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Optimization of DNA isolation from four species of *Rhododendron* from Europe

Caprar M.[1, 2], Cantor Maria[2]*, Sicora Oana[1], Copaci Cristina [1], Sicora C.[1]

[1)]Biological Research Center, Botanical Garden "Vasile Fati" Jibou, Parcului Street, no.14, 455200 Jibou, Romania; [2)]University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Horticulture, Mănăștur Street, no 3-5,4000472 Cluj-Napoca, Romania;

*Corresponding author. Email: marcantor@yahoo.com

Abstract DNA isolation is a procedure used to obtain genetic material from different organisms in order to use it in subsequent molecular analysis. The quantity and quality of the material are very important as the result from the next steps depend on it, respectively the widely used techniques of molecular biology such as RAPD, RFLP, AFLP, sequencing and others. The work shows the results obtained in isolating DNA from leaves with different extraction kits from four European species of *Rhododendron*, *Rhododendron luteum*, *Rhododendron ferrugineum, Rhododendron myrtifolium* and *Rhododendron hirsutum.* All the extraction protocols followed have three main stages: the destruction of the cellular integrity and the release of DNA in a homogenate called cell lysate, the purification of DNA from RNA, protein and other metabolic products, obtaining DNA in the desired concentration and known purity. The experiment used 3 DNA extraction kits aiming at both quantity and quality of the DNA, as well as the cost and complexity related to each kit.

Key words

rhododendron, isolate DNA Kits, protocols, DNA concentration

Rhododendrons, including azaleas, are a well-known, diverse and popular group of plants with a long and rich horticultural history. The centers of diversity for the rhododendrons are in the Himalayas and South East Asia where they form important components of mountain ecosystems. Also they naturally grow, albeit with less diversity, in North America, Europe and elsewhere in Asia, in moist frequently mountain ecosystems [2].

Rhododendrons in general prefer to grow in regions of high rainfall, high humidity and a temperate climate, also having a preference for acidic soils. The genus has a great diversity of forms, ranging from low creeping plants a few centimeters tall to trees of 30 meters. In total over 1000 species are recognized and the genus is subject to ongoing taxonomic debate. Many species of rhododendrons are valued for their horticultural and medicinal properties [4]. In some communities they are used for firewood, timber, teas, honey, wine and jams, as well as being valued for their narcotic potential, other species are a source of insecticides.

DNA extraction is an important step in the field of plant molecular biology. The high level of polysaccharide in the cell walls and several secondary metabolites affect DNA purification and the obtaining of high quality of DNA. Rhododendrons contain phenolic compounds which negatively influence the DNA purification [1].

For total genomic DNA isolation several methods have been used. Young leave collected in the field were dried in silica gel. Prior DNA isolation leaf tissue was grounded to powder in liquid nitrogen. This step probably inhibits the phenolic compounds which inhibits the extraction of good quality DNA. For DNA extraction a modified CTAB method was used in many papers [3]. From the commercial DNA extraction kits, DNeasy Plant Mini kit from Quiagen was used with good results for DNA isolation of *Rhododendron ferrugineum* species [5].

Material and Methods

The biological material used in the analysis was collected in June, in 2011-2012 from several locations in the Alps for the species *Rhododendron hirsutum* and *Rhododendron ferrugineum*. From the reservations Lendorf (Austria) and Kolacznia (Poland), biological material was collected for the species *Rhododendron luteum* (Table 1). Samples were taken for *Rhododendron myrtifolium* from Rodna and Piatra Craiului Mountains. A distance of a few meters between individuals was respected. Young, healthy leaves were used, without rust spots or other parasites, which were immediately placed in tubes filled with silica gel. After sampling, the leaves were stored at-80 °C until use for DNA isolation.

Because CCB Jibou doesn't have a source of liquid nitrogen, a suitable alternative method was attempted for isolating DNA, bypassing the stage of grinding in the liquid nitrogen.

