RESEARCHES REGARDING MOLECULAR MARKER ASSISTED SELECTION FOR COMMON BUNT (TILLETIA SPP.) RESISTANCE IN WHEAT (TRITICUM AESTIVUM L.)

(PhD THESIS SUMMARY)

PhD SUPERVISOR
PROF. DR. CONSTANTIN BOTEZ

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INTRODUCTION

Wheat (*Triticum aestivum* L.) is from the group of cereals the most cultivated plant on large surfaces. Because of its special importance in the diet of people, wheat crop is spread over all continents. Among the pathogens that cause disease in wheat, species of fungi that attack the ear of wheat represent pathogens of major importance. Mycosis affects both wheat production and the quality of harvested wheat seeds.

Common bunt of wheat is a wheat disease caused by two species of *Tilletia* (*Tilletia foetida* and *Tilletia carries*). This disease is transmitted through infected seeds or soil contaminated with spores. Common bunt is now found by seed treatment with fungicides. Common bunt resistance is based on major resistance genes that have been identified, currently having a number of 15 known resistance genes (Hoffmann and Metzger, 1976). In previous studies, the attempts to find genetic markers linked to resistance genes to *Tilletia* species led to the identification of several specific markers linked to resistance genes, some of which are used in selection (Laroche et al., 2000).

The objective aimed at in this PhD thesis was to identify genetic markers for resistance in common wheat (*Triticum aestivum* L.) to *Tilletia* species causing common bunt of wheat which can be used in selection. In this approach, the main research objective was to test some markers existent in literature (RAPD and SSR markers), as well as to identify new markers for in order to show the molecular polymorphism in cultivars and lines used in this thesis.

CHAPTER I

GENERAL ASPECTS REGARDING WHEAT IMPORTANCE AND CULTIVATION

Common wheat (*Triticum aestivum* L.) belongs to the order Gramineae, family Gramineae (*Poaceae*) *Triticum* L. genus that includes a large number of species, subspecies, varieties and cultivars. The *Triticum* genus forms a polyploid series that includes diploid, tetraploid and hexaploid species, all having the basic number of chromosomes *2n* = 7. (Feldman, 2001). Common wheat is of major economic importance, being cultivated on large areas worldwide, and it is used both in human food and for animal feeding.

1.1. WHEAT GENOME

Common wheat (*Triticum aestivum* L. em. Thell) is an allohexaploid (*2n = 6x = 42*) composed of three distinct but closely related genomes (A, B, D) coming from three genitor species, each with the basic number of chromosomes (n = 7). The three genomes come from *Triticum Urartu* (Genome A), a species unknown of *Sitopsis* (Genome B) and from *Triticum tauschi* comes Genome D (Ganal and Röder, 2007). Wheat genome is made of 16×10⁶ chromosome pairs, being nearly 100 times larger than the *Arabidopsis* genome, 40 times larger than the genome of rice and about 6 times larger than the maize genome (Amuruganathan and Earle, 1991).

1.2. GENERAL ASPECTS OF MAIN WHEAT PATHOGENS

Wheat crop is very sensitive to the attack of infectious diseases caused by a large number of pathogens: viruses, bacteria, fungi, and harmful to insects such as the Hessian fly and the Russian wheat aphids that can transmit viruses to the wheat. There have been identified over 40 fungi, 32 viruses, 81 bacterial diseases that attack wheat plants at different stages of development (Bobes et al., 1977).

Among the ear of wheat diseases, currently the most common is dwarf bunt (*Tilletia controversa*) and common bunt (*Tilletia spp.*), next to fusariosis (*Fusarium* Head Blight) that cause considerable production losses. In addition to these, there are frequently reported parasitic fungi: yellow rust, brown rust, black rust, flying pitch, blackening of cereals, wheat mildew, septoriosis, alternariosis (McIntosh, 1998).
CHAPTER II

STUDY ON TILLETTIA SPECIES FOR COMMON BUNT OF WHEAT

2.1. SPECIES OF TILLETTIA GENUS. MORPHOLOGY. TAXONOMY

Tilletia genus is part of the family Tilletiaceae, order Ustilaginales, Bazidiomycetes class. Tilletia species causing common bunt are T. laevis Kühn (synonym Tilletia foetida (Wall.) Liro) and Tilletia carries (DC.) Tul. Tilletia carries (DC.) Tul. (synonym Tilletia tritici (Bjerk.) Wint (Dumitraș and Bontea, 1991).

Common bunt is also called smelly pitch or wearing pitch and is one of the most widespread and damaging diseases of wheat. Tilletia species (T. foetida and T. tritici) are very similar to each other differing only in morphology, in terms of teliospores’ shape: the spores of T. tritici species have reticulated walls whereas the spores of T. foetida have smooth walls (Saari et al., 1996). The pathogen agent of the common blunt is hosted in wheat but may be fount in other Triticum species too; this pathogen agent is found in species that are sensitive to one or more races of common bunt, as well as in most species susceptible to dwarf bunt.

2.2. BIOLOGY OF COMMON BUNT

Common bunt is spread by infected seed increase. During cropping, spores are spread on the surface of seeds and in soil and survive from one season to another. At threshing time, bunt seeds break out and the spores are scattered in the atmosphere and on the soil’s surface, as well as on seeds. The infection is initiated when the teliospores from the surface of seeds or soil germinate at the same time with the seeds. By the germination of teliospores occurs (by their cariogamia) the reductional division consisting of one or two meiotic divisions, even before germination. By germination, it is formed a promycelium that is non-septate, hyaline and multinucleated, on which appears at the endings an indefinite number of basidsiospores (primary sporidiae). From the primary sporidiae or from the conidia, by copulation are developing dicariotic infection filaments that penetrate successively the hypocotile axis by all plant tissues and form toxic chlamydospores. Chlamydospores are black, of spherical shape and replace the content of the grains in the ear.

2.3. PHYSIOLOGIC RACES OF COMMON BUNT

Phatogen races both for commun blunt and dwarf blunt have been identified. Depending on the reaction to bunt of certain cultivars called differential cultivars and based on the gene-for-gene relation that occurs between fungi and the host plant, there have identified and defined a total of 30 races for T. tritici, 16 races for T. laevis and 17 races for T.controversa (Goates, 1996). In addition to the races listed here, many other pathotypes were also identified (Hoffmann, 1982).

In Romania, the first research on the physiological specialization of species of common bunt and dwarf bunt made by Sâvulescu in 1957 and Sandu-Ville (1934) revealed 20 races for Tilletia laevis, 9 races for Tilletia triticoides (Gassner) Sâvulescu, 8 races for Tilletia tritici and 7 races for Tilletia controversa. The spreading of the bunt races depends on the cultivated wheat varieties; the maintainance in crop of vertically resistant varieties leads to new and more virulent races, because bunt races can make unlimited new combinations of more aggressive virulence genes (Kendrick and Holton, 1961).

The presentation of virulence for bunt races to each known resistance genes shows that the most effective bunt resistance genes are genes Bt8, Bt11 and Bt12, against which no common bunt race has shown virulence, as well as genes Bt13, Bt14 and Bt15, against which present virulence only one, respectively or two bunt races. Also, it is to be noticed the highest virulence of Tilletia tritici (T-30) race, shown to seven resistance genes.

