TAGGING OF THE SPECIFIC GENOME AND CHROMOSOME(S) OF HEXAPLOID WHEAT TRIGGERING CHROMOSOME ELIMINATION IN WHEAT x *IMPERATA CYLINDRICA* SYSTEM OF DOUBLED HAPLOIDY BREEDING

THESIS

By

VINEETA KAILA
(A-2009-40-08)

Submitted to

CHAUDHARY SARWAN KUMAR
HIMACHAL PRADESH KRISHI VISHVAVIDYALAYA
PALAMPUR – 176 062, INDIA

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Dr. H. K. Chaudhary
Professor and Head

Department of Crop Improvement
College of Agriculture
CSK HP Krishi Vishvavidyalaya
Palampur-176062 (H.P.) INDIA

CERTIFICATE – I

This is to certify that the thesis entitled **"Tagging of the specific genome and chromosome(s) of hexaploid wheat triggering chromosome elimination in wheat x *Imperata cylindrica* system of doubled haploidy breeding"** submitted in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy (Agriculture)** in the discipline of **Plant Breeding & Genetics** of CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur is a bonafide research work carried out by **Ms. Vineeta Kaila (Admission No. A-2009-40-08)** daughter of **Mrs. Raj Kaila and Dr. Om Prakash Kaila** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

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

(H.K. Chaudhary)
Major Advisor

Place: Palampur
Dated: June, 2013

CERTIFICATE- II

This is to certify that the thesis entitled, **"Tagging of the specific genome and chromosome(s) of hexaploid wheat triggering chromosome elimination in wheat x *Imperata cylindrica* system of doubled haploidy breeding"** submitted by **Ms. Vineeta Kaila (Admission No. A-2009-40-08)** daughter of **Dr. Om Prakash Kaila** to the CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur in partial fulfillment of the requirements for the degree of **Doctor of Philosophy (Agriculture)** in the discipline of **Plant Breeding & Genetics** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

Dr. H.K. Chaudhary Chairperson Advisory Committee	Dr. Sanat Sujat Singh External Examiner
Dr. R.K. Chahota Member	Dr. (Mrs.) Usha Rana Member
Dr. K.S. Rana Member	Dr. J.K. Sharma Deanôs nominee

_____
Head of the Department

_____
Dean, Postgraduate Studies

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CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur- 176062 (H.P)

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Name of the student	:	Vineeta Kaila
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ABSTRACT

The present research endeavour entitled as ñtagging of the specific genome and chromosome(s) of hexaploid wheat triggering chromosome elimination in wheat x *Imperata cylindrica* system of doubled haploidy breedingò was undertaken at Molecular Cytogenetics & Tissue Culture Lab and Experimental Farm of the Department of Crop Improvement, CSK HP Agricultural University, Palampur, Himachal Pradesh, India during 2010- 11 and 2011- 12 with the objective of identifying the wheat genomes and chromosomes responsible for elimination of whole *I. cylindrica* chromosome complement in wheat x *I. cylindrica* hybrids during early embryogenesis. Upon hybridization of different species of wheat *viz., Triticum monococcum, T. tauschii* and *T. durum* with *I. cylindrica,* haploid embryos were recovered in *T. tauschii* and *T. durum* only which revealed that the D and B genomes of hexaploid wheat are triggering the paternal chromosome elimination process. The possibility of enhancing the efficiency of embryo formation *via* increasing the exogenous supply of 2,4-D was also exploited and 0.03 % and 0.04 % of 2,4-D concentration were found optimum for haploid induction in *T. durum* and *T. tauschii*, respectively. The induction of haploid embryos in these species was also confirmed by cross-sectional studies of fertilized ovules derived after their hybridization with *I. cylindrica*. For further resolution of the genetic control of paternal chromosome elimination in wheat x *I. cylindrica* crosses, cytogenetic stocks *viz.,* complete set of monosomic and nullisomic lines derived from óChinese Springô cultivar of hexaploid wheat and D genome substitution lines of tetraploid wheat cultivar óLangdonô were crossed with *I. cylindrica.* Statistical analysis of embryo formation frequency in monosomics revealed the role of 4D, 1D, 6B and 6D chromosomes as the absence of these chromosomes resulted in reduction of haploid embryo formation frequency from control as well as mean. These results were also supported by data obtained from nullisomics and substitution lines. Morphological characterization of monosomic series was carried out on the basis of 24 traits to identify visual markers for differentiation of aneuploids from disomic plants. Eight morphological descriptors were identified for differentiation of nullisomics, that is, seed size, plant height, spike length, number of tillers, florets per spike, seeds per spike, days to flowering and 100 seed weight. Whereas, two descriptors were identified in case of monosomics *viz.,* spike shape for 5A and days to flowering for 2B monosomic.

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(Vineeta Kaila) **(H.K. Chaudhary)**
Student **Major Advisor**
Date: **Date:**

_____
Head of the Department

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(Vineeta Kaila) **(H.K. Chaudhary)**
Student **Major Advisor**
Date: **Date:**

_____
Head of the Department

_____
Dean, Postgraduate Studies

1. INTRODUCTION

The success of any plant breeding endeavour depends upon the prevalence of genetic diversity present in the germplasm of the targeted plant species. During green revolution era, most of the wheat improvement efforts were focused on accumulation of desirable alleles manifesting traits of interest that resulted in development of dwarf, input responsive and high yielding varieties hence, ensuring food and livelihood security of million of people. However, the replacement of wheat landraces with more uniform varieties having common parents, eroded and narrowed the genetic base that not only resulted in yield plateau but also increased vulnerability of the crop to new diseases and insect pests. To overcome such undesirable consequences of wheat improvement programmes and prepare the wheat cultivars for future challenges and changing climatic scenario, there is need to widen the genetic base of the adopted germplasm by incorporating novel genes from distant sources. The wide hybridization mediated genetic upgradation programmes involving wild species provide opportunities for combination of diverse genomes, shuffling and reassembling of desirable alleles thereby creating enormous amount of genetic variation (Goodman et al. 1987; Sain et al. 2002). Nevertheless, various pre- and post- fertilization barriers such as pollen- stigma incompatibility and instability of alien genome in hybrids are often encountered during such hybridization endeavours. The instability of alien chromosomes after normal fusion of gametes may result in partial or complete uniparental chromosome elimination. In cereals, a partial somatic elimination of chromosomes of one parental species has been reported in wide crosses of *Triticum aestivum* x *Hordeum bulbosum* (Barclay 1975), *Avena sativa* x *Zea mays* (Riera-Lizarazu et al. 1996) and *H. lechleri* x *H. vulgare* (Linde-Laursen and von Bothmer 1999). Complete uniparental chromosome elimination occurs in some interspecific crosses involving closely related species of same genus like *H. vulgare* x *H. bulbosum* (Kasha and Kao 1970) as well as among species of different genera *viz., T. aestivum* x *Z. mays* (Laurie and Bennett 1989), *T. aestivum* x *Pennisetum glaucum* (Ahmad and Comeau 1990) and *T. aestivum* x *Sorghum bicolor* (Inagaki and Mujeeb-Kazi 1995).

In wheat, frequent loss of whole paternal chromosome complement has been observed when it is crossed with various distant species of Gramineae genera like *H. bulbosum* (Barclay 1975), *Z. mays* (Laurie and Bennett 1989), *Coix lachryma-jobi* (Mochida and Tsujimoto 2001) and *Imperata cylindrica* (Chaudhary et al. 2005). Such wide crosses have been successfully utilized for development of haploids and doubled haploids in wheat. Though chromosome elimination assists in the acceleration of wheat improvement programmes *via* achieving instant homozygosity in just two years which leads to instant fixation of desirable characters of recombinants as well as reduction in the number of generations required for development of cultivars yet, it also restricts the possibilities of alien introgressions into the genetic background of wheat. In hybrid cells, the two parental genomes are restricted to separate domains throughout cell cycle and such separation is reported to be under genotypic control. This separation leads to the elimination of either of the two genomes. Several explications have been proposed to account for such uniparental chromosome elimination process. Some hypotheses state the role of host specific nucleases in degradation of alien chromosomes (Davies 1974) and others affirm absence of functional kinetochores in the alien chromatin responsible for their elimination (Komeda et al. 2007) depicting the role of maternal and paternal parents, respectively. However, the actual mechanism of chromosome elimination occurring in wide hybrids of various crop species remains poorly understood. The genetic resolution of chromosome elimination in wide hybrids will assist in successful retention of alien chromosomes in crop species and recovery of intergeneric hybrids with desirable gene combinations.

Among the various available chromosome elimination- mediated approaches of doubled haploidy breeding in wheat, wheat x *I. cylindrica*- mediated system has proved to be the most efficient in terms of haploid induction frequency not only in bread wheat (Chaudhary et al. 2005) but also in the wide hybrids like triticale x wheat and wheat x rye derivatives too where maize mediated system failed to recover haploid embryos (Kishore et al. 2011). Several other features of this grass like genotype non- specificity, abundance of pollen availability, synchronous flowering with wheat and its availability in the surroundings of wheat fields render it as a suitable pollen source than maize which needs to be raised as an off-season crop under polyhouse conditions for synchronizing its

flowering with wheat. The wild perennial weedy grass, *I. cylindrica* possess various desirable traits like tolerance to drought, alkaline and acidic soils, extreme temperature and various other strategic traits for survival (Galinato et al. 1999). The possibility of retaining some of the chromosomes or chromosome segments of *I. cylindrica* in the genetic background of wheat brings anticipation of enhancing the genetic diversity among wheat germplasm for resistance to various biotic and abiotic stresses.

Keeping in view, the genetic control of host genome in elimination of alien chromosomes and the significance of understanding the mechanism of chromosome elimination in wheat x *I. cylindrica* hybrids, the present investigation entitled as ñTagging of the specific genome and chromosome(s) of hexaploid wheat triggering chromosome elimination in wheat x *Imperata cylindrica* system of doubled haploidy breedingò was undertaken with the following objectives:

To

i. identify the genome (A, B or D) of the hexaploid wheat (AABBDD) responsible for the elimination of *I. cylindrica* chromosomes in the wheat x *I. cylindrica* system and

ii. locate the specific chromosome(s) of the identified wheat genome triggering the elimination process.

2. REVIEW OF LITERATURE

Hybridization is a strong evolutionary force which can potentially reshape the genetic composition of populations and create novel genotypes that facilitate adaptation to new environments. This is particularly true for wide hybridization where completely different set of genetic complements combine and interact in a utterly novel way to generate previously unobserved phenotypes that may be superior in expression of both qualitative and quantitative traits (Stebbins 1950). However, the success of such alien introgressive programmes depends mostly on the pairing and stable inheritance of alien chromosome(s) in the hybrids and subsequent generations (Repellin et al. 2001). On the contrary, the rapid and complete uniparental chromosome elimination following interspecific or intergeneric hybridization provides opportunity of induction of haploids and ensuring development of complete homozygous lines in just two years. Doubled haploidy breeding serves as a useful tool in crop improvement which leads to instant fixation of desirable characters of recombinants enhanced selection efficiency and development of mapping populations. Nevertheless, the expediency of doubled haploids developed through uniparental chromosome elimination does not surmount the predicament laid down by elimination of chromosomes in the introgression of desirable traits into the crop species from an alien source. In order to mitigate this hindrance, there is dire need to understand the genetic mechanism underlying the chromosome elimination process and further seek ways to allow retention of the alien chromosomes or chromosome segments.

Keeping the above in view, the present investigation was carried out to study the mechanism of chromosome elimination in wheat x *I. cylindrica* hybrids which will open new vistas for the genetic upgradation of wheat either through successful introgression of desirable traits of *I. cylindrica* or enhancement of the haploid induction efficiency of the doubled haploidy breeding system. The literature pertinent to the investigation was reviewed under following heads:

2.1 Doubled haploid induction in wheat

Doubled haploids provide opportunity of accelerating the genetic upgradation endeavours in crop plants by offering means to rapidly advance selected lines to complete homozygosity and increase the efficiency of selection (Campbell et al. 2000; Claudio et al. 2003; Sharma et al. 2005). The existence of haploid plant was first discovered in *Datura stramonium* and wheat by Blakeslee et al. (1922) and Gaines and Aase (1926), respectively.

The earliest possibilities of haploid induction in wheat were investigated by various Chinese workers following androgenesis- mediated system of haploid production (Chu et al. 1973; Ouyang et al. 1973; Wang et al. 1973). They formulated appropriate protocols and successfully developed doubled haploid lines. Later, Craig (1974) developed haploid plants from *in vitro* regeneration of anthers in wheat. Likewise, other approaches of haploid induction in wheat *viz.,* irradiated pollen culture (Natarajan and Swaminathan 1958), un-pollinated ovary culture (Zhu and Wu 1979) and isolated pollen culture (Wei 1982) were exploited with only limited success. The response of the afore mentioned androgenesis mediated techniques had several restrictions such as, less recovery of green plantlets (Wang et al. 1973), influence of donor plant environment (Lu et al. 1991) and genotype specificity (Fedak et al. 1997). The advent of chromosome elimination- mediated techniques provided practically feasible, easy, result oriented and efficient approaches for induction of haploid embryos in wheat.

2.2 Chromosome elimination approaches

In wide hybrids, genome elimination and nuclear instability are common but variable phenomenon. In some hybrids the chromosomes are lost slowly over generations whereas, in some cases the elimination is so rapid that the chromosomes are eliminated during the initial period of embryogenesis (Humphreys 1978). Similarly, in some wide hybrids the alien chromosomes are only partially eliminated while in others complete genome of the one parent is lost (Bennett et al. 1976). Under natural circumstances, the hybrids that encounter rapid elimination of entire alien genome result in aborted embryos in few days after fertilization. However, when such embryos are rescued on artificial medium they develop into haploid plantlets with chromosome complement of only one parent (Subrahmanyam and Kasha 1973). In wheat, several chromosome elimination-mediated approaches of haploid induction are available which are mentioned ahead:

2.2.1 Bulbosum technique

The first report of uniparental chromosome elimination in *H. vulgare* x *H. bulbosum*, popularly known as the ôBulbosum techniqueô was made by Stephan (1969). During early embryogenesis, chromosomes of the wild relative are preferentially eliminated from the cells of developing embryos leading to the formation of the haploid embryos of *H. vulgare*. The endosperm is frequently formed, but its development is usually disturbed. The embryos are hence excised from developing caryopsis at 12-14 days and cultured *in vitro* on artificial medium. The bulbosum method was the first haploid induction method to produce large number of haploids across most barley genotypes. Kasha and Kao (1970) presented evidence to explain that the *H. vulgare* monoploids are developed as a result of genetically controlled elimination of *H. bulbosum* chromosomes and not by parthenogenesis since, haploids of only *H. vulgare* were obtained when it was used as a male parent in the wide hybridization.

The potential of *H. bulbosum* in terms of haploid induction in barley was extended to bread wheat by Barclay (1975). Haploid wheat plantlets were obtained when ôChinese Springô variety of *T. aestivum* was crossed with *H. bulbosum* (Zenkteler and Straub 1979). Nevertheless, this method was unsuccessful with other wheat varieties due to the effect of dominant crossability inhibitor alleles *Kr1, Kr2, Kr3* and *Kr4* located on 5B, 5A, 5D and 1A (Riley and Chapman 1967; Krolow 1970; Sitch et al. 1985; Zheng et al. 1992) which prevent the entry of *H. bulbosum* pollen tube into the ovary of wheat. Jalani and Moss (1980) reported that the crossability inhibitor genes had little effect on pollen germination and time taken for the pollen tubes to reach the micropyle and it was the number of pollen tubes reaching the micropyle which was affected by the *Kr*-genes. Similarly some additional factors affecting crossability between 'Chinese Spring' wheat and *H. bulbosum* were also found on chromosomes 3A, 3B and 3D (Miller et al. 1983). This system was hence useful to a limited extent in wheat due to the sensitivity of the *H. bulbosum* pollen to the crossability inhibitor genes.

2.2.2 Wheat x maize system

This system of doubled haploidy breeding is widely used throughout the world for the production of haploids and doubled haploids in wheat. This was Zenkteler and Nitzsche (1984), who for the first time reported that embryos were frequently formed

when hexaploid wheat was pollinated with maize. Though, Laurie and Bennett (1986) pioneered in exploiting the wide hybrid for induction of wheat haploids. They reported that the embryos developing in the wheat x maize hybrids were capable of producing haploid wheat plants. Laurie and Bennett (1988a) developed the first *in vitro* method to rescue the haploid wheat embryos from the wheat x maize crosses and subsequently numerous studies were conducted to evaluate the feasibility of this technique for the development of true breeding doubled haploid lines. This intergeneric hybridization of wheat with maize resulted in the formation of haploid wheat embryos because the maize chromosomes in the hybrid zygote are rapidly eliminated in the first three cycles of cell division. The cytological investigation of wheat x maize hybrids proved that the maize pollen germinated and grew into the wheat embryo sac where the fertilization of wheat egg by maize pollen led to development of a hybrid zygote with 21 wheat and 10 maize chromosomes. The hybrid zygotes were karotypically unstable as a result of which maize chromosomes fail to attach to the spindle microtubules and move towards the poles during cell divisions due to progressive loss of centromere activity resulting in elimination of maize chromosomes after 3-4 mitotic cell divisions (Laurie and Bennett 1989).

The maize chromosome elimination system in wheat is insensitive to crossability inhibitor genes (Laurie and Bennett 1989) and it enables the production of large number of haploids from any genotype including those recalcitrant to androgenesis and bulbosum technique (Inagaki et al. 1998; David et al. 1999; Cherkaoui et al. 2000; Chaudhary et al. 2002; Singh et al. 2004; Pratap et al. 2006). Comeau et al. (1992) also suggested development of wheat haploid plants through ovule culture after hybridization with maize pollen.

Zhang et al. (1996) comparatively analyzed the embryogenesis in wheat x maize hybrids and self- pollinated wheat plants using paraffin sectioning. They reported that development of embryo is not accompanied by the formation of an endosperm and the endosperm nuclei remain free in the cytoplasm, fail to advance into the cellular stage and degenerate later. Pratap et al. (2006) evaluated the comparative efficiency of anther culture and maize mediated system of haploid induction in wheat and triticale genotypes. They reported that haploid plantlet formation was significantly higher through maize mediated approach as compared to androgenesis in both wheat and triticale genotypes.

2.2.3 Wheat x pearl millet

Pearl millet (*Pennisetum glaucum*) was successfully utilized for induction of haploids in hexaploid wheat, though the regenerated plants exhibited variation in chromosome number (Ahmad and Comeau 1990). Out of the three plants recovered, one was having somatic chromosome number of 42 while two others were having 21 and 22 chromosomes. The plant with 22 chromosomes was having 21 chromosomes of wheat and one chromosome of pearl millet which was subsequently eliminated during gamete formation. Inagaki and Mujeeb-Kazi (1995) compared haploid induction efficiency in hexaploid wheat when crossed with maize, pearl millet and sorghum. The seeds and embryos recovered from wheat x pearl millet were of reduced size than that of wheat x maize. Unlike earlier workers, haploids recovered from wheat x pearl millet were devoid of pearl millet chromosomes. Pearl millet was also suggested as an alternative pollen source for haploid induction in bread wheat, durum wheat and hexaploid triticale (Inagaki and Hash 1998).

2.2.4 Wheat x *Imperata cylindrica*

Imperata cylindrica, a wild weedy perennial grass (2n = 2x = 20) does not require repeated sowings and grows widely in the vicinity of wheat fields under natural conditions in almost all parts of the world wherever wheat is cultivated. Furthermore, it is a winter season plant that coincides well for flowering with that of wheat and triticale naturally. Chaudhary et al. (2005) identified the *I. cylindrica* as an efficient alternative pollen source for doubled haploid production performing superior over maize- mediated system of haploid induction. Pratap et al. (2005) reported that among various species of Graminae family *viz., Zea mays, Sorghum bicolour, Pennisetum americanum, Setaria italica, Festuca arundinaceae, Imperata cylindrica, Cynodon dactylon, Lolium temulentum* and *Phalaris minor* tested for haploid plant production in wheat, *I. cylindrica* produced significantly more haploid embryos over others. Additionally, *I. cylindrica is* also genotype non-specific and hence can be hybridized with any variety of wheat, triticale or their derivatives.

Cytological investigation of the *I. cylindrica*- mediated chromosome elimination approach revealed that there is no endosperm formation in wheat x *I. cylindrica* hybrids and the elimination of *I. cylindrica* chromosomes takes place during the first zygotic

division itself thus, allowing the production of haploid embryo- carrying seeds (Komeda et al. 2007). The pollen of *I. cylindrica* produces significantly higher number of haploids than maize in wheat, triticale, wheat x rye and triticale x wheat derivatives (Chaudhary 2007; 2008a; b; 2009; 2010a; b; 2011; 2012; 2013; Kishore et al. 2011; Chaudhary et al. 2013a; Chaudhary 2013).

Although the wheat x *I. cylindrica* system emerged as most efficient chromosome elimination mediated technique for induction of haploids however, the recovery of doubled haploids was inadequate. Recently, Tayeng et al. (2012) reported that the *in vivo* application of colchicine (2000 ppm) enhances the doubled haploid production efficiency in wheat x *I. cylindrica*- mediated chromosome elimination approach of doubled haploidy breeding. Similarly, the protocol was further improved by eliminating emasculation step and going for direct pollination with *I. cylindrica.* The morphological marker, that is, absence of endosperm in haploid embryo- carrying seeds developed from wheat x *I. cylindrica* hybridization can be used quite efficiently to exploit the asynchronous behaviour of anthesis within wheat spikes (Chaudhary et al. 2013b). The direct pollination saves labour, time and results in efficient recovery of haploids and doubled haploids in precious segregants during the end of the season when anthers dehisce very fast.