Table 1

Plant material collection points

No	Specie	Location	Altitude	Geographical Coordinates	Date	Country
1.	*Rh.ferrugineum*	Breuil-Cervinia	2020	45°55′49″N/7°37′36″E	2011	Italy
2.	*Rh.ferrugineum*	Breuil-Cervinia	1870	45°54′46,87″N/7°36,55′80″E	2011	Italy
3.	*Rh.ferrugineum*	Groβglockner	1900	47°03′45,12″N/12°49′22.17″E	2011	Austria
4.	*Rh.ferrugineum*	Zumdorf	1530	46°36′03,91″N/8°80′49.77″E	2011	Switzerland
5.	*Rh.ferrugineum*	Klausen pass	1952	46°52′05,36″N/8°51′16.71″E	2011	Switzerland
6.	*Rh.ferrugineum*	Edelweisspitze	2002	47°07′24,53″N/12°48′38.11″E	2011	Austria
7.	*Rh.ferrugineum*	Furka Pass	1720	46°35′21,11″N/8°29′27.58″E	2011	Switzerland
8.	*Rh.ferrugineum*	Furka Pass	1600	46°35′43,77″N/8°29′49″E	2011	Switzerland
9.	*Rh.ferrugineum*	Furka Pass	1920	46°35′15,80″N/8°29′01.42″E	2011	Switzerland
10.	*Rh.ferrugineum*	Flüela Pass	2100	46°44′42,73″N/9°59′24.87″E	2011	Switzerland
11.	*Rh.ferrugineum*	Flüela Pass	2000	46°46′50,92″N/9°55′32.92″E	2011	Switzerland
12.	*Rh.ferrugineum*	Col d'Izoard	2270	44°49′21″N/6°43′53″E	2012	France
13.	*Rh.ferrugineum*	Col d'Izoard	2070	44°49′55″N/6°43′25″E	2012	France
14.	*Rh.ferrugineum*	Col du Galibier	2330	45°02′49″N/6°23′51″E	2012	France
15.	*Rh.ferrugineum*	Col du Glamdon	1590	45°15′18″N/6°11′20″E	2012	France
16.	*Rh.ferrugineum*	Col du Lautaret	2100	45°02′17″N/6°24′04″E	2012	France
17.	*Rh.ferrugineum*	Col de la Cayolle	2179	44°15′15″N/6°45′09″E	2012	France
18.	*Rh.ferrugineum*	Col de la Bonnette	2100	44°19′41″N/6°51′47″E	2012	France
19.	*Rh.ferrugineum*	Val – d'Isere	2060	45°27′13″N/7°02′05″E	2012	France
20.	*Rh.ferrugineum*	Petit Saint Bernand	2010	45°38′54″N/6°51′13″E	2012	Italy
21.	*Rh.ferrugineum*	Col du Thule	2026	45°42′07″N/6°54′24″E	2012	Italy
22.	*Rh.hirsutum*	Klausen pass	1660	46°52′07″N/8°51′08″E	2012	Switzerland
23.	*Rh.hirsutum*	Triglave	1650	46°23′41″N/13°58′24″E	2012	Slovenia
24.	*Rh.luteum*	Lendorf	670	46°50′07″N/13°25′24″E	2012	Austria
25.	*Rh.luteum*	Kolacznia	195	$50^{0}18^{'}20$N/$22^{0}16^{'}73$ E	2012	Poland
26.	*Rh.myrtifolium*	Piatra Craiului	1948	$45^{0}34^{'}255$N/$25^{0}14^{'}$E	2012	Romania
27	*Rh.myrtifolium*	Rodnei	1825	$47^{0}34^{'}255$N/$24^{0}35^{'}$E	2012	Romania
28	*Rh.ferrugineum*	Pirinei	1900	$42^{0}34^{'}255$N/$0^{0}54^{'}$E	2012	Spain

After harvesting, the plant material was kept in a freezer at -80 °C. For the testing stage of DNA isolation, three mini commercial isolation kits were purchased, respectively: InnuSpeed Plant DNA Kit - Analytikjena [6], DNeasy Plant Mini Kit - Qiagen [8]; Isolate DNA Kits - Bioline [7].

In general, all three work protocols have three main stages, namely the destruction of the cellular integrity and release of DNA into a homogenate called lysate, DNA purification from RNA and other metabolic products, respectively obtaining DNA into the desired concentration.

