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A Method of DNA Extraction from Plants for Metagenomic Analysis Based on the Example of Grape *Vitis amurensis* Rupr.

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Abstract—A new method for extracting DNA from plants is proposed, using the example of wild grapes *Vitis amurensis* Rupr. for further preparation of libraries for metagenomic analysis. The method is based on the isolation of DNA by an inexpensive CTAB method with an additional stage of DNA purification using silica spin columns (CTAB-spin method). A comparative analysis of the results of metagenomic analysis of endophytes on DNA isolated using the proposed CTAB-spin method and using the commercial kit ZymoBIOMICS DNA Miniprep (Zymo Research) was performed. It was found that when using the CTAB-spin method, the number of sequences of the *16S* rRNA site and the diversity of bacterial genera were 2.8 and 1.2 times greater, respectively, than when using the ZymoBIOMICS kit. At the same time, the number of sequences of the internal transcribed spacer 1 (*ITS1)* and the biodiversity of endophytic fungi did not differ significantly during DNA extraction by two methods. Thus, the proposed method of DNA isolation for metagenomic analysis is an available and effective alternative to commercial kits for the isolation of plant DNA for new-generation sequencing methods.

Keywords: grapes, DNA, metagenome, new-generation sequencing (NGS), bacteria, fungi, endophytes, *Vitis amurensis*

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Next generation of sequencing (NGS) is a massively parallel sequencing technique providing ultra-high throughput, scalability and rate [1]. NGS allows for simultaneous and independent sequencing of billions of nucleic acid fragments. The technique is used for nucleotides sequencing in the genomes or target regions of DNA or RNA. NGS has revolutionized biological sciences, enabling laboratories to solve a wide range of applied problems and to study biological systems at a new level that goes beyond the capabilities of traditional DNA sequencing techniques [2].

In the last decade, the number of publications on NGS has increased by dozens of times, indicating that this method is in demand. The rapid advances in the NGS technology and the simultaneous development of bioinformatics tools have allowed both small and large research teams to assemble draft genome sequences de novo for any organism of interest [3]. In addition to using NGS for large-scale whole genome sequencing [4], these technologies can be used for high-throughput whole transcriptome sequencing [5], whole exome sequencing [6], directed or candidate gene sequencing [7, 8], genome methylation sequencing [9], and Metagenomic Next Generation Sequencing (mNGS) [10].

mNGS is the sequencing of all nucleic acids in a sample, which may contain mixed microbial populations. mNGS makes it possible to identify microbes and to determine their proportions in samples. The possibility of sequencing and identification of nucleic acids from many different taxa for metagenomic analysis provides a new powerful research platform for simultaneous identification of genetic materials from absolutely different kingdoms of organisms [11]. mNGS applications have a wide range of possibilities, including the diagnosis of infectious diseases, tracking outbreaks, infection control surveillance, the detection of mutations and pathogens, and the study of microbial communities that inhabit various plants and animals [12, 13].

As a rule, the success of mNGS sequencing largely depends on the quality of isolated DNA. Plant genomic DNA is usually extracted using expensive commercial kits such as DNeasy Plant Mini Kit (50 samples, Qiagen, Germany) [14], QIAamp® Fast DNA Stool Mini Kit (50 samples, Qiagen, Germany) [15], PowerSoil® DNA Isolation Kit (50 samples, MoBio Inc., United States) [16], and ZymoBIOMICS DNA miniprep kit (50 samples, Zymo Research, United States) [17].

The aim of this work was to compare the endophytic communities of bacteria and fungi of the wild grape *Vitis amurensis* Rupr. by mNGS sequencing of DNA isolated from different grape organs using the method developed in the present work and the commercially available ZymoBIOMICS DNA miniprep kit.

MATERIALS AND METHODS

Plant material and surface sterilization conditions. The tissues of two wild grapevines *V. amurensis* (young stems of 7–8 cm in length with three healthy leaves and mature berries) were collected in an unprotected natural area near the city of Vladivostok (Russia) in September, 2021. Plant samples were delivered to the laboratory within 30 min.

