# DEVELOPMENT AND IDENTIFICATION OF ALIEN-ADDITION LINES OF *Aegilops triuncialis* IN WHEAT

**Thesis**

Submitted to the Punjab Agricultural University
in partial fulfilment of the requirements
for the degree of
**MASTER OF SCIENCE**
in
**PLANT BREEDING**
(Minor Subject : Biotechnology)

by
**Ranvir Singh Gill**
(L-92-A-209-M)

**Department of Plant Breeding**
**College of Agriculture**
PUNJAB AGRICULTURAL UNIVERSITY
LUDHIANA-141 004
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Dedicated

to my

Loving Parents

"TO WHOM I OWE MUCH MORE THAN I CAN SAY"

2-1-96. Dean/Head Deptt of Plant Breeding, PAU LDH.

# CERTIFICATE I

This is to certify that the thesis entitled, "Development and Identification of Alien Addition Lines of *Aegilops triuncialis* in Wheat", submitted for the degree of Master of Science in the subject of Plant Breeding (Minor subject: Biotechnology) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Mr. Ranvir Singh Gill** (L-92-A-209-M) under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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**(DR H.S. Dhaliwal)** 4/10/95
MAJOR ADVISOR
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# CERTIFICATE II

This is to certify that the thesis entitled, "Development and Identification of Alien Addition Lines of *Aegilops triuncialis* in Wheat" by **Mr. Ranvir Singh Gill** (L-92-A-209-M) submitted to the Punjab Agricultural University, Ludhiana, in partial fulfilment of the requirements for the degree of Master of Science in the subject of Plant Breeding (Minor subject: Biotechnology) has been approved by Student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.

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RSGill

***(RANVIR SINGH GILL)***

Title of the thesis : Development and Identification of Alien-Addition Lines of *Aegilops triuncialis* in Wheat

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## ABSTRACT

The $BC_1F_2/BC_2$ and $BC_2F_1/BC_2F_2/BC_3$ seeds of an intergeneric cross of *Triticum aestivum cv. WL 711* (♀) x *Aegilops triuncialis* (♂) were investigated to develop alien addition lines of *Aegilops triuncialis* in wheat. The seeds of various backcross derivatives exhibited low germination, high sterility and low seed set due to the formation of unbalanced gametes and action of 'cuckoo' chromosome. A total of twelve monosomic and four disomic addition lines were identified by cytological investigations of fifty three plants, which were allocated to different homoeologous groups by using various markers. On the basis of grain characteristics, two monosomic addition plants having amber, bold grains, could be allocated to group seven. All other alien addition lines having slender, smaller and red grains could be allocated to homoeologous group three and further, to chromosome 3C ('Cuckoo' chromosome). The presence of homoeologous group one was also ruled out by SDS-PAGE of HMW glutenin subunit analysis. On the basis of disease reaction of various resistant $BC_2F_2$ plants, red grain colour and chromosome number, the alien chromosome present in these plants could be allocated to 3U having genes for yellow rust resistance, other chromosome probably 6U having genes for leaf rust resistance and unknown chromosome carrying resistance to both of the rusts. 'Cuckoo' chromosome was the major hinderance during the investigation due to which fourteen possible alien addition lines could not be obtained. The exploitation of a gene 'IgCI' which acts as dominant suppressor of 3C was suggested to obtain alien addition lines of all homoeologous groups. Alternatively, the exploitation of 'cuckoo chromosome' for stable transfer of desired characters, by producing translocations between 'cuckoo' chromosome and desired chromosome was suggested.

H.S.Dhaliwal
**(Major Advisor)**

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# CONTENTS

# CHAPTER I

## INTRODUCTION

Wheat is one of the most commonly consumed cereal of the world and ranks next to rice. The production of wheat in India increased from nearly 11 million tonnes in 1961 to 59.1 million tonnes in 1993-94 (Statistical Abstract of India, 1993-94) and from 1.7 million tonnes to 13.33 million tonnes in Punjab in the same period.

This increased production is largely attributed to the development of high yielding varieties through various breeding programmes. The burgeoning human population necessitates that this trend of improvement should be maintained.

However, in the recent decades, the genetic variability of cultivated wheats has been greatly eroded and the diminished pool of useful variability has been extensively exploited by modern plant breeding practices thus increasing its vulnerability to different diseases and other stresses.

To stabilize wheat production, minimize the use of pesticides and to improve the quality of wheat products, it is necessary to transfer the genes from diverse germplasm of related wild species. The development of alien addition lines of the donor species is a pre-requisite to such transfers.

Alien addition lines of a number of species including *Secale cereale, Hordeum vulgare, Aegilops* species, *Agropyron* species and *Haynaldia* etc., have been developed (Driscoll 1983, Sharma and Gill 1983, Islam and Shephard 1988, Vansiffhout 1989).

The introduction of alien genetic material into hexaploid wheat has been further facilitated by cytological and molecular techniques for rapid and accurate identification and characterization of alien chromatin in derivatives of wheat-alien species crosses. A number of morphological and or phenotype markers were employed for this purpose. The modern techniques which include heterochromatic banding patterns (Gill and Kimber 1974, Gill *et al*. 1988), and biochemical markers mainly isozymes (Driscoll and Sears 1971) are very useful. More recently the Restriction Fragment Length Polymorphism (RFLP) markers (Sharp *et al*. 1988, **Apples** and Dvora'k 1982) and RAPD-markers (Williams *et al*. 1990, Kings *et al*. 1993) have been found useful in increasing the efficiency of characterization.

Among the related wild species, the species of *Aegilops* possess vast variability for disease resistance and tolerance to other stresses (Dhaliwal and Gill 1983).

*Aegilops triuncialis* has been found a promising source for resistance to rusts, leaf spot diseases and powdery mildew (Dhaliwal *et al*. 1993). Also, it has been found to carry some new alleles for high molecular weight glutenin subunits (Singh *et al*. 1994).

The intergeneric crosses between wheat and *Aegilops triuncialis* were in different segregating generations at the Biotechnology Centre, Punjab Agricultural University, Ludhiana. The present investigation was, therefore, undertaken to:

a) Develop the alien addition lines of *Aegilops triuncialis* in *Triticum aestivum* cv. WL711 and

b) To allocate the alien chromosomes of different addition lines to particular homoeologous groups by using various markers.

CHAPTER II

## REVIEW OF LITERATURE

To stabilize wheat production at enhanced level of production, it is imperative to broaden the gene pool of cultivated wheats through transfer of genes from diverse germplasm of related wild species. The development of alien-wheat addition lines of the donor species is a prerequisite to this transfer.

A brief review of literature pertaining to various aspects of development and identification of alien addition lines is presented under the following sub-heads:

1. Development of alien addition lines.
2. Basic studies related to the development.
3. Characterization of alien addition lines by using:
    - a) Morphological and disease resistance markers.
    - b) Cytological markers
    - c) Biochemical markers, and
    - d) Molecular markers.

### 1. DEVELOPMENT OF ALIEN ADDITION LINES

In 1876 Wilson, for the first time presented some completely sterile ears of probable wheat-rye hybrids for the consideration of Botanical Society of Edinburgh. This made a beginning for plant breeders to expand the gene pool of wheat by introducing variation from related wild species and exotic grasses of Triticeae.

In wheat, the wheat like plants with alien characters were being studied in 1920's, for example, hairy neck character of rye (Leighty and Taylor, 1924). However, the first unequivocal addition lines were reported by Florell (1931), who isolated cytologically confirmed disomic addition lines from back cross progenies of a hexaploid wheat x rye $F_1$ hybrid.

O'Mara (1940) published, what is now regarded as standard method of producing alien addition lines via an amphiploid crossed with wheat, followed by repeated backcrossing.

Since then, a number of alien addition lines of various species (listed by Driscoll 1983, Sharma and Gill, 1983, Islam and Shephard 1988) have been produced by a variety of methods:

1. Via $F_1$ hybrids (Florell, 1931).
2. Via amphiploid production (O'Mara, 1940).
3. Via bridging species (Hyde, 1953) and
4. Via induced chromosome elimination method (Islam *et al.* 1975).

## 2. BASIC STUDIES

Sears 1953 made nullisomic analysis in wheat for determining the position of various genes on different chromosomes. However, due to reduced vigour and fertility only two nullisomic lines were maintained and for the remaining 19 chromosomes monosomic analysis was employed. During the production of monosomics the transmission rate of univalent chromosome through male gametes was estiamted to be 4 per cent.

Bowden (1959) suggested that many relatives of hexaploid wheat (*Triticum aestivum*) are known to carry interesting genes. These genes might be useful in broadening the genetic base of *Triticum aestivum*. The genus *Aegilops* is closely related to wheat and many of its species can be easily crossed with wheat. The amphiploids and later on derived chromosome addition lines should be the first step towards incorporating such genes into cultivars.

Riley (1960) studied the meiotic behaviour, sterility and fertility of alien addition lines of rye in wheat. He found that lines with single pair of rye chromosomes added to full complement of wheat were less regular at meiosis. This was due to the failure of rye chromosomes to pair which led to the loss of the rye chromosomes in addition lines, thereby returning to euploid state. No addition line was found as fertile as the wheat variety, although marked differences in fertility of different addition lines were observed.

Alston and Jones (1968) found that the transmission of univalents in pentaploids of *Triticum aestivum* and *Triticum durum* differed when backcrossed to *T.aestivum* and *T.durum* genotypes. A deviation was observed in normal binomial distribution of alien chromosomes when *T.durum* was used as a recurrent parent. The various causes of deviation from normal distribution were postulated.

Makino (1974) backcrossed a natural pentaploid (produced from a cross between *T.spelta* and *T.durum*) to durum parents and observed the transmission rate of D-genome chromosomes through female gamete to be 2.64 per cent for natural pentaploids and 1.23 per cent for synthetic pentaploids. Various causes of low transmission of alien chromosomes were made.

Makino (1976) developed all the seven possible monosomic addition lines of *Aegilops umbellulata* in *Triticum durum*. On selfing these addition lines, the transmission rate of univalent varied from 4.5 to 25.9 per cent through female gametes and 0.9 to 3.1 per cent through male gametes.

Yashvir and Kesawan (1978) studied the transmission and cytological behaviour of three D genome monosomic addition lines and one dimonosomic addition line in *Triticum durum*; and their selfed progenies. The frequency of monosomic addition lines in its selfed progenies was 14.7, 27.5 and 40 per cent and frequency of monosomic addition in the progeny of double monosomic was 8.4 per cent. But no disomic addition plant was obtained in the progeny of double monosomic addition line indicating zero per cent male transmission.

Ornella *et al.* (1984) based on studies of a range of wheat/rye addition lines and substitution lines by C-banding revealed that both wheat and rye chromosomes affect each other's homoeologous pairing.

The strongest reduction of wheat pairing was produced by chromosome 5R of rye and the weakest diminution was produced by 3R. The reduction in pairing of rye chromosomes by wheat chromosomes was from 6.9 to 48.4 per cent in different addition lines.

Miller (1984a) during the development of alien addition lines of rye in wheat found that disomic addition lines were not entirely stable. They lost the added chromosome during successive generations reverting to the euploid state. Any 43 chromosome addition in the progeny of disomic addition lines in the next generation was found to give rise to 42 chromosome plants. The addition lines became less frequent in successive generations and thus required cytological analysis after every generation for maintenance.

Lacadena and Wranenco (1985) studied the nucleolar organizer activity of wheat-*Aegilops umbellulata* addition lines and concluded that U genome was predominant with respect to nucleolar competition in Triticeae.

Yachevskaya (1985) selected the plants with 43-50 chromosomes from $F_2$ of a cross between *Triticum aestivum* - *Agropyron intermedium* to study the inheritance of chromosomes in different classes. In the progeny of monosomic addition lines the alien chromosome was retained in 35 per cent plants while 5 per cent had disomic chromosome additions. The selection of plants with 43-46 chromosomes was found to be the best choice for obtaining addition lines as plants with higher chromosome number gave no addition lines.

Mukai (1985) found that alloplasmic wheat addition lines carrying an alien chromosome of *Agropyron intermedium* when crossed with Chinese Spring, the $F_1$ hybrids germinated poorly. The germinated grains had short roots whose meiotic chromosomes contained many aberrations. The

*Agropyron intermedium* chromosome was found to be responsible for such observations.

Fedak (1985) reviewed various useful species of *Agropyron*, *Aegilops* and *Triticum* for enhanced levels of tolerance to drought, cold and salinity and of increased photosynthesis and high grain protein content. Also, most of the species were crossed with *Triticum aestivum* and in some cases addition lines were developed.

Similarly, Gill *et al*. (1987) screened different accessions of wheat and related species for resistance to rusts and powdery mildew. Different accessions of *Triticum aestivum* as well as *Aegilops* showing resistance to some diseases were identified.

Dhaliwal *et al*. (1987) identified that chromosomes 6R and 7R of rye and 1H and 4H of barley carried gene(s) for resistance to Karnal bunt, after screening of previously characterized addition lines under artificial conditions.

Chrzastek (1987) found that in wheat-rye addition lines the frequency of 42 chromosomes increased with each generation of selfing. The frequency of euploid plants in the progeny of 43 chromosome plants was higher than for 44 chromosomes. Moreover, the progeny of monosomic and disomic addition lines was found to contain mainly plants with 42, 43 and 44 chromosomes, the percentage of these being different in different lines.

Blanco and Simeone (1987) during the production of alien addition lines of *Dasypyrum villosum* in *Triticum durum* observed 7.5 to 27.7 per cent transmission frequency of different addition lines. Each chromosome was found to have a specific effect on morphology and fertility. Also monotelosomic addition plants derived from a high frequency of abnormal chromosome divisions were isolated.

Lukasjewskii (1988) made a comparison of the different approaches used for development of disomic addition lines i.e., by use of multiple monosomic addition lines as a source of disomic additions, backcross of heptaploids to octaploids and using haploids obtained through crosses to *Hordeum bulbosum*. The second approach was found to require least labour.

Xue *et al.* (1988) made cytological studies of barley chromosome additions in wheat. The average frequency of bivalents and univalents at metaphase-I of pollen mother cells was 20.6 and 0.66 per cell respectively. The studies showed that fertile alloplasmic wheat barley addition lines were produced but the progenies were unstable having somatic chromosome numbers from 41 to 44 with only 4.3 per cent of progenies with 2n = 43.

Sharma (1989) based on studies of transmission of whole and telo-chromosomes in alien addition lines of *Aegilops trachycaullus* in wheat found that pollen carrying an extra chromosome was at selective disadvantage. Also the plants with 2n = 42 were more vigorous as compared to the plants with 2n = 43.

Dyck *et al* (1990) used chromosome 5B method to promote homoeologous pairing for transferring resistance from *Aegilops caudata* to common wheat. In the initial cross, monosomic 5B of wheat was used and the resulting resistant plants were taken for a series of backcrossing. The selection was also practised from similar crosses with *Aegilops triuncialis* and the resistant plants were found to be chromosome addition lines.

Claesson *et al*. (1990) proposed that the crossability of *Aegilops triuncialis* with wheat can be increased to 67 per cent from 32 per cent (Ogzen, 1983) by using *Triticum aestivum* as a male parent rather than female parent.

Singh *et al*. (1992) during the production of crosses of WL711 x *Aegilops triuncialis* and WL 711 x *Aegilops ovata* for transfer of disease resistance observed low seed set (25 and 9% respectively) in both crosses. The hybrids were completely male and partially female sterile. The meiotic pairing in both the crosses was also very low.

Dhaliwal *et al.* (1993) under the project "Evaluation and cataloguing of wild and tetraploid progenitor species of cultivated wheats" at Punjab Agricultural University evaluated more than 14000 accessions of germplasm of cultivated wheats and related wild species for resistance against leaf and stripe rusts powdery mildew foliar diseases, Karnal bunt etc. Among the polyploid *Aegilops* species, *Aegilops triuncialis*, *Aegilops ovata* and *Aegilops triaristrata* were the promising sources. A high proportion of lines of *Aegilops triuncialis* were resistant to leaf rust (95%), stripe rust (85%) and powdery mildew (80%).

The *Aegilops triuncialis* was also found to be a good source for transferring new alleles of HMW glutenin sub units for improving the quality of wheat (Singh *et al.* 1994).

Bai *et al.* (1994) found that the backcrossing of $F_1$ hybrids to wheat was more difficult (seed set 0-7.4%) than to produce $F_1$ hybrids (seed set 12.5 - 78.33%) during development of *Aegilops triaristratum* wheat addition lines. The low fertility of $F_1$ hybrids was due to low chromosome pairing. Gametes with complete or nearly complete genomes from $F_1$ were only viable.

**Cuckoo Chromosomes**

The tendency of certain alien chromosomes to be preferentially transmitted during backcross to wheat has been recognized as an appreciable hindrance in the production of wheat alien addition lines.

Mann (1975) obtained partially male and female sterile plants having monosomic addition or substitution during a series of backcrossing programme carried for production of cytoplasmic substitution lines of *Triticum aestivum* into cytoplasm of *Aegilops longissima* and *Aegilops sharonensis*. The selfed progenies of these lines were fertile. It was found that the male and female gametes having a particular *Aegilops* chromosome were functional while the gametes lacking it were non-functional; thereby suggesting an apparent gametocidal action of particular *Aegilops* chromosome leading to its preferential transmission.

Similarly Endo and Tseunawaki (1975) during the production of cytoplasmic substitution lines of *Triticum aestivum* cv. Selkrik and 'King' II and *Triticum spelta* with the cytoplasm of *Aegilops triuncialis* observed partial male sterility (25%) and high female sterility. The sterility was associated with preferentially transmitted sub-terminal chromosome (i chromosome of *Aegilops triuncialis*) through the gametes of both sexes. The selfed progeny of cytoplasmic substitution lines was fertile and was having 2n=43 having i as an extra chromosome.

Endo (1978) made an approach to develop alien-addition lines of *Aegilops triuncialis* in common wheat. But he failed to obtain chromosome addition lines of alien chromosome from different homoeologous groups and was able to obtain addition lines of one type only. Further, a study was conducted on gametocidal action of *Aegilops* chromosome in common wheat. A subterminal chromosome of both *Aegilops triuncialis* and synthetic *triuncialis* was found to cause severe sterility in all cultivars of wheat except a wheat cultivar 'Norin 26'. Sterility was revealed gametophytic in nature and was due to a particular chromosome of *Aegilops triuncialis* or synthetic *triuncialis* in monosomic state. Most of the gametes especially egg cells lacking that chromosome could not function in fertilization leading to the preferential transmission of chromosome to the offspring. The *Aegilops triuncialis* and synthetic *triuncialis* chromosomes were demonstrated to be very similar to each other with respect to structure and gametocidal action.

Using monosomic lines of Chinese Spring the *triuncialis* chromosomes was found to carry all the markers of homeologous group three. So the *triuncialis* chromosome was allocated to homoeologous group three. The gametocidal genes of *Aegilops triuncialis* were suggested to originate from *Aegilops caudata* (C genome).

Similar types of genes that provide 'Cuckoo' quality to chromosomes leading to preferential transmission have been reported in *Aegilops caudata* (Endo and Katayama 1979) and *Aegilops cylindrica* (Endo 1979).