Rather (2012) exploited the potential of pollen preservation in *I. cylindrica* for undertaking the doubled haploidy breeding programmes in wheat at places where *I. cylindrica* is not growing naturally. The preservation of *I. cylindrica* pollen was done under varied preservation regimes (-80^{o}C, -20^{o}C and 4^{o}C) and -20^{o}C was identified as most effective preservation regime for storage of pollen for at least a month with only 50% reduction in viability. Rather et al. (2013) further paved way for enhancement of *I. cylindrica* – mediated haploid induction protocol by means of identifying and utilizing most efficient genotype of *I. cylindrica* exhibiting highest recovery of haploid embryos.

2.3 Genetic mechanism of chromosome elimination

Several attempts have been made in order to understand the genetic mechanism underlying the chromosome instability and their subsequent loss in wide hybrids. Though various speculations have been made regarding the mechanism involved in uniparental

chromosome elimination following wide hybridization, yet the mechanism still remains poorly understood. Nonetheless it is certain that the process of chromosome elimination is not random and under genetic control of either or both parents. Gupta (1969) compared the mitotic cycle of *Nicotiana plumbaginifolia* and *N. tabacum* x *N. plumbaginifolia* hybrids and suggested that the distribution of heterochromatin and late replication in *N. plumbaginifolia* chromosomes caused their instability in the hybrids. In barley and bulbous barley hybrids, the chromosome instability was considered to be influenced by genetic factors as the haploid induction frequency is high in the embryos which initially contained a ratio of 1 *vulgare* to 1 *bulbosum* genomes. Whereas, triploid embryos containing 1 *vulgare* to 2 *bulbosum* genomes were relatively stable and the most stable endosperm tissues examined had a ratio of 1 *vulgare* to 4 *bulbosum* genomes (Subrahmanyam and Kasha 1973). The genetic control mechanism of chromosome elimination in above wide hybrids was also investigated by Ho and Kasha (1975) who crossed seven primary trisomic lines of *H. vulgare* chromosomes with tetraploid *H. bulbosum* and concluded that both chromosomes 2 and 3 of *H. vulgare* were responsible for elimination of bulbosum chromosomes. They further used monotelotrisomics for chromosomes 2 and 3 to conclude that both arms of chromosome 2 and the short arm of chromosome 3 are responsible for elimination of *H. bulbosum* chromosomes.

Davies (1974) proposed that the rapid or gradual loss of uniparental genomes in interspecific hybrids is resultant of degradation of the alien chromosomes by the host genome- specific nucleases. According to him, loss of only specific chromosomes could be either due to absence of recognition/ restriction sites specific for the degrading enzymes on the retaining chromosomes or protection of the host genome protects itself by DNA modification prior to the action of nucleases.

Bennett et al. (1976) reported that the failure of chromosomes to initiate or complete either congregation at metaphase or migration to the poles led to the loss of chromosomes in *H. vulgare* x *H. bulbosum* hybrids. They also indicated that chromosome elimination in *Hordeum* hybrids may be caused by a disturbed control of protein metabolism in hybrid seeds and *H. bulbosum* chromosomes are selectively eliminated since they are less efficient than *H. vulgare* chromosomes in forming normal attachments to spindle protein. Similarly, Kasha (1976) considered the elimination of genomes as one step process that is brought about by multipolar cell division.

Orton and Tai (1977) also suggested the role of spindle organizer in chromosome elimination. As per their hypothesis, the genetic similarity of parental spindle organizers precedes normal chromosome behaviour whereas, if the spindle organizers are from different sources and exhibit differences, then they are liable to function independently even within the same protoplast. As a result of this individual functionality, both spindle organizers will establish themselves as pole and attract their chromosomes, resulting in formation of micronuclei and elimination of genomes. Likewise, Bennett et al. (1981) put forward that the main cause of chromosome elimination in wheat x maize system is failure of centromeres of maize chromosomes to attach to spindle microtubules which results from variation in the initial size of the centromeric regions of the parental chromosomes. The variation in chromosome size and hence, failure of centromeric activity resulting in the loss of alien chromosomes were also observed in various crosses such as wheat x *H. bulbosum* (Barclay 1975; Bennett et al. 1976; Finch and Bennett 1982), tetraploid wheat x maize (OôDonoughue and Bennett 1988), hexaploid wheat x sorghum (Laurie and Bennett 1988b), *H. vulgare* x maize (Laurie and Bennett 1988c) and between wheat x pearl millet (Laurie 1989). Whereas, Schwarzacher et al. (1987) reported that the spatial separation of parental genomes in wide hybrids, alignment of either genome towards periphery and centromere positions is not just a packaging phenomenon determined by chromosome size *per se* but is under genetic control.

Laurie and Bennett (1989) reported that the maize chromosomes are lost during the post zygotic mitosis due to their inability to align on the equatorial plate during metaphase or move towards poles during anaphase. They also reported that the micronuclei containing maize chromatin were observed until four celled stage however, no maize chromatin was reported in eight celled stage which suggests that the maize chromosomes are completely lost during first three zygotic divisions.

The differences between the eliminated and retained chromosomes with respect to centromeres size and tendency to occupy peripheral location during mitosis revealed that centromeric activity was hampered in *H. marinum* x *H. vulgare* cross. The tissue specific elimination of alternative whole parental genomes was the result of specific suppression of genes involved in centromere function by DNA methylation (Finch 1983). Whereas, specific pattern of elimination of chromosomes derived from chromosome orders of genomes in *Hordeum lechleri* x barley hybrids was observed by Linde-Laursen and von Bothmer (1999).

Kim et al. 2002 suggested that the chromosomes of *H. bulbosum* did not undergo direct peripheral localization but they went through the nuclear extrusion and budding process in *H. vulgare* x *H. bulbosum* hybrids. The phenomenon of genome elimination by nuclear extrusions following spatial separation was also established in wheat x pearl millet by Gernand et al. (2005) and later in *H. vulgare* x *H. bulbosum* (Gernand et al. 2006). They suggested that heterochromatization of pearl millet chromosomes in micro nuclei takes place before final step of haploidization.

Mochida et al. (2004) observed the process of chromosome elimination using laser confocal microscopy. They reported that spindle fibers fail to attach to the maize kinetochore which causes delayed chromosome movement to the poles and hence, elimination. Similarly, the cytological investigation of wheat x *I. cylindrica* hybrids also confirmed that elimination of *I. cylindrica* chromosomes occurs due to missegregation of its chromosomes during first zygotic division owing to lack of functional kinetochores. The *I. cylindrica* chromosomes remained in the cytoplasm till telophase but after two celled stage, no *I. cylindrica* chromosomes were observed (Komeda et al. 2007).

In oat x maize hybrids, Jin et al. 2004 suggested that the CENH3 proteins, a histone H3 variant encoded by the oat *CenH3* gene are incorporated into both oat and maize chromosomes. The oat CENH3 is sufficient to organize a kinetochore on a maize chromosome and hence, retaining one or few maize chromosomes. A supporting study revealed that centromere activity is epigenetically specified by incorporation of CENH3 sequences that replaces conventional H3 in centromeric nucleosomes (Henikoff and Dalal 2005).

Ravi and Chan (2010) reported that line carrying CENH3 histone variant can induce haploids in *Arabidopsis thaliana* without requiring wide hybridization. This system has potential to develop both maternal and paternal haploids depending upon usage of variant line in the cross. A major breakthrough was made by Sanei et al. (2011) by explaining that missegregated chromosomes in a classic barley interspecies cross (*H. vulgare* x *H. bulbosum*) fail to assemble kinetochores and the microtubule attachment sites that mediate chromosome inheritance.

In a different study, the aberrant phenomenon such as breakage, bridge formation, and non-disjunction was revealed by pearl millet chromosomes leading to their elimination. A model was proposed indicating that the retention of cohesin protein in the pearl millet sister chromatids is responsible for non-disjunction, chromosomal bridges as well as breakage (Ishii et al. 2010). Furthermore, the ability of plants to conserve their genome by distinguishing host DNA from foreign DNA *via* specific recognition and subsequent elimination of foreign DNA through endonuclease activation and genome specific fragmentation was also considered one of the mechanisms of elimination of alien chromosomes (Houben et al. 2011).

2.3.1 Influence of wheat genetic background on haploid induction

There are several reports of haploid induction to be influenced by both maternal and paternal genetic backgrounds. The earliest reports in this regard were from Suenaga and Nakajima (1989) who suggested that the maize genetic background itself is responsible for elimination of chromosomes whereas, the later workers contradicted with their results. Laurie and Reymondie (1991) crossed 19 winter and spring wheat genotypes with maize F_1 hybrid. They suggested that the embryo formation was significantly higher in spring wheats as compared to winter wheat ecotypes, but no significant variation was found between varieties within the spring or winter wheats. In durum wheat, Almouslem et al. (1998) suggested that the variable response for maize- mediated haploid induction was influenced by maternal genotypes. Likewise, Sharma et al. (2005) reported the influence of genetic backgrounds of winter and spring wheat on haploid induction parameters in wheat x maize system.

Niroula and Thapa (2009) reported that the efficiency of polyhaploid induction in wheat through wheat x maize system is determined by wheat genotypes. Moradi et al. (2009) reported that the percentage of haploid regeneration and doubled haploid production is dependent on the wheat genotypes and independent of maize genotypes.

On the contrary, Lefebvre and Devaux (1996) were of the opinion that both maternal and paternal genotypes have influence over haploid induction. Their results were in concordance with Chlyah et al. (1999) who studied the effect of different wheat

as well as maize genotypes on haploid induction and reported that haploid induction parameters are influenced to a greater extent by genetic makeup of both wheat and maize. Chaudhary et al. (2002) observed a significant influence of wheat parents and their interactions with maize genotypes for seed formation frequency and that of maize genotypes alone for embryo formation frequency in wheat x maize hybridization programme. Bouatrous et al. (2010) also reported the influence of parental genotypes on haploid induction parameters in durum x maize crosses.

In wheat x *I. cylindrica* cross only little work has been done so far yet, Rather and his associates (2013) reported that in wheat x *I. cylindrica* hybrids, the individual effects of wheat and *I. cylindrica* genotypes as well as their interactions are influencing the haploid induction parameters. They also suggested that the diversity present among *I. cylindrica* genotypes can be exploited to hasten the embryo formation frequency in this wide hybridization- mediated approach of doubled haploidy breeding.

2.3.2 Role of wheat genomes and chromosomes in paternal chromosome elimination

The efficiency of haploid induction following maize mediated system in common wheat is usually higher than durum wheat, suggesting the role of one genome more than the other. Inagaki et al. (1997) reported that the D- genome chromosomes in triticale genetic background have the effect on increasing polyhaploid induction frequency through elimination of maize chromosomes in triticale x maize crosses. They found out that hexaploid triticales produced embryos at low frequencies (0.0 to 5.4%) whereas, higher frequencies of embryo formation were recovered in triticale substitution lines with 2D and 4D chromosomes.

In a similar study, Ito et al. (1997) used chromosome substitution lines for 5A and 5B to study the influence of these chromosomes on haploid induction frequency using wheat x maize system. The chromosome substitution lines CS (Cap 5A) and CS (T. spt 5A) showed lower frequency of haploid formation whereas, CS (Mara 5B) showed no significant deviation in comparison to Chinese Spring. They concluded that chromosomes 5B does not reduce the production frequency of haploid wheat plants although, 5A does so.

Inagaki and Hash (1998) investigated the potential of pearl millet for induction of haploids in bread wheat, durum wheat and hexaploid triticale and reported a significant reduction in embryo formation frequency owing to the absence of D genome chromosomes. Similarly, Almouslem et al. 1998 investigated the haploid induction frequency in three important cytogenetic stocks LDN 5D(5B), LDN *Ph1 ph1b*, and Cappelli *ph1c ph1c* and reported that the substitution of 5D chromosome in place of 5B enhanced the haploid formation frequency.

Dogramaci-Altunetepe and Jauhar (2001) used 14 ôLangdonô (LDN) D-genome disomic substitution lines of durum wheat and crossed them with maize. They observed that the substitution line 5D(5B) was most successful for both embryo formation and haploid plantlet production. Similarly, the effects of all homoeologous group- 5 chromosomes on induction of haploidy in both durum wheat and bread wheat were investigated by Jauhar et al. (2008). They used disomic substitution lines of durum wheat, 5D(5A) and 5D(5B) and nullisomic-tetrasomic lines of bread wheat, N5A-T5D, N5D-T5A, N5A-T5B, N5B-T5A, N5B-T5D and N5D-T5B for production of haploids by crossing them with maize and reported that the overall production of haploids was low in the cytogenetic stocks however the substitution line 5D(5B) produced higher number of haploids and hence, indicating the role of 5D chromosome in the uniparental elimination process. Mujeeb-Kazi et al. (2006) produced haploid plants in durum wheat x maize crosses with a haploid plant production frequency ranging from 0 ï 12.3 % across 44 genotypes whereas, synthetic hexaploid (SH) wheats derived from each of the 44 cultivars x *Ae. tauschii* accessions gave a haploid frequency range from 2.5 to 42.6% providing unequivocal evidence that the D genome of *Ae. tauschii* significantly influenced and enhanced haploid production efficiency in SH wheats.

Keeping all the above in view, it is quite evident that the mechanism underlying the elimination of uniparental chromosomes is yet unresolved though, it is certain that the chromosome instability is under genetic control. Based on the influence of wheat genetic background on elimination of alien chromosomes, the present investigation was hence carried out to establish the role of wheat genome(s) and chromosome(s) in triggering the elimination of paternal chromosomes in wheat x *I. cylindrica* hybrids.

3. MATERIALS AND METHODS

Uniparental chromosome elimination is consequence of intergenomic conflict arising due to the combination of genetically unrelated genomes following wide hybridization. In several wide hybrids involving wheat as maternal parent, preferential chromosome elimination suggests the genetic control of wheat chromosomes on elimination process. Wheat x *Imperata cylindrica* is one of the recently reported chromosome elimination approach that is efficiently being used for the production of wheat haploids and doubled haploids (Chaudhary et al. 2005). *I. cylindrica* is a perennial grass that possesses desirable traits like tolerance to various abiotic stresses like drought, alkaline and acidic soil conditions and extreme temperature. The elucidation of genetic mechanism of chromosome elimination in wheat x *I. cylindrica* system will be quite useful for exploitation of this wild grass as a potential genetic resource for genetic upgradation of bread wheat. The understanding of the mechanism will not only allow the retention of certain desirable traits from *I. cylindrica* but will also help us in enhancing the haploid induction efficiency of the system through chromosomal alterations. Hence, to explicate the genetic control of chromosome elimination process in wheat x *I. cylindrica* hybrids, the present investigation entitled as ñTagging of the specific genome and chromosome(s) of hexaploid wheat triggering chromosome elimination in wheat x *Imperata cylindrica* system of doubled haploidy breedingò was carried out at Molecular Cytogenetics & Tissue Culture Lab and Experimental Farm of the Department of Crop Improvement, CSK HP Agricultural University, Palampur, Himachal Pradesh, India during 2010-11 to 2012-13. The hybridization of diploid, tetraploid and hexaploid species and their cytogenetic stocks with *I. cylindrica* as pollen source was undertaken at Experimental Farm of the Department of Crop Improvement situated at 76º3ôE longitude and 32º6ô N latitude at an elevation of 1290.8 m (a.m.s.l). Whereas, further studies related to the haploid embryos obtained from wheat x *I. cylindrica* hybridization was carried out at Molecular Cytogenetics & Tissue Culture Lab of the Department of Crop Improvement. The experimental material used and methods employed are described as under:

3.1 Materials

3.1.1 Tagging of wheat genome triggering the chromosome elimination process

Bread wheat (2n = 42) is an allohexaploid (AABBDD) possessing three different sets of homologous chromosomes coming from three different diploid progenitors *viz., Triticum monococcum* (AA), *Aegilops speltoides* (BB) or its close relative and *T. tauschii* (DD). Hence, the material for identification of the specific (A, B or D) genome(s) playing role in triggering the chromosome elimination process and thereby, leading to induction of haploid embryos included *T. monococcum* (2n = 2x = 14), *T. tauschii* (2n = 2x = 14) and *T. durum* (2n = 4x = 28). The tetraploid species used in the investigation represents both A and B genomes (Plate 1).

3.1.2 Tagging of specific wheat chromosome(s) triggering chromosome elimination process

Since wheat is an allohexaploid with basic chromosome number (x = 7) hence, there are 21 pairs of homologous chromosomes which are further categorized into 7 homoeologous groups, that is, from 1A to 7A, 1B to 7B and 1D to 7D. In order to identify the specific chromosome(s), complete set (21 lines) of monosomic and nullisomics series based on ócChinese Springô cultivar of hexaploid wheat were used. In addition to this, 11 tetraploid wheat D genome substitution lines derived from ócLangdonô variety of tetraploid wheat (*T. durum*) were also used (Table 3.1).

3.1.3 Pollen Source

The *I. cylindrica* (Plate 2), a perennial grass growing naturally in the surroundings of wheat fields in Experimental Farm of the Department of Crop Improvement was used as pollen source for hybridization with the above mentioned wheat germplasm.

3.2 Methods

3.2.1 Raising of germplasm

The material used in the present investigation was sown in staggered manner to provide enough time period for performing hybridization with *I. cylindrica*. The late flowering species, *T. tauschii* was sown during second fortnight of October till the end of December. The other material was sown during first week of November till second week of January. The seeds of monosomic lines were allowed to germinate in petriplates placed

a) *Triticum monococcum* (AA)b) *Triticum tauschii* (DD) c) *Triticum durum* (AABB)

Plate 1 Diploid and tetraploid species of wheat used for hybridization with *Imperata cylindrica*

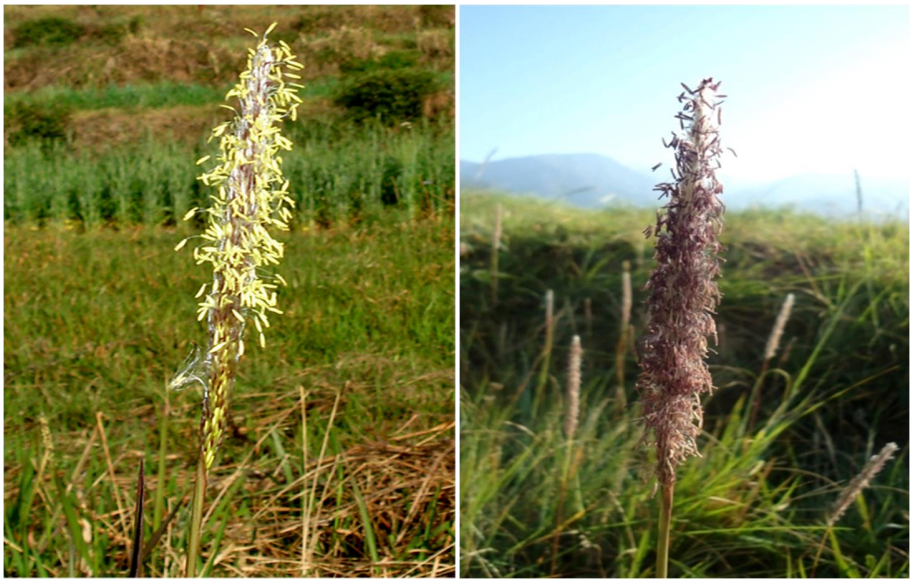


Plate 2 *Imperata cylindrica*, an efficient pollen source used for producing haploids in wheat

at 25^{o}C temperature in growth chamber and then transplanted in field after excising their roots for cytological detection of monosomics and nullisomics from normal disomics. Similarly, the substitution lines exhibiting low germination were also allowed to germinate in petriplates at the optimum temperature (25^{o}C) and then transplanted to the field.

Table 3.1 Different D genome substitution lines of tetraploid wheat derived from 'Langdon' cultivar

S. No	Substitution line	Annotations
1.	1A(1D)	1A chromosome pair is replaced by 1D chromosome pair
2.	2A(2D)	2A chromosome pair is replaced by 2D chromosome pair
3.	3A(3D)	3A chromosome pair is replaced by 3D chromosome pair
4.	4A(4D)	4A chromosome pair is replaced by 4D chromosome pair
5.	1B(1D)	1B chromosome pair is replaced by 1D chromosome pair
6.	2B(2D)	2B chromosome pair is replaced by 2D chromosome pair
7.	3B(3D)	3B chromosome pair is replaced by 3D chromosome pair
8.	4B(4D)	4B chromosome pair is replaced by 4D chromosome pair
9.	5B(5D)	5B chromosome pair is replaced by 5D chromosome pair
10.	6B(6D)	6B chromosome pair is replaced by 6D chromosome pair
11.	7B(7D)	7B chromosome pair is replaced by 7D chromosome pair
12.	Langdon	Pure line with no chromosomes from D genome

3.2.2 Haploid induction

The haploid induction in wheat germplasm using *I. cylindrica* as pollen source was carried out as per the standard protocol given by Chaudhary et al. (2005) (Plate 3). The protocol involved the following steps:

i) Emasculation

The optimum stage for emasculation varied depending upon the temperature and duration of flowering season. During initial period of flowering, the spikes that were fully out of the boot leaf encompassing immature green coloured anthers and stigma in receptive stage were considered optimum for emasculation. Whereas, during end of flowering season, the spikes totally covered by boot leaf were considered optimum for emasculation as the dehiscence of anthers was rapid during such period of flowering.

