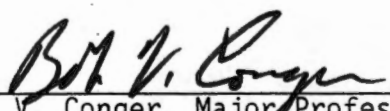


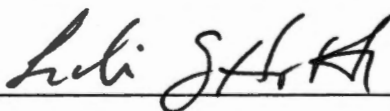
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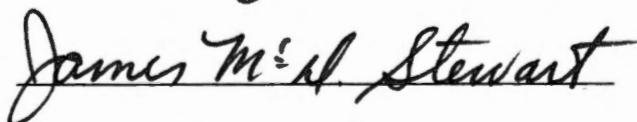
I am submitting herewith a thesis written by Janet Norwood Reed entitled "Cytogenetic Analyses of Tall Fescue (Festuca arundinacea Schreb.) Plants Regenerated From Tissue Cultures." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant and Soil Science.



Bob V. Conger, Major Professor

We have read this thesis
and recommend its acceptance:





Accepted for the Council:



The Graduate School

CYTOGENETIC ANALYSES OF TALL FESCUE (FESTUCA ARUNDINACEA
SCHREB.) PLANTS REGENERATED FROM TISSUE CULTURES

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Janet Norwood Reed

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ABSTRACT

The primary objective of this study was to compare the cytogenetic variability present in tall fescue (Festuca arundinacea Schreb.) plants regenerated from embryo-derived calli of ethylmethane sulfonate (EMS)-treated (TEM) and non-EMS treated (REG) caryopses and from control plants grown from seed. Secondary objectives were to relate meiotic abnormalities with chiasma frequency, pollen stainability, phenotypic characteristics, and chromosome numbers.

Panicles of tall fescue were collected in May of 1982 and 1983 and fixed in Carnoy's solution. The anthers were dissected from the florets, placed on a microscope slide, and the contents pressed out. After staining with 0.5% acetocarmine, the pollen mother cells were scored for chromosome number, chiasmata frequency, and meiotic aberrations. Pollen from mature anthers was placed on a microscope slide, stained with Newton's Iodine Solution, and examined for viability. Phenotypic characteristics such as date of maturity, growth habit, and leaf morphology of plants exhibiting abnormalities were noted.

All control plants had $2n=42$ chromosomes and no meiotic abnormalities were observed. Nearly 48% of the regenerated plants derived from EMS-treated embryos, and nearly 42% of the plants derived from nontreated embryos exhibited aberrations, the most common being lagging chromosomes, chromosomal bridges, and micronuclei in spores. A low frequency of aneuploids, one

monosomic ($2n=41$), and two trisomics ($2n=43$) were identified. Univalents associated with the aneuploids were responsible for lagging chromosomes and micronuclei in those plants. Inversion heterozygosity and "sticky" chromosome associations accounted for chromosomal bridges.

Meiotic analyses should be performed on all plants regenerated from tissue cultures to aid in the identification of novel genotypes. Meiotically aberrant plants have the potential for possible use in genetic and/or breeding studies.

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CHAPTER I

INTRODUCTION

The potential of plant cell and tissue culture technology in the improvement of important agricultural plants has been the subject of numerous symposia, books, and reviews. One of the early primary uses was seen as that of cloning selected genotypes. This requires that the regenerated plants be genetically identical to the parent. However, phenotypic variants have been frequently observed. This variability is highly undesirable for cloning and may even preclude the use of certain in vitro procedures for that purpose in certain plant species. However, from a positive standpoint, it represents a potentially powerful tool for creating genetic variation for plant breeding programs. Tissue culture-induced variability has been termed "somaclonal variation" by Larkin and Scowcroft (38). These authors have also reviewed the uses, potential uses, and origin of somaclonal variation in numerous crops.

The tissue culture cycle per se, i.e., the establishment of an undifferentiated cell mass from an explant and the eventual regeneration of plants after several cell generations, produces large amounts of genetic variability in some plants. However, both radiation and chemical mutagens have been used in addition to tissue culture cycles to enhance variability in sugarcane (30) and alfalfa (63).

While evidence suggests that phenotypic changes are not associated with gross chromosomal changes in some species (38) variability in chromosome number and morphology has been found in several others (6, 25, 70, 71). Chromosomal changes include polyploidy, aneuploidy, chromosomal rearrangements, deficiencies, and dicentric chromosomes.

Regeneration from callus cultures has now been reported for all major cereals and several forage grasses (20). However, the objective of most studies was to recover and establish plants. In comparison to actual reports of plant regeneration in these species, the cytogenetics of the culture systems and regenerated plants has received limited attention. Notable exceptions include the studies by McCoy et al. (48) with oats (Avena sativa L.) and McCoy and Phillips (47) with maize (Zea mays L.) in which extensive meiotic analyses were conducted on a large number of plants. The superiority of meiotic to mitotic analyses for detecting deletions, duplications, and translocations was also emphasized.

Tall fescue (Festuca arundinacea Schreb.) is a self-incompatible, outcrossing, allohexaploid possessing a wide range of inherent variability. However, phenotypic variation for growth habit, size and color of leaves, and other characters beyond that occurring in plants grown from seed has been observed in plants regenerated from tissue cultures in our laboratory. Therefore the primary objective of this research was to compare meiotic abnormalities in pollen mother cells (PMC's) of field established tall

fescue plants (1) grown from seed, (2) regenerated from embryo-derived callus, and (3) regenerated from embryo-derived callus in which the caryopses had been previously treated with ethyl methane sulfonate (EMS). Specific criteria studied included determination of chromosome number, chiasmata frequency, chromosomal aberration frequency, and pollen stainability.

CHAPTER II

LITERATURE REVIEW

I. TALL FESCUE

Festuca arundinacea Schreb. (tall fescue, cv. Ky 31) is a broad-leaved, cool-season perennial bunchgrass grown in the temperate zones of North America, South America, Europe, and Africa (13). Tall fescue grows well over a wide range of soil and climatic conditions. It has been one of the most widely used grass species for pasture, turf, and conservation purposes in the United States since its discovery in the 1940's (13). The importance of tall fescue as a forage grass is evidenced by its increased usage in the United States since 1949. At that time tall fescue was grown on approximately 16,000 hectares. By 1973, it was grown on approximately 12 to 14 million hectares (14). It is estimated that 1.4 million hectares are currently grown in Tennessee (15).

Several workers have studied the cytogenetics of tall fescue. A chromosome number of $2n=42$ was reported by Levitsky and Kuzminā (40); 21 bivalents were observed by Peto (60). Crowder (22) studied the meiotic cycle in tall fescue and concluded that it was either an auto- or autoallohexaploid. Subsequently, Malik and Thomas (45) proposed that tall fescue consisted of three genomes and designated them as AABB'B'. They considered

the BB and B'B' genomes to be very closely related with the AA genome being more distally related. These authors were the first to suggest the possibility of genetic control of diploid pairing in tall fescue.

Tall fescue is now considered a typical allohexaploid ($2n=6x=42$) that consists of three genomes with the designation AABBCC (33, 34). The A genome arose from F. pratensis (diploid meadow fescue) (45). The B genome is thought to have arisen from Lolium since tall fescue and Lolium cross readily and the hybrids form chiasmata during chromosome pairing (11). The C genome is probably from another Festuca species and contains the genes responsible for diploid-like meiosis (33).

Although tall fescue is a vigorous, productive, and persistent pasture grass, there are problems with palatability and forage quality in late spring and summer. Efforts are being made to improve quality and other traits through conventional breeding (13). More recently, the use of tissue culture techniques to supplement conventional breeding methods has received increased attention. Kasperbauer et al. (36) showed callus induction and plant regeneration in annual ryegrass X tall fescue F_1 hybrids. Dale (24) obtained regenerated plants of tall fescue through meristem tip culture and Lowe and Conger (43) demonstrated plant regeneration from callus using mature embryos as the explant source.

II. CYTOGENETIC ANALYSES OF CULTURED CELLS AND PLANTS

Changes in DNA content and chromosome number of in vitro cultured plant cells have been observed since the early development of plant tissue culture methodology. Mitra et al. (49) reported the occurrence of haploid, tetraploid, and polyploid nuclei, multinucleate cells and chromosomal bridges in cultured carrot cells. Various chromosomal aberrations were also described in species with low chromosome numbers such as Haplopappus ($2n=4$) and Crepis ($2n=6$) (50, 67, 71).

Other in vitro changes include morphogenic and growth characteristics of calli, varying abilities of cultures to produce secondary metabolites, and ability of cells to regenerate (25, 70).

Although some cultured cells show chromosome aberrations, the regenerated plants do not always reflect these changes. Crepis is considered to be genetically stable in culture, although karyotypic changes due to chromosomal aberrations are present in cultured cells (25). Haplopappus has yet to be regenerated from callus or callus cultures, so stability in the regenerated plants is unknown (29, 71).

Balzan (5) found a high frequency of tetraploid and hypertetraploid cells and various anomalies such as stickiness, bridges, and lagging chromosomes in Zea callus cultures. Since a very low frequency of aberrations occurred in the regenerated

plants, this species is regarded to be genetically stable in vitro (27, 47).

