**CORE Organic Cofund**


***"SCOOP: Developing intercropping systems with camelina to increase the yield and quality parameters of local underutilized crops"***

**Deliverable report**

# D2.1. Evaluation of genetic variations of camelina and companion crops

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**Tasks performed by AUP**

Molecular markers play an essential role in the assessment of genetic diversity. They are an important tool for the discovery and practical use of polymorphisms. Over the past thirty years, numerous molecular marker techniques have been developed, based on practically the entire spectrum of molecular cellular mechanisms. Molecular markers based on the most variable regions in the genome, namely microsatellites and mobile genetic elements, are widely used (Monden et al., 2014). Microsatellites are the simplest tandemly repeated sequences resulting from multiple consecutive repetitions of a short repeat unit (up to 13 bp), so that a long line of monotonous repeats is produced. Such sequences are thought to be produced primarily by replication errors, the DNA polymerase 'slipping' so that it goes back and replicates a given short section multiple times. Therefore, in closely related species and even in individuals of the same species, the length of such tandem sequences is different. This is the basis of various diagnostic and genotyping techniques. Other highly variable regions of great importance for estimating genetic diversity are the transposable elements. Retroelements are mobile genetic elements in high copy numbers in eukaryotic genomes (Kalendar et al. 2010). They underlie genome variation and are relevant to the evolution of species, so they can be used to characterize their genetic potential at all systematic levels (Kalendar and Schulman, 2014).

In the present study, an assessment of the genetic diversity analysis of *Camelina sativa* plants with ISSR and iPBS primers was performed. The efficiency of the primers used was evaluated based on the intensity and number of amplified fragments in the generated profiles in selected representatives.

A total of 48 genotypes of camelina (*Camelina sativa*) have been used for the assessment of genetic diversity. The analyses were done in the Department of Crop Sciences and the Department of Microbiology and Environmental Biotechnology in the AUP. Young leaves of camelina varieties were used for DNA isolation, and triturated in liquid nitrogen. DNA extraction was performed with a CTAB protocol. Sample concentrations were determined on an agarose gel by comparison with standard concentrations of lambda DNA (Thermo Scientific Life Sciences, Cat. No SD0011, Lithuania). Four concentrations were prepared: I. - 50 ng/µL, II - 20 ng/µL, III - 10 ng/µL and IV - 5 ng/µL from a 300 ng/µL lambda DNA solution to serve to compare concentrations of the isolated DNA samples.

iPBS primers designed by (Kalendar et al., 2010) were used (Table 1). PCR reactions were performed in 10 µL reaction mixture. Each reaction mixture contained 15 ng template DNA, 1 µL of 10 x Green PCR buffer (Thermo Fisher Scientific), 1 µM primer, 1 µL of 2 mM dNTP MIX (Thermo Fisher Scientific), and 0.1 µL of 5 U DreamTaq DNA polymerase (Thermo Fisher Scientific). PCR amplifications were performed under the following conditions: an initial denaturation step at 94°C for 2 min, followed by 35 cycles at 95°C for 15 s., at 40-60°C (depending on the nucleotide sequence and Tm of the primers) for 1 min and at 68°C for 1 min., followed by a final extension of 72°C for 5 min (Andeden et al., 2013).

The results of the PCR products were visualized by electrophoresis in a 1.5% agarose gel (1x TAE) stained with SafeView (NBS Biologicals, UK) at 70 V. A GeneRuler 100 bp DNA Ladder was used for fragment length comparison.

**Results**

A total of 48 iPBS of C. sativa varieties were tested to evaluate the effectiveness and select the most suitable iPBS primers for genotyping representatives of the *Brassicaceae* family. The primers tested were described as the most efficient for the study of genetic diversity in camelina. Some of the iPBS primers have been described in various studies as informative for camelina as well (Orhan and Kara, 2023).