Wheat spikes were emasculated by removing anthers with the help of forceps without disturbing lemma and palea. The immature central florets, apical and basal spikelets of each spike were removed. The emasculated spikes were covered with butter paper bags for maintaining humidity and avoiding pollination with foreign pollen. In awned cultivars, the awns were clipped off for easy bagging of spikes. The emasculated wheat spikes were marked with duly labelled jewel tags containing information regarding date of emasculation and name of the genotype.

ii) Pollination

Preferably next morning, emasculated spikes were pollinated with the fresh pollen of *I. cylindrica* using camel hair brush. The pollen of *I. cylindrica* was available in abundance during the morning hours from 7.30 to 9.30 am. The pollinated spikes were covered immediately with butter paper bags and tagged accordingly.

iii) *In vivo* hormonal application

The uppermost internodes of the hexaploid wheat culms were injected with a solution of 0.01 percent solution of 2, 4-D for three consecutive days at 24 hrs, 48 hrs and 72 hrs after pollination using a syringe fitted with a fine hypodermic disposable needle. The injection holes were sealed by using petroleum jelly. The diploid and tetraploid wheat species were injected with variable concentration of 2,4-D in order to find out the optimum 2,4-D dose for efficient haploid induction.

iv) Embryo rescue

After 18-20 days of pollination, the crossed spikes were harvested. The embryo carrying pseudoseeds were identified using light source (Bains et al. 1998). The embryo was seen floating in the fluid (aqueous solution) instead of solid endosperm as found in selfed seeds. The embryo carrying pseudoseeds were then washed thoroughly using *Tween- 20* under tap water to avoid any sort of infection or contamination. The embryos were then excised in laminar air flow chamber after surface sterilization of pseudoseeds using 0.1 percent $HgCl_2$ for 3-5 minutes and two washings with autoclaved distilled water to remove $HgCl_2$. These excised embryos were transferred to the test tubes containing MS medium supplemented with essential amino acids.

Plate 3 General protocol followed for haploid induction (Chaudhary et al. 2005)

v) Growth conditions

Cultured embryos were given cold treatment at 4°C temperature in dark for first 24 hours. After that they were incubated in the dark in the Plant Growth Chamber at 25±1°C for about a week till the regeneration of shoots and roots is initiated. The regenerated plantlets were then shifted to the other section of the Plant Growth Chamber at 25± 1°C with 10/14 hrs light/dark profile for proper development of plants.

vi) Root induction

The green haploid plantlets developed through embryo culture were then transferred to rooting medium (liquid) for profuse rooting. Rooting medium comprising half strength of MS salts 1 mg/ l each of NAA (napthalene acetic acid), IBA (indole butyric acid) and devoid of sucrose and agar. M- shaped filter paper were immersed in test tubes for providing support and continuous supply of nutrients to plants through capillary action.

3.2.2.1 *Triticum monococcum* x *I. cylindrica* hybridization

The spikes that have emerged out of boot leaf were considered optimum for emasculation. Emasculation was carried out by clipping off the upper one third part of the florets in the spikelets. The anthers become visible mostly in one and occasionally in two florets of a spikelet. All the three anthers were removed carefully using a fine forceps without injuring the stigma or rupturing the anthers. Small butter paper bags were used for bagging as the culms of *T. monococcum* were very slender.

One day after emasculation, the emasculated spikes of *T. monococcum* were pollinated with *I. cylindrica* pollen using camel brush. Pollinated spikes were covered with butter paper bag followed by tagging and labelling.

Different concentration of growth hormone (2,4-D) ranging from 0.01 to 0.05 percent were used for *in vivo* application. The pollinated spikes of *T. monococcum* were harvested at 9-11 days after pollination and the seeds obtained were then screened against light source for presence and absence of embryos.

3.2.2.2 *Triticum tauschii* x *I. cylindrica* hybridization

The spikes in which one third of the spikelets were out of the boot leaf were found optimum for emasculation in *T. tauschii.* The central florets were removed allowing only two florets on each spikelet borne on either side of the spike. With the help of scissor the

florets are clipped off at or nearer to centre exposing the uninjured anthers. With the help of fine forceps, the anthers were removed without damaging the stigma. Bagging was done immediately to avoid undesirable pollination.

The next day, pollination was carried out by dusting the pollen of *I. cylindrica* over the clipped florets with the help of camel brush. The pollinated spikes were bagged carefully to avoid dehydration of the pseudoseeds and insect attack.

The *in vivo* hormonal dosage were manipulated from the standard protocol of Chaudhary et al. (2005) to estimate the most optimum concentration of 2,4-D for efficient recovery of haploid embryos. Various doses of 2,4-D *viz*., 0.01, 0.02, 0.03, 0.04 and 0.05 percent were applied *in vivo* to the uppermost internodes of pollinated *T. tauschii* plants in the form of injection consecutively for 3 days after pollination during 2010-11. During 2011-12 the most optimum dose was injected to *T. tauschii* plants to recover higher number of haploid embryos.

The harvesting of pollinated spikes of *T. tauschii* was done after 9-11 days. The fluid filled pseudoseeds were removed from the spikes carefully without damaging them. The pseudoseeds were washed and transferred to laminar air flow hood where the embryos were excised under aseptic conditions and cultured on artificial medium.

3.2.2.3 *Triticum durum* x *I. cylindrica* hybridization

The emasculation procedure in durum wheat is somewhat similar to that of hexaploid wheat. However, the central florets are to be removed carefully without damaging the primary and secondary florets. The emasculated spikes were covered with butter paper bags for maintaining humidity and avoiding pollination with foreign pollen after clipping the awns at the top.

The receptivity of stigma is very crucial in durum wheat for proper seed set upon pollination with *I. cylindrica* pollen. Hence, pollination with *I. cylindrica* pollen was done when the stigma feathers were fully expanded one or two days after emasculation. Pseudoseed formation was severely hampered when pollination was done on too young or too old florets. The *in vivo* hormonal concentration was again manipulated to work out the optimum dose of 2,4-D for proper seed and embryo formation. Various doses of 2,4-D *viz*., 0.01, 0.02, 0.03, 0.04 and 0.05 percent were applied *in vivo* to the uppermost internodes of the *T. durum* plants in the form of injection consecutively for 3 days after pollination during 2010-11. During 2011-12, the most optimum dose of 2,4-D was

injected to the plants for proper seed set and recovery of higher number of haploid embryos. After 13-15 days of pollination, the crossed spikes were harvested and the embryo carrying pseudoseeds were screened against light and washed thoroughly. The haploid embryos were excised and cultured on artificial medium under aseptic conditions.

The genotype ôKWS 29ô was also pollinated with *I. cylindrica* under similar conditions and the embryo formation obtained was used as standard check for comparing embryo formation recovered in *T. monococcum, T. tauschii* and *T. durum.*

3.2.2.4 *Triticum aestivum* aneuploids x *I. cylindrica* hybridization

The monosomic and nullisomic lines derived from óChinese Springô variety of hexaploid wheat were pollinated with *I. cylindrica* after emasculation at optimum stage. The application of 0.01 percent 2,4-D solution was given to the uppermost internode of pollinated spikes at 24, 48 and 72 hours after pollination. The crossed spikes were harvested at 15 to 17 days after pollination. The embryo carrying pseudoseeds were screened, washed thoroughly and haploid embryos were excised and cultured on artificial medium under aseptic conditions. óChinese Springô variety of hexaploid wheat in which the monosomic series has been developed, was pollinated with *I. cylindrica* pollen in the similar manner and the embryo formation frequency obtained was used as for comparison.

3.2.2.5 D- genome substitution lines x *Imperata cylindrica* hybridization

Similar to tetraploid wheat (*T. durum*), the D- genome substitution lines were hybridized with *I. cylindrica*. The *in vivo* hormonal concentration was standardized in the first year (2010-11) and the optimum dose of 2,4-D was utilized for application under *in vivo* conditions in the next year (2011-12). The ôLangdonô variety of *T. durum* used for the development of D- genome substitution lines was also pollinated with *I. cylindrica* pollen under similar conditions and the haploid induction frequency obtained was used as control.

3.2.3 Media used for the embryo culture

3.2.3.1 Medium for embryo regeneration

Murashige and Skoog (MS) medium supplemented with essential amino acids (Table 3.2 and 3.3) was used for the rescue of haploid embryos of wheat (Murashige and Skoog 1962).

3.2.3.2 Medium for rooting

Liquid rooting medium consisted of half strength MS salts, 1mg napthalene-3-acetic acid (NAA) per litre and 1mg indole-3-butyric acid (IBA) per litre was used for inducing profuse rooting in regenerated plants (Table 3.4).

Table 3.2 Composition of MS medium stock solutions

S. No	Quantity	Strength	Salts	Quantity	Use ml/l	Amount in culture (mg)
I	(2 L)	X 20	Ammonium Nitrate	66.0 g	50	1650
			Potassium Nitrate	76.0 g		1900
			Potassium Dihydrogen Phosphate	6.8 g		170
			Boric acid	0.248 g		6.2
			Manganese Sulphate	0.892 g		22.3
			Zinc Sulphate	0.344 g		8.6
			Potassium Iodide	0.033 g		0.825
			Copper Sulphate *	1.0 ml		0.025
			Cobalt chloride *	1.0 ml		0.250
			Sodium Molybdate *	1.0 ml		0.025
II	(1/2 L)	X 50	Calcium Chloride	11 g	20	440
III	(1/2 L)	X 50	Magnesium Sulphate	9.25 g	20	370
IV	(1 L)	X 100	Ferrous Sulphate	2.78 g	10	27.8
			Disodium EDTA	3.728 g		37.28
V	(1 L)	X 100	Thiamine HCl	0.010 g	10	0.1
			Nicotinic acid	0.050 g		0.5
			Pyridoxine HCl	0.050 g		0.5
			Glycine	0.200 g		2
VI			Myoinositol			100
VII			Sucrose			30000
VIII			Agar			8000
IX			Glutamine			150
X			Kinetin **			0.5

* Dissolve 100 mg of copper sulphate and cobalt chloride and 1 g of sodium molybdate in 100 ml of water separately. Then take 1 ml each and add to stock solution I.

** Dissolve 0.1g in 5ml 1N solution of NaOH and then add 9.5 ml distilled water

Table 3.3 Composition of MS medium (1 litre)

S. no.	Constituents	Quantity
1	Sucrose	30 g
2	Agar- Agar	8 g
3	Myoinositol	0.10 g
4	Glutamine	0.15 g
5	Kinetin	0.50 ml
6	Stock solution I	50 ml
7	Stock solution II	20 ml
8	Stock solution III	20 ml
9	Stock solution IV	10 ml
10	Stock solution V	10 ml

Table 3.4 Composition of rooting medium (1 litre)

S. no.	Constituents	Quantity
1	Glutamine	0.15 g
2	Myoinositol	0.10 g
3	IBA*	1 ml
4	NAA*	1 ml
5	Stock solution I	50 ml
6	Stock solution II	20 ml
7	Stock solution III	20 ml
8	Stock solution IV	10 ml
9	Stock solution V	10 ml

*Dissolve 100 mg in 5 ml 0.5 N NaOH and 95 ml distilled water (Heat slightly in test tube till complete dissolution)

3.2.4 Cytological analysis

The cytological investigation was carried out for detection of aneuploids (monosomics and nullisomics) from normal disomics in monosomic series developed from ócChinese Springô cultivar of hexaploid wheat (Plate 4) and confirmation of haploidy status of plantlets derived from hybridization of *T. durum*, substitution lines, monosomics and nullisomics with *I. cylindrica*. For cytological confirmation of monosomics and nullisomics from disomics, the seeds were allowed to germinate on Whatman filter paper at optimum temperature till the roots emerging from the seeds attained a length of about 2 cm. In case of confirmation of haploidy status, the roots were excised from wide hybridization- derived regenerated plants. The overall procedure followed for cytological analysis is given below:

a) Cold Treatment

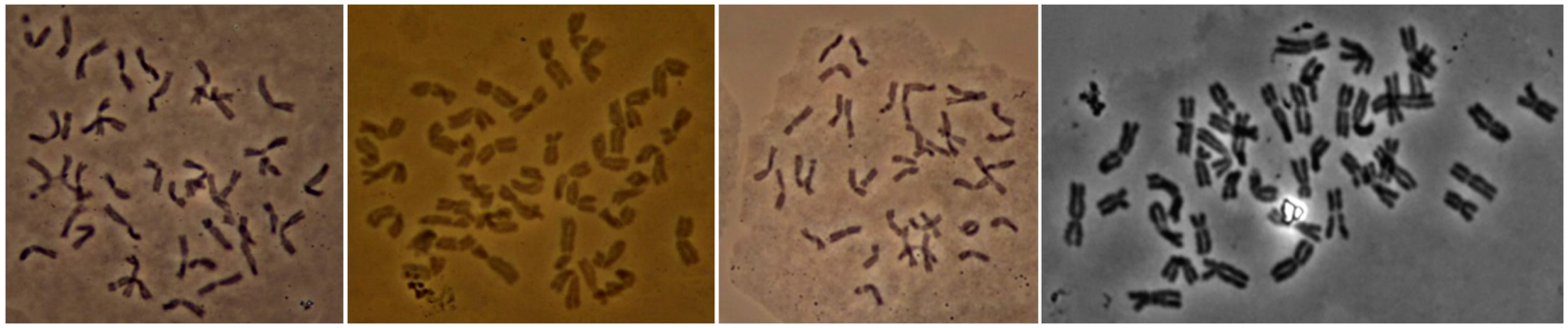
The roots (1 cm) of germinated seeds of each monosomic line of hexaploid wheat were excised and then transferred into vials containing 5 ml distilled water. The roots from the regenerated plantlets were also excised after washing off the media and then transferred into vials containing distilled water. The vials were placed in ice box filled with ice. The roots were then subjected to cold treatment by placing the ice box at 4^{o}C for 18-20 hours.

b) Fixing of roots

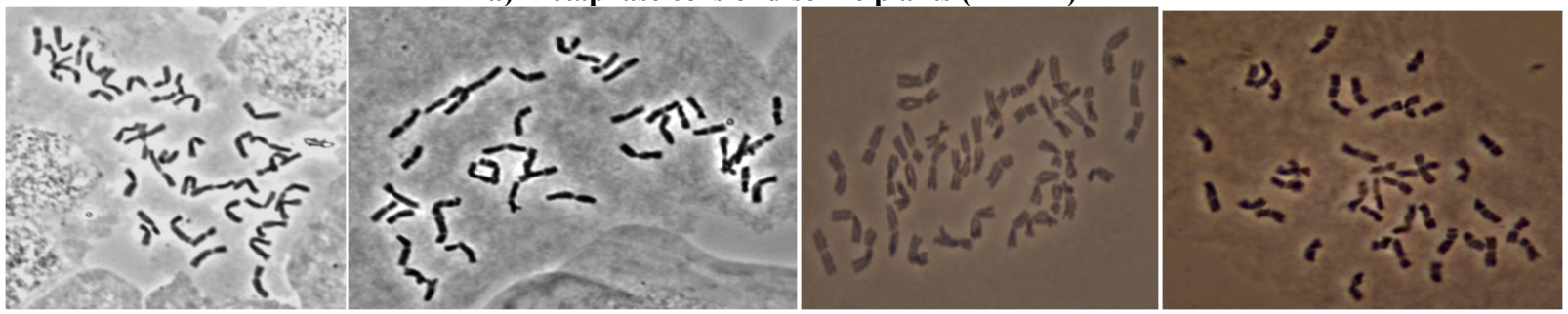
The roots were fixed in freshly prepared solution of absolute ethanol and glacial acetic acid (3:1). Fixed stocks were kept at room temperature and after 4-5 days of fixation of roots, the cytological investigation was initiated.

c) Slide Preparation

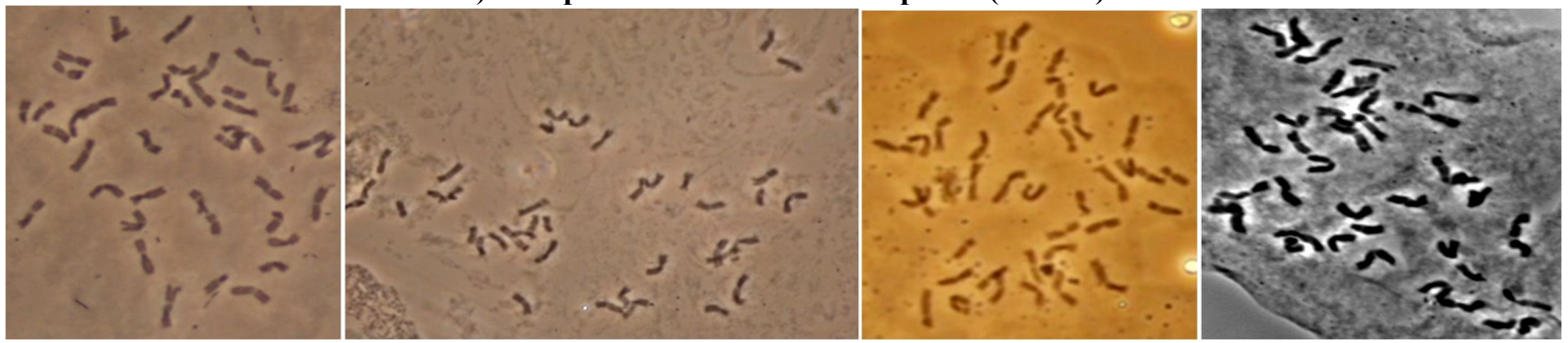
The roots were first stained by placing them in 0.2 percent acetocarmine solution for 15 - 20 minutes. The slide preparation was done by placing the fixed root on the Whatman filter paper and the root cap was removed by using the surgical blade. Immediately after this, the blunt side of the scalpel was used to squeeze the root so as to take out the meristematic cells. The meristematic cells were placed on the clean slide and a drop of 45 percent acetic acid was poured on it. Taping with fine wooden stick was exercised after placing a cover slip inclined on one side with a sterilized razor blade.



a) Metaphase cells of disomic plants (2n = 42)



b) Metaphase cells of monosomic plants (2n = 41)



c) Metaphase cells of nullsomic plants (2n = 40)

Plate 4 Cytological identification of disomic and aneuploid plants in monosomic series of hexaploid wheat

Proper taping is a crucial and highly useful in separating and spreading the cells on the slide. The slide was subjected to warm treatment over a spirit lamp for few seconds and immediately squashed by placing the slide in a folded filter paper and pressing by thumb on the area of cover slip. The prepared slide was observed in the phase contrast microscope (OLYMPUS CX 31) and fluorescent microscope (OLYMPUS BX 61). Chromosomes were counted from good metaphase spreads.

d) Fixation of slides

Slides with good metaphase spreads were placed on the dry ice with the cover-slip facing upside for 15 minutes. After dry ice treatment, the cover slip was removed using blade and immediately placed in the 45 percent glacial acetic acid for 15 minutes. Thereafter, the slides were removed from 45 percent acetic acid and kept for drying in slide stand. The slides were then kept overnight in the desiccator containing silica gel. The dried slides were kept in the slides box, properly sealed and preserved in deep freezer (-20^{o}C).