Aneuploid and tetraploid pollen mother cells have been found in regenerated plants of Lilium, long considered the most stable species in culture (9, 69). Apium is also considered to be stable, although aneuploid and polyploid cells have been observed in the regenerated plants (72). Brachycome is the only stable Compositae species found to date. Mitotic analyses of regenerated plants revealed no aberrations and all plants were genetically and phenotypically identical to the parents (29).

It is now accepted that the variability in regenerated plants is less than that observed in the cultures from which they were derived (25, 26). Lupi et al. (44) reported that aneuploid cells are selected against in favor of diploid cells in developing Triticum plants (diplontic selection). Novak and Vyskot (54) concluded that selection was in favor of diploid cells during organogenesis of Nicotiana. Thus, it is evident that specific chromosome constitutions are selectively favored during the course of plant regeneration (57).

Genetically and morphologically stable plants are the exception rather than the rule (25, 38, 70, 71). Sacristan and Melchers (66) obtained a high percentage of aneuploids in regenerated plants of Nicotiana. Also, in this species, mixoploid, aneuploid, and polyploid plants were recovered from suspension cultures (56) and tetraploids were recovered by Prat (61) from

protoplasts. Haploid and diploid plants of Nicotiana were obtained by Novak and Vyskot (54) when haploids were used as the experimental material. Chaleff and Keil (18) observed a high frequency of tricotyledonous seedlings in the progeny of in vitro produced picloram-resistant mutants of tobacco when compared to the progeny of nonresistant mutants

Shepard et al. (68) used leaf protoplasts of potato (Solanum tuberosum) to produce plants that exhibited variation in many characters: (1) growth habit, (2) maturity date, (3) tuber uniformity, (4) fruit production, (5) tuber skin color, and (6) disease resistance.

Cichorium (2n=18) plants regenerated in vitro from callus were mainly mosaics with chromosome numbers ranging from haploid, hypodiploid to diploid, and hyperdiploid with no tetraploids or hypertetraploids found (16). Cytophotometric data correlated highly since the majority of nuclei contained 1C to 2C amounts of DNA (16). These data agree with previous investigations on regenerated plants of Triticum and Lilium (9, 10) since the majority of regenerated plants were aneusomatic, i.e., consisted of both euploid and aneuploid cells (16).

Browsers and Orton (12) and Bennici (8) described aneuploid and mixoploid regenerated plants from suspension cultures of Apium and Pelargonium, respectively. In vitro regenerated Arachis plants from anther-derived callus showed chromosome numbers ranging from haploid to octoploid (4) while Cappadocia and Ramula (17) obtained

diploid plants from anther-derived callus and tetraploids from stem internode cultures in Lycopersicon.

In monocotyledonous plants, Malnassy and Ellison (46) isolated tetraploids from callus cultures of asparagus and Novak (55) recovered aneuploids and tetraploids from callus cultures of Allium. An extensive study was conducted on in vitro regenerated Haworthia plants by Ogihara (57). Meiotic and mitotic analyses revealed aneuploids, tetraploids, reciprocal and non-reciprocal translocations, paracentric inversions, and sub-chromatid aberrations (57). There was great variation in leaf shape of the regenerated plants while growth vigor, leaf color, stomata number, pollen fertility, and results of an esterase zymogram showed no significant differences between regenerated and nonregenerated plants (57).

Several workers have conducted cytological investigations on tree species regenerated in vitro. Lester and Berbee (39) described aneuploids, polyteny, mosaics, and variation in phenotypes of Populus. Patel and Berlyn (59) reported an increase in chromosomal aberrations such as bridges, micronuclei, lagging chromosomes, and nuclear fragmentation in buds of Pinus. Cytophotometric studies showed that DNA content increased with time in culture and the regenerated buds contained 8C and higher levels (59).

Numerous nonagronomic plants regenerated in vitro exhibit genetic and phenotypic variability, but only a limited number of

agronomic species have been examined for tissue culture-induced variability. Morphological variations and cell-to-cell differences in chromosome number were found in sugarcane plants derived from callus (31). Chromosome numbers of the variant plants ranged from $2n=94$ to $2n=120$. Another population of regenerated plants had $2n=ca. 106$. However, this variability is comparable to that found in the source cultures (31). In a later experiment, Liu and Chen (41) related the morphological variation in sugarcane plants derived from callus to chromosomal mosaicism.

There are few reports of cytogenetic analyses of regenerated cereal plants. Bennici and D'Amato (10) reported chromosomal mosaicism in Triticum. Phenotypic variants in Oryza were reported by Nishi et al. (53) and Henke et al. (32). Orton (58) reported a reduction in polyploidy, aneuploidy, and chromosomal rearrangements in regenerated plants of Hordeum compared to the preceding callus and suspension cultures. Ruiz and Vazquez (65) disagreed with Orton (58) citing discrepancies in chromosome counts as the basis for higher numbers of aneuploids in comparison to their results (65). Mix et al. (51) recovered aneuploid, haploid, diploid, triploid, and tetraploid plants from microspore callus of Hordeum. Cummings et al. (23) reported a heteromorphic bivalent, aberrant chromosome behavior and phenotypic variability in regenerated plants of Avena. A high frequency of partial chromosome loss resulting in heteromorphic bivalents, trisomy, monosomy,

inversions, and interchanges in regenerated plants of Avena was reported by McCoy et al. (48).

Cytogenetic analyses of in vitro regenerated forage and range grasses are even fewer in number than the cereals. Ahloowalia (1, 2) reported mosaics, polyploidy, and wide variation in plant morphology in Lolium plants regenerated from triploid ($2n=21$) callus. Chen et al. (19) reported a low frequency, three out of 83, of tetraploids in regenerated plants of Andropogon ($2n=60$). Thus, cytogenetic analyses of in vitro regenerated agronomic species, especially the forages, is lagging behind other plant species.

Feulgen microspectrophotometry on embryo-derived calli of tall fescue showed predominantly cells with $2C$ nuclei (21). Cells with DNA contents of $4C-8C$ and higher were present in very low frequency but may indicate the possibility of aneuploidy. Kasperbauer et al. (36) reported that short-term cultures of F_1 embryos from diploid Lolium multiflorum X hexaploid Festuca arundinacea produced plants with expected normal chromosome number of $2n=4x=28$, but polyploid plants ($2n=8x=56$) were recovered from older cultures.

III. ETHYLMETHANE SULFONATE IN TISSUE CULTURES

Chemical mutagens have been widely used to create genetic and cytogenetic changes in whole plants and more recently have been used in tissue cultures (70). Nabors et al. (52) obtained Nicotiana plants with resistance to various levels of NaCl by

treating suspension cultures with a 1.5% solution of ethylmethane sulfonate (EMS). Reisch and Bingham (63) isolated Medicago plants resistant to ethionine by treating liquid suspension cultures with EMS and ethionine. The plants exhibited variation in growth, morphological characters, and chromosome numbers. Most plants retained the original diploid number; however, a tetraploid, a hexaploid, and several aneuploids were isolated. Morphological abnormalities were associated with chromosome numbers other than diploid (63).

The preceding summary shows that genetic and phenotypic variability occurs in most plant species cultured in vitro and that variability might also be induced by using mutagens. Most of the forage grasses have been successfully cultured (1, 2, 3, 20) but further analyses of the regenerated plants is rare. Cytogenetic analysis would identify novel genotypes for use in crop improvement programs and identification of aberrant genotypes could provide material for studying genetic and biochemical processes.

CHAPTER III

MATERIALS AND METHODS

I. SOURCE OF EXPERIMENTAL MATERIAL

Inflorescences, leaf bases and root tips of tall fescue (Festuca arundinacea Schreb. cv. Ky 31) plants were used as the experimental material for this research. The plants used were from three sources. One group of plants grown from seed served as controls. Another group of plants were regenerated from callus cultures obtained from mature embryos that were plated on Murashige and Skoog (MS) medium containing 9 mg/liter of 2,4-dichlorophenoxy acetic acid (2,4-D) (43). This group of plants will hereafter be referred to as REG plants. The third group of plants were regenerated from callus obtained from mature embryos which were previously treated with ethylmethane sulfonate (EMS). These embryos were presoaked in distilled water for 48 hours, treated with 0.02, 0.04, 0.06, and .12M concentrations of EMS for 8 hours at ambient temperature and washed overnight in running tap water. The treated embryos were plated on Schenk and Hildebrandt (SH) medium containing 4.4 mg/liter of dicamba as outlined by Lowe (42). These regenerated plants will be referred to as TEM plants (treated-embryo).

II. MEIOTIC ANALYSES OF MICROSPOROCYTES

Collection and Preparation of Material

Whole immature panicles were collected in May of 1982 and 1983 from field-grown tall fescue plants. The panicles were fixed in Carnoy's solution (6 parts 95% ethanol:3 parts chloroform:1 part glacial acetic acid) for at least 48 hours at 8°C. If long term storage was required (over 2 weeks), the material was transferred to 70% ethanol. With the aid of a stereo microscope, the anthers were dissected from each floret, placed on a glass microscope slide in a drop of 0.5% acetocarmine, cut in half cross-wise, and the contents pressed out with a dissecting needle. The debris was removed and a coverslip was added. Spreading and flattening of the cells was facilitated by tapping the coverslip with the pointed end of the dissecting needle followed by firm downward pressure with the thumb. The slides were made semi-permanent by ringing the coverslip with Hoyer's medium.

