrose level. Therefore, it appears that most medium supplements or culture conditions may only offer trivial benefits in orchardgrass anther culture whereas the explant may be providing the primary stimulus required to induce embryo production. Perhaps exploring new source plant genotypes may provide more advances in orchardgrass anther culture compared to evaluating additional medium supplements.

At least one androgenetic plant from 6 week cold pretreated orchardgrass anthers was recovered following culture for 6 to 8 weeks on SH-30 supplemented with 9% sucrose. Chromosome doubling (endoreduplication) was observed in the cells of this regenerant resulting in a mixoploid plant. Furthermore, there were up to five additional plants recovered which possibly arose from microspores; all cells from these plants contained the somatic chromosome number and a gametic

origin could not be confirmed. This suggests that chromosome doubling may have occurred sometime during the androgenetic development of these plants. Peroxidase isozyme electrophoretic banding patterns from these plants did not differentiate between the somatic or gametic nature of these plants. However, other isozyme systems need to be examined in order to clearly determine an origin of these plants. Therefore, if these plants have a gametic origin and possess desirable traits, they could be utilized as polyhaploid parent plants in a conventional breeding program.

One of several plants regenerated from orchardgrass pistil culture was a mixoploid with a possible embryo sac origin. This also suggests that endoreduplication resulted after embryo induction from the embryo sac. However, other than a slow growth habit this plant appeared normal.

Orchardgrass pistils cultured in vitro initiated embryos directly from ovary and style tissues. These primary embryos then disorganized after continued culturing and reinitiated secondary embryos in a cyclic fashion which suggests long-term totipotency. The nature of orchardgrass pistil embryogenesis likens itself to morphological research on embryo initiation and development.

Excised orchardgrass ovules cultured on SH medium containing 0 to 30 μM dicamba or SH-30 containing 0 to 5 mg/l kinetin failed to initiate any embryos. Since embryogenesis was observed from pistil explants this suggests that some endogenous compounds and/or developmental cell-types are absent in ovule but present in ovary and style

tissues. Furthermore, one orchardgrass ovule removed from a pistil precultured for 4 weeks and then recultured on SH-30 initiated a callus mass which then initiated embryos. Perhaps the preculture of pistils provided the stimulus to ovules in situ for induction of embryogenesis. The exact origin of this callus mass is unknown; however, it is either from nucellar to embryo sac tissues.

Polyhaploid plant regeneration from orchardgrass anther or pistil culture may provide the ability to obtain homozygous or near homozygous genotypes which might be applicable in conventional plant breeding. The culture techniques and polyhaploid identification methods discussed in this dissertation provides a basis in which such plants might be more fully utilized in plant breeding.

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