3.2.5 Histological studies

The histological studies of ovaries fertilized with *I. cylindrica* pollen and harvested at different days after pollination was undertaken to check the development of embryos different maternal species and cytogenetic stocks used in the present investigation. The fertilized ovaries were fixed in mixture of absolute ethyl alcohol and glacial acetic acid (3:1) and stored at 4^{o}C till use. The general protocol followed for histological study depicted in Plate 5 is given below:

i) Requirements

1) Labelling of tissue and pre- dehydration step

a) Paper slips
b) Lead pencil
c) Muslin cloth
d) Thread
e) Rubber bands
f) Scissor

2) Dehydration

a) Ethyl alcohol

b) t- butyl alcohol

c) Eosin (1%)- Dissolve 1g Eosin in 20 ml distilled water + 80 ml Ethyl alcohol

d) Liquid Paraffin

e) Paraffin (38-56^{o}C)

f) Paraffin wax (58-60^{o}C)

3) Paraffin embedding

a) Melted Paraffin wax (58-60^{o}C melting point)

b) Aluminum moulds

c) Marker

d) Blade/ Scalpel for trimming of paraffin blocks having embedded ovules

4) Section cutting

a) Microtome + accessories

b) Water bath

c) Hot plate and glass slides

d) Doubled distilled water

5) Staining

a) Xylene (100%)

b) Ethyl alcohol

c) Flowing tap water

d) Doubled distilled water

e) Safranine (1%) - Dissolve 2g Safranine in 200 ml of 2- Methoxy ethanol

f) Fast green (1%)- Dissolve 1g Fast green in 100 ml Ethyl alcohol

g) Clove oil (100%)

h) Clove oil : Ethyl alcohol : Xylene (1: 1: 1)

i) Xylene (100%)

j) DPX mountant

k) Silica gel (desiccator)

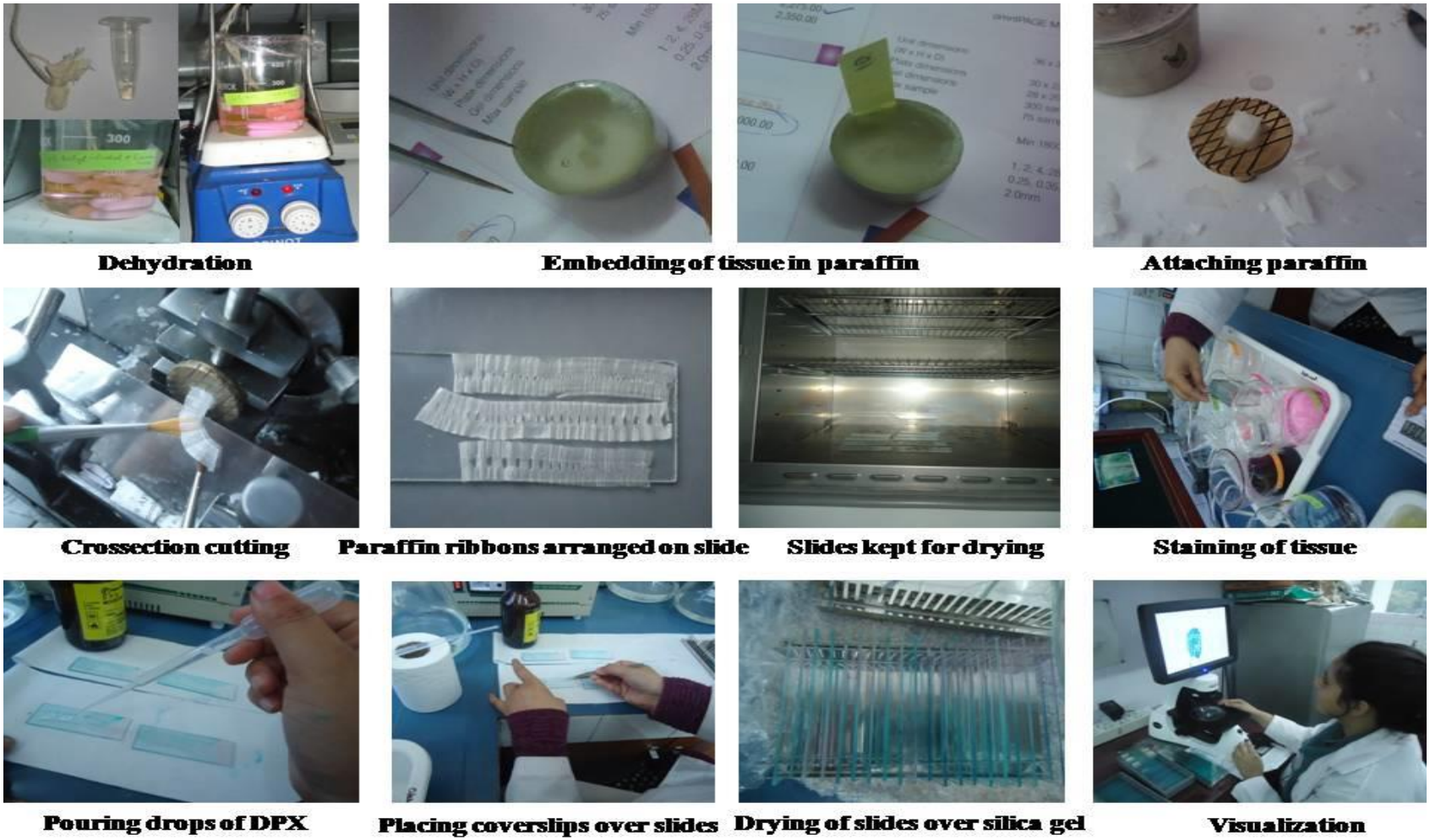


Plate 5 General protocol followed for histological studies of fertilized ovaries of different wheat species and cytogenetic stocks crossed with *I. cylindrica*

ii) Procedure

A) Dehydration

1) The ovules were placed over a muslin cloth along with a labeled paper slip. The tissue and its label were tied tightly using thread or rubber band leaving thread tail.
2) The preserved ovules were placed in muslin cloth separately in similar fashion and attached with a wooden stick.
3) The tissue were dipped in 85% Ethyl alcohol solution and kept on a stirrer for 24 hours. The beakers were covered properly.
4) The tissues were then shifted into 80% Ethyl alcohol for 4 hours over stirrer.
5) After that the tissues were placed in 90% Ethyl alcohol over stirrer for 4 hours.
6) Later the tissues were shifted to 100% Ethyl alcohol over stirrer for 4 hours.
7) After 4 hours the tissues were dipped in 100% t-butyl alcohol and placed on stirrer for 24 hours.
8) On the following day, the tissues were transferred to t- butyl alcohol and Eosin mixture kept on stirrer for 4 hours. The t- butyl alcohol and Eosin mixture was prepared by dissolving 1-2 drops of 1% Eosin in 300 ml of t-butyl alcohol.
9) The next step was transfer of tissue into t- butyl alcohol and liquid paraffin (1:1) solution for 24 hours over a stirrer.
10) The next day, tissues were dipped into paraffin (melting point = 38-56^{o}C) inside incubator at 50^{o}C for 24 hours.
11) At last the tissues were given paraffin treatment (melting point = 58-60^{o}C) inside incubator at 60^{o}C for 24 hours.

B) Embedding of the tissue

1) The tissues were taken out from the incubator and embedded into paraffin blocks made by pouring paraffin (melting point = 58-60^{o}C) in aluminum moulds.
2) The tissues were labeled by inserting a paper strip labeled with information into the uncongealed paraffin.

3) Paraffin was allowed to solidify. The moulds were allowed to loosen by cooling and the paraffin blocks were easily taken out.
4) The ovules were carefully separated from each other and blocks were trimmed properly.
5) The blocks were then attached to a metallic slab with the help of melted paraffin.

C) Section cutting

1) Thin paraffin sections (0.5 µm) in the form of long continuous ribbons containing tissue were cut using microtome.
2) The paraffin sections were placed in an open container filled with distilled water inside water bath at 45^{o}C to remove the folds of the paraffin ribbons.
3) The fully stretched paraffin sections were placed on a glass slide. Labelling was done with diamond cutter pencil and then the slides were placed inside incubator at 50^{o}C till the paraffin melted and tissue was adhered to the glass slide.

D) Staining of the tissue

The slides were placed in a slide basket and stained by placing them in the following solutions sequentially:

1) 100% Xylene for 15-17 minutes
2) 100% Ethyl alcohol for 5 minutes
3) 70% Ethyl alcohol for 5 minutes
4) 30% Ethyl alcohol for 5 minutes
5) Washed under flowing tap water for 5 minutes
6) Doubled distilled water for 5 minutes
7) 1% safranine solution for 10 minutes
8) Washed under flowing tap water for 5 minutes
9) Doubled distilled water for 5 minutes
10) 50% Ethyl alcohol for 5 minutes
11) 95% Ethyl alcohol + 0.5% picric acid for 1 second
12) 95% Ethyl alcohol + 4 drops of ammonia for 2 seconds
13) 95% Ethyl alcohol for 5 minutes

14) 100% Ethyl alcohol for 5 minutes
15) 100% Ethyl alcohol for 5 minutes
16) 1% fast green solution for 4 seconds
17) 100% Clove oil for 10 seconds
18) Solution of clove oil: absolute alcohol: Xylene (1:1:1) for 20 seconds
19) Xylene for 10 seconds
20) At the end of staining process, two to three drops of DPX mountant followed by coverslips (24 x 60 mm) were placed over the slides
21) The slides were then kept over silica gel for about 7 days.

E) Visualization

The slides were visualized under phase contrast microscope (Olympus CX 31 and AMG Evos) after one week.

3.2.6 Recording of observations

3.2.6.1 Haploid induction parameters

The data were recorded with respect to number of florets pollinated, number of pseudoseeds and number of embryos recovered to work out the pseudoseed and embryo formation frequency as follows:

$$\text{Pseudoseed formation frequency (\%)} = \frac{\text{Number of pseudoseeds formed}}{\text{Total number of wheat florets pollinated}} \times 100$$

$$\text{Embryo formation frequency (\%)} = \frac{\text{Number of pseudoseeds carrying embryo}}{\text{Total number of wheat florets pollinated}} \times 100$$

3.2.6.2 Morphological characterization of aneuploids

The morphological characterization of the monosomic lines was carried out in order to know the effect of deletion of one or two chromosomes on the morphology of the plants and for utilization of such morphological markers in easy identification of aneuploids from the normal disomic plants. The morphological characterization of monosomic lines was done on the basis of following characteristics:

1. Growth habit: The growth habit of plants was recorded on visual basis from initial growth stages till maturity.
2. Pigmentation: Pigmentation on stem, leaves, glumes, auricle and ligule was recorded from seedling stage till maturity.

3. Neck hairiness: Observations were recorded as presence or absence of neck hairiness.
4. Presence of awns: Monosomic lines were characterized with respect to presence or absence of awns on visual basis.
5. Spike shape: The spike shape was recorded as per the spike shapes given by Briggle and Reitz (1963).
6. Density of spike: The density of spike was recorded as compact or lax spikes on visual basis.
7. Waxiness: Waxiness of plants was observed as presence or absence of waxy appearance.
8. Seed shape: The shape of seeds was observed on visual basis and seed shape was assigned from oval, ovate and elliptical categories.
9. Seed colour: The aneuploids were observed for seed colour using Sodium hydroxide (NaOH) test. The seeds were dipped in 5% NaOH solution for 60-90min. The seeds that turned straw yellow were considered as white grains and those turning dark brownish as red grains.
10. Seed hardiness: Seed hardiness was observed as hard or soft seeds using mechanical test.
11. Seed size: The size of seed was observed on visual basis and were classified into small, medium and large categories.
12. Plant height (cm): The height of plants was measured in centimetres from ground level to the tip of the tallest tiller.
13. Spike length (cm): The spike length was measured in centimetres from base to the tip of the spike.
14. Number of tillers: The number of tillers were counted for individual plant.
15. Florets per spike: Total number of florets present in one spike was recorded on individual plant basis.
16. Seeds per spike: Total number of seeds set on a spike was counted for individual plant.
17. Days to flowering: Days to flowering was recorded as the duration from transplanting till initiation of flowering.

18. Flag leaf: The habit of boot leaf was observed on visual basis from booting to heading stage.
19. Spike colour: The spike colour was observed on visual basis during flowering stage.
20. Auricle colour: The colour of auricle was observed on visual basis throughout the crop duration.
21. Ligule colour: The visual characterization of plants for ligule colour was also done throughout the crop duration.
22. Seed hairiness: Presence of hairs on the chalazal end of seeds was recorded visually.
23. Leaf colour: The colour of leaves was recorded throughout the crop duration *via* visual examination.
24. 100 seed weight (g): The weight of 100 seeds was measured and recorded in grams.

3.2.7 Statistical analysis

The statistical analysis was carried out with respect to the embryo formation frequency (%) recovered after hybridization of different monosomic and nullisomic lines of hexaploid wheat and D- genome substitution lines of tetraploid wheat with *I. cylindrica*. As per Rangaswamy (2006), Z- test of significance was used to test the difference among embryo formation frequency (%) of each aneuploid line and that of control. The embryo formation frequency (%) of each monosomic and nullisomic line was also tested against mean embryo formation frequency of all the monosomic and nullisomic lines, respectively.

Similarly, Z- test was also used to test the difference of embryo formation frequency of D- genome substitution lines from the control and mean embryo formation frequency of all the substitution lines. The Z value for comparison of two proportions was estimated using following formula:

$$Z = \frac{p_1 - p_2}{\sqrt{p\frac{q_1 q_2}{n_1} + \frac{q_1 q_2}{n_2}}}$$

Where,

p_1= embryo formation frequency (%) of genotype

p_2= embryo formation frequency (%) of control

q_1= 1-p_1

q_2= 1-p_2

n_1= number of florets of the genotype pollinated with *I. cylindrica* pollen

n_2= number of florets of the control pollinated with *I. cylindrica* pollen

For comparison with mean,

p_2= mean embryo formation frequency (%) of all the genotypes

n_2= mean number of florets pollinated with *I. cylindrica* pollen

4. RESULTS AND DISCUSSION

Uniparental chromosome elimination occurring in several wide hybrids of plant species acts as boon or bane for the plant breeders. Like any other morphological or biochemical characteristics of an individual, such processes are also genetically controlled. The present investigation was carried out after extensive hybridization of diploid, tetraploid species and cytogenetic stocks of wheat to find out the role of genetic background (genomes and chromosomes) of wheat in triggering the elimination of *I. cylindrica* chromosomes in wheat x *I. cylindrica* hybrids. The investigation will open new vistas in respect of the successful retention of the desirable traits of the weedy grass, *I. cylindrica* in wide hybrids of wheat. Moreover, it will also be very helpful in enhancing the haploid induction efficiency of the wheat x *I. cylindrica*- mediated chromosome elimination approach of doubled haploidy breeding. The results obtained during the investigation are presented and discussed hereunder:

4.1 Identification of wheat genome(s)

Bread wheat is an allohexaploid comprising three different genomes *viz.,* AA, BB and DD derived from three different sources. The resolution of the genetic mechanism involved in instability and hence elimination of alien chromosomes in wide hybrids of wheat x *I. cylindrica* was made possible through analysis of haploid induction data obtained in species representing different genomes of hexaploid wheat upon hybridization with *I. cylindrica*. The results obtained upon hybridization of different species of wheat with *I. cylindrica* are given here under:

4.1.1 *Triticum monococcum* x *I. cylindrica*

The spikes of *T. monococcum* crossed with *I. cylindrica* and supplemented with 0.01 percent of 2,4-D during *rabi* 2010-11 resulted in pseudoseed formation frequency of 35.50 percent (Table 4.1). The pseudoseeds obtained were devoid of aqueous solution as found in *T. aestivum* x *I. cylindrica* derived pseudoseeds. The pseudoseeds recovered from *T. monococcum* x *I. cylindrica* hybridization were also devoid of embryos.

4.1.2 *Triticum tauschii* x *I. cylindrica*

The pseudoseed and embryo formation frequency obtained in *T. tauschii* x *I. cylindrica* during *rabi* 2010-11 was 53.20 and 6.80 percent, respectively when supplemented with 0.01 percent 2,4-D solution (Table 4.1). The fluid filled pseudoseeds were small in comparison to those of hexaploid wheat and hence, screening of floating embryos in the pseudoseeds against light source was difficult. The embryos were rescued from the surface sterilized pseudoseeds under aseptic conditions in laminar air flow chamber.

4.1.3 *Triticum durum* x *I. cylindrica*

Triticum durum, an allotetraploid species of wheat was hybridized with *I. cylindrica* to know the role of B genome in the elimination of *I. cylindrica* chromosomes. During *rabi* 2010-11, the hybridized spikes of *T. durum* resulted in recovery of 50.35 percent pseudoseeds and 2.08 percent haploid embryos when supplied with 0.01 percent 2,4-D solution (Table 4.1). The morphology of pseudoseeds was comparable with those of hexaploid wheat but the embryo formation was quite low.

4.1.4 *Triticum aestivum* x *I. cylindrica*

The hexaploid wheat variety, KWS 29 was used as control to evaluate the embryo formation frequencies of the diploid and tetraploid species. During *rabi*2010-11, the hybridization of ôKWS 29ô with *I. cylindrica* produced 60.75 and 38.26 percent pseudoseeds and haploid embryos, respectively (Table 4.1).

Table 4.1 Embryo formation frequency (%) in diploid and tetraploid species of wheat after pollination with *Imperata cylindrica* pollen at 0.01 percent 2,4-D concentration during *rabi* 2010- 11

Wheat Species	Florets pollinated	Pseudoseeds obtained	Pseudoseed formation frequency (%)	Embryos formed	Embryo formation frequency (%)
T. monococcum	3386	1202	35.50	0	0
T. tauschii	2308	1228	53.20	157	6.80
T. durum	2598	1308	50.35	54	2.08
T. aestivum **(KWS 29) Control**	609	370	60.75	233	38.26

4.1.5 Manipulations in the concentration of 2,4-D

The external supply of auxins during early embryogenesis promotes suitable development of both pseudoseeds and embryos. Due to poor pseudoseed set and size, different concentration of 2,4-D solution *viz.,* 0.01, 0.02, 0.03, 0.04 and 0.05 percent were used for injection into the spikes of wheat species for three consecutive days at 24 hours after pollination with *I. cylindrica*. The results obtained through alterations in 2,4-D concentration have been discussed here under:

4.1.5.1 *Triticum monococcum* x *I. cylindrica*

The pseudoseed formation frequency in *T. monococcum* using 0.01, 0.02, 0.03, 0.04 and 0.05 percent 2,4-D solution was 34.92, 35.54, 35.42, 34.71 and 35.77 percent, respectively. The pseudoseed number and size was not affected much by change in 2,4-D concentration (Plate 6) and no embryos were recovered from the *T. monococcum* x *I. cylindrica*- derived pseudoseeds at any concentration of 2,4-D solution used (Table 4.2).

4.1.5.2 *Triticum tauschii* x *I. cylindrica*

The effect of increased concentration of 2,4-D was revealed on both pseudoseeds (Plate 7) and embryo formation frequency in *T. tauschii*. The pseudoseed formation frequency at 0.01, 0.02, 0.03, 0.04 and 0.05 percent 2,4-D concentration was 55.56, 57.50, 58.06, 58.33 and 52.31 percent, respectively. Whereas, the embryo formation frequency was 6.67, 11.25, 13.98, 17.86 and 3.08 percent, respectively (Table 4.3). From these results, it is evident that maximum haploid embryo formation in *T. tauschii* upon hybridization with *I. cylindrica* occurs at 0.04 percent 2,4-D solution (Fig. 4.1).

4.1.5.3 *Triticum durum* x *I. cylindrica*

In *T. durum,* the pseudoseed formation frequency and size increased with an increase in concentration of 2,4-D used for injection of the pollinated spikes (Plate 8). The pseudoseed formation enhanced from 50.68 to 56.98 percent when 2,4-D concentration was increased from 0.01 to 0.05 percent, respectively. The embryo formation frequency was also affected by change in the concentration of 2,4-D solution (Fig. 4.2). The embryo formation frequency obtained at 0.01, 0.02, 0.03, 0.04 and 0.05 percent 2,4-D was 5.41, 11.72, 13.46, 7.26 and 8.14 percent, respectively (Table 4.4). Thus, maximum haploid embryos were recovered from *T. durum* x *I. cylindrica* crosses at 0.03 percent concentration of 2,4-D.

Table 4.2 Embryo formation frequency (%) in *Triticum monococcum* pollinated with *Imperata cylindrica* pollen at different concentration of 2,4-D

2,4-D concentration (%)	Florets pollinated	Pseudoseeds obtained	Pseudoseed formation frequency (%)	Embryos formed	Embryo formation frequency (%)
0.01	126	44	34.92	0	0
0.02	121	43	35.54	0	0
0.03	144	51	35.42	0	0
0.04	121	42	34.71	0	0
0.05	123	44	35.77	0	0

Table 4.3 Embryo formation frequency (%) in *Triticum tauschii* pollinated with *Imperata cylindrica* pollen at different concentration of 2,4-D

2,4-D concentration (%)	Florets pollinated	Pseudoseeds obtained	Pseudoseed Formation frequency (%)	Embryos formed	Embryo formation frequency (%)
0.01	90	50	55.56	6	6.67
0.02	80	46	57.50	9	11.25
0.03	93	54	58.06	13	13.98
0.04	84	49	58.33	15	17.86
0.05	65	34	52.31	2	3.08

Table 4.4 Embryo formation frequency (%) in *Triticum durum* pollinated with *Imperata cylindrica* pollen at different concentration of 2,4-D

2,4-D concentration (%)	Florets pollinated	Pseudoseeds obtained	Pseudoseed formation frequency (%)	Embryos formed	Embryo formation frequency (%)
0.01	148	75	50.68	8	5.41
0.02	145	78	53.79	17	11.72
0.03	104	57	54.81	14	13.46
0.04	124	68	54.84	9	7.26
0.05	86	49	56.98	7	8.14

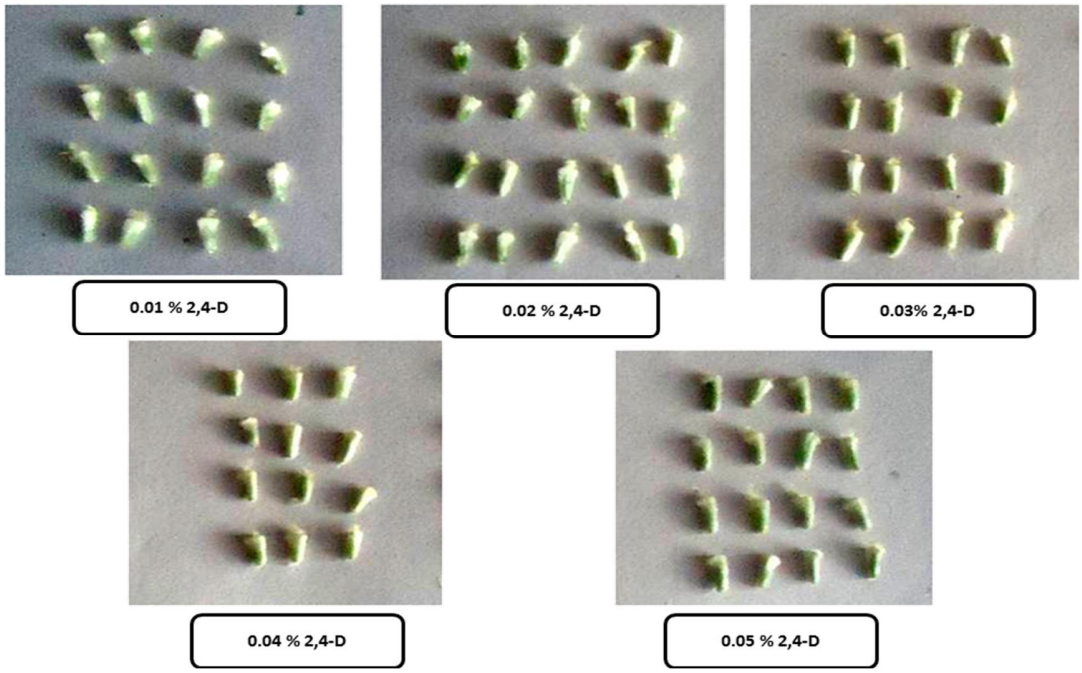


Plate 6 Pseudoseeds obtained in *Triticum monococcum* after using different concentration of 2,4-D injected into the spikes pollinated with *Imperata cylindrica* pollen