For testing, plant material was used from all four species of *Rhododendron* from the same location and individual for each species. Extraction protocols were followed exactly except for the ones that stipulated using liquid nitrogen, one of the objectives of the study was finding a method that does not involve the use of liquid nitrogen in the process of cellular wall destruction. The quantity of processed plant material was 85 mg for all 4 species of *Rhododendron*. The differences between the three kits are presented in the table below (Table 2).

Table 2

Working protocols for isolation kits

InnuSpeed Plant DNA Kit – Analytikjena	Dneasy Plant Mini Kit – Qiagen	Isolate DNA Kits – Bioline
Starting material up to 100 mg Homogenization: plant material is cut into small pieces and add to Lyses Tube Pwith 100 µl H_2O Lyses Tube is added to SpeedMill Homogenization time:3 minutes (program for very hard plant material) The next steps according to the protocol are: Lyses of starting material and pre-filtration Binding of DNA on Spin Filter Washing of the bound DNA Elution of DNA	Starting material up to 100mg Homogenization: frozen plant material is cut into small pieces and grind to a fine powder with precooled (-80 ^{o}C) mortar and pestle The next steps according to the protocol are: Lyses and precipitation Centrifugation through QIAshredder Binding of DNA Washing of DNA Elution of DNA	Starting material up to100 mg Homogenization: frozen plant material is cut into small pieces and grind to a fine powder with precooled (-80 ^{o}C) mortar and pestle The next steps according to the protocol are: Lyses Precipitation Binding of DNA on Spin Column Washing of the bound DNA Elution

After isolation, the DNA was quantified by the spectrophotometric method using Nanodrop2000 (Table 3).

Table 3

DNA concentration in the 4 species of *Rhododendron* using three isolation kits

Commercial kit	Species								Price per sample (lei)
	Rhododendron ferrugineum		*Rhododendron luteum*		*Rhododendron myrtifolium*		*Rhododendron hirsutum*		
	Concentration (ng/µl)	Absorption ratio 260/280	Concentration (ng/µl)	Absorption ratio 260/280	Concentration (ng/µl)	Absorption ratio 260/280	Concentration (ng/µl)	Absorption ratio 260/280	
InnuSpeed Plant DNA Kit	262.2	2.07	77.5	1.85	121.7	1.95	127.5	1.96	27.2
Dneasy Plant Mini Kit	8.4	1.60	9.7	1.80	23.6	1.98	16.2	1.70	19.0
Isolate DNA Kits	95.8	2.10	87.5	2.09	112.5	2.01	74.5	1.90	17.0

Analyzing Table 3, it shows that the best results in terms of DNA isolation from all four species of *Rhododendron* were obtained using DNA Plant Kit InnuSpeed but also the costs by sample are the biggest. For effective isolation activity, taken in consideration that in the future we intend to process a large number of samples, a second experimental stage was started, namely the combination of the first experimental kit (InnuSPEEDDNA Plant Analytikjena) with the last, respectively (Isolate DNA Kit-Bioline).

Modified Bioline Protocol

Frozen leaves (stored in at -80° C) were cut into small pieces that were kept for several hours at-80 °C. They were grinded until a fine powder was

obtained. The mortar and pestle used for the grinding were also stored at-80 °C for 24 hours (before the grinding).About 85 mg of grinded plant material was weighed. This material was placed in 2 ml screw cap tubes which were previously weighed together with 1gram of chrome steel beads (1.3 mm diameter). For each sample 400 ml Lysis Buffer was added. The prepared samples were placed in SpeedMill Plus (analytikjena) homogenizer program for hard plant material (three minutes).After homogenization, samples are centrifuged at 10,000 xg for 1 minute after which the supernatant is carefully transferred to a 2 ml Eppendorf tube. The samples are incubated in a water bath at 65 °C for 30 minutes. Occasionally samples are mixed by vortexing. Add 100 µl Precipitation Bufferand vortex for 5 seconds. Incubate on ice for 5 minutes. Centrifuge at maximum speed (14500 xg) for 5 minutes. The supernatant is transferred to Spin Column PD1 collection tubes. Centrifuge at 10,000 xg for 1 minute. Remove PD1filtercolumns and keep the filtered solution. Approximate the volume of the filtered solution for the next step. Add 0.5 Binding Buffer PD volumes to the filtered solution and mix by pipetting. Transfer the sample to Spin Column PD2. Centrifuge at 10,000 x g for 2 minutes. Spin Column PD2 is placed on new collection tubes. Add 700 µl Wash Buffer PD. Centrifuge at 10,000 x g for 1 minute. Discard the filtered solution and re-use collection tubes. Add 700 µl PD Wash Buffer and centrifuge at 10,000 xg for 1 minute. Discard the solution filtered. Centrifuge at maximum speed (14,500 xg) for 2 minutes. SpinColumn PD2 is placed on 1.5 ml Elution tubes and 100 µl Elution Buffer is added directly on the filtration column membrane. Incubate at room temperature for a minute. Centrifuge at 6000 x g for 1 minute to elute the DNA. Pipette the eluted DNA solution (from the elution tube) and pass it back on the filtration column membrane. Centrifuge at 6000 x g for 1 minute. The isolated DNA can be used in subsequent experiments.