Determination of Chromosome Number

Counts were made of well-spread chromosomes in pollen mother cells (PMC's) in late diakinesis and metaphase I. The types and numbers of chromosome configurations present in each treatment group were noted. More than 20 plants per treatment group were analyzed. The number of cells counted per plant ranged from 10-71 with 17 being the median, 19 the mean, and 16 the mode.

Chiasmata Frequency

Bivalents from at least 10 cells per plant were scored as having either one or two chiasmata. The mean number of chiasmata per cell was determined by adding the two categories and the mean number of chiasmata per bivalent was obtained by dividing the total chiasmata per cell by the number of bivalents. The mean number of chiasmata per bivalent was computed for each group of plants analyzed. The treatments were compared by a two-tailed t test to determine if any differences were significant.

Aberration Frequency

Abnormalities in pollen mother cells (PMC's) in all stages of meiosis were observed. Lagging chromosomes, "sticky" chromosome bridges, and inversion bridges with fragments were scored during anaphase II and telophase II only because of the greater ease in obtaining spreads with cells in these stages. Micronuclei were counted in uninucleate microspores after tetrad formation was completed. The frequency of aberrations per plant was computed as the percentage of cells with abnormalities. At least 300 PMC's in anaphase II and telophase II and 500 uninucleate microspores were observed per plant. The mean number of plants with aberrations was computed for each of the three different treatments.

III. MITOTIC ANALYSES OF SOMATIC TISSUE

Leaf Bases

Actively growing young tillers were collected from field- or greenhouse-grown tall fescue plants in the fall of 1981 and 1982 and in the spring of 1982. The outermost leaves were removed with the aid of a stereo microscope leaving the youngest leaf and the shoot apical meristem intact. The upper and extreme lower portions of the leaves were discarded leaving a section of tissue approximately 1-1.5 cm in length. The sections were pretreated for 2 to 3 hours in a .05% solution of saponin saturated with monobromonaphthalene and then fixed in Farmer's solution (3 parts 95% ethanol:1 part glacial acetic acid) for at least 3 hours, but preferably overnight (7).

The tissue was macerated by treating with a 0.5% pectinase (Sigma Chemical Co., St. Louis, MO), 0.2% cellulase (Yakult Pharmaceutical Ind., Japan) solution for 1 to 2 hours depending on size, age, and tenderness of tissue. This step was simplified by using size 00 embedding capsules (Polysciences Inc., PA) to hold the tissue and enzyme solution. The tissue was removed from the enzyme solution, tapped onto a glass microscope slide and mixed in one drop of modified carbol fuchsin. A coverslip was added and tapped with the blunt or pointed end of a dissecting needle. The slide was then placed between blotter paper and the cells further spread and flattened by the application of direct,

downward pressure with the thumb. The coverslip was sealed with melted Paraplast (Sherwood Medical Industries, St. Louis, MO). Chromosome counts were made on at least five cells per plant.

Root Tips

Approximately two weeks prior to the collection of root tips, whole plants or several tillers were root-pruned and re-potted. Very young, tender root sections approximately 2 cm in length were removed and immediately placed in 0.1% colchicine for 3 to 4 hours at ambient temperature after which they were rinsed and placed in cold (4-5°C) glacial acetic acid for 20 minutes. The root tips were hydrolyzed with 1N HCl at 60°C for 15 minutes and then stained with Schiff's Reagent. Staining was hastened and intensified by heating the reagent to 60°C immediately before the roots were added. The squash was made in 45% acetic acid and Paraplast was used to make the slide semi-permanent. At least five cells per plant were counted.

IV. POLLEN STAINABILITY

Pollen stainability gives an estimate, not an exact measurement, of fertility (28). A preliminary study was conducted on 20 randomly selected plants from each treatment group whereby at least 200 pollen grains per plant were counted and the mean pollen stainability calculated. Subsequent effort focused on aneuploid plants or plants in which meiotic irregularities were observed.

Panicles with mature anthers collected in mid to late spring were either analyzed immediately or stored in water under refrigeration (2°C) for later use. The pollen to be analyzed immediately was shaken onto a microscope slide in one or two drops of 0.5% acetocarmine or Newton's Iodine Solution (1 gm I₂ and 1 gm KI in 100 ml of 80% ethanol). A coverslip was added and the slide placed between blotter paper whereby gentle pressure was applied to remove air bubbles and excess stain.

Panicles that were stored for later use were removed from refrigeration and kept at ambient temperature until the anthers began to dehisce. Alternatively, the anthers were removed, placed on a slide, and the pollen grains very carefully teased out. The grains were stained as before and the absence or presence of starch was indicated by translucent or darkened grains. The mean percent pollen stainability of the three treatments were compared in pairs by the two-tailed t test to establish the significance of differences observed.

V. PHENOTYPIC ANALYSES

The time of flowering in the REG and TEM plants was rated on a scale of 1-5 with one indicating that inflorescences had barely begun to emerge from the flag leaf and five indicating inflorescences with dehiscing anthers.

Leaf width, color, vigor, and growth habit of REG and TEM plants, especially aberrant plants, were noted in early to late spring.

CHAPTER IV

RESULTS

I. MEIOTIC ANALYSES

The normal progression of meiosis in tall fescue from prophase through a pollen grain undergoing mitosis is illustrated in Figure 1. Chromosomes during prophase are shown in Figure 1A. Twenty-one bivalents regularly form at metaphase I and migrate simultaneously to the poles in anaphase I (Figures 1B and 1C, respectively). A cell wall formed in the dyad after completion of telophase I is illustrated in Figure 1D. Twenty-one chromosomes congregate at the equatorial plate in metaphase II, (Figure 1E), and the chromatids disjoin 21:21 at anaphase II (Figure 1F). Cytokinesis takes place again after telophase II (Figure 1G). Intact tetrads can be seen if the preparation is handled carefully. The uninucleate stage of microspore development follows with the formation of a pollen grain with one pore (Figure 1H). Formation of a single pore is the usual situation for monocotyledonous plants.

The number of plants studied, number of aneuploids, percent aberrant plants, mean pollen stainability, and mean chiasmata per bivalent for each treatment in this investigation are presented in Table 1. All control plants studied had the normal chromosome number of $2n=42$. No meiotic irregularities were noted and mean pollen stainability was approximately 98%.

FIGURE 1. Normal progression of meiosis in tall fescue.

- A. Prophase (1400 x)
- B. Metaphase I (720 x)
- C. Anaphase I (660 x)
- D. Telophase I (1000 x)
- E. Metaphase II (1300 x)
- F. Anaphase II (820 x)
- G. Telophase II (1500 x)
- H. Pollen grain undergoing mitosis (1300 x)

