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Genetic similarity of four strawberry cultivars in respect to *Verticillium* wilt susceptibility under *in vitro* selection

Genetyczne podobieństwo czterech odmian truskawki w odniesieniu do podatności na wertycyliozę w warunkach selekcji *in vitro*

Summary. *Verticillium* wilt caused by pathogenic soil fungus – *Vertcillium dahliae* Kleb. is one of the most serious strawberry diseases not treated chemically. The strawberry is a species more or less susceptible to *Verticillium* sp., hence the need to select new genetically resistant cultivars. In this study, susceptibility of four strawberry cultivars to *Verticillium* wilt was examined under *in vitro* selection conditions as regards the genetic similarity of selected resistant plants. Microplants of 4 strawberry subclones obtained by *in vitro* cloning of each cultivar were inoculated *in vitro* with liquid mycelial homogenate of the pathogen. Development of disease symptoms was observed after 15, 30, 45, 60 and 75 days post inoculation. Results revealed differences of susceptibility to *V. dahliae* which depended on the subclone. The least susceptible to *Verticillium* wilt turned out to be 'Plena' subclone, followed by the 'Elsanta', 'Feltar' and 'Teresa' subclones. The highest genetic similarity of 59% was found between 'Elsanta' and 'Teresa' subclones, which after 75 days from inoculation were also characterized by a very similar percentage of resistant plants.

Key words: tissue culture, *Fragaria* × *ananassa*, *Verticillium dahliae*, UPGMA, pathogenesis, ISSR markers

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INTRODUCTION

Verticillium wilt is a dangerous soil disease of strawberries caused by the polyphagous fungus *Verticillium dahliae* Klebahn belonging to the mitospore fungi (*Fungi imperfecti*) [Kurze et al. 2001, Masny and Żurawicz 2008, Żebrowska 2010, Sowik et al. 2016]. The pathogen was first described in 1913 by Klebahn [Rataj-Guranowska and Drapikowska 2015]. Meszka and Bielenin [2009] report that losses in strawberry plantations caused by *Verticillium* wilt can reach up to 80%. The first symptom on infected plants is the loss of turgor, followed by yellowing and wilting of the initially older leaves located on the edges of the crown, which is the most characteristic symptom of *Verticillium* disease [Sowik et al. 2015]. The youngest leaves growing from the center of the crown remain healthy for the longest time [Profic-Alwasiak 2000]. The last stage of the disease is the complete destruction of the root system, which results in tissue necrosis and dieback of the plant [Sowik et al. 2015]. The disease spreads along the plantation with contaminated agricultural equipment, through the transfer of pathogen-containing soil, contaminated plant material or through irrigation systems. The low effectiveness of treatments such as crop rotation and restrictions on the use of harmful chemicals for soil fumigation (containing methyl bromide and chloropicrin) make combating *Verticillium* disease on strawberry plantations very difficult or even impossible. The use of pesticides additionally increases production costs, and the harmful substances contained in them can accumulate in the soil, poisoning the environment. An alternative for producers is the cultivation of varieties with high tolerance or resistance to diseases. Obtaining such plants by conventional methods consists in crossing material with a known, high resistance to a given factor and subjecting the F_1 generation seedlings obtained in this way to selection pressure of the pathogen [Żurawicz 1997]. However, it is a long and laborious process. Thanks to traditional methods of breeding and selection, many cultivars with a larger size have been obtained tolerance to pathogens. Among the Polish cultivars, the cultivar 'Grandarosa' grown at the Institute of Horticulture in Skierniewice deserves attention, showing greater tolerance to *Verticillium* wilt compared to the cultivars 'Honeoye' and 'Elsanta' [Masny et al. 2015]. The use of modern biotechnological methods in resistance breeding, such as *in vitro* cultures and molecular diagnostics, significantly shortens the process of selecting more tolerant forms to this dangerous soil pathogen. The advantage of selection under *in vitro* culture is the possibility of evaluating a very large number of plants in a small laboratory space, independent of the seasons. The selected plants obtained in this way can be used for further crossings as parent plants and a source of resistance genes for progeny plants. Selection *in vitro* significantly speeds up the breeding process compared to the conventional breeding method, while ensuring reliable results regarding the resistance reaction in selected forms, which persists in field conditions and in subsequent generations.

