PROTECTION OF GENETIC RESOURCES OF POMOLOGICAL PLANTS AND SELECTION OF GENITORS WITH TRAITS VALUABLE FOR SUSTAINABLE FRUIT PRODUCTION

Journal of Fruit and Ornamental Plant Research vol. 12, 2004 Special ed.

ASSESSMENT OF DIVERSITY OF WILD RASPBERRIES (*Rubus idaeus* L.) IN LITHUANIA

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(Received August 10, 2004/Accepted January 20, 2005)

A B S T R A C T

Twenty genotypes of wild red raspberry (*Rubus idaeus* L.) from the germplasm collection of the Botanical Garden of Vilnius University were analyzed for genetic diversity on the basis of random amplified polymorphic DNA markers. Initial screening with 44 oligonucleotide primers identified 36 primers corresponding to 285 RAPD loci. 80% of the loci detected were polymorphic. Each primer produced a mean of 7.9 bands. Indices of genetic distance between the genotypes ranged from 0.176 to 0.318. Eight of the primers produced ten or more informative PCR products. These primers promise to be useful in the further analysis of the *R. idaeus* germplasm collection.

Data from RAPD fingerprints were subjected to factor analysis by the principal components method. The adaptive value of 230 polymorphic RAPD loci was assessed. Correlations between soil conditions and the results obtained by factor analysis were elaborated by calculating the Pearson correlation and its p-value. The only significant correlation found was with soil acidity at the sites from which the genotypes were collected ($r = -0.65$). In the genotypes studied, adaptive polymorphism in the gene coding for superoxide dismutase did not seem to be determined by the soil characteristics at the sites from which the genotypes were collected.

Key words: *Rubus idaeus*, germplasm collection, genotype, RAPD, genetic diversity, adaptive polymorphism

INTRODUCTION

The wild red raspberry (*Rubus idaeus* L.: Rosaceae) grows widely all over the Northern hemisphere. *Rubus idaeus* has been an important source of genes for breeding new raspberry varieties (Alice et al., 2001; Marshal et al., 2001). In the development of improved varieties, the main qualities selected for are hardiness, productivity, disease resistance, fruit size, and firmness. Scientists have also started using *R. idaeus* as a model plant to develop and apply genetic techniques in the study of perennials, particularly members of the family Rosaceae (Graham et al., 2003).

Discrete, spatially separated populations of *R. idaeus* are adapted to local conditions, which may result in effective reproductive isolation in the absence of geographical barriers (Marshal et al., 2001; Graham et al., 2003). These isolated populations may be a source of unique genetic information which enables them to thrive in their specific habitats. This genetic information may be useful in breeding for plants which can cope with recent ecological changes, such as pollution, global warming, and habitat destruction. The conservation of wild raspberry populations is important to prevent the loss of these valuable coadapted gene complexes.

In Lithuania, *R. idaeus* grows in many different habitats, such as forests, empty lots, clearings, parks, and roadsides. *R. idaeus* produces the best-developed colonies in nutrient-rich and well-lit habitats with normal moisture regimes. In 2001, a program was undertaken to collect clonal samples of wild raspberries in order to study their genetic diversity (Labokas et al., 2002). The collection at the Botanical Garden of Vilnius University now contains over one hundred genotypes from all over Lithuania. Modern molecular marker techniques are essential in effectively evaluating this germplasm collection as a source of useful genetic material for raspberry breeding programs (Howell and Newbury, 1994; Chwedorzewska et al., 2002; Lund et al., 2003).

This study represents a first step toward the better understanding of the value of Lithuanian wild raspberries as potential sources of valuable genes. The aims of this study were: 1) to identify which set of RAPD primers provides the most useful information and is most convenient to use in evaluating genetic variation among a large number of samples; and 2) to evaluate the role of the loci identified and of superoxide dismutase (SOD) isozyme polymorphism in the adaptation of *R. idaeus* to the conditions prevailing at the site from which the genotypes were originally collected

MATERIAL AND METHODS

Plant and soil samples. Clonal samples of *R. idaeus* were collected from the wild during the growth season in 2001. Each clonal sample consisted of three plants with one or two canes each. The samples were added to the field

T a b l e 1 . Original habitats and soil conditions at the sites from which the genotypes of *Rubus idaeus* L. were collected