Table 1. Characteristics of primers used in the study

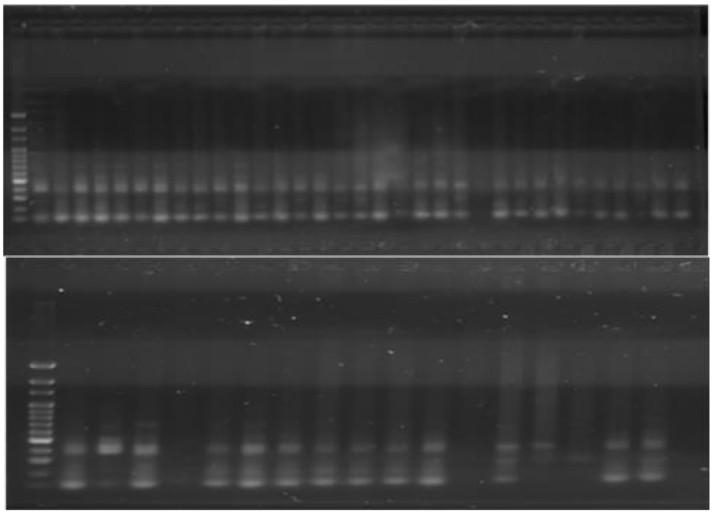
| **iPBS primers** | Nucleotide sequence | **Annealing temperature** | **Reference** |
|---|---|---|---|
| 2400 | CCCCTCCTTCTAGCGCCA | 60℃ | Kalendar et al., 2010 |
| 2242 | GCCCCATGGTGGGCGCCA | | |
| 2395 | TCCCCAGCGGAGTCGCCA | | |
| 2237 | CCCCTACCTGGCGTGCCA | | |
| 2228 | CATTGGCTCTTGATACCA | 55℃ | |
| 2232 | AGAGAGGCTCGGATACCA | | |
| 2249 | AACCGACCTCTGATACCA | | |
| 2240 | AACCTGGCTCAGATGCCA | | |
| 2221 | ACCTAGCTCACGATGCCA | | |
| 2241 | ACC TAG CTC ATC ATG CCA | | |
| 2229 | CGACCTGTTCTGATACCA | | |
| 2243 | AGT CAG GCT CTG TTA CCA | | |
| 2238 | ACC TAG CTC ATG ATG CCA | | |
| 2386 | CTGATCAACCCA | 40℃ | |
| 2074 | GCTCTGATACCA | | |
| 2276 | ACCTCTGATACCA | | |
| 2277 | GGCGATGATACCA | | |
| 2375 | TCGCATCAACCA | | |
| 2272 | GGCTCAGATGCCA | | |
| 2390 | GCAACAACCCCA | | |
| 2394 | GAG CCT AGG CCA | | |
| **ISSR** | Nucleotide sequence | **Annealing temperature** | **Reference** |
| UBC 807 | AGA GAG AGA GAG AGA GT | 55℃ | (Denduangboripant et al., 2010). |
| UBC808 | AGAGAGAGAGAGAGAGC | | (Andeden et al., 2013) |
| UBC810 | GAGAGAGAGAGAGAGAT | | |
| UBC823 | TCTCTCTCTCTCTCTCC | | |
| UBC826 | ACACACACACACACACC | | |
| UBC890 | VHVGTGTGTGTGTGTGT | | |

While ISSR markers have been used for a long time and have proven their effectiveness in assessing the genetic diversity of members of the Brassicaceae family, the iPBS markers developed by Kalendar et al., 2010 were used a little later in 2015 by a Turkish team on Fabaceae plants (Certel et al., 2023). After that, more studies were conducted to evaluate the genetic diversity with the iPBS molecular marker technique (Ozturk et al., 2020, Haliloglu., et al., 2022), the informative results obtained confirm the great potential of the retrotransposin-based iPBS technique for evaluation and research of the representatives of the *Brassicaceae* family.

In the present study, the reactions performed show that both molecular marker techniques (iPBS) lead to the generation of clear profiles with different numbers, intensities and lengths of the fragments. Under the above reaction conditions, the number of amplified fragments in the iPBS reactions was from 1 to 10 and in the ISSR reactions from 3 to 8.

Most of the iPBS reactions were performed with a selected representative of camelina varieties and in over 90% of the reactions performed with the selected conditions profiles suitable for genotyping were established, but in 5 of the reactions with primers iPBS 2074, iPBS 2375, iPBS 2390, iPBS 2386, iPBS 2232 and iPBS 2242 more than 5 fragments are amplified. 3 to 7 fragments were generated in the 6 ISSR reactions with a camelina variety representative under the indicated PCR conditions. The analysis of the obtained results indicated not so high level of genetic diversity in the tested genotypes (Fig. 1, 2 and 3), however, the distribution of this diversity was heterogeneous.

