SUMMARY

of the doctoral thesis entitled:

USE OF MOLECULAR BIOLOGY TECHNIQUES TO DETERMINE VARIETAL PURITY IN FIELD CROPS GROWN IN ROMANIA

Ph.D-student: VASILE Valentina

Scientific coordinator: *Professor, PhD. CORNEA Călina Petruța*

KEY-WORDS: *varietal purity; wheat; maize; SSR markers; KASP; genetic diversity*

Agriculture has a great importance to humanity and it is no wonder that the interest and development of research in this field has increased. The development of molecular biology techniques, especially those using molecular markers or PCR based technology, have offered new opportunities for agricultural research in recent decades. However, climatic changes continues to generate food insecurity due to reduced harvests, related price increases, thus affecting the global economic situation.

Given the important role played by our country in the grain production of the European Union, it becomes imperative that investments in agriculture, in human and material resources involved, as well as in the evolution of scientific research in the field, should become a priority.

Identification of plant varieties and assessment of varietal purity plays an important role both in seed quality control and also in plant breeding, registration and protection.

In terms of seed quality control it is important to maintain or to confirm the genetic purity of a variety, and the use of molecular biology techniques is much more useful than when using techniques involving the phenotypic approach. Techniques based on morphological identification involve intensive effort and the purity and variety of various types of seeds can sometimes be difficult to assess. Thus, the use of molecular biology techniques in variety testing can greatly simplify the process of seed control, increasing objectivity, efficiency and at the same time reducing the material effort and time required for field testing.

The purpose of this research study was to evaluate the varietal purity and implicitly the genetic diversity of the main crops grown in Romania by using molecular biology techniques. In that regard, the present research study aimed to evaluate the varietal purity and genetic diversity in different wheat cultivars and maize inbred lines

grown in Romania using molecular biology techniques based on the use of SSR markers .

In order to achieve the purpose of this research study, the following objectives were achieved:

1. Selection of a molecular biology technique that uses reliable markers so that the results can be obtained quickly and the technology to be easy to use, not generating high costs, not requiring a large amount of DNA and prior information about the plant genome.

2. Selection of the working method and the best reaction conditions for the selected SSR markers, a method that allows obtaining satisfactory results under repeatability and reproducibility conditions.

3. Selection of the most informative SSR markers to allow highlighting the genetic polymorphism associated with the biological material used.

4. Varietal purity assessment in different wheat cultivars and maize inbred lines grown in Romania using the selected SSR markers.

5. Genetic diversity assessment in different wheat cultivars and maize inbred lines grown in Romania using the selected SSR markers.

6. Highlighting the fact that quality requirements are ensured in the production of certified seeds in Romania.

As a result of achieving the proposed objectives, it will be possible to make a more accurate assessment of the varietal purity in seeds for the selected cultivars and it will be possible to highlight the presence of a diversified germplasm in Romanian crops which maintains Romania's status as an important grain producer in Europe.

The present work was structured in two main parts, namely part I presenting the **bibliographic study** and part II presenting the **personal research**.

The first part of the work includes two chapters in which is presented a summary of the current state of research on varietal purity assessment in field crops using biomolecular techniques, as well as a summary of the data related to molecular markers and molecular biology techniques based on their use.

In **chapter I** entitled "**State of research on determining varietal purity in field crops using biomolecular techniques**" are summarized information on the current status and trends regarding methods for assessing varietal purity in plants, namely, the determination of varietal purity by protein electrophoresis and the determination of varietal purity by using molecular markers, as well as regulations regarding how to determine varietal purity and the use of molecular biology techniques in main crops. Given Romania's status as an important producer and exporter of wheat and maize, the researches in the present study focused on these two crops. Thus, a summary of molecular biology techniques used in wheat and maize is presented here.

