Fusarium Head Blight resistance in wheat

using the in vitro androgenic approach

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Aan mijn ouders

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Bibliographic abstract

Fusarium head blight (FHB) in wheat in North-West Europe is mainly caused by the fungus *Fusarium culmorum*. Toxin contamination of the seeds is one of the greatest hazards of the pathogen. Introducing haploidisation and in vitro selection into the wheat breeding process might accelerate the release of new FHB-resistant cultivars. For anther culture ability, inheritance studies on crosses between wheat cultivars revealed that additive effects accounted for the majority of the genetic variation and that up to six green plants per 100 anthers could be achieved. Testing different types of plant material on medium containing FHB-toxins showed that the toxin Deoxynivalenol inhibited growth of all types. The FHB-resistance of the doubled haploid (DH) genotypes, obtained after colchicine treatment of anther culture derived haploids, was assessed in the field for four consecutive years. Several DH-lines were identified that showed a resistance greater than the most resistant parent, thus offering good possibilities for practical wheat breeding.

List of abbreviations

ADON	Acetyl-deoxynivalenol
AR	Agarose Ring
CIF	Callus Induction Frequency
CIRA	Callus Induction per Responding Anther
CPI	Culture Plate Insert
CPRO	Centre for Plant Breeding and Reproduction Research
2,4-D	2,4 Dichlorophenoxyacetic acid
DAPI	4',6-Diamidino-2-phenylindole
DH	Doubled Haploid
DLO	Agricultural Research Service (Dienst Landbouwkundig Onderzoek)
DON	Deoxynivalenol
EIF	Embryo Induction Frequency
ERA	Embryos per Responding Anther
FDA	Fluorescein Diacetate
FHB	*Fusarium* Head Blight
GCA	General Combining Ability
IAA	Indole-3-Acetic Acid
MCS	Multicellular Structures
MS	Murashige & Skoog Medium
NGC	Nederlands Graan Centrum, Stichting
NIV	Nivalenol
P2	Potato-2 medium
RA	Responding Anthers
REML	Residual Maximum Likelihood
SCA	Specific Combining Ability

Contents

Account

Most of the results presented in this thesis have been published elsewhere, or will be published in the near future. This thesis is based on the following publications:

Bruins MBM, Karsai I, Schepers J & Snijders CHA (1993) Phytoxicity of deoxynivalenol to wheat tissue with regard to in vitro selection for Fusarium head blight resistance. Plant Science 94: 195-206.

Bruins MBM & Snijders CHA (1993) Effect of sucrose concentration on viability and sporophytic development of mechanically isolated wheat (*Triticum aestivum* L.) microspores. in: Li ZS & Xin ZY (eds.), Proceedings of the eighth International Wheat Genetics Symposium, 20-25 July 1993, Beijing, China, pp 679-683.

Bruins MBM & Snijders CHA (1995) Inheritance of anther culture derived green plantlet regeneration in wheat (*Triticum aestivum* L.). Plant Cell, Tissue and Organ Culture 43: 13-19.

Bruins MBM, Rakoczy-Trojanowska M & Snijders CHA (1996) Isolated microspore culture in wheat (*Triticum aestivum* L.); the effect of co-culture of wheat or barley ovaries on embryogenesis. Cereal Research Communications 24(4): 401-408.

Stellingen

1. Succes in antherencultuur van tarwe wordt, zoals veel auteurs beweren, niet bepaald door de hoeveelheid embryo's en calli die gevormd worden, maar meer door het aantal antheren dat een androgenetische respons geeft.
   Dit proefschrift

2. In tarwe is voldoende genetische variatie tegen *Fusarium* aarziekte aanwezig.
   Dit proefschrift

3. Om de resistentie tegen *Fusarium* aarziekte zo compleet mogelijk te maken verdient het aanbeveling de resistentie-genen uit de verschillende resistentiebronnen te accumuleren in de toekomstige tarwecultivars.
   Dit proefschrift

4. *Fusarium* aarziekte is een van de meest gevaarlijke pathogenen op tarwe en mais, twee van de drie belangrijkste voedselgewassen in de wereld.
   Dit proefschrift

5. Research on *Fusarium* Head Blight concerns us all.
   This thesis

6. Indien door gebrek aan financiering onderzoek wordt stopgezet, hoeft dit niet te wijten te zijn aan de kwaliteit van het betreffende onderzoek, maar kan ook een gevolg zijn van een zekere 'modegevoeligheid' van de financierende instanties.

7. De uitspraak "Hoe mooier het jaarverslag, des te lager de winst" gaat gelukkig niet altijd op.
   Marc van Opijnen, Intermediair 32 (37), 13 september 1996.

8. Bij het schrijven van een nieuw onderzoeksvoorstel kan het uitvoeren van een octrooirecherche, naast het uitvoeren van de gebruikelijke literatuurrecherche, de aanvrager een hoop ellende besparen.

9. Leden van een amateurvereniging onderscheiden zich vaak niet van professionals door een verminderde inzet of een lagere kwaliteit van het geboden product, maar veel meer door het feit dat zij er niet voor betaald worden.

10. Kunst is de vertekening van de werkelijkheid die ons meer de werkelijkheid doet realiseren.

11. Wie van tuinieren houdt, geeft blijk van een positieve kijk op de toekomst.

12. Nothing is more revealing than movement
    Martha Graham, 1935.

Stellingen behorende bij het proefschrift: "*Fusarium* Head Blight resistance in wheat using the in vitro androgenic approach", door M.B.M. (Marcel) Bruins.

Wageningen, 31 maart 1998.

Chapter 1: General introduction

Underlying thesis presents the development of an in vitro method to improve resistance to *Fusarium* Head Blight (FHB) resistance in wheat (*Triticum aestivum* L.). This important disease results in serious yield and quality losses throughout the world and can only be controlled by means of resistance. However, selection for resistance is at present only feasible under field conditions, and the development of new resistant cultivars, especially when using sources of exotic germplasm containing resistance genes, takes many years. The development of an efficient in vitro selection technique, combined with the routine application of androgenesis and subsequent doubling of the chromosomes, would make the development of resistant varieties more feasible.

This thesis describes the experiments to develop such methodologies in relation to eachother, since if in vitro selection could be applied to the haploid stage of androgenesis, such a combination would result in a very considerable gain in efficiency and development time of resistant varieties.
In this first chapter the present knowledge about the disease in wheat, and the state of the art for both in vitro selection for disease resistance and in vitro androgenesis are discussed.

1.1 *Fusarium* Head Blight in wheat

At present, wheat is grown on about 230 million hectares in the world, being the most widely grown food crop in the world. Wheat diseases, both in temperate and tropical regions, can be severe, and because wheat is one of the most important food sources in the world, it is important to find control measures. For economic and environmental reasons, host plant resistance is the most appropriate and sustainable disease control method (Dubin & Rajaram 1996). FHB is mainly caused by the fungi *Fusarium culmorum*, (W.G. Smith), perfect state unknown and *Fusarium graminearum* Schwabe, with perfect state *Gibberella zeae* (Schw.) (Mesterhazy 1995), while in the cooler regions of North-Western Europe, *F. culmorum* dominates. The inoculum of the fungi is seed-borne or soil-borne and infected plant material of cereals and grasses can be used as such. The fungi can also cause seedling blight and root, crown and foot rot. Light brown watersoaked spots on the glumes of the spike are the first symptoms of the disease. Subsequently, in a short period, the infected spikelets dry up and look like ripe spikelets in an otherwise green ear. Infection of the crop with these fungi can cause considerable yield losses, more than infection by other fungal species like *Septoria* glume blotch (*Stagonospora nodorum* Berk.)(Tvaruzek *et al.* 1996). It was also found that gluten from wheat kernels, damaged by *Fusarium*, contained a lower proportion of

glutenins than did healthy kernels. However, no qualitative and quantitative differences in gliadins attributable to *Fusarium* damage were apparent. The effect on loaf volume appeared to be cultivar dependent, ranging from cultivars that were virtually unaffected to cultivars that showed a drastic decline in loaf volume (Dexter *et al.* 1996). Infection with the pathogen may also contaminate the seeds with toxins, e.g. deoxynivalenol (DON), nivalenol (NIV) and acetyl-deoxynivalenol (ADON), with the isomers 3-ADON and 15-ADON. Contents of DON of up to 48 mg kg^{-1} wheat seed (Snijders & Perkowski 1990) and up to 500 mg kg^{-1} maize seed (Lew *et al.* 1991) have been found. These mycotoxins have phytotoxic effects on wheat plants or parts thereof. Besides phytotoxic effects, these toxins are also hazardous to humans and animals.

1.1.1 *Toxicity*

DON, ADON and NIV belong to the group of trichothecene mycotoxins, which are a group of over 148 structurally similar fungal metabolites produced by species of *Fusarium* and related fungi, and are capable of producing a wide range of toxic effects. DON is found worldwide in crops and is an important safety issue because of its toxicity and because it is a very common contaminant of grain. DON is in some reports the only detected toxin (Snijders & Perkowski 1990), but in any case the most abundant toxin in grain (Abbas *et al.* 1989, Chelkowski 1989, Langseth & Elen 1996, Logrieco *et al.* 1990, Maier & Oettler 1996) compared with ADON and NIV, which are however, more toxic. The two isomers of ADON are about twice as toxic as DON (Mirocha *et al.* 1989) and NIV is even about 10 times more toxic than DON (Joffe 1986). Because DON is by far the most important safety issue, the research reported in this thesis focused on DON. Mycotoxins in the trichothecene class are true secondary metabolites and are produced when the fungus suffers a limitation of a specific nutrient, in this case nitrogen (Miller & Blackwell 1986).

At the cellular level in humans, as well as in animals and plants, the main toxic effect is the inhibition of protein synthesis via binding to the ribosome. In animals, moderate to low ingestion of toxin can cause a large number of not clearly defined effects, which are associated with reduced performance and immune function. The most evident effect at low dietary concentrations is the reduction in food intake (anorexia), while higher doses cause vomiting. It is also known that DON can alter brain neurochemicals. Animals that are fed on low to moderate doses are able to recover from the initial weight losses, while higher doses induce more long-term changes in the feeding behaviour. At lower dosages of DON, hematological, clinical and immunological changes are also temporary and decrease when compensatory and adaptation mechanisms are established. Due to differences in the metabolism of DON, swine are more sensitive to DON than mice, poultry and ruminants, with males being more sensitive than females (Rotter *et al.* 1996).

DON alters the functioning of the normal immune system, where the toxin can be immunosuppressive or immunostimulatory, depending on the dose and duration of the exposure.
Immunosuppression can be explained by the inhibition of translation and immunostimulation can be related to interference with normal regulatory mechanisms.

In vivo, DON suppresses the normal immune response to pathogens and at the same time induces auto-immune like effects which are similar to human immunoglobulin A (IgA) nephropathy. Other effects include the superinduction of cytokinine production by T-helper cells (in vitro) and the activation of macrophages and T-cells to produce a proinflammatory cytokinine wave that is analogous to that found in lipopolysaccharide-induced shock (in vivo). These effects have been intensively studied in mice, but several investigations suggest that immunotoxic effects are also likely in domestic animals (Rotter *et al.* 1996).

Similar effects can also occur in humans. Immediate symptoms of poisoning with a member of the trichothecene toxin group are: vomiting, irritation of the skin, diarrhea, food refusal, hemorrhages, neural malfunctioning, miscarriages and eventually death (Joffe 1986, Kuiper-Goodman 1985). Also the suppression of the immune system is an important side effect that should not be disregarded (Miller & Atkinson 1987). Chronic intake of small amounts of these toxins, direct skin contact with infected plants (Snijders *et al.* 1996) and even inhalation of the spores of these fungal species should be avoided (Trenholm *et al.* 1989).

Tolerable daily intakes for DON were estimated by Kuiper-Goodman (1985) for adults at 3.0 μg DON kg^{-1} body weight and for infants at 1.5 μg kg^{-1} body weight, respectively. Current guidelines for tolerable daily intake of DON are in Canada 2 ppm in uncleaned soft wheat and 1 ppm for infant foods. In the USA, tolerance limits are 1 ppm for bran, flour and germ intended for human consumption (Trucksess 1995). As an example we calculated that, based on these guidelines, the wheat crop in 1979 and 1982 in the Netherlands contained DON-concentrations well above these limits, clearly underlining the need for more resistant cultivars, with lower contamination levels of the grain.

1.1.2 *Resistance*

From a crop husbandry point of view, no practical chemical treatments are effective against this disease in wheat (Bai & Shaner 1994, Milus & Parsons 1994). Fungicide treatment of seeds did generally improve germination and emergence but efficacy is dependant on cultivar and temperature at germination (Gilbert & Tekauz 1995). Attempts to avoid FHB by varying planting date were not succesfull (Wiersma *et al.* 1996) and it seems that the most efficient way to prevent this disease is to grow resistant cultivars. In wheat, a wide range of genetic variation for FHB-resistance has been found, from resistant to very susceptible, but immunity has not yet been found. The number of genes involved have been estimated from one to six (Liao & Yu 1985a, Snijders 1990a). These resistance genes are thought to be located on the 4A, 4D, 5A, 7A and the 7B chromosome of bread wheat. The effects of the individual genes can be quite large and several publications indicate an additive inheritance, with the dominance effect also being statistically significant (Liao & Yu 1985a &1985b, Snijders 1990a). Takeda & Wu (1996) performed both diallel analysis and top cross analysis and showed that the direction and degree of dominance varied with parental combinations of diallel and top-crosses, clearly indicating that the genetic system behind the resistance

is not simple. Also on the fungal side, the genetic structure is not completely elucidated. Miedaner *et al.* (1996) tested 42 isolates of *F. culmorum* on a winter rye population. All isolates appeared to be pathogenic, but differed in their ability to cause disease (aggressiveness). Significant isolate-environment interaction was found, but accounted only for 14% of the total variance, and the correlations for aggressiveness across environments ranged from 0.6 to 0.8 (p=0.01). However, the aggressiveness of the isolates did not depend on their geographic origin (Eeuwijk *et al.* 1995), year of isolation, host species habitat or host organ from which they were isolated. It appeared that *F. culmorum* isolate IPO 39-01, which was used in most of the CPRO-DLO research and which was isolated in 1966, was still one of the three most aggresssive isolates. On the basis of RAPD's, cluster and principle coordinate and PCR marker-based analyses, Miedaner *et al.* (1996) concluded that aggressiveness of *F. culmorum* was inherited as a complex trait.

Breeders could employ 'passive' and 'active' resistance mechanisms in their attempts to create resistant varieties. 'Passive' resistance encompasses components as plant height, presence of awns, spikelet density and escape mechanisms, whereas 'active' mechanisms comprises physiological processes (Crute *et al.* 1985) e.g. resistance against initial or kernel infection or to spread of the pathogen (Schroeder & Christensen 1963), resistance to the toxins by decomposing them (Miller *et al.* 1985, Miller & Arnison 1986, Snijders & Perkowski 1990) and tolerance. Tolerance is generally defined as the ability of the plant to endure the effects of levels of parasitic infection and disease, which, if they occurred at equivalent levels in other plants of the same or similar species, would cause greater impairment of growth or yield and is in this way an active resistance mechanism (Clarke 1986, Mesterhazy 1995). Genotypes with awns appeared to be more susceptible to head blight when tested under natural epidemic conditions in the field, but this trait did not influence head blight severity in artificial inoculations. Dwarf genotypes were more severily infected by head blight than tall genotypes under natural conditions, but genotypes of different plant height classes were similarly susceptible after artificial inoculations. A high positive correlation between head blight resistance and plant height or spike length was found (Liao & Yu 1985b). If selection of dwarf and awned genotypes cannot be avoided, the higher susceptibility caused by awns and dwarfness under natural epidemic conditions can be decreased by a higher level of physiological resistance like preventing or slowing down the initial infection, as variability in physiological resistance is available. In later generations, traits like percentage of seed infection or tolerance can be studied by additionally measuring yield reduction and grain weight. Stability of disease reaction appears to be connected with resistance level, the most resistant genotypes are the most stable, and the most susceptible ones tend to have more unstable reactions in different epidemic conditions (Mesterhazy 1995).

Visual assessment of the head blight infection after artificial inoculation is the most frequently used way to screen for resistance (Mesterhazy 1995) and is, on the basis of heritability estimates, considered better than screening for yield reduction (Snijders 1990c). Significant transgression of *Fusarium* resistance of the

parental lines was observed in progenies and were considered to be genetically fixed (Snijders 1990b, Takeda & Wu 1996). Accumulation of resistance genes is therefore possible and this offers opportunities for wheat breeding. The resistance can be transferred from unadapted genotypes to commercial cultivars in a backcross breeding programme.

Resistance to FHB appears to be based on several, probably minor genes and these resistance genes can be combined and/or can be transferred to genotypes with good agronomical values like high yield or quality. This is usually carried out using a backcross programme. Using doubled haploids can accelerate the transfer of the resistance genes by homozygotisation of the F_1-lines with the desired resistance genes and backcross them again. The best lines from the first backcross generation can after haploidisation produce genotypes with the desired agronomical traits and resistance. Such a recurrent haploid selection programme is in theory an efficient procedure to transfer quantitative traits to cultivars and shortens the process of the creation of new cultivars in self-pollinated species like wheat (Foroughi-Wehr & Friedt 1984, Foroughi-Wehr & Wenzel 1990, Gallais 1988, Griffing 1975).

1.2 In vitro androgenesis

1.2.1 Introduction

Androgenesis is the outgrowth of the male reproductive cell into a haploid plant, and with gynogenesis, the female reproductive cell grows out. Androgenesis and gynogenesis are abnormalities occurring in natural fertilisation processes. Haploid plants formed in this way have been found in more than 100 angiosperm species and since we have learned how to stimulate this process, gametogenesis has become a valuable tool in breeding programmes. In most species, such as the cereals, androgenesis is the most efficient way of producing haploid plants (Palmer *et al.* 1996), however in some species gynogenesis is more efficient, e.g. in sugar beet (Pedersen & Keimer 1996) and in onion (Keller & Korzun 1996).

In normal pollen development, the young microspores originate from the pollen mother cells after meiotic divisions. The term microspore is used for male reproductive cells that contain only one nucleus. Normally the microspores undergo a pollen mitosis in which the nucleus divides asymmetrically and binucleate pollen is formed. In species with trinucleate pollen, one of the nuclei divides once again in the so called second pollen mitosis. This process is called gametophytic development. In androgenesis in microspores, symmetric divisions of the nucleus take place. This process is called sporophytic development. To induce this change in development, usually a form of stress is needed.

Androgenesis can be induced in two ways: with anther culture and with microspore culture.
In anther culture, the complete anther with immature microspores and pollen grains is placed on a nutrient medium. A form of stress, such as low temperature or starvation, was already applied to the plant beforehand or is applied later after transfer of the anthers to the medium. Part of the pollen grains start

to divide in a sporophytic way and form an embryo or callus. After a certain period, four to six weeks in wheat, the haploid embryos and/or callus burst out of the anther. The induction frequency (= percentage embryos per 100 excised anthers) differs from crop to crop and from genotype to genotype. The callus or the embryos can be made to regenerate into plants. Some species like *Nicotiana tabacum* or *Datura innoxia* have a fast outgrowth to plants. It takes three to four months from anther inoculation to a mature plant. With *Paeonia* the first step of anther inoculation until the appearance of the first plantlets takes four to six months (Sunderland 1974).

With microspore culture, the immature microspores are isolated from the anther and are placed in or on nutrient medium. The isolation can be carried out in two ways: mechanically or by the so called 'shed pollen' technique. Stress is not applied to the anthers, but to the isolated microspores. After isolation and stress treatment, the microspores are cultured under non-stress conditions. Embryogenesis takes place in the same way as in anther culture.

Most research has been carried out on anther culture and this technique is most widely used in practice. Anther culture is an attractive method to produce haploid plants, because the procedure is relatively simple, the induction into sporophytic divisions is relatively easy and in some species high induction frequencies occur. One of the advantages of anther culture above microspore culture can be the in some species berieficial influence of the anther wall on sporophytic outgrowth (Ouyang 1986).

1.2.2. History of androgenesis

In 1953 for the first time it was achieved to induce haploid callus from mature pollen grains of *Ginkgo biloba* (Tulecke 1953). In the years thereafter pollen of more and more species were cultured. However, scientists were only capable of producing undifferentiated callus, that could not be induced to form shoots or roots. In 1964, the first embryos were formed in anther culture of *Datura innoxia* and haploid plants were regenerated (Guha & Maheshwari 1964). In the following years, success in the field of anther culture was achieved in a number of important crops: in 1967 in tobacco, 1968 in rice and in the 1970-ties in barley, wheat, triticale, rye, maize, *Brassica* spp., tomato, potato, *Capsicum*, *Asparagus*, eggplant, and *Pelargonium* (Sunderland 1974, Wenzel *et al.* 1985). At the moment it is possible to produce haploid plants via androgenesis in more than 247 species, belonging to more than 88 genera in 34 families (Sangwan & Sangwan-Norreel 1990) and these numbers are still increasing.

The first doubled haploid (DH) wheat cultivars that were accepted on variety lists were Jinghua no. 1 in China (Hu 1986) and Florin in France (De Buyser *et al.* 1987). In China a large number of DH wheat cultivars has been released in agriculture.

In microspore culture, the first successes were obtained with the 'shed pollen' technique, usually preceded by a preculture in the anthers. Plants were produced in tobacco (Nitsch & Norreel 1974, Sunderland & Roberts 1977), rice (Chen *et al.* 1980, 1981), wheat (Wei 1980, 1982), barley (Wei *et al.* 1986) and maize (Coumans *et al.* 1989, Pescitelli *et al.* 1989, 1990). For most crops, mechanical isolation

of microspores has not yet been studied thoroughly. Most research and subsequent success on isolated microspore culture of cereals has been carried out on barley. Also in maize and rice the technique proved to be relatively succesful, whereas for wheat only a few succesful reports exist (Jähne & Lörz 1995).

1.2.3. Applications of androgenesis

With androgenesis haploid plants are formed. Doubling of the number of chromosomes can take place spontaneously in culture, or can be induced by chemical agents (colchicine, oryzaline). The DH-plants are extremely useful in plant breeding research. Important applications are:

- *The fast production of homozygous plants.*
  The doubled haploid plants are completely homozygous and find their equivalent in inbreeding lines. A long inbreeding process is no longer necessary, and a breeding programme can be shortened substantially.
- *Genetic analysis at crossings.*
  Recessive alleles in the DH-material are no longer masked by dominant alleles, and in this way DH-plants present a more accurate picture of the variation in the parental gametes. These recessive alleles can be selected for directly, because of their homozygous state.
- *Genetic analysis at the DNA-level.*
  The DNA of both sets of homologues is the same and this facilitates genetic studies with DH-lines, e.g. molecular marker research and linkage studies (Bohuon *et al.* 1996, Dion *et al.* 1995, Kjaer *et al.* 1995, Lefebvre & Palloix 1996, Melchinger 1990). Specific traits can more easily be transferred.
- *Genetic modification through transformation or in vitro selection.*
  Microspores or microspore derived embryos of cereals can be subjected to an in vitro selection system (Fadel & Wenzel 1993, Sari-Gorla *et al.* 1994) or can be transformed by direct DNA-transfer (Chair *et al.* 1996, Harwood *et al.* 1995, Jardinaud *et al.* 1995, Loeb & Reynolds 1994, Ritala *et al.* 1995) or by an *Agrobacterium* vector system (Creissen *et al.* 1990). The *Agrobacterium* system, however, is still not working optimally in gramineous species (Jähne *et al.* 1995).

Although there are many advantages, there are still many problems that limit the use of the technique:

- Up till now, the method has been restricted to a few families like the *Solanaceae*, *Cruciferae* and the *Gramineae*. Specifically species in the *Leguminosae* and the *Compositae* family are still quite recalcitrant (Sangwan & Sangwan-Norreel 1990, Theiler-Hedtrich & Hunter 1996). Also in woody angiosperms and in gymnosperms, progress is slow (Baldursson & Ahuja 1996).
- There is a strong genotype effect, some genotypes being much more amenable to androgenesis than others. This limits the application to a restricted number of genotypes of a species.
- Up tot 100-900 embryos per anther can be achieved (Dunwell & Cornish 1985), but estimates of the number of microspores per anther range from 10,000 to 17,000 or more (Chuong & Beversdorf 1985,

Pan *et al.* 1991) indicating that the frequency of embryo formation is relatively low. This means that on average only a small percentage of the microspores undergoes androgenic development, and of this small part, only a small proportion regenerates into mature green plants, due to loss of plants during the regeneration process. Sometimes no plants regenerate at all (Regner 1996).

- It is stated that there is premature and possibly unwanted selection pressure at the microspore level (Devaux 1992, Foisset & Delourme 1996).
- In certain species structurally and physiologically abnormal pollen embryos occur (Lashermes *et al.* 1994, Schumann *et al.* 1991).
- In certain species the regenerants are genetically instable (Logue 1996).
- In certain species a large number of non-haploids is formed. This latter phenomenon can be caused through fusion of nuclei, outgrowth of anther wall cells, or through endopolyploidisation (Chen *et al.* 1984, Chu *et al.* 1978, Hidaka & Omura 1989, Lee & Chen 1987, Rose *et al.* 1987, Vuteva & Zagorska 1990).
- In cereals albino regenerants are a major problem. 20-90% Albino regenerants are reported in wheat (Andersen *et al.* 1987, Zhou & Konzak 1992), 30-99% in barley (Powell 1988, Kasha *et al.* 1990), 20-60% in rice (Chen 1986, Quimio & Zapata 1990) and 70-80% in triticale (Charmet & Bernard 1984).

1.2.4. Anther culture in wheat

The method used for anther culture of wheat is comparable to that used in most other crops. In the following section, the method for wheat will be described in more detail, and the factors important for the success of androgenesis are identified:

- *The genotype of the donor material.*

  Embryo formation and plant regeneration are to a great deal genotype dependent. In wheat heritabilities for both traits were estimated at 0.6 to 0.7 (Lazar *et al.* 1984). The genes responsible for in vitro androgenesis are located on the nuclear genome and not on the cytoplasmic genomes (Bullock *et al.* 1982). Genotypes with the 1B/1R wheat-rye translocation chromosome all have a higher *in vitro* response (Foroughi-Wehr & Zeller 1990, Henry & De Buyser 1985, Müller *et al.* 1990). For regeneration, regions on chromosomes 2D and 5A are involved in green plant regeneration (De Buyser *et al.* 1992, Szakacs *et al.* 1988) while regions on chromosomes 1B (long arm) and 5B are involved in albino plant regeneration (Agache *et al.* 1989, De Buyser *et al.* 1992).
- *Growth of the donor material.*

  The growing conditions of the donor plants preceding the moment of anther harvest have a large influence on embryo formation. In wheat, anthers from field plants in general give a better response than anthers from plants grown in the greenhouse (De Buyser & Henry 1986, Ouyang 1986). However, the growing conditions of field plants can not be controlled, and this may explain why some authors report a higher and more reliable response from greenhouse plants (Bjornstad *et al.* 1989).

As no microscopic difference in morphology or developmental stage can be found between microspores of different seasons, it is stated that the growing conditions probably influence the endogenous status of the donor plants (Wenzel & Foroughi-Wehr 1984). Critical factors are: light intensity, photoperiod, nutrient supply and CO_2 concentration.

- *Developmental stage of the microspores.*

  Embryos can be obtained from microspores that are in the meiotic stages until the binucleate stage in pollen development. In wheat the late uninucleate stage gives the highest number of embryos (He & Ouyang 1984).

- *Stress factors.*

  To achieve the shift from a gametophytic to a sporophytic development, various stress treatments are possible: low temperature (4-7°C), high temperature (32-35°C), starvation, centrifugation, spraying with ethrel, radiation, lowering of the atmospheric pressure or osmotic stress (Wenzel & Foroughi-Wehr 1984, Sangwan-Norreel *et al.* 1986). However, the reported results achieved with these methods are in many cases not reproducible by other research groups or contradictory (Sunderland 1974, 1983). Most applied in anther culture of wheat is a low temperature treatment: the cut wheat spikes are stored on tap water for a period ranging from 3 to 14 days at a temperature of 4°C to 7°C (Datta & Wenzel 1987, Lazar *et al.* 1985). However, several publications report an inhibitory effect of this cold pretreatment on anther response and callus productivity (Ghaemi *et al.* 1995, Karimzadeh *et al.* 1995, Marsolais *et al.* 1984, McGregor & McHughen 1990).

- *Induction medium.*

  The optimal medium varies between species and also within the species. For wheat the Potato-2 medium (P2), to which a potato extract has been added, gives good results (Chuang *et al.* 1978). Other gramineous species give high embryo yields on N6-medium (Chu 1978), B5-medium (Gamborg 1970), or MS-medium (Murashige and Skoog 1962). The good results of media with potato extract (P1, P2, P4) are ascribed to the presence of a large diversity of amino acids (Hughes 1958). A disadvantage of media with added organic components is a lower reproducibility of results.

  Besides agar, also agarose is used to gellify the medium (Aldemita & Zapata 1991). Gelrite (Johansson & Calleberg 1989), PEG (Mohmand & Nabors 1991) en Ficoll-400 (Datta *et al.* 1986) are used to increase the viscosity of the medium. At these higher viscosities, the anthers float on top of the medium and in this way a better oxygen supply is assured. Also fluid media, without gellifying agents are used (Henry & De Buyser 1981, McGregor & McHughen 1990).

  As carbohydrate source, usually sucrose is used in a relative high concentration, e.g. for wheat anther culture 9%. Maltose also gives good results, especially with barley (Last & Brettel 1990, Orshinsky *et al.* 1990).

  In the media in general the composition of the anorganic nutrients (macro- and micro salts) does not vary much. $Na_2FeEDTA$ in almost all media is used in a concentration of 10^{-4} M (Nitsch & Nitsch 1969).

For anther culture of most cereals, the growth regulators 2,4-D or 6-Benzylamino-purine (BAP) are added (Bhaskaran & Smith 1990, Hoekstra *et al.* 1992). Of the many tested additives, only glutamin (Henry & De Buyser 1981), myo-inositol, kinetin and serin appear to have a positive effect (De Buyser & Henry 1986, Hassawi *et al.* 1990).

- *Induction conditions.*

  For anther culture induction temperatures between 25°C and 35°C appear to be optimal (Huang 1987). Ouyang *et al.* (1983) reported that a higher induction temperature at the start of the induction (8 days 32°C), followed by growth at 25°C, gives in certain cases a higher embryo yield.

  The influence of light is still not clear, although higher embryo yields seem to be achieved in complete darkness as compared to induction in the light (Bjornstad *et al.* 1989).

- *Regeneration medium and -conditions.*

  The mostly used regeneration medium is a hormone free MS medium, but for wheat the 190-2 medium (Zhuang & Xu 1983) is also frequently used. The sucrose concentration in these two regeneration media is decreased to 2% to 3%. Liang *et al.* (1987) developed a medium on which induction of sporophytic development as well as regeneration was possible. Pauk *et al.* (1991) developed a three and four step system in which recalcitrant calli can be brought to regeneration by transferring them to media with other hormone concentrations. In this way 4% to 14% more regenerants can be produced. The addition of silver nitrate in the medium, as an inhibitor of ethylene formation, enhances shoot formation (Purnhauser *et al.* 1987). In wheat, regeneration usually takes place at a 16 hour light period and at a temperature of 25°C till 27°C.

- *Doubling of the chromosomes.*

  Colchicine is most frequently used for chromosome doubling. Usually the meristem of the plants is submerged for a few hours in a colchicine solution of 0.05%. Attempts were made to treat *in vitro* material with colchicine, with mixed succes (Barnabas *et al.* 1991, Szakacs & Barnabas 1995, Zhuang & Xu 1983). For humans, colchicine is carcinogenic and therefore, to protect laboratory staff, also other mitosis inhibitors (oryzalin, trifluralin) were tested for chromosome doubling. However, these appeared less effective (Hassawi & Liang 1991). In maize amiprophos methyl (APM) and pronamide showed an efficient genome doubling (Wan *et al.* 1991).

1.2.5. Isolated microspore culture in wheat

Anther culture has several advantages over isolated microspore culture, however, in some cases microspore culture is preferred above anther culture. Considerations to choose microspore culture are:

- In general, isolated microspore culture proceeds a lot faster and takes less work, compared to anther culture. For research purposes it is important that the effect of certain treatments in an experiment can be seen immediately.
- There is no negative effect of the anther wall that can deteriorate and have a detrimental influence

(Huang 1986).