iPBS 2074 Correlation coefficient = 0.954



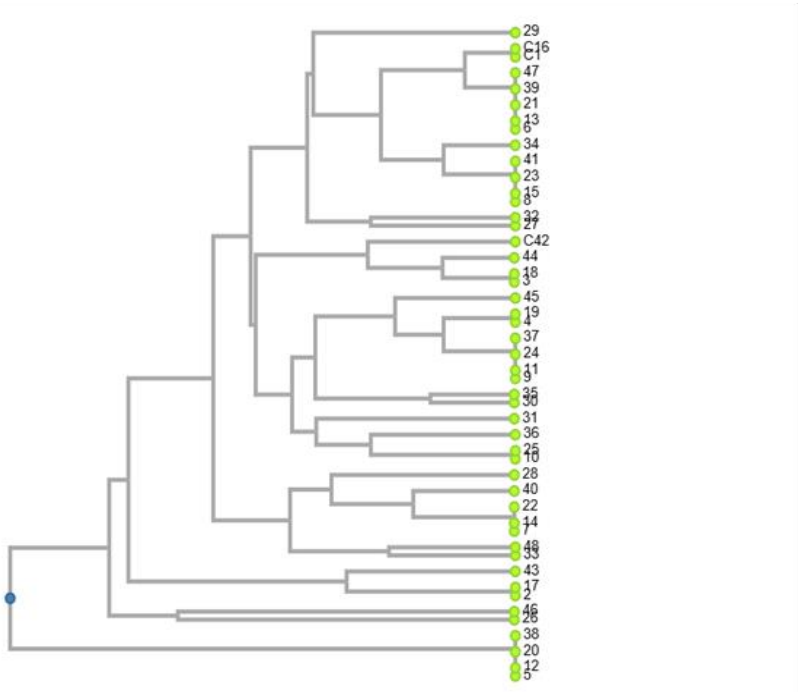
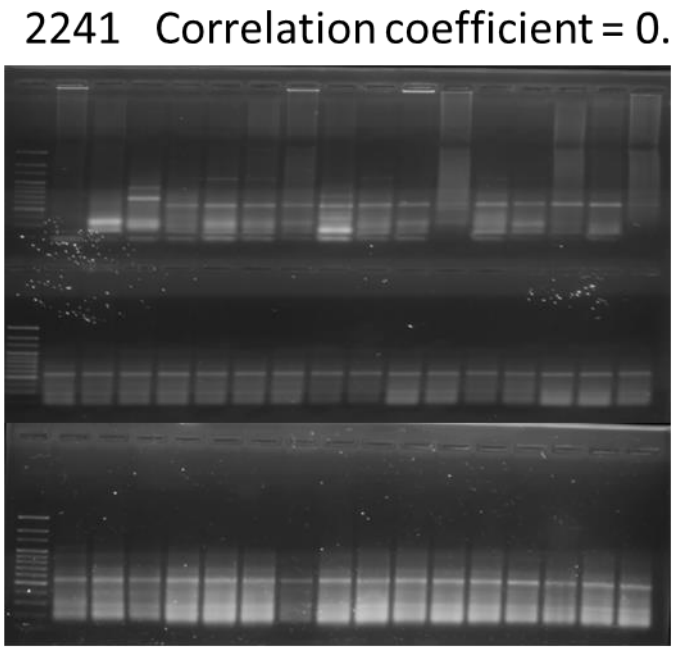


Figure 1. Agarose gel electrophoresis and dentrogram of genetic diversity profiles of camelina varieties generated with iPBS 2074 molecular marker techniques.



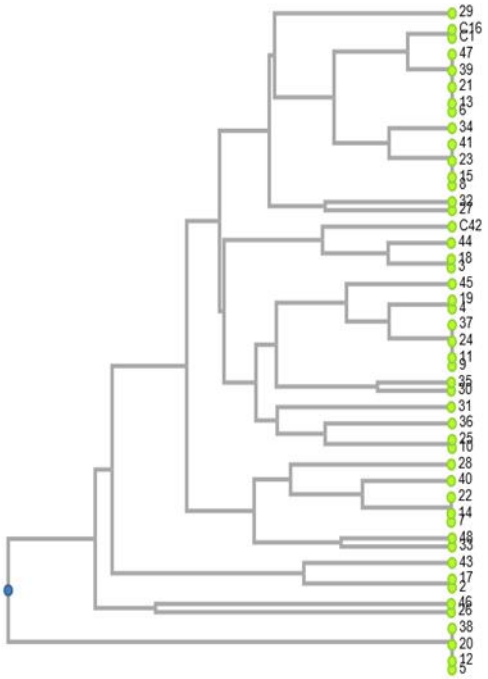




Figure 2. Agarose gel electrophoresis and dentrogram of genetic diversity profiles of camelina varieties generated with iPBS 2241 molecular marker techniques.

