TEZĂ DE DOCTORAT

Assessment of the potential to use monogenic and polygenic resistance for apple scab in breeding programs

(SUMMARY OF THE Ph.D. THESIS)

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INTRODUCTION

According to recent studies, it is estimated that by 2050 the global population will reach approximately 10 billion people. In this context, it is essential to identify modern solutions through which agriculture can provide sufficient quantities of food, of superior quality, and at lower costs. The apple (*Malus domestica*) is one of the most important fruit crops in the world, playing a crucial role in fruit growing due to its economic, nutritional, and cultural value.

In the 20th century, breeding programs for fruit species focused on obtaining cultivars with exceptional aesthetic and taste traits. Currently, most of these apple breeding programs are aimed at developing cultivars with sustainable resistance to major diseases that have a significant economic impact (Hofer *et al.,* 2021), including apple scab (*Venturia inaequalis*).

Therefore, research aimed at obtaining top apple cultivars with durable resistance and superior fruit quality is fundamental for a future in which apple production will be more environmentally friendly and resilient.

This doctoral thesis proposes the development of hybrid apple populations that exhibit resistance or tolerance to apple scab, one of the most damaging diseases of apples, caused by the fungus *Venturia inaequalis*, aligning with current breeding requirements.

STRUCTURE OF THE DOCTORAL THESIS

The doctoral thesis titled: Assessment of the potential to use monogenic and polygenic resistance for apple breeding programs, consists of 149 pages, including 10 chapters, 58 figures, and 32 tables. The thesis is structured into two main parts. The first part, titled Current State of Knowledge, represents the up-to-date documentation on the chosen topic. It is organized into 2 chapters and spans 29 pages, accounting for 23% of the total thesis. The second part represents the Personal Contribution, which is presented in 97 pages and includes 8 chapters, 47 figures, and 29 tables, representing 77% of the total thesis. This section details the experiments conducted during the 2021-2024 period. The bibliography consists of 156 references, both domestic and foreign, as well as four web citations.

Current state of knowledge:

Chapter 1. Apple cultivation worldwide and in Romania presents recent aspects related to the importance of apples, as well as the situation of apple cultivation both globally and in Romania.

Chapter 2. General considerations regarding apple resistance to scab describes, on one hand, the taxonomy, symptomatology, and evolution of the pathogen *Venturia*

inaequalis, and, on the other hand, provides detailed information on apple resistance to this pathogen as well as the implications of Marker-Assisted Selection (MAS) in apple breeding.

Personal contribution:

Chapter 3. Aims and objectives of the research presents the central goal of the doctoral thesis, as well as the objectives proposed to achieve it.

Chapter 4. Specifics of the natural environment of the research describes both the relief, hydrology, and climate elements of the environment in which the research was conducted over a period of 10 years and during the experimental period.

Chapter 5. Study 1 - Identification and selection of genitors for use in an apple breeding program presents the criteria for selecting genitors for the apple breeding program, as well as the working protocols for the selection stage using the MAS method, accompanied by the results obtained.

Chapter 6. Study 2 - Performing artificial crosses and obtaining hybrid apple plants describes the organization of artificial pollination experiments, as well as the results obtained in this regard.

Chapter 7. Study 3 - Evaluation and selection of apple hybrids using Marker-Assisted Selection (MAS) presents the work steps as well as the results obtained in order to identify the presence/absence of genes of interest for resistance to apple scab (*Rvi6*, *Rvi2*, *Rvi5*).

Chapter 8. Study 4 - Establishment of the hybrid field describes aspects related to the planting of hybrids that have inherited apple scab resistance genes, as well as their behavior in natural apple scab infections.

AIMS AND OBJECTIVES OF THE RESEARCH

The central aim of the doctoral thesis was to obtain hybrid apple populations exhibiting resistance or tolerance to one of the most harmful diseases of apples, apple scab, caused by the fungus *Venturia inaequalis*, in accordance with current breeding requirements.