The tested plants can be selected under aseptic *in vitro* culture conditions for both biotic factors (resistance to fungal pathogens) and abiotic factors (drought, substrate salinity, heavy metals, low temperature). Selection in *in vitro* cultures can only be carried out on traits manifested at the cell, callus or seedling level. In order to select microplants for biotic stresses, the selection factor may be toxic metabolites of the pathogen, most often post-culture filtrate. In the case of selection for abiotic factors, various additions to the medium are used, e.g. during selection for resistance to drought or salinity, compounds increasing the osmotic pressure in the culture, such as NaCl, $AlCl_3$, PEG and

others, are used [Dziadczyk et al. 2003, Michalik 2009, Chandra et al. 2010, Rai et al. 2011, Shokaeva et al. 2011]. It was experimentally found that selection *in vitro* is a useful method in breeding work, and the results obtained are similar to the results of selection in field conditions [Żebrowska 2010]. Thanks to selection under *in vitro* cultures, many varieties and breeding clones of strawberry, tolerant or resistant to *Alternaria alternata* [Takahashi et al. 1992], *Botritis cinerea* [Orlando et al. 1997, Shokaeva et al. 2011], *Colletotrichum acutatum* [Damiano et al. 1997, Maas 2004, Hammerschlag et al. 2006], *Fusarium oxysporum* [Toyoda et al. 1991], *Phytophthora cactorum* [Maas et al. 1993, Sowik et al. 2001, Shokaeva et al. 2011, Marecki and Żebrowska 2021], *P. fragariae* [Maas et al. 1993], *P. nicotianae var. parasitica* [Amimoto 1992], *Rhizoctonia fragariae* [Orlando et al. 1997] and on *Verticillum dahliae* [Sowik et al. 2001, Sowik et al. 2003, Żebrowska et al. 2006, Żebrowska 2010, 2011]. Selection *in vitro* can also be successfully used in research on the mechanism of inheritance of resistance to pathogens [Żebrowska et al. 2006]. Strawberries resistant or tolerant to the factor used during selection are then acclimated to natural growing conditions and subjected to further analysis.

A great support in modern strawberry breeding is molecular diagnostics using molecular markers to help select the desired traits based on the DNA analysis of the individual. Accurate phenotypic and molecular characterization of parental forms from a set of genetic resources enables the creation of an appropriate crossbreeding program and the avoidance of undesirable crossbreeding effects [Korbin 2007]. There are many markers that allow detecting variability in plants at the DNA level [Korbin 2011]. Among the various molecular techniques to assess the genetic similarity between strawberry genotypes, the markers AFLP (Amplified Fragment Length Polymorphism), RAPD (Randomly Amplified Polymorphic) DNA and ISSR (Inter Simple Sequence Repeat) are most often used [Whitaker 2011]. In research work on diversity in strawberry ISSR markers were used by Korbin et al. [2002], Arnau et al. [2003], Kuras et al. [2004], Hussein et al. [2008], Debnath and Ricard [2009], Morales et al. [2011] and Nunes et al. [2013].

Thanks to the use of molecular markers in strawberry research, the most significant progress has been achieved in the field of resistance breeding. An important step in this field was the identification of the *Rpf1* locus – the single dominant gene for resistance to the fungus *Phytophthora fragariae* var. *fragariae*, causing strawberry red root rot. Seven RAPD markers associated with the *Rpf1* locus were identified during the study of the F_1 population from the cross-breeding program of resistant varieties, the closest of which is located 3.0 cM from the gene. These markers have found wide application in modern resistance breeding [Whitaker 2011, Chandler et al. 2012].

Genetic factors determining strawberry resistance to *Verticillium* wilt are not fully understood. In the domestic and foreign literature, there is no clear answer whether one or many genes are responsible for immunity. Within this species there are only forms more or less susceptible to *Verticillium* wilt. Strawberry defense mechanisms are also not fully understood and explained. In order to explain them, many detailed studies on the interaction between the plant and the pathogen are needed. In response to biotic stress, strawberry exhibits similar mechanisms as in other plants. It is assumed that this plant recognizes pathogens and responds to them appropriately based on innate immunity, which includes all the cellular and molecular mechanisms that the plant has to defend itself against the pathogen [Amil-Ruiz et al. 2011].

Regarding the importance of *in vitro* cultures and molecular diagnostics in modern resistance breeding, this study was undertaken to recognize the susceptibility of four

strawberry cultivars to *Verticillium dahliae*. *In vitro* selection was used for this evaluation with the molecular analyses at DNA level of resistant forms.

MATERIAL AND METHODS

The research material consisted of four strawberry cultivars: 'Elsanta' = ['Gorella' × 'Holiday'], 'Feltar' = [('Senga Tigaiga' × 'Merton Dawn') S_1], 'Plena' = ['Senga Sengana' × 'Merton Dawn'] and 'Teresa' = ['Redgauntlet S_1' × 'Senga Sengana S_1'] from the collection of the Department of Genetics and Horticultural Plant Breeding at the Experimental Farm in Felin belonging to the University of Life Sciences in Lublin. The Dutch cultivar 'Elsanta' in field conditions is susceptible to diseases of the root system caused by fungi of the genus *Verticillium* sp. The susceptibility of other cultivars to this pathogen has not been thoroughly investigated so far.