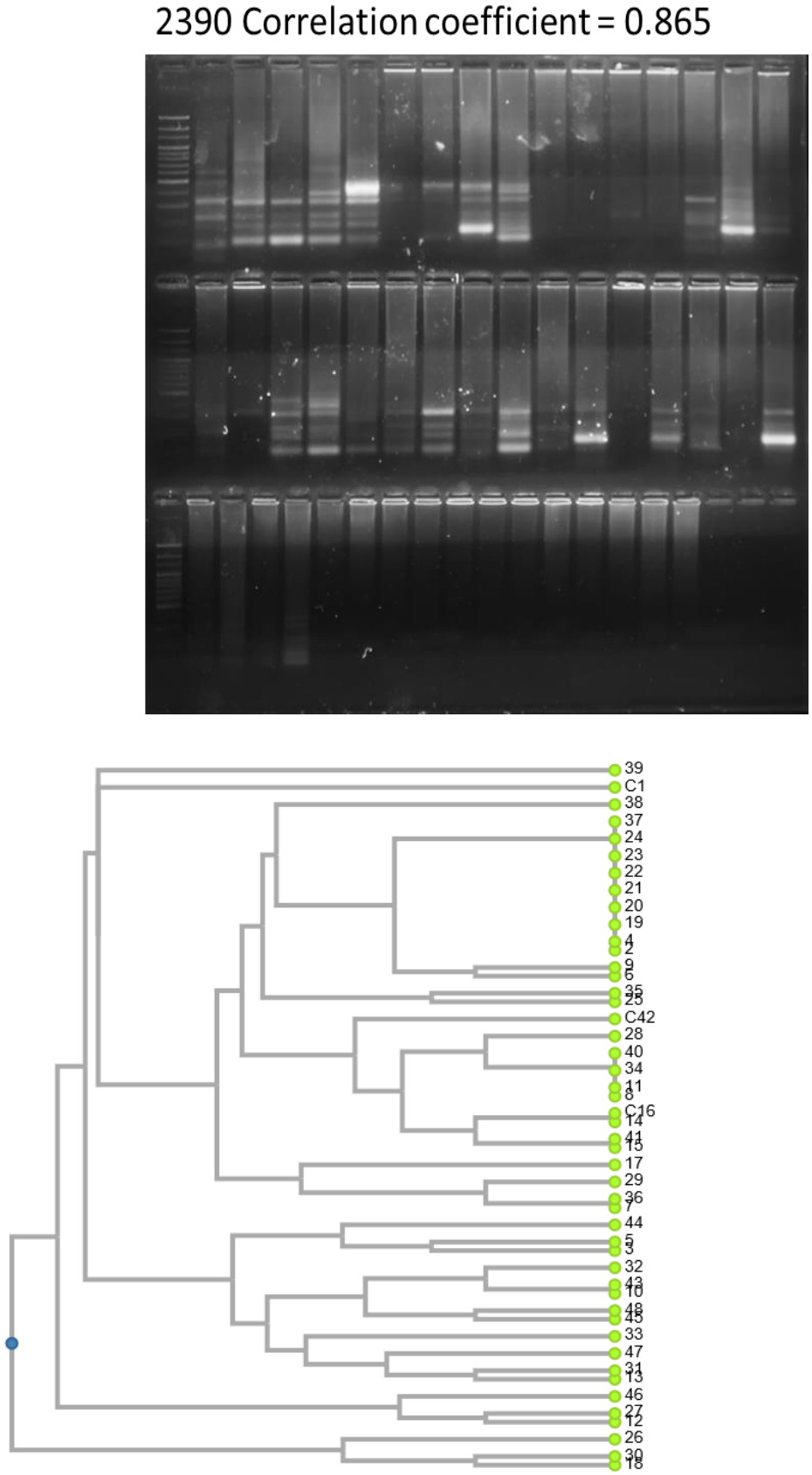



Figure 3. Agarose gel electrophoresis and dentrogram of genetic diversity profiles of camelina varieties generated with iPBS 2390 molecular marker techniques.

For the purpose of molecular analyses, a total of 10 primers that successfully amplified microsatellite sequences in the genome of the untreated and radiation-treated camelina variants were used. The resulting bands have a distinct polymorphism, which corresponds to a different character of the distribution of the nucleotide sequences. Table 2 summarizes the results of the ten iPBS primers. The total number of products is 65, of which a total of 31 polymorphic bands and 34 monomorphic bands. The percentage of polymorphism was calculated to be 47.69%.

Table 2. Number of PCR products obtained using 10 iPBS primers.

| Name of the iPBS primer | Primer Sequence | Tm C | Total bands | Polymorphic bands | Polymorphism ration, % | Shannon index |
|---|---|---|---|---|---|---|
| 2074 | GCTCTGATACCA | 40.5 | 6 | 3 | 50 | 0.35 |
| 2087 | GCAATGGAACCA | 43.5 | 6 | 3 | 50 | 0.30 |
| 2241 | ACCTAGCTCATCATGCCA | 55.5 | 7 | 3 | 42.85 | 0.38 |
| 2238 | ACCTAGCTCATGATGCCA | 55.5 | 5 | 3 | 60 | 0.32 |
| 2243 | AGTCAGGCTCTGTTACCA | 54.9 | 8 | 5 | 62.5 | 0.41 |
| 2393 | TACGGTACGCCA | 47.1 | 4 | 1 | 25 | 0.19 |
| 2390 | GCAACAACCCCA | 47.6 | 9 | 6 | 66.66 | 0.36 |
| 2394 | GAGCCTAGGCCA | 48.5 | 9 | 3 | 33.33 | 0.26 |
| 2375 | TCGCATCAACCA | 45.2 | 5 | 2 | 40 | 0.33 |
| 2276 | ACCTCTGATACCA | 42.7 | 6 | 2 | 33.33 | 0.24 |
| | | | 65 | 31 | 47.69 | |

Transcriptional analyses of fatty acids gene expression (FAD6) in camelina sole crop and intercropping system in organic farming.

