AN ABSTRACT OF THESIS OF

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Title: Production of Haploid Plants in Selected Winter Wheat Genotypes through Anther Culture and Intergeneric Crosses with Maize.

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A higher level of efficiency in the production of doubled-haploids must be achieved if this procedure is to be beneficial in plant improvement. Of equal importance is the development of protocols, which are not genotypic specific and result in progeny that represent a random sample of gametes from the parental lines.

To address these issues seven diverse winter wheat genotypes and a spring wheat, previously shown to be responsive in haploid plant production from anther culture, represented the experimental material. Two methods of generating haploid plants were employed. These were anther culture and the intergeneric hybridization of wheat with maize. Three induction media (MN6, P2 and Liang's) in liquid and semi-solid forms were employed with anther culture a direct culture. A modified floret culture along with different concentrations (20 and 100 ppm) and mode of application of 2,4-D were examined for the wheat x maize approach, Measurements included callus and embryo production and haploid plantlet regeneration.

Two winter genotypes 'Gene' and 'Yamhill' did not respond to the anther culture methodology. For responsive genotypes MN6 was superior to P2 and Liang's medium for the traits measured. In contrast 'Chris' the spring wheat was more responsive for plantlet

regeneration on P2 medium. No differences in haploid plantlet regeneration for the traits were observed between liquid and semi-solid medium forms.

For the wheat x maize approach, direct culture was superior to the modified floret culture for embryo and haploid plantlet production. No differences were found in either 2,4-D concentration or whether 2,4-D was applied through tiller injection or placed as droplets on the florets.

The wheat x maize approach was superior to anther culture for both embryo and green haploid plant production based on the number of florets used. Albino plants were produced in all responsive genotypes with anther culture. In contrast, no albino plants were regenerated with the wheat x maize approach. All genotypes were responsive for both embryo production and haploid plant regeneration in the intercrossing approach. In addition no regeneration distortion was observed for specific population employing this protocol.

The wheat x maize approach was superior to anther culture for embryo production. This was also true for green haploid production based on the number of florets used. A higher proportion of albino plants was also observed when anther culture was employed. A further attribute of the wheat x maize approach was that all genotypes tested were responsive to embryo and haploid production. It was also revealed that no segregation distortion was present in three doubled haploid populations developed from wheat x maize crosses.

PRODUCTION OF HAPLOID PLANTS IN SELECTED WINTER WHEAT GENOTYPES THROUGH ANTHER CULTURE AND INTERGENERIC CROSSES WITH MAIZE

by

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Maria Graciela Mendoza Agurto. Author

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IN DEDICATION TO:

MY BELOVED MOTHER
GRACIELA AGURTO DE MENDOZA

THE MEMORY OF MY BELOVED FATHER
GERVASIO M. MENDOZA

An example of faith, love, and sacrifice and a constant inspiration in my life.

DEDICADA A:

MI QUERIDA MADRE:
GRACIELA AGURTO DE MENDOZA

LA MEMORIA DE MI AMADO PADRE
GERVASIO M. MENDOZA

Un ejemplo de fe, amor y sacrificio y una inspiracion constante en mi vida.

PRODUCTION OF HAPLOID PLANTS IN SELECTED WINTER WHEAT GENOTYPES THROUGH ANTHER CULTURE AND INTERGENERIC CROSSES WITH MAIZE

1. INTRODUCTION

Haploids are sporophytic plants that contain the gametic chromosome number. When they arise from diploid species they contain a single genome and are described as monoploids in contrast to haploids derived from polyploid species, containing two or more genomes and are called polyhaploids. Following chromosome doubling, haploid plants become doubled-haploids (DHs).

The doubled-haploid methodology offers several advantages to plant improvement programs. Perhaps the most important is the rapid approach to homozygosity. Since haploid plants carry only one set of alleles at each locus, upon doubling, homozygous and homogeneous lines are available. This allows for i) evaluation of qualitative and quantitative traits, ii) avoids the masking of recessive genes, iii) evaluate possible environment x genotype interactions, and iv) identify superior parental combinations. Other benefits are detecting genetic linkages and determine recombination values (Snape, 1988) and for molecular genome identification obtain reproducible DNA polymorphism.

Skepticism of the application of doubled-haploids in breeding programs has been expressed by some scientists. The main issue is that haploidization process involves only one cycle of meiotic recombination. Many traits of agronomic importance, such as yield, are under polygenic genetic control. In such cases, one cycle of recombination is insufficient if linkages are present, thus not all the potential genetic variation derived from a cross will be available

(Hu, 1986). To overcome this potential disadvantage, different strategies have been proposed. These include the delay of the haploidization process until F2 or F3 generation and/or practice selection prior to haploid production (Hu, 1996). Zhang et al. (1984) developed a method composed of one cycle of haploidization followed by sexual hybridization between different doubled-haploid genotypes followed by another cycle of haploidization of the selected hybrids. By using this method, some polygenic traits have been successfully improved. An example is the release of the cold-resistant rice cultivar 'Huan Han Zao' (Hu, 1996).

Though the theoretical and practical usefulness of haploid plants has been recognized since 1922 when Blakeslee found spontaneous haploid plants in *Datura stramonium,* their exploitation remained restricted for many years because of the very low frequency with which they occur in nature. With the discovery of the colchicine to double chromosome number and more recent developments in biotechnology, has facilitated the making doubled haploid plants has become widely employed.

Several techniques have been developed in cereal crops for the induction of haploid plants. These can be classified in two systems, 1) the gametophyte system which includes anther, microspore and unpollinated ovary-ovule culture *in vitro,* and 2) the intergeneric crossing system which capitalizes on the phenomenon of chromosome elimination followed by *in vitro* embryo rescue. The anther, as well as microspore culture methods, are potentially the most efficient methods for induction of haploid plants as thousands of microspores are available per anther or floret compared to only one ovule (haploid) expected per floret with intergeneric crossing methodologies.

In wheat (*Triticum aestivum* L.), the induction of haploid plants from microspores through anther culture is at present the method utilized in most laboratories around the world. Since the initial production of anther derived haploid plants in 1973 (Ouyang et al, 1973, De Buyser and Henry, 1980), continuous efforts have been made to study the methodological and

theoretical aspects of this process. Results from these investigations have established that the androgenetic process is under control of a complex genetic system involving both additive and non-additive nuclear genes as well as cytoplasmic genetic factors (Agache et al., 1988; Lazar et al, 1984b; Deaton et al, 1987; Ouyang et al., 1983). Expression of these genes is strongly influenced by environmental factors such as anther donor plant environment (Bjornstad et al., 1989; Jones and Petolino, 1987; Simmons, 1989), pollen developmental stage (He and Ouyang, 1984), culture incubation temperature (Ouyang et al., 1986; Huang, 1987; Simmons, 1989), medium formulation (Chu and Hill, 1988; Marburger et al., 1987; McGregor and McHughen, 1990, Ouyang, 1986). Although adjusting cultural factors has contributed to making substantial improvements in the response of wheat anthers, some obstacles which restrict the application of this technique in breeding programs still remain. One major limitation is that the response is strongly genotype dependent (Han-Min et al., 1990; Jones and Petolino, 1987; Lazar et al., 1984; Ouyang, 1983). Another important constraint is the occurrence of large numbers of albino plants. Approximately 20-90% albino plants may be produced from anther cultures of wheat (Andersen et al., 1987; Zhou and Konzak, 1991). Although various studies have been carried out to investigate the causes of albinism in cereal crops, no definite conclusion about the mechanisms responsible for this phenomenon has been drawn (Day and Ellis, 1984; Huang, 1987; Marsolais et al., 1983; Wang et al., 1973; Zhou, 1996; Ziegler et al., 1990).

The intergeneric crossing system emerged as an alternative approach for the production of haploid in wheat when the 'bulbosum technique' was successfully applied to barley (Barclay, 1975). Later, a high frequency of haploid plants was obtained through the chromosome elimination process through crosses of wheat cultivar ''Chinese Spring'' used as female with pollen from tetraploid *Hordeum bulbosum*. Subsequent studies have shown that crossability of wheat with tetraploid *H. bulbosum* is controlled by two dominant genes Kr1 and Kr2 which reduce the ability of the pollen tube to penetrate the ovary wall and the micropyle at the base of

stigma preventing double fertilization (Snape et al., 1979). Most American and European wheat genotypes carry these genes making them non-crossable with *H. bulbosum*, or show poor crossability, thus the 'bulbosum technique' was restricted to a few wheat genotypes (Sitch and Snape, 1987a).

A more recent technique, which also capitalizes on the process of chromosome elimination after intergeneric crosses of wheat with maize (*Zea mays* L.), was found to be insensitive to the action of dominant alleles Kr1 and Kr2 (Laurie and Bennett, 1987). Wheat haploid plants have been recovered from wheat x maize crosses by spikelet culture (Laurie and Bennett 1988b), treating spikes with 2,4-D (Laurie and Reymondie, 1991) injecting 2,4-D into the upper internode (Suenaga and Nakajima, 1989), and using detached tiller culture (Riera-Lizarazu and Kazzi, 1990). The use of this technique has attracted the interest of plant breeders as a valuable alternative approach to the production of wheat haploid plants. One of the reasons is that all wheat genotypes appear responsive of producing haploids. Also the production of albino plants among haploid regenerants is not a constraint in contrast to anther culture.

Even though the deployment of the doubled-haploid method offers several benefits to breeding programs, breeders are cautious when incorporating these techniques, unless adequate levels of success are assured in order to balance inputs invested in these technologies to be useful in plant improvement programs. Snape et al., (1986) proposed three criteria to be fulfilled by any doubled-haploid system. These are i) easy, consistent production of large numbers of doubled-haploid of all genotypes in the breeding program, ii) doubled-haploid should be genetically normal and stable, and iii) the doubled-haploid population should contain a random sample of parental gametes.

In this context, the present study addressed the desire to obtain information regarding the fulfillment of important criteria.

The specific objectives of this thesis research were: i) to evaluate the production efficiencies of wheat haploid plants obtained through anther culture and wheat x maize crosses employing diverse genotypes of wheat, ii) to test the anther culture response of these genotypes in three induction media and investigate if a genotype x media interaction is present iii) to explore different protocols for the wheat x maize method, and iv) to assess the occurrence of random distribution of parental gametes.

2. LITERATURE REVIEW

Two main methodologies are employed used for the production of doubled-haploid plants (DHs) in hexaploid wheat. These include the androgenetic route, via anther and microspore culture, and intergeneric crossing which creates haploids through chromosome elimination by hybridizing wheat with other species of the Gramineae.

Anther culture is become the most widely used technique in the production of haploids in wheat. Consistent progress has been made in the improvement of this technique in the last three decades. However, constraints such as a genotype specificity and high rate of albino production have limited the efficiency in the production of doubled-haploid plants.

Crosses of wheat with other species of the Gramineae such as *Hordeum bulbosum* L. (Inagaki., 1986; Sitch and Snape, 1986a), *Zea mays* (Laurie and Bennett, 1988; Suenaga and Nakajima, 1989; Inagaki et al., 1990), *Zea mays* spp. mexicana (Ushiyama et al., 1991), *Pennicetum americanum* (Ahmad and Comeau, 1990; Matzk and Mahn, 1994), have succeeded in producing wheat haploid plants. Among these intergeneric crosses methodologies, the wheat x maize crosses approach is becoming the most popular.

2.1. ANTHER CULTURE

Since the successful development of the first haploid embryos from microspores of *Datura innoxia* in 1964 by Guha and Maheshwari, anther culture has proven to be one of the most popular methods for obtaining haploid plants in different crops. Fuji, (1970) reported the first work on wheat anther culture, in which six species were tested. Callus was obtained from *Triticum aegilopoides*, *T. dicoccoides* but anthers of *T. aestivum* did not respond (Hu., 1996).

The first wheat anther-derived plants were obtained in the early 1970s by three different research groups, Ouyang et al., (1973), Chu et al., (1973), and De Buyser et al. (1987).

Anther culture has become routine in several laboratories worldwide. Particular achievements have been made in countries like People's Republic of China (PRC) where prior to 1991 21 cultivars were developed and released using anther culture. The amount of land planted to these cultivars has reached more than million hectares. Current wheat breeding laboratories are combining anther culture with conventional breeding procedures as a routine method for wheat improvement (Hu, 1996). Other active programs where this methodology has being extensively investigated include Canada (Marsolais et al., 1983; Lazar et al., 1984b; Simmons, 1989; McGregor and McHughen, 1990; Chu et al., 1990) and USA (Andersen et al., 1987; Liang et al., 1987; Baenziger et al., 1991; Zhou and Konzak, 1991; Navarro-Alvarez et al., 1994).

The anther culture technique involves the growing in vitro of immature anthers in a synthetic medium in order to induce embryo/callus formation from the microspore pollen grain (Ouyang, 1986). This step is followed by the regeneration of haploid plantlets and subsequent development of doubled-haploid plants (DHs) by chromosome doubling with colchicine. Based on the number of male gametes participating in the androgenetic process, anther culture has a great potential because more haploids are expected per spike than when haploidization is achieved via female gametophyte (Jensen, 1986). However, when measured at microspore level, even in responsive genotypes, the process has to be considered rare. Comparing two different genotypes for their culturability, Henry et al. (1984) showed that percentages of multicellular pollen grains at 17 days of culture were low even for embryogenic lines. In fact, 10 or 100 embryos produced by 100 anthers are considered rare event, since their frequencies are equal to 0.00005 or 0.0005, respectively, if one assumes that there are 2000 microspores per anther. The authors emphasized the potential of androgenesis in wheat by showing that the numbers of embryos per spike would be 250 if all the embryos observed at 17 days had grown. The induction of wheat haploids via anther culture is determined by three independent components: 1)

induction of callus or embryos, 2) regeneration of plantlets, and 3) production of green plantlets. The efficiency of this technique is measured by the frequency of these three components (De Buyser and Henry, 1986).

2.1.1. **Genetic Factors**

Investigations reported in the literature have indicated variation in performance of genotypes regarding their ability to produce callus and/or embryos and plantlet regenerants (Marsolais et al., 1983; Ouyang, 1983; Lazar et al., 1990; Simmons, 1989; Orlov et al., 1993). The anther culture response in most studies has shown to be more dependent upon genotype than on culture conditions (Lazar et al., 1990; Jones and Petolino, 1987a; Han-Min et al., 1990).

Crosses made on responsive and non-responsive wheat genotypes to anther culture, including reciprocals, have revealed that the androgenetic response of the F_1 hybrids was intermediate between the two parental lines (Kurdika et al., 1986). This suggests that the trait is heritable and it is mainly transmitted by nuclear genes (Bullock et al., 1982; Henry and De Buyser, 1985; Agache et al., 1988). Lazar et al., 1984b) used diallel analysis with five spring wheat cultivars including reciprocal crosses to estimate genetic parameters involved in the embryo or callus and plantlet induction rates. They found that variation is mainly due to genetic factors with preponderance of additive effects, though dominance effects (specific combining ability) and cytoplasmic effects were present. Picard and De Buyser et al., (1977) have also reported dominance effects in studies from crosses between doubled-haploid lines when used as parents. From the studies of Lazar et al. (1984b), estimates of narrow sense heritability values for both individual cultivars and families gave high values ($h = 0.6$-0.7) despite some interaction with environmental factors.

Other investigators have observed the polygenic nature of the androgenetic process. Picard et al. (1990), using F1-F2 regression analysis and coefficient of regression as a direct

estimate of narrow sense heritability, found b=0.77 (P<0.01) for embryo production. Deaton et al. (1987) using three spring-wheats and their F1; F2, and backcrosses found that mainly additive effects influence callus induction. Working with 49 genotypes of wheat Moieni and Sarrafi (1994) found high narrow-sense heritability values for embryo/callus and plant regeneration response. A study conducted by Agache et al. (1988) using F1's derived from substitution lines in spring wheat, observed transgressive segregants for embryo/callus and plantlet production indicating that two or more genes were involved in the genetic control of these traits. Dominance effects have been also reported by Deaton et al. (1987) and cytoplasmic effects by Zhou and Konzak (1991), although nuclear genes were found to be predominant in these studies. Genetic studies also demonstrated that the response for callus or embryo induction and plant regeneration are under independent control (Deaton et al., 1987; Agache et al., 1988).

Chromosome substitution lines studies in the cultivar 'Chinese Spring' have located genes affecting haploid production. Factors associated with the increase of embryo production have been located in both arms of chromosomes 3A, 5A, and 7D; whereas those associated with the decrease of embryo/callus production were located on 1B, 2A ,2D, 5B (De Buyser et al., 1992). Genes on chromosome 3A have been reported to increases the total (green + albino) plant regeneration response. In contrast genetic factors on 3B, 3D, and 6D resulted in a decrease in such a response (Szakacs et al., 1988). Henry and De Buyser (1985) observed that genes responsible for green plantlet regeneration response of wheat embryos were located on the 1RS chromosome arm present in several translocated 1BL/1RS cultivars. This finding corroborated similar results using the cultivar Salmon (Jensen, 1986). Other studies have reported green plant regeneration increases associated with chromosomes 1BS, 2D, 5A, 5B (De Buyser, 1992; Szakack, 1988). It has been found that chromosome arm 1BL possess at least one gene increasing the frequency of albino plants (De Buyser et al., 1992).

2.1.2. **Cultural Factors**

Although it has been well documented that genetic factors play a predominant role in the androgenetic process of wheat, environmental factors have been also observed to interact with the response of the genotype of the donor plant. Often these interactions have been found to have a significant effect in the production of embryo/callus and/or regeneration of green plantlets and consequently in the production of haploid plantlets.

Temperature: Variation in temperature before or during anther culture has been reported to influence callus or embryo production and regeneration frequencies. Results from different studies suggest that: 1) small variations in temperature (2-3 °C) can lead to drastic variations in embryo/callus production (Ouyang, 1986). 2) exposure of anthers to high temperatures (29°C - 32°C) increases callus or embryo production (Huang, 1987; Ouyang et al., 1983; Li et al., 1988). 3) cold pre-treatments of anther donor plants are sometimes unnecessary (Marsolais et al., 1983; Sesek et al., 1994), and 4) variation and intensity of cold treatments lead to significant differences in callus production (Lazar et al., 1985a).

Light: The influence of light on the androgenetic response of wheat is still unclear. Ouyang et al. (1983) found no significant response to light during the callus induction process. Bjornstad et al. (1989) working with two cultivars of spring wheat, found that high light intensity during the induction phase strongly suppressed the callus development in both genotypes, but stimulated plantlet regeneration in the less non-responsive genotype. Weak diffuse light did not inhibit callus induction. From a study by Ziegler et al. (1990) using four cultivars of spring wheat, it was found that regeneration of green plantlets was improved by incubation of anthers in the dark; whereas incubation under light yielded more than 90% albino plants.

Age of Donor Plants: Age of the anther and maturity of pollen at the time of the culturing is the most critical factor in the induction of callus or embryos and consequently in the production of haploid plants. Success depends upon the accuracy in selecting spikes containing the appropriate stage of the microspores. Although it has been observed that haploid plantlets

can be obtained from microspores at various developmental stages from early meiosis to late binucleate stage, the most productive anthers are those that contain uninucleate microspore just before mitosis, (Chu, 1978; He and Ouyang, 1984). They divided the microspore stage into early-uninucleate (including early stage I and early stage II, mid uninucleate, late uninucleate and pre-mitosis stage. The response of anther culture the variation of pollen stage was sensitive, and the production of callus decreased sharply if anthers at stages other than mid or late uninucleate stage were used (Hu, 1996).

Induction Medium: The culture medium for the induction of callus and /or embryos from the anthers is one of the most significant factors in the success of the androgenetic system. The basic culture medium for cereal crops generally contains inorganic salts and organic substances, mainly from the group B vitamins. However, other constituents have been determined to be of particular importance.

Concentration of **nitrogen** of both ammonium and nitrate forms are critical in the induction media. Studies performed by Feng and Ouyang (1989) in wheat anther culture revealed that $NO3^-$ in the induction medium affected callus induction and regeneration independent of $NH4^+$, but their effects were additive. They also observed that $KNO3^-$ concentration influenced green plant regeneration; the higher the $KNO3^-$ level used, the higher the green plant regeneration, and that a medium without $NO3^-$ or $NH4^+$ gave poor regeneration response.

Only two plant **hormones,** 2,4-Dichlorophenoxiacetic Acid (2,4-D), an auxin, and Kinetin, a cytokinin, are routinely added to the anther culture induction medium of wheat, though other auxins such as Naphtalenacetic Acid (NAA) or Indolacetic Acid (IAA) are occasionally used. Authors agree that the suitable concentration of 2,4-D and Kinetin are 1-3 mg/l and 0.5 mg/l, respectively (Ouyang, 1986). Studies carried out by Chinese research groups have revealed that concentrations of 2,4-D lower than 1mg/l led to low pollen callus yield, and concentrations higher than 3mg/l not only decreased the yield, but reduced the quality of the pollen callus (Ouyang et al., 1973; Research Group 301, 1977a). These research groups have also investigated

the role of the Kinetin in the andogenetic process. They found that this hormone did not have a prominent effect in the induction of callus, but had a primary effect in the inhibition of callus formation from somatic cells of the cut end of anther filament.

Also **sugars**, are critical components in wheat anther culture. They have two roles, as a carbon source and as an osmotic regulator. High concentrations of sugar (12%) were found to be beneficial for embryo/callus induction (Dunwell, 1985). Sucrose is the most common form of sugar used for any tissue culture media as well as for those designed for callus or embryo production of cereals. Recent studies have indicated that other sugars can give substantial increases in embryo/callus production in wheat anther culture. Studies performed by Last and Brettel, (1990) Orsinky et al., (1990) and Zhou et al., (1991), showed that maltose was superior to sucrose for both callus induction and green plant regeneration for a range of genotypes. The use of maltose also allowed green plantlets to be recovered from genotypes that failed to produce plants when anthers were cultured in a medium with sucrose. In an experiment carried out by Chu et al., (1990) monosaccharides, especially 0.21 M glucose in liquid medium resulted in embryo production frequencies 2-10 times higher that in the medium with 0.21 M sucrose. This response was genotype dependent. Organic growth compounds, such as **Myoinositol** and **Glutamine**, have proven beneficial to wheat anther culture (Chu et al., 1991).

Several induction media have been formulated for cereals. The initial experiments in wheat anther culture involved the use of **MS** (Murashigue and Skoog, 1962) and Miller media. Subsequently, other media such as **C1**, **C17** and **WM2S**, have been utilized. However, the main breakthrough in the increase of the callus/embryo production frequencies was achieved when two kinds of media, both characterized by their lower levels of N, were identified. The first group of media, based on the use of potato extract are: *P1* (Anonymous, 1976) and *P2* (Chuang et al., 1978). Anthers cultured in *P1* have yielded callus induction rates as high as 33.1% (Lazar et al., 1990), 40.7% (Chuang et al., 1978) and 43.2% (Anonymous, 1976a). In *P2*, production frequencies of callus as high as 51.4% and 77.3% depending on the genotypes (Sesek et al.,

1994). The second group are the synthetic media N6 (Chu et al., 1975). Chu et al., (1990) obtained high embryo induction values ranging from 40 to 750 embryos per 100 anthers and plantlet regeneration frequencies of 40 to 90 plantlets per 100 embryos using modified N6 (*MN6*) medium supplemented with glucose (0.21M), for culturing anthers of 13 genotypes of winter wheat and two spring wheat.

Solidifying agent: Agar products derived from seaweeds known as *Gracilaria* or *Gelidium*, have traditionally been the most common gelling agents. It is both, cheap and readily available. However it has been observed to have negative effects on callus formation likely due to the release of impurities from the agar into the tissue culture medium (Wernicke and Kohlenbach, 1976). The effects of various agars in wheat anther culture (Bacto Agar, Difco, Type A, Type E, Gum Agar, Sigma; Purified Agar, Difco; Phytagar, Gibco) were tested by Simonson and Baenziger (1992). Embryo production was significantly lower in Phytagar, while plant production was significantly higher in Bacto Agar and Type E Agar. Experiments conducted utilizing liquid medium without agar appeared to circumvent the problem of inhibitory substances released by the agar resulting in a positive effect on the initiation of callus (Jones and Petolino, 1987; Zhou and Konzak, 1989). Liquid medium can have drawbacks since embryos or calli often sink into the liquid resulting in fewer regenerants due to detrimental anaerobic condition (Chen, 1986; Zhou and Konzak, 1989). **Ficoll**, a non-ionic, synthetic polymer of sucrose, which increases surface density when added to the liquid cultures, has been found to significantly benefit the anthers, response in wheat anther cultures compared to liquid medium alone (Calleberg and Johansson, 1996). Jones and Petolino (1987) found no difference in embryo production from cultured anthers of soft winter wheat on liquid medium supplemented with Ficoll compared with a normal liquid medium. More embryos produced on Ficoll-containing medium were capable of plantlet regeneration compared to those produced in liquid medium alone. According to the work of Zhou and Konzak (1989), more than 85 % of the calli produced in anther culture resulted in plant regeneration on Ficoll supplemented medium compared to only

39 to 57 % in medium lacking Ficoll. Because of the high cost of Ficoll and problems related to the floating cultures, less expensive alternatives have been tested. Culturing anthers of cultivar Pavon, Simonson and Baenziger (1992), considered whether inexpensive starches could replace Ficoll. They found significantly greater embryoid–initiation frequencies on P1 media with maize (0.67) and wheat starch (1.50) than with Ficoll (0.47). Similar frequencies (0.51, 0.50 and 0.52 respectively) were observed on 85D12 medium.

Regeneration Medium: Although plantlets occasionally regenerate directly in induction medium, usually embryos must be transferred to a medium with lower concentrations of hormones and sugar to induce differentiation. The choice of regeneration medium is not as strict as the choice of induction medium for wheat anther culture (Ouyang, 1986). Regeneration rate of plantlets from pollen callus are determined by 1) culture conditions in the process of pollen callus formation, 2) callus age, and 3) genotype of the anther-donor plants (Ouyang et al., 1983). The most important difference between the requirement of pollen callus induction and that of plantlet regeneration is in the auxin and sucrose level, both must be lower as compared to the induction medium (Ouyang, 1986). The sucrose concentration usually used in regeneration medium is 3%, and concentrations above 3% will significantly reduce the regeneration rate (Zhuang and Xu, 1983). According to Ouyang et al., (1986) the most suitable combinations of auxin/cytokinin for regeneration medium might be IAA/kinetin 0.2-2/0.2-2 mg l^{-1} or NAA/Kinetin 0.5/0.5 mgl^{-1}

As with the induction media, different laboratories use different regeneration media. Modified **MS**, **Miller**, **N6**; and **C17** are popular, and similar. MS medium is the most often used. The media **190-2**, developed specifically for wheat anther culture by Zhuang and Xu (1983), seems to be more effective (Andersen et al., 1987; Ziegler et al., 1990; Pauk et al., 1991).