2.4. SYMPTOMS OF COMMON BUNT INFECTION ON PLANTS
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The symptoms are fully manifested (Figure 1) and visible in plant maturity by turning the entire content of wheat grains in a mass of black spores. Wheat plants infected with *Tilletia* spores is influenced by several factors, among which soil reaction and type, the amount of teliospores, temperature, light and humidity are the most important (Dumitraș and Bontea, 1991; Ghibu, 2002).

![Fig. 1. Symptoms of common bunt infection on wheat - ear – right side (Source: Matanguihan et al., 2011). Wheat kernels (grains) infected with common bunt spores – left side (Original)](image)

2.5. STRATEGIES FOR COMMON BUN CONTROL

**Chemical control.** Over time, bunt has been controlled by the intensive treatment with fungicides of the seed material; due to this method, losses have been significantly reduced (Nagy and Moldovan, 2007). If these fungicides are applied regularly, losses are completely eliminated because they are effective both on the spores on seeds and on spores on the soil (Dumitraș and Bontea, 1991).

**Use of resistant varieties.** The cultivation of bunt resistant varieties may be the best way to control the disease when sources of resistance are available. As in the classic breeding, there has not been any interest for this disease, many current varieties of winter wheat grown in Europe have been identified as highly susceptible to bunt (*Tilletia tritici* and *Tilletia laevis*). But there were also identified genotypes with resistance in some varieties (Dumalasová and Bartoš, 2006; Wächter et al., 2007). Following tests carried out, at European level are mentiones varieties of Czech origin (Bill cultivar, with a degree of infection under 15% in artificial infection), (Dumalasová and Bartoš, 2007), European varieties Tjelvar şi Stava (*Bt8, Bt9, Bt10*) with a very low level of infection of 0,6% and 0,7% respectively (Dumalasová and Bartoš, 2006b). Tests carried out have shown other resistant genotypes, for example, 0028162-II wheat line with an average of the control level of 0,1% (Huber and Buerstmayr, 2006). In Romania, there were identified more bunt resistant wheat lines, with source of resistance from *Triticum monococum* and *Triticale* (Onică and Săulescu 2008). Also, some varieties showed increased resistance in all tests performed: Flamura 85, Arieșan, Apulum, Transilvania, Voyage şi Estica (Ghibu, 2002).
CHAPTER III

GENERAL ASPECTS OF WHEAT RESISTANCE TO COMMON BUNT

3.1. GENETIC BASIS OF DISEASES RESISTANCE IN PLANTS

3.1.1. Types of plant resistance

Depending on the host plant’s response to pathogen attack, resistance was divided into two groups: specific resistance and non-specific resistance.

- **Specific resistance** is expressed in early development stages of plants (seedling stage) and is characterized by the race-cultivar specificity. Specific resistance is generally monogenic and dominant and is quickly defeated by pathogens, so it is less sustainable.

- **Non-specific resistance** is characterized by sensitivity of plants in the seedling stage but the resistance is manifested in the adult plant stage, when plants are inoculated with a mixture of races of the pathogen. These varieties retain a good level of resistance, very sustainable. Non-specific resistance was detected in wheat varieties that have no major gene or have major genes that became ineffective.

3.2. GENETIC CONTROL OF WHEAT RESISTANCE TO COMMON BUNT

Common bunt resistance is controlled by major genes for resistance but many of them have been defeated by the virulence of the pathogen’s races (Hoffman, 1982). There has been identified a number of 15 major resistance genes (noted from Bt1 to Bt15), which provide specific resistance (Hoffman and Metzger, 1976; Goates, 1996). These are found alone or in combination with other genes in varieties resistant or tolerant to bunt belonging to *Triticum* species. Bt8 gene is responsible for the resistance of most varieties but some varieties have the Bt12 gene in combination with other genes. Another resistance gene (*Bt-Z*), originating from a translocation from *Agropyron intermedium*, was used to control common bunt in the Soviet Union (Mozgovoi et al., 1987).

In terms of type of resistance, the resistance of wheat to *Tilletia* spp is a specific resistance, monogenic controlled, based on the gene-for-gene relation between wheat resistance genes and antivirulent genes of the pathogen (Hoffman, 1982). Although resistance genes are known from a long time, lasting durability can be achieved only by introgression of several resistance genes in a variety (Hoffman and Metzger, 1976).

Resistance to common and dwarf bunt is controlled by the same resistance genes (Hoffmann and Metzger, 1976). A cultivar resistant to a common bunt race will also be resistant to a dwarf bunt race with the same or less virulence genes.

3.3. RESISTANCE SOURCES TO COMMON BUNT IN *TILLETIA* SPECIES

Although seed treatment with fungicides is a very effective control method, there are still necessary wheat bunt resistant cultivars, especially to control the disease in organic agriculture, to produce organic crops and as an alternative less expensive to the treatment with fungicides (Saari et al., 1996). The basic control method of wheat bunt remains the creation of resistant varieties. Common bunt resistance was identified in several species of *Triticum* and *Aegilops* and other related species: *Triticum, Aegilops* and *Agropyron*, Emmer wheat, *Triticale, Triticum spelta*, Durum wheat (Rubiales et al., 2001, Mamluk, 1998). Sources of resistance to bunt were identified in *Hordeum chilense*, a wild diploid barley species from Chile and Argentina.

Currently, origin centers of wheat resistance to common bunt have been investigated and there were identified sources of wheat resistance to bunt in Serbia, Iran and Montenegro in some wild varieties (Bonmann et al., 2006).
CHAPTER IV
MOLECULAR MARKERS AS NEW TOOLS USED IN BREEDING FOR PLANTS RESISTANCE TO DISEASE

A molecular marker is represented by the genetic differences between individual organisms or species (Collard et al., 2005). Compared with morphological or biochemical markers, molecular markers correspond to polymorphisms found at the DNA level. The analysis of these polymorphisms by molecular biology techniques address to the entire genome and to the regions that are translated or not into proteins, regardless the environmental conditions.

4.1. TYPES OF MOLECULAR MARKERS

The types of genetic markers used in genetics and plant breeding can be grouped as follows:
- phenotypic markers
- isoenzymatic markers
- protein markers
- RFLP markers
- Markers based on PCR (RAPD, AFLP, SSR, ISSR)
- Other types of molecular markers (STS, SCAR, CAPS, ESTs, DArT) that exist and are being used.

Recently, one group of molecular markers used is represented by a single nucleotide polymorphism (Single nucleotide Polymorphism - SNPs).

4.2. USING MOLECULAR MARKERS IN PLANT BREEDING FOR DISEASE RESISTANCE

Breeding for disease resistance has begun by incorporating major genes for resistance into new varieties and by using specific resistance (oligogenic) of the vertical resistance. The practice has shown that the cultivation incorporating a major gene conferring unsustainable resistance is defeated by the emergence of new physiological races of the parasite. For wheat resistance to common bunt, the insertion of Bt resistant genes into specific races to cultivated varieties was the most commonly used method to improve disease control.

4.2.1. MARKER ASSISTED SELECTION (MAS)

Apart from their fundamental interest, the molecular markers are especially important in the field of selection.

The selection based on phenotype or on morphological markers is used in the conventional amelioration. Such a selection is often less efficient due to the number of genes that are responsible for a certain character and to the degree to which the environment bears upon the expression of these genes.

Molecular marker assisted selection (MAS) is an indirect selection method that requires the selection of the plants bearing the parts involved in the expression of the characters of interest (resistant plants) by the molecular markers (Landjeva et al., 2007). At the moment, marker assisted selection is focused on:

1. the selection of the economically important characters for which the selection based exclusively on phenotype is difficult to render or costly;
2. the selection of the quantitative characters for which the phenotype-based selection would be difficult to render;
3. pyramidion of the resistance genes.