Preparation of the starting material

In order to obtain the starting material for *in vitro* selection, each cultivar was cloned under aseptic *in vitro* culture conditions from the ends of young runners 3–4 cm long, collected in the number of 50 pieces from plants of each donor cultivar. These ends were the primary explants used to establish the first stage of the culture. The collected plant material was rinsed on sieves under running water for 15 minutes. It was then subjected to surface decontamination for 15 minutes in a 1% sodium hypochlorite solution prepared in sterile distilled water with the addition of a wetting agent. Decontaminated primary explants after washing them three times in sterile distilled water under aseptic conditions under a laminar flow chamber (POLON KL-21) were individually plated on Murashige and Skoog (MS) medium [Murashige and Skoog, 1962]. The medium was supplemented with 1 mg dm^{-3} IAA (indolyl-3-acetic acid), 1 mg dm^{-3} BAP (6-benzylaminopurine) and 0.01 mg dm^{-3} GA_3 (gibberellic acid, tertiary derivative), solidified 7,5 g dm^{-3} agar, with a fixed pH of 5.7. The medium was decontaminated in an autoclave for 20 min at 121°C and 0.1 MPa pressure. The test tubes with primary explants were transferred to the cultivation room (phytotron) where, under controlled environmental conditions (photoperiod 16 h day/8 h night, temperature 20°C, lighting intensity 30 μmol m^{-2} s^{-1}), the first stage of *in vitro* culture was carried out for 6 weeks by monitoring and removing infections (Fig. 1a). As the explants multiplied, they were separated into individual microshoots and transferred to fresh medium of the same composition (2nd stage of culture). The second stage of culture was completed after obtaining a minimum of 300 microshoots for each cultivar. The obtained microshoots were rooted on MS medium without growth regulators. In this way, four subclones, i.e.: 'Elsanta', 'Feltar', 'Plena', 'Teresa' were obtained *via in vitro* propagation from the strawberry cultivars tested. Each subclone represented 1 cultivar and included 300 microplants for *in vitro* selection.

Preparation of the selection factor

In order to evaluate the susceptibility of the obtained subclones to *Verticillium dahliae* under *in vitro* selection, the selection factor was prepared. The selection factor used for *in vitro* infection (inoculation) of microplants was a pure culture of the pathogen – *Verticillium dahliae* Kleb. from the Bank of Plant Pathogens in Poznań, catalog number 1093.

The pathogen was cultivated on a Potato Dextrose Agar (PDA) medium, which was supplemented with 300 g dm^{-3} of potatoes, 20 g dm^{-3} of agar and 20 g dm^{-3} of glucose in the distilled water solution. The medium was decontaminated in a pressure autoclave at the temperature of 121°C, under the pressure of 0.1 MPa for 20 min. Then, 100 mg dm^{-3} of streptomycin was added to the medium under the laminar airflow chamber, thoroughly mixed and poured into aseptic Petri dishes with a diameter of 11 cm, leaving the medium to solidify. The pathogen was inoculated under a laminar flow chamber. The pathogen was cultured on a previously prepared PDA medium in the dark for 3 weeks at a temperature of 18–20°C (Fig. 1b).

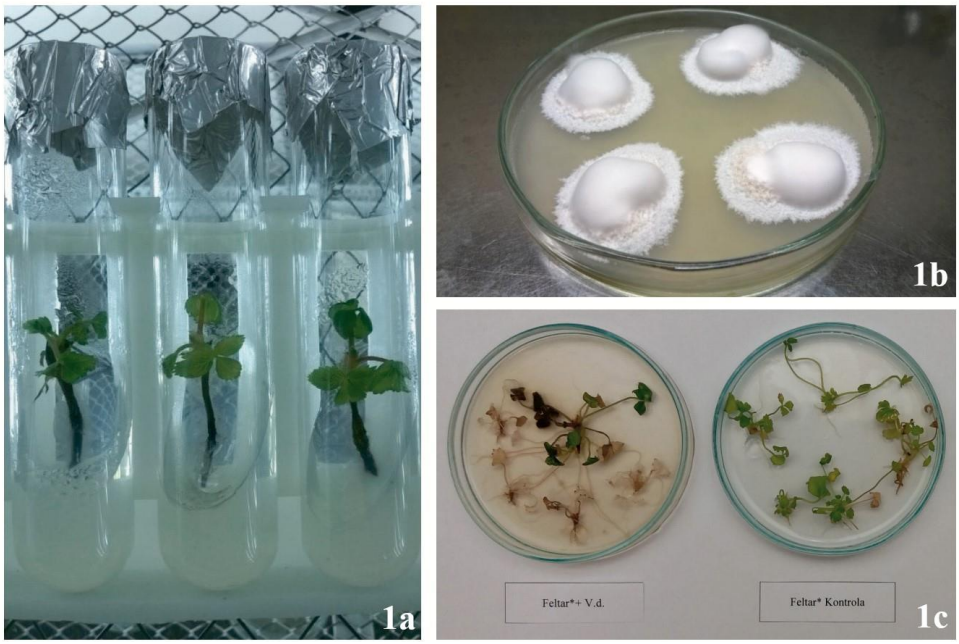

Fig.1. a) First stage of in vitro strawberry subclones culture; b) three-week-old culture of *Verticillium dahliae* on PDA medium; c) differences between main ('Feltar'* + V.d.) and control ('Feltar'* Kontrola) samples after 75 dpi

Preparation of inoculum to infect plants

A three-week-old culture of the pathogen was flooded under aseptic conditions with 50 ml of sterile distilled water. The mycelial suspension obtained in this way was transferred to a larger, sterile glass vessel with a volume of 900 ml. The suspension was then homogenized and diluted with sterile distilled water in a ratio of 1 : 10 (V/V) proportion to obtain the appropriate conidia density (10^5 ml^{-1}).