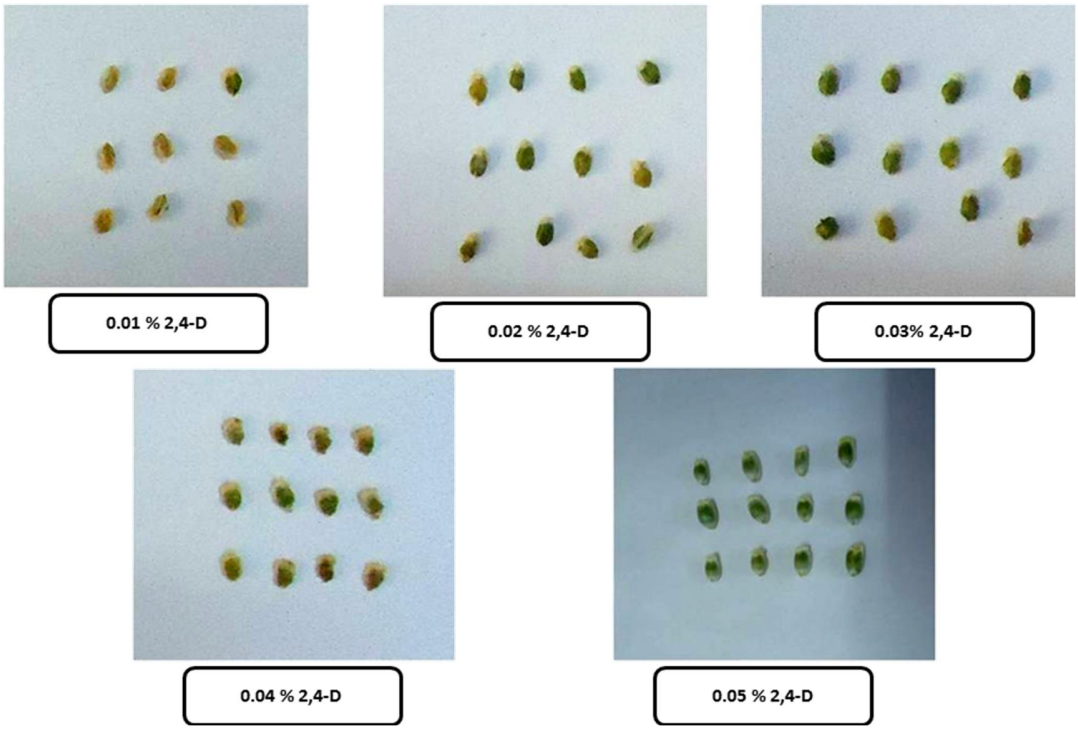


Plate 7 Pseudoseeds obtained in *Triticum tauschii* after using different concentration of 2,4-D injected into the spikes pollinated with *Imperata cylindrica* pollen

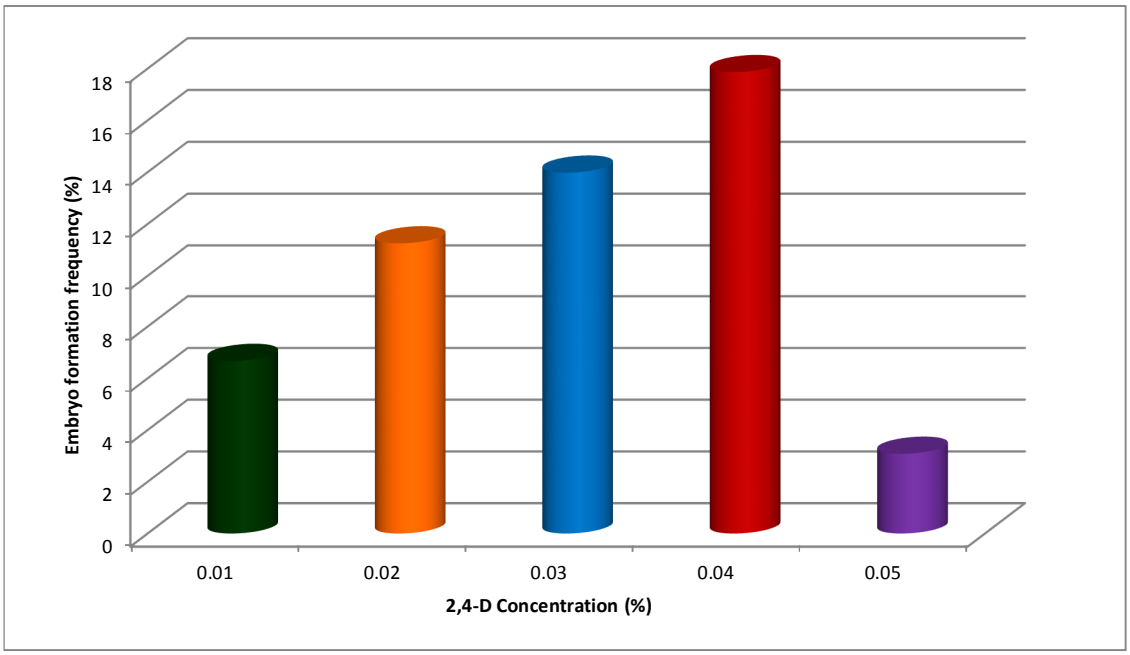


Fig. 4.1 Graphical representation of embryo formation frequency recovered in *Triticum tauschii* after pollination with *Imperata cylindrica* using different concentration of 2,4-D

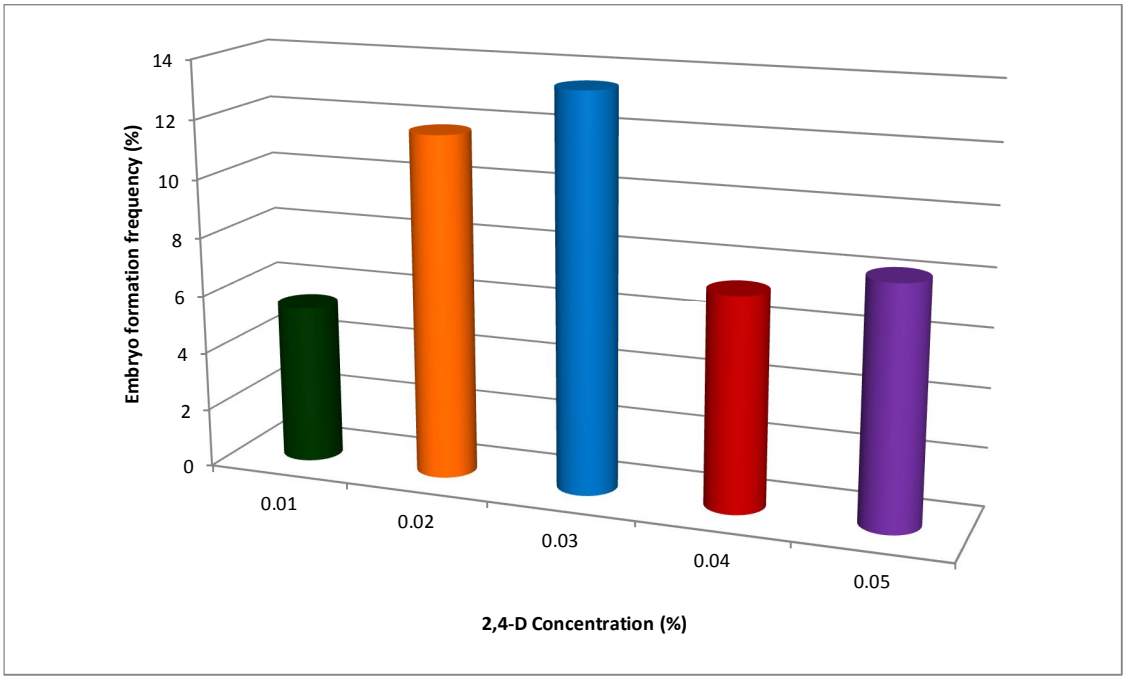


Fig. 4.2 Graphical representation of embryo formation frequency obtained in *Triticum durum* after pollination with *Imperata cylindrica* using different concentration of 2,4-D

4.2 Haploid induction efficiency at optimum dose of 2,4-D

After evaluating the most suitable dose of 2,4-D solution for haploid induction to be injected into the pollinated spikes of different species of wheat during *rabi* 2010-11, the particular concentration of 2,4-D identified as the most optimal dose was used for supplementing the production of haploids in *T. monococcum, T. tauschii* and *T. durum* after pollination with *I. cylindrica* during *rabi* 2011-12 and the results obtained are given here under:

4.2.1 *Triticum monococcum* x *I. cylindrica*

Though there was no considerable effect of 2,4-D concentration on pseudoseed and embryo formation frequency in *T. monococcum* during *rabi* 2010-11 yet, the spikes of *T. monococcum* were again crossed with *I. cylindrica* using 0.01 percent 2,4-D solution for injection. The pseudoseed formation recovered was 37.89 percent but no embryos were found in the harvested pseudoseeds (Table 4.5). Histological investigation of ovules of *T. monococcum* also revealed that no embryos were formed when fertilized with *I. cylindrica* pollen (Plate 9).

4.2.2 *Triticum tauschii* x *I. cylindrica*

The pollinated spikes of *T. tauschii* when injected with the optimum dose of 2,4-D (0.04%) produced pseudoseeds to the tune of 58.36 percent. The frequency of haploid induction was also significantly enhanced by supplementing the pollinated spikes of *T. tauschii* with optimum dose of 2,4-D and the embryo formation frequency obtained was as much as 15.20 percent (Table 4.5). The developing embryos were also observed in cross-sections of *T. tauschii* ovules fertilized with *I. cylindrica* pollen (Plate 10). The embryos were rescued under sterile conditions on artificial medium supplemented with kinetin to promote organogenesis. However, the embryos were too small in size as compared to hexaploid or tetraploid wheat and hence, failed to regenerate.

4.2.3 *Triticum durum* x *I. cylindrica*

Upon using the optimum concentration of 2,4-D solution for injection into the pollinated spikes of *T. durum* (0.03%), the pseudoseed and haploid induction efficiency of *T. durum* was enhanced to the extent of 54.79 and 11.03 percent, respectively (Table 4.5). The development of embryos in *T. durum* when pollinated with *I. cylindrica* was

also confirmed through cross-sectional studies of the fertilized ovules (Plate 11). The haploid embryos were successfully regenerated into haploid plantlets. The cytological investigation of the root tip cells derived from regenerated *T. durum* plantlets also revealed the haploid nature of the *I. cylindrica*- mediated haploid durum plants (Plate 12).

Table 4.5 Embryo formation frequency (%) in diploid and tetraploid species of wheat after pollination with *Imperata cylindrica* pollen at optimum concentration of 2,4-D during *rabi* 2011- 12

Wheat Species	2,4-D concentration (%)	Florets pollinated	Pseudoseeds obtained	Pseudoseed formation frequency (%)	Embryos formed	Embryo formation frequency (%)
T. monococcum	0.01	3394	1286	37.89	0	0.00
T. tauschii	0.04	2178	1271	58.36	331	15.20
T. durum	0.03	3245	1778	54.79	358	11.03
T. aestivum (KWS 29) Control	0.01	489	304	62.17	184	37.62

The effect of change in the concentration of 2,4-D solution used for injection into the pollinated spikes of different species of wheat is depicted in Fig. 4.3. From the figure, it is quite clear that the production of haploids in *T. tauschii* and *T. durum* can be enhanced to a great extent through manipulation in the 2,4-D concentration. These results were in concordance with Laurie and Bennett (1988a) and Almouslem et al. (1998). The lack of ability of *T. monococcum* to produce haploid embryos divulged that there is no role of A genome in eliminating the chromosomes of *I. cylindrica*. The role of B and D genome of hexaploid wheat in the *I. cylindrica*- mediated chromosome elimination approach of haploid induction is very clear from the fact that haploid embryos were successfully induced in *T. durum* and *T. tauschii*. However, the contribution of D genome seems to be more than B genome of hexaploid wheat in view of the fact that higher haploid induction frequency in *T. tauschii* was observed in contrast to *T. durum*. These results are in correspondence with Inagaki et al. (1997), Inagaki and Hash (1998) and Mujeeb-Kazi et al. (2006) who found that D- genome chromosomes enhance the haploid production in durum wheat and triticale in maize- mediated system of doubled haploidy breeding.

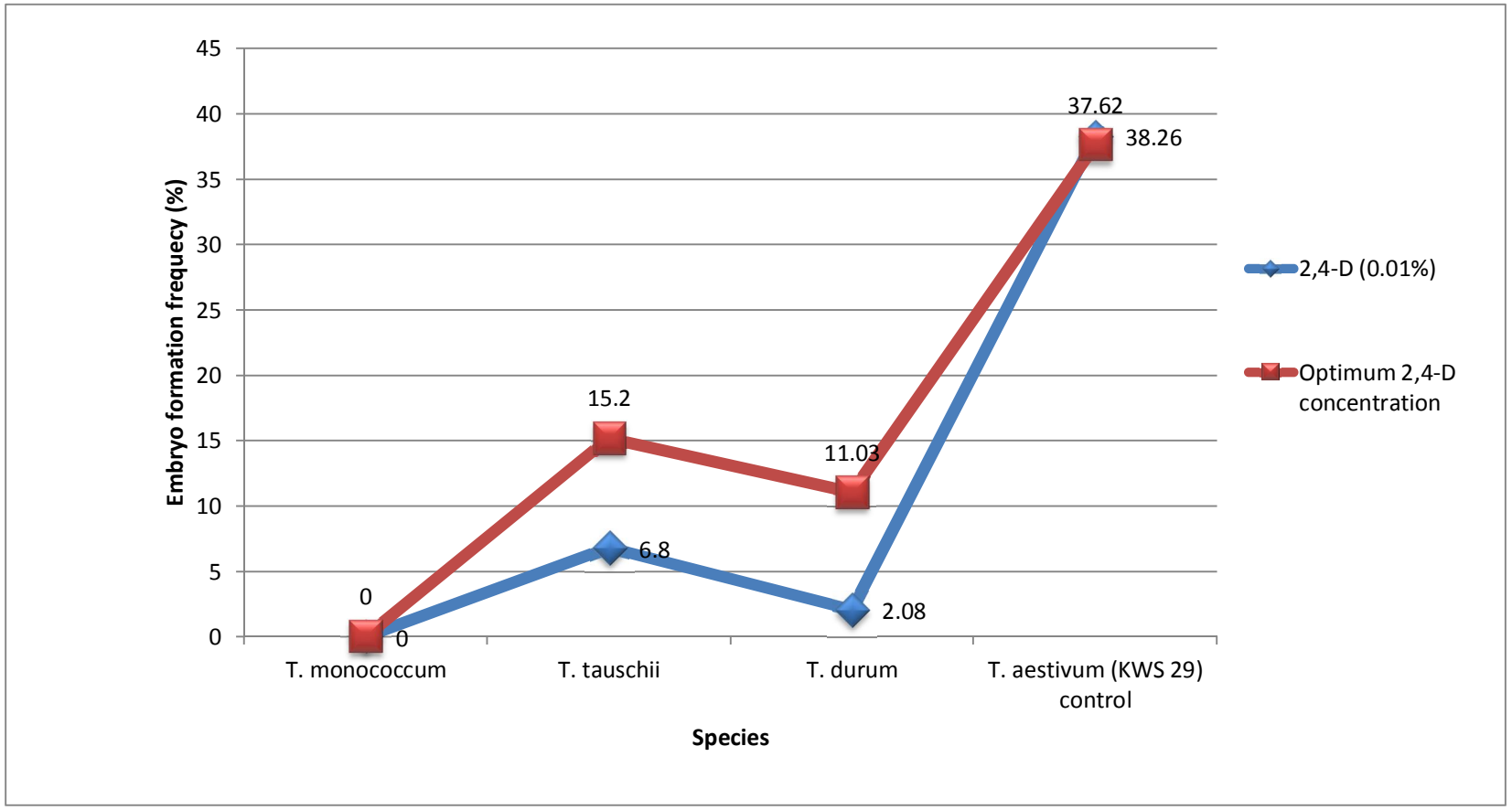


Fig. 4.3 Graphical representation of increase in embryo formation frequency of different species of wheat after pollination with *Imperata cylindrica* at optimum concentration of 2,4-D

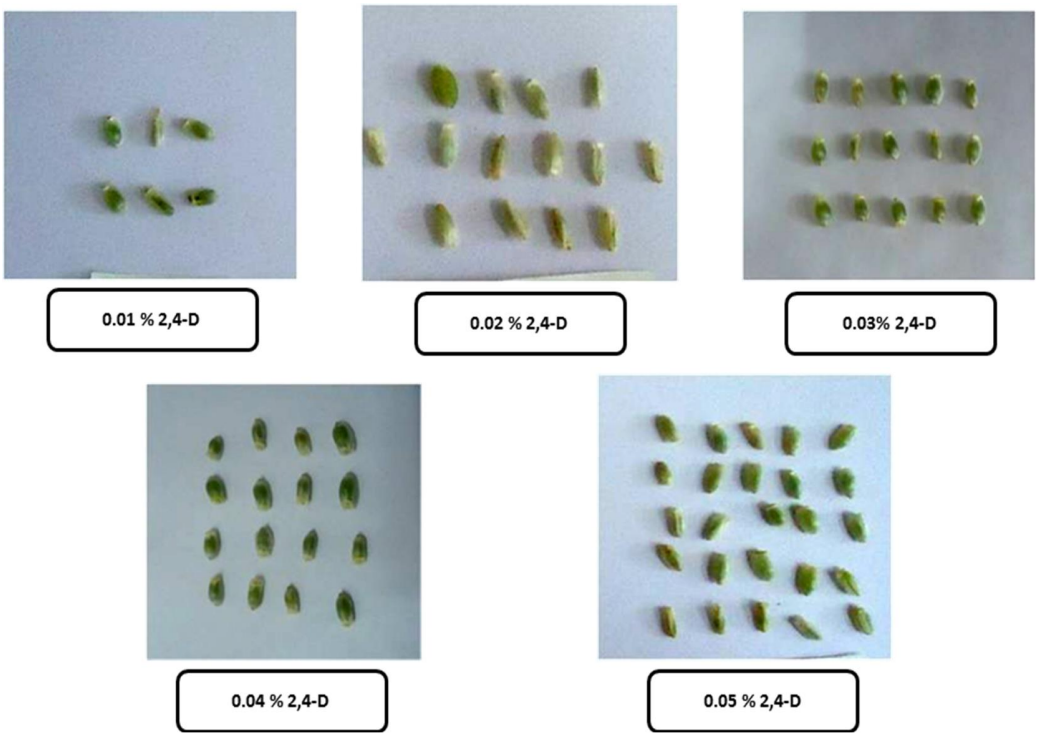


Plate 8 Pseudoseeds obtained in *Triticum durum* after using different concentration of 2,4-D injected into the spikes pollinated with *Imperata cylindrica* pollen

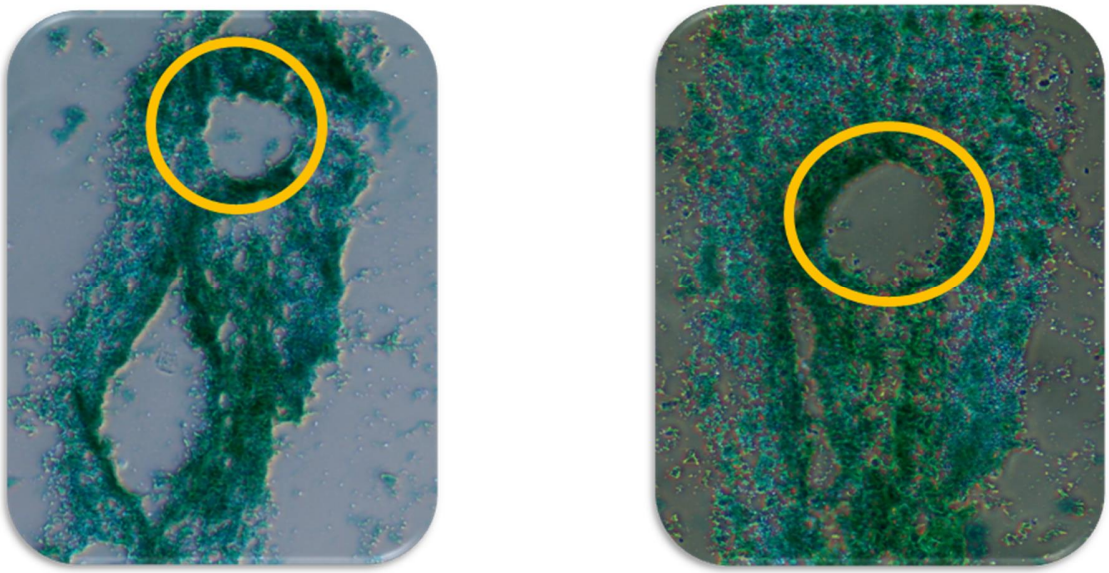


Plate 9 Histological studies of fertilized ovaries of *Triticum monococcum* revealing no embryo formation upon hybridization with *Imperata cylindrica*

Plate 10 Histological studies of fertilized ovaries of *Triticum tauschii* revealing the development of embryos upon hybridization with *Imperata cylindrica*

