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Analyses of Somaclonal Variation in Hexaploid Wheat (*Triticum aestivum* L.)

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ANALYSES OF SOMACLONAL VARIATION IN HEXAPLOID WHEAT (*Triticum aestivum* L.)

by

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بِسْمِ اللّٰهِ الرَّحْمٰنِ الرَّحِيْمِ

* مَثَلُ الَّذِيْنَ يُنْفِقُوْنَ اَمْوَالَهُمْ
فِيْ سَبِيْلِ اللّٰهِ كَمَثَلِ حَبَّةٍ اَنْبَتَتْ سَبْعَ
سَنَابِلَ فِيْ كُلِّ سُنْۢبُلَةٍ
مِّائَةُ حَبَّةٍ ۗ وَاللّٰهُ يُضٰعِفُ لِمَنْ يَّشَآءُ ۗ
وَاللّٰهُ وَاسِعٌ عَلِيْمٌ *

The parable of those
Who spend their substance
In the way of Allah is that
Of a grain or corn : it groweth
Seven ears, and each ear
Hath a hundred grains.
Allah giveth manifold increase
To whom He pleaseth :
And Allah careth for all
And He knoweth all things.

Dedicated

to

the Lovely Memory
of My Sister

HANAA

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Z. N. Hashim

Zahra Noori Hashim

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ABSTRACT

Analysis of Somaclonal Variation

in Hexaploid Wheat (Triticum aestivum L.)

by

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Department: Plant Science

Somaclonal variation, to provide germplasm for crop improvement, must be screened, selected and characterized. Immature wheat, Triticum aestivum L., (PCYT-10) embryos (10-12 days old) were cultured on Murashige and Skoog (MS) medium containing 0.5 mg L^{-1} 6-furfurylaminopurine (kinetin) and 2, 3, or 4 mg L^{-1} of 1-methoxy-3,6-dichlorobenzoic acid (2,4-D). Dicamba, at 2 and 3 mg L^{-1} and 0.2 mg L^{-1} 2,4-D, produced 12.7%, 30.3%, and 28.2% of the somaclones, respectively. No plantlets were produced from other treatments. Variants were characterized by cytology, biochemistry and morphology. Somaclones showed significant differences in length and width of flag leaf, plant height, number of tillers, spike length, awn length, and number of seeds per main head when compared to parental controls for two salfed recurrent generations. Number of spikelets per main head in the second generation showed no significant difference from controls. Stability

and segregation of somaclones for measured traits indicated that genetic changes had occurred which could enhance wheat germplasm.

Leaf isozymes of somaclones (SC_1 and SC_2) showed no variation in glutamine oxaloacetate transaminase (GOT) (E.C. 2.6.2.1.), leucine aminopeptidase (LAP) (E.C. 3.4.11.1), or esterase (EST) (E.C. 3.4.99) bands in 28% of the somaclones, 28% with light, and 44% missing a fast movind band. Approximately 30% of the normal group set no seeds. Mutants with the missing band were stable through the fourth-selfed generation, whereas, variants with the light EP band were still segregating. Plants with the missing EP band were morphologically normal compared to the parents except rachis internodes were longer than those of the parents.

There was no correlation between the missing EP band and a missing chromosome in some mutants. The mutant may have been due to a point mutation, deletion, or activation of a repressor gene. Variants exhibited a wide range of protein density and missing or extra bands.

Relative amounts of DNA per telophase nucleus were affected by inorganic salts and sucrose levels. Ploidy level increased with time within single-strength MS, but not within double MS medium. Calli grown on the modified double MS medium exhibited a higher number of shoots than those grown on modified MS.

Individual variants with desirable characteristics with high seed production, high protein levels, supernumerary spikelets, and larger flag leaves could be incorporated into a wheat improvement program.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

The ultimate aim of plant breeders is to increase production and to improve the quality of cultivars to meet agricultural requirements. This can be achieved by selecting from increased genetic variability. Conventional plant breeding methods have selected from a finite amount of genetic variability to develop current crop cultivars, but these methods are laborious and time consuming. During the last few years, substantial attention has been focused on the importance of plant tissue culture for crop improvement. Protocols for tissue culture and subsequent plant regeneration have now been established for many species such as alfalfa (Medicago sativa L.) (Saunders and Bingham, 1972), barley (Hordeum vulgare L.) (Saalbach and Koblitz, 1977), oat (Avena sativa L.) (McCoy et al., 1982), potato (Solanum tuberosum L.) (Shepard et al., 1980), sugarcane (Saccharum officinarum L.) (Larkin and Scowcroft, 1982), and wheat (Triticum aestivum L.) (Ahloowalia, 1982). Several reports (Hanzel et al., 1985; Maddock et al., 1983; Orton, 1979; Schaeffer et al., 1979) have shown that efficiency and response of plants to tissue culture depend on genotype as well as media components.

With wheat in particular, it has been possible to produce shoots and plantlets by using immature embryos as explants in certain media (Ahloowalia, 1982; Karp and Maddock, 1984; Maddock et al., 1983; Ozias-Akins and Vasil, 1982; Lapitan et al., 1984; Larkin et al., 1984). These

reports, however, have shown relatively high frequencies of genetic variability and morphological abnormalities in plants regenerated from wheat tissue cultures. The total somaclonal variation in these studies was underestimated, since most of the biochemical variations cannot be detected by morphological and/or cytological analyses.

Electrophoretic protein patterns are characteristic of the genotype and are independent of growth conditions (Lee and Ronalds, 1967). Thus, electrophoresis of seed proteins could provide another tool to measure genetic alterations induced by tissue culture.

Allozyme techniques have been useful in analyzing genetic variation in cultivated plant populations. A perusal of the literature indicates considerable information is now available about genetic correlation of wheat isozymes. Chromosomal locations of genes that encode multiple molecular forms of more than 20 wheat enzymes have been identified (Hart, 1983). Comparison of banding patterns from parental wheat plants and those produced from *in vitro* grown plants should provide clues to the type of variant induced in the tissue cultured wheat. For example, if certain protein bands are missing in variant plants, it might suggest that point mutations, deletions or certain types of chromosomal aberrations have occurred. If, however, banding patterns in the variants from *in vitro* grown wheat plants have shifted to different locations from what was observed in parental lines, then perhaps only point mutations have occurred. Examination of zymogram phenotypes of parental lines and tissue culture regenerated plants should confirm this hypothesis. Meiotic analysis conducted on regenerated plants that exhibit variant zymogram phenotypes should also reveal chromosomal aberrations among suspect chromosomes. The overall objectives of this

study were to identify and to characterize somaclonal variations in wheat plants, and to determine whether observed variants are genetically stable. More specifically the objectives were to:

1. Optimize the auxin level in in vitro culture medium for callus induction and regeneration of a specific wheat cultivar.

2. Identify and record types of mutations by comparing, in parental and first and second generation in vitro grown wheat plants, the following isozymic systems: Esterase (EST), glutamine oxaloacetate transminase (GOT), leucine aminopeptidase (LAP), and endopeptidase (EP).

3. Compare electrophoretograms of storage seed proteins, in parental and regenerated plants.

4. Detect chromosomal aberrations by mitotic analyses of regenerated plants that exhibit variant zymogram phenotypes.

Review of Literature

In recent years, considerable attention has been focused on the tremendous potential of tissue culture for genetic manipulation and plant improvement. Successful regeneration of mature, vigorous, and healthy plants is the first step required to apply this technique in crop production.

Currently, necessary tissue culture procedures are well established for many species, and plants have been regenerated with varying degrees of success. Regeneration of mature plants from legumes, M. sativa L. and Trifolium spp., have been achieved from cell suspension cultures, protoplasts and calli (Phillips and Collins, 1980; Reisch and Bingham, 1981; Saunders and Bingham, 1972). Vegetatively propagated crops such as potato have been regenerated from tissue culture. Shepard and his

colleagues have derived many thousands of protoclones from Russet Burbank leaf protoplasts (Shepard et al., 1980; Secor and Shepard, 1981). Sugarcane is vegetatively propagated and has been successfully regenerated from callus tissue (Larkin and Scowcroft, 1982, 1983; Liu, 1981). Several investigators have established the regeneration of whole plants normally reproduced by seeds such as maize, Zea mays L., (Green and Phillips, 1975; Torne et al., 1980; Vasil et al., 1984), barley (Dale and Deambrogio, 1979), oat (Cummings et al., 1976), rice, Oryza sativa L., (Nishi et al., 1968; Tamura, 1968), and sorghum, Sorghum bicolor (L) Moench (Masteller and Holden, 1970).

In wheat, a substantial number of reports have focused on regeneration of whole plants from tissue culture (Ahloowalia, 1982; Bennici and D'Amato, 1978; Larkin et al., 1984; Maddock et al., 1983). Wheat calli have been initiated from different explants such as mature seeds (Conger, 1981), immature embryos (Ozias-Akins and Vasil, 1983), coleoptilar nodes (McHughen, 1983), stem and rachis segments (Conger, 1981), inflorescences near meiosis or at earlier stages of development (Chin and Scott, 1977; Gosch-Wackerle et al., 1979), immature leaf bases (Ahuja et al., 1982), and mesocotyls (Yurkova et al., 1982). Immature wheat embryos, however, are the ideal sources for initiation of embryogenic callus cultures (Maddock et al., 1983; Larkin et al., 1984; Ozias-Akins and Vasil, 1982, 1983; Sears and Deckard, 1982). Five hundred and fifty one regenerants were derived from callus culture initiated from 30 immature embryos of the wheat cultivar "Millewa" (Davies et al., 1986).

For years, Murashige and Skoog's (MS) (1962) culture medium has been the most widely used nutrient medium for regenerating plants.

Other media that consist of diverse nutrient compositions are more or less modifications of the MS medium. Most of these media produced abnormal calli that resulted in a reduction of regenerants. However, doubling MS medium components supplemented with vitamins increased the number of regenerants from different wheat cultivars (Ozias-Akins and Vasil, 1983; Carman et al., 1987a,b). The double MS medium supplemented with vitamins was shown to produce normal wheat calli with normally dividing cells (Hashim et al., 1986).

The addition of hormones induces an increase in growth rates or organogenesis of the callus. In the late 30's, it was reported that an auxin, indoleacetic acid (IAA), enhanced the growth of callus cultures from popular, Populus deltoides Marsh., and carrot, Daucus carota L., (White, 1943). Naphthaleneacetic acid (NAA), another auxin, and N6-Benzyladenine (BA), a cytokinin, have also been used by many investigators (Daykin et al., 1976; Economou and Read, 1982; Rao et al., 1973; Staba, 1980). The most commonly used auxin is 2,4-dichlorophenoxyacetic acid (2,4-D). It increases the frequency of callus initiation (Bayliss and Dunn, 1979; Hanzel et al., 1985; Fedak, 1984; Maddock et al., 1983). However, 1-methoxy-3,6-dichlorobenzoic acid (dicamba) and 4-amino-3,5,6-trichloropicolinic acid (picloram) were superior for callus induction in annual ryegrass, Lolium multiflorum Lam., and tall fescue, Festuca arundinacea Schreb., over 2,4-D (Conger et al., 1982). Hanning and Conger (1982) reported that dicamba induced callus on orchardgrass, Dactylis glomerata L., leaf sections. Collins et al. (1978) reported that picloram was more effective than 2,4-D in wheat and other callus cultures. Papenfuss and Carman (1987) reported

that dicamba was superior to 2,4-D in inducing plant regeneration from wheat calli.

Somaclonal variation refers to the increased genetic variation detected in plants regenerated from cell and tissue culture (Larkin and Scowcroft, 1981). This variation seems to be the rule rather than the exception in many tissue culture regenerated plants species. Some of the variation may preexist in the explant as residual heterozygosity (Barbier and Dulieu, 1980). However, plants derived from protoplast cultures (Lorz and Scowcroft, 1983) and dihaploid cultures (De Paepe et al., 1981; Prat, 1983) showed extensive somaclonal variations that eliminated residual heterozygosity as a major source of variation.

The genetic changes that have been reported are mainly alterations in chromosome number and structure. These include deletions, duplications, inversions, translocations, aneuploidy and polyploidy. Other genetic changes included gene amplifications and depletions, point mutations, increased recombination frequencies, activation of transposable elements, cytoplasmic genome rearrangements, and changes in the relative amount of DNA. Plants with different doses of genes will possess different morphological, physiological and biochemical characteristics. Extensive reviews of the subject have been reported by Evans et al. (1984), Larkin and Scowcroft (1981, 1983), Maliga (1984), Orton (1983a), and Skirvin (1978).

Chromosome instability has been reported in many taxa, such as Daucus carota (Smith and Street, 1974), Nicotiana sp. (Nuti Ronchi et al., 1981) and Solanum sp. (van Harten et al., 1981). Polyploidy plants have been recovered from many regenerated plant species (Griesbach, 1987; Newell et al., 1984; Skirvin, 1978). In 1963, Steward found

haploid cells and giant cells with nuclei containing several times the normal chromosome number in carrot diploids grown in liquid culture. Mixoploids and mosaics, that transmit as genetic traits to the next generation, have been reported in high percentages in regenerated tobacco plants (Nuti Ronchi et al., 1981; Ogura, 1976). Aneuploidy also has been frequently reported in regenerated plants (Bayliss, 1980; Bennici et al., 1976; Reisch and Bingham, 1981). Monosomy and trisomy were reported in regenerated oat plants (McCoy et al., 1982).

Chromosomal abnormalities, other than numerical changes, have also been detected in regenerated plants. For example, rearrangements may be responsible for the genetic variations in cultured tissue. Jelaska et al. (1978) noted that chromosomes from cultured Vicia faba L. cells possessed altered Giemsa C-banding patterns. Scowcroft et al. (1985) showed a significant reduction in C-banding levels of the mitotic chromosomes of Triticale somaclones. Heteromorphic pairs (deletions or translocations) have been observed in oat (Cummings et al., 1976; McCoy et al., 1982) and maize somaclones (Green et al., 1977), while chromosome breakage and reunion, translocations, multicentrics were found in barley regenerants (Foroughi-Wehr et al., 1979; Orton, 1980). The meiotic chromosome behaviour of ryegrass somaclones suggested the presence of deletions, inversions and reciprocal translocations (Ahloowalia, 1978).