2.1.3. **Main constraints related to anther culture in wheat**

Albinism is a major problem related to the process of androgenesis in cereals. Albino plants are those which are chloroplast-deficient. These plants remain in the vegetative state unable to produce female or male gametes even under heterotrophic conditions (Chen, 1986). The mechanism of albinism in plant regeneration is complex. Both genetic as well as physiological factors, including internal and external environments of the explants, have been reported to influence the development of albino plants. In studies of crosses involving parents known to produce albino plants, Bullock et al. (1982) observed a high proportion of albinos in the F2 suggesting that this trait is under genetic control. Investigations performed by De Buyser et al. (1992), using aneuploid stocks of "Chinese Spring" wheat lines, revealed that the 1BL chromosome arm was associated with albino plant frequencies. Wang et al. (1973) found chromosomal abnormalities associated with an increase of albino plant frequencies.

Cultural factors are also responsible for the presence of albino plants. The temperature during the initial stage of the in vitro microspore division has been reported to influence the proportion of regenerant plants that are albinos (Marsolais et al., 1983; Huang, 1987). Experiments designed to test the effect of different light conditions on callus induction and plant regeneration revealed a significant decrease in albino plantlets when incubation of anthers took place under dark conditions, (Bjornstad et al., 1989; Ziegler et al., 1990). Other reports have noted an increase of the medium osmolalities of the liquid regeneration medium using Ficoll, higher maltose concentrations or hydrolysis of sucrose to glucose and fructose, significantly decreased the frequencies of albino regenerants (Orsinky et al., 1990; Han-Min et al., 1990; Zhou et al., 1991).

2.2. WHEAT x MAIZE CROSSES

Overcoming barriers to interspecific hybridization has given rise to a discipline generally referred to as 'wide-crossing'. Among other important uses, the 'wide-crossing' technique has proven to be of great value to cereal geneticists and tissue culture researchers as an alternative to anther culture in the production of DH plants.

Barclay (1975), was the first to report that wheat DHs could be produced from an intergeneric crossing between the cultivated hexaploid wheat *Triticum aestivum* L. (2n=6x=42) and a wild diploid perennial barley *Hordeum bulbosum* L. (2n=2x=14). In this procedure, known as the 'bulbosum technique', the female gamete of wheat is fertilized by the *H. bulbosum* pollen. Zygote and embryo induction is high, however the chromosomes of *H. bulbosum* are eliminated leaving the wheat genome in the embryos. The embryos resulting will not survive due to absence of endosperm. If they are rescued and cultured in vitro, plantlets from these monoploid embryos (n=3x=21 ABD) may regenerate. Chromosome doubling either naturally or with colchicine, will give fertile flowers bearing homozygous offsprings (2n=42=42 AABBDD) (Jensen, 1977).

Subsequently, extensive work has been conducted using this technique in the production of wheat DHs. Studies have revealed that most of the American and European wheat genotypes show little or no crossability with *H. bulbosum*. This is due to the presence of incompatible dominant genes Kr_1 and Kr_2 in wheat germplasm (Falk and Kasha, 1981; Suenaga et al, 1991). Chromosome substitution studies have shown that materials carrying alleles Kr_1 and Kr_2 are located in the long arms of chromosomes 5B and 5A, respectively. These lines showed reduced crossability with many alien species, including rye and *H. bulbosum* with Kr_1 having greatest effect (Sitch and Snape, 1986a). These Kr genes act by inhibiting alien pollen tube growth through the ovary wall and the micropile at the base of the stigma, thus preventing double fertilization (Snape et al., 1980).

Using crosses involving wheat (*T. aestivum*) var. 'Chinese Spring' and maize (*Zea mays*) var. ''Seneca 60'' , Laurie and Bennett (1987, 1988) found that pollen tubes reached the embryo sac in about 80% of the wheat florets. The embryo and occasionally an endosperm or both are developed in 20-30% of the florets. These observations indicated that action of the dominant Kr alleles for crossability did not operate in wheat x maize crosses. This was later illustrated by Laurie (1989a) when using crosses between the wheat var. ''Highbury''' carrying dominant alleles for crossability Kr1 Kr2 with the maize var. ''Seneca 60'' and with *H. bulbosum*. He reported mean fertilization frequencies of 50% and 0.5% with the respective genera. Cytological analysis of wheat x maize zygotes at metaphase revealed the presence of 21 wheat and 10 maize chromosomes as expected. Since maize chromosomes have poorly defined centromeres, their failure to attach to the spindle tubules, result in being lost during the first division cycles (Laurie and Bennett, 1989). Thus embryos with cells containing an haploid complement of wheat chromosomes (n=3x=21 ABD) are produced from which polyhaploid homozygous plants (2n=6x=42 AABBDD) can be derived by chromosome doubling (Laurie and Bennett, 1988; Suenaga and Nakajima, 1989). Since in wheat x maize crosses action of dominant alleles Kr1 and Kr2 is not a problem, wheat researchers have became interested in this approach as an alternative to the 'bulbosum technique' for haploid production.

The production of DH plants through chromosome elimination via crosses of wheat x maize involves three main phases: 1) intergeneric hybrydization 2) embryo formation and rescue, and 3) plant regeneration. The efficiency of this methodology is measured in terms of frequencies of each of these components. Embryo formation is the component of principal interest since it represents the frequency of potential plants. Most studies directed to improving the efficiency of this method have been focused on maximizing the frequency of embryo induction and the frequency of embryos that can be recovered as plants. The main

factors which have been found to be related to the production of haploid plants by the wheat x maize crosses can be integrated in two main groups: genetic and cultural factors.

2.2.1. **Genetic Factors**

Few studies have been conducted concerning the consequence of different wheat and maize parental materials in the production of DHs.

Laurie and Reymondie (1991), working with 19 winter and spring cultivars of hexaploid wheat found no significant variation among genotypes in the frequency of embryo production, although spring wheat cultivars showed a slightly higher rate of 47.8% embryo production compared to 39.5% obtained for winter wheat. Similarly, Giura (1994), from results obtained from crosses over a three years period using 49 randomly selected F_2 and F_3 populations of winter wheat, found only slight genotypic differences in 1991 and 1993 and no differences in 1992 (Personal communication). However, Suenaga et al. (1991), working with 47 wheat cultivars and lines from different countries, reported a wide variation in the frequency of embryo production (0.9 – 35 %) among genotypes. All the cultivars investigated showed crossability and produced embryos after they were crossed with the maize hybrid B14 / CI64. To date all wheat genotypes tested have shown an ability to produce haploids and consequently DH plants using the wheat x maize protocol.

Suenaga and Nakajima (1989) reported significant variation in embryo formation (18.8 to 31.9 %) using five cultivars of maize as pollen donors. Similar results were reported later by Suenaga et al. (1991) using 52 maize cultivars and inbred lines and three F1 hybrids. They noted considerable variation in the frequency of embryo induction (1.6 to 36.0 %). Likewise, Matzk and Mahn (1994) from an experiment with 17 diploid and three tetraploid maize genotypes observed frequencies of embryo formation varying from 17 to 36%. Like

wheat genotypes, all maize genotypes have shown crossability with all the wheat materials used and embryos have been successfully developed to provide DH plants.

2.2.2. **Cultural Factors**

Crossing procedures: Laurie (1989b) using crosses of 'Highbury'' x 'Seneca 60', found two factors, which significantly affected the frequency of fertilization. These were a lower fertilization frequency when the wheat glumes were cut, and also the age of the florets. Besides, pollinations made one day before or after anthesis gave the highest embryo production frequency.

Hormone treatment: The effect of exogenous auxins on embryo development has been demostrated by Matzk and Mahn, 1994. From studies performed to analyze the effect of auxins on the induction of embryos originating from wheat x maize crosses, 2, 4-dichlorophenoxyacetic acid (2;4-D) has been reported as a determining factor in the induction of embryos (Laurie and Bennett, 1988; Suenaga and Nakajima, 1989; Inagaki and Tahir, 1992). Concentrations of 10 ppm (Kisana, 1993; Laurie and Reymondie, 1991), 20 ppm (Giura, 1994) or 100ppm (Suenaga and Nakajima, 1989; Riera-Lizarazu and Kazzi, 1990) injected in small amounts (about 5 ml.) in the upermost internode and/or on the florets of wheat plants 1-2 days after pollination, have been found to improve embryo induction frequencies.

Regeneration Media: Different laboratories have used different media for the regeneration of wheat haploid plantlets from developed embryos. The most commonly reported are, 1) MS half strength (Murashigue and Skoog, 1962), 2) Gamborg's B5 (Gamborg et al., 1968), 3) Modified B5 (Jensen, 1977, and 4) Difco Orchid media. No specific studies have been reported to compare the effect of different media on the regeneration of wheat haploid plants.

2.3. ANTHER CULTURE vs. WHEAT x MAIZE CROSSES

As has been pointed out by Baenziger et al. (1991), despite the progress made on the theoretical and methodological aspects of haploidization in wheat, there is still lack of information concerning critical parameters required for an efficient utilization of DH systems in plant breeding. In the comparison of methodologies for haploid production in their efficacy to a breeding program, three important criteria are suggested by Snape et al. (1986), these include, 1) easy, consistent production of large numbers of doubled-haploids from all genotypes in the breeding program, 2) doubled-haploids should be genetically normal and stable, and 3) such populations should contain a random sample of parental gametes.

2.3.1. Criterion 1: '*Efficient production of DHs from all genotypes*'

In anther culture, due to the lack of response from some genotypes, the first condition, 'easy, consistent production of large numbers of doubled-haploid from all genotypes in the breeding program' has not been achieved. A number of studies have demonstrated that anther culture is a genotype dependent trait with some genotypes showing null response to haploid induction through this system (Ouyang, 1983; Lazar, 1984; Andersen et al., 1987; Sesek et al., 1988; Simmons, 1989; Orlov et al., 1993). As a consequence, some desirable germplasm can not be used for the induction of haploid plants. Fewer investigations have been conducted employing the wheat x maize approach when compared to the abundant information accumulated for anther culture. Currently, studies with wheat x maize crosses have revealed variation in embryo production between hexaploid and tetraploid wheat cultivars (Amrani et al., 1993), winter and spring genotypes (Laurie and Reymondie, 1991). From those studies, no differences were detected between genotypes within those categories. However, Suenaga et al., (1991) reported considerable differences in embryo production among 47 wheat cultivars from different

countries. Although the effect of wheat genotypes in the production of haploid plants by wheat x maize crosses has not been well established, all wheat genotypes tested to date, have shown ability to produce embryos and consequently haploid and doubled-haploid plants using this methodology.

Anther culture and wheat x maize crosses were compared for the production of wheat doubled-haploid plants by Kisana et al. (1993). They used five crosses to compare both methodologies simultaneously under same growing conditions. From data obtained from anther culture, differences were detected among the crosses for callus formation and plantlet regeneration involving anther culture. On average 5.8% of the anthers produced callus, 2.8 % of the callus regenerated plantlets, and the production efficiency obtained was 0.38 green plantlets per 100 cultured anthers. No significant differences were found in embryo formation and plant production among the five wheat-maize crosses. Using wheat x maize crosses, on the average 10.3% of the florets pollinated produced embryos, 46.5% of the embryos regenerated plants and the overall production was 4.8 plants per 100 florets pollinated.

2.3.2. Criterion 2: *'DH lines should be genetically stable'*

Haploidization systems offer to the breeding programs of self-pollinated crops the opportunity of obtaining homozygous or near homozygous lines from heterozygous individuals in a short time frame. The major advantage of this process is the time saved in developing a new cultivar. Additionally, doubled-haploid lines can provide unique and valuable material for genetic analysis since the lack of heterozygosity reduces the number of phenotypic classes in comparison to F_2 populations and consequently simplifies segregation studies. The simplification of genetic effects caused by the absence of dominance effects as well as the production of stable/homogeneous and replicable material makes DH lines prime material in quantitative genetic studies (Choo et al. 1985). Before exploiting these rapid advancement systems for quantitative genetics and linkage studies as well as for breeding, it is important to determine if the

genetic variation exhibited by the derived near or completely homozygous lines adequately and accurately reflects the allelic variation present in the parental material. The fidelity of genetic variation in the derived population will be first influenced by the occurrence of mutation during the developmental process, particularly gametoclonal variation with respect to anther culture, and secondly by the influence of gametic and other forms of 'unconscious' selection (Snape et al., 1988).

According to Evans et al., (1984) the term gametoclonal variation describes '*phenotypically variant plants regenerated from gametophytic cells*'. It has been established as early as 1974 that in vitro androgenesis generates this kind of variation (Picard et al.,1986; Evans et al., 1984; Baezinger et al.,1991). Gametoclonal variation can be of nuclear or cytoplasmic nature. In general, polyploid species such as wheat, triticale, oat, and potato exhibit greater levels of somaclonal and gametoclonal variation than diploid species (Huang, 1996). Polyploids have a greater genetic buffering capacity, which may allow greater tolerance to gametoclonal variation. In wheat, it has shown that two or more cycles of in vitro anther culture can permanently alter the wheat chromosomes. Results over several years indicated that about 90% of the DH lines were genetically uniform and about 10% have abnormal chromosome complements, with a range of changes in both chromosome number and structure (De Buyser and Henry, 1986; Hu, 1986)

Variation in chromosome number: Polyploidy is the most frequently observed chromosomal abnormality in anther/microspore cultures and regenerated plants. Aneuploidy is less prevalent probably because of the deleterious effects of genetic inbalance.

Regenerants with different levels of ploidy or polyploids (7x, 8x, 9x, 11x, 12x), mixoploids and aneuploids (nullisomics, monosomics, trisomics, and tetrasomics) have been obtained from anther culture of winter and spring types of intervarietal hybrids of common wheat (Shaeffer et al., 1979, Hu et al., 1986). Using the wheat cultivar 'Orofen', Hu (1983) reported that from cytological analysis of root of 472 anther derived d plants obtained by anther culture,

85% were euploids (haploid and diploids) and 9.3% were aneuploids (7x, 8x, 9x 11x and 12x), and mixoploids.

Variation in chromosome structure: From the same study conducted by Hu (1983) a doubled haploid plant (2n=42) with a pair of 1B long arm deletion was detected. The morphological characteristics of this plant expressed uniformly and seed set normally. Seeds from the second haploid generation were also obtained and it was determined that 1B deletion was stable. Later investigations conducted by the same author revealed the occurrence of dicentric chromosomes, translocations, deletions and telosomics

Biometrical Variation: Variation in agronomic traits especially of wheat DH lines derived from anther culture has been well documented. Field trials conducted by several research groups worldwide have shown a range of variation among doubled-haploid lines for quantitative traits such as yield, height, heading date, and quality (Picard et al., 1986). Baenziger et al. (1989) conducting multi-year, multi-location field trials of doubled haploid lines (DHLs) and single-seed descent derived lines (SSDLs) resulting from the cultivars 'Chris' and Kitt, found greater variation among DHLs than SSDLs. In the same study, 70 DHLs averaged significantly lower grain yield than the 50 SSDLs. In 'Chris' the DHLs yielded less, but were not significantly lower for grain yield than the SSDLs. In both cultivars, the genetic component of variance for yield of the DHLs was significantly larger than of the SSDLs, indicating the presence of gametoclonal variation.

Differential response was found by Mitchell et al. (1992) over two years at two locations when employing 40 F1 anther culture-derived haploid (ACLs) lines, and 40 F4-derived random lines obtained by single seed descent (SSDLs) lines. In general, the mean of SSDLs was consistently higher than the mean of ACLs for plant height and kernel weight. The ACLs exhibited greater genetic variance for grain protein with a larger proportion of high protein lines than SSDLs. No differences between methods for harvest index and heading date were found.

For grain yield, the response varied with the year. The SSDLs had higher mean yield in the year affected by severe drought conditions.

Winzeler et al. (1987), reported superior performance in protein content and in the response to various diseases as 'powdery mildew' (*Erysiphe graminis var. tritici*), 'stripe rust' (*Puccinia striiformis*) and 'septoria nodorum blotch' (*Septoria nodorum*) in DH lines of spring wheat compared to lines produced by the pedigree system.

Two reports were found in the literature concerning an assessment of variability generated from wheat x maize crosses. Laurie and Snape (1990) assessed the agronomic performance of nine DH lines of ''Chinese Spring'' (CS), six DH lines of 'Hope,' and 14 DH lines of the single chromosome substitution lines of CS Hope 5A, and their parents. No significant variation was detected in either population of the 'Chinese Spring' DH lines and neither DH population differed from its parent. The Hope DH lines differed significantly for tiller biomas, spikelet number per ear, ear grain weight and 50 grain weight. However, differences were attributed to the poor performance of one of the six DH lines. The other five lines resembled the parent and no variation was detected when the aberrant line was excluded from the analysis. 'Chinese Spring' (Hope 5A) DH lines showed significant variation for time of ear emergency time, but this was probably due to genetic heterogeneity in the parental stock. In general, no definite conclusions can be drawn from this study and the authors pointed out that the size of the population was too small to determine if the variation observed accurately reflected the frequency of induced mutations.

More conclusive results were revealed from larger DH populations developed by Suenaga and Nakajima (1993) to evaluate six agronomic traits in second and third generations of doubled-haploid lines (DH2 and DH3, respectively). The DH populations were obtained from cultivar 'Fukuho' through wheat x maize crosses. Field data indicated that out of 110 DH lines, 15 revealed visible variations such as extreme dwarfism, low seed fertility, alteration in spike type, and these differences were transmitted to the DH3 lines. The range of DH3 were larger

with DH3 lines when compared to DH2 lines, except for spike number/plant. Analysis of variance showed the presence of heterogeneity and heterozigosity in the DH2 and DH3 plants, respectively. These results indicated the occurrence of gametoclonal variation and also that most of the variation detected was caused by a process following the chromosome doubling, which in the case of maize crosses is the colchicine treatment rather than to the 2,4-D treatment or to the *in vitro* culture.

A study on mitocondrial DNA (mtDNA) and chloroplast DNA (cpDNA) of anther derived DH lines from five and six generation of selfing of cultivar 'Moisson' was performed by Picard et al. (1986). Isolated DNA, using restriction patterns and different restriction enzymes followed by agarose gel electrophoresis revealed a stability in both organelle genomes. Absence of reciprocal effects found from a diallel study with the same material, corroborated these results. Similar findings were obtained by Rode et al. (1985). Previous studies performed by Picard et al. (1980b) showed that gliadins composition remain constant during different generations of multiplication.

2.3.3. Criterion 3: ***'The DH population should contain a random sample of the parental gametes'***

Absence of segregation distortion due to haploidization process has been considered to be the third condition for successful use of doubled-haploid lines in quantitative and linkage studies as well as in breeding programs (Snape et al., 1986). Segregation distortion can be defined as a "deviation from the expected Mendelian proportion of individuals in a given genotypic class within a segregating population' (Lyttle T.W., 1991). Segregation distortion has been documented in a wide range of organisms, including plants in which species or hybrids exhibit preferential dysfunction of gametes carrying one allelic class. This can occur in pollen, in megaspores or both (Xu et al., 1997). Distorted segregation can be

detected with almost any kind of genetic marker, including morphological mutants, isozyme, and DNA markers.

Consequences of segregation distortion are important in genetic analysis in particular in genetic mapping. One of the assumptions underlying MAPMAKER, the most commonly used software in genetic map construction (Lander et al., 1987), is the absence of distorted segregation. However, non random distribution of gametes has been already reported in DH lines in particular in those derived from androgenetic systems in cereals especially in barley (Powell et al., 1986; Graner et al., 1991; Heun et al., 1991), maize (Bentolila et al., 1992), rice (Xu et al., 1997), and in maize, rice grass, and wheat (Foisset and Delourme, 1996).

Few reports are available in the literature regarding the study of occurrence of gametophytic selection among doubled-haploid lines derived from anther culture (ACDH) in wheat. To investigate if gametophyte selection takes place in anther culture, De Buyser et al. (1992) conducted a study using monosomic lines for chromosome 1D of "Chinese Spring" (CSM1D). From the cultured anthers of CSM1D, haploid plants with 20 and 21 chromosomes were obtained at relative frequencies of 6.1% and 93.9 %, respectively, indicating a reduction of nullihaploids due to a strong selection for euhaploid gametes. Results of segregation for morphological makers in ACDH lines have been compared to the segregation data in other types of progenies, such as lines obtained by alternative methods of haploidization, F_2, back crosses (BC), or single-seed descent (SSD). Bjorsntad et al (1993) investigated if segregation distortion takes place in SSD and ACDH lines from six crosses of spring wheat. They found seven cases of segregation distortion in both populations. A selection pressure occurring during the *in vitro* process of anther culturability has been postulated as the cause of segregation distortion (Foisset and Delourme, 1996).

Segregation of parental gametes has been also investigated in DH populations derived from *intergeneric crosses* in cereal crops. In barley, a study was conducted by Powell et al., (1986) to assess if segregation distortion takes place among doubled-haploid lines derived from

anther culture (ACDH) and *H. bulbossum* (HbDH) crosses, for five genetic markers (raquilla hair type, DDT reaction, mildew reaction, plant height, and C hordein polymorfism). They reported significant departure from the expected 1:1 ratio for three of five genes analyzed in the ACDH lines. Whereas, the HbDH derived lines from the same F1 exhibited deviant segregation only for raquilla hair type marker. Using a different hybrid Powell et al. (1986) reported absence of segregation distortion for seven markers among DH lines obtained through the *H. bulbosum* technique. An absence of segregation distortion was confirmed by Schon et al., (1990) who demonstrated a mendelian segregation at 23 loci on HbDH lines.

In wheat, only one study was found in the literature concerning the occurrence of this phenomenon in DH lines derived from wheat x maize crosses. Suenaga and Nakajima (1993) analyzed F2 plants and 203 DH lines derived from the cross Fukuho-komuji x Oligo Culm for eight genetic markers including high molecular weight-glutenin, NaCl soluble proteins (bands 1, 3 and 4), grain esterase (band 5), GA-insensitivity, glume color and glume pubescence. From their results, no deviations from the expected segregation were observed among the DH lines except for glume pubescence.

3. MATERIALS AND METHODS

3.1. Study 1 ANTHER CULTURE

3.1.1. Plant material and procedures.

Seven cultivars and elite lines of winter wheat (*Triticum aestivum* L.) ('Stephens', 'Hoff', 'Gene','Yamhill',OR 3870039, OR 870025, and OR 889176), were used as anther donors. The spring cultivar ''Chris',' previously reported as being responsive to anther culture, was included as a control. A description of the experimental germplasm is provided in Appendix Table 1. Following 5-6 weeks of vernalization at 5±1 °C, twelve plants per donor parent were planted at three planting dates at two weeks intervals starting on January 10, 1995. Two plants of each genotype were grown in 1/2-gallon pots containing a soil mixture of pumice rock, river loam and peat moss (2:1:1) lime and 0.35 grs of fertilizer (13-13-13) per pot. The pots were randomly assigned to benches in the greenhouse. The material was illuminated with high pressure sodium lamps (1100 uE / mol^2 / s) using an 18 hr photoperiod and 20/15 ° C day/night temperature. Water was added to avoid any moisture stress.

3.1.2. Methodology

3.1.2.1. *Collection and cold pre-treatment*: The first four tillers of each plant were collected at the 'boot' or Feekes stage 10, when the top of the spike was midway between the penultimate and flag leaf. This stage coincides with the mid-late uninucleate stage of the microspores, the most adequate stage for anther culture. Tillers were given a two day, pre-cold treatment by putting them in a container with tap water and storing in a refrigerator at 4±1° C.

3.1.2.2. *Cytological examination*: After the cold pre-treatment and prior to culturing, the stage of microspores was checked under light microscopy. This was performed by removing one to two anthers from a primary or secondary floret and macerating. They were then stained with 4% acetocarmine (W/V) in 50% (v/v) acetic acid (Last and Bretell, 1990). When the majority of the pollen grains were judged to be at mid to late uninucleate stage the spike was selected.

3.1.2.3. *Surface Sterilization*: The spikes were then surface sterilized using a 20% v/v commercial bleach solution for 10 min and rinsed four times in sterile water.

3.1.2.4. *Embryo/callus Induction*: After surface sterilization apical and basal spikelets were removed from each spike as well as the central florets from the remaining florets. Three anthers from the outermost florets of each spikelet were dissected and cultured on 10x35 mm petri dishes containing 3 ml of one of the three induction medium. Anthers from one half of each spike were plated in liquid medium and the other half in a semi-solid medium. Semi-solid medium consisted of a mixture of 3 Gelrite : 1Agar, and replenished with fresh liquid medium every two weeks. Number of anthers dissected from each spike and cultured in each petri dish with either liquid or semi-solid medium was recorded as well as the number of florets and spikes used. The three induction media used for this study were :

Chu N6 medium modified according to Li et al. (1988) and known as MN6 medium, with filter sterilized vitamins, with 0.5 mg/l of 2,4-D, 0.5 mg/l of kinetin and 90g/l of sucrose

Potato 2 (P2) medium (Chuang et al., 1978) with 1.5 mg/l of 2,4-D, and 0.5 mg/l o kinetin.The potato extract was prepared using an unknown cultivar.

85 D12-*3* or *Liang's* medium (Liang et. al, 1987) with 1.5 mg/l of NAA, 1m/l of kinetin, and 75 g/l of sucrose.

Detailed components of these media are presented in Appendix Table 2.

The MN6 and P2 media were selected as being the best media noted in the literature and from a previous experiment performed in this laboratory (unpublished). *Liang's* medium was included in this experiment for its direct plant regeneration property. (Liang et al., 1987). Prior to

the addition of the gelling agent, the media were adjusted to pH 5.5 and pH 5.8 for liquid and solid, respectively. The gelling agent used for the three different media was a mixture of Gelrite and Agar (3:1 g/l). According to Kelko, 1982, Gelrite is a gellan gum produced by the bacterial strain S-60 of *Pseudomonas elodea*. Chemically it is a polysaccharide comprised of uronic acid, rhammose and glucose (Calleberg and Johansson, 1996). After anthers were cultured, the petri-dishes were sealed with strips of parafilm and incubated at constant temperature (29°C) under dark conditions. Following 30 days of culturing, anthers were checked periodically to detect formation of embryos or callus and to add fresh liquid media when necessary. Anthers showing embryos or callus were isolated in individual petri-dishes and recorded as responsive.