Grape samples were washed with soap and running water; 0.2 g of each grape organ was weighed under sterile conditions. The weighed samples were then washed in 70% ethanol for 2 min, in 10% hydrogen peroxide solution for 1 min, and then with sterile water five times.

DNA isolation. *V. amurensis* grape samples from the two grapevines collected in July and September, 2021, were used in the work (four biological replicates altogether). DNA was isolated from 11 grape samples (four leaf and four stem samples from the two grapevines, two berry samples, and one seed sample from one grapevine) using CTAB protocol with a spin column and commercial ZymoBIOMICS DNA miniprep (Zymo Research, United States). One biological experiment was performed in two analytical replicates.

A surface-sterilized grape sample was weighed at 30, 50, and 100 mg and put into a sterile mortar to be ground. The mortar and the pestle were sterilized by autoclaving (121°C, 0.21 MPa, 20 min).

After the sample had been ground in the mortar, the ZymoBIOMICS DNA miniprep kit was used for DNA isolation according to the manufacturer's protocol. In case of the CTAB protocol with a spin column, 800 μL of CTAB buffer (100 mM Tris pH 7.5, 1.4 M NaCl, 40 mM EDTA pH 7.5, 1% cetyltrimethylammonium bromide, CTAB) was added to the ground sample, following by stirring until a homogeneous state and incubation for 1 h at 60°C in the Gnome thermostat (DNA Technology, Russia). Then, 300 μL of chloroform was added, followed by gentle stirring and centrifugation for 10 min at 4°C and 16100 *g* (5415R, Eppendorf, Germany). Then, 420 μL of the supernatant was taken into separate tubes, with the addition of 950 μL of 96% ethanol, incubated overnight at –20°C, and then centrifuged for 10 min at 4°C and 16100 *g*. The supernatant was removed and the precipitate was dried until complete evaporation of ethanol at room temperature (30 min).

The precipitate was dissolved in 100 μL of distilled water. It should be noted that the resultant DNA solution can already be used for amplification of individual genes in case of gene-specific PCR [18] or for DNA methylation analysis [19]. However, the attempts to use it for obtaining the libraries of the *16S* and *ITS*1 gene regions failed; no characteristic PCR products were observed and hence the stages of DNA purification on silica membrane spin columns (e.g., C1002-50 IC-XL, Zymo Research) were added. Two columns were used for a single sample; 50 μL of the resultant aqueous DNA solution was applied to each column and centrifuged for 30 s at 4°C and 6900 *g*. Then, 200 μL of DNA Purification Solution (GuSCN, 5 M; EDTA, pH 8.0, 0.1 M) was added, followed by centrifugation under the same conditions. After the removal of the supernatant, 700 μL of a washing solution (80% ethanol; 10 mM Tris-HCl, pH 7.5) was added to the precipitate, followed by centrifugation. The columns were then transferred to new 1.5-mL tubes and centrifuged for 1 min at 4°C and 6900 *g* to remove the washing solution. Afterwards, the columns were again transferred to new 1.5-mL tubes and left on a laboratory bench for 5 min at room temperature to evaporate the remaining washing solution. Then, 50 μL of elution solution (5 mM Tris-HCl, pH 7.5) was applied to the first column, followed by incubation for 1 min on the bench at room temperature and centrifugation for 30 s at 6900 *g*; the resultant eluate (i.e., the elution solution that had passed through the first column) was then used to wash the first column again. The first column was removed and the second column was placed into the same tube and washed twice with the eluate. As a result, the DNA from the two columns was purified and eluted into 50 μL of the elution solution.

DNA quality and quantity were assessed with a P300 spectrophotometer (IMPLEN, United States).

Library preparation and sequencing. DNA samples were sent to Eurogen (Russia) for Illumina high-throughput sequencing. The library preparation protocol for sequencing is described in the 16S Metagenomic Sequencing Library Preparation manual (section no. 15044223 Rev. B; Illumina). The regions of bacterial *16S* rRNA were amplified using primers 515F (5'GGT AAT ACG KAG GKK GCD AGC) and 806R (5'RTG GAC TAC CAG GGT ATC TAA) modified for *Vitis* sp. plants [20]. The regions of the *ITS1* intergenic spacer of endophytic fungi were amplified using primers ITS1f (5'CTT GGT CAT TTA GAG GAA GTA A) and ITS2 (5'GCT GCG TTC TTC ATC GAT GC) [21].