To achieve the goal of this doctoral thesis, the following objectives were set:

a. Identification of apple genitors to be used in breeding programs aimed at obtaining hybrids with resistance/tolerance to apple scab.

b. Performing artificial pollinations between selected genitors based on the traits that are desired to be transferred to the hybrids.

c. Identifying hybrids that have inherited resistance genes to apple scab using molecular markers through the MAS method.

d. Establishing the hybrid field and monitoring the behavior of the hybrids plants under natural infection conditions with the *Venturia inaequalis* fungus.

SPECIFICS OF THE NATURAL ENVIRONMENT OF THE RESEARCH

The experiments conducted within this doctoral thesis, both those carried out in the field and those performed in the laboratory, took place at the Fruit Research and Development Station Bistrița (FRDS Bistrița), located in the northern part of Romania, on the Bistrița hills. During the course of the studies, temperatures and precipitation were analyzed in two stages. The first stage took place between 2021-2022, when the data were analyzed in the context of artificial pollinations, and the second stage occurred between 2023-2024, when the data were studied in relation to the planting and development of the apple hybrids in the field.

In May, the period when artificial pollinations took place, the average annual temperature was 13.3°C (2021) and 15.3°C (2022). More specifically, the temperature recorded on pollination days ranged between 3.9°C and 10.4°C in 2021, and between 5.9°C and 8.2°C in 2022.

For the years when the hybrids were transferred to the field, the monthly average values ranged between -0.6°C (January) and 21.4°C (August) in 2023, and between -0.3°C (January) and 24.4°C (July) in 2024. The hybrids were planted in the field in April, with temperatures of 8.4°C (2023) and 6.4°C (2024).

The total precipitation in the years when artificial pollinations were carried out was 847.5 mm (2021) and 838.6 mm (2022), while in the years when the hybrids were planted in the field, it was 765.7 mm (2023) and 743.2 mm (2024).

STUDY 1 - IDENTIFICATION AND SELECTION OF GENITORS FOR USE IN AN APPLE BREEDING PROGRAM

Purpose of the study

The purpose of Study 1 is to evaluate different apple cultivars with specific genetic characteristics, in order to obtain cultivars with enhanced resistance to apple scab, determined by the presence of the *Rvi6* (*Vf*) gene. Therefore, this study focuses on selecting resistant and more productive apple cultivars that meet market requirements and contribute to reducing pesticide use in apple production by obtaining hibryds that are resistant or tolerant to apple scab infection.

Materials and methods

The biological material used consisted of 23 apple cultivars ('Alex', 'Auriu de Bistrița', 'Bistrițean', 'Dany', 'Doina', 'Fiesta', 'Florina', 'Fuji Kiku', 'Gala Mitchgla', 'Generos', 'Golden Delicious', 'Goldprim', 'Idared', 'Jonathan', 'Katja', 'Lena', 'Pinova', 'Red Cap', 'Reinette du Canada', 'Salva', 'Starkrimson', 'Williams Pride', and 'Yellow'), each with different characteristics and traits.

The method used was Marker-Assisted Selection (MAS), which plays an important role in apple breeding by allowing the rapid and precise identification of genes of interest, thus accelerating the development of new cultivars with desired traits.

The genitors were amplified using a total of four pairs of primers, including two dominant primer pairs (AM19, $U1_{400}$) and the other two codominant primers (AL07 and VFC), capable of distinguishing homozygous genotypes from heterozygous ones.

Results and conclusions

Molecular analysis showed that the apple cultivars that have the *Rvi6* (*Vf*) gene in a dominant state are: 'Salva', 'Bistrițean', 'Williams Pride', 'Florina', 'Lena', 'Pinova', 'Goldprim', 'Dany', 'Doina', and 'Alex'. Therefore, these cultivars have a heterozygous genotype (*Vfvf*). The rest, including 'Idared', 'Jonathan', 'Golden Delicious', 'Starkrimson', 'Auriu de Bistrița', 'Fuji Kiku', 'Red Cap', 'Katja', 'Gala Mitchgla', 'Fiesta', 'Yellow', and 'Reinette du Canada', possess the *vf* gene, but in a recessive state, thus presenting a homozygous recessive genotype (*vfvf*).