Other chromosomal irregularities, such as ring chromosomes, micronuclei, acentric and centric fragments have been observed in garlic, Allium sativum L., somaclones (Novak, 1980). Anaphase bridges were detected in tissue culture derived Haplopappus gracilis (Nutt.) Gray. plants (Singh et al., 1975). Such chromosomal abnormalities will

lead to loss or duplication of genetic material and the appearance of variant somaclones. Also gene function and expression will be affected.

In addition, morphological abnormalities have been well characterized in many plant species. Reisch and Bingham (1981) observed phenotypic changes in regenerated alfalfa plants. They found considerable increases in the frequency of variants when the culture tissues were treated with mutagenic compounds. Likewise, morphological variations were found among regenerated oat plants (Cummings et al., 1976). Cytological examinations of those plants showed aberrant chromosome configurations. Regenerated sugarcane plants have also expressed leaf and stalk morphological changes (Heinz et al., 1977; Larkin and Scowcroft, 1981; Liu and Chen, 1976). In maize somaclones, both endosperm and seedling heritable mutations have been scored. Plants regenerated from the same maize subculture showed different mutation rates with an overall average of 1.0 per somaclone for simply inherited mutations (Edallo et al., 1981). Numerous variations were reported among the progeny of barley somaclones (Deambrogio and Dale, 1980). Rice plants derived from homozygous donors showed dramatic variations (Oono, 1981). The regenerants were examined for many characters including chlorophyll mutations, panicle lengths, plant heights, heading dates, seed yields, and 1,000 grain weights. Rice lines with increased grain numbers, panicle lengths and 1,000 grain weights were selected. Among the somaclones, were also plants with reduced height and some with high NaCl tolerance. Considerable variations for spike length, fertility and plant height also have been observed among Triticale somaclones (Jordan and Larter, 1985).

Gene amplification has been demonstrated in many higher organisms during differentiation or as responses to environmental conditions. Some genes amplify themselves, i. e., the number of gene copies per haploid genome increases. This might increase mRNA and protein production from that gene. In the flax variety Stormont Cirrus, Cullis and Goldsborough (1980) observed changes in the DNA as a response to different environmental conditions. Similarly, there is evidence of ribosomal RNA gene amplification and depletion in maize, rye, Secale cereale L., tobacco, Nicotiana tabacum L., and vine crops (Flavell, 1975; Seigel, 1975).

If such a phenomenon occurs in plant tissue culture, it may account, at least in part, for the production of variant somaclones. Although not documented, several cases in plant cell cultures are consistant with gene amplification and deamplification. Many selection cycles were required to obtain high level resistance to salinity in tobacco cultures (Nabors et al., 1980) and resistance to high toxin levels in maize cultures (Gengenbach and Green, 1975). Depletion of DNA sequences has been observed in soybean, Glycine max (L.) Merr., somaclones. One third of the ribosomal genes were lost after prolonged growth on medium with maltose as the carbon source (Jackson, 1980). Dolezel and Novak (1984a), working with Allium sativum L., observed an accumulation of cells with higher nuclear DNA content, ranging as high as 64C, depending on the hormone treatment. They attributed the DNA accumulation to uninterrupted DNA synthesis and/or an endogenous pool of DNA precursors. Quantitative and qualitative heritable changes in the nuclear DNA have been found in dihaploid plants of Nicotiana sylvestris derived by consecutive cycles of androgenesis (De Paepe et al., 1982).

In Triticale, among the plants regenerated from immature cultures, one showed 80% reduction in the 2.5 kb fragment. This fragment is the location of the nucleolar organizer (Scowcroft et al., 1985).

Chloroplast and mitochondrial genomes also are affected by somaclonal variation. Plants regenerated from immature embryos of maize with TMS-cytoplasm grown on toxin selected and unselected media have shown heritable changes that were attributed to an altered mitochondrial genome (Brettell et al., 1980). Restriction endonuclease patterns of high molecular weight mitochondrial DNA (mtDNA) of the maize somaclones confirmed mtDNA changes. Although the regenerants exhibited N-type cytoplasm plant characters, they possessed mtDNA sequences different from that of N-type cytoplasm plants or their parents' T-type cytoplasm (Kemble et al., 1982). Similar results have been reported by Gengenbach et al. (1981). Somatic hybrids of Nicotiana tabacum and Nicotiana knightiana showed extensive rearrangements in the mitochondrial genome. Restriction endonuclease patterns of mtDNA of eight hybrids were different from both parents due to genetic recombinations (Nagy et al., 1981). N. tabacum cybrids showed mtDNA sequences different from their parents or a mixture of the parents. The new DNAs resulted from recombinations occurring during the time in tissue culture (Belliard et al., 1979). Maternal inheritance and restriction enzyme analyses of chloroplast DNA suggested that mutations have occurred in chloroplast DNA of tomato, Lycopersicum esculentum Mill., somaclones (Evans and Sharp, 1983; Evans et al., 1984).

Somaclones of different species have shown dramatic biochemical changes (Skirvin, 1978). Different levels of free amino acids, proteins and sugars have been found in Citrus grandi L. callus lines (Chaturvedi

et al., 1974). Also of clones derived from Dioscoreu dehoidesa Wall. cell culture, seventeen showed significant variability in accumulation of steroid saponins. Four clones synthesize more disogenin, while 13 clones synthesize more yamogenin (Karanova et al., 1986). Somaclones exhibiting chlorophyll deficient mutants have been recovered from N. tabacum (Dulieu and Barbier, 1982; Lorz and Scowcroft, 1983), and tomato, (Evans and Sharp, 1983). The chlorophyll and carotenoid variants appearing in tomato were shown to be genetically stable and heritable (Evans and Sharp, 1983). Alcohol dehydrogenase-1 (ADH-1) mutants have been isolated and extensively analyzed from maize somaclones (Brettell and Jeppesen, 1985; Brettell et al., 1986; Scowcroft et al., 1985). Electrophoretic patterns of prolamin of all regenerated triticale plants (x Triticosecale Wittmack) cv Carman, were similar to the parental banding patterns with variation in the intensity of the bands (Jordan and Larter, 1985).

Many independent reports have shown high frequencies of genetic variability and morphological abnormality in plants regenerated from wheat tissue cultures. Numerical and structural chromosome changes were observed among wheat somaclones (Karp and Maddock, 1984; Nakamura et al., 1981; Lapitan et al., 1984). Regenerated plants from immature embryos of wheat included variants for leaf size, plant height, stem thickness, spike shape, pollen fertility and seed set (Ahloowalia, 1982). Moreover, phenotypic variations were observed among plants that were derived from immature embryos and inflorescences of wheat (Maddock et al., 1983). Larkin et al. (1984) reported extensive somaclonal variation for morphological and biochemical characteristics among 142 regenerants of wheat. Variations were observed in awns, plant height,

heading time, grain color, tiller number, glume color, gliadin and a-amylase regulation. More recently, the progeny of regenerants of the wheat cultivar Millewa were assayed for Adh-1 loci mutants. Seventeen regenerants exhibited altered ADH-1 zymograms. Cytogenetic analyses of these somaclones showed that 13 regenerants were aneuploid and 4 regenerants possessed the 4A isochromosome, a 3BS/4A translocation or a 7BS/4A translocation (Scowcroft et al., 1985; Davies et al., 1986). B-amylase mutants also have been isolated and identified by the isoelectric focusing technique (Scowcroft et al., 1985).

Some somaclonal variations seem to be superior over the source plant, as was recognized in sugarcane (Heinz and Mee, 1971). Since then, several useful variants have been recovered from tissue cultures, such as those with high sucrose yield (Heinz et al., 1977; Liu, 1981) and disease resistant plants (Larkin and Scowcroft, 1982). Evans et al. (1984) reviewed somaclonal and gametoclonal variation and discussed the potential of these variations as novel raw materials for basic genetic studies and plant breeding.

Identification of variants is an important step in a breeding program. Chromosomal abnormalities have been identified by cytological examinations, however, there are some other abnormalities, such as single base mutations that cannot be recognized by cytological techniques (Evans et al. 1984; Larkin and Scowcroft, 1982). Variants may result from the heritably altered expression of genes. The expression may be altered by amplification, deletion, or translocation of a gene (Brown, 1981). While large changes in chromosome structure are readily detected cytologically, the frequently occurring, less dramatic changes, such as gene mutations, require other methods of

detection. Electrophoretic isozymes have been widely used as markers in plant systematic, genetic and evolutionary studies (Brown and Allard, 1969; Cardy and Kannenberg, 1982; Torres et al., 1978a,b). Isozyme analyses of plants grown in tissue culture, however, have been used in relatively few studies. Orton (1983b) examined phosphoglucomutase (PGM) and shikimic acid dehydrogenase (SDH) in celery, Apium graveolens L., callus culture to investigate relationships between changes in zymogram phenotype and chromosome number or structure. Clones from one reciprocal cross always exhibited heterozygous phenotypes at both isozyme loci, but 25.8% of clones from the other exhibited loss of the PGM locus while remaining 100% heterozygous at the SDH locus.

CHAPTER II

MORPHOLOGICAL ANALYSES OF WHEAT SOMACLONES

Introduction

Numerous reports have focused on the regeneration of plantlets from wheat tissue culture (Ahloowalia, 1982; Bennici and D'Amato, 1978; Larkin et al., 1984; Maddock et al., 1983). Various tissues have been used to initiate wheat callus, e.g., scutellar nodal tissue from mature embryos (Conger, 1981), immature embryos (Ozias-Akins and Vasil, 1983), coleoptilar nodes (McHughen, 1983), stem and rachis segments (Conger, 1981), inflorescences near meiosis or at earlier stages of development (Chin and Scott, 1977; Gosch-Wackerle et al., 1979), immature leaf bases (Ahuja et al., 1982), and mesocotyls (Yurkova et al., 1982). However, efficient plant regeneration has been obtained by the initiation of embryogenic callus cultures from immature embryos and inflorescences (Davies et al., 1986; Larkin et al., 1984; Maddock et al., 1983; Ozias-Akins and Vasil, 1982, 1983; and Sears and Deckard, 1982). Degeneration of plantlets from wheat tissue culture has been improved by manipulating media components. Murashige and Skoog's (MS) (1962) medium supplemented with vitamins has been the most widely used nutrient medium for regenerating plants. The addition of hormones induces an increase in the growth rates or organogenesis of the callus. 2,4-D is the most commonly used auxin in wheat callus induction (Larkin et al., 1984; Maddock et al., 1983; Ozias-Akins and Vasil, 1982; Sears and Deckard, 1982). However, Pappenfuss and Carman (1987) reported that

dicamba was more effective than 2,4-D in inducing callus and plant regeneration from immature wheat embryos in the dark.

Somaclonal variation seems to be the rule rather than the exception in many plant species. In wheat, the genetic changes that have been reported are mainly alterations in chromosome number and structure (Ahloowalia, 1982; Bennici and D'Amato, 1978; Karp and Maddock, 1984; Lapitan et al., 1984). Morphological analysis largely has been restricted to the initial regenerants but not their progeny (Ahloowalia, 1982; Maddock et al., 1983). The most extensive somaclonal variation analyses in hexaploid wheat were reported by Larkin et al., 1984. They evaluated families of somaclones (SC_3) of the cv `Yaqui 50E' in the field for variations in awns (present or absent), plant height, heading time, fertile tiller number, and grain and glume color. More recently, wheat regenerants have been evaluated for their economic traits (Ryan et al., 1987).

In this chapter, experiments involving callus induction, plant regeneration from immature wheat embryos and the morphology of the primary regenerants and their progeny are recorded.

Nomenclature

In this chapter, SC_1 will represent the primary regenerant that has been derived from immature wheat embryo cultures. An SC_2 family is the progeny of selfed seed of a single SC_1 plant. An SC_3 family is the progeny of selfed seed of a single SC_2 plant. This nomenclature was introduced by Larkin et al. (1984) and has been adopted by others (Davies et al., 1986; Scowcroft et al., 1985).

Material and Methods

Spring wheat plants, cv PCYT-10 (a CIMMYT selection, Ciano X Gallo, EBWN 7582) were grown in a greenhouse under ambient conditions and utilized as sources of immature embryos for callus induction. Prior to anthesis, the main spikes of parental plants were bagged (Fig. 1) to prevent contamination from stray pollen. Immature embryos were obtained from caryopses 10-12 days after anthesis. Twelve caryopses were removed from each of the main spikes and surface-sterilized for 15 min in 10% chlorox solution containing two drops of Tween 20 in 100 mL and then rinsed four times with sterile distilled water. A few seeds were left on the main spikes until maturity and were used for further analysis. Embryos were excised and placed with the coleorhiza axis in contact with the medium. Eight embryos were placed in each Petri dish. A total of 142 embryos/treatment were used in this experiment.

The basal culture medium consisted of MS inorganic salts modified by a 25 percent reduction in manganese and addition of 0.15 mg L^{-1} asparagine, 0.5 mg L^{-1} nicotinic acid, 0.05 mg L^{-1} pyridoxine-HCL, 0.5 mg L^{-1} thiamine-HCL, 0.1 mg L^{-1} 6-furfurylaminopurine (kinetin), and 20 g L^{-1} sucrose. The medium was adjusted to pH 5.8 with 1 N NaOH before autoclaving, solidified with 7.5 g L^{-1} agar, and autoclaved at 121 ^{o}C for 15 min. Three different levels of either 2,4-D (0.2, 1.7, or 3.4 mg L^{-1}) or dicamba (2, 3, or 4 mg L^{-1}) were added to induce calli. 2,4-D was added before adjusting the pH while dicamba was filter-sterilized and added to the autoclaved medium. After 7-8 weeks in the dark, calli were

Figure 1. Parental (PCYT-10) plants bagged at anthesis to prevent contamination from stray pollen.

subcultured onto the same modified MS medium. Calli exhibiting germinated embryoids were transferred to medium containing 0.1 mg L^{-1} kinetin to produce shoots and roots grown under lighted conditions at 29 ^{o}C. Plantlets were transplanted to 100-mm pots in a mixture of peat moss:vermiculite (1:1) and placed under a plastic cover for 1 week in a growth chamber maintained at 26 ^{o}C, 16/8 h day/night and 300 mol m^{-2} s^{-1} (Fig. 2). Pots were then transferred to a greenhouse with a temperature regime of 25/18 ^{o}C day/night. High pressure sodium lamps (1,000 W) provided a 16/8 h day/night regime with an irradiance of 800 mol m^{-2} s^{-1} (400-700 nm) at plant height. Leaf samples were taken from the regenerated plants (SC_1) for isozyme analyses.