Molecular marker assisted selection is considered very useful in the amelioration of cultivated species’ resistance to disease (Langridge et al., 2001) because:

- Identifying the resistance genotype by field testing is costly, requires a long timeframe and is highly influenced by the environmental conditions. Due to the markers, the environmental bias in selection is eliminated.
The selection can be rendered without appeal to inoculation tests, thus avoiding the errors associated with the use of these procedures and enabling resistance breeding in areas where the pathogen is inexistent.

- The selection scheme is speeded up as those making the selection may infer the presence of the gene by the presence of the marker before its phenotypical expression.
- The markers linked to different resistance genes offer the possibility of combining more genes (the genes’ pyramid) when the selection of certain genes in the presence of other genes is difficult to render.

CHAPTER V
THE AIM AND THE OBJECTIVES OF RESEARCH WORK

The attempts of the previous studies to find genetic markers linked to the resistance to *Tilletia spp.* led to the identification of several RAPD markers linked to the resistance genes (Laroche et al., 2000). Their utility has been confirmed for the identification of the varieties that bear this gene, and thus their importance resides in the fact that they can be used in the selection process to identify the wheat varieties and lines that are resistant as an alternative to the treatment of seeds with fungicides.

Consequently, the present research aims at identifying the molecular markers for some of the wheat genes resistant to common bunt. The research is based on the use of preexistent markers and on their testing for the wheat varieties and lines cultivated locally. It sought then to identify new markers that can be used in the selection for resistance to common bunt.

Therefore, our research aimed to reach the following objectives:

1. Identify unspecific molecular markers by using RAPD (Random Amplified Polymorphic DNA) techniques for the wheat genes resistant to common bunt;
2. Transform these dominant markers into specific co-dominant SCAR (Sequence Characterized Amplified Regions) markers;
3. Obtain $F_2$ segregating generations by the self-pollination of $F_1$ plants resulted from the crossing of resistant parental varieties with sensitive ones;
4. Characterize the phenotype of $F_2$ generation plants using tests of artificial infection;
5. Test some SSR specific markers in order to emphasize the molecular polymorphism;
6. Assign the identified markers to genes resistant to common bunt using the bulk segregation method;
7. Establish a chain relationship between the identified markers and the genes resistant to common bunt.

CHAPTER VI
MATERIAL AND METHODS

6.1. PLANT MATERIAL USED TO IDENTIFY MARKERS FOR WHEAT RESISTANCE TO COMMON BUNT

6.1. Plant material used to identify markers for wheat resistance to common bunt

1. Analyzed germplasm represented by parental forms of wheat lines is represented by 13 varieties of autumn wheat belonging to segregating generations, of which 5 are field-resistant to bunt and 8 are sensitive to the disease. This biological material is taken from NARDI Fundulea, Bucharest, where the lines and varieties have been obtained. The following five lines of autumn wheat are known to be resistant to common bunt, exhibiting their resistance repeatedly in tests: 99419G4-1A/1-1, 00274G2-31, 00281G2-11, 00399G2-11, 01450G1-1. The winter wheat varieties that are sensitive to common bunt are: Farmec, Delabrad, Glosa, Boema, Jiana, Crina, Dropia and the line F96869G1-108 also exhibited sensitivity to common bunt in repeated tests done at NARDI Fundulea (Săulescu N., personal communication).
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2. There have also been analyzed two sets of dihaploid lines:
   - 28 lines resulted from the GP 369 combination (P99419G4-1AI1-1/00356G8-1), marked from 9.1 to 9.28;
   - 41 lines resulted from the GP 384 combination (P99419G4-1AI1-1/98047G14-2INC1), marked from 4.1 to 4.41.

3. The F2 segregating populations obtained through the crossing of bunt-resistant parental forms with bunt-sensitive ones have also been used as biological material.

6.2. USED METHODS

   In order to be employed in the selection, the molecular markers identified need first to be assigned to the resistance genes. Different segregating populations are thus analyzed in order to emphasize the co-segregation of markers with the resistance gene (or with the character of interest for which the gene is responsible) (Landjeva et al., 2007). The analyses are based either upon the co-segregation of the markers with the resistance gene, or upon other analysis methods:
   - the use of the lines obtained through backcrossing (RIL lines);
   - the use of nearly isogenic lines (NIL);
   - the use of the bulk segregation method (Bulk Segregant Analysis);
   - the use of dihaploid lines.

   In this thesis, two methods have been approached for the identification of molecular markers:
   - the co-segregation of the marker with the resistance gene;
   - the bulk segregation method (Bulked Segregant Analysis - BSA).

6.2.1. Co-segregation methods

   The co-segregation method is based upon the idea that the marker and the resistance gene segregate together while the presence of the gene is emphasized by the presence of the marker.

6.2.2. Bulked Segregant Analysis Method (BSA)

   This method involves effecting two blends of DNA derived from two extreme phenotypes (one resistant and one sensitive) taken from the individuals of a segregating population (F2 or double haploids). The DNA blends (bulks) are analyzed using molecular markers to test the polymorphism between them (Michelmore et al., 1991). The polymorph markers obtained are then used to establish the chained distance to the gene of interest in the segregating populations (Landjeva et al., 2007).

6.2.3. Isolation of genomic DNA

   The isolation of DNA was done using the protocol described by Lodhi et al. (1994) and modified by Pop et al. (2003) – protocol that was based on the use of CTAB (cetil trimetilammonium bromide), adding citric acid, PVP and Dieca, and using fresh leaves.

6.2.4. Isolation of plasmid DNA

   The isolation of plasmid DNA has been done through the method of the alkaline lysis. The plasmid DNA thus resulted is of a high quality and in a quantity large enough to be used in further analysis.

6.2.5. Determining the quantity and the quality of isolated nucleic acids (DNA)

   The DNA can be quantified in general using the following methods: the gel electrophoresis and the spectrophotometric quantification of DNA. The quality of the extracted DNA must be appropriate for further analysis. The quality of the isolated DNA is of utmost importance for different analyses such as restraining enzymes digestion or the PCR amplification of the matrix DNA.

   The crosscheck of the quantities and quality of the DNA solution was done using the spectrophotometric method. In the process of quality assessment, the DNA was sometimes migrated in agarose gel (0,8%) as well.
6.3. MOLECULAR ANALYSIS METHODS

6.3.1. Analysis with specific markers for resistance gene Bt10

The primer pair FSA and RSA is specific for the resistance gene Bt10 and it marks a polymorph fragment of 275 bp (Laroche et al., 2000). These primers were tested both for the parental lines and for the dihaploid lines of wheat.

6.3.2. Amplification of the DNA fragments by polymerase chain (PCR)

The amplification of the DNA fragments through the polymerase chain reaction is based on the capacity of the DNA polymerase enzyme to synthesize DNA fragments in the presence of primers with nucleotide sequence and of the four synthetic nucleotides (dNTPs).

6.3.3. The nucleic acids electrophoresis (DNA)

The DNA fragments amplified through PCR were separated through electrophoresis in agarose or polyacrylamide gel. Their visualization and examination was achieved through a UV light source and the images were captured and stocked with the Alpha Innotech Image arapparatus.