The purpose of the study is an evaluation of the transcription of the fad6 gene involve in the synthesis of very long fatty acid (VLFA) in seeds of camelina grown on certified organic field ain sole crop and in intercrop with legumes peas (*Pisum sativum*) and vetch (*Vicia sativa).* In higher plants, polyunsaturated fatty acids (PUFAs) are synthesized by a variety of FAD (fatty acid desaturases). FAD is a ubiquitous enzyme family and is responsible for introducing double bonds into the hydrocarbon chains of fatty acids (Singh et al., 2002; Zhu et al. 2018). They play an essential role in fatty acid metabolism and maintain biological membranes in most creatures. Biosynthesis of α-linolenic acid is converted from linoleic acid by FAD genes. Linoleic acid and α-linolenic acid are so-called essential fatty acids (EFAs) in human bodies because of their inability to synthesize these compounds (Zhu et al. 2018; Damude and Kinney, 2008). Therefore, FAD6 is the key enzyme for producing linoleic acid and is also the speed-limiting enzyme for routes of ω-6 and ω-3.

***Real-time PCR analysis***

Total RNA was extracted from 100 mg leaves of control camelina plants and leaves from camelina intercropped plants. First-strand cDNAs were reversetranscribed from 1 μg each

total RNA using a cDNA Synthe-sis Kit (). Gene-specific primers for fad6 gene (fad6-F – 5'-ATCACATAAGCCCAAGCATACCG-3' and fad6 – 5'-TCGTCTTCATCAACCGCCATTT-3') expression by Primer 5 software (Applied Biosystems, Foster City, CA, USA). Each 25 µlPCR reaction contained 12.5 µl 2× SYBR Green Mastermix (- iTaq Univer SYBR Green SMX 500, BioRad, Hercules City, CA, USA),0.5 µl 10 µM each primer, 1 µl of each first-strand cDNAtemplate, and 10.5 µl dd$H_2O$. The PCR reaction was per-formed in a Real-time PCR detector (Bio-RadLaboratories, Hercules City, CA, USA) with Opticon Monitor3 software (Bio-Rad Laboratories). The following thermal cycling profile was used: 95 °C for 10 min; 35 cycles of 95°C for 15 s and 56°C for 1 min; and 95°C for 15 s,60°C for 1 min, and 95 °C for 15 s. Actin expression was used as an internal control to normalize all data. Experiments were carried out with three independent biological replicates each containing three technical replicates. The statistical assess-ment was calculated from three biological replicates. The foldchange in mRNA expression was estimated using thresholdcycles, by the 2−ΔΔCT method (Livak and Schmittgen, 2001).

***Fad6 gene expression in the development seeds of camelina***

Two PCR reactions were performed with 2 sample pairs of primers. The purified RNA does not contain DNA; therefore, the obtained amplification curves are not due to contamination and the RNA is of the necessary quality to run the PCR reactions. Actin was chosen as the "housekeeping" gene because it is expressed equally in all stages of camellia seeds development. As a result, *fad6* expression in camelina K3 variety grown intercropping with vetch was 5-fold higher compared to control self-grown plants (Fig. 3). It was observed the correlation between levels and *fad6* gene expression across seed formation of K3 in the nine different cultivation plots. Possitive correlation was detected in C18:3 and C20:1 fatty acid.

These results are proven also by the FAD6 expression - in K3 grown with vetch was 5-fold higher compared to control of pure stand plots. It was observed a correlation between levels and *fad6* gene expression across seed formation for K3 in the nine different cultivation plots. Possitive correlation was detected in **α**-linolenic acid C18:3 and eicosenoic acid C20:1.