The libraries were then purified and mixed in equimolar proportion using a set of SequalPrep™ normalizing plates (ThermoFisher, Cat no. A10510-01). The quality control of the resultant library pools was performed using a fragment analyzer, and quantification was performed using qPCR.

The library pool was sequenced using an Illumina MiSeq System (2 × 250 paired ends) with a MiSeq v2 reagent kit (500 cycles). FASTQ files were obtained using bcl2fastq v2.17.1.14 transformation software

(Illumina). The phage PhiX library was used to control sequencing parameters. Most of the reads related to the phage DNA were removed during demultiplexing.

The bacterial and fungal sequences were deposited in NCBI with registration numbers PRJNA813962 and PRJNA874841, as well as in the database of the Laboratory of Biotechnology at the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of the Russian Academy of Sciences, Russia (https://biosoil.ru/downloads/biotech/Vitis%20metagenom/2021-09=Vitis_amurensis_endophytes_-16s; https://biosoil.ru/downloads/biotech/Vitis%20metagenom/2021-09=Vitis_amurensis_endophytes_ITS).

Computational analysis. Paired-end NGS reads were preprocessed using QIIME 2 [22] and DADA2 [23]. As a result of preprocessing, paired-end reads were merged; chimeric sequences and the remaining phage PhiX DNA sequences were removed from the high-throughput sequencing data. The taxonomic identification of DNA sequences was performed with the QIIME 2 Scikit-learn algorithm [24] using pretrained classifiers based on Silva 138 database (99% OTUs from the 515F/806R sequence region) for endophytic bacteria [25] and UNITE database (99% OTUs from the ITS1f/ITS2 sequence region) for endophytic fungi [26]. The chloroplast and mitochondrial DNA sequences and sequences that were not identified to the rank of Phylum, as well as archaeal and eukaryotic DNA sequences, were excluded from the analysis.

The results were processed using the R programming language. The phyloseq library [27] and the tidyverse package [28] were used for data prefiltration and preparation. The taxa for the bar chart and UpSet visualization chart were filtered on the basis of a relative abundance >0.1% for each biocompartment. The bar charts combined taxonomic ranks that were relatively rare (<0.1% for each factor) into a single group referred to as "other." The data on alpha diversity based on the Shannon diversity index and beta diversity based on the Bray–Curtis dissimilarity statistics were obtained using Vegan (available online: https://cran.r-project.org/web/packages/vegan/vegan.pdf) [29]. The Bray–Curtis dissimilarity data were converted into an even sampling depth and presented as ordination diagrams using nonmetric multidimensional scaling (NMDS). The Wilcoxon Rank Sum Test was performed to analyze the data on alpha diversity between the groups. Statistical checking of the data on beta diversity was performed using the Permanova test (999 permutations) included in the Vegan package [29]. The ggplot2 [28] and ComplexHeatmap [30] libraries were used for graphical representation of the results.

RESULTS AND DISCUSSION

Isolated DNA quantity and quality. Initially, DNA was isolated from 30, 50, and 100 mg of a surface-sterilized *V. amurensis* grape leaf by the two methods described above. Eventually, all DNA samples were dissolved in 50 μL of the elution solution; therefore, both the concentrations and quantities of isolated DNA could be directly compared by different methods.

It turned out that the concentration and quantity of isolated DNA was 2.2–3.5 times higher when using the CTAB-spin method than when using ZymoBIOMICS (Table 1). Moreover, the 50-mg sample showed the best results in both methods; therefore, 50-mg samples were used further in the work. The DNA was isolated from 50 mg of grape stems, berries and seeds, and it was shown that the CTAB-spin method was also more efficient (Table 1). It should be noted that the 260/280 and 260/230 ratios of absorbance were 1.9–2.0 and 0.4–0.6, respectively, in all samples isolated by the CTAB-spin method and ZymoBIOMICS. It is known that the optimal 260/280 and 260/230 ratios for working with nucleic acids are 1.8–2.0 and 2.0–2.2, respectively. Thus, both methods made it possible to obtain fairly pure DNA preparations with respect to protein impurities (260/280), but DNA samples contained quite a lot of impurities of other substances with absorption at 230 nm. Perhaps, the high absorbance at 230 nm is due to the presence of carbohydrates, which is fairly typical of work with plant tissues. Additional purification on spin columns slightly increased the 260/230 ratio but, at the same time, the DNA concentration decreased; hence, it was decided to work with DNA samples without additional purification.