3.1.2.5. *Plantlet regeneration*: Induced callus of about 2mm diameter and/or embryos were transferred to 25x100 mm culture tubes containing *190-2* regeneration medium (Zhuang and Xu, 1983) with 0.5mg/l kinetin and 0.5 mg/l naphthalene acetic acid (NAA) 30g/l of glucose and 7 g/l of Agar (Appendix Table 2). Tubes with cultured embryos and /or callus were placed under dark conditions at 20 ± 1° C. After three weeks of culture, embryos and/or callus were examined periodically to observe if shoot regeneration occurred. When shoots originated from embryos, the number of shoots counted per anther were based on the number of embryos capable of producing shoots. When shoots originated from callus, the number of shoots counted per anther were based on the number of isolated calli showing shoots regardless of the number of developing shoots originating from each callus. Only callus attached to anthers were recorded in the liquid medium. If anthers produced more than one callus, they were isolated and recorded separately. Every callus was isolated by culturing in individual 10x35 mm petri dishes containing the same type of medium from which they were removed. When regenerated shoots reached about 2 cm in length, they were placed under continuous cool white fluorescent lamps (80 uEmol /m^2/ sec) at 20+1 ° C.

When winter wheat plantlets reached the top of the tubes (about 7 cm) they were transferred to a vernalization chamber for 6 weeks at 5±1 ° C using 8 hr photoperiod. After

vernalization winter plantlets as well as the non-vernalized spring plantlets were planted in 6 cm giffy pots containing vermiculite, covered with a float row cover, and then placed in the greenhouse under a misting device until they were well rooted. Chromosome counts were then made from root tips of a random sample of seedlings from each genotype following the protocol used by Haunold (1968).

3.1.3. **Experimental design and statistical analysis**:

The experiment was set up as 7x3x2 factorial in a split-split-plot design with a minimum of six replications with individual spikes considered as replications. The main plots were genotypes (factor A with eight levels), the induction media were the sub-plots (factor B with three levels) and the medium forms were the sub-sub-plots (factor C with two levels). Weighted Analysis of Variance (ANOVA) were performed using General Linear Models (GLM) tests in SAS (SAS Institute.1995) because of the different number of spikes and anthers per wheat genotype. Differences among genotype means for the same medium and between media mean values for the same genotype were compared using the Least Square Means (LSM) procedure (Steel and Torrie, 1980). Frequencies of embryo/calli production, haploid plant regeneration (total, green and albino), and haploid plant production (total, green and albino) obtained were expressed as follows:

Embryo-calli production= (n^o of embryos obtained / n^o of anthers cultured) x 100

Haploid plant regeneration = (n^o of plants regenerated/ n^o of embryo-calli cultured) x 100

Haploid plant production = (n^o of plants produced / n^o of anthers cultured) x 100

Frequencies were converted to Square Root + 0.5 (Steel and Torrie, 1980) to normalize the frequency distributions before statistical analysis.

3.2. Study 2 **WHEAT x MAIZE CROSSES**

Two experiments were performed using the wheat x maize approach.

3.2.1 Comparison of 'Modified Floret culture' vs 'Direct culture'

3.2.2 2,4-Dichlorophenoxyaceticacid (2,4-D) assays

3.2.1 *Comparison of 'Modified Floret Culture' vs 'Direct Culture'*

3.2.1.1. **Plant material and growing conditions**

The winter wheat genotypes used as female parents in the wheat x maize cross method were the same used as anther donors in the anther culture technique (Study 1). Wheat plants were grown under same growing conditions as described in Study I during the winter season of 1993-1994. The maize hybrid B14 / CI64, reported by previous studies as a good pollinator in the production of wheat haploid plants, was used as the pollen donor (Suenaga and Nakajima,1989). Four seeds of this genotype were sown every four days in 1-4 gallon pots containing the same soil mixture and fertilizer used for the wheat plants and supplemented with 20-40 gr. of osmote fertilizer (14-14-14). Pots were placed in a controlled growth chamber illuminated with cool white fluorescent (300-350 uEmol / m^2/ sec) using 18 hr photoperiod at 29/26 ° C day/nigh temperature regime and water added to maintain moisture.

3.2.1.2. **Methodology**:

3.2.1.2.1.*Crossing procedures*: Wheat spikes were emasculated two to four days before anthesis leaving the glumes intact and removing the apical basal and central florets. After

emasculation spikes were covered with the flag leaf and a polyethylene bag to avoid desiccation. Pollinations were made with pollen from hybrid maize B14/CI64 within 5-15 minutes of pollen release, and when most wheat florets were at anthesis (feathery stigma stage).

3.2.1.2.2._*Embryo Induction*: Two protocols were assayed. The first protocol was the '***Modified Floret Culture'*** which was mainly based on the floret culture procedure reported by Laurie (1992) with one modification. Immediately following pollination, spikes were placed in a flask containing an aqueous solution of 100 mg/l of 2,4-D for 48 hr. Florets were then removed and surface sterilized in 20% v/v commercial bleach and rinsed in sterile distilled water. After surface-sterilization, florets were cultured in 35x 60 mm petri dishes containing 10 ml of Murashigue and Skoog (MS) basal medium (Murashigue and Skoog, 1962) supplemented with 0.1 mg/l of 2,4-D, 60 g/l of sucrose and 7 g/l of Sigma agar (Appendix Table 3) After three weeks of incubation at 20°C under continuous light provided by cool white fluorescent lamps (120 u E mol/m^2/s) florets were dissected under sterile airflow conditions, the caryopses removed and embryos recovered with the aid of a stereoscopic microscopy. The second protocol was the '***Direct Culture'*** which was a combination of the procedures reported by Suenaga and Nakajima (1989) and Laurie et al. (1990). One day after pollination 5 ml of a 100ppm 2,4-D solution was injected into the base of the uppermost internode of the wheat plant using a small hypodermic syringe. Following the hormone treatment, spikes were covered with glassine bags and left on the plant for 2-3 weeks. Spikes were then collected and placed in a flask containing tap water to avoid desiccation. Caryopses removed from florets were surface-sterilized with a 20% v/v commercial bleach solution. After surface sterilization caryopses were dissected and embryos rescued under a stereoscopic microscopy and sterile airflow conditions.

3.2.1.2.3._*Plantlet Regeneration*: Embryos obtained from the 'modified floret culture' and 'direct culture' were cultured in 25x10 test tubes containing Modified B5 (MB5) regeneration medium (Jensen, 1986) made with filter sterilized vitamins and supplemented with 30/l of glucose and 7g/l of Sigma agar (Appendix Table 7). The medium was adjusted to pH 5.8

before adding agar. Cultured embryos were placed under dark conditions at room temperature (20±1 °C) until they germinated in one to six weeks. When coleoptiles were about 2 cm of length they were placed under continuous light conditions provided by cool white fluorescent lamps (80 uEmol / m^2 / sec) at 20±1 °C. When seedlings reached the top of culture tubes (about 7 cm) they were recorded as complete regenerated haploid plantlets and transferred to a vernalization chamber at 5±1 °C using 8-hr photoperiod for 5 weeks. Following vernalization, haploid seedlings were removed from culture tubes and planted in 6-cm peat pots containing vermiculite. Small pots with haploid plantlets were placed in trays, covered with a float row cover, and transferred to the greenhouse and placed under a misting device to provide a high humidity conditions until they were well rooted. Chromosome counts were then made from tip roots of a sample of plantlets from each genotype, following the technique previously described.

3.2.1.3. **Experimental Design and Statistical Analysis**

The experiment was set up as a 7x2 factorial in a split-plot design with six to nine replications using individual spikes as replications. The main plots were the genotypes (factor A with seven levels) and the sub-plots were the intergeneric cross protocol (factor B with two levels). Weighted analysis of variance was performed using General Linear Model test in SAS. Frequencies were expressed as follows:

Seed Set = (Number of seeds formed / number of florets pollinated) x 100

Embryo induction = (n° of embryos found / n° of seeds dissected) x 100

Embryo production = (n° of embryos found / n° of florets pollinated) x 100.

Haploid plant regeneration = (n° of plants regenerated / n° of embryos cultured) x 100.

Haploid plant production efficiency = (n° plants produced / n° of florets pollinated) x 100

Frequencies of embryo induction were converted to arcsin $\sqrt{x}$ (Steel and Torrie, 1980) to normalize the frequency distributions before statistical analysis.

3.2.2. *2,4-Dichlorophenoxyacetic acid (2,4-D) assay*

3.2.2.1._**Plant material, growing conditions and methodology**

The winter-wheat genotypes used as female parents in this experiment were the same as used in the anther culture experiment and the first intergeneric crossing experiment. The maize parent was inbred line A188. This experiment was conducted during the spring and summer of 1995. Planting and growing conditions were the same as described for the first experiment (Study 2.1). The methodology used was the same as the 'direct protocol' previously described with two modifications regarding the hormone 2,4-D which included: 1) Two concentrations: 20 ppm and 100 ppm. , and 2) Two modes of application: tiller injection and dropping solution in florets. These treatments were applied 24 hrs. after pollination.

3.2.2.2. **Experimental design and statistical analysis**

The experiment was set up as a 7x2x2 factorial in a split-plot design with three to nine replications. The main plots were genotypes (factor A with seven levels) and the sub-plots included hormone 2,4-D concentration (factor B with two levels) and 2,4-D mode of application (factor C with two levels) in a 2x2 factorial arrangement. Differences among genotypes and between hormone doses within genotypes were tested using the Least Square Means (LSM) procedure (Steel and Torrie, 1966). Frequencies of embryo induction, embryo production, haploid plantlet regeneration and haploid plantlet production were converted to arcsin $\sqrt{x}$ (Steel and Torrie, 1980) to normalize the frequency distributions before statistical analysis.

3.3. ANTHER CULTURE vs. WHEAT x MAIZE CROSSES

3.3.1. *Criterion* : *'Efficient production of DHs of all genotypes'*

3.3.1.1. Comparison in a F1generation

3.3.1.1.1. Plant material and growing condition

Forty-seven F1 wheat plants of the cross 'Yamhill' x OR 3870039 (Appendix Table 1) were used as anther donors for the anther culture method and as female parents for the intergeneric crossing technique with maize. After 5-6 weeks of vernalization at 4±1°C, plants were grown under controlled greenhouse conditions as described in the previous experiments. Five planting dates at two weeks interval were employed, starting on January 15, 1996. Pots containing two F1 plants were randomly placed on a bench. At least two spikes were assigned at random to anther culture and wheat x maize cross methods. The development of haploid plantlets through anther culture and wheat x maize techniques were carried out in parallel from March to August of 1996.

The *anther culture* technique was applied according to the methodology described previously in Study 1. *MN6* and 190-2 media (Appendix Tables 2 and 5) were used for the induction of embryonic calli from the anthers and for the regeneration of shoots from the embryos, respectively.

For the *wheat x maize crosses*, a combination of protocols reported by Suenaga and Nakajima (1989), and Laurie et al (1990) and previously described as 'direct protocol' in Study 2. The male parental source was a pool of pollen from inbred A188 and 'Early Sun Glow', an early sweet maize. The 2,4-D hormone was applied to the pollinated spikes by tiller injection at 100 ppm concentration 24 hours after pollination. Spikes were harvested between 12 and 15 days

after pollination for embryo rescue. Jensen Modified B5 (MB5) medium (Appendix Table 7) was also used as plantlet regeneration medium for the embryos.

Tubes containing haploid plantlets developed by both methodologies were placed in a vernalization chamber at 5±1°C for six weeks. Subsequently they were planted in 6 cm peat pots containing vermiculite, placed in trays covered with a float row cover, and left in the greenhouse under a misting device for approximately 7-14 days for rooting and acclimatization. When haploid plantlets were at the two to three-tiller stage they were removed from the soil and a colchicine treatment applied for chromosome doubling. Roots were washed and cut back to 5 cm length and immersed in small tubes containing 4 ml of a 0.5 % colchicine solution for 12 hours under continuous light. Roots were then washed in running water for five hours, then plantlets were transplanted to potted soil, placed in the greenhouse and grown to maturity under the same controlled conditions as described in the previous experiments. At maturity, plants, which produced seed, were recorded as doubled-haploid plants.

3.3.1.1.2. **Experimental design and statistical analysis**

A Complete Randomized Block Design (CRB) using plants as blocks and individual spikes as replications was employed. Weighted Analysis of Variance (ANOVA) was performed using the General Linear Model (GLM) procedure in SAS because of the different number of spikes and florets used for the anther culture and wheat x maize cross methodologies.

The criterion used for the comparison of the anther culture and wheat x maize cross techniques was the efficiency in the production of haploid plants which was based on the frequencies or percentage of green-plantlets obtained.

Frequencies of embryogenic calli and plantlets (green and albino) produced, were expressed as percentage of total florets used in the anther culture and the total number of florets pollinated in the wheat x maize crosses technique. To improve the normality,

percentage data for callus production and green plants production based on the number of florets was transformed by Square Root ±0.5. The arcsine x $^{1/2}$ transformation was applied to percentages of plant regeneration (total and green) taken from the number of callus cultured.

3.3.1.2 **Comparison in seven genotypes of winter wheat**

The comparison of haploid production efficiencies between anther culture and wheat x maize crosses was based on the experimental data results obtained from:

Study 1: Anther culture (*MN6* medium)

Study 2: Wheat x maize crosses ('direct culture')

Crosses of wheat x hybrid maize B14 / CI64

Crosses of wheat x inbred maize A188 (2,4-D assay)

The criterion used for the comparison of haploid production was based on the observed mean percentage of green-plantlets obtained in the anther culture experiment and in the two experiments using wheat x maize crosses.

Frequencies of embryogenic calli and plantlets (green and albino) produced were expressed as percentage of total florets used in the anther culture and the total number of florets pollinated in the wheat x maize crosses technique.

3.3.2. **Criterion : *'Random distribution of parental gametes in DH populations.'***

3.3.2.1. **Plant material and growing conditions**

Three crosses involving parents carrying alternate alleles for the presence or absence of awns were made during crossing season in April 1995 in the experimental fields of Oregon State University located at Hyslop Crop Science Laboratory near Corvallis, Oregon. The three crosses were: 'Yamhill' (awless) x OR 380039 (awned),' Cross 2: ''Yamhill' (awnless) x 'Hoff'

(awned),' and Cross 3: 'OR 887196 (awnless) x OR 3870039 (awned)'. Pedigrees are presented in Appendix Table 1.

Forty-seven, 36, and 41 vernalized F1 plants from Cross 1, Cross 2 and Cross 3, respectively, were grown in 1997 in potted soil in a greenhouse under the same controlled conditions as described in Study 3.1.

From Cross 1, DH lines were developed from F1 plants using the anther culture and wheat x maize cross methods from F1 plants. The anther culture method was as described in Study 1. The induction medium used was *MN6* supplemented with 90 gr. of maltose and solidified with Gelrite/Agar 3:1. The medium for regeneration was 90-2 supplemented with 30 gr of sucrose and 7gr of Agar.

From Cross 2 and Cross 3, doubled haploid lines were developed from F1 plants only by wheat x maize method.

The wheat x maize method employed in this study was as describe in Study 2 using a combination of protocols reported by Suenaga and Nakajima (1989) and Laurie et al., (1990) and described in Study 2 as 'direct protocol'. The maize pollen used was a pool of pollen from Inbred A188 and 'Early Sun Glow."

3.3.2.1. **Analysis of the Experiment**

In doubled haploid populations derived from crosses between homozygous parents carrying alternated alleles for monogenic markers, 1:1 segregation ratios are expected. Deviations from this ratio can imply the occurrence of gametic selection. Chi square tests and associated probability levels (p-values) to measure the goodness-of-fit, were applied to the progeny obtained from the three crosses involving the monogenic marker controlling the presence of awns.

4. RESULTS

The results regarding haploid production in selected wheat genotypes are presented according to the main two studies, anther culture and wheat x maize intergeneric crosses. A comparison of anther culture and wheat x maize methods in haploid production efficiencies and random distribution of parental gametes following haploidization are also provided.

4.1. Study 1 **ANTHER CULTURE**

Appendix Tables 5, 6 and 7 provides information for anther response, embryo/callus production, plantlet regeneration, and haploid plantlet production efficiency (HPE) for six genotypes of wheat using three induction media.

Genotypes 'Gene' and 'Yamhill' were not responsive to the media tested, therefore the statistical analysis of variance was based on data from the five responsive genotypes and the control cultivar 'Chris'. Square root + 0.5 transformation (Steel and Torrie, 1980), was applied to improve the normality of the percentage data. Results of the analysis of variance, with transformed (Table 1) and non-transformed data (Appendix Table 8) were similar, therefore tables of means presented are from original percentage data. Single degree of freedom comparison tests among means obtained from transformed data are also provided (Tables 2 to 8 and 10 and 11).

Since a high correlation (r=0.88) was encountered between anther response and embryo/callus production, only analysis of embryo/callus induction frequencies was chosen to be analyzed, as well as frequencies regarding haploid plant regeneration and production.

Table 1. Mean Squares (Type III) from the analysis of variance for wheat genotype, induction medium, and medium form for anther response, embryo/callus production, haploid plantlet regeneration and production frequencies of six responsive genotypes of wheat (*Triticum aestivum* L.) obtained by anther culture.

SOURCE of Variation	DF	MS Type III[1]							
		Anther response	Embryo/callus Production	Haploid Plantlet Regeneration			Haploid Plantlet Production		
				Total	Albino	Green	Total	Albino	Green
Genotype	5	1.6	1.7	29.0**	13.1*	30.5**	52.1*	0.7*	0.8*
p (G)	50	1.1*	1.4	11.6	5.8	6.5	32.8	0.3	0.2
Media (M)	2	25.4**	24.8**	139**	56.2**	35.1**	319**	2.2**	0.9*
G x M	10	0.4	0.5	9	4.8	12.1*	25.5	0.3	0.6**
Medium Form (F)1		10.0**	10.5**	41	47.6**	0.1	133*	1.5*	0.1
G x F	5	0.5	0.5	2.5	1.5	0.2	20.2	0.1	0.1
M x F	2	0.9	1.1	13.7	6.4	2.9	8.3	0	0
G x M x F	10	1.8*	1.9*	11.4	5	3.4	36.8	0.2	0.2
Error	147	0.7	0.8	9.8	5.8	4.3	29.9	0.2	0.2

*, ** *Significant at the 0.05 and 0.01 probability levels respectively*

[1] *Mean Squares type III taken from percentage data transformed by Square Root + 0.5*

4.1.1. **Genotype Effect**

Analysis of variance performed on the six responsive genotypes revealed no differences in *anther response* and *embryo/callus production* frequencies (Table 1). Genotype difference was found for the regeneration frequencies of haploid plantlets (total, albino and green), which varied from 7.6 to 28.5% among the genotypes evaluated with 'Hoff' and 'Stephens' showing the highest regeneration frequencies (Table 2). The frequency of albino plantlets regenerated from embryo/callus of the responsive genotypes ranged from 5.0% for 'Hoff' to 15.7% observed in 'Stephens'. Marked differences were observed in green-plantlet regenerating frequencies among genotypes and a genotype x medium interaction was also detected (Table 1).

The *haploid plantlet production* (total, albino and green) was also influenced by the genotype in similar way to the plantlet regeneration frequencies (Table 1). From the data presented in Table 3, production of total plantlets ranged from a low (0.4%) observed in OR 870025 to the highest (2.6%) noted in cultivar 'Chris' (control). Production frequencies of albino plantlets ranged from the lowest (0.4%) elicited by OR 870025 to the highest (1.1%) observed in 'Stephens'. Production of green plantlets obtained for cultivars 'Chris' (1.6%) and 'Hoff' (0.8%) were the highest among all genotypes evaluated.

From the genotypes tested, 'Stephens', OR 3870039, and OR 870025 had higher frequencies of albino plantlets than green plantlets. Only genotype 'Hoff' and the control 'Chris' yielded higher frequencies of green than albino plantlets (Fig 1).

4.1.2. **Induction Medium Effect**

Differences between the three-induction medium tested were found for all traits (Table 1). The effect of the media was primarily evident on the type of response induced. Modified N6 (MN6) medium induced only embryonic callus from anthers in all genotypes. This induced callus

Table 2. Comparison of observed genotype mean values and least square means with standard errors (in parenthesis) for regeneration frequencies of haploid plantlets (total, albino and green) obtained from embryo/callus of six responsive genotypes of wheat *(Triticum aestivum* L.) through anther culture in three induction media.

Wheat Genotype	Means Embryo/calli		HAPLOID PLANTLET REGENERATION		
			Total	Albino	Green
			%	%	%
Chris (C)	35	34	**20.0** (17.0 + 6.7) **bc**	**12.2** (7.2 + 4.9) **b**	**8.2** (10.0 + 4.1)
Hoff	41	27	**28.5** (36.3 + 6.7) **a**	**5.1** (3.6 + 4.9) **b**	**19.1** (31.9 + 4.1)
Stephens	42	29	**22.2** (32.2 + 6.1) **ab**	**15.7** (21.9 + 4.4) **a**	**6.5** (10.2 + 3.7)
OR 3870039	38	52	**14.4** (12.4 + 6.1) **c**	**5.7** (6.5 + 4.4) **b**	**3.4** (2.5 + 3.7)
OR 887196	40	20	**16.2** (16.5 + 5.3) **c**	**6.6** (7.3 + 3.9) **b**	**7.0** (6.8 + 3.3)
OR 870025	36	24	**7.6** (5.3 + 6.8) **c**	**10.1** (8.7 + 4.6) **b**	**0.0** (0.0 + 3.4)

Means followed by the same letter within traits among genotypes (columns) are not significantly different at the 0.05 probability level based on the Least Square Mean (LSM) test..

P values obtained from single degree of freedom comparison means of percentage data transformed using Square Root + 0.5

Table 3. Comparison of observed mean values and least square means with standard errors (in parenthesis) for production frequencies of haploid plantlets (total, albino and green) obtained from anthers of six responsive genotypes of wheat (*Triticum aestivum* L.) through anther culture in three induction media.

Wheat Genotype	Means	Anthers cultured	HAPLOID PLANTLET PRODUCTION Total %	Albino %	Green %
Chris (C)	35	1321	**2.6** (2.4 + 0.6) **a**	**1.1** (0.7 + 0.4) **ab**	**1.6** (1.6 + 0.4)
Hoff	41	1347	**1.2** (1.1 + 0.6) **ab**	**0.2** (0.1 + 0.3) **b**	**0.8** (1.0 + 0.4)
Stephens	42	1217	**1.5** (2.3 + 0.5) **a**	**1.1** (1.6 + 0.3) **a**	**0.4** (0.6 + 0.4)
OR 3870039	38	1329	**1.5** (1.5 + 0.5) **ab**	**1.0** (1.1 + 0.3) **ab**	**0.3** (0.2 + 0.4)
OR 887196	40	1235	**0.7** (0.7 + 0.4) **b**	**0.3** (0.4 + 0.3) **b**	**0.3** (0.3 + 0.3)
OR 870025	36	1409	**0.4** (0.4 + 0.5) **b**	**0.4** (0.4 + 0.3) **b**	**0.0** (0.0 + 0.4)

Means followed by the same letter between genotypes within traits (columns) are not significantly different at the 0.05 probability level based on the Least Square Means (LSM) test.
P values obtained from percentage data transformed using Square Root + 0.5

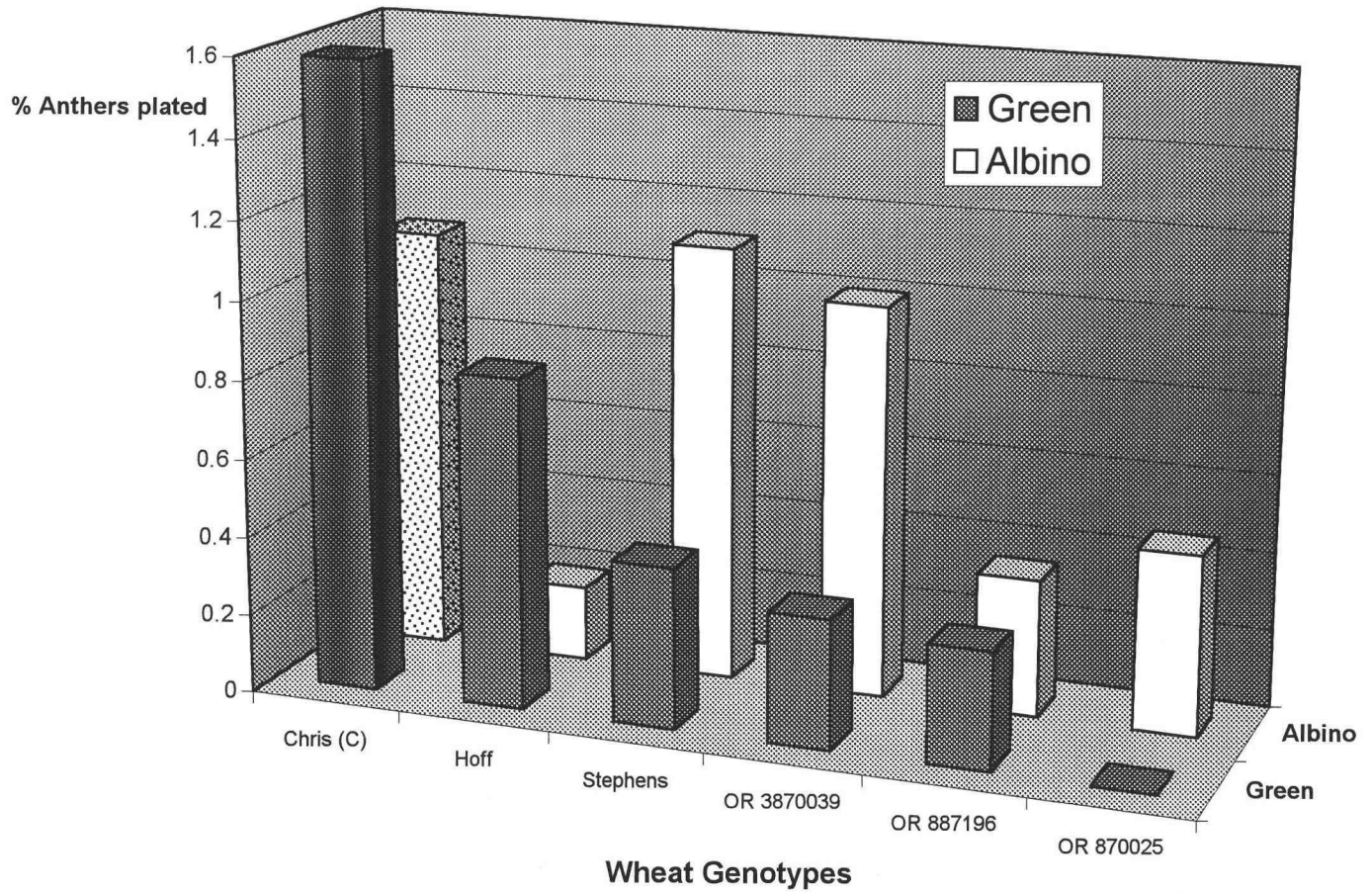

Fig. 1 Production of green and albino haploid plantlets obtained from six responsive genotypes of wheat (*Triticum aestivum* L.) through anther culture

was whitish and compact, consisting of fused embryoidal structures. No embryos and /or embryoids were produced without an intermediate callus phase on this medium. Potato 2 (*P2*) medium induced two types of response, masses of no embryonic callus, and embryos/embryoids without an intermediate callus phase. *Liang's* medium induced primarily well-differentiated embryos and some embryoids, but no callus was detected from anthers cultured. No identifiable somatic embryos were produced and callus generated by microspores was released through ruptured anther walls. No callus was observed from the anther walls and occasionally callus formation was detected from anther filaments, which was easily identified and discarded.