Miller *et al*. (1982) obtained only one addition line of *Aegilops sharonensis* in *Triticum aestivum* after a series of backcrossing. This was due to preferential transmission of *Aegilops* chromosome. The chromosome was investigated in detail by C-banding and genomic *in situ* hybridization and was allocated to homoeologous group four designated as $4S^{l}$.

Miller (1984b) studied the action of Cuckoo chromosomes in monosomic additions of *Aegilops sharonensis* to wheat. Meiosis was normal in anthers and ovules with alien chromosome as univalent at metaphase-I. Abnormality was observed in the first embryo sac mitosis and first pollen grain mitosis in about half ovules and other microspores of each addition plants. At the abnormal metaphases 'cuckoo' chromosomes were found to act with wheat chromosomes leading to the formation of small, lethal, acentric segments in those gametophytes lacking a particular alien chromosome.

Tsujimoto and Tsunewaki (1985) located a gene designated as Igc1 on chromosome 3B of wheat cultivar Norin-26. The gene has been reproted to act as dominant suppressor of gametocidal gene on chromosome 3C. In the presence of gene, the 3C chromosome did not show any preferential transmission and was distributed in normal frequency as of other alien-chromosomes. So, the use of this gene for obtaining alien-addition lines from *Aegilops* species having 'cuckoo' chromosome (*Aegilops caudata*, *Ae.triuncialis*) was suggested.

Miazga *et al*. (1988) analyzed the monosomic and disomic rye addition lines in cv. Grana at Metaphase-I. The frequency of rod and ring bivalents was found to depend upon the added chromosomes. There were more univalents in all the addition lines except those disomic for 4R chromosomes which was found to be transmitted more often and stably among addition lines.

Endo (1988) reported the occurrence of chromosome mutations in the progeny of certain alien addition and substitution lines of *Triticum aestivum* having the alien chromosomes from *Aegilops cylindrica, Ae.triuncialis, Ae.sharonensis* and *Ae.longissima*. The mutations were studied by chromosome banding and were found mostly deletions and translocations, which occurred at various sites of chromosomes. The monosomic alien additions of *Aegilops cylindrica* or *Ae.triuncialis* chromosome induced mutations almost exclusively in gameotyophytes lacking the alien chromosome leading to sterility of those gametes. However, the alien chromosomes in disomic condition rarely caused sterility and chromosomal abnormality; but they were found to induce high rates of various chromosomal mutations in certain genetic backgrounds.

Further, it was reported that on crossing a disomic addition line of *Triticum aestivum* (having alien chromosome of group 3 of *Aegilops triuncialis*) with *Trititum aestivum* cv. Burgas in which 1B chromosome pair was substituted by 1R of rye, a high rate of chromosomal mutations in rye chromosome were found which included four translocations with wheat chromosomes. It was suggested that such chromosomal mutations can also occur in other chromosomes possessing useful genes and such chromosomal mutations induced by *Ae.triuncialis* or *Ae.cylindrica* chromosomes can be exploited by breeding programmes.

The transmission frequency of a preferentially transmitted chromosome of *Aegilops sharonensis* ($4S^l$) was found to be 97.8 per cent by King *et al*. (1990).

King and Koebner (1991) exploited the phenomena of preferential transmission of chromosome $4S^l$ of *Aegilops sharonensis* to eliminate segregation of height in semi-dwarf wheat varieties. The translocation involving short arm of *Aegilops sharonensis* and $Rht_2$ carrying arm of chromosome 4D in *Triticum aestivum* were produced. The translocation were found to be stable and preferentially transmitted both by pollen and egg; thus ensuring homozygosity of $Rht_2$.

Similarly King *et al*. (1992) used $4S^l$ chromosomes of *Aegilops sharonensis* for the transfer of *Gli*-U1 and *Glu*-U1 genes. Translocation were produced between long arm of $4S^l$ carrying gene for preferential transmission and short and long arms of 1U of *Ae.umbellulata*; carrying Gli U1 and Glu U1 respectively.

The selection of translocation was practised on the basis of an aminopeptidase isozyme ($4S^l$ short arms) and GliU 1 and Glu-U1. All these non-wheat protein and isozymes were electrophoretically different from their wheat equivalents which aided in selection. The translocation were found stable and preferentially transmitted.

## 3. CHARACTERIZATION OF ALIEN ADDITION LINES

Once addition lines either monosomic or disomic are isolated, it is necessary to determine the identity of alien chromosomes for allocating it to a particular homoeologous group.

Alien chromosomes present in aneuploid strains of wheat have been distinguished from one another by their karyotype, their effects on plant phenotypes and by meiotic pairing with particular cytogenetic stocks, (Hart 1979). Many related species have Karyotype with chromosomes morphologically distinct from those of wheat. The wheat chromosomes have either median or sub-median chromosomes whereas many alien species for example, *Aegilops aucheri*, *Aegilops heldreichii* and *Aegilops sharonensis* have chromosomes with subterminal centromere (Chenneverriah 1960).

The heterochromatic banding patterns mainly C-banding has been used as a major identification criteria for characterizing alien addition lines of a number of species (Gill and Kimber, 1974). *In situ* hybridization with highly repeated DNA sequences as probes has also been used (Jones and Flavell 1982).

A number of protein structural genes particularly those coding for isozymes are highly effective for the identification of alien-chromosomes as well as for determining their homoeology (Driscoll and Sears 1971).

The availability of large number of different categories of molecular markers has revolutionalized the genetic analysis. Restriction fragment length polymorphism (RFLPs) has shown to be one of the most powerful tools for detection and evaluation of genetic variation in germplasm collection of plants and monitoring and characterization of alien genetic transfers during different breeding programmes (Helentjaris *et al*. 1985, Sharp *et al*. 1988).

The randomly amplified polymorphic DNA (RAPD) markers using polymerase chains reactions (PCR) (Williams *et al*. 1990) has also been found useful in characterizing alien introduction in wheat (King *et al*. 1993).

### (a) Characterization of Alien-addition lines on the basis of phenotype/morphological markers and disease analysis

One of the earliest reports of a phenotype effect of a specific alien-chromosome in a wheat background was made by Florell (1931) who described the association of 'hairy neck' character of rye with a single specific chromosome of rye added to wheat. Riley and Chapman (1958) extended this idea to show that distinct phenotypes could be associated with other individual rye chromosomes.

Since then,a number of alien addition lines (Table 1) have been identified by using different morphological markers.

Also information on homoeology of alien chromosomes has been made on the basis of information regarding location of genes controlling a particular morphological character.

Similarly, different alien addition lines have been characterized (Table 1) on the basis of reaction to a particular disease; linked to a specific alien chromosome.

## Chromosomal location of genes controlling grain morphological characteristics (colour and size)

Sears (1944) made nullisomic analysis in *Triticum aestivum* var. Chinese Spring and studied the association of various morphological characters with different chromosomes. He located one of the three complex loci responsible for grain colour (Nelson-Ehle 1911) on chromosome 3D. Nelson-Ehle (1911) had established that red vs. white seed colour in wheat (*Triticum aestivum* L.) was conditioned by alleles at three different loci, with red being dominant. Metzger and Sialbagh (1970) based on monosomic analysis of 'Red Bobs' and 'Kharkof's wheat (*Triticum aestivum*) further ascertained that three homeologous loci located on chromosomes 3A, 3B and 3D

Table 1: Various addition lines identified by phenotypes or morphological markers and disease reaction

| Alien species | Wheat cultivar | Alien chromosome | Cytological, morphological and disease resistace markers | Reference |
|---|---|---|---|---|
| *Secale cereale* cv. King-II | Hold fast winter wheat | I<br><br>II<br>III<br>IV<br>V1 | 22", 21"+t"L<br>HP on long arm<br>Red grains and Yr on long arm<br>22", 21"+t"α Pm on α arm<br>Purple straw on long arm<br>22", 21"+tα" Juvenile tussocky habit on αarm. | Riley and Chapman (1958) |
| *Secale cereale* cv. Rosein | Cornell wheat selection 82 AI.2.4.7 | 2R | 21"+t"α, Lr. 25 and Pm 27 on long arm | Driscoll and Jenson(1963) |
| *Secale cereale* cv. Petkus | Norms 40 winter wheat | 1 | 21" + t"α.<br>Lr on α arm | *Mukade *et al.* (1970) |
| *Secale cereale* cv. Imperial | Chinese Spring | 1R<br>2R<br>3R<br>5R | 22", Sr<br>22", Awn promotion<br>22", Sr 27 on short arm<br>22", HP on long arm | Sears and Driscoll (1971) |
| *Secale cereale* cv. Weser | Chinese Spring | 5R | 21"+1', 21"+t"L. HP on long arm<br>22", | **Chang *et al.*(1973) |
| *Agropyron elongatum* 2n=70 | Thatcher Spring wheat | $Ae^4$ | 22", Blue Aleurone | Knott(1958) |
| *Agropyron elongatum* 2n=70 | Chinese Spring | 3el | 22", Lr 24 and Sr. | Schlehuber and Sebesta (1958) |

| | | | | |
|---|---|---|---|---|
| *Agropyron elongatum* | Thatcher Spring wheat | 6el<br>7el | 22", 21" + t"$\alpha$<br>Sr 26 on $\alpha$ arm<br>22", 21" + t" $\alpha$<br>Yellow flour on $\alpha$arm<br>Lr 19 and Sr 25 | Knott(1961) |
| *Agropyron elongatum* (2n=70) | Rescue | Ae4<br>Ae5<br>Ae6 | 22", Blue Aleurone<br>22", Nodding head<br>22", Wsm | Larson *et al.*(1973) |
| *Agropyron intermedium* | Norin-27<br>Wintr wheat | 5 Agi. | 22", Wsm | Cauderon *et al.*(1973) |
| *Agropyron intermedium* | Winter wheat | - | 22", Sr Lr, on $\alpha$\arm, Yr on ßarm | Weinheus(1973) |
| *Agropyron Intermedium* | C-115093 | Ai4 | 22", Wsm | Wells, *et al.*(1974) |
| *Agropyron glaucum* | Nambukangi winter wheat | - | 22"+1', Lr | Mukade, *et al.*(1974) |
| *Agropyron elongatum* 2n=14 | Chinese Spring | 2E<br>VI<br>7E | 22", 21"+t"L. awn promotion on long arm<br>22", 21"+t"L. Wax suppressor and tenacious glumes on long arm<br>22", 21"+t"$\alpha$ Purple straw on $\alpha$-arm | Dvorak and Knott(1974) |
| *Agropyron trichophorum* 2n=28 | Blue Baart<br>Spring Winter | IV | 21"+t" Blue aleurone | Chapman and Miller, (1978) |
| *Agropyron elongatum* 2n=70 | Chinese Spring | 4E | Sr 21"+1' | Bains *et al.*(1992) |
| *Aegilops umbellulata* | Chinese Spring | 6U | 22", Lr 9 on Long arm | Sears and Kimber(1956) |
| *Aegilops umbellulata* | Chinese Spring | 7U or G | 22" late flowering on long arm short ears | Kimber(1966) |

| | | | | |
|---|---|---|---|---|
| *Aegilops umbellulata* | Chinese Spring | 5U<br>2U or D<br>E | 22", short plant and head<br>22", Awn promotion on long arm<br>22", short plant and head | Kimber 1966 |
| *Aegilops comosa* | Chinese Spring | 2M | 22", 21"+t"s.<br>Yr 8 on short arm | Riley and Chapman 1968 |
| *Aegilops bicornis* | Holdfast winter wheat | $3S^b$<br>7 | 22", red grain and brittle rachis<br>Purple straw on ß arm | Riley and Chapman, 1968 |
| *Aegilops variabilis* | Chinese Spring<br>Chinese Spring/ POSO | Chromosome<br>A<br>C<br>E<br>G<br>H<br>N<br>O | 22" Black glume<br>21"+1′ Open head<br>21"+1′ Open head, emerges side of sheath<br>22". Waxless awn promotion<br>21"+1′ Short plant open head<br>21"+1′ Awn promotion nodding head<br>22" Open head, short plant, CCn | Jewell and Driscoll, 1974 |
| *Aegilops ventricosa* | Chinese Spring | 6MV | 22", Resistance to *Heterodera avenae* | Rivoal *et al.*, 1986. |
| *Aegilops squarrosa* | *Triticum durum* cv. PBW-114 | 2D<br>3D and<br>6D<br>6D<br>1D and 3D | Genes for susceptibility to Lr and Sr. vitorous grains<br>Genes for resistance to Kb<br>Round grains<br>Small grains and narrow leaves | Dhaliwal, *et al.* 1990 |
| *Haynaeldia villosa* | Ningmai 7317 Spring Wheat | $V_2$ | 22", Pm | Liu *et al.*, 1988. |

Sr - Stem rust
Yr - Yellow rust
Lr - Leaf rust
KB - Karnal bunt
Wcm - Wheat curl mite
Wsm - Wheat Streak mosaice virus
PM - Powdery mildew
CCN - Cereal cyst nematode
Hp - Hairy peduncle

control grain colour in wheat. The genes were designated as $R_2$, $R_3$ and $R_1$ respectively.

Bocklhurst (1977) in an experiment with two cultivars of wheat studied the variation in grain size. It was found to depend upon rate of accumulation of dry matter which in turn was governed by the number of endosperm cells formed. The cell number in endosperm seemed to be regualted by the supply of assimilates to the developing grains.

Gleadow *et al.* (1982) studied the grain growth in six wheat lines which represented a wide range of mature grain weight. It was found that within the large grained group, the differences were primarily a function of endosperm cell number as starch granules per cell were nearly equal. There was no significant difference between the lines in size of either A-type or B-type granules although the ratio of B-type to A-type granules differed significantly between the lines.

Chozecki *et al.* (1983) studied the effect of individual chromosomes on large differences in grain size by making reciprocal monosomic analysis between Chinese Spring and a large grained variety, Spica. Chromosomes 1A, 1D and 7A were associated with effects on grain size of over 10 per cent each with no compensating effects on grain number. Chromosomes 1D and 7A influenced all the grains within ear, while 1A effected only the grains in the second and third florets.

The grain size differences were correlated with endosperm cell number and specific effects on the pattern of starch granules growth. Chromosome 7A affected the number of A-type starch granules (amyloplasts) per endosperm, while 1A and 1D effected total number of starch granules per endosperm and the volume of A-type granules.

### (b) Cytological markers

Various cytogenetic techniques such as Karyotype analysis and heterochromatic banding patterns have been used for characterizing alien genetic material in wheat background during development of alien addition and translocation lines.

Gill and Kimber (1974) identified all the seven disomic additions of imperial rye chromosomes and classified these on the basis of their relatedness with wheat chromosomes.

Makino (1981) identified alien addition lines of *Aegilops squarrosa* and *Aegilops umbellulata* and *Triticum speltoides* in *Triticum durum* on the basis of cytological studies and allocated different addition lines to seven homoeologous groups.

Islam and Shephard (1981) identified six out of the seven possible disomic addition lines of barley in wheat by isozyme studies and allocated these to different homoeologous groups on account of homeology with wheat chromosome. They used N-banding technique to confirm authenticity of individual addition lines.

Jewell and Driscoll (1983) characterized nine alien addition lines of *Aegilops variablis* in *Triticum aestivum* on the basis of N-banding patterns.

Liu *et al* (1986) identified different wheat rye addition lines on the basis of Giemsa C-banding and allocated these to different homoeologous groups.

Fernandez and Jouve (1988) used a combination of morphological, biochemical and cytological markers (C-banding) to identify *Hordeum chilense* chromosome addition lines in *Triticum durum*. Information on homology of alien chromosomes was also given on the basis of previously assigned morphological markers to different chromosomes and isozymes markers.

Bhullar *et al.* (1988) characterized D-genome monosomic addition lines in *Triticum durum* on the basis of karyotype analysis.

Gill *et al.* 1988 proposed a standard nomenclature for the designation and description of heterochromatic bands (C-bands and N-bands) on the metaphase chromosome maps of *Triticum aestivum* cv. Chinese Spring.

Friebe and Heun (1989) used C-banding technique for the identification of *Triticum ovatum* addition lines in wheat.

Dhaliwal *et al.* (1991) developed a complete set of monosomic addition lines of *Aegilops squarrosa* in *Triticum durum* by using Giemsa C-banding.

Mukai and Gill (1991) used *in situ* hybridization to detect barley chromatin added to wheat. Using this technique the behaviour of specific barley chromosomes was analyzed at interphase and prophase cells and also a genomic clone of $\alpha$-amylase gene containing a specific dispersed repeat sequence was used to identify barley chromosome additions to wheat

Melz *et al.* (1991) identified the alien addition lines of wheat to rye by C-banding and polyacrylamide gel electrophoresis and allocated these to seven homoeologous groups of wheat.

Friebe *et al.* (1991) identified alien chromatin of *Agropyron intermedium* controlling wheat streak mosaic virus resistance and of *Aegilops speltoides* for green bug resistance in wheat by using C-banding technique and *in situ* hybridization.

Hueros *et al.* (1991) identified six alien addition lines of *Aegilops longissima* chromosomes in wheat by using C-banding technique.

Baum (1991) identified monosomic and double monosomic addition lines of wheat in rye by using C-banding technique and molecular markers.

### (c) Biochemical markers: (i) Isozyme studies

The isozyme variation has been used in the identification and characterization of alien chromosome(s) in derivatives of wheat alien species hybrids. Also the homoeology of alien chromosomes can be made by identifying the genes they express that are orthologous to *Triticum aestivum* isozyme genes whose chromosomal locations are known (Hart and Tuleen (1983a).

The first usage of an isozyme gene as chromosomal marker during the development of alien-addition lines was reported by Driscoll and Sears (1971) who practised selection for an esterase present in amphiploid of *Triticum aestivum* cv. Chinese Spring x Rye cv. Imperial but not found in any of the six available addition lines. This resulted in the selection of seventh possible disomic addition line now designated as 7R.

A series of seven disomic addition lines to Chinese Spring were identified by Tang and Hart (1975) on the basis of zymogram phenotypes of different isozymes. The zymogram phenotypes produced by one or more of the Imperial chromosomes was found to differ sufficiently from Chinese Spring so as to provide an evidence for absence or presence of rye chromosomes in Chinese Spring.

Also the alien chromosomes were allocated to different homoeologous groups based on the information of genes controlling a particular isozyme linked to specific wheat chromosome.

Hart (1979) by using gene mapping technique constructed an extensive map of *Triticum aestivum* in which the chromosomes were marked with different isozyme loci.

Four barley isozyme loci orthologous to wheat loci were identified and located in Betzes barley chromosomes by Hart *et al* (1980) which played an important role in the development and identification of Chinese Spring - *Hordeum vulgare* cv. Betzes addition lines series by Islam and Shephard (1981). Zymogram phenotypes of different isozymes showed that four disomic addition lines could be unambiguously distinguished from one another and from other three addition lines. Confirmation of the authenticity of isozyme studies was made by meiotic analyses and N-banding.