The heterozygous genotypes (*Vfvf*), indicating the presence of the *Rvi6* (*Vf*) gene in the dominant state, showed amplification bands in the gel at the following base pairs: 570 bp (AL07), 526 bp (AM19), 338 bp (U1*400*), and 286 bp (VFC). On the other hand, the recessive *vf* gene indicated those samples with a homozygous recessive genotype, showing amplification in the gel at the following base pairs: 823 bp (AL07), 484 bp, and 646 bp (VFC).

Based on the results obtained, ten cultivars were selected: 'Auriu de Bistrița', 'Salva', 'Bistrițean', 'Generos', 'Florina', 'Idared', 'Golden Delicious', 'Starkrimson', 'Jonathan', and 'Williams Pride'.

STUDY 2 - PERFORMING ARTIFICIAL HYBRIDIZATIONS AND OBTAINING HYBRID APPLE PLANTS

Purpose of the study

The purpose of the study was to perform hybrid combinations between the selected genitors and subsequently obtain a variability of hybrid plants (F1) that

combine both resistance to apple scab and superior fruit qualities, so that, in the end, new apple cultivars with superior qualities compared to those currently available in cultivation could be obtained.

Materials and methods

In 2021 and 2022, at FRDS Bistrița, intraspecific hybrid combinations were made to obtain F1 apple hybrids. A total of 20 intraspecific combinations were made using 10 apple cultivars, of which four were native ('Auriu de Bistrița', 'Salva', 'Bistrițean', and 'Generos') and six were foreign ('Florina', 'Jonathan', 'Golden Delicious', 'Starkrimson', 'Idared', and 'Williams Pride').

For artificial hybridization, seven essential stages were followed: genitor selection, choosing the trees from the orchard, identifying the inflorescences in the crown, flower isolation and emasculation on the female tree, pollen collection from the male tree, artificial pollination, monitoring flower setting, and finally, harvesting the hybrid fruits.

Results and conclusions

For interpreting the results, the following were quantified: the number of hybrid seeds obtained and the germination percentage from each combination. Finally, the percentage of hybrids that passed the first selection stage, i.e., those that were sufficiently developed to be subsequently tested molecularly, was calculated.

Using the cultivars 'Florina' as the male genitor, five hybrid combinations were made, resulting in a total of 888 hybrid seeds. After germination, healthy hybrid plants suitable for transplanting into individual pots were selected. Thus, of the 888 hybrid seeds, 425 hybrid plants (47.9%) emerged, which were later transplanted into containers for proper development. Throughout the vegetation period, the plants were carefully monitored for development, and by the end of the period, 337 plants (79.3%) were prepared for testing. In these pollinations, the best results were obtained from the hybrid combinations 'Starkrimson' x 'Florina' (98.0%), followed by 'Auriu de Bistrița' x 'Florina' (96.9%).

For the hybrid combinations in which resistance to apple scab was transferred from the femele genitor 'Salva', a total of 651 hybrid seeds were obtained, of which 165 hybrid seedlings (25.4%) were later transplanted into individual pots. From the hybrids selected for molecular testing, a total of 97 hybrids (58.8%) were chosen. Among the hybrid combinations where the femele genitor was 'Salva', the highest percentage of hybrids transplanted into individual pots was found in the combination 'Salva' x 'Jonathan' (84.8%), followed closely by the hybrid combination 'Salva' x 'Starkrimson' (81.8%).

For the hybrid combinations in which the cultivars 'Generos' was used as the male genitor, a total of 730 hybrid seeds were obtained. Of these, 227 hybrids (31.1%) emerged, which were subsequently moved for optimal development into individual pots. The best percentages of hybrids suitable for pot planting were obtained in two combinations: 'Auriu de Bistrița' x 'Generos' (38.5%) and 'Jonathan' x 'Generos' (37.5%).

In the hybrid combinations where the cultivars 'Generos' was used as the female genitor, 473 hybrid seeds were obtained, of which 156 hybrid plants (33%) emerged and developed optimally for transplanting into individual containers. These plants were from three of the combinations, with the top two being the combination 'Generos' x 'Idared' (53.6%), followed closely by the combination 'Generos' x 'Jonathan' (46.0%).

