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Breeding a New Apple Hybrid Population with the *Vf* Gene Through Marker-Assisted Selection

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RESEARCH ARTICLE

Abstract

Apple scab, caused by *Venturia inaequalis*, is one of the most damaging pathogens affecting apple species. Cross combinations were made between the Salva cv. (female parent), a valuable local cultivar known for its fruit quality and used as a donor of the V*f* resistance gene, and Jonathan (male parent), a variety widely cultivated but susceptible to scab. The V*f* gene was first identified in *Malus floribunda* Clone 821, which was subsequently transferred to commercial varieties through various breeding programs. To confirm the presence of the V*f* gene, the progeny from this cross was tested using Marker-Assisted Selection (MAS) with one dominant primer pair (AM19) and two codominant primers (AL07 and VFC) to distinguish between homozygous and heterozygous genotypes. From the crossing, 67 hybrids were obtained, of which 35.8% (24 hybrids) were classified as resistant (heterozygous - *Vfvf*), while 64.2% (43 hybrids) were classified as susceptible (recessive homozygotes - *vfvf*). This population of hybrids resistance to *Venturia inaequalis* represents a good starting point in obtaining cultivars with resistance to scab.

Keywords: *Malus domestica*; marker; monogenic resistance; scab; *Vf* gene.

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INTRODUCTION

The apple is one of the most important fruit species due to its ability to adapt to various climatic conditions and soils, ensuring stable and profitable yields. Additionally, apples are appreciated for their nutritional value and versatility in the food industry, which contributes to their constant demand on the market (O'Rourke, 2021). One of the most problematic diseases of apple trees is apple scab, caused by the fungus *Venturia inaequalis*. This is one of the most common and harmful diseases of apples, affecting leaves, fruits, and branches, significantly reducing the quality and yield of the tree (Patocchi et al., 2020). The spread of the fungus *V. inaequalis* is similar to its host species *Malus domestica* (CABI, 2022), but is particulary problematic in temperate climats due to the fact that high humidity favours infection. In Romania, the regions most affected by apple scab are areas with more humid climate and frequent rainfall, such as Transylvania, especially the central and northern counties of the region (Preda et al., 2020). Control of apple scab involves a combination of preventive measures and chemical treatments. Prevention primarily includes the selection of resistant varieties, alongside other phytosanitary hygiene practices (Chatzidimopoulos et

al., 2020). To control scab, sometimes as many as 20 treatments are necessary throughout a season, which leads to a high degree of environmental pollution (Sokolova and Moročko-Bičevska, 2022).

Apple breeding trends to focus on developing new varieties with increased genetic resistance to scab. The goal is to reduce the need for chemical treatments while simultaneously improving fruit productivity and quality. Emphasis is placed on using natural genetic resources and modern technologies, such as selective breeding and biotechnology, to create varieties that are durable and adapted to various climatic conditions (Zelmene et al., 2022). The Marker-Assisted Selection (MAS), is one modern technology that allows for the identification of genes of interest. This method involves using genetic markers to quickly and precisely identify plants possessing the desired genes, thus accelerating the breeding process (Khajuria et al., 2018). The most widely used gene for apple scab resistance is *Vf* (Rvi6), derived from a wild apple species, *Malus floribunda* clone 821. This gene has been widely used in breeding programs to develop commercial apple varieties capable of withstanding this disease (Iancu et al., 2023).

The aim of this study was to evaluate the results of a controlled hybridization process within apple species between two highly valued cultivars. Additionally, the transmission of the *Vf* resistance gene in the progenies is verified through molecular methods, and the behavior of the selected hybrid plants regarding apple scab infection was observed.

