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Oryginalny artykuł naukowy
Original research paper

Ocena wzrostu i stabilności genetycznej agrestu (*Ribes grossularia* L.) rozmnażanego *in vitro* oraz *ex vitro*

Assessment of growth and genetic stability of gooseberry (*Ribes grossularia* L.) propagated *in vitro* and *ex vitro*

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Celem badań była ocena fenotypowa i genetyczna roślin 15 genotypów agrestu, rozmnożonych *in vitro* oraz *ex vitro* przez sadzonki zielne, uprawianych drugi rok warunkach polowych. Silniej rosły krzewy rozmnożone *in vitro*. Ich wysokość i liczba pędów były istotnie większe dla 11 genotypów, a szerokość dla 12 genotypów agrestu. Krzewów, na których pojawiły się owoce było znacznie więcej u roślin mnożonych tradycyjnie. Przeprowadzono analizę stabilności genetycznej klonów pochodzących z kultur *in vitro* pięciu odmian agrestu. Analizowano 13–15 roślin z *in vitro* oraz rośliny mateczne. Liczba produktów generowanych przez pary starterów AFLP wahała się od 33 do 108. Najwyższą całkowitą liczbę produktów amplifikacji uzyskano w wyniku reakcji AFLP dla roślin 'Hinnonmaki Rot' (300), a najmniejszą dla odmiany 'Hinsel' i 'Resika' (262). Zmienność genetyczna w roślinach agrestu *in vitro* wahała się od 1,03% dla 'Captivator' do 10,3% w przypadku 'Hinsel'. Stabilność genetyczną oceniano także przy użyciu markerów ISSR. Wykorzystano rośliny 5 genotypów pochodzące z rozmnażania tradycyjnego oraz *in vitro*. Uzyskano łącznie 2294 produktów amplifikacji, z czego 2,8% było polimorficznych. Wielkość otrzymanych produktów wynosiła od 250 do 2900 pz, w zależności od startera i odmiany. Analiza ISSR-PCR wskazała na różny stopień polimorfizmu – od 0 dla 'Hinnonmaki Rot' i 'Resica' do 11,6% dla odmiany 'Hinsel'.

Słowa kluczowe: AFLP, agrest, DNA, ISSR, kultury *in vitro*, polimorfizm

The aim of the study was to evaluate phenotypically and genetically, 15 gooseberry genotypes plants propagated *in vitro* and *ex vitro* by cuttings grown in the second year in field conditions. The outcome of this was that *in vitro* propagated shrubs were shown to grew more strongly. Their height and the number of shoots were significantly higher for the 11 genotypes and a width of 12 genotypes for gooseberry. However, fruit abundance was greater in much more traditionally multiplied plants. Beyond the aforementioned, Genetic stability analysis of clones derived from *in vitro* cultures of five gooseberry varieties was performed. Herein, 13-15 plants with *in vitro* and mother plants were analyzed. The number of products generated by the AFLP primer pairs ranged from 33 to 108. The highest total number of amplification products was obtained as a result of the AFLP reaction for the plants ‚Hinnonmaki Rot' (300), and the lowest for the varieties ‚Hinsel' and ‚Resika' (262). Genetic variability in gooseberry *in vitro* plants ranged from 1.03% for ‚Captivator', to 10.3% for ‚Hinsel'. Genetic stability was also assessed using ISSR markers. Plants of five genotypes derived from conventional and *in vitro* reproduction were used. Accordingly, a total of 2294 amplification products were obtained, of which 2.8% were polymorphic. The size of the obtained products was from 250 to 2900 bp, depending on the starter and variety. ISSR-PCR analysis showed different degrees of polymorphism - from 0 for the 'Hinnonmaki Rot' and 'Resica' to 11.6% for the ‚Hinsel' variety.

Key words: AFLP, gooseberry, DNA, ISSR, *in vitro* cultures, polymorphism

Introduction

The aim of the study was morphological and phenotypical traits of gooseberry plants propagated in vitro and ex vitro in the second year of field cultivation. The genetic stability of the 5 genotypes in comparison with parental plants was analysed using the AFLP and ISSR techniques.

