Original scientific paper
DOI: /10.5513/JCEA01/25.4.4233
Journal of Central European Agriculture, 2024, 25(4), p.955-965

Characteristics of Slovak amaranth varieties based on coding regions polymorphism and endophytic bacteria prevalence

Charakteristika Slovenských odrôd láskavca pomocou polymorfizmu kódujúcich oblastí a endofytických baktérií

Dagmar MORAVČÍKOVÁ[1], Matúš KYSEĽ[2], Silvia FARKASOVÁ[3], Andrea HRICOVÁ[4], Veronika ŠTEFÚNOVÁ[1], Jana ŽIAROVSKÁ[1] (✉)

[1] Slovak University of Agriculture in Nitra, Faculty of Agrobiology and Food Resources, Institute of Plant and Environmental Sciences; Tr. A. Hlinku 2, 94976, Nitra, Slovak Republic

[2] Department of Dendrobiology, Institute of Forest Ecology, Slovak Academy of Sciences - Zvolen, Vieska nad Žitavou 178, 951 52 Slepčany, Slovak Republic

[3] Slovak University of Agriculture in Nitra, Research Centre of AgroBioTech; Tr. A. Hlinku 2, 94976, Nitra, Slovak Republic

[4] Slovak Academy of Sciences, Centre of Plant Biology and Biotechnology, Institute of Plant Genomic and Biology, Akademická 969, 949 01, Nitra, Slovak Republic

✉ Corresponding author: jana.ziarovska@uniag.sk

Received: January 12, 2024; accepted: July 19, 2024

ABSTRACT

Amaranthus is one of the underutilised crops with the potential to overcome some of the current challenges of modern agriculture under highly unstable environmental conditions. Here, two Slovak varieties of amaranth – Zobor (*A. hypochondriacus* × *A. hybridus*) and Pribina (*A. cruentus*) were characterised by Conserved DNA-Derived Polymorphism (CDDP), P450-based analogue (PBA) markers and Tubulin-Based Polymorphism (TBP) and by its most abundant endophytes. CDDP and TBP profiles showed a very similar distribution of the generated fragments for both of the analyzed varieties. PBA profiles generated the lowest value of polymorphism. A wide range of amplicons was generated by all of the techniques used in the study with the length of PCR fragments ranging from 80 up to 2292 bp. The variability of the endophytic composition of the Pribina variety showed the following three most abundant genera: Endobacter, Dichotomicrobium and Burkholderia. Abiotrophia, Endobacter and Limimonas were most abundant in the Zobor variety.

Keywords: Amaranthus, Zobor variety, Pribina variety, coding regions markers, endophytes

ABSTRAKT

Láskavec patrí k málo využívaným druhom s potenciálom prekonať niektoré aktuálne výzvy moderného poľnohospodárstva, ktorých súčasťou sú veľmi nestabilné podmienky prostredia. V štúdii sú hodnotené dve slovenské odrody láskavca – Zobor (*A. hypochondriacus* × *A. hybridus*) a Pribina (*A. cruentus*), ktoré boli charakterizované polymorfizmom odvodeným z konzervatívnych oblastí DNA (CDDP), markérmi analógov P450 (PBA, polymorfizmom odvodeným od variability oblastí tubulínu (TBP) a pomocou najpočetnejšími endofytov, ktoré obsahujú. Profily CDDP, ako aj TBP ukázali veľmi podobnú distribúciu generovaných markérových odtlačkov pre obe analyzované odrody. Profily PBA poskytli najnižšiu hodnotu polymorfizmu. Široké spektrum amplikónov bolo generované všetkými technikami použitými v štúdii s rozsahom dĺžky PCR fragmentov od 80 do 2292 bp. Variabilita endofytického zloženia odrody Pribina vykazovala ako najpočetnejšie rody Endobacter, Dichotomicrobium a Burkholderia. Abiotrofia, Endobacter a Limimonas boli najpočetnejšie pre odrodu Zobor.

Kľúčové slová: Amaranthus, odroda Zobor, odroda Pribina, markéry kódujúcich oblastí, endofyty

INTRODUCTION

In today's agriculture, the unpredictability of climate change, such as more frequent dry and higher temperatures, poses significant challenges. In response, researchers are turning their attention to underutilized plant species, intrigued by their unique qualities and potential to thrive in such conditions (Mayes et al, 2011). Amaranthus spp. stands out as a notable example thanks to its nutrient-rich and climate-resilient traits, which are typically characterised by high genetic variation, environmental adaptability and phenotypic plasticity (Rastogi and Shukla, 2013; Jamalludin et al, 2022). Amaranth is a member of the Amaranthaceae family and consists of more than 60 species comprised of three subgenera - vegetable amaranth (Albersia), cultivated grain amaranth (*Amaranthus*) and weedy amaranth (Acnida) (Mosyakin and Robertson, 1996).

Different breeding strategies are being applied to amaranth to meet new environmental challenges (Anadruha et al, 2023). The preference for polymorphism in the profile of amaranth genetic resources clearly indicates that the sequential divergence is relatively high (Bardini et al, 2004; Li et al, 2013; Poczai et al, 2013; Bošeľová and Žiarovská, 2016) and breeding approaches hold the potential to yield favorable outcomes. Mutant breeding of the amaranth and its introduction into crop production has been significantly accelerated and made more effective by the maintenance breeding of mutant lines and new varieties of amaranth in the form of field trials. The agronomic indicator, the weight of thousands of seeds and the biochemical indicator, the nutritional value of the seeds, were improved in two Slovak amaranth varieties – Pribina and Zobor (Hricová et al, 2011; Kečkešová et al, 2013).