STUDY 3 - EVALUATION AND SELECTION OF APPLE HYBRIDS USING THE MARKER ASSISTED SELECTION (MAS) METHOD

Purpose of the study

The purpose of this study was to evaluate the hybrids obtained from the eight hybrid combinations made following artificial pollinations in 2021, using MAS selection with the help of molecular markers. Two experiments were organized for this purpose: **Experiment 1** - **Molecular analysis of hybrids to identify the *Rvi6* (*Vf*) gene**, and **Experiment 2 - Molecular analysis of hybrids to identify the *Rvi2* (*Vh2*) and *Rvi5* (*Vm*) genes**.

Materials and methods used in Experiment 1 (identification of the *Rvi6* gene) and Experiment 2 (identification of the *Rvi2* and *Rvi5* genes).

The biological material used consisted of several F1 hybrid apple populations obtained from artificial pollinations made in five hybrid combinations using the cultivar 'Florina' as the male genitor ('Auriu de Bistrița' x 'Florina', 'Jonathan' x 'Florina', 'Starkrimson' x 'Florina', 'Idared' x 'Florina'), and from three hybrid combinations where 'Salva' was used as the female genitor ('Salva' x 'Auriu de Bistrița', 'Salva' x 'Jonathan', 'Salva' x 'Starkrimson'). All the hybrids were tested using the MAS method, utilizing primers specific for each gene. For the *Rvi6* (*Vf*) gene, the primers AL07 - codominant (Khajuria *et al.,* 2004), AM19 - dominant (Tartarini *et al.,* 1999), and VFC - codominant (Afunian *et al.,* 2004) were used. For *Rvi2* (*Vh2*), the primer OPL19 (Bus *et al.,* 2005) was used, and for the *Rvi5* (*Vm*) gene, the primer OPB12 (Cheng *et al.,* 1998) was used.

Results and conclusions regarding Experience 1 (Identification of the *Rvi6* gene)

As a result of applying the MAS method, the number of hybrids that inherited the *Rv*i6 (*Vf*) gene varied depending on the hybrid combination and the origin of the resistance source (either from the female genitor or the male genitor).

Therefore, in the combinations where the source of resistance came from the male genitor ('Florina'), the following results were obtained. Thus, from the 'Auriu de Bistrița' x 'Florina' combination, a total of 187 hybrids were tested, of which 84 inherited the *Rvi6* resistance gene (*Vf*). The next hybrid combination tested was 'Jonathan' x 'Florina' with a number of seven hybrids, of which four presented the gene of interest. In the case of the 'Golden Delicious' x 'Florina' hybrid combination, a total of 14 hybrids were tested, of which only three inherited the *Rvi6* resistance gene. For the 'Starkrimson' x 'Florina' hybrid combination, a total of 49 hybrids were tested. Of these, more than half (29 hybrids) showed the gene. The last hybrid combination tested was 'Idared' x 'Florina'. A total of 31 hybrids were molecularly tested for the identification of the *Rvi6* (*Vf*) gene. Among them, 12 inherited the resistance gene. In a graphical representation, the situation above is shown as follows: The highest percentage comes from the hybrid combination 'Jonathan' x 'Florina' (57.1%), followed closely by the 'Starkrimson' x 'Florina' combination (51.1%). On the opposite end, with the lowest percentage of hybrids inheriting the *Rvi6* (*Vf*) gene, was the 'Golden Delicious' x 'Florina' combination (21.4%).

From the combinations where the source of resistance came from the female genitor ('Salva'), the following results were obtained: in the 'Salva' x 'Auriu de Bistrița' combination, 12 hybrids were tested, of which six inherited the *Rvi6* resistance gene (*Vf*); in the 'Salva' x 'Jonathan' hybrid combination, 67 hybrids were tested, of which 24 presented the *Rvi6* (*Vf*) gene; in the 'Salva' x 'Starkrimson' hybrid combination, 18 hybrids were tested. Among them, four hybrids inherited the *Vf* resistance gene. The percentages of inheritance for this gene ranged between 22.2% in the 'Salva' x 'Starkrimson' combination and 50.0% in the 'Salva' x 'Auriu de Bistrița' combination. Between these two values, there is the percentage of 35.8% obtained in the 'Salva' x 'Jonathan' combination.