Sample number	Collecting coordinates		Habitat description	Characteristics of the soil of original site				
	latitude [north]	longitude [west]		pH_{KCl}	humus content [%]	total nitrogen [%]	available p_2o_5 [mg/kg]	available k_2o [mg/kg]
1JL	54°17′08″	23°40′14″	narrow belt between lakeside and roadside	7.12	6.20	0.48	123.30	130.70
2JL	54°21′27″	24°13′46″	cutting of spruce-wood	4.12	5.90	0.23	106.70	112.60
3JL	56°06′24″	22°51′10″	alderwood glade	6.14	5.75	0.37	116.30	230.10
4JL	55°24′29″	23°47′17″	oakwood glade	7.02	7.48	0.42	59.20	132.20
5JL	54°17′08″	23°40′14″	cutting of sprucewood	3.56	4.32	0.20	24.60	53.80
6JL	55°25′43″	23°24′14″	cutting of mixed forest, on peat	4.01	28.52	1.93	90.10	708.20
7JL	54°13′49″	23°46′45″	margin of broad-leaved forest	4.85	1.80	0.13	145.50	103.00
8JL	55°35′14″	21°07′46″	sandy coastal lagoon	4.75	0.97	0.02	65.70	28.50
9JL	54°43′46″	25°29′03″	cutting of pine-wood, on peat	3.42	24.74	1.10	158.80	428.20
10SS	-	-	peat bog	6.78	9.57	0.73	386.90	416.00
11JL	54°20′57″	24°45′20″	mature pinewood	5.46	3.94	0.26	95.00	254.10
12JL	54°33′45″	24°42′17″	lakeside, mixed forest	5.48	3.33	0.22	64.60	136.80
13JL	55°24′26″	24°32′39″	cutting of mixed forest	5.62	8.12	0.58	252.50	101.60
14JL	55°37′00″	24°05′55″	field margin, river bank	6.60	4.58	0.22	129.20	260.80
15JL	54°32′45″	24°09′32″	cutting of mixed forest	4.01	5.06	0.28	59.60	80.20
16JL	54°45′06″	25°18′31″	electric power line, mixed forest	5.15	7.48	0.48	25.40	53.00
17JL	54°48′43″	24°28′47″	electric power line, broadleaf forest	5.97	2.78	0.24	68.60	67.90
18JL	54°28′16″	25°08′02″	peat bog	4.75	28.96	3.06	161.00	238.60
19JL	54°47′48″	25°27′03″	pinewood	4.02	1.80	0.04	130.50	22.30
20JL	54°51′40″	25°12′39″	narrow belt between fen and dry sprucewood	3.05	5.28	0.13	17.80	55.40

collection at the Botanical Gardens of Vilnius University and managed according to standard horticultural practices. The original habitats of the genotypes are shown in Table 1. During collection, soil samples were taken from the rhizosphere and were analyzed at the Chemical Analyses Laboratory of the Institute of Botany according to standard methods. Soil pH was measured by potentiometry. Available phosphorus was measured by photometry after soluble phosphates were extracted with 0.2 M HCl. Available potassium was measured by flame photometry after soluble potassium was extracted with 0.2 M HCl. To determine total nitrogen, soil samples were first boiled in concentrated sulfuric acid. The ammonium ions produced were then detected by photometry with alkaline sodium salicylate and hypochlorite. Humus content was measured by oxidation with potassium dichromate and sulfuric acid.

DNA extraction and PCR amplification. DNA was purified from fresh young leaves with the Genomic DNA Purification Kit (#K0512, Fermentas). Each amplification was performed in 25 µl containing 1×PCR buffer (Fermentas), 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of the primer, 1.0 u of Taq DNA polymerase (Fermentas), and 50 ng of *R. idaeus* DNA. Forty-four oligonucleotide primers were used for analysis, fourteen primers from Fermentas, and Random Primer kits 270, 380, and 470 from Carl Roth GmbH+Co. RAPD reactions were carried out in a Thermo Cycler (Biometra) under conditions described earlier (Žvingila et al., 2002).

Amplification products were analyzed by electrophoresis in 1.6% TBE agarose gel. All reactions were repeated at least twice. Only clear and reproducible DNA bands were scored.