Material and Methods

The research material consisted of plants representing 8 cultivars and 7 breeding clones of gooseberry that were propagated in vitro and ex vitro from cuttings in the second year of cultivation in the field. The experiment was arranged in an experimental random block system, in 3 replicates, 5 plants per plot. The height and width of shrubs and the number of new shoots were measured, and fruit formation was recorded. The growth parameters were analysed using one-way analysis of variance (Statistica 13.1), separately for each genotype and parameter, and compared using Tukey's test at p=0.05.

Genetic variability was analysed for five gooseberry cultivars: 'Captivator', 'Hinnonmaki Rot', 'Hinsel', 'Invicta', and 'Resika'. For each cultivar, 13-15 plants propagated in vitro in comparison with parent plants were analysed.

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Genetic variability was analysed in two replicates using the AFLP technique according to Zabeau and Vos (1993).

Additionally, plants from cuttings and from in vitro propagation representing five cultivars were analysed for genetic stability using ISSR markers. For each gooseberry cultivar, young leaves were sampled from 10 cuttings, and 10 in vitro derived plants. Genomic DNA was isolated from approx. 100 mg of triturated plant tissue. Isolation was carried out in duplicate using commercial kits: DNeasy® Plant Mini Kit (Qiagen), NucleoSpin® 96 Plant kit (Macherey-Nagel), and DNA Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp.). Isolated DNA samples were analysed spectrophotometrically (Epoch, BioTek). The concentration of genomic DNA was calculated and assessed for purity based on the 260/280 nm ratio. DNA amplification for each genotype was performed twice with each of the 5 selected primers. The total number of ISSR-PCR products, the presence and number of polymorphic products, and the level of polymorphism in the studied genotypes were calculated.

Results

The growth parameters measured for 15 assessed gooseberry genotypes indicated that bushes propagated in vitro were more vigorous (Tab. 1). The height and number of shoots from in vitro propagated plants were significantly greater for 11 genotypes compared to those propagated by conventional cuttings, while the width of bushes was significantly greater for 12 genotypes. The percentage of bushes with fruits was higher in ex vitro plants in11 gooseberry genotypes.

The number of products generated using the AFLP primer pairs ranged from 33 to 108 (mean 52.2). The total number of amplification products was the highest for 'Hinnonmaki Rot' plants (300) and the lowest for 'Hinsel' and 'Resika' (262). Genetic variability analysed by AFLP for in vitro propagated plants differed for individual varieties and ranged from 1.03% for 'Captivator' to 10.3% for 'Hinsel'. In 'Hinsel' all 5 pairs of AFLP-PCR primers generated polymorphic products. For other varieties, polymorphic products were obtained in reactions with 2–3 pairs of primers (Tab. 2, Fig. 1).

Tabela 1
Table 1

Wysokość, szerokość, liczba pędów i procent roślin z owocami u krzewów agrestu rozmnożonych *in vitro* oraz *ex vitro* (n=15).

Height, width, number of shoots and percentage of plants with fruit in gooseberry bushes propagated *in vitro* and *ex vitro* (n=15).