Results and Discussions

Combining the two work protocols (DNA extraction from plant material using SpeedMill-InnuSPEED and Isolate plant DNA Mini Kit- Bioline) and the use of beads steel has yielded good results in terms of the amount of DNA extracted as well as its quality, as verified in subsequent experiments (PCR). From Table 4 it is observed that the DNA concentration from *Rhododendron ferrugineum* species varied between 92 and 470ng/µl, for *Rhododendron myrtifolium* extracted DNA concentration was 550 ng/µl for the sample from Piatra Craiului Mountains and 742.2ng/µl for the sample from Rodnei Mountains. The results were good also for *Rhododendron hirsutum,* with the concentration of 550.0ng/µl from the Klausen Pass sample and 392.5 ng/µl the sample from Triglave (Alps). Sufficiently good results were obtained in the case of *Rhododendron luteum* species were the DNA concentration was 63,4ng/µl for the Lendorf (Austria) sample and 125.6 ng/µl for the sample from Kolacznia (Poland). Concentration reading was taken with Nanodrop 2000 (Fig. 1).

Table 4

The concentration of isolated DNA from the various samples of *Rhododendron*

No.	Species	Location	Altitude	Isolation efficiency and concentration (ng/µl)	Absorption ratio 260/280	Country
0	1	2	3	4	5	6
1.	*Rh.ferrugineum*	Breuil-Cervinia	2020	92.9	1.65	Italy
2.	*Rh.ferrugineum*	Breuil-Cervinia	1870	146.3	2.09	Italy
3.	*Rh.ferrugineum*	Groβglockner	1900	297.1	1.89	Austria
4.	*Rh.ferrugineum*	Zumdorf	1530	198.1	2.12	Switzerland
5.	*Rh.ferrugineum*	Klausen pass	1952	185.8	2.05	Switzerland
6.	*Rh.ferrugineum*	Edelweisspitze	2002	192.1	2.04	Austria
7.	*Rh.ferrugineum*	Furka Pass	1720	185.7	2.01	Switzerland
8.	*Rh.ferrugineum*	Furka Pass	1600	215.8	2.06	Switzerland
9.	*Rh.ferrugineum*	Furka Pass	1920	325,3	2.14	Switzerland
10.	*Rh.ferrugineum*	Flüela Pass	2100	184.5	1.86	Switzerland
11.	*Rh.ferrugineum*	Flüela Pass	2000	380.0	1.98	Switzerland
12.	*Rh.ferrugineum*	Col d'Izoard	2270	74.6	1.77	France
13.	*Rh.ferrugineum*	Col d'Izoard	2070	222.4	2.11	France
14.	*Rh.ferrugineum*	Col du Galibier	2330	182.0	1.85	France
15.	*Rh.ferrugineum*	Col du Glamdon	1590	337,9	2.12	France
16.	*Rh.ferrugineum*	Col du Lautaret	2100	277.5	1.98	France
17.	*Rh.ferrugineum*	Col de la Cayolle	2179	272.0	2.03	France