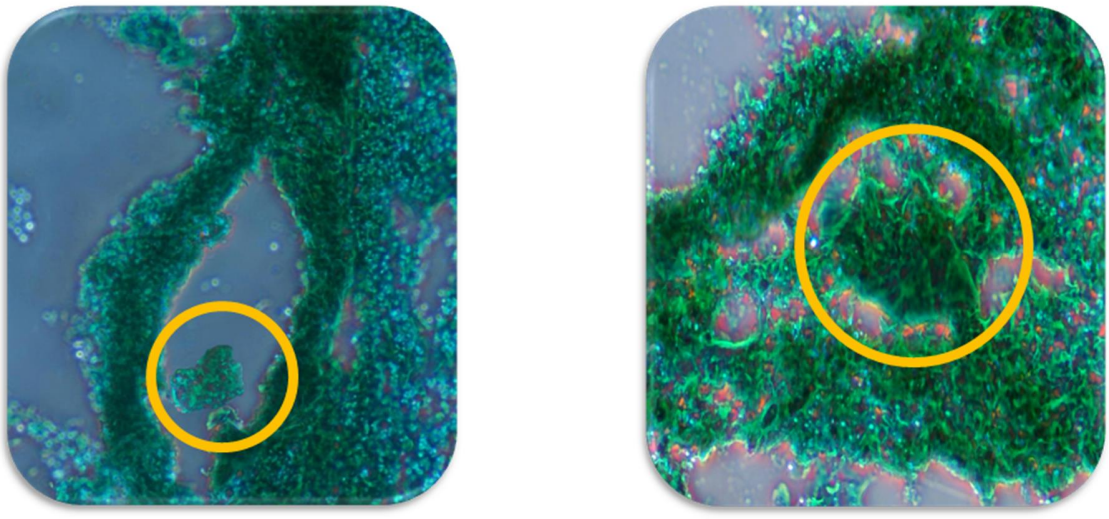


Plate 11 Histological studies of fertilized ovaries of *Triticum durum* revealing the development of embryos upon hybridization with *Imperata cylindrica*

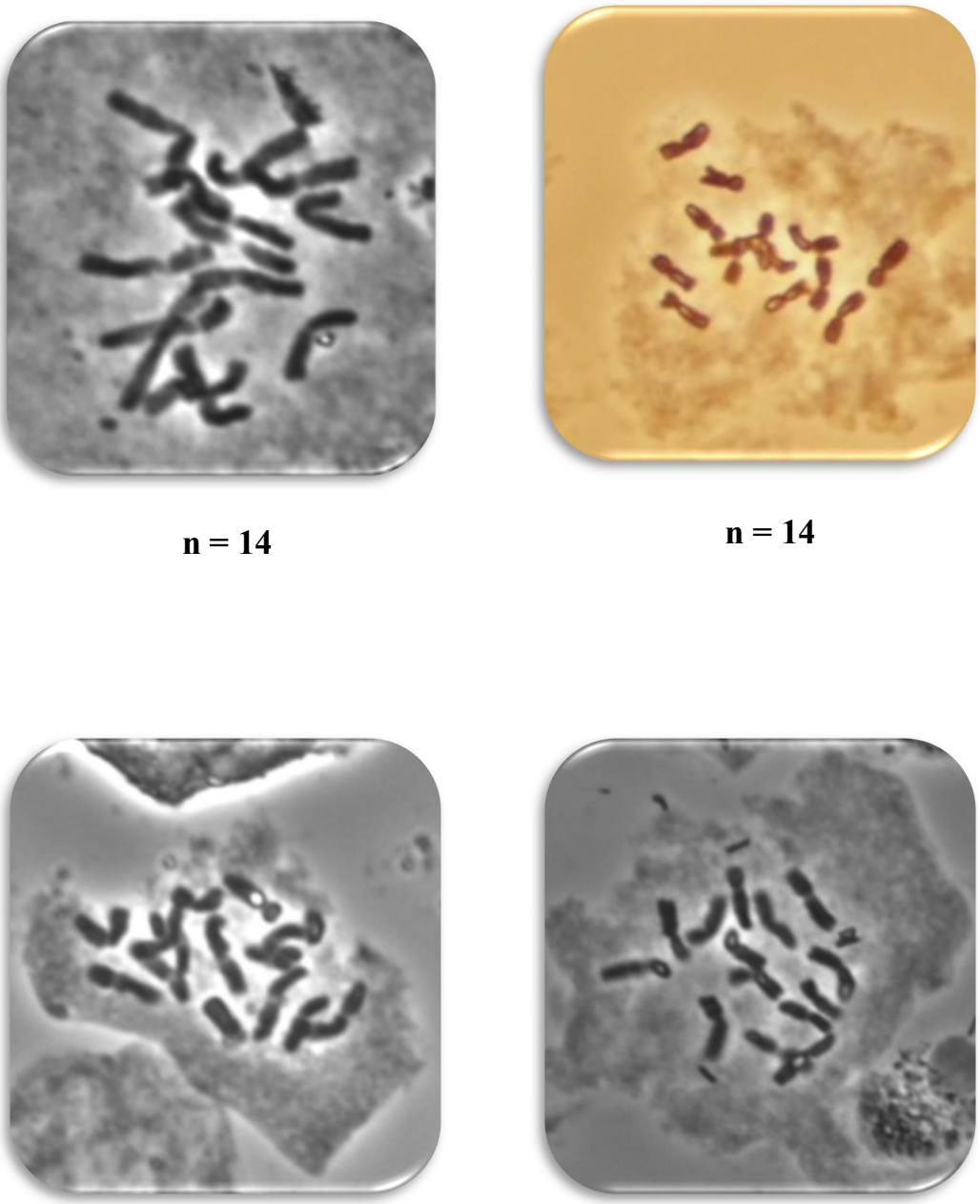


Plate 12 Cytological investigation (metaphase) of regenerated *Triticum durum* plantlets revealing their haploid nature

4.3 Identification of wheat chromosome(s)

Subsequent to the recognition of the genomes that are controlling the instability and elimination of alien chromosomes in wheat, further resolution is required in respect of the tagging of specific chromosome(s) of wheat triggering the chromosome elimination process. The task was accomplished by investigating the haploid induction efficiency in series of monosomic and nullisomic lines of hexaploid wheat and D-genome substitution lines of tetraploid wheat (*T. durum*) after crossing them with *I. cylindrica*. The results obtained are mentioned here under:

4.3.1 Monosomic lines of hexaploid wheat x *I. cylindrica*

The pseudoseed formation frequency obtained after hybridization of complete set of monosomics series of hexaploid wheat with *I. cylindrica* ranged from 52.72 to 76.02 percent with a mean value of 67.49 percent (Table 4.6). The embryo formation frequency varied from 6.84 to 24.73 percent with the line having missing 4D chromosome exhibiting lowest embryo formation frequency followed by the lines having missing 1D, 6B and 6D chromosomes (Fig. 4.4). The mean value of embryo formation frequency over all the monosomic series was 17.31 percent whereas, that of control (Chinese Spring) was 26.33 percent. The data obtained on embryo formation frequency was subjected to Z test of significance to compare the performance of monosomic lines with mean and control. The statistical analysis revealed significant deviations of all monosomic lines from control except 5B monosomic. The monosomic lines 5B, 6B, 1D, 4D and 6D also exhibited significant difference from mean. The 5B monosomic had significantly higher embryo formation frequency than mean whereas, the rest monosomic lines had embryo formation less than mean (Table 4.6).

The embryo formation frequency of the monosomic lines having significantly lesser embryo formation frequency than mean were subsequently compared with each other to understand the extent of role played by each chromosome in haploid induction *via* chromosome elimination. As evident from Table 4.7, it is quite clear that chromosome 4D exhibited maximum influence on the haploid embryo formation followed by 1D. The influence of 6B and 6D chromosomes was at par but significantly less than that of 4D and 1D chromosomes.

Table 4.6 Embryo formation frequency (%) obtained by crossing complete set of the monosomic lines of hexaploid wheat with *Imperata cylindrica* using optimum concentration of 2,4-D (0.01%)

Genotype**	Florets pollinated	Pseudoseeds obtained	Pseudoseed formation frequency (%)	Embryos formed	Embryo formation frequency (%)	Z^1	Z^2
1A	681	359	52.72	130	19.09*	-3.57	0.86
2A	595	444	74.62	107	17.98*	-4.01	0.32
3A	634	390	61.51	125	19.72*	-3.17	1.14
4A	529	359	67.86	100	18.90*	-3.41	0.72
5A	440	244	55.45	90	20.45*	-2.50	1.31
6A	946	702	74.21	177	18.71*	-4.10	0.74
7A	693	439	63.35	129	18.61*	-3.85	0.63
1B	895	670	74.86	159	17.77*	-4.59	0.24
2B	777	518	66.67	154	19.82*	-3.30	1.25
3B	550	370	67.27	93	16.91*	-4.49	-0.19
4B	765	577	75.42	138	18.04*	-4.27	0.37
5B	659	403	61.15	163	24.73@	-0.74	3.37
6B	787	583	74.08	106	13.47*@	-7.05	-2.05
7B	555	368	66.31	97	17.48*	-4.20	0.08
1D	823	569	69.14	82	9.96*@	-9.56	-4.17
2D	841	558	66.35	148	17.60*	-4.62	0.15
3D	527	338	64.14	90	17.08*	-4.35	-0.11
4D	863	584	67.67	59	6.84*@	-12.12	-6.32
5D	805	612	76.02	142	17.64*	-4.55	0.17
6D	687	482	70.16	92	13.39*@	-6.89	-2.04
7D	891	608	68.24	172	19.30*	-3.70	1.03
Control (Chinese Spring)	1052	792	75.29	277	26.33		
Mean			67.49		17.31		

*PÒ0.05 when compared with control;
@PÒ0.05 when compared with mean;
**Alphabets designate the genotype having the particular chromosome absent e.g. 1A means chromosome no. 1 of the A- genome is absent in the monosomic line
[1]Calculated values of Z for comparison of embryo formation frequency of genotypes with the control;
[2]Calculated values of Z for comparison of embryo formation frequency of genotypes with mean.

Fig. 4.4 Graphical representation of embryo formation frequency of different monosomic lines of hexaploid wheat after pollination with *Imperata cylindrica* using 0.01 percent 2,4-D

The haploid embryos generated out of the crossing of complete set of monosomic series with *I. cylindrica* were excised under aseptic conditions and were allowed to undergo direct organogenesis to produce haploid plantlets. Cytological investigation of the regenerated haploid plantlets was carried out and the plants with n = 21 and n - 1 = 20 chromosomes were recovered (Plate 13). The development of haploid embryos in monosomic lines was also confirmed through histological studies (Plate 14). From these results, it is vivid that the loss of chromosomes 4D, 1D, 6B and 6D result in significant decrease of haploid embryo formation frequency. Hence, it can be stated that these chromosomes are triggering the elimination of paternal chromosomes in wheat x *I. cylindrica* hybrids during early embryogenesis. The contribution of 4D chromosome being highest is followed by 1D, 6B and 6D chromosomes. Similarly, Inagaki et al. (1997) reported that 4D chromosome enhances the haploid induction frequency in triticale when pollinated with maize pollen and Jauhar et al. (2008) enunciated that the nullisomy for 5B combined with 5D tetrasomy can enhance the haploid induction frequency.

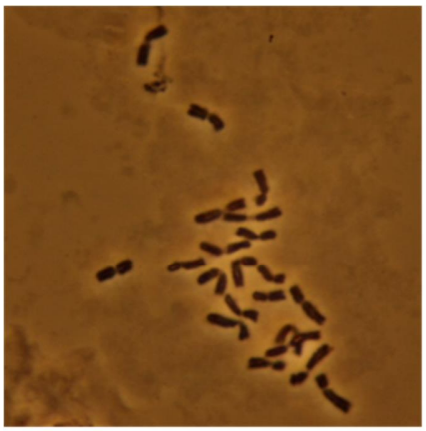
Table 4.7 Comparison of embryo formation frequency of monosomic lines deviating from mean using Z- test

Z-value	**4D** (6.84)	**1D** (9.96)	**6B** (13.47)	**6D** (13.39)
4D (6.84)	-	2.31*	4.45*	4.20*
1D (9.96)		-	-2.19*	-2.06*
6B (13.47)			-	-0.04
6D (13.39)				-

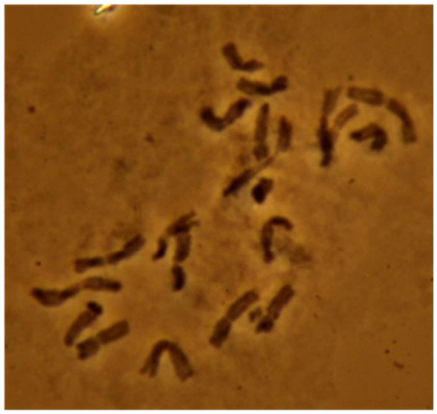
*P0.05
Figures in parentheses represent the embryo formation frequency of the monosomic line

4.3.2 Nullisomic lines of hexaploid wheat x *I. cylindrica*

The pseudoseed formation frequency obtained after hybridization of nullisomic series of hexaploid wheat with *I. cylindrica* ranged from 17.39 to 55.26 percent with mean 42.43 percent (Table 4.8). The pseudoseed frequency was very less in nullisomic 1A (18.44%), 1B (17.39%) and 2B (18.62%) as they exhibited certain degree of sterility. The embryo formation frequency varied from 0 to 12.35 percent with the 1B nullisomic exhibiting the lowest followed by 4D, 2B and 1A nullisomics (Fig. 4.5). The mean value of haploid induction frequency over all the nullisomic lines was 7.73 percent. The

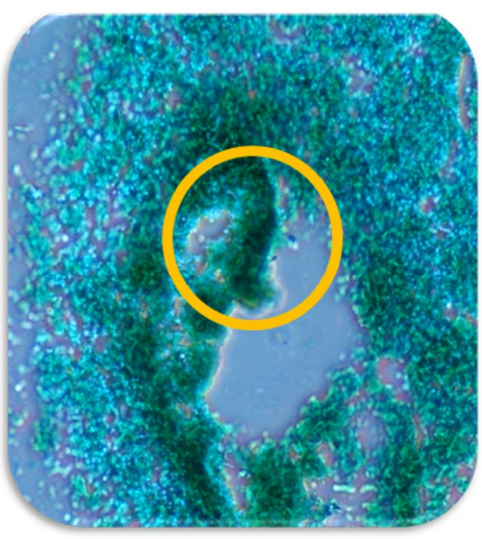


n = 21



n = 20

Plate 13 Metaphase spreads of regenerated haploid and nullihaploid plantlets derived from monosomic lines x *I. cylindrica* hybridization



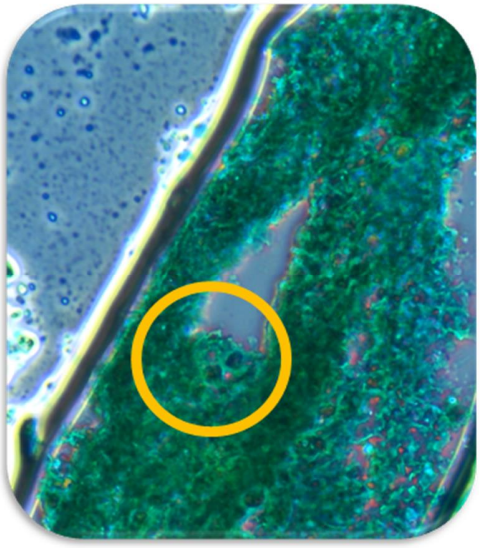


Plate 14 Histological studies of fertilized ovaries of monosomic lines of *Triticum aestivum* revealing the development of embryos upon hybridization with *Imperata cylindrica*

statistical analysis revealed that the embryo formation frequency of nullisomic lines was significantly less than the disomic line (Chinese Spring). The comparison of embryo formation frequency with mean revealed that 1A, 1B, 2B and 4D nullisomics exhibited significant differences (Table 4.8). All the nullisomic lines exhibiting significant difference from mean except 4D nullisomic exhibited sterility and hence resulted in poor pseudoseed set. Though, nullisomic lines were very weak and it was not possible to perform large number of crosses yet, it was revealed that 4D chromosome of hexaploid wheat is triggering the elimination of *I. cylindrica* chromosomes. Cytological investigation of the regenerated haploid plantlets derived from embryo rescue confirmed the haploid nature (n = 20) of the plants (Plate 15).

4.3.3 D genome substitution lines of tetraploid wheat x *I. cylindrica*

The D genome substitution lines of tetraploid wheat when crossed with *I. cylindrica* produced pseudoseeds ranging from 41.49 to 71.30 percent whereas, the embryo formation frequency ranged from 6.87 to 15.54 percent with mean embryo formation over all the lines equivalent to 10.81 percent. The control (Langdon) exhibited embryo formation frequency to the tune of 10.24 percent (Table 4.9). The embryo formation frequency was highest in 4B(4D) followed by 1B(1D), 1A(1D) and 4A(4D) (Fig. 4.6). When compared with control, five lines showed significant deviation *viz.,* 1A(1D), 4A(4D), 1B(1D), 4B(4D) and 6B(6D). The former four lines exhibited significantly higher embryo formation frequency whereas the later exhibited lesser embryo formation frequency in comparison with control. The substitution lines 1B(1D), 4B(4D) and 6B(6D) also exhibited significant differences from mean (Table 4.9).

From the above results, it is evident that the substitution of chromosomes 4D in place of 4A and 4B chromosomes increased the haploid embryo formation frequency over control. Inagaki et al. (1997) also reported that substitution with 4D chromosome in triticale enhanced the haploid embryo formation in triticale x maize crosses. Similarly, the haploid embryo formation frequency also enhanced significantly over control when 1D chromosome substituted 1A and 1B chromosomes. However, replacement of 6B chromosome by 6D resulted in the significant decrease of embryo formation frequency from mean. This shows that chromosomes 4D, 1D and 6B augment the haploid embryo formation by triggering the elimination of *I. cylindrica* chromosomes. The haploid status of the regenerated plantlets was confirmed through cytological investigation of their excised roots (Plate 16).

Table 4.8 Embryo formation frequency (%) obtained by crossing complete set of nullisomic lines of hexaploid wheat with *Imperata cylindrica* using optimum concentration of 2,4-D (0.01%)

Genotype**	Florets pollinated	Pseudoseeds obtained	Pseudoseed formation frequency (%)	Embryos formed	Embryo formation frequency (%)	Z^1	Z^2
1A	385	71	18.44	13	3.38*@	-13.99	-2.31
2A	464	209	45.04	54	11.64*	-7.29	1.77
3A	369	159	43.09	34	9.21*	-8.44	0.67
4A	370	189	51.08	30	8.11*	-9.28	0.18
5A	221	104	47.06	22	9.95*	-6.74	0.86
6A	184	85	46.20	16	8.70*	-7.10	0.37
7A	560	302	53.93	41	7.32*	-10.88	-0.21
1B	46	8	17.39	0	0.00*@	-19.39	-4.72
2B	247	46	18.62	8	3.24*@	-13.08	-2.26
3B	275	126	45.82	26	9.45*	-7.58	0.71
4B	271	122	45.02	22	8.12*	-8.49	0.17
5B	190	105	55.26	23	12.11*	-5.21	1.52
6B	219	107	48.86	10	4.57*	-11.11	-1.46
7B	162	83	51.23	20	12.35*	-4.79	1.50
1D	321	132	41.12	15	4.67*	-12.05	-1.52
2D	276	119	43.12	33	11.96*	-6.04	1.66
3D	273	120	43.96	30	10.99*	-6.58	1.30
4D	163	63	38.65	2	1.23*@	-15.60	-3.51
5D	178	75	42.13	20	11.24*	-5.53	1.21
6D	215	99	46.05	10	4.65*	-10.97	-1.41
7D	200	98	49.00	19	9.50*	-6.69	0.67
Control (Chinese Spring)	1052	792	75.29	277	26.33		
Mean			42.43		7.73		

*PÒ0.05 when compared with control;

@PÒ0.05 when compared with mean;

**Alphabets designate the genotype having the particular chromosome pair absent e.g. 1A means pair of chromosome no. 1 of the A- genome are absent in the nullisomic line

[1]Calculated values of Z for comparison of embryo formation frequency of genotypes with the control;

[2]Calculated values of Z for comparison of embryo formation frequency of genotypes with mean