Morphological characteristics were recorded and compared with parental plants. At maturity, the height of plants was measured from the soil surface to the base of the main head. Flag leaf length was measured from base to tip and width was measured at the middle of the leaf blade. Main head length was measured from base to top of the upper spikelet excluding the awns. Number of spikelets and seeds per each main head were counted. Four awns were measured from each main head. Number of tillers per plant was also counted. These parameters were recorded for parental, SC_1, and SC_2. Two seeds from each main head and tiller of each SC_1 plant were used to produce the SC_2 plants. The seeds from the SC_2 variant families were grown in the greenhouse for further genetic analyses.

The first generation of plants was arranged in a completely randomized design with each hormone level as a treatment. The second generation was a completely randomized-nested design, in which the seeds

Figure 2. Primary wheat regenerants grown under high moisture atmosphere to harden them off.

from the donor plants served as the control and each regenerant progeny was compared with its donor parent. All data were subjected to standard analysis of variance and means were separated by the F test. Treatments (parent control and tissue culture) were partitioned into single degrees of freedom, namely, control versus tissue culture, dicamba versus 2,4-D, and 2 mg L^{-1} versus 3 mg L^{-1} of dicamba. Regression analyses were conducted on data from the first generation.

Results and Discussion

Culture and Regeneration

Calli were initiated on the upper surface of embryos after two weeks on the modified MS medium supplemented with 2,4-D (0.2, 1.7, or 3.4 mg L^{-1}) or dicamba (2, 3, or 4 mg L^{-1}) (Fig. 3). By visual appearance, the quality of calli differed within a Petri dish. Both hard, compact and soft, friable calli were obtained from all culture media. However, toxic symptoms and brown coloration were observed on calli grown in media containing 1.7 or 3.4 mg L^{-1} 2,4-D or in 4 mg L^{-1} dicamba. Calli obtained from medium containing 3.4 mg L^{-1} 2,4-D did not grow when subcultured or transferred to regeneration medium, thus, no plants were obtained from this treatment. Calli initiated on medium containing 1.7 mg L^{-1} 2,4-D were not regenerable when transferred to auxin-free medium. The calli produced roots only. Plant regeneration was very low (1.4%) from this treatment, thus, these regenerants were not included in the statistical analyses.

With few exceptions, calli showed reddish brown coloration and growth suppression when grown on medium supplimented with 4 mg L^{-1} dicamba. The calli that survived this treatment were dividing rapidly

Figure 3. Calli from immature wheat (PCYT-10) embryo.

even when transferred to regeneration medium, but lost their regeneration ability and a few produced abundant roots (Fig. 4). Thus, no plantlets were regenerated from this treatment.

Medium containing 0.2 mg L^{-1} 2,4-D produced the highest percentage of regenerable calli. Plants were regenerated from approximately 39% of explants cultured on that medium. Of the immature embryos cultured on media containing 2 or 3 mg L^{-1} dicamba, only 24%, and 35% gave regenerable calli, respectively.

Efficient plant regeneration from immature embryo cultures has been reported by several independent researchers (Shimada, 1978; Gosch-Wackerle et al., 1979; Ozias-Akins and Vasil, 1982; Larkin et al., 1984). Media components such as inorganic salts, vitamins, and growth regulators, subculture intervals, and culture conditions such as temperature and photoperiod were slightly different in these experiments. High levels of 2,4-D induced embryogenic calli. To develop embryoids from the calli, 2,4-D concentration should be reduced gradually. This can be achieved either by prolonged growth of the calli on the same medium without subculturing or by transfer of the calli to a medium containing lower concentrations of 2,4-D (Ho and Vasil, 1983). High levels of dicamba and low levels of kinetin have been reported to give better production of embryogenic calli than 2,4-D (Carman et al., 1987a; Papenfuss and Carman, 1987). Ozias-Akin and Vasil (1982) cultured immature embryos of different wheat cultivars on MS medium supplemented with a range of 2,4-D levels (1 to 8 mg L^{-1}) and reported that 2 mg L^{-1} 2,4-D was the optimum concentration for scutellar callus growth and for the inhibition of precocious germination of the original

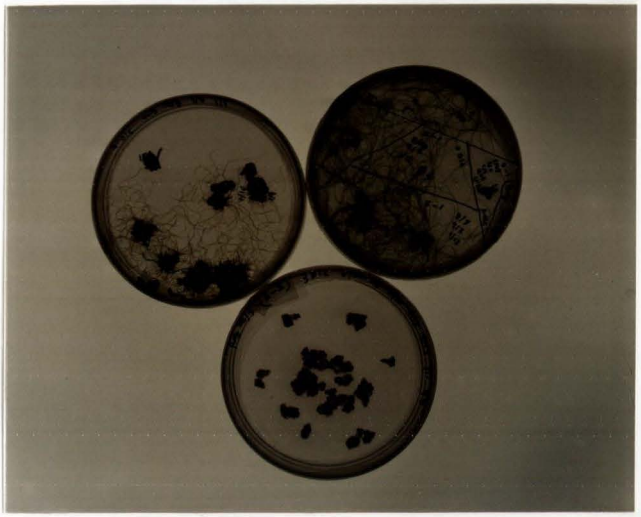

Figure 4. Calli that lost its regeneration ability.

embryo. However, 1 mg L^{-1} 2,4-D was reported to be the ideal concentration by others (Sears and Deckard, 1982; Larkin et al., 1984).

IAA and zeatin, at 1 mg L^{-1} each, were beneficial for shoot regeneration; with shoot primordia being observed on 75% of the calli, and 40% of these developed into shoots (Gosch-Wackerle et al., 1979). Larkin et al. (1984) used 0.5 mg L^{-1} IAA and 1 mg L^{-1} BAP for shoot initiation. They were able to obtain as many as 140 regenerants from a single embryo of the CIMMYT line Yaqui 50E after three months in culture, and up to 231 regenerants after eight months.

Inorganic salt concentrations in the medium have a direct effect on calli quality and growth (Carman et al., 1987a). After 2-4 weeks on Maddock et al. (1983), or Sears and Deckard's (1982) media, calli cells showed an increase in the relative amount of nuclear DNA at telophase stage but not on Ozias-Akins and Vasil's (1983) medium (Hashim et al., 1986). Compared with the other two media, Ozias-Akins and Vasil's medium contained double amounts of inorganic salts. Other components of the medium are important for successful plant regeneration and will be discussed later.

Light is important for plant growth and development (Mohr 1964). Plant tissue culture also requires light to regulate morphogenetic processes (Murashige, 1974). Light duration, illuminance, and quality have been reported to influence callus induction, and root and shoot formation in many plant species (Economou and Read, 1987). In wheat, plant regeneration was increased for some genotypes when the culture was incubated under a diffuse light source (Gosch-Wackerle et al., 1979; Stein et al., 1986). However, continuous darkness increased the number

of somatic embryos per callus in other genotypes (Carman et al., 1987b; Papenfuss and Carman, 1987).

In this study, a low level of kinetin with a high level of dicamba or 2,4-D was used to produce embryogenic calli in the dark. Although the embryos were cultured on the same medium for 7 to 8 weeks to reduce the auxin concentration before subculturing, plant regeneration was not as high as that reported in the literature. In addition to the toxic effect of the high level of auxin in some of the treatments, the amount of inorganic salts might not have been sufficient to support actively dividing callus cells. Higher plant regeneration might be obtained by increasing the concentration of inorganic salts in the medium.

Approximately 72% of the regenerants survived the transplanting process. Lack of appropriate connection between the roots and the shoots might be partially responsible for unsuccessful transplanting. Also, it was impossible to control the moisture level under the plastic covers. Some of the regenerants were a target for fungal infection due to high moisture levels in the pots. Misting frames could perhaps overcome this problem and increase the number of surviving regenerants.

Morphology of Regenerants

Statistical analyses of mophological traits showed significant differences among primary regenerants and between them and their parents (Table 1). Compared to the parental population of PCYT-10, regenerants from all treatments were shorter and exhibited increased tillering. For example, somaclones from 0.2 mg L^{-1} 2,4-D and from 2 and 3 mg L^{-1} dicamba showed 97, 44 and 40% increase in the number of tillers, respectively. The number of tillers produced per plant via the tissue

Table 1. Mean values of morphological traits of the first generation wheat regenerates and the PCYT-10 control.

Treatments	Flag Leaf Length (mm)*	Flag Leaf Width (mm)	Plant Height (mm)	Main Spike Length (mm)	No. of Spikelets	No. of Seeds per Main Spike	No. of Tillers Including Main Head	Awn Length (mm)
PCYT-10 (Control)	221.13	7.97	620.10	82.23	13.10	34.83	3.83	73.29
3 mg/L Dicamba	73.88	4.43	198.88	30.88	4.49	4.28	5.35	39.87
2 mg/L Dicamba	72.17	3.75	185.28	32.78	4.72	3.72	5.53	40.55
0.2 mg/L 2,4-D	90.57	5.49	262.30	42.12	6.63	5.03	7.53	51.08

Table 1. (continued)

Analysis of Variance

Pr > F**

Treatments	Flag Leaf Length (mm)*	Flag Leaf Width (mm)	Plant Height (mm)	Main Spike Length (mm)	No. of Spikelets	No. of Seeds per Main Spike	No. of Tillers Including Main Head	Awn Length (mm)
Treatments	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0013	0.0001
Control vs. Tissue Culture	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0057	0.0001
Dicamba vs. 2,4-D	0.6540	0.6738	0.1766	0.0120	0.0102	0.9307	0.1575	0.0517
3 mg/L Dic vs. 2 mg/L Dic	0.1490	0.0050	0.0025	0.0088	0.0026	0.3952	0.0852	0.0106

*mm = millimeter

**Probability that an F value would occur by chance.

culture technique was significantly greater than those observed on the parental control. Auxin type and the level of dicamba treatments showed no significant effect on the number of tillers produced.

Generally, calli initiated on media supplemented with dicamba regenerated short, compact plants. Mature wheat plants regenerated from calli grown on 2 and 3 mg L^{-1} dicamba were reduced in height by 70 and 68%, respectively, while those from 0.2 mg L^{-1} 2,4-D showed less reduction (58%). An exception, was one particular plant regenerated from 0.2 mg L^{-1} 2,4-D, which was 45 mm tall with a flag leaf 40 mm long and 100 mm wide (Fig. 5). Although the primary regenerants from the dicamba treatments were reduced in height, these reductions were not significantly different from those observed with the auxin type. However, primary regenerants were significantly shorter than the parental controls. Although plant height is subject to environmental as well as genetic effects, it is the sum total of both factors. If plants are grown under controlled environmental conditions, the height phenotypes expressed may be attributed to the genetic effects. In wheat, plant height is under polygenic control (Worland et al., 1980), thus, changes occurring at any of these loci may give rise to a line with altered height. Therefore, a wide range of plant heights might be expected among regenerants.

Variability has been noted among the regenerants for flag leaf size (Fig. 6). The pattern of reduction for plant height was also observed for length and width of the flag leaf. For example, regenerants from both 2 and 3 mg L^{-1} dicamba showed about 67% reduction in flag leaf length and 44% and 53% reduction in width, respectively. Length and width of the flag leaf amongst regenerants from 0.2 mg L^{-1}

Figure 5. Shortest regenerant from 0.2 mg L^{-1} 2,4-D treatment.

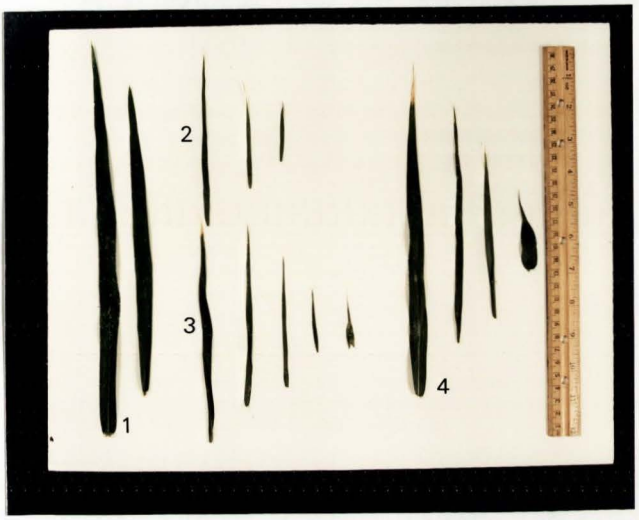

Figure 6. Variation in flag leaf size: 1) Parental, 2) Regenerants from 3 mg L^{-1} dicamba, 3) 2 mg L^{-1} dicamba, and 4) 0.2 mg L^{-1} 2,4-D.

2,4-D was reduced from the parental controls by 59% and 31%, respectively. Auxin type showed no significent difference in either flag leaf width and length. Cereal grain yield is influenced by inflorescence and flag leaf size. In wheat, there is a high positive correlation between flag leaf area and grain yield (Monyo and Whittington, 1973). Moreover, the larger the flag leaf area in wheat the higher the carbon exchange rate (Blum, 1985; Shimshi and Ephrat, 1975).

Somaclones from all the treatments showed a reduction in main head length as well as the number of spikelets per main head (Fig. 7). Regenerants from 2 and 3 mg L^{-1} dicamba treatments showed more than a 50% reduction in both main head length and the number of spiklets per main head, whereas the regenerants from 0.2 mg L^{-1} 2,4-D treatment showed approximately 49% in both traits. The effect of auxin type and dicamba concentrations, however, were less pronounced. Two regenerants from 3 mg L^{-1} dicamba had two spikes on the same stem (Fig. 8). Also, many regenerants from 2,4-D treatment had two spikelets on the same rachis node (Fig. 9). These two characters were not found among any of the parental control plants that were grown in the greenhouse. However, the last character was first observed in cross progenies from Spelta X vulgare (Nilsson-Leissner, 1925). Two different kinds of additional spikelets were recorded in the progenies of these crosses. Some arose singly by the side of a normal spikelet, and then were arranged at right angles to it, and some grew from the rachis immediately below the points of insertion of the normal spikelets, and were arranged parallel to them. Also, the replacement of one or two flowers in one or several

Figure 7. Main heads: 1) Parental, 2) 0.2 mg L^{-1} 2,4-D, 3) 2 mg L^{-1} dicamba, and 4) 3 mg L^{-1} dicamba.