The characterization of plant varieties by DNA markers is a well-established approach that provides a wide range of different techniques that can be used depending on the research objective. Specific conserved DNA markers of coding regions are used to analyze intraspecific variability at the level of individual varieties, genetic diversity, DNA fingerprinting and germplasm conservation (Igwe et al, 2021; Chňapek et al, 2022; Vivodík et al, 2023). Among these, three are well established due to their universality for plant application to plant genomes - Conserved DNA-Derived Polymorphism (CDDP), P450-based analogue (PBA) markers and Tubulin-Based Polymorphism (TBP). Conserved genes, which are used as primer binding sites for primers in the CDDP technique represent protein sequences of conserved amino acids and, when amplified in PCR with short universal or degenerate primers generate length polymorphisms that provide information about the individual differences of the accession analyzed (Poczai et al, 2013; El Dessoky et al, 2020; Igwe et al, 2021). PBA polymorphism is generated in PCR analysis by primers that bind to the CYP or heme-binding sites and differences in the random distribution of cytochrome P450 regions in plants are obtained by universal primers (Rana et al, 2005; Poczai et al, 2013; Ravi et al, 2020).

The TBP technique uses single degenerate primer pairs complementary to conserved regions of the tubulin exons that anneal and amplify intervening introns in PCR for genotyping plant genetic resources (Poczai et al, 2013; Braglia et al, 2020). So far, all of them have only been applied to amaranth genetic resources in a few studies (Popa et al, 2010; Žiarovská et al, 2022), but have been summarized as suitable for describing genetic diversity and length polymorphism differences.

Another factor, that has been identified in recent decades as an important and variety-specific trait for yield and stress tolerance is endophytes (Chaudhary et al, 2022; Watts et al, 2023). Advanced techniques for analyzing microbial communities present exciting opportunities for gathering and enriching data in specialized research areas. These methods enable the identification of potentially beneficial or harmful species, the localization of potential sources of contamination and pollution, and the creation of microbial profiles reflecting the health status of individuals or entire populations (Hallmann and Berg, 2006; Caporaso et al, 2012; Shi et al, 2014; Gonzalez-Teuber et al, 2017). Current studies focus on the descriptive analysis of phylogenetic relationships of selected groups, the screening of habitants of landscape

and environmental interest, or the promotion of a rapid and cost-effective method at present (Hallmann and Berg, 2006; Hardoim et al, 2008; Santoyo et al, 2016). Symbiotic endophyte-plant relationships are considered natural and physiological for higher plants and each individual contains one or more endophytic fungal or bacterial species typical of its morphological parts (Strobel and Daisy, 2003; Deshmukh et al, 2015). It has been widely reported that the endophyte-plant relationship often results in specialized traits within plants due to the presence of endophytes (Yadav and Meena, 2021). Individual relationships range from a specific defence strategy against diseases and pathogens-antimicrobial, anticancer, antioxidant, anti-inflammatory and insecticidal up to the facilitation of plant growth through enhanced nutrient acquisition and stress tolerance (Manganyi and Ateba, 2020).

In the study, our focus was analyzing the polymorphism of the Pribina and Zobor varieties of Slovak amaranth. We used various DNA markers from coding regions to assess their specificity. Additionally, we aimed to identify the primary endophyte profiles associated with these two specific amaranth cultivars.

MATERIAL AND METHODS

Coding regions markers analysis

Amaranth varieties Zobor and Pribina were cultivated on Murashige and Skoog Medium (1962) for 8 weeks in controlled conditions at 30 ± 3 °C, 40 ± 3% of relative humidity and a photoperiod of 16 h light/8 h darkness. Cold LED light lamps provided the light intensity. All treatments were performed in triplicate, each replication contained ten seeds per culture container. Bulked total genomic DNA was isolated from plants by a modified methodology by Rogers and Bendich (1994) – CTAB method. PCR mixtures (Table 1) was established under reaction conditions (95 °C/5 min, [95 °C/1 min, 50 C/1 min, 72 °C/2 min] x44, 72 C/10 min + cooling 12 °C).

The list of primers used in the analyses included 5 CDDP primer combinations, 3 PBA primer combinations and 2 TBP primer combinations (Table 2). Screening analyses of PCR success were performed on 1.5% agarose gel electrophoresis. Image analysis of documented images (GeneSnap, SynGene) was performed using GelAnalyzer 2010.

The output data were also presented by positional polymorphism according to the equation created for interpretation purposes (Figure 1). The positional polymorphism is defined as the proportion of polymorphic fragments from the total number of fragments based on the size range of the selected marker (1 kb DNA ladder or 100 bp DNA ladder, both Bioline), where p_{ij} is the positional polymorphism value, c_{ij} is the number of positions corresponding to polymorphic fragments, n_i is the total number of fragments, i indicates the marker system used, and j denotes the observed factor (variety, template, primer).

A total of 5 CDDP primer combinations were used in the analysis, where WRKY-F was combined in all other R"X" primers.

Table 1. PCR mixture composition

PCR (10 µL)	PBA	TBP	CDDP
Master Mix (2x MyTaq)	5 µL	5 µL	5 µL
Primer 100 mMol/dm^{-1} (Forward + Reverse)	0.6 µL + 0.6 µL	1 µL + 1 µL	0.8 µL + 0.8 µL
DNA (30 ng)	2 µL	3 µL	1.4 µL
PCR Water	1.8 µL	0 µL	2 µL

Notes: CDDP – Conserved DNA-derived polymorphism; PBA – Cytochrome P450-based analogues; TBP – Tubulin-based polymorphism.