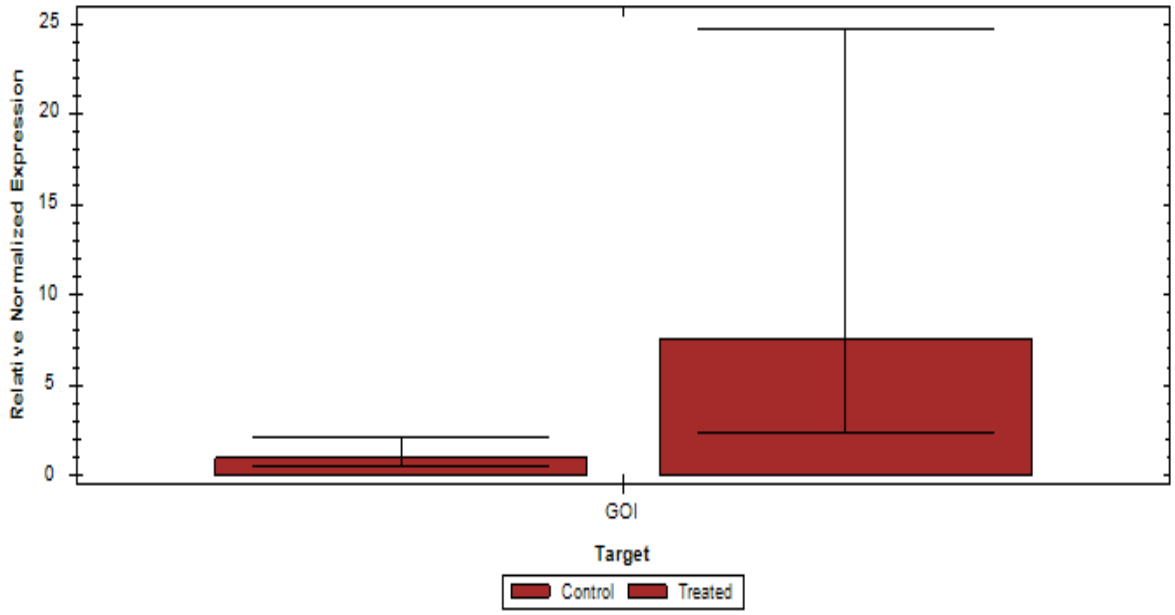



Figure 3. Comparison of fad6 gene expression level between sole crop of the local Bulgarian landrace of camelina (K3) compared to its intercropping with vetch.

**Tasks performed by UWM**

The plant material for the analysis of genetic diversity of camelina (*Camelina sativa*) consisted 52 genotypes. First, an in vitro collection was established in the Plant Biotechnology Laboratory of the Department of Genetics, Plant Breeding and Bioresource Engineering Engineering (UWM) in order to secure the available plant materials. For this purpose, a dozen or so seeds of each genotype were sterilized. The sterilization process included (1) a pre-sterilization step in 70% ethanol for 20 seconds, (2) a main sterilization step in 1.5% sodium hypochlorite for 7 minutes, and (3) a three-step rinse in sterile water for 5, 10, and 15 minutes. The sterilization method turned out to be very effective, achieving a culture sterility level of 95.24%. Additionally, the average germination capacity of *C. sativa* seeds was observed to be 85.71%, which indicates that the sterilization conditions used were highly suitable for the initiation of in vitro camelina culture.

In the next stage, the DNA of each of the 52 camelina genotypes was isolated twice (in two biological replicates), using leaves taken directly from plants grown in previously established in vitro cultures. The universal Milligan method was used for DNA extraction with some modifications. The quantity and quality of DNA were assessed using a NanoDrop 2000 spectrophotometer and a Qubit 4 fluorometer and were additionally confirmed by electrophoresis. The proposed isolation method turned out to be very efficient and suitable for isolating DNA from *C. sativa* plants. The isolates were characterized by high DNA content, on average 680.58 ng/µl, and very good quality - both ratios (260/80 and 260/230) were within generally accepted ranges indicating the absence of DNA contamination.

The RAPD method was used to analyze the genetic diversity of *C. sativa*. PCR reaction conditions standardly used in the laboratory also turned out to be suitable for camelina. The PCR reaction was performed in a PCR-RAPD reaction mixture containing 10 ng of DNA for 37

cycles (94°C for 30 s, 40°C for 2 min, 72°C for 2 min) with initial denaturation (94°C for 10 min) and final extension (72°C for 8 minutes). A total of 60 RAPD primers were tested, and 32 primers generating clear and repeatable bands, and additionally characterized by a high level of polymorphism of the obtained RAPD products, were selected for the proper analysis. All DNA samples were genotyped twice to ensure reproducibility of results. In the case of the primers selected for proper analysis, an average of 9.38 RAPD products with a high level of polymorphism were obtained (average 88.28%).

The analysis of the obtained results indicated not so high level of genetic diversity of the tested genotypes (Figs. 2.1 - 2.3), however, the distribution of this diversity was heterogeneous. In the case of 43 genotypes, greater genetic similarity was observed (group 1), and the remaining 9 genotypes showed clear genetic differences (groups 2 and 3) to the remaining genotypes tested. Both the PCoA analysis (Fig. 2.2.) and the analysis of the values of individual genetic differentiation coefficients between the studied *C. sativa* genotypes, the results of which were illustrated in the form of a dendrogram (Fig. 2.3.), made it possible to group the genotypes into three groups depending on the degree of their genetic differentiation.