Extraction and assay of superoxide dismutase (SOD, EC 1. 15. 1. 1. 1). Leaf discs of twenty plants were homogenized in pre-chilled 50 mM potassium buffer, pH 7.8 (1 g plant material to 1 ml buffer). The homogenate was centrifuged at 12,500 rpm for 15 min at 4ºC. On the same day, the supernatant was analyzed by native polyacrylamide gel electrophoresis at 4ºC with a 4% stacking gel and a 9% separating gel in Tris-glycine buffer, pH 8.3, at 200 V and 40 mA. 20 µl of crude leaf extract was loaded in each lane. SOD isozymes were visualized by NBT reduction. After electrophoresis, the gels were incubated in a staining solution for one hour at 37ºC in the dark (Fridovich, 1978).

Data analysis. DNA bands were scored as either present or absent using BioDocAnalyse software (Biometra). Banding patterns were compared to each other using TREECON for Windows (Van de Peer and de Wachter, 1994). Genetic distance was calculated according to the formula described by Nei and Li (1979). A dendrogram was constructed using the distance matrix of Nei and Li and the neighbor-joining method. Data from RAPD fingerprints were subjected to factor analysis by the principal components method. Only polymorphic fragments were analyzed using the SAS V8 program package. Correlations between soil characteristics and the results obtained by factor

analysis were elaborated by calculating the Pearson correlation and its p-value using the CORR procedure. The RAPDs which correlate best with adaptation to soil acidity were identified using STEPDISC SAS. The least significance level for the selection of single RAPD was used 0.01.

RESULTS

Table 2. Genetic diversity parameters established with informative RAPD primers in twenty genotypes of *Rubus idaeus*

RAPD primer	Sequence 5'→3'	Total bands[1]	Poly-morphic bands	Size range of DNA fragments [bp]	Genotype specific bands [bp]	Number of finger-prints[2]
A3	GACAGACAGA	11	8	190-1065	1 (590)	14
A4	CGGGTACCAA	11	10	355-1035	2 (470, 530)	18
A5	GGGAACCCGT	9	8	270-1100	1 (795)	10
A6	GACCCGTCCC	7	4	520-1080	0	12
A7	GAAACGGGTG	7	7	250-730	0	16
B6	TGCTCTGCCC	6	5	300-1100	0	13
B7	GGTGACGCAG	8	6	480-1570	0	12
MP2	AGTCGTCCCC	8	8	605-2700	0	16
MP3	CCATCCCCCA	7	6	430-1370	0	11
MP4	GGTGAACGCT	7	6	545-1125	0	14
MP5	GTCATGCCTGG	5	3	610-1540	2 (610, 1090)	4
MP7	TCGGCACGCA	6	5	700-2060	1 (910)	10
Roth 270-1	GTCTCGTCGG	7	7	430-1155	0	12
Roth 270-3	GTGTAGGGCG	8	8	410-3275	1 (880)	10
Roth 270-5	GCCCTCTTCG	10	7	540-2200	0	12
Roth 270-6	CAGGGGCATC	9	5	515-2450	0	10
Roth 270-7	GAGACCTCCG	6	5	850-1800	0	7
Roth 270-8	GGCCTTCAGG	8	6	500-1330	1 (805)	12
Roth 270-9	GCTCTCACCG	7	7	420-2500	2 (420, 580)	12
Roth 270-10	TGCACGGACG	7	7	780-2980	1 (2980)	13
Roth 380-1	ACGCGCCAGG	6	3	520-2370	0	7
Roth 380-2	ACTCGGCCCC	11	10	830-2530	0	20
Roth 380-3	GGCCCCATCG	13	10	400-2010	2 (700, 820)	20
Roth 380-4	CGCGAGGTGC	7	5	220-1720	1 (450)	11
Roth 380-6	CCCGACTGCC	9	6	685-1100	3 (685, 890, 900)	7
Roth 380-7	GGCAAGCGGG	7	6	470-1140	0	11
Roth 380-8	CGCACCGCAC	6	6	500-1440	1 (1000)	10
Roth 380-9	ACGGCGGCTC	6	2	530-1300	0	4
Roth 470-1	GCCCCTCTTG	6	5	560-1180	0	13
Roth 470-3	CTGTCGGCTC	11	10	450-2930	0	15
Roth 470-4	GGACCGCTAG	7	6	630-1450	0	10
Roth 470-6	GCACGTGAGG	9	6	630-2370	0	13
Roth 470-7	CTATCGCCGC	6	6	610-2530	3 (610, 630, 800)	8
Roth 470-8	GAGAGGGAGG	11	9	380-2550	1 (590)	19
Roth 470-9	CCGGGGTTAC	10	7	670-2710	1 (670)	13
Roth 470-10	CGCAGACCTC	6	6	745-1860	0	11