Chapter II entitled "**Molecular markers and molecular biology techniques based on their use**" includes both information related to the classification and importance of genetic markers, being included here traditional markers and molecular markers, as well as information related to molecular markers used in molecular biology techniques. Information related to non PCR based markers as well as information related to PCR based markers are summarized here, listing ten of the most commonly used PCR markers (markers: RAPD, AFLP, RAMP, ISSR, SCAR, ScoT, CAPS, SNP, KASP and SSR). Given the fact that SSR markers were used in the present research study, a subchapter related to the importance of using molecular biology techniques and SSR markers in varietal purity and genetic diversity assessment in field crops was included in chapter II.

Since, according to others research studies and information presented in the bibliographic study, molecular biology techniques using SSR markers present numerous advantages in terms of simplicity, efficiency, accessibility and reproducibility, they were selected in the present research study for varietal purity and genetic diversity assessment in wheat and maize.

The second part of the work includes **chapters III** and **IV**, where there are included information related to personal researches on varietal purity and genetic diversity assessment in various wheat cultivars and maize inbred lines grown in Romania.

Chapter III entitled "**Varietal purity and genetic diversity assessment in wheat cultivars grown in Romania**" includes a brief introduction on the experimental design, information on the materials and working method, results and discussion on the use of SSR markers in varietal purity and genetic diversity assessment in the selected wheat cultivars and partial conclusions resulting from the molecular data analysis.

In the subchapter "**Materials and working methods**" are included information about:

- **Wheat plant material selection**

The plant material used in the research study was represented by 40 seeds samples (C1-C40) from the Central Laboratory for the Quality of Seeds and Planting Material (LCCSMS), from various harvesting campaigns, as well as from seeds collections. Of these, 34 samples were represented by certified wheat cultivars, the rest being seeds from uncertified cultivars, such as spelt wheat (*Triticum spelta* L.), *Triticum monococcum* , *emmer* wheat (*Triticum dicoccon* Schrank), seeds belonging to another genus, such as triticale seeds *(× Triticosecale* Wittmack) and naked oat (*Avena nuda* L.), samples from the LCCSMS seeds collection.

For 14 of the 34 certified cultivars, seeds from two harvest campaigns (2019 and 2020) were included in the study, and 4 of the 14 cultivars were also compared with

the standard seeds. Thus, during the research study, a total of 58 samples were analysed from these two harvesting campaigns.

In order to highlight the fact that the conditions regarding the maintenance of varietal purity are respected, seven seeds samples belonging to the same certified wheat cultivar, multiplied in 2022 in different locations in districts Călărași, Ialomița, Suceava and Tulcea, were also included in the study, these seven samples being compared with the standard seeds.

- **Selection of DNA isolation method**

DNA extraction was performed using the NucleoSpin Plant II extraction kit (Macherey - Nagel). The selection of the lysis buffer was established following an experimental plan that consisted in the use of three lysis buffers: a CTAB-based buffer (PL1), a buffer containing SDS (PL2) and a lysis buffer (CF) of an extraction kit from food and feed (NucleoSpin Food).

- **SSR markers used in experiments**

In the present study, a number of 22 SSR markers were selected and used to assess the varietal purity and genetic diversity of some wheat cultivars grown in Romania. Among them, 14 SSR markers (DuPw167, DuPw217, DuPw004, DuPw115, DuPw205, Xgwm155, Xgwm413, Xgwm003, Xgwm372, Xbarc184, Xbarc347, Xbarc074, Xgwm052 and Xgwm095) are recommended by an international accreditation body (ISTA) for varietal purity assessment of wheat cultivars, and the remaining SSR markers (Xwmc603, Xwmc596, Xwmc418, Xbarc170, Xgwm469, Xwmc474, Xwmc533, Xgwm71) were selected from the literature to be tested for the chosen purpose.

- **The experimental plan regarding the reaction conditions selection**

For the 14 SSR markers (DuPw167, DuPw217, DuPw004, DuPw115, DuPw205, Xgwm155, Xgwm413, Xgwm003, Xgwm372, Xbarc184, Xbarc347, Xbarc074, Xgwm052 and Xgwm095) the experimental plan consisted in choosing for each marker both a PCR reaction mix, as well as the optimal annealing temperature, so that the resulting amplification products can be well highlighted, without the appearance of non-specific amplification products, allowing a good interpretation of the obtained results. For this purpose, three PCR reaction mixtures were tested.