Genotyp Genotype	Wysokość [cm] Height [cm]		Szerokość [cm] Width [cm]		Liczba pędów [szt.] Number of shoots [pcs.]		Rośliny z owocami [%] Plants with fruits [%]	
	In vitro	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*	*In vitro*	*Ex vitro*
Biały Triumf	*26,9 b	19,9 a	30,6 b	18,3 a	6,2 a	6,9 a	0	33,0
Captivator	46,3 a	48,1 a	54,7 b	49,8 a	14,0 b	7,7 a	86,7	93,3
Hinnonmaki Rot	36,1 a	36,2 a	51,9 a	53,7 a	14,2 b	10,7 a	53,3	100
Hinsel	51,3 b	37,5 a	43,3 a	54,3 b	9,8 a	11,3 a	6,7	53,3
Invicta	38,2 b	30,9 a	44,1 b	37,7 a	10,1 a	7,9 a	20,0	66,6
Kamieniar	31,6 b	25,0 a	37,1 b	25,6 a	10,1 b	5,7 a	13,3	60,0
Pax	32,2 a	31,7 a	42,3 b	31,3 a	11,1 b	5,5 a	0	26,6
Resika	47,6 a	52,6 b	39,7 a	46,7 b	12,1 b	7,5 a	0	53,3
AGR-2/2	37,1 b	30,1 a	45,5 b	37,5 a	16,0 b	6,1 a	20,0	41,7
AGR-2/33	37,8 b	24,5 a	49,0 b	25,5 a	12,8 b	4,3 a	60,0	23,1
AGR-86	48,9 b	30,6 a	60,5 b	47,8 a	12,7 b	7,0 a	20,0	0
AGR-101	52,0 b	22,5 a	68,5 b	25,9 a	24,9 b	4,0 a	33,3	57,1
AGR-102	41,7 b	26,4 a	60,7 b	28,3 a	15,9 b	5,0 a	26,6	44,4
AGR-108	52,2 b	37,6 a	47,8 b	33,2 a	10,2 a	8,0 a	0	0
AGR-117	60,8 b	38,8 a	74,1 b	44,7 a	14,5 b	8,5 a	6,7	0

**średnie oznaczone tą samą literą nie różnią się istotnie p=0,05 wg testu Tukeya*
**means followed by the same letter, were not significantly acc. to Tukey's test.*

The concentration of DNA was in the range of 7.8 to 55.1 ng/µl and the 260/280 nm ratio was from 1.61 to 2.00. A total of 2,294 amplification products were obtained in reactions with five starters for five analysed genotypes (Tab. 3), of which 64 products (2.8%) were polymorphic. The size of the amplification products was from 250 to 2,900 bp, depending on the primer and variety. The number of generated PCR products differed depending on the type of primer, and for the 'Invicta' variety it ranged from 42 for the primer 834 to 168 for the primer 849. The ISSR-PCR analysis revealed different levels of polymorphism, from 0 for 'Hinnonmaki Rot' and 'Resika', to 11.6% for the 'Hinsel' cv. (Tab. 3, Figs. 2, 3).

Tabela 2
Table 2

Analiza markerów AFLP w roślinach 5 odmian agrestu pochodzących z kultur *in vitro* oraz roślin matecznych.

Analysis of AFLP markers in plants of 5 gooseberry cultivars obtained from *in vitro* and donor plants.

Odmiana Cultivar	Startery Primers	Liczba produktów amplifikacji Number of amplification products		Polimorfizm % Polymorphism %
		Całkowita Total	Polimorficznych Polymorphic	
'Captivator'	Pst-AT/Mse-CG	71	0	0
	Pst-TA/Mse-GA	103	2	1,94
	Pst-AGC/Mse-CT	37	0	0
	Pst-CAG/Mse-TG	43	0	0
	Pst-GTC/Mse-AC	37	1	2,70
	Ogółem: Total:	291	3	1,03
'Hinsel'	Pst-AT/Mse-CG	60	10	16,66
	Pst-TA/Mse-GA	62	3	4,83
	Pst-AGC/Mse-CT	47	8	17,02
	Pst-CAG/Mse-TG	56	4	7,14
	Pst-GTC/Mse-AC	37	2	5,40
	Ogółem: Total:	262	27	10,30
'Hinnonmaki Rot'	Pst-AT/Mse-CG	59	0	0
	Pst-TA/Mse-GA	108	0	0
	Pst-AGC/Mse-CT	38	0	0
	Pst-CAG/Mse-TG	58	1	1,72
	Pst-GTC/Mse-AC	37	3	8,10
	Ogółem: Total:	300	4	1,33
'Invicta'	Pst-AT/Mse-CG	58	1	1,72
	Pst-TA/Mse-GA	79	1	1,03
	Pst-AGC/Mse-CT	41	1	2,43
	Pst-CAG/Mse-TG	68	0	0
	Pst-GTC/Mse-AC	42	0	0
	Ogółem: Total:	288	3	1,04
'Resika'	Pst-AT/Mse-CG	54	1	1,85
	Pst-TA/Mse-GA	36	0	0
	Pst-AGC/Mse-CT	33	4	12,2
	Pst-CAG/Mse-TG	92	0	0
	Pst-GTC/Mse-AC	47	2	4,25
	Ogółem: Total:	262	7	2,67