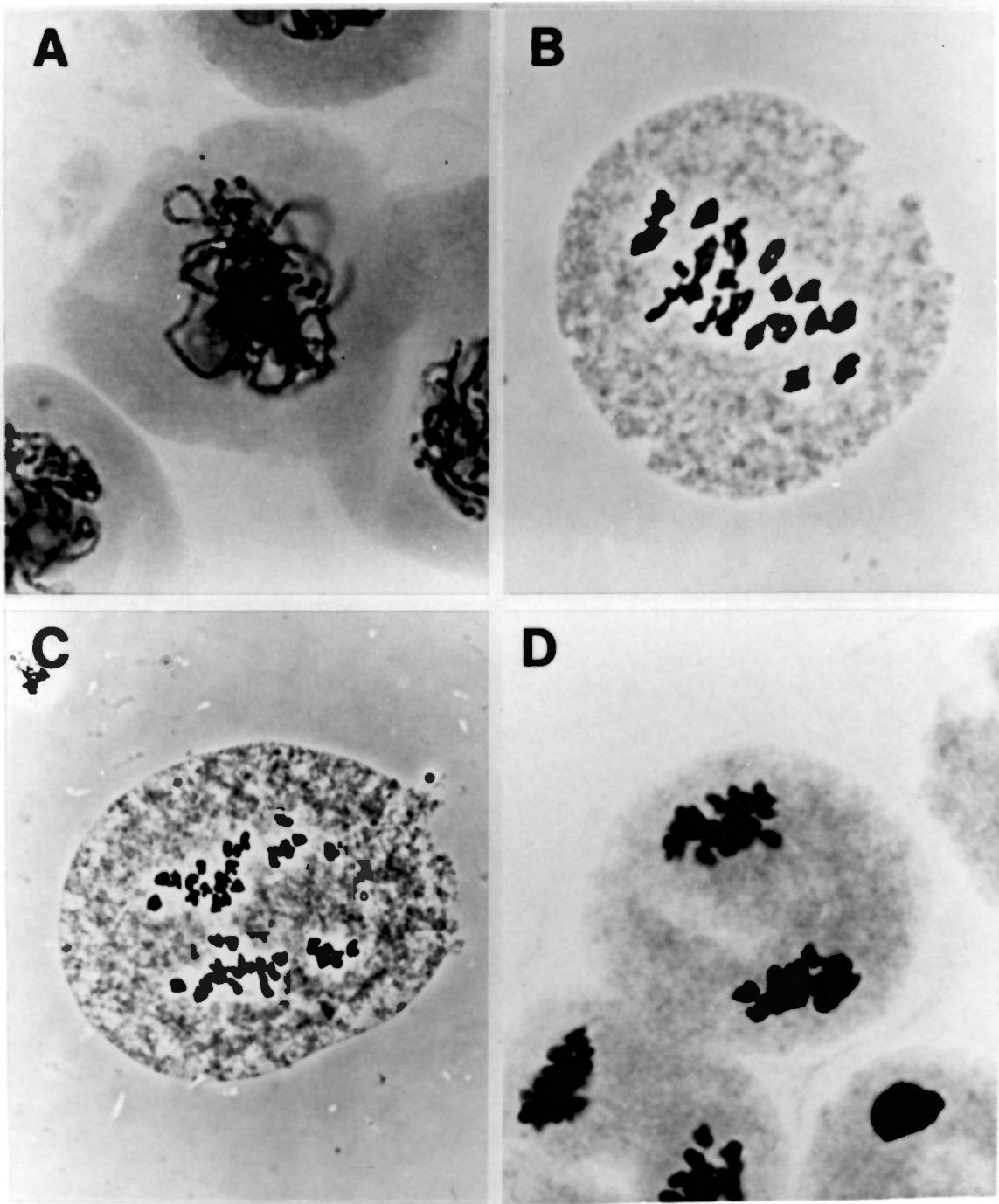


FIGURE 1

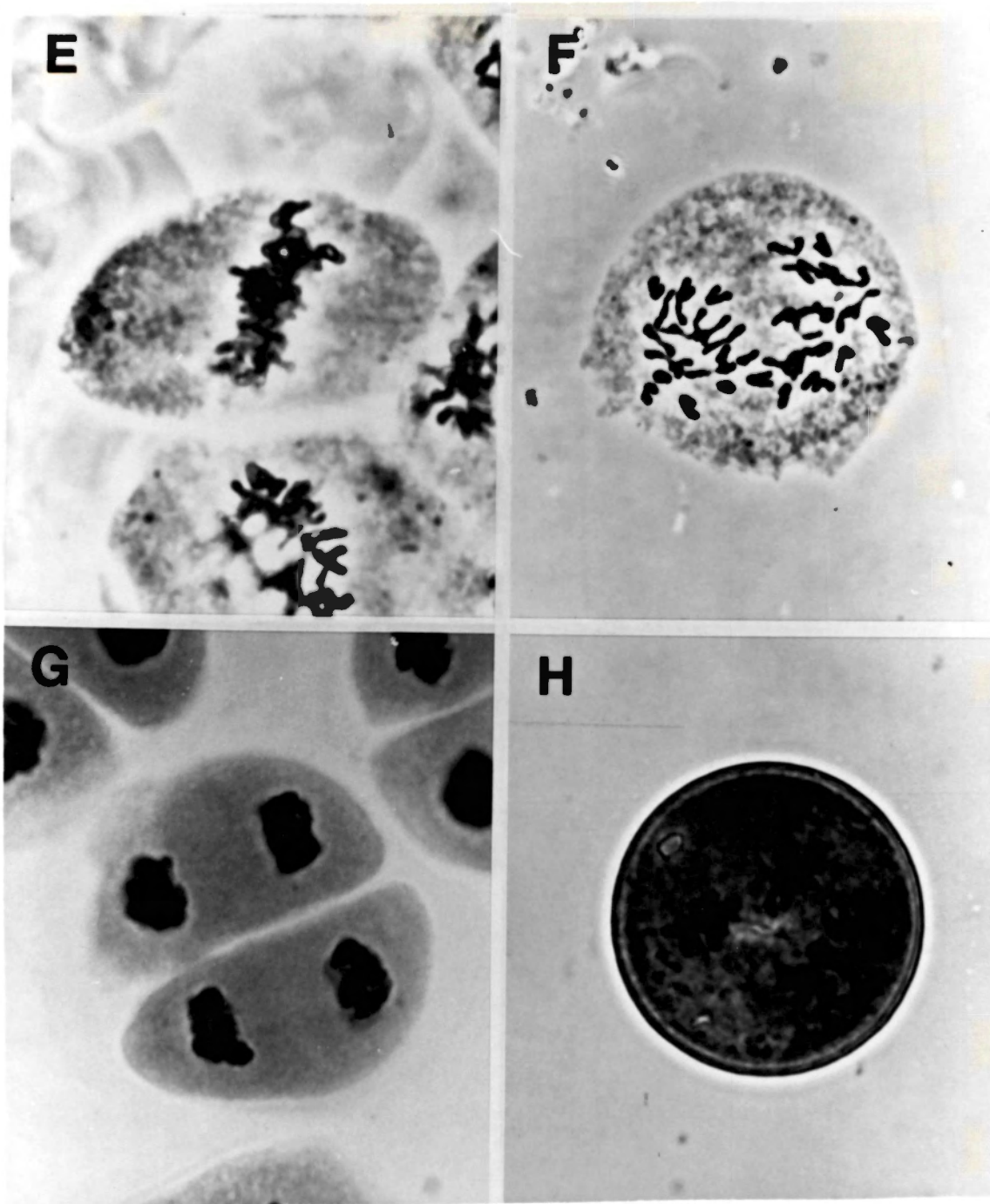


FIGURE 1 (Continued)

TABLE 1. Number of Plants Studied, Number of Aneuploids, Percent Aberrant Plants, Mean Pollen Stainability, and Mean Chiasmata Per Bivalent for Control, REG and TEM Plants

Treatment Group	Number Plants Studied	Percent Aberrant Plants*	Number of Aneuploids	Mean Chiasmata Per Bivalent	Mean Percent Stainability of Pollen
Control	21	0.0	0.0	1.47	98.0
REG	31	41.9	2 (2n+1)	1.51	96.1
TEM	21	47.6	1 (2n-1)	1.56	95.0

*Plants with laggards, micronuclei, or bridges.

Approximately 42% of the REG plants exhibited meiotic abnormalities such as lagging chromosomes, "sticky" bridges, bridges with associated acentric fragments and micronuclei in spores. Nearly 48% of the TEM plants showed abnormalities. The range of pollen stainability in the control plants was 94.5%-100% while the REG and TEM plants had ranges of 80.1%-99.2% and 81.2%-99.7%, respectively. At the 0.10 level of significance there were differences in the pollen stainabilities of the control versus REG plants and control versus TEM plants. However, there was no significant difference between the stainabilities of the REG and TEM plants when tested at the same level.

The mean number of chiasmata per bivalent, an estimate of crossing-over, was slightly higher in the TEM plants than in the REG or control plants. The mean chiasmata per bivalent ranged from 1.32-1.55 in the control plants and the range was from 1.36-1.64 in the REG plants. The highest mean in the REG plants (1.64) was that of a trisomic ($2n+1=43$). The range of mean chiasmata per bivalent in the TEM plants was 1.46-1.68 and the lowest mean (1.46) was associated with a monosomic ($2n-1=41$). There was no significant difference in the mean chiasmata per bivalent between the control and REG plants or between the REG and TEM plants when tested at the 0.10 level of significance. There was a significant difference in mean chiasmata per bivalent between the control and the TEM plants when tested at the same level.

Acentric fragments were observed in 9.8% of all the regenerated plants studied. A higher percentage (12.9%) of plants with acentric fragments occurred in the REG plants compared to the TEM plants (4.7%). Figure 2A shows a chromosome squash of a PMC with the normal chromosome number of 42 (21 bivalents). A trisomic, monosomic, and acentric fragment are illustrated in Figures 2B, 2C, and 2D, respectively. Figure 3 represents the meiotic behavior of the univalent. The univalent can be seen in diakinesis as a separate, distinct piece of chromatin (Figure 3A), and in metaphase II and anaphase II it usually lies to the side of the plate (Figures 3B and 3C). Figure 3D shows a highly condensed univalent in an equatorial view of anaphase II. Univalents often show retarded movement, referred to as lagging, in anaphase or telophase (Figure 3E) and may end up as micronuclei in the microspores (Figure 3F).

Types of meiotic aberrations observed in tall fescue plants regenerated from tissue cultures are shown in Figure 4. A PMC with two nucleoli persisting in diakinesis is shown in Figure 4A. Lagging chromosomes ("laggards") and micronuclei in spores were the most common abnormalities. "Laggards" were observed in both the first and second division. The number of "laggards" in cells varied between one and several (Figure 4B). In some cases, the lagging chromosome(s) were highly condensed while in others they were less condensed. Micronuclei in tetrads and spores occurred in varying numbers and were randomly distributed between the spores in any given tetrad (Figure 3F). The sizes of the micronuclei were usually consistent.

FIGURE 2. Metaphase I chromosomes in tall fescue pollen mother cells.