- Also for in vitro selection experiments, single cells are preferred to multicellular structures as a more uniform selection pressure (e.g. toxin tolerance) is secured and the microspores are not shielded by the anther wall (Gustafson *et al.* 1995).
- In some species like barley and rape seed, larger numbers of embryos can be produced compared to anther culture (Palmer *et al.* 1996).
- In isolated microspore culture, all formed plants truly originate from microspores, whereas in anther culture there is the possibility that plants regenerate from the outgrowth of cells of the anther wall.

The isolation of the microspores can be carried out in two ways: mechanically or by the so called 'shed pollen' technique. With mechanical isolation the anther wall is artificially ruptured and the microspores are released. With the 'shed pollen' technique the anthers are placed in a medium in which the anthers burst open after a few days and the microspores are released. Shed microspore culture has as advantage over mechanical isolation that it avoids complicated isolation and purification procedures and possible damage to the microspores. However, the shed microspore technique is not completely comparable to mechanical isolation as the microspores are still in contact with the anther for a certain period. It is suggested that in liquid medium the floating anthers produce a number of conditioning factors (Köhler & Wenzel 1985) and the shed microspore technique shows more resemblance to anther culture than to isolated microspore culture. With the shed pollen technique in wheat, green regenerants were obtained, however too low to be of use for in vitro selection experiments (Kasha *et al.* 1990). Isolating the microspores mechanically proved to be more successful (Gustafson *et al.* 1995, Mejza *et al.* 1993, Touraev *et al.* 1996, Tuvesson & Ohlund 1993).

With microspore culture the stress is not applied to the anthers, but to the isolated microspores e.g. starvation, low or high temperature (Mejza *et al.* 1993, Touraev *et al.* 1996). After isolation and the stress procedure, the microspores are cultured under non-stress conditions. Such a procedure has been shown to be effective for tobacco (Garrido *et al.* 1991) or barley (Hoekstra *et al.* 1992). A part of the microspores will divide sporophytically and will form callus and/or embryos. In some species the carbohydrate concentration is higher than in anther culture, e.g. for microspore culture of cabbage, lily or tulip 13% sucrose proved to be optimal (De Buyser & Henry 1986, Bulk *et al.* 1994).

Conditioning the culture medium with immature ovaries (Touraev *et al.* 1996) and centrifugation over a 20% maltose solution (Mejza *et al.* 1993) or a Percoll gradient (Touraev *et al.* 1996) provided a more homogeneous and viable microspore population and a higher number of embryos, whereas a sucrose centrifugation step proved to have a detrimental effect (Gustafson *et al.* 1995). Also the density of the microspores appeared to be important, with optimal microspore densities ranging from 10-20 x 10^3 microspores ml^{-1} (Touraev *et al.* 1996), 50-100 x 10^3 microspores ml^{-1} (Mejza *et al.* 1993, Tuvesson & Ohlund 1993) to 200 x 10^3 microspores ml^{-1} (Gustafson *et al.* 1995).

As conclusion it can be stated that green wheat plants have been regenerated from isolated microspore culture, as has been reported in several publications, and nowadays also for recalcitrant wheat genotypes isolated microspore culture can be successful (Touraev *et al.* 1996). However, the technique is not yet efficient enough to compete with anther culture for the production of doubled haploids that can be used in a breeding programme.

1.2.6. Androgenesis in practical breeding practice

For breeding companies, the main advantage of anther culture is the production of doubled haploid, homozygous lines. The gain in time in the breeding process can be substantial, especially in crops with a relatively long growing season (winter wheat) and with biannual and perennial crops. In crops with a short growing season, other methods can be more attractive, e.g. the Single Seed Descent (SSD) method. An example for the gain in time in winter wheat is given in Table 1. The table shows that the minimal gain in time is two years. This gain in time compared to conventional breeding methods has also been described for spring and winter barley (Laubach, 1991). For the French DH-cultivar Florin it took seven years from the first cross between the parental lines until acceptance for the variety list. With the conventional pedigree method such material would have been in the F_9-generation. For Florin it meant a gain in time of four years.

As donor generation for the use of anther culture in a practical breeding programme usually F_1- or F_2-material is used. A disadvantage of taking F_1-seed as donor material is that the maximum of non-desired genes, e.g. susceptibility to certain diseases is included, whereas when a F_2-population is used as donor material, part of the undesired genes are discarded, but also a part of the gain in time, compared to a conventional breeding programme, is lost. An extra problem is that the first field generation (DH_0-lines) tends to flower relatively open as a result of the high level of inbreeding, showing a relatively high level of cross pollination. In this way a large part of the acquired homozygosity can be lost again.

The amount of work needed for the production of DH-lines is shown in Table 2. For the production of 500 DH-lines of barley, Luckett & Smithard (1990) needed no more than 23 days of anther culture, but only with a good responding genotype (proportion responding anthers = 0.68). This importance of the proportion of responding anthers shows again the genotype dependency. When averages are taken of a randomly chosen collection of parental lines and crosses, like it was used for the *Fusarium*-resistance research programme at CPRO-DLO, the proportion of responding anthers is lower compared with a case when only one, good responding, genotype is used (Table 2).

A good economic evaluation of anther culture into the conventional breeding programmes is difficult to make and the decision to incorporate anther culture is therefore generally based on personal preferences. Most companies with a cereal breeding programme have experimented with anther culture of wheat or barley or have contracted the method out to specialised institutions or other companies, e.g.

the Resistenzlabor der Saatenunion, Leopoldshöhe, Germany or Florimond Desprez, Cappelle en Pévèle, France. Ding *et al.* (1995) estimated the costs of producing anther culture derived DH-lines from Australian wheat germplasm to range from $A 1.40 to $A 85.52 per DH-line for the genotypes with the highest and lowest cultureability indices, respectively. If selfed seeds were to be produced for shipment to the customer, the costs would have to be raised by $A 9.00. Brennan & Kahn (1989) estimated the total cost of producing a single, pure breeding line of wheat in a conventional breeding programme in Australia to be about $A 18.00. Ding *et al.* (1995) added the extra costs of $A 9.00 for seed production to the costs for the production of the DH-lines, and stated that 27 of the 66 wheat genotypes appeared to be capable of competitive homozygous line production. However, the main benefit of using androgenesis in a breeding programme is the gain in time (Brennan 1989) and therefore the DH-technique is competitive for most of the genotypes tested.

Table 1. Comparison of the Doubled Haploid (DH) method and the conventional breeding method for winter wheat.

Year	Month	DH-method	Conventional method
0	May/July	Select parents	
0	Aug/Sept	Vernalisation of the seedlings + growth in the greenhouse	
0	Nov	Crosses for F_1-seed	
0	Dec	Harvest seed + vernalisation	
1	Feb/March	To greenhouse	To the field
1	April	Excision of the anthers	
1	July	Embryo formation	Harvest F_2-seeds
1	Sept	Doubling and regeneration	
1	Oct	DH_0 to field	Plant F_2-seeds
2	July/Aug	Harvest DH_0	Harvest F_3-seeds
3	July/Aug	Select and harvest DH_1-rows	Harvest F_4-seeds
4	July/Aug	Harvest preliminary yield trials of the DH_2	Harvest F_5-seeds
5	July/Aug	Harvest of extended yield trials of the DH_3	Select and harvest F_6-earrows
6	July/Aug	Harvest of extended yield trials of the DH_4	Harvest preliminary yield trials

(data Baenziger *et al.* 1984).

Table 2. Comparison of a few important factors in the production of doubled haploid (DH) lines.

Factor	Luckett & Smithard 1990	CPRO-DLO data*
Number of days anther culture	23	28
Average number of spikes per day, used for DH-production	30	27
Average number of anthers per spike	30	70
Total number of excised anthers	21,000	53,000
Proportion of responding anthers	0.68	0.03 (0.73)
Number of green regenerants per 100 responding anthers	8	2.4
Number of green regenerants	1136	45
Proportion of fertile regenerants	0.44	0.93
Number of fertile DH-lines	500	42

* = Average over 24 genotypes, (between brackets: best genotype).

1.3. In vitro selection

The basis for plant breeding is the availability of sufficient genetic variation and discriminating selection procedures. In case there is not enough variation within the crop, mutation techniques, crossing with wild species and genetic modification can be used to widen the available variation. Also the introduction of in vitro culture has revealed a new source of variation: somaclonal variation, i.e. variation induced by in vitro culture of plant material (Larkin & Scowcroft 1981). Variation which is observed among plants that were regenerated from cultured gametes is referred to as gametoclonal variation (Evans *et al.* 1984). These types of variation can be caused by changes in structure and number of chromosomes, mitotic recombination, (point)mutations or the transposition, methylation, amplification or deletion of DNA in the genomes of the nuclei, chloroplasts or mitochondria. When these alterations are of a genetic nature, they are stable and will be transmitted to the offspring, but when these alterations are of an epigenetic nature, it will not be transmitted to the offspring and therefore hardly usefull in plant breeding for new cultivars. Gametoclonal variation is dealt with in more detail in chapter 5.

Somaclonal and gametoclonal variation are used in plant breeding for improvement of traits that are of agronomical importance, e.g. disease resistance, and have together with in vitro selection been used to produce new cultivars (Monti & Moore 1992). However, whether the use of soma- or gametoclonal

variation is succesfull, depends on the frequency of occurrence of specific and stable variants, and whether the selection of these variants can be carried out efficiently. Advantages of in vitro selection for disease resistance are that a larger number of genotypes can be screened under controlled conditions and the limited amount of space that is needed to screen all these genotypes. Selection for disease resistance might be improved by the application of a selection pressure (e.g. toxins) to cells or tissues in culture.

When focusing on using selection pressures combined with somaclonal or gametoclonal variation, two main directions can be distinguished, selection with abiotic or biotic selective agents. For resistance or tolerance to abiotic stress, the focus is on salt, drought, herbicides and low temperature (Barakat & Abdellatif 1996, Burgutin *et al.* 1996, Calleberg 1996, Sari-Gorla *et al.* 1992). Within the group of biotic selective agents, in vitro selection for disease resistance is most focused on.

Possible selective agents for disease resistance can be toxins, culture filtrate (Hammerschlag 1988), or the pathogen itself (Sacristan & Hoffmann 1979, Sun *et al.* 1986). Using the pathogen itself often proved to be difficult due to problems with the growth of the pathogen itself and creating an uniform selection pressure in vitro culture (Daub 1986, Sacristan 1982). However, promising results were achieved and resistant plants were found in this way in tobacco (Murakishi & Carlson 1982, Toyoda *et al.* 1989). Culture filtrates have been used in those cases where the filtrate shows phytotoxic activity, but no well characterized toxins are known. In the case of *Fusarium oxysporum* f.sp. *lilii*, a pathogen on lily, careful HPLC-analysis of the culture filtrate revealed the pathogenic compound: fusaric acid (Löffler 1990). Using culture filtrate, wheat plants could be produced that were more resistant to FHB or *Helminthosporium sativum*, respectively, than the donor material it was derived from (Ahmed *et al.* 1996, Guo *et al.* 1991). The combination of somaclonal or gametoclonal variation with in vitro selection for toxin tolerance, has already proven to be succesfull in finding disease resistance in a number of crops. For wheat, in this way obtained resistance to *Pseudomonas syringae* pv. *syringae* and to *Helminthosporium sativum* was reported (Pauly *et al.* 1987, Chawla & Wenzel 1987, Chawla & Kole 1990). Fadel & Wenzel (1993) tested a mixture of *F. culmorum* and *F. graminearum* toxins on wheat anthers and anther culture derived embryos from parents with different levels of resistance and stated that there were fair possibilities to improve the FHB-resistance level in wheat. For selection with toxins in cell cultures, it is important that the toxin acts at the cellular level and that resistant genotypes in vivo also show toxin tolerance in vitro. This is usually the case with host specific toxins, that play a primary role in pathogenesis (Gengenbach *et al.* 1977, Larkin & Scowcroft 1983, Rines & Luke 1985). The mode of action and the properties of a toxin determine whether or not it is suitable for use as a selective agent. Also non-specific toxins can be of interest for in vitro selection. Toxin resistant cells may be selected from genotypes that are sensitive to the toxin. Plants that are resistant to these toxins usually do not show a complete resistance to the pathogen. The FHB-produced toxins DON and ADON are considered to be non-specific toxins and are regarded as aggressiveness factors,

i.e. they increase the extent of the disease symptoms and colonization, but are not involved in the primary interaction that determines compatibility (Al-Heeti 1987, Desjardins 1992, Manka *et al.* 1985, Snijders & Krechting 1992).

1.4. Scope of this thesis

No chemical control is effective against FHB and as the toxic effects on man and animal can be quite severe, FHB-resistance should have a high priority in wheat breeding programmes. Variation for resistance is available, but the resistance genes known up to now are mostly located in exotic genotypes and time-consuming backcrossing is needed. Introducing a haploid step can accelerate the transfer of the resistance genes to cultivars. Applying an in vitro selection pressure has several advantages over in vivo selection as more genotypes can be screened in less space and in a shorter period. The aim of this research was to develop an in vitro selection method for *Fusarium*-resistance to be used on the gametophytic phase in wheat and to apply this to populations of wheat segregating for FHB-resistance and to resistant and susceptible wheat lines. To achieve this goal, first a genetic analysis was carried out on the anther culture ability, on plant regeneration and, after doubling, on seed set of a 7x7 complete diallel in wheat (Chapter 2). Green plant regeneration was then studied in more detail (Chapter 3). Two of the toxins produced by *Fusarium culmorum*: deoxynivalenol (DON) and 3-acetyl deoxynivalenol (3-ADON), were chosen to act as selective agents on seedlings, coleoptile segments, anther derived callus and anther derived embryos (Chapter 4). After regeneration the doubled haploid plants were transferred to the greenhouse for maturation and seed set. The offspring of the doubled haploids was tested in four subsequent years in the field for their reaction to *Fusarium* (Chapter 5). Finally, research on optimisation of microspore culture of wheat was carried out (Chapter 6). The impact of the results in this thesis on practical wheat breeding and on wheat research and recommendations for future research are discussed in Chapter 7.

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Chapter 2: Genetic analysis of anther culture in wheat (*Triticum aestivum* L.): androgenic response, regeneration and seed set based on a 7x7 complete diallel cross

Abstract

Inheritance of androgenic ability in wheat (*Triticum aestivum* L.) anther culture was studied by diallel analysis. Seven parental cultivars, differing for their androgenic response and *Fusarium* Head Blight (FHB) resistance, together with the 42 F_1-combinations of the complete diallel were evaluated for several androgenic traits in five replicates. A total number of 130,000 anthers was cultured of which 14% responded. Average embryo induction frequency was 21% and the number of produced embryos per responding anther was 1.7. Diallel analysis of anther culture response, regeneration and seed set of the DH_0-regenerants was based on the model of Gardner and Eberhart. For percentage Responding Anthers (RA), Embryo Induction Frequency (EIF) and Embryo induction per Responding Anther (ERA), genetic effects explained 62%, 65% and 65% of the total variation, additive effects explained 63%, 75% and 82% of the genetic variation and narrow sense heritabilities were 0.39, 0.48 and 0.53, respectively. A total number of 17,819 embryos was transferred to MS regeneration medium, of which on average 30% regenerated into plantlets, of which 11% was green. Except for two F_1-combinations, of all 42 F_1-combinations green plants could be recovered. Significant genetic differences were found. For percentage green regenerants, percentage albino regenerants and percentage embryos with only root formation genetic effects explained 38%, 48% and 21% of the total variation, additive effects explained 30%, 65% and 37% of the genetic variation and narrow sense heritabilities were 0.11, 0.32 and 0.08, respectively.

After doubling with colchicine, the doubled haploid regenerants (DH_0) were evaluated for their seed set. A total number of 500 embryos produced 1,964 plants and 27,185 spikes. Of the embryos, plants and 9,310 spikes that were analysed, 89%, 84%, 50% showed seed set, respectively. Significant genetic differences were found and for percentage seed set on DH_0-plants the genetic effect explained 78% of the total variation, the additive effect explained 4% of the genetic variation and narrow sense heritability was 0.01. On the DH_0-plants, over 226,000 seeds were produced, with an average of 116 seeds per DH_0-plant. The DH_1-offspring will be tested further in the field for agricultural traits, such as their resistance to FHB.

Introduction

In wheat breeding programmes in North-Western Europe, *Fusarium* head blight resistance is an important selection trait. As most resistant germplasm cannot compete with North-Western European wheat varieties and is regarded as exotic, recurrent backcrossing is necessary to introgress the resistance. The efficiency of breeding programmes using recurrent selection could be improved by introducing a haploid step and in this way shorten the process of the creation of new cultivars in self-pollinated species like wheat (Foroughi-Wehr & Friedt 1984, Gallais 1988, Griffing 1975). Selected plants will have acquired the desired resistance in a homozygous form. In gramineous species, in vitro androgenesis seems to be the optimal method to produce haploid plant material. For barley, the method of isolated microspore culture is to be preferred over anther culture, as more embryos can be produced (Hoekstra *et al.* 1992). In wheat, maize, rye, rice and all grasses, anther culture is still a more efficient way to produce large amounts of haploid embryos (Bruins *et al.* 1996, Gustafson *et al.* 1995), with up to 1002 wheat embryos per 100 anthers (Otani & Shimada 1993), and 357 green wheat plants per 100 anthers (Orshinksy & Sadasivaiah 1994). Using isolated microspore culture in wheat, the best genotype produced only 5.5 embryos and 1.6 green plants per 100 anthers (Tuvesson & Öhlund 1993).

The diallel method has proven to be an efficient way to analyse the genetic background of certain agronomically important characters. In an diallel a number of parental genotypes are crossed reciprocally in all possible combinations, and the resulting F_1-progenies are analysed for the characteristics of interest. Several research groups have published methods for analysing diallel data (Gardner & Eberhart 1966, Griffing 1956, Hayman 1954, Mather & Jinks 1977, Morley Jones 1965, Walters & Morton 1978). Singh & Paroda (1984) stated after evaluating all these methods, except those of Hayman (1954) and Mather & Jinks (1977), that the method of Gardner & Eberhart (1966) would be superior to other methods as it has several advantages above the others:

1. The model assumes arbitrary gene frequencies at all loci between the parents, and is therefore equally applicable to a fixed set of both homozygous varieties as well as those mating at random.
2. The variety and cross means can be predicted, and if certain heterosis effects are negligible, the predicted variety cross means have smaller standard errors than the observed variety cross means.
3. Heterosis effects are further sub-divided to provide additional information on the varieties involved. The estimates obtained are particularly useful in making predictions and choosing breeding materials and breeding methodologies.
4. An analysis of variance with appropriate F-tests is provided for various types of gene action involved.
5. The variety effects, as presented by Gardner and Eberhart, depend only on additive X additive gene action, regardless of the gene frequencies or correlated gene distribution (Sokol & Baker 1977).
6. Heterosis can easily be calculated from the estimates obtained in this model.

In wheat, in vitro androgenic potential is mainly genetically controlled (De Buyser & Henry 1979, He & Ouyang 1984, Jähne & Lörz 1995), with predominantly additive effects (Bullock *et al.* 1982, Chevrier *et al.* 1990, Tuvesson *et al.* 1989), but also dominance effects (Deaton *et al.* 1987), epistasis (Agache *et al.* 1988) and even cytoplasmic or maternal effects were found (Ekiz & Konzak 1991 a,b & c, Sági & Barnabás 1989).

Green plant regeneration using F_1-combinations of highly androgenic responsive genotypes crossed with genotypes with a high green plant regeneration was studied earlier (Bruins & Snijders 1995). In this study, it was found that genotypic effects accounted for the majority of the total variation. Additive and dominant gene action, but no reciprocal differences were detected.

The percentage fertile DH_0-plants resulting from anther culture in wheat tends to vary greatly, ranging from 21% (Barnabás *et al.* 1991) to 96% (Shimada *et al.* 1994).

To combine a high *Fusarium* resistance with a good androgenic response, seven wheat varieties were selected which differed strongly for their *Fusarium* resistance and their response in anther culture. Our investigation is the first to use a complete 7x7 diallel to study the genetic background of androgenic response, regeneration and seed set on the same set of wheat genotypes.

Materials and Methods

Anther culture

Based on preliminary experiments, seven wheat genotypes were selected, namely three cultivars Praag 8, Frontana, Ringo Sztar, and four breeding lines PF 8049, Ft 83-326, SVP 72017-17-5-10-1 and SVP 73016-2-4 (Table 1). Sources of these lines are given in Snijders (1990a, 1990b). Of these genotypes, four had a high FHB-resistance, one a moderate resistance and two were susceptible. One genotype had a high response in anther culture, two a moderate response and four a low response (Table 1). Reciprocal crosses between these seven parental genotypes were made. The whole set of 42 F_1-combinations and seven parents was sown in five replicates in time (Table 2). Each replicate contained two to four plants per F_1-combination or parent, so in total 10 to 15 plants of each genotype were tested. F_1-plants and parents were vernalised for eight weeks at 4°C, prior to transplant to an accurately controlled phytotron chamber. Plants were grown under a 16 h photoperiod and a temperature regime of 15°C (light) and 10°C (dark). Tillers were harvested in the mid-boot stage, i.e. 43 on the Zadoks growth scale (Zadoks *et al.* 1974). Per replicate three to six tillers per plant were harvested, the total number of tested tillers per F_1-combination or parent varied from 23 to 60. Harvest of the tillers from the donor plants and subsequent anther culture was carried out according to Bruins *et al.* (1993). Six weeks after incubation, anther response, callus and embryo formation were measured.

The following calculations were made:

RA = Percentage Responding Anthers that produced at least one embryogenic or non-embryogenic structure.

CIF = Callus Induction Frequency = Number of formed embryogenic and non-embryogenic structures / total number of plated anthers.

EIF = Embryo Induction Frequency = Number of formed embryogenic structures / total number of plated anthers.

CIRA = Callus Induction frequency per Responding Anther.

ERA = Embryo induction frequency per Responding Anther.

Table 1. Parental genotypes used for the diallel cross, type of material, *Fusarium* Head Blight Index and percentage responding anthers. *Fusarium* Head Blight-Index measured as the weighed mean of five years (1987-1991) of the percentage head blight infection per ear in the field.

Parent-number	Genotype	Type	*Fusarium* Head Blight-index	Responding Anthers (%)
1	SVP 72017-17-5-10-1	winter	3.3	0.2[1]
2	Praag 8	winter	2.1	0.8
3	Ft 83-326	spring	0.9	1.9
4	Frontana	spring	1.6	1.9
5	Ringo Sztar	winter	9.6	9.4
6	PF 8049	spring	52.2	5.6
7	SVP 73016-2-4	winter	43.2	4.3

[1] Data derived from genotype SVP 72017-17-5-10.

Data were analysed using a square root transformation to improve normality of the distribution. Diallel analyses were carried out according to the model described by Gardner and Eberhart (1966). Genotypic effects were subdivided according to Hayman (1954) into:

additive genetic effects:

a) = variation between the mean effects of each parental line,

and non-additive genetic effects:

b) = heterosis = variation in the reciprocal sums not ascribable to (a)

c) = average maternal effects of each parental line, and

d) = variation in the reciprocal differences not ascribable to (c).

The heterosis-component (b) was according the model of Gardner and Eberhart (1966) further subdivided

into: b_1 = average heterosis, b_2 = variety heterosis and b_3 = specific heterosis. These components are similar to the three components described by Hayman (1954), where b_1) = mean dominance variation, b_2) = further dominance deviation due to the r^{th} parent and b_3) = remaining discrepancy in the rs^{th} reciprocal sum. Narrow sense heritability estimates were calculated as the proportion of additive effects on the genetic variation.

Regeneration of the anther culture derived embryos

Embryogenic structures larger than 1 mm were transferred to hormone free MS medium (Murashige & Skoog 1962), and regeneration of the embryos was scored as the number of embryos that regenerated into green shoots, albino shoots, embryos that regenerated only roots or did not regenerate at all. Data of the percentages green shoots and embryos with roots only, were analysed using a square root transformation to improve the normality of the distribution. Diallel analyses were carried out according to the model described by Gardner & Eberhart (1966).

Seed set on the DH_0-regenerants

Green plantlets were transferred to culture tubes and, after reaching the two-three leaf stage, to soil and covered individually. After two weeks, the transparent plastic covers were removed and two weeks later all plants were treated with colchicine for chromosome doubling. For this, the plants were removed from the soil, the roots were washed and the growth meristems of the plantlets were soaked for 12 hours in a 0.05% colchicine solution, rinsed with tap water for three hours and replanted in soil. After another two weeks, the plants were vernalised for 8 weeks at 4°C. After vernalisation, plants were grown to maturity and seed set was scored. Per plant the number of spikes, spikelets and seeds in the outer two flowers of each spikelet were assessed. Percentage seed set was measured on the first five spikes per plant as the number of seeds in the outer two flowers of each spikelet, divided by the double number of spikelets. Diallel analysis was carried out according to the model described by Gardner & Eberhart (1966).

Results

Anther culture

As indicated in Table 3, correlations between Callus Induction Frequency (CIF) and Embryo Induction Frequency (EIF) and between Callus Induction frequency per Responding Anther (CIRA) and Embryo induction frequency per Responding Anther (ERA) were very high (0.97 in both cases). Therefore, besides percentage Responding Anthers, only Embryo Induction Frequency and Embryo induction frequency per Responding Anther will be discussed. Other significant correlations included % Responding Anthers with Embryo Induction Frequency and Embryo Induction Frequency with Embryo induction frequency per

Responding Anther. Table 2 shows the Responding Anther, Embryo Induction Frequency, and Embryo induction frequency per Responding Anther values averaged over all 49 genotypes per replicate. Mean percentage Responding Anthers varied between the five replicates from 11.3% to 13.8%, Embryo Induction Frequency from 18.5% to 26.7% and Embryo induction frequency per Responding Anther from 1.67 to 2.17, respectively. Table 4 shows the androgenesis data for the complete diallel averaged over the five replicates in time. Genetic effects were highly significant for all measured variates. The Half-Sib families of Ringo Sztar, in which this cultivar was either used as a male or female parent, showed the highest values for anther culture response and embryo induction frequency, which confirmed the earlier results from 1990, as already indicated in Table 1. Comparison of the mean values indicated that genotype Ft 83-326, either used as a male or female parent, had the highest values for Embryo induction frequency per Responding Anther.

Significant Best-Parent heterosis, where the androgenic value of the F_1-combination outperformed the value of the best parent, was found for 3 (7%), 9 (21%) and 6 (14%) of the 42 F_1-combinations for percentage Responding Anther, Embryo Induction Frequency and Embryo induction frequency per Responding Anther, respectively (Table 4; see *).

Table 5 shows the results of the ANOVA. Complete balancing of the replicates appeared to be difficult as a result of incomplete germination of the F_1-seeds. In nine occasions seeds of a F_1-combination failed to germinate, resulting in 235 degrees of freedom instead of 245. The replicate effects were low but significant for RA, and were highly significant for Embryo Induction Frequency and Embryo induction frequency per Responding Anther (Table 5). Replicate 5 (July-Oct) produced significantly less responding anthers and less embryos. Replicates 1 & 5 produced significantly less embryogenic structures per responding anther (Table 2). Genetic effects and additive effects (component a) were highly significant for all three variates. Narrow sense heritabilities for percentage Responding Anthers, Embryo Induction Frequency and Embryo induction frequency per Responding Anther were 0.39, 0.48 and 0.53, respectively. When the seven parents were compared to the 42 F_1-combinations (average heterosis) only low significance for percentage Responding Anthers was found, accounting for only 1.2% of the total variation. Variety heterosis was highly significant for all three variates, but accounted only for 8%, 5% and 4% of the total variation of percentage Responding Anthers, Embryo Induction Frequency and Embryo induction frequency per Responding Anther, respectively. No maternal effects (component c) were observed and small but significant reciprocal differences, not ascribable to maternal effects (component d) were found.

Table 2. Androgenic values of a 7x7 full diallel in wheat, averaged over the 42 F_1-combinations and seven parent cultivars as measured in five replicates in time.

	Androgenic response					Regeneration			
Replicate	Excision dates[1]	Excised Anthers	RA[2] (%)	EIF (%)	ERA	Placed Embryos	% green plants	% albino plants	% only roots
1	23/06-22/09	21,786	13.8 a	24.7 a	1.72 b	2926	3.9 ab	26.6 bc	18.5 a
2	20/10-16/12	18,501	13.0 ab	26.5 a	2.09 a	3705	2.9 b	21.5 d	16.6 a
3	10/01-15/04	21,283	12.7 ab	26.7 a	2.17 a	2849	2.8 b	28.3 ab	16.8 a
4	31/03-23/06	29,734	13.3 ab	25.6 a	1.89 ab	3366	3.0 ab	31.1 a	9.5 b
5	12/07-11/10	39,183	11.3 b	18.5 b	1.67 b	4973	4.8 a	24.6 cd	7.8 b
Mean		**26,097**	**13.5**	**20.9**	**1.70**	**3564**	**3.5**	**26.5**	**13.8**

[1] Excision dates refer to the first and last date of anther excision.

[2] Replicate means not followed by the same letter are significantly different at the 0.05 level of probability.

Table 3. Correlation matrix of the anther culture variates of a 7x7 full diallel in wheat, based on replicate means. Degrees of Freedom = 47.

CIF	0.90**							
EIF	0.79**	0.97**						
CIRA	0.32*	0.66**	0.78**					
ERA	0.18	0.54**	0.69**	0.97**				
green	0.20	0.13	0.08	-0.08	-0.14			
albino	0.46**	0.56**	0.55**	0.45**	0.40**	-0.19		
root	-0.32*	-0.40**	-0.42**	-0.28	-0.28	-0.34**	0.09	
seed set	-0.14	-0.22	-0.19	-0.16	-0.06	-0.29	0.03	-0.02
	RA	CIF	EIF	CIRA	ERA	green	albino	root

*, ** = significant at the 0.05 and 0.01 level of probability, respectively.

Genotype 5 (Ringo Sztar) had significantly the highest GCA values for percentage Responding Anthers and Embryo Induction Frequency (Table 6), and is a very suitable parent to transmit favourable alleles responsible for a high androgenic production. Genotype 3 (Ft 83-326) has significantly the highest GCA value for Embryo induction frequency per Responding Anther, and is a suitable parent for transferring alleles for a good androgenic embryo production per responding anther. Highly significant differences in SCA values were found (Table 4; see underlined data). Estimated SCA values varied from: -0.69 (genotype 2 = Praag 8) to 0.69 (cross 5x7) for percentage Responding Anthers, from -1.33 (genotype 2 = Praag 8) to 1.41 (cross 4x6) for Embryo Induction Frequency and from -0.31 (genotype 4 = Frontana) to 0.25 (cross 1x7) for Embryo induction frequency per Responding Anther. Significant SCA values were found for 6 (12%), 6 (12%) and 5 (10%) of the 49 entries for percentage Responding Anthers, Embryo Induction Frequency and Embryo induction frequency per Responding Anther, respectively. In six of these 17 occasions a parent had a significant SCA-value. A significant positive SCA-value indicates that this specific combination yielded a higher value as can be expected from the GCA values, a negative sign indicates the opposite. With genotypes 6 (PF 8049) and 7 (SVP 73016-2-4), when used as a male parent, all significant SCA-values were positive, and with genotypes 2 (Praag 8) and 5 (Ringo Sztar), when used as a male parent, all significant SCA-values were negative (Table 4). With genotype 4 (Frontana) the significant SCA value for RA was positive, the other two values were negative.

Table 4. Percentage Responding Anthers (RA), Embryo Induction Frequency (EIF) and Embryo Induction per Responding Anther (ERA) of 42 F_1 combinations and seven parents of a 7x7 complete diallel in wheat, averaged over five replicate means in time. 1-7: see Table 1.