Figure 8. Regenerant plant exhibiting 2 spikes on the same stem.

Figure 9. Regenerant plant exhibiting supernumerary spikelets.

spikelets by whole spikelets or the growing of certain spikelets into whole branches supplied with up to 7 normal spikelets were observed.

The term supernumerary spikelets, often referred to as ear branching, embraces additional sessile spikelets at a rachis node and additional spikelets on an extended rachilla, as all of these are controlled by the same genes (Pennell and Halloran, 1983, 1984). The expression of this character and its stability is strongly influenced by environmental conditions such as photoperiod and temperature (Koric, 1973; Pennell and Halloran, 1984; Sharman, 1967). Several researchers have shown that supernumerary spikelets in wheat is inherited in a recessive manner (Koric, 1973, 1978; Sharman, 1967; Pennell and Halloran, 1983). More recently, inheritance and the chromosomal location of the genes that controlled supernumerary spikelets in tetraploid wheat have been identified using trisomy and substituted lines (Klindworth et al., 1987; Williams et al., 1987).

Number of seeds in the main head was reduced by more than 80% in all of the regenerants. This reduction was due to partial and/or complete sterility in some of the regenerants. Approximately 56% of the main heads obtained from 3 mg L^{-1} dicamba were sterile, whereas fertility was considerably higher in the regenerants from 2 mg L^{-1} dicamba and 0.2 mg L^{-1} 2,4-D. No seeds were set on 18% of 2,4-D regenerants and 22% of 2 mg L^{-1} dicamba regenerants. However, some seeds were produce by the tillers of most of these somaclones. Although auxin type and dicamba level had no significant effect on the number of seeds, the primary regenerants produced fewer seeds per main head than did the parental controls. Other variations, such as seed size and color have also been observed among the somaclones. Some variants

produced large and/or white seeds compared to the parental control (Fig. 10). Further analyses are required to confirm the stability of these variants.

The parental plants exibited long awns with an average length of 73 mm. Compared with the parents, all the somaclones had shorter awns with a reduction of about 45% in dicamba regenerants and 30% in 2,4-D regenerants. No awnless plants were recovered from any of the treatments. Awns contribute about 40-80% of the total spike carbon exchange rate and about 10-20% of spike transpiration (Blum, 1985). Thus, larger awns on the ear indicate plants are more adaptive to hot, dry environments (Blum, 1985, 1986).

Regression analyses showed a positive correlation between all of the measured traits except the number of tillers. Negative correlation has been found between number of tillers, flag leaf length, plant height, spike length, number of spikelets and number of seeds per spike. Low positive correlation (0.04) was found between the number of tillers and flag leaf width (Table 2).

Statistical analyses of second generation plants were complicated. Each family was compared with plants that were obtained from the parental donor. Generally, fewer variations occurred among progenies of the regenerants (SC_2) compared to that observed among the primary regenerants (SC_1) (Table 3). This was expected, since the primary regenerants were exposed to different environments during the process of transferring the plantlets from the medium to the growth chamber and then to the greenhouse, whereas the SC_2 where grown in the greenhouse.

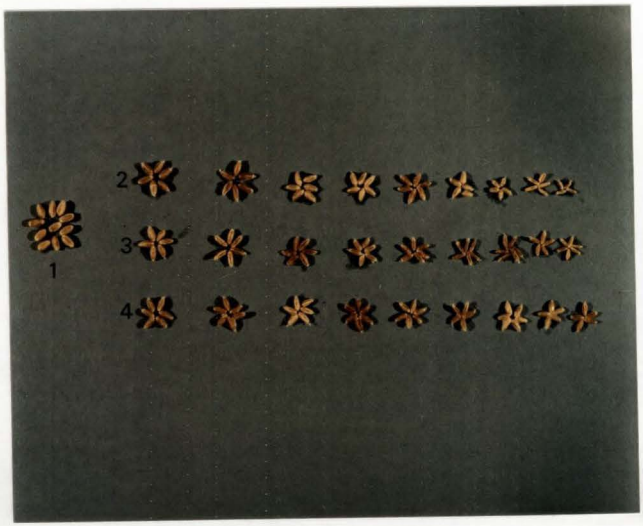

Figure 10. Observed variation in seed size and color in the PCYT-10 somaclone families: 1) Parental, 2) 3 mg L^{-1} dicamba, 3) 2 mg L^{-1} dicamba and 4) 0.2 mg L^{-1} 2,4-D.

Table 2. Correlation between all of the measured variables from the first generation of regenerated wheat plants.

Treatments	Flag Leaf Length	Flag Leaf Width	Plant Height	Main Spike Length	No. of Spikelets	No. of Seeds per Main Spike	No. of Tillers Including Main Head
Flag dent width	0.49						
Plant height	0.59	0.57					
Main spike length	0.61	0.71	0.92				
No. of spikelets	0.60	0.70	0.91	0.96			
No. of seeds per main spikes	0.60	0.56	0.90	0.88	0.87		
No. of tillers	-0.11	0.04	-0.11	-0.04	-0.03	-0.20	
Awn length	0.55	0.57	0.75	0.81	0.75	0.66	0.06

Table 3. Mean values of morphological of the second generation wheat regenerates and the PCYT-10 control.

Treatments	Flag Leaf Length (mm)*	Flag Leaf Width (mm)	Plant Height (mm)	Main Spike Length (mm)	No. of Spikelets	No. of Seeds per Main Spike	No. of Tillers Including Main Head	Awn Length (mm)
PCYT-10 (Control)	120.48	11.66	325.94	84.18	15.27	22.78	4.31	48.84
3 mg/L Dicamba	131.64	10.85	363.31	76.70	14.89	20.05	3.28	50.04
2 mg/L Dicamba	91.53	10.32	283.65	75.93	14.81	15.00	5.02	43.90
0.2 mg/L 2,4-D	144.48	12.15	356.76	87.63	15.26	23.99	4.81	54.03

Table 3. (Continued)

Analysis of Variance

Pr > F**

Variables Source	Flag Leaf Length (mm)*	Flag Leaf Width (mm)	Plant Height (mm)	Main Spike Length (mm)	No. of Spikelets	No. of Seeds per Main Spike	No. of Tillers Including Main Head	Awn Length (mm)
Treatments	0.0001	0.0001	0.0001	0.0001	0.7531	0.0001	0.0001	0.0001
Control vs. Tissue Culture	0.6549	0.0582	0.2947	0.0024	0.5841	0.0049	0.8232	0.7520
Dicamba (Dic) vs. 2,4-D	0.0028	0.1299	0.0001	0.0001	0.7774	0.5274	0.0001	0.4389
3 mg/L Dic vs. 2 mg/L Dic	0.0001	0.0001	0.0001	0.0001	0.4389	0.0001	0.4798	0.0001
Parent (medium)	0.0001	0.1396	0.0003	0.0001	0.9999	0.0049	0.0479	0.0001

*mm = millimeter

**Probability that an F value would occur by chance.

All the SC_2 were significantly different from the parental plants for plant height (Table 3). Progenies of plants regenerated from 0.2 mg L^{-1} 2,4-D and 3 mg L^{-1} dicamba showed an increase in plant height of 9.4% and 11.5%, respectively. However, progenies from 2 mg L^{-1} dicamba showed 13.0% reduction in plant height. To study the stability of plant height, all the seeds of one individual regenerant SC_1 from 3 mg L^{-1} dicamba, which was very short compared to the parent plants, were grown in the greenhouse. The progenies were segregated over a wide range of plant heights. Some were as short as their parent and others were similar to the donor (PCYT-10 control) plant (Fig. 11), while seeds from the shortest regenerant produced uniformly tall offspring.

Significantly differences were observed in flag leaf size of primary regenerants as compared to their parental controls (Table 3). The significant effects were due to auxin type, dicamba level and parental media interaction. Families from 0.2 mg L^{-1} 2,4-D regenerants showed an increase in flag leaf size of 26.1% and 4.2% in length and width, respectively (Table 3). Families from 3 mg L^{-1} dicamba regenerants showed a 15.5% increase in flag leaf length and 7.0% reduction in flag leaf width. Progenies from 2 mg L^{-1} dicamba showed a reduction of 20.1% and 11.5% in flag leaf length and width, respectively. Families from both 2 and 3 mg L^{-1} dicamba regenerants exihibited about 9% reduction in main head length compared to the parental plants, while progenies from 0.2 mg L^{-1} 2,4-D showed an increase of 4% in main head length.

There were no significant differences among parents and the progenies of all regenerants in the number of spikelets per main head. However, the progenies from 0.2 mg L^{-1} 2,4-D showed an increase of 5.3%

Figure 11. SC_2 family segregating for plant height.

in the number of seeds per main head. This might be due to the presence of supernumerary spikelets among these plants. Families from 2 and 3 mg L^{-1} exhibited 34.2% and 12.0% reduction in the number of seeds per main head.

The number of tillers was significantly different among the progenies from all the regenerants and their parents (Table 3). This difference was due to auxin type. Approximately 24% reduction in the number of the tillers was observed among the progenies of 3 mg L^{-1} dicamba regenerants compared to the parent plants. Families from 0.2 mg L^{-1} 2,4-D and 2 mg L^{-1} dicamba showed an increase of 11.6% and 16.5%, respectively, in the number of tillers compared to the parents. Awns length differences were attributed to dicamba concentration parental media interaction. Awns were slightly longer in the progenies of 3 mg L^{-1} dicamba regenerants compared to the parents (2.5%). Awn length was approximately 10% shorter in the progenies of 2 mg L^{-1} dicamba and 10% longer in the progenies of 2 mg L^{-1} 2,4-D regenerants compared to the parents.

Individual variants with desirable characteristics such as supernumerary spiklets and large spikes and flag leaves that we have observed in somaclones could be incorporated into a wheat improvement program. While these desirable characteristics may already exist within the wheat germplasm, tissue culture appears to increase the frequency with which they appear. Therefore, by using appropriate selection techniques, wheat breeders could broaden the germplasm pool in a shorter time.

CHAPTER III

ISOZYME ANALYSES OF WHEAT REGENERANTS

Introduction

Somaclonal variation refers to increased genetic variation detected in plants regenerated from cell and tissue culture (Larkin and Scowcroft, 1981). Some of the variation may preexist in the explant as residual heterozygosity (Barbier and Dulieu, 1980). However, plants derived from protoplast cultures (Lorz and Scowcroft, 1983) and dihaploid cultures (De Paepe et al., 1981) showed extensive somaclonal variations that eliminated residual heterozygosity as a major source of variation. Recent reviews illustrating the scope of somaclonal variation include those of Larkin and Scowcroft (1981, 1983), Evans et al. (1984), and Larkin et al. (1985).

Although somaclonal variations are not favorable in tissue culture, some variant plants seem to be superior over the source plant, as was recognized in sugarcane (Heinz and Mee, 1971). Since then, several useful variants have been recovered from tissue cultures, such as those with high sucrose yield (Heinz et al., 1977; Liu, 1981) or disease resistance (Larkin and Scowcroft, 1982). More recently, Evans et al. (1984) reviewed somaclonal and gametoclonal variations and discussed their potential as novel raw materials for basic genetic studies and plant breeding.

Identification of variants is an important step in a breeding program. Variants have been identified by cytological and morphological analyses. These analytical tools are satisfactory for major chromosomal

changes. However, for less dramatic genetic changes, e.g., single base mutations, gene amplifications, deletions, or transposable elements other methods of detection are required (Brown, 1981; Evans et al., 1984; Larkin and Scowcroft, 1982).

Biochemical data obtained through isozyme electrophoresis can be used to reveal these minor genetic changes. Compared to other plant characters, isozymes are simply inherited and relatively free of environmental influence. While many morphological markers are recessive and only expressed in the homozygous condition, isozymes are expressed in both homozygous and hetrozygous conditions. In addition, isozymes can be easily analyzed and rapidly screened for with a minimum sacrifice of plant material. Thus, isozymes have been used widely as markers in plant systematic, genetic and evolutionary studies (Brown and Allard, 1969; Cardy and Kannenberg, 1982; Torres et al., 1978a,b). Although A- and B-amylases and alcohol dehydrogenase-1 variants have been reported in wheat regenerants (Larkin et al., 1984; Ryan and Scowcroft, 1987; Davies et al., 1986), electrophoretic analyses for isozymes of plants regenerated through tissue culture have received relatively little attention.

In wheat, the genetic control of several isozymes is now very well understood and the chromosomal location of their structural genes have been identified (Hart, 1978, 1979, 1983; Hart and Langston, 1977). For example, three triplicate sets of GOT structural genes have been identified in Chinese Spring wheat by aneuploid genetic analyses. These genes are located in the 3q, 6p and 6q chromosome arm groups of Chinese Spring (Hart, 1975). Two additional GOTs have been identified, one is located in the chromosomes of homoeologous group 7. A total of five

individual GOT groups have been reported in wheat (Jaaska, 1981). Biochemical analyses of genetically controlled GOT-3 variants indicated that this isozyme group is active as a dimer (Hart and Langston, 1977). Aneuploid analysis of etiolated seedling showed that there are three aminopeptidases in wheat (Hart, 1973; Hart and Langston, 1977). The structural genes for these isozymes are located in the 6p chromosome arms of Chinese Spring.

A total of 33 EST isozymes, both minor and major, were detected in several wheat tissues at different stages of development. EST structural genes, that control 22 of these isozymes, were located in the chromosome arms of homoeologous group 3q. Other EST genes were located in the chromosomes of homoeologous group 6 and in chromosomes 2B and 2D (Nakai, 1976).

One duplicate and three triplicate sets of EST genes have been identified by several researchers (Barber et al., 1968, 1969; Jaaska, 1980; May et al., 1973; Hart, 1983). These genes were located in the 3p, 3q, 6q, and 7p groups. One of the enzymes is active as a dimer and three as monomers.

There are 4 EP isozymes in Chinese Spring. The structural genes of these isozymes are located in the chromosomes of homoeologous 7q group (Hart and Langston, 1977).

Objectives of this portion of the study were to identify, by isozyme analyses, the somaclonal variations in wheat plants regenerated from tissue-cultured immature embryos and to determine whether the observed variants were genetically stable.