6.3.4. RAPD markers analysis

In order to test the polymorphism regarding their resistance between the resistant and sensible parental lines and varieties and in order to discover the RAPD polymorph markers, we used a number of 32 primers chosen randomly (Operon primers).

6.3.5. Cloning and sequencing the RAPD polymorph bands

In order to be able to continue using them, the RAPD polymorph amplification products are isolated from the migration gel in view of purifying and obtaining the sequences. After the migration and analysis of the gel, eight representative bands were isolated and reamplified with the help of the universal primers, as follows:
- 3 bands obtained from the amplification with the primer OPC 04 (550 bp)
- 5 bands obtained from the amplification with the primer OPA 17 (850 bp)

The cloning of the bands separated and isolated from the agarose gel had as purpose their sequencing and based on the nucleotide sequence the construction of specific primers which amplify this band. The DNA fragments recovered were linked in the vector pJET1.2/blunt and were transformed in the bacteria *Escherichia coli*, the stem GM2163 using the protocol indicated by the producer. For the cloning of the bands we used the cloning kit CloneJetTM PCR Cloning Kit (Fermentas). It contains the cloning vector pJET1.2/blunt which contains a lethal gene. In the moment of the insertion of the fragment desired in the cloning site, this gene is inactivated.

After the identification of the transformed clones, they were cultivated overnight on the LB medium with ampicillin, which were used for the isolation of the plasmid DNA.

Of the positive colonies, the extraction of the plasmatic DNA was achieved based on the protocol of the alkaline lysis. The plasmid DNA extracted was sequenced in the Macrogen laboratories, Holland.

6.3.6. Microsatellite markers (SSRs) analysis

In the present thesis we tested for polymorphism a set of microsatellite markers located on different wheat chromosomes in the regions where they are located and some resistance genes (Bt) present in the analyzed germplasm. The markers were selected based on the information existent in the literature as regards their linkage with some resistance wheat genes at the common bunt. The set of primers for the markers was synthesized in the Mycrosynth Company, Switzerland, and comprises 11 pairs of primers.
CHAPTER VII

OBTAINED RESULTS

7.1. GENETIC ANALYSIS OF THE WHEAT RESISTANCE TO THE COMMON BUNT IN THE F₂ SEGREGANT POPULATIONS

For obtaining the F₂ generation in 2007, the hybridizations were achieved between the resistant parental forms and the sensitive one (resistant x sensitive). The wheat autumn lines: 99419G4-1A/1-1, 00274G2-31, 00281G2-11, 00399G2-11, 01450G1-1 were used as father forms in the cross-breeding. The varieties and lines of autumn wheat, sensitive to *Tilletia spp.* - Farmec, Delabrad, F96869G1-108, Glosa, Boema, Jiana, Crina, Dropia were used as mother forms in the cross-breeding. The F₁ hybrids obtained were seeded in the field in the autumn of 2008 in view of obtaining the segregate F₂ population.

Before the planting of seeds, they were artificially infected with *Tilletia caries* and *Tilletia foetida* spores. The results of the artificial infection (fig. 2) were assessed in the parental varieties and lines and in the following 10 descendencies from the populations F₂: 99419G4-1A/1-1 x Glosa, 99419G4-1A/1-1 x Boema, 99419G4-1A/1-1 x Dropia, 00274 G2-31 x Glosa, 00274 G2-31 x Dropia, 00281G2-11 x Boema, 00399G2-11 x Boema, 01450G1-1 x Glosa, 01450G1-1 x Boema, 01450G1-1 x Dropia.

The experiments of artificial inoculation with spores of (*Tilletia caries* and *Tilletia foetida*) were organized in the field, in the agricultural space of the USAMV Cluj-Napoca botanical garden, during the period 2008-2009. The experiments were achieved in completely randomized blocks with three repetitions. A number of approximately 250 seeds were planted in the field of every descendency F₂ and from the parental forms, a number of 20 seeds. The spores were obtained by harvesting the ears naturally infected in the field from Şimnic Agricultural Research Development Station. Both the eight F₂ descendencies and the parental forms were infected.

The resistance was evaluated by reporting the number of plants attacked to the total number of plants. The variance analysis was used for interpreting the data. For the $\chi^2$ analysis we tested a population formed of 12 F₂ descendencies, the populations tested in the number of 10 and two variants represent the repetitions made. In addition, we performed a genetic analysis for the evaluation of the manner of transmission of the resistance to common bunt. Knowing the manner of transmission of the resistance to the common bunt is an important objective for identifying the resistance genes and the genetic markers to be used in the selection assisted by molecular markers.

![Fig. 2. Symptoms of the infection with bunt on the ears, ear infected with bunt (left), healthy ear (right)](image-url)
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Reaction of the parental varieties and lines to the artificial infection

The average of the infection percentage with bunt in the parental varieties and lines (%)

<table>
<thead>
<tr>
<th>Resistance gene</th>
<th>Line</th>
<th>Average of the infection percentage (%)</th>
<th>Variety (line)</th>
<th>Average of the infection percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>?</td>
<td>99419G4-1A/1-1</td>
<td>0</td>
<td>Delabrad</td>
<td>50</td>
</tr>
<tr>
<td>Bt5</td>
<td>00274 G2-31</td>
<td>0</td>
<td>F96869G1-108</td>
<td>50</td>
</tr>
<tr>
<td>Bt11</td>
<td>00281G2-11</td>
<td>0</td>
<td>Glosa</td>
<td>undetected</td>
</tr>
<tr>
<td>Bt8, Bt10</td>
<td>00399G2-11</td>
<td>0</td>
<td>Boema</td>
<td>undetected</td>
</tr>
<tr>
<td>Bt11</td>
<td>01450G1-1</td>
<td>0</td>
<td>Jiana</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Crina</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dropia</td>
<td>100</td>
</tr>
</tbody>
</table>

The resistant parental wheat lines used as parents in the hybridizations constantly manifested resistance to *Tilletia* spp. Without symptoms of bunt infections, these have proven to be resistant in other tests carried out in the previous years as well (table 1). In the sensitive varieties and lines, the infection degree varied between 33.3%-100%. For example, the Dropia variety is known as very sensitive to the species *Tilletia* (Oncică Fraga, 2007) the infection percentage was of 100% at the dose of 5 g spores/1000 g seeds.

The reaction of segregant F₂ descendencies to artificial infection

The analysis of variance was achieved in order to test the differences as regards the infection percentage between the F₂ descendencies resulted from the 12 cross-breedings (table 2). The data regarding the infection percentage used for the analysis of variance were transformed in angles = arc sin √%.

