Comparative analysis of the DNA isolated from thyme leaves using different methods

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Сравнительный анализ ДНК, выделенной из листьев чабреца разными способами

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Background. The base for a molecular analysis is DNA of high quality. For DNA isolation, different kits or classical methods are used. For mass analysis, isolation with kits is a very expensive process. So, the objective of our investigation was to find a cheap method for high-quality DNA isolation from leaves of various thyme cultivars. **Materials and methods.** Leaves cut from thyme accessions (*Thymus mastichina* L. cv. 'Svetliachok', *T. striatus* Vahl. cv. 'Jubileiniy', *T. vulgaris* L. cv. 'Fantasia', and *T. vulgaris* cv. 'Jalos'.) maintained *ex situ* in the collection of the Nikita Botanical Gardens were used as the material for the analysis. Light microscopy was used to study leaf anatomy and localize essential oil on leaf cross sections. Essential oil was extracted on Ginsberg devices, and phenolic content was measured with The Folin–Ciocâlteu reagent (FCR). Commercial kits (DiamondDNA™, PureLink® Plant Total DNA Purification Kit) and classical methods (CTAB, CTAB with 2% polyvinylpyrrolidone) were used for DNA isolation. DNA quality was evaluated spectrophotometrically, with electrophoresis (horizontal, automated system Agilent 4200 TapeStation) and PCR. **Results.** The analysis showed that the leaf blade mesophyll of four thyme cultivars had inclusions with essential oil. The content of essential oil and phenolic compounds was measured biochemically. Since the plants were characterized by the presence of secondary metabolites, DNA was isolated by different methods. Spectrophotometry demonstrated that the classical CTAB method and CTAB with 2% PVP provided the best results. Using an automated electrophoresis system, the presence of high-molecular-weight DNA (more than 52000 bp) in significant amounts was detected in the samples isolated with DiamondDNA™ kit and CTAB + 2% PVP. **Conclusion.** Among the tested kits and methods, CTAB + 2% PVP provided thyme DNA suitable for PCR and, presumably, for genome library preparation. The low cost of reagents for this technique makes it applicable for future mass analysis of plant material.

Key words: *Thymus*, cultivars, leaves, essential oil, phenols, DNA isolation.

Актуальность. Основой молекулярного анализа является ДНК высокого качества. Для ее выделения используют разные наборы или классические способы. Массовый анализ ДНК, при ее выделении с помощью наборов является дорогостоящим процессом. Поэтому целью нашего исследования был поиск относительно недорогого способа выделения ДНК хорошего качества из листьев разных сортов чабреца. **Материалы и методы.** Для исследований использовали листья растений чабреца (*Thymus mastichina* L. сорт 'Светлячок', *T. striatus* Vahl. сорт 'Юбилейный', *T. vulgaris* L. сорт 'Фантазия', *T. vulgaris* сорт 'Ялос'), культивируемые в коллекции Никитского ботанического сада. С использованием световой микроскопии на поперечных срезах листьев изучали анатомическое строение органов, локализацию включений с эфирным маслом. Эфирное масло извлекали на аппаратах Гинзберга, фенольные соединения определяли с помощью реагента Фолина – Чокальтеу. ДНК выделяли с применением коммерческих наборов и классических способов с цетилтриметиламмоний бромидом (ЦТАБ) и поливинилпирролидоном (ПВП). Качество ДНК оценивали спектрофотометрически, с помощью электрофореза и ПЦР. **Результаты.** В мезофилле листовой пластинки четырех сортов чабреца выявляли включения с эфирным маслом. Содержание эфирного масла и фенольных соединений варьировало между сортами. Спектрофотометрический анализ показал, что классический способ с ЦТАБ и добавлением 2% ПВП позволяет получить ДНК хорошего качества длиной более 52000 пн. **Заключение.** Среди исследованных наборов и способов, ЦТАБ + 2% ПВП обеспечивал необходимый выход и качество ДНК, подходящей не только для ПЦР, но и, предположительно, для подготовки геномных библиотек. Относительно недорогие реагенты для этого метода делают его пригодным для будущего массового анализа растительного материала.