- A. Normal spread of 21_{II} ($2n=42$) (1000 x)
- B. A monosomic with 20_{II} and 1_I ($2n=41$), arrow indicates univalent (1300 x)
- C. A trisomic with 21_{II} & 1_I ($2n=43$), arrow indicates univalent (1900 x)
- D. 21_{II} and an acentric fragment ($2n-42$), arrow indicates acentric fragment (830 x)

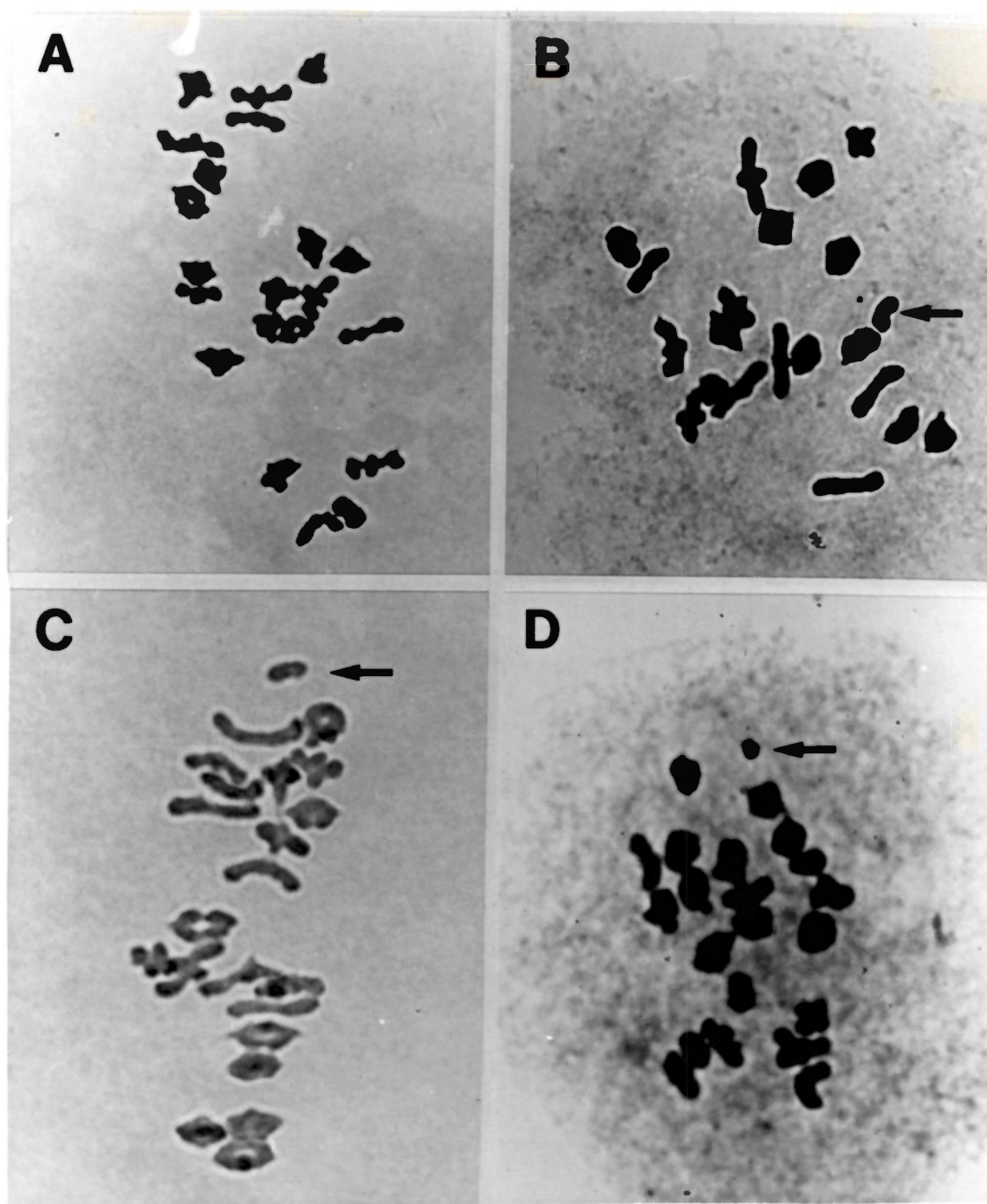


FIGURE 2

FIGURE 3. Behavior of the univalent during meiosis.

- A. Univalent in diakinesis (arrow) (960 x)
- B. Univalent in anaphase II-polar view (arrow) (720 x)
- C. Univalent in metaphase II (arrow) (1100)
- D. Univalent in anaphase II-equatorial view (arrow) (800 x)
- E. Laggard in telophase II (arrow) (1200 x)
- F. Micronuclei in a tetrad (arrow) (880 x)

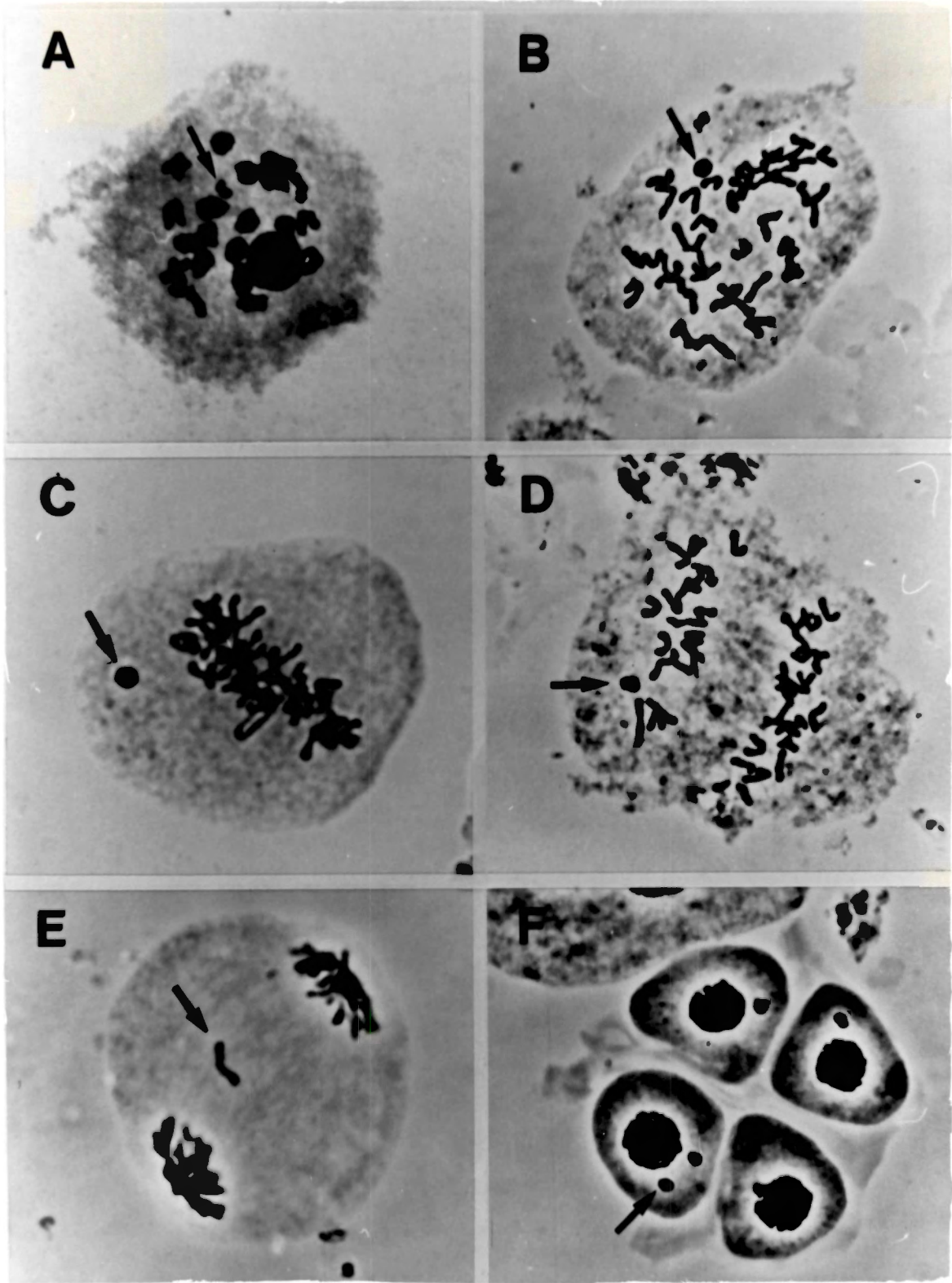


FIGURE 3

FIGURE 4. Meiotic aberrations observed in tall fescue plants regenerated from tissue cultures.