	Genotype ♂ / ♀	1	2	3	4	5	6	7	MEAN
RA	1	7.8	8.2	10.8	16.0	21.5	10.1	5.3	**11.4**
	2	8.8	6.9	9.9	16.2	23.1	18.2*	10.2	**13.3**
	3	8.8	10.8	8.6	9.3	16.1	14.9*	8.4	**11.0**
	4	9.6	13.6	10.9	12.7	16.9	16.5	9.5	**12.8**
	5	18.8	24.5	18.7	17.6	21.7	21.0	26.3*	**21.2**
	6	9.6	11.2	10.7	15.1	19.6	10.1	9.7	**12.3**
	7	10.8	8.6	11.9	2.2	15.1	10.0	17.7	**12.3**
	MEAN	**10.6**	**12.0**	**11.6**	**14.2**	**19.1**	**14.4**	**12.4**	**13.5**
EIF	1	4.9	6.7*	15.4	23.5*	30.4	11.1	4.2	**12.2**
	2	7.5*	2.9	18.6	22.6*	33.2	26.4*	10.5	**15.6**
	3	11.9	17.8	27.5	20.9	30.3	39.1*	12.0	**21.8**
	4	9.7	24.3*	25.6	19.6	42.4	49.1*	10.9	**24.1**
	5	26.8	36.7	47.2	36.5	48.0	38.4	43.7	**39.3**
	6	10.0	17.1	21.3	40.0*	41.7	23.9	11.2	**22.1**
	7	11.7	8.2	22.9	17.8	21.9	12.8	21.5	**16.2**
	MEAN	**11.0**	**14.4**	**24.7**	**25.3**	**34.9**	**27.0**	**14.5**	**20.9**
ERA	1	0.7	0.9*	1.5	1.7	1.6	1.3	1.0	**1.2**
	2	0.9*	0.5	2.3	1.4	1.5	1.5	1.1	**1.3**
	3	1.6	2.0	3.2	3.4*	2.1	2.8	1.8	**2.4**
	4	1.1	1.9*	2.7	1.7	2.8*	3.0*	1.3	**2.0**
	5	1.5	1.6	2.7	2.3	2.5	2.2	1.8	**2.1**
	6	1.2	1.6	2.0	2.8	2.2	2.7	1.2	**1.9**
	7	1.2	1.1	2.1	1.6	1.5	1.6	1.4	**1.5**
	MEAN	**1.2**	**1.3**	**2.3**	**2.1**	**2.0**	**2.1**	**1.3**	**1.7**

* = Best-Parent heterosis at the 0.05 level of probability.

__ = Estimated SCA-effect significantly different from zero at the 0.05 level of probability.

Regeneration of the anther culture derived embryos

Of the 27,271 formed embryos, 17,819 (65%) were larger than 1 mm and were transferred to hormone free MS-medium for regeneration. Of these transferred embryos, 30% regenerated into plantlets (Table 2), of which 11% was green, 27% regenerated into albino plantlets, 14% of the embryos only regenerated into roots and 56% of the embryos did not regenerate at all. On average, 0.5 green plants per 100 anthers were produced.

Correlation data showed that the percentage green regenerants had only one significant correlation: the negative correlation with percentage embryos with only root formation (Table 3). None of the other androgenic traits showed a correlation with percentage green regenerants. Correlation data of percentage albino regenerants were positive for the anther culture response variables percentage Responding Anthers, Embryo Induction Frequency and Embryo induction frequency per Responding Anther. All correlations were

positive and significant at the 0.01 level of probability. Percentage embryos with only root formation had significant negative correlations with percentage Responding Anthers and Embryo Induction Frequency (Table 3).

Table 5. Analysis of variance (mean squares, according to the method of Gardner and Eberhart 1966), for percentage Responding Anthers (RA), Embryo Induction Frequency (EIF) and Embryo Induction per Responding Anther (ERA), of a 7x7 full diallel in wheat, averaged over five replicates in time.

			Mean squares		
Code	Factor	d.f.	RA	EIF	ERA
	Replicates	4	1.00*	7.34***	0.39***
	Genotypes	48	2.33***	9.30***	0.31***
a	varieties	6	11.66***	55.43***	1.99***
b	heterosis	21	1.31***	3.27***	0.08**
b_1	average	1	2.17*	2.92	0.01
b_2	variety	6	2.33***	5.63***	0.14**
b_3	specific	14	0.81**	2.28*	0.07*
c	maternal	6	0.74	1.78	0.03
d	reciprocal differences	15	0.66*	2.30*	0.05
	Genotypes x replicates	183	0.35	1.18	0.04
	Total	235	0.77	2.95	0.10

*, **, *** significant at the 0.05, 0.01 and 0.001 level of probability, respectively
d.f. = degrees of freedom.

Table 7 shows the regeneration data of the anther culture derived embryos of the 42 F_1-combinations and parents. Percentages green regenerants varied from 0.0% to 13.5% (cross 7x2), percentages albino regenerants varied from 9.3% (cross 1x7) to 45.0% (cross 2x5) and percentages embryos that had only root regeneration varied from 7.2% (cross 4x5) to 21.3% (cross 7x2).

For percentage green plants, genotypes 2 (Praag 8), 5 (Ringo Sztar) and 7 (SVP 73016-2-4) had on average the highest values, when used as a male or female parent. The two reciprocal F_1-combinations 2x7 and 7x2 showed significantly a higher green plant regeneration as compared to the rest of the 42 F_1-combinations, both with over 13% green plants (Table 7). One F_1-combination (3x1) and genotype 4

(Frontana) failed to produce green regenerants. For percentages albino regenerants, genotypes 3 (Ft 83-326) and 5 (Ringo Sztar) had on average the highest values when used as a male or female parent. For percentages embryos that gave only root regeneration, genotype 2 (Praag 8) had the highest values. Significant Best-Parent Heterosis was found for 10 (24%), 1 (2%) and 6 (14%) of the 42 F_1-combinations for percentage green plants, percentage albino plants and percentage embryos with only root formation, respectively (Table 7, see *).

Table 6. Estimated GCA-effects of each wheat parent as used in the 7x7 full diallel, averaged over the five replicates on the basis of square root transformed data, except for the percentage albino plants and the percentage seed set on the DH_0-regenerants. A positive sign indicates that this genotype will easily transmit the characteristic to its offspring, a negative sign indicates the opposite. 1-7: see Table 1.

Parent-number	RA	EIF	ERA	% green	% albino	seed set
1	-0.30	-1.17*	-0.22*	-0.37	-5.55	-2.1
2	-0.08	-0.52	-0.15*	0.19	0.68	-3.4
3	-0.28	0.30	0.24**	-0.55	6.44	-0.8
4	0.05	0.50	0.15*	-0.01	-3.59	-1.0
5	0.96**	1.53**	0.06	0.47	7.05	-3.0
6	0.06	0.44	0.07	0.00	-0.73	-0.4
7	-0.42	-1.08*	-0.14	0.27	-4.30	9.5

*, ** Significantly different from zero at the 0.05 and 0.01 level of probability, respectively.

Table 8 shows the results of the ANOVA for regeneration. Replicate effects were significant for percentage albino plants and percentage embryos with only root formation, caused by replicate 4 (April-June) in which significantly more albino regenerants and by replicates 4 & 5 in which significantly lower percentages embryos with root regeneration were produced (Table 2). Genetic effects were significant for percentage embryos with only root formation and highly significant for percentage green and percentage albino regenerants. Additive effects were highly significant for all three variates and narrow sense heritabilities for percentage green plants, percentage albino plants and percentage embryos with only root formation were 0.11, 0.32 and 0.08, respectively. Average heterosis was not significant, variety heterosis (component b_2) was only significant for percentage green plants and specific heterosis (component b_3) was significant for percentage green plants and percentage albino plants. No maternal and reciprocal differences were detected for regeneration.

Table 7. Percentages green regenerants, albino regenerants and calli with only root-regeneration of seven parents and 42 F_1-combinations used in the 7x7 full diallel averaged over five replications in time. 1-7: see Table 1.

	Genotype ♂ ♀	1	2	3	4	5	6	7	MEAN
% green	1	3.8	2.2	2.9	0.8	4.5	1.5	3.7	**2.8**
	2	4.5	2.3	2.3	6.2*	2.4	4.0	13.4*	**5.0**
	3	0.0	0.5	0.3	1.7*	3.4	1.6	0.9	**1.2**
	4	1.8	1.8	1.3*	0.0	3.5	2.1	5.0*	**2.2**
	5	2.7	2.3	2.2	9.1*	3.9	3.2	5.8*	**4.2**
	6	3.2	5.4	4.6	5.3	2.6	7.0	1.6	**4.2**
	7	1.7	13.5*	0.9	3.2*	10.6*	2.6	0.7	**4.7**
	MEAN	**2.5**	**4.0**	**2.1**	**3.8**	**4.4**	**3.1**	**4.4**	**3.5**
% albino	1	19.1	19.7	22.5	23.7	24.9	18.8	9.3	**19.7**
	2	21.7	20.2	31.8	24.8	45.0	26.2	15.5	**26.5**
	3	34.9	34.4	37.9	20.7	41.4	29.2	38.2	**33.8**
	4	20.4	31.0*	32.1	18.4	23.7	25.4	14.5	**23.6**
	5	30.8	38.7	38.8	26.9	38.9	40.2	26.5	**34.4**
	6	17.5	20.7	32.8	18.0	31.5	21.3	26.7	**24.1**
	7	22.3	19.5	29.8	25.1	24.0	27.8	15.2	**23.4**
	MEAN	**23.8**	**26.3**	**32.2**	**22.5**	**32.8**	**27.0**	**20.8**	**26.5**
% root	1	19.8	14.8	11.2	10.4	13.9	19.7	18.0	**15.4**
	2	20.0	20.3	17.8	19.3	9.0	16.7	15.6	**17.0**
	3	15.8	17.3	11.0	16.1*	12.1*	10.5	14.2*	**13.9**
	4	7.5	13.3	17.9*	13.8	7.2	8.2	8.5	**10.9**
	5	15.5	11.9	8.6	7.9	8.1	8.5	15.2*	**10.8**
	6	17.6	20.4	10.8	13.6	13.9	20.6	15.8	**16.1**
	7	14.7	21.3*	8.0	11.8	9.9	9.7	11.5	**12.4**
	MEAN	**15.8**	**17.0**	**12.2**	**13.3**	**10.6**	**13.4**	**14.1**	**13.8**

* = Best-Parent heterosis at the 0.05 level of probability.

__ = Estimated SCA-effect significantly different from zero at the 0.05 level of probability.

For percentage green plants, genotypes 3 (Ft 83-326) and 5 (Ringo Sztar) and for percentage albino plants genotypes 1 (SVP 72017-17-5-10-1) and 5 (Ringo Sztar) showed the highest and lowest GCA values, respectively (Table 6). However, none of the GCA values were found to be significant. Estimated SCA values varied from: -1.06 (genotype 4 = Frontana) to 1.24 (cross 2x7) for percentage green plants, from -9.04 (cross 3x4) to 10.47 (cross 2x5) for percentage albino plants and from -0.85 (cross 4x1) to 0.98 (cross 4x3) for percentage embryos with only root formation. Significant SCA values were found for 7 (14%), 5 (10%), and 1 (2%) of the 49 genotypes for percentage green plants, percentage albino plants and percentage embryos with only root formation, respectively (Table 7, see underlined data). In two of these 13 cases a parent had a significant negative SCA-value for percentage green plants. Also negative SCA-values had F_1-combinations 5x2 for percentage green plants and 1x7, 2x7 and 3x4 for percentage albino plants. The rest of the significant SCA-values were positive.

Table 8. Analysis of variance (mean squares, according to the method of Gardner and Eberhart 1966), for percentage green regenerants, percentage albino regenerants, embryos with only root regeneration and percentage seed set averaged over five replicates in time.

			Mean squares				Mean squares
Code	Factor	d.f.	% green	% albino	% root	d.f.	% seed set
	Replicates	4	1.63	596.44***	22.71***	1	27.3
	Genotypes	48	2.79***	314.61***	76.89*	44	733.2*
a	varieties	6	6.65***	1656.47***	4.87***	6	220.1
b	heterosis	21	3.24**	133.61*	1.52	20	897.0*
b_1	average	1	1.79	200.49	0.72	1	1976.4*
b_2	variety	6	3.90**	33.55	1.00	5	838.5*
b_3	specific	14	3.06**	171.72**	1.80	14	840.8**
c	maternal	6	1.94	104.51	1.15	6	570.3
d	recipro diff	15	0.95	115.30	0.69	12	798.6**
	Genotypes x replicates	183	1.18	75.02	1.11	32	276.7
	Total	235	1.51	132.84	1.58	77	534.4

*, **, *** significant at the 0.05, 0.01 and 0.001 level of probability, respectively.

d.f. = degrees of freedom.

Table 9 shows the regeneration data of the 49 combinations. It can be seen that for the number of regenerants per 100 plated embryos, genotypes 2 (Praag 8), 3 (Ft 83-326) and 5 (Ringo Sztar) showed the highest mean values when used as a male or as a female parent. For the percentage green plants per total number of regenerated plants the highest values were observed for genotypes 4 (Frontana), 5 (Ringo Sztar) and 7 (SVP 73016-2-4) when used as a male parent and for genotypes 2 (Praag 8), 6 (PF 8049) and 7 (SVP 73016-2-4) when used as a female parent. Genotype 3 (Ft 83-326) and its Half-Sib families had the lowest percentage green plants and relatively more albino regenerants than the other genotypes, which was confirmed by extremely low green/albino ratios. For green plants per 100 plated anthers on average genotype 5 (Ringo Sztar), when used as a male or as a female parent, and more specific F_1-combination 5x4 showed the highest values. For green plants per 100 produced embryos, the highest values were observed for genotypes 4 (Frontana), 5 (Ringo Sztar) and 7 (SVP 73016-2-4) when used as a male parent and for genotypes 2 (Praag 8), 5 (Ringo Sztar) and 7 (SVP 73016-2-4) when used as a female parent. Green/albino ratios were highest for F_1-combinations (2x7), (7x2) and (7x5).

Seed set of the DH_0-regenerants

As a result of a large spread in the maturation of the DH_0-plants the replications could not be distinguished for percentage seed set. Table 10 shows the seed set values of the 49 genotypes, averaged over all five replications. On average 10 embryos per F_1-combination lead to a mature green plant, 40 DH_0-plants, 555 spikes and over 4600 seeds were derived from one F_1-combination or parent. 62% of the flowers was sterile, averaged per parent ranging from 43% to 76%. The variation in seed set between the individual F_1-combinations was much larger: 8% to 98%. In total 27,185 spikes were formed, of which only the first five ears per plant, in total 9,310 were analysed for their seedset. Of the 9310 analysed spikes, 50.2% was sterile. On average 487 and 46 seeds were found per fertile entry and analysed fertile spike, respectively. Parent 5 and its Half Sib families showed the highest values for the number of embryos, DH_0-plants, DH_0-spikes and total number of seeds. For percentage sterile flowers, 3 out of 42 (7%) showed Best Parent Heterosis for the lowest percentage sterile flowers, all when genotype 1 was used as a male parent (Table 10, see *). Genotype 3 and F1 combinations 3x2 and 3x7 show the lowest percentages sterile flowers, with 13%, 8% and 5% sterile flowers respectively.

Calculations showed that the percentage seed set was not significantly correlated with any of the other variables (Table 3). Table 8 shows the results of the ANOVA for percentage seed set. The replicate and additive effects were not significant, whereas a small but significant genotype effect was detected. Narrow sense heritability for percentage seed set was 0.01. A small but significant genotypic effect was found. Average, variety and specific heterosis components were all significant for seed set percentage. No significant maternal effects were detected. Reciprocal differences, however, were highly significant but no significant GCA values were found for percentage seed set (Table 6). Estimated SCA values (data not shown) varied from: -13.4 to 13.4, however none of them was significant.

Of the green plants surviving colchicine doubling, 5.3% of the plants stayed vegetative and 18.2% of the plants produced only sterile spikes.

Table 9. Regeneration of seven parents and 42 F_1-combinations used in the 7x7 full diallel, averaged over five replications. 1-7: see Table 1.

Genotype ♂ ♀	1	2	3	4	5	6	7	**MEAN**
Regenerants per 100 plated embryos								
1	23.5	25.2	23.8	22.5	30.2	21.6	24.7	**25.1**
2	28.0	31.0	35.3	30.7	43.1	30.7	22.5	**33.0**
3	34.1	35.9	39.3	25.0	46.0	28.2	37.2	**36.4**
4	21.8	32.2	34.9	17.5	27.4	25.9	16.9	**26.7**
5	32.8	41.3	41.3	33.3	42.4	39.8	32.7	**37.9**
6	20.9	22.6	32.7	20.4	33.3	26.9	27.3	**27.8**
7	23.7	31.5	30.2	23.6	34.2	29.3	16.2	**26.2**
MEAN	**27.3**	**33.3**	**35.7**	**25.7**	**36.7**	**29.2**	**25.5**	**31.2**
Percentage green plants / total number of regenerated plants								
1	17.9	11.4	10.2	3.9	13.6	6.7	21.6	**11.0**
2	13.5	11.5	7.9	22.6	6.5	15.6	32.8	**13.9**
3	0.0	1.3	1.3	6.7	7.4	5.7	1.4	**3.8**
4	7.7	4.6	4.0	0.0	17.5	4.7	16.1	**7.8**
5	9.9	5.3	5.0	27.7	9.9	5.6	18.7	**11.1**
6	15.6	19.1	10.4	20.9	8.9	24.1	5.9	**14.0**
7	7.7	39.3	3.8	10.8	34.1	10.3	6.2	**17.7**
MEAN	**9.4**	**10.2**	**5.1**	**16.5**	**13.0**	**10.3**	**14.9**	**11.1**
[Green plants / albino plants] ratio								
1	0.2	0.1	0.1	0.04	0.2	0.1	0.3	**0.1**
2	0.2	0.1	0.1	0.3	0.1	0.2	0.5	**0.2**
3	0.0	0.01	0.01	0.1	0.1	0.1	0.01	**0.04**
4	0.1	0.1	0.04	0.0	0.2	0.1	0.2	**0.1**
5	0.1	0.1	0.1	0.4	0.1	0.1	0.2	**0.1**
6	0.2	0.2	0.1	0.3	0.1	0.3	0.1	**0.2**
7	0.1	0.7	0.04	0.1	0.5	0.1	0.1	**0.2**
MEAN	**0.1**	**0.1**	**0.1**	**0.2**	**0.2**	**0.1**	**0.2**	**0.1**
Green plants per 100 plated anthers								
1	0.1	0.1	0.3	0.2	0.8	0.1	0.3	**0.3**
2	0.2	0.1	0.4	1.0	0.7	0.8	0.5	**0.5**
3	0.0	0.1	0.1	0.3	0.7	0.4	0.1	**0.2**
4	0.2	0.2	0.2	0.0	1.2	0.4	0.2	**0.4**
5	0.6	0.5	0.6	2.2	1.2	0.5	1.5	**1.0**
6	0.3	0.5	0.6	0.9	0.9	0.8	0.1	**0.6**
7	0.1	0.7	0.2	0.3	1.5	0.3	0.1	**0.5**
MEAN	**0.2**	**0.3**	**0.3**	**0.7**	**1.0**	**0.5**	**0.4**	**0.5**
Green plants per 100 produced embryos								
1	2.2	1.3	1.5	0.6	2.2	0.9	3.1	**1.6**
2	2.3	1.8	1.5	4.5	1.6	3.0	4.0	**2.7**
3	0.0	0.2	0.3	1.0	1.9	0.8	0.3	**0.8**
4	1.3	0.8	0.8	0.0	2.8	0.7	1.7	**1.2**
5	2.0	1.3	1.2	5.5	2.3	1.2	3.4	**2.4**
6	2.5	2.7	2.4	2.0	1.6	2.8	1.0	**2.1**
7	0.9	7.6	0.7	1.5	7.0	1.7	0.7	**2.8**
MEAN	**1.6**	**1.9**	**1.1**	**2.5**	**2.7**	**1.6**	**2.3**	**2.0**

Table 10. Fertility data over five replications of seven parents and 42 F_1-combinations used in the 7x7 full diallel. 1-7: see Table 1.

Genotype ♂ ♀	1	2	3	4	5	6	7	**MEAN**
Number of different anther culture derived embryos that lead to a mature green plant								
1	48	--	3	3	23	2	3	**14**
2	2	1	3	10	7	9	9	**6**
3	--	1	3	3	6	5	1	**3**
4	2	4	4	--	15	7	5	**6**
5	11	7	7	25	53	6	36	**21**
6	3	7	6	4	9	22	--	**9**
7	3	12	3	1	43	4	10	**11**
MEAN	**12**	**5**	**4**	**8**	**22**	**8**	**11**	**10**
Total number of DH_0-plants derived from one F_1-combination or parent								
1	139	--	4	5	75	4	13	**34**
2	3	3	10	24	60	20	82	**29**
3	--	1	5	7	29	23	3	**10**
4	5	25	15	--	64	16	30	**26**
5	39	26	16	133	226	26	156	**89**
6	15	22	24	7	48	110	--	**32**
7	18	66	4	7	169	10	21	**42**
MEAN	**31**	**20**	**11**	**31**	**96**	**30**	**44**	**40**
Total number of DH_0-spikes within one combination or parent								
1	1268	--	34	71	1238	48	132	**465**
2	39	53	36	366	1172	220	1022	**415**
3	--	13	39	24	317	143	65	**100**
4	39	354	53	--	692	146	669	**326**
5	467	370	147	1494	3245	94	2454	**1182**
6	149	265	199	37	578	957	--	**364**
7	339	1284	44	152	3279	115	370	**798**
MEAN	**384**	**390**	**79**	**357**	**1503**	**246**	**785**	**555**
Total number of seeds, harvested on DH_0-plants, within one combination or parent								
1	13616	--	14	383	21446	332	574	**6061**
2	91	39	230	4430	4641	5916	7184	**3219**
3	--	355	1232	309	790	343	1864	**816**
4	923	4835	399	--	2597	1489	2045	**2048**
5	10733	4719	3003	5807	30064	802	18668	**10542**
6	3062	1510	3777	484	3044	6767	--	**3107**
7	3151	8307	192	219	18689	916	1858	**4762**
MEAN	**5263**	**3294**	**1264**	**1939**	**11610**	**2366**	**5366**	**4624**
Number of sterile flowers of the five biggest spikes / double number of spikelets from the same spikes								
1	70	--	98	79	50	62	96	**76**
2	88	n.d.	93	46	71	45	64	**68**
3	--	8	13	82	78	71	5	**43**
4	28*	48	45	--	67	70	86	**57**
5	37	50	62	74	55	68	73	**60**
6	28*	83	66	66	61	61	--	**61**
7	46*	67	68	77	72	78	73	**69**
MEAN	**49**	**51**	**64**	**71**	**65**	**65**	**66**	**62**

* = Best-Parent heterosis at the 0.05 level of probability (best parent is considered the parent with the lowest percentage sterile flowers).
-- = No green regenerants or no plants could be regenerated to maturity, n.d. = not determined.

Discussion

Anther Culture

Anther culture response in Table 4 was higher than in previous experiments, as indicated in Table 1. In the preliminary experiments, the method for anther culture was apparently at that time sub-optimal. However, ranking of the parental genotypes stayed similar, with the highest percentages responding anthers in genotype Ringo Sztar.

In agreement with all previous reports on diallel studies of anther culture in wheat (Lazar *et al.* 1984, Ekiz & Konzak 1994a) and barley (Hou *et al.* 1994, Powell 1988), highly significant genotypic differences for anther culture ability were found in this study. Also the predominance of the additive effects found in this study, accounting for 63% to 82% of the total genetic variation, is in agreement with other genetic studies in wheat (Bullock *et al.* 1982, Hou *et al.* 1994, Lazar *et al.* 1984, Tuvesson *et al.* 1989) or Triticale (Balatero *et al.* 1995). However, Ekiz and Konzak (1994b) testing a 4x4 complete diallel in wheat, found dominance and epistatic effects to be a major source of genetic variation, and Andersen *et al.* (1987) testing 215 wheat cultivars found the interactions between genotypes and replicates to be dominating, accounting for 45% to 50% of the variation. In our study significant SCA effects were found for percentage Responding Anthers, Embryo Induction Frequency and Embryo induction frequency per Responding Anther, indicating non-additive gene effects, e.g dominance or epistasis. Deaton *et al.* (1987) analysed three spring wheat parents with their F_1, F_2 and backcross generations and found dominance effects for callus induction frequency. Balatero *et al.* (1995) stated that the three parameter additive dominance (AD) model fitted well for embryo induction, indicating absence of epistatic effects. When the model was extended to include estimates of epistatic effects (six-parameter model), using the method of Jinks & Jones (1958), no significant epistatic effects were obtained, confirming the adequacy of the three parameter AD model (Balatero *et al.* 1995).

Genetic effects explaining the majority of the total variation do not imply rapid introgression of androgenic traits, due to genotype x environment variation (Jones & Petolino 1987), dominance and epistatic effects and large random variation components (Deaton *et al.* 1987). Heritability estimates of androgenic traits might therefore produce more information on the efficiency of the introgression process. Narrow sense heritabilities in this study were 0.39, 0.48 and 0.53 for percentage Responding Anthers, Embryo Induction Frequency and Embryo induction frequency per Responding Anther, respectively, so progress can be made. Other authors found narrow sense heritabilities of 0.62 to 0.70 for percentage Responding Anthers in wheat (Lazar *et al.* 1984) and 0.28 or 0.24 for percentage Responding Anthers in barley (Dunwell *et al.* 1987, Powell 1988, respectively). Deaton *et al.* (1987) found in only one out of three wheat crosses a significant narrow sense heritability of 0.94 for RA. As shown by Hou *et al.* (1994) testing

F_2- and F_3-combinations of barley, progress in anther culture response by crossing and selection is possible, but the choice of the genotypes has to be made carefully.

In the present study best-parent heterosis for percentage Responding Anthers, Embryo Induction Frequency and Embryo induction frequency per Responding Anther was found for 7%, 21% and 14% of the 42 F_1-combinations, respectively (Table 4), indicating trangression of traits. In the study of Charmet & Bernard (1984), testing a 7x7 complete diallel in hexaploid Triticale also best-parent heterosis was found for embryogenesis. Ouyang (1986) testing 17 wheat crosses found in 71% of the F_1-combinations best-parent heterosis for pollen callus induction frequency. However, Abd El-Maksoud & Bedö (1993) studying four wheat cultivars and their hybrids found no significant best-parent heterosis.

Low but significant reciprocal differences were found for percentage Responding Anthers and Embryo Induction Frequency. This would indicate a small role for cytoplasmic factors for these two traits. Lazar *et al.* (1984) and Charmet & Bernard (1984) also found significant reciprocal effects, but in the latter study these were caused by one line with *Triticum timopheevi*-cytoplasm. If this line with *Triticum timopheevi*-cytoplasm was excluded, no significant reciprocal effects were detected.

Whether the reciprocal effects found in the underlying study are truly of cytoplasmic origin can be analysed with the F_2-generation. In case the reciprocal effects are still present in the F_2-generation, cytoplasmic inheritance is most likely the cause of the differences, as was demonstrated by Ouyang (1986) with 17 crosses of wheat and by Hou *et al.* (1994) in a 4x4 complete diallel of barley. However, Goodenough (1984) states that in higher eukaryotes cytoplasmic inheritance of genetic traits is usually synonymous with maternal inheritance. In our study, no maternal effects (component c) were found and small but significant reciprocal differences, not ascribable to maternal effects (component d) were found for percentage Responding Anthers and Embryo Induction Frequency (Table 5), again indicating towards cytoplasmic factors.

Regeneration of the anther culture derived embryos

On average, 30% of the embryos regenerated into plantlets, with the highest percentage for the F_1-combination 3x5, with 46 regenerants per 100 embryos. Three other publications on wheat reported regeneration frequencies of 49 plants per 100 embryos for the genotype with the highest regeneration frequency (Agache *et al.* 1988, Lazar *et al.* 1984, Lazar *et al.* 1985). In the underlying study, 11% of the regenerants was green, with the highest percentage for F_1-combination 7x2, with 39.3 green plants per 100 regenerants. On average 0.5 green plants per plated 100 anthers were produced. Lazar *et al.* (1985) found 7 green plants per 100 anthers. The main reason for this difference is probably that in our study no selection for a high green plant regeneration was made between the parental lines. Genotypes in this study were merely chosen for their anther culture abilities and their *Fusarium* Head Blight resistance.

In this study additive components were significant to highly significant and explained 30 to 65% of the

variation for the three regeneration traits (Table 8). Ekiz & Konzak (1994a) found additive effects and epistasis for plant regeneration and green plant frequency, but did not give the percentage variation explained by these effects. Balatero *et al.* (1995) tested three reciprocal crosses in triticale and found that, as well as for embryo induction, also for plant regeneration the simple three parameter additive dominance (AD) model was sufficient. However, in not all crosses significant additive and dominance effects were found and the authors explained this with a higher sensitivity of plant regeneration to environmental factors.

In Table 9 it can be seen that of the parental lines, genotype 6 (PF 8049) had the highest green/albino ratio. This genotype also ranked relatively high for percentage green plants, green plants per 100 plated anthers and green plants per 100 produced embryos. However, this genotype did not transmit this trait to its offspring as the means of the F_1-combinations, where PF 8049 is one of the parents, did not show these high values. A high percentage green regenerants is apparently not always inherited to the offspring. This was confirmed by the fact that the narrow sense heritability for green plant regeneration in this study was low: 0.11. Charmet & Bernard (1984) testing triticale, found a broad sense heritability of 0.54 for green plant regeneration and 0.27 for green plant production. F_1-combinations 2x7, 7x2 and 7x5 had higher green/albino ratios, while their parents had a lower green/albino ratio, and low percentages green plants. This indicated heterosis, and was confirmed by the fact that the heterosis component for green plant regeneration was significant at the 0.01 level of probability.

No correlation was found between percentage green regenerants and percentage Responding Anthers, Embryo Induction Frequency or Embryo induction frequency per Responding Anther (Table 3). This was in agreement with other cereal studies on the correlation between embryo induction and plant regeneration (Balatero *et al.* 1995, Charmet & Bernard 1984, Deaton *et al.* 1987, Foroughi-Wehr *et al.* 1982). Anther culture response and green plant regeneration appear to be under genetically independent control mechanisms. Balatero *et al.* (1995) state that the inheritance of plant regeneration might be more complex due to a higher sensitivity to environmental factors. In our study the environment for donor plant growth consisted of an accurately controlled phytotron chamber in which the changes of environment could be neglected. There were highly positive correlations between percentage albino regenerants and percentage Responding Anthers, Callus Induction Frequency, Embryo Induction Frequency, Callus Induction frequency per Responding Anther and Embryo induction frequency per Responding Anther. This means that genotypes that produce well in anther culture also tend to have high percentages albino regenerants. As the majority of the regenerants is albino, this is not surprising. The percentage embryos with only root formation had a significant negative correlation with percentage Responding Anthers, Callus Induction Frequency and Embryo Induction Frequency.

Significant SCA values were detected for all three investigated regeneration traits, indicating dominance

and/or epistasis. Studying a population of 38 DH-lines developed from the F_1 between 2 wheat parents, Agache *et al.* (1988) also found epistatic control of regeneration ability of the total number of plants. Unfortunately no statistical data were given on green plant regeneration.

In this study no maternal effects (component c) or reciprocal differences not ascribable to maternal effects (component d) were detected for regeneration. This is confirmed by Balatero *et al.* (1995) who also found no significant reciprocal differences stated that there was no indication for maternal effects. In the underlying study no significant GCA effects were found. This is in contrast with Charmet & Bernard (1984), who found significant GCA-, SCA- and reciprocal effects for green plant regeneration and for plant yield. The GCA/SCA ratio was significant for green plant regeneration, indicating predominantly additive gene action. However, the authors concluded that non-additive gene action and cytoplasmic influences or nucleo-cytoplasmic interaction were also involved, as both SCA and reciprocal effects were significant. As earlier mentioned, one line with *T. timopheevi* cytoplasm was responsible for the reciprocal effect. The maternal effects appeared to be more important than specific reciprocal effects and the authors concluded that cytoplasmic influences were involved (Charmet & Bernard 1984).

Heterosis was significant for percentage green plants and percentage albino plants, with a significant variety heterosis for percentage green plants and significant specific heterosis for percentage green plants and percentage albino plants. Significant heterosis for regeneration was also described in other publications (Charmet & Bernard 1984, Ekiz & Konzak 1994a). Balatero *et al.* (1995) tested three crosses of hexaploid triticale and found overdominance for regeneration as the green plant regeneration efficiencies exceeded those of the parents.

For green plants per 100 regenerants and for green plants per 100 embryos, genotype 2 (Praag 8) mainly contributed in a positive way, when used as a female parent and genotype 4 (Frontana) mainly contributed in a positive way when used as a male parent. Genotypes 5 (Ringo Sztar) and 7 (SVP 73016-2-4) contributed in either case in a positive way to a higher number of green plants. This indicates the need of carefull parent selection and also as which parent (male or female) a genotype is to be used.