Results and conclusions regarding Experience 2 (Identification of the *Rvi2* and *Rvi5* genes)

In this experiment, a total of 165 hybrids were tested for the identification of two major genes, *Rvi2* (*Vh2*) and *Rvi5* (*Vm*). These hybrids came from six hybrid combinations: 'Auriu de Bistrița' x 'Florina', 'Jonathan' x 'Florina', 'Golden Delicious' x 'Florina', 'Starkrimson' x 'Florina', 'Idared' x 'Florina', and 'Jonathan' x 'Salva'.

From the 'Auriu de Bistrița' x 'Florina' combination, a total of 78 hybrids were tested. The *Rvi2* (*Vh2*) gene was transmitted to 49 hybrids, while the *Rvi5* (*Vm*) gene was transmitted to only 14 hybrids. In the case of the 'Golden Delicious' x 'Florina' combination, three hybrids were tested. The Rvi2 (*Vh2*) gene was identified in two hybrids, while the *Rvi5* (*Vm*) gene could not be identified because no amplifications were obtained. From the 'Starkrimson' x 'Florina' hybrid combination, 46 hybrids were tested, of which 37 inherited the *Rvi2* (*Vh2*) gene and 28 inherited the *Rvi5* (*Vm*) gene. In the 'Idared' x 'Florina' hybrid combination, 11 hybrids were tested, of which only one hybrid inherited the *Rvi2* (*Vh*2) gene and five inherited the *Rvi5* (*Vm*) gene. In the 'Salva' x 'Jonathan' hybrid combination, 23 hybrids were tested, with 12 inheriting the *Rvi2* (*Vh2*) gene and 13 inheriting the *Rvi5* (*Vm*) gene.

The percentage of hybrids that inherited the two resistance genes varied depending on the gene and hybrid combination. Thus, the *Rvi2* (*Vh2*) gene was transmitted in the range of values from 80.4% ('Starkrimson' x 'Florina'), followed by the 'Golden Delicious' x 'Florina' combination (66.7%), while the lowest percentage was recorded in the 'Idared' x 'Florina' combination (9.1%).

For the *Rvi5* (*Vm*) gene, the percentages ranged between 60.9% ('Starkrimson' x 'Florina') and the lowest percentage in the 'Auriu de Bistrița' x 'Florina' combination (17.9%).

It was observed that the highest percentage of hybrids having both resistance genes in their genome was found in the 'Starkrimson' x 'Florina' hybrid combination (60.9%), while the lowest percentage was observed in the 'Auriu de Bistrița' x 'Florina' combination (16.7%).

Study 4 - ESTABLISHMENT OF THE APPLE HYBRID FIELD

Purpose of the study

The purpose of this study is to continue the selection of apple hybrids, aiming to evaluate their behavior against apple scab attack under natural infection conditions. Additionally, the processes of fruiting and the quality of the obtained fruits are monitored.

Materials and methods used in Study 4 – Establishment of the hybrid field

The establishment of the hybrid field is the immediate step following the primary selection of hybrids resulting from artificial pollinations.

The planting took place in the spring of 2023 (for the hybrids resulting from pollinations with the 'Florina' cultivar, used as the male genitor, and those from pollinations with 'Salva', used as the female genitor), as well as in the spring of 2024

(for those from pollinations with the 'Generos' cultivar, used as both the male and female genitor).

Results and conclusions regarding Study 4 – Establishment of the hybrid field

Following the first selection stage, a total of 490 apple hybrids were planted in the field, obtained from 15 hybrid combinations. Thus, five types of combinations were identified from which the hybrids originate. The largest number of hybrids (194) and the most hybrid combinations (6) belong to the sensitive x resistant category. In this case, the female genitors ('Jonathan', 'Golden Delicious', 'Starkrimson', 'Idared') are sensitive to apple scab, while the male genitors ('Florina', 'Generos') are resistant to apple scab. The next category, based on the number of hybrids (111), is tolerant x resistant, while the category with the most hybrid combinations (94) is resistant x sensitive.