Differences in *embryo/callus production* by anthers cultured on the three media were observed (Table 1). Since no genotype x medium interaction was detected from the analysis of variance, main media effects are presented here. From Table 4 it can be observed that anthers cultured on *MN6* medium gave higher embryo/callus induction frequencies than those cultured on either *P2* or *Liang's* media. No differences were found in the production of embryo/callus from anthers cultured on these latter media.

Plantlet regeneration responses of the embryo/callus (total, albino and green) were also influenced by the medium on which the anthers were cultured (Table 1). It can be observed from Appendix Table 6 that embryo/callus from all six responsive genotypes were capable of plantlet regeneration (albinos and/or green) when they were derived from anthers cultured on *MN6* medium. Five genotypes regenerated plantlets when embryo/callus formed on *P2*. Only 'Chris' regenerated plantlets from embryos formed on anthers cultured on *Liang's* medium. No interactions were detected when regeneration frequencies of total and albino plantlets were analyzed. In Table 5 it can be observed that embryo/callus induced from anthers cultured on *MN6* medium gave the highest frequency of regeneration of total plantlets (32.5%) representing a two-fold increase over *P2* (17.2%) and a ten-fold increase over *Liang's* medium (3.0%). Embryos from all genotypes, except the control cultivar 'Chris', were not capable of regenerating plantlets

Table 4. Comparison of observed mean values and least square means with standard errors (in parenthesis) for anther response and embryo/callus production frequencies obtained from six responsive genotypes of wheat (*Triticum aestivum* L.) through anther culture in three induction media (MN6, P2 and Liang's).

Induction Medium	Anthers cultured	Anther response	Embryo/callus production
	n	%	%
MN6	3702	**5.5** (5.6 ± 0.5) **a**	**5.5** (5.7 ± 0.5) **a**
P2	3179	**1.5** (1.6 ± 0.4) **b**	**1.9** (1.8 ± 0.5) **b**
Liang's	3232	**1.3** (1.5 ± 0.5) **b**	**1.6** (1.8 ± 0.5) **b**

Means followed by the same letter among media and within traits (columns) are not significantly different at the 0.05 probability level based on the Least Square Mean (LSM) test.
P values obtained from single degree of freedom comparisons among means of percentage data transformed by Square Root + 0.5

Table 5. Comparison of observed mean values and least square means with standard errors (in parenthesis) for regeneration frequencies of haploid plantlets (total, albino and green) obtained from embryo/callus of six responsive genotypes of wheat (*Triticum aestivum* L.) through anther culture in three induction media (MN6, P2 and Liang's).

Induction Medium	Embryo/callus cultured +	HAPLOID PLANTLET REGENERATION		
		Total	Albino	Green
		%	%	%
MN6	131	**32.5** (32.5 ± 3.9) **a**	**18.4** (18.8 ± 2.8) **a**	**13.0** (17.1 ± 2.3)
P2	74	**17.2** (18.1 ± 3.9) **b**	**5.5** (5.5 ± 2.9) **b**	**7.8** (9.5 ± 2.4)
Liang's	43	**3.0** (6.2 ± 4.6) **c**	**2.3** (3.9 ± 3.4) **b**	**0.8** (3.9 ± 2.8)

Means followed by the same letter among media within traits (columsn), are not significantly different at the 0.05 probability level, based on the least square Mean (LSM) test.
P values taken from single degree of freedom comparison among means of percentage data transformed by Square Root + 0.5

when originated from anthers cultured on *Liang's* induction medium (Appendix Table 6). Regeneration frequencies of albino plantlets were also higher from embryo/callus produced on

MN6 medium (18.4%) compared to the frequencies obtained from those originated from *P2* (5.5%) or *Liang's* (2.3%) media (Table 5). For the regeneration of green plantlets a separate analysis of genotypes means on each medium was performed due to a genotype x medium interaction detected (Table 1). Data from Table 6 and illustrated in Fig 2, indicate that all winter wheat genotypes experienced an increase in the regeneration frequencies of green plantlets when embryos were derived from *MN6* medium over embryo/callus originated from the other two media. Only embryo/callus from the cultivar 'Chris' showed a decrease in the regeneration of green plantlets when derived from *MN6* medium. Embryo/callus from this cultivar had a higher green-plantlet regenerating frequency when originated from *P2* medium.

The effect of the induction media on the overall *production of haploid plantlets*, measured by the percentage of haploid plantlets obtained from the total number of anthers cultured, was similar to plantlet regeneration frequencies. As seen from Table 7, differences in the production of total plantlets were observed. Anthers cultured on *MN6* medium gave the highest frequency of plantlets, twice the frequency obtained from *P2*, and a five-fold increase over the production obtained in *Liang's* medium. The production of albino plantlets was also higher when anthers were cultured on *MN6* medium when compared to *P2* medium.

The media had an influence on *production efficiency of green plantlets*, and a genotype x medium interaction was also observed (Table 1). From Table 6, can be noted that all genotypes, except cultivar 'Chris', did not yield green plantlets when anthers were cultured on *Liang's* medium. Anthers from four of six genotypes tested yielded green plantlets when cultured on *P2* medium with five genotypes responding when anthers were cultured on *MN6* medium (Fig 3). As seen from Table 8, except for OR 870025which only produced albino plantlets, all winter genotypes showed higher production frequencies of green plantlets when anthers were cultured

Table 6. Comparison of observed mean values and least square means (in parenthesis) for regeneration frequencies of green plantlets from embryo/callus obtained from six responsive genotypes of wheat (*Triticum aestivum* L) through anther culture in three induction media (MN6, P2 and Liang's).

Induction Medium	Emb/Call n	WHEAT GENOTYPE					
		Hoff	Stephens	OR 887196	OR 3870039	OR 870025	Chris (C)
		%	%	%	%	%	%
MN6	131	34.5 (56.9 ± 6.5) **a**	22.7 (23.3 ± 6.2) **a**	14.1 (11.8 ± 5.5) **a**	8.1 (7.7 ±5.3) **a**	0.0 (0.0 ± 5.8) **a**	2.8 (3.2 ± 5.7) a
P2	74	21.4 (23.5 ± 5.7) **b**	1.5 (3.7 ± 3.3) **b**	8.3 (9.3 ± 6.1) **a**	0.0 (0.0 ± 6.4) **a**	0.0 (0.0 ± 5.9) **a**	17.6 (20.9 ± 6.9) **b**
Liang's	43	0.0 (0.0 ± 0.00) **b**	0.0 (0.0 ± 5.6) **b**	0.0 (0.0 ± 5.6) **b**	0.0 (0.0 ± 7.8) **a**	0.0 (0.0 ± 7.9) **a**	4.9 (5.9 ± 6.8) a

Means followed by the same letter among media within genotypes (columns) are not significantly different at the 0.05 probability level .based on the LSM test.

P values obtained from single degree of freedom comparison among means of percentage data transformed by Square Root + 0.5

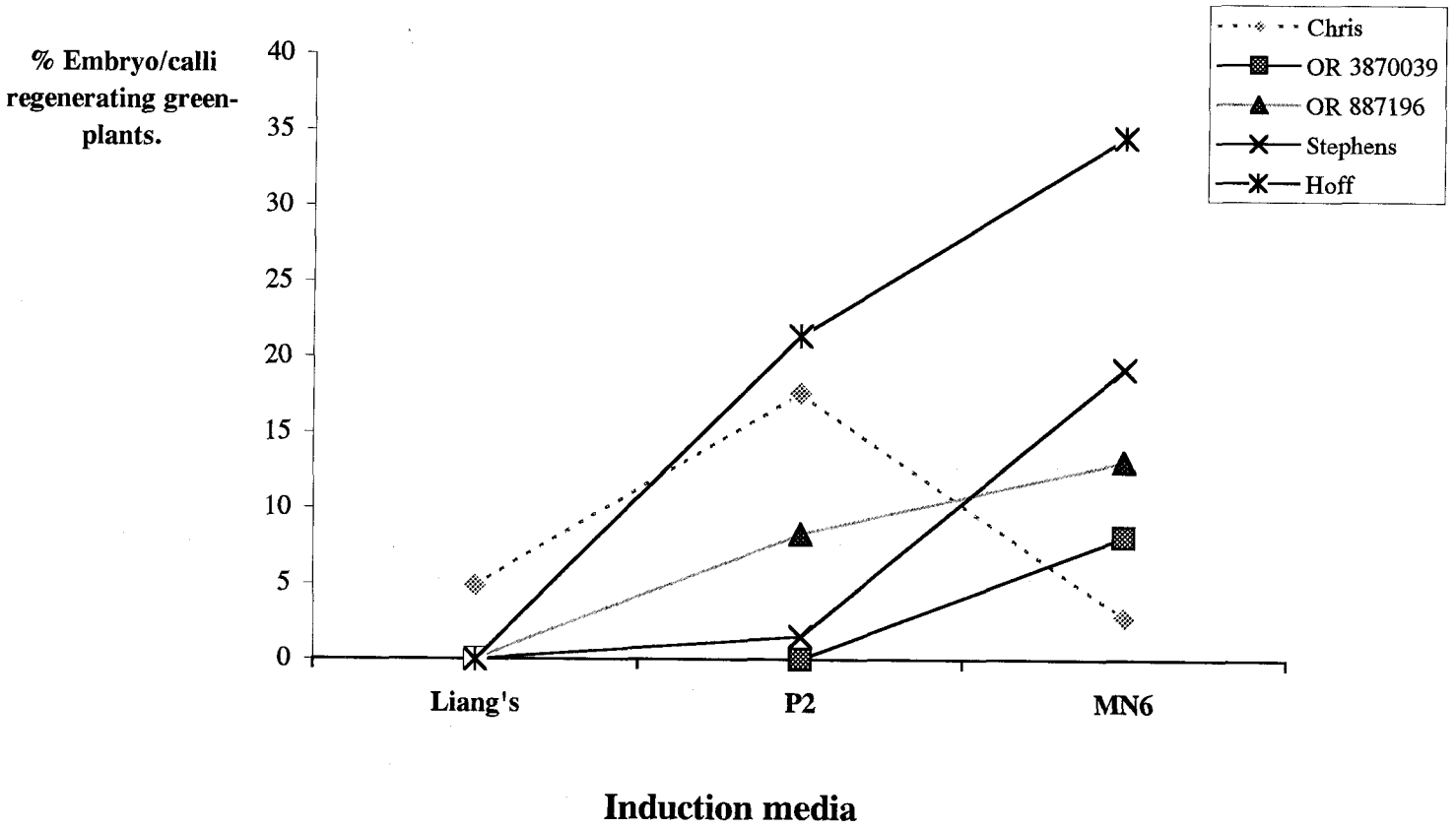

Fig. 2 Mean values for regeneration frequencies of green haploid plantlets from embryo/calli of five genotypes of wheat (Triticum aestivum L.) obtained through anther culture in **three induction media**

Table 7. Comparison of observed mean values and least square means with standard errors (in parenthesis) for production efficiencies of haploid plantlets (total albino and green) obtained from six responsive genotypes of wheat (*Triticum aestivum* L.) through anther culture in three induction media (MN6, P2 and Liang's).

Induction Medium	Anthers cultured	HAPLOID PLANTLET PRODUCTION		
		Total	Albino	Green
		%	%	%
MN6	3702	**2.1** (2.3 ± 0.3) **a**	**1.3** (1.3 ± 0.2) **a**	**0.8** (0.9 ± 0.2)
P2	3179	**1.3** (1.3 ± 0.3) **b**	**0.5** (0.3 ± 0.2) **b**	**0.7** (0.8 ± 0.2)
Liang's	3232	**0.4** (0.6 ± 0.4) **c**	**0.2** (0.3 ± 0.2) **b**	**0.1** (0.3 ± 0.3)

Means followed by the same letter within traits (columns) are not significantly different at the 0.05 probability level based on the Least Square Means (LSM) test.
P values obtained from single degree of freedom comparisons among means of percentage data transformed by Square Root + 0.5

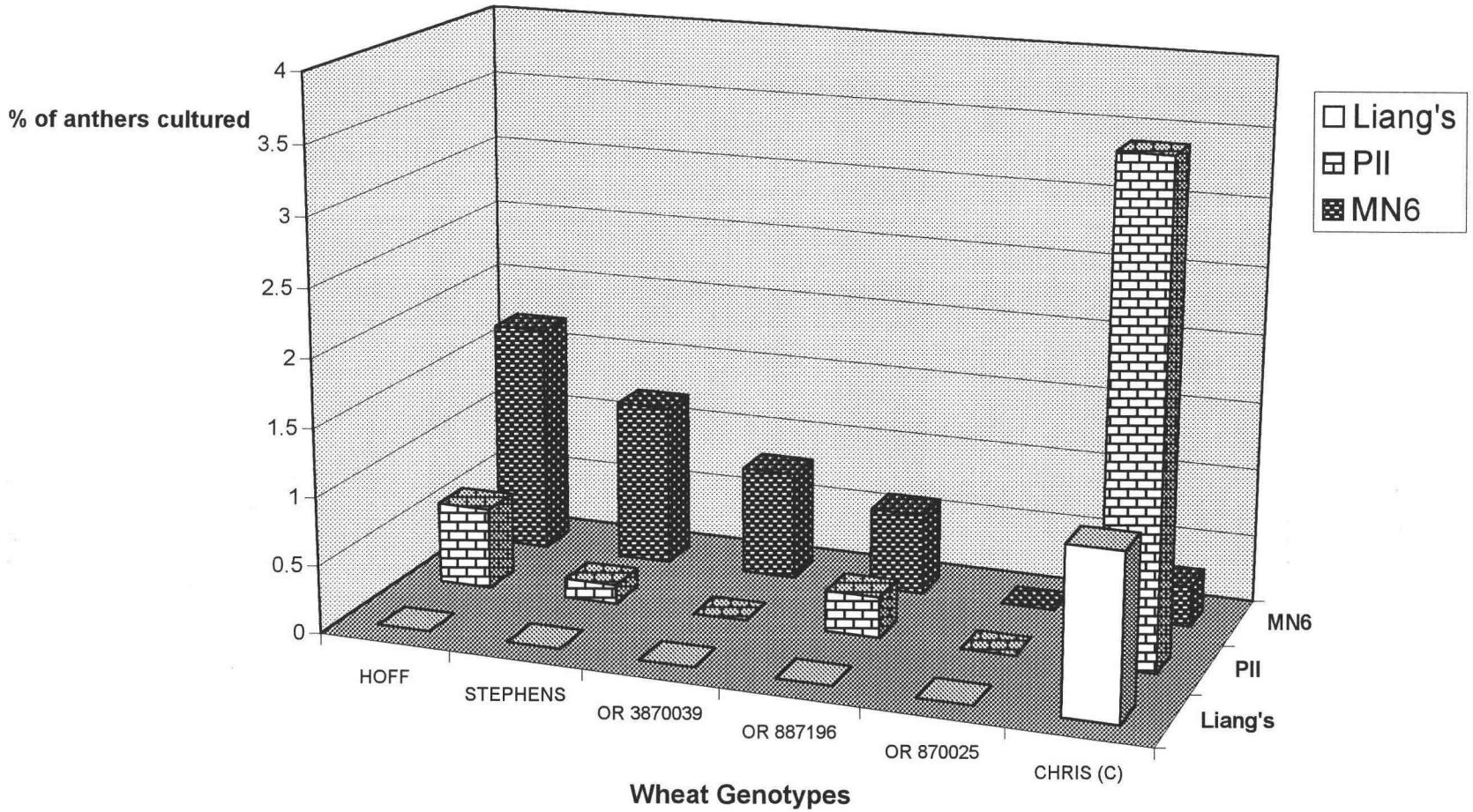

Fig. 3 Effect of three induction media on the production frequencies of green haploid plantlets of six responsive genotypes of wheat (*Triticum aestivum* L.) through anther culture

Table 8. Comparison of observed mean values and least square means with standard errors (in parenthesis) for haploid production efficiencies (HPE) of green plantlets obtained from anthers of six responsive genotypes of wheat (*Triticum aestivum* L) through anther culture in three induction media. (MN6, P2 and Liang's).

InductionAnthers MEDIUM plated		WHEAT GENOTYPES					
		OR 870025	OR 887196	OR 3870039	Stephens	Hoff	Chris (C)
	n	%	%	%	%	%	%
MN6	3702	0.0 (0.0 ± 0.6) **a**	0.6 (0.6 ± 0.5) **a**	0.8 (0.8 ± 0.5) **a**	1.2 (1.4 ± 0.6) **a**	1.7 (2.3 ± 0.3) **a**	0.3 (0.2 ± 0.6) **al**
P2	3179	0.0 (0.0 ± 0.6) **a**	0.3 (0.3 ± 0.6) **a**	0.0 (0.0 ± 0.6) **a**	0.1 (0.2 ± 0.5) **a**	0.6 (0.6 ± 0.6) **b**	3.6 (3.6 ± 0.6) **b**
Liang's	3232	0.0 (0.0 ± 0.8) **a**	0.0 (0.0 ± 0.5) **a**	0.0 (0.0 ± 0.8) **a**	0.0 (0.1 ± 0.4) a	0.0 (0.2 ± 0.7) **b**	1.2 (1.2 ± 0.7) **a**

Means followed by the same letter among media and within genotypes (columns), are not significantly different at the 0.05 probability level based on the Least square means (LSM) test..
P values obtained from single degree of freedom comparisons among means of percentage data transformed by Square Root + 0.5

on *MN6* medium. These frequencies varied from 0.06 to 1.7%. Cultivar 'Chris' produced the lowest frequency of green plantlets on this medium. Green-plantlet production from anthers of the winter genotypes were lower when cultured on *P2* than when cultured on *MN6* medium; however cultivar 'Chris' gave a higher frequency of green plantlets on the latter medium (Fig. 3).

4.1.3. **Induction medium form effect**

The form in which the medium was used (liquid or semi-solid) influenced most of the traits studied (Table 1). When considering the *anther response* and *embryo/callus induction* frequencies, a genotype x medium x medium-form interaction was detected; therefore, a separate analysis was performed. Since *MN6* medium generated data for all genotypes tested it was selected for this analysis. The analysis of variance (Table 9) showed an interaction between genotype and medium forms. This result indicated that genotypic effect on the induction of embryo/callus was dependent on the form of the induction medium. A comparison of genotypes means for embryo/callus produced on *MN6* medium showed that anthers for most of the genotypes had an increase in frequencies when cultured on *MN6* medium in the liquid form (Table 10, illustrated in Fig 4). These differences were significant only for OR 3870038. From Fig 4, it can be also noted that anthers from 'Chris' showed an opposite response exhibiting a better embryo/callus induction frequencies when cultured on semi-solid medium.

For *plant regeneration* response, as can be observed from Table 11, the frequency regeneration of total plantlets was superior in liquid medium (40.7%) over semi-solid medium (22.0%). Regeneration of albino plantlets were higher when anthers were cultured on liquid medium (25.0%) than when they were cultured on semi-solid cultures (9.2%). However, the green-plant regeneration response did not varied from liquid (14.8%) to semi-solid medium (12.6%). The same pattern of response was reflected on the final plant production efficiencies. As seen in Table 11, anthers cultured on liquid medium yielded higher frequencies of total (1.6%)

Table 9. Analysis of variance for production frequencies of embryogenic callus obtained from anthers of six responsive genotypes of wheat (*Triticum aestivum* L.) on MN6 induction medium in two forms: liquid and semi-solid, through anther culture.

SOURCE OF VARIATION	Df	MEAN SQUARES [(1)] TYPE III	F
Genotype (G)	5	1.66	0.8
P (G)	45	1.08	1.7
Medium Form (F)	1	6.69	5.7*
G x F	5	3.42	2.9*
Error	28	1.16	

* *Significant at the 0.05 probability level.*

[(1)] *From percentage data transformed by Square Root + 0.5*

Table 10. Comparisons of observed mean values and least square means (in parenthesis) for **embryo/callus production** frequencies obtained from six responsive genotypes of wheat (*Triticum aestivum* L.) through anther culture using three induction media in two forms: semi-solid and liquid

Medium Form	WHEAT GENOTYPE					
	OR 870025	**OR 887196**	**OR 3870039**	**Hoff**	**Stephens**	**Chris (C)**
	%	%	%	%	%	%
Semi-solid	**2.8** (2.6 ± 2.2) **a**	**1.6** (3.3 ± 1.9) **b**	**2.7** (1.6 ± 1.5) **c**	**3.5** (3.2 ± 1.7) **f**	**8.0** (9.8 ± 2.3) **g**	**8.8** (8.2 ± 1.9) **h**
Liquid	**5.8** (5.8 ± 1.4) **a**	**6.8** (6.8 ± 1.5) **b**	**9.5** (10.3± 1.7) **e**	**5.5** (5.5 ± 1.5) **f**	**7.2** (6.4 ± 1.6) **g**	**2.9** (3.8 ± 1.7) **h**

Means followed by the same letter within genotypes (columns) are not significantly different at the 0.05 probability level.
Comparisons between medium forms are made within genotypes (columns).
P values obtained from single degree of freedom comparisons among means of percentage data transformed by Square Root + 0.5.

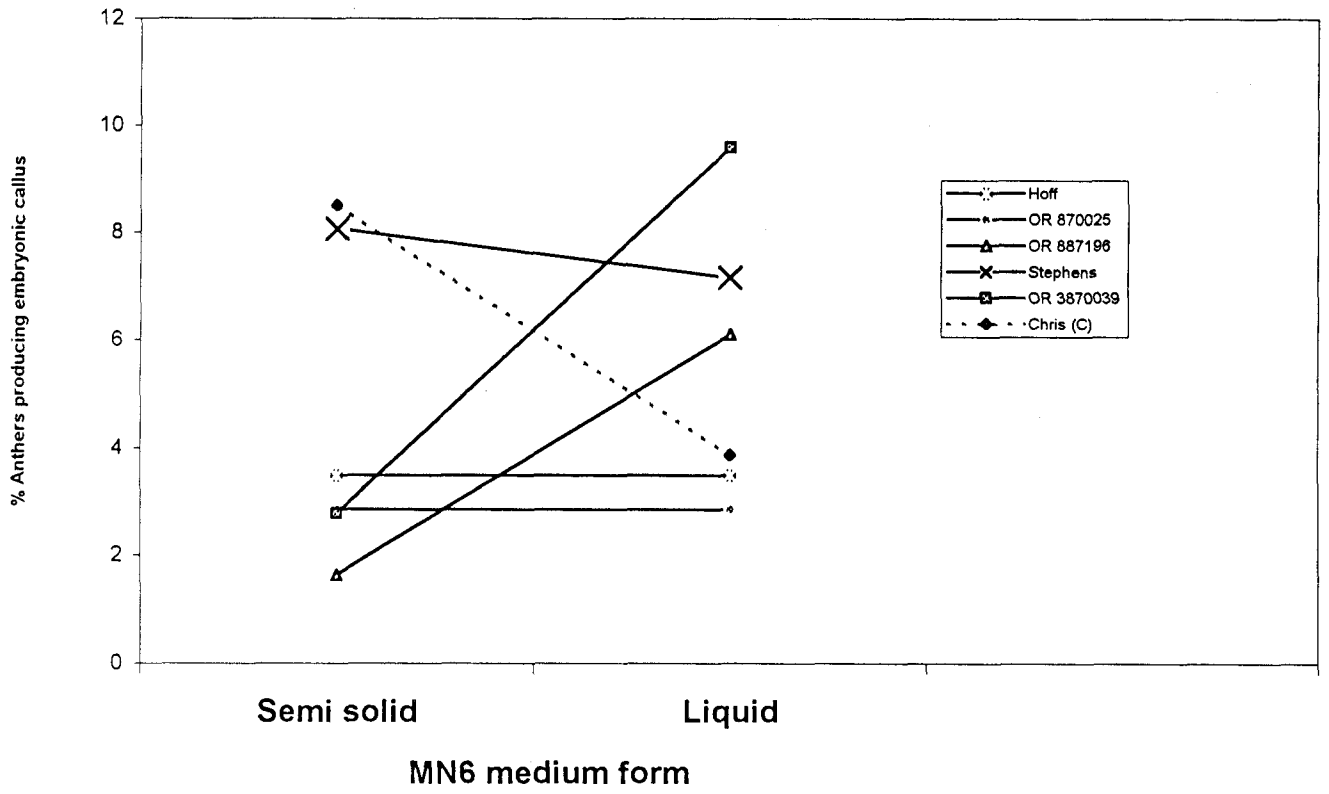

Fig. 4 Effect of the induction medium form on **embryogenic callus production** of six responsive genotypes of wheat (*Triticum aestivum L.*) through anther culture in MN6 induction medium.

Table 11. Comparison of observed mean values and least square means (in parenthesis) for regeneration and production of haploid plantlets obtained from six responsive genotypes of wheat (*Triticum aestivum* L) through anther culture using three induction media in two forms: semi-solid and liquid.

Medium Form	Means	**HAPLOID PLANTLET REGENERATION**			**HAPLOID PLANTLET PRODUCTION**		
		Total	**Albino**	**Green**	**Total**	**Albino**	**Green**
	n	%	%	%	%	%	%
Semi-solid	105	**22.0** (19.1 ± 6.7) **a**	**9.2** (10.0 ± 5.0) **d**	**12.6** (9.5 ± 4.5) **f**	**0.9** (0.8 ± 0.5) **g**	**0.8** (0.5 ± 0.2) **i**	**0.4** (0.5 ± 0.2) **k**
Liquid	128	**40.7** (42.5 ± 5.4) **c**	**25.0** (24.3 ± 3.9) **e**	**14.8** (16.6 ± 3.9) **f**	**1.6** (1.7 ± 0.5) **h**	**0.8** (1.0 ± 0.2) **j**	**0.7** (0.8 ± 0.2) **k**

Means followed by the same letter within traits (columns) are not significantly different at the 0.05 probability level.
Comparisons between medium forms are made within traits (columns).
P values obtained from single degree of freedom comparisons among means of percentage data transformed by Square Root + 0.5

and albino plantlets (0.8%) than anthers cultured on semi-solid medium (0.9% and 0.4% respectively). Green-plant production frequencies, were not affected by the change of medium form. The production obtained on liquid medium (0.7%) did not differ significantly from the production of green plantlets obtained on the medium in semi-solid form (0.5%) (Table 11).