[1]Total number of RAPD band detected which are reproducible and useful as molecular markers.
[2]Number of fingerprints that can be distinguished within the group of twenty genotypes of *R. idaeus* with different RAPD primers

A total of forty-four random primers were used to analyze the RAPD patterns of twenty genotypes of *R. idaeus*. Thirty-six informative primers

produced a total of 285 scorable RAPD bands (Tab. 2). All of these primers identified polymorphic loci among the genotypes examined. RAPD bands ranged from approximately 190 to 3275 bp. Of the 285 loci, 81% were polymorphic. The total number of scorable bands per primer ranged from five (MP5) to thirteen (Roth 380-3). Some genotype-specific products were also identified. Two primers (Roth 470-7 and Roth 380-6) amplified three genotype-specific bands each. On the basis of molecular data, a matrix of relative genetic distances (Gdxy) was computed following the procedure of Nei and Li (1979). Genetic distances ranged from 0.176 to 0.318. A dendrogram of the genetic relationships among the twenty genotypes studied was drawn using Gdxy values according to the Neighbor-Joining method (Fig. 1).

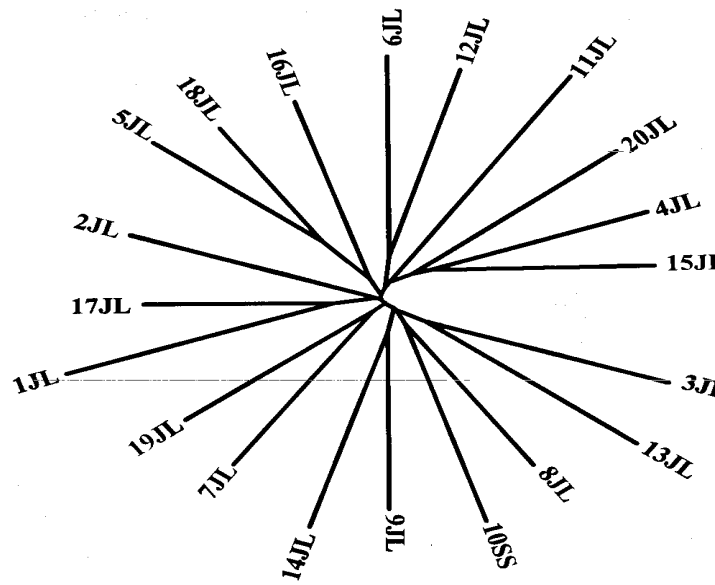

Figure 1. Neighbor joining dendrogram drawn from the genetic distance matrix (Nei and Li, 1979) of the twenty *Rubus idaeus* genotypes presented in Table 1

The principal component method of factor analysis was then used to evaluate correlations between the distribution of the RAPD loci among the twenty genotypes and nitrogen, phosphorus, potassium, humus content and soil acidity at the sites from which the genotypes were collected (Tab. 3). The only significant correlation was with soil acidity ($r = -0.65$). About 22 RAPD markers contributed to the correlation between DNA polymorphism and soil acidity. STEPDISC identified the nine RAPD bands which contributed the most: Roth 380-3 (1550 bp), Roth 380-3 (1390 bp), Roth 470-7 (800 bp),

MP2 (605 bp), MP4 (1015 bp), A4 (470 bp), A4 (530 bp), A7 (730 bp), and B6 (1000 bp).

T a b l e 3 . Correlation of factor analysis estimates with soil characteristics

Variable	Nitrogen	P_2O_5	K_2O	Humus	Acidity
Factor1	-0.01	-0.35	-0.18	-0.03	-0.65*
Factor2	-0.03	0.22	-0.07	-0.03	-0.26
Factor3	-0.01	-0.08	-0.05	-0.05	-0.30

*Significant at a level of 1%

Isozymes are another class of molecular markers widely used in plant studies. The usefulness of isozymes as markers of adaptation to local conditions has been the subject of numerous studies (Nevo, 1983; Li et al., 1999; Ren et al., 1999; Martinez et al., 2001). In our study, the correlation between superoxide dismutase (SOD) isoenzyme patterns and soil acidity at the sites from which the genotypes were collected, was also investigated. At least eight SOD activity bands were identified. According to the number and mobility of the bands, six main SOD enzyme spectra were identified (Fig. 2).