From the obtained homogenate, four 100 ml inoculum portions were prepared in smaller, sterile glass vessels with a volume of 250 ml. The inoculum prepared in this way was a liquid homogenate of the pathogen's live mycelium to infect microplants.

***In vitro* inoculation of microplants in the pathogen's live mycelium homogenate**

Well-rooted microplants with at least 4 leaves were the starting material for selection. Subclonal plants were infected under aseptic conditions by immersing them

in the inoculum for 1 minute, after previously damaging the roots by cutting them with a scalpel to a length of about 1.5 cm. 100 ml of the homogenate was used to inoculate 25 microplants. Infected microplants were placed on Petri dishes with previously prepared agar medium without minerals and sucrose (7.5 g of agar dissolved in 1000 ml of distilled water, pH 5.7). Five microplants were placed on one Petri dish. The experiment was set up in triplicate for each subclone. One repetition consisted of 100 plants. Observations were carried out for 75 days. A control sample was also prepared for each subclone. Well-rooted plants at the stage of at least 4 leaves, after prior damage to the roots (by cutting them to a length of about 1.5 cm with a scalpel), were immersed in 100 ml of sterile distilled water for 1 minute (mock inoculation). The plants were then placed in sterile Petri dishes with agar medium. Five plants were placed on one Petri dish. For each subclone, the control consisted of 100 plants. The plates with infected microplants and the control were placed in a culture room under controlled environmental conditions to observe the development of disease symptoms within 75 days post inoculation.

Observation of the course of pathogenesis

The development of disease symptoms in the form of progressive leaf chlorosis was carried out in 5 observation periods: 15, 30, 45, 60 and 75 days post inoculation (dpi) – Figure 1c. According to the methodology provided by Sowik et al. [2001], Żebrowska [2011] and Sowik et al. [2015] the degree of plant infection was assessed using a five-point rating scale of susceptibility (description is given in the legend to the Table 1).

Analogous observations were carried out on control microplants, observing the development of leaf chlorosis and their death as a result of mock inoculation.

The susceptibility of the tested subclones to the selection factor was determined using the disease index (DI) for the extent of infection development using the McKinney [1923] disease index. The extent of infection (DI in %) was determined, which was used to observe the course of pathogenesis in individual subclones infected with *V. dahliae*. The disease index was calculated according to the formula:

$$DI = (\Sigma vn)/(NV) \times 100$$

where: DI – disease index,
v – numerical value of the infection class,
n – number of plants in a given observation period in a given class,
N – total number of infected plants in a given sample,
V – numerical value of the highest class.

The numerical data were statistically analyzed by analysis of variance with the Statistica 13.1 program (Statistica 13.1. Stat-Soft.Polska. 2020). The significance of the differences between the values of the examined features was estimated with the Student's t-test and the Duncan's multiple range test at the significance level of $\alpha = 0.05$.

Molecular analyses

In this research molecular analyses at DNA level of resistant forms selected *in vitro* from four strawberry subclones were done to assess their genetic similarity in respect to *Verticillium* wilt susceptibility. After the *in vitro* selection, from 10 randomly selected microplants of each subclone that survived the selection pressure of the pathogen and did not show symptoms of infection (completely resistant), DNA was isolated using the CTAB method described by Gawel and Jarret [1991]. ISSR markers were used to assess genetic similarity at the DNA level of resistant plants. Sixteen markers derived from Sigma-Aldrich

were used in this experiment. DNA purity and concentration were determined using a Thermo Scientific NanoDrop 2000 spectrophotometer. DNA amplification was performed in the TProfessional Basic Gradient Biometry thermal cycler in a final reaction volume of 15 µL for each reaction, which contained 1.5 µL PCR buffer, 1.2 µL dNTP (10 mM dNTP MIX), 0.7 µL oligonucleotide primer, 0.9 µL $MgCl_2$ (25 mM), 0.15 µL of Taq DNA polymerase (Dream Taq DNA polymerase 5 U / µL) and 3 µL of template DNA. Each of the 35 cycles of the polymerase chain reaction (PCR) consisted of 3 stages: 45 seconds at 94°C (DNA denaturation), 1 min at the temperature of primer annealing, 2 min at 72°C (DNA elongation). The resulting DNA amplification products were separated on a 1.5% agarose gel containing 0.1% ethidium bromide under 1 × TBE buffer. The electrophoretic separation of the amplified DNA fragments was carried out for 90 min at 100V. Ethidium bromide-stained DNA fragments were visualized on the gel under ultraviolet (UV) light. The electrophoresis results were digitized using with the Gene Snap Syngene software and then processed with the GeneTools Syngene program. The dendrogram showing the genetic similarity of resistant plants at the DNA level was generated with the Past3 program using the UPGMA (Unweighted Pair Group with Arithmetic Mean) method.