For the rest of the SSR markers, the optimization consisted only in making a temperature gradient in order to choose the optimal annealing temperature, as well as variations in the primers concentration and PCR reaction times.

- **The final reaction conditions selected**

Are included here the final reaction conditions for the 22 SSR markers, conditions that were selected following the data analysis obtained in the PCR reaction optimization experimental plan.

- **KASP markers used in experiments**

To demonstrate that the results obtained with SSR markers are reproducible, 3 KASP markers (gene *1-FEH w3* associated marker, BS00023119 (6A) marker and KASP

marker BS00060097 (4A)) were also used in the present research study, these markers being used for the first time at national level for the evaluation of the varietal purity of wheat cultivars.

In the subchapter "**Results and discussion**" are presented informations about the results and discussion related to: the selection of the DNA isolation method for the tested wheat cultivars, the optimization of the reaction conditions for the tested SSR markers, the analysis of the PCR products obtained with the 22 SSR markers tested, the varietal purity and genetic diversity assessment of wheat cultivars by using SSR markers, as well as results and discussion related to the use of the KASP technique in the assessment of varietal purity and genetic diversity of the selected wheat cultivars.

Following the data analysis obtained as a result of using molecular biology techniques in order to assess the varietal purity and genetic diversity of the selected wheat cultivars, a series of **partial conclusions** could be stated.

The selected DNA isolation method for the wheat cultivars tested allowed good amplification results, the extracted DNA meeting the acceptability criteria regarding the optimal concentration and purity for the amplification reaction with SSR markers. Amplification of the extracted DNA was proven for all the extraction conditions tested, and the selected extraction method can also be used for other cereal species.

The working method chosen for varietal purity and genetic diversity assessment in wheat cultivars, using SSR markers meets the conditions regarding **robustness, reproducibility** and **repeatability**.

All the selected SSR markers showed amplification products under the selected reaction conditions, and the use of a Hot Start enzyme increased the **specificity** of the method.

Following the molecular analyses, it was observed that out of the **22 SSR markers**, **21** showed a certain degree of polymorphism on the selected wheat samples.

The highest degree of polymorphism was observed for **Xwmc596** marker (7 alleles), followed by markers **Xwmc603** and **Xwmc474** (6 alleles); Xgwm71, Xbarc347 and Xbarc074 (5 alleles); Xgwm469, DuPw004, Xgwm155 and Xbarc184 (4 alleles); Xwmc418, Xbarc170, Xwmc533, DuPw167, DuPw115, Xgwm413 and Xgwm372 (3 alleles); DuPw217, DuPw205, Xgwm003 and Xgwm095 (2 alleles). Marker Xgwm052 showed the lowest degree of polymorphism (one allele), but may be considered for future studies on other wheat cultivars. Thus, all the selected SSR markers demonstrated that they have potential in varietal purity assessment, managing to distinguish between different wheat varieties, but also between different species and genera.

The molecular analysis also showed that in the two harvesting campaigns (2019 and 2020) no contamination of the varieties was observed, thus demonstrating that **the requirements regarding the quality assurance of certified seeds production**

are maintained. No contamination was observed either when comparing the seeds from the 4 certified wheat cultivars with the standard seeds, nor when comparing the seeds belonging to the same certified wheat cultivar, multiplied in different locations with the standard seeds.

The use of a new technique in wheat cultivars varietal purity assessment, namely the **KASP technique**, showed that the results obtained are **reproducible** as no differences were observed between the results obtained using SSR markers and those obtained using KASP markers. Thus, the use of KASP markers: BS00023119_6A, BS00060097 and the *1-Feh-w3* associated marker, showed that between the cultivars tested in the two campaigns (2019 and 2020) and compared with the standard seeds and between the seeds multiplied in different locations and compared with the standard seeds, there are no differences.

In this way, it was possible to demonstrate that is minimized the risk that the method based on using SSR markers will generate false positive or false negative results, but also the fact that are respected the conditions in terms of maintaining varietal purity, important factor in breading programmes and seeds certification process.