- A. Two nucleoli present during diakinesis in the monosomic 23II² 194 (1200 x)
- B. Lagging chromosomes in anaphase II (1100 x)
- C. "Sticky" chromosome association after telophase I
Note cytokinesis (1100 x)
- D. "Sticky" chromosome association after telophase I
Note cytokinesis (1100 x)
- E. Bridge and fragment in anaphase I (arrow denotes fragment) (1200 x)
- F. Multiple bridges during telophase I (1000 x)
- G. Bridge in telophase (1100 x)
- H. Bridge and fragment in telophase II (arrow denotes fragment) (1100 x)
- I. Bridge in teleophase I (1100 x)
- J. Bridges in telophase II (1100 x)
- K. Bridge and micronuclei in tetrad (1300 x)
- L. Bridge and laggard in teleophase II (1200 x)

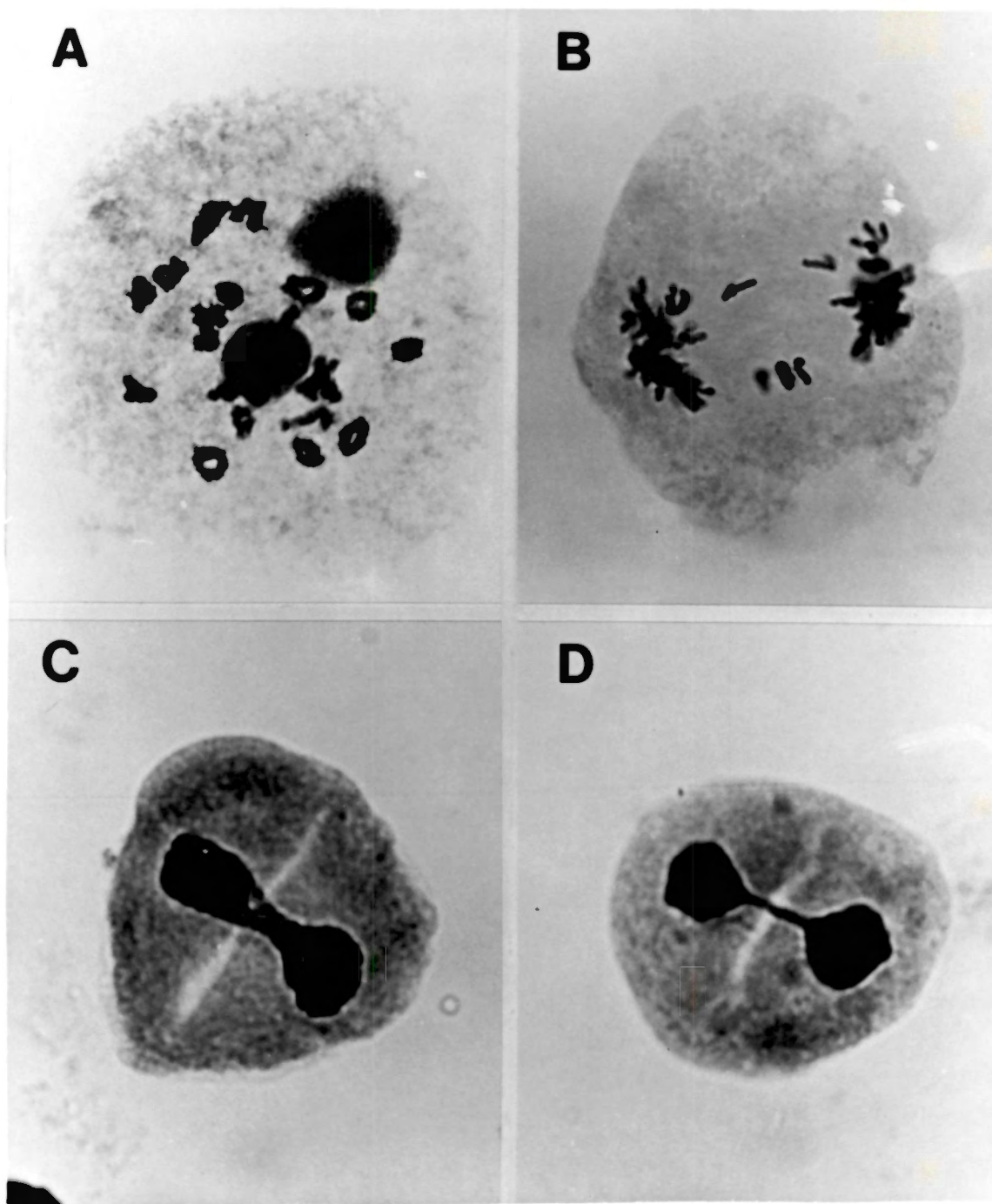


FIGURE 4

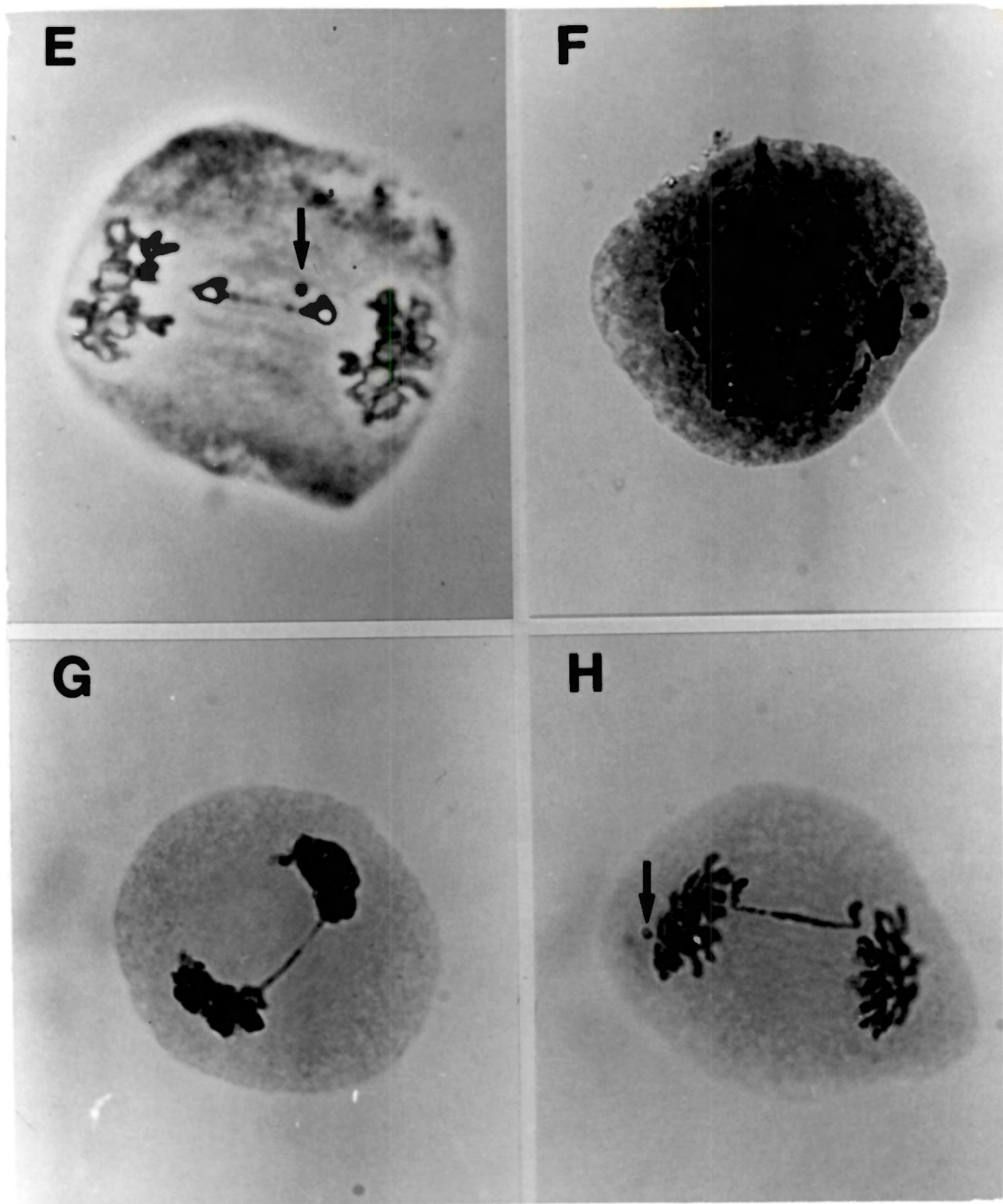


FIGURE 4 (Continued)