Materials and Methods

Plant Material

Spring wheat plants, cv PCYT-10 (a CIMMYT selection, Ciano X Gallo, EBWN 7582) were used in this study. Parental plants along with SC_1, SC_2, or SC_3 were grown in the greenhouse under controlled conditions and used for isozyme analyses.

Sample Preparation

The youngest, fully expanded leaf from parental, SC_1, SC_2, and selected SC_3 wheat plants were weighed and then macerated with a prechilled morter and pestle in a 1:5 ratio of extraction buffer (w/v) at 4^{o} C. The extraction buffer consisted of 0.1 M tris(hydroxymethyl)aminomethane, 0.1 M KCl, 0.005 M EDTA, 0.04 M 2-mercaptoethanol, and 0.1 M sucrose (pH 7.5). A few grains of sea sand were used with the grinding mixture. One hundred uL of the crude extract were loaded onto the gel. The samples were electrophoresed in the cold room until the tracking dye (Bromophenol Blue) moved out of the separating gel. The gel was stained for one of the following isozymic systems: Esterase (EST), glutamine oxaloacetate transminase (GOT), leucine aminopeptidase (LAP), and endopeptidase (EP).

Polyacrylamide Gel Electrophoresis

The PAGE protocol of Davis (1964), with minor modification, was used in this study. The separating gel consisted of 1 part of 3.02 M TRIS-HCl (pH 8.9), 2 parts of 3.94 M of acrylamide and 0.05 M N,N'-methylenebisacrylamide (BIS), 1 part distilled water, 4 parts of 0.01 M ammonium persulfate (APS), and 8 uL of N,N,N,N'-tetramethyl-

ethylenediamine (TEMED). The stacking gel consisted of 2 parts of 0.41 M TRIS-HCl (pH 6.9), 4 parts of 1.41 M acrylamide and 0.16 M (BIS), 8 parts of 1.17 M sucrose, 2 parts of 0.01 M (APS), and 2 uL of (TEMED). The running buffer, that was used for both upper and lower buffer tanks, consisted of 0.038 M glycine and 0.004 M TRIS (pH 8.3). Fresh buffer with 10 uL of 0.1 % bromophenol blue was used for the upper tank for each run. The buffer of the lower tank was replaced after the sixth run.

Isozyme Staining

Immediately after electrophoresis, the gel was stained with one of the following histochemical reaction mixtures (Eduardo-Vallejos, 1983):

Endopeptidase (EP). The gel was soaked 3x for 30 min each in 50 ml of 0.1 M Tris-0.1 M Maleic acid-NaOH pH (5.8) at 2-4 ^{o}C prior to staining. Thirty mg of Black K (Brentamine fast black K salt) were dissolved in 0.1 ml of dimethyl formaldehyde (DMF) and the solution added to 75 ml of Tris-maleate-NaOH buffer. Then 30 mg of BANA (N-Benzoyl-DL-Arginine B-Naphthylamide Hydrochloride) were added to the staining mixture and the gel was incubated in the dark at 37 ^{o}C for 1-2 hours. The gel was then washed with water and either photographed and/or dried.

Leucine Aminopeptideas (LAP). The gel was soaked in Tris-maleate-NaOH as above prior to staining. Thirty mg of Fast Blue B Salt (o-Dianisidine, Tetrazotized Zinc Chloride complex) were dissolved in 1.0 ml of ethanol and added to 75 ml of Tris-Maleate-NaOH buffer pH (5.8). Twenty mg of L-Leucyl-B-napthylamide, HCl were dissolved in 1.0 ml of

acetone and added to the stain-buffer mixture. The gel was then incubated in the dark at 37 ^{o}C for 1-2 hours.

Glutamine Oxaloacetate Transaminase (GOT). The gel was stained for 3-5 min at room temperature in the following reaction mixture: 0.75 mg pyridoxal-5-phosphate, 300 mg aspartic acid, 150 mg A-ketoglutaric acid, and 225 mg fast blue BB monosodium salt (Diazotized 4'-amino-2',5'-diethoxy-benzamilide zinc chloride salt) in 75 ml of 0.2 M Tris-HCl pH (8.0). The gel was then washed immediately and photographed and/or dried.

Esterases (EST). Thirty mg of A-napthyl acetate and 10 mg of B-napthyl acetate were dissolved separately in 1.0 ml acetone. Then they were added to 90 ml distilled water and 10 ml of 1.0 M Tris-HCl pH (7.0). One hundred mg of fast blue RR (Diazotized product of 4-benzoyl amino-2,5-dimethoxyaniline zinc chloride) were added to the stain mixture. The gel was stained at room temperature for 2-3 hours. The gel was then washed and photographed and/or dried.

Results and Discussion

Consistent GOT, LAP, and EP zymograms were obtained by using crude extract of the youngest fully expanded leaf. However, leaf EST zymograms were affected by the stages of plant development. Thus, no particular segregation patterns of EST zymogram were recognized in the primary regenerants or their progenies.

Although 100 regenerants and their progenies (583) were analysed electrophoretically, none of them showed any LAP or GOT variants (Figs. 12 and 13). However, one regenerant, which was derived from 3 mg L^{-1} dicamba, gave rise to a family with altered EP zymograms (Fig. 14).

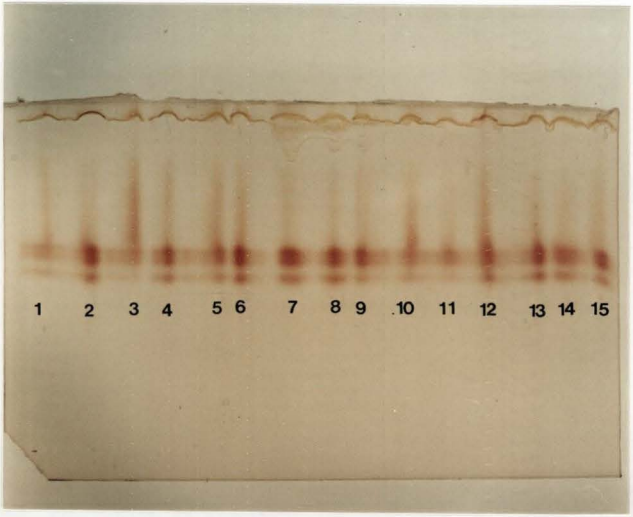

Figure 12. Leucine aminopeptidase (LAP) zymogram of wheat regenerates. Lane # 5 is parental.

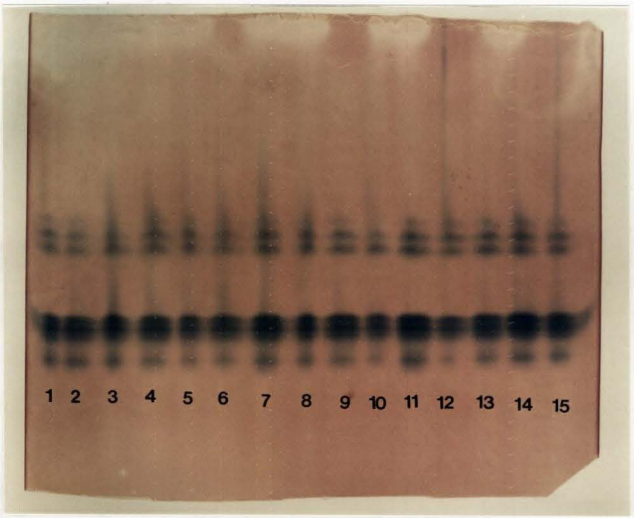

Figure 13. Glutamine oxalacetate transaminase (GOT) zymogram of wheat regenerates. Lane # 5 is parental.

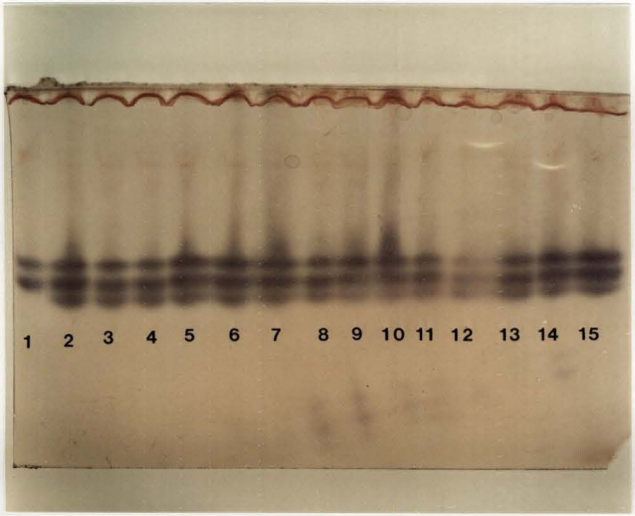

Figure 14. Endopeptidase (EP) zymogram of wheat regenerants exhibiting an EP variant. Lane # 5 is parental and lane # 1 is the variant.

This particular regenerant had a partially sterile main head that produced only 7 viable seeds. Two progenies exhibited normal EP zymograms compared to the parent. One of these somaclones was morphologically normal and produced seeds at maturity, but the other somaclone was very compact with small leaves, many tillers and did not set any seeds.

Three progenies showed one missing EP band. Morphological analysis of these somaclones indicated a looseness of the spikes that showed an expansion of the rachis internodes in the EP variant (Fig. 15). All the regenerants with the EP missing band were stable through the fourth-selfed generation.

The remaining progenies exhibited a reduction in the intensity of the fast moving EP band as was shown by densitometry (Figs. 16 and 17). No morphological differences were noticed between these somaclones and the parental control. The progeny of these somaclones segregated in the same pattern explained above. All sister regenerants derived from the same embryo explant or from embryos obtained from the same doner plant displayed a wild type EP phenotype. This indicated that the mutation had occurred during culture.

Nulli-tetra analyses of Chinese Spring wheat showed that the structural genes for EP isozymes are located in the long arms of group 7 chromosomes (Hart and Langston, 1977). Zymogram phenotypes of EP isozymes vary with the dosage of specific group 7 chromosome and chromosome arm. For example, a wheat plant that is nullisomic for 7A or ditelo-7AL will not express EP-2. Moreover, EP-3 and EP-4 are not expressed by a plant lacking either chromosome 7B or chromosome arm 7BL.

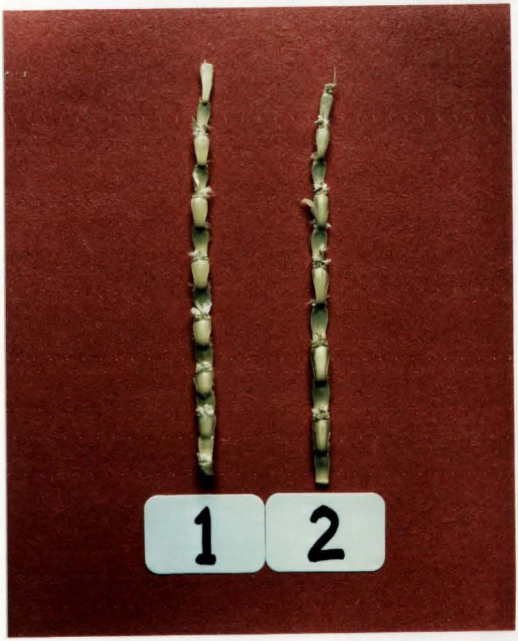

Figure 15. Morphological variant showing elongated rachis internodes. 1) Parent and 2) EP variant.

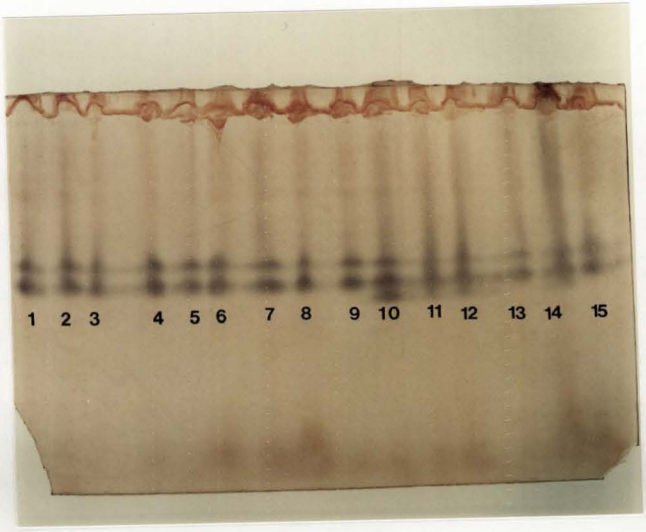

Figure 16. Endopeptidase (EP) zymogram of wheat regenerants exhibiting EP variants. Lanes # 1-9, 13 and 15 with one missing band, lane # 10 parental and lanes # 11, 12, and 14 with light band.

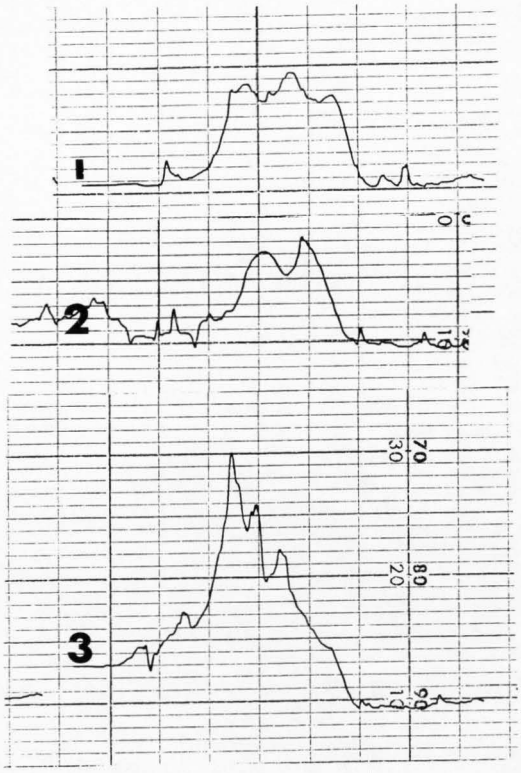

Figure 17. Densitometric phenograms of: 1) control, 2) variant with missing EP band, and 3) variant with light EP band.

EP-1 is not expressed by a plant lacking either chromosome 7D or chromosome arm 7DL.