Table 2. List of used primers

Primer code	Primer sequence	Length	Technique
WRKY-F	5´-TggCgSAAgTACggCCAg -3´	18	CDDP
WRKY-R1	5´-gTggTTgTgCTTgCC-3´	15	CDDP
WRKY-R2	5´-gCCCTCgTASgTSgT-3´	15	CDDP
WRKY-R2B	5´-TgSTgSATgCTCCCg-3´	15	CDDP
WRKY-R3	5´-gCASgTgTgCTCgCC-3´	15	CDDP
WRKY-R3B	5´-CCgCTCgTgTgSACg-3´	15	CDDP
CYP1A1F	5´-gCCAAgCTTTCTAACAATgC-3´	20	PBA
CYP1A1R	5´-AAggACATgCTCTgACCATT-3´	20	PBA
CYP2B6F	5´- gACTCTTgCTACTCCTggTT-3´	20	PBA
CYP2B6R	5´-CgAATACAgAgCTgATgAgT-3´	20	PBA
CYP2C19F	5´-TCCTTgTgCTCTgTCTCTCA-3´	20	PBA
CYP2C19R	5´-CCATCgATTCTTggTgTTCT-3´	20	PBA
TBPfex1	5´- AACTgggCBAARggNCAYTAYAC-3´	23	TBP
TBPrex1	5´-ACCATRCAYTCRTCDgCRTTYTC -3´	23	TBP
TBPfin2	5´-gARAAYgCHgAYgARTgYATg-3´	21	TBP
TBPrin2	5´-CRAAVCCBACCATgAARAARTg-3´	22	TBP

Notes:
Letter „S“ indicates the presence of a nitrogenous base cytosine (C) or guanine (G);
Letter „B“ indicates the presence of a nitrogenous base cytosine (C), guanine (G) or thymine (T);
Letter „R“ indicates the presence of a nitrogenous base guanine (G) or adenine (A);
Letter „N“ indicates the presence of all 4 nitrogenous bases;
Letter „Y“ indicates the presence of a nitrogenous base cytosine (C) or thymine (T);
Letter „D“ indicates the presence of a nitrogenous base guanine (G), adenine (A) or thymine (T);
Letter „H“ indicates the presence of a nitrogenous base cytosine (C), adenine (A) or thymine (T);
Letter „V“ indicates the presence of a nitrogenous base cytosine (C), guanine (G) or adenine (A).
A total of 5 CDDP primer combinations were used in the analysis, where WRKY-F was combined in all other R”X” primers.
Primers designed as in: Yamanaka et al. (2003); Bardini et al. (2004), Collard and MacKill (2009).

$$p_{ij}\ (\%) = \frac{c_{ij}}{n_i} \text{ x } 100$$

Figure 1. Formula for calculation of position polymorphism

Analysis of endophytic bacteria

Extraction of the genomic DNA from Zobor and Pribina varieties grown in vitro was performed by a column method using the GeneJet™ Plant Genomic Extraction Kit (Thermo Scientific). Selective PCR analysis was performed according to time and temperature regimes in 2 separate reactions (primers – Table 3).

PCR1: 98 °C, 30 sec [98 °C, 5 sec; 72 °C, 50 s] x40 with final polymerization at 72 ° C, 120 s + cooling at 12 °C. PCR2: 98 °C, 30 sec [98 °C, 5 sec; 72 °C, 20 sec; 72 °C, 20 s] x15, [98 °C, 5 s; 69 °C, 20 sec; 72 °C, 20 s] x15, [98 °C, 5 s; 66 °C, 20 sec; 72 °C, 20 s] x15 with final polymerisation at 72 °C, 120 s + cooling at 12 °C.

Table 3. Structure of 16S rRNA primers

Label	Orientation	Variable part	Length	Primer part	Length
i341-04N	Forward	5´-AgACgAAg	8	gCCTACgggNggCWgCAg -3´	18
i341-15N	Forward	5´-AgATCAAg	8	gCCTACgggNggCWgCAg -3´	18
i341-16N	Forward	5´-ACTAgCAg	8	gCCTACgggNggCWgCAg -3´	18
i799-03N	Reverse	5´-ACTgTATAC	8	MgggTATCTAATCCKgTT -3´	18
i799-04N	Reverse	5´-ATCACCTAC	8	MgggTATCTAATCCKgTT -3´	18
i799-16N	Reverse	5´-ATAgATTAC	8	MgggTATCTAATCCKgTT -3´	18

Notes:
Letter „H“ indicates the presence of a nitrogenous base cytosine (C), adenine (A) or thymine (T);
Letter „K“ indicates the presence of a nitrogenous base thymine (T) or guanine (G);
Letter „M“ indicates the presence of a nitrogenous base cytosine (C) or adenine (A);
Letter „N“ indicates the presence of all 4 nitrogenous bases;
Letter „V“ indicates the presence of a nitrogenous base cytosine (C), guanine (G) or adenine (A);
Letter „W“ indicates the presence of a nitrogenous base adenine (A) or thymine (T).