Comparison of microbial communities in DNA samples isolated by different methods. A total of 3 108 452 and 3 559 302 paired-end reads were obtained for the *16S* rRNA and *ITS1* amplicons, with an average of 282 587 and 323 573 paired-end reads per sample, respectively. As a result of high-throughput sequencing data preprocessing with QIIME 2 and DADA2, a total of 574 207 *16S* rRNA sequences in 22 samples was left for analysis. After the procedures of bioinformatics quality control, 2 753 016 sequences in 22 samples altogether were identified for *ITS1* (Fig. 1).

According to comparative analysis, the number of *16S* rRNA sequences of endophytic bacteria was 2 times higher in the samples isolated by the CTAB-spin method (Fig. 1a). The endophytic bacterial community was represented by 11 taxa at the class level with relative representation above 0.1%. In the samples isolated by the two DNA extraction methods, bacterial classes were the same but the percentages of these classes considerably varied (Fig. 1a). For example, the percentage of the class Alphaproteobacteria was 11% in the samples isolated using a commercial kit and almost 22% in the samples isolated by the CTAB-spin method. The class Bacilli had a percentage of 2.7% in the samples isolated by the former method and 18% in the samples isolated using ZymoBIOMICS DNA miniprep. Representatives of three classes:

Table 1. The concentration of DNA isolated from the leaves, stems, berries and seeds of *Vitis amurensis* grapes by different methods*

Isolation technique	Grape organ	DNA concentration, μg/μL	DNA quantity, μg
30 mg, ZymoBIOMICS	Leaf	0.006 ± 0.003[c]	0.30 ± 0.15[c]
50 mg, ZymoBIOMICS	Leaf	0.012 ± 0.005[bc]	0.60 ± 0.25[bc]
50 mg, ZymoBIOMICS	Stem	0.005 ± 0.002[c]	0.25 ± 0.10[c]
50 mg, ZymoBIOMICS	Berry	0.005 ± 0.003[c]	0.25 ± 0.15[c]
50 mg, ZymoBIOMICS	Seeds	0.004 ± 0.002[c]	0.20 ± 0.10[c]
100 mg, ZymoBIOMICS	Leaf	0.007 ± 0.004[c]	0.35 ± 0.20[c]
30 mg, CTAB-spin	Leaf	0.021 ± 0.005[ab]	1.05 ± 0.25[ab]
50 mg, CTAB-spin	Leaf	0.026 ± 0.007[a]	1.30 ± 0.35[a]
50 mg, CTAB-spin	Stem	0.028 ± 0.009[a]	1.40 ± 0.45[a]
50 mg, CTAB-spin	Berry	0.031 ± 0.011[a]	1.55 ± 0.55[a]
50 mg, CTAB-spin	Seeds	0.030 ± 0.010[a]	1.50 ± 0.50[a]
100 mg, CTAB-spin	Leaf	0.024 ± 0.006[ab]	1.20 ± 0.30[ab]

* The results are presented as the standard error of the mean. The mean values in the columns, followed by the same letter, did not differ by Student's *t*-test. $p < 0.05$ was considered as statistically significant.

Acidobacteriae, Chlamydiae and Oligoflexia, were absent in the samples isolated using the commercial kit but identified in the samples isolated by the method proposed in the present work at 0.13, 0.37 and 0.13%, respectively. In addition, biodiversity of the genus composition of endophytic bacteria was richer in samples with DNA isolated by the CTAB-spin method (Fig. 1c). When DNA was isolated using a commercial kit, there were 76 genera in the genus composition of endophytic bacteria; if DNA was isolated by our method, the endophytic community included 91 genera; 17 and 2 genera were unique for the samples isolated by the CTAB-spin method and by ZymoBIOMICS DNA miniprep, respectively (Fig. 1c).