4.2. Study 2. **WHEAT x MAIZE CROSSES**

4.2.1 **Comparison of 'Modified floret culture' and 'Direct culture'**

Pollination of wheat genotypes OR 870025, OR 3870039, OR 887196, 'Yamhill', Gene, 'Hoff' and 'Stephens' with pollen of hybrid maize B14/CI64 resulted in the production of fertilized seeds, haploid embryos and green haploid plantlets (Appendix Table 9). The analysis of variance did not revealed significant effects for wheat genotype or genotype x culture technique; however, marked differences were found between 'modified floret culture' and 'direct culture' techniques assayed for all traits (Table 12). These differences were first evident in the morphology of the seed. Seeds recovered from florets developed on the plant were oval shaped, larger (2.5-4mm), lighter green, and had a healthier appearance than those derived from the 'modified floret culture'. The latter were rounded, watery, and smaller (1.5-3mm). 'Modified floret culture' also had a high rate of bacterial contamination (194 of 865 florets cultured were lost), decreasing drastically the seed set frequencies compared to the 'direct culture' technique (Table 13).

Embryo induction frequencies were also greatly influenced by the use of the two techniques. Embryos and/or embryoids were recovered from all wheat genotypes using both techniques; however the frequency of embryos induced on seeds derived from florets left on the plantlet when 'direct culture' was used, were significantly higher (Table 13). The morphology of

Table 12. Mean Squares from the analysis of variance for wheat genotype and culture technique ('modified floret culture' and 'direct culture') on seed set, embryo induction, haploid plant regeneration and production frequencies obtained from six genotypes of winter wheat (*Triticum aestivum* L) through crosses of wheat x maize (B14 / CI64).

Source of variation	MEAN SQUARES TYPE III			HAPLOID PLANTLETS	
	df	Seed Set [1]	Embryo Induction[2]	Regeneration[1]	Production[1]
Genotypes (G)	5	109	10	470	10
p(G)	31	336	5	1097	37
Technique(T)	1	16442**	31*	28334**	504.9**
G x T	5	699	2	1206	36
Error	31	334	7	1007	23

*, ** *Significant at the 0.05 and 0.01 probability levels.*

[1] *Mean Squares from original percentage data.*

[2] *Mean Squares from percentage data transformed by arcsin* $\sqrt{x}$

Table 13. Comparison of observed mean values and least square means with standard errors (in parenthesis) for seed set, embryo induction, regeneration and production frequencies of haploid plantlets obtained from six genotypes of winter wheat (*Triticum aestivum* L.) through wheat x maize crosses using two techniques: 'modified floret culture' and 'direct culture'.

Wheat x maize TECHNIQUE	**SEED SET**			**EMBRYO INDUCTION**			**HAPLOID PLANTLETS**				
							REGENERATION			**PRODUCTION**	
	Spikes	Pollinated florets		Disected seeds			Cultured embryos			Pollinated florets	
			%		%			%			%
'Modified floret culture'	36	865	**50.1** (50.7 ± 3.1)	405	**11.0**	(10.8 ± 2.1)	49	**33.7**	(33.7 ± 5.9)	865	**2.7** (5.4 ± 2.1)
'Direct culture'	36	902	**82.3** (81.6 ± 3.1)	693	**16.3**	(16.4 ± 2.1)	108	**64.5**	(64.5 ± 5.9)	902	**8.7** (8.5 ± 2.1)

embryos produced was also distinct. The 'modified floret culture' generated more embryoid-like structures than well-differentiated embryos. 'Direct culture' produced larger and well-differentiated embryos and one embryo with no endosperm was normally found in all fertilized seeds; however, the presence of one abnormal endosperm with the appearance of a disintegrated tissue was also found in one seed. Multiple embryos (two, three and four) were recovered in nine of 902 dissected seeds derived from this protocol or 'direct culture. No seeds with multiple embryos, or abnormal endosperm were found from the 'modified floret culture'. Self-pollinated seeds were occasionally found when both techniques were used, but due to their plump greenish-white appearance and normal, white compact endosperm they were easily distinguished from seeds derived from wheat x maize fertilization. Immature embryos induced by 'modified floret culture' and 'direct culture' techniques regenerated plantlets within one to five weeks of incubation in all wheat genotypes tested. However, mean regeneration frequency of embryos produced by 'direct culture' was higher (64.5%) than the mean frequency of embryos derived from 'modified floret culture' (33.7%). Consequently, the final haploid production efficiency obtained using 'direct culture' was twice as high as the one obtained through the ' modified floret culture' (Fig 5).

Fifty-two cultured embryos were rescued from the 'modified floret culture,' 26 did not experience any organogenesis, five produced callus and/or roots and 19 (36.5 %) regenerated complete grown plantlets. From 109 embryos derived from the 'Direct culture', seven showed no sign of organogenesis, 16 produced callus and/or roots, four induced only root growth, three initiated shoots which did not achieve complete morphogenesis, and 74 (67%) regenerated complete plantlets (Appendix Table 10).

From the 93 haploid plantlets obtained from this experiment, 50 were treated with colchicine for chromosome doubling. Of these colchicine-treated plantlets, 37 (74%) survived to complete maturity. The remaining 43 plantlets which did not receive the colchicine treatment were left in the greenhouse until maturity. No seeds were produced by these plantlets indicating a

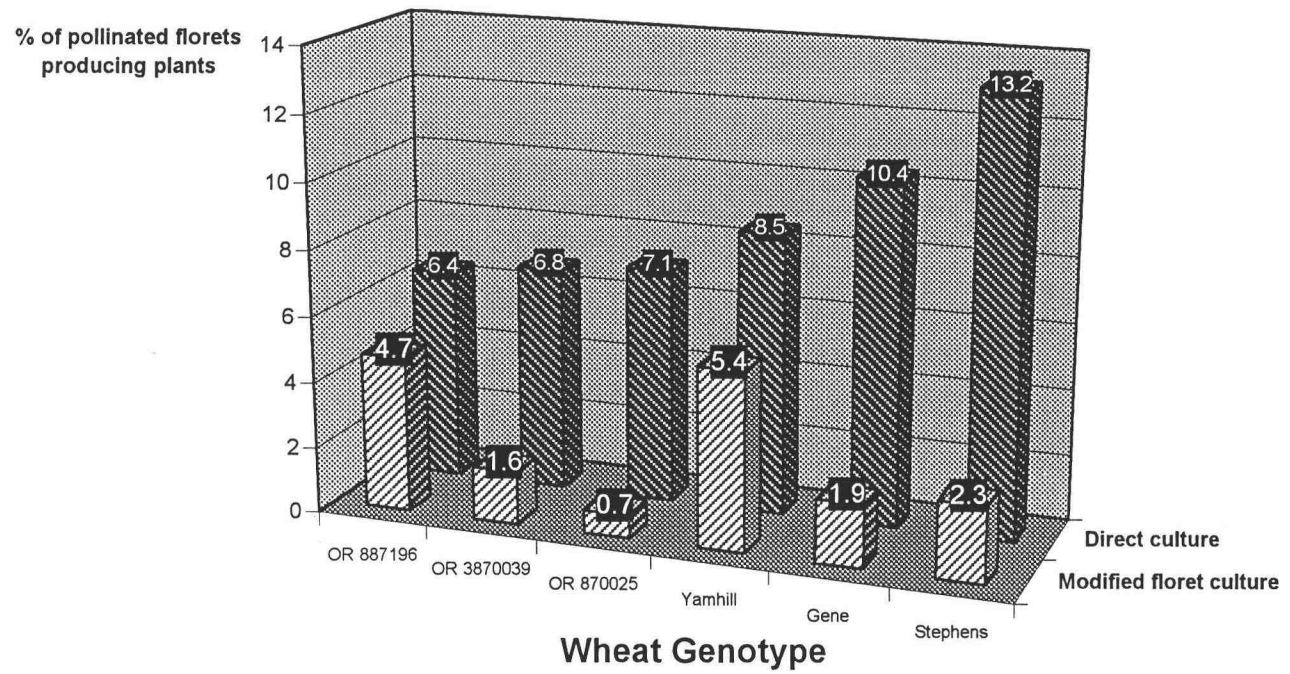

Fig. 5. Haploid production efficiencies (HPE) obtained from six genotypes of winter wheat (*Triticum aestivum* L.) through wheat x maize crosses using two techniques: 'modified floret culture' and 'direct culture'.

lack of fertility as a result of their haploid condition. Chromosome counts performed on root tips from a sample of 20 seedlings revealed the presence of 21 chromosomes.

4.2.2. **2,4-Dichlophenoxyacetic acid (2, 4-D) assay**

Appendix Table 11 indicates that all seven winter-wheat genotypes evaluated produced fertilized seeds with haploid embryos capable of regenerating green-plantlets after being fertilized with pollen from inbred maize A188. The analysis of variance revealed differences among genotypes for all traits (Table 14). No variation was found when the hormone 2,4- D was applied in two different doses (20 and 100 ppm) or in two modes of application (floret spraying and a combination of floret spraying with tiller injection). However, a significant genotype x dose interaction was detected for *embryo induction and production* frequencies and on the plantlet production efficiency level. A separate analysis of the response of each genotype at the two dose levels for embryo production as well as for plantlet production was performed.

As seen from Table 15 and illustrated in Fig 6 four genotypes, 'Stephens', OR 870025, OR 887196, and OR 3870039 had an increase in embryo induction frequencies with an increased dose of 2,4-D (20 to 100 ppm), though this increase was significant only for genotypes 'Stephens' and OR 887196 (Table 15). 'Yamhill' had similar embryo induction frequencies at both dose levels while the cultivar 'Gene' showed a slight decrease in frequencies at a higher dose (100 ppm) though this increase was not significant.

Marked differences among genotypes for the capacity of the embryos to *regenerate plantlets* was found. No interaction was observed from the analysis for this trait. As can be seen from Table 16, regeneration frequencies of green plantlets varied from 22.1 to 55.8 % among genotypes. 'Hoff' and inbred line OR 887196 were the highest among the genotypes in the plantlet regeneration capacity, 55.8 % and 50.2% respectively, though these frequencies were not

Table 14. Mean Squares (Type III) from the analysis of variance for wheat genotype, dose of hormone 2,4-D (20 and 100 ppm) and mode of application ('tiller injection' and 'florets dropping') on embryo induction, embryo production, haploid plantlet regeneration, and production frequencies of seven genotypes of winter wheat obtained through wheat x maize cross method using inbred line A188 as maize pollen donor.

SOURCE of Variation	MEAN SQUARES (TYPE III)					
		SEED SET[1]	EMBRYO		HAPLOID PLANTLET	
	df		Induction[2]	Production[2]	Regeneration	[2]Production[2]
GENOTYPE (G)	6	105	798.4**	858.4**	3944.4**	424.5*
p (G)	59	69.5*	154.5*	147.1**	1203.1	78.6
2,4- DOSE (D)	1	0.4	186.2	186.9	1260.2	0.1
G x D	6	58.9	69.1*	266.3**	680.3	157.1*
2,4-D MODE(M)	1	81	0.2	27.1	715.6	7.8
D x M	1	22.3	63.5	106.6	1540.7	27.1
G x M	6	51.5	123.8	136.4	419.85	64.1
G x D x M	6	21.7	50.8	45.4	1572.5	46.4
Error	139	44.6	110	91.3	1046.5	57.8

*, ** *Significant at the 0.05 and 0.01 levels of probability.*

1 Mean Squares from original percentage data.

2 Mean Squares from percentage data transformed by arcsine $\sqrt{x}$

Table 15. Comparison of observed mean values and least square means with standard errors (in parenthesis) for embryo induction frequencies obtained from seven genotypes of winter wheat (*Triticum aestivum* L.) through crosses of wheat x maize using two dose levels of hormone 2,4-D (20 and 100 ppm) and inbred maize A 188 as pollen donor.

2,4-D Dose	WHEAT GENOTYPE						
	Stephens	GENE	YAMHILL	HOFF	OR 870025	OR 887196	OR 3870039
(ppm)	%	%	%	%	%	%	%
20	4.3 (3.6 ± 2.5) **a**	14.9 (14.4 ± 2.6) **c**	14.3 (14.2 ± 2.2) **d**	10.2 (10.5 ± 2.6) **e**	2.5 (4.5 ± 2.6) **f**	11.6 (12.0 ± 1.9) **g**	2.5 (4.5 ± 2.6) **j**
100	9.1 (9.2 ± 2.4) **b**	13.8 (12.4 ± 2.5) **c**	15.0 (14.3 ± 2.4) **d**	10.9 (10.6 ± 2.5) **e**	9.7 (9.2 ± 2.4) **f**	22.5 (23.0 ± 2.0) **i**	9.7 (9.2 ± 2.4) **l**

Means followed by the same letter within wheat genotypes (columns) are not significantly different at the 0.05 probability level.
Comparison between dose means are made within wheat genotypes (columns)
P values obtained from single degree of freedom comparison among means of percentage data transformed by arcsine $\sqrt{x}$.

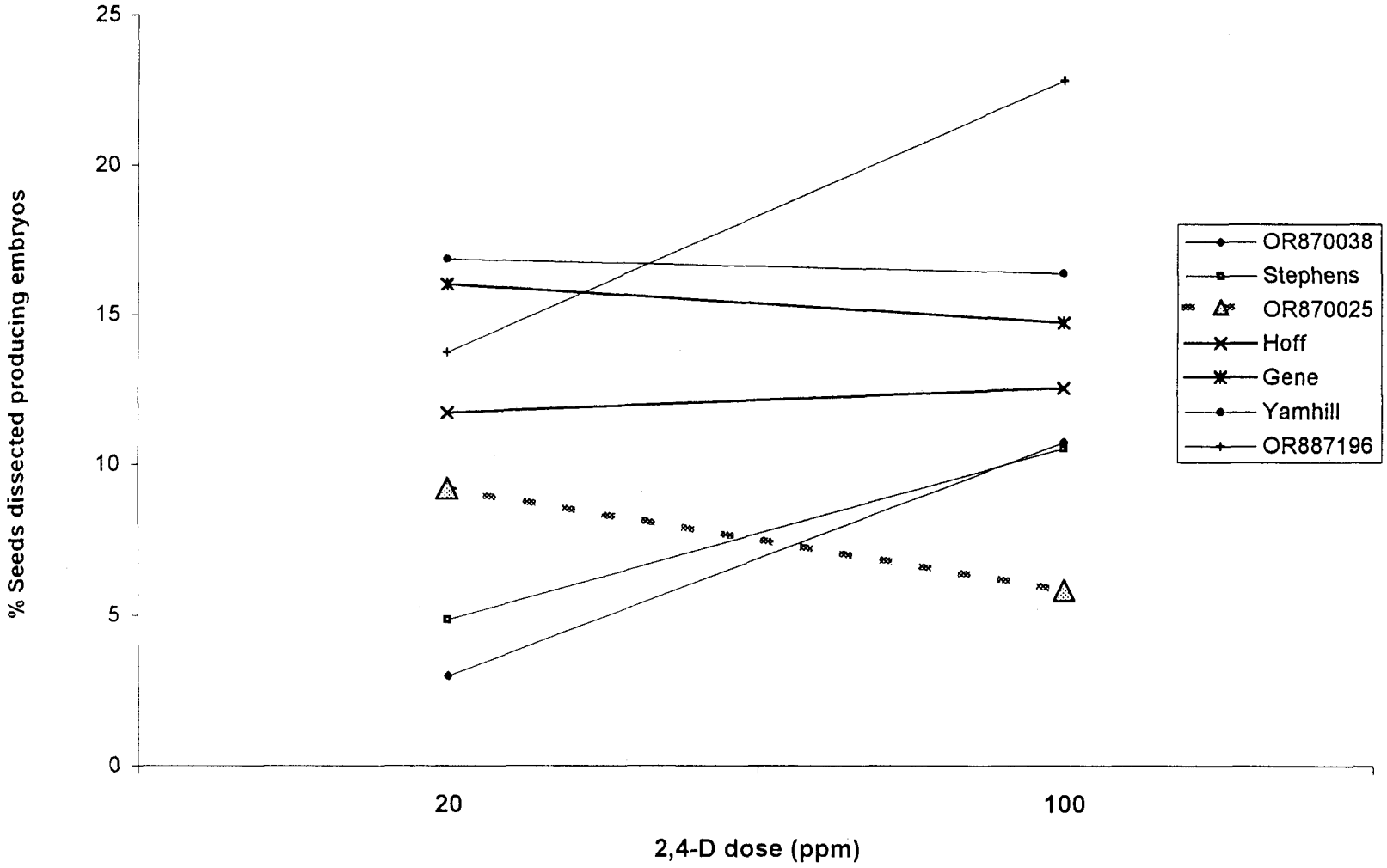

Fig. 6 Effect of two doses of hormone 2,4-D (20 and 100 ppm) on **embryo induction** frequencies of seven genotypes of wheat (*Triticum aestivum* L.).

Table 16. Comparison of observed mean values and least square means (in parenthesis) for regeneration frequencies of haploid plantlets obtained from embryos of seven genotypes of winter wheat (*Triticum aestivum* L.) trough wheat x maize cross method using inbred line A188 as maize pollen donor.

WHEAT GENOTYPE	Number of means	Embryos cultured	Haploid Plantlet Regeneration (green)
	n	n	%
Hoff	27	69	**55.8** (55.7 ± 7.2) **a**
OR 887196	44	144	**50.2** (51.6 ± 5.8) **a**
Gene	30	122	**48.2** (47.9 ± 7.6) **a b c**
Yamhill	33	145	**45.3** (45.4 ± 6.5) **a b c**
OR 3870039	23	46	**35.4** (39.2 ± 8.3) **a b c**
Stephens	30	47	**34.9** (31.6 ± 8.3) **b c d**
OR 870025	37	57	**22.1** (22.5 ± 6.1) **d**

Means followed by the same letter are not significantly different at the 0.05 probability level. P values obtained from single degree of freedom comparisons among means of percentage data transformed by arcsine $\sqrt{x}$

significantly superior to most of the genotypes evaluated except for those frequencies obtained for OR 870025 and 'Stephens'.

The analysis of variance also revealed a significant effect of genotype in the final *haploid plantlet production* efficiency i.e. the percentage of haploid plantlets produced by the total number florets pollinated (Table 14). But since a genotype x dose effect was also encountered, a separate analysis of genotype means for each level of 2,4-D was performed. Single degree of freedom comparison tests among 2,4-D dose means for the genotypes was performed (Table 17 and Fig.7). These tests revealed significant variation only for two genotypes ('Hoff' and OR 887196) with different response patterns to the two levels of 2,4-D. In fact at a higher dose level (100 ppm) inbred line OR 887196 achieved a two-fold increase from 4.2 to 10.2 % for this trait. However, 'Hoff' had a significant decrease in plant production efficiency from 6.1 to 3.8 % at the 100 ppm dose compared to 20 ppm dose of 2,4-D.

4.3. Study 3. **ANTHER CULTURE vs. WHEAT x MAIZE CROSSES**

4.3.1. **Criterion: '*Efficient production of DHs of all genotypes*'**

4.3.1.1. **Comparison of haploid production in a F1 population**

A comparison of the production of embryo/embryonic callus and haploid plantlets through anther culture and the wheat x maize crossing methodology are summarized in ANOVA Table 18 and Table 19. Data were obtained from 47 F_1 donor plantlets from the cross 'Yamhill' x OR 3870039 from which 82 spikes were used for anther culture and 91 spikes for the pollinations with maize inbred line A188.

TABLE 17. Comparison of observed mean values and least square means (in parenthesis for production efficiencies of haploid plantlets (HPE) of seven genotypes of wheat obtained through wheat x maize cross method using two dose levels of hormone 2,4-D (20 and 100 ppm) and inbred line A188 as maize pollen donor.

Wheat Genotype	2,4-D Dose (ppm)	Means n	Florets pollinated n	Haploid Plantlet Production Efficiency (HPE) %
Yamhill	20	17	548	7.9 (7.4 ± 1.2) **a**
	100	16	503	6.3 (6.8 ± 1.2) **a**
Gene	20	15	488	7.0 (7.5 ± 1.4) **a**
	100	15	491	7.0 (7.0 ± 1.4) **a**
Stephens	20	14	340	2.4 (2.1 ± 1.3) **a**
	100	16	396	4.1 (4.0 ± 1.2) **a**
OR 3870039	20	10	289	1.0 (2.1 ± 1.7) **a**
	100	13	394	4.6 (4.3 ± 1.4) **a**
OR 870025	20	18	565	3.1 (3.2 ± 1.2) **a**
	100	20	625	1.8 (1.8 ± 1.0) **a**
Hoff	20	13	375	**6.1** (6.0 ± 1.4) **a**
	100	14	390	3.8 (3.8 ± 1.4) **b**
OR 887196	20	22	546	**4.2** (4.8 ± 1.1) **a**
	100	22	554	**10.2** (11.3 ± 1.1) **b**

Means followed by the same letter, within genotypes are not significantly different

P values obtained from single degree of freedom comparisons among means of percentage data transformed by arcsin $\sqrt{x}$

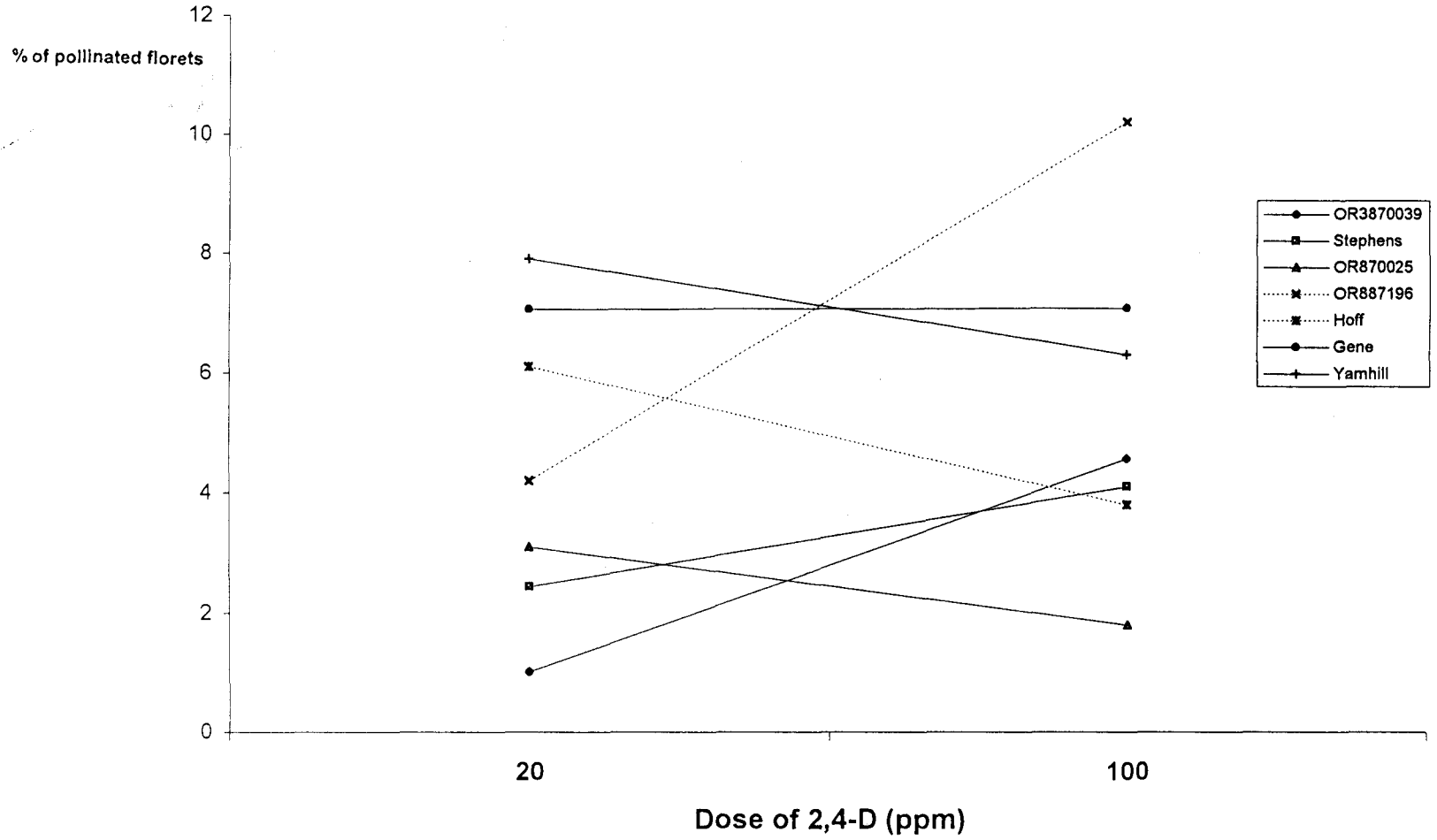

Fig 7. Comparison of observed mean values for **production efficiencies** of haploid plantlets (HPE) obtained from seven genotypes of winter wheat (*Triticum aestivum* L.) through wheat x maize cross method at 20 and 100 ppm levels of hormone 2,4-D.

`Table 18 Mean Squares (Type III) from the analysis of variance for embryo production, plant regeneration (total and green), and production efficiencies of green haploid plantlets obtained from a F1 population of winter wheat (Yamhill x OR 3870039) through two haploidization methods: anther culture and wheat x maize crosses.

Source of Variation	MEAN SQUARES TYPE III		HAPLOID PLANTLET		
	df	EMBRYO PRODUCTION	REGENERATION	PRODUCTION	
			Total	Green	Green
Block	46	44	16816	13748	16
Method	1	0.4	1496020***	2293455***	1839***
Block x Method	45	55**	25963**	12413	13
Error	92	18	12432	9868	1

**** Significant at the 0.001 probability level* [1] *From data transformed using Arcsin x ½* [2] *From data transformed using Sqrt + 0.5*

Table 19. Comparison of observed mean values and least square means (in parenthesis) for embryo production, haploid plantlet regeneration (total and green) and production frequencies of green haploid plantlets obtained in a F1 population of winter wheat (Yamhill x OR 3870039) using two haploidization methods: anther culture and wheat x maize crosses.

HAPLOID METHOD	Spikes used	Florets used	EMBRYO PRODUCTION	HAPLOID PLANTLETS REGENERATION		HAPLOID PLANTLETS PRODUCTION	
				Total	Green	Green	
	n	n	%	%	%	%	n
Wheat x Maize	91	2245	10.8 (10.8 ± 0.8) **a**	82.5 (82.5 ± 3.0) a	81.5 (81.7 ± 2.2) a	8.8 (8.3 ± 0.3) **a**	**2.17** / spike
Anther Culture	82	2100	11.9 (11.5 ± 0.8) **a**	28.9 (29.9 ± 3.1) b	7.7 (7.7 ± 2.3) **b**	1.0 (1.0 ± 0.3) **b**	**0.26** / spike

Means followed by the same letter within columns are not significantly different at the 0.05 probability level

Embryo production: In the anther culture method 6300 cultured anthers excised from a total of 2100 florets gave a production of 250 embryonic calli equivalent to the 11.9 % of florets used. In the wheat x maize cross methodology, 243 embryos were dissected from 2245 florets pollinated with maize pollen, representing 10.8% of florets pollinated. Frequencies in embryo/callus formation produced by the two methods were not different, as shown by the analysis of variance (Table 18), and from simple mean comparison tests (Table 19).