Agarose gel electrophoresis (1.5%) was used to verify amplification and PCR products purified with AMPure XP Beads were stored at -20 °C in a plate (Amplicon). Ligation of indices (specific nucleotide triplets not found in the primer sequence of interest) and adapters followed Illumina sequencing ligation protocols (Klindworth et al. 2013). Q5 High-Fidelity DNA Polymerase coupled index pairs to PCR plates and adapter ligation was provided by the TrueSeq DNA PCR-Free LT Kit provided by the Illumina® MiSeq ligation protocol. Magnetic particles (sample purification beads) were cooled to 4 °C before use and the TrueSeq DNA PCR-Free LT Kit was set at -20 °C. As a part of the purification process, we selected adapters that were correctly bound, guided by the qPCR calibration curve and alignment of DNA concertation (0.715 ng.μL^{-1}, 4.17 mmol.dm^{-3}) for sample sequence conversion (Figures 2 and 3). The last step before sequencing involved removing any inhibitors using the PCR purification kit (Jena Bioscience).

$$\frac{ng.\mu L^{-1}\mu \times 10^{6}}{660 \text{ x average library fragment length}} = \text{Molarity}$$

Figure 2. The formula for converting molarity (Quibit)

The prepared library was sequenced on the MiSeq Illumina® platform using the MiSeq Kit V3 600 cycles kit, following the Illumina 16S Metagenomic Sequencing Library Preparation Guide.

Sequencing data were then processed using Seed 2 software (Větrovský and Baldrian, 2013), filtering out sequences shorter than 300 base pairs, sequences with quality scores below 30 (Q < 30), and chimeric sequences. Identification of endophytic bacterial species was performed using the SILVA database (Yilmaz et al., 2014). Further data processing involved additional manipulation of large data files at the user level, using the Python 3.6 programming language as the primary editing tool.

$$\frac{399 \text{ (control oligonucleotide length)}}{\text{average library fragment length}} \times \overline{pmol.dm^{-3}} = \text{Molarity}$$

Figure 3. The formula for converting molarity (qPCR)

RESULTS

DNA markers fingerprint-based analysis of amaranth

The selected DNA marker techniques resulted in high conservatism of the observed segments (Table 4). Analyzed groups of primers for used marker techniques showed that along the high conservatism of binding sites for individual primers, the PBA markers appear to have

the least positional/length polymorphism in the genomes of Slovak amaranth varieties (37.01%). All of the obtained amplicon profiles were reproducible (Figure 4).

Figure 4. CDDP profile of WRKYF+R2 primer combination for triplicate of Zobor variety

The position/length polymorphism of the remaining 2 techniques was 60.53% (TBP) and 66.67% (CDDP). The effect of variety on polymorphism was negligible with a maximum deviation of 1.61%.

Electrophoretic separation of the amplified products was performed on agarose gels and analyzed using GelAnalyzer 20.1 software. The first category of amplified fragment characteristics consists of those generated by the TBPex1-F+R, TBPin1F+R and WRKYF+R1 primer combinations, where 6 or 7 fragments were amplified (Table 5).

The second category includes combinations of CYPA1F+R and WRKYF+3b with approximately 13 fragments per fingerprint profile. The next category includes primer combinations that generated 14-15 fragment sizes (CYP2BF+R, CYP2CF+R and WRKYF+3). Primer combinations WRKYF+R2 and WRKYF+R2b separately amplified 11 fragment sizes (WRKYF+R2b) and 17 fragment sizes (WRKYF+R2). The impact of the variety on the frequency of fragment sizes is primarily observed in the Zobor variety. However, this influence is not specifically associated with a particular marker system or primer combination.

Table 4. Polymorphism obtained in individual marker technique primer combinations used in the characterization of Slovak amaranth varieties

CDDP	Polymorphism	PBA	Polymorphism	TBP	Polymorphism
Total	66,7%	Total	37%	Total	60,5%
Primer	Polymorphism	Primer	Polymorphism	Primer	Polymorphism
WRKY-R1	10,5%	CYPA1	23,7%	TBPex1	61,1%
WRKY-R2	54,9%	CYP2B6	48,8%	TBPin2	60%
WRKY-R2b	70%	CYP2C19	58,7%	Variety	Polymorphism
				Pribina	15,4%
WRKY-R3	48,9%	Variety	Polymorphism	Zobor	15,4%
WRKY-R3b	15,8%	Pribina	26,7%		
Variety	Polymorphism	Zobor	26,3%		
Pribina	22,5%				
Zobor	22%				

Notes: CDDP – Conserved DNA-derived polymorphism; PBA – Cytochrome P450 based analogues; TBP – Tubulin-based polymorphism.

Table 5. Fingerprint characteristics of DNA techniques used in the study

Variety	Marker technique	Primer pair	Number of amplicons	Average length of amplicon/bp	Minimum length of amplicon/bp	Maximum length of amplicon/bp
Pribina	TBP	TBPin2F+R	7	350	81	892
Pribina	PBA	CYPA1F+R	13	764	83	1960
Pribina	PBA	CYP2BF+R	15	756	83	1870
Pribina	PBA	CYP2CF+R	17	759	83	1986
Pribina	CDDP	WRKYF+R1	7	1048	86	2008
Pribina	CDDP	WRKYF+R2	17	1058	96	2292
Pribina	CDDP	WRKYF+R2b	8	1042	92	2019
Pribina	CDDP	WRKYF+3	17	983	84	2101
Pribina	CDDP	WRKYF+3b	13	928	144	1961
Zobor	TBP	TBPex1F+R	6	1151	81	2221
Zobor	TBP	TBPin2F+R	6	362	81	904
Zobor	PBA	CYPA1F+R	10	667	83	1960
Zobor	PBA	CYP2BF+R	14	640	82	1760
Zobor	PBA	CYP2CF+R	14	661	83	1986
Zobor	CDDP	WRKYF+R1	6	387	80	830
Zobor	CDDP	WRKYF+R2	16	872	91	2292
Zobor	CDDP	WRKYF+R2b	11	1111	90	2129
Zobor	CDDP	WRKYF+3	13	816	80	1691
Zobor	CDDP	WRKYF+3b	13	934	89	1884

Primers designed as in: Yamanaka et al. (2003); Bardini et al. (2004), Collard and MacKill (2009).