The data on metagenomic sequencing of the *ITS1* intergenic spacer sequences of endophytic fungi show that DNA extraction and purification by both methods has similar results with respect to the number of sequences (1214521 and 1230181 sequences) (Fig. 1b). The percentage of the classes of endophytic fungi varied, but not as significantly as in case of bacterial community. The class Dothideomycetes in the samples isolated by the CTAB-spin method accounted for 52% of the total number of endophytic fungi; when using the commercial kit, the percentage of this class was 66% (Fig. 1b). The percentage of the class Tremellomycetes was 41% and 27% in the samples isolated by the CTAB-spin method and the commercial kit, respectively (Fig. 1b). The fungi of the genera *Acremonium* and *Acrospermum* were detected in the samples isolated by the proposed method but were not detected in the samples isolated using ZymoBIOMICS DNA miniprep (Fig. 1d).

Alpha diversity (i.e., local community diversity) was measured by calculating two estimates: the Shannon Diversity Index and the number of bacterial genera. According to the data we obtained, the median value of the Shannon index, which is a measure of entropy that increases depending on the number of genera in a sample, was statistically significantly higher in the samples of endophytic bacteria with DNA isolated by the CTAB-spin method, compared to DNA isolated using the commercial kit (Fig. 2a). In the samples of endophytic fungi, Shannon index values were not significantly different when comparing the two methods of DNA isolation (Fig. 2b). In addition, the number of bacterial and fungal genera detected in the samples isolated by the CTAB method with modifications was higher compared to the samples obtained using the commercial kit (Figs. 1c, 1d).

Beta diversity (the comparison of microbial communities on the basis of their composition) estimates the distance or the degree of dissimilarity between each pair of samples. Beta diversity values were calculated using Bray–Curtis dissimilarity and presented as ordination diagrams using the NMDS method. In the diagram of beta diversity in the samples of endophytic bacteria, the samples were grouped into separate clusters depending on the DNA extraction technique (Fig. 2c) and were statistically significantly different according to the Permanova test. However, the samples of endophytic fungi were localized within the same cluster regardless of the DNA extraction technique, which confirmed the results of the Permanova test (Fig. 2d).

Due to the rapid development of modern DNA sequencing techniques, NGS methods are used for scientific and diagnostic purposes more frequently, placing new high demands on the quantity and quality of isolated DNA.

Today, most of the existing plant DNA extraction techniques do not make it possible to obtain DNA that can be used for NGS; therefore, in the present work