Plantlet regeneration: The capacity of plantlet regeneration from the embryos and embryonic callus derived from anther culture and wheat x maize crosses was found to be different (Table 19). From the 250 embryonic calli originated by anther culture and cultured, 67 (26.8%) regenerated haploid plantlets. Of these haploid plantlets 21 (31.4%) were green and 46 (68.6 %) were albino plantlets. In the crosses of wheat x maize, from the 243 embryos cultured, 198 (82.5 %) produced green plantlets. Only one albino plantlet was found among the progeny when the wheat x maize cross methodology was used.

Haploid production efficiency: Only 1.0 % of the florets used yielded green haploid plantlets with anther culture. In contrast when the florets were pollinated with maize inbred line A188, the production efficiency of green plantlets improve to 8.8 % (Table 19).

.3.1.2 **Comparison of haploid production in seven genotypes of winter wheat**

Embryo/callus production: Table 20 shows that from 1158 florets used in the anther culture experiment using *MN6* induction medium, 107 embryogenic callus were produced representing a mean frequency of 9.0 % of the florets used, with frequencies varying from 0.0 to 18.0 % among the seven winter wheat genotypes evaluated. 'Stephens' showed the highest frequency (18.0 %) and cultivars Gene and 'Yamhill' did not showed any embryogenic callus

TABLE 20. Observed number and percentages (in parenthesis) of embryogenic calli and embryos obtained in seven genotypes of winter wheat (*Triticum aestivum L.*) using two haploidization methods: anther culture and wheat x maize crosses.

Wheat Genotype	ANTHER CULTURE			WHEAT x MAIZE					
				Wheat x B14 / C164			Wheat x A188		
	Florets used	Embryo-calli produced		Florets pollinated	Embryos produced		Florets pollinated	Embryos produced	
	n	n	%	n	n	%	n	n	%
OR 3870039	184	21	(11.4)	190	19	(10.0)	683	49	(7.1)
OR 887196	140	17	(12.0)	130	18	(13.8)	1100	188	(17.0)
OR 870025	175	22	(12.6)	162	14	(8.6)	1290	66	(5.1)
Stephens	130	23	(18.0)	127	18	(14.1)	736	56	(7.6)
Hoff	182	5	(14.0)	–	–	–	765	79	(10.3)
Yamhil 1	86	0	(0.0)	140	21	(15.0)	1051	158	(15.0)
Gene	261	0	(0.0)	178	26	(14.6)	979	144	(14.7)
TOTAL	1158	107	**(9.0)**	927	116	**(12.5)**	6604	740	**(11.2)**

production. For the wheat x maize crosses experiments all the seven wheat genotypes produced embryos including Gene and 'Yamhill' with frequencies varying from 8.6 to 15.0% when crossed with hybrid maize B14 / C164 and from 5.1 to 17.0 % when crossed with inbred maize A188. Considering all genotypes, the mean frequencies of embryo production obtained with the anther culture averaged 9.0% and when wheat genotypes were crossed with hybrid maize B14/CI64 or inbred maize A188, mean frequencies were 12.5% and 11.2% respectively (Table 20).

Plantlet regeneration: As seen in Table 21, in the anther culture experiment 39.0% of the embryogenic calli from five genotypes regenerated plantlets. From these plantlets, 55.0% were albinos and 45.0% were green-plantlets. The mean frequency of green-plant regeneration was 18% from the total number of embryogenic calli cultured. Frequencies of plant regeneration (including green and albinos) varied from 23.1 to 59.9%. The highest percentage of albino plantlets (100%) was regenerated in OR 870025 followed by 'Stephens' (38.5%). The lowest percentage of albinos was observed in 'Hoff' (7.7%). In the wheat x maize crosses only green-plantlets were regenerated, and with higher frequency values than those obtained from the anther culture experiment. These frequencies varied from 50% to 77.7% with a mean frequency of 63.8 % when wheat genotypes were crossed with hybrid maize B14/C164. When the pollinator was the inbred maize A188, 342 of 740 (46.2%) regenerated green plantlets with frequencies that ranged from 37.8 to 50.6%.

Plantlet production: From the data presented in Table 22 and Fig 8, for the anther culture from 1158 florets, 42 plantlets (albino and green) were produced at a mean frequency of 4.0 %. The total-plantlet production frequencies varied from the lowest (3.0%) obtained for OR 870025 to the highest (12.5%) resulted from 'Stephens'. From these plantlets, 19 were green, which represented a frequency of 2.0% of the total number of florets used. The production of green plantlets from the five responsive genotypes ranged from 0% corresponding to OR 870025 which only yielded albino plantlets, to the highest frequency (4.9%) for the cultivar 'Hoff'.

TABLE 21. Observed number and percentages (in parenthesis) of haploid plantlets regenerated from embryogenic calli and embryos obtained from seven genotypes of winter wheat (*Triticum estivum* L.) using two haploidization methods: anther culture and wheat x maize crosses.

	ANTHER CULTURE (MN6)					WHEAT x MAIZE				
WHEAT GENOTYPE	**HAPLOID PLANTLET REGENERATION**					**HAPLOID PLANTLET REGENERATION**				
						Wheat x B14/CI64			**Wheat x A188**	
	Embryos	**Total**		**Albino**	**Green**	**Embryos**	**Green**		**Embryos**	**Green**
	n	n	%	n %	n %	n	n	%	n	n %
OR 3870039	20	5	(23.1)	3 (15.0)	2 (10.0)	19	14	(73.6)	49	22 (44.9)
OR 887196	17	5	(29.0)	2 (11.0)	3 (14.1)	18	9	(50.0)	188	80 (42.5)
OR 870025	22	6	(26.2)	6 (26.2)	0 (0.0)	14	8	(57.1)	66	25 (37.8)
Stephens	22	14	(59.9)	9 (38.5)	5 (22.0)	18	14	(77.7)	56	28 (50.0)
Hoff	25	12	(46.6)	3 (7.7)	9 (34.5)	-	-	-	79	37 (46.8)
Yamhill	0	0		0	0	21	14	(66.6)	58	80 (50.6)
Gene	0	0		0	0	26	17	(65.3)	144	70 (48.6)
Total	**107**	42	**(39.0)**	23 **(21.0)**	19 **(18.0))**	116	74	**(63.8)**	740	342 **(46.2)**
		42		(55.0)	(45.0)					

TABLE 22. Observed numbers and percentages (in parenthesis) for production frequencies of green haploid plantlets obtained from seven genotypes of winter wheat (*Triticum aestivum* L.) using two haploidization methods: anther culture and wheat x maize crosses.

	ANTHER CULTURE (MN6)			WHEAT x MAIZE					
	HAPLOID PLANTLET PRODUCTION			HAPLOID PLANTLET PRODUCTION					
				Wheat x B14/C164			Wheat x A188		
Wheat Genotype	Florets Used	Green-Plants		Florets Pollinated	Green Plantlets		Florets Pollinated	Green Plantletss	
	n	n	%	n	n	%	n	n	%
OR 3870039	184	2	(1.0)	190	14	(7.3)	683	22	(3.2)
OR 887196	140	3	(2.0)	130	9	(6.9)	1100	80	(7.2)
OR 870025	175	0	(0.0)	162	8	(4.9)	1290	25	(1.9)
Stephens	130	5	(3.8)	127	14	(11.2)	736	28	(3.8)
Hoff	182	9	(4.9)				765	37	(4.8)
Yamhill	86	0	(0.0)	140	14	(10.0)	1051	80	(7.6)
Gene	261	0	(0.0)	178	17	(9.5)	979	70	(7.1)
Total	1158	19	**(2.0)**	927	74	**(8.0)**	6604	342	**(5.2)**

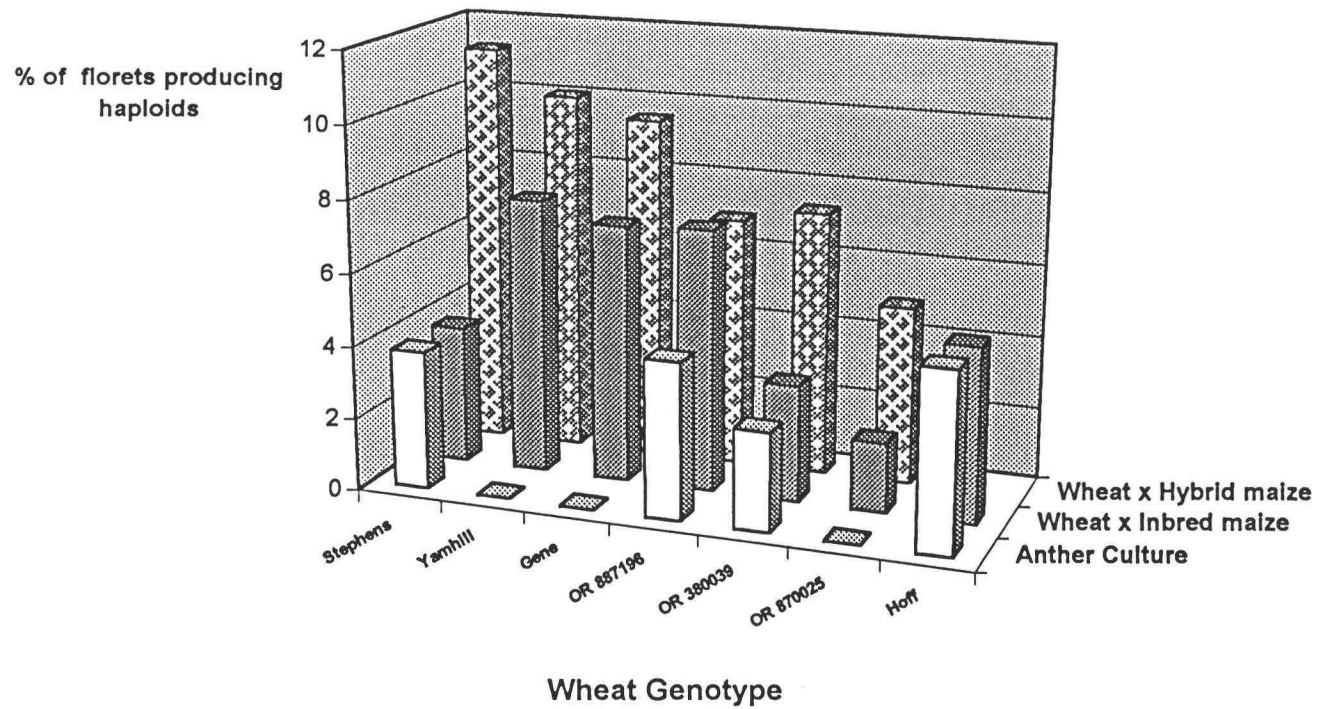

Fig. 8 Comparison of observed mean values for percentages of **green haploid plantlets** obtained from seven genotypes of winter wheat (*Triticum aestivum* L.) through two haploidization methods: anther culture and wheat x maize crosses.

When the wheat x maize crossing method was employed, 8.0% and 5.2% of the florets produced green, haploid plantlets when crossed with hybrid maize B14 / C164 and inbred maize A188, respectively. Production frequencies varied from 4.9% by OR 870025 to 11.2% for 'Stephens' when the hybrid maize B14/C164 was used as pollinator and from 1.9% obtained from OR 870025 to 7.6% observed for 'Yamhill' when the inbred maize A188 was the pollen donor.

4.3.2. **Criterion: *'Random distribution of parental gametes in DH populations'***

Three crosses between winter wheat parents carrying alternate alleles for the awned vs awnless spike trait were used to evaluate possible distortion of genetic segregation ratio.

Table 23 presents the total number of doubled-haploid plantlets with the number of awned and awnless plantlets in the progeny of each cross, as well as the Chi-Square (x^2) values and respective probability values for goodness-of-fit based on the predicted 1:1 segregation ratio.

From the cross Yamhill' x OR 3870039, two populations of doubled-haploids were developed, one through anther culture and another through wheat x maize cross method. With the anther culture method 75 F2 doubled haploid lines were generated. From those 58 were albinos and 17 were green. Since the albino plantlets did not reached maturity due to the lack of chlorophyll, the presence and absence of awns was not scored, from these lines. Goodness of fit test was applied to the remaining haploid population (green plants), but since the size of this population was too small, no conclusions can be drawn.

Using the wheat x maize cross method, three populations of 94, 81, and 120 F_2 doubled-haploid (DH) plantlets were developed from F1 plantlets derived from the crosses, ''Yamhill' x OR 3870039','Yamhill' x 'Hoff'' and 'OR 887196 x OR 3870030', respectively. A good fit to expected 1:1 ratio was found +at the loci controlling the presence of awns in the of F_2 DH lines derived from the three crosses.

Table 23 Segregation for presence and absence of awns, and Chi-square values with probability levels (P-value) obtained in doubled-haploid populations developed through anther culture and wheat x maize methods from Cross 1 (Yamhill x OR 3870039) and through wheat x maize crosses method from Cross 2 (Yamhill x Hoff) and Cross 3 (OR 887196 x OR 3870039).

WHEAT CROSS	Haploidization Method	Awns	Actual number	Expected number 1:1	$X^2=(a-e)^2/e$	P	
Yamhill x OR3870039	**Anther Culture**[1]	Albinos	59				
		awned	12	8.5	2.88	0.05-0.10	
		awnless	5	8.5			
	Wheat x Maize	awned	49	47	0.08	0.5-0.95	n.s
		awnless	45	47			
Yamhill x Hoff	**Wheat x Maize**	awned	38	40.5	0.15	0.5 –0.95	n.s
		Awnless	43	40.5			
OR887196 x OR 3870039	**Wheat x Maize**	awned	58	60	0.08	0.5-0.95	n.s
		Awnless	62	60			

5. DISCUSSION

The conventional approach for the genetic improvement of self-pollinated crop plants such as wheat is through the development of homozygous lines. As a consequence, selection for quantitatively inherited traits must be delayed until F5 or later generations when a near-homozygous condition is achieved. Also at such time possible genotype x environment interactions can be evaluated. Thus, for winter wheat 10 to 12 years may be required before a new cultivar is available for release to growers.

The 'haploidization' technique combined with chromosome doubling to generate doubled–haploid plants (DHs) constitutes an important development in plant breeding since complete homozygous plants can be achieved in a single generation. The time required to obtain a high level of homozygosity and consequently to develop a new cultivar can be reduced. The assurance and acceleration of a high level of homozygosity, has other important implications to breeding of self-pollinated crops. Two main advantages are, doubled- haploid providing the elimination of any bias that may be attributed to heterozygosity, and environmental effects being more precisely estimated since lack of segregation allows for replication of uniform material. Other advantages for the doubled-haploid approach are 1) quantitative traits can be analyzed and selected earlier in the breeding program, 2) additive variance is more important in the DH method than in selfed F2 progeny for traits controlled by additive genes, 3) doubled-haploid lines are of value in recurrent selection (RS) because they increase genetic progress during RS, 4) the method is valuable in predicting parent value in crosses, 5) doubled-haploids have been valuable for detecting linkage associated with quantitative traits, including the use for calculating recombination values between linked genes, 6) smaller population sizes are required to obtain a desired genotype which is equal to the square root of the required F2 population size, and 7) for

molecular genome identification, particularly for QTL analysis to obtain reproducible DNA polymorphisms.

Some important questions have been raised regarding the limitations of the use of haploidy. If haploid plants are produced from the F1 generation there will not be any opportunity to obtain desired recombinants in case of linkages. Another limitation to haploid production could be the availability of controlled growth facilities with competent technical assistance. These latter factors involve more costly investments compared to the conventional procedures if a high degree of efficiency is not reached.

Two main approaches for the production of doubled haploids in wheat are used. These involve anther culture, the oldest and most popular technique, and a more recent intergeneric crossing technique, which capitalizes on the phenomenon of chromosome elimination in wheat x maize crosses, followed by embryo rescue.

Plant breeding is a long-term investment; and breeders are cautious in incorporating novel technologies in their programs unless adequate levels of success are assured. Three criteria to be fulfilled by any haploidization method include 1) easy, and consistent production of large numbers of doubled-haploids of all genotypes, 2) doubled-haploids should be genetically stable, and 3) doubled-haploid populations should contain a random sample of the parental gametes.

This investigation focused on factors, which enhance the efficiency of doubled-haploid production and compared anther culture with wheat x maize methods in terms of their efficiencies for the criteria previously noted.

5.1. Study 1. **ANTHER CULTURE**

5.1.1. **Genotype effect**

A marked genotype effect in the response to anther culture was found among the eight wheat genotypes. This corroborates previous studies (Ouyang et al., 1973; Shaeffer et al.,1979; Marsolais et al., 1982; Jones and Petolino, 1987a; Simmons, 1989; Orlov et al., 1993; Sesek et al., 1994). The variability was primarily expressed in the *production of embryo/callus* from the anthers. Anthers of six wheat genotypes produced embryo/callus, however, cultivars Gene and 'Yamhill' were non-responsive. Thus, statistical analysis was performed from data obtained from the six responsive genotypes. No differences were detected in *embryo/callus* production in the responsive material. Variability among donor plants of the same genotype in anther response and embryo/callus production was observed. Similar observation has also been reported by other researchers (Picard and De Buyser, 1977; De Buyser and Henry, 1980; Andersen et al., 1987). In a study conducted by Zhou and Konzak (1992), using four spring cultivars, 'Chris', Yecora Rojo, Edwall and WA 7176, observed that within genotype variation accounted for 25 to 34 % of the total variation. The presence of this variability constitutes a problem in anther culture since it leads to fluctuating results over time with the same genetic material. Such interactions may be caused by the plant material being at slightly different physiological conditions related to plant growth environment (i.e. temperature, light, humidity etc.) or in vitro conditions (i.e. substrates, temperature, medium osmolalities, etc. (Andersen et al., 1987).

In contrast to embryo/callus response, differences in the frequencies of *plant regeneration* were observed among the responsive genotypes over all the media tested. A wide variation in green and albino plant regeneration frequencies was found among genotypes. Frequencies of *albino* regenerants compared to green plants were also found to be higher in most genotypes except for 'Hoff' and the control cultivar 'Chris'. The 'capacity' of these genotypes to

produce more albino than green plants was maintained in two media *MN6* and *P2*. This suggests that this trait was mainly under genetic control, and corroborates the findings of Ouyang et al. (1983), Andersen et al. 1987, Chu et al. (1990), and Navarro-Alvarez et al. (1994). The lack of variation among genotypes in embryo/callus production in contrast with the variability found in the plant regeneration responses could again be explained by different genetic factors associated to these traits, as previously suggested by Deaton et al., (1987) and Agache et al. (1988).

5.1.2. **Induction medium effect**

The induction medium was a major determinant of the variability of anther response, embryo/callus production, plant regeneration, and final production efficiency. The effect of the media in *embryo/callus* production was expressed primarily in the type of response. *MN6* medium induced callus formation during the early stages of culturing; which then developed embryo-like fused structures in later stages of development. No embryos or embryoids were formed without an intermediate callus phase on this medium. This was true for all genotypes evaluated on *MN6*. On Potato 2 (*P2*) medium, two types of induced response were observed: masses of soft, non-organized callus (non-embryonic) and embryos or/and embryoids formed directly from the anthers without an intermediate callus phase. *Liang's* medium primarily yielded well-differentiated embryos and some embryoids directly from the anthers. No callus (either embryonic or non-embryonic) was detected from anthers plated on *Liang's* medium among all genotypes. Since the pattern of response was uniform over the genotypes tested within media, components in the induction media appeared to be responsible for the different types of induced response. This is supported by an investigation by Ball et al, (1993). They suggested that the type and concentration of some auxins in the medium regulate the specific pathways of microspore development in anther culture. It has also been found that IAA induced direct embryogenesis, whereas 2,4-D favored rapid cell proliferation and callus

induction (Armstrong et al., 1987). As seen in Appendix Table 2, both media (*MN6* and *P2*) contain 2,4-D as growth regulator. However, in *Liang's* medium, only embryos were found. This lack of callus formation in this medium may be explained by the absence of 2,4-D, which is replaced with NAA.

MN6 medium was superior to *P2* and *Liang's* for *embryo/callus production* for all genotypes except for cultivar 'Chris'. The embryo/callus mean production frequency on *MN6* was about three times higher than *P2* and *Liang's* media. This superiority was also manifested in the plant regeneration and consequently in the final plant production frequencies of haploid plants. Embryo/callus from all six responsive genotypes were capable of regenerating plants (green and/or albinos) when derived from *MN6* medium with frequencies that were two and ten times higher compared to the frequencies obtained from embryo/callus produced on *P2* and *Liang's* media, respectively. Embryo/callus from four of the six genotypes regenerated plants was formed on *P2* medium. However, embryos produced in *Liang's* medium were not capable of plant regeneration except those from 'Chris'. Since the same medium for plantlet regeneration was used (190-2) to culture all embryo/calli obtained from the three induction media (*MN6*, *P2* and *Liang's*), differences in regeneration response could be attributed to these induction media in which the anthers were plated.

The *regeneration of green plants* was also strongly influenced by the media and a genotype x media interaction was detected indicating that green-plant regeneration performance of the different genotypes was dependent on the medium on which the anthers were plated. Individual analysis of means indicated a positive effect for *MN6* medium with all responsive genotypes except cultivar 'Chris' which showed an opposite response, by giving higher green-plant regeneration frequencies when anthers were cultured on *P2* or *Liang's* media.

These data indicated that for most of the genotypes tested, *MN6* medium is more suited for anther culture. Extra additives in *MN6* medium such as glutamine or myoinositol or the use of maltose as carbon source could be responsible for the enhanced effects of this particular medium in the responses to anther culturability of these genotypes. Myoinositol has proven to substantially increase the number of calli in barley anther culture when added to the medium depending on the concentration (Kasha, 1989).

Studies demonstrated that the response of wheat embryo/callus to regenerate is influenced by the sugar in the induction medium (Orsinky et al., 1990; Zhou et al., 1991; Ball et al., 1992). In experiments to compare different sugars on anther culture responses, maltose was found to be superior to sucrose for callus induction and green plant regeneration for a range of wheat genotypes (Last and Brettell, 1990; Orsinky et al., 1990). The use of maltose also allowed green plants to be recovered from genotypes that failed to produce plants when anthers were cultured on medium with sucrose. In the present study maltose was added to *MN6* medium and sucrose to *Liang's* and *P2* media. It was observed that embryo/callus from genotype OR 870025, which was not capable of plant regeneration on *Liang's* or *P2* did respond on *MN6* medium. This observation supports those reported by Last and Brettel (1990) and Orsinky et al. (1990). It has been demonstrated that sucrose inhibits embryogenic development of cultured barley microspores because it is rapidly hydrolyzed to glucose and fructuose by invertase which is present in a relatively high active state. In contrast sugars such as maltose or cellobiose are hydrolized relatively slowly by less active gluscosidases. In these cases the intracellular concentrations of hexoses (glucose and fructuose) does not reach inhibitory levels (Hu, 1996).

Regarding the opposite response of cultivar 'Chris' in *P2* and *Liang's* media compared to all other genotypes, might be explained by the fact that the formulation of these particular media was mainly based on the response of this well-known responsive cultivar (Liang et al., 1987; Lazar et al., 1985., Han-Min, 1990).

5.1.3. Induction medium-form effect

All the investigations conducted to compare solid versus liquid medium have used agar or agar-derived compounds as a solidifying agent for the medium. Studies have shown that in general callus formation and green plant yield decreased with increased agar concentration (Lazar et al., 1985; McGregor and McHughen, 1990). It has been suggested that agar contains inhibitory substances that hamper the formation of callus and embryoids from anthers (Dunwel, 1985; Lazar et al.,1985; Mc Gregor and Mc Hughen, 1990). Addition of other gelling agents may be beneficial to the cultures as substitutes of agar to avoid the inhibitory effects of this compound.

In the present experiment a semi-solid treatment was used, Gelrite, an agar substitute gelling gum. Gelrite is produced by a bacterial strain S-60 of *Pseudomonas elodea* and is a self-gelling hydrocolloid that forms rigid, brittle, transparent gels in the presence of soluble salts. Chemically it is a polysacharid comprised of uronic acid, rhamnose, and glucose (Kelco, 1982). Gelrite was used in a mixture with agar in a proportion of 3 Gelrite :1 Agar, to diminish the inhibitory effect of agar. This mixture was combined with regular additions of liquid medium to provide fresh nutrients to the anthers and allow better access to them during the incubation period.

From an analysis performed on data obtained from *MN6* medium, a genotype x medium form interaction was detected indicating that genotype response on the *embryo/callus* production varied with the condition of the medium on which the anthers were cultured. Individual comparisons of means between the two medium forms showed an increase in the embryo/callus production frequencies of four genotypes and a decrease in embryo/callus production in the control cultivar 'Chris', when anthers were cultured on liquid medium. However, these differences were only significant for OR 3870039. Considering that differences found between medium forms for most genotypes, it seems that

the variation detected for embryo/callus production response was caused by the variation found in OR 3870039.

The *total plant regeneration* response (albino + green) was found to be favored by the liquid medium. Likewise, a marked increase in the frequencies of albino regenerants was observed. These results support observations reported by Ouyang (1986) and Jones and Petolino (1987b) who found that albinism was increased on liquid cultures compared to solid cultures. The causes of this phenomenon have not been elucidated. However, regeneration of *green plants* were not significantly affected by the variation of the medium form in the present study.

The effect of the variation of the medium form on the plant regeneration response was reflected on the final *plant production efficiency* of the anthers. In fact, production of *total* and *albino* plants in liquid medium was about two times more than on semi-solid medium. However, no significant differences in *green-plant production* efficiencies were found between the two medium forms. Since the most important component in the process of haploidization is the productivity of green plants. a mixture of 3 Gelrite : 1 Agar combined with liquid medium additions, appears to be an advantage. This was true even though no differences in the embryo/callus induction or green plan-productivity was observed in the present study.

5.1.4. **General performance in anther culturability of eight genotypes of wheat.**

This discussion is based on results obtained for the seven winter wheat genotypes and one spring genotype, the cultivar 'Chris', evaluated on *MN6* induction medium since most complete data was generated from this medium. Limited information to evaluate the androgenetic performance of agronomically useful germplasm is available in the literature. Most investigations on anther culture are based mainly on responsive genotypes. Frequencies obtained

in this study will be compared to that reported in literature involving valuable germplasm for breeding programs.