Seed set on the DH_0-regenerants

Of two combinations no green plants could be regenerated and of three combinations no seeds were obtained. Before doubling with colchicine, the majority of the plants showed the haploid phenotype: small thin leaves and a 'grassy, bushy' structure. Flow cytometry experiments indicated that all plants with such a phenotype had the haploid chromosome number as compared to well-known cultivars used to serve as control samples (results not presented). Percentages spontaneous doubled haploids in wheat range from 4.3% to 42% (Gustafson *et al.* 1995, Li *et al.* 1988, Loschenberger *et al.* 1995, Ziegler *et al.* 1990) and was 63% in winter barley (Devaux 1987).

Although a high percentage of the variation could be explained by genetic effects (78%), only a small

amount of the genetic effects could be contributed to additive effects (4%). Narrow sense heritability for percentage seed set was therefore extremely low (0.01) and progress for this trait will be slow.

In this study, the haploid regenerants were doubled with colchicine in the conventional way, and showed a relatively high percentage of 84% fertile DH_0-plants and 116 seeds per DH_0-plant. Other publications in which the haploid regenerants were doubled in the conventional way, report of 21% fertile DH_0-plants and 31 seeds per DH_0-plant (Barnabas *et al.* 1991). In the latter study, the authors found that applying colchicine in the induction phase increased the number fertile plants up to 69% and the number of seeds per plant increased up to 58. Gustafson *et al.* (1995) found fertility rates of 87% for spontaneously doubled haploid wheat regenerants derived from microspore culture and 27% for colchicine doubled wheat regenerants from the same experiments. Devaux (1987) reported 70.7% fertility and on average 97 seeds per plant on colchicine treated anther culture derived barley regenerants, and 73% fertility and 188 seeds per plant on spontaneously doubled haploids, derived from anther culture. It is clear that colchicine treatment has a detrimental effect on seed set, as compared to spontaneous doubling. In our case, 84% of the plants contained one or more seeds, however, not more than half of the analysed spikes set seed. Seed set of monocotyledoneous species after an in vitro phase is a major problem. Due to the in vitro process, fertility of the doubled haploids is reduced. He *et al.* (1993) tested haploid cell suspensions, derived from anther culture and found only 2 out of 14 DH-plants to be fertile. Ahmed & Sagi (1993) achieved only 1 fertile plant out of 1000 regenerated shoots, derived from embryogenic cell suspsensions. Shimada *et al.* (1994), testing Japanese wheat cultivars, found only 11 out of 285 (3.8%) regenerated doubled haploids to be fertile. Lanaud (1987) studied doubled haploids in cocoa (*Theobroma cacao*) and it appeared that the DH-plants usually had a lower fertility than the parents. A lack of differentiation of the ovules was the main reason for the lower fertility, however, these unfavourable characters were not observed in the cross progeny of the DH-plants. Authors stated that the lower fertility was caused by homozygosity, forced on a normally allogamous plant. Ovule fertilities were 70% to 77% and pollen fertilities 60% to 88%.

No correlation was found between the seed set percentage with any of the measured anther culture or regeneration traits. Genetic control of the measured traits appears to be independent. This is confirmed by Takacs *et al.* (1994) who found no correlation between in vitro seed set and the haploid plant regeneration frequency. They also concluded that the genetic components were independent. Introducing anther culture in a conventional breeding programme can quickly provide the breeder with genotypes that have a higher embryo production. However, the final measure of efficiency of the anther culture method is the number of green plants that can be regenerated from a certain number of cultured anthers. Best parent heterosis for green plant regeneration was found in 10 out of 42 F_1-combinations and therefore carefull parent selection is of eminent importance. Heritabilities for anther culture are more promising than for green plant regeneration, and therefore more emphasis should be paid to parents which, after crossing, result in F_1-hybrids with a good green plant regeneration.

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Chapter 3: Inheritance of anther culture derived green plant regeneration in wheat (*Triticum aestivum* L.)

Abstract

A study was set up to determine the inheritance and combining ability of the factors anther culture response and green plant regeneration. Reciprocal crosses were made between cultivar Ringo Sztar, showing high anther culture response and the cultivars Ciano 067 and Benoist H77022, showing a high level of green plant regeneration. Averaged over all genotypes, 23.0% of the anthers responded and a callus induction frequency of 77.8% was observed. Of all the embryos, 43.0% developed into plantlets, 25.6% of the regenerants being green, the result being that 3.3 green plants per 100 anthers were formed. Genotypic effects accounted for 57.7%, 86.3% and 77.5% of the total variance of anther culture response, callus induction frequency and embryo induction frequency, respectively. Additive and dominant gene action was detected for all characteristics, including green plant regeneration. No reciprocal differences were found for anther culture response, embryo induction frequency and green plant regeneration, indicating no cytoplasmic effects. A small but significant reciprocal difference was found for callus induction frequency. Embryo production was primarily correlated with anther culture response and not with the number of embryos produced per plated anther or per responding anther. Possible mechanisms for the inheritance of green plant regeneration are discussed.

Introduction

Gramineous species have proven to be recalcitrant crops with regard to in vitro androgenesis techniques such as anther- and microspore culture. In general, many genotypes of most of the species respond poorly. In particular green plant regeneration is low, either because regeneration of albino plantlets occurs or no regeneration at all. The occurrence of high numbers of albino plants has been frequently reported. In wheat 97% albino plants for cultivar Edwall were found by Zhou & Konzak (1989) and 88% averaged over four German spring wheat cultivars by Ziegler *et al.* (1990). High percentages of non-regenerating embryos (excluding albino's) were also reported in wheat, e.g. 90% by De Buyser & Henry (1979) and 80% by De Buyser *et al.* (1989). However, some wheat genotypes showed high levels of expression for certain other androgenic traits, e.g. anther culture response, callus and embryo induction frequencies, green plant regeneration and may therefore be used in crossing programmes to try to improve androgenesis in general in agronomical important cultivars. Wheat cultivars that are thought to express androgenic traits at high levels are e.g. Ciano 067, Pavon 076 and Dirkwin, which showed callus induction frequencies of 115, 334 and 479 calli per 100 anthers and a green plant yield of 70, 72 and 357 green plants per 100 anthers, respectively (Ouyang *et al.* 1983; Zhou *et al.* 1991; Orshinsky & Sadasivaiah 1994). Cultivar Gernard 81 produced up to 1002 embryos per 100 anthers (Otani & Shimada 1993).

Optimization of anther culture conditions (i.e. growth conditions of the donor plants, culture medium and pretreatments of the anthers), has improved the efficiency of anther culture through the years. Foroughi-Wehr & Friedt (1984) reported in the barley genotype Igri 2.4 green plants per 100 anthers. Cistué *et al.* (1994) reported ten years later in the same genotype up to 1800 green plants per 100 anthers. However, previous studies have indicated that androgenic traits are also under strong genetic control. Heritabilities were estimated in various studies. Lazar *et al.* (1984b) found in wheat narrow sense heritabilities for callus production frequency and regeneration frequency of 0.6-0.7, and Ekiz & Konzak (1994a) estimated in wheat narrow sense heritabilities of 0.68, 0.54 and 0.43 for callus induction, green plant percentage and green plant yield, respectively. A relatively high heritability indicates that introgression of that androgenic trait will show rapid progress. However, significant environmental variances (Lazar *et al.* 1984a) and relatively high error variances (Deaton *et al.* 1987) may slow down the introgression process.

Nuclear, as well as cytoplasmic or maternal effects are known to influence anther culture efficiency. In addition, interactions between several traits were found (Lazar *et al.* 1984b; Andersen *et al.* 1987). Extensive deletions in the plastid genome are primarily suggested to be the cause for microspore derived albino plants in wheat and barley (Day & Ellis 1984, 1985).

This study was carried out to investigate the inheritance of green plant regeneration in relation to anther culture response in wheat. A wheat genotype with a high anther culture response, but low green plant regeneration was reciprocally crossed with two wheat genotypes with relatively low anther culture

response, but high green plant regeneration. Genotypes that combine high values of both traits are presumed to be suitable genotypes for in vitro selection experiments (Bruins *et al.* 1993). For that reason, one of the genotypes in this study (Ringo Sztar) was chosen for its high *Fusarium* Head Blight (FHB) resistance level in the field. In vitro selection for FHB-resistance with highly responsive and regenerative genotypes at the haploid level would be an efficient way of producing homozygous FHB-resistant genotypes.

Materials and methods

Three wheat cultivars (Ringo Sztar, Ciano 067 and Benoist H77022), known for their extreme response in anther culture and green plant regeneration were used in this study. Ringo Sztar had previously shown to have a high anther culture response (Bruins *et al.* 1993). 18-20 Responding anthers and 60-70 calli per 100 anthers were found in several experiments. However, plant regeneration percentages were extremely low: 0.3 green plants and 1.1 albino plants per 100 embryos. For comparison: the means for plant regeneration, averaged over 23 genotypes from the same experiment, including Ringo Sztar, amounted to 3.4 green plants per 100 embryos and 33 green plants per 100 regenerated plants. Tuvesson *et al.* (1989) found in their experiments that the parents Ciano 067 and Benoist H77022 gave 19 and 24 embryos per 100 anthers, and had relatively high regeneration frequencies of 53 and 32 green plants per 100 regenerated plants, respectively.

Seeds of these three parent cultivars were sown and vernalized for eight weeks. Plants were grown in the greenhouse with a 14 h photoperiod and a temperature regime of 15°C (light) and 10°C (dark). Reciprocal crosses were made between cultivar Ringo Sztar and the other two parents. Seeds of the four F_1-combinations were sown together with the parental cultivars and vernalized for eight weeks. Plants were grown in the greenhouse with a 14 h photoperiod for five weeks and a temperature regime of 15°C (light) and 10°C (dark). After that, plants were transferred to the field. At least ten F_1-plants per combination were tested. The first three heads of each plant were chosen for anther culture. Anther culture was carried out according to Bruins *et al.* (1993), in short: excision of the anthers at the mid-uninucleate stage, tillers were surface sterilized spraying with 70% ethanol, the anthers were plated on P2 medium and culture conditions were 28°C in the dark. After six weeks of culture, the following factors were assessed: the number of responding anthers per spike that produced at least one embryogenic or non-embryogenic structure (watery callus) and the number of embryogenic structures and non-embryogenic structures per spike. The following calculations were made: number of responding anthers/number of plated anthers x 100% (=anther culture response; RA), total number of embryogenic and non-embryogenic structures/number of plated anthers x 100% (=callus induction frequency; CIF), number of embryogenic structures/number of plated anthers x 100% (=embryo induction frequency; EIF), total number of

embryogenic and non-embryogenic structures per responding anther (CIRA), number of embryogenic structures per responding anther (ERA).

Embryos larger than 1 mm were transferred for regeneration to MS medium (Murashige & Skoog 1962), supplemented with 3% sucrose, 1 mg l^{-1} silver nitrate, 160 mg l^{-1} glutamine and 0.5 mg l^{-1} thiamine. After two to three weeks, regeneration of the embryos was assessed. Plated embryos were subdivided into four classes: green shoots, albino shoots, only roots or no regeneration. All data were transformed by taking the square root to improve the normality of the distribution. The transformed data were analyzed on the basis of predicted means from Residual Maximum Likelihood (REML) Variance Component Analysis (Genstat 5 Committee 1993).

Results

In vitro androgenic development could be induced in all parents and F_1-combinations (Table 1). Highly significant differences for anther culture response were found among entries. Ringo Sztar, which was chosen because of its good anther culture response, proved to have significantly the highest anther culture response of all three parents with 32.3% responding anthers. Ciano 067 had the lowest androgenic response (4.2%) and Benoist H77022 showed an intermediate response (13.8%). The F_1-combinations Ringo Sztar x Ciano 067 and Ciano 067 x Ringo Sztar showed intermediate anther culture responses (18.4% and 11.8%, respectively) between the two parents, indicating additive inheritance. The anther culture response values for the reciprocals were not significantly different from each other, implying no cytoplasmic effects. Anther culture response values of the F_1-combinations Benoist H77022 x Ringo Sztar and Ringo Sztar x Benoist H77022 were not significantly different from parent Ringo Sztar, indicating dominant inheritance, and were not significantly different from each other. Ranking for callus induction frequency and embryo induction frequency was similar to anther culture response (Table 1). For green plant regeneration, Ciano 067 showed the highest (39.7%), Benoist H77022 an intermediate (17.3%) and Ringo Sztar the lowest frequency (2.8%)(Table 2), a ranking identical to previous results and literature (Tuvesson *et al.* 1989). The reciprocal F_1-combinations Ringo Sztar x Ciano 067 and Ciano 067 x Ringo Sztar were not different from each other. These combinations also showed intermediate green plant regeneration (20.4% and 25.0%, respectively) between the two parents, indicating additive inheritance. The reciprocal F_1-combinations Benoist H77022 x Ringo Sztar and Ringo Sztar x Benoist H77022 showed a comparable low green plant regeneration (1.2% and 1.6%, respectively) as parent Ringo Sztar. The absence of reciprocal differences for anther culture response, embryo induction frequency and green plant regeneration indicates no role for cytoplasmic factors for these three traits.

Table 1. Anther culture response of three wheat parents and four F_1-crosses*.

Genotype	Number of plated anthers	% Responding anthers	% Callus Induction Frequency	% Embryo Induction Frequency	Callus Induction/ responding anther	Embryo Induction/ responding anther
Ciano 067	2270	4.2a	19.9a	14.4a	3.25a	2.33a
Ringo Sztar x Ciano 067	2598	18.4b	69.0c	45.7b	3.48a	2.29a
Ciano 067 x Ringo Sztar	3666	11.8b	37.8ab	22.7ab	2.89a	1.75a
Ringo Sztar	1726	32.3c	117.7d	72.9c	3.61a	2.24a
Benoist H77022 x Ringo Sztar	1959	36.0c	118.7d	72.6c	3.67a	2.21a
Ringo Sztar x Benoist H77022	4014	33.1c	121.6d	74.3c	3.58a	2.18a
Benoist H77022	2211	13.8b	61.9bc	44.6b	2.88a	2.03a
Mean		23.0	77.8	48.9	3.38	2.13

* Treatment means not followed by the same letter are significantly different at the 0.05 level of probability as determined by REML Variance Component Analysis after square root transformation.

Table 2. Plant regeneration from anther derived embryos of three wheat parents and four F_1-crosses.

Genotype	Number of plated embryos	green shoots (%)	albino shoots (%)	roots only (%)	green plants per responding anther	green plants per 100 anthers
Ciano 067	290	39.7	26.9	7.9	0.74	5.1
Ringo Sztar x Ciano 067	759	20.4	32.5	8.3	0.30	6.0
Ciano 067 x Ringo Sztar	661	25.0	28.3	7.4	0.36	4.5
Ringo Sztar	601	2.8	36.9	7.5	0.03	1.0
Benoist H77022 x Ringo Sztar	809	1.2	34.9	8.5	0.01	0.5
Ringo Sztar x Benoist H77022	1687	1.6	39.3	6.7	0.02	0.7
Benoist H77022	683	17.3	11.3	10.7	0.27	5.3
Mean	-	**11.0**	**32.0**	**7.9**	**0.14**	**3.3**

For callus induction frequency a low but significant reciprocal difference shows that Ringo Sztar as a female parent favours callus induction more than as male parent. No large differences between parents and/or F_1-combinations for the percentage of embryos that regenerated only roots was found, and it varied from 6.7% to 10.7%. Averaged over all genotypes, of the 18,444 anthers, 23.0% had responded with embryos or non-embryogenic structures. On these responding anthers 9028 embryos and 5322 non-embryogenic structures were found, most anthers producing more than one structure. The 5490 embryos that were larger than 1 mm were transferred to regeneration medium. Eleven percent of them developed into green plants, 32.0% into albino plants and 57.0% did not regenerate or developed only roots. Overall, of the embryos regenerated into plantlets, 25.6% was green. Per 100 anthers on average 3.3 green plants were formed.

Table 3 shows correlations between the different androgenic characters. Combinations with a significant high positive correlation were: percentage responding anthers with callus- and embryo induction frequency; callus induction frequency with embryo induction frequency (0.98; not shown); callus induction per responding anther with embryo induction per responding anther; and non-embryogenic structures with albino regenerants. Combinations with a significant high negative correlation were: anther culture response with green plant regeneration; callus- and embryo induction frequency with green plant regeneration (-0.96 and -0.96; not shown); and non-embryogenic structures and albino regenerants with root regeneration. Variance analysis showed that genotypic components for anther culture response, callus induction frequency and embryo induction frequency accounted for 57.7%, 86.3% and 77.8% of the total variance, respectively.

Table 3. Correlation matrix of different traits. Presented are the values of r. *=P<0.05, **=P<0.01.

CIF	0.95**						
EIF	0.94**						
CIRA	0.17						
ERA	0.08	0.89**					
% Non-embryos	0.22	0.68	0.27				
% green	-0.98**	0.07	0.41	-0.49			
% albino	0.55	0.58	0.04	0.99**	-0.49		
% root	-0.16	-0.57	-0.08	-0.91**	0.14	-0.86*	
% No regeneration	0.75	-0.47	-0.51	-0.08	-0.49	-0.10	0.39
	RA	CIRA	ERA	Non-Embryos	% green	% albino	% root

Discussion

Average anther culture response and callus induction frequency values in this experiment of 23.0% and 77.8%, respectively, were higher than anther culture response and callus induction frequency values reported in most other publications on anther culture of wheat: 20.3% and 41.0% (Barnabas *et al.* 1991); 7.8% and 20.0% (Abd El-Maksoud & Bedö 1993); 18.0% and 57.4% (He *et al.* 1993), for anther culture response and callus induction frequency, respectively. The regeneration frequency was 12.8 plants per 100 plated anthers, of which 25.6% was green, whereas Tuvesson *et al.* (1989) reported a percentage of 23.4 plants per 100 plated anthers of which 15.3% was green. However, Ouyang *et al.* (1983) produced 72 green plants per 100 anthers in cultivar Ciano 067, whereas under our conditions with the same cultivar, only 5.1 green plants per 100 anthers were produced.

The majority of the total variance of the androgenic traits could be explained by genotypic effects. In this study, additive and dominant gene action were found for anther culture response and callus- and embryo induction frequency. Previous publications indicated that androgenic traits were mainly controlled by nuclear genes, with the additive gene action being predominant (Zhou & Konzak 1992). Dominant gene action was reported in wheat by Lazar *et al.* (1984b).

The absence of reciprocal differences for several androgenic traits, found in our experiments, is in agreement with other publications on wheat and barley where no indication for reciprocal effects (Bullock *et al.* 1982; Zhou & Konzak 1992) or small reciprocal effects (Foroughi-Wehr *et al.* 1982; Lazar *et al.* 1984b) were reported. However, other reports on barley and wheat indicated significant reciprocal effects

for callus induction, green plant percentage and green plant yield (Powell 1988; Ekiz & Konzak 1994a). In the study of Ekiz & Konzak (1994a) the reciprocal effects were mainly caused by two cross combinations. Sagi & Barnabas (1989), using alloplasmic lines, found significant cytoplasmic effects for anther culture response. For plant regeneration no cytoplasmic effects could be detected. Ekiz & Konzak (1991 a,b,c) found significant reciprocal differences and explained the absence of such reciprocal differences in other publications by a narrow base of cytoplasm genetic variation or by relatively low levels of anther culture response, caused by the methods used. In our study a narrow base of cytoplasm genetic variation is unlikely because of the distant relationship between the three parents. Ringo Sztar is a Hungarian cultivar and Ciano 067 and Benoist H77022 are CIMMYT and French cultivars, respectively. Besides this, the levels of callus induction frequency in the present study, up to 122 calli per 100 anthers, are similar to those found by Ekiz & Konzak (1991c) for genotypes Chris and Edwall (123 and 133 calli per 100 anthers, respectively).

Ouyang (1986), on the other hand, stated that pollen callus induction frequency is controlled mainly by genes of the diploid anther wall tissue and not by genes of haploid pollen cells. Such maternal effects occurred when pollen lines derived from F_1-hybrids with great heterosis for pollen callus yield were used again as anther donors. The pollen callus induction frequencies were much lower than the induction frequencies of the F_1-hybrids, showing the disappearance of heterosis.

As the pollen population in our study was formed in the anthers of F_1-plants, it consisted of a segregating F_2-population, and possible mechanisms of inheritance for green plant regeneration can be speculated upon, with the assumption that there is no gametic selection. The genetic constitution of the gametes is likely to play a role, however parental effects cannot be excluded. The intermediate percentages of green plant regeneration per 100 embryos or per responding anther in the F_1-combinations between Ringo Sztar and Ciano 067 indicate segregation and that it is unclear whether the genetic constitution of the F_2-pollen population or of the F_1-maternal tissue caused the intermediate reaction. The low percentages of green plant regeneration per 100 embryos, per responding anther or per 100 anthers in the F_1-combinations between Ringo Sztar and Benoist H77022, comparable to parent Ringo Sztar, is most likely to be caused by the genetic constitution of the F_1-plant, which could be caused by a maternal effect.

The number of embryogenic structures per responding anther (ERA) was not significantly different between the genotypes and varied from 1.81 to 2.75. This is in agreement with other publications were the ERA value varied not significantly from 1.9 to 2.4 (Barnabás *et al.* 1991) or from 2.2 to 2.7 (Takács *et al.* 1994). Using four tetraploid *Triticum turgidum* genotypes on nine different media combinations, the ERA value was found to vary from 1.2 to 1.9, with one exception of a genotype producing 2.3 embryos per responding anther (Ghaemi *et al.* 1994). This ERA value appears to be an independent character of the medium used and which might be under genetic control.

The correlation between anther culture response and callus induction frequency or embryo induction frequency was high (r=0.95 and 0.94, respectively), similar to the results that were found by Pauk *et al.*

(1991). In their report 6 parents and 10 F_2-populations were tested on two media and the correlations between anther culture response and callus induction frequency were r=0.92 and r=0.91 for the two media, respectively. This correlation indicates that the production of embryos and non-embryogenic structures is mainly dependent on anther culture response and not on the number of structures per plated anther. No correlation was found between anther culture response and callus induction per responding anther or embryo induction per responding anther. This, combined with the fact that the number of embryogenic structures per responding anther was not significantly different between the genotypes, suggests that embryo production is predominantly related to anther culture response and not to the number of formed embryos per responding anther. In several previous reports anther culture efficiency was subdivided into three components: 1) Callus Induction Frequency; 2) Plantlet Regeneration Frequency (= number of structures producing green or albino plantlets/number of calli x 100%; 3) Green Plantlet Yield (= number of green plants produced/number of anthers cultured x 100%)(Ouyang *et al.* 1983; Konzak & Zhou 1991). Considering the strong correlation between anther culture response and callus induction frequency, found in our set of data and in previous publications where also both parameters were assessed (Chapter 2, Knudsen *et al.* 1989, Pauk *et al.* 1991), while no reports of no correlation are known, and the absence of significant differences for calli or embryos per responding anther, suggests that in this experiment the genetic variance for anther culture response was larger than for callus induction frequency. Therefore, another component can be formulated which can be added to the above mentioned three, or in this study even replace the first component, namely: Anther culture response (the number of anthers giving one or more structures).

This set of genotypes showed a high negative correlation between anther culture response, callus induction frequency, or embryo induction frequency with green plant regeneration. This is not so surprising, as the genotypes in the present study were selected for their extreme response in green plant regeneration; Ringo Sztar giving large numbers of embryos, but mainly albino regenerants and the other two parents giving less embryos but much higher percentages green regenerants. In contrast, the majority of other publications on the genetic basis of androgenic traits in wheat reported no significant correlation coefficients between different anther culture response components and regeneration components, indicating that these two groups of components may be controlled by different genetic factors (Agache *et al.* 1988; Ekiz & Konzak 1994b).

The F_1-combinations Ringo Sztar x Ciano 067 and Ciano 067 x Ringo Sztar combine a relatively high anther culture response with a relatively high percentage of green plant regeneration. The combination of these two traits in one genotype might provide a suitable genotype for in vitro selection experiments. The inclusion of a cultivar with a relatively high level of resistance against Fusarium head blight, Ringo Sztar, ensures the genetic variation needed for such in vitro selection experiments.

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Chapter 4: Phytotoxicity of deoxynivalenol to wheat tissue with regard to *in vitro* selection for *Fusarium* head blight resistance.

Abstract

Four types of wheat plant material i.e. seedlings, coleoptile segments, anther derived callus and anther derived embryos, were tested at different concentrations of deoxynivalenol (DON) and 3-acetyldeoxynivalenol (3-ADON). DON inhibited growth of all types of plant material. Seedling growth response to 4×10^{-5} M DON of a large set of genotypes, did not differentiate between tolerant and sensitive genotypes according to observed *Fusarium* Head Blight (FHB) resistance level in the field. In general coleoptile segments showed a growth reduction at 10^{-5} M DON. A concentration of 10^{-4} M DON appeared to be the optimum concentration to differentiate between haploid wheat calli for DON- tolerance. However, growth analysis data of 40 callus clones did not show any correlation with the known FHB-resistance levels of the original donor genotypes and -populations. Regeneration of the anther derived embryos in the embryo selection experiment was decreased 100-fold on DON-containing medium. Averaged across the callus and embryo selection experiments, green plant regeneration showed a decrease of approximately 20-fold on medium containing toxin.

Introduction

Fusarium Head Blight (FHB) is a fungal disease of wheat (*Triticum aestivum* L.) occurring in both temperate and sub-tropical regions. FHB is predominantly caused by *Fusarium graminearum* Schwabe and *F. culmorum* (W.G. Smith) Sacc.. Even low infection levels lead to significant yield losses and contamination of grain with mycotoxins. Genetic variation for FHB-resistance is widely present in the available gene pool. However, the resistance is quantitative and probably based on several minor genes, making it difficult to select for. Complete resistance has not yet been discovered. For estimating the amount of fungal biomass, visual scoring of FHB is an adequate tool. This estimation can be used as a reliable parameter of the resistance level in large scale screening of varieties or breeding lines
(Snijders 1990 a-c, Snijders & Krechting 1992). For early selection in segregating populations, a laboratory method is highly desirable. The large number of individuals that can be screened and the limited space that is needed makes the application of *in vitro* selection an attractive approach.

During the last two decades many studies have been carried out to obtain plants with increased levels of disease resistance using toxic metabolites produced by the pathogen as selection agent, mostly applied at callus level (Bulk, 1991). Selection for resistance with toxic compounds was not successful in a considerable number of studies, possibly also because fundamental knowledge about the role of toxic components in pathogenesis, the mode of action and properties of toxins and their interaction with host cells was lacking in many cases. Van den Bulk (1991) lists disease resistant plants of various crops obtained by *in vitro* selection. Most successes were obtained with host-specific toxins. Successful *in vitro* selection for *Fusarium* resistances is limited to *F. oxysporum* in tomato, potato and alfalfa, using culture filtrate containing toxic metabolites or pure fusaric acid. It should be noted that for most of these toxic metabolites their role in the pathogenesis has not been elucidated yet, and that these studies were aimed at taking advantage of somaclonal variation occurring in tissue cultures. Somaclonal variation is a rather undirected way of creating variation as many factors can affect its nature and frequency. In the study reported here segregating populations of wheat have been used as the main source of variation.

Fusaric acid was used to select microcalli (Wenzel et al. 1984) or embryogenic calli (Chawla & Wenzel, 1987) of barley for resistance against fusaric acid, in order to obtain resistance to the *Fusarium* disease causing seedling blight, root rot and head blight. Plants could be regenerated from the surviving resistant calli and testing by leaf bio-assay revealed that many were resistant to the toxin. However, it was not demonstrated whether barley plants with an increased resistance to *Fusarium* were obtained.

Both *Fusarium graminearum* and *F. culmorum* produce the non-macrocyclic trichothecenes deoxynivalenol (DON) and 3-acetyldeoxynivalenol (3-ADON). There are strong evidences that trichothecene production may be involved in the pathogenicity of *Fusarium* spp. (Desjardins et al. 1989, Snijders & Perkowski 1990, Beremand *et al.* 1991). Even in low concentrations (< 10^{-5} M) these toxins are highly toxic to plant and animal tissue as they inhibit eukaryotic protein synthesis by blocking the peptidyl transferase

step (Carter *et al.* 1980). DON and 3-ADON are non-specific toxins and are regarded as aggressiveness factors, i.e. they increase the extent of disease symptoms and colonization but are not involved in the primary interaction that determines compatibility (Snijders & Krechting 1992, Al-Heeti 1987, Desjardins 1992, Manka et al. 1985). Plants tolerant to these toxins do not show complete resistance, but an increased resistance (Snijders & Krechting 1992). Our study reported here is aimed at the development of a system to select for FHB-resistance in segregating wheat material using DON as the selective agent. Experiments were carried out on seedling, coleoptile, anther derived callus and embryo level, and compared with available information from field experiments.

Materials and Methods

Plant material of 41 wheat genotypes and segregating populations was used in the experiments. The FHB-resistance of these genotypes, given as the weighted mean of five years (1987-1991), is given in Table 1 (Snijders 1990c). FHB-resistance was assessed in the field as the percentage head blight per individual plant. All genotypes used are part of the *Fusarium* resistance breeding programme at the CPRO-DLO. No preliminary selection for *in vitro* response was carried out.

Toxins

Deoxynivalenol (DON) and 3-acetyldeoxynivalenol (3-ADON) were obtained from Sigma and J.D. Miller, Plant Research Centre Agriculture Canada, Ottawa, Canada. DON and 3-ADON were dissolved in 2 ml ethanol (70%), diluted with 2 ml H_2O, sterilized by membrane filtration (0.22 μm OPTEX, Millipore) and added to the medium.

Effect at seedling level

Seeds of 18 winter wheat genotypes were disinfected by dipping in 70% ethanol for a few seconds and then immersing in a 1% calcium hypochlorite solution for 10 minutes, followed by several rinses in sterile distilled water. Seeds were incubated on wet filter paper under sterile conditions at a temperature of 16°C. After 48 hours, germinated seeds were transplanted to 180x16 mm culture tubes with 12.5 ml MS medium (Murashige & Skoog 1962), solidified with 8 g l^{-1} purified agar (Oxoid) and containing 0 M, 1.7×10^{-5} M, 2.7×10^{-5} M, 3.4×10^{-5} M and 4.1×10^{-5} M DON (5, 8, 10 and 12 mg l^{-1} respectively). Incubation took place at 16°C with a day length of 16 hours. Each experiment had a randomized block design with 4 replicates, representing the four levels in the incubator. Coleoptile and root lengths were measured every 24 h for 20 days.

Table 1. Resistance of 41 wheat (*Triticum aestivum* L.) genotypes, expressed as *Fusarium* head blight index[a], and inclusion (*) in selection experiments at seedling (S), coleoptile (CO), callus (CA) and embryo (E) level. Genotypes are presented in descending order of FHB-resistance.

		FHB-index	Selection level			
Genotype	Code[b]	(%)	S	CO	CA	E
Ft 83-326[c]	I	0.9	-	*	*	*
Frontana[c]		1.6	-	*	-	*
Ning 8343[c]	II	1.7	-	*	*	*
Praag 8	III	2.1	-	*	*	*
SVP 72017-17-5-10-1		3.3	-	*	-	-
SVP 72107-17-5-10	IV	7.6	*	*	*	*
SVP 77076-1		7.9	*	-	-	-
Arina		8.4	*	-	-	-
SVP 77076-4		8.6	*	-	-	-
SVP 77076-38		9.5	*	-	-	-
Nobeokabozu komugi[c]	V	9.6	-	-	-	*
Ringo Sztar	VI	9.6	-	-	*	*
Kraka		11.1	-	-	-	*
Kaluzskaja 9	VII	11.6	-	-	*	*
SVP 77078-30		13.3	*	*	-	-
SVP 72003-4-2-4		14.9	*	*	-	-
Saiga		18.2	*	*	-	-
SVP 77079-15		18.3	*	-	-	-
SVP 75059-28		21.3	*	*	-	-
Arminda		24.2	*	-	-	-
Obelisk		37.8	-	-	-	*
SVP 75059-32	VIII	39.9	*	*	*	*
SVP 73030-8-1-1		42.7	*	*	-	-
SVP 73016-2-4	IX	43.2	*	*	*	*
SVP 75059-46		47.8	*	-	-	-
SVP 73012-1-2-3		48.7	*	*	-	-
CWW 4055/3	X	49.6	-	*	*	-
PF 8049[c]		52.2	-	-	-	*
Nautica		56.8	*	-	-	-
SVP 72005-20-3-1	XI	57.5	*	*	*	*
Pf 80271[c]		59.2	-	-	-	*
BR 14 (Pf 79780)[c]		59.6	-	-	-	*
SVP 77071-2-6		69.2	*	-	-	-
SVP C8703 F2 (IVxXI)		segregating	-	-	*	-
SVP C8709 F2 (IVxIX)		segregating	-	-	*	*
SVP C8726 F2 (VIIIxIX)		segregating	-	-	*	*
SVP 88001 F2 (IIIxII)		segregating	-	-	-	*
SVP 88002 F2 (XxI)		segregating	-	-	*	*
SVP 89002 F1 (IVxVI)		segregating	-	-	*	*
SVP 89002 F2 (IVxVI)		segregating	-	-	*	*
SVP 89004 F2 (VIIxV)		segregating	-	-	*	-

[a] Weighed mean of five years (1987-1991). Infection measured as the percentage head blight per ear in the field.
[b] Parental code used for F_1- and F_2-populations.
[c] Spring wheat.