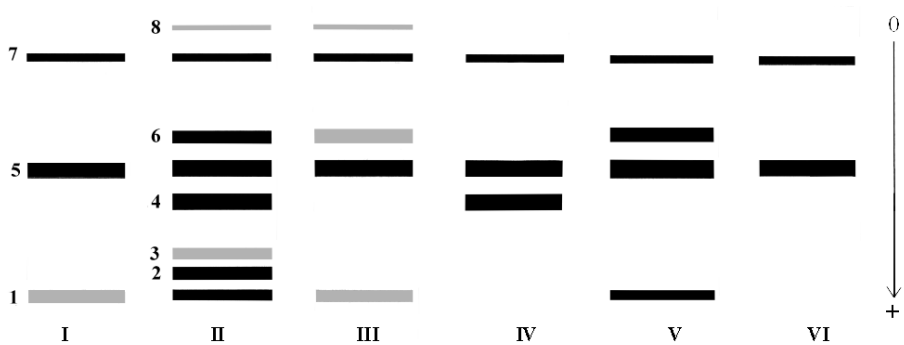

Figure 2. Superoxide dismutase (SOD) isozyme profiles (I – VI) in the leaves of *Rubus idaeus* L.

Profile	Genotypes
I	1JL, 3JL, 10SS, 12JL
II	4JL
III	14JL
IV	11JL, 13JL, 16JL, 17JL
V	18JL, 15JL
VI	2JL, 19JL, 20JL, 8JL, 5JL, 6JL, 7JL, 9JL

DISCUSSION

Knowledge of the level of genetic variation in plant germplasm collections is important to effectively utilize and conserve the species. Using molecular markers to manage germplasm collections can help plant breeders solve practical problems (Masojć, 2002). RAPD has a number of advantages that enable efficient detection of DNA polymorphisms (Williams et al., 1990). Even though RAPD is relatively fast, assessing genetic diversity in large number of samples with many primers is costly and time-consuming. Because not all RAPD markers are equally informative, it is necessary to find a small set of primers specific for highly polymorphic amplification products which lend themselves to easy and reliable analysis (Thompson and Nelson, 1998).

We evaluated the usefulness of forty-four oligonucleotide primers in identifying RAPD markers in twenty genotypes of *R. idaeus*. Eight primers either did not produce any PCR products at all, or produced complicated RAPD patterns unsuitable for further analysis. The amplification products produced by the remaining 36 informative primers were polymorphic in the genotypes studied. Each primer yielded an average of 7.9 bands per genotype. Eight primers identified ten or more RAPD bands which were scorable, reproducible, and useful as molecular markers (Tab. 2). The level of polymorphism among the loci identified with these eight primers was 80.7%. We recommend using this set of eight primers to further analyze the *R. idaeus* germplasm collection.

Among the primers tested, two were especially informative: Roth 380-2 and Roth 380-3. With each of these primers, each of the genotypes studied could be identified by a unique combination of bands, a genotype-specific fingerprint, as it were (Tab. 2).

The Neighbor Joining dendrogram constructed from the Gdxy values shows the level of divergence among the genotypes studied (Fig. 1). Genetic distances did not correlate with the geographic distance between the sites from which the genotypes were collected ($r = 0.0017$). The pattern of branching was mainly independent of geography. For example, the two largest clusters include twelve genotypes sampled from an area that covers a large part of Lithuania. In the Tayside region of Scotland, genetic diversity within and between colonies of wild raspberry correlated with geographical distance (Graham et al., 1997). Our results suggest that geographical distance is not the only factor determining genetic differentiation. Differences in ecological conditions may also play a role. We therefore checked for a correlation between RAPD polymorphism and the soil properties at the original habitats of the genotypes. A significant correlation was found between RAPD polymorphism and soil acidity ($r = -0.65$). Curvilinearity testing indicated that the relationship was linear (Fig. 3). 64.5% of the variation among samples

was explained by the first three principle components identified by principle component analysis. This indicates that the genotypes studied had adapted to the acidity of the soil at the sites from which they were collected. The adaptive role of RAPD polymorphism was also demonstrated by Nevo et al. (1998), Fahima et al. (1999), and Semagan et al. (2000). Our results also indicate that the non-coding part of the plant genome can be affected by natural selection.