Isozyme analysis of EP in the present study indicated that the mutants are either homozygous or heterozygous null for EP-1. However, mitotic root tip chromosome analyses showed that some of the variants with missing EP bands exhibited 41 chromosomes and some had the normal chromosome allotment of 42. Thus, there was no correlation between the missing EP band and the missing chromosome (Fig. 18). The mutant may represent a deletion, translocation, the activation of a repressor gene, point mutation at the active site of the enzyme, or the presence of a transposable element. The last possibility is least likely, because the mutant is stable up to the fourth-selfed generation under greenhouse conditions. Also the active site of the isozyme is very conservative (Markert, 1975).

Structural changes in chromosomes in regenerated plants are very well documented (Larkin and Scowcroft, 1981; McCoy et al., 1982; Lapitan et al., 1984). Thus, the mutant observed in this study could have resulted from a deletion, inversion or translocation in the long arm of chromosome 7D. Further studies with N- or C-banding, meiotic analyses, or `arm ratio' measurements could confirm which one of these changes is involved.

Although chlorophyll analyses were not conducted, differences in the intensity of green color of the leaves were observed among the somaclones during isozyme sample preparation. These variations could be due to nonlethal chlorophyll mutants. Such mutants could be used as genetic markers for other traits which do not have distinguished phenotypes.

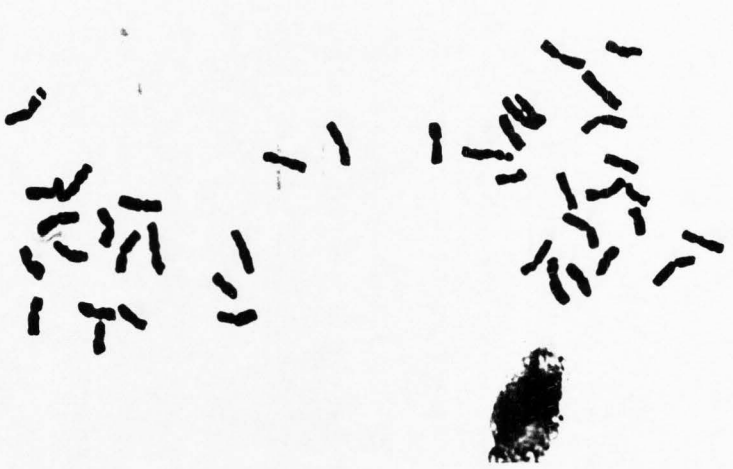

Figure 18. Mitotic metaphase chromosomes from EP variant (missing band) showing 2n = 41.

The above isozymes were selected for this study because of the limited source for the samples. Since morphological analyses had to be conducted on these plants, only a small portion of the leaf was used for isozyme analyses to reduce plant injury. Isozymes, which are active only under specific environmental conditions, were eliminated from this study. For example, the alcohol dehydrogenase isozyme is induced in wheat roots and in germinating grains by partial anaerobiosis (Hart, 1980; Jaaska and Jaaska, 1980). All the seeds from the primary regenerants were replanted in the greenhouse to conduct progeny test. Thus, isozymes such as A- and B-amylases and peroxidase, which required grain tissue, were eliminated from this study (Marchylo et al., 1976).

The non-denaturing discontinuous electrophoresis system of Davis (1964) was adopted for isozyme separation. Riboflavin was replaced by (APS) and TEMED in the stacking gel. This minor change gave faster and better gel polymerization than the standard formula. Better GOT isozymes separation was obtained when the samples were electrophoresed on long gel of 10% acrylamide concenteration.

CHAPTER IV

PROTEIN ANALYSES OF WHEAT SOMACLONES

Introduction

As world population continues to increase, the demand for food and protein supplies will also increase (Wittwer, 1979). Plant breeding and selection programs have long been used to improve and increase protein production in many cultivated crops to meet this demand. However, the development of improved cultivars, through standard plant breeding techniques, takes seven to ten years and may be difficult in some crops due to the complicated genetic control of protein biosynthesis. Moreover, the centers of origin of original germplasm for most agronomic crops have been exploited.

Plant biotechnology, such as tissue culture, has potential application in crop improvement and development (Larkin and Scowcroft, 1983; Scowcroft et al., 1985). This technique can reduce the time of developing new and improved agronomic crops by one-third to one-half. Moreover, tissue culture requirements for many crops have been established, and regenerant production has been increased. The regenerants display a considerable amount of somaclonal variation, including heritable changes at the molecular and chromosomal levels (Baertlein and McDaniel, 1987; Evans et al., 1984; Larkin and Scowcroft, 1981, 1983; Larkin et al., 1984; Orton, 1983b; Skirvin, 1978). These somaclones are a rich source of desirable germplasm for broadening plant genetic resources.

One wheat cultivar has produced as many as 200 regenerants from one explant under specific tissue culture conditions (Larkin et al., 1984). Among these regenerants, substantial somaclonal variants have been reported (Karp and Maddock, 1984; Larkin et al., 1984; Lapitan et al., 1984). More specifically, some of the somaclones exhibit changes in the electrophoretic patterns of the gliadin proteins (Larkin et al., 1984; Scowcroft et al., 1985).

Wheat proteins are the best known among the proteins of cereal grains. Extraction, separation, and identification of these proteins have been summarized in several extensive reveiws (Garcia-Olmedo et al., 1982; Lasztity, 1984; Wrigley and Shephard, 1973; and Wrigley et al., 1982). Electrophoretic protein patterns are characteristic of the genotype and are independent of growth conditions (Lee and Ronalds, 1967; Wrigley, 1970). Thus, the variant wheat somaclones will reveal genetic changes at the molecular level. Identification of novel variants for these agronomically important characters will expand the genetic resources for wheat improvement. In this study, quantitative and qualitative analyses of salt soluble (globulins) wheat seed proteins were conducted on parental and SC_2 endosperm in an attempt to elucidate valuable information about the complexity and heritability of these proteins.

Materials and Methods

Plant Material

Spring wheat plants, cv PCYT-10 (a CIMMYT selection, Ciano X Gallo, EBWN 7582) were used in this study. Proteins extracted from mature caryopses of parental and (SC_2) plants were examined

electrophoretically. Two to 20 individual seeds per SC_2 family, a total of 520 samples, were analyzed.

Sample Preparation

Individual seeds were cut transversely and the embryo half stored for later growth and analysis. The endosperm portion was ground using a mortar and pestle and the ground samples were mixed with 0.5 N NaCl, pH 7.5 for 1 h at room temperature. The ratio of endosperm tissue to NaCl solution was 10 mg to 0.1 mL. Cell debris was pelleted by centrifugation in a Fisher micro-centrifuge model 235B for 2 min at room temperature. The supernatant was then mixed with the treatment buffer in a ratio of 4:3 and boiled for 1 min in a boiling water bath. The buffer consisted of 0.13 M tris-Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol.

Electrophoresis was carried out on a standard 10% discontinuous SDS-polyacrylamide slab gel (Laemmli, 1970). One hundred uL of the sample mixture were loaded onto the gels. The gels were stained with 0.13% Coomassie Blue R-250 in 50% methanol and 10% acetic acid over night. After staining, gels were destained in 50% ethanol and 10% acetic acid. Destaining solution was changed several times until clear background was obtained.

Gels were scanned using a Bio-Rad densitometer scanner model 1650 and Bio-Rad flat bed chart recorder model 1321. Quantitative estimations of individual proteins were obtained by measuring the height of the peaks. The data were subjected to cluster analysis to measure a relatedness coefficient for each somaclone in comparison to other somaclones and the parent plants.

Results and Discussion

Electrophoretic analyses of salt soluble seed proteins showed changes in protein bands of the SC_2 somaclones compared to the parents (Fig. 19). Changes included the appearance of new proteins, deletion of some bands and intensity alteration of others.

Differences in total protein content of the seeds from somaclones and parents were judged by band intensity as measured by densitometry. Some of the somaclones showed overall darker banding patterns compared to the parents. An increase in intensity of a specific protein band may suggest gene amplification or the presence of a new band with the same migration distance as the original band, thereby intensifying the band. This latter suggestion can be tested by two dimensional electrophoresis, i.e., a single band on a one dimensional gel may be separated to more than one on a two dimension gel (Wrigley, 1970). By contrast, a decrease in band intensity might indicate a deletion of one of the proteins that has the same migration distance on the gel. Genetic analyses of such variants are complicated since the proteins are synthesised in the triploid endosperm by codominant genes (Wrigley, 1970). Gene amplification, duplication, or deletion will all effect the intensity of specific protein bands.

The SC_2 of somaclones were evaluated for the presence or absence of one or more protein bands (Fig. 19). Approximately 0.7, 0.3, 11.4, 9.4, 6.7, 0.3, 3.0, and 5.0% of the plants from 0.2 mg L^{-1} 2,4-D treatment were missing eight bands, numbers 1, 3, 4, 5, 6, 8, 9, and 10, respectively. No somaclones from this group were missing two bands, numbers 2 or 7. About 3.2, 13.7, 4.2, 3.2, 2.1, and 7.4% of SC_2 from 3

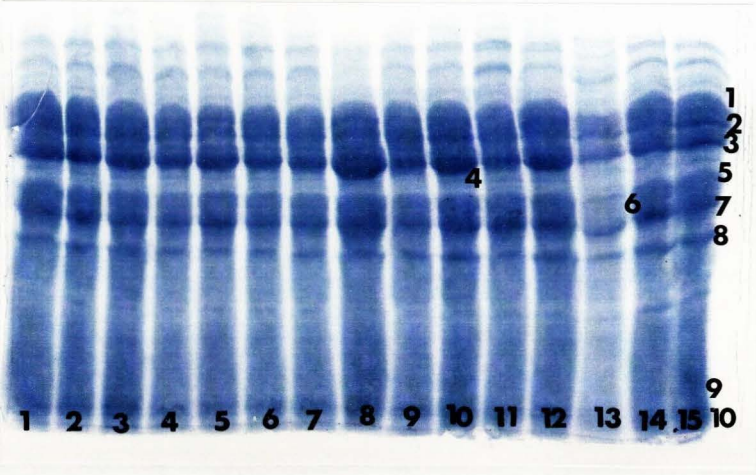

Figure 19. Seeds from SC_2 somaclones exhibiting missing protein bands and altered intensity in others. Lane # 5 is parental.

mg L^{-1} dicamba treatment were missing six bands, numbers 2, 4, 5, 6, 9, and 10, respectively. None of the plants tested in this treatment were missing four bands, numbers 1, 3, 7, or 8. Moreover, 1.8, 1.8, 17.9, 8.9, 10.7, and 1.8% of SC_2 somaclones from the 2 mg L^{-1} dicamba treatment exhibited six missing bands, numbers 2, 4, 5, 6, 9, and 10, respectively. None of the somaclones from this treatment were missing specific bands, numbers 1, 3, 7, or 8.

Members of one SC_2 family exhibited deletion of more than one protein band. The segregation for these bands was independent. For example, three different members of one family from 0.2 mg L^{-1} 2,4-D treatment showed, respectively: one member was missing band number 4, another was characterized by the absence of band number 5, and a third was missing two bands, numbers 4 and 5. Members of another family from the same treatment exhibited, respectively: deletion of one band, number 4 or number 6, and still another one was missing two bands, both number 4 and 6. Also some of the somaclones were characterized by the appearance of new protein bands. These bands were minor and showed low intensities as was shown by densitometry. These results are in agreement with the simple codominant inheritence of protein bands (Mecham et al., 1978). Heritable changes in gliadin patterns were observed amongst wheat somaclones (Larkin et al., 1984). These changes included deletion of specific bands, the presence of new bands, or changes in band intensity.

In some cases, plants regenerated from the same immature embryo exhibited the same changes in banding patterns of salt soluble proteins. This might indicate that the genetic change had occurred early in the culture.

Cluster analysis and the resulting phenogram, based upon protein variation, were very complicated due to the large sample size (see Fig. A1). Approximately 70% of the parents (the immature embryo donors) was grouped together. The other 30% was divided between two different small groups. Within these two small groups, the donors were surrounded by somaclones originating from them.

In some families of somaclones, all members clustered together (Fig. A1), whereas in other families, members were clustered in separate groups. This latter distribution of the somaclones indicated that genetic changes at the protein level had occurred during the tissue culture growth phase. Replicated samples that were loaded on different gels exhibited similar banding patterns and were clustered in the same groups. This eliminated the effect of variation in protein intensities and banding patterns sometimes encountered during electrophoresis and staining conditions.

The nutritional value of wheat depends on the amount of protein and amino acids presence. Generally, these two characteristics are influenced by environment as well as heredity. Genes that code for wheat storage proteins have been identified and are very well studied (Garcia-Olmedo et al., 1982; Wrigley et al., 1982). However, selection for these traits by conventional plant breeding has been discouraging because it is time consuming and may lead to reduction in total yields. Somaclonal variation appears to provide variants with desirable protein levels. Using electrophoretic techniques, we have, in a very short time, identified individuals with high protein content and some with new

protein bands compared to their parents. Further analysis, however, is required to confirm their stability and to test the quality of these variants for milling and baking.

CHAPTER V

CYTOPHOTOMETRIC ANALYSES OF SOMACLONAL VARIATION IN WHEAT CALLI

Introduction

Genetic variability and morphological abnormality have been reported in plants regenerated from tissue culture (Bayliss, 1980; Constantin, 1981; D'Amato, 1978; Dolezel and Novak, 1984a,b; Larkin and Scowcroft, 1981; Larkin et al., 1984; Scowcroft et al., 1985). Certain plant growth regulators, especially 2,4-Dichlorophenoxyacetic acid (2,4-D), may induce mitotic aberrations, endomitosis or endoreduplication in tissue cultured cells (Chen and Chen, 1980; Dolezel and Novak, 1984a; Ghosh and Gadgil, 1979). However, Dolezel and Novak (1984b), working with the Tradescantia stamen hair system, a system reknowned for its high sensitivity to chemical mutagens (Sparrow and Schairer, 1971), found no alteration in the frequency of somatic mutations following direct application of the culture medium to Tradescantia inflorescences regardless of the hormonal composition. Bayliss (1980) likewise found no evidence that plant growth regulators induced mitotic aberrations in callus cultures, but rather that they induced unorganized growth of the callus tissue that is controlled by growth regulators.