The measurements obtained from a particular culture tube with a specified genotype within a replicate constitute repeated measurements on the same object. The measurements in time in this experiment can be modelled by a simple function like a logistic curve, as it concerned a growth experiment with a clearly defined lower limit, being the length of the coleoptile or root after 2 days germination, which was 2 mm, and an upper limit, the maximum attainable length within the tube: 115 mm for coleoptile length, 65 mm for root length (Keen et al. 1986, Rowell & Walters 1976). Subsequently the parameters *K*, (the slope parameter) and *t50* (the point of inflexion of the curve, the time at which the coleoptile or root has a length equal to half of the tube) of these curves were analyzed in an ordinary analysis of variance as new characteristics describing the behaviour of the objects in time.

Effect at coleoptile tissue level

Coleoptile growth of seedlings of which the root growth in the DON-containing medium is completely inhibited seems more like a tolerance reaction of the plant to inhibition of root activity. To evade this problem, the effect on coleoptile tissue, directly incubated in a DON-containing solution, was studied. The method used was based on that of Wang & Miller (1988). Wheat seeds were surface sterilized as described above. Seeds were sown in moist sterile sand in 200 ml jars which were placed in a dark incubator for 3 days at 22-25°C, the temperature depending on the known germination speed of the genotype. Using a device consisting of three razor blades mounted 3 mm and 4 mm apart on a handle, a 4 mm coleoptile section was cut from each seedling after the apical 3 mm had been discarded. DON was added to a buffer (1.794 g l^{-1} KH_2PO_4; 1.019 g l^{-1} citric acid monohydrate; pH 5.6) at 0 M, 10^{-6} M, 10^{-5} M 10^{-4} M, and 10^{-3} M. DON was initially sterilized in 2 ml ethanol. After evaporation of the ethanol, a stock solution was made with sterile buffer. Ten coleoptile sections were incubated in a 10 ml tube containing 2 ml of DON-containing sterile buffer with 2% sucrose, or in buffer with 2% sucrose without DON (control). The segments floated on the medium and were well provided with oxygen. The tubes were then incubated in the dark at 25°C on a horizontal rotary shaker at 50 rpm and an amplitude of 1.9 cm for 20 h. At the end of this period, the size of the segments was measured using an image analyzer system. Three replicates were used. Data were analyzed for differences in mean growth of the sections between treatments and the control. Similar experiments were performed with 3-ADON.

Production of anther derived callus and embryos

Anthers were cultured according to the method of Ouyang (1986). Basically tillers from field grown plants were collected in the mid-boot stage, i.e. 43 on the Zadoks growth scale (Zadoks et al. 1974), when the boots are swollen but the flag sheath has not yet opened. Anthers from spikelets centrally positioned within the head were squashed in drops of acetocarmine. Heads containing microspores in the mid- to late uni-nucleate stage were sterilized with 0.1% $HgCl_2$ solution for 8 minutes, followed by four rinses with sterile water. For callus induction, anthers were aseptically excised and placed on Potato-2 medium (P2)

(Chuang et al. 1978) in 6 cm Petri dishes. The cultures were incubated in the dark at 29°C. After 30 days, embryos were transferred from the induction medium to callus propagation medium (MS medium supplemented with 160 mg l^{-1} L-glutamine, 0.5 mg l^{-1} thiamine, 2 mg l^{-1} 2,4-D, 3% sucrose and 0.6% agar (Daichin), pH 5.8); ten days later this was repeated for newly emerged embryos. The calli were subcultured every three weeks, until sufficient material was available for the selection experiments.

Determination of the differentiating DON-concentration at callus and embryo level.

To define the differentiating DON-dose for selection, two experiments, each including three different DON-concentrations were performed, namely 0 M, 10^{-5} M, 3×10^{-5} M and 12×10^{-5} M DON (0, 3, 9, 36 mg l^{-1}), and 0 M, 3×10^{-5} M, 9×10^{-5} M and 27×10^{-5} M DON (0, 9, 27, 81 mg l^{-1}), respectively. Callus from the FHB-susceptible winter wheat cultivar Kraka was used. Five callus pieces were plated per Petri dish (Ø 6 cm). In experiment 1 each replicate consisted of calli from the same clone. In experiment 2 each replicate consisted of calli derived from anthers of the same head. The experiment consisted of a complete randomized design with seven or eight replicates (Petri dishes). Incubation was as described above. Initial callus fresh weight and final callus fresh weight were measured per Petri dish after three weeks of incubation for experiment 1 and and after four weeks for experiment 2. Values were expressed as a percentage of the growth on control medium.

Effect at callus level.

Each callus clone tested was derived from one embryo. The calli were obtained as described above. As the callus clones did not all grow at the same rate, the number of subcultures (2-3) and the number of calli per callus clone involved in the experiment were different. Half the number of calli per callus clone was placed on control medium, the other half on DON-containing medium. Initial weight of the callus pieces used for the selection experiments was about 30 mg. Selection media had the same composition as the medium for callus propagation, except that they were solidified with 0.6% SeaPlaque agarose (FMC) instead of agar.

DON was sterilized in 70% ethanol from which a stock solution was prepared and added to the sterile medium. The media were checked for DON-degradation with time, using the analysis method described in Snijders & Krechting (1992). After four weeks of incubation the change in callus fresh weight was measured for each individual callus piece. In this experiment an image analyzer was used to measure also the area of a 2-dimensional projection of each callus, relative to a fixed area x. For two genotypes this was done at weekly intervals and for five other genotypes only at the beginning and end of the experiment.

Effect at embryo level.

Embryos produced in anther culture were directly transferred to DON-free regeneration medium or regeneration medium with 10^{-4} M DON. To both media 10 mg l^{-1} $AgNO_3$ was added to inhibit ethylene

action (Purnhauser et al. 1987). Regeneration was expressed as the number of embryos that had formed green or albino shoots or roots after eight weeks. Regenerated plants from anther derived callus and embryo selection experiments were transferred to soil and cultivated under a 16 h light period and a temperature regime of 15°C (day) and 10°C (night).

Results

Effect at seedling level

For coleoptile and root growth the parameters K_r and *t50* were analyzed per experiment for both control and treated seedlings. In all experiments genotype and medium effects were significant. There were no genotype x medium interactions indicating that the growth of the genotype on the control medium was related to the growth on DON-containing medium. Expressing K_r, the slope parameter, and *t50*, point of inflexion of the curve of the coleoptile growth on medium with 4.1×10^{-5} M DON as a percentage of the growth on control medium, K_r averaged 52% and varied for the genotypes between 39% and 69%; *t50* averaged 154% and varied between 131% and 167%. In general the coleoptile growth rate on DON-containing medium was 50% slower. Figure 1 illustrates coleoptile growth for the genotypes SVP 72017-17-5-10 and SVP 72005-20-3-1 and difference in *t50* between control and DON-containing medium. Maximum root length on DON-containing medium was, for all genotypes, never more than 4 mm. There was no correlation between reaction of seedling growth on DON-containing medium and FHB-resistance as described in Table 1.

Effect at coleoptile tissue level

The results of the effect of DON and 3-ADON on the growth of coleoptile sections are given in Table 2. For both DON and 3-ADON there was a considerable reduction in growth for concentrations 10^{-5} M and higher. For some genotypes a complete growth reduction was obtained at 10^{-4} M or 10^{-3} M toxin. Only one wheat genotype, SVP 72017-17-5-10-1, a line selected from SVP 72017-17-5-10, showed a relatively high trichothecene tolerance. Coleoptile segments of this line were the least affected by the two toxins at all four concentrations, except at 10^{-3} M DON where the genotype CWW 4055/3 showed a higher tolerance to the toxin.

Table 2. Growth of wheat (*Triticum aestivum* L.) coleoptile tissue in the presence of DON and 3-ADON, expressed as a percentage of the growth on control medium (DON-free). Genotypes are presented in descending order of FHB-resistance (see Table 1). - = not measured.

Genotype		Growth (%)			
	DON	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M
Ft 83-326		114	29	28	3
Frontana		103	24	17	7
Ning 8343		83	-	0	15
Praag 8		108	5	16	4
SVP 72017-17-5-10-1		149	60	34	20
SVP 72017-17-5-10		104	19	0	0
SVP 77078-30		71	14	18	20
SVP 72003-4-2-4		107	22	2	4
Saiga		86	28	19	0
SVP 75059-28		80	24	6	0
SVP 75059-32		93	12	2	0
SVP 73030-8-1-1		69	18	12	16
SVP 73016-2-4		89	24	20	10
SVP 73012-1-2-3		116	33	26	14
CWW 4055/3		120	40	21	27
SVP 72005-20-3-1		74	32	19	9
Mean		**98**	**26**	**15**	**9**
	3-ADON	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M
Ft 83-326		79	38	15	2
Frontana		56	36	16	10
Ning 8343		96	47	22	10
Praag 8		85	36	15	10
SVP 72017-17-5-10-1		163	94	31	23
SVP 72017-17-5-10		92	38	3	0
SVP 77078-30		91	34	13	13
SVP 72003-4-2-4		93	32	7	1
Saiga		109	46	14	0
SVP 75059-28		94	35	19	4
SVP 75059-32		97	20	5	4
SVP 73030-8-1-1		99	28	13	12
SVP 73016-2-4		79	30	8	5
SVP 73012-1-2-3		93	20	7	6
CWW 4055/3		83	39	5	3
SVP 72005-20-3-1		98	26	8	9
Mean		**94**	**37**	**13**	**7**

Determination of the differentiating DON-concentration at callus and embryo level

The callus induction frequency (number of calli/number of cultured anthers x 100%) for Kraka was 9%. Of the calli derived from Kraka, 51% gave embryogenic callus. The reduction in callus weight gain during incubation, expressed as percentage of the control, has been measured for five DON- concentrations (Figure 2) in two experiments.

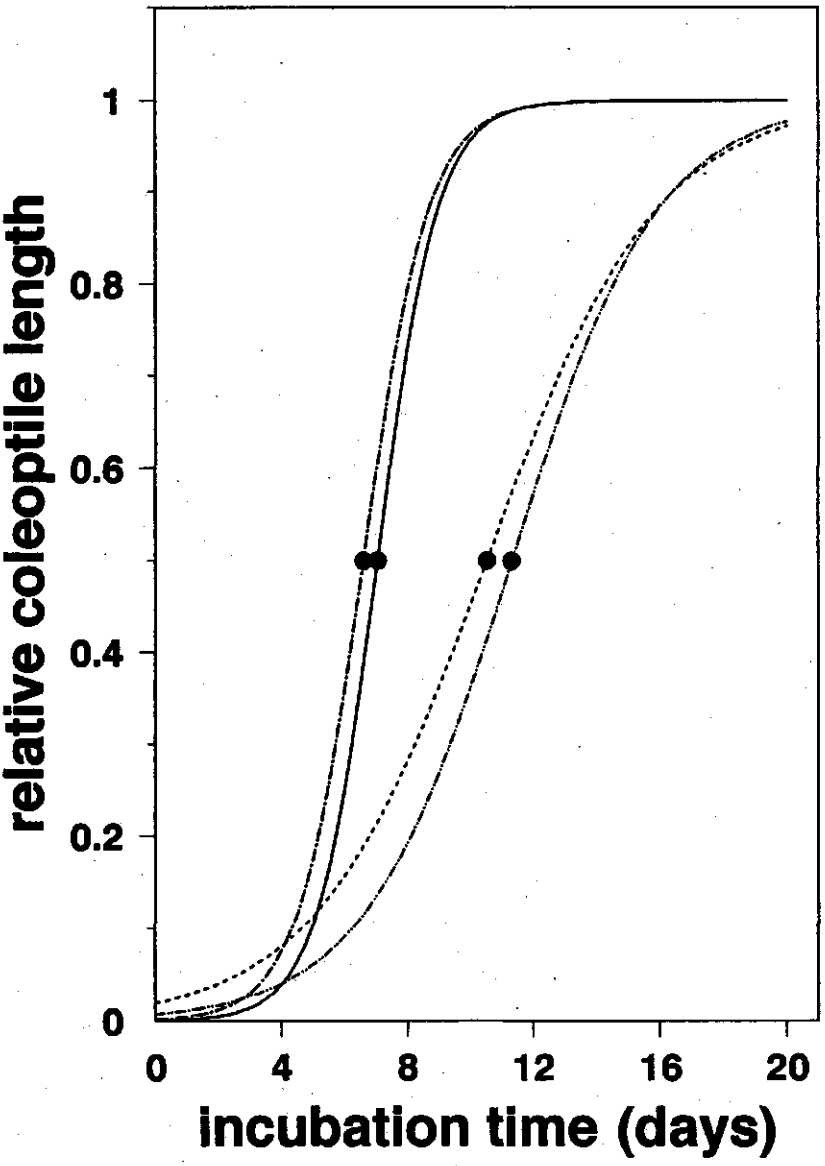

Figure 1. Seedling coleoptile growth of the wheat lines SVP 72017-17-5-10 and SVP 72005-20-3-1 on control medium and medium containing 4.1×10^{-5}M DON as a function of days of incubation. Coleoptile growth is expressed as the fraction of the distance to the top of the tube covered by the coleoptile. –·–·= SVP 72005-20-3-1 control, ——= SVP 72017-17-5-10 control, – – = SVP 72005-20-3-1 DON, –·–·= SVP 72017-17-5-10 DON. The point of inflexion of the curve *t50* is indicated by ●.

Taking into account the standard errors of the means, in experiment 1 there was no significant difference between the concentrations of 10^{-5} M and 3×10^{-5} M DON. At 12×10^{-5} M DON, the reduction in callus weight gain averaged 48.6% and ranged from 30.0% to 76.5% of the control. None of the calli died at this concentration. In experiment 2 the reduction in callus fresh weight gain at 27×10^{-5} M DON averaged 49.3% of the control. So, even this highest concentration of DON used, did not kill the calli. Based on the means of each experiment the response of callus fresh weight gain to DON-concentration can be described by an exponential curve (Figure 2).

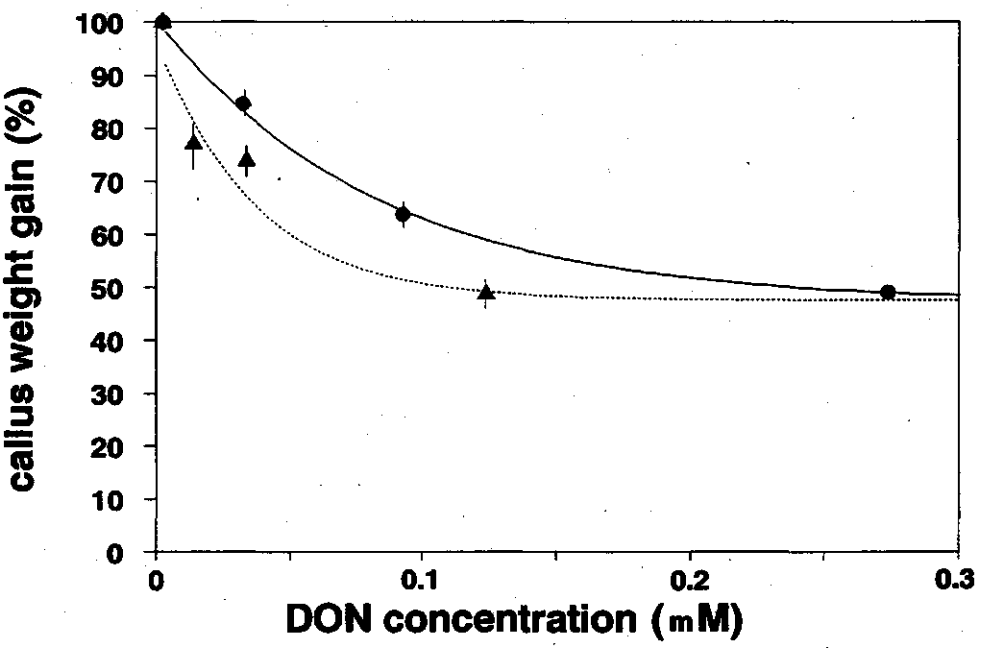

Figure 2. Reduction of weight gain of wheat (*Triticum aestivum* L.) callus after culture on DON-containing medium. Values have been expressed as a percentage of the growth on control medium (DON-free). The data presented are the means, and regressions for experiment 1 (▲---) and experiment 2 (●——). Vertical lines on the points represent the standard errors.

The coefficient of determination (R^2) was 79% for experiment 1 and 99% for experiment 2 (Steel & Torrie 1981). As the lowest DON-concentrations with a clear effect are located between 9×10^{-5} M and 12×10^{-5} M DON, a concentration of 10^{-4} M DON was chosen to evaluate the reactions of different wheat genotypes.

Effect at callus level

The reaction of 40 callus clones derived from 15 genotypes differing in FHB-resistance was measured at the differentiating DON-concentration determined, i.e. 10^{-4} M. In total 1624 callus pieces were measured with an average of 40 callus pieces per callus clone.

Anther culture response varied greatly with genotype. The highest percentage of responding anthers (number of anthers giving one or more calli/total number of anthers) was for the Hungarian winter wheat variety Ringo Sztar (18.6%). This genotype also had the highest callus induction frequency (63.8%). Table 3 shows the reduction of callus weight gain and callus area gain of the DON-treated calli, expressed as a percentage of the control. Testing more than one callus clone per parental line showed that results were similar, with the exception of genotype SVP 73016-2-4, which showed growth data varying from 17% to 100%. For segregating material there was, as expected, a large variation in growth of the different callus clones within a population. Although in most cases only two clones of a population could be tested, still a large variation for growth was observed e.g. SVP C8703 F_2 no.9 and no. 9.3. Growth of clone 9 was inhibited by the toxin (20%) whereas clone 9.3 was stimulated (110%). Clones SVP C8726 F_2 no.3A and no. 3D.3.2 showed a similar variation (202% and 18% respectively). Different callus clones derived from one embryo, e.g. SVP 88002 F_2 no. 8A, were all strongly inhibited by the toxin. On the control medium, the growth of calli did not differ significantly between genotypes.

For two spring wheat genotypes, Ning 8343 and FT 83-326, the area changes of calli were measured at weekly intervals (Figure 3). For both genotypes the increase in callus area per unit time on DON- and control-medium was linear. This linearity implies that for measuring changes in area, measuring the initial and final area will be sufficient. Based on the data of all individual callus pieces, there was a linear correlation between change in weight and change in area for the control (r=0.62) and for DON (r=0.66). Based on the means per genotype (Table 3), the correlation coefficient is 0.97. Weight change is a more reliable and simple criterium than area change for selection on DON-tolerance and is thus to be preferred.

The regeneration of the calli was poor. Out of 812 callus pieces on control medium, only 11 green regenerants could be obtained; two from Praag 8 no.7, one from SVP 73016-2-4 no. 46.2.1 and eight from SVP 88002 F_2 no.3-5. As a result of selection on toxin medium, only one green regenerant could be obtained, namely from the genotype Praag 8 no.7.

Table 3. Effect of 10^{-4} M DON on the reduction of callus fresh weight gain of 40 wheat (*Triticum aestivum* L.) callus clones and on the reduction of callus area gain of seven callus clones. Results have been expressed as a percentage of the growth on control medium (DON-free).

Genotype	Number of calli	Weight gain change (%)	Area gain change (%)
Cultivars and lines			
Ft 83-326	40	45	31
Kaluzskaja 9 no. 28.1.1	42	9	
Kaluzskaja 9 no. 28.2.3	56	20	
Ning 8343	30	47	54
Praag 8	32	43	38
Praag 8 no. 7	20	47	
Ringo Sztar 9.1.1	42	23	
Ringo Sztar 51.2.2	42	24	
SVP 72005-20-3-1	16	37	32
SVP 72017-17-5-10	16	40	44
SVP 73016-2-4	32	100	106
SVP 73016-2-4 no. 22	10	43	
SVP 73016-2-4 no. 22.2	10	84	
SVP 73016-2-4 no. 46.2.1	56	17	
SVP 75059-32	16	32	25
SVP 75059-32 no. 20	20	25	
Segregating material			
SVP C8703 F2 no. 9	30	20	
SVP C8703 F2 no. 9.3	154	110	
SVP C8709 F2 no. 8.1	40	5	
SVP C8709 F2 no. 8.2	30	1	
SVP C8726 F2 no. 3A	56	202	
SVP C8726 F2 no. 3D.3.2	56	18	
SVP 88002 F2 no. 1	20	66	
SVP 88002 F2 no. 3.5	30	40	
SVP 88002 F2 no. 3.7	56	9	
SVP 88002 F2 no. 6.3	42	18	
SVP 88002 F2 no. 8A1	56	17	
SVP 88002 F2 no. 8A1.15	42	13	
SVP 88002 F2 no. 8A2	56	11	
SVP 88002 F2 no. 8A3	42	8	
SVP 88002 F2 no. 8A4	42	5	
SVP 88002 F2 no. 8A5	42	9	
SVP 88002 F2 no. 8A6	42	17	
SVP 89002 F1 no. 2.1	42	30	
SVP 89002 F1 no. 9A.1	42	39	
SVP 89002 F2 no. 10.1.1	42	61	
SVP 89004 F2 no. 12.1.1	42	98	
SVP 89004 F2 no. 14.1.1	42	110	
SVP 89004 F2 no. 14.1.8	56	20	
SVP 89004 F2 no. 14G	42	125	

Effect at embryo level

Table 4 shows the results of anther culture for 23 wheat genotypes. High-responding genotypes from the callus selection experiments again gave a high response to anther culture, up to a maximum percentage responding anthers of 20.2% for Ringo Sztar. The Brazilian spring wheat line PF 8049 showed the highest callus induction frequency of 66.7%. For eight genotypes more than 100 embryos were plated. More than 900 embryos of Ringo Sztar were plated. In general, regeneration of the embryos was poor. On a total of 1336 embryos plated on toxin-free regeneration medium, 45 embryos with green primordia or green shoots were obtained. On ninety embryos albino shoots developed and on 104 embryos only roots. Of the 1184 embryos plated on toxin-containing regeneration medium, green shoots were obtained on two embryos and roots only on two other embryos.

Table 4. Anther culture response of 23 wheat (*Triticum aestivum* L.) genotypes and regeneration of the embryos formed in the embryo selection experiment. AE = number of anthers excised, AR = % responding anthers, CIF = callus induction frequency, ET = number of embryos tested, ALB = % calli with albino shoots, GS = % calli with green shoots, R = % calli with roots only.

Genotype	AE	AR	CIF	ET	ALB	GS	R
BR 14 (Pf 79780)	1764	2.8	5.3	102	4	16	20
CWW 4055/3	1041	0.8	0.9	7	0	0	0
Frontana	1289	1.9	4.0	18	10	0	10
Ft 83-326	2708	1.9	3.7	103	26	0	8
Kaluzskaja 9	984	0.8	2.3	20	0	8	17
Kraka	3342	0.3	0.3	10	0	0	0
Ning 8343	3185	3.7	6.6	108	9	0	26
Nobeokabozu komugi	1142	1.9	3.9	11	0	0	17
Obelisk	2844	0.3	0.3	5	0	0	0
Pf 80271	2534	1.0	1.8	24	5	5	0
PF 8049	2310	5.6	14.2	352	5	7[a]	16[b]
Praag 8	3570	0.8	1.1	40	0	0	0
Ringo Sztar	2480	9.4	20.2	909	10	3[a]	5
SVP 72005-20-3-1	321	0.3	0.3	2	0	0	0
SVP 72017-17-5-10	1292	0.2	0.5	5	0	0	0
SVP 73016-2-4	1955	4.3	4.8	181	4	7	10
SVP 75059-32	803	1.5	2.7	17	0	0	13
SVP C8709 F2	246	0.8	0.8	3	0	0	0
SVP C8726 F2	432	0.5	0.9	6	0	0	0
SVP 88001 F2	1076	2.7	4.1	16	0	0	0
SVP 88002 F2	1035	1.0	1.7	18	22	0	22
SVP 89002 F1	2295	6.6	12.3	331	3	2	3
SVP 89002 F2	1518	2.2	3.0	232	0	0	0
Total	40166			2520			
Average		2.6	5.0		7	2	4

[a] one green shoot on DON-containing medium.

[b] two calli with only roots on DON-containing medium.

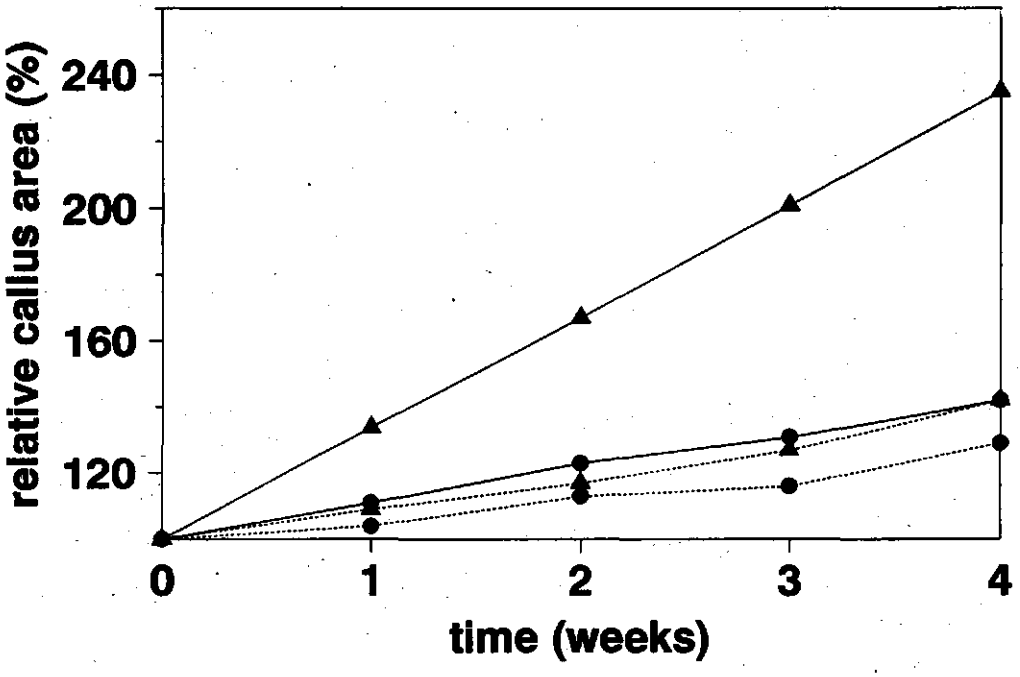

Figure 3. Area changes of wheat (*Triticum aestivum* L.) calli during culture on a DON-containing (10^{-4} M) and control (DON-free) medium for two spring wheat genotypes during a 28 day culture period. Values have been expressed as a percentage of the initial area. An image analyzer was used to measure the area of a 2-dimensional projection of the callus, relative to a fixed area.
(▲—— = Ft 83-326 control, ●—— =Ning 8343 control, ▲···= Ft 83-326 DON, ●···= Ning 8343 DON).

Discussion

The primary aim of this study was to develop an efficient *in vitro* selection system for screening large numbers of genotypes for their FHB-resistance. This FHB-resistance is thought to be based on several minor genes, and accumulation of these genes in a variety is highly desired. In a breeding programme the *Fusarium* resistance genes of a donor are brought into a background with high agronomic value by repeated backcrossing. The efficiency of a backcross programme would be highly increased with the

production of doubled haploids of a BC_i (i= 1,2,3..), especially when an *in vitro* selection at the haploid level would be possible. Also accumulation of resistance genes in a donor could be obtained in an efficient way with use of anther culture of F_1 or F_2 material derived from crosses between two partially resistant genotypes. In field experiments transgression for FHB-resistance was found in F_2-material derived from crosses between two partially resistant genotypes with different genetic backgrounds (Snijders 1990a,b).

Wheat seedling growth has been shown to be completely inhibited at a concentration of 10^{-4} M DON (Shimada & Otani 1990, Snijders 1988). Snijders (1988) suggested that a DON-concentration between 1.5×10^{-5} M and 2.5×10^{-5} M would be optimal to screen seedlings for tolerance to DON. Wakulinsky (1989) observed significant inhibition of germination and a reduction of wheat seedling growth rate at 3.4×10^{-5} M DON and 3.2×10^{-5} M 3-ADON. Particularly root growth was strongly inhibited, which is in agreement with our results, the observations of Shimada & Otani [24] and those of Bottalico *et al.* (1980) of tomato seedling root growth at 2×10^{-5} M DON. Wakulinsky [26] concluded that based on coleoptile growth of seedlings on DON-containing medium it was possible to differentiate between three genotypes according to FHB-level in the field. However, in our study seedling growth response of a large set of genotypes covering the whole range of resistance did not correlate with FHB-resistance level in the field. Shimada & Otani (1990) concluded the same for nine spring wheats.

Growth of wheat coleoptile tissue was strongly inhibited at 10^{-4} M and 10^{-3} M DON and 3-ADON. This is in accordance with the observations of Wang & Miller [18]. Only one wheat genotype, 'SVP 72017-17-5-10-1, showed a trichothecene tolerance, as observed for other genotypes in Wang & Miller (1988). The DON-tolerant winter wheat line has a high level of resistance to FHB. The parental line it was selected from, SVP 72017-17-5-10, showed inhibition of DON-translocation from chaff to kernel resulting in a low colonization level by *F. culmorum* (Snijders & Krechting 1992). Wang & Miller (1988) demonstrated a high trichothecene tolerance in Frontana, which was not found in our study. The fact that the Frontana accessions were obtained from different sources might explain this discrepancy.

DON had a clear effect on callus growth rate and regeneration. In 85% of the callus clones in the selection experiment a growth reduction was observed. Regeneration in the embryo selection experiment was decreased 100-fold on DON-containing medium. Averaged across the callus and embryo experiments, green plant regeneration was decreased 20-fold on medium containing toxin. Ahmed *et al.* (1991) tested wheat calli from diploid embryos for tolerance to toxic metabolites of *F. graminearum* and *F. culmorum* via a double-layer culture technique and observed that regeneration ability of the tolerant calli was lower than that of the unselected calli. Guo *et al.* (1991) observed that *F. graminearum* culture filtrate inhibited the induction of callus and plantlets in anther culture and finally could kill the calli. Also maize embryos grown on medium with water suspensions, water filtrate or chloroform extract from maize contaminated with *F. graminearum* showed a lower weight gain compared to medium with a water suspension from non-contaminated maize and led to inhibition and deformation of embryo organs (Brodnik et al. 1978). The early phase of embryo development depends on the intensity of respiration and mobilization of

carbohydrates, which in turn depend on the activity of enzymatic processes in embryogenic cells. A low DON-concentration blocks protein synthesis and thus enzym synthesis, which would explain the observed callus and embryo growth reduction.

The growth analysis data of the 40 callus clones did not show any correlation with the FHB-resistance levels of the original donor populations ($r = 0.028$). This raises the question whether callus selection for *in vitro* tolerance to DON is possible. However, Guo *et al.* (1991) observed that somaclonal variation and induced variation after gamma-ray irradiation resulted in callus pieces tolerant to *F. graminearum* culture filtrate. Tolerant calli were not colonized by *F. graminearum*. Plantlets grown from these calli were tolerant to the culture filtrate and regenerated plants showed a high FHB-resistance even surpassing the resistance of the highly resistant Chinese cultivar Sumai #3.