Tabela 3
Table 3

Analiza markerów ISSR w roślinach 5 odmian agrestu pochodzących z kultur *in vitro*, *ex vitro* oraz roślin matecznych.

Analysis of ISSR markers in plants of 5 gooseberry cultivars obtained from *in vitro*, *ex vitro* and donor plants.

Odmiana Cultivar	Starter Primer	Wielkość produktów(pz) The size of products (bp)	Liczba produktów amplifikacji Number of amplification products		Polimorfizm % Polymorphism %	
			Ogółem Total	Polimorficzne Polymorphic		
‘Captivator’	822	650 – 1300	63	0	0	1,4
	825	280 – 1200	112	7	6,4	
	830	500 – 1000	105	0	0	
	848	500 – 1000	84	0	0	
	849	500 – 2900	147	0	0	
‘Hinnonmaki Rot’	823	450 – 1100	84	0	0	0,0
	825	280 – 1300	126	0	0	
	834	280 – 1050	105	0	0	
	840	450 – 1500	105	0	0	
	853	700 – 1400	63	0	0	
‚Hinsel’	825	250 –1300	105	4	3,8	11,6
	834	500 – 1200	67	5	7,5	
	848	500 – 2500	75	17	22,7	
	849	400 – 1380	95	11	11,6	
	853	400 – 1500	64	10	15,6	
‘Invicta’	821	550 – 2000	47	5	10,6	2,3
	825	250 – 1200	90	5	5,6	
	834	260 – 750	42	0	0	
	849	400 – 2000	168	0	0	
	853	600 – 1500	84	0	0	
‘Resika’	821	350 – 2000	105	0	0	0
	822	650 – 1250	63	0	0	
	825	250 – 1300	84	0	0	
	834	500 – 1200	126	0	0	
	849	500 – 1200	84	0	0	