0	1	2	3	4	5	6
18.	*Rh.ferrugineum*	Col de laBonnette	2100	201.2	2.03	France
19.	*Rh.ferrugineum*	Val – d'Isere	2060	287.7	2.12	France
20.	*Rh.ferrugineum*	Petit Saint Bernand	2010	196.8	1.98	Italy
21.	*Rh.ferrugineum*	Col du Thule	2026	125.0	1.60	Italy
22.	*Rh.hirsutum*	KlausenPass	1660	550.0	2.15	Switzerland
23.	*Rh.hirsutum*	Triglave	1650	392.5	2.13	Slovenia
24.	*Rh.luteum*	Lendorf	670	63.40	2.10	Austria
25.	*Rh.luteum*	Kolacznia	195	125.6	2.01	Poland
26.	*Rh.myrtifolium*	Piatra Craiului	1948	550.0	2.12	Romania
27.	*Rh.myrtifolium*	Rodnei	1825	747.2	2.15	Romania
28.	*Rh.ferrugineum*	Pirinei	1900	470.0	2.11	Spain

To establish the integrity of the DNA, an electrophoresis was made with agarose gel (1%, 1 x TAE). For visualization, Midori Green was used (Fig. 2).

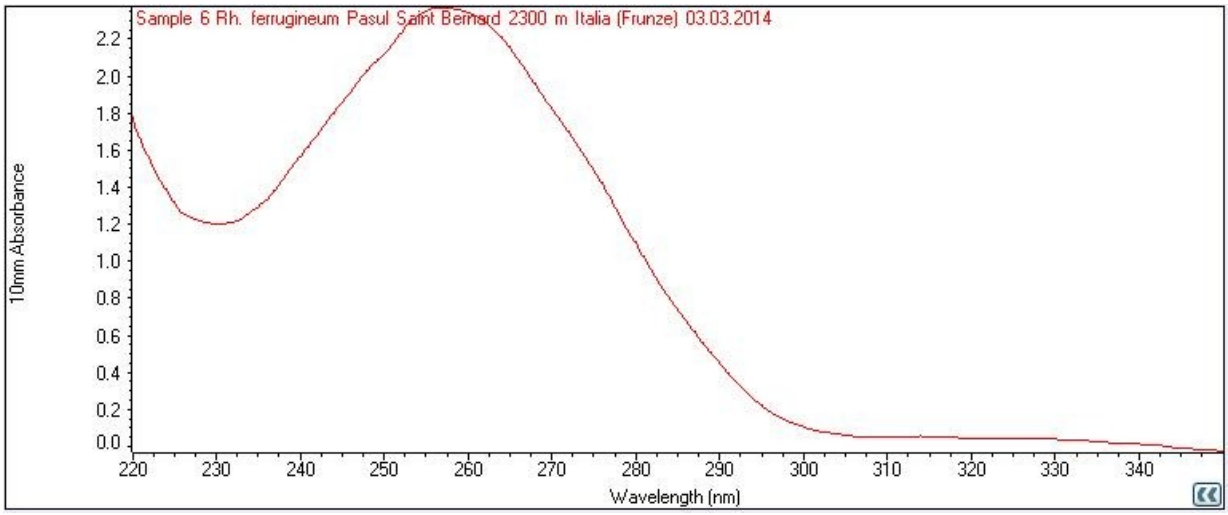

Fig. 1 DNA Qunatification extracted with Isolate DNA Kit Bioline (with own adaptation)

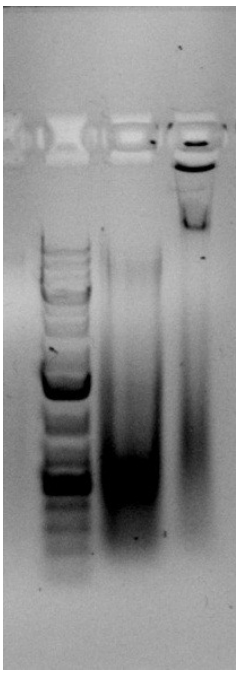

Fig.2. Genomic DNA migrated in agarose gel

Conclusions

For optimal isolation of DNA from "hard" species, such as *Rhododendron* the preparation stage of plant material is very important. Mechanical disruption (grinding) of the plant material with beads homogenizer is more easily achieved than grinding with liquid nitrogen and the quality of DNA obtained is better. A further disadvantage of the grinding method with the use of liquid nitrogen is the need to have a continuous source of liquid nitrogen. After combining the two protocols, the results on the quality and quantity of isolated DNA were very good making the method used successfully in the future. From the economic efficiency point of view the adjustments made by us considerably decreased the cost price on each sample.

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