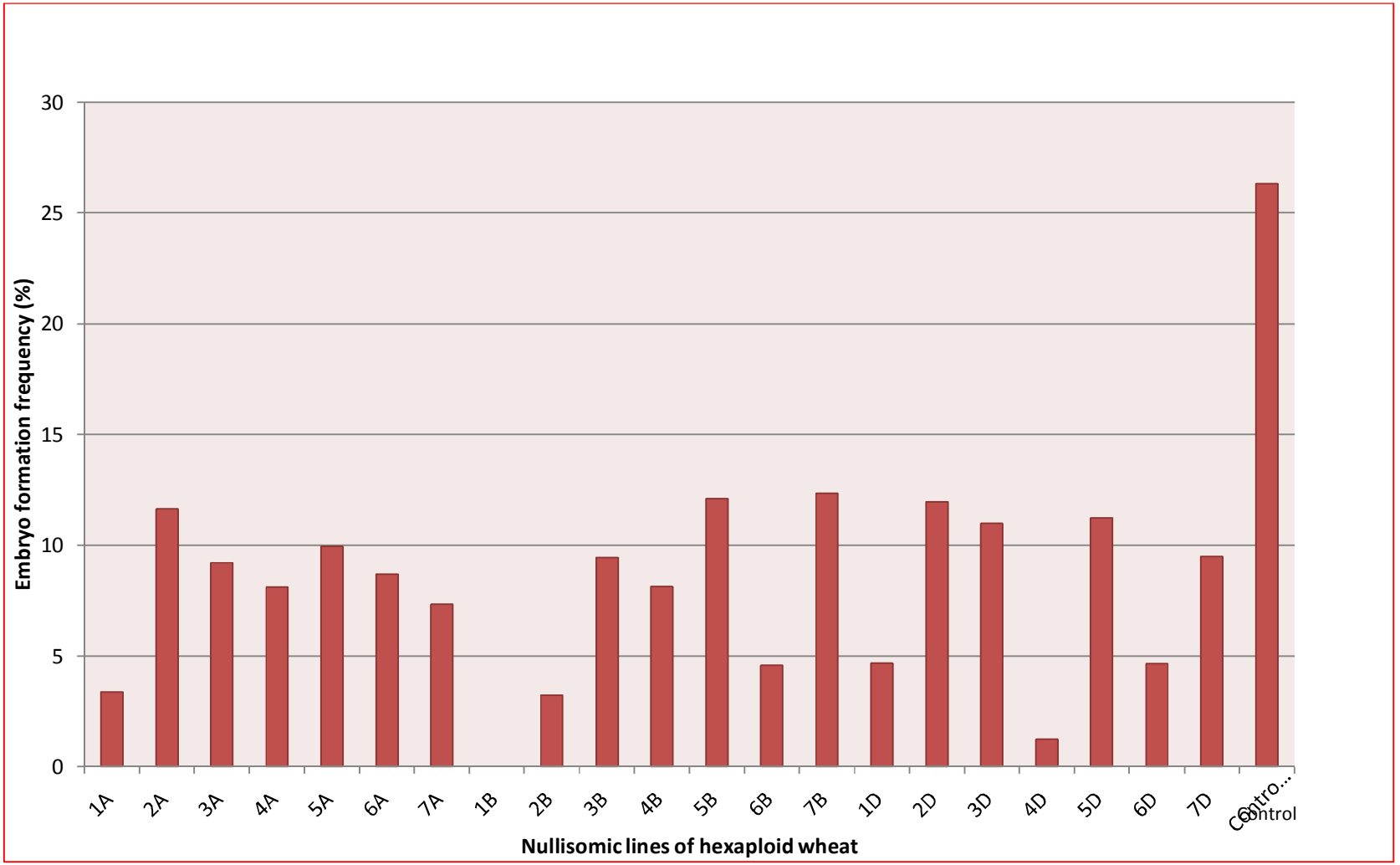


Fig. 4.5 **Graphical representation of embryo formation frequency of different nullisomic lines of hexaploid wheat after pollination with *Imperata cylindrica* using 0.01 percent 2,4-D**

Table 4.9 Embryo formation frequency (%) obtained by crossing different D- genome substitution lines of tetraploid wheat with *Imperata cylindrica* using optimum concentration of 2,4-D (0.03%)

Genotype**	Florets pollinated	Pseudoseeds obtained	Pseudoseeds formation frequency (%)	Embryos formed	Embryo formation frequency (%)	Z^1	Z^2
1A(1D)	527	250	47.44	74	14.04*	2.08	1.62
2A(2D)	532	255	47.93	40	7.52	-1.77	-1.90
3A(3D)	570	304	53.33	53	9.30	-0.59	-0.85
4A(4D)	588	297	50.51	81	13.78*	2.02	1.55
1B(1D)	850	544	64.00	124	14.59*@	2.74	2.13
2B(2D)	554	395	71.30	55	9.93	-0.19	-0.49
3B(3D)	502	263	52.39	39	7.77	-1.57	-1.73
4B(4D)	592	379	64.02	92	15.54*@	2.93	2.40
5B(5D)	555	365	65.77	52	9.37	-0.54	-0.80
6B(6D)	568	392	69.01	39	6.87*@	-2.29	-2.36
7B(7D)	511	212	41.49	52	10.18	-0.04	-0.34
Control (Langdon)	879	559	63.59	90	10.24		
Mean			57.02		10.81		

*P0.05 when compared with control;
@P0.05 when compared with mean;
**Figures in parentheses represent the chromosome substituted in place of the chromosome written outside the parentheses;
[1]Calculated values of Z for comparison of embryo formation frequency of genotypes with the control;
[2]Calculated values of Z for comparison of embryo formation frequency of genotypes with mean.

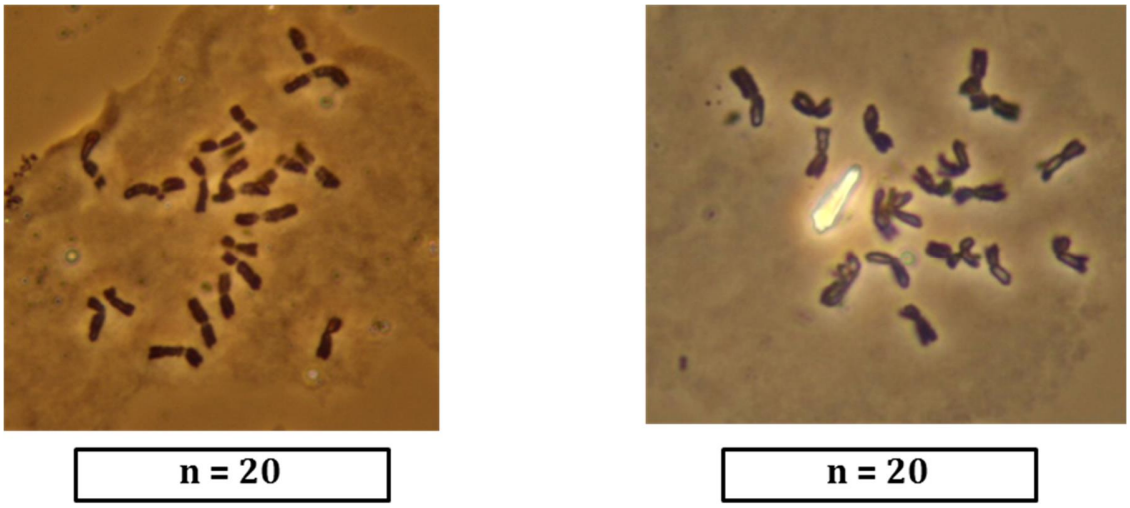


Plate 15 Metaphase spreads of regenerated nullihaploid plantlets derived from nullisomic lines x *I. cylindrica* hybridization

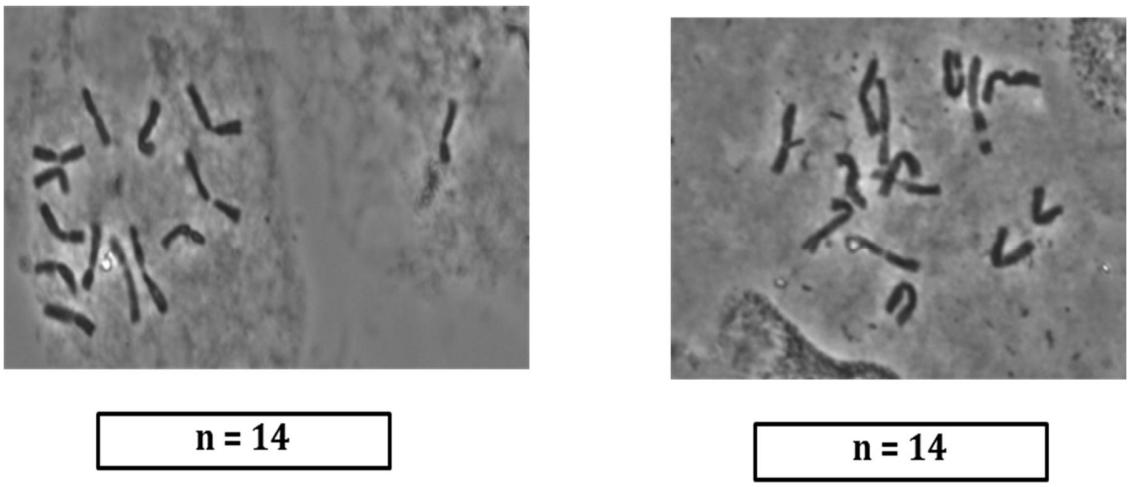


Plate 16 Metaphase spreads of regenerated plantlets derived from substitution lines x *I. cylindrica* cross

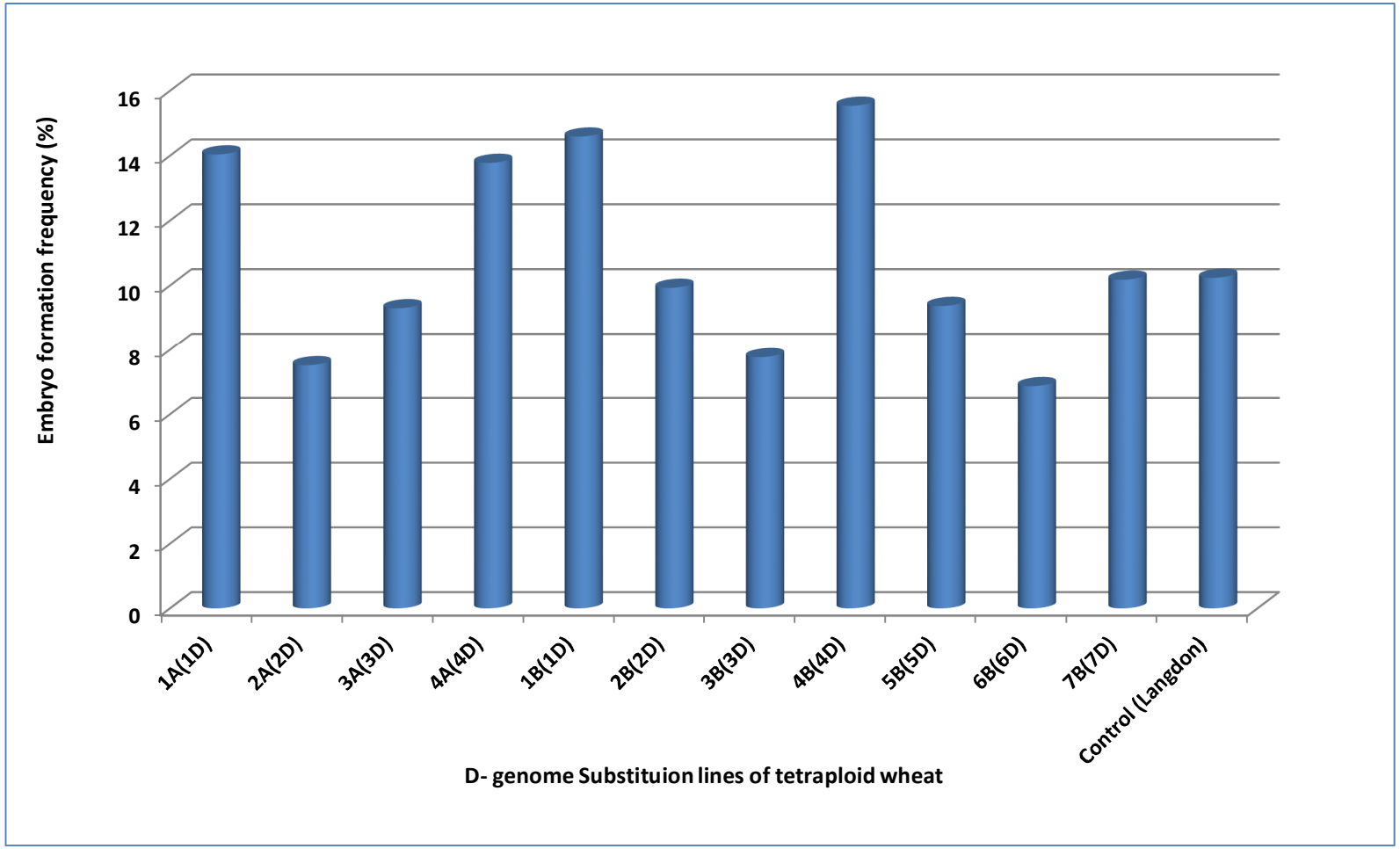


Fig. 4.6 **Graphical representation of embryo formation frequency of D-genome substitution lines of tetraploid wheat after pollination with *Imperata cylindrica* using 0.03 percent 2,4-D**

4.4 Morphological characterization of aneuploids

The 20 paired and one unpaired chromosome in monosomic plants of wheat are divided unequally during meiosis hence giving rise to two types of gametes with chromosome numbers ónô and ón - 1ô, which upon selfing result in mixture of disomics, monosomics and nullisomics in the progeny. In order to undertake monosomic analysis for identification of genetic control of any character, the aneuploids need to be screened in every generation. Cytological identification of monosomics and nullisomics is the most commonly used method which is not only time consuming but also requires good expertise for preparation of excellent metaphase spreads. In order to identify a visual marker for easy and quick identification of monosomic, nullisomic and disomic plants, morphological characterization of monosomic series of hexaploid wheat was carried out during the present investigation. The results obtained with respect to the morphological characterization of 24 characters depicted in Table 4.10 are mentioned hereunder:

4.4.1. Growth habit

All the plants of complete set of monosomic series (monosomic, nullisomic or disomic) exhibited spreading type of growth and the differentiation of aneuploids from normal plants was not possible.

4.4.2 Pigmentation

No specific patterns of pigmentation on any plant part was observed during all the developmental stages of monosomic series of hexaploid wheat.

4.4.3 Neck hairiness

All the three groups *viz.,* monosomics, nullisomics and disomics were devoid of neck hairiness. Hence, the trait was not a suitable morphological marker for identification of aneuploid plants.

4.4.4 Presence of awns

All the plants of whole monosomic series were devoid of awns.

4.4.5 Spike shape

Most of the monosomic, nullisomic and disomic plants had clavate spike shape however, the 5A monosomic and nullisomic plants had fusiform spike shape (Plate 17). This morphological character hence acted as a descriptor for identification of 5A monosomic or nullisomic plants.

4.4.6 Density of spike

The spikes of all the plants belonging to monosomic series were more or less uniformly lax and hence, it was not possible to differentiate the aneuploids or normal plants on the basis of spike density.

4.4.7 Waxiness

The monosomic, nullisomic and disomic plants were observed for leaf, culm or spike waxiness during all growth stages but, no waxiness was observed in any case.

4.4.8 Seed shape

All the plants had ovate seed shape in general.

4.4.9 Seed colour

The visual observation for seed colour of different aneuploids and disomic plants was difficult hence, the seed colour was identified using 5% sodium hydroxide test which revealed that the monosomics, nullisomics and disomics have red seed colour (Plate 18).

4.4.10 Seed hardiness

The aneuploids and disomics had soft seeds and the trait was unable to make a distinction between the three classes of plants.

4.4.11 Seed size

Although, the seeds of both aneuploids and disomic class did not belong to any one class however, in general monosomics and disomics were having large to medium seed size with most being in medium category. Similarly, the nullisomic plants of entire series were mostly having small and shriveled seeds (Plate 19).

4.4.12 Plant height

The height of plants from base to the tip of the spike in different monosomic lines ranged from 71.40 to 94.10 cm with the minimum height in 5B monosomic and maximum height in 3A monosomic. The average plant height of disomic plants was 92.37 cm whereas, the nullisomic plants had shorter stature with the plant height ranging from 48.80 to 83.40 cm.

Table 4.10 Morphological characterization of different aneuploids of bread wheat for phenotypic differentiation from disomics

Line	Type*	Growth Habit	Pigmentation	Neck Hairiness	Awns	Spike shape	Spike Density	Waxiness	Seed shape	Seed colour	Seed hardiness	Seed size	Plant height (cm)
1A	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	83.10
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	69.66
2A	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	87.80
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	75.40
3A	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	94.10
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	83.40
4A	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	94.00
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	71.80
5A	M	Spreading	Absent	Absent	Absent	Fusiform	Lax	Absent	Elliptical	Red	Soft	Medium	91.00
	N	Spreading	Absent	Absent	Absent	Fusiform	Lax	Absent	Ovate	Red	Soft	Small	70.10
6A	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	88.40
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	65.60
7A	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	85.80
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	59.00
1B	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	81.00
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	57.00
2B	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	74.80
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	55.70
3B	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	79.40
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	62.80

Contd…./-

4B	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	84.20
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	57.30
5B	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	71.80
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	48.80
6B	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	90.60
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	73.70
7B	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	80.10
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	57.50
1D	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	85.60
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	65.20
2D	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	87.20
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	65.30
3D	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	79.50
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	60.50
4D	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	83.40
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	69.60
5D	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	90.40
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	69.80
6D	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	79.80
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	66.30
7D	M	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	89.30
	N	Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Small	58.40
Disomic		Spreading	Absent	Absent	Absent	Clavate	Lax	Absent	Ovate	Red	Soft	Medium	92.37

Contd…./-

Line	Type*	Spike length (cm)	Number of tillers	Florets/ spike	Seed/ spike	Days to flowering	Flag leaf	Spike colour	Auricle Colour	Ligule Colour	Seed Hairyness	Leaf Colour	100 Seed Weight
1A	M	5.90	3-4	65-75	47-58	155	Drooping	Light Green	Off White	Off White	Hairy	Green	2.43
	N	4.34	1-2	55-70	18-21	158	Drooping	Light Green	Off White	Off White	Hairy	Green	1.87
2A	M	5.46	3-4	63-70	65-68	151	Drooping	Light Green	Off White	Off White	Hairy	Green	3.9
	N	3.76	1-3	50-54	38-45	155	Drooping	Light Green	Off White	Off White	Hairy	Green	1.52
3A	M	7.52	3-5	75-82	57-65	150	Drooping	Light Green	Off White	Off White	Hairy	Green	2.69
	N	5.76	1-2	45-53	39-43	156	Drooping	Light Green	Off White	Off White	Hairy	Green	1.13
4A	M	7.67	3-4	72-80	62-67	151	Drooping	Light Green	Off White	Off White	Hairy	Green	3.23
	N	5.77	2-3	50-64	41-45	153	Drooping	Light Green	Off White	Off White	Hairy	Green	1.75
5A	M	9.80	4-5	90-95	43-44	149	Drooping	Light Green	Off White	Off White	Hairy	Green	3.46
	N	7.34	2-3	65-72	30-36	155	Drooping	Light Green	Off White	Off White	Hairy	Green	1.78
6A	M	7.86	3-5	75-85	56-58	175	Drooping	Light Green	Off White	Off White	Hairy	Green	4.04
	N	6.90	2-3	50-70	38-45	181	Drooping	Light Green	Off White	Off White	Hairy	Green	2.09
7A	M	7.52	2-4	76-82	61-65	172	Drooping	Light Green	Off White	Off White	Hairy	Green	3.52
	N	7.00	2-3	60-70	40-55	180	Drooping	Light Green	Off White	Off White	Hairy	Green	1.54

Contd…/-

1B	M	7.60	3-5	80-85	54-59	150	Drooping	Light Green	Off White	Off White	Hairy	Green	3.73
	N	7.10	2-3	55-65	20-22	157	Drooping	Light Green	Off White	Off White	Hairy	Green	1.58
2B	M	6.06	2-4	65-75	59-63	183	Drooping	Light Green	Off White	Off White	Hairy	Green	3.74
	N	4.40	2-3	50-65	22-25	186	Drooping	Light Green	Off White	Off White	Hairy	Green	1.55
3B	M	8.66	3-6	78-85	66-71	161	Drooping	Light Green	Off White	Off White	Hairy	Green	3.24
	N	6.70	2-4	60-75	55-63	164	Drooping	Light Green	Off White	Off White	Hairy	Green	1.42
4B	M	8.24	4-6	90-95	56-61	155	Drooping	Light Green	Off White	Off White	Hairy	Green	2.74
	N	7.00	3-4	68-75	38-42	159	Drooping	Light Green	Off White	Off White	Hairy	Green	1.27
5B	M	6.82	4-5	70-78	67-68	155	Drooping	Light Green	Off White	Off White	Hairy	Green	2.81
	N	5.60	2-3	62-65	46-53	160	Drooping	Light Green	Off White	Off White	Hairy	Green	1.19
6B	M	8.52	2-5	80-85	63-65	156	Drooping	Light Green	Off White	Off White	Hairy	Green	3.22
	N	6.36	2-3	58-65	37-42	162	Drooping	Light Green	Off White	Off White	Hairy	Green	1.65
7B	M	7.26	3-5	65-80	51-58	153	Drooping	Light Green	Off White	Off White	Hairy	Green	2.80
	N	6.40	1-2	50-55	47-49	159	Drooping	Light Green	Off White	Off White	Hairy	Green	1.26
1D	M	7.50	3-5	70-85	40-42	150	Drooping	Light Green	Off White	Off White	Hairy	Green	3.26
	N	6.30	1-3	45-55	26-32	154	Drooping	Light Green	Off White	Off White	Hairy	Green	1.59

Contd…/-

2D	M	7.40	5-7	70-83	44-49	150	Drooping	Light Green	Off White	Off White	Hairy	Green	3.39
	N	5.96	1-3	60-67	28-31	155	Drooping	Light Green	Off White	Off White	Hairy	Green	1.46
3D	M	7.30	3-6	70-84	57-63	151	Drooping	Light Green	Off White	Off White	Hairy	Green	3.49
	N	6.60	2-3	55-70	37-41	157	Drooping	Light Green	Off White	Off White	Hairy	Green	1.51
4D	M	7.44	4-7	65-76	56-58	160	Drooping	Light Green	Off White	Off White	Hairy	Green	3.33
	N	6.60	1-3	60-70	34-41	164	Drooping	Light Green	Off White	Off White	Hairy	Green	1.57
5D	M	7.56	3-5	75-80	54-68	162	Drooping	Light Green	Off White	Off White	Hairy	Green	2.80
	N	6.60	2-3	65-68	42-47	171	Drooping	Light Green	Off White	Off White	Hairy	Green	1.08
6D	M	7.26	3-5	60-75	62-66	150	Drooping	Light Green	Off White	Off White	Hairy	Green	3.55
	N	7.24	1-3	65-70	35-48	156	Drooping	Light Green	Off White	Off White	Hairy	Green	1.79
7D	M	7.24	2-6	72-75	58-64	150	Drooping	Light Green	Off White	Off White	Hairy	Green	2.79
	N	5.44	1-4	45-54	46-51	153	Drooping	Light Green	Off White	Off White	Hairy	Green	1.17
Disomic		7.33	3-8	75-86	55-67	143	Drooping	Light Green	Off White	Off White	Hairy	Green	3.48

*M= Monosomic; N= Nullisomic

4.4.13 Spike length

The average spike length in different monosomic lines ranged from 5.46 to 9.80 cm. However, most of the monosomics did not deviate much from the disomic plants which exhibited spike length of 7.33 cm. The 5A monosomic plants were having maximum spike length. On the other hand, the nullisomics had spikes with relatively reduced size ranging from 3.76 to 7.34 cm.