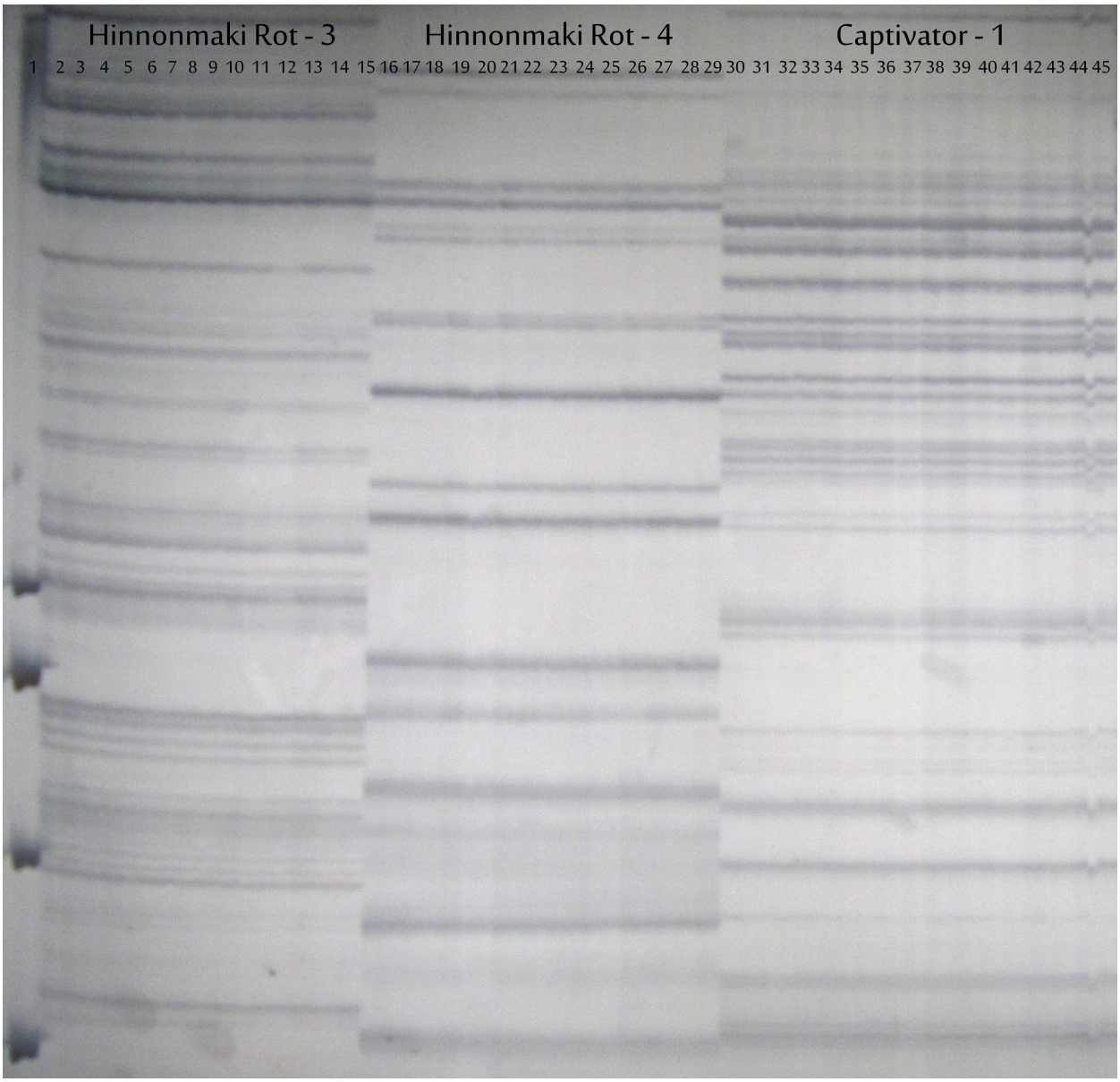
Discussion

The morphological parameters of gooseberry bushes representing different cultivars and breeding clones in the second year of cultivation in the field indicated that the type of propagation, by cuttings and by in vitro shoot cultures influenced the growth, shape and vigour of plants, as well as the time necessary to reach the fructification phase. Some researchers have reported that plants propagated in vitro are characterised by greater vigour and a more dynamic increase in biomass (Dubois et al. 1988; Howard et al. 1989; Drew and Smith 1990). This was also confirmed in our study, which revealed that in most genotypes the gooseberry shrubs from plants derived in vitro were significantly taller, wider and had more numerous shoots compared to plants propagated by cuttings. Our observations are novel for gooseberry and consistent with reports on the growth of other soft fruit plants propagated in vitro.

Many reports have been published on the use of in vitro techniques for the propagation of various species of plants from the genus *Ribes* (Podwyszyńska et al. 2006; Sedlák and Paprštein 2012; Podwyszyńska and Pluta 2019). However, only a few of them concern the possibility of effective in vitro propagation of gooseberry (Welander 1985, Wainwright and Flegmann 1986, Reed and Hummer 2002). Kucharska et al. (2020) reported the role of meta-Topoline (mT) used instead of benzylaminopurine (BAP) in the development of an efficient method for the micropropagation of 14 gooseberry genotypes.