Ключевые слова: *Thymus*, сорта, листья, эфирное масло, фенолы, выделение ДНК.

Introduction

Essential oils are volatile oily liquids with a characteristic strong smell that accumulates during secondary metabolism in the inflorescences at higher concentration as well as in other vegetative organs, epidermal structures and different internal tissues but in smaller amount (Rehman et al., 2016; Sharifi-Rad et al., 2017). In plants, they are utilized for fertilization, protection from fungal attacks or as antimicrobial components, irritant or repellant (Naeem et al., 2018). Because of their biological activity, such as antimicrobial and anti-inflammatory, essential oils have been used for medical purposes since ancient times (Sharifi-Rad et al., 2017). Currently they are also used in perfumes, cosmetics and food industry (Dhifi et al., 2016; Pashtetskiy et al., 2017). In the Nikita Botanical Gardens (NBG), the collection of aromatic and medicinal plants is represented by 108 genera, 315 species, subspecies, forms, cultivars and hybrids belonging to 34 families. Plants of the families Lamiaceae Martinov and Asteraceae Bercht. & J. Presl are most widely represented in the collection, amounting to 24 and 20% of the total number of accessions, respectively. The Lamiaceae family has 26 genera and 75 species. Among them, the genus *Thymus* L. includes 12 species: *Thymus* sp., *T. nitens* Lamott., *T. pulegioides* L., *T. striatus* Vahl., *T. mastichina* L., *T. helendzhicus* Klokov & Des.-Shost., *T. comosus* Heuffel ex Iriseb., *T. kotschyanus* Boiss. et Hohen., *T. vulgaris* L., *T. serpyllum* L., *T. roegneri* K. Koch, and *T. richardii* subsp. *nitidus* (Guss.) Jalas (Marko et al., 2018). Breeding of essential oil plants in the NBG includes the development of drought-tolerant, high-oleic cultivars, different chemotypes, and high-quality perfumery plants with a high mass fraction of the main components in essential oil (Plugatar, Shevchuk, 2019). Their ornamental characteristics are also taken into consideration. Further breeding implies knowledge of the genetic differences between cultivars. The base for a molecular analysis is DNA of high quality (Tiwari et al., 2107; Bulavin et al., 2019a). For DNA isolation, different kits or classical methods are used. For mass analysis, isolation by kits is a very expensive process (Afsharzadeh, Abbasi, 2016; Fu et al., 2017). So, the objective of our investigation was to search for a cheap technique for the isolation of DNA with good yield and quality.

Materials and methods

Leaves of *Thymus* plants (*T. mastichina* cv. 'Svetliachok', *T. striatus* cv. 'Jubileiniy', *T. vulgaris* cv. 'Fantasia', and *T. vulgaris* cv. 'Jalos'.), grown *ex situ* on the collection plot of essential oil plants at the Nikita Botanical Gardens, were harvested for the investigation in March and May of 2020.

For the anatomical studies, the middle parts of leave blades were excised and processed on a freezing microtome (MZ-2, Ukraine) equipped with a lab cooling system OL-ZSO 30 (Inmedprom, Russian Federation). Sections were stained with methylene blue and examined using a CX41 light microscope (Olympus, Japan) equipped with an SC 50 camera (Olympus, Germany) and CellSens Imaging Software version 1.17.

Essential oil from the thyme leaves was isolated by the method of hydrodistillation on Ginsberg devices (Isikov et al., 2009; Logvinenko, 2017) with subsequent measurement of its volume. The content of phenolic compounds in leaves was measured with the Folin–Ciocâlteu reagent (AppliChem GmbH, Germany) (Gerzhikova, 2002).

