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DNA ISOLATION FROM DRY SAMPLES OF BROOMRAPE - THE EFFECT OF ISOLATION METHOD AND SAMPLE STORAGE ON DNA YIELD AND QUALITY

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ABSTRACT

Accurate characterization of broomrape races is of a great importance for creation of sunflower hybrids resistant to this parasite. In molecular diversity studies, it is necessary to isolate sufficient quantity of good quality DNA. As collecting and transporting fresh broomrape tissue and seed samples for molecular analyses can be problematic, we have tested the possibility of DNA isolation from dry tissue of mature broomrape plants, with the aim to find optimal method for sample storage and isolation of sufficient quantity of DNA for multiple PCR reactions needed for diversity studies. Mid parts of dry broomrape stalks were collected and stored in four different ways. Ten DNA isolation protocols were tested and the obtained results compared. The best results regarding DNA yield and quality were obtained with dry samples stored at room temperature in ventilated space. DNA suitable for RAPD analysis was isolated with three of ten tested methods. As protocol of Somma (2006) is labour intensive and produced the lowest DNA yield, only DNA isolation with DNeasy® Plant Mini Kit, Qiagen and protocol by Rogers and Bendich (1985) could be recommended for future studies.

Key words: broomrape, DNA isolation, RAPD, dry samples, sample storage.

INTRODUCTION

Broomrape (*Orobanche cumana* Wallr.) is a holoparasitic angiosperm. It contains no chlorophyll, and infests sunflower (*Helianthus annuus* L.) roots. In contaminated fields it leads to reduction of sunflower yield between 50 and 90% (Ciuca et al., 2004). As such, broomrape is considered to be one of the most important sunflower weeds. It is widely spread in Eastern and Southern parts of Europe (Ciuca et al., 2004). The best way of managing broomrape is by creating broomrape resistant sunflower hybrids. However, in recent years new broomrape races have emerged.

Accurate characterization of