To obtain preliminary results regarding the behavior of the hybrids under apple scab attack, studies were conducted as early as the first year of planting in the hybrid field.

As a result, the highest leaf infection rate was observed in the 'Idared' x 'Florina' combination (76.9%) of hybrids, followed by the 'Starkrimson' x 'Florina' hybrid combination (40.9%). The lowest percentage of infected hybrids was found in the 'Auriu de Bistrița' x 'Florina' hybrid combination (38.1%).

In the case of hybrid combinations with the 'Salva' cultivar used as the female, all three hybrid combinations had a certain percentage of infected plants. The hybrid combination with the lowest percentage of infected plants was 'Salva' x 'Auriu de Bistrița' (16.7%). On the opposite end, the hybrid combination 'Salva' x 'Jonathan' had the highest percentage of infected plants (30.4%).

Out of the total hybrids planted in the selection field, 40.5% (hybrids from combinations with the 'Florina' cultivar as the male genitor) and 27.3% (hybrids from combinations with the 'Salva' cultivar as the female genitor) were infected with apple scab.

It can be observed that in the case of both hybrid combinations where the female genitor is 'Auriu de Bistrița', the lowest percentage of infected hybrids was reported. This can be explained by the fact that the female genitor ('Auriu de Bistrița') is known for its tolerance to apple scab infections, and thus it is possible that this tolerance is partially transmitted to its hybrids (Bivolariu *et al.,* 2021).

INNOVATIVE CONTRIBUTIONS OF THE THESIS

The doctoral thesis addresses an important topic in apple breeding, providing innovative contributions that meet the current requirements for developing apple

cultivars resistant to apple scab, a highly economically damaging disease caused by the fungus *Venturia inaequalis*. The first part of the thesis focuses on an updated review of the specialized literature on apple scab and the use of molecular markers in apple breeding, thus constituting a solid foundation for apple breeding programs.

An important part of the research was the identification of the most suitable genitors for the apple breeding program. Over 20 cultivars, both native and foreign, with relevant phenotypic and genotypic characteristics, such as the presence of scab resistance genes, were studied. This allows for the production of hybrids with disease resistance and high-quality fruit. Among the notable cultivars are 'Auriu de Bistrița', 'Salva', 'Bistrițean', 'Generos', 'Florina', 'Idared', 'Golden Delicious', 'Starkrimson', 'Jonathan', and 'Williams Pride'.

Equally, the study explores the importance of artificial pollination, which contributes to the production of viable hybrid seeds. A notable example was the 'Auriu de Bistrița' cultivar, which, although a progeny of the 'Golden Delicious' cultivar, showed good results in artificial pollinations.

Another innovative aspect of the research is the pyramiding of resistance genes *Rvi2* (*Vh2*), *Rvi5* (*Vm*), and *Rvi6* (*Vf*) into a single genotype, which represents an important step in improving resistance to apple scab. The results of this study significantly contribute to the creation of new apple cultivars with superior characteristics to existing ones.

The creation of a solid genetic base, which includes approximately 500 hybrid hybrids, represents an essential contribution of the research in this doctoral thesis. Additionally, for FRDS Bistrița, an important achievement was the use of the MAS (Marker-Assisted Selection) method to identify plants with scab resistance genes. This apple breeding program brings new results and fills a gap in the field, as no new elite plants have been obtained in the Bistrița area for developing a high-performing cultivar in the past 15 years.

The hybrids obtained in this research meet current requirements and can represent an important foundation for future studies in the field. The thesis also fostered cooperation between several specialized institutions, such as FRDS Bistrița, UASVM Cluj, and RIFG Pitești Mărăcineni, and some of the results are included in the Guide "Efficienting the genetic improvement of fruit tree and shrub cultivars." The results of the research have both national and international significance, considering the global importance of the issue of apple resistance to apple scab, a disease present in all regions where the *Malus* genus is cultivated.

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