The genetic diversity analysis for the 40 samples showed the ability of the selected SSR markers to detect the genetic diversity between different wheat cultivars, but also between wheat and other species. The selected SSR markers were able to distinguish common wheat from *Triticum spelta* L., *Triticum monococcum*, *Triticum dicoccon* Schrank, but also from cereals belonging to another genera such as triticale (*×Triticosecale* Wittmack) and *Avena nuda* L.. Thus, the analysis of the statistical data generated by NTSYSpc 2.10e software application and the dendrogram generated by using the UPGMA algorithm and the Dice coefficient could highlight the presence of a diversified germplasm in certified wheat cultivars in Romania.

The SSR markers selected in the present research study proved to be useful in varietal purity and genetic diversity assessment and the polymorphic ones can also be used in future wheat breeding programs. Future use of a larger number of SSR markers may increase the specificity of the method.

In **chapter IV** entitled "**Varietal purity and genetic diversity assessment in maize inbred lines grown in Romania**" are presented information related to the materials and methods used in the experimental study, the results and discussion regarding the use of SSR markers in varietal purity and genetic diversity assessment and partial conclusions on the use of SSR markers in varietal purity and genetic diversity assessment in the selected maize inbred lines.

The subchapter "**Materials and methods**" includes information on:

- **Maize plant material selection**

The maize plant material was represented by seeds from 13 maize inbred lines (LC1-LC13) grown in Romania, obtained from the Central Laboratory for the Quality of

Seeds and Planting Material (LCCSMS). The tested seeds belonged to the pre-basic generation 2 (PB G2) and basic (B) seeds classes. To check if the varietal purity is preserved, for 3 of the maize inbred lines, seeds from both the PBG2 and the B categories were analysed in parallel. In order to demonstrate that SSR markers prove their efficiency in varietal purity assessment of the maize inbred lines, two maize hybrids (H1 and H2) were also introduced into the study.

- **DNA extraction from maize seeds**

The optimization at this stage consisted of DNA extraction from maize seeds using the PL1 buffer of the NucleoSpin Plant II kit, the variations included being: DNA extractions from seeds, embryos and plant material resulting from seed germination for 3 of the selected samples.

- **SSR markers used in experiments**

8 SSR markers (umc1545, umc1448, umc1117, umc1061, phi109275, phi102228, phi083, phi015) were selected to assess the varietal purity and genetic diversity of the 13 maize inbred lines.

- **Testing the reaction conditions for varietal purity assessment of selected maize inbred lines**

The experimental plan included optimizations consisting in the variations of the primers concentration, the final reaction volume and the creation of a temperature gradient for all SSR markers in order to choose the optimal annealing temperature.

The subchapter "**Results and discussion**" included results and discussion regarding the selection and optimization of the DNA isolation method, optimization of the reaction conditions for the selected SSR markers, analysis of the products obtained with the selected SSR markers, results and discussion regarding the varietal purity assessment of the maize inbred lines and maize hybrids following the use of SSR markers, as well as the genetic diversity analysis of the selected maize inbred lines.

Regarding the selection and optimization of the DNA isolation method, seeds grinding and DNA extraction with PL1 lysis buffer proved to be the most efficient and rapid extraction method. The selected isolation method allowed obtaining good amplification results with all 8 SSR markers tested, genomic DNA meeting the acceptability criteria regarding the optimal concentration and purity for the PCR reaction.

Under the selected reaction conditions for the experimental study, all 8 SSR markers showed amplification products.

Following **the molecular analyses,** it was observed that all the selected markers showed a certain degree of polymorphism on the selected maize inbred lines. Thus, **phi015** marker proved to be the most polymorphic among the 8 selected SSR markers (5 alleles), followed by **umc1545** , **umc1448** and **umc1117** markers (4 alleles), phi102228 and phi083 markers (3 alleles) and markers umc1061 and phi109275 markers (2 alleles).

The tested SSR markers proved their efficiency in both **varietal purity assessment of maize inbred lines, as well as in the case of checking the varietal purity of the selected maize hybrids** (H1 and H2).