The infection percentage with bunt in the F₂ segregant descendencies

<table>
<thead>
<tr>
<th>Crit. no.</th>
<th>Population F₂</th>
<th>No. plants</th>
<th>Average of the infection percentage (%)</th>
<th>Angles =arc sin √%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99419G4-1A/1-1 x Glosa</td>
<td>39</td>
<td>43.6</td>
<td>41.3</td>
</tr>
<tr>
<td>2</td>
<td>99419G4-1A/1-1 x Boema</td>
<td>28</td>
<td>35.7</td>
<td>36.7</td>
</tr>
<tr>
<td>3</td>
<td>00274 G2-31 x Glosa</td>
<td>50</td>
<td>28</td>
<td>31.9</td>
</tr>
<tr>
<td>4</td>
<td>00274 G2-31 x Dropia</td>
<td>59</td>
<td>47.4</td>
<td>43.5</td>
</tr>
<tr>
<td>5</td>
<td>00399G2-11x Boema</td>
<td>56</td>
<td>33.9</td>
<td>35.6</td>
</tr>
<tr>
<td>6</td>
<td>01450G1-1 x Glosa</td>
<td>37</td>
<td>24.3</td>
<td>29.5</td>
</tr>
<tr>
<td>7</td>
<td>01450G1-1 x Boema</td>
<td>16</td>
<td>62.5</td>
<td>52.2</td>
</tr>
<tr>
<td>8</td>
<td>01450G1-1 x Dropia</td>
<td>49</td>
<td>18.4</td>
<td>25.4</td>
</tr>
<tr>
<td>9</td>
<td>99419G4-1A/1-1 x Glosa - R</td>
<td>21</td>
<td>38.1</td>
<td>38.1</td>
</tr>
<tr>
<td>10</td>
<td>00274 G2-31 x Glosa - R</td>
<td>35</td>
<td>40</td>
<td>39.2</td>
</tr>
</tbody>
</table>
The results of the analysis of variance and of the experimental values F (table 3) indicate the fact that there are real significant differences between the eight F2 descendencies as regards the percentage of plants infected after the artificial inoculation with bunt spores. From the table of the variance analysis we notice there are real significant differences between all the analyzed descendencies, and compared to the parental line used as control (table 4). In table 4 we present the differences between the average of the infection percentage in the sensitive parent used as control variant and the segregant variances F2. It results that the difference between the genotypes as regards the reaction to the common bunt is due to the resistance genes (Coța et al., 2009).

Table 3

<table>
<thead>
<tr>
<th>Variation source</th>
<th>GL</th>
<th>$s^2$ (arc sin $\sqrt{%}$)</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>7</td>
<td>224.88*</td>
<td>2.85 (2.76)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P=5%</td>
</tr>
<tr>
<td>Repetitions</td>
<td>2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>14</td>
<td>78.80</td>
<td></td>
</tr>
</tbody>
</table>

*indicates the significance at the probability level of 0.5

The significance of differences regarding the average of the bunt infection percentage in the sensitive control parent and the descendencies F2.

<table>
<thead>
<tr>
<th>F2 population</th>
<th>Average of the attacked no. of plants (%)</th>
<th>Difference</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive parent taken as control</td>
<td>53.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>00450G1-1 x Boema</td>
<td>63.1</td>
<td>9.68</td>
<td>-</td>
</tr>
<tr>
<td>00274G2-31 x Dropia</td>
<td>46.5</td>
<td>-6.82</td>
<td>-</td>
</tr>
<tr>
<td>99419G4-1A/1-1 x Glosa</td>
<td>42.8</td>
<td>10.52 (15.54)</td>
<td>*</td>
</tr>
<tr>
<td>99419G4-1A/1-1 x Boema</td>
<td>33.6</td>
<td>19.72 (21.52)</td>
<td>**</td>
</tr>
<tr>
<td>00399G2-11 x Boema</td>
<td>33</td>
<td>20.32 (21.52)</td>
<td>**</td>
</tr>
<tr>
<td>00274G2-31 x Glosa</td>
<td>27.7</td>
<td>25.62 (29.93)</td>
<td>***</td>
</tr>
<tr>
<td>00450G1-1 x Glosa</td>
<td>25.7</td>
<td>27.62 (29.93)</td>
<td>***</td>
</tr>
<tr>
<td>00450G1-1 x Dropia</td>
<td>17.2</td>
<td>36.12 (29.93)</td>
<td>***</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DL 5%</th>
<th>DL 1%</th>
<th>DL 0.1%</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.54 (7.2%)</td>
<td>21.52 (13.6%)</td>
<td>29.93 (25%)</td>
</tr>
</tbody>
</table>

Compared with the control variant from table 4, we notice that all the experimental variants are very significant.

The effect of the inoculation dose on the infection degree

In the experiments carried out we used the dose of 5 g spores/1000g seeds, and in four of the descendencies we repeated the inoculation with the second dose (of 8 spores/1000 g seeds) with the purpose of insuring a total infection. When testing the resistance to *Tilletia spp* the results are influenced a lot by the testing conditions that influence the infection manifestation, by the necessary environment conditions for the disease manifestation: high soil humidity, soil temperatures of 5-10 °C.
favorable for the spores’ development; if the seeds are not totally infected the disease is not manifested and the plants are characterized as resistant.

The tests carried out in the present thesis through the analysis of variance emphasized significant differences in the reaction of the inoculated plants with the two spore doses between the two doses used; the differences were non-significant between the F² wheat descendance (table 5).

The results of the variance analysis regarding the percentage of plants infected with bunt in the two inoculation doses

<table>
<thead>
<tr>
<th>Variation sources</th>
<th>GL</th>
<th>$s^2$</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repetitions</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose</td>
<td>1</td>
<td>111.8*</td>
<td>7.98 (4.60) P = 5%</td>
</tr>
<tr>
<td>Genotype</td>
<td>3</td>
<td>21.3</td>
<td>0.22</td>
</tr>
<tr>
<td>Interaction dose x genotype</td>
<td>3</td>
<td>67.5</td>
<td>0.70</td>
</tr>
<tr>
<td>Error</td>
<td>14</td>
<td>95.9</td>
<td></td>
</tr>
</tbody>
</table>

7.1.2. Inheritance of the wheat resistance to common bunt to the segregant descendants of the F₂ population

In the present study, based on the knowledge regarding the resistance genes from the genealogy of the parental forms, resistance was supposed to be controlled by only one resistance gene.

The analysis of phenotype segregation in the F₂ generation. The observed number of resistant and sensitive plants, the segregation ratio expected for a resistance gene ($\chi^2$).

<table>
<thead>
<tr>
<th>Nr.</th>
<th>The wheat parentage F₂ tested</th>
<th>No. plants</th>
<th>Ratio to be expected (res:sens)</th>
<th>Chi square ($\chi^2$)</th>
<th>Values $\chi^2$ la P=5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>00399G2-11x Boema</td>
<td>14 8</td>
<td>3:1</td>
<td>1.5</td>
<td>0.22</td>
</tr>
<tr>
<td>2</td>
<td>00274 G2-31 x Glosa</td>
<td>22 17</td>
<td>3:1</td>
<td>7.18</td>
<td>0.007</td>
</tr>
<tr>
<td>3</td>
<td>99419G4-1/1-1x Glosa</td>
<td>18 10</td>
<td>3:1</td>
<td>1.7</td>
<td>0.19</td>
</tr>
<tr>
<td>4</td>
<td>99419G4-1/1-1 x Boema</td>
<td>36 14</td>
<td>3:1</td>
<td>0.24</td>
<td>0.62</td>
</tr>
<tr>
<td>5</td>
<td>00274 G2-31 x Dropia</td>
<td>31 28</td>
<td>3:1</td>
<td>15.8</td>
<td>0.00007</td>
</tr>
<tr>
<td>6</td>
<td>99419G4-1/1-1/ Dropia</td>
<td>37 19</td>
<td>3:1</td>
<td>2.37</td>
<td>0.12</td>
</tr>
<tr>
<td>7</td>
<td>00281G2-11 x Boema</td>
<td>28 9</td>
<td>3:1</td>
<td>0.0085</td>
<td>0.92</td>
</tr>
<tr>
<td>8</td>
<td>01450G1-1 x Glosa</td>
<td>6 10</td>
<td>3:1</td>
<td>12</td>
<td>0.0005</td>
</tr>
<tr>
<td>9</td>
<td>99419G4-1/1-1x Glosa - R</td>
<td>40 9</td>
<td>3:1</td>
<td>1.14</td>
<td>0.28</td>
</tr>
<tr>
<td>10</td>
<td>00274 G2-31 x Glosa - R</td>
<td>13 8</td>
<td>3:1</td>
<td>1.92</td>
<td>0.16</td>
</tr>
<tr>
<td>11</td>
<td>00399G2-11x Boema - R</td>
<td>21 14</td>
<td>3:1</td>
<td>4.18</td>
<td>0.04</td>
</tr>
<tr>
<td>12</td>
<td>00450G1-1 x Glosa - R</td>
<td>13 8</td>
<td>3:1</td>
<td>1.06</td>
<td>0.30</td>
</tr>
</tbody>
</table>