RESULTS

The course of pathogenesis after *in vitro* infection of microplants with a liquid homogenate of *Verticillium dahliae* mycelium together with the values of the disease index (DI) for the tested strawberry subclones and control plants were presented in Table 1–4.

Symptoms of disease on microplants developed gradually, becoming visible 15 days post inoculation. In all subclones inoculated in the mycelial homogenate, the disease symptoms (leaf chlorosis) occurred faster and more violently than in the case of control plants (Tabs 1–4), and the values of the disease index were significantly higher at subsequent observation dates. Only in the 'Feltar' subclone, no significant differences in the values of this indicator were found between the third and fourth observation dates. After 15 dpi (1st observation period), the lowest disease index was found in the 'Plena' subclone (DI = 2.35%), with the highest percentage of microplants without symptoms of *Verticillium* disease (91.16%). The further course of pathogenesis in this subclone was the mildest among all the tested subclones, with the lowest values of the disease index at subsequent observation dates, reaching significantly the lowest mean disease index after 75 dpi (DI = 12.84%) with the highest percentage of microplants without disease symptoms (39.30%) – Table 3. In the remaining subclones, the pathogenesis proceeded with higher values of the disease index at subsequent observation dates, reaching the mean values of this index not significantly different from each other after 75 dpi for the subclones 'Elsanta', 'Feltar' and 'Teresa' (respectively; DI = 29.85%; 33.38% and 34.02%) – Tables 1, 2, 4. The most similar course of pathogenesis was observed in subclones 'Feltar' and 'Teresa', in which the highest values of the disease index (DI = 14.03% and 9.56%, respectively) and the highest percentage of microplants with three and four leaves infected were observed already in the first observation period (Tabs 2 and 4). Analyzing the percentage of microplants without disease symptoms 75. dpi, it was found that in the 'Elsanta' and 'Teresa' subclones it was very similar and amounted to about 9%, while in the 'Feltar' subclone it was slightly higher, at about 15% , and in the 'Plena' subclone – the highest (as stated above). The control subclones of all tested cultivars (with the exception of the 'Plena' control subclone) had significantly lower mean values of the disease index in relation to the subclones inoculated with *V. dahliae* (Tabs 1–4).

Table 1. Contribution of microplants (%) with leaf chlorosis and disease index (DI) values (%) of the strawberry 'Elsanta' subclone

Subclone	Scale of susceptibility (0–4)	Days post inoculation (dpi)					Mean DI (%)
		15	30	45	60	75	
Elsanta	0	79.67	46.67	27.33	17.33	8.33	$29.85^{a2)\ a3)}$
	1	17.33	33.33	34.67	27.00	16.67	
	2	1.67	10.67	20.67	27.00	28.00	
	3	0.67	5.33	8.33	14.33	19.33	
	4	0.67	4.00	9.00	14.33	27.67	
DI (%)		$6.00^{e1)}$	$19.44^{d1)}$	$30.11^{c1)}$	$39.64^{b1)}$	$54.06^{a1)}$	
Elsanta control	0	97.00	94.00	93.00	92.00	90.00	$7.35^{b2)}$
	1	3.00	6.00	5.00	3.00	4.00	
	2	0.00	0.00	2.00	2.00	2.00	
	3	0.00	0.00	0.00	3.00	3.00	
	4	0.00	0.00	0.00	0.00	1.00	
DI (%)		$0.25^{d1)}$	$4.5^{c1)}$	$5.75^{c1)}$	$10.25^{b1)}$	$14.00^{a1)}$	

Scale of susceptibility: 0 – no leaf chlorosis; 1 – chlorosis involving one leaf (25%);
2 – chlorosis involving two leaves (50%);
3 – chlorosis involving three leaves (75%); 4 – chlorosis involving four or more leaves (100%).
[1)] Duncan's test for Disease index values of subclones at days post inoculation.
[2)] Student's t-test for mean values of disease index of main and control sample.
[3)] Duncan's test for mean values of disease index between main samples of subclones tested.
Values followed by the same letter do not differ significantly at $P \leq 0.05$.

Table 2. Contribution of microplants (%) with leaf chlorosis and disease index (DI) values (%) of the strawberry subclone 'Feltar'

Subclone	Scale of susceptibility (0–4)	Days post inoculation (dpi)					Mean DI (%)
		15	30	45	60	75	
Feltar	0	59.67	33.00	23.00	16.67	14.67	$33.38^{a2)\ a3)}$
	1	23.67	34.33	33.33	28.33	24.33	
	2	11.33	18.00	20.33	22.00	18.00	
	3	3.00	9.00	12.00	16.00	15.00	
	4	2.33	5.67	11.33	17.00	28.00	
DI (%)		$14.03^{d1)}$	$26.25^{c1)}$	$34.44^{b1)}$	$42.08^{b1)}$	$50.08^{a1)}$	
Feltar control	0	96.00	95.00	95.00	94.00	94.00	$6.65^{b2)}$
	1	3.00	3.00	2.00	2.00	1.00	
	2	1.00	2.00	2.00	2.00	1.00	
	3	0.00	0.00	1.00	1.00	2.00	
	4	0.00	0.00	0.00	1.00	2.00	
DI (%)		$3.25^{d1)}$	$4.25^{cd1)}$	$5.50^{c1)}$	$8.50^{b1)}$	$11.75^{a1)}$	

Explanation as in Table 1.