Delbris *et al.* (1981) on the basis of two biochemical markers CM4 (a protein component from chloroform:methanol extract) and Alpha a-b (Alkaline phosphatase isozymes) identified *Aegilops ventricosa* addition lines to *Triticum aestivum* and allocated these to group $4M^v$ on the basis of previous information on gene location for these markers. Isozymes were used as chromosome markers to characterize two Chinese Spring - *Elytriga elongata* chromosome addition lines by Hart and Tuleen (1983b). The selection was done on the basis of Est-1 and leaf peroxidase (lpx-2) loci which are othologous to loci located on chromosome 3p and 5q respectively. Then selection was practised for Glutamic acid oxaloacetate transminase (GOT-3) isozyme orthologous to 3q of Chinese Spring which lead to the successful isolation of putative 3 and 5 *Elytriga elongata* addition lines.

Hart and Tuleen (1983c) based on zymogram phenotypes of thirteen isozymes identified four *Aegilops longissima* addition lines in Chinese Spring and allocated them the homoeologous groups $2S^l$, $1S^l$, $5S^l$ and $3S^l$.

Salinas *et al*. (1985) characterized five different disomic addition lines based on peroxidase (per), $\alpha$-amylase ($\alpha$-amy), and acid phosphatase (acp) and esterase (est) zymogram phenotypes of Chinese Spring, betzes barley and presumptive betzes addition lines to Chinese Spring.

Six *Agropyron intermedium* addition lines and one ditelosomic addition line in Chinese Spring were also identified by Figueras *et al.* (1986) on the basis of zymogram phenotypes of different isozymes. Also homoeology was studied between wheat and alien chromosomes and chromosomes L1, L2, L3, L4, L5 and L7 were found homoeologous to wheat chromosomes 7, 3, 1, 4, 2 and 6 respectively.

Pietro *et al.* (1986) used a combination of cytological techniques and electrophoretic patterns of different isozymes to identify *Triticum searsii* alien addition lines in Chinese Spring. Ten *Triticum searsii* enzyme structural genes and four morphological markers were used to allocate alien chromosomes to different homoeologous groups.

Forester *et al.* (1987) developed and described six wheat *Agropyron intermedium* addition lines on the basis of phenotype and biochemical markers. An account of homoeology of each alien chromosome was also given. Fernandez and Jouve (1988) used nine isozymes as chromosomal markers to identify *Hordeum* chilense chromosome addition in *Triticum durum* along with morphological and cytological markers. An account of homoeology of alien chromosomes was also made.

Hart and Gale (1989) proposed a detailed genetic map of *Triticum aestivum* in which the different biochemical and molecular loci known so far have been mapped to different chromosome arms.

The homoeologous group two has genes for peroxidase (per-2) which is expressed in young leaves. Homoeologous groups three and four has genes for esterase and acid phosphatase, respectively.

Morris *et al.* (1990) used combination of cytological techniques and biochemical markers to identify six *Elymus trachycaulus* ($H^t$ genome) disomic addition lines in Chinese Spring. Also the alien chromosomes were allocated to different homoeologous groups by using different morphological and biochemical markers.

Zhong *et al* (1990) made isozyme studies of previously characterized disomic rye addition lines in wheat showing resistance and susceptibility to powdery mildew. Several new isoperoxidases were found linked with both resistant and susceptible lines.

Gill *et al.* (1991) provided a detailed genetic map of *Triticum tauschii* where different biochemical loci recognized so far have been shown marked to different chromosomes. Further, it was found that the two accessions used for construction of map which were most divergent for restriction fragment length polymorphism among seven accessions tested were polymorphic only for five isozymes out of fifteen isozymes tested.

Jouve and Diaz (1993) recognized a new form of esterase (Est-6) isozyme. The expression was controlled by Est-A6, Est-B6, Est-D6 of wheat and Est-R6 gene of rye located on homoeologous group 3A, 3B, 3D and 6R respectively. The expression was reported in young leaves.

Schmidt *et al.* (1993) tested six leaf and three seed isozymes for their ability to identify *Triticum aestivum* - *Aegilops markgraffi* amphiploid and six presumptive alien addition lines. Four leaf isozymes and three seed isozymes were found useful in differentiating amphiploid and five addition lines, but the leaf peroxidases did not form any *Aegilops* specific bands among addition lines.

### (ii) Protein Analysis

The wheat storage proteins mainly comprise glutenins and gliadins. Glutenins can be further classified as high molecular weight glutenin subunits (HMW) and low molecular weight subunits (LMW) (Payne and Corfield, 1979).

Payne *et al.* (1980) reported that all HMW glutenin subunits are under the control of homoeologous group one of wheat. The subunit composition is variable (Beitz *et al.* 1973) and each variety has 3 to 5 subunits for HMW out of which 1 subunit (1 AX) is controlled by chromosome 1A, one or 2 subunits by 1B (Bx, By) and 2 subunits (Dx and Dy) by 1D. Lawrence and Shephard (1981a) reported that genes controlling HMW glutenin subunits are located at three complex loci - one each on the long arms of chromosomes 1A, 1B and 1D.

Lawerence and Shephard (1981b) reported that two subunits controlled by genes on 1D are coded by different genes at each of the two loci that are tightly linked. Similar results were drawn for 1B. It was then concluded that subunits may be controlled by non-allelic closely linked genes.

Wide variability among the electrophoretic patterns of both high and low molecular weight subunits was observed by Payne *et al.* (1981).

Blackman and Payne (1987) proposed the technique of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for HMW glutenin studies as more versatile, reproducible requiring small samples for testing.

Vallega and Waines (1987) investigated different accessions of *Triticum turgidum* var. *dicoccum* of diverse origins for variation in HMW glutenin subunits and identified various new alleles which were not present in *Triticum aestivum*. Similar type of variability was observed by Calvin and Ornella (1990) in *Aegilops* species having some new alleles not present in cultivated wheats.

Giura and Manisa (1988) identified six addition lines of homoeologous group one from *Aegilops ovata*, *Aegilops triuncialis*, *Aegilops variablis*, *Aegilops columnaris* and *Aegilops triaristratum* (4x and 6x). The analysis was done on basis of morphological markers and high molecular weight subunits.

Singh *et al*. (1994) carried out half seed analysis by SDS-PAGE to analyse HMW glutenin subunits from *Aegilops* umbellulata (U), *Aegilops caudata* (C), *Aegilops triuncialis* (UC) and *Aegilops ovata*; along with the *Triticum aestivum* as a check.

The 'x' subunits of both *Aegilops umbellulata* and *Ae.caudata* was found to possess subunits with higher molecular weight than the corresponding 'Ax' subunit of wheat, thus, showing different banding pattern. Both the species also had 'y' subunits which is lacking in 6 x wheats. Both the 'x' and 'y' subunits of *Aegilops umbellulata* and *Ae.caudata* were present in *Aegilops triuncialis*.

### (d) Molecular markers

In the recent years, molecular biology has provided new tools for rapid and detailed genetic analysis among different organisms. The most important of these are DNA markers, which are now available for a range of cytogenetic applications particularly those involved in alien wheat chromatin manipulation, its introgression into desired species followed by characterization.

Flavell (1978) characterized rye chromosome addition or telocentric addition lines in Hold Fast Wheat-King-II by using highly repetitive rye specific sequences as probes.

Apples and Dvora'k (1982) demonstrated the use of restriction fragment length polymorphism (RFLP's) for the first time in wheat by using ribosomal spacer probe (PTA 250) to study polymorphism in different cultivars of Triticeae. Also the probe hybridized to *Triticum dicoccoides*, showing variability in banding pattern of *Triticum dicoccoides* and *Triticum aestivum* suggesting RFLP's as tool for identifying alien chromatin during production of addition and translocation lines.

Apples *et al*. (1986) again studied the structure of ribosomal DNA (rDNA) spacer region from representatives of different genomes. The results indicated that main spacer region of rDNA units is sufficiently rapid to allow each genome to be clearly identified when alien transfers from these species are made.

The species specific sequences have been found as desirable markers for studying relationships among different species and for tracing out alien chromatin transferred to different species (Kanamthawat *et al*. 1987). Two types of approaches are suggested either using total genomic DNA as probe which is made specific to species by blocking with DNA sequences common to other species or species specific probes can be obtained from isolated and cloned DNA sequences.

Berry *et al*. (1988) isolated a specific rye probe (pbw 98) from chromosome 5R of rye and another probe (pwu 46) that cross hybridizes to all rye chromosomes. These probes have been suggested as useful markers in tracing out rye chromatin transferred to different species.

Sharp *et al* (1988) identified fourteen RFLP probes from wheat-cDNA library each of which was assigned to specific arm of seven homoeologus groups of wheat. The probes also hybridized to various species of *Aegilops*, *Agropyron*, *Haynaldia* and *Hordeum*. So these probes were found as useful markers to identify different chromosomes/chromosome arms from various Triticeae species during alien genetic transfer. Also the probes were useful in allocating different alien chromosomes to a particular homoeologous group.

Hart and Gale (1989) proposed a detailed genetic map of wheat where various molecular and biochemical loci recognized so far were shown marked to particular chromosome.

Friebe *et al.* (1991) identified alien chromosomes of *Agropyron intermedium* for wheat streak mosaic virus resistance and that of *Aegilops speltoides* for green bug resistance in wheat by cytogenetic analysis and *in situ* hybridization developed by Mukai and Gill (1991). The latter workers have used the technique of *in situ* hybridization for locating barley chromatin added to wheat by using a genomic clone for $\alpha$-amylase gene as a probe.

Baum (1991) identified monosomic and double monosomic addition lines of wheat in rye by cytological studies and southern hybridization using ribosomal spacer probe PTA 250-4 as a marker.

Jahoor *et al.* (1991) identified some RFLP markers for barley by screening Pst-1 based genomic DNA library from barley. Probes were assigned to discrete barley chromosomes by using previously characterized wheat-barley addition lines and were suggested as useful markers to identify barley chromosomes added to wheat during production of alien addition and translocation lines.

Xin *et al.* (1991) screened two cDNA probes to detect *Thinopyrum intermedium* chromosome addition lines carrying resistance to barley yellow dwarf viruses in wheat. A specific band for *Thinopyrum intermedium* and its derivatives was found in southern hybridization and it was also possible to determine the possible size of alien fragment by comparing relative density of specific band.

Cannel *et al.* (1992) identified twenty three cDNA probes and two genomic DNA probes mapped to particular chromosome arms in barley as useful markers for characterizing alien-genetic transfer of barley in recipient backgrounds.

Devos and Gale (1992) made an evaluation of the use of random-amplified polymorphic DNA (RAPD) as genetic marker system in wheat. Reproducible amplifcation products were obtained from varietal homozygous single chromosome recombinant lines and wheat alien chromosome addition line with genomic DNA using selected primers. Thus the applicability of this technique in genetic analysis of alien transfers was suggested.

King *et al.* (1993) used random amplified polymorphic DNA for the first time in wheat to identify

*Thinopyrum bessarabicum* chromosome segments in *Triticum aestivum*. The results were confirmed by *in situ* hybridization.

Talbert *et al*. (1994) proposed an alternate technique to random amplified polymorphic DNA (RAPD) by suggesting the use of "sequence tagged site" polymerase chain reaction products (PCR) as markers. Thirty seven primers sets were sequenced and designed and most of them were found to amplify sequences that mapped to specific chromosomes of wheat. Further nine out of sixteen primer sets tested revealed polymorphism among twenty hexaploid wheat genotypes when PCR products were digested with restriction enzymes. So this was proposed as a useful technique in identifying alien genetic transfers as well as imparting alien chromosomes to different homoeologous groups.

A sequence derived from rye $\alpha$-secalin gene was used as primer in PCR by Lee *et al* (1994) to characterize various wheat and triticale genetic stocks. A particular fragment was found to be present only in rye, wheat and triticale lines carrying a particular rye chromosome 2R.

William *et al*. (1994) developed RAPD markers by using polymerase chain reaction (PCR) for the amphiploid of *Triticum aestivum* and *Thinopyrum bessarabicum* love. Some of the primers showing specific bands to *Thinopyrum* spp. were proposed as useful markers for the identification of *Thinopyrum bessarabicum* chromatin in wheat.

Koebner and Martin (1994) explored the RAPD-PCR technique for the detection of rye chromatin in wheat. It was found that although a large number of primers gave differential rye PCR products in hybrids tested but majority of patterns were non-reproducible. Those that were reliable did not amplify rye product from template carrying only 1 pair of chromosome.

D'Ovidio *et al.* (1994) selected some useful clones from partial genomic libraries of *Triticum urartu*, *Aegilops squarrosa* and cDNA library of *Triticum durum* which were found to hybridize both to cultivated and wild species revealing considerable polymorphism. These were mapped to specific chromosomes and were proposed as useful markers for the analysis of alien addition lines of these species.

Bai *et al.* (1994) isolated alien addition lines of *Triticum triaristratum* (UUMMMnMn genome) showing resistance to different races of leaf and stem rust in wheat by cytology and disease reaction studies. The alien chromosomes were allocated to homoeologous groups 5, 2 and 7 through RFLP analysis using a set of homoeologous group specific wheat cDNA probes, but their allocation to U, M and Mn genomes could not be made.

CHAPTER III

## MATERIAL AND METHODS

The present investigation was carried out at the Biotechnology Centre, Punjab Agricultural University, Ludhiana in 1993-94 and 1994-95.

The material comprising the seeds of $BC_1F_2/BC_2$ generation of an intergeneric cross of widely adapted hexaploid wheat variety WL711 (as female) and *Aegilops triuncialis* (CU) Acc.3549 (as male) was provided by Dr Harjit Singh, Geneticist, Biotechnology Centre, PAU, Ludhiana.

The $BC_2/BC_1F_2$ seeds of four fertile plants with following identification numbers were used in the study:

| Identification No. | No.of seeds | Generation |
|---|---|---|
| 930923 | 5 | $BC_2$ |
| 25-5-1 | 4 | $BC_1F_2$ |
| 25-5-3 | 8 | $BC_1F_2$ |
| 930924 | 41 | $BC_2$ |

During 1993-94 the seeds from each plant were sown on moist-filter papers in petri dishes. The root tips of seedlings were fixed in 3:1 (alcohol:acetic acid) solution for mitotic analysis following standard procedure.

When the coleoptile was 4-5 cms long the plants were transplanted in bread boxes which were then placed in glass house. The screening for rust resistance was done under controlled conditions by inoculating the plants with single spore isolates of leaf rust. The plants were then transplanted in fields and observations for response to disease reaction were also made at adult plant stage.

The spikes of $BC_1F_2/BC_2$ plants were fixed in 6:3:1 Carnoy's solution for meiotic analysis at prebooting stage.

During this season all the plants were backcrossed to WL711 and data on number of pollinations/plant were taken from some of plants to determine seed set.

The $BC_2F_1/BC_3$ seeds were harvested individually from fertile $BC_1F_2/BC_2$ plants in April 1994 and the progeny of various plants were given new identification numbers as:

| Identification number during 1993-94 | Identification number during 1994-95 | No. of seeds | Generation |
|---|---|---|---|
| 930923 | Sterile | - | - |
| 25-5-3 | Sterile | - | - |
| 25-5-1-1 | 940478 | 6 | $BC_2/F_1$ |
| 930924-2 | 940520 | 8 | $BC_3$ |
| 930924-7 | 940521 | 10 | $BC_3$ |
| 930924-8 | 940522 | 10 | $BC_3$ |
| 930924-9 | 940523 | 3 | $BC_3$ |
| 930924-16 | 940524 | 6 | $BC_3$ |
| 930924-17 | 940525 | 6 | $BC_3$ |
| 930924-18 | 940526 | 10 | $BC_3$ |
| 930924-20 | 940527 | 10 | $BC_3$ |
| 930924-21 | 940528 | 6 | $BC_3$ |
| 930924-24 | 940529 | 2 | $BC_3$ |
| 930924-26 | 940580 | 4 | $BC_3$ |
| 930924-28 | 940581 | 3 | $BC_3$ |
| 930924-38 | 940582 | 10 | $BC_3$ |
| 930924-40 | 940583 | 2 | BC3 |
| 930924-41 | 940584 | 2 | $BC_3$ |

In the next season (1994-95) the seeds were again germinated on moistened filter papers and fixations of root tips was done for mitotic studies in 3:1 farmers fixative.

The distal halves of some of seeds were kept for SDS-PAGE and the embryonic halves were germinated for cytological analyses.

Plants were then transferred to bread boxes and then to fields after 20-25 days. At pre-booting stage again fixations were done in 6:3:1 Cornoy's fixative for meiotic studies.

The spikes of some selfed $BC_2$ ($BC_2F_2$) plants (65-6-2, 65-6-3, 65-6-4, 65-6-6, 65-6-7, 65-7-1, 65-7-2, 76-7-3, 65-7-5 and 65-7-6) were also fixed for meiotic studies.

After cytological studies, the DNA was isolated from the leaves of certain plants and also isozymes studies were made. During 1995, all the plants were allowed to self-pollinate and $BC_3F_2/BC_2F_3$ seeds were harvested individually.

**Mitotic Studies**

The seeds were germinated in petri dishes on moist filter paper (moistened by fungicide solution) for 3-4 days.

The root tips were collected when they were 1-2 cms long and were pre-treated for 3 hours with 0.05 per cent colchicine.

The root tips were then put in Farmers' fixative (3 parts 95% ethanol : 1 part glacial acetic acid) and were kept at room temperature for 15-20 days.

For mitotic studies the fixative was drained off and root tips were immersed in 2 per cent acetocarmine solution for 1-3 hours. Then the roots in carmine solution were made to boil for few seconds. The intensely stained root tips were squashed on glass slide in 45 per cent acetic acid, the root cap region was cut off and then a very fine section of root tip was cut by sharp edged razor blade. A drop of 45 per cent acetic acid was put over it and squashes were made with gentle tapping of cover slip.

The observations were made under light microscope and desired slides were photographed.

**Meiotic Studies**

The spikes were collected at prebooting stage in Carnoy's fixative. (6 parts 95% ethanol : 3 parts choloroform : 1 part glacial acetic acid) in the morning.

After 48 hours the spikes were transferred to 70 per cent ethanol and were stored in a refrigerator.

For meiotic studies the anthers were excised from the spikelets and were squashed on a glass slide in the presence of 2 per cent acetocarmine. The chromosome pairing was studied at different meiotic stages and desired slides were photographed.

**Photography**

The photography was done with 'Nickon' Photomicroscope by using 'Orwo' 125 ISI film at 40x magnification. Light intensity was kept constant at six volts and filters used were either $ND_2$ or $ND_4$ or both. Exposure time was automatically adjustable according to light and filter being used.

**Disease reaction**

The first leaf of 7-8 days old seedlings was inoculated following the standard techniques of inoculation. The inoculum of brown rust race 77A-1 was used for inoculation. After 12-15 days, the seedling reaction were recorded according to key developed by Mains and Jackson (1926).

The observations on field reaction to prevalent races of yellow rust and leaf rust were made at adult plant stage following Modified Cobb's Scale (Peterson *et al.* 1948).

### Backcrossing

The spikes of various plants were emasculated one day prior to anthesis. Central florets and poorly developed apical and basal spikelets were removed at the time of emasculation. The emasculated heads were covered with glycine envelops to prevent uncontrolled pollination.