Endophytic bacteria of Pribina and Zobor varieties

The filtered metagenomic data is divided into 2 distinct datasets, reflecting variations in sequence quality. The filtered metagenomic data is divided into 2 distinct datasets, reflecting variations in sequence quality. The first dataset comprises 353 bacterial sequences associated with the Pribina variety. Despite this count, we identified 20 endophytic bacterial phylum from the Pribina variety, encompassing 15 unique bacterial phyla. Conversely, the sequencing data from the Zobor variety consisted of 341 bacterial sequences, with 17 distinct bacterial phylum identified.

The variability of the endophytic composition of the variety Pribina showed the following five most abundant genera: Endobacter, Dichotomicrobium, Burkholderia, Taonella and Spartobacteria (Table 6). Compared to the Zobor variety, the number of identified sequences was much lower for the Pribina variety. The analysis shows that both varieties have most of the identified genera belonging to the genus *Endobacter* sp.

Table 6. A top 5 of the most frequently occurring endophytic bacterial genera in the Pribina and Zobor varieties of amaranth

PRIBINA variety		ZOBOR variety	
Genera	NS	Genera	NS
Endobacter[1]	313	*Abiotrophia*[1]	1.039
Dichotomicrobium[1]	10	*Endobacter*[2]	862
Burkholderia[1]	9	*Limimonas*[2]	424
Taonella[1]	3	*Candidatus Carsonella*[2]	265
***Spartobacteria* Incertae Sedis**[3]	2	*Pleomorphobacterium*[2]	128
Total numbers (%)	95.47	Total numbers (%)	76.22
Genera with 1 sequence	14	Genera with 1 sequence	17

Notes:
NS – number of sequences,
Underlined genera are part of the same branch of *Rhodospirillales*.
Bold-highlighted genera do not appear in any other filtrated profile of bacterial colonization of test varieties of amaranth.
[1] branch of *Firmicutes*,
[2] branches of *Proteobacteria*,
[3] branches of *Bacteroidetes*.

DISCUSSION

The TBP, PBA, and CDDP marker systems are markers of coding regions of DNA and gene families (Poczai et al, 2013).

The high frequency of intraspecific polymorphisms in plant material (*Solanaceae*, *Rosaceae* and *Libiatae*) showed that PBA markers effectively reflect genetic diversity (Yamanaka et al, 2003). The profile specificity of PBA fingerprints and individual plant species was previously documented (Bošeľová and Žiarovská, 2016). Amplified PBA fragment sizes within 400 -1000 bp reflect both functional and genomic region differences and confirm the effectivity of P450 gene analogues as genetic markers for plant genetic diversity studies (Yamanaka et al, 2003).

PBA is more likely to be used to detect genomic variability than isozyme, RAPD, AFLP, or ISSR markers as they do not focus on the common border of coding and non-coding regions but on neutral regions or specific parts of the genome. This makes them effective tools for characterizing genetic resources and assessing the genetic variation of species (Schuler and Werck-Reichhart, 2003). PBA marker was applied by Žiarovská et al. (2022) in the analysis of the same amaranth varieties growing under cadmium stress conditions, with the result that different profiles were generated for stressed plants.

The TBP method is based on the presence of the first intron in the coding sequence of β-tubulin, the length of which can vary depending on the β-tubulin family of isotypes it defines (Bardini et al., 2004). TBP has less than a quarter of the information on markers detected by SSR analysis, as most of the DNA sequences of each analysed clone represent an intron sequence. Fragments longer than 300 bp correspond to the expected amplification product of the first intron including small portions of exons (Bardini et al, 2004). Fragments longer than 1,200 bp are well reproduced and can be used to differentiate cultivars.

However, their origin is unclear and fragments should be further investigated. Also in our study, the positional polymorphism of the TBP marker technique varied in the expected values between the amaranth varieties analyzed. Rabokon et al (2018) suggest that these are DNA homoduplex and/or heteroduplex complexes visible

on polyacrylamide gels. It is premature to suggest that fragments above 1,200 bp represent duplexes until we have the necessary information about their behaviour under stress. We have studied several transcription factor genes associated with developmental and physiological processes (WRKY – 80%), where the percentage of polymorphic fragments for the chrysanthemum population exceeds the polymorphism of markers of the ISSR and SRAP system (Li et al, 2013; Poczai et al, 2013). Of the 5 primer combinations used by Collard and Mackill (2009), 2 were specified for the *Thinopyrum* genome (WRKY-R1, WRKY-R2) and 1 for the *Triticum* genome (WRKY-R1) (Guo et al, 2016). Application of TBP markers has not been widely applied to amaranth species. Popa et al. (2010) used the TBP method to analyse possible polymorphism in the β-tubulin gene on 6 genotypes of Amaranthus. The results showed an intraspecific polymorphism of the *A. cruentus* variety Alegria, while no polymorphism was detected in the other species studied.