Table 3. Contribution of microplants (%) with leaf chlorosis and disease index (DI) values (%) of the strawberry subclone 'Plena'

Subclone	Scale of susceptibility (0–4)	Days post inoculation (dpi)					Mean DI (%)
		15	30	45	60	75	
Plena	0	91.16	84.36	78.58	63.62	39.30	12.84 $^{a2) b3)}$
	1	7.14	11.56	13.26	17.34	13.39	
	2	1.70	3.74	5.44	11.56	12.58	
	3	0.00	0.34	2.38	5.10	16.25	
	4	0.00	0.00	0.34	2.38	17.94	
DI (%)		2.35$^{e1)}$	4.36$^{d1)}$	7.05$^{c1)}$	19.97$^{b1)}$	36.45$^{a1)}$	
Plena control	0	98.85	95.93	94.55	90.89	88.06	2.96$^{a2)}$
	1	1.15	3.22	2.87	1.97	0.85	
	2	0.00	0.85	1.43	3.74	2.55	
	3	0.00	0.00	1.15	2.55	4.77	
	4	0.00	0.00	0.00	0.85	3.77	
DI (%)		0.29$^{c1)}$	1.09$^{c1)}$	1.96$^{cb1)}$	3.44$^{b1)}$	8.01$^{a1)}$	

Explanation as in Table 1.

Table 4. Contribution of microplants (%) with leaf chlorosis and disease index (DI) values (%) of the strawberry subclone 'Teresa'

Subclone	Scale of susceptibility (0–4)	Days post inoculation (dpi)					Mean DI (%)
		15	30	45	60	75	
Teresa	0	70.33	31.67	19.33	12.33	9.33	34.02$^{a2) a3)}$
	1	21.33	42.33	37.67	28.00	20.00	
	2	5.67	12.00	19.33	27.67	28.67	
	3	1.00	6.00	9.67	10.67	10.67	
	4	1.67	8.00	14.00	21.33	31.33	
DI (%)		9.56$^{e1)}$	26.58$^{d1)}$	36.31$^{c1)}$	44.67$^{b1)}$	53.00$^{a1)}$	
Teresa control	0	97.00	97.00	96.00	95.00	93.00	5.40$^{b2)}$
	1	2.00	2.00	1.00	2.00	1.00	
	2	1.00	1.00	3.00	1.00	2.00	
	3	0.00	0.00	0.00	2.00	3.00	
	4	0.00	0.00	0.00	0.00	1.00	
DI (%)		2.50$^{c1)}$	2.50$^{c1)}$	3.75$^{c1)}$	6.50$^{b1)}$	11.75$^{a1)}$	

Explanation as in Table 1.

Table 5. DNA polymorphism of the strawberry subclones using ISSR primers

Sequence (5′–3′)	Number of loci			
	total	polymorphic	%P	size range (bp)
VBVACACACACACACAC	7	7	100.0	400–2000
HVHTGTTGTTGTTGTTGT	6	5	83.3	600–3000
BDBCACCACCACCACCAC	8	8	100.0	300–1500
GAAGAAGAAGAAGAAGAA	5	5	100.0	1000–1500
ATGATGATGATGATGATG	6	3	50.0	300–3000
GATAGATAGATAGATAGATA	7	7	100.0	700–3500
GACAGACAGACAGACAGACA	6	5	83.3	300–2500
AGTGAGTGAGTGAGTG	7	7	100.0	300–2500
Mean	6.5	5.9	–	–
Totality	52	47	89.58	300–3500

Explanation of symbols: H = A + T + C, B = G + T + C, D = G + A + T, V = G + A + C.
%P – percentage of polymorphism.