Figures 2.1 – 2.3 on the next pages

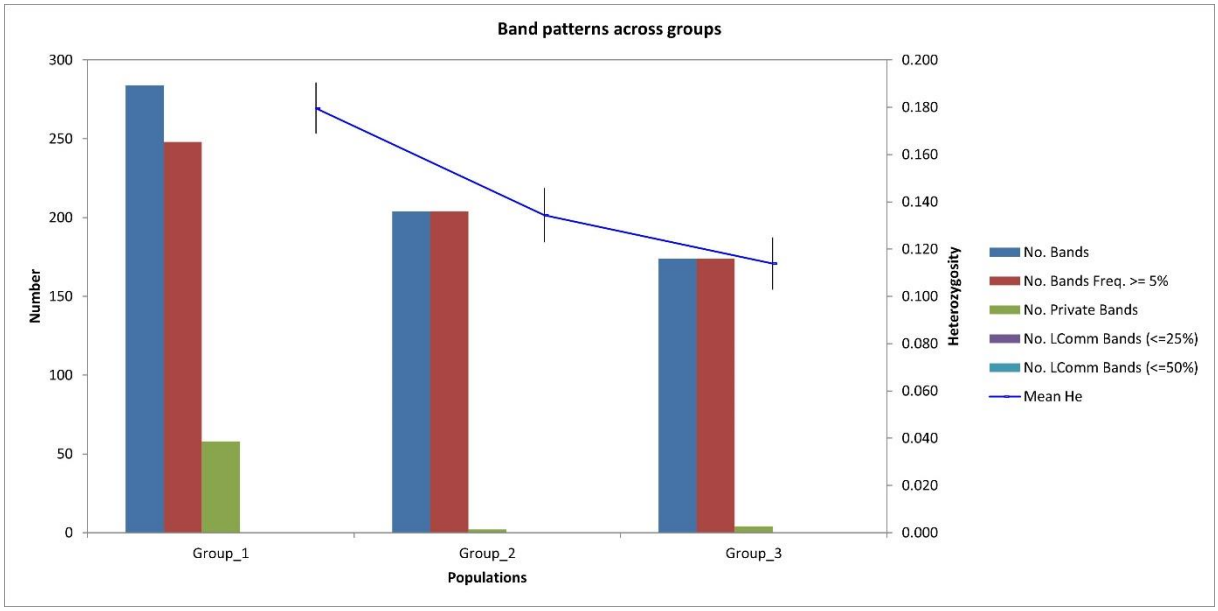



Fig. 2.1. Basic parameters of genetic diversity of *C. sativa*

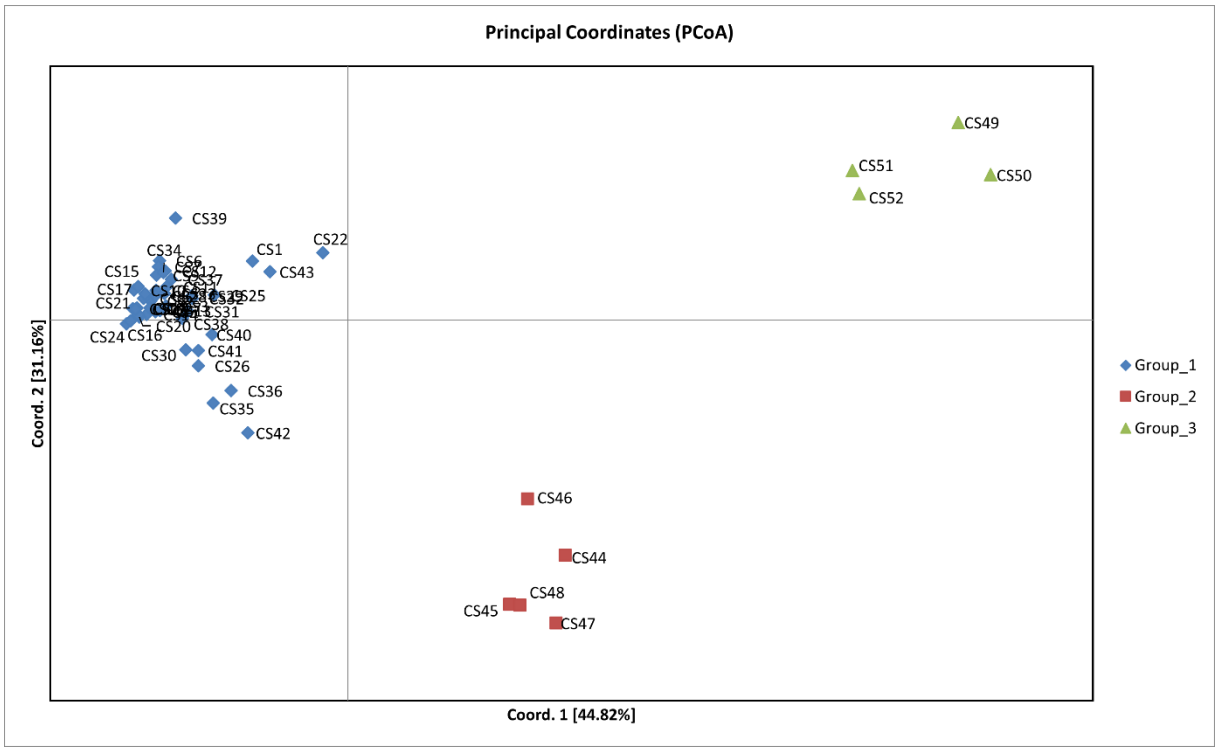