Although controversy exists concerning the role of growth media on the plant genetic and morphological stability in in vitro cultured plant tissues, several excellent reviews (Bayliss, 1980; Constantin, 1981; D'Amato, 1978; Larkin and Scowcroft, 1981; Larkin et al., 1984;

Scowcroft et al., 1985) concluded that plant tissue does generate genetic and heritable variability. A number of possible mechanisms has been proposed for the origin of these changes, namely, karyotype changes, cryptic changes associated with chromosome rearrangement, transposable elements, somatic gene rearrangements, gene amplifications and deletions, somatic crossing over and sister chromatid exchanges (Larkin and Scowcroft, 1981).

Dolezel and Novak (1985), working with Allium sativum L., observed an accumulation of cells with higher nuclear DNA content, ranging as high as 64C depending on the hormone treatment. They attributed the accumulation to uninterrupted DNA synthesis and/or an endogenous pool of DNA precursors. A wide range in DNA content was detected in diploid Nicotiana tabacum callus, ranging from a low of only 5 pg of DNA per cell to a high of 40 pg (Berlyn, 1983), while the diploid value was 7.8 pg. Karlsson and Vasil (1986) reported that embryogenic cell suspension cultures of Panicum maximum Jacq. and Pennisetum purpureum Schum., maintained in Murashige and Skoog's (1962) (MS) medium containing coconut water and 2,4-D, were comprised predominantly of normal diploid cells. Carman et al. (1987a) reported that medium based on double the MS inorganic salt concentration significantly improved somatic embryogenesis in wheat over other media. The objective of this study was to measure the relative amounts of DNA from wheat root tips and calli of 10- to 12-day-old immature embryos grown in three different media and also to study the effect of these media on shoot production and plant regeneration.

Materials and Methods

Spring wheat, Triticum aestivum L. cv PCYT-20, was grown in a kidman sandy loam (Calcic haploxerolls):peat moss:vermiculite (3:1:1) in 150 x 165 mm plastic pots under greenhouse conditions with a temperature regime of $25^{o}/18^{o}$ C day/night. High pressure sodium lamps (1000 watts) provided a 16/8 h day/night regime with an irradiance of 800 m^{-2} s^{-1} (400-700 nm). Flowering spikes were tagged at pollination and fertilization. Ten to 12 days after anthesis, young embryos were excised and randomly assigned to the media of Maddock et al. (1983), Ozias-Akins and Vasil (1983), or Sears and Deckard (1982) (Table 4). After 2, 4, 6, or 8 weeks of growth on the various media, calli samples were collected and prepared for Feulgen cytophotometry (Berlyn and Miksche, 1976). Microspectrophotometric measurements were made on 100 anaphase or early telophase nuclei at 508 and 550 nm. Standards for DNA measurements were obtained from wheat meristem root tips. After 18 weeks, calli from different media were evaluated for number of shoots or roots only or production of plantlets. All data were analyzed in a randomized complete block design.

Results and Discussion

Nuclear DNA content of immature wheat embryo calli cells increased when grown on Maddock's or Sears and Deckard's media at two and four weeks, but decreased at six and eight weeks (Table 5). At eight weeks, all treatments approached the DNA level of wheat root tips. Maddock's medium increased the nuclear DNA content to about 0.5- to 2.5-fold, while Sears and Deckard's medium induced a 3- to 3.5-fold increase after

Table 4. Components of 3 media used for induction of embryogenic callus from immature wheat embryos.

Component	Medium* A	B	C
MS Inorganic Salts	2X	1X	1X
mM Concentrations			
MYO-Inositol	0.56		0.56
L-Asparagine		1.00	
Sucrose	58.43	58.43	87.64
uM Concentrations			
Nicotinic Acid	4.06		4.06
Pyridoxine.HCl	2.43		2.43
Thiamine.HCl	0.30	1.50	0.30
2,4-D	4.52	4.52	4.52
MG/L Concentrations			
Casein Hydrolysate	100		
Difco Bacto Agar	10,000	7,000	6,000
Coconut Water (V/V)			10%
pH	5.75	5.80	5.80

*Medium a (Ozias-Akins and Vasil, 1983); Medium B (Sears and Deckard, 1982); Medium C (Maddock et al., 1983).

Table 5. Nuclear DNA content (+ standard error S.E.) in wheat (PCYT 20) root tips and calli of immature embryos grown in three different media.

Age of Culture (Weeks)	Medium*	DNA per Nucleus (pg) ± S.E.**
2	A	11.1 ± 1.3
	B	32.3 ± 3.4
	C	15.6 ± 4.6
4	A	9.9 ± 3.1
	B	37.6 ± 2.4
	C	24.7 ± 2.8
6	A	10.5 ± 1.7
	B	17.1 ± 2.1
	C	16.1 ± 1.7
8	A	12.2 ± 2.2
	B	13.8 ± 2.6
	C	13.2 ± 2.5
Root Tips		10.9

* Medium A after Ozias-Akins and Vasil (1983), medium B after Sears and Deckard (1982), and medium C after Maddock et al. (1983).

**Mean of 50-100 telophase cell nuclear content.

two and four weeks, respectively. Immature wheat embryo calli cells grown on the Ozias-Akins and Vasil's medium contained nuclear DNA contents not significantly different from those observed in the wheat root tips. Since the Ozias-Akins and Vasil's medium contained 2X the amount of MS medium inorganic salts, it is hypothesized that these additional nutrients provided the rapidly dividing cells with sufficient nutrition so that the nuclear DNA content was similar to the DNA of root tips.

The two-week lag period in the increase in DNA content with Maddock's medium compared to the Sears and Deckard's medium may be attributed to the additional energy provided by the high level of sucrose. At four weeks, however, the sucrose supply may be approaching a deficit level, and the lower inorganic salt effect is being expressed. Sears and Deckard's medium lacked the additional sucrose or the extra inorganic salt concentrations and the effect of the 2,4-D was expressed at two weeks. After six and eight weeks, nearly all of the 2,4-D appears to have been utilized by the cells, and the nuclear DNA contents were approaching that observed in the wheat root tips.

After 18 weeks without subculturing, the number of plantlets produced on the three media was significantly different ($P < 0.10$), with the highest number being produced on medium "A" (Figs. 20 and 21). Furthermore, significant differences ($P < 0.10$) in the number of embryoids producing shoots or roots only were observed between the three media, with the highest shoot production on medium "A" and roots on "B" (Figs. 22 and 23). Testing the effect of genotype and culture medium on embryogenic callus induction, Carman et al. (1987a) concluded that a

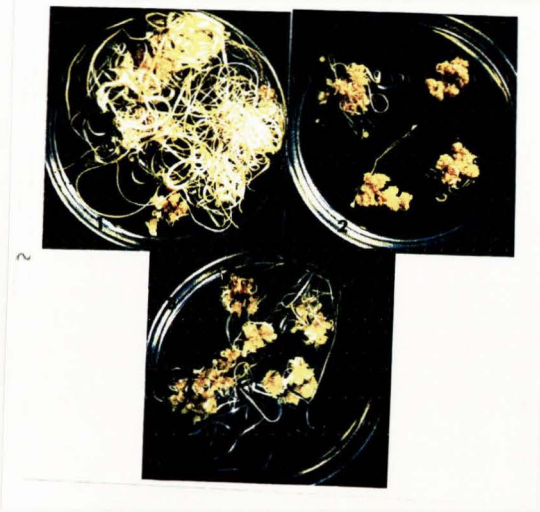

Figure 20. Wheat (PCYT-10) calli grown for 18 weeks on: 1) Ozias-Akins and Vasil, 2) Sears and Deckard, and 3) Maddock et al. media. Note the differences in shoot development.

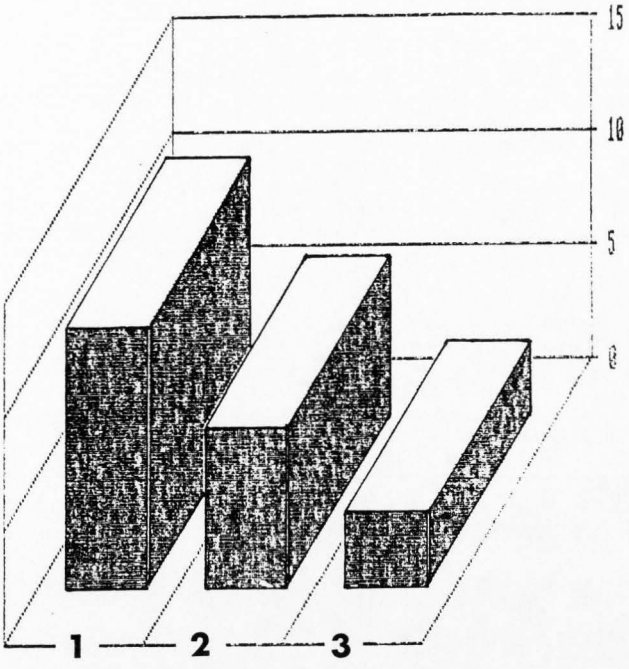

Figure 21. Average number of plantlets produced per immature embryo grown on: 1) Ozias-Akins and Vasil, 2) Maddock et al., and 3) Sears and Deckard media.

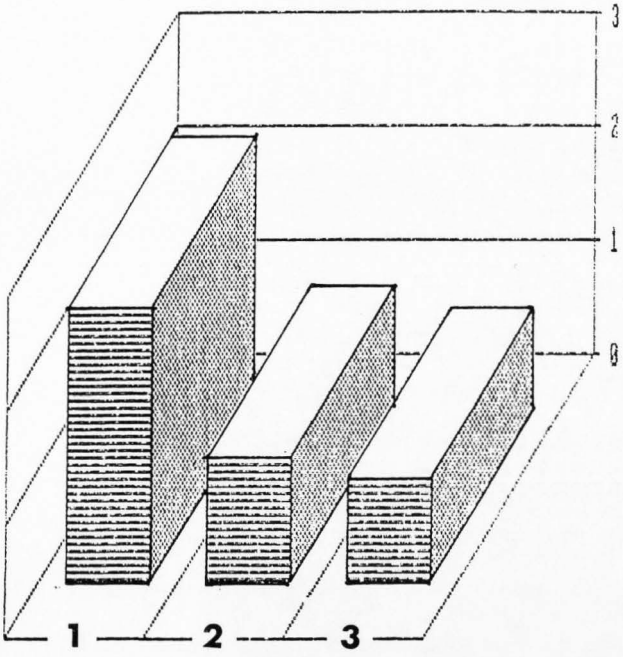

Figure 22. Average number of shoots produced per immature embryo grown on: 1) Ozias-Akins and Vasil, 2) Maddock et al., and 3) Sears and Deckard media.

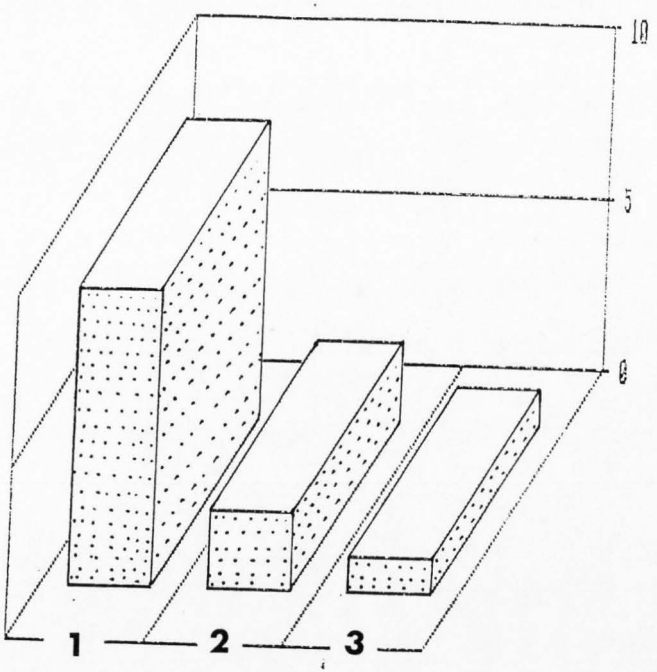

Figure 23. Average number of roots per immature embryo grown on: 1) Sears and Deckard, 2) Maddock et al., and 3) Ozais-Akins and Vasil media.

medium based on double the Murashige and Skoog (MS) inorganic salt concentration was significantly better than other media.

The nutrient resources available for plants, namely, CO_2, H_2O, and inorganic ions, are of low energy status. In fact, assimilation of these inorganic nutrients requires energy. In green plants, this energy can be provided through photosynthesis by converting light energy into chemical energy in the form of carbohydrates. In wheat grain, for example, the plant sap surrounding the embryo (cavity sap) contains 10-50 mM sucrose, small amounts of hexoses and high concentrations of oligosaccharides (Ho and Gifford, 1984). These carbohydrates are essential for kernel growth rate and size.

Under In vitro conditions, carbon can be provided by adding sucrose in the media (George and Sherrington, 1984). The amount of sucrose is essential to satisfy the energy requirement of the actively dividing cells. The amount of sucrose in media "A" and "B" is approximately equal to that in the cavity sap (58.43 mM). Medium "A" contained myo-inositol which provided another source of energy. However, medium "C" contained myo-inositol and approximately double the amount of sucrose (87.64 mM). The abnormality in the amount of DNA in cells grown in medium "B" may result from low energy levels.

The potassium ion (K^+) is an essential element for higher plants. Concentration of this element in the cavity sap of wheat kernels ranges from 10 to 40 mM (Ho and Gifford, 1984). The K^+ concentration in MS medium was 20 mM. Thus, doubling the MS inorganic salts should have provided an optimal level of K^+ for the cultured immature embryos.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Plantlets were regenerated from immature wheat (PCYT-10) embryos cultured on MS medium supplimented with 0.5 mg L^{-1} kinetin and 2, or 3 mg L^{-1} of dicamba or 0.2 mg L^{-1} of 2,4-D. Morphological analyses of these somaclones and their progenies showed significant differences in flag leaf size, plant height, number of tillers, spike length, awn length, and number of seeds per main head when compared to the parental controls. However, number of spikelets per main head in the second selfed generation showed no significant difference from the parental controls. Some of the somaclones had supernumerary spikelets and some produced white seeds.

Leaf isozyme analyses of the somaclones and their progenies reveal the existence of stable endopeptidase (EP) mutant. Members of the family with (EP) mutant were characterized by either one missing band or the presence of lighter band in this position compared to the parental controls. Other members of the same family showed normal (EP) zymograms, however, some of them did not survive. Somaclones with the missing (EP) band were morphologically normal except the rachis internodes were longer when compared with that of the parents.