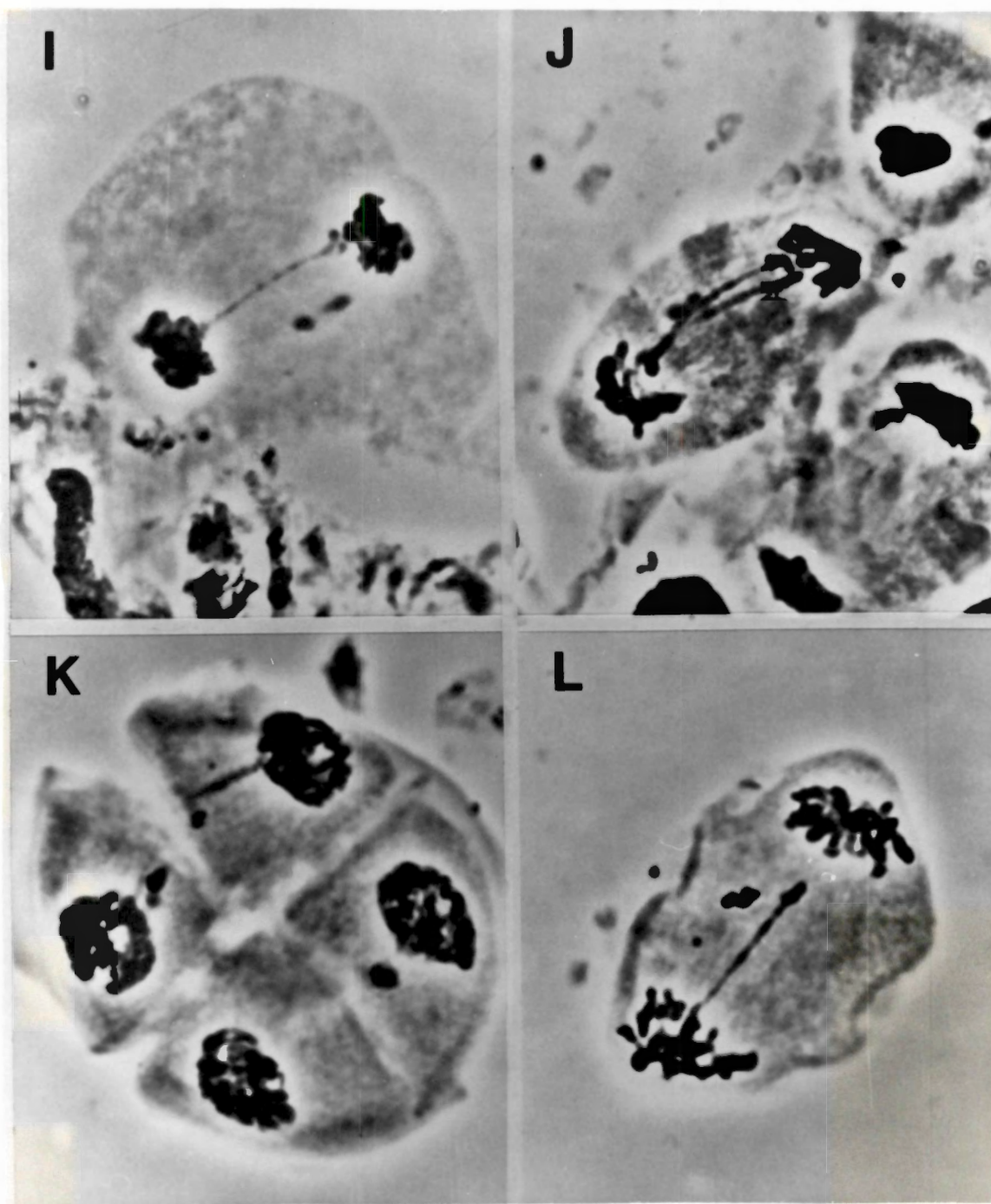


FIGURE 4 (Continued)

Chromosomal bridges were present in both anaphase and telophase of both the first and second divisions. In some cases, bivalents and chromatids showed difficulty in separating during anaphase I and II. These associations are referred to as "sticky" bridges (22). Some "sticky" bridges involved large amounts of chromatin resembling "dumbbells" (Figure 4C and 4D). Other "sticky" bridges took the form of fine, thread-like connections between the early formations of nuclei (Figures 4F, 4G, 4I, 4J, 4K, 4L). Distinct, acentric fragments were seen in conjunction with some bridges (Figures 4E, 4H). These bridges and their associated fragments occurred in low frequency and were scored as inversion bridges.

A more detailed analysis of 9 out of 13 aberrant genotypes in the REG plants is shown in Table 2. In general, the level of difficulty in obtaining chromosome counts and aberration frequencies increased with increasing meiotic irregularities, therefore, not all aberrant genotypes were completely analyzed.

One trisomic exhibited many aberrations while the other had few meiotic abnormalities. Regenerated (REG) plants with acentric fragments tended to have high percentages of meiotic aberrations, in some cases, higher than that observed in the trisomics. High percentages of "sticky" bridges between chromosomes were common in plants with acentric fragments.

Pollen stainability was high (above 95%) for all REG plants studied except for RT 65 and RT 74. The other two REG

TABLE 2. Aberrant Genotypes in Plants Regenerated from Callus Derived from Nontreated Embryos (REG)

Plant Number	Chromosome Configuration	Mean Chiasmata per Bivalent	Percent Pollen Stainability of Cells	Aberration Frequency (Percent of PMC's with)			
				Inversion Bridges	Micronuclei in Spores	Laggards	"Sticky" Bridges
RT 21	21 _{II} & 1 _I [†]	1.54	96.2	0.0	32.2	48.6	2.5
RT 51	21 _{II} & 1 _I [†]	1.64	96.2	0.0	6.5	19.5	0.8
RT 84	21 _{II} ^{††}	1.59	96.2	0.0	55.2	61.1	0.3
RT 75	21 _{II} ^{††}	----	95.7	0.0	10.2	19.8	6.3
RT 65	21 _{II} ^{††}	1.42	80.1	0.0	13.6	31.5	7.3
RT 74	21 _{II} ^{††}	----	87.8	0.0	25.5	44.8	1.1
RT 14	21 _{II}	----	98.0	0.0	8.8	26.2	2.2
RT 23	21 _{II}	1.41	95.7	0.0	2.1	8.0	0.0
RT 42	21 _{II}	----	98.8	0.0	1.5	6.1	0.5

[†]Trisomic (2n+1=43).

^{††}Acentric fragment present.

plants with acentric fragments showed high pollen stainability even though one plant, RT 84, exhibited the highest percentages of aberrations of all the REG plants analyzed.

Analyses of 8 of the 10 aberrant genotypes in the TEM group are shown in Table 3. The monosomic exhibited a high percentage of aberrations but pollen stainability remained high (above 97%). In contrast to the findings in the REG plants with acentric fragments, the only TEM plant with an acentric fragment, 23II² 171, exhibited a very low percentage of meiotic abnormalities. However, the pollen stainability was reduced (86.4%).

II. POLLEN STAINABILITY

Pollen stainability in the TEM plants was above 93% with the exception of 23II² 171 (acentric fragment) and 23I¹ 283. Low frequencies of aberrations were found in 23I¹ 283 but the pollen stainability was 89.2%. One TEM plant, 23II² 352, had high frequencies of aberrations yet the chromosome number was normal and the pollen stainability was higher than that of the other TEM plants. Another TEM plant, 23I¹ 349, had no aberrations, but the percent pollen stainability was low (82%). This indicates the possibility that factors other than chromosomal or cytogenetic aberrations played a role in the fertility of these plants.

TABLE 3. Aberrant Genotypes in Plants Regenerated from Callus Derived From Treated Embryos (TEM)

Plant Number	Chromosome Configuration	Mean Chiasmata per Bivalent	Percent Pollen Stainability of Cells	Aberration Frequency (Percent of PMC's with)			
				Inversion Bridges	Micronuclei in Spores	Laggards	"Sticky" Bridges
23II ² 194	20II & 1I [†]	1.46	97.5	0.6	24.3	33.8	0.6
23II ² 171	21II ^{††}	1.52	86.4	0.0	4.1	0.6	0.0
23I ¹ 371	21II	1.50	93.8	1.2	14.4	10.2	5.7
23II ¹ 322	21II	1.57	95.1	0.0	0.4	0.0	0.2
23I ¹ 283	21II	1.51	89.2	0.0	7.1	11.7	0.0
23I ¹ 416	21II	----	94.4	0.3	6.4	10.2	0.8
23I ¹ 301	21II	----	98.5	0.0	2.8	5.9	1.6
23II ² 352	21II	1.55	96.9	0.0	23.3	47.7	0.6

[†]Monosomic (2n-1=41).

^{††}Acentric fragment present.

III. MITOTIC ANALYSES

Shoot meristems from 53 TEM and REG plants were studied, although chromosome numbers were confirmed on only 10 plants. All 10 plants, 8 REG and 2 TEM, had the normal $2n=42$ chromosome number. Meiotic analyses showed acentric fragments in two of the plants analyzed mitotically, but no evidence of fragmentation was seen in the mitotic figures. Extreme stickiness in one REG plant, RT 7, prevented an accurate chromosome count in meiosis, but a somatic count of 42 chromosomes was obtained.

IV. PHENOTYPIC ANALYSES

In general, the REG plants matured later than the TEM plants. At the last observation, May 25, 1983, 11.4% of the REG plants were at level 3 of maturity compared to 7.0% of the TEM plants. On the first date of observation, April 25, 1983, 6.6% of the TEM plants were at maturity level 1, 2, or 3. In contrast, none of the REG had reached level 1. The monosomic, 23II² 194, was the earliest maturing of all plants analyzed. Plants with acentric fragments and/or many aberrations were slower to begin anthesis.