Verification of the varietal purity of the two selected maize hybrids could only be confirmed with 3 of the 8 SSR markers used in the study. Thus, although the marker phi109275 did not initially show a high polymorphism, it was able to establish with certainty the varietal purity of the H2 hybrid. The umc1117 marker was able to establish with certainty the varietal purity of both hybrids, and phi083 marker was able to establish with certainty the varietal purity of the H1 hybrid.

The statistical data analysis included the creation of a binary matrix as well as the generation, with the help of an online application, of a dendrogram that showed the genetic diversity between the maize inbred lines introduced into the study.

After analyzing the statistical data generated by the UPGMA algorithm, using Dice coefficient and the similarity matrix data, could be observed a high genetic similarity between maize inbred lines LC1(PBG2) and LC2 (B), which proves that the **varietal purity is kept between seeds categories**.

Since between maize inbred lines LC3 (PBG2) and LC13 (B), as well as between lines LC4 (B) and LC10 (PBG2), the evaluation of the amplification products with the eight SSR markers revealed a degree of similarity between 25-37 %, two hypotheses can be issued: either a contamination of the original line, or an improvement of the original inbred line desired by the breeder.

The molecular analysis performed demonstrated that SSR markers proved to be useful for varietal purity and genetic diversity assessment, being able to differentiate between related maize lines and to place maize inbred lines into groups based on genetic similarity.

The varietal purity and genetic diversity assessment between different maize inbred lines can play an important role in breeding process and in obtaining new hybrids, therefore, the results obtained can be a starting point for the evaluation of other maize inbred lines and the selection of lines with desired traits in maize hybrids breeding programs.

The results obtained both in chapter III and in chapter IV will be able to be used in the future in the development and validation of new methods for varietal purity assessment, in germplasm improvement and/or conservation programs .

At the end of the thesis were included a series of **general conclusions**, as well as **the novelty aspects of the research study**.

The results obtained in the present research study bring in their entirety a novelty in the field on a national level.

The novelty of the research topic consists in the fact that, on a national level, there are no validated and/or accredited methods that allow the varietal purity

assessment of the planting material through molecular biology techniques based on the use of SSR markers.

Another novelty aspect of the research theme consists in the fact that the KASP technique is used for the first time at the national level in varietal purity assessment of wheat cultivars.

The results obtained in this research study provide both up-to-date information on the genetic diversity of wheat and maize planting material grown in Romania, as well as information that may be used in the future in the development of new methods for varietal purity assessment or in breeding programs and/or germplasm conservation.

Since the methodology regarding the use of SSR markers in varietal purity assessment of wheat and maize planting material is not fully defined in national and international legislation, the present study may in the future be a source of information for the development of an appropriate methodology that can be applied both nationally as well as internationally.

The results obtained in the present research study will be able to be used in the future for the validation and accreditation of methods that will allow the varietal purity assessment of wheat and maize seeds and planting material, and the working methodology will be able to be introduced in the future in the specific national legislation.

The research activity was carried out both within **the Faculty of Biotechnologies** of USAMV**,** Bucharest, as well as within **the Laboratory of Electrophoresis and Genetically Modified Organisms** of LCCSMS. Research activity on the KASP technique was carried out in the **Phenotyping and Genotyping Laboratory** of INCDA Fundulea.

The present thesis contains a number of 202 pages, including a number of 13 tables and 59 figures. The work includes 6 annexes. In annex I and II are presented the lists of tables and figures. In Annex III are presented information about the DNA concentration and purity ratios for the wheat cultivars used in the study. Annex IV and V present the data included in the similarity matrix resulting from the application of the UPGMA algorithm and Dice coefficient to the wheat cultivars (annex IV) and maize inbred lines tested (annex V). Annex VI presents the molecular results obtained for the SSR markers used in the present study to assess the varietal purity of the selected wheat cultivars. The bibliography contains 213 bibliographic references published in specialized journals and web sources.

The results obtained in the present research study were disseminated through 4 articles published both in WOS and BDI indexed journals, as well as through scientific communications at international events.