From Figure 2 it can be concluded that a DON-concentration higher than 27×10^{-5} M will not result in a further reduction of callus weight, although this concentration did not kill the calli. Menke-Milczarek & Zimny (1991) observed that diploid calli from immature embryos died at 34×10^{-5} M DON. The regeneration capacity of the calli (forming shoots of about 2 mm) in their study decreased exponentially with increasing DON-concentration, comparable with Figure 2 in our study. Their data confirmed that a concentration of 10^{-4} M DON is the optimum concentration to differentiate wheat calli for DON-tolerance. A higher concentration of DON would not lead to improvement of differentiation.

The lower sensitivity of calli compared to the coleoptile tissue bioassay could be explained by the fact that the coleoptile segments are incubated in a liquid solution with DON, surrounding the whole tissue. In callus culture only a relatively small part of the tissue surface is in contact with the medium. No facts are known on the mode of DON-penetration into calli. This problem might be overcome by using a single cell selection system e.g. microspore culture. In that way a more homogenous selection pressure can be assured for each individual genotype.

Regeneration of the haploid wheat material in our experiments proved to be a severe bottle neck. In the callus selection experiment only 0.7% of the callus pieces regenerated green shoots and in the embryo selection experiment 1.9%. High percentages of albino shoots and embryos with only root formation were observed.

Regenerants from the callus and embryo selection experiments are currently growing. Offspring of these plants together with the parents and F_2 populations will be tested in the field for *Fusarium* resistance. After artificial inoculation, the resistance level of these doubled haploid lines will give additional information on the possibilities to use *in vitro* selection for FHB-resistance in wheat.

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Chapter 5: Resistance and gametoclonal variation of doubled haploid wheat lines with regard to *Fusarium* head blight

Abstract

In four consecutive years crosses were made between *Fusarium* Head Blight (FHB) resistant and susceptible cultivars and breeding lines. Parents, F_1- or F_2-populations were used as donor material for anther culture and were together with the anther culture derived doubled haploid (DH) wheat lines tested in the field for their FHB-resistance. Date of flowering and percentage of infection were measured, three and four weeks after artificial inoculation. For two years also straw length was measured. The phenotypes within the DH-lines proved to be stable and homogeneous and no visible segregation occurred. In most cases the F_1-, F_2-populations and anther culture derived doubled haploid (DH) lines had infection levels intermediate between the two parents, indicating an additive inheritance. However, in some cases the F_2-population or the DH-lines showed a significant lower infection level than the most resistant parent. Two causes for this transgression were identified: segregation and gametoclonal variation. In cases where the F_2-populations were significantly more resistant than the two parents, it was concluded that accumulation of resistance genes of the partially resistant parents had occurred.

When DH-lines, directly derived from cultivars or breeding lines, were more resistant than the parental lines, it was attributed to gametoclonal variation. A strong correlation was observed for the infection percentage after three weeks, with that after four weeks. No evidence for the occurrence of additional genetic variation obtained after a long callus phase was found, as compared to direct regeneration of the microspores via embryogenesis.

Introduction

Fusarium Head Blight (FHB) of wheat (*Triticum aestivum* L.) is a fungal disease that causes severe losses in humid and semi humid wheat-growing areas of the world. In the Netherlands, FHB is mainly caused by *Fusarium culmorum*. The pathogen can cause root-, foot- and crown rot, reduced kernel setting and kernel weight, leading to yield reduction. FHB-invaded kernels show destroyed starch granules, storage proteins and cell walls. The pathogen can also produce mycotoxins in the crop, of which the toxins in the trichothecene group are the most notorious. Deoxynivalenol (DON), acetyl-deoxynivalenol (ADON), with the isomers 3-ADON and 15-ADON, and Nivalenol (NIV) are members of the trichothecene group. These toxins inhibit protein synthesis via binding to the ribosome and are through this capacity able of producing a wide range of toxic effects, including skin irritation, diarrhea, vomiting, hemorrhages, miscarriages and eventually death (Rotter *et al.* 1996).

No chemical treatment is effective in preventing this pathogen. Resistant cultivars are the most suitable method in eliminating this problem. Crossing resistant genotypes and using the F_1- and F_2-populations as donor material for anther culture, might provide the breeder with genotypes that have accumulated the resistance genes in a homozygous way. Accumulation of resistance genes was already proven for two genotypes with each two different resistance genes by Ginkel *et al.* (1996), and for a 10x10 half diallel of winter wheat lines (Snijders 1990d). Previous studies indicated that at least 1 to 6 genes are involved in the resistance (Gu 1983, Liao & Yu 1985a, Snijders 1990b, Yu 1982, Zhang & Pan 1982, Zhou *et al.* 1987). In most studies an additive inheritance was found (Liao & Yu 1985b, Snijders 1990c). However, also non-additive effects were detected (Li & Yu 1988, Liao & Yu 1985b) and of the non-additive effects dominance of resistance predominated over recessiveness (Snijders 1990c).

Another way of creating variability, besides crossing and mutation techniques, is the use of somaclonal variation, i.e. variation induced by in vitro culture of plant material (Larkin & Scowcroft 1981). A clone can be defined as a population of cells or organisms that is derived from one single cell or from a common ancestor by means of mitosis (Webber 1903). Induction of somaclonal variation occurs either during plant development or during the in vitro cell culture, in the mitotic process. Related to this is the variation between derivatives of cultured gametic cells, i.e. cultured cells, their regenerants or the progeny thereof, and which is referred to as gametoclonal variation (Evans *et al.* 1984). These gametoclonal variants can be the result of meiotic or mitotic divisions (Huang 1996). However, when referring to gametoclonal variation three different types of variation are recognized besides the variation resulting from segregation and independent assortment:

1. New genetic variation which is induced as a result of the cell culture procedures.
2. New variation at the haploid level which is induced by the chromosome doubling procedure.
3. New variation induced at the diploid level, resulting in heterozygosity (Morrison & Evans 1987).

Somaclonal and gametoclonal variation have provided practical breeders with many usefull variants to be

selected and were also used to produce new cultivars (Baenziger *et al.* 1991, Grunewaldt & Dunneman 1990, Monti & Moore 1992).

The role of the chromosome doubling procedure should not be underestimated. In this study for doubling of the chromosome number, colchicine was used, which is known for its ability to cause additional mutations (Franzke & Rose 1952, Luckett 1989). Suenaga & Nakajima (1993) stated that most of the variation detected in 15 of the 110 doubled haploid (DH) lines derived from one wheat variety was caused by the colchicine treatment, rather than by the in vitro culture procedure. This is contradicted by Sariah *et al.* (1993) who claimed that the variability between the anther culture derived DH-lines of barley was mainly caused by the in vitro methods and to the possibility of the parental line not being completely homozygous. Koba *et al.* (1991) stated that the variation between doubled haploid wheat lines is mainly caused by mutations occurring during anther culture and Snape *et al.* (1992) did not find any evidence for gametoclonal variation in their DH-lines of wheat.

This study was conducted to see in which way the resistance was expressed in the doubled haploid lines, whether or not the resistance could be accumulated in a homozygous way and to what extent gametoclonal variation influences the level of FHB-resistance of the DH-lines derived from varieties.

Material and methods

Plant material

Crosses were made between resistant and susceptible wheat cultivars or breeding lines or between two resistant cultivars or breeding lines. Resistant cultivars and breeding lines, with FHB-indices lower than 10.0, as presented by Snijders (1990a) were in descending order of resistance: Ft 83-326 (S), Ning 8343 (S), Praag 8 (W), Ning 7840 (S), SVP 72017-17-5-10 (W) and Ringo Sztar (W). The growing type of the genotype is presented in brackets with S for Spring wheat and W for Winter wheat. The other cultivars and breeding lines: SVP 73016-2-4 (W), CWW 4055-3 (W), SVP 75059-32 (W), SVP 72005-20-3-1 (W) and PF 8049 (S) were considered to be moderate susceptible to susceptible with ascending FHB-indices from 30.0 to 51.8.

Anther culture and seed set

Parents, F_1- and F_2-populations were used as donor material for anther culture. Anther culture and the subsequent regeneration were performed as described in Bruins *et al.* (1993). Green regenerated plantlets were transferred to culture tubes and after reaching the two-three leaf stage, transferred to soil and covered individually with a transparent cover. After two weeks, the covers were removed and two weeks later, all plants were treated with colchicine for chromosome doubling. For this, plants were removed from the soil, roots were washed and the growth meristems of the plantlets were soaked for 12 hours in a

0.05% colchicine solution, rinsed with tap water for three hours and replanted to soil. All DH_0-plants were regenerated from toxin-free regeneration medium, except for three plants from the callus selection experiments in 1990 (Bruins *et al.* 1993). These three plants were regenerated from calli that had been subjected to the toxin DON, present in the subculture medium. Of these three green plants, only one green DH_0-plant set seeds after colchicine treatment.

Field experiments

All DH_0-plants, from toxin and control medium, were grown to maturity, selfed, seeds were harvested and were sown together with their parents and F_1- or F_2-populations. Numbers of seeds that were sown for a parent, F_1-(or F_2-)population or DH-line were 50, 100 and 50, respectively. As some of the parents were of the winter type, all parental lines and DH_1-seedlings were vernalised for 8 weeks at 4°C. Plants were transferred to the field and planted in rows of one meter, with an interrow distance of 30 cm and a distance between the plants of 15 cm. As the occurrence of natural epdemics is unpredictable, plants were artificially inoculated. Lines were inoculated with a hand sprayer at the first day of flowering of each individual row with a spore suspension of *Fusarium culmorum* with a density of 250.000 spores ml^{-1}. Inoculation took place until run-off, i.e. until the spore suspension ran off the spikes. This inoculation was repeated after four days. *F. culmorum* isolate IPO 39-01 was used, which was found to have the highest toxigenic potential (Snijders 1990b). Computerised overhead mist irrigation was used for a period of two weeks after inoculation, to ensure a high humidity and a good initial infection of the pathogen. No plants were placed under the water outlets of the irrigation system. In all four years the first date of flowering and the FHB-levels, three and four weeks after the artificial inoculation (FHB3 and FHB4, respectively), were visually assessed. In 1991 and 1993 also the straw length of the plants was measured.

Four DH_0-genotypes of Ringo Sztar did not produce enough seeds on the DH_0-plants, so they were multiplied in 1993 and the DH_2-genotypes were tested in 1994 for their FHB-resistance. In 1992, some of the DH_1(F_2 88002) lines showed a much lower infection level as compared to the parents, so these combinations were retested with their parents in 1994. In the field experiment of 1994 the cultivars Wang Shui Bai and Ning 7840 (also known as Nanjing 7840) were included in the tests as both cultivars were described to be highly resistant against FHB (Bai & Shaner 1996, Ginkel *et al.* 1996). Statistical analysis of the head blight data was based on the average genotype means. For all four years, correlations were calculated between FHB3 and FHB4, straw length and flowering date. Six of the eleven parents and breeding lines were tested in more than one year: five in three years and one in two years, and in this way serving as multi-year replications. For the genotypes tested in more than one year, correlations were calculated between FHB-data in the various years.

Sister line crossing programme

In the field experiment of 1992, the Praag 8-7 sisterlines, of which some of the calli had been subjected

to toxin stress, appeared to be highly resistant. There was significant variation among sisterlines subjected to toxin stress and control lines, that were all derived from the same anther culture derived embryo. Reciprocal crosses were made between the Praag 8-7 sisterlines with the highest levels of resistance, derived from control and from toxin containing medium, and these lines were also reciprocally backcrossed to the parent Praag 8-7. The parent Praag 8-7, DH_1-, DH_2-, backcross (BC)-lines and $F_1(DH_1)$-populations were tested in 1994 for their FHB-resistance.

Results

In Table 1 the results from the field experiment in 1991 are shown, and it can be seen that for FHB3 and FHB4 in all five crosses the percentage of FHB-infection of the F_2-populations was intermediate or equal to one of the two parents (dominance). In all except one case, the infection level of the DH_1-lines was intermediate or not statistically different from the original parents, used in the cross to produce the F_2-populations. Only $DH_1(F_2$ 88001)-7 was more susceptible than the most susceptible parent (Praag 8). For flowering date only one DH_1-line differed significantly from the parents. Four out of nine DH_1-lines had significantly a different straw length as compared to the parental or F_2-populations: two of them were shorter and two were significantly longer. The observed characteristics of the plants plants within each DH_1-line were very stable and homogeneous and no visible segregation occurred.

In the field experiment of 1992, the DH_1-lines directly derived from a cultivar or breeding line were investigated (Table 2A). In most cases the DH_1-lines showed a similar infection level as the two donor lines Praag 8 and SVP 73016-2-4. For FHB3 however, in six out of 18 cases, the DH_1-line had a significantly lower infection level, and for FHB4, three DH_1-lines had significantly a lower infection level as compared to the homozygous parents used for doubled haploid regeneration. In the case of Praag 8, for FHB3 five of ten DH_1-lines and for FHB4 three out of ten DH_1-lines were significantly more resistant than the resistant donor genotype Praag 8. For FHB3, plant numbers 2 & 3 from the 1C1-line were significantly more resistant than plant numbers 2 & 3 from the 1T2-line, which were regenerated from calli selected on toxin containing medium. These inter-callusline differences were not detected for FHB4. The DH_1(SVP 73016-2-4-3)-lines were all equally susceptible to FHB as the susceptible donor genotype, except for DH_1(SVP 73016-2-4-3)-4 which was for FHB3 less infected and for DH_1(SVP 73016-2-4-3)-1 which was for FHB4 more infected.

In Table 2B doubled haploids of F_2-plants of two cross combinations have been investigated. With F_2(88002), for FHB4, two DH_1-lines were significantly different from their parents CWW 4055-3 and Ft 83-326: one more resistant and the other one more susceptible. The F_2(89002)-population and the DH_1-lines derived thereof showed all a lower infection level than the parental genotypes, for FHB3 as well as for FHB4. The DH_1-lines were significantly more resistant as the F_2-populations, they were derived from.

Table 1. Five wheat crosses analysed for flowering date, straw length and *Fusarium* Head Blight infection (% infected spikelets) in 1991, three (FHB3) and four weeks (FHB4) after artificial inoculation, with the parental genotypes (P_1 & P_2), the F_1- & F_2-populations and the F_1- and F_2-derived DH_1-lines.

Genotype	Flowering date (June)[1]	FHB3 (%)	FHB4 (%)	Length[2] (cm.)	No. of plants
P_1: SVP 72017-17-5-10	23.3	13.5	29.7	97.2	91
P_2: SVP 72005-20-3-1	25.3	36.9	63.3	103.1	50
F_2 C8703	24.9	20.0	49.1	102.3	81
DH_1(F_2 C8703)-7	23.2	28.2	60.9	91.8	45
DH_1(F_2 C8703)-8	24.5	10.0	28.9	115.0	54
P_1: SVP 72017-17-5-10	23.3	13.5	29.7	97.2	91
P_2: SVP 73016-2-4	27.2	61.6	84.8	99.8	97
F_2 C8709	26.0	20.5	49.2	98.5	64
DH_1(F_2 C8709)	24.5	33.5	69.6	75.5	105
P_1: SVP 75059-32	22.3	29.4	59.4	85.0	55
P_2: SVP 73016-2-4	27.2	61.6	84.8	99.8	97
DH_1(F_2 C8726)-2	33.5	36.7	75.2	104.3	47
DH_1(F_2 C8726)-3	25.5	42.6	70.4	93.3	50
P_1: Praag 8	28.3	2.5	7.2	141.3	46
P_2: Ning 8343	17.3	0.8	9.4	89.8	41
F_2 88001	22.3	2.1	6.7	123.6	105
DH_1(F_2 88001)-7	23.0	5.2	9.8	122.5	32
P_1: Ft 83-326	22.3	7.2	15.3	126.1	47
P_2: CWW 4055-3	31.0	66.2	84.2	86.5	50
F_2 88002	24.5	23.1	53.2	109.4	90
DH_1(F_1 88002)-1	23.0	20.9	48.9	96.9	59
DH_1(F_1 88002)-2	24.4	31.1	67.5	98.4	40
DH_1(F_2 88002)-6	26.3	22.1	85.9	85.8	100

[1]: Flowering date in June, e.g. 33 = 3rd of July.

[2]: Straw length in cm.

Underlined values: Significantly higher or lower values (p=0.05) than highest or lowest value of parental- and donor genotypes.

Table 2. DH-regenerants directly derived from homozygous lines (A), or from F_2-populations (B), analysed in 1992 for their *Fusarium* Head Blight infection (% infected spikelets), three (FHB3) and four weeks (FHB4) after artificial inoculation, together with their Donors (D) or Parents (P_1 & P_2).

	Genotype	Flowering date (June)[1]	FHB3 (%)	Range FHB3	FHB4 (%)	Range FHB4	No. of plants
A	D: Praag 8	12.5	4.4	0-20	9.4	0-30	24
	DH_1(Praag 8-7 1C1)-1	11.0	8.9	0-30	18.9	5-60	9
	DH_1(Praag 8-7 1C1)-2	11.0	0.6	0-1	6.3	0-10	8
	DH_1(Praag 8-7 1C1)-3	13.0	0.6	0-1	3.3	1-10	9
	DH_1(Praag 8-7 1C1)-4	13.0	1.2	0-5	5.3	1-10	8
	DH_1(Praag 8-7 1C1)-5	13.0	3.7	0-10	10.7	1-20	9
	DH_1(Praag 8-7 1C1)-6	12.8	2.4	0-5	10.1	1-20	9
	DH_1(Praag 8-7 1T2)-1	12.4	2.8	0-10	8.6	1-30	15
	DH_1(Praag 8-7 1T2)-2	11.6	1.7	0-10	5.8	0-10	18
	DH_1(Praag 8-7 1T2)-3	11.9	1.6	0-10	6.9	0-30	18
	DH_1(Praag 8-7 2C4)	12.5	4.3	0-10	16.4	1-35	50
	D: SVP 73016-2-4	10.2	45.7	15-70	76.4	60-90	22
	DH_1(SVP 73016-2-4-3)-1	11.0	51.7	30-80	84.2	80-90	6
	DH_1(SVP 73016-2-4-3)-2	10.0	41.7	30-60	71.7	60-80	6
	DH_1(SVP 73016-2-4-3)-3	10.0	34.2	15-40	75.0	60-90	6
	DH_1(SVP 73016-2-4-3)-4	10.0	31.4	10-50	67.1	40-80	7
	DH_1(SVP 73016-2-4-3)-5	10.0	45.7	20-60	68.6	40-90	7
	DH_1(SVP 73016-2-4-3)-6	10.0	47.1	20-60	68.6	60-80	7
	DH_1(SVP 73016-2-4-3)-7	10.6	38.6	20-50	70.0	60-80	7
	DH_1(SVP 73016-2-4-3)-8	11.0	42.9	30-60	67.1	40-90	6
B	P_1: CWW 4055-3	15.8	26.9	0-70	44.0	0-80	24
	P_2: Ft 83-326	0.0	52.5	15-100	70.2	20-100	24
	D: F_2 88002	3.4	35.5	5-100	70.2	5-100	51
	DH_1 (F_2 88002-3-5)-1	8.3	13.3[3]	5-20	26.7	20-35	3
	DH_1 (F_2 88002-3-5)-2	3.0	33.7	10-70	52.1	25-90	12
	DH_1 (F_2 88002-3-5)-3	3.0	40.8[2]	10-80	62.9	40-85	12
	DH_1 (F_2 88002-3-5)-4	3.0	36.2	5-70	63.3	25-95	12
	DH_1 (F_2 88002-3-5)-5	1.0	58.3	30-90	83.8	60-100	12
	DH_1 (F_2 88002-3-5 1C2)-1	1.0	51.7[2]	20-70	61.7	30-80	6
	DH_1 (F_2 88002-3-5 1C2)-2	1.0	54.3[2]	30-90	81.4	70-90	7
	DH_1 (F_2 88002-3-5 2C2)-1	1.0	38.8	10-60	70.6	60-90	8
	DH_1 (F_2 88002-3-5 2C2)-2	1.0	25.7[3]	20-30	65.7	40-80	7
	DH_1 (F_2 88002-3-5 2C4)-1	1.0	22.0	0-60	52.0	20-80	5
	DH_1 (F_2 88002-3-5 2C4)-2	2.1	31.4	5-60	65.0	15-90	7
	DH_1 (F_2 88002-3-5 3C1)-1	5.3	21.2	0-100	28.1	0-100	8
	DH_1 (F_2 88002-3-5 3C1)-2	5.0	11.2[3]	0-30	25.0	5-60	5
	P_1: SVP 72017-5-10	6.2	49.2	5-80	64.2	20-100	24
	P_2: Ringo Sztar	1.0	39.6	10-60	67.5	60-80	24
	D: F_2 89002	3.6	12.4	1-60	29.2	5-70	48
	DH_1(F_2 89002-14)-1	3.0	8.5[4]	0-20	21.6[4]	5-50	19
	DH_1(F_2 89002-14)-2	3.6	9.8[4]	1-25	22.1[4]	5-40	19
	DH_1(F_2 89002-14)-3	3.5	6.2[4]	0-30	16.6[4]	0-60	19

C = derived from control medium, T = derived from toxin containing medium
Values: Sign. higher or lower values (p=0.05) than highest or lowest value of parental- and donor genotypes.
[1]: Flowering date in June, e.g. 3 = 3rd of June.
[2]: Significantly different from parent CWW 4055-3, but not significantly different from donor F_2 88002
[3]: Significantly different from donor F_2 88002, but not significantly different from parent CWW 4055-3
[4]: Significantly different from donor population F_2(89002).

Table 3. Doubled haploid regenerants from a cultivar and two breeding lines together with their Donor genotypes (D), analysed in 1993 for flowering date, straw length and *Fusarium* Head Blight infection (% infected spikelets), three (FHB3) and four weeks (FHB4) after artificial inoculation.

Genotype	Flowering date (June)[1]	FHB3 (%)	FHB4 (%)	Length[2] (cm.)	No. of plants
D: Ringo Sztar	29.3	3.9	7.1	91.0	15
DH_1 (Ringo Sztar)-1	31.7	3.5	5.9	91.6	21
DH_1 (Ringo Sztar)-3	31.9	14.8	25.4	73.2	37
DH_1 (Ringo Sztar)-5	30.6	3.2	7.3	83.9	54
DH_1 (Ringo Sztar)-6	31.1	3.9	6.8	90.1	62
DH_1 (Ringo Sztar)-8	31.2	2.0	3.5	84.3	57
DH_1 (Ringo Sztar)-11	31.7	2.8	7.8	80.0	47
DH_1 (Ringo Sztar)-19	32.4	1.5	3.1	88.3	55
D: SVP 73016-2-4	39.3	34.5	59.0	83.5	19
DH_1 (SVP 73016-2-4)-10	41.2	29.2	54.4	87.5	43
D: PF 8049	26.3	6.9	13.3	93.4	17
DH_1 (PF 8049)-2	25.6	8.2	17.2	90.1	32
DH_1 (PF 8049)-7	26.1	7.7	19.1	92.2	34

[1]: Flowering date in June, e.g. 39 = 9th of July.

[2]: Straw length in cm.

Underlined values: Significantly higher or lower values (p=0.05) than highest or lowest value of parental- and donor genotypes.

As an example, in table 2 the ranges are given in FHB3- and FHB4-level of the parents, the F_2 populations and the DH_1-lines. Ranges varied greatly, and therefore for one cross combination also the frequency distributions were drawn (Figure 1). It appeared that the moderately resistant parental line SVP 72017-17-5-10 had a greater range than the moderately resistant parent Ringo Sztar. The F2 89002 population showed an intermediate range between its two parents. For the three DH-lines the range for FHB3-values was smaller than for FHB4.

In the field experiment of 1993 DH1-lines of cultivar Ringo Sztar and of the breeding lines SVP 73016-2-4 and PF 8049 were investigated. For FHB3 and for FHB4, two of the seven DH_1-lines derived from the moderately resistant cultivar Ringo Sztar had significantly a lower and one a higher level of infection than the parent itself (Table 3). For the three DH_1-lines from the two other (susceptible) donor genotypes, one gave for FHB4 a significantly higher infection level as compared to the parent, the rest was not significantly different. For flowering date, most of the DH_1-lines differed from their parents. For the straw length of the DH_1(Ringo Sztar)-lines, five out of seven were significantly shorter than the donor parent and the DH_1(SVP 73016-2-4)-10-line was significantly taller than its donor.

Table 4: Doubled haploid (DH) regenerants derived from homozygous lines (A), or from F_2-populations (B), analysed in 1994 together with the Parental- (P_1 & P_2) and Donor genotypes (D), for flowering date and *Fusarium* Head Blight infection (% infected spikelets), three (FHB3) and four weeks (FHB4) after artificial inoculation.

	Genotype	Flowering date (June)[4]	FHB3 (%)	FHB4 (%)	No. of plants
	Wang Shui Bai	26.0	16.0	29.3	15
	Ning 7840	23.0	40.5	65.5	10
A	P: Praag 8	36.0	42.9	58.4	45
	C: DH_1(Praag 8-7-1C1)-3	36.0	36.4	52.2[3]	97
	T: DH_1(Praag 8-7-1T2)-2	36.0	39.0	55.0	54
	DH_2(Praag 8-7-1C1)-3	36.0	39.1	56.7[1]	147
	DH_2(Praag 8-7-1T2)-2	36.0	39.4[1]	56.5[1]	176
	PxC = F_1[Praag 8 x DH_1(1C1)]	36.0	40.0	52.1	31
	CxP = F_1[DH_1(1C1)-3 x Praag 8]	36.0	41.4	60.1[1]	42
	PxT = F_1[Praag 8 x DH_1(1T2)-2]	36.0	55.0[2]	74.0[2]	20
	TxP = F_1[DH_1(1T2)-2 x Praag 8]	36.0	42.1[1]	57.4	53
	CxT = F_1[DH_1(1C1)-3 x DH_1(1T2)-2]	36.0	36.7	49.2	6
	TxC = F_1[DH_1(1T2)-2 x DH_1(1C1)-3]	36.0	35.3	49.1	17
	D: Ringo Sztar	26.0	51.6	86.0	74
	DH_2(Ringo Sztar)-3	29.0	86.6	98.1	49
	DH_2(Ringo Sztar)-4	27.0	79.3	96.2	82
	DH_2(Ringo Sztar)-7	26.0	52.3	79.6	42
	DH_2(Ringo Sztar)-VI-33-1	26.0	77.9	95.8	12
B	P_1: CWW 4055	46.0	67.9	88.1	26
	P_2: Ft 83-326	29.0	77.6	84.8	29
	D: F_2 (88002)	33.0	68.3	81.6	23
	DH_2 (F_2 88002-3-5)-2C4	34.8	51.1	66.8	79
	DH_2 (F_2 88002-3-5)-3C1	35.8	38.0	54.4	71

C = derived from control medium
T = derived from toxin containing medium.
Underlined values: Significantly higher or lower values (p=0.05) than highest or lowest value of parental- and donor genotypes.
[1]: Significantly different from DH_1(Praag 8)-7-1C1.
[2]: Significantly different from all four DH_1- and DH_2-genotypes.
[3]: Significantly different from the two DH_2-genotypes.
[4]: Flowering date in June, e.g. 36 = 6th of July.

In the field experiment of 1994, the FHB-levels were much higher than in previous years (Table 4). All previously resistant cultivars, including Wang Shui Bai and Ning 7840, showed considerably higher infection levels than the multi-year FHB-index as presented in Snijders (1990a). This increase in infection was most evident in the genotypes Praag 8 and Ringo Sztar. Praag 8 showed a 10-17 times and Ringo Sztar a 12-13 times increase in 1994, as compared to previous years. Relative ranking for FHB-infection of the different genotypes, however, stayed the same for the four years.

Table 5. Correlation coefficients (r) between FHB-values of genotypes that were tested in more than one year.

Year	FHB3	FHB4	FHB3 & FHB4
1991 vs. 1992	0.10	0.39	0.37
1992 vs. 1993	--	--	0.54
1993 vs. 1994	--	--	--
1991 vs. 1993	--	--	--
1991 vs. 1994	0.30	0.67	0.54
1992 vs. 1994	0.75	0.83	0.82
All four years	--	--	0.26

--: Insufficient data for correlation calculations.

In 1994 two DH_2-populations were tested. From the four Ringo Sztar DH_2-populations, no data for the DH_1-generation were available, due to insufficient seed set on the DH_0-plants. For the 88002 DH_1- and DH_2-lines it appeared that, except for the almost equal FHB4-infection levels of the DH_1- and DH_2-lines derived from F_2 88002-3-5-2C, the infection levels of the DH_1-lines in 1992 were all consistently lower than of the DH_2-lines in 1994.

For the Praag 8 sisterline crossing programme it was observed in 1994 that the selected DH_1- and DH_2-lines derived from control medium were more resistant than the DH_1- and DH_2-lines derived from toxin-containing medium. Similar results were obtained for DH_1-lines in 1992. In the case of Ringo Sztar, for FHB3, all significant differences concerned a higher infection level. With FHB4, all four DH_2-lines differed significantly from the parent, one of them was more resistant than the donor Ringo Sztar. The DH_2-lines derived from the F_2(88002)-population were both for FHB3 and for FHB4 more resistant than the parents and the F_2-donor population.

Correlations calculated for all four years between FHB3 and FHB4, straw length and flowering date revealed, as expected, to be high between FHB3 and FHB4, with a correlation coefficient 'r' ranging from 0.88 to 0.99. Correlations of FHB3 and FHB4 with straw length were lower, in 1991 ranging from -0.50 to -0.66, and in 1993 from -0.23 to -0.20, respectively. Correlations of FHB3 and FHB4 with flowering date were highest in 1993, ranging from 0.73 to 0.71, and lowest in 1994, ranging from -0.22 to -0.32, respectively. Correlations between FHB3- and FHB4-infection levels for genotypes that were tested in more than one year are shown in Table 5. Relatively high values were found between FHB4-infection levels in 1991 and in 1994 and for FHB3- and FHB4-infection levels in 1992 and 1994.

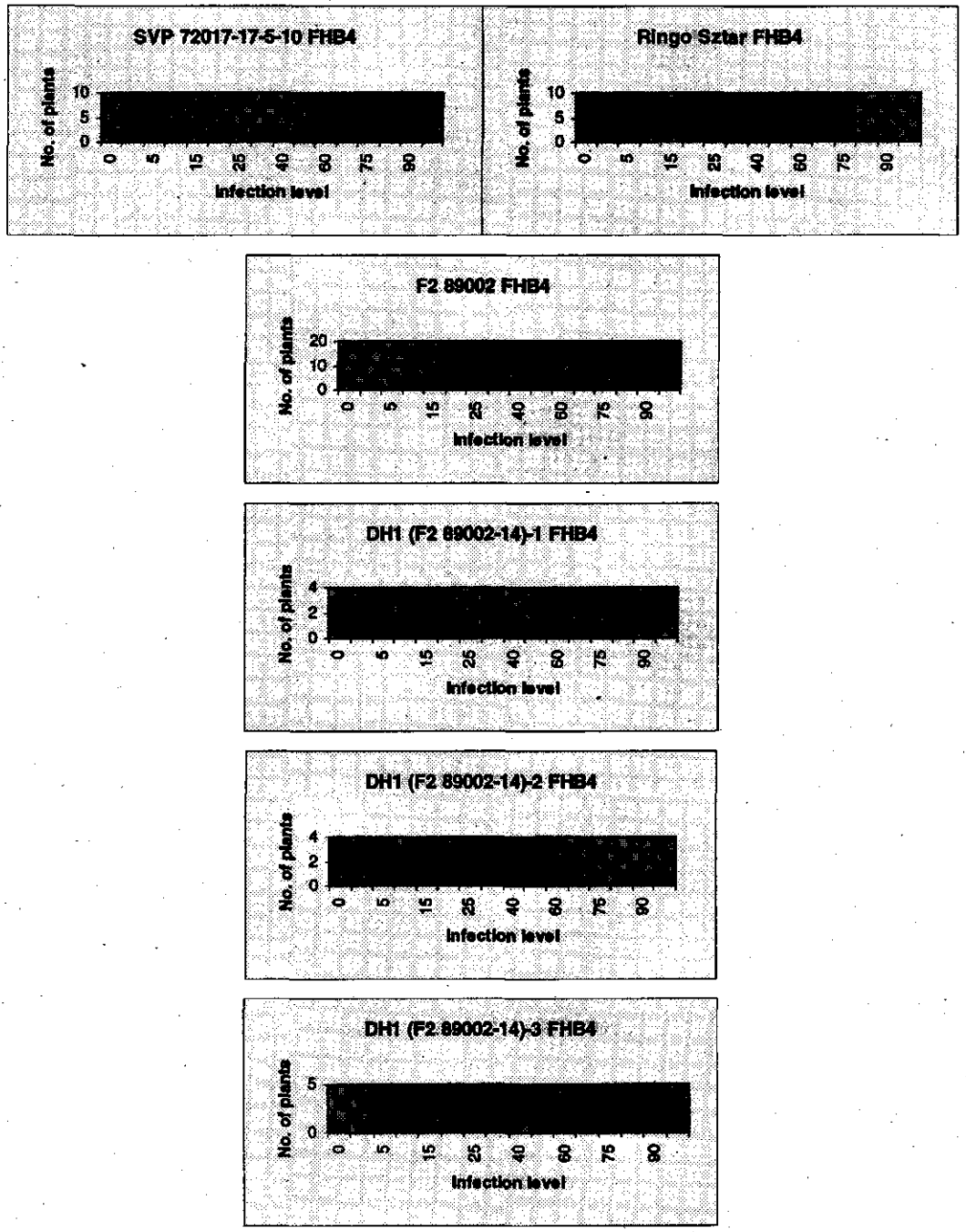

Figure 1. Range in FHB4-values for the two parents, the F_2-donor population and three DH_1-lines.