Cytological analysis showed a missing chromosome in some of the (EP) mutants, however, others exhibited normal chromosome number. Thus, there was no correlation between the missing chromosome and the missing band.

Extensive variations for salt soluble seed proteins were observed among the somaclones. Individual variants exhibited missing or extra protein bands at different positions on the gel. Differences were also observed in the relative quantity of each of the resolved proteins as was measured by the densitometer.

Somaclones with desirable agronomic traits such as high seed production, large flag leaves and awns, number of tillers, and high protein content could be incorporated into wheat improvement program.

Cytophotometric analyses of immature wheat embryo calli cells showed that the relative amounts of DNA were affected by the levels of sucrose and inorganic salts in the mediun. An increase in the relative amount of DNA of telophase cells with time within single-strength MS medium was observed but not the double MS. Number of regenerants was higher from calli grown on double MS compared to that grown on the standard MS. With cytophotometry, it was possible to measure the amount of somaclonal variation induced by different culture media at the DNA level. Some of these variations can not be detected in the regenerants since cells with drastic chromosome aberrations lose their regeneration ability.

At this point, the author would like to suggest some research ideas for further study:

1. C- and/or N-banding of the EP mutant.
2. mRNA and cDNA analyses, DNA hybridization and sequencing.
3. Restriction Fragment Length Polymorphisms to identify variants.
4. Chlorophyll analysis of the somaclones.
5. Immunocytochemistry and electron microscopy of somaclones.
6. Field testing of the somaclones for yield quality and quantity.

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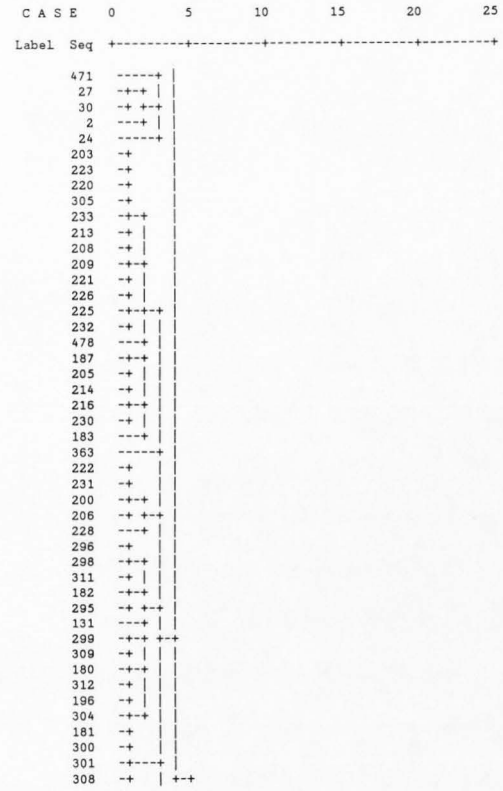
APPENDIX

Figures

Figure A1. Phenograms of somaclone seed proteins.

1-299 0.2 mg L^{-1} 2,4-D
300-355 2 mg L^{-1} Dicamba
356-451 3 mg L^{-1} Dicamba
453-490 Parental

Figure A1. (continued)



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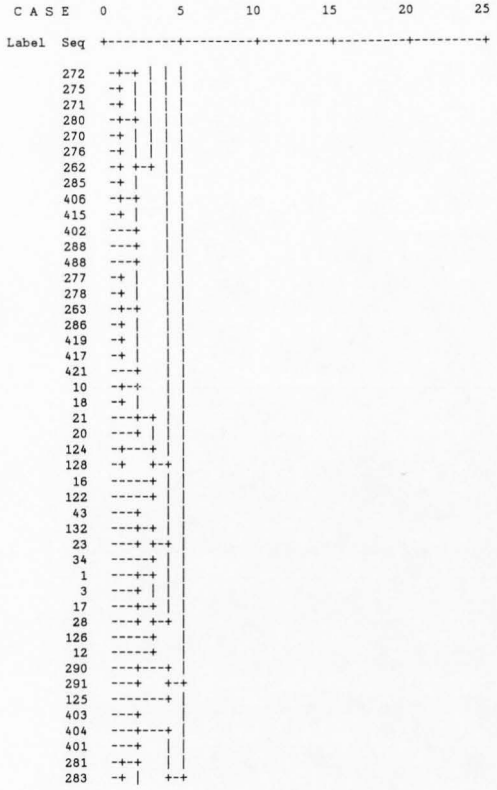

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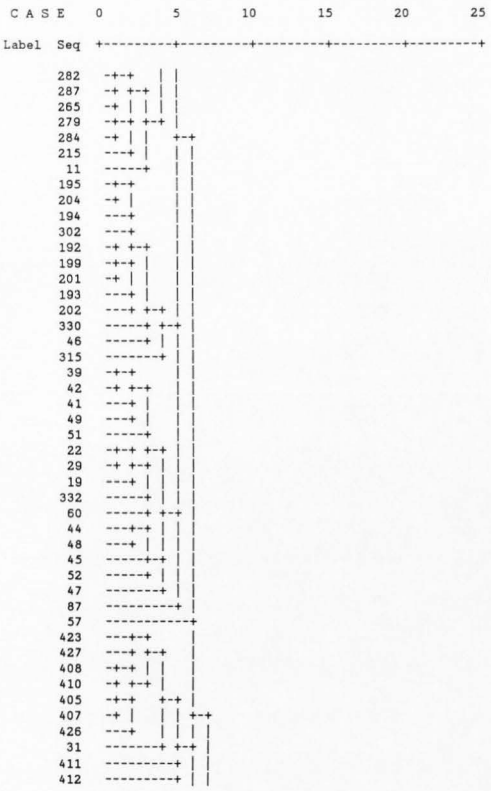

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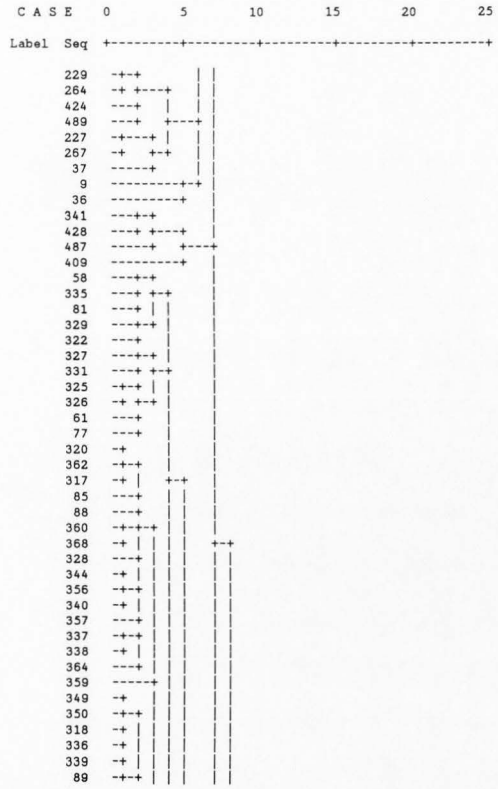

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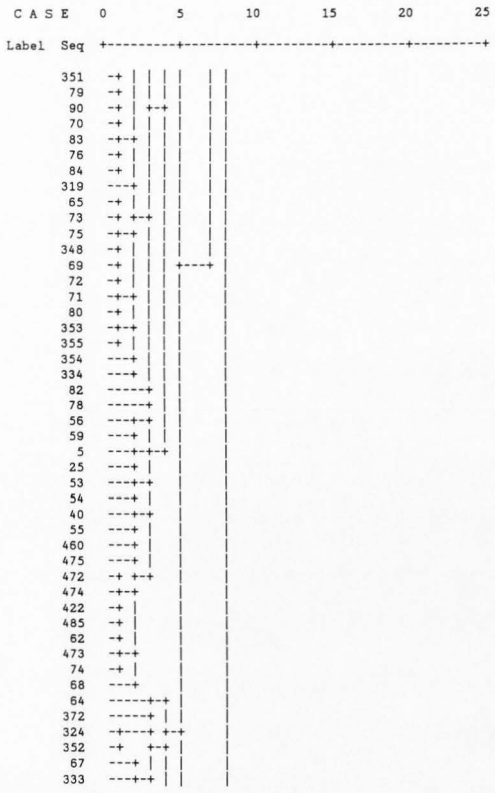

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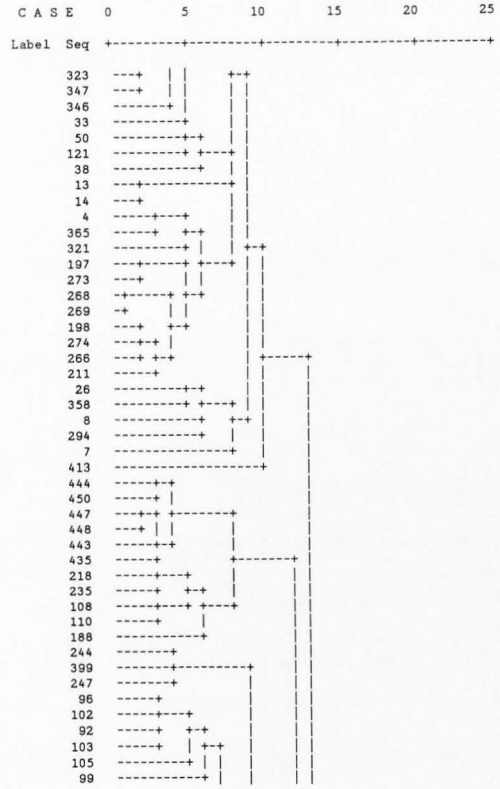

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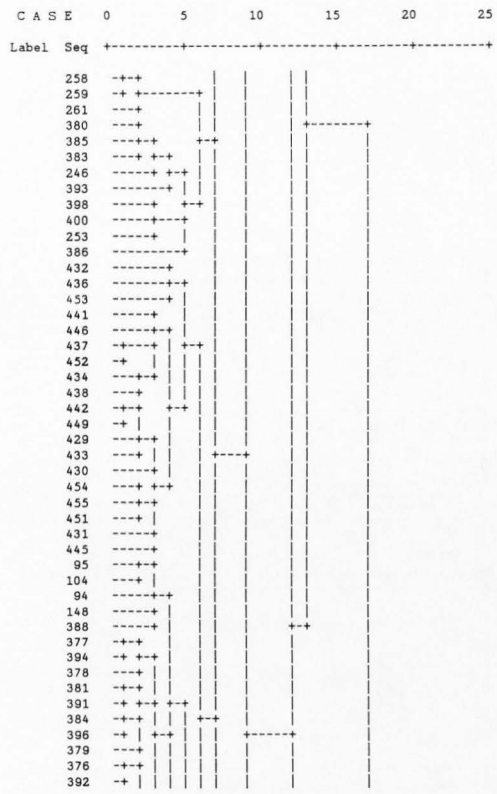

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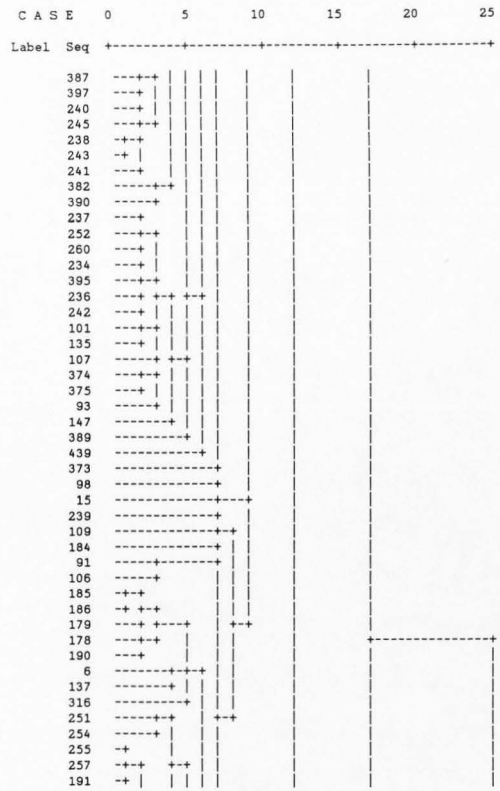

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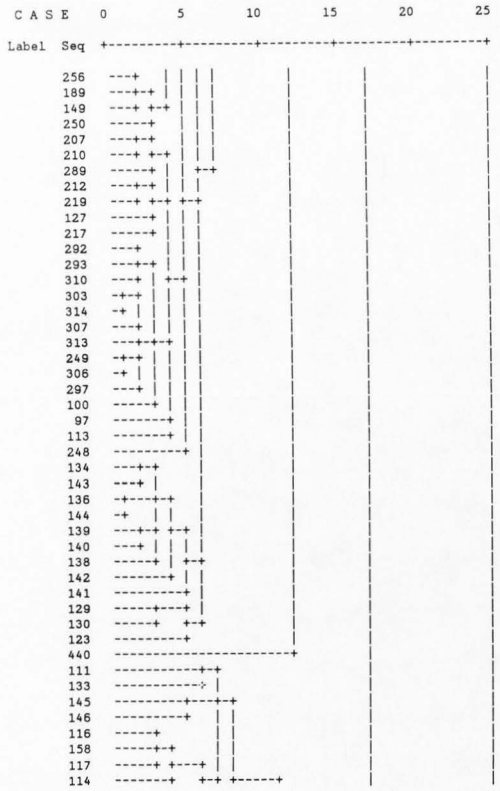

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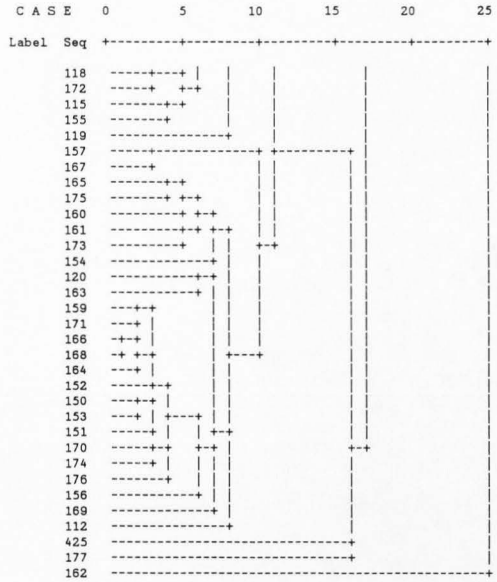

VITAE

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