Fig. 2.2. PCoA plot showing the genetic diversity of the tested *C. sativa* genotypes

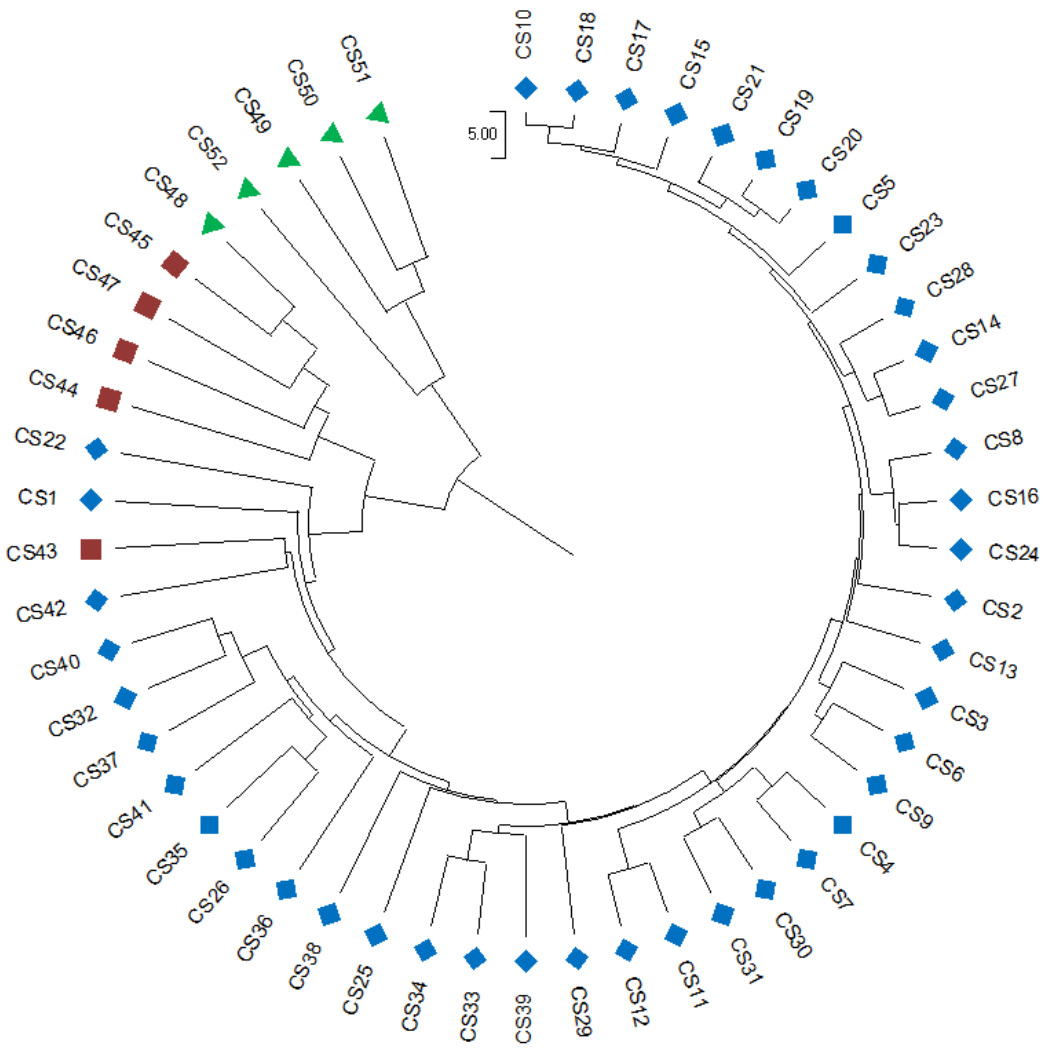



Fig. 2.3. Dendrogram of genetic diversity of the studied *C. sativa* genotypes