The primers of the CDDP marker system itself are short degenerate oligonucleotide sequences of functional gene sequences. The typical fragment size range for CDDP markers is between 200 and 1,500 bp (Collard and Mackill, 2009). The results presented in our study contain a total of 223 amplified fragments in the range corresponding to the expected length. When examining the averages of fragment sizes (ranging from 180 to 385 base pairs) and their deviations (ranging from 135 to 665 base pairs), we observed a distinctive pattern in the fingerprint generated for the Zobor variety. Specifically, the primer combination WRKY-R1 exhibited a remote positioning, capturing the fewest number of fragments, with the longest fragment spanning only 830 base pairs.

The analysis of endophytes shows that both varieties have a high percentage of the identified genera belonging to the genus *Endobacter* sp. This genus is not usually identified in endophytic settlements (Ramírez-Bahena et al. 2013), although it does not outgrow other growth endogenous bacteria by growth conditions (Bac*Dive*, 2019). The only reasonable cause for a fundamental change in the endophytic composition could be related to the reduced quality of sequencing data. Molecular community fingerprints reveal the presence of rDNA genes in the total yield of plant nucleic acids including cell organelles that also contain 16S rDNA genes. Identification of individual PCR fragments in libraries of random clones may fail due to high levels of cellular organelles. Exclusion of 16S rDNA amplicons from extra-nuclear DNA of eukaryotic origin is provided with a primer (799F, *Escherichia coli*) that does not bind to chloroplast DNA (Chelius and Triplet, 2001). The 968F/1401R primer combination captured more chloroplast amplicons from potato DNA extracts than the 799F/1401R combination because it amplified extra-nuclear DNA genes (Hallmann and Berg, 2006). With the genus *Endobacter* sp. (phylum *Furmicutes*) it was also obtained the genus *Abiotrophia* sp. (phylum *Proteobacteria*), which contains up to 60% of the sequences of the Zobor variety. The most abundant genus is the genus *Abiotrophia* sp. With more than a quarter belonging to the ZOBOR variety. These two endophytic genera are similar in terms of growth conditions (Ramirez-Bahena et al, 2013).

The position of the genus *Endobacter* sp. in the colonization of the variety Zobor is probably influenced by the position of the genus *Limimonas* sp. The Bacterial genus *Endobacter* sp. is more acidophilic, and the genus *Limimonas* sp. is characterized by halophilic potential (Ramirez-Bahena et al, 2013; Bac*Dive*, 2019). It is not possible to determine which of the factors were involved in the colonization of the host organism, but in conjunction with radiation, it appears that salinity, then the temperature and final acidity/alkalinity are most involved in colonization and the colonization by the thermophilic genera *Oceanotoga* sp. and the *Orientia* sp. did not vary significantly between varieties (BacDive, 2019).

Potentially harmful bacteria may latently colonize plants, without affecting the host organism and its microbial populations. If such a condition is not permanent and the bacteria are only delayed in the attenuation phase, the activator of their pathogenicity and antagonism mechanisms may be the later stage of plant growth,

the achievement of critical density *„quorum sensing“* or response to stress stimulus. These mechanisms are very similar and sometimes the expression of one metabolite or the total number of bacteria is sufficient (Hardoim et al, 2008; Santoyo et al, 2016).

CONCLUSION

The binding sites of the WRKY primers within the CDDP marker technique are evenly and sparsely distributed among the analyzed Slovak amaranth varieties, regardless of the primer or genotype. In particular, the WRKY-R2 combination showed an increased number of fragments compared to other combinations, while the WRKY-R2b primer combination showed a positional polymorphism.

Based on fragment parameters and positional polymorphisms, fragments larger than 1000 base pairs (PBA) and 1200 base pairs (TBP) show potential for genotype differentiation. However, the differentiation of TBP markers proved to be more challenging than PBA markers. The genus *Endobacter* sp. is the most abundant genus in both varieties. Its position on the Zobor variety may be suppressed in favor of endophytic bacteria of the genus *Abiotrophia* sp. The presence of the genus *Burkholderia* sp. may be related to the ability of pathogenic bacteria to alter gene expression in the environment depending on the microbial density. However, this strategy could be limited by the growth and culture conditions that influence the success of amaranth colonization by endophytic bacteria.

ACKNOWLEDGEMENT

This study was supported by the project VEGA 2/0013/22 Amaranth plasticity in response to heavy metals: multi-scale analysis from ecophysiological to molecular aspects.

REFERENCES

Anuradha, Kumari, M., Zinta, G., Chauhan, R., Kumar, A., Singh, S., Singh, S. (2023) Genetic resources and breeding approaches for improvement of amaranth (*Amaranthus* spp.) and quinoa (*Chenopodium quinoa*). Frontiers in Nutrition. 10:1129723. DOI: https://doi.org/10.3389/fnut.2023.1129723

BacDive (2019) The Bacterial Diversity Metadatabase. Available at: https://bacdive.dsmz.de/ [Accessed 12 September 2023].

Bardini, M., Lee, D., Donini, P., Mariani, A., Giani, S., Toshi, M., Lowe, C., Breviario, D. (2004) Tubulin-Based Plymorphism (TBP): A New Tool, Based on Functionally Relevant Sequence, to Assess Genetic Diversity in Plant Species. Genome, 47 (2), 281-291. DOI: https://doi.org/doi:10.1139/G03-132

Bošeľová, D., Žiarovská, J. (2016) Direct PCR as the Platform of *Hedera helix*, L. Genotyping without the Extraction of DNA. Journal of Central European Agriculture, 17 (4), 841-949. DOI: https://doi.org/10.5513/JCEA01/17.4.1795

Braglia, L., Gavazzi, F., Morello, L., et al. (2020) On the applicability of the Tubulin-Based Polymorphism (TBP) genotyping method: a comprehensive guide illustrated through the application on different genetic resources in the legume family. Plant Methods, 16, 86.