For DNA isolation, intact young leaves were cut from *ex situ* cultivated plants. Samples were transferred to mortars, frozen in liquid nitrogen, and homogenized using a pestle. The tissue powders, preventing thawing, were transferred to test tubes with a lysing buffer. Isolation procedure was carried out according to the protocols of the manufacturers of the DiamondDNA™ (DiamondDNA, Russian Federation), PureLink® Plant Total DNA Purification Kit (Thermo Scientific™, USA), isolation with CTAB-method (trimethylammonium bromide) (Rogers et al., 1985; Doyle J.J, Doyle J.L., 1987) and CTAB-method with 2% polyvinylpyrrolidone (PVP). DNA quantity and quality were analyzed with NanoPhotometer NP80 (Implen, Germany) at wavelengths A_{260}, A_{280}, by electrophoresis in 1% agarose gel with 1×TBE buffer and using the Agilent 4200 TapeStation automated electrophoresis system (Agilent Technologies, Germany). For assessment of DNA quality, PCR was also performed with BioMaster HS-Taq PCR kit (BiolabMix, Russian Federation) according to the manufacturer's protocol using primer OPA18 (5'AGGTGACCGT3') for RAPD assay. PCR were carried out in the C1000™ Thermal Cycler (Bio-Rad, Singapore). The reaction conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of incubation at 94°C for 30 s, annealing at 32°C for 30 s and incubation at 72°C for 30 s, and with a final extension step at 72°C for 5 min. The amplified fragments were analyzed by electrophoresis in 1.5% agarose gel with 1×TBE buffer at 100 V during 1 h using universal power supply PowerPacTM (Bio-Rad, Singapore). The agarose gel was imaged with E-box documentation system (Vilber Lourmat, France).

Results and discussion

For a molecular analysis, different vegetative and generative organs of a plant are used in wet or dry state. Selection depends on the aim of investigation. In our works, we used wet leaves, because these organs were for a long period on plants grown *ex situ*, and smaller sample weight was needed for investigation. It is well known that aromatic plants contain secondary metabolites. Thymes contain more than 1% of the essential oil with phenols, such as camphene, β-myrcene, 1,8-cineole, camphor, β-caryophyllene, caryophyllene oxide, thymol, carvacrol, *p*-cymne, borneol, sabinene, and γ-terpinene. Essential oil composition varies depending on the growth place. Tannins, bitters, gums, organic acids, flavonoids and some salts are also detected in the plant material (Karomatov, Asadova, 2017).

Firstly, an anatomical investigation was performed. The analysis showed that leaf blades of four thyme cultivars ('Svetliachok', 'Fantasia', 'Jubileiniy' and 'Jalos') were characterized by differentiated mesophyll (Fig. 1), and both mesophyll cells had inclusions with essential oil inside. The amounts of essential oil and phenolics in the leaf blades of the thyme cultivars was measured biochemically (Table 1).

According to our data, we ranged plants from higher to smaller values of the leaf essential oil content in the following order: 'Svetliachok' → 'Jubileiniy' → 'Fantasia' → 'Jalos'. The descending order of the phenolic content in plant leaves was as follows: 'Svetliachok' → 'Jalos' → 'Jubileiniy' → 'Fantasia'. Since the plants were characterized by the presence of secondary metabolites, DNA was isolated by different methods. Below, the $A_{260/280}$ wavelength ratio data are shown. Spectrophotometric analysis demonstrated that the classical CTAB method using CTAB buffer with 2% PVP gave the best results (Table 2). PureLink® Plant Total DNA Purification Kit isolated DNA with lower concentration values for