The segregation in F₂ in the ratio 3:1 resistant: sensitive for the reaction to the bunt infection shows that the resistance of the parental forms used in the cross-breedings is conferred by only one resistance.
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gene, except for progenies derived from five crosses (00399G2-11x Boema, 99419G4-1A/1-1 x Boema, 00281G2-11 x Boema, 00274G2-31 x Glosa – R, 01450G1-1 x Glosa - R). The segregation mode of the gene could not be evaluated in these ones, probably because of the limited number of available plants (that could be evaluated); in this way, they were more affected by the error that the others (table 6).

7.2. RESULTS REGARDING QUANTITY AND QUALITY OF ISOLATED DNA SAMPLES

By using the isolation protocol based on using the CTAB we can appreciate the fact that the samples present a high concentration and purity.

7.3. RESULTS OF ANALYSIS WITH SPECIFIC MARKERS FOR THE RESISTANCE GENE BT10 (FSD and RSA)

In the analyzed parental lines, the fragment of 275 bp specified by Laroche et al. (2000) was not emphasized at any of the analyzed lines (and in the individual lines either). In exchange, it was emphasized a polymorph fragment of 420 bp possibly associated with the resistance gene BT10. The fragment of 420 bp was noticed at the line 23- 99419G4-1A/1-1 şi 25 - 00281G2-11.

Fig. 3. The amplification products obtained with the specific primers FSD and RSA in the individual lines 23.1-23.10- individual plants from line 99419G4-1A/1-1, 24.1-24.10 –line 00274G2-31, 25.1-25.8– line 00281G2-11, 26.1-26.10 – line 00399G2-11, 27.1-27.9- line 01450G1-1, 15- Farmec, 16 – Delabrad, 17 - F96869G1-108, 21 – Crina.

The results of the analysis with specific rye primers (F3/R3) (Katto et al., 2004)

Fig. 4. The amplification products obtained with specific primers for rye (F3/R3), at dihaploid lines GP 369 and the proveniences of references: 17- line F96869G1-108 and 21 – Crina variety. L-marker 100 bp.
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The majority of the dihaploid lines analyzed obtained from the hybrid combination GP 369, and one of the reference proveniences (21 – Crina variety) presented the band of 1400 bp specific to the rye genome (fig. 4), which means that in their genealogy the rye also participated. The presence of certain sequences from the rye genome at the provenience considered sensitive indicates the fact that among the genes taken from the rye there are no resistance genes to bunt.

The absence of the band in the other reference provenience considered, also sensitive (17- line F96869G1-108), makes us believe that the genetic material coming from rye has not integrated genes with common resistance to bunt. We notice the amplification product, very rich in lines 9.7, 9.8, 9.9, 9.10, and the presence of other non-specific bands.

7.4. THE RESULTS OF THE ANALYSIS WITH RAPD Markers

Of the 32 random primers tested, a number of 9 primers were polymorph. A high polymorphism was obtained with the following primers as well: UBC 570, Mic 07, Mic 13, OPH 20 (Coța et al., 2007). A part of the polymorph primers were eliminated since the identified polymorphism was not reproducible.

The polymorphism identified by the RAPD markers tested in the parental forms and in the testes dihaploid lines is synthesized in the following manner:

- **The primer OPA 17** identified the fragment of 850 bp in the resistant parental line 00399G2-11 and in the F₂ population 00399G2-11 x Boema as well;
- **The primer OPC 10** identified the fragment of 900 bp in the resistant parental line 99419G4-1A/1-1 and in the dihaploid lines GP369;
- **The primer OPA 16** identified the fragment of 900 bp in the resistant parental line 00274G2-31 and in the dihaploid lines as well;
- **The Mic 14 primer** identified the fragment of 630 bp in the resistant parental line 99419G4-1A/1-1 and in the dihaploid lines as well;
- **The primer OPC 04** identified the fragment of 550 bp only in the resistant parental lines in the resistant lines with the number 1 -99419G4-1A/1-1 and 3- 00281G2-11.
- **The primer 70.04** identified the fragment of 750 bp in the resistant parental line 00281G2-11 and in the F₂ population 00281G2-11 x Boema as well.

The analysis of the molecular polymorphism identified by the polymorph RAPD markers in the dihaploid lines

Among the primers that identified a polymorphism in the parental lines (OPA 16 and Mic 14) they were then tested in all the dihaploid lines analyzed in order to see the manner in which the polymorphism is identified in these lines (Coța et al., 2007).
In the case of the decamer primer Mic 14 the polymorph band of about 630 bp, compared with the sensitive reference proveniences (17 and 21), is rather weak as intensity and most of the times present in the dihaploid lines obtained both from the combination GP 384, and by the combination GP 369 (figure 5).

The analysis of the molecular polymorphism identified by the marker RAPD OPA17

The co-segregation method. The primer OPA 17 emphasized in the parental lines and varieties a polymorph fragment (of approximately 850 bp), the fragment was present in the parental line 00399G2-11 (it possesses the resistance gene Bt8 or Bt10) which was absent in all the sensitive varieties. The line 00399G2-11 has in its genealogy the variety landrace PI178383 which is an important source of resistance to bunt.

As a consequence, the fragment of 850 bp could be considered a “candidate” market which marks the resistance genes (Bt8 or Bt10).

The analysis of bulk segregation in the F2 plants of the population 00399G2-11 x Boema. Through the analysis of the bulk segregation with the primer RAPD OPA17 it was emphasized the resistance polymorph fragment of approximately 850 bp characteristic to the resistant parental line 00399G2-11 which was also encountered in the F2 plants.

For making the DNA mixture from the resistant and sensitive plants we selected 5-7 plants of each category whose DNA samples were mixed. The analysis of these mixtures with the primer OPA17 emphasized the fragment of approximately 850 bp which was identified in the mixture of resistant plants. This polymorph fragment was not emphasized in the mixture of sensitive plants or in the sensitive parental form – Boema variety (fig. 6).

The polymorph band amplified by this primer (of approximately 850 bp) was confirmed through the analysis of the bulk segregation, as a consequence it was selected to be cloned and sequenced.
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Fig. 6. Amplification products obtained with the primer OPA17 in the following genotypes: Pr – resistant parental line 00399G2-11, Ps – sensitive variety Dropia, Br-resistant mixture Bs – sensitive DNA mixture, plants of the F₂ generation (1-16).