Caporaso, J.G. et al. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME Journal, 6 (8), 1621-1624. DOI: https://doi.org/10.1038/ismej.2012.8

Chelius, M.K., Triplett, E.W. (2001) The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L. Microbial Ecology, 41 (3), 252-263. DOI: https://doi.org/10.1007/s002480000087

Collard, B.C.Y., MacKill, D.J. (2009) Conserved DNA-Derived Polymorphism (CDDP): A Simple and Novel Method for Generating DNA Markers in Plants. Plant Molecular Biology Reports, 27 (4), 558-562. DOI: https://doi.org/doi:10.1007/s11105-009-0118-z

Deshmukh, S., Verekar, S., Bhave, S. (2015) Endophytic fungi: A reservoir of antibacterials. Frontiers in Microbiology, 5, 715.

González-Teuber, M., Vilo, C., Bascuñán-Godoy, L. (2017) Molecular characterization of endophytic fungi associated with the roots of *Chenopodium quinoa* inhabiting the Atacama Desert, Chile. Genomics Data, 11, 109-112. DOI: https://doi.org/10.1016/j.gdata.2016.12.015

Guo, J., Yu, X., Yin, H., Liu, G., Li, A., Wang, H., Kong, L. (2016) Phylogenetic relationships of *Thinopyrum* and *Triticum* species revealed by SCoT and CDDP markers. Plant Systematics and Evolution, 302 (9), 1301-1309. DOI: https://doi.org/10.1007/s00606-016-1332-4

El Dessoky, D.S., Attia, A.O., Ismail, I.A., Alotaibi, S.S., Aljuaid, B.S. (2020) Molecular assessment of genetic stability using CDDP and DNA-barcoding assays in long-term micropropagated rose plant. Pakistan Journal of Biological Science, 23, 1176–1183.

Hallmann, J., Berg, G. (2006) Spectrum and Population Dynamics of Bacterial Root Endophytes. In: Microbial Root Endophytes [online]. Berlin: Springer, pp. 15-31. DOI: https://doi.org/10.1007/3-540-33526-9_2

Hardoim, P.R., Van Overbeek, L.S., Van Elsas, J.D. (2008) Properties of bacterial endophytes and their proposed role in plant growth. In: Bacterial Endophytes of Rice – Their Diversity, Characteristics and Perspectives [online]. Groningen: University of Groningen, pp. 463-471. DOI: https://doi.org/10.1016/j.tim.2008.07.008

Hricová, A. Kečkešová, M., Gálová, Z., Libiaková, G., Gajdošová, A. (2011) Investigation of Protein Profile Changes in Amaranth Seeds after Radiation Mutagenesis. Chemiské listy, 105 (7), 542-545 (In Slovak).

Chaudhary, P., Agri, U., Chaudhary, A., Kumar, A., Kumar, G. (2022) Endophytes and their potential in biotic stress management and crop production. Frontiers in Microbiology, 13, 933017.

Chňapek, M., Mikolášová, L., Vivodík, M., Gálová, Z., Hromadová, Z., Ražná, K., Balážová, Ž. (2022) Genetic diversity of oat genotypes using SCoT markers. Biology and Life Sciences Forum, 11 (1), 29. DOI: https://doi.org/10.3390/IECPS2021-11926

Igwe, D.O., Ihearahu, O.C., Osano, A.A., et al., (2021) Genetic diversity and population assessment of *Musa* L. (Musaceae) employing CDDP markers. Plant Molecular Biology Reporter, 39, 801-820.

Jamalluddin, N., Massawe, F.J., Mayes, S., Ho, W.K., Symonds, R.C. (2022) Genetic diversity analysis and marker-trait associations in Amaranthus species. PLoS ONE, 17 (5), e0267752. DOI: https://doi.org/10.1371/journal.pone.0267752

Kečkešová, M., Palenčárová, E., Gálová, Z., Gažo, J., Hricová, A. (2013) Nutritional Quality of Grain Amaranths (*Amaranthus* L.) Compared to Putative Mutants Lines. Journal of microbiology, biotechnology and food sciences, 2 (1), 1716-2174.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O. (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Research, 41 (1), e1-e1. DOI: https://doi.org/10.1093/nar/gks808

Li, T., Guo, J., Li, Y., Ning, H., Sun, X., Zheng, C. (2013) Genetic Diversity Assessment of Chrysanthemum Germplasm Using Conserved DNA-Derived Polymorphism Markers. Scientia Horticulturae, 162, 271-277. DOI: https://doi.org/10.1016/j.scienta.2013.08.027

Manganyi, M.C., Ateba, C.N. (2020) Untapped potentials of endophytic fungi: A review of novel bioactive compounds with biological applications. Microorganisms, 8, 1934

Mayes, S., Massawe, F.J., Alderson, P.G., Roberts, J.A., Azam-Ali, S.N., Hermann, M. (2011) The potential for underutilized crops to improve security of food production. Journal of Experimental Botany, 63, 1075–1079. DOI: https://doi.org/10.1093/jxb/err396

Mosyakin, S.L., Robertson, K.R. (1996) New infrageneric taxa and combinations in Amaranthus (*Amaranthaceae*). Annales Botanici Fennici, 33, 275–282.