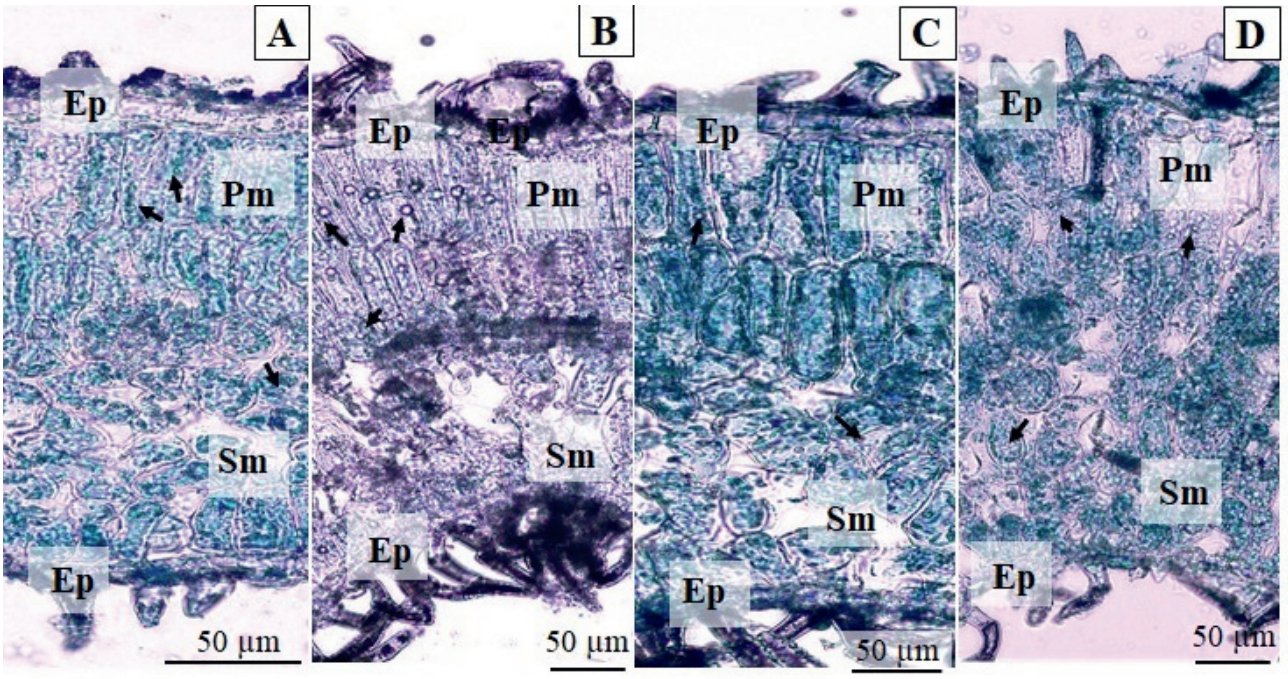

Fig. 1. Transversal section fragments of the thyme leaves from four cultivars: A – Svetliachok, **B** – Fantasia, **C** – Jubileiniy, **D** – Jalos; **Ep** – epidermis, **Pm** – palisade mesophyll, **Sm** – spongy mesophyll. Arrows indicate inclusions with essential oil

Рис. 1. Фрагменты поперечных срезов листьев четырех сортов чабреца: A – Светлячок, **B** – Фантазия, **C** – Юбилейный, **D** – Ялос; **Ep** – эпидерма, **Pm** – столбчатый мезофилл, **Sm** – губчатый мезофилл. Стрелками показаны включения с эфирным маслом

Table 1. Essential oil and phenolic compound content in the leaves of thyme cultivars

Таблица 1. Содержание эфирного масла и фенольных соединений в листьях разных сортов чабреца

Cultivar	Essential oil content, %/100 g	Phenolic compound content, mg/100 g	Main component
Svetliachok	1.18	1072 ± 29.45	1,8-cineole
Fantasia	0.18 ± 0.08	689 ± 13.8	thymol
Jubileiniy	0.25 ± 0.04	818 ± 10.33	thymol
Jalos	0.16 ± 0.01	914 ± 9.29	linalool

all cultivars. In the samples isolated with DiamondDNA™ kit (Fig. 2A), DNA fragmentation was detected.

Since *T. mastichina* cv. 'Svetliachok' was characterized by a high accumulation of essential oil and phenols, investigation of the DNA samples was performed additionally on the automated electrophoresis system. Figure 3 shows the satisfactorily DNA state based on the DNA Integrity Number (DIN) value. The presence of high-molecular-weight DNA (more than 52,000 bp) in significant amounts was detected in the samples isolated with DiamondDNA™ kit and CTAB + 2% PVP. Its concentrations were 14.6 ng/µl (molecular size 57,509 bp) and 11.7 ng/µl (more than 60,000 bp), respectively. CTAB with 2% PVP gave DNA with concentrations 7.86 ng/µl (58,025 bp) and 14.6 ng/µl (58,025 bp). Using the CTAB method, high-molecular-weight DNA was obtained in the concentration of 2.53 ng/µl, but in the samples isolated with PureLink® Plant Total DNA Purification Kit, high-molecular-weight DNA was not detected.