7.5. CONVERSION OF RAPD MARKERS INTO SCAR (SEQUENCE CHARACTERIZED AMPLIFIED REGIONS) SPECIFIC MARKERS

7.5.1. Isolation of RAPD polymorphic bands from electrophoresis gel

The polymorph bands obtained with the primers OPA 17 and OPC 04 were isolated from the gel. The polymorph DNA fragments obtained with the primers RAPD OPA 17 and OPC 04 were cloned. In order to convert the polymorph band of approximately 600 bp obtained with the primer OPC 04 and the 800 bp band obtained with the primer OPA 17 in the SCAR markers, the cloning of polymorph bands was necessary. In the image below (fig. 7) we present the bands recovered from the polymorph amplification product (of 550 bp) amplified with the primer OPC 04.

Fig. 7. The reamplification of recovered bands obtained with the primer OPC 04

7.5.2. Cloning and sequencing the polymorph RAPD bands

These bands were cloned in the Escherichia coli (GM2163) bacterium. We analyzed the colonies that integrated the cloned fragment, which survived on the selective medium with ampicillin (Fig.8).

Fig. 8. Bacterial colonies on the selective medium which integrated the cloned fragment.
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The isolation of the plasmid DNA. We selected three random colonies from every plate, the isolated plasmid DNA was quantified and then diluted (at 30 ng/µl) in order to be used in the PCR amplification. In the image below we see the amplified plasmids which integrated the polymorph fragment of 850 bp and the fragment of 550 bp (fig. 9). After checking the plasmids, the DNA could be used for sequencing. The sequencing of cloned fragments was carried out for us in the Macrogen laboratories, Holland (fig. 10).

![Fig. 9. The amplification of the DNA fragment cloned with pJET 1F and pJET1R primers](image)

| NNNNNCNCNNATAGGAGAGCGGCCGCCAGATCTTCCGAGATGCTCGAGTTTTTCAGC |
| AAGATAGTTATGAACATAGCTAGTCTAGATCTTCTTACGATCTCCAA |
| ATTAATCGGATCCACAGTGAGCGGAGCAGACAGCAGCCATGCGGCCAGCCATT |
| GTATACATAAGCGTTGGTTAACCCGTTTTGTGCCTTTACTGTTTTCTTGGT |
| GTTTGTGTCTCCTTTTTTTCCCTTTCTTTTATTTTATACTTTACCTTTATACAGC |
| ATTTGGGTTACATTTTTCTTTACCTTTTTCGTTCTATCTGTGTGCTTTCTCATAAAAAATG |
| TCGCAGATTTTGGTCAAAAACAAATAGTCCTATCCATTAACTTCAAGCTA |
| CCATCAAATAGAATTGACATATTTGGATCCCTCATTAAACAAATTTGTTAAAAA |
| ATACAAGAAAGTGCGTGAGGTTTTAATATGAATCCAGATTCTTTATACATG |
| ACTAACACTTTTTTATTGATATGAGATGTAGAAAAGTCTTTTGTTTTATTTATCA |
| TTCTAGAGATCTCCTCCTACAAATTCCCCCCACCAGCAAATCTCTCCTGCTAAC |
| ATAGAAATGACTACTTNTTTTAGATCCCCACTTAAACAAATTTGTTAAAAAAATACA |
| CNATTCTAGAGGTTGTCAATCTAGAATCCAGATTCTTTTCTCTTTGATCATCCCCCTTACTAAC |
| ACTTTTTTCTTTTGGATATGAGATGTAGAAAAGTCTTTTGTCTTTACTTCAACAGC |
| GGTATCTTTCCAGAGATCTCCTCCTATTTTTGTCTACGAAAATCTCGC |

Fig. 10. The nucleotid sequence resulted from the sequencing of the 850 bp band

7.6. RESULTS OF ANALYSIS WITH MICROSatELITe MARKERS

The microsatellite markers located on the chromosome 1B, 2B and 2D in wheat were used to identify the polymorphism between the parental forms. Of the 11 markers tested only two markers emphasized polymorphism in the parental lines: Xgwm633 and Xgwm114.

7.6.1. Assigning the microsatellite molecular markers to common bunt resistance genes

The co-segregation method. The Xgwm633 marker emphasized the band of 200 bp in the resistance line 1-99419G4-1A/1-1 which was not present in any of the sensitive lines and varieties. In exchange, in the varieties and resistant line another fragment of approximately 250 bp was emphasized.
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The marker Xgwm114 emphasized the band of 120 bp in several resistant lines which had or not the gene Bt11 and two bands of 160 bp and 180 bp in the sensitive varieties. The marker Xgwm114 is specific to gene Bt11. This marker confirmed the presence of the gene in the genotype of the line that carrier this gene (wheat line 00281G2-11) through the polymorph fragment of 120 bp. Nevertheless the fragment appeared in other resistant lines as well (carriers of other resistance genes) and also in two of the sensitive lines.

The fact that the polymorph marker appeared in the lines which were not known as having the Bt11 gene shows that these lines also have this resistance gene.

The polymorphism identified by the markers was tested in the segregant populations F2 as well. With the marker Xgwm633 we tested the population resulted from the cross-breeding of the lines 99419G4-1A/1 where we emphasized the resistance gene with the sensitive Glosa variety. With the marker Xgwm114 we tested the population resulted from the cross-breeding of the line 00274G2-31 (with the gene Bt5 and which emphasized the polymorph band of 120 pb) with the sensitive Glosa variety.

Bulk Segregant Analysis – BSA method

The analysis of population F2 99419G4-1A/1-1 x Gloss with marker Xgwm633. This population is formed of a number of 51 plants, each plant was individualized phenotypically for the resistance to common bunt. The DNA was isolated in all the F2 plants and submitted to the PCR analysis using the primer Xgwm633 and it was tested genotypically with this marker. The marker Xgwm633 is known as being linked with the QTL loci with resistance to bunt (Fofana et al., (2008). In the analyses carried out in the F2 plants, the marker emphasized the two polymorph fragments and the parental lines of 200 bp and 230 bp. The resistance gene present in the wheat line 99419G4-1A/1-1is not known but it is supposed to be the gene Bt8 or Bt10.

Through the Bulked Segregant Analysis with the marker Xgwm633 the presence of the two polymorph fragments was confirmed, the one of 200 bp for the DNA mixture from the resistance plants and the one of 230 bp for the mixture coming from the sensitive plants.

The analysis of population F2 00274G2-31 x Gloss with the Xgwm114 marker

This population was formed of a number of 52 plants. The polymorph fragment for resistance of 120 bp was also found in F2 population in many individual genotypes which were phenotypically resistant and in the F2 individual plants as well, which were phenotypically sensitive. In addition the marker for the sensitivity area was identified in many plants F2 which were or not phenotypically sensitive.

The bulk segregation analysis. The mixtures were carried out the same way as for the marker Xgwm633 in the analyzed population.

The marker Xgwm114 showed in the DNA mixture of the sensitive plants the same sensitivity polymorph fragments of 160 bp and 180 bp and in the sensitive parental line as well. And in the DNA mixture from the resistant plants besides the polymorph fragment of resistance at the dimension of 120 bp, we also emphasized the two sensitivity fragments characteristic to the sensitive parental form. This thing can be explained by the fact that in choosing the plants for creating the mixtures besides the resistant plants, we also selected a sensitive plant but which does not manifest sensitivity phenotypically.

We must mention the fact that the fragment of 120 bp specific to the resistance line was also reported by Cichy and Goates (2009), (Cichy K., personal communication, April 20, 2010). However, in the sensitive genotypes they identified only one fragment with the dimension of 110 bp.