Discussion

The intermediate reactions to FHB of most of the F_2-populations and DH_1-lines indicate an additive inheritance, which is confirmed by other studies (Liao & Yu 1985b, Snijders 1990c). Also in barley, additive genetic factors control *Fusarium* Head Blight resistance. However, Takeda & Wu (1996) found that dominance effects were still statistically significant and in some varieties also maternal effects were found.

Cultivars have a much higher level of homozygosity than F_1- or F_2-populations and, therefore, it is expected that DH_1-lines derived from pollen of an F_1- or F_2-population differ more from each other than DH_1-lines derived from pollen of a cultivar. Looking at the variation between different DH_1-lines derived from F_2-populations and from a cultivar or breeding line showed that significant differences occurred for flowering date, FHB3, FHB4 and straw length. These differences appeared on either side of the average value, e.g. DH_1-lines flowering significantly earlier and later than the parental genotype were observed. However, with the measurements in this study it could not be concluded whether DH_1-lines derived from F_2-populations show more variation between the lines than those derived from a cultivar or breeding line. The number of tested DH_1-lines per donor genotype was in several cases relatively low, so more research is needed to substantiate the above mentioned assumption.

In 1992, the F_2(89002)-population showed a significantly lower infection level than the two parents (Table 2). It can be assumed that the resistance genes of both partially resistant parents (Ringo Sztar and SVP 72017-17-5-10) have accumulated in the F_2-population. The three DH_1(F_2 89002)-lines all showed a significantly lower infection level than the average infection level of the F_2(89002)-population and it was concluded that in these DH_1-lines segregation in favour of a higher level of resistance occurred. DH-lines surpassing the better parent and also heterotic F_1-populations have been described in previous publications (Caligari *et al.* 1987).

Besides variation based on segregation and independent assortment, also new variation induced by the in vitro culture procedure (Type 1, Morrison & Evans 1987) was found. In 1992 and 1994, some DH_1(Praag 8-7)-lines were significantly more resistant and some significantly more susceptible than the parental cultivar. In 1993 for FHB3 and FHB4, two of the seven DH_1-lines derived from cultivar Ringo Sztar, had significantly a lower level of infection and one had a significantly higher level of infection than the parent itself (Table 3). As the donors Ringo Sztar and Praag 8 are commercial cultivars, and expected to be homozygous, the transgression indicates the occurrence of gametoclonal variation in the narrow sense, i.e. variation introduced by the in vitro procedure (Type 1). Whether the gametoclonal changes were stable or not has to be concluded from the offspring of the DH-lines. Some of the DH_2-lines of Praag 8, tested in 1994, showed a resistance level similar to DH_1-lines. So it can be concluded that for this genotype the changes were of a genetic nature.

As colchicine was used to double the chromosome number, variation induced by the chromosome

doubling procedure (Type 2) might also be one of the sources of the observed variation. Variation induced at the diploid level, resulting in heterozygosity (Type 3) is less likely to be one of the reasons of the interline variation, as the plants within all the DH-lines were homogeneous and no visible segregation had occurred.

The DH-lines tested in 1991 and 1992 were regenerated from a callus selection programme (Chapter 4). In this programme several callus subcultures were used to ensure an adequate amount of calli for in vitro selection experiments on toxin tolerance (Bruins *et al.* 1993). The lines tested in 1993 and 1994 were derived from the direct embryo induction selection programme in which no callus-phase was used. A long callus-phase increases the chance of mutations and a direct embryo regeneration system could prevent this (Murigneux *et al.* 1993). Therefore, it might be expected that the lines produced via direct regeneration from the embryos in 1993 and 1994 show less variation for the investigated traits than the callus derived lines in 1991 and 1992. When looking at the number of altered traits it appeared that 13%, 34%, 44% and 57% of all measured traits on the DH-lines were significantly different from the parental values in 1991, 1992, 1993 and 1994, respectively. Therefore, in this study it could not be concluded that a longer callus phase increases the level of variation, more the opposite. This conclusion can also be drawn from the DH_1-lines from F_2(88002-3-5), tested in 1992. The first five lines were regenerated without a long callus phase, whereas the other eight DH_1-lines of that genotype had undergone a long callus phase. No large differences for the traits tested could be detected between the two groups of DH_1-lines.

The range in FHB-values, as indicated in Table 2, showed for some genotypes a relatively high range. This might indicate 'escapes', e.g. genotypes that escaped a thorough inoculation. From the frequency distributions (Figure 1) it can be concluded that these extreme values occurred only rarely. From a breeding point of view, genotypes varying into highly susceptible genotypes are not agronomically of importance and have to be discarded.

In 1993, the shortest DH_1-line, DH_1(Ringo Sztar)-3, was also the most susceptible one. The relation between straw length and resistance has been described in earlier publications (Mesterhazy 1995, Liao & Yu 1985b) which stated that shorter genotypes were more severely infected, however only under natural conditions. However, after artificial inoculation, genotypes of different height classes were similarly susceptible (Mesterhazy 1995). DH_1(Ringo Sztar)-3 was significantly shorter than the second shortest DH_1-line: DH_1(Ringo Sztar)-11. This latter line however, was significantly more resistant than its parent. Correlation coefficients between straw length and FHB3 or FHB4 were all negative: -0.50 and -0.66 in 1991 and -0.23 and -0.20 in 1993, respectively. As the correlation between the infection levels and straw length is negative in both years, this would indicate a trend that shorter genotypes are more susceptible than longer ones. Liao & Yu (1985b) found a high positive correlation between resistance to *Fusarium* head blight and spike length. Praag 8 had a relatively long spike and was one of the most resistant parents in this and other studies (Bürstmayer *et al.* 1996). Genotypes with a shorter spike in this study, like CWW

4055 appeared to be susceptible to highly susceptible. However, more research has to be carried out in order to elucidate this view.

The FHB-infection levels in 1994 were significantly higher than in the other three years. Previously highly resistant cultivars like Praag 8 (Bürstmayer *et al.* 1996), showed high infection levels in 1994. As the concentration of fungal spores, the fungal isolate and the other experimental conditions were exactly the same, a significant influence of the climate is proposed. Weather data for the years 1991-1994 over the months May, June and July for location 'De Bilt' were retrieved from the national weather station KNMI in the Netherlands. Maximum and minimum temperature at 1,5 meter, duration and amount of rain, sun hours and evaporation data were compared with FHB-infection levels. It appeared that maximum and minimum temperature were highest in July 1994, maximum temperature being 4°C higher than the second highest value of 23.6°C in July 1991. Correlation data showed that maximum and minimum temperature in July had a high positive correlation ($0.88<r<0.99$) with FHB3- and FHB4-levels for all genotypes tested. Evaporation was highest in July 1994 with 123,6 mm against the second highest value 109.5 in May 1992 and correlation studies revealed that evaporation had a high positive correlation ($0.91<r<0.99$) with FHB3- and FHB4-levels for all genotypes tested. Amount and duration of rainfall was highest in July 1993, but as the field plots were artificially irrigated with an overhead mist irrigation, rainfall was not considered a discriminating factor. It can be concluded that warm and moist conditions most favour growth and spread of FHB. This is also confirmed by other publications on this subject (Daamen *et al.* 1991, Logrieco *et al.* 1988, Parry *et al.* 1995, Sutton 1982, Wiersma *et al.* 1996). A strong year effect was found by Nijs *et al.* (1996) who compared fungal infections in cereals, grown in the Netherlands in 1991 and 1993. They found that in 1993 a higher number of samples was infected with *Fusarium* (83%) than in 1991 (34%). Similar year effects were observed earlier (Bedő *et al.* 1992, Snijders 1990a) and were explained by weather conditions.

Offspring of the DH_1-lines could provide information about the stability of the resistance. DH_2-plants of several genotypes were tested in 1994. The DH_2(Praag 8)-lines showed similar infection levels as compared to the corresponding DH_1-lines. For Ringo Sztar no DH_1-data were available. For the F_2-88002 population it appeared that the infection levels of the DH_2-lines in 1994 were almost all higher than the levels for the DH_1-lines in 1992. However, as the infection levels of all genotypes were higher in 1994 as compared to previous years, no conclusion can yet be drawn about the stability of the resistance in different generations. In order to elucidate more the genetic basis of this resistance, offspring of the DH-lines has to be tested in several years for their FHB-resistance, to see whether the resistance is stably transmitted or not.

In all four years minimum and maximum temperatures were lowest in May and highest in July. If temperature is one of the determining environmental factors for FHB-resistance, than early genotypes which have been growing in lower temperature regimes than late genotypes, would show a lower infection

level. Correlations between flowering date and FHB-infection levels ranged from -0.44 for FHB4 in 1992 to 0.73 for FHB3 in 1993 and were negative in 1992 and 1994 and positive in 1991 and 1993. Ringo Sztar, being one of the earliest flowering genotypes, proved to be one of the most resistant ones, whereas Praag 8, flowering moderately late, was also one of the most resistant genotypes. No clear conclusion could be drawn in our experiments for the relation between earliness and resistance. The relatively high correlation coefficients between FHB3 or FHB4 values of genotypes that tested in more than one year would indicate the absence of genotype x year interactions, but more research is needed to validate this assumption.

The Praag 8 sisterline crossing programme, in which resistant DH_1-lines derived from Praag 8 were crossed, showed that for FHB3 the DH_1- and DH_2-lines regenerated on control medium were more resistant than the DH_1- and DH_2-lines regenerated on toxin medium. For FHB4 this was only the case for the DH_1-lines. Similar results were found in 1992 where for FHB3 the DH_1(Praag 8-7)-1C1 plant numbers 2 & 3 were more resistant than the three DH_1-lines derived from toxin-containing medium. However, in 1992 four weeks after inoculation this difference had disappeared. The significant difference that occurred in several cases between the homozygous parents and the DH-lines indicates that the toxin stress could be effective, however, the 1C1-lines, not selected by toxin stress, were even more resistant than 1T2-lines, due to gametoclonal variation.

In this study, for several traits, transgression in DH_1-lines from F_2-populations and gametoclonal variation in DH_1-lines from homozygous parents were shown. Both methods can provide the commercial wheat breeder with agronomically interesting genotypes which are more resistant to *Fusarium* Head Blight.

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Chapter 6: Isolated microspore culture in wheat (*Triticum aestivum* L.): the effect of co-culture of wheat or barley ovaries on embryogenesis.

Abstract

In this study the effects of different ovary co-culture methods on sporophytic development of wheat microspores were investigated. Mechanically isolated microspores of greenhouse grown donor plants of wheat (*Triticum aestivum* L.) were co-cultured with wheat or barley ovaries either in agarose rings or in culture plate inserts. The viability of the microspores was better in co-culture with wheat ovaries from one cultivar as compared to co-culture with a mixture of wheat ovaries from four cultivars. Co-culture of the microspores with ovaries in culture plate inserts had no significant effect on viability of the microspores, but had, in comparison with culture in agarose rings, a large positive effect on the percentage swollen microspores. No clear genotypic effect could be detected. A significant correlation between the number of swollen microspores and the number of multicellular structures was found.

Introduction

The main goal of our microspore culture studies is to exploit the advantages of selection at the cellular level with regard to *Fusarium* head blight resistance in wheat (*Triticum aestivum* L.), for which the *Fusarium*-toxin deoxynivalenol (DON) also known as vomitoxin, could be used as selection agent (Snijders & Schepers unpublished results). Selection using haploid tissue is preferred, as all genes, whether dominant or recessive, are expressed, and regenerated doubled haploids will give homozygous offspring. Selection with microspore-derived tissue was studied earlier, and large differences for growth on toxin containing medium were detected (Bruins *et al.* 1993). However, the regeneration capacity of green plantlets after the selective stage was too low to be of use for in vitro selection purposes.

The use of isolated microspore culture has several advantages over anther culture: in certain species a higher production of embryos and green regenerants per anther can be achieved, the method is less laborious, and regeneration from anther tissues is excluded. Also, in case of in vitro selection experiments, single cells are preferred to multicellular structures or callus, as selection pressure will be more uniform.

In barley, cultures using mechanically isolated microspores resulted in higher percentages of embryos per anther as compared to the shed pollen technique (Hoekstra *et al.* 1992). Improvements of protocols for barley isolated microspore culture, e.g. maltose as carbohydrate source (Scott & Lyne 1994), conditioning of the media with ovaries or anthers (Kasha *et al.* 1990a), starvation of the anthers in mannitol (Roberts-Oehlschlager & Dunwell 1990) or addition of phenyl acetic acid (PAA) (Ziauddin *et al.* 1992) have been found to be only to a minor extent effective for wheat isolated microspore culture (data not presented).

Using the shed pollen technique in wheat microspore culture, calli and green regenerants have been obtained (Kasha *et al.* 1990b), but at a rate too low to be of use for in vitro selection. Mechanical isolation of the microspores and subsequent sporophytic divisions also resulted in microcalli, embryos and green plants (Mejza *et al.* 1993, Tuvesson and Öhlund 1993). The protocol of Mejza *et al.* (1993) consisted of a pretreatment of the spikes of 2 days at 25°C or 7 days at 5°C. Microspores were isolated with a blender and centrifugated over a 20% maltose solution to remove the dead microspores. Culture of the microspores was done in CHB-2 medium (Chu *et al.* 1990) at 25°C in the dark and embryos and green plants were produced. In preliminary experiments in our laboratory, when the microspores were cultured without ovaries, percentages viable microspores were lower and fewer swollen microspores were observed as compared to co-culture with ovaries (Bruins & Snijders 1993), in agreement with Mejza *et al.* (1993). When the wheat microspores were cultured with wheat ovaries in direct co-culture, or with the ovaries cultured in agarose rings, higher percentages viable microspores were observed. Also the percentages of swollen microspores, the first stage of sporophytic development, were higher. Centrifugation of the isolated microspore slurry over a 20% maltose solution showed a relative higher number of viable microspores in a microspore population. However, this protocol did not lead to plant regeneration, and adjustments

needed to be made. Culture plate inserts were tested in comparison to agarose rings. In this chapter, the effect of different pretreatments of the ovary donor spikes, as well as ovary co-culture methods of the microspores on viability and embryogenesis of mechanically isolated wheat microspores will be described.

Materials and methods

Plant material

Plants of the wheat cultivars, Chris, Ciano 067, Frontana and Ringo Sztar, and one barley cultivar, Igri, were used in this study. Chris, Ciano 067 and Ringo Sztar were chosen for their high in vitro androgenic abilities (Bruins *et al.* 1993), whereas Frontana was included because of its consistent high resistance against *Fusarium* Head Blight (Bürstmayer *et al.* 1996, Ginkel *et al.* 1996). All genotypes were vernalized at 4°C (10 h photoperiod) for 8 weeks prior to transplanting to the greenhouse. Plants were grown in potting compost, fertilized every month with an artificial fertilizer (12-10-18 NPK). No pesticides were used. Supplemental light was provided by HPI/T lamps to extend daylength to 14 hours in the early spring period. Greenhouse temperature conditions were 15°C (light) and 10°C (dark). Spikes were collected in the mid boot stage, when the microspores were in the mid- or late uninucleate stage. The flag leaf was removed and the wheat spikes were stored with the basal ends in tap water and were given a pretreatment in the dark for two days at 25°C.

Isolation and culture of the microspores

After pretreatment, the leaf sheath was disinfected with 70% ethanol and air dried in a laminar flow cabinet. A total of 40-60 anthers of the central part of each spike were excised under sterile conditions, transferred to 1.0 ml filter-sterilized liquid CHB-2 medium (Chu *et al.* 1990) and squeezed gently with the backside of a syringe to isolate the microspores. Subsequently the slurry was filtered through a nylon sieve of 88 μm, the filtrate was collected in 50 ml centrifuge tubes and centrifuged at 1000xg for five minutes. The pellet was washed two times with 50 ml of medium and centrifuged as above, resuspended in 5 ml of medium, placed on top of a 45 ml 20% maltose solution and centrifuged at 2500xg for 5 minutes, to remove the dead microspores. Usually this did not lead to clear bands of microspores in the upper part of the solution as described by Mejza *et al.* (1993), so the upper 10-15 ml was collected and centrifuged at 1000xg for final pelleting. The microspores were cultured in co-cultivation with wheat or barley ovaries either in 6-well plates, or in agarose rings in Petri dishes. In case of the 6-well plates 0.5 ml of liquid medium was pipetted into the well (diameter 3.5 cm). Ovaries were harvested from the same spikes that were used for microspore culture. Twelve wheat or barley ovaries per well were placed in the medium and covered by a culture plate insert (CPI) (Millicel-CM PICM 030 50, pore size 0.4 μm). Inside the culture plate insert, 0.5 ml of the microspore solution was pipetted (Figure 1A). For the Petri dishes (diameter 6 cm), a

ring of 0.5 ml medium with 1% agarose (diameter 2 cm) was poured at the bottom of the dish. After solification of the medium twenty ovaries were positioned with the basal end in the agarose ring. One ml of microspore solution was pipetted inside the agarose ring (AR)(Figure 1B). Microspores were also cultured without ovaries.

Microspore density was measured at each experiment without adjustment to a fixed density. Viability of the microspores was assessed one day after isolation by the fluorochromatic assay with FDA (fluoresceïn diacetate) and the developmental stage was assessed with DAPI (4',6-diamidino-2-phenylindole) staining to certify that the microspores were in the mid- and late uninucleate stage.

Swelling of the microspores precedes sporophytic divisions in microspore culture of many species e.g. barley and maize (Hoekstra *et al.* 1993, Pretova *et al.* 1993). Therefore in these experiments the percentages swollen microspores were assessed, two days after incubation (Figure 1C). Data of the percentages swollen microspores were transformed by taking the square root to improve normality of the distribution. Microspores were cultured in CHB-2 medium and incubated in the dark at 28°C. Macroscopically visible embryos (Figure 1F), developed from microspores, were transferred for regeneration to MS medium (Murashige & Skoog 1962) supplemented with 3% sucrose and solidified with 0.30% Gelrite (Kelco). All data were analyzed on the basis of predicted means from Residual Maximum Likelihood (REML) Variance Component Analysis (Genstat 5 Committee 1993). In the statistical analyses, density was considered a covariate. After analysis, the data for swollen microspores were back-transformed to actual percentages. Twenty anthers from at least five spikes of every microspore isolation replication of tested spikes were placed on the potato-based P2 anther culture medium (Chuang *et al.* 1978) to determine the quality of the donor material as measured by anther culture. Anther culture was carried out according to *Bruins et al. (1993).*

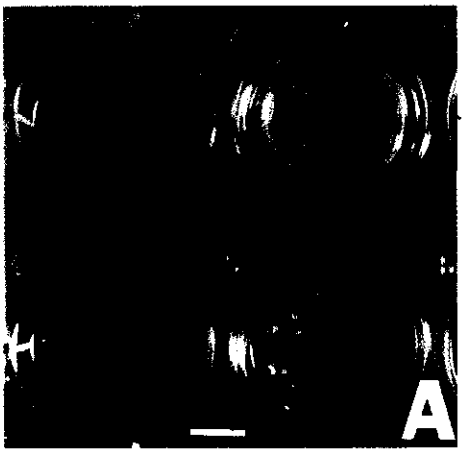
Effect of ovary source and culture method on the sporophytic development of the microspores.

All wheat spikes were pretreated for two days at 25°C and four different types of ovary co-culture were tested:

- wheat ovaries from one cultivar (the same cultivar that was used for microspore culture),
- a mixture of wheat ovaries from all four cultivars, three ovaries from each cultivar.
- barley ovaries excised from pretreated donor spikes (4°C for 7 days)
- barley ovaries, excised from non-pretreated donor spikes.

The four ovary sources were each tested in three replications with microspores pooled from at least three spikes per replication. Microspores were cultured in agarose rings or in culture plate inserts.

Figure 1. (pagina 109) A) Culture of the microspores in a Culture Plate Insert (upper right corner) (Bar=1cm). B) Culture of the microspores inside an Agarose Ring with ovaries (Bar=0.5cm). C) Swollen microspore (Bar=25μm). D) Multinucleate structure stained with DAPI (Bar=25μm). E) Multicellular structure (Bar=25μm). F) Microspore derived embryo (Bar=100μm).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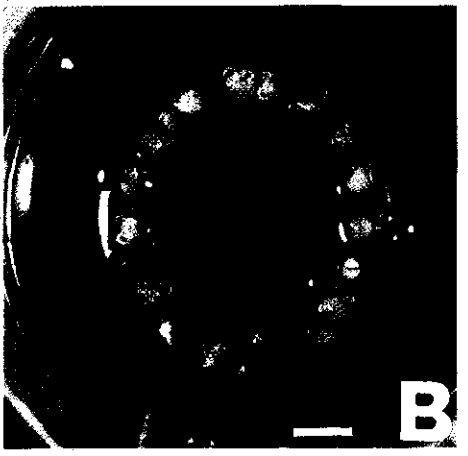
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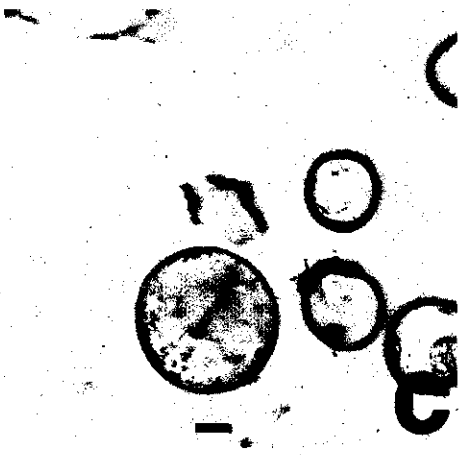

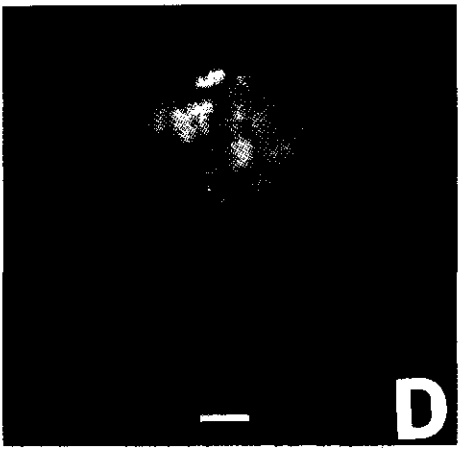
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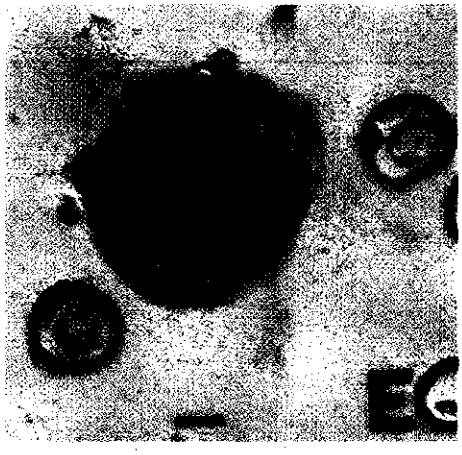

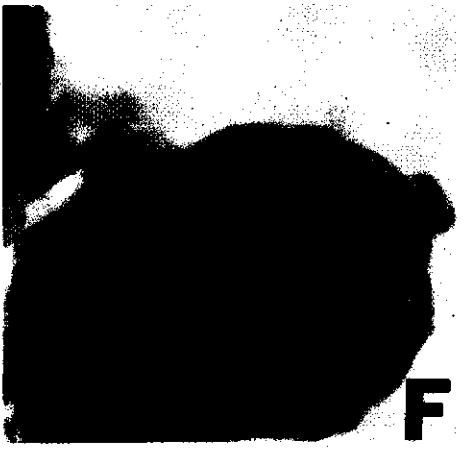
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Results and discussion

Effect of ovary source and culture method on the sporophytic development of the microspores.

Table 1 shows the percentages viable microspores in co-culture with wheat or barley ovaries after one day of incubation. Only in one case, that of co-culture with ovaries from pretreated barley spikes, was a significantly higher number of viable microspores found in the CPI method in comparison with the AR method. Co-culture with wheat ovaries from one cultivar showed significantly higher numbers of viable microspores when compared to co-culture with a mixture of ovaries from four cultivars, irrespective of the culture method. Pretreatment of the barley spikes at 4°C for seven days, has only in the CPI method a pronounced positive effect on viability. There was no consistent genotype effect.

Table 1. Percentage viable microspores after one day of incubation, of four different wheat cultivars in co-culture with wheat (WH) or barley (BA) ovaries. AR = Agarose Ring, CPI = Culture Plate Insert.

		Ovary co-culture type[a]				
	Genotype	**WH ovaries, one cultivar**	**WH ovaries, mix of cultivars**	**BA ovaries, pretreated**	**BA ovaries, non-pretreated**	**Mean**
AR	Frontana	75 abcd	56 def	72 abcd	79 abc	**70 kl**
	Ringo Sztar	51 def	66 bcd	68 bcd	65 bcde	**62 l**
	Chris	89 a	62 cde	65 bcde	63 bcde	**69 kl**
	Ciano 067	88 ab	52 def	79 abc	-	**72 kl**
	Mean	**76 hi**	**59 j**	**71 i**	**72 hij**	**69 q**
CPI	Frontana	82 abc	75 abcd	90 a	28 f	**69 kl**
	Ringo Sztar	90 a	67 bcd	91 a	62 cde	**78 k**
	Chris	84 ab	80 abc	89 a	62 cde	**79 k**
	Ciano 067	83 abc	53 def	63 bcde	42 ef	**60 l**
	Mean	**85 h**	**69 ij**	**83 h**	**53 j**	**72 q**

[a] = Means not followed by the same letter are significantly different at the 0.05 level of probability as determined by REML Variance Component Analysis. Four levels of means are presented: genotype × co-culture type × culture method (a-f), co-culture type × culture method (h-j), genotype × culture method (k-l) and culture method (q).

Table 2 shows the back-transformed percentages of swollen microspores in co-culture with wheat or barley ovaries. In the AR method, only a few swollen microspores were observed. Except when using non-pretreated barley ovaries, significantly higher percentages of swollen microspores were found when the microspores were cultured in the CPI method, as compared to the corresponding AR method. Co-culture with wheat ovaries showed a significantly higher percentage of swollen microspores in comparison with the corresponding co-culture treatment with barley ovaries (Table 2). Mejza *et al.* (1993) tested wheat microspores in co-culture with wheat, barley and maize ovaries and found the highest positive effect on the development of microspore derived embryos with barley ovaries, while co-culture with wheat ovaries showed a slightly lower positive effect, and co-culture with maize ovaries did not result in any sporophytic divisions.

Table 2. Percentage swollen microspores after two days of incubation, of four different wheat cultivars in co-culture with wheat (WH) or barley (BA) ovaries. AR = Agarose Ring, CPI = Culture Plate Insert.

		Ovary co-culture type[a]				
	Genotype	**WH ovaries, one cultivar**	**WH ovaries, mix of cultivars**	**BA ovaries, pretreated**	**BA ovaries, non-pretreated**	**Mean**
AR	Frontana	9.7 def	1.4 fghi	0.0 i	0.0 i	**1.4 x**
	Ringo Sztar	0.0 i	5.2 defgh	0.9 hi	0.8 ghi	**0.4 x**
	Chris	5.2 defgh	5.3 defgh	0.0 i	0.9 ghi	**1.2 x**
	Ciano 067	5.1 def	10.3 d	1.0 efgh	-	**7.3 w**
	Mean	**2.9 rs**	**5.3 r**	**0.3 t**	**0.5 st**	**2.0 y**
CPI	Frontana	50.2 bc	29.9 c	7.7 ab	19.6 def	**34.8 u**
	Ringo Sztar	9.7 de	42.0 bc	9.8 de	5.2 defg	**15.2 v**
	Chris	59.2 ab	79.8 a	51.3 bc	10.5 def	**52.3 u**
	Ciano 067	36.1 c	9.7 def	6.0 efgh	0.5 hi	**7.8 w**
	Mean	**37.2 p**	**37.3 p**	**23.0 q**	**4.8 rs**	**23.0 z**

[a] = Means not followed by the same letter are significantly different at the 0.05 level of probability as determined by REML Variance Component Analysis. Four levels of means are presented: genotype × co-culture type × culture method (a-i), co-culture type × culture method (p-t), genotype × culture method (u-x) and culture method (y-z).

Averaged over all four genotypes, pretreatment of the barley ovaries in the CPI method had a positive effect on the percentage of swollen microspores, in comparison with no pretreatment of the barley spikes. Only with Frontana and Chris a significantly higher number of swollen microspores was found in the CPI

method with barley pretreated ovaries. In the CPI method, averaged over the four culture methods, only Ciano 067 did not show a significantly higher number of swollen microspores. Correlation between percentage of viable microspores and percentage of swollen microspores was relatively high ($r=0.62$, $p<0.01$).

No multicellular structures (MCS) or embryos were produced in the AR method. Table 3 shows the number of MCS produced in the CPI method. All genotypes, except Ciano 067, produced multinucleate (Figure 1D) and multicellular structures (Figure 1E). Chris produced the highest number of MCS, in co-culture with a mixture of wheat ovaries, whereas Frontana was the only genotype that produced MCS in case of non-pretreated barley ovaries. The 16 MCS with Chris (Table 3) were produced at a density of 42 x 10^3 microspores ml^{-1} (data not shown) so one MCS could be produced per 2600 microspores. Correlation of the number of MCS with the percentage of viable microspores was low and non-significant ($r=0.18$), but the correlation of the number of MCS with the percentage swollen microspores was relatively high ($r=0.57$, $p<0.05$).

In the CPI-method, two embryos were formed with Frontana in co-culture with its own ovaries (Table 3) and two were formed in co-culture with pretreated barley ovaries. Four embryos were formed by Ringo Sztar in co-culture with pretreated barley ovaries. No plants could be regenerated from any of the embryos.

Table 3. Total number of multicellular structures of four different wheat cultivars formed in co-culture with wheat (WH) or barley (BA) ovaries in the CPI-method. In parentheses the number of formed embryos.

	Ovary co-culture type			
Genotype	**WH ovaries, one cultivar**	**WH ovaries, mix of cultivars**	**BA ovaries, pretreated**	**BA ovaries, non-pretreated**
Frontana	6 (2)	0	6 (2)	10
Ringo Sztar	2	0	6 (4)	0
Chris	10	16	5	0
Ciano 067	0	0	0	0
Mean	**4.5**	**4**	**4.3**	**2.5**

The density of the microspores varied from 21 to 67 x 10^3 microspores ml^{-1}. No effect of the density on the viability or percentage of swollen microspores could be detected using the co-variate analysis. Mejza *et al.* (1993), however, did find a density effect in isolated microspore culture of wheat with better results with 50 x 10^3 microspores ml^{-1} as compared to 100 x 10^3 microspores ml^{-1}. Other authors also found a density effect in isolated microspore culture of wheat (Gustafson *et al.* 1995) with an optimal density of

200 x 10^3 microspores ml^{-1}. The used range of 21-67 x 10^3 microspores ml^{-1} in the present study might have been too narrow to detect a density effect in the experiments.