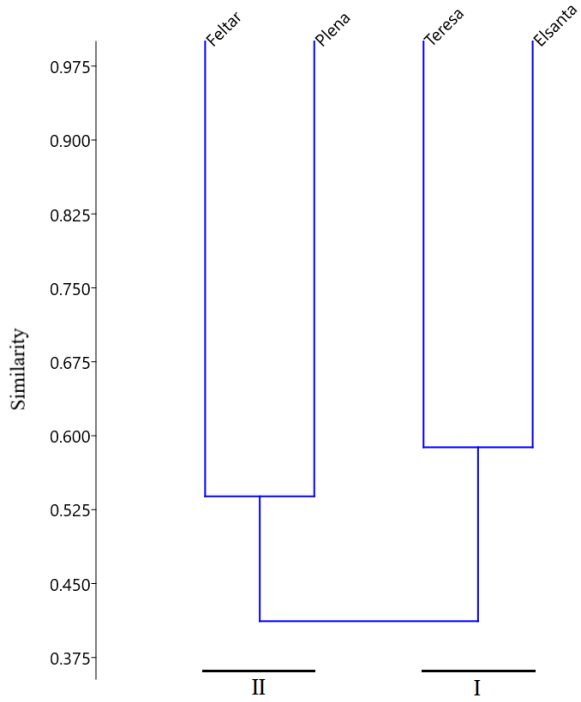

Fig. 2. Genetic similarity of four strawberry subclones

DNA amplification products were observed with the use of eight primers. Results of amplification were given in Table 5. The analysis of the UPGMA dendrogram plotted on the basis of the similarity matrix made it possible to determine the genetic similarity at the DNA level between the plants selected from the subclones, which did not show disease symptoms and were considered resistant. In this research, eight of the 16 tested ISSR primers, generated amplification products with a percentage of polymorphism of 89.58%. A single primer participated in the synthesis of 3 to 8 polymorphic products (average 5.9). Of the 52 DNA fragments generated, 47 were polymorphic. Cluster analysis of the UPGMA dendrogram (Fig. 2) showed the presence of two main groups of genetic similarity. The first group (I) and the second group (II) were similar to each other in 41%. The first group (I) consisted of 'Teresa' and 'Elsanta' subclones with genetic similarity of 59%. The similarity in the second group (II), which included 'Feltar' and 'Plena' subclones, was 53%.

DISCUSSION

Strawberry is a species more or less susceptible to *Verticillium* sp., hence the need to select new genetically resistant cultivars. Nowadays, it is one of the most important directions in the breeding of this species. Using modern biotechnological methods, such as *in vitro* cultures or molecular diagnostics, resistance breeding has now become more effective compared to conventional methods. The use of tissue cultures for strawberry (*Fragaria* × *ananassa* Duch.) resistance selection in this work is justified by both domestic and foreign publications. Sowik et al. [2008] and Żebrowska [2010], using selection for the pathogenic fungus *Verticillium dahliae* under *in vitro* culture, tested the resistance of strawberry cultivars and clones. Żebrowska [2010] wrote that the susceptibility of the tested strawberry cultivars under *in vitro* conditions was similar to the susceptibility of these cultivars under field conditions, and selection *in vitro* can be successfully used in breeding programs.

Standard MS medium recommended for strawberry micropropagation was used to propagate the tested cultivars under *in vitro* culture in order to obtain the starting material for selection. A similar medium was used by Żebrowska [2011]. Sowik et al. [2015] used the basic medium according to Boxus with the addition of macroelements according to the composition of Knop's medium and microelements and vitamins according to the composition of the MS medium with the addition of glycine (1 mg dm^{-3}), inositol (100 mg dm^{-3}), glucose (40 g dm^{-3}), IBA (1 mg dm^{-3}), BA (0.1 mg dm^{-3}), GA_3 (0.1 mg dm^{-3}).

The pathogen *Verticillium dahliae* was cultured on PDA medium, similarly to Żebrowska et al. [2006], Żebrowska [2010, 2011]. Sowik et al. [2001] maintained the *V. dahliae* pathogen on 2% malt extract and Czapek – Dox medium. All the above-mentioned authors used 3–4 week-old cultures of pathogens to obtain the inoculum. The 1 : 10 V/V dilution of the inoculum used in this study was justified by the Sowik et al. [2001] experiment in which cultivar differences in the selection factor were most visible in plants inoculated in mycelium homogenate diluted 10 and 1000 times.

Analyzing the course of pathogenesis after infection with *V. dahliae*, it can be concluded that the intensity of microplant dieback varied in subsequent observation dates and dependent on the tested subclone, which indicates the genetic background of the immune response of a given subclone to the pathogen. On plants inoculated with

V. dahliae, a gradual chlorosis of the leaves occurred, leading to the death of the entire microplants *in vitro*. Chlorosis appeared first on the oldest leaves, with the passage of time covering the youngest leaves. The appearance of symptoms in this order is confirmed by studies conducted by Jęcz and Korbin [2010] and Żebrowska [2010]. The first symptoms of *Verticillium* disease, such as loss of turgor and yellowing of leaves, were observed on microplants of all the subclones studied in the first observation period, i.e. 15 days post inoculation. In the subsequent observation dates, all the subclones studied continued to develop the disease, which varied depending on the genotype. Żebrowska [2011] observed similar differences in the development of disease symptoms in the 'Teresa' and 'Filon' subclones. Seventy-five days post inoculation, a significant proportion of microplants were severely affected and died. However, in all tested subclones there were plants that survived the pressure of the pathogen. Similar selection results were achieved by Sowik et al. [2008], Żebrowska [2010, 2011]. Disease index values differed significantly, except for the 'Plena' subclone, between the main and the control sample. The obtained results suggest that the pathogenic effect of *V. dahliae* was the factor causing a marked increase in the dieback of microplants in the main sample. The 'Teresa' subclone turned out to be the most susceptible to *V. dahliae*. Slightly lower susceptibility was observed in 'Feltar' and 'Elsanta' subclones, respectively. The lowest susceptibility to *V. dahliae* was shown by the 'Plena' subclone. None of the analyzed subclones showed complete resistance to the selection factor. The obtained results may suggest that there is no single mechanism of resistance to *V. dahliae* in the strawberry species and the resistance is specific for each cultivar. Shaw et al. [1996], as well as Żebrowska et al. [2006] write that the relative tolerance to *V. dahliae* on the one hand appears to be polygenic and controlled by additive genes, and on the other hand may be controlled by a single, partially dominant gene. This is confirmed by selection works towards resistance to pathogens. Both domestic and foreign authors divide the tested cultivars in terms of susceptibility to the selection factor into resistant, moderately susceptible and susceptible. The cultivar 'Elsanta' is considered to be very susceptible to diseases caused by soil-borne pathogens. This is written by Meszka et al. [2005], who assessed the susceptibility of several strawberry cultivars in field conditions and included the 'Elsanta' cultivar in the group of genotypes characterized by medium or high susceptibility to *Verticillium* wilt. During four years of observation, the infection index of this cultivar ranged from 26.6% to 42%. The susceptibility results obtained in this experiment for the 'Elsanta' subclone (29.85%) are confirmed in the above-mentioned studies.

Żebrowska [2011] as a result of the research on susceptibility to *Verticillium* wilt, included the 'Teresa' cultivar in the group of moderately susceptible genotypes. The percentage of microplants with total chlorosis after 75 dpi reached 76.27%. In the case of the 'Plena' subclone, the highest tolerance to *V. dahliae* was observed. In this experiment, this subclone showed the lowest percentage of microplants completely infected after 75 days from inoculation, amounting to 17.94%, and the lowest average disease index (DI = 12.84%) among the tested subclones. This selection result may be related to the pedigree of the 'Plena' cultivar, which comes from the 'Senga Sengana' cultivar known for its low susceptibility to *Verticillium* wilt [Meszka et al. 2005]. Different results for susceptibility to *Verticillium* wilt in the breeding lines of the Plena cultivar from self-pollination ('Plena S_1') and from free pollination ('Plena o.p.') were obtained by Żebrowska et al. [2006]. Both tested breeding lines showed a high percentage of plants completely infected on day 75 post inoculation, which reached the value of 91.50% in the breeding line 'Plena S_1' and 93.00% in the breeding line 'Plena o.p.'.

The molecular analysis of microplants that survived the pressure of the pathogen without disease symptoms, with the use of ISSR markers, applied in the current research, is justified in the work of Mahmoud et al. [2017]. The PCR-based ISSR technique is simple and a rapid technique used to detect genetic variation. This technique has been used in the study of the strawberry genome [Korbin 2007]. Strawberry genetic diversity using ISSR markers was studied by Korbin et al. [2002], Debnath et al. [2008] and Morales et al. [2011].

The choice of the molecular analysis method was made on the basis of the analyzed literature data. The optimal primer sequences used for the course of the reaction were highly specific to their complementary sequences in the DNA template. Our results of the molecular analyses were in accordance with those obtained by Kaleybar et al. [2018], where polymorphism of strawberry DNA amplification products was of 96.5%.

The dendrogram generated on the basis of the molecular analyzes determined the genetic similarity between the tested strawberry cultivars. Cluster analysis confirmed the earlier results obtained during *in vitro* selection. These subclones, which were characterized by a very similar contribution of plants without disease symptoms after selection ('Teresa' and 'Elsanta'), also showed the greatest genetic similarity at the DNA level. The 'Feltar' and 'Plena' subclones, characterized by a lower genetic similarity, were also less similar to each other in terms of the contribution of plants without disease symptoms after the selection was completed. In this research the genetic similarity between cultivars tested ranged from 41% to 59%. These results are consistent with those presented by Morales et al. [2011] and Kaleybar et al. [2018], in which genetic similarity between strawberry cultivars ranged from 30% to 88% and 31% to 70% (respectively).

CONCLUSIONS

The *in vitro* selection procedure used in this study for resistance to *Verticillium dahliae* can be recommended to assess the differentiation of strawberry cultivar susceptibility to this pathogenic factor. Using this procedure, it is possible not only to estimate the average susceptibility of a cultivar to the pathogen, but also to observe the course of pathogenesis in the days following inoculation. This is important for understanding the resistance mechanisms that are gradually activated in the plant as a reaction to infection and for determining the time needed by the plant to launch a defensive reaction against the pathogen. ISSR markers can be successfully used to assess the genetic similarity of selected strawberry cultivars towards resistance to *V. dahliae*. This is evidenced by the high agreement of the results of molecular analyzes with the valuation assessment of strawberry subclones obtained during the selection under *in vitro* conditions. Considering the above, it can be concluded that selection with the use of *in vitro* cultures can be applied as a method of research on the genetics of resistance to pathogens in strawberry.

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