MATERIALS AND METHODS

Controlled pollinations of the apple species were conducted at the Fruit Research and Development Station Bistrița (FRDS Bistrița), Romania, in 2021. The biological material consisted of two apple cultivars: Salva (female genitor, a valuable local cultivar in terms of fruit quality, donor of the *Vf* gene) and Jonathan (male parent, a cultivar widely cultivated in Romania and globally, with a late harvesting period - winter cultivar), as well as their resulting F1 hybrids from the controlled pollinations. To rapidly identify the origin of the progenies, the hybrid combination of the two mentioned cultivars and each hybrid was assigned an identification code from the outset. Thus, it will be referred to the combination of Salva x Jonathan, named 21.06 (Figure 1).

Figure 1. Apple cultivars used as genitors in controlled pollinations Salva x Jonathan (21.06).

The controlled pollination followed the specific steps outlined in the literature, quantifying the results in terms of the number of hybrid seeds obtained, suitable hybrids for planting in individual pots, and ultimately, the most important observation: the percentage of hybrids that inherited the *Vf* resistance gene, identified through modern selection methods (MAS). The obtained hybrids were selected, and those that did not exhibit symptoms of apple scab on the leaves underwent selection, using three pairs of specific markers: two dominant and one codominant, the latter being able to distinguish between homozygous and heterozygous genotypes (Table 1).

DNA extraction and amplification were carried out as follows: fresh leaf samples were collected from each descendant and placed in paper bags labelled according to the hybrid identifier. The plant material was grounded in liquid nitrogen, and 100 mg of each sample was transferred into 1.5 ml extraction tubes and stored at -20 °C. DNA was isolated using the Invisorb Spin Plant Mini Kit from Invitek Molecular (Bioline), following the manufacturer's protocol. A total of 50 µl of DNA was obtained from each sample. The concentration and purity of the DNA were measured using a NANODROP 2000c spectrophotometer.

Table 1. List of primers used in the experience for identification *Vf* gene

No.	Marker	Primer sequences 5' to 3'	No.	Primer type	Fragment	References
1	AL07	F-TGGAAGAGAGATCCAGAAAGTG	22	Codominant	570 (*Vf*)	Khajuria et al., 2014
		R-CATCCCTCCACAAATGCC	18		823 (*vf*)	Tartarini et al., 1999
2	AM19	F-CGTAGAACGGAATTTGACAGTG	22	Dominant	526 (*Vf*)	Khajuria et al., 2014
		R-GACAAAGGGCTTAAGTGCTCC	21			Tartarini et al., 1999
3	VFC	F: GGTTTCCAAAGTCCAATTCC	20	Cominant	286 (*Vf*) 484 (*vf*)	Afunian et al., 2004
		R: CGTTAGCATTTTGACTTGAC	20		646 (*vf*)	

The DNA amplification mix was prepared in 0.2 ml tubes using MyTaq Red Mix (Bioline), with a final reaction volume of 25 µl per tube for each sample. DNA amplification was carried out using an Eppendorf Mastercycler ep gradient S.

The cycling parameters used for primers AL07 and AM19 were as follows: initial denaturation at 95 °C for 1 min; 35 cycles x (denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 2 min) and final extension at 72 °C for 10 min. The cycling parameters used for primer VFC were as follows: initial denaturation at 94 °C for 4 min; 35 cycles x (denaturation at 94 °C for 1 min; annealing at 58 °C for 1 min; extension at 72 °C for 1 min). The final extension cycle was applied at 72 °C for 7 min.

A 10 µl aliquot of the amplified products was separated on a 1.5% agarose gel via electrophoresis in 1X TAE buffer for 50 min. A 100 bp DNA Ladder RTU was used as the size marker. The bands were visualized using RedSafe Nucleic Acid Staining Solution and analyzed under UV light with the Quantity One 1-D Analysis Software system.

For the interpretation of the molecular analysis results regarding the electrophoretic profile of each amplified sample, the presence of the *Vf* gene was marked with a plus (+), and its absence with a minus (–). The Salva cultivar was used as a positive control, while the Jonathan served as a negative control.

RESULTS AND DISCUSSIONS

The genitors were initially tested to confirm the presence of the *Vf* gene. Polymerase Chain Reaction (PCR) analyses confirmed that the Salva cultivar is heterozygous (*Vfvf)*, while the Jonathan cultivar is homozygous recessive (*vfvf*).