The emasculated spikes were pollinated five to six days following emasculation with fresh pollen of WL711. Spikes of WL711 undergoing anthesis were removed and the upper portion of the florets was clipped. These were then exposed to the sun for a few minutes. When the anthers extruded out, the pollen was then dusted on the top of emasculated spikes. The pollinated spikes were then again covered with glassine envelopes.

### High Molecular Weight Subunit Studies

### Method

One dimensional Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis method as described by Smith and Payne (1984) with little modifications was used to characterize various alien addition lines on the basis of HMW glutenin subunit composition.

### Modifications

- The concentration of acrylamide and bisacrylamide were changed.

- The pH of separating gel was reduced from 8.8 to 8.6.
- Time period for running gel was adjusted to 14 hour at 8 mA current.

**Purification of the chemicals**

Although the chemicals of analytical grade from Sisco were used, but still these chemicals needed purification. The chemicals were purified by the methods given by Hames and Rickwood (1982) as below:

**Acrylamide**: The acrylamide was purified by dissolving 60 g of acrylamide in one litre of chloroform at 50°C and filtered the solution while hot without suction through No.1 Whatman filter paper. The supernatant was stored at -20°C for recrystalisation. The crystals were collected by filtration and then dried and stored in the refrigerator.

**Bisacrylamide**: The bisacrylamide was purified by dissolving 10g of bisacrylamide in one litre of acetone at 50°C and filtered the solution while hot through No.1 Whatman filter paper. The supernatant was stored at -20°C for recrystalisation. The crystals were collected by filtration and then dried and stored in the refrigerator.

**Sodium dodecyl sulphate (SDS)**

SDS was purified by dissolving 200g of the SDS in 3 litre of boiling ethanol at 50°C and filtered while hot.

The supernatant was kept at 4°C for 2-3 days for recrystalisation. The crystals were separated by filtering and then crystals were dried and stored. Then the stock solutions of required reagents were prepared as given in Appendix.

**SDS-PAGE-HMW glutenin subunit analysis**

Half seed of each palnt was ground using pliers with smooth surface. The crushed mixture was transferred to a glass tube. The extraction solution was prepared by mixing 4 ml distilled water, 1.7 ml of 3x sample buffer and 0.3 ml of 2-mercapto ethanol. The amount of extraction solution added to each tube was calculated by multiplying a factor 0.025 with their respective seed weight. After adding extraction solution the contents were mixed on vortex mixture for 2-3 minutes and the contents were allowed to stand for about 1 hour at room temperature.

**Preparation of separating and stacking gels**

The whole electrophoresis apparatus was disinfected by dipping into 1N NaOH solution for 3-4 hour. It was washed thoroughly with tap water and then rinsed with distilled water. The glass plates were dried and fixed in the gel casting apparatus. The sealing of the plates was done through rubber seal around the space.

To prepare separating gel 7.25 ml of 35% acrylamide, 2.08 ml of 2% bisacrylamide, 5.1 ml distilled water and 9.4 ml Tris pH 8.6 were mixed in 150 ml capacity flask. This mixture was degassed. After degassing 0.25 ml of 10% SDS, 0.94 ml of freshly prepared 1% Ammonium persulphate and 30 µl of N, N, N', N' tetramethylethylenediamine (TEMED) were added and the solution was thoroughly mixed. The solution was immediately poured into the gel moulds upto the depth of about 1 cm below the position of the sample wells. The surface of polymerizing gel was overlayered with 1-2 ml of water using a syringe. The separating gel was left to polymerise for about 15-30 minutes.

The stacking gel was prepared by mixing 1.75 ml of 35 per cent acrylamide, 0.75 ml of 2 per cent bisacrylamide, 15.1 ml. of distilled water and 2.5 ml of tris pH 6.8. After degassing, 1.94 ml of 1 per cent Ammonium per sulphate, 0.20 ml of 10 per cent SDS and 40 µl of TEMED were added and solution was thoroughly mixed. The water was poured off from the top of the separating gel and the surface was washed with 2-5 ml of stacking gel solution and the mould was filled with the solution up to the top of the plates. The stacking gel was left to polymerise. After about 1 h. the comb was carefully removed without disturbing the wells and the wells were washed twice with the running buffer.

**Sample Loading**

The samples were incubated at 100°C for 2 minutes before loading. The plates with gel were then placed in the electrophoresis apparatus containing the running buffer. The air bubbles from the lower surface of the gel were removed. Then upper reservoir was filled with running buffer. The samples were loaded by drawing the supernatant into a microliter syringe and introduced 30 $\mu$l into a sample well.

After loading samples the electrodes were connected to the power pack with the anode (+) connected to the bottom reservoir and the cathode (-) connected to the upper reservoir. The current was 8 mA and the gel was allowed to run overnight for 14 hr.

**Staining**

After termination of the electrophoresis the glass plates were removed and carefully separated and the stacking gel was removed from the separating gel by cutting the interface with a scalpel. The separating gel was then placed in the staining solution which contained 125 ml 10 per cent w/v Trichloroacetic acid, 375 ml of distilled water and 25 ml of filtered Commassie blue dye. The staining solution was slowly agitated at a speed of 8- rpm for 24-48 hr on an orbital shaker. The stained gel was destained with water for 1-2 days. After destaining, the gels were photographed.

## ISOZYME STUDIES

Horizontal starch gel electrophoresis was used for studying isozymes of peroxidase, esterase and acid phosphatase. For the studies, the starch was obtained from Sisco Research Laboratories, Bombay and was hydrolysed partially. For this 200 g of unhydrolysed starch in 400 ml acetone was taken in a conical flask and kept at 38±1°C for overnight. Then 4 ml of concentrated HCl was added and starch was incubated at 39°C for one hour and thirty five minutes. Acetone was decanted off and the starch was washed with distilled water in a Bucher's funnel. Finally, the starch was washed with acetone and dried in an oven at 40°C.

Then the stock solutions of reagents required for the studies were prepared as given in Appendix.

### Sample preparation

2 grams of young leaves were taken and crude leaf extracts were prepared by homogenizing the definite amount of leaves in definite volume of 0.1 M. Tris Hcl (pH 7.5) buffer. The extracts were filtered through muslin cloth

into glass vials and vials were kept in freezer till further use.

**Gel preparation**

A solution of hydrolysed starch (12%) and gel buffer was carefully homogenized in one litre side arm conical flask. It was heated with continuous stirring till the starch became translucent and less viscous. The cooked starch was degassed with the help of vacuum pump and poured into gel mould. The mould was covered with a glass slab in such a way that no air bubble gets entrapped into the gel. For proper setting of the gel it was allowed to stand overnight. Before use, the gel was kept in refrigerator for 30 min.

**Sample application**

Slots were made in the middle of the gel. The extracts were absorbed on 5 x 5 mm Whatman no.3 chromatographic paper wicks. The excess extract was removed on ordinary filter paper and the wicks were inserted into the slots. Electrophoresis was carried out using ADCO, power supply and systronic migration chamber for 5-6 hours at 300v and 30 mA. The wicks were removed after 15 minutes to reduce streaking. The gel was run till borate buffer front reached near the anodal end.

**Slicing**

When borate buffer front reached the anodal end the gel slab was taken out of the migration chamber and one tier of strips removed at a time. The slicing was done using a thin slicing wire.

**Staining**

**Peroxidase: (Veech, 1969)**

One gram of the benzidine was dissolved by heating in 1 litre of 0.2 M acetate buffer and 3.6 ml of acetic acid in one litre distilled water. The solution was cooled and filtered. Then250 ml of benzidine solution, five ml of three per cent $H_2O_2$ was added at the time of incubation for staining the gels. After 5 minutes of incubation blue bands started turning brown slowly which were recorded and photographed.

**Esterase** (Harris and Hopkinson 1976).

Gels were incubated in 100 ml of 0.1M phosphate buffer, pH 6.5 containing 40 ml Fast blue B and four ml of one per cent stock solution of α-naphthyl acetate and ß-naphthyl acetate for four hours. The esterase bands appeared as reddish brown spots on the gel.

**Acid phosphatase** (Rendel and Stormont, 1964).

Gels were incubated in 100 ml of 0.2M acetate buffer containing 100 mg Sodium α-naphthyl phosphate and 40 mg sodium ß naphthyl phosphate, 100 mg Fast blue BB salt, 250 mg Polyvinyl pyrolidone, one g sodium chloride and 10 drops of 10 per cent stock solution of magnesium chloride in 0.2 M acetate buffer at 35°C for six hours. Acid phosphatase bands appeared as pinkish brown spots on gel.

**Isolation of DNA**

The DNA was isolated from plants identified as monosomic and disomic alien addition lines by cytological studies along with their parents. Dellaporta *et al.* (1983) method was used for DNA isolation. Various Stock Solutions listed in Appendix required for DNA isolation were prepared.

**Steps**:

1. The fresh green leaves weighing approximately 2.5 to 3 gm were collected in clean polythene bags and brought to the laboratory.
2. The leaves were cut into small pieces and were grounded into fine powder with the help of liquid nitrogen in a pestle-mortar.
3. The fine powder was then transferred into centrifuge tubes which contained 10-15 ml of preheated (65°C) extraction buffer. The contents were mixed well by gentle shaking for 2 min.

4. The samples were incubated at 65°C for 10-15 minutes and shakings were given by inversions after every 5 minutes.
5. Five ml of 5M Potassium acetate was added after cooling the samples to room temperature. The samples were mixed by vigorous shaking and placed on ice for 30 minutes.
6. The contents were centrifuged for 20 minutes at 3000 rpm (4°C).
7. The solution was filtered through autoclaved muslin cloth and the supernatant was collected in fresh centrifuge tubes.
8. To the supernatant, 2/3 volume of isopropenol (app. 12.5 ml) was added followed by incubation at 4°C for 30 minutes.
9. The samples were centrifuged for 5 minutes at 3000 rpm (4°C).
10. A clear pellet was formed at the bottom of each tube. The Isopropanol was discarded and pellet was washed in 70 per cent alcohol. The pellet was then dissolved in 5 ml of T.E. buffer.
11. To the solution 5$\mu$l of RNase (preheated to 80°C) was added followed by incubation of samples at 37°C for 10 minutes.

12. To the contents 1/10 volume (app. 0.5 ml) of sodium acetate and 2.5 ml of absolute alcohol were added.
13. The contents were mixed by gentle shaking and incubated for 30 minutes on Ice.
14. The samples were centrifuged for 5 minutes at 3000 rpm (4°C).
15. A clear pellet was formed at the bottom. The pellet was washed with 70 per cent ethanol.
16. Centrifugation was done for 5 minutes at 3000 rpm (4°C). Ethanol was discarded and DNA pellet was dried in a vacuum desiccator.
17. The pellet was dissolved in 1 ml of TE buffer and samples were transferred to 2 ml eppendoff's tubes.

**Purification**

18. 1 ml of satured phenol chloroform was added to each tube and contents were mixed properly.
19. Centrifugation was done at 3000 rpm for 8 minutes.
20. The upper part from tubes was transferred to fresh 2 ml eppendoff's tubes.
21. Equal volume of chloroform: Isoamyl alcohol (24:1) was added to the tubes. The contents were mixed and centrifuged at 3000 rpm for 8 minutes.

22. The upper part was taken and transferred to fresh 2 ml appendoff's tubes. To this 2/3 volume of absolute alcohol and 1/10 volume of sodium acetate was added and the samples were kept on Ice for 30 minutes.

23. The samples were centrifuged at 3000 rpm for 5 minutes.

24. The supernatant was discarded and pellet was dried in a vacuum desiccator.

25. The pellet was dissolved in 200 ml of T.E. buffer.

26. The agarose gel (0.8%) was casted off to see the quality of DNA. 10 μl of DNA (dissolved in TE) + 3μl of loading dye (Bromophenol blue) was added to the wells and samples were run at 50 v for 2 hours.

27. The gel was stained with Ethidium bromide (EtBr) for 15 min., followed by destaining with water. DNA was visualized under transilluminator and gel was photographed.

**Restriction enzyme digestion**

On visualization of DNA under transilluminator, it was found that fewer samples were showing degradation. Only good quality DNA was taken for restriction. The pre-requisites were:

1. DNA samples

2. Restriction enzymes
3. Reaction buffer
4. Autoclaved distilled water
5. BSA (Bovin serum album)
6. RNase and;

The constitution of the digestion mixture should be as follows:

(a) the total volume for digestion should be app. 2x the volume of DNA.

(b) The BSA and reaction buffer should be approx 1/10 of the total volume.

(c) The enzyme added should not exceed 10 per cent of the total volume.

In the present investigation, the digestion was done with two restriction enzymes by making following cocktail:

| Components of digestion mixture | Enzyme used<br>Hind III<br>$\mu$l | <br>EcoRI<br>$\mu$l |
|---|---|---|
| Enzyme | 4 | 4 |
| Restriction enzyme buffer | 6 | 9 |
| B.S.A. | 4 | 4 |
| RNase | 2 | 2 |
| DNA | 40 | 30 or 40 |
| Water | 3 | 4 |
| Total volume | 59 | 63 or 73 |

**Steps**

1. The DNA was added to a sterile eppendoff's tube.
2. Then appropriate amount of autoclaved distilled water was added to the tubes.
3. The cocktail of reaction buffer, BSA and RNase was added.
4. At the end enzyme was added to the mixture.
5. Vortexing was done at slowest speed for few seconds.
6. Samples were incubated for overnight at 37°C in a water bath.
7. The DNA was precipitated by using 2 volumes of ethanol and 1/10 volume of 3M sodium acetate (pH 5.5). The samples were mixed thoroughly. The samples were then placed in refrigerator for 1 hour and spun in microcentrifuge for 10-15 minutes.
8. The ethanol was removed and samples were dried by inverting tubes on filter paper.
9. The samples were then dissolved in 20-30 $\mu$l of T.E.
10. To the samples 5 $\mu$l of loading dye (bromophenol blue) was added. The samples were run on a 0.8 per cent agarose gel (2 gm agarose in 250 ml of TBE) for 16 hours at 50 volts.

11. Then gel was stained with ethidium bromide for 10-15 minutes followed by destaining with water.
12. The gel was then visualized under U/V transilluminator and photographed.

**Making blots**

After ensuring the proper digestion of DNA by restriction enzymes, it was transferred to a nylon membrane by Southern blotting. The stock solutions of various reagents required were prepared as listed in Appendix.

**Steps**:

1. The gel was treated with 500 ml of 0.25 M HCl for 15 minutes at room temperature with gentle shaking. The solution was drained off and the gel was gently rinsed with autoclaved distilled water.
2. To denature DNA the gel was treated with denaturing solution (.4N NaoH + 0.6 M Nacl) for 30 minutes at room temperature. During this, the gel was shaken gently by placing the tray on very slow speed shaker.
3. The denaturing solution was discarded. The gel was treated with neutralizing solution (0.5 M Tris + 1.5 M Nacl. pH 7.5), for 30 minutes at room temperature. The gel was shaken gently.

4. The nylon membrane was trimmed to the size of gel and a cut was given at its lower left corner. The membrane was handled only after wearing gloves.
5. The membrane was soaked in autoclaved water for 1-2 minutes.
6. The electrophoresis chamber was used for southern transfer. Its tanks were filled with 20 x SSC. Two 3 mm chromatographic paper sheets were placed, the one end of whose was made to dip in 20 x SSC and other end was placed over flat central surface of the chamber. These were made wet by 20 x SSC and all the air bubbles between glass surface and wicks were removed by rolling a test tube over the sheets.
7. Two sheets of chromatographic paper were placed over the wicks (on flat central surface of chamber). These were made wet by 20 x SSC and all the air bubbles were removed).
8. The lower left corner of the gel was also trimmed to keep proper orientation with the membrane and gel was placed face down on the bed of chromatography paper. All the air bubbles were removed.

9. The membrane was placed over the gel by matching lower left cut corners.

10. Two sheets of 3 mm chromatographic paper (exactly to the size of membrane) were placed over the membrane and were made wet by 20 x SSC. All the air bubbles were carefully removed.

11. All the areas except that covered by membrane were covered with plastic wraps to prevent evaporation and easier capillary movement only through the gel.

12. A stack of filter paper towels was put over the chromatographic membranes and 500 ml bottle was placed on the top of towels. The filter paper towels were changed regularly at an interval of 5-6 hours and process was continued for 36 hours.

13. The stacks were removed the membrane was taken out with the help of autoclaved forceps; and was treated with 0.2M Tris + 2 x SSC to remove excess salts.

14. The membrane filter was placed in a folder of chromatographic paper and was baked at 80°C for 2 hours. It was then marked with lead pencil and again wrapped in filter paper sheets for storage.

15. The gel was stained with Ethidium bromide for 10-15 minutes followed by destaining with water. It was

then visualized under U/V transilluminator to ensure proper transfer of DNA from gel to membrane.

# CHAPTER IV

## RESULTS

The results of the investigation 'Development and Identification of alien-addition lines of *Aegilops triuncialis* in wheat are given in this Chapter.

### 1. DEVELOPMENT OF ALIEN ADDITION LINES

#### Percentage germination of seeds

Seed germination from different crosses during 1993-94 is shown in Table 2 and during 1994-95 in Table 3. The percentage germination during 1993-94 ranged from 25 per cent to 60 per cent with an average of 48.38 per cent. During 1994-95, the germination percentage ranged from 0 to 64.7 with an average of 38.8 per cent.

The plant number 940522 had highest germination percentage and plant numbers 940520, 940582, 940583 and 940584 also had good germination. Plant numbers 940578, 940521, 940524, 940525, 940528 and 940580 had lower germination percentage, as compared to the average.

#### Fertility

The total number of seeds planted during 1993-94 and the plants that formed seeds are listed in Table 4. All the plants from cross 930923 and 25-5-3 were sterile.

Table 2: Germination percentage of different crosses during 1993-94

| S.No. | Cross No. | Total number of seed sown | Seeds germinated | %age germination |
|---|---|---|---|---|
| 1. | 930923 | 5 | 3 | 60 |
| 2. | 25-5-1 | 4 | 2 | 50 |
| 3. | 25-5-3 | 8 | 2 | 25 |
| 4. | 930924 | 41 | 24 | 58.5 |

Average germination = 48.38%

Table 3: Germination percentage of different crosses during 1994-95

| Cross No. | No. of seeds sown | Germinated | Germination percentage |
|---|---|---|---|
| 940478 | 10 | 3 | 30 |
| 940520 | 4 | 2 | 50 |
| 940521 | 30 | 9 | 30 |
| 940522 | 17 | 11 | 64.7 |
| 940523 | 2 | 0 | 0 |
| 940524 | 20 | 6 | 30 |
| 940525 | 6 | 2 | 33.3 |
| 940527 | 20 | 8 | 40 |
| 940528 | 6 | 2 | 33.3 |
| 940580 | 15 | 5 | 33.3 |
| 940582 | 10 | 6 | 60 |
| 940583 | 2 | 1 | 50 |
| 940584 | 2 | 1 | 50 |

Average germination = 38.8%

Table 4: Percentage of fertile plants during 1993-94

| Cross No. | Plant sown in field | Fertile | Fertility (%) |
|---|---|---|---|
| 930923 | 3 | 0 | 0 |
| 25-5-1 | 2 | 1 | 50 |
| 25-5-3 | 2 | 0 | 0 |
| 930924 | 24 | 16 | 66.6 |

Average fertility = 29.15%

Table 5: Seed set percentage during 1993-94

| Plant identification number | No.of pollinations per plant | No.of seeds per plant | Percentage seed set |
|---|---|---|---|
| 25-5-1-1 | 175 | 25 | 14.3 |
| 940524-8 | 46 | 17 | 38.2 |
| 940524-17 | 84 | 16 | 19.1 |
| 940524-18 | 87 | 14 | 16.2 |
| 940524-26 | 113 | 24 | 21.3 |
| 940524-28 | 62 | 4 | 6.5 |
| 940524-38 | 108 | 10 | 9.3 |

Average seed set = 17.84%

The average fertility of plants from different crosses was found to be 29.15 per cent.