A 4% level of *responding anthers* (anthers producing embryo/callus) out of the total number of anthers cultured has been proposed as the criterion for genotype fitness for plant breeding (Orlov et al., 1993). Andersen et al. (1987) reported that only 37 of 215 cultivars of winter type (17%) had a level of responding anthers above 5% using *P2* as culture medium. Foroughi-Wehr and Zeller, (1990), from a study of 50 winter and 25 spring genotypes observed only two of the winter population had responsive anthers above the 4% level and for spring genotypes none of them exceeded this value. From an investigation reported by Orlov et al. (1993) with anthers from 30 genotypes of common wheat (including winter and spring genotypes), cultured on P1 medium, 43% had a number of responding anthers above 4%. In this study, the mean percentages of responding anthers found among the responsive genotypes of winter wheat varied from 4.2 to 7.5% (Appendix Table 5). These values indicate that all these genotypes exceeded the minimum 4% level with inbred line OR 3870039 and cultivar 'Stephens' showing the highest frequencies among the winter material.

The results from the present study revealed that five of the seven winter wheat genotypes tested (71%) exceeded the minimum level of 4%. Though the range of the genotypes evaluated was smaller than previous studies cited, these results are encouraging

Green-plant regeneration frequencies obtained in this study varied from 0 - 44.4% with a mean frequency of 26.8%. These frequencies were higher than those reported by Andersen et al., (1987). From their work with anthers from 215 winter wheat cultivars cultured on *P2* medium, the averaged green plant regeneration frequency was 19%. However, from an experiment designed by Chu et al., (1990) to test the effect of glucose and sucrose in a double layer *MN6* regeneration medium for this trait, they found frequencies varying from 0-63% in medium supplemented with glucose and from 0-15 % in medium with sucrose. The frequencies obtained

when glucose was used were higher than the frequencies observed in the present research. However it should be noted that the sugar source was sucrose rather than glucose as in this study.

The mean frequency of *albino* regenerants (18.4%) was higher than the mean frequency of green plants regenerated (13.0%). From the six responsive genotypes, four (OR 870025, 'Stephens', OR 3870039 and control 'Chris') regenerated more albino than green plants. It should be noted that with the exception of control 'Chris', all responsive genotypes maintained the same performance (regarding albino or green regeneration) in *P2* medium. OR 870025 were not taken into consideration because no plants were regenerated from embryos produced on the *P2* medium. These results suggest that a genetic control be mainly involved in the response to regenerate albino plants from these genotypes. However, it is also necessary to mention that the frequency of albino regenerants was lower in *P2* medium. No albino plants were regenerated with the cultivar 'Hoff' and a much-reduced frequency was observed for the control 'Chris' in the *P2* medium. These results suggested that some components in *P2* medium might favor the production of green plants. Furthermore, it was observed that liquid *MN6* medium regenerated more albino plants compared to semi-solid medium. Though this effect did not markedly influence the final production of green plants, there is an indication that the liquid condition of the medium may promote the occurrence of albino plants. This observation is in concordance with a previous observation reported by Zhou et al., (1991).

From these observations, it is evident that although the proportion of green/albino plants appears to be mainly under genetic control, cultural factors also influenced the expression of these genes, confirming other reported results (Ouyang et al, 1983; Andersen et al., 1987; Bjornstad et al., 1989; Zhou and Konzak, 1991; Navarro-Alvarez et al., 1994). In this study, cultural factors related to the medium form (liquid or semi-solid) or the induction medium by itself resulted in changing the frequency of albinos in certain genotypes.

The *final mean production efficiency* of green haploids obtained from the five winter wheat material in *MN6* medium was 0.6%. This frequency was low compared with the frequency

1.3 % obtained by Andersen et al. (1987) and those reported by Orlov et al. (1993) which ranged from 0-19%. One genotype in the latter study yielded 50% green plants.

It was observed that the variation introduced by the medium overcame the variation incorporated by the wheat genotypes suggesting that medium conditions played a critical role in the productivity of the wheat material in this study and genotype effect was minimized by the influence of the medium. This was confirmed by the analysis of variance based on data from *MN6* medium. From these results no genotype effect was observed probably because of a 'neutralizing' effect of *MN6* medium. However, a marked effect of the genotype was shown on the production of haploid plants on *P2* medium.

In the last decade, valuable information has been gained about the beneficial influence of critical components such as the carbon source in the responses to anther culture. Some researchers have reported increases in green plant regeneration rates on spring wheat materials with the addition of maltose to the induction medium compared to sucrose (Orsinky, 1990; Zhou et al., 1991). Chu et al., 1990, in an experiment performed to compare the effect of different monosacharides on 15 genotypes of winter wheat, found outstanding embryo induction frequencies ranging from 40-750 embryos per 100 culture anthers in a liquid medium with 0.21 M of glucose. These frequencies were 2-10 higher than that in the medium with 0.21 with sucrose depending on the genotypes used. These authors reported that in one wheat genotype (F1 SC8828) more than 360 plants could be regenerated from 100 anthers. However the authors did not indicate if those plants were identified as being genotipically different.

An important observation was that from all responsive genotypes cultivars 'Hoff' and cultivar 'Chris' only the two hard red winter wheat cultivars tested showed the highest green-plant regeneration and production frequencies. These results support observations reported by Zhou and Konzak (1992) who in a survey of over 300 wheat genotypes found that red and hard grain wheat genotypes produced higher percentages of green plants than soft white wheats. These findings have significant implications to the use of anther culture in breeding

programs since it could help researchers in selecting wheat materials for anther culturability, saving work and inputs from *in vitro* examinations of germplasm with less androgenetic potential.

5.2. Study 2. **WHEAT x MAIZE CROSSES**

5.2.1 **Comparison of 'Modified floret culture' and Direct culture'**

The present study was planned to assess the response of seven genotypes of winter wheat, using the best protocols reported in the literature, and to explore new protocols.

An experiment was conducted to compare two techniques, the first was a three step procedure, noted as 'modified floret culture', and the second technique was the two step procedure, identified as 'direct culture.' No differences were detected among the wheat genotypes. However, marked differences were found between the two techniques for all the traits measured. The difference was first evident in the seed production. Due to the high incidence of bacterial contamination in the 'modified floret culture', 22.4% of the florets were lost, drastically decreasing the seed set frequencies. It was observed that the bacterial contamination was promoted by evaporation and high humidity conditions found inside the petri-dishes, which was induced by the illumination from the fluorescent lamps. Cultures which were distant from the light source had less bacteria contaminants. This observation suggested that wheat plants might carry the bacteria, which develops the symptoms only if conditions such as high humidity are optimal for disease development. This constraint was not encountered when the 'direct culture' was used.

Differences were also present in the morphology of the seeds. Caryopsis recovered from florets left on the plant to develop using the 'direct culture' technique were oval shaped, larger

(2-4 mm), lighter green in color and had a healthier appearance than those from the 'modified floret culture' The latter were rounded, watery and smaller (1-3 mm).

The morphology of embryos was also markedly influenced by the use of the two techniques. The floret culture generated smaller (0.1-0.8) and embryo-like structures compared to the 'direct culture' that produced bigger (0.3-1.5) and mostly well-differentiated embryos. Since no differences in seeds or embryos morphology were reported from previous studies using either 'floret culture' or 'detached tiller culture', it seems likely that longer exposure of the ovaries to 2,4-D when using the 'modified floret culture' was related to the differential in seed and embryo morphologies. Also, a decrease in the mean frequency of embryo induction and in the efficiency of embryo production was found when the 'modified floret culture' was used.

Regeneration of plants and consequently the final haploid production efficiency was decreased when the 'modified floret culture' was used. This suggested that cutting the tillers to put them in a flask with a 100 ppm 2,4-D solution for 24 hr. and then culturing the florets on Murashigue and Skoog (MS) medium with 2,4-D (0.1 mg/L) , could have caused a detrimental effect on the ovaries.

Comparing the data results from the 'direct culture' with other studies conducted using a similar protocol, the mean *embryo production* frequency was higher (12.0 %) to those reported by Suenaga and Nakajima in 1989 (7.3%), Kisana et al. in 1993 (10.3%), and lower than those reported by Suenaga in 1991 (15.2%) and Laurie and Reymondi in 1991 (32.8 %). *Plant regeneration* frequencies obtained in the direct culture in this study varied from 50.0 to 78.5 % among the wheat genotypes with a mean frequency of 64.8%. These are were higher than those reported by Amrani et al (12.1– 50.3%), Kisana et al., (46.5%) and similar to the mean frequency reported by Laurie and Reymondi (1991) from their work with 14 genotypes of winter wheat (61.4 %). The final *production efficiencies of haploid plants* obtained with the 'direct culture' in this experiment, varied from 6.4 to 13.2 % with a mean frequency of 8.0 %. Comparing these results with those found in literature, these frequencies were higher to those reported by Amrani

et al. in 1993 (0.6 to 9.6 %), Kisana et al., in 1993 (4.8%) Suenaga and Nakajima in 1990 (4.4 %). They were similar to those reported by Inagaki and Tahir in 1991 (9.5 %) from their study with 20 wheat genotypes and lower compared to the mean production efficiency reported by Laurie and Reymondi (1991) from their work with 14 genotypes of winter wheat (21.4 %).

5.2.2. **2, 4-Dichlorophenoxyacetic acid (2,4-D) assay.**

Suenaga and Nakajima (1989) established that wheat haploid plants can be produced by leaving the seeds on the plant when 2,4-D was injected into to the stems after pollination with maize. Since then most of the studies have been performed using this protocol. Because different parental genotypes of wheat or maize and different concentrations of 2,4-D have been applied in those experiments, it is not clear which factors are responsible for improved haploid production.

From a study planned to evaluate the influence of different levels of the auxin 2,4-D for the productivity of haploid plants by wheat x maize crosses, it was reported that 2,4-D was most efficient in promoting embryo induction when high levels of 2,4-D (50-100 ppm) were applied (Matzk and Mahn, 1994). However, the highest embryo and haploid production reported in literature was obtained with the application of a lower level of the auxin (10 ppm) by Laurie and Reymondie (1991).

Regarding the mode of application of the hormone solution, the stem injection procedure one day after pollination used by Suenaga and Nakajima (1989) has been the most frequently used. This practice can be an easy task when wheat plants are vigorous with thick stems. However, often application of 2,4-D by stem injection becomes a difficult procedure when the stems are too narrow.

The present study was performed to investigate if using a low and a high dose of 2,4-D (20 and 100 ppm) and two modes of hormone application by injecting the uppermost internode ('stem injection') or placing a drop of the hormone into each floret ('floret

dropping') influence the responses in the process of haploid production through wheat x maize crosses. From the analysis of variance, no differences were found among the 2,4-D treatments. However, a genotype x dose interaction was detected in most of the traits under study.

Considering the mode of application of the hormone, it was observed that applying a drop of 2,4-D solution on each floret had similar effect to applying the solution by tiller injection on the embryo production, plant regeneration, and final haploid productivity. This offers the option of using different applications at the researcher's convenience, in particular when the injection task becomes difficult.

The analysis of variance revealed differences due to the two doses of 2,4-D. Also a genotype x dose interaction was detected. This indicated that the response of the genotypes was dependent on the dose level of 2,4-D and the trait measured. Considering *embryo production*, most of the genotypes did not exhibit differences with 2,4-D doses, but two OR 887196 and cultivar 'Stephens' had a significant increase in frequencies at a higher dose level (100 ppm). In contrast, OR 870025 was favored by the lower dose (20 ppm) though the difference was not significant. No variation was induced for *plant regeneration* frequencies by the two dose levels. However, the final production of haploid plants was influenced by the concentration of 2,4-D. Although most genotypes produced similar frequencies of haploids, OR 887196 had a higher frequency at the 100 ppm dose, while 'Hoff' produced significant higher frequency of haploid plants at 20 ppm. These results suggested some genotypes are affected by the change of concentration of 2,4-D being more sensitive to higher or lower levels of the hormone.

The analysis of variance revealed significant variation for wheat genotype for most of the traits. This is in contrast with the previous experiment using wheat x maize cross (Study 2.1), where no variability was found among the same genotypes. Previous studies have also reported different results regarding the genotype effect on the haploid production

through wheat x maize crosses. Laurie and Reymondi (1991), reported differences between winter and wheat genotypes, but not within the spring or winter wheat categories. Amrani et al (1993) found differences in haploid production between tetraploid and hexaploid wheats but no differences were detected within genotypes of the same ploidy level. In contrast, Suenaga et al. (1991) detected marked variation among 49 wheat genotypes from different countries. Whether this variation was controlled by genetic or physiological factors is not known.

When comparing the performance of the seven genotypes of winter wheat from the present wheat x maize cross studies (Study 2.1 and Study 2.2) it appears that physiological factors had an important contribution in the variation encountered in these experiments. Both experiments were primarily performed at different seasons of the year. In Study 2.1, the plants were grown during the winter-spring season and in Study 2.2 during the spring-summer season. A lower performance in haploid plant production of the genotypes was noted in the second wheat x maize study. It can also be observed that the same genotypes performed differently when the studies are compared. In Study 2.1, cultivar 'Stephens' showed the highest production efficiency and inbred line OR 887196 the lowest performance; however, in the second experiment, OR 887196 ranked on top of the plant production and cultivar 'Stephens' produced one of the lowest efficiencies. A similar situation was reported by Matzk and Mahn (1994). These authors suggested a seasonal variation caused the opposite performance of one of their genotypes evaluated in two experiments (one in spring and one in summer). They also mentioned that in a separate experiment, the pollen viability, rate of fertilization and embryo development were largely influenced by the temperature regime. These findings suggested that seasonal variations and temperature, may play an important role in the physiological condition, and consequently in the expression of the wheat plants for haploid production.

Another factor that could have influenced the production efficiency in this experiment compared to the previous experiment, was the use of different maize pollen donors. From two studies conducted to evaluate the genotype effect of the maize parents in wheat x maize method, marked variation was found among pollinators. Hybrid maize B14 / CI64 was superior among four genotypes tested by Suenaga and Nakajima, (1989) and one of the best among 55 maize parents used by Suenaga et al., (1991) in haploid production efficiencies. The use of B14 / CI64 as pollen source in Study 2.1 may have also contributed to obtaining higher efficiencies in that study, compared to Study 2.2 where inbred maize A188 was used.

5.3. Study 3. **ANTHER CULTURE vs. WHEAT x MAIZE CROSSES**

5.3.1 **Criterion**: *'Efficient production of DHs of all genotypes'*

The first and most important criterion for choosing a particular doubled-haploid system is the frequency of doubled haploid plants generated. This frequency will depend on two components, namely the frequency of haploid production and the frequency of doubling of the haploids. However in many species chromosome doubling is no longer a problem with the use of colchicine. High rates of doubling (90-95%) have been achieved in bread wheat, therefore comparisons between systems will depend essentially on the frequency of haploid production.

Meaningful comparisons between haploidization systems can be only be made when they are used with the same genotypes. Only one study has been published where such a comparison has been made in wheat between anther culture and wheat x maize crosses (Kisana et al., 1993). Five different three way crosses were compared for their haploid production frequencies using anther culture and wheat x maize cross method. From their results, the anther culture produced 0.6 plants per 100 anthers in contrast to 4.7 haploid plants per 100 florets when the wheat x maize

cross approach was employed. Even though the measuring system (number of anthers and number of florets) used to compare the efficiencies was not the same, the superiority of wheat x maize on those genotypes was evident. Other ways to compare frequencies between anther culture and intergeneric crossing for cereals are needed. Since each floret has three anthers, expressing frequencies on floret basis could be a more useful criterion. In this study haploid production efficiencies obtained through both methodologies were compared according to their production frequencies or percentages taken from the total number of florets used in the anther culture and/or pollinated in the wheat x maize crosses.

5.3.1.1. **Comparison in a F1 population**

From the analysis of variance and from individual mean comparison marked differences between the two methods were found for most of the traits evaluated in the 'Yamhill' x OR 3870039 F1 population. Considering the *embryogenic callus production* efficiency (percentages of embryos and/or embryogenic callus based on the total number of florets used), the frequency of embryogenic callus obtained with the anther culture (11.9%) did not differ significantly from the frequency of embryos obtained with the wheat x maize crosses method (10.8%). However the plant regeneration response was strongly influenced by the method from which the embryos were derived. In fact, the 82.5 % of embryos derived from the wheat x maize crosses regenerated plants, while 28.9 % of the embryogenic callus produced by anther culture were capable of plant regeneration. Since the regeneration frequency found with the anther culture is in the range of those frequencies reported by the other studies (Bedo et al., 1988; Han-Min et al., 1990; Orsinky et al., 1990; Ziegler et al., 1990; Zhou and Konzak; 1989) the low frequency can not be attributed to more or less accurate culture conditions but to the method itself. Furthermore, when considering the green plant regenerating frequencies the differences between the two methods become even stronger since a high percentage of the total number of plants regenerated from the

anther culture (68.%) were albinos and a lower percentage were green (31.4%). However, from the wheat x maize crosses all the plants regenerated were green, except one.

It is worthy to note, that the *plant regenerating* frequencies obtained from this F1 population (81.5 %), were markedly higher to those obtained using inbred cultivars and lines in Study 2.1 (63.8 %) and Study 2.2 (46.2 %), and similar to the best frequency reported to date (81.0%) when detached tiller culture was employed (Riera-Lizarazu and Kazi 1990). One reason for this higher frequency could be attributed to the hybrid vigor from the plants in the F1 compared to the homozygous condition of the genotypes evaluated in the other two experiments condition. Also the embryo rescue time could have played a role in their plant regeneration response. Embryos derived from F1 population (Study 3.1) were rescued within two weeks (12-14 days after pollination). Most of the embryos derived from the previous experiments (Study 2.1 and Study 2.2) were rescued between 14-21 days after pollinations. These latter embryos were very large, with non-typical aspect and with a deformed scutellum. This suggests that leaving the seeds with embryos for longer than optimal time in the plant could have been detrimental to the embryo probably because of the lack of nourishment, since no endosperm is present in the seeds fertilized with maize pollen, or if present it is an abnormal endosperm.

When considering the final *haploid production efficiencies*, 198 (8.8%) green plants were produced from 2245 florets pollinated with maize, and only 19 green plants (0.9%) from 2100 florets used in the anther culture. The superiority of the wheat x maize cross method in this population was also evident in terms of number of spikes used since an average of 2.2 and 0.3 green haploids were obtained using wheat x maize and anther culture methods, respectively.

5.3.1.2. **Comparison in seven genotypes of winter wheat**

When the anther culture method was used, five of seven wheat genotypes induced embryogenic callus formation, the exception being cultivars Gene and 'Yamhill' where no embryogenic callus was formed. Since no response was observed with these two genotypes on any of the media tested, they can be considered as recalcitrant to anther culturability. With the wheat x maize method, all seven genotypes successfully produced embryos either when crosses were made with hybrid maize B14 / CI64, or with inbred line A188. The overall mean frequency of embryogenic callus production obtained from anther culture (9.0%) was lower that the mean embryo-production frequencies obtained with the wheat x maize cross method using either B14 / CI64 (12.5%) or A188 (11.2%) as pollinators

The disadvantage of anther culture was also evident at plant regeneration level since the mean frequency of plant regenerated from embryos derived from anthers was lower (39.0%) compared to the frequencies obtained with the wheat x maize crosses with hybrid maize B14 / CI64 (63.8%) and with inbred maize A188 (46.2%). Furthermore, from the total number of plants regenerated in the anther culture, a high frequency of albino plants was obtained, and only 20% of the embryos regenerated green plants. This value, compared to the green-plant regeneration frequencies obtained with the wheat x maize cross method, was markedly lower since albino plants were not observed among the regenerants when this method was used.

The superiority from the wheat x maize crosses was reflected in the final haploid *production frequencies of green haploid plants*. The frequencies of green haploids obtained from anther culture was much lower for all genotypes when compared with the wheat crosses method when hybrid maize B14 / CI64 was used as pollinator (Study 2.1). The difference between anther culture and wheat x maize crosses method was smaller when inbred maize A188 was used as pollen donor (Study 2.2). Nevertheless, the average green-plant production frequency obtained from this experiment with A188 (5.2%) was twice as higher as the frequency obtained with the anther culture method.

Other important factors contributed to the low performance of the anther culture compared to the wheat x maize method in the present study. First, a restriction in response to the anther culturability from two cultivars Gene and 'Yamhill'. These genotypes instead, showed the best performance in haploid plant productivity with the wheat x maize cross method. Secondly, a low regeneration response was also found from embryo/callus derived from anther culture. This phenomenon has been frequently observed in most of anther culture experiments. Pauk et al (1991) found that the frequency of plantlet regeneration can be enhanced in unresponsive calli, but the efficacy of this technique varied with the wheat cultivars. Another important constraint encountered in the present study was a high rate of albinos, which greatly decreased the quality of the regenerated plants from the anther culture. Though much information has been gained regarding the manipulation of physiological factors (environmental and culture medium conditions) to improve either the response to anther culturability or the proportion of green/albino plants among regenerants, the involvement of genetic factors in both components still play important roles in the haploid productivity of the wheat materials.

Overall results obtained, either when an F1 population was used or when the homozygous wheat genotypes were evaluated, indicated a superior performance of the genotypes in haploid production when the wheat x maize method was used for the production of haploid plants corroborating results obtained by Kisana et al (1993).

5.3.2. **Criterion**: *'Random distribution of parental gametes in the DH populations'*

Despite the advancement of theoretical and practical use of anther culture and doubled haploid methodologies in wheat, there is still little information concerning critical parameters necessary for applying doubled haploid technology in an efficient manner in plant breeding programs. One of the parameters a breeder would like to know is if the doubled

haploid population accurately represents the gametic array of parental materials. Differential gamete selection, if present must be unrelated to, or in favor of agricultural fitness. For quantitative genetic and linkage studies, however, doubled-haploids must be derived from a random sample of gametes.

Deviation from expected Mendelian ratios have been reported by several authors for a range of genetic markers in populations derived from anther culture in cereal crops. In barley, most of the studies performed on doubled haploid lines derived from anther culture (ACDH) have shown segregation distortion. Such distortions have also been reported among anther derived DH lines in rice, maize, rice-grass and wheat. In wheat, the few studies available regarding this phenomenon in doubled-haploid populations derived from anther culture show different results.

Segregation of parental markers has also been investigated in doubled haploid populations derived from intergeneric crosses. From investigations conducted on doubled-haploid populations derived from crosses of barley with *H. bulbosum* it has been shown that there is likely no gametic selection when using this intergeneric crossing system Powell et al., 1986; Shon et al., 1990).

This kind of information on doubled-haploid lines in wheat derived from crosses of wheat x maize has been found from one study using eight genetic markers Suenaga and Nakajima, 1993). From this study, random distribution of parental gametes has been observed for all except one trait. The present study experiment was conducted to analyze random distribution of gametes in F2 doubled-haploid populations developed by anther culture and the wheat x maize cross method derived from 'Yamhill' x OR 3870039.

The doubled-haploid population developed through anther culture elicited a high percentage of albino regenerants. These albino plantlets did not reach maturity due to lack of clorophyll, so the presence or absence of awns could not be scored on these plants. The X^2 test applied to the green plantlets suggested a deviation form the expected ratio 1:1 in this

population. Segregation distortion can be due to a linkage between factors controlling the awn type and factors controlling anther culture responsiveness. From the anther culture experiment (Study1) cultivar 'Yamhill' (awnless) was a non-responsive wheat, and OR 3870039 (awned) a responsive genotype. Similar results were reported by Thompson et al. (1991), indicating the presence of distorted segregation in favor of the responsive parent allele at four of ten marker loci among microspore-derived barley plants. The selection could have also occurred at the gametophyte level, caused by a selection pressure exerted by the *in vitro* process. Linkage between genes controlling albinism and awn type can not be excluded as a cause of the deviant segregation for this trait. Since the population of green plants was too small, no conclusive evidence for the occurrence of segregation distortion for the awn type. It should also be taken into consideration the presence of albino plants as a factor that contributes to the disruption of genetic ratios in anther culture doubled-haploid populations.

For the F2 doubled-haploid population developed through wheat x maize cross method from the same cross, X^2 test showed a good agreement with the expected ratios 1:1 for the locus controlling the presence and absence of awns. Similar results were obtained from other two populations of doubled-haploid obtained from two different crosses ('Yamhill' x 'Hoff' and OR 887196 x OR 3870039) and developed through wheat x maize cross method.

In summary, based on the overall outcome given by the present research using the two most important methodologies for haploid production in wheat, the wheat x maize approach showed superiority compared to the anther culture method in the range of the wheat genotypes evaluated. This superiority was expressed in the following manner:

In regard to the production efficiencies of wheat haploid, a parameter of first priority from a plant breeder's point of view, higher success was obtained with the wheat x maize cross method either when F1 plants were used as plant donors or when the seven inbred lines were the donor materials. In addition to the lower efficiency in haploid production, anther culture showed

a genotype restriction with two genotypes having null response to anther culturability. This suggests the presence of a genetic control in the response to anther culture. In the wheat x maize approach all genotypes were capable of haploid production, and the variability detected among the genotypes appeared to be caused by seasonal variation more than by genetic factors in this investigation. Since it has not been shown that genetic factors are involved in the genotype responses in wheat x maize crosses, this advantage could be added to this intercrossing methodology compared to anther culture.

Another important constraint involved in the anther culture and also detected in this work was the high rate of albino regenerants. Though more information has been gained in the past years for the improvement of green/albino ratios, it still is a constraint in the use of anther culture. Another limitation in anther culture, is the high proportion of embryo/callus with no plant-regeneration 'ability'. This kind of embryo/callus is frequently observed during the anther culture process. Although adjustment of cultural conditions may improve the regeneration responses, efficacy of that improvement has been shown to be dependent on the wheat genotype.

Finally, when considering another parameter of importance for plant breeders, the occurrence of a random distribution of parental gametes in the haploid population is critical. This study revealed an absence of segregation distortion in the doubled-haploid population derived from wheat x maize crosses. This is probably due to the pathway, the phenomenon of chromosome elimination, which avoids the selection pressure from the *in vitro* culture during the process of embryogenesis after fertilization, in contrast to anther culture where the embryo/callus formation takes place under *in vitro* conditions.

Taking into consideration all these aspects, to date, wheat x maize crosses seem to be a more reliable and convenient approach to generate haploid plants in hexaploid wheat and as the more sound approach to be employed in plant improvement programs.

6. CONCLUSIONS

There are two important haploidization systems available to wheat breeders, anther culture and intergeneric cross of wheat x maize. However, before incorporating these systems into a breeding strategy, it is important to know the levels of efficiency each system offers the plant breeder.

The objectives of this study were 1) to investigate the haploid production efficiencies that can be obtained through anther culture and wheat x maize cross method employing seven genotypes of winter wheat, 2) to test the anther culture response of these genotypes in three induction media and investigate if genotype x media interactions are present, 3) to explore protocols of wheat x maize cross method, 4) to compare green haploid production efficiencies between anther culture and wheat x maize cross method, and 5) to investigate if segregation distortion take place during the process of production of doubled-haploid through anther culture and wheat x maize cross method.