Murashige, T., Skoog, F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant, 15 (3), 473-497. DOI: https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Popa, G., Cornea, C. P., Ciuca, M., Babeanu, N., Popa, O., Marin, D. (2010) Studies On Genetic Diversity In Amaranthus Species Using The RAPD Markers. Analele Universitati din Oradea – Fascicula Biologie, 17 (2), 280-285.

Pozcai, P., Varga, I., Laos, M., Cseh, A., Bell, N.E., Valkonen, J.P.T., Hyvönen, J. (2013) Advances in Plant Gene-Targeted and Functional Markers: A Review. Plant Methods, 9 (1), 6. DOI: https://doi.org/10.1186/1746-4811-9-6

Rabokon, A.N., Pirko, Y.V., Demkovych, A.Y., Blume, Y.B. (2018) Comparative analysis of the efficiency of intron-length polymorphism of β-tubulin genes and microsatellite loci for flax varieties genotyping. Cytology and Genetics, 52 (1), 1-10. DOI: https://doi.org/10.3103/S0095452718010115

Ramírez-Bahena, M.H., Tejedor, C., Martín, I., Velázquez, E., Peix, A. (2013) *Endobacter medicaginis* gen. nov., sp. nov., isolated from alfalfa nodules in an acidic soil. International Journal of Systematic and Evolution Microbiology, 63 (5), 1760-1765. DOI: https://dx.doi.org/10.1099/ijs.0.041368-0

Rana, M.K., Munjal, V.D., Sonika, S., Bhat, K.V. (2005) Utility of P450-based-analogue (PBAs) markers for diversity analysis of linseed (*Linum usitatissimum* L.) cultivars. Indian Journal of Plant Genetic Resources, 18 (2), 194–199.

Rastogi, A., Shukla, S. (2013) Amaranth: A new millennium crop of nutraceutical values. Critical Reviews in Food Science and Nutrition, 53, 109–125. DOI: doi.org/10.1080/10408398.2010.517876

Ravi, R.S.D., Siril, E.A., Nair, B.R. (2020) The efficiency of Cytochrome P450 gene-based markers in accessing genetic variability of drumstick (*Moringa oleifera* Lam.) accessions. Molecular Biology Reporter, 47 (4), 2929–2939.

Rogers, S.O., Bendich, A.J. (1994) Extraction of total cellular DNA from plants, algae and fungi. In: Plant Molecular Biology Manual, vol. 2(D1). Dordrecht: Kluwer Academic Publishers, p. 1-8.

Santoyo, G., Moreno-Hagelsieb, G., Del Carmen Orozco-Mosqueda, M., Glick, B.R. (2016) Plant growth-promoting bacterial endophytes. Microbiology Research, 183, 92-99. DOI: https://doi.org/10.1016/j.micres.2015.11.008

Schuler, M.A., Werck-Reichhart, D. (2003) Functional genomics of P450s. Annual Reviews in Plant Biology, 54 (1), 629-67. DOI: https://doi.org/10.1146/annurev.arplant.54.031902.134840

Shi, Y., Yang, H., Zhang, T., Sun, J., Lou, K. (2014) Illumina-Based Analysis of Endophytic Bacterial Diversity and Space-Time Dynamics in Sugar Beet on the North Slope of Tianshan Mountain. Applied Microbiology and Biotechnology, 98 (14), 6375-6385. DOI: https://doi.org/10.1007/s00253-014-5720-9

Strobel, G., Daisy, B. (2003) Bioprospecting for microbial endophytes and their natural products. MMBR, 67, 491–502.

Větrovský, T., Baldrian, P. (2013) Analysis of soil fungal communities by amplicon pyrosequencing: current approaches to data analysis and the introduction of the pipeline SEED. Biology and Fertility of Soils, 49 (8), 1027-1037. DOI: https://doi.org/10.1007/s00374-013-0801-y

Vivodík, M., Balážová, Ž., Chňapek, M., Hromadová, Z., Mikolášová, L., Gálová, Z. (2023) Genetic relationship of soybean (*Glycine max* L.) genotypes using SCoT markers. Journal of Microbiology, Biotechnology and Food Sciences, 13 (1), 1–3.

Watts, D., Palombo, E.A., Jaimes Castillo, A., Zaferanloo, B. (2023) Endophytes in Agriculture: Potential to Improve Yields and Tolerances of Agricultural Crops. Microorganisms, 11 (5), 1276.

Yadav, G., Meena, M. (2021) Bioprospecting of endophytes in medicinal plants of Thar Desert: An attractive resource for biopharmaceuticals. Biotechnology Reporter, 30, e00629.

Yamanaka, S., Suzuki, E., Tanaka, M., Takeda, Y., Watanabe, A.J., Watanabe, A K.N. (2003) Assessment of Cytochrome P450 Sequences Offers A Useful Tool for Determining Genetic Diversity in Higher Plant Species. Theoretical and Applied Genetics, 108(1), 1-9. DOI: https://doi.org/10.1007/s00122-003-1403-0

Yilmaz, P. et al. (2014) The SILVA and 'All-Species Living Tree Project (LTP)' Taxonomic Frameworks. Nucleic Acids Research, 42 (D1), D643-D648. DOI: https://doi.org/10.1093/nar/gkt1209

Žiarovská, J., Štefúnová, V., Hricová, A. (2022) PBA polymorphism profiles of Slovak Amaranth varietes growing under cadmium In: Vliv abiotických a biotických stresoru na vlastnosti rostlin 2022. Praha: Česká zemědělská univerzita, 2022, s. 137-140 (In Slovak).