The linkage analysis

The SSR markers tested in this study were used to calculate the linkage distance between the resistance markers and genes. The results regarding the segregation for resistance and the two polymorph SSR markers (Xgwm633 and Xgwm114) in the F2 segregant populations are presented in table 7.
The PhD thesis summary

The segregation of F₂ plants for the resistance to bunt and the polymorph markers

Table 7

<table>
<thead>
<tr>
<th>Marker Xgwm633</th>
<th>Population 99419G4-1A/1-1xGlosa</th>
<th>Marker Xgwm114</th>
<th>Population 00274G2-31xGlosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-B-</td>
<td>A-Bb</td>
<td>aa-B-</td>
</tr>
<tr>
<td>22</td>
<td>7</td>
<td>14</td>
<td>5</td>
</tr>
</tbody>
</table>

The linkage analysis with the marker Xgwm633 in the analyzed population showed that the marker is linked at a rather big distance with the resistance gene of 32 cM. This big distance is due to the fact that the marker Xgwm633 is linked with an associated QTL with resistance to common bunt in the analyzed population, resistance controlled by polygenes.

The linkage analysis with the Xgwm114 marker in the analyzed population showed that the marker is linked to the resistance gene Bt11 at a distance of 8 cM. The smaller recombination value which was evaluated between the marker Xgwm114 and the gene Bt11 (8 cM) suggests the fact that this marker can be a candidate in the selection programs for the wheat resistance to common bunt, qualitative resistance ensured by the gene Bt11.

CONCLUSIONS

- The markers known for certain wheat genes for common bunt resistance in the analyzed wheat lines and varieties were tested by using the markers specific to the Bt10 gene in order to identify the presence of this gene in the analyzed germplasm. The analysis with the specific marker of 275 bp represented by the primers FSD and RSA for the gene Bt10 has not emphasized this fragment in the lines with this gene either. In exchange these primers emphasized other polymorph fragment of 420 bp in the parental line 99419G4-1A-1, polymorph band that was also identified in the dihaploid lines resulted and a band of 1450 bp in the majority of analyzed dihaploid lines. As a consequence, these bands can be taken into account as markers for the gene Bt10 in the analyzed germplasm.

- The markers RAPD and SSR identified were assigned to the wheat genes with resistance to common bunt based on two analysis methods: the method of co-segregation of the marker with the resistance gene and the BSA- bulked segregant analysis method.

- In the analysis with the RAPD markers of a number of 32 markers tested, nine markers identified polymorphism between the resistant parental varieties and the sensitive ones. For the subsequent analyses, we selected among these primers those that emphasized a repeatable polymorphism, and these were the primers OPA17 and OPC04.

- Based on the method of co-segregation the RAPD markers identified were assigned to the resistance genes in the following manner:
  - The OPA17 marker emphasized a RAPD polymorph fragment (of approximately 850 bp) in the parental line 00399G2-11 (with the resistance genes Bt8 or Bt10) which was absent in all the sensitive varieties (lines). The wheat line 00399G2-11 has in its genealogy the landrace variety PI178383 that represents an important source of resistance to bunt. As a consequence the fragment of 850 pb could be considered a “candidate” marker that marks the resistance genes (Bt8 or Bt10). This fragment was cloned and sequenced. The sequence resulted is available to build the specific primers of SCAR type.
  - The primer OPC 04 emphasized the polymorph fragment of approximately 550 bp only in the resistance parental line 99419G4-1A-1 with the genes Bt8 and Bt9, Bt10. Taking into account that this fragment was repeatable and was not present in any of the
sensitive lines and varieties, it is considered a candidate marker for the resistance genes present in line 99419G4-1A/1-1.

- Based on the bulked segregation method, we analyzed the presence of the 850 pb marker with the primer OPA17 in the F2 population 00399G2-11x Boema. The polymorphism emphasized with this primer (the fragment of 850 bp) was confirmed through the bulked segregation analysis. Thus, this polymorph marker can be assigned to the resistance genes (Bt8 or Bt10) and used in the selection for the wheat resistance to common bunt.

- We attempted the conversion of two of the markers emphasized by the RAPD primers (OPA17 and OPC04) into specific markers of SCAR type. The polymorph fragments were cloned and sequenced. Based on the data obtained after sequencing the polymorph RAPD markers we can build specific primers which are co-dominant and which will be used in the analysis with the SCAR markers.

- In the analysis with the microsatellite markers we used in this study the SSR markers which control the resistance to bunt, represented by 11 pairs of SSR primers. The SSR markers which emphasized polymorphism between the resistant and sensitive parental forms were the markers Xgwm633 and Xgwm114.

- Based on the co-segregation method, the SSR markers were assigned to the resistance genes in the following manner:
  - The Xgwm633 marker is associated with the QTL loci of resistance to bunt, in the analyses carried out, the band of 200 bp was emphasized at the resistant line 1- 99419G4-1A/1-1 which was not present in any of the sensitive lines and varieties.
  - The Xgwm114 marker emphasized the band of 120 bp in several resistant lines which had or not the Bt11 gene and two bands of 160 bp and 180 bp in the sensitive lines. With this marker, the presence of the gene was confirmed in the genotype of the wheat line carrying this gene (00281G2-11 şi 01450G1-1). This marker emphasized the presence of the resistance Bt11 in other genotypes carrying these genes as well (Bt8, Bt5, Bt8). These two markers identified were used for the analysis of the resistance in the F2 populations.

- Through the bulking segregation method with SSR markers the SSR markers were assigned to the resistance genes in the following manner:
  - The Xgwm633 marker was emphasized in the two polymorph fragments and in the parental lines of 200 bp for resistance and 230 bp for the sensitivity area. The resistance gene present in the wheat line 99419G4-1A/1-1 is not known, but it is supposed to be the gene Bt8 or Bt10. With this marker we confirmed the presence of the two polymorph fragments, the one of 200 bp for the DNA mixture from the resistant plants and the one of 230 bp for the mixture coming from the sensitive plants. As a result this market can be assigned to a resistance gene present in this line.
  - The Xgwm114 marker has shown in the DNA mixture of the sensitive plants the same polymorph fragments of 160 bp and 180 bp as in the sensitive parental line. In the DNA mixture from the resistant plants, besides the polymorph fragment of resistance at the dimension of 120 bp, the two sensitivity fragments characteristic to the sensitive parental form were also emphasized.

- The linkage analysis between these two SSR markers, the markers and genes that they mark, carried out in the F2 population did not emphasize a strong linkage with the resistance lines.
  - The marker generated by the Wmc114 primer identified a stronger linkage (8 cM) than the other Xgwm633 marker (32 cM). Thus, this polymorph marker can be used in the selection assisted by molecular markers for the wheat resistance to the common bunt, qualitative resistance ensured by the Bt11 gene. And thus it can be used in the indirect selection for resistance to common bunt ensured by the Bt11 gene.
  - The big distance of linkage between the Xgwm633 marker and the resistance gene is explained by the fact that in the analyses carried out in this study, this marker is associated with a QTL of wheat resistance to common bunt.
The results obtained after the artificial infection with common bunt spores in the 10 populations analyzed have shown that there are significant differences between the tested populations as regards the resistance to common bunt.

The analysis of phenotypic segregation in the F2 populations has shown that the resistance of parental lines is controlled by only one resistance gene.

SELECTIVE BIBLIOGRAPHY