When microspores were cultured without ovaries, percentages of viable microspores were much lower and ranged from 14 to 37%, and no swollen microspores were observed (data not shown). The induction of sporophytic divisions appears to be difficult without conditioning the medium with anthers or ovaries (Köhler & Wenzel 1985), co-culture of the microspores with ovaries (Mejza *et al.* 1993) or pre-culture of the microspores in the anthers (Cho & Zapata 1990). Apparently, isolated microspores are lacking the beneficial substances provided by anther or ovary tissues, necessary for induction. Several researchers observed that during the early stages of development the pollen derived embryoid was connected with the anther wall by a tube-like structure which later developed into a suspensor-like or multilayered cell bar of the callus adhered to the anther wall (Chen 1983). In this way the anther wall tissue may offer the essential nutrients to pollen grains during de-differentiation. Furthermore, the anther wall tissue absorbs, stores and transforms the exogenous substances in the medium, and thus acts as a metabolite pool for the pollen grains (Zhong & Liang 1980).

Anther culture, from the same spikes as used for isolated microspore culture, showed average percentages responding anthers ranging from 3.0% for Ciano 067 to 22.6% for Ringo Sztar. This was comparable to and even higher than previous results (Bruins *et al.* 1993), so the growth conditions of the donor plants were considered to be adequate for succesfull anther culture. From the anther culture derived embryos, green plants could be regenerated.

As mentioned by Tuvesson & Öhlund (1993), it appears also in this study that the limiting step of wheat isolated microspore culture was the formation of embryos from the multicellular structures, since these multicellular structures can be produced in all genotypes, except Ciano 067. However, Ciano 067 is known for its low androgenic response (Bruins & Snijders, 1995). Isolated microspore culture of wheat still does not produce the amount of embryos that can be produced in anther culture of wheat, but it can be a useful technique in developmental and transformation studies. In conclusion, it can be stated that culture plate inserts might prove to be a promising method in isolated microspore culture of wheat, and probably worthwhile to investigate its effect for other recalcitrant species in microspore culture.

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Chapter 7: General discussion

Anther culture in the practical breeding process

It can be questioned whether or not to introduce anther culture in the practical breeding process of wheat. Many studies were dedicated to compare several breeding methods in wheat. For the production of (near-)homozygous lines in wheat and barley, research groups focused on the Single Seed Descent (SSD) method, the *Hordeum bulbosum* method (HB) and anther culture (AC). In wheat, in a field experiment, a comparison was made between AC-, SSD- and HB-lines, all from the same cross. No significant differences could be found between lines produced by the three methods (Henry *et al.* 1988). This was confirmed by Picard *et al.* (1986) who found that the variance of the doubled haploid (DH) lines was in the same order of magnitude as that of the SSD- and bulk-lines for earliness, height and reactions to *Puccinia striiformis* and *Erysiphe graminis*. Also in other crops, e.g. Brussels sprouts or oilseed rape, no clear differences could be detected between AC- and SSD-derived lines (Chen & Beversdorf 1990, Kubba *et al.* 1989, Pink *et al.* 1987). However, other publications do report of differences between these selection methods. In barley, comparisons were made between AC-, Pedigree-, SSD- and HB-derived lines, and it appeared that AC-lines in most cases had a lower mean grain yield and 1000 grain weight, but had a higher number of grains/spike (Morden *et al.* 1989). Devaux (1987) compared AC- and HB-lines of winter barley and found that anther culture seemed to be slightly more efficient in comparison with the *H. bulbosum* method. An advantage of anther culture would be that the DH-method tends to preserve existing linkages, and therefore produces a higher proportion of lines exceeding the better parent, than the SSD method (Caligari *et al.* 1987). According to Snape (1988), DH-lines have practical and computational advantages over SSD-populations in calculations of recombination frequencies when many loci are segregating. A disadvantage of DH-populations would be that they are technically more difficult to produce and population size for most crosses is likely to be small, whereas SSD-populations are relatively cheap and easy to produce.

For practical breeding purposes anther culture has already proven its importance. The first anther culture derived wheat cultivars were accepted on variety lists in 1986 and 1987. Since then, several cultivars have been released using the in vitro androgenesis technique. Using an F_1-population, the breeder chooses to accept a maximum of undesired genes, e.g. susceptibility to certain diseases, whereas if an F_2-population is used, part of the undesired material is already discarded, but also a part of the gain in time is lost. The androgenesis technique can also be used at the end of a breeding programme to instantly purify near cultivar lines. Breeders usually take the F_1-population as donor population for anther culture as the gain

in time is more important compared to the extra effort to get rid of the undesired genotypes.

As the in vitro androgenesis technique is working for most of the wheat genotypes (Touraev *et al.* 1996) and also the costs of the DH-technique are competitive to that of other techniques producing (near)homozygous lines (Brennan 1989, Brennan & Kahn 1989, Ding *et al.* 1995) there seem to be enough reasons for implementing the DH-technique in practical wheat breeding programmes.

Fusarium Head Blight

Toxicity

In 1993 three percent of the tested cereal samples collected in the Netherlands contained deoxynivalenol in levels of over 500 μg kg^{-1} (Nijs *et al.* 1996). DON appears to be a very stable compound, during both storage and processing of food, and does not degrade at high temperatures (Scott 1991). On average, baking and cooking reduced the amount of DON in wheat or wheat products by 40% (Abbas *et al.* 1988, Besling *et al.* 1983, Carvajal *et al.* 1987, Isohata *et al.* 1986, Young *et al.* 1984). This means that wheat products that are made from DON-containing wheat will still contain DON. Snijders (1990) calculated that in the Netherlands, in the period 1979-1986, in several years the estimated daily intake of DON was about equal to the limit of tolerance as advised in Canada and the USA.

Selection

As natural head blight infections are only occurring irregularly, visual evaluation after artificial inoculation is the most important way to screen for resistance in the early years of a breeding programme. Other more precise and labour consuming assessments like tolerance or 1000-kernel-weight can be applied later in the breeding programme. Although Bai & Shaner (1996) state that a mixture of local isolates would be an appropriate inoculum to screen for FHB-resistance, other authors found no indications for *Fusarium* strain specific resistance, indicating that one isolate would be sufficient to screen different wheat populations for their FHB-resistance. Any aggressive isolate can be used for testing resistance to FHB, without special regard to the origin or the host source of the isolate (Eeuwijk *et al.* 1995, Miedaner *et al.* 1996, Snijders 1994).

Selection for a higher level of physiological resistance can decrease an eventual higher susceptibility caused by awns and dwarfness. In later generations, traits like percentage of seed infection or tolerance can be identified by additionally measuring yield reduction. Stability of disease reaction appears to be connected with resistance level, the most resistant genotypes are the most stable, and the most susceptible ones tend to have more unstable reactions in different epidemic conditions (Mesterhazy 1995).

Prerequisites for in vitro selection level are that the toxins act at the cellular level and the resistant genotypes show toxin tolerance in vitro. Only a few doubled haploid genotypes, derived from cultivar Praag 8 survived the toxin treatment, subsequent regeneration and seed set. These proved to be more resistant

than the parent Praag 8. Other lines, however, from the same parent that were regenerated without toxin stress, proved to be even more resistant, also after selfing. No toxin stress was applied, so it can be concluded that the variation for this trait in a large part of this research was created by gametoclonal variation, which was present after plant regeneration. As expected, after crossing, transgression was observed in the F_2-populations, also in this study, so there are possibilities for the breeders to use anther culture in a breeding programme for improving the resistance. Accumulation of resistance genes can be achieved. Even when the accumulation takes place in unadapted genotypes this does not render the selected variants worthless. The resistance can be transferred to commercial cultivars in a breeding programme.

Plant breeders should be aware of the complexity of the resistance to *Fusarium*. Every resistance type and its components may have its own genetic background, which up to now seem to be based on oligo- or polygenic mechanisms. The problem with the few genetic analyses made to date is that they only recognize head infection severity without differentiating between components. This means that the genetic background is largely unknown.

In breeding for *Fusarium* head blight a few factors may be considered. Highly resistant cultivars are known and although these cultivars are of exotic origin, the FHB-resistance genes can be introduced in breeding programmes to introgress the resistance and accumulate the resistance genes into commercial varieties.

Agricultural production has grown tremendously in the last 30 years and the world produces enough food to provide every person with more than 2700 Calories per day, which is normally sufficient to ensure that every person has access to adequate food, if distribution is more or less equal.

These agricultural production gains are based on the widespread use of new high-yielding cultivars, new technologies and the increased reliance on food imports. Net cereal imports in developing countries in the 70ies tripled from 20 million tonnes to 67 million tonnes per year. However, still more than 800 million people in the developing world suffer from chronic undernutrition. Over the past few years, the world grain stocks have dwindled to dangerously low levels, showing the vulnerability of the food supplies in the world where the population is expected to reach 7 billion people by the year 2010. It was calculated that approximately 25% of the world's food crops are affected by mycotoxins annually (Council for Agriculture Science and Technology 1989). Not only wheat is affected with *Fusarium* head blight. *F. graminearum* is the causal pathogen of ear rot in maize, and with this, two of the three most important food crops in the world are threatened by *Fusarium* Head Blight. Outbreaks of toxicosis associated with the consumption of mold-contaminated wheat and corn, related to the presence of *Fusarium* Head Blight have been reported in Japan, India and China (Kuiper-Goodman 1994, Beardall & Miller 1994). It is strikingly clear that, amongst other factors, also resistant cultivars are of the utmost importance, ensuring that the little food supplies that reach the undernourished, are of a good and non health-threatening quality.

Research on *Fusarium* Head Blight concerns us all.

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Summary

Wheat (*Triticum aestivum* L.) belongs to the three most important food crops in the world. In certain years, the crop can suffer considerable damage as a result of *Fusarium* Head Blight (FHB), especially as no chemical control is effective against this disease. This disease is mainly caused by the fungi *Fusarium culmorum* and *F. graminearum* but in the cooler regions of North-Western Europe, *F. culmorum* predominates. The pathogen causes a wide range of different damage, of which toxin contamination of the seeds is among the most threatening. These toxins, of which deoxynivalenol (DON), nivalenol (NIV) and acetyldeoxynivalenol (ADON), with the isomers 3-ADON and 15-ADON are most notorious, are capable of inhibiting protein synthesis and are, therefore, extremely hazardous to man and animal. For economic and environmental reasons, host plant resistance is the most appropriate and sustainable disease control method and should be given a high priority in any wheat breeding programme.

Androgenesis is the outgrowth of the male reproductive cell into a haploid plant. Up until now, for more than 250 plant species haploid plants have been produced via in vitro androgenesis. However, when using the in vitro androgenesis technique there are still specific problems to be solved for the individual crops. The applications and advantages of in vitro androgenesis are for example rapid production of haploid plants evoking a shorter breeding regime, easier genetic analyses both at crossings and at the DNA level and possibilities for genetic modification and in vitro selection. In vitro selection has been used efficiently to find agronomically altered traits and to produce new cultivars. With the use of toxins as selective agent new resistances have been found in wheat, e.g. against *Pseudomonas syringae* pv. *syringae* or *Helminthosporium sativum*. A general overview on *Fusarium* Head Blight (FHB), on toxicity of the toxins produced by this pathogen, on in vitro androgenesis and in vitro selection is presented in Chapter 1.

In Chapter 2, the results of a 7x7 full diallel on the inheritance of androgenic ability in wheat anther culture are presented. Seven parental cultivars, differing in both androgenic response and FHB-resistance, together with the 42 F_1-combinations of the complete diallel were evaluated for several androgenic traits in five replicates. In total 130,000 anthers were cultured, of which 14% responded. Diallel data were analysed by the model of Gardner and Eberhart and it appeared that most of the genetic variation could be explained by additive genetic effects. A total of 17,819 embryos were transferred to MS regeneration medium, of which on average 30% regenerated into plantlets. Of them 11% was green. Except for two combinations, green plants were recovered from all 42 F_1-combinations. Significant genetic differences were found and genetic effects explained 38%, 48% and 21% of the total variation for the percentage of green regenerants, the percentage of albino regenerants and the percentage of embryos that formed only roots, respectively. Additive effects explained 30%, 65% and 37% of the genetic variation and narrow

sense heritabilities were 0.11, 0.32 and 0.08, respectively. Replicate effects were highly significant for the factors percentage albino regenerants and percentage embryos with only root formation. No significant General Combining Ability (GCA) effects were found. Variety heterosis was only significant for the percentage green regenerants and specific heterosis was significant for percentage green- and percentage albino regenerants. No reciprocal effects were found. Large significant differences in Specific Combining Ability (SCA) values were observed, with 13.5% of the F_1-combinations outyielding the best parent.

About 2,000 plants were doubled with colchicine and 84% of the doubled haploid (DH) plants could be grown to seed set. For seed set, genetic effects explained 78% of the total variation, but additive effects where responsible for only 4% of the genetic variation and, therefore, the narrow sense heritability was low (0.01). According to heritabilities, for embryo production progress can be rapid, for green plant regeneration it will be important to choose the parents very carefully and for seed set, progress is more hard to make. No correlation between embryo production, plant regeneration or seed set could be found. Over 200,000 seeds were formed on the DH-genotypes.

A low green plant regeneration is considered to be one of the main bottlenecks for efficient use of the in vitro androgenesis technique in wheat. To study the inheritance of anther culture response and green plant regeneration more specifically, reciprocal crosses were made between the wheat cultivars Ringo Sztar, Ciano 067 and Benoist H77022, each of which had both a good response in anther culture and a high frequency of green plant regeneration (Chapter 3). It was found that, averaged for all genotypes, 23.0% of the anthers responded and a callus induction frequency of 77.8% was observed. Furthermore it appeared that of all the embryos, 43.0% developed into plantlets, 25.6% of the regenerants being green, resulting in 3.3 green plants per 100 anthers. It was also found that genotypic effects accounted for 57.7%, 86.3% and 77.5% of the total variance of anther culture response, callus induction frequency and embryo induction frequency, respectively. Additive and dominant gene actions were detected for all androgenesis and regeneration characteristics and no reciprocal differences were found, indicating the absence of cytoplasmic effects. It was concluded that embryo production was primarily correlated with anther culture response and not with the number of embryos produced per plated anther or per responding anther.

Advantages of in vitro selection compared to in vivo selection are that a larger number of genotypes can be screened under controlled conditions and that a limited amount of space is needed to screen all genotypes. For wheat, resistance against several diseases was reported through in vitro selection with the help of toxins as selective agent. In order to elucidate the phytotoxicity of FHB-produced toxins, effects were studied on four types of wheat plant material i.e. seedlings, coleoptile segments, anther derived callus and anther derived embryos, using different concentrations of DON and 3-ADON (Chapter 4). It appeared that DON inhibited growth of all types of plant material and that the seedling growth response to 4×10^{-5}

M DON of a large set of genotypes did not differentiate between tolerant and sensitive genotypes according to the observed FHB-resistance level in the field. In general, coleoptile segments showed a growth reduction at 10^{-5} M DON, whereas a concentration of 10^{-4} M DON appeared to be the optimum concentration to differentiate between haploid wheat calli for DON-tolerance. However, growth analysis data of 40 callus clones did not show any correlation with the known FHB-resistance levels of the original donor genotypes and populations. Regeneration of the anther derived embryos in the embryo selection experiment was decreased 100-fold on DON-containing medium. Averaged across the callus and embryo selection experiments, green plant regeneration showed a decrease of approximately 20-fold on medium containing the toxin.

Most of the resistance genes against FHB that are known up to now, are located in for European standards considered exotic wheat genotypes and introduction of these genes into varieties requires extensive backcrossing. A haploid step could accelerate the transfer of the genes to cultivars. The final aim of our research was selection for high FHB-resistance in the field and, therefore, crosses were made between resistant and susceptible genotypes for four consecutive years (Chapter 5). Parents, F_1- or F_2-populations were used as donor material for anther culture and were, together with the anther culture derived doubled haploid (DH) wheat lines, tested in the field for their FHB-resistance. Percentage infection was measured three and four weeks after artificial inoculation. Besides infection, also date of flowering and, during two years, straw length was scored. The observed plant traits within the various DH-lines were stable, homogeneous and no visible segregation occurred. In most cases, the F_1-, F_2-populations and the DH-lines were for infection level intermediate between the two parental infection levels, indicating an additive inheritance. However, the infection levels of some of the doubled haploids were significantly lower than the levels of the most resistant parent. In cases where the F_2-populations were significantly more resistant than the two parents, it was concluded that accumulation of resistance genes of the partially resistant parents had occurred. In cases where the DH-lines, derived from cultivars were significantly more resistant than the cultivars, it was attributed to gametoclonal variation. No evidence was found that a longer callus phase might lead to a higher level of variation. In 1994 infection levels were substantially higher than in the three previous years, probably due to higher maximum temperatures in the inoculation period.

Microspore culture has several advantages over anther culture, e.g. for in vitro selection experiments, single cells are preferred to multicellular structures as a more uniform selection pressure is secured. The possibility of using isolated microspore culture of wheat for in vitro selection experiments are described in Chapter 6. Experiments were carried out to optimise the isolation and culture of isolated microspores of this recalcitrant crop. It was found that the viability of the microspores was better when co-cultured with wheat ovaries from one cultivar as compared to co-culture with a mixture of wheat ovaries from four

cultivars. Furthermore it appeared that co-culture of the microspores with ovaries in culture plate inserts had no significant effect on viability of the microspores, but had, in comparison with culture in agarose rings, a large positive effect on the percentage of swollen microspores. A significant correlation between the number of swollen microspores and the number of multicellular structures was found.

For future research it will be necessary to analyse the progenies of the diallel-derived DH-regenerants for their FHB-resistance for several generations. It has to be elucidated whether or not the resistance levels of the highly resistant DH-genotypes will also be found in their offspring. Because in some DH-lines accumulation of resistance genes appears to have occurred, these lines will be very useful in breeding for introgression of this trait into commercial varieties.

Wheat and maize, two of the three most important food crops in the world, are affected by *Fusarium* head blight. Research on FHB resistance and resistant cultivars are of the utmost importance to ensure an adequate food supply around the world.

Samenvatting

Een algemeen beeld van *Fusarium* aarziekte, de toxiciteit van de door het pathogeen geproduceerde toxines, in vitro androgenese en in vitro selectie op veranderde eigenschappen wordt gegeven in hoofdstuk 1. Tarwe (*Triticum aestivum* L.) behoort tot de drie meest belangrijke voedselgewassen in de wereld. In sommige jaren kan het gewas zware schade oplopen als gevolg van *Fusarium* aarziekte, o.a. door het feit dat er geen effectieve chemische bestrijding van de ziekte mogelijk is. De ziekte wordt voornamelijk veroorzaakt door de schimmels *Fusarium culmorum* en *F. graminearum*, maar in het koelere noord-west Europa komt vnl. *F. culmorum* voor.
Het pathogeen veroorzaakt schade op verschillende manieren, waaronder besmetting van de zaden met toxines. Deze toxines, waarvan deoxynivalenol (DON), nivalenol (NIV) en acetyldeoxynivalenol (ADON), met de isomeren 3-ADON en 15-ADON, het meest berucht zijn, zijn in staat de eiwit-synthese te blokkeren en zijn hierdoor zeer gevaarlijk voor mens en dier. Uit economisch en milieu-oogpunt is waardplant-resistentie het meeste geschikte en duurzame controle-middel en zou een hoge prioriteit moeten krijgen in ieder tarweveredelingprogramma.

Androgenese is de uitgroei van een mannelijke geslachtscel tot een haploide plant. Tot nu toe is het mogelijk gebleken om in meer dan 250 plantensoorten haploide planten te produceren via in vitro androgenese. Bij een groot aantal van deze gewassen, waaronder tarwe, zijn er nog steeds een aantal specifieke problemen die opgelost dienen te worden. Enkele voordelen van in vitro androgenese zijn: een snelle productie van haploide planten, resulterend in een kortere veredelingscyclus, eenvoudiger genetische analyses bij kruisingen en op DNA-niveau en mogelijkheden voor genetische modificatie en in vitro selectie. In vitro selectie is al vele malen op een efficiënte manier toegepast om te selecteren op eigenschappen die van agronomisch belang zijn en om nieuwe cultivars te produceren. Met behulp van toxines als selectieve agentia zijn er in het verleden in tarwe nieuwe resistenties gevonden, o.a. tegen *Pseudomonas syringae* pv. *syringae* en tegen *Helminthosporium sativum*.

In hoofdstuk 2 worden de resultaten van een 7x7 volledig kruisingsprogramma (diallel) weergegeven. Gekeken werd naar de overerving van androgenese vermogen in tarwe antherencultuur. Hiervoor zijn zeven ouderlijnen, die van elkaar verschilden in androgenetische respons en resistentie tegen *Fusarium* aarziekte, samen met de 42 F_1-combinaties van de complete diallel geëvalueerd op hun androgenetische respons in vijf herhalingen. In totaal werden meer dan 130.000 antheren uitgelegd, waarvan 14% een respons gaf, d.w.z. tenminste een embryo of callus produceerde. Het bleek dat het grootste deel van de genetische variatie in androgenetische respons verklaard kon worden door additieve effecten.
Bijna 18.000 embryos werden overgezet op MS-regeneratie medium waarvan gemiddeld 30% tot plant

regenereerden, waarvan 11% groen waren. De rest van de regeneranten waren albino, een probleem dat veelvuldig optreedt bij regeneratie van plantensoorten uit de Gramineeën familie. Op twee F_1-combinaties na, konden van alle 42 F_1-combinaties groene planten geregenereerd worden. Er werden significante genetische verschillen gevonden en genetische effecten verklaarden 38%, 48% en 21% van de totale variatie voor het % groene regeneranten, het percentage albino regeneranten en het percentage embryos die alleen maar wortels regenereerden, respectievelijk. De additieve effecten verklaarden respectievelijk 30%, 65% en 37% van de genetische variatie en de waarden voor de erfelijkheidsgraad (heritability) in nauwe zin waren respectievelijk 0,11, 0,32 en 0,08. Herhalingseffecten waren alleen significant voor het % albino regeneranten en voor het % embryos met alleen maar wortelvorming. Er werden geen significante algemene combinatie geschiktheids effecten gevonden. Er werden geen reciproke effecten gevonden, maar wel significante verschillen in specifieke combinatie geschiktheid, waarbij 13,5% van de F_1-combinaties een hogere score hadden dan de beste ouder.

Van ca. 2000 haploide planten werd het chromosoomaantal met colchicine verdubbeld en hiervan zette 84% zaad. Voor zaadzetting verklaarden de genetische effecten 78% van de totale variatie, echter additieve effecten verklaarden slechts 4% van de genetische variatie en hierdoor was de erfelijksheidsgraad in de nauwe zin laag (0,01). Afgaande op de erfelijkheidsgraden kan door selectie op embryo productie via selectie een snelle progressie geboekt worden, voor en hoog percentage groene plant regeneratie bleek de keuze van de ouders zeer belangrijk. Er werd geen correlatie gevonden tussen embryo productie, plant regeneratie en zaadzetting. Er werden meer dan 200.000 zaden gevormd op de verdubbelde haploide planten.

Een lage regeneratie frequentie van groene planten bij tarwe wordt als één van de voornaamste beperkingen gezien voor een efficiënte toepassing van de in vitro androgenese techniek in de tarweveredeling. Om de overerving van antherencultuur respons en groene plant regeneratie in tarwe nader te bestuderen werden reciproke kruisingen gemaakt tussen de tarwecultivars Ringo Sztar, Ciano 067 en Benoist H77022. Alledrie cultivars vertoonden een hoge respons in antherencultuur en een relatief hoge frequentie van groene plant regeneratie (hoofdstuk 3).

Gemiddeld over alle genotypen bleek 23% van de antheren een respons te geven, d.w.z. vormde tenminste een embryo of callus. Er werd een callus inductie frequentie van 78% gevonden en het bleek dat van alle embryo's 43% tot planten regenereerden, en 26% van de regeneranten bleek groen te zijn Dit resulteerde in 3,3 groene planten per 100 uitgelegde antheren.

Genotype effecten verklaarden 58%, 86% en 78% van de totale variantie voor antherencultuur respons, callus inductie frequentie en embryo inductie frequentie respectievelijk.

Additiviteit en dominantie werden voor alle waargenomen androgenese en regeneratie-eigenschappen gevonden. Er werden geen reciproke verschillen gevonden, duidend op de afwezigheid van cytoplasmatische effecten. Er kon geconcludeerd worden dat de embryo productie voornamelijk afhangt

van antheren cultuur respons en niet van het aantal embryos dat per uitgelegde of responderende anthere gevormd werd.

Voordelen van in vitro selectie boven in vivo selectie zijn dat een groter aantal genotypen onder gecontroleerde omstandigheden op een kleiner oppervlak getoetst kan worden. Zo zijn m.b.v. in vitro selectie met toxines als selectieve agentia bij tarwe al verschillende nieuwe resistenties gevonden. Om de fytotoxiciteit van de door *Fusarium*-soorten geproduceerde toxines meer op te helderen werd het effect van deze toxines op vier typen plantmateriaal bestudeerd: zaailingen, coleoptiel-segmenten, calli en embryos uit antherencultuur (hoofdstuk 4). Deze typen plantmateriaal werden bij verschillende concentraties van DON en 3-ADON bestudeerd. Het bleek dat DON de groei van alle vier typen plantmateriaal remde. De in het veld geobserveerde verschillen tussen vatbare en resistente genotypen werden niet terug gevonden in verschillen in de zaailing-groei van een groot aantal genotypen bij $4x10^{-5}$ M DON.
In het algemeen bleek dat coleoptiel-segmenten een groeireductie te zien gaven bij 10^{-5} M DON, terwijl een concentratie van 10^{-4} M DON optimaal was om bij haploide tarwe calli te differentiëren op DON-tolerantie. Echter, groeianalyse-data van 40 callusklonen vertoonden geen correlatie met de bekende *Fusarium* aarziekte resistentieniveau's van de oorspronkelijke donor genotypes en -populaties. Regeneratie van de embryos uit antherencultuur werd met een 100-voud geremd op DON-bevattend medium. Gemiddeld over de callus- en embryo-selectie experimenten bleek dat groene plant regeneratie met een 20-voud verminderd werd op DON-bevattend medium.

De meeste resistentie-genen tegen *Fusarium* aarziekte die tot nu toe bekend zijn, zijn aanwezig in voor Europese begrippen exotische tarwegenotypes en de introductie van deze genen in N.W. Europese cultivars vergt tijdsrovende terugkruisingen. Een haploide stap in het selectieprogramma kan de overbrenging van de genen naar cultivars aanzienlijk versnellen. Omdat het uiteindelijke selectiedoel een hoge *Fusarium* aarziekte resistentie in het veld was, werden er kruisingen gemaakt tussen resistente en vatbare genotypen. Ouderlijnen, F_1- en F_2-populatie's werden gebruikt als donormateriaal voor antherencultuur en deze ouderlijnen, F_1- en F_2-populatie's werden samen met de uit antherencultuur voortgekomen verdubbelde haploide tarwelijnen (DH) in vier opeenvolgende jaren in het veld op hun *Fusarium* aarziekte resistentie getest (hoofdstuk 5). Drie en vier weken na kunstmatige inoculatie werd het infectieniveau gemeten. Naast infectieniveau werden ook nog bloeidatum en, gedurende twee jaren, strolengte en gebaardheid waargenomen. De planten binnen de verschillende DH-lijnen waren zeer stabiel en homogeen en er was geen uitsplitsing zichtbaar.

In de meeste gevallen vertoonden de F_1- en F_2-populaties en de DH-lijnen een intermediair infectieniveau t.o.v. die van de beide ouders, duidend op een additieve overerving. Echter, in sommige gevallen waren de infectieniveau's van de DH-lijnen significant lager dan die van de meest resistente ouder. In de gevallen

waar de F_2-populaties significant resistenter waren dan de twee ouders lijkt dit toegeschreven te kunnen worden aan accumulatie van de resistentie-genen van de partieel resistente ouders. In de gevallen waarin de DH-lijnen, afgeleid van cultivars, significant resistenter waren dan de cultivar zelf ligt gametoclonale variatie meer voor de hand. Er werden geen aanwijzigingen gevonden dat een langere callusfase leidt tot een hoger niveau van variatie binnen de regeneranten. De infectieniveau's in het veld waren in 1994 hoger dan in de drie voorgaande jaren, waarschijnlijk door de hogere temperatuur in de inoculatie-periode.

Er zijn verschillende voordelen van geïsoleerde microsporencultuur boven antherencultuur te noemen bijvoorbeeld bij het gebruik van in vitro selectie hebben individuele cellen de voorkeur boven multicellulaire structuren, omdat bij in vitro selectie met individuele cellen een homogenere selectiedruk te bereiken is. De mogelijkheid om geïsoleerde microsporecultuur van tarwe verder te optimaliseren, om uiteindelijk te kunnen gebruiken voor in vitro selectie experimenten wordt beschreven in hoofdstuk 6 bestudeerd. Er werden experimenten uitgevoerd om de isolatie en de cultuur van de microsporen van dit recalcitrante gewas te optimaliseren. Het bleek dat de vitaliteit van de microsporen in co-cultuur met tarwe-ovaria van één cultivar hoger was dan in co-cultuur met tarwe-ovaria van vier cultivars. Verder bleek dat co-cultuur van de microsporen met de ovaria in "cultuurplaat wels" geen significant effect had op de vitaliteit van de microsporen. Deze opkweekmethode had, in vergelijking met cultuur van de tarwe-ovaria in agarose-ringen, een duidelijk positief effect op het percentage gezwollen microsporen. Er werd een significante correlatie gevonden tussen het aantal gezwollen microsporen en het aantal meercellige structuren.

Voor toekomstig onderzoek zal het nodig zijn om de verdubbelde haploide nakomelingschappen uit de eerdergenoemde diallel te analyseren. Er zal onderzocht moeten worden of en hoe de hogere resistentie niveau's die gevonden zijn in sommige verdubbelde haploide genotypen naar hun nakomelingen overerven. In verschillende verdubbelde haploide lijnen lijkt accumulatie van resistentie-genen opgetreden te zijn en derhalve zouden deze geselecteerde lijnen als kruisingsouder zeer bruikbaar kunnen zijn voor de inkruising van *Fusarium* aarziekte resistentie in commerciële cultivars.

Tarwe en mais, twee van de drie meest belangrijke voedselgewassen in de wereld, worden allebei aangetast door *Fusarium* aarziekte. Onderzoek naar *Fusarium* aarziekte resistentie en resistente cultivars is van het grootste belang voor een adequate voedselvoorziening in de wereld.

Curriculum vitae

Marcellinus Bernardus Maria (Marcel) Bruins werd geboren op 19 augustus 1964 te Stokkum. In 1983 behaalde hij het ongedeeld VWO diploma aan het Ludger college te Doetinchem. In hetzelfde jaar startte hij met de studie Plantenveredeling aan de toen nog Landbouwhogeschool, tegenwoordig Landbouwuniversiteit te Wageningen. In juni 1989 sloot hij deze studie met goed gevolg af, met specialisaties Plantenveredeling en Plantenziektenkunde. In januari 1990 werd op het Centrum voor Plantenveredelingsonderzoek (CPO), later het DLO-Centrum voor Plantenveredelings- en Reproduktie Onderzoek (CPRO-DLO), begonnen aan het onderzoek naar *Fusarium* resistentie in tarwe. Dit project werd gefinancierd door de Stichting Nederlands Graan Centrum (NGC).

Marcellinus Bernardus Maria (Marcel) Bruins was born on 19th August 1964 in Stokkum, The Netherlands. In 1983 he graduated from the Ludger college in Doetinchem. In the same year he started his study Plant Breeding at the Agricultural University in Wageningen. In June 1989 he graduated from the University with the specialisations Plant Breeding and Plant Pathology. In January 1990 he started working at the Centre for Plant Breeding Research (CPO), which in 1991 merged into the DLO-Centre for Plant Breeding and Reproduction Research (CPRO-DLO). The topic of his work was *Fusarium* resistance in wheat. This project was financed by the Netherlands Grain Centre (NGC).

Nawoord

Het is er dan toch nog van gekomen: Het proefschrift is af. Phew!
Tijdens de jaren dat ik er aan gewerkt heb, hebben velen hun bijdrage aan dit proefschrift geleverd en ik wil eenieder dan ook hartelijk bedanken voor alle steun en hulp. Zonder volledig te kunnen zijn wil ik toch graag een paar mensen met name noemen.

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