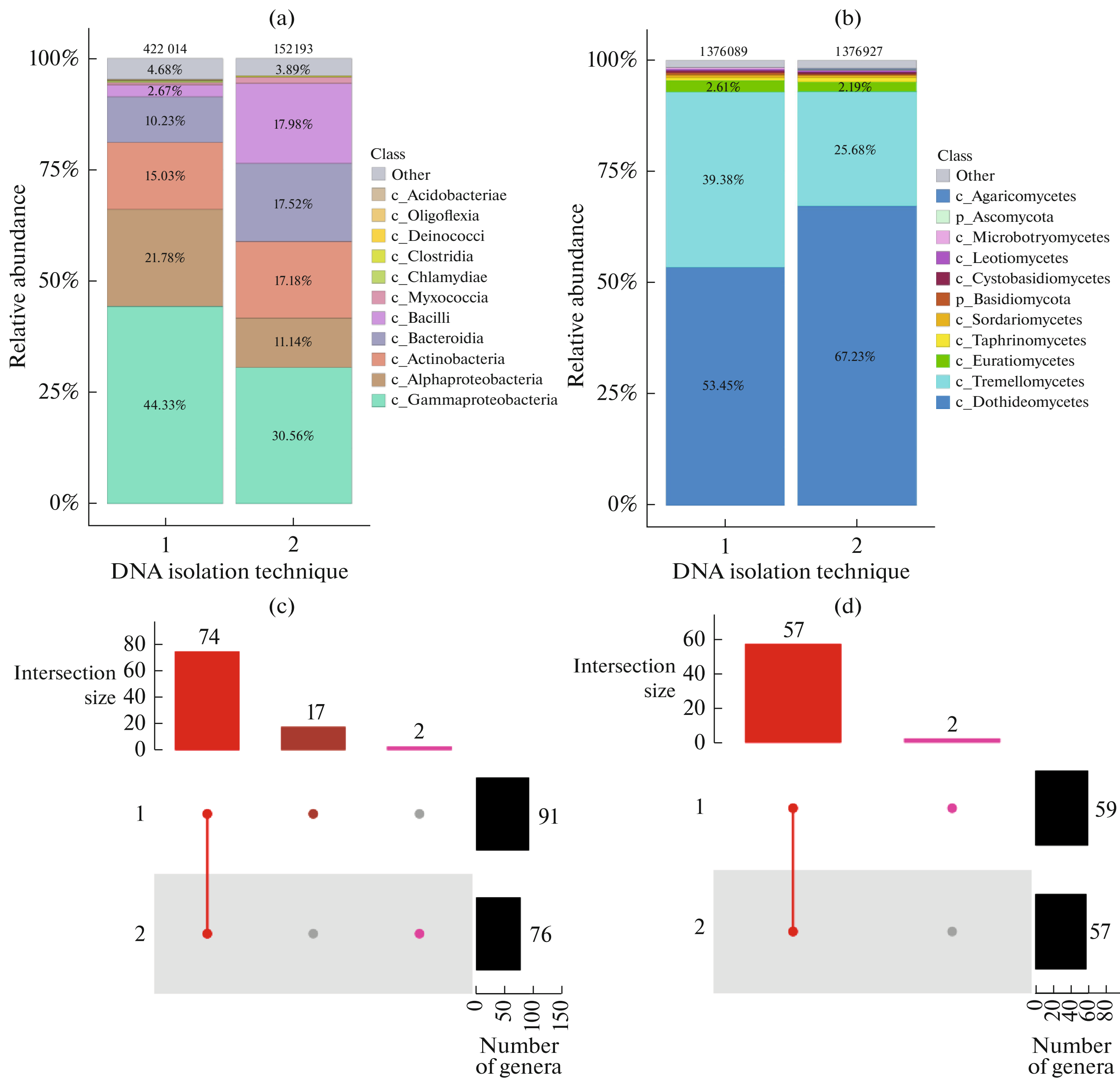

Fig. 1. Comparative analysis of the composition of endophytic bacteria (a, c) and fungi (b, d) by next generation sequencing (NGS) of the samples isolated by two techniques: CTAB-spin method (1) and ZymoBIOMICS DNA miniprep (2). Taxonomic bar charts at the class level for the composition of the bacterial and fungal community of *Vitis amurensis* (a, b, %); (c, d, the number of genera), stratification diagrams (UpSet) at the genus level showing the overlapping taxa in the samples isolated by the two techniques (1, 2). The taxa were filtered on the basis of relative abundance >0.1% for each biocompartment. The filtered taxa on the bar charts were classified as "other" and removed from the UpSet diagram. The number of DNA sequences is indicated above the taxonomic columnar sections.

we modified the existing technique based on CTAB extraction using additional stages of spin column-based purification, or the CTAB-spin method. The calculated cost of the approach proposed in this work was six or more times lower compared to the application of commercial kits.

The final analysis of diversity of endophytic bacteria and fungi using DNA isolated by the CTAB-spin method showed high values for the number of reads and certain genera, which exceeded the values obtained using DNA isolated using the well-known Zymo Research kit.