The isolated DNA samples were also amplified with primer OPA 18 (Fig. 4). The amplification results show clear PCR products with different sizes (bp), suggesting the acceptability of all investigated DNA samples for PCR amplification.

For DNA isolation, kits utilizing liquid sorbent technology (DiamondDNA), silica-based membrane technology in the form of spin column (PureLink), classical CTAB-method, and CTAB method + 2% PVP were used. According to the manufacturer's protocol, kits provide the purified genomic DNAs with $A_{260/280}$ ratio more than 1.7, usually ranging in the interval of 1.8–2.0, which is an evidence of the pure DNA (Vega-Vela, Chacón-Sánchez, 2011). It should be noted, that kits and protocols are usually aimed for model plants (peas, wheat, etc.). The studied essential oil plants were characterized by secondary metabolite accumulation at various concentrations. It is well known that identifying secondary metabolites in the plant cells and their further presence in so-

Table 2. **Quality of the DNA isolated by different methods from the leaves of thyme cultivars**
Таблица 2. **Показатели качества ДНК, выделенной разными способами из листьев чабреца**

Parameter / Isolation method	Plant material weight (mg)	$A_{260/280}$	DNA concentration (ng/µl)
Svetliachok			
DiamondDNA™	29.67 ± 1.2	1.87 ± 0.15	226.3 ± 79.06
PureLink®	100.67 ± 2.33	1.7 ± 0.04	10.38 ± 4.26
CTAB	102.67 ± 0.33	2.02 ± 0.02	66.2 ± 3.63
CTAB +2% PVP	102.67 ± 1.77	1.99 ± 0.33	122.53 ± 41.56
Fantasia			
DiamondDNA™	30.66 ± 0.67	1.83 ± 0.09	42.35 ± 6.25
PureLink®	100.33 ± 1.2	1.58 ± 0.07	8.27 ± 0.42
CTAB	102 ± 1.15	1.91 ± 0.09	89.7 ± 81.81
CTAB +2% PVP	103 ± 1	1.84 ± 0.05	23.83 ± 5.91
Jubileiniy			
DiamondDNA™	29.66 ± 0.33	1.9 ± 0.09	61.48 ± 13.06
PureLink®	99.67 ± 2.02	1.61 ± 0.06	7.7 ± 1.17
CTAB	101.33 ± 1.86	1.79 ± 0.11	67.75 ± 61.29
CTAB +2% PVP	100.33 ± 0.33	1.97 ± 0.03	157.73 ± 123.58
Jalos			
DiamondDNA™	28.66 ± 0.33	1.9 ± 0.09	124.2 ± 26.07
PureLink®	101.33	1.64 ± 0.05	8.28 ± 2.29
CTAB	101 ± 1	1.9 ± 0.12	58.75 ± 32.47
CTAB +2% PVP	102.67 ± 0.67	1.96 ± 0.03	56.43 ± 15.61