Leaf blade characteristics of the REG and TEM plants varied in color from yellow-green to deep-green to blue-green. Some leaves were very narrow and others were very wide. Growth habits varied from very weak to very vigorous with tall, upright

and flat, spreading or compact genotypes observed. The monosomic, 23II² 194, had a vigorous, upright growth habit and many panicles. The leaves were yellow-green and medium in width. This plant was similar in appearance to the plant with the acentric fragment. One trisomic, RT 21, displayed reduced vigor and very few panicles. This low, spreading plant had wide yellow-green leaves. The other trisomic, RT 51, was very vigorous, tall and upright with wide yellow-green leaves. Two of the REG plants with acentric fragments, RT 65 and RT 74, had very fine leaf texture i.e., the leaves were very narrow. One of these plants, RT 65, had a weak compact growth habit and medium green leaves, in contrast to RT 74, in which a vigorous, spreading growth habit and yellow-green leaves were observed. Another REG plant with an acentric fragment, RT 75, was very weak with yellow-green, medium width leaves. Blue-green, medium width leaves and a vigorous, upright growth habit was observed in RT 84, another REG plant with an acentric fragment.

CHAPTER V

DISCUSSION

Meiotic analyses of tall fescue plants regenerated from tissue cultures revealed a low frequency of monosomy and trisomy, and a higher frequency of other meiotic abnormalities. The primary monosomic identified by my research regularly formed 20 bivalents and one univalent. Jauhar (33, 34) isolated several monosomic plants that were grown from seed. Chromosome pairing was usually restricted to 20 bivalents and one univalent, but one monosomic formed many multivalents. Jauhar (34) presumed that pairing was disturbed due to the lack of a critical chromosome. It is assumed that the missing chromosome in the monosomic identified by my research did not carry the genes for control of diploid-like pairing.

The trisomics identified by this research are assumed to be primary trisomics. No multivalents were observed and the chromosome configurations were usually 21 bivalents and one univalent. The univalents were predominantly rod-shaped, thereby eliminating the possibility of a secondary trisomic (62).

Nearly 10% of the regenerated plants analyzed had an acentric fragment in addition to the regular chromosome complement during metaphase I. These could arise from chromosomal breakages during prophase; however, no obvious heteromorphic bivalents were seen. Small supernumerary chromosomes with a median centromere

have been reported in tall fescue (22) but no centromere could be distinguished in fragments of the present study.

The behavior of the monosomic univalent was similar to that observed for most univalents (37). The high frequency of lagging chromosomes and micronuclei in the monosomic can be accounted for by the presence of the unpaired univalent. The appearance of two lagging chromosomes, frequently observed in anaphase II, can be explained if the univalent divided at anaphase II but not anaphase I. If not incorporated into the daughter nuclei, two micronuclei would be seen in one spore if the resultant chromatids migrated together to the same pole in telophase II. Likewise, one micronucleus would be seen in each of two spores if the chromatids migrated separately to different poles.

The behavior of the trisomic univalents was similar to that in the monosomic. However, while it appeared that in the monosomic, the univalent was not incorporated into the telophase II nuclei, the reverse was true for one of the trisomics, RT 51. In this case, the percent of micronuclei in spores was much lower than the percent of "laggards." This can be explained if it is assumed that some of the "laggards" became part of the telophase II nuclei. The other trisomic, RT 21, exhibited high percentages of "laggards" and micronuclei which is not unusual given the behavior of the univalent. Observations of number of "laggards" in anaphase II and micronuclei per spore indicate that the univalent divided once, either in anaphase I or anaphase II, or it divided twice, once in anaphase I and again in anaphase II.

The trisomic, RT 51, identified in this study possessed the highest chiasmata frequency of all the plants studied. This is consistent with the findings of Jauhar (35) who noted a significantly higher chiasmata frequency in trisomics of tall fescue in comparison to the sister disomics. There is evidence that chiasmata frequency in tall fescue is under polygenic control (34). Jauhar (34) surmised that genes controlling chiasmata in triplicate dose may have been responsible for the differences observed. However, no such conclusions can be drawn from my research because of the low number of observations made.

Khush (37) described several sources of aneuploids. It is likely that the present aneuploids were induced either by the tissue culture cycle or by EMS. Analyses of control plants showed only chromosome complements of $2n=42$. Other sources of variation in chromosome number include endoreduplication and nuclear fragmentation while in the callus state (16). The small numerical changes in chromosome number in the present plants rule out endoreduplication since this phenomenon is usually associated with much larger changes in chromosome number. Nuclear fragmentation (amitosis) during mitotic cell divisions in callus is a possible source of chromosome number change in the present plants (10).

Plants with chromosomal bridges were classified as aberrant types in this study. Crowder (22) observed several types of

"sticky" associations in many seed-grown tall fescue plants and used the phrase "sticky" bridges. The descriptions and photographs by Crowder (22) of the different types of "sticky" bridges matched very closely the anomalies observed in this study. One kind of association involved chromatin that was very thinly stretched and was assumed to result from a physiological change in the surface properties of the chromosome.

Inversion bridges and their associated acentric fragments were observed in low frequencies in tall fescue plants regenerated from tissue cultures. When one chromosome of a homologous pair has a reversed segment (an inversion), and the homologue is structurally normal, an inversion heterozygote is formed (62). Pairing difficulties are encountered in prophase when homologous chromosomes synapse and one of the chromosomes, either the normal or the inverted one, forms a reverse loop. Crossing over within the loop causes a dicentric chromosome and an acentric fragment to form. During anaphase I separation, the two centromeres of the dicentric chromosome are pulled to opposite poles and the acentric fragment to neither pole. The end result are chromosomes which are deficient for, or have duplications of some loci (64). The pairing and separation behavior just described occurs in the case of a heterozygous, paracentric inversion (62). Observations of dicentric chromosomes and bridges and fragments in PMC's of tall fescue lead me to believe that some of the regenerated plants studied are heterozygous for a paracentric inversion. The

occurrence of high percentages of micronuclei in plants with normal chromosome numbers can also be accounted for if there is heterozygosity for an inversion (48).

In conclusion, in vitro culture of tall fescue induces meiotic irregularities and changes in chromosome number. The chromosomal variability obtained in vitro is not increased significantly by the previous treatment of embryos with EMS. Cytogenetic analyses of regenerated plants is recommended to identify novel genotypes that may be further used in genetic or breeding studies.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The primary objective of this study was to determine the cytogenetic variability present in tall fescue plants grown from seed and regenerated from tissue cultures. The secondary objective was to compare the cytogenetic variability in regenerated plants derived from EMS-treated embryos with the variability in plants regenerated from nontreated embryos. Cytological investigation included determination of chromosome number, chiasma frequency, pollen stainability, and identification and calculation of the frequency of meiotic aberrations.

Cytogenetic analyses of pollen mother cells from inflorescences of plants regenerated from tissue cultures revealed aneuploid chromosome numbers and meiotic irregularities. In some cases, reduction in pollen stainability was associated with meiotic abnormalities. Chiasma frequency in one of the trisomics was higher than for any other plant analyzed. Morphological differences were present in the aneuploids.

All control plants grown from seed had the normal chromosome number of $2n=42$ and no meiotic aberrations were observed. There was greater phenotypic variation among plants regenerated from tissue cultures than among control plants grown from seed. Nearly 48% of the regenerated plants derived from EMS-treated embryos exhibited aberrations while nearly 42% of plants derived

from nontreated embryos showed irregularities. There were no significant differences in chiasmata formation and pollen stainability between the REG and TEM groups.

Tissue culture of tall fescue has been shown to induce chromosomal changes. Cytogenetic analyses should be performed on all regenerated plants to identify aneuploids that may be used in the establishment of a monosomic or trisomic series. Further studies of the present aneuploids should be conducted to determine heritability of chromosome number and aberrations and to aid in the understanding of regulatory mechanisms that control chromosome pairing.

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APPENDIX

TABLE 4. Additional Analyzed Tall Fescue Plants Regenerated From Tissue Cultures with Normal Chromosome Number of $2n=42$

REG	EMS
RT 25	23II ² 197
RT 22	23II ¹ 138
RT 83	23I ¹ 349
RT 30	23II ² 300
RT 76	23I ¹ 371
RT 15	23I ² 285
RT 36	23II ¹ 322
RT 41	23I ¹ 283
RT 8	23II ¹ 262
RT 20	23I ² 267
RT 17	23I ¹ 158
RT 12	23I ² 311
RT 23	23I ¹ 416
RT 13	23I ¹ 301
RT 27	23II ² 119
RT 52	23II ¹ 143
RT 42	23II ² 352
RT 14	
RT 75	

VITA

Janet Norwood Reed was born on July 14, 1954, in Paris, Tennessee. She is the daughter of Mr. and Mrs. W. E. Norwood of Knoxville, Tennessee. She was graduated from Farragut High School in Knoxville, Tennessee in 1972. She served in the United States Army from 1976 to 1979 and attended Cameron University from 1977 to 1979 while stationed in Lawton, Oklahoma. She attended The University of Tennessee, Knoxville from 1979 to 1981 where she received her Bachelor of Science degree in Agriculture in June of 1981. She began the Master of Science program in Plant and Soil Science in June of 1981 and plans to receive said degree in December 1983. She is married to Robert Edward Reed, Jr. of Knoxville, Tennessee.