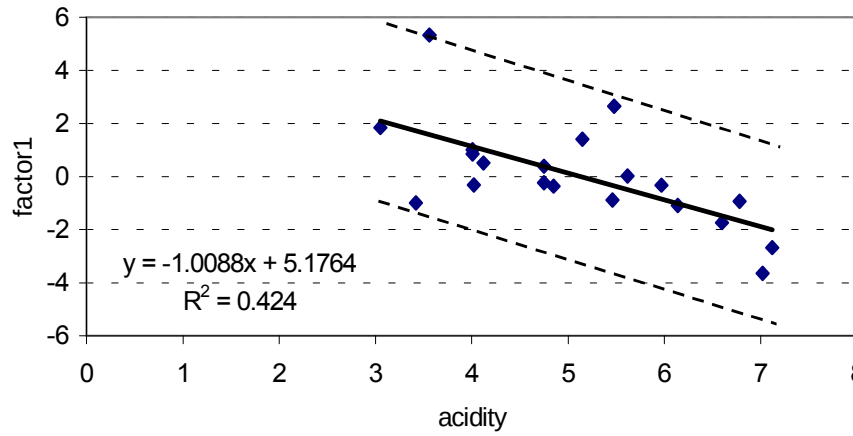

Figure 3. Scatter plot of *Rubus idaeus* genotypes based on values from factor analysis (Factor 1) and soil acidity at the sites from which the genotypes were collected. The dashed lines show the upper and lower limits of the 95% confidence range

SOD protects plant cells from toxic superoxide radical produced in response to biotic and abiotic stress (Scandalios, 1993). Soil acidity is a major environmental stress factor which limits plant growth (Vergeer et al., 2003). In our study, six types of SOD spectra were detected (Fig. 2). However, we did not find any adaptive dependence between SOD spectra and the acidity of the soil at the sites from which the genotypes were collected.

CONCLUSSIONS

1. Of the forty-four primers tested, eight produce highly polymorphic, clear and reproducible RAPD patterns, and can be recommended for analyzing genetic diversity in a large number of samples of *Rubus idaeus*.
2. Some of the RAPD markers detected correlated with the acidity of the soil at the sites from which the genotypes were collected. They can be considered to be examples of polymorphism which promotes adaptation to

local conditions. Our results suggest that these RAPD loci could be used as molecular markers of adaptation to local conditions.

Acknowledgement. The study was supported by the Nordic Gene Bank and the Lithuanian National PGR Program "Genofondas''.

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OCENA RÓŻNORODNOŚCI DZIKO ROSNĄCYCH GENOTYPÓW MALINY WŁAŚCIWEJ (*Rubus idaeus* L.) NA LITWIE

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S T R E S Z C Z E N I E

Analizowano różnorodność genetyczną dwudziestu genotypów maliny właściwej (*Rubus idaeus* L.) z kolekcji Ogrodu Botanicznego Uniwersytetu Wileńskiego, za pomocą polimorficznych markerów DNA. Wstępna ocena z użyciem 44 oligonukleotydowych starterów, pozwoliła na identyfikację 36 starterów korespondujących z 285 loci RAPD. Osiemdziesiąt procent wykrytych loci było polimorficznych. Każdy starter generował średnio 7,9 fragmentów DNA. Wskaźniki dystansu genetycznego między genotypami wynosiły od 0,176 do 0,318. Używając ośmiu starterów uzyskano dziesięć lub więcej polimorficznych produktów reakcji PCR. Te startery mogą być przydatne do dalszych analiz genotypów *R. idaeus* zgromadzonych w kolekcji.

Dane uzyskane za pomocą analizy RAPD poddano analizie czynnikowej metodą składników głównych. Oceniono wartość adaptacyjną 230 polimorficznych loci RAPD. Wzajemne zależności między warunkami glebowymi i wynikami uzyskanymi przez analizę czynnikową opracowano przez obliczenie współczynnika korelacji Pearson'a i jej wartości p. Istotną współzależność stwierdzono tylko dla kwasowości gleby w miejscach, z których pobierano genotypy ($r = -0.65$). W badanych genotypach, polimorfizm adaptacyjny genów kodujących dysmutazę nadtlenkową nie wydawał się być zależny od właściwości gleby.

Słowa kluczowe: *Rubus idaeus*, kolekcja zasobów genowych, genotyp, RAPD, różnorodność genetyczna, polimorfizm adaptacyjny