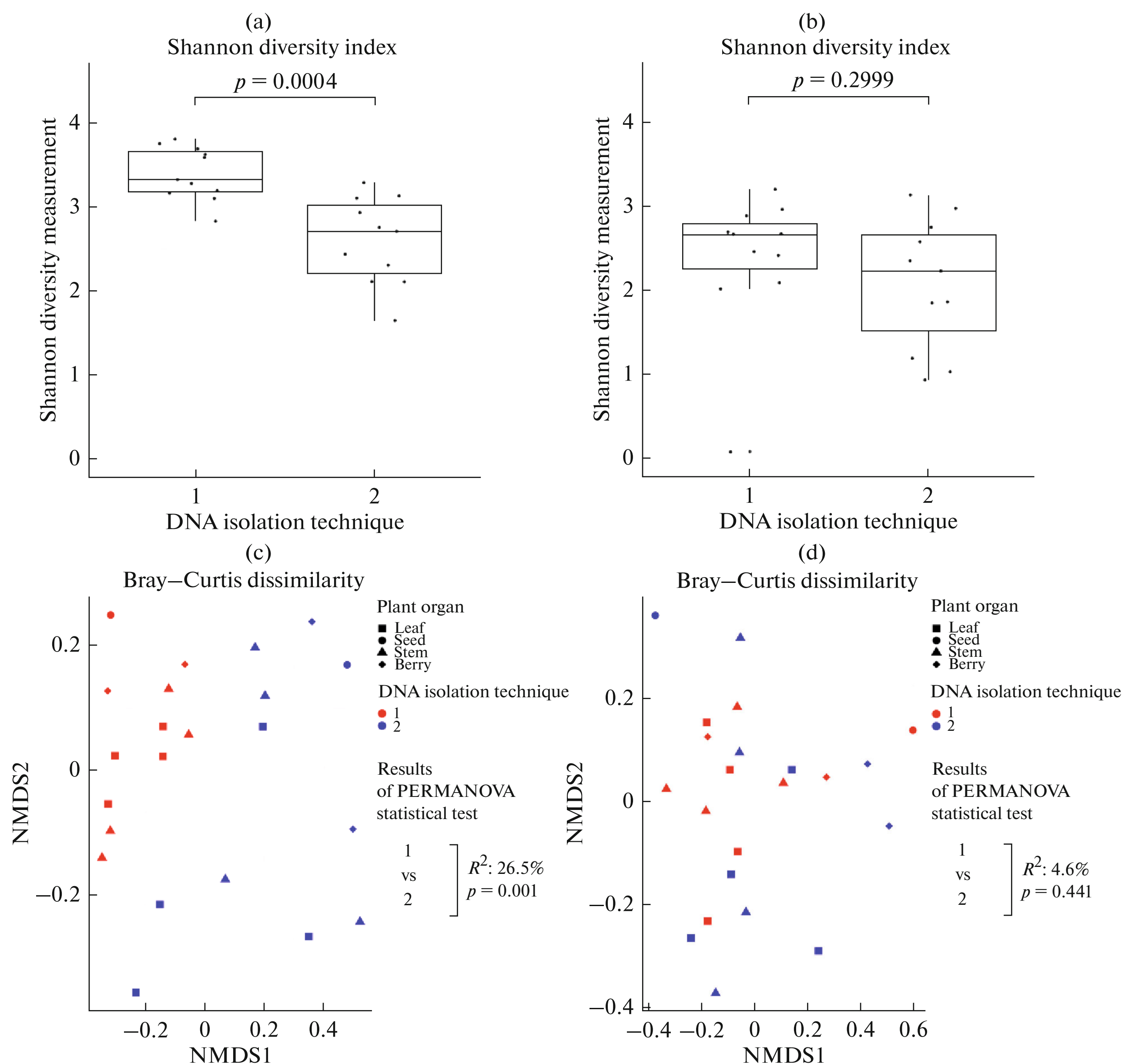

Fig. 2. The Shannon alpha diversity and Bray–Curtis beta diversity of endophytic bacteria (a, c) and fungi (b, d) as a result of next generation sequencing (NGS) of the samples isolated by two techniques: CTAB-spin method (1) and ZymoBIOMICS DNA miniprep (2). The data on beta diversity are presented using non-metric multidimensional scaling (NMDS).

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Thus, this work presents a detailed protocol for efficient DNA extraction from plant cells for subsequent NGS analysis on the basis of CTAB extraction followed by spin column-based purification. Its efficiency has been confirmed by the results of metagenomic analysis with high values of the samples. This protocol will be useful for studies in molecular biology and plant biotechnology using NGS methods.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflicts of interest. This article does not contain any studies involving ani-

mals or human participants performed by any of the authors.

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