During 1994-95, only 3 plants (940583-2, 940525-1, 4) out of 42 $BC_3$ plants were sterile leading to an average of 92.8 per cent fertility. However, among the selfed progeny ($BC_2F_2$ plants) marked differences in sterility ranging from completely fertile to completely sterile plants (Table 10) were observed.

**Seed set**

The total number of pollinations made during 1993-94 and the number of seeds formed in some of crosses is listed in Table 5. The percentage seed set of different crosses ranged from 6.5 per cent to 38.2 per cent with an average seed set of 17.84 per cent.

The plant number 940524-8 showed good seed set, plants 25-5-1-1, 940524-17, 940524-18, 240524-26 had average seed set and plant numbers 940524-28, 940524-38 had poor seed set.

**Cytological studies**

The mitotic chromosome number of various plants was determined from their root tip cells. Four monosomic addition plants and two plants with chromosome number 44 were identified by these studies. The authenticity of mitotic results was confirmed by study of meiotic

Table 6a: Alien addition lines identified by mitotic studies

| Plant No. | Chromosome number | Meiotic chromosome configuration | | |
|---|---|---|---|---|
| | | Diakinesis | Metaphase | Anaphase |
| 940580-5 | 44 | - | 21"+2′ | - |
| 940522-12 | 43 | - | 21"+1′ | - |
| 940582-11 | 44 | 22" | 22" | - |
| *940582-14 | 43 | - | - | - |
| 940582-6 | 43 | - | 21"+1 | 21+21+1 |
| 940582-3 | 43 | 21"+1′ | 21"+1′ | - |

*Plant did not survive in glass house.

Table 6b: Alien addition lines identified by meiotic studies

| Plant No. | Chromosome number | No. of PMC's studied | Meiotic chromosome configuration | | |
|---|---|---|---|---|---|
| | | | Diakinesis | Metaphase | Anaphase |
| 940527-1 | 43 | 10 | - | 21"+1' | 22+20+1 |
| 940527-5 | 43 | 7 | 21"+1' | 21"+1' | - |
| 940527-9 | 44 | 10 | - | 22" | 21+21+2 |
| 940522-3 | 43 | 6 | 21"+1' | 21"+1' | - |
| 940522-17 | 43 | 9 | 21"+1' | 21"+1' | 21+21+1 &<br>20+22+1 |
| 940580-3 | 43 | 12 | - | 21"+1' | 23+20 |
| 940580-9 | 44 | 14 | 22" | 22" | 22+22 |
| 940521-3 | 43 | 12 | - | 21"+1' | 22+21 |
| 940521-8 | 43 | 15 | 21"+1' | 21"+1' | 20+19+4 &<br>21+21+1 |
| 940524-6 | 44 | 15 | 22" | 22" | 22+22(44) |
| 940584-4 | 43 | 11 | - | 21"+1' | 21+21+1 |

chromosome configurations. Three monosomic addition lines were confirmed by these studies as the plant number 940582-14 did not survive under glasshouse conditions. From 44 chromosome plants one plant (940582-11) was identified as disomic addition line and the other plant (940580-5) was a double monosomic addition. (Table 6a).

In addition to this, eight new monosomic addition lines and three disomic addition lines were identified from the study of meiotic chromosome associations (Table 6b). The analysis was made by analysing 10-15 PMC's per plant at different meiotic stages viz., diakinesis, metaphase and anaphase (Fig. 1.1 to 14.2).

### Frequency of alien addition lines

The data obtained on $BC_2F_2$/$BC_3$plants with chromosome number varying from 42 to 45 along with their percentage is shown in Table 7.

It is evident that as many as fifty three plants involving eight $BC_3$ progenies and two $BC_2F_2$ progenies were studied for their chromosome number. Plant number 940584 had only one plant in its progeny and was a monosomic addition line. The progeny of plant numbers 940580 and 940582 were able to retain the alien chromosomes in higher frequency as comapred to the progeny of other $BC_3$ plants.

Table 7: Frequencies of plants with varying chromosome number in $BC_3/BC_2F_2$ generation of a cross *Aegilops triuncialis* x *T.aestivum* cv. WL 711

| Plant Identification number in $BC_2$ | 2n chromosome number of $BC_2$ plants | Plant identification number in $BC_3/BC_2F_2$ | 2n chromosome number in $BC_3$ progeny | | | | |
|---|---|---|---|---|---|---|---|
| | | | 42 | 43 | 44 | 45 | Total plants |
| 930924-7 | - | 940521($BC_3$) | 5(55.5)* | 2(22.2) | 1(11.11) | 1(11.11) | 9(16.9) |
| 930924-8 | 43 | 940522 ($BC_3$) | 5(62.5) | 3(37.5) | -(-) | -(-) | 8(15.09) |
| 930924-20 | 44 | 940527 ($BC_3$ | 5(62.5) | 2(25) | 11(12.5) | -(-) | 8(15.09) |
| 930924-16 | 44 | 940524 ($BC_3$ | 3(75) | -(-) | 1(25) | -(-) | 4(7.5) |
| 930924-2 | - | 940520 ($BC_3$) | 1(100) | -(-) | -(-) | -(-) | 1(1.8) |
| 930924-26 | 46 | 940580 ($BC_3$) | 2(40) | 1(20) | 2(40) | -(-) | 5(9.4) |
| 930924-38 | 45 | 940582 ($BC_3$) | 2(33.3) | 3(50) | 1(16.67) | -(-) | 6(11.3) |
| 930924-41 | 45 | 940584 ($BC_3$) | -(-) | 1(100) | -(-) | -(-) | 1(1.8) |
| 930924-26 | 46 | 65-6 ($BC_2F_2$) | 2(33.3) | -( - ) | -(-) | 4(66.6) | 6(11.3) |
| 930924-26 | 46 | 65-7 ($BC_2F_2$) | 3(60) | -(-) | -(-) | 2(40) | 5(9.4) |
| | | Total | 28(52.8) | 12(22.6) | 6(11.3) | 7(13.2) | 53 |

*Figures in parentheses indicate percentages.

In the selfed progeny ($BC_2F_2$), the plant number 65-6 was having higher frequency of alien chromosomes.

In general, as many as twenty eight (52.8%) plants out of fifty three plants studied were euploid. Twelve plants (22.6%) were identified as monosomic addition lines and six (11.3%) plants had 44 chromosomes, out of which four plants were disomic additions and two plants were double monosomic addition lines as confirmed by meiotic chromosome configuration.

**Stability of alien addition lines**

In the $BC_3$ progeny of a plant characterized as monosomic addition line in $BC_2$ (plant no. 930924-8 in $BC_2$ and 940522 in $BC_3$) (Table 7) comprising eight plants, the alien chromosome was retained by three (37.5%) plants, while five (62.5%) plants reverted to 42 chromosome wheat plants. Thus, the female transmission of the alien chromosome was 37.5 per cent.

In the $BC_3$ progeny of plant characterized as disomic addition in $BC_2$ (plant no. 930924-16 in $BC_2$ and 940524 in $BC_3$) (Table 7), the alien chromosome was retained by only one plant out of four plants. Also no monosomic addition line was obtained in the progeny as three plants had 42 chromosomes.

## PLATE-A: CHROMOSOME NUMBER, PAIRING AND DISTRIBUTION IN VARIOUS STAGES OF CELL DIVISION IN DIFFERENT ALIEN ADDITION LINES

| | |
|---|---|
| Fig.1.1 | Pairing behaviour of plant number 940582-3, showing 21" + 1' at Metaphase. |
| Fig.1.2 | Root tip cell of plant number 940582-3, showing 2n = 43. |
| Fig.2.1 | PMC of plant number 940524-6, showing 22 + 22 distribution at Anaphase. |
| Fig.2.2 | Chromosome pairing behaviour of plant number 940524-6, showing 22" at Metaphase. |
| Fig.3.1 | Chromosome distribution in plant number 940580-9, showing 22 + 22 distribution at Anaphase. |
| Fig.3.2 | Pairing behaviour of plant number 940580-9, showing 22" at Metaphase. |

1.1

1.2

2.1

2.2

3.1

3.2

PLATE-A

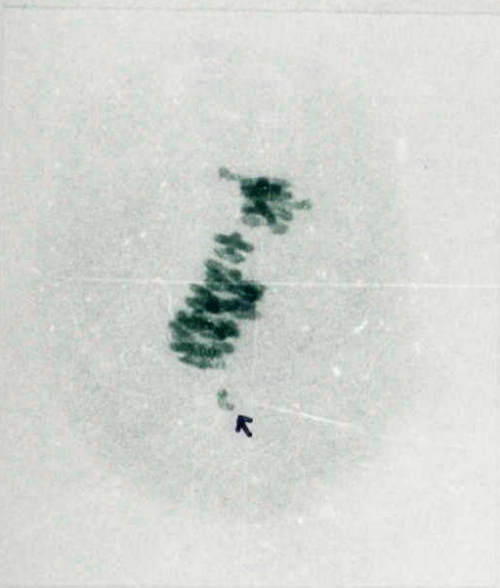
* → shows univalent

## PLATE-B: CHROMOSOME NUMBER, PAIRING AND DISTRIBUTION IN VARIOUS STAGES OF CELL DIVISION IN DIFFERENT ALIEN ADDITION LINES

| | |
|---|---|
| Fig.4.1 | Chromosome pairing behaviour in plant number 940580-3, showing 21" + 1′ at Metaphase. |
| Fig.4.2 | Chromosome distribution in plant number 940580-3, showing 20 + 23 distribution at Anaphase. |
| Fig.5.1 | Pairing behaviour of plant number 940522-12, showing 21" + 1′ at Metaphase. |
| Fig.5.2 | Root tip cell of plant number 940422-12, showing 2n=43. |
| Fig.6.1 | Chromosome distribution in plant number 940521-8, showing 21 + 21 + 1 at Anaphase. |
| Fig.6.2 | PMC of plant number 940521-8, showing 20 + 19 + 4 distribution at Anaphase. |

4.1

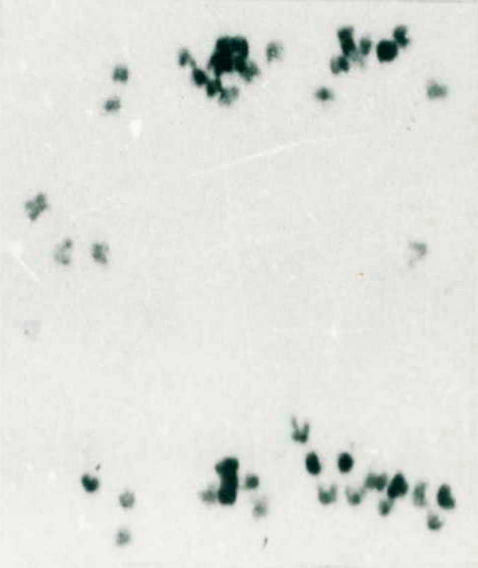
4.2



5.1

5.2

6.1



6.2

PLATE-B

## PLATE-C: CHROMOSOME NUMBER, PAIRING AND DISTRIBUTION IN VARIOUS STAGES OF CELL DIVISION IN DIFFERENT ALIEN ADDITION LINES

Fig.7.1 Root tip cell of plant number 940582-6, showing 2n = 43.

Fig.7.2 Chromosome distribution in plant number 940582-6, showing 21 + 21 + 1 distribution at Anaphase.

Fig.8.1 PMC of plant number 940527-1, showing 21" + 1' at Metaphase.

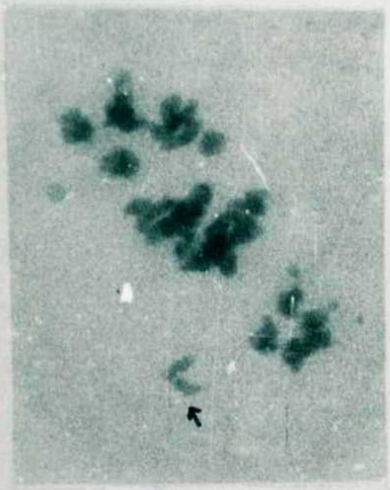
Fig.8.2 Chromosome distribution at Anaphase of plant number 940527-1, showing 20 + 22 + 1 distribution.

Fig.9.1 Plant number 940522-17, showing 21 + 21 + 1 distribution at Anaphase.

Fig.9.2 Chromosome distribution of plant number 940522-17, showing 20 + 22 + 1 distribution at Anaphase.

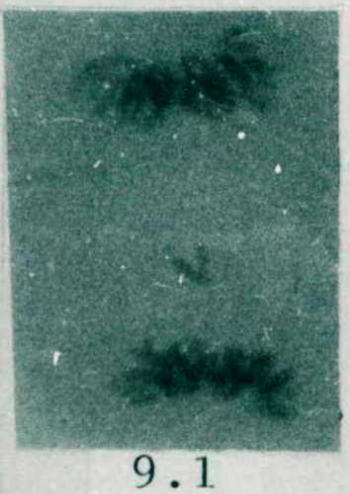
7.1

7.2

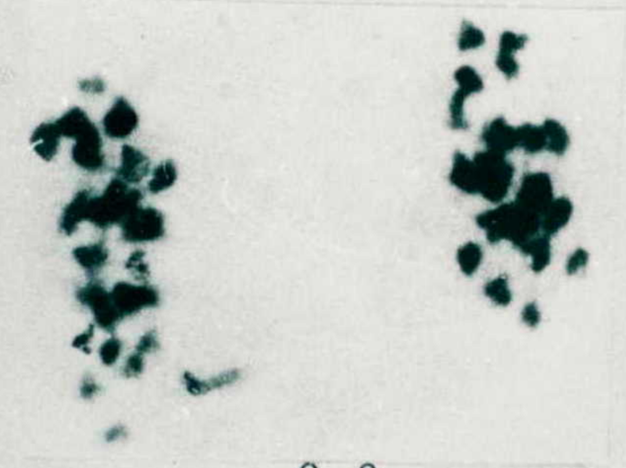


8.1

8.2.



9.1



9.2

PLATE-C

## PLATE-D: CHROMOSOME NUMBER, PAIRING AND DISTRIBUTION IN VARIOUS STAGES OF CELL DIVISION IN DIFFERENT ALIEN ADDITION LINES

Fig.10.1 Chromosome pairing behaviour in plant number 940527-5, showing 21" + 1′ at Metaphase.

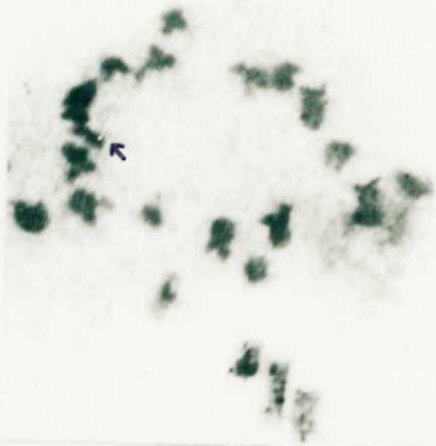
Fig.10.2 PMC of plant number 940527-5, showing 21" + 1′ at Diakinesis.

Fig.11.1 PMC of plant number 940582-11, showing 22" at Diakinesis.

Fig.11.2 Root tip cell of plant number 940582-11, showing 2n=44 chromosomes.

Fig.12.1 & Fig.12.2 Chromosome pairing behaviour in plant number 940522-3, showing 21" + 1′ at Metaphase.

10.1

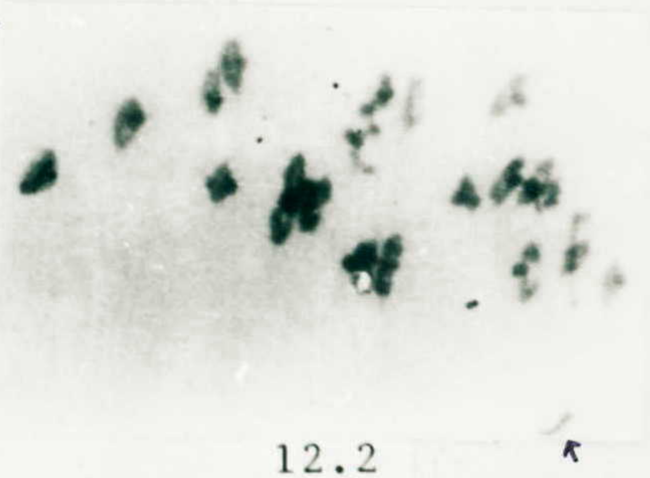


10.2

11.1

11.2

12.1



12.2

PLATE-D

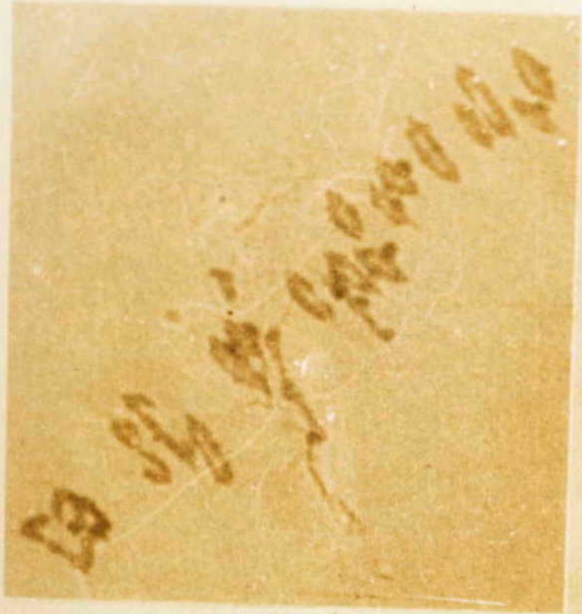
## PLATE-E: CHROMOSOME NUMBER, PAIRING AND DISTRIBUTION IN VARIOUS STAGES OF CELL DIVISION IN DIFFERENT ALIEN ADDITION LINES

Fig.13.1 Pairing behaviour in plant number 940527-9, showing 22" at Metaphase.

Fig.13.2 Chromosome distribution at Anaphase in plant number 940527-9, showing 21 + 21 + 2 distribution.

Fig.14.1 Chromosome distribution at Anaphase in plant number 940521-3, showing 22 + 21 distribution at Anaphase.

Fig.14.2 Chromosome pairing behaviour of plant number 940521-3, showing 21" + 1′ at Metaphase.