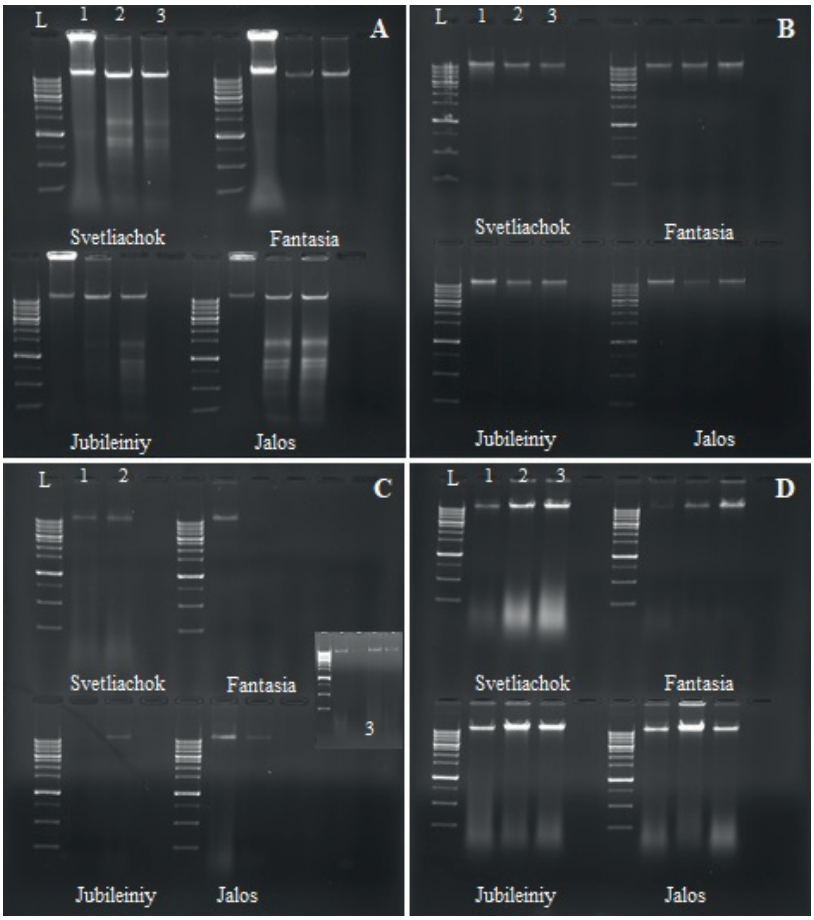

Fig. 2. Electrophoregrams of DNA samples extracted from the leaves of four thyme cultivars by different methods: **A** – DiamondDNA™ kit, **B** – PureLink® Plant Total DNA Purification Kit, **C** – CTAB, **D** – CTAB + 2% PVP; **1 – 3** – samples, **L** – ladder (10,000 bp, Sky-High 250 b – 10 kb, Biolabmix)

Рис. 2. Электрофореграммы образцов ДНК, выделенных из листьев четырех сортов чабреца разными способами: **A** – набор DiamondDNA™, **B** – набор PureLink® Plant Total DNA Purification Kit, **C** – ЦТАБ, **D** – ЦТАБ + 2% ПВП; **1 – 3** – образцы, **L** – маркер (10 000 пн, Sky-High 250 b – 10 kb, Биолабмикс)

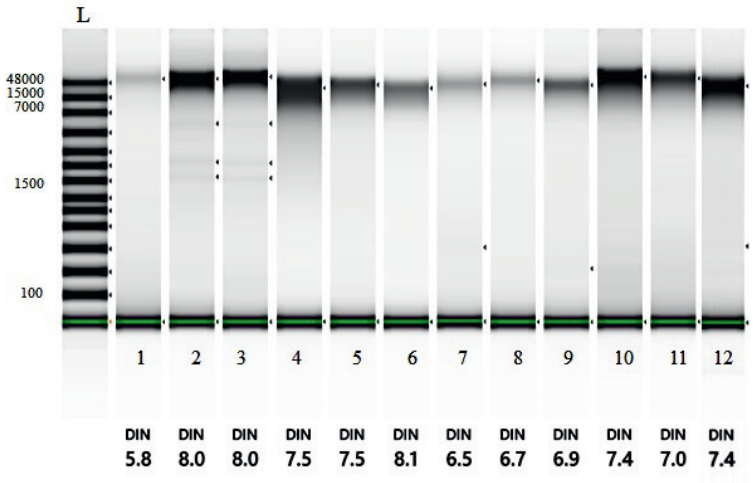

Fig. 3. Electrophoregrams of DNA samples isolated from the leaves of cv. Svetliachok: **L** – ladder (Genomic DNA Ladder, Agilent Technologies), **1 – 3** – DiamondDNA™ kit, **4 – 6** – PureLink® Plant Total DNA Purification Kit, **7 – 9** – CTAB, **10 – 12** – CTAB + 2% PVP