4.4.14 Number of tillers

Ample amount of variability existed for this character within and among various monosomic lines. However, in general the number of tillers in disomic, monosomic and nullisomic plants ranged from 3 to 8, 3 to 7 and 1 to 4, respectively. Most of the disomic plants exhibited higher number of tillers whereas, only few monosomic plants had tillers as high as 7. The number of tillers in nullisomics was lesser than monosomic and disomic plants.

4.4.15 Florets per spike

The monosomic lines varied for florets per spike from 60-75 to 90-95 whereas, the disomic plants had 75-86 florets per spike. On the contrary, the nullisomics had reduced number of florets per spike ranging from 45-53 to 68-75. The monosomic line 4B exhibited maximum number of florets per spike (90-95).

4.4.16 Seeds per spike

The range of seeds per spike in monosomic plants ranged from 42-44 in 1D monosomic to 67-68 in 5B monosomic. The normal disomic plants had 55-67seeds per spike. Conversely, the nullisomic lines exhibited reduced seed set with 1A and 3B nullisomic exhibiting seed set of 18-21 and per spike 55-63 per spike, respectively. Nullisomic lines 1A, 1B and 2B had poor seed set due to sterility as also reported by Singh (2003).

4.4.17 Days to flowering

The initiation of flowering was earliest among disomic plants at 146 days and the flowering initiation in monosomic plants was delayed by two to three days. The monosomic plants varied for days to flower initiation from 149 to 183 days. While in

nullisomic plants, flowering was further delayed and the duration of flower initiation varied from 153 to 186 days. Most of the monosomics and nullisomics initiated flowering in 150 to 155 days except for 2B monosomic and nullisomic plants which flowered in 183 and 186 days after sowing, respectively.

4.4.18 Flag leaf

All the three categories of plants exhibited drooping type of flag leaf and the trait was unable to distinguish aneuploids and the disomic plants (Plate 20).

4.4.19 Spike colour

There was no variation present among the plants for this trait and both the disomic as well as aneuploid plants were having uniformly light green spikes.

4.4.20 Auricle colour

No differences were identified for this trait. The auricles were off white in colour in both disomic and aneuploid plants (Plate 21).

4.4.21 Ligule colour

Ligule colour was also not able to distinguish between the three categories of plants due to uniformity of ligule colour (off white) among all the plants (Plate 21).

4.4.22 Seed hairiness

The hairiness of seed was a predominant trait of all the plants. None of the monosomic or nullisomic line revealed absence of hairs on the seed.

4.4.23 Leaf colour

The leaf colour of all the plants was uniformly green barring from the minor variation that arises during different growth stages. Hence, it was not able to differentiate the monosomic or nullisomic plants from the disomics.

4.4.24 100 seed weight

The weight of 100 seeds of monosomic series varied form 2.43 g to 4.04 g with the disomic seeds too falling within this range with a 100 seed weight of 3.48 g. The nullisomic plants however, exhibited less seed weight corresponding to 1.08 g to 2.09 g per 100 seeds.

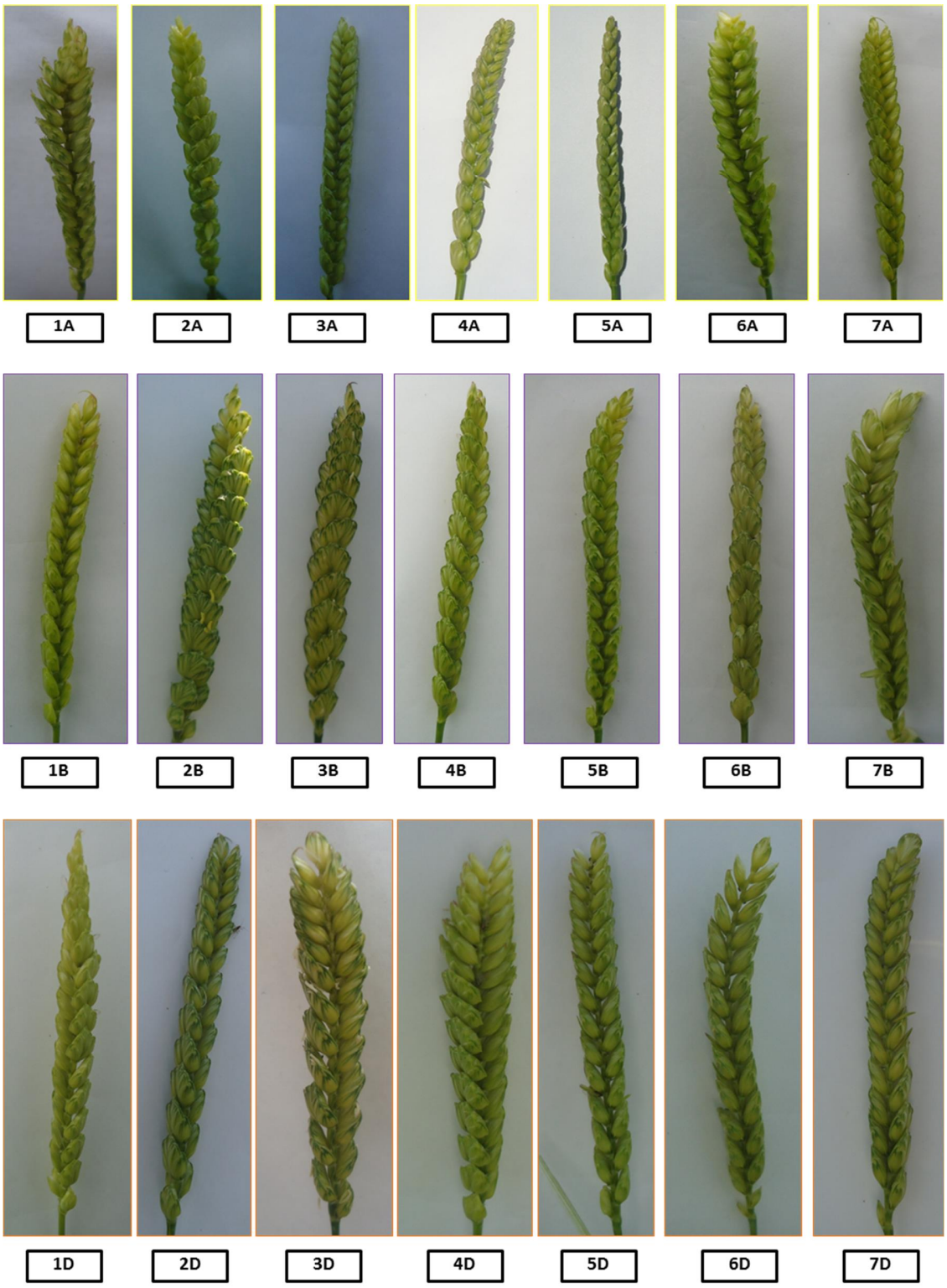


Plate 17 Spikes of monosomic series derived from 'Chinese Spring' cultivar of bread wheat

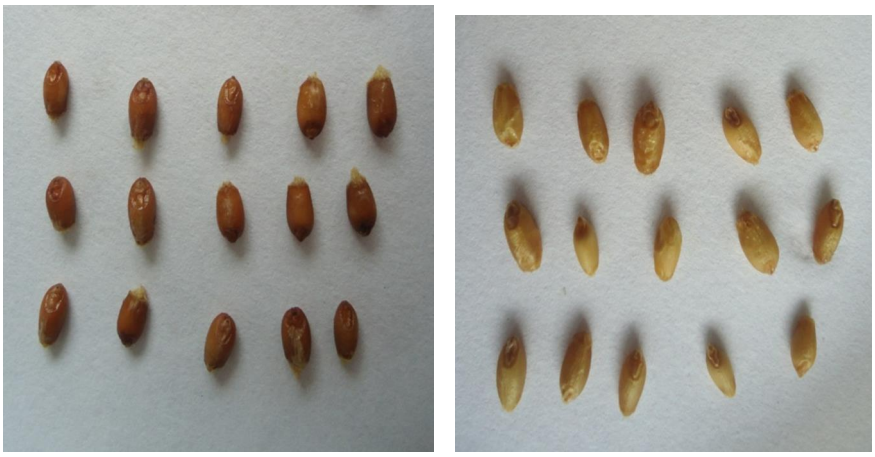


Plate 18 Seed colour observed (red seeds of all monosomic lines on left hand side and white seeds of local wheat cultivar on right hand side) after dipping the seeds in 5% NaOH solution for 60-90 minutes

Plate 19 Shape and size of the seed harvested from monosomic, nullisomic and disomic lines

Plate 20 Flag leaf of monosomic, nullisomic and disomic plants exhibiting drooping character

Plate 21 Off white auricle and ligule of monosomic series derived from 'Chinese Spring' cultivar of hexaploid wheat

From these results, it is clear that the loss of only one chromosome however had not much of influence on the morphology of monosomic plants and hence, they were not clearly distinguishable from the disomics except for two characters *viz.,* spike shape and days to flowering. The spike shape was clear morphological descriptor for identification of 5A monosomic as well as nullisomic plants whereas, the days to flowering was recognized as a morphological descriptor for identification of 2B aneuploids. Moreover, the morphological characterization of aneuploids revealed that the nullisomics could be easily differentiated from their monosomic or disomic counterparts on the basis of reduced plant height and vigour. Eight out of 24 characters *viz.,* seed size, plant height, spike length, number of tillers, florets per spike, seeds per spike, days to flowering and 100 seed weight were able to distinguish the nullisomics from monosomic and disomic plants.

5. SUMMARY AND CONCLUSIONS

The present research investigation entitled as ñTagging of the specific genome and chromosome(s) of hexaploid wheat triggering chromosome elimination in wheat x *Imperata cylindrica* system of doubled haploidy breedingò was designed with the objective to identify the genome (A, B or D) of the hexaploid wheat (AABBDD) responsible for the elimination of *I. cylindrica* chromosomes in the wheat x *I. cylindrica* system and locate the specific chromosome(s) of the identified wheat genome triggering the elimination process.

The materials used for identification of specific genome(s) of allohexaploid wheat that promote the uniparental chromosome elimination in wheat x *I. cylindrica* hybrids comprised of two diploid and one tetraploid species of wheat *viz., T. monococcum* (AA), *T. tauschii* (DD) and *T. durum* (AABB), respectively. These species were extensively hybridized with *I. cylindrica* with the intention of sighting the potential of each genome for induction of haploid embryos. The induction of haploids in all the three species was undertaken as per the standard protocol given by Chaudhary et al. 2005 during *rabi* 2010-11 and 2011-12.

T. monococcum failed to induce haploid embryos whereas, varied frequency of haploid embryos was recovered from *T. tauschii* and *T. durum.* The performance of *T. tauschii* and *T. durum* in respect of haploid production was low in comparison to the hexaploid wheat hence, manipulation in the standard protocol of *I. cylindrica*- mediated haploid induction at 2,4-D application stage was carried out during *rabi* 2010- 11 to investigate the possibility of enhancing their haploid production. Five concentration of 2,4-D from 0.01 to 0.05 percent were applied to pollinated spikes for three consecutive days after pollination at 24 hours interval. The optimum dose of 2,4-D observed for highest percentage of haploid embryo recovery in *T. tauschii* and *T. durum* was 0.04 and 0.03 percent, respectively. In the following year that is, *rabi* 2011-12, the three species were again crossed with *I. cylindrica* and the pollinated spikes were supplemented with optimum dose of 2,4-D.

Upon hybridization of *T. monococcum* with *I. cylindrica,* pseudoseed formation took place however, haploid embryos were not recovered. The cross-sectional study of fertilized ovules also revealed that the pseudoseeds produced were devoid of embryos. This suggests that the genome AA does not play any role in induction of haploid embryos following *I. cylindrica*- mediated chromosome elimination approach.

As far as *T. tauschii* is concerned, small pseudoseeds and embryos were recovered upon pollination with *I. cylindrica* pollen. However, the 9 to 11 days old embryos failed to regenerate on artificial medium. The development of embryos inside the pseudoseeds of *T. tauschii* was also confirmed *via* histological study of the fertilized ovaries.

The wide hybridization between *T. durum* and *I. cylindrica* resulted in formation of pseudoseeds more or less comparable with those derived from *T. aestivum* x *I. cylindrica* hybridization. The 13 to 15 days old haploid embryos were excised and transferred to artificial medium for their regeneration into complete green plantlets. The haploid nature of the *T. durum* x *I. cylindrica*- derived embryos was confirmed cytologically. The cross-sections of fertilized ovaries of *T. durum* also confirmed development of embryos upon pollination with *I. cylindrica* pollen. These results indicated that two genomes of hexaploid wheat *viz.,* B and D play an important role in the triggering the elimination of *I. cylindrica* chromosomes. Furthermore, it was observed that the embryo formation frequency of *T. tauschii* was higher than *T. durum* which suggests that the role of D genome of hexaploid wheat in haploid induction was higher than that of B genome. These results were in concordance with Inagaki et al. (1997), Inagaki and Hash (1998) and Mujeeb-Kazi et al. (2006).

For the sake of identification of specific chromosome(s) of hexaploid wheat controlling the uniparental chromosome elimination, different cytogenetic stocks *viz.,* complete set of monosomic and nullisomic series of hexaploid wheat and D- genome substitution lines of tetraploid wheat were used. The haploid embryo formation frequency in monosomic lines x *I. cylindrica* crosses exhibited significant differences when compared with both the control and the mean. The embryo formation frequency of all the monosomic lines except 5B monosomic was significantly reduced as compared to the normal disomic plants. Four chromosomes whose absence revealed significant reduction in the embryo formation frequency of the monosomic lines from mean were 6B, 1D, 4D and 6D. The relative contribution of the four chromosomes was estimated using Z test of

significance and it was found out that the loss of 4D chromosome resulted in significantly higher reduction of haploid production followed by 1D whereas, the effect of 6B and 6D was at par with each other but lesser than that of 4D and 1D chromosomes.

The haploid embryos of monosomic lines were rescued and successfully regenerated on artificial medium. The cytological investigation of regenerated plantlets revealed that both the normal and nulli-haploids were recovered. The development of embryos was also observed during cross-sectional study of the fertilized ovaries.

The hybridization of nullisomics and *I. cylindrica* was cumbersome due to reduced vigour and sterility of nullisomic plants. The embryo formation frequency was significantly reduced in nullisomics, especially in certain lines that exhibited greater degree of male and female sterility. From the nullisomic analysis, chromosome 4D was identified as a key player in the reduction of haploid embryo formation frequency. The haploid embryos were regenerated to develop into complete plantlets having slow growth and reduced vigour. The haploid nature and development of embryos in nullisomic lines was confirmed through cytological and histological investigations.

Among D- genome substitution lines, haploid embryo frequency of four lines *viz.,* 1A(1D), 4A(4D), 1B(1D) and 4B(4D) was significantly higher than the control which suggested that haploid embryo formation was enhanced by the substitution of 1 D chromosome in place of 1A or 1B and of 4D for 4A or 4B. These results signify the role of 1D and 4D chromosomes in elimination of *I. cylindrica* chromosomes. Moreover, the substitution line 6B(6D) exhibited significant reduction in the embryo formation frequency in comparison to control and mean which implies that the substitution of 6D chromosome in place of 6B has detrimental effect on haploid induction. The haploid embryos recovered from hybrids of substitution lines x *I. cylindrica* were regenerated into green plantlets. The cytology of roots excised from regenerated plants confirmed the haploid nature of the plantlets.

In all, the extensive hybridization of cytogenetic stocks of tetraploid and hexaploid wheat revealed that four chromosomes *viz.,* 4D, 1D, 6B and 6D in the similar order trigger the elimination of paternal chromosomes in wheat x *I. cylindrica* hybrids. These results were somewhat in correspondence with Inagaki et al. (1997) especially with respect to role of chromosome 4D.

The morphological characterization of monosomic, nullisomic and disomic plants was carried out on the basis of 24 morphological traits in order to identify visual markers for easy and quick differentiation of aneuploids from the disomic plants. The nullisomic plants having a missing pair of chromosome exhibited a general reduction in vigour and hence were easily distinguishable the monosomics or disomics on the basis of eight out of 24 characters *viz*., seed size, plant height, spike length, number of tillers, florets per spike, seeds per spike, days to flowering and 100 seed weight. On the other hand, the loss of only one chromosome did not made much impact on plant morphology hence, their differentiation from disomics was not possible except in monosomic 5A and 2B. The 5A monosomic had fusiform spike whereas, 2B monosomic had delayed flowering as compared to disomic plants.

Conclusions

The results obtained during the whole investigation are concluded as:

- The manipulation in the concentration of 2,4-D solution affects the pseudoseed and haploid embryo formation in *T. tauschii* and *T. durum,* however, no significant effect was observed on haploid induction parameters in *T. monococcum*.
- The optimum dose of 2,4-D to be injected into the pollinated spikes for efficient haploid induction was 0.04 percent in *T. tauschii* and 0.03 percent in *T. durum.*
- Paternal chromosome elimination in wheat x *I. cylindrica* hybrids is the outcome of B and D genome of hexaploid wheat. The relative effect of D genome is higher than that of B genome of hexaploid wheat.
- The specific chromosomes 4D, 1D, 6B and 6D trigger the elimination of *I. cylindrica* chromosomes through their individual as well as interactive effects.
- The identified chromosomes triggering chromosome elimination process showed different effects on haploid induction frequency. The relative contribution of 4D chromosome being the highest followed by 1D whereas, the effect of 6B and 6D was at par with each other but lesser than that of 4D and 1D.

- The morphological characterization of aneuploids revealed that the nullisomics could be easily differentiated from their monosomic or disomic counterparts on the basis of their reduced vigour whereas, the monsomics were almost similar for various morphological traits with disomics except 5A monosomic (different for spike shape) and 2B monosomic (different for days to flowering). Eight out of 24 characters *viz.,* seed size, plant height, spike length, number of tillers, florets per spike, seeds per spike, days to flowering and 100 seed weight were able to distinguish the nullisomics from monsomic and disomic plants.

Implications

- Alteration and manipulation in the chromosomes 4D, 1D, 6B and 6D triggering the chromosome elimination of *I. cylindrica* chromosomes can be used for:
    - Successful introgression of the desirable traits of *I. cylindrica* into the genetic background of wheat
    - Increasing the haploid induction efficiency of the doubled haploidy breeding system.
- Durum wheat improvement programmes can be hastened through *I. cylindrica*-mediated chromosome elimination approach of doubled haploidy breeding using 0.03 percent 2,4-D solution for injection into the spikes.

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Brief Biodata of the Student

Name	**:**	Vineeta Kaila
Father's Name	**:**	Dr. Om Prakash Kaila
Mother's Name	**:**	Mrs. Raj Kaila
Date of Birth	**:**	18th March, 1986
Permanent Address	**:**	Ward No. 4, Ghumarwin, Distt. Bilaspur, H.P.

Academic Qualifications:

Qualification	Year	School/ College	Board/ University	Marks (%)	Division	Major Subject
10th	2001	D.A.V. Public School, Palampur	CBSE	62.00	1st	English, Maths, Science, Social Science and Hindi
12th	2003	D.A.V. Public School, Palampur	CBSE	71.40	1st	English, Physics, Biology, Chemistry, Phy. Edu.
B.Sc.(Ag)	2007	CSK HPKV, Palampur	CSK HPKV	79.70	1st	Agriculture & allied sciences
M.Sc.(Ag)	2009	CSK HPKV, Palampur	CSK HPKV	81.20	1st	Plant Breeding & Genetics
Ph.D (Ag)	2013	CSK HPKV, Palampur	CSK HPKV	82.00	1st	Plant Breeding & Genetics

Thesis tittle in M.Sc. : Studies on genetic diversity among different species of *Trifolium* using morphological traits and molecular markers

Fellowships/Scholarship/Gold medal/Awards/Any other Distinction:

1.	2007	Received University **Gold Medal** for securing highest CGPA during B Sc Agriculture programme
2.	2008	Honoured by **CAPAA** for being **Gold Medalist** of B. Sc. Agriculture Programme
3.	2004- 2011	University merit scholarship holder during B.Sc, M.Sc. and Ph.D
4.	2011- 2013	**DST INSPIRE Fellow** for Doctoral programme from 2011 to 2013
5.	2011 & 2012	Qualified **National Eligibility Test-** 2011 & 2012 for Professorship organized by Agricultural Scientists Recruitment Board recognized by UGC/ CSIR
6.	2009	Received **Best Poster Award** in National seminar on ódesigning crops for changing climateôheld during Oct, 2009 at Birsa Agricultural University, Ranchi, India

Publications:

Research Papers: 6

Scientific Popular Articles: Presented in National Symposia: 11 & book chapter: 1

Visits Abroad: China- For participating and presenting paper in the 4th Asian Chromosome Colloquium, 2010 held at Beijing, China.