13.1

13.2

14.1

14.2

PLATE-E

## 2. CHARACTERIZATION OF ALIEN ADDITION LINES

To allocate the alien chromosomes to different homoeologous groups, the various morphological, disease resistance, biochemical, and molecular markers were used.

### (i) Morphological markers

Various addition lines identified by cytological studies were examined in the field to associate them with any of the salient morphological marker. The characters studied were shape of leaves, spikes, glumes, lemma etc. All the addition lines were similar in gross plant morphology and did not differ from the recurrent wheat parent WL 711.

### (ii) Disease reaction

The response of different plants to race 77A-1 of leaf rust at seedling stage under controlled conditions and to prevalent races of leaf rust and yellow rust under field conditions during 1993-94 is shown in Table 8. Seven plants 25-5-1, 25-5-3-5, 930924-18, 930924-22, 930924-26, 930924-27, 930924-41 were found resistant to race 77A-1 and 5 plants 930923-1, 25-5-3-8, 930924-9, 930924-10, and 930924-23 had mesothetic reaction. All other plants were susceptible. Among the checks WL 711 and Agra local were heavily susceptible (Score $4^-$ and $3^+$ respectively), whereas

Table 8: Reactions of plants to leaf rust race 77A-1 under controlled conditions and to prevalent races of leaf rust and yellow rust under field conditions (1993-94)

| Plant genotypes | Reaction to race 77A-1 | Field reaction | |
|---|---|---|---|
| | | Leaf rust | Yellow rust |
| WL711 | 4⁻ | 80S | 90S |
| *Aegilops triuncialis* | 0 | 0 | F or 0 |
| 940923-1 | x | - | - |
| " -2 | - | - | - |
| " -4 | - | - | - |
| 25-5-1-1 | 1⁻ | 20S | 20S |
| " -2 | - | 40S | 40S |
| 25-5-3-5 | 0 | - | - |
| " -8 | x | - | - |
| 930924-2 | 4⁻ | 90S | - |
| 930924-4 | 3⁺ | 80S | - |
| 930924-5 | 3⁺ | 90S | - |
| 930924-7 | 3⁺ | 90S | 40S |
| 930924-8 | 3⁺ | 90S | - |
| 930924-9 | x | R | 0 |
| 930924-10 | x | 80S | - |
| 930924-15 | 4⁻ | - | - |
| 930924-16 | - | 80S | - |
| 930924-17 | 4⁻ | - | - |
| 930924-18 | 1⁻ | R | 0 |
| 930924-20 | 3⁺ | 90S | 40S |
| 930924-21 | 3+ | 90S | - |
| 930924-22 | 0 | - | - |
| 930924-23 | x | 60S | 40S |
| 930924-24 | 3⁺ | 90S | 40x |
| 930924-26 | 0 | 10S | 10S |
| 930924-27 | 2⁻ | - | 0 |
| 930924-28 | 3⁺ | 60S | 10S |
| 930924-34 | 3⁺ | - | - |
| 930924-35 | 3⁺ | 90S | - |
| 930924-38 | 3⁺ | 90S | - |
| 930924-40 | 3⁺ | 80S | - |
| 930924-41 | 0; | R | 40S |
| Agra local | 3⁺ | 60S | - |

0 to 2 = Resistant, 3 to 4 susceptible, x or MS = Mesothetic or heterogenous, F = Free from rust

+ or - indicate slightly higher (more susceptible) or lower reaction than the mean of class.

- Means that the data is not taken in field because either plant were sterile or plants were heavily infested by LR that data for YR could not be taken.

*Aegilops triuncialis* was found to show high level of resistance (0;) to the race.

Under field conditions, the data on leaf rust was recorded only on fertile plants. Four fertile plants 25-5-1, 930924-18, 930924-26, 930924-41 were able to maintain resistance to prevalent races of leaf rust. Another plant 930924-9 showing mesothetic reaction under controlled conditions was found to show high level of resistance to rust under field conditions. The checks WL 711 and Agra local were again heavily infected by leaf rust (80S and 60S) and *Aegilops triuncialis* maintained the resistance. (0;).

Among these leaf rust resistant plants also 5 plants 25-5-1-1, 930924-9, 930924-18, 930924-26 and 930924-27 (sterile) were resistant to yellow rust. Another plant 930924-28 showing susceptibility to leaf rust was found resistant to yellow rust. Among checks WL711 and *Aegilops triuncialis* were having scores of 90s and 0 respectively.

The chromosome number of various resistant fertile plants is given in Table 9.

During 1994-95, the data on disease resistance under controlled conditions was not recorded due to lack of timely availability of inoculum. Under field conditions, all the $BC_3$ plants were found heavily susceptible to both

Table 9: Chromosome constitution of fertile resistant plants

| Plant No. | Chromosome number (2n) of fertile plants | |
|---|---|---|
| | 1993-94 | 1994-95 |
| 25-5-1-1 | 50 | ♠ |
| 930924-9 | 44 | - |
| 930924-18 | N.S. | - |
| 930924-26 | 46 | 42-44 |
| 930924-28 | 44 | - |
| 930924-41 | 45 | 43 |

NS = not studied
♠ = Plant did not survive in glass house or field.
\- = Seeds did not germinated.

the rusts. However, in the selfed progeny ($BC_2F_2$) of plant number 930924-26, eight plants showing ressitance to either leaf rust, yellow rust or both were identified. The reaction of various $BC_2F_2$ plants to race 77A-1 under controlled conditions and to prevalent races of leaf rust and yellow rust along with their chromosome number, seed colour and data on sterility is shown in Table 10.

Two plants 65-6-2 and 65-7-6 were resistant to both leaf rust and yellow rust. Plant number 65-6-4 was resistant to leaf rust only while plant numbers 65-6-6, 65-6-7, 65-7-1, 65-7-2, 65-7-3 were resistant to yellow rust only. Only plant number 65-6-4 was completely fertile while plant number 65-6-6 was compeltely sterile. Other plants were having low to very low fertility.

### (iii) Biochemical studies

#### (a) High molecular weight subunit analysis

The high molecular weight glutenin subunit studies of half seeds of some plants was done to characterize the alien addition lines of homoeologous group one. A total of twenty plants (940580-20, 940522-1, 940522-4, 940522-3, 940522-5, 940522-7, 940522-9, 940522-11, 940522-12, 940522-17, 940524-17, 940524-14, 940521-10, 940521-15, 940521-16, 940521-23, 940582-3, 940582-6, 940582-11, 940582-12) were studied along with their parents.

Table 10: Showing reactions of $BC_2F_2$ plants to race 77A-1 of leaf rust and prevalent races of leaf rust and yellow rust, seed colour, sterility observation and their chromosome number

| Plant Identification Number | Seedling reaction to race 77A-1 of leaf rust | Field reaction | | Sterility observations | Chromosome number | Seed colour |
|---|---|---|---|---|---|---|
| | | Leaf rust | Yellow rust | | | |
| 65-6-2 | 0 | F | F | LF | 45 | Red |
| 65-6-4 | 0 | F | S | Ft | 42 | Red |
| 65-6-6 | 4 | S | F | St | 45 | Red |
| 65-6-7 | 4 | S | F | LF | 45 | Red |
| 65-7-1 | 4 | S | F | VLF | 42 | Red |
| 65-7-2 | 4 | S | F | VLF | 42 | Red |
| 65-7-3 | 4 | S | F | VLF | 45 | Red |
| 65-7-6 | 0 | F | F | LF | 45 | Red |

F=Free, S=Susceptible, LF=Low fertility, St=Sterile

0-2=resistant, 4=Susceptible Ft=Fertile VLF=Very low fertility

The results of studies are shown in Fig. 15. None of the plants had *Aegilops triuncialis* specific bands and were similar in banding pattern to the recurrent *Triticum aestivum* parent.

Six of the twenty plants (940522-3, 940522-12, 940522-17, 940582-3, 940582-6, 940582-11) were identified as alien addition lines by cytological studies.

**(b) Isozyme markers**

The isozymes of peroxidase esterase and acid phosphatase were analysed to allocate the addition lines to groups 2, 3, and 4 respectively. The polymorphism between the parental species for various enzymes was studied before their use for the allocation of addition lines. Each sample was applied twice to avoid any experimental error.

Fig. 16.1 shows the zymogram patterns of peroxidase isozymes. Five anodal and three cathodal bands were obtained, thus forming a total of eight bands. However, the banding pattern did not show any polymorphism among the parents.

The isozyme patterns of esterase and acid phosphatase are shown in figures 16.2 and 16.3 respectively. Esterase isozyme showed three anodal bands and acid phosphatase showed two anodal bands. Again, the

Fig.15 High molecular weight glutenin SDS-PAGE patterns of $BC_3$ progeny of *Aegilops triuncialis* x *Triticum aestivum* cv. WL 711 and the parents.

Representation of lanes:

| F-1 | Lane no. | Sample | Lane | Sample no. | Lane no. | Sample |
|---|---|---|---|---|---|---|
| | 1. | WL 711 | 6. | 940522-3 | 11. | 940522-12 |
| | 2. | WL 711 | 7. | 940522-5 | 12. | 940522-17 |
| | 3. | 940580-20 | 8. | 940522-7 | 13. | *Ae.triuncialis* |
| | 4. | 940522-1 | 9. | 940522-9 | 14. | *Ae.triuncialis* |
| | 5. | 940522-4 | 10. | 940522-11 | | |
| F-2 | 1. | WL 711 | 6. | 940521-15 | 11. | 940582-11 |
| | 2. | WL 711 | 7. | 940521-16 | 12. | 940582-12 |
| | 3. | 940524-17 | 8. | 940521-23 | 13. | *Ae.triuncialis* |
| | 4. | 940524-14 | 9. | 940582-3 | | |
| | 5. | 940524-10 | 10. | 940582-6 | | |

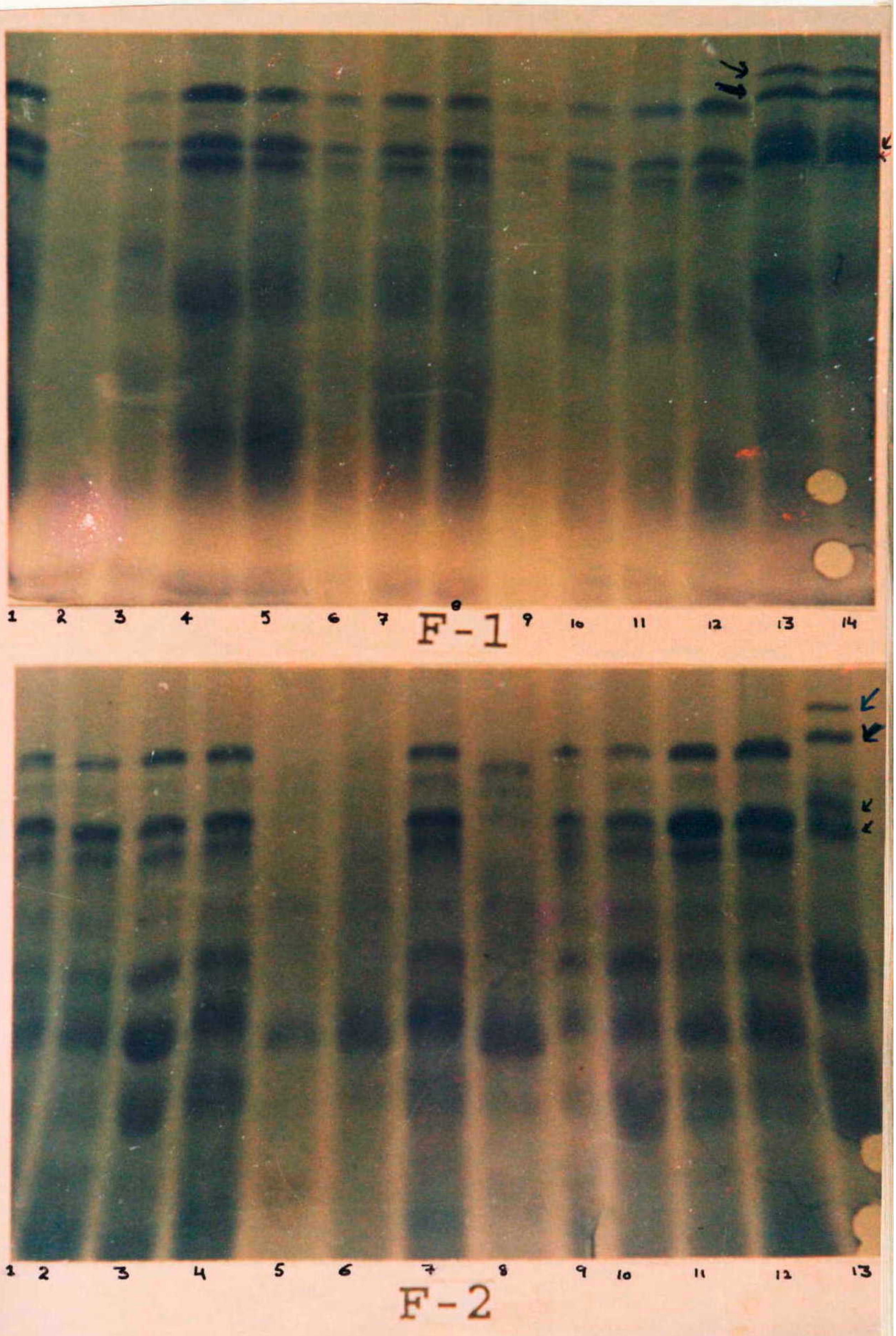
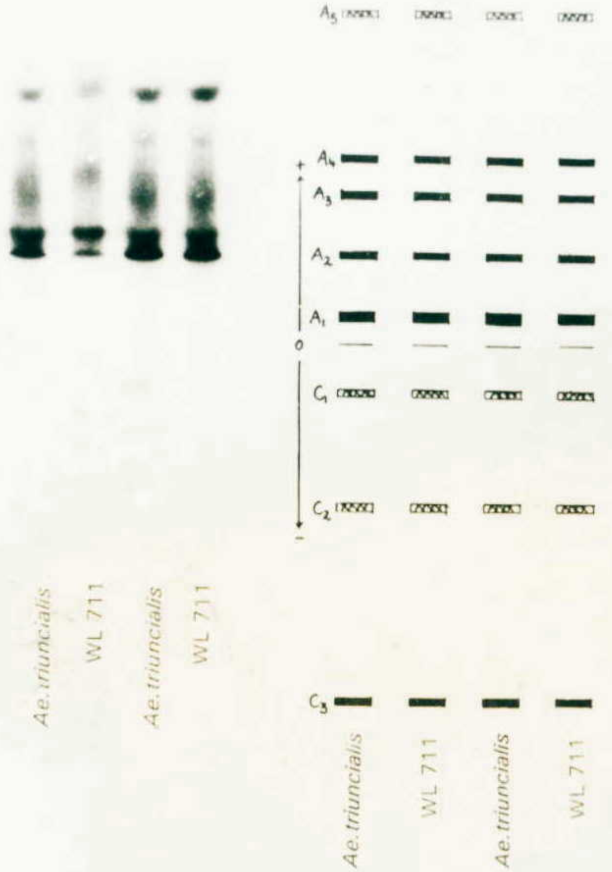


Fig. 15

→ :- Indicates Ae. triuncialis specific bands.

Fig. 16.1 Peroxidase banding pattern of parental species



PEROXIDASE BANDING PATTERN OF PARENTAL SPECIES

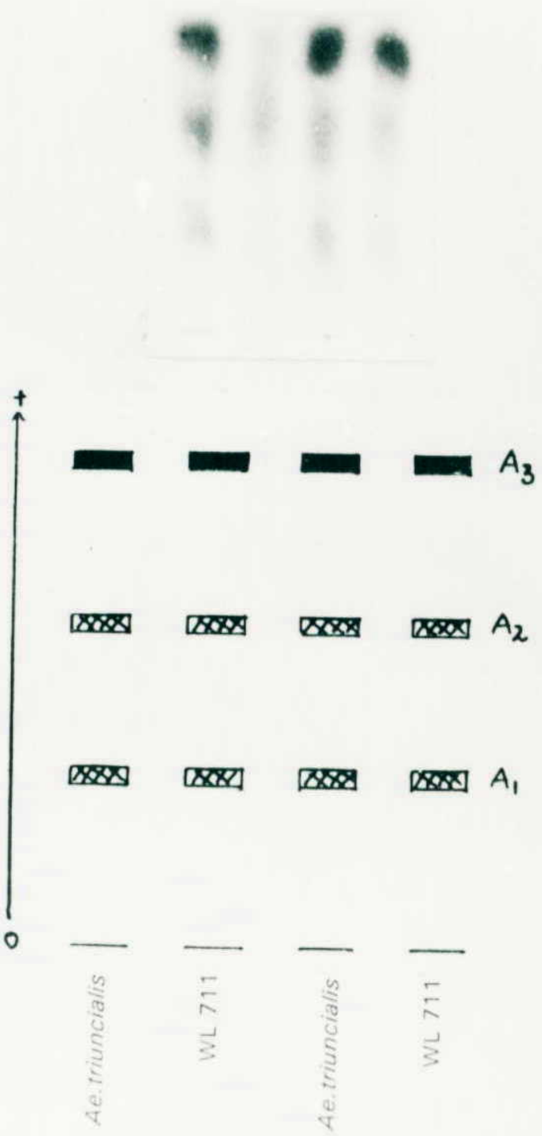
Fig 16·1

INTENSITY OF BANDS

■ HIGH
▨ MEDIUM
▨ LOW

Fig. 16.1

Fig.16.2 Esterase banding pattern of parental species

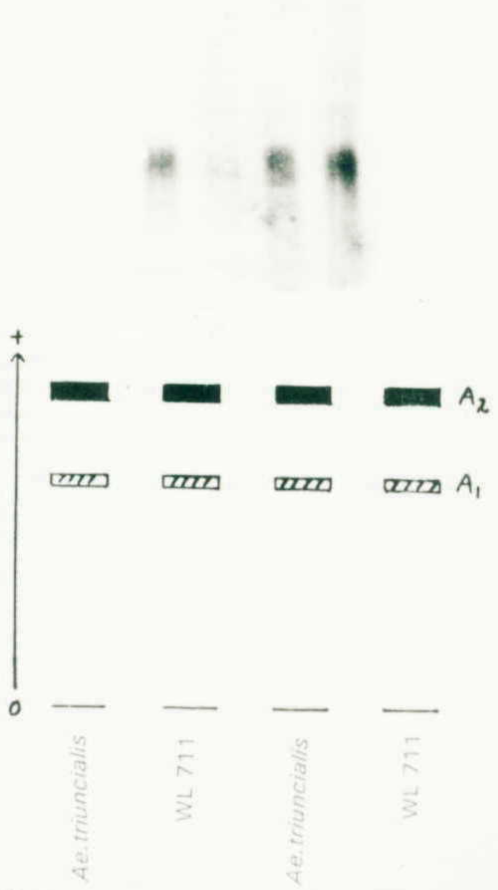


ESTERASE BANDING PATTERN OF PARENTAL SPECIES

Fig 16·2

Fig. 16.2

Fig. 16.3 Acid phosphatase banding pattern of parental species



ACID PHOSPHATASE BANDING PATTERN OF PARENTAL SPECIES

Fig16·3

Fig. 16.3

isozymes studied did not show any polymorphism among the parents.