Рис. 3. Электрофореграммы образцов ДНК, выделенных из листьев чабреца сорта Светлячок: **L** – маркер (Genomic DNA Ladder, Agilent Technologies), **1 – 3** – набор DiamondDNA™ kit, **4 – 6** – набор PureLink® Plant Total DNA Purification Kit, **7 – 9** – ЦТАБ, **10 – 12** – ЦТАБ + 2% ПВП

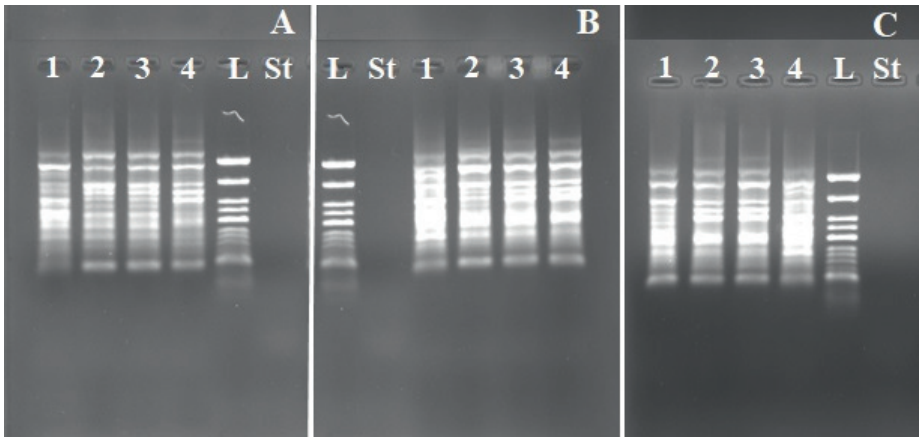

Fig. 4. Electrophoregrams of PCR products amplified with OPA18 primer: L – ladder (1,500 bp, Step50 plus, Biolabmix), **St** – negative control without DNA template; **A** – DiamondDNA™ kit, **B** – PureLink® Plant Total DNA Purification Kit, **C** – CTAB + 2% PVP; **1** – Svetliachok, **2** – Fantasia, **3** – Jubileiniy, **4** – Jalos

Рис. 4. Электрофореграммы продуктов ПЦР с праймером OPA18: L – маркер (1500 пн, Step50 plus, Биолабмикс), **St** – отрицательный контроль без ДНК-матрицы; **A** – набор DiamondDNA™ kit, **B** – набор PureLink® Plant Total DNA Purification Kit, **C** – ЦТАБ + 2% ПВП; **1** – Светлячок, **2** – Фантазия, **3** – Юбилейный, **4** – Ялос

lution with DNA may lead to failure of molecular analysis. For example, essential oil accumulation in leaf blades influence the DNA quality (Bulavin et al., 2019b), phenolic compounds and terpenes can reduce the yield and purity of extracted DNA due to its degradation (Kanauja et al., 1999; Vega-Vela, Chacón-Sánchez, 2011). These undesirable phenomena are eliminated by adding PVP in the range 1–5% (Hills, van Staden, 2002; Sá et al., 2011; Arruda et al., 2017; Lucas et al., 2019) to the CTAB extraction buffer and further overnight rehydration of the PVP. Our results also demonstrated that DNAs isolated from four thyme cultivars with the CTAB method + 2% PVP had robust values of $A_{260/280}$ ratio despite the presence of essential oils and phenols in the plant tissues. Applicability of DNA for molecular analysis was also confirmed by PCR with a random primer. Agarose gel electrophoresis of PCR products showed that no inhibition of Taq DNA polymerase activity was observed and clear marker fragments were detected.

Conclusion

Using any techniques for DNA isolation should be based on the type of the plant material. The obtained results demonstrated that the quality of the DNA isolated from thyme leaves depended on the presence of secondary metabolites, their amount, and the isolation method. Among the tested kits and methods, CTAB + 2% PVP provided thyme DNA suitable for PCR. It is also assumed that such DNA is suitable for genome library preparation. The low cost of reagents for this technique makes it applicable for future mass analysis of plant material.

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