Somaclonal variation is often observed in long-term *in vitro* cultures of berry plants. Molecular markers obtained using different techniques

such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR), fingerprinting, simple repeat sequences (SSR), as well as amplified length polymorphism fragments (AFLP), are used to study somaclonal variability, but also to identify genotypes, study varietal identity and determine relatedness within many species of fruit plants, including the genus *Ribes* (Lanham and Brennan 2001, Brennan et al. 2008, Cavanna et al. 2009). In our study, we decided to apply the AFLP technique because it was reported as the most effective in the detection of DNA polymorphism in gooseberry (Lanham and Brennan, 1999a), and has been widely used in research on other plant species (Bahulikar et al. 2004, Meudt



Rys. 1. Fragment elektroforegramu produktów AFLP-PCR; 1 – marker wielkości DNA 50 pz (50 bp DNA Ladder, Thermo Fisher Scientific); 2-15 – reakcja DNA roślin odmiany Hinnonmaki Rot ze starterami Pst-AGC/Mse-CT (2-14 – rośliny z kultur *in vitro*, 15 - roślina mateczna); 16-29 - reakcja DNA roślin odmiany Hinnonmaki Rot ze starterami Pst-CAG/Mse-TG (16-28 – rośliny z kultur *in vitro*, 29 - roślina mateczna); 30-45 - reakcja DNA roślin odmiany Captivator ze starterami Pst-AT/Mse-CG (30-44 – rośliny z kultur *in vitro*, 45 - roślina mateczna)

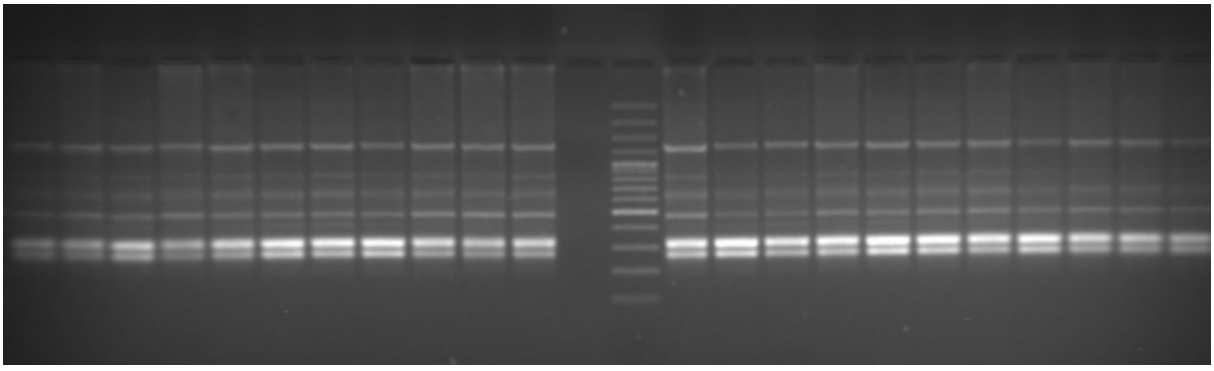
Fig. 1. Fragment of electrophoretic banding pattern of AFLP- PCR; 1 – 50 bp DNA Ladder (Thermo Fisher Scientific); 2-15 – reaction of DNA of Hinnonmaki Rot plants with Pst-AGC/Mse-CT primers (2-14 – *in vitro*-derived plants, 15 - donor plant); 16-29 - reaction of DNA of Hinnonmaki Rot plants with Pst-CAG/Mse-TG primers (16-28 – *in vitro*-derived plants, 29 - donor plant); 30-45 - reaction of DNA of Captivator plants with Pst-AT/Mse-CG primers (30-44 – *in vitro*-derived plants, 45 - donor plant)

and Clarke 2007, Kumar et al. 2013, Costa et al. 2016).