### (iv) Molecular markers

The genomic DNA was isolated from the leaves of sixteen plants including eleven monosomic addition plants, four disomic addition lines and a double monosomic plant, along with the parents. The quality and quantity of DNA was checked by running the DNA samples on 0.8 per cent agarose gel. Hind-III lambda marker was used as standard to calculate the concentration of the isolated DNA. A good quality of DNA was obtained from thirteen plants (940522-12, 940582-11, 940582-6, 940527-1, 940527-5, 940527-9, 940522-3, 940522-17, 940580-3, 940580-9, 940521-3, 940521-8, 940524-6 and the parents.

Then fifteen microgram of genomic DNA from each of the eleven addition lines (940522-12, 940582-11, 940582-6, 940527-1, 940527-5, 940522-3, 940580-3, 940580-9, 940521-3, 940521-8, 940524-6 and the parents, WL 711 and *Aegilops triuncialis* was digested with ECORI and Hind-III restriction enzymes. The sampels were separated by electrophoresis on 1 per cent agarose gel and blotted on to a nylon membrane. The results of digestion by restriction enzymes are shown in Fig.17.

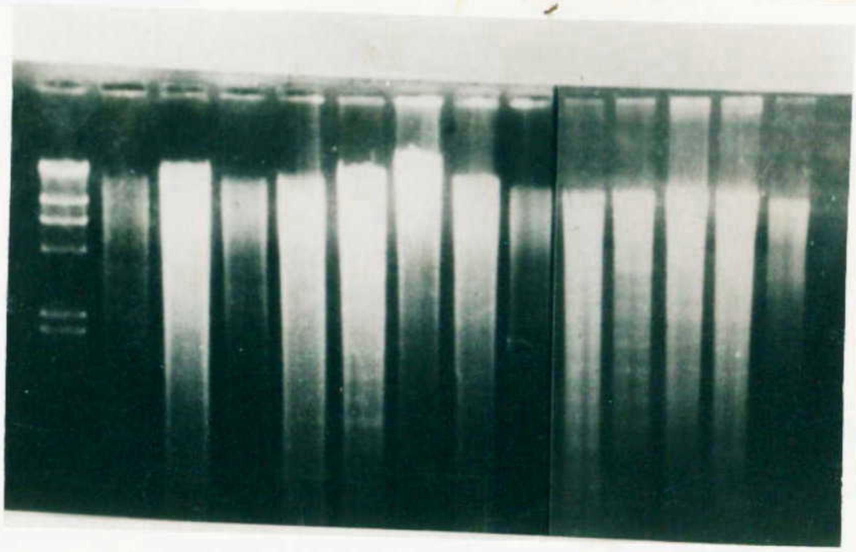
Fig.17 DNA digested with ECOR-I and Hind-III restriction enzymes

G1: ECOR-I digestion patterns.

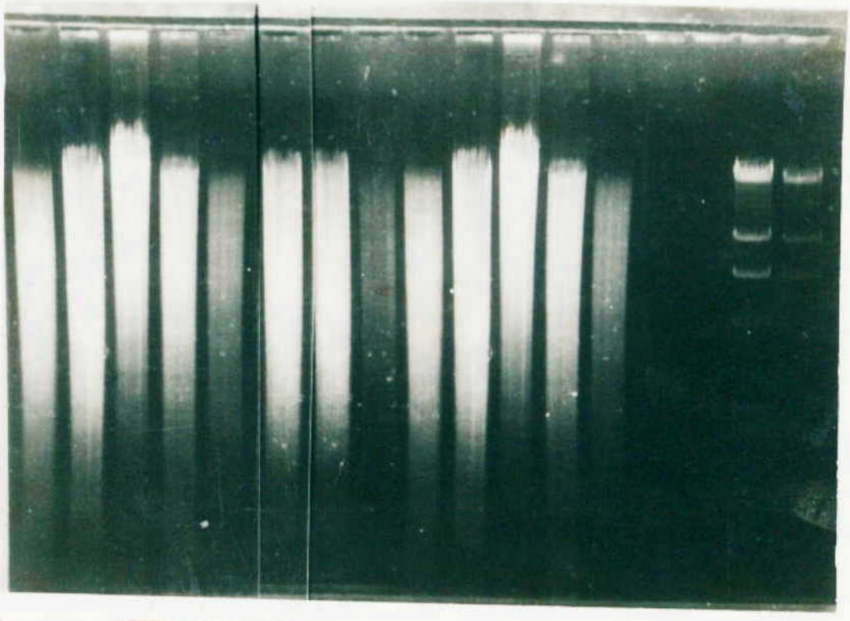
| Lane no. | Sample | Lane no. | Sample | Lane no. | Sample |
|---|---|---|---|---|---|
| 1. | Lambda marker | 6. | 940527-5 | 11. | 940521-8 |
| 2. | 940522-12 | 7. | 940522-3 | 12. | 940524-6 |
| 3. | 940582-11 | 8. | 940580-3 | 13. | *Ae.triuncialis* |
| 4. | 940582-6 | 9. | 940580-9 | 14. | WL 711. |
| 5. | 940527-1 | 10. | 940521-3 | | |

G-2 Hind-III digestion pattern

| | | | | | |
|---|---|---|---|---|---|
| 1. | 940522-12 | 6. | 940522-3 | 11. | 940524-6 |
| 2. | 940582-11 | 7. | 940580-3 | 12. | *Ae.triuncialis* |
| 3. | 940582-6 | 8. | 940580-9 | 13. | WL 711 |
| 4. | 940527-1 | 9. | 940521-3 | 14. | Lambda marker |
| 5. | 940527-5 | 10. | 940521-8 | 15. | Lambda marker |



G-1



G-2

Fig. 17

### (v) Grain characteristics

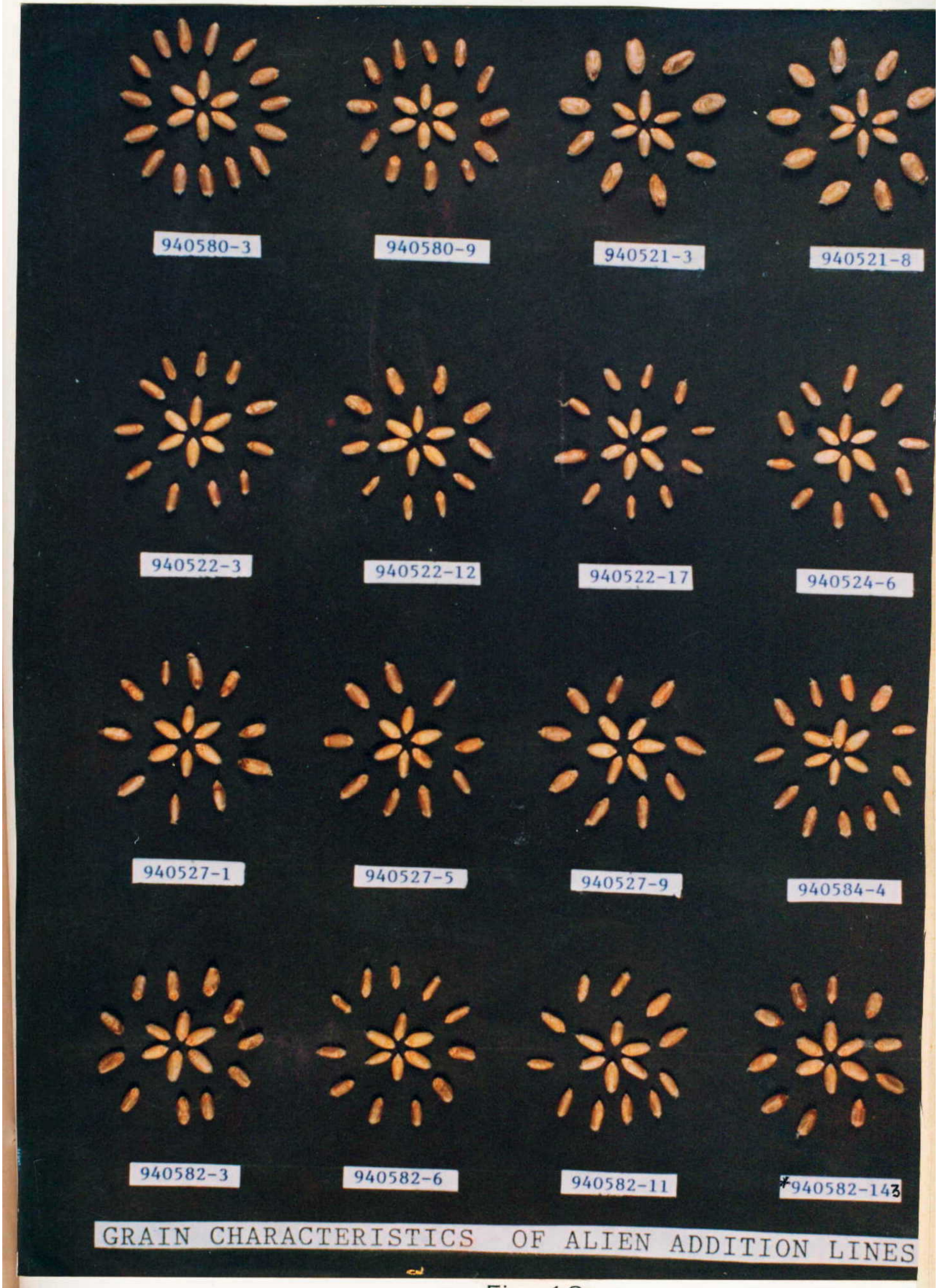
The $BC_3F_2$ grains of various addition lines were observed for seed colour, size and overall appearance (Fig.18).

The grains of plant number 940521-3 and 940521-8 were amber and bolder while the grains of all other plants identified as alien addition lines were red, small and slender as compared to their recurrent parent WL 711.

Among the red grained plants, the grains of plants identified as monosomic addition plants (940580-3, 940522-3, 940522-12, 940522-17, 940527-1, 940527-5, 940584-4, 940582-3 and 940582-6 were shrivelled whereas the grains of disomic addition plants (940580-9, 940527-9, 940524-6, 94052-11) were normal in appearance.

Fig. 18: Grain colour and size characteristics of different alien addition lines

*Plant number 940582-143 also represents plant number 940582-3, samples were repeated to make the symmetry.



GRAIN CHARACTERISTICS OF ALIEN ADDITION LINES

Fig. 18

## CHAPTER V

# DISCUSSION

*Aegilops triuncialis* possesses a multiple resistance to various wheat diseases (Dhaliwal *et al*. 1993) and novel genes for the improvement of protein quality (Singh *et al*. 1994).

The present investigation was, therefore, planned to develop the alien-addition lines of *Aegilops triuncialis* in wheat, as the development of alien addition lines of the donor species is considered to be a pre-requisite for the transfer of such useful variability into cultivated species.

The $BC_1F_2/BC_2$ seeds of an inter-generic cross of widely adapted hexaploid wheat variety WL 711 (as female) x *Aegilops triuncialis* (as male) were taken for investigation during 1993-94. The various findings of investigation are discussed under following sub-heads:

1. Development of alien addition lines.
2. Characterization of alien addition lines.

## 1. DEVELOPMENT OF ALIEN ADDITION LINES

The $BC_1F_2/BC_2$ seeds exhibited poor germination with an average germination percentage of 48.38 during 1993-94 (Table 2). Similarly, their $BC_2F_1/BC_3$ progeny had poor germination with an average of 38.8 per cent (Table 3). The low germination percentage is mainly attributable to degeneration of hybrid endosperm. Some of the seeds of $BC_1F_2/BC_2/BC_3/BC_2F_1$ plants were small and shrivelled. Similar type of seeds were obtained by Bai *et al.* (1994) in the $F_1$ and $BC_1$ progeny during the production of *Aegilops triaristratum* addition lines in wheat and inviability of hybrid endosperm was reported as the cause. It was found that the abnormal development of hybrid endosperm caused necrosis at the upper region of scutellum near the tip of coleoptile and then in rest of the embryo, until the embryo died. Same process might have occurred in the present case in shrivelled seeds causing the embryos to eventually die.

The viable $BC_1F_2/BC_2$ plants also exhibited high sterility. The progeny of plant number 25-5-3 ($BC_1F_2$) and 930923 ($BC_2$) was completely sterile. Also low seed set with an average of 17.84 per cent was observed on $BC_1F_2/BC_2$ plants which also showed low fertility of $BC_1F_2/BC_2$ plants. Bai *et al.* (1994) reported 0.7 to 14 per cent seed set in $BC_1$ of a cross of *Triticum triaristratum* with wheat. Also low seed set in $BC_1$ was reported as compared to $F_1$ (12.5% to 78.33%). Similar results were obtained in our investigation as the seed set on $F_1$ was reported to be 25 per cent (Singh *et al.* 1992). The possible cause of low seed set on $BC_1$ than $F_1$

was explained by Bai *et al.* (1994), on the basis of cytological studies. It was reported that only unreduced gametes from restitution divisions plus some gametes with partial genomes from $F_1$ were only involved in the production of viable backcrossed seeds.

The low fertility of $BC_1F_2/BC_2$ seeds may be due to the presence of higher number of *Aegilops triuncialis* chromosomes as univalents (the chromosome number varied from 42 to 50) which failed to pair during meiosis leading to the formation of unbalanced gametes. Riley (1960) also reported low fertility to be associated with meiotic irregularities due to pairing failure of rye chromosomes in wheat-rye addition lines. Bai *et al* (1994) also reported low chromosome pairing as the cause of low fertility.

'Cuckoo' chromosomes may be the other possible cause of low fertility as reported by Endo and Tsunewaki (1975) and Endo (1978). Also majority of the addition lines characterized later carried 'cuckoo' chromosome. The sterility has been shown to be associated with preferentially transmitted sub-terminal chromosome of group three of *Aegilops triuncialis* through the gametes of both sexes. Sterility was brought by the fact that when a particular *Aegilops triuncialis* chromosome was present in monosomic state most of the gametes especially the egg cells lacking that chromosome were sterile and could not function in fertilization.

The sterility in the lines without 'cuckoo' chromosome was due to the abnormality in the first embryo sac mitosis and first pollen grain mitosis (Miller 1984, b). In the abnormal metaphases the 'cuckoo' chromosomes led to the formation of small, lethal, acentric fragments of wheat chromosomes in the gametophytes lacking it, thus making them unviable.

However, a remarkable increase in the fertility was obsrved in $BC_3$ progeny. This might be due to the elimination of chromosomes of *Aegilops triuncialis* at very higher rate as majority of the $BC_3$ plants were euploid. In the selfed, ($BC_2F_2$) progeny, again high sterility was observed (Table 10). The low chromosome pairing in some of plants having 45 chromosomes and the substitutions, in plants with 42 chromosomes might be the possible cause for the low fertility of these plants.

On the basis of cytological studies of 53 $BC_2F_2/BC_3$ plants, twenty eight (52.8%) were found to be euploid. Only twelve (22.6%) plants were monosomic addition lines and four (7.5%) were disomic additions (Table 7). The possible cause of low transmission of alien chromosomes may be the preferential elimination of univalents during megasporogenesis and microsporogenesis, such that the female gametes with fewer alien chromosomes would be more viable. Similar results were obtained by Riley (1960) during the production of wheat-rye addition lines. He found that lines with single pair of rye chromosomes added to full complement of wheat were less regular at meiosis which was due to the failure of rye chromosomes to pair, leading to their preferential elimination. So, the higher frequency of plants were found to return to euploid state.

In the $BC_3$ progeny of a plant characterized as monosomic addition in $BC_2$, Table 7 (plant no. 930924-8 and 940522 in $BC_2$ and $BC_3$ respectively). The alien chromosome was retained by three (37.5%) out of eight plants. So the transmission rate of single alien chromosome via female gamete was 37.5 per cent. However, the differential transmission rates via male and female gametes were not studied as the spikes were backcrossed to recurrent parent and no selfed seed was obtained. Blanco and Simeone (1987) reported 7.5 per cent to 27.5 per cent transmission rate of alien chromosome in the backcrossed progeny of a cross *Dasypyrum villosum* x *Triticum durum*. Makino (1976) reported 4.5 per cent to 25.9 per cent transmission rate of alien chromosomes by female gamete and 0.9 to 3.1 per cent via male gamete in the selfed progeny of monosomic addition lines of *Aegilops umbellulata* in *Triticum durum*. However, the transmission rate of a preferentially transmitted chromosome of *Aegilops sharonensis* has been reported to be quite high (97.8%) by King *et al*. (1993). Xue (1988) obtained very low rate of retention (4.3%) of barley chromosomes in the selfed progeny of barley chromosome additions to wheat.

In the $BC_3$ progeny of previously characterized $BC_2$ disomic addition plant, Table 7 (plant no. 930924-16 and 940524 in $BC_2$ and $BC_3$ respectively), only one plant was able to retain the alien chromosome in disomic form while no monosomic addition plant was obtained. However, maximum frequency of plants was expected here with 2n = 44. The causes of deviation of results are unexplainable. However, the data may be showing biased results due to small number of plants in the $BC_3$ progeny. Cytological maintenance of alien-addition lines year after year is necessary as suggested by Miller (1984, a) as supported by the present investigation and the results of Yachevskaya (1985), Chrzastek (1987) and Xue (1988), showing higher unstability of alien addition lines.

## 2. CHARACTERIZATION OF ALIEN ADDITION LINES

The markers used for characterizing alien-addition lines included biochemical, morphological, disease ressitance and molecular markers.

Among the biochemical markers, the high molecular weight glutenin subunit analysis was done by the half seed technique described by Blackman and Payne (1987) for characterizing alien chromosomes belonging to homoeologous group one (Payne *et al*. 1980, Lawrence and Shephard 1981). *Aegilops triuncialis* was found to express specific bands of 'x' and 'y' subunits which are different from those of Glu 1A, 1B and 1D subunits of *Triticum aestivum*. (Singh et al. 1994). A total of twenty samples were tested out of which six plants were later identified as addition lines cytologically. However, none of the plant showed *Aegilops triuncialis* specific band (Fig.15). So, these results ruled out the existence of alien chromosome of homoeologous group one among the plants tested.

The isozymes have also been shown as useful markers for the identification of alien addition lines as well as for allocating the alien chromosomes to particular homoeologous group in a number of species (Driscoll and Sears 1971, Tang and Hart 1975, Islam *et al*., 1981, Forester *et al*. 1987, Zhong *et al*. 1990). So, the isozymes of peroxidase, esterase and acid phosphatase were selected to characterize any of the addition lines belonging to homoeologous groups 2, 3 and 4 respectively as the location of genes for these isozymes has been reported on respective chromosomes (Gill *et al.* 1990).