As a result of the hybridization process from the combination 21.06 (Salva × Jonathan), a total of 87 seeds were obtained, of which 79 seedlings (90.8%) were suitable for transplanting into individual pots. Among the sprouted hybrids, 67 (84.8%) were selected for molecular testing (Table 2).

Table 2. Progenies of Salva x Jonathan (21.06) cross combination investigated for scab resistance

Hybrid Code	Hybrid combination	AL07		AM19	VFC		Genotype
		Vf	*vf*	*Vf*	*Vf*	*vf*	
21.06.01	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.02	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.03	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.04	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.05	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.06	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.07	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.08	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.09	Salva x Jonathan	-	+	-		+	*vfvf*
21.06.10	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.11	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.12	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.13	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.14	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.15	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.16	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.17	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.18	Salva x Jonathan	-	+	-	-	+	*vfvf*

Hybrid Code	Hybrid combination	AL07		AM19	VFC		Genotype
		Vf	*vf*	*Vf*	*Vf*	*vf*	
21.06.19	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.20	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.21	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.22	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.23	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.24	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.25	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.26	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.27	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.28	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.29	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.30	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.31	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.32	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.33	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.34	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.35	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.36	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.37	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.38	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.39	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.40	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.41	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.42	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.43	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.44	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.45	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.46	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.47	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.48	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.49	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.50	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.51	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.52	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.53	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.54	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.55	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.56	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.57	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.58	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.59	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.60	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.61	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.62	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.63	Salva x Jonathan	-	+	-	-	+	*vfvf*
21.06.64	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.65	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.66	**Salva x Jonathan**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
21.06.67	Salva x Jonathan	-	+	-	-	+	*vfvf*
Pozitive control (C+)	**Salva**	**+**	**+**	**+**	**+**	**+**	***Vfvf***
Negative control	**Jonathan**	**-**	**+**	**-**	**-**	**+**	***vfvf***

Thereafter, all 67 hybrids obtained from controlled crossbreeding were tested by molecular analysis: 35,8% (24 hybrids: 21.06.02, 21.06.06, 21.06.08, 21.06.19, 21.06.21, 21.06.22, 21.06.23, 21.06.28, 21.06.30, 21.06.36, 21.06.40, 21.06.43, 21.06.45, 21.06.47, 21.06.49, 21.06.50, 21.06.51, 21.06.53, 21.06.56, 21.06.59, 21.06.60, 21.06.64, 21.06.65, 21.06.66) were classified as resistant (with heterozygous genotype *Vfvf)* since they presented a signal of amplification of 526 bp (AM19), 870 bp (Al07) and 286 bp (VFC). The remaining 64.2% (43 hybrids) were shown to be homozygous recessive, making it possible to amplify a specific band with a size of 823 bp (AL07) and 484 bp, 646 bp (VFC) corresponding to recessive gene (*vf*).

The 24 hybrids that inherited the *Vf* gene were transplanted into the hybrid field (Figure 2). According to phenotypic observations of each hybrid, an encouraging finding was noted: none of the hybrids in the field were infected with apple scab during the first year of cultivation, even though a nearby commercial apple orchard showed symptoms of scab.

Figure 2. The planting area of hybrids that have inherited the *Vf* resistance gene.

CONCLUSIONS

Following the screening for the *Vf* resistance gene, the overall results revealed that out of a total of 67 apple progenies, 24 (38.5%) were classified as resistant, exhibiting a heterozygous genotype (*Vfvf*), while the remaining 43 (64.2%) were classified as sensitive to scab, possessing a homozygous recessive genotype (*vfvf*). Thus, the selected hybrids were used as biological material for establishing the hybrid field at FRDS Bistrița. Additionally, MAS has once again proven to be an effective technique for identifying the presence of the *Vf* gene at an early stage of hybrids selection.

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Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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