DNA-based markers generated in RAPD and ISSR-PCR assays were used to characterize the genotypes collected in the gene bank and used in breeding projects at the Research Institute of Horticulture for plants such strawberries, apples, red and black currants, and gooseberry (Korbin et al. 2002). Studies on the polymorphism of 12 gooseberry varieties conducted by Lanham and Brennan (1999a, 1999b, 2001) have revealed that the use of ISSR primers failed to differentiate 3 varieties. In our research, the ISSR primers allowed us to distinguish five analysed gooseberry genotypes. The highest level of polymorphism was obtained for the 'Hinsel' variety, and each of the five tested primers showed the presence of polymorphic products (Tab. 3, Fig. 3). Such a high polymorphism of the 'Hinsel' variety, compared to other studied genotypes, proves its genetic instability.

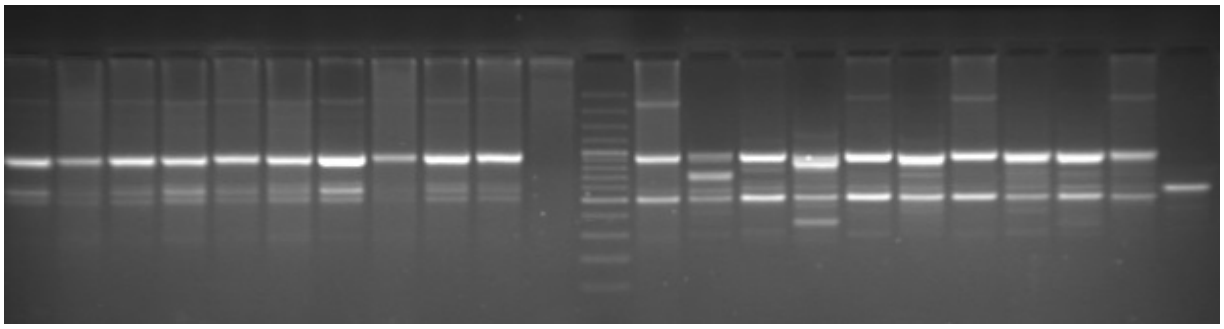
Conclusions

1. The height, width and number of shoots measured for shrubs propagated in vitro compared to those propagated by cuttings were significantly greater for most of the analysed gooseberry genotypes.
2. The AFLP technique was found effective in detecting the degree of somaclonal variation in gooseberry generated during in vitro propagation.
3. 'Captivator', 'Invicta', 'Hinnonmaki Rot' and 'Resika' cultivars are genetically stable when cultured in vitro. The developed method for gooseberry micropropagation produces genetically stable plants.



Rys. 2. Elektroforegram produktów PCR z zastosowaniem startera 825 dla odmiany Hinnonmaki Rot, ścieżka 1 i 14: roślina mateczna, 2–11: sadzonki z kultur *in vitro*, 12: kontrola negatywna, 13: GeneRuler™ 100bp DNA Ladder Plus (Thermo Fisher Scientific), 14–24 sadzonki wegetatywne

Fig. 2. Electrophoretic banding pattern of PCR products using primer 825 for cultivar Hinnonmaki Rot, lane 1 and 14: donor plant, 2–11: seedlings from cultures *in vitro*, 12: negative control, 13: GeneRulerTM 100bp DNA Ladder Plus (Thermo Fisher Scientific), 14 – 24 vegetative seedlings



Rys. 3. Elektroforegram produktów PCR z zastosowaniem startera 848 dla odmiany Hinsel, ścieżka 1–10: sadzonki z kultur *in vitro*, 11: kontrola negatywna, 12: GeneRuler™ 100bp DNA Ladder Plus (Thermo Fisher Scientific), 13: roślina mateczna, 14–23 sadzonki wegetatywne

Fig. 3. Electrophoretic banding pattern of PCR products using primer 848 for cultivar Hinsel, lane 1–10: seedlings from cul- tures *in vitro*, 11: negative control, 12: GeneRulerTM 100bp DNA Ladder Plus (Thermo Fisher Scientific), 13: donor plant, 14 – 23: vegetative seedlings

4. The 'Hinsel' cv has a higher degree of polymorphism when cultured in vitro.

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