The parental species were tested to study polymorphism for the isozymes. However, the parental species failed to show polymorphism for all the three enzymes tested. (Fig.16.1, 16.2 and 16.3). This indicates that both the parents had identical alleles for the expression of these enzymes. So this technique could not be used for characterizing the alien addition lines. Gill *et al.* (1991) also obtained polymorphism only among five enzymes, out of fifteen among the two accessions of *Triticum tauschii* that were most divergent for RFLP's. Similarly, Schmidt *et al.* (1993) did not obtain any *Aegilops* specific band for peroxidase isozyme on testing the ability of different isozymes to characterize *Triticum aestivum - Aegilops marksgraffi* addition lines.

Various aneuploid series of Chinese Spring have been extensively used for mapping the loci of isozymes of different enzymes inspite of the lack of polymorphism among related species.

The gross morphology of alien addition lines did not differ from each other and from the recurrent parent, suggesting that either the genetic control of various morphological traits of recurrent parents was epistatic to those of *Aegilops triuncialis* or the addition lines carried only particular chromosome not affecting any of the morphological traits examined.

Thirteen addition lines had smaller, red and slender grains while the grains of other two plants (940521-3, 940521-8) were amber and bold. Fig. 18. Nelson-Ehle (1911) had established that red vs. white colour in wheat was conditioned by alleles at three different loci, (the red being dominant) and these alleles have been allocated to the chromosomes of homoeologous group three by Sears (1944) and Metzger and Sialbagh (1974).

Similarly, the alien addition lines of *Aegilops bicornis* in holdfast winter wheat and that of *Aegilops squarrosa* in *Triticum durum* with red grains have been allocated to group three by Riley and Chapman (1968) and Dhaliwal *et al.* (1991), respectively.

So the various addition lines with red-grain colour could be allocated to homoeologous group three.

Further, the shrivelled appearance of the grains of monosomic addition lines and normal appearance of disomic plants confirmed the presence of group 3C having 'cuckoo' chromosome which has been reported to cause grain shrivelling when present in monosomic state (Endo 1978, Endo 1988).

The grain size in wheat has been found to be controlled by number of endosperm cells and size (Gleadow *et al*. 1983). The chromosomes 1A, 1D and 7A have been found to be associated with grain size in wheat by Chozecki *et al*. (1983) as they govern number and pattern of growth of endosperm cells. So, the lines with amber and bold grains could have alien chromosomes from homoeologous group 1 or 7. However, the high molecular glutenin weight subunit analysis of these plants did not reveal the presence of *Aegilops triuncialis* specific bands (Fig.19). So, these lines could be allocated to group 7.

On the basis of response to disease reaction to race 77A-1 under controlled conditions and to prevalent races of leaf rust and yellow rust under field conditions, several plants showing resistance to either both diseases or a particular disease were identified (Table 8) during 1993-94. As the recurrent parent was heavily infected by rust diseases while the donor species showed high level of resistance, it showed that the resistance was due to the

Fig.19: High molecular weight glutenin SDS-PAGE patterns of alien-addition lines having bold grains along with the parental species.

Representation of lanes:

| Lane number | Sample |
|---|---|
| 1 | WL 711 |
| 2 | WL 711 |
| 3. | 940521-3 |
| 4. | 940521-3 |
| 5. | 940521-8 |
| 6. | 940521-8 |
| 7. | *Ae. triuncialis* |

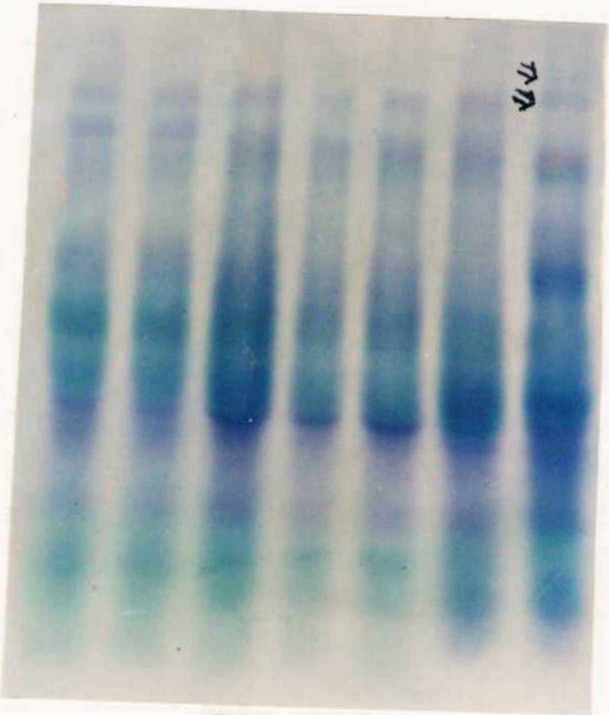


Fig. 19

⇒ Indicates Ae. triuncialis specific bands presence of alien chromosomes and their expression was dominant in *Triticum aestivum* background.

However, during 1994-95, the progeny of only two resistant plants (930924-26, 930924-41) was obtained in field conditions. All the $BC_3$ plants in the progeny of these plants were susceptible to both of the rusts. This was due to the fact that the alien chromosomes carrying the genes for disease resistance had been eliminated in the $BC_3$ progeny, as is clearly indicated by the chromosome number of resistant $BC_2$ plants and their $BC_3$ progeny in Table 9.

However, in the $BC_2F_2$ progeny of a plant 930924-26, eight resistant plants showing resistance to either leaf or yellow rust or both were identified (Table 10). Also these plants had red-grain colour which was due to the presence of *Aegilops triuncialis* chromosome of group three. The data on chromosome number and resistance to leaf and yellow rust in these plants suggests that there are certain additions and substitutions of other chromosomes of *Aegilops triuncialis*.

The plant numbers 65-7-1 and 65-7-2 with 42 chromosome number had resistance to yellow rust only. So, these were probably the substitution lines of chromosome 3U. The low fertility of these plants further supports substitution. The plant numbers 65-6-6, 65-6-7 and 65-7-3 with 45 chromosomes had resistance to yellow rust only. So these could be the disomic addition of 3C (red grains) along with the monosomic addition of 3U. Plant numbers 65-6-2 and 65-7-6 also had 45 chromosomes but resistance to both leaf and yellow rust. So, in these plants an unknown chromosome carrying resistance to both the rusts was present along with the disomic addition of 3C ('cuckoo' chromosome). The plant no. 65-6-4 had 42 chromosomes and resistance to leaf rust only. So, it could be a double substitution line for chromosome 3C and probably 6U as the gene *Lr* 9 for leaf rust has been reported on chromosome 6U of *Aegilops umbellulata* by (Sears and Kimber 1956).

For molecular characterization of addition lines the DNA was isolated. Good quality DNA was obtained from thirteen addition lines out of sixteen. The DNA degradation observed in other three addition lines might be due to heavy infection of rusts. Further, the southern blots were made by restricting the DNA by ECOR-I and Hind III restriction enzymes to characterize addition lines by the RFLP markers using chromosome specific probes provided by Sharp *et al.* (1988). However, due to the non-availability of either radioactive or non-radioactive label, the southern hybridization of blots could not be accomplished.

The 'cuckoo' chromosome was the major hinderance due to which the extraction of fourteen possible alien addition lines of *Aegilops triuncialis* could not be accomplished. Also, among the disease resistant plants no monosomic addition line without the 'cuckoo' chromosome could be obtained as the 'cuckoo' chromosome was retained along with the chromosomes governing disease resistance. The disease resistant $BC_2F_2$ plants with 45 chromosomes may be crossed further to obtain monosomic addition lines. Endo (1978) was also unable to obtain alien-addition lines of *Aegilops triuncialis* in wheat belonging to different homoeologous groups as all the lines obtained were of group 3C only. Miller *et al*. (1982) also obtained only one addition line of *Aegilops sharonensis* in wheat of homoeologous group four ($4S^1$), the chromosome also having 'cuckoo' characteristics.

**Implications of alien addition lines**

Once the addition lines of alien species become available, these may be effectively used for the genetic analysis and chromosome manipulations of cultivated wheats. The characterized alien addition lines in which homoeologous group of the alien chromosomes is identified, can be used to determine the role of each alien chromosome for their usefulness.

The donor species (*Aegilops triuncialis*) taken during the present investigation possesses several desirable traits including high level of disease resistance (Dhaliwal *et al*. 1993) and genes for improvement of protein quality (Singh *et al*. 1994). Thus, the development of alien-addition lines of *Aegilops triuncialis* in wheat would have been of immense importance for plant breeders for transferring desirable characters into cultivated wheats.

Since *Aegilops triuncialis* lacks 'ph' suppressing locus, the transfer of genes into wheat background could be a difficult task. So the use of monosomic '5B' of *Triticum aestivum* as practiced by Dyck *et al*. (1990) or '5B' suppressing system of *Aegilops speltoides* could be more appropriate for transferring desirable genes of *Aegilops triuncialis* into *Triticum aestivum*.

Moreover, the interference of 'cuckoo' chromosomes has been recognized as a major hinderance in development of alien addition lines of fourteen possible chromosomes of *Aegilops triuncialis* in wheat during the present investigation. To overcome this problem, Tsujimoto and Tsunewaki (1985) have identified a gene 'Igcl' on chromosome 3B of wheat cultivar Norin-26. The gene is reported to act as dominant suppressor of gametocidal gene of 3C. This gene can be exploited for obtaining the alien addition lines of all homoeologous groups of *Aegilops triuncialis* as all chromosomes show normal distribution during the interspecific cross in the presence of this gene.

**Uses of 'cuckoo' chromosome**

The transmission rate of 'cuckoo' chromosomes has been reported to be very high. King *et al.* (1990) have reported upto 97.8 per cent transmission of a preferentially transmitted chromosome of *Aegilops sharonensis* ($4S^1$). So this chromosome may be exploited for the stable transfer of desirable characters into recipient species. Endo (1988) has shown that chromosome (3C) of *Aegilops triuncialis* when present in disomic state induces high rates of translocation. For example, on crossing a disomic addition line carrying chromosome 3C of *Aegilops triuncialis* with *Triticum aestivum* cv. 'Burgas' in which 1B chromosome pair has been substituted by 1R of rye high frequency of translocations in 1R with wheat chromosomes were observed. This phenomena might be exploited for transferring the desirable genes from alien addition lines into recipient backgrounds as an alternate to the induced translocations.

For example, King and Koebner (1991) used $4S^1$ (preferentially transmitted chromosome of *Aegilops sharonensis*) to eliminate segregation in height among semi dwarf wheats. Translocations were produced between $4S^1$ and Rht2 gene carrying chromosome arm of 4D of wheat. The translocation were stable and preferentially transmitted by gametes of both sexes. Similarly, King *et al.* (1993) exploited the same $4S^1$ chromosome for transferring Glu-U1 and Gli-U1 genes of *Aegilops umbellulata* into wheat.

Thus, the high frequency of alien addition lines of group 3C obtained in the present investigation could find their use in transferring the desired characters from donor species into recipient backgrounds as discussed above.

# SUMMARY AND CONCLUSIONS

The present investigation was aimed to develop alien addition lines of *Aegilops triuncialis* in wheat cv. WL 711 as the donor species possess several desirable traits including high level of disease resistance (Dhaliwal *et al*. (1993) and genes for the improvement of protein quality (Singh *et al*. 1994).

The $BC_1F_2/BC_2$ and $BC_2F_1/BC_3/BC_2F_2$ seeds of an intergeneric cross of *Triticum aestivum* cv. WL 711 (as female) and *Aegilops triuncialis* (as male) were investigated during 1993-94 and 1994-95 respectively.

Poor germination of $BC_1F_2/BC_2$ (48.38% average) and $BC_2F_1/BC_3/BC_2F_2$ seeds (38.8% average) was observed which was attributable to the degeneration of hybrid endosperm in some of shrunken seeds. Also the $BC_1F_2/BC_2$ seeds exhibited low fertility and low seed set due to the low pairing of alien chromosomes which led to the formation of unbalanced gametes and due to the action of 'cuckoo' chromosome. However, the fertility of $BC_3$ plants was quite high which might be due to the higher rate of elimination of alien chromosome in $BC_3$ progeny as majority of plants were euploid.

On the basis of cytological analysis of fifty three plants, twelve monosomic and four disomic addition lines were identified, whereas majority of the plants (52.8%) had lost the alien chromosomes which might be due to preferential elimination of univalents during gametogenesis. Various monosomic and disomic addition lines were characterized on the basis of biochemical, morphological and disease resistance markers. The various findings can be summarized as below:

1. Various addition lines exhibited similarity in gross morphology to one another as well as to the recurrent parent.
2. On the basis of grain characteristics, two monosomic addition lines having amber and bold grains could be allocated to group seven.
3. All other monosomic and disomic addition lines having slender, smaller and red grains could be allocated to group three.
4. The alien addition lines with red grain colour could be further allocated to chromosome 3C on the basis of shrivelled appearance of grains of monosomic addition lines, indicating the presence of 'cuckoo' chromosome.

5. The presence of homoeologous group one of *Aegilops triuncialis* among various addition lines was also ruled out by SDS-PAGE, HMW glutenin subunit studies.
6. The parents might have identical alleles for the expression of peroxidase, esterase and acid phosphatase enzymes, as they did not show any polymorphism.
7. Among the disease resistant plants, none of the alien addition lines could be obtained due to the interference of 'cuckoo' chromosome. The resistant plants had either substitution or multiple additions. However, possibility was there as plants with multiple additions (2n = 45) could be further backcrossed to recurrent parent.
8. On the basis of red grain colour of resistant plants, their chromosome number and disease reaction, the plants carrying disease resistance could be allocated to chromosome 3U having genes for yellow rust resistance only, other chromosome probably 6U having genes for leaf rust resistance only and a third unknown chromosome having genes for both leaf and yellow rust.

'Cuckoo' chromosome was the major hinderance during the investigation due to which fourteen possible alien addition lines could not be obtained. The exploitation of a gene 'IgCl', present in cv. Norin-26, which acts as dominant suppressor of cuckoo chromosome of *Aegilops triuncialis* is suggested.

Alternatively, exploitation of disomic addition lines of group 3C of *Aegilops triuncialis* ('cuckoo' chromosome) is suggested for the stable transfer of desired characters from related species. This can be achieved by producing translocations between 'cuckoo' chromosome arm having genes for preferential transmission and the chromosome arm of species having desired character to be transferred. Such translocations have been reported to have preferential and stable transmission to successive generations.

# Appendix

**List of solutions used during HMW glutenin subunit studies, Isozyme studies, DNA Isolation and preparation of blots.**

1. **Acetate buffer**: 0.2 M buffer was prepared by mixing 16.405 g of sodium acetate and 3.6 ml of glacial acetic acid in one litre of distilled water and pH was adjusted to 6.5.
2. **Acrylamide 35%**: 35 g of purified acrylamide was dissolved in double distilled water to make 100 ml volume.
3. **Bridge buffer**: 0.3 M sodium borate buffer, pH 8.65 was prepared by dissolving:

   10.55 g boric acid

   40.0 g sodium hydroxide pellets

   in one litre of distilled water and pH was adjusted to 8.65.
4. **Chloroform: Isoamyl solution**: mixed in ratio of 24:1.
5. **Denaturing solution**: (0.4 N NaOH + 0.6 M NaCl): 16 g of NaOH and 35.064 g of NaCl were dissolved in 700 ml distilled water and volume was made one litre.
6. **Dibasic solution**: 0.2 M Disodium hydrogen phosphate (pH 9.2) 14.19 g/500 ml of distilled water.

   Then 68.5 ml of mono, 31.5 ml of dibasic and 100 ml of distilled water were added and pH was adjusted to 6.5.

7. **Electrophoresis buffer (pH 8.3)**: 75.5 g glycine, 15 g Tris and 5 g SDS were dissolved in distilled water and final volume was made to 5 litre.
8. **Ethanol**: Absolute and 70%.
9. **Ethidium bromide**: 1 g of Ethidium bromide was dissolved in 100 ml of water. The solution was stirred on magnetic stirrer until dye was mixed properly. The tube was wrapped in aluminium foil and stored at -20°C.
10. **Extraction buffer**: Composition:
    - (a) 100 mM Tris HCl (7.88 g/500 ml; pH 8.0)
      7.88 g of tris HCl were dissolved in 100 ml of distilled water and pH was adjusted to 8.0
    - (b) 50 mM EDTA (9.306 g/500 ml, pH 8.0):
      9.306 g of EDTA were dissolved in 100 ml distilled water. pH was adjusted to 8.0.
    - (c) 1.25% SDS
    - (d) 50 mM NaCl
      6.25 g of SDS and 14.61 g of NaCl were dissolved in 200 ml of distilled water. The final volume was made to 500 ml by mixing all the solutions and adding distilled water.
    - (e) 3.8 g of sodium metabisulphate was added just before use and solution was autoclaved.

11. **Isopropanol**
12. **0.25 M HCl**: 25 ml HCl + 1075 ml distilled water.
13. **Monobasic solution**: 0.2M sodium dihydrogen phosphate (pH 4.3) 15.6 g/500 ml of distilled water).
14. **Neutralizing solution**: (0.5 M Tris + 1.5 M NaCl; pH 7.5):

    60.57 g of tris and 87.66 g of NaCl were dissolved in 700 ml distilled water. pH was adjusted to 7.5 and final volume was made to 1 litre.
15. **Phenol chloroform**: Equal amounts of phenol and chloroform were mixed. Mixture was equilibrated by extracting several times with 0.1 M tris HCl (pH 7.6). The equilibrated mixture was stored under equal volume of 0.01M tris HCl (pH 7.6) in dark glass bottles.
16. **Phosphate buffer**: 0.1M buffer of pH 6.5 was prepared by mixing solutions of mono and dibasic salts of sodium.
17. **5M Potassium acetate**: 49.07 g was dissolved per 100 ml of distilled water.
18. **RNAse enzyme**
19. **3x sample buffer**: 10 ml glycerol, 6.25 ml 1M Tris pH 6.8, 2 g SDS (purified), 20 mg pyronine-G (tracking dye) and 12.05 ml distilled water were mixed to 50 ml measuring cylinder and stored as stock solution.

20. **3M sodium acetate**: 24.60 g was dissolved per 100 ml of water.

21. **10% SDS**: 10 gm purified SDS was dissolved in distilled water to make 100 ml volume.

22. **20 x SSC**: 173 g of NaCl and 88.2 g sodium titrate were dissolved in 800 ml water. pH was adjusted to 7.0 with 10 N solution of NaOH. Final volume was made one litre with distilled water. Solution was dispersed into aliquotes and was sterilized by autoclaving.

23. **Staining dye**: 2 g Brilliant Commassie blue-R was dissolved in 95% ethanol and final volume was made to 50 ml and stored in dark.

24. **TAE buffer 1X**

25. **T.E. buffer**

26. **1M Tris pH 6.8**: 12.11 g Trisma base was dissolved in 50 ml of distilled water. The pH was adjusted to 7.0 with concentrated HCl and then accurately with 1N HCl to 6.8. The final volume was made to 100 ml.

27. **1M Tris pH 8.6**: 12.11 g of Trisma base was dissolved in distilled water. The pH was adjusted to 8.6 with concentrated HCl and it was allowed to cool at room temperature. There was slight increase in pH after cooling at room temperature which was again adjusted to 8.6 and the final volume was made to 100 ml.

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*Original not seen.