Based on the results obtained, the following conclusions are drawn:

6.1. Study 1. **ANTHER CULTURE**

1. In the anther culture experiment, marked genotype variability for anther response, embryo/callus production, plant regeneration (total, green and albino) and haploid production efficiencies were revealed in the seven winter wheat materials evaluated. This genotype variability was greater for green-plant regeneration. Two cultivars, 'Gene' and 'Yamhill', showed no embryo/callus induction response on any of the three anther culture induction media tested.

2. Synthetic medium *MN6* was superior to *P2* and *Liang's* induction media for all the traits measured and five responsive genotypes of winter wheat. The spring cultivar 'Chris' was more responsive on the *P2* media. Embryos derived from anthers cultured on *MN6* and *P2* medium were capable of plant regeneration, but no shoots were regenerated from embryos derived from anthers cultured on *Liang's* medium, except for the control cultivar 'Chris'.

3. The use of two different medium forms (liquid and semi-solid), did not significantly influenced the production efficiency of green plants. This suggests that the use of a mixture of 3 Gelrite: 1 Agar combined with liquid medium additions did not cause a positive or negative effect on the productivity of plants and could be taken into consideration as an alternative to the liquid medium for practical use and to diminish the risk of contamination in the cultures involved in the handling of liquid cultures.

4. The final efficiency of green haploid production in anther culture among the seven genotypes of winter wheat (including the non- responsive genotypes) varied from 0.0 to 1.7% of the total number of anthers cultured, with a mean frequency of 0.8%.

5 The highest frequency in green plant production was displayed by cultivar 'Hoff,' a Hard Red Winter-cultivar.

6. In four of the six responsive genotypes of winter wheat a higher proportion of albino plants compared to green plants was produced.. t. Regeneration frequencies of albino plants significantly varied among genotypes suggesting the presence of a genetic control for this trait.

6.2. Study 2 WHEAT x MAIZE CROSSES

7 All wheat genotypes evaluated produced embryos and haploid plants in all the experiments using wheat x maize cross haploidization method.

8. Using wheat x maize crosses, "direct culture' technique was superior to 'modified floret culture' in embryo production, plant regeneration and plant production. High incidence of bacterial contamination during the *in vitro* floret culture largely contributed to the loss of seeds, and consequently embryos when using the 'modified floret culture technique.' Exposure of the spikes by immersing the cut tillers in a 2,4-D solution for 48 hours seems to have a detrimental effect on the embryogenetic process.

9. The response to two different concentrations of the hormone 2,4-D was dependent on the wheat genotypes. Most genotypes were insensitive to changes in dosage. OR 887196, exhibited a substantial increase in haploid production at the higher dose level (100 ppm) in contrast to the cultivar 'Hoff' where a lower dose (20 ppm) favored haploid production.

10. No significant variation was caused by the use of two modes of 2,4-D application ('tiller injection' and 'florets dropping') for all the traits under study. This result offers the option of using any of both treatments at the researcher's convenience.

11 A within wheat genotype variation was revealed in the experiment conducted to evaluate the 2,4-D treatments in wheat x maize crosses. Seasonal variation may be responsible for this effect.

6.3. Study 3. **ANTHER CULTURE vs. WHEAT x MAIZE CROSSES**

12. When the 'Yamhill' x OR 3870039 F1 population was used to compare the two methods, a higher success was obtained with the wheat x maize cross method for all the traits under study except for embryo production, compared with the anther culture. The green haploid production efficiency (based on the number of florets used) obtained with anther culture was significantly lower (1.0%) than the efficiency obtained with the wheat x maize crosses (8.8%). In terms of number of spikes used, 0.26 green haploids per spike were produced with anther culture and 2.2 green haploid per spike with the wheat x maize cross method. In the anther culture, a very high occurrence of albino plants (68.0% of the total plants regenerated) was encountered. Only one albino plant was observed with the wheat x maize cross approach.

13. Results suggested that gamete selection did not take place during the process of haploidization through wheat x maize crosses.

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APPENDIX

Appendix Table 1. Pedigree and brief description of wheat genotypes (*Triticum aestivum* L.) used in the anther culture and wheat x maize cross studies.

Wheat Genotype	Pedigree	Origin	Grain	Growth	Agronomic Characteristics
Chris (Control)	Kenya 58/Newtahatch// Thatcher/3/Frontana/Thatcher	MAES-USDA	Hard-red	Spring	Awnless, medium in height and maturity.
Stephens	North Desprez/Pullman 101	OSU, 1977	Soft-white	Winter	Awned, standard height,high yielding, mid-late maturity. Very Satisfactory quality traits
Yamhill	Heines VII/Redmond (Alba)	OSU, 1969	Soft-white	Winter	Awnless, medium height, high yielding, late maturity. good milling and baking qualities.
Gene	Cleo/PCH//ZZ	OSU,	Soft-white	Winter	Awless medium height, high yielding,late Maturity. Very satisfactory quality traits
Hoff	Probstorfer-Extrem/Tobari 66	OSU, 1992	Hard-red	Winter	Awned, semi-dwarf, high yielding with Good milling and baking properties.
OR 870025	G1-1228/IBIS//YMH/HYS	OSU	Soft white	Winter	Awned, medium in height
OR 887196	TJB 368-251/BUC	OSU	Hard-white	Winter	Awnless, medium height, early maturity.
OR 3870039	VPM/MOS951//2*HILL	OSU	Soft-white	Winter	Awned, medium in height

Appendix Table 2. Components of three basal induction media for wheat anther culture: Modified N6 (MN6))[1], 85D12 (Liang's)[2], and Potato 2 (P2)[3].

COMPONENTS	MN6 mg/l	Liang's mg/l	P2 mg/l
Macroelements			
KNO_3	1415	1400	1000
$Ca(NO_3)_2.4H_2O$	-	-	100
$(NH_4)_2SO_4$	232	NH_4NO_3 300	-
KH_2PO_4	200	400	200
$CaCl_2.2H_2O$	83	150	KCl 35
$MgSO_4.7H_2O$	93	73	125
Microelements			
$MnSO_4.4H_2O$	5	11	
H_3BO_3	5	6	
KI	0.4	0.8	
$CuSO.H_2O$	0.0125	0.025	
$CoCl_2.6H_2O$	0.0125	0.025	
$Na_2MoO_4.2H_2O$	0.0125	-	
$Fe_2SO4.7H_2O$	32	27.8	27.8
Na_2EDTA	32	37.3	37.3
Vitamins			**Potato Extract[3]**
Glutamine	1000	-	-
Myo-inositol	300	-	-
Thiamine-Hcl	2.5	0.5	1.0
Glycine	1.0	6.0	-
Pyrodixine	0.5	0.5	-
Nicitinic Acid	0.5	1.5	-
Ascorbic Acid	0.5	-	-
Biotin	0.25	2.0	-
Calcium panthotenate	0.25	-	-
Hormones			
2,4-D	0.5	-	1.5
NAA	-	1.5	-
Kinetin	0.5	1.0	0.5
Sugar	Maltose 90gr	Sucrose 60gr	Sucrose 60gr
Gelrite/Agar: Semi-Solid	3/1	3/1	3/1
Liquid	-	-	-

[1] *Chu et al., 1990.* [2] *Liang et al., 1987.* [3] *Chuang et al., 1978*

Appendix Table 3. Componets of 190-2[1] and Modified B5 (MB5)[2] **regeneration media** for embryo/callus and embryos derived from wheat anther culture and wheat x maize crosses respectively.

MEDIUM COMPONENTS	190-2 mg/L	MB5 mg/L
Macroelements		
KNO_3	1000	2500
$Ca(NO_3)_2.4H_2O$	100	-
$(NH4)_2SO_4$	200	134
KH_2PO_4	300	$NaH_2PO_4.H_2O$ 150
$MgSO_4.7H_2O$	200	250
KCl	40	$CaCl_2$ 150
Microelements		
$MnSO_4.4H_2O$	8	10
$ZnSO_4$ .7H2O	3	2
H_3BO_3	3	3
KI	0.5	0.75
$CuSO_4.5H_2O$	-	0.025
$CoCl_2.6H_2O$	-	0.025
$Na_2MoO_4.2H_2O$	-	0.25
Iron		
$Na_2EDTA.2H_2O$	37.3	37.3
$FeSO_4.7H_2O$	27.8	27.8
Vitamins		
Glycine	3	-
Thiamine HCl	0.5	0.8
Pyrodixine	0.5	0.8
Nicotinic Acid	0.5	0.8
Hormones		
Kinetin	0.5	-
NAA	0.5	-
Sucrose	30 gr.	30 gr.
Agar	7gr.	7gr.

[1] *Zhuang and Jia, 1973.* [2] *Jensen, 1985.*

Appendix Table 4. Components of **Murashige and Skoog** (1962) **basal medium** used for the 'modified floret culture' technique in the wheat x maize cross method (Study 3. 2.1)

MEDIUM COMPONENTS	**mg/L**
Macroelements	
NH_4NO_3	1900
KNO_3	1650
$CaCl_2.H_2O$	440
KH_2PO_4	170
$MgSO_4$	370
Microelements	
$MnSO_4.7H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
Na_2MoO_4	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Iron	
$Na_2MoO_4.2H_2O$	37.3
$FeSO_4.7H_2O$	27.8
Vitamins	
Inositol	100
Glycine	2
Nicotinic Acid	0.5
Pyrodixine	0.5
Thiamine.HCl	0.1
Casein Hydrolysate	1gr.
Hormone 2,4-D	**0.1**
Sucrose	60gr.
Agar	7 gr.

Appendix Table 5. Observed mean values and least square means with standard errors(in parenthesis) for anther response and embryo/callus production frequencies obtained from six responsive genotypes of wheat (*Triticum aestivum L.*)through anther culture using three induction media (MN6, P2 and Liang's).

Wheat Genotype	Induction Medium	Anthers cultured	Responding anthers	Embryo/callus Production
		n	%	%
	MN6	536	6.0 (6.2 ± 1.1)	6.0 (5.0 ± 1.3)
Chris	P2	340	3.9 (3.7 ± 1.3)	4.6 (3.8 ± 1.5)
(Control)	Liang's	445	2.9 (3.6 ± 1.3)	4.7(4.2 ± 1.5)
	MN6	546	4.6 (4.6 ± 1.2)	4.6 (4.6 ± 1.4)
Hoff	P2	427	1.1 (0.9 ± 1.1)	1.1 (0.9 ± 1.2)
	Liang's	374	0.5 (0.1 ± 1.3)	0.0 (0.1 ± 1.3)
	MN6	526	4.9 (4.3 ± 1.1)	4.9 (4.1 ± 1.3
OR870025	P2	546	0.0 (0.4 ± 0.1)	0.0 (0.4 ± 1.3)
	Liang's	337	0.9 (0.6 ± 1.5)	0.9 (0.6 ± 1.7)
	MN6	420	4.2 (4.2 ± 1.0)	4.6 (4.5 ± 1.2)
OR887196	P2	363	0.8 (0.7 ± 1.2)	1.0 (0.8 ± 1.3)
	Liang's	452	0.3 (1.1 ± 1.1)	0.3 (1.0 ± 1.2)
	MN6	390	7.5 (7.9 ± 1.2)	7.5 (7.5 ± 1.4)
Stephens	P2	402	1.0 (1.1 ± 1.0)	1.3 (1.5 ± 1.2)
	Liang's	425	0.9 (1.0 ± 1.3)	0.9 (1.7 ± 1.5)
	MN6	554	6.2 (6.6 ± 1.0)	6.1 (6.4 ± 1.8)
OR3870039	P2	503	2.8 (2.5 ± 1.2)	3.7 (3.1 ± 1.3)
	Liang's	272	3.3 (2.8 ± 1.6)	3.3 (3.4 ± 1.8)

[1] *Since no anther response and consequently no embryo/callus were produced from anthers of Gene and Yamhill, no data are presented for these two wheat genotypes.*

Appendix Table 6. Observed mean values and least square means (in parenthesis) for regeneration frequencies of haploid plantlets (total, albino and green) obtained from six genotypes of wheat (*Triticum aestivum L.*) through anther culture using three induction media (MN6, P2 and Liang's).

Wheat Genotype	Induction Medium	Emb/call[2] cultured	HAPLOID PLANTLET REGENERATION		
			Total %	Albino %	Green %
	MN6	34	29.5 (27.1 ± 9.3)	28.9 (25.8 ± 7.0)	2.8 (3.2 ± 5.7)
Chris	**P2**	35	18.6 (17.9 ± 4.3)	0.8 (-4.2 ± 8.3)	17.7 (20.9 ± 6.9)
(control)	**Liang's**	27	10.2 (6.8 ± 11.1)	5.3 (6.6 ± 8.8)	4.9 (5.9 ± 6.8)
	MN6	25	46.6 (62.8 ± 10.2)	7.7 (5.6 ± 7.5)	34.5 (56.9 ± 6.5)
Hoff	**P2**	5	28.5 (28.8 ± 9.2)	0.0 (0.3 ± 6.7)	21.4 (23.5 ± 5.7)
	Liang's	3	0.0 (0.0 ± 2.2)	0.0 (0.0 ± 5.6)	0.0 (0.0 ± 2.8)
	MN6	22	26.2 (24.8 ± 10.3)	26.2 (20.7 ± 6.9)	0.0 (0.0 ± 5.8)
OR**870025**	**P2**	0	0.0 (1.8 ± 9.6)	0.0 (0.3 ± 6.7)	0.0 (0.0 ± 5.8)
	Liang's	3	0.0 (-0.7 ± 3.0)	0.0 (2.4 ± 2.2)	0.0 (0.0 ± 7.8)
	MN6	17	29.0 (23.4 ± 8.9)	12.0 (12.4 ± 6.5)	14.1 (11.8 ± 5.5)
OR**887196**	**P2**	3	25.0 (22.3 ± 9.9)	8.3 (4.2 ± 7.0)	9.3 (9.3 ± 6.1)
	Liang's	2	0.0 (3.8 ± 9.1)	0.0 (5.2 ± 6.0)	0.0 (-0.5 ± 5.6)
	MN6	23	59.9 (61.6 ± 10.0)	38.5 (38.2 ± 7.4)	22.0 (23.3 ± 6.2)
Stephens	**P2**	7	17.2 (22.3 ± 8.6)	15.6 (18.5 ± 6.3)	1.5 (3.8 ± 5.3)
	Liang's	3	0.0 (12.6 ± 10.8)	0.0 (2.1 ± 7.8)	0.0 (3.5 ± 6.6)
	MN6	21	23.1 (23.7 ± 8.7)	15.0 (10.0 ± 6.4)	10.0 (7.7 ± 5.3)
OR**3870039**	**P2**	6	13.8 (15.3 ± 9.8)	13.1 (7.7 ± 7.1)	0.0 (0.0 ± 6.0)
	Liang's	5	0.0 (-1.7 ± 1.0)	0.0 (1.8 ± 3.5)	0.0 (0.0 ± 6.0)

[1] *Since no embryo/callus was obtained from anthers of Gene and Yamhill, no plants were regenerated and no data was obtained for these two wheat genotypes* [2] *Emb/call: number of embryo/callus cultured*

Appendix Table 7 Observed mean values and least square means (in parenthesis) for production frequencies of haploid plantlets obtained from six responsive genotypes of wheat (*Triticum aestivum L.*) through anther culture using three induction medium (MN6, P2 and Liang's)

Wheat Genotype	Medium	Anthers cultured	HAPLOID PLANTLET PRODUCTION		
			Total	Albino	Green
	MN6	536	1.9 (1.7 + 0.8)	1.7 (1.5 + 0.5)	0.3 (0.2 + 0.6)
Chris P2	340	3.8	(3.3 + 1.3)	0.2 (0.2 + 0.6)	3.6 (3.6 + 0.6)
(Control)	Liang's	445	2.3 (2.2 + 1.0)	1.2 (1.2 + 0.6)	1.2 (1.2 + 0.7)
	MN6	546	2.2 (2.4 + 0.9)	0.4 (0.3 + 0.6)	1.7 (2.3 + 0.6)
Hoff	P2	427	1.1 (0.9 + 0.8)	0.0 (0.0 + 0.5)	0.6 (0.6 + 0.6)
	Liang's	374	0.0 (0.0 + 0.9)	0.1 (0.0 + 0.6)	0.0 (0.2 + 0.7)
	MN6	526	1.1 (0.8 + 0.8)	1.2 (0.8 + 0.6)	0.0 (0.0 + 0.6)
OR870025	P2	546	0.0 (0.1 + 0.8)	0.0 (0.1 + 0.5)	0.0 (0.0 + 0.6)
	Liang's	337	0.0 (0.1 + 0.1)	0.0 (0.1 + 0.7)	0.0 (0.0 + 0.8)
	MN6	420	1.3 (1.3 + 0.8)	0.7 (0.7 +0.5)	0.6 (0.6 + 0.5)
OR887196	P2	363	0.8 (0.6 + 0.9)	0.3 (0.1 + 0.5)	0.3 (0.3 + 0.6)
	Liang's	452	0.0 (0.4 + 0.8)	0.0 (0.8 + 0.6)	0.0 (0.1 + 0.5)
	MN6	390	3.9 (4.8 + 0.9)	2.7 (3.4 + 0.5)	1.2 (1.4 + 0.6)
Stephens	P2	402	0.8 (1.0 + 0.7)	0.7 (0.7 + 0.5)	0.1 (0.2 + 0.5)
	Liang's	425	0.0 (1.0 + 0.9)	0.0 (0.8 + 0.6)	0.0 (0.1 + 0.4)
	MN6	554	2.1 (2.5 + 0.7)	1.1 (1.5 + 0.5)	0.8 (0.8 + 0.5)
OR3870039	P2	503	1.7 (1.8 + 0.8)	1.5 (1.7 + 0.5)	0.0 (0.0 + 0.6)
	Liang's	272	0.0 (0.1 + 1.1)	0.0 (0.1 + 0.1)	0.0 (0.0 + 0.8)

1 Since no embryo/callus was obtained from anthers of cultivars Gene and Yamhill, no data for haploid plants was obtained for these genotypes.

Appendix Table 8. Mean square values (Type III) from the analysis of variance for wheat genotype, induction medium, and medium form on anther response, embryo/callus production, haploid plantlet regeneration and haploid plantlet production frequencies of six responsive genotypes of wheat (*Triticum aestivum* L.) obtained through anther culture.

SOURCE	DF	MEAN SQUARES TYPE III							
		Responding anthers	Embryo/callus Production	Haploid Plantlet Regeneration			Haploid Plantlet Production		
				Total	Albino	Green	Total	Albino	Green
Genotype (G)	5	38.6	49.9	3512.4*	1154.4*	3126.3*	17.6*	8.9*	9.5*
p (G)	50	22.9	32.8	1168	558.9	630.5*	7.4	3.5	3.1
Medium (M)	2	389.2**	364.1**	14126**	4715**	2816.6**	44.8**	21.8**	6.1*
G x M	1	9.3	13	917.4	508.2	1135.9	11.4	4.2	9.7*
Medium Form (F)	10	141.8**	180.1**	3768.1	4170.9	93.1	31.8	12.9	4.7
G x F	5	15.8	19.9	288.3	149.5	23.3	10.5	2.2	3.8
M x F	2	4.8	10.3	1380.4	515	269.3	0.1	0	0.4
G x M x F	10	37.1*	45.6	1052.1	514	316.2	8.2	2.6	4
Error	147	20.5	20.5	1052.1	563	399.6	8.7	3.4	4.5

*, ** *Significant at the 0.05 and 0.01 probability levels respectively.*

[1] *Mean Squares taken from original percentage data.*

Appendix Table 9. Observed mean values and least square means (in parenthesis) for seed set, embryo induction, embryo production, haploid plant regeneration and production frequencies obtained from seven genotypes of winter wheat (Triticum aestivum L.) through wheat x maize cross method using two techniques:'Modified floret culture' and 'Direct culture' with hybrid B14/CI64 as maize pollen donor.

		WHEAT GENOTYPES					
		OR 870025 %	OR 3870039 %	OR 887196 %	Yamhill %	Gene %	Stephens %
Seed Set	MFC	49.9 (49.9 + 8.1)	49.5 (49.5 + 6.9)	60.8 (60.8 + 7.4)	59.1 (59.1 + 8.01)	36.4 (36.4 + 6.9)	49.0 (49.0 + 6.9)
	DC	86.5 (86.5 + 8.1)	77.1 (77.1 + 6.9)	78.7 (78.7 + 7.4)	70.6 (70.6 + 8.1)	90.4 (90.4 + 6.9)	87.9 (87.9 + 6.9)
Embryo Induction	MFC	5.4 (5.4 + 5.6)	7.6 (7.6 + 4.7)	18.6 (18.6 + 5.1)	16.0 (16.0 + 5.6)	7.7 (7.7 + 4.7)	11.2 (11.2 + 4.7)
	DC	11.9 (11.9 + 5.6)	12.7 (12.7 + 4.7)	14.3 (14.3 + 5.1)	20.1 (20.1 + 5.6)	18.9 (18.9 + 4.7)	20.0 (20.0 + 4.7)
Embryo Production	MFC	2.0 (2.0 + 3.5)	13.1 (13.1 + 3.7)	13.1 (13.1 + 3.7)	10.3 (7.9 + 4.9)	2.5 (2.5 + 3.5)	7.2 (7.2 + 3.5)
	DC	10.1 (10.1 + 3.5)	10.3 (10.3 + 3.7)	10.3 (10.3 + 3.7)	12.8 (12.8 + 3.9)	16.0 (16.5 + 3.5)	15.5 (15.5 + 3.5)
Plant Regeneration	MFC	8.3 (8.3 + 14.8)	42.8 (42.8 + 13.7)	23.6 (23.6 + 14.2)	60.0 (60.0 + 16.2)	42.8 (42.8 + 13.7)	25.0 (26.2 + 13.1)
	DC	50.0 (50.0 + 14.8)	78.5 (78.5 + 13.1)	55.0 (55.0 + 14.2)	67.3 (67.3 + 16.2)	66.7 (66.7 + 13.7)	66.7 (66.7 + 13.7)
Plant Production	MFC	0.6 (0.6 + 1.9)	1.6 (1.6 + 1.7)	4.7 (4.7 + 1.9)	5.4 (4.5 + 2.1)	1.9 (1.9 + 1.7)	2.3 (2.3 + 1.8)
	DC	7.1 (7.1 + 1.9)	6.8 (6.8 + 1.7)	6.4 (6.4 + 1.9)	8.5 (5.4 + 2.1)	10.4 (10.4 + 1.7)	13.2 (13.2 + 1.8)

MFC: 'Modified floret culture' DC: 'Direct culture'

Appendix Table 10. Observed values for organogenesis and regeneration of green haploid plantlets obtained from embryos of seven genotypes of winter wheat (Triticum aestivum L.) derived from crosses of wheat x maize using two techniques: 'modified floret culture' and 'direct culture' with hybrid B14/CI64 as maize pollen donor.

WHEAT GENOTYPE	WHEAT x MAIZE TECHNIQUE	Means (spikes)	Embryos cultured	Embryos lost	No Regeneration	Callus	Roots	Callus/roots	Incomplete Growing	Complete Green Plants
		n	n	n	n	n	n	n	n	n
Stephens	MFC	7	8		4		1			3
	DC	7	18	1		4			1	12
Yamhill	MFC	5	8		4					4
	DC	5	21	2	4	3				14
OR 887196	MFC	6	19	2	8		1		1	7
	DC	6	12	1		2				9
OR 3870039	MFC	7	7		3	2				2
	DC	7	19		2			2	1	14
Gene	MFC	7	5		2		1			2
	DC	7	26	1	1	3	1	3		17
OR 870025	MFC	5	8	1	5			1		1
	DC	5	13	1		3				8
Total	MFC	37	55	3	26	2	2	1	1	19
	FC	37	109	8	7	10	3	6	2	74
	MFC: Modified floret culture DC: Direct culture									

Appendix Table 11. Observed mean values and least square means with standard errors (in parenthesis) for **seed set, embryo induction** and **embryo production** frequencies obtained from seven genotypes of winter wheat (*Triticum aestivum L.*) through wheat x maize crosses method using two dose levels of hormone 2,4-D (20 and 100 ppm) and inbred maize A188 as pollen donor parent.

Wheat Genotype	2,4-D dose	Florets pollinated	Seed Set		Seeds	Embryo Induction[1]	Embryo Production[2]
		n	%		n	%	%
Stephens	20	340	87.7	(88.0 ± 1.8)	293	4.8 (4.1 ± 2.2)	4.3 (3.6 ± 2.5)
	100	396	86.5	(90.6 ± 1.6)	327	11.3 (10.7 ± 2.7)	9.1 (9.2 ± 2.4)
Gene	20	488	92.3	(91.2 ± 1.9)	446	16.0 (15.6 ± 2.9)	14.9 (14.4 ± 2.6)
	100	491	90.2	(89.7 ± 1.8)	478	14.7 (13.1 ± 2.8)	13.8 (12.4 ± 2.5)
Yamhill	20	548	86.2	(86.4 ± 1.6)	469	16.8 (16.8 ± 2.5)	14.3 (14.2 ± 2.2)
	100	503	88.6	(90.1 ± 1.7)	444	16.3 (15.6 ± 2.7)	15.0 (14.3 ± 2.4)
Hoff	20	375	94.5	(92.7 ± 1.8)	345	11.7 (11.9 ± 2.9)	10.2 (10.5 ± 2.6)
	100	390	87.3	(88.9 ± 1.8)	338	12.5 (12.1 ± 2.8)	10.9 (10.6 ± 2.5)
OR 870025	20	565	93.0	(85.9 ± 1.6)	449	9.2 (9.3 ± 2.5)	7.2 (7.5 ± 2.2)
	100	625	81.3	(85.0 ± 1.5)	499	5.8 (5.2 ± 2.3)	3.9 (3.5 ± 2.1)
OR 887196	20	546	88.2	(88.3 ± 1.4)	533	13.7 (14.2 ± 2.2)	11.6 (12.0 ± 1.9)
	100	554	90.2	(90.4 ± 1.5)	470	22.8 (23.9 ± 2.3)	22.5 (23.0 ± 2.0)
OR 3870039	20	289	86.4	(89.0 ± 2.3)	239	3.0 (4.9 ± 3.5)	2.5 (4.5 ± 2.6)
	100	394	86.4	(86.2 ± .9.0)	335	10.7 (10.1 ± 2.9)	9.7 (9.2 ± 2.4)

[1] *Embryo induction frequencies : based on percentages taken from the total number of seeds dissected.*
[2] *Embryo production frequencies: based on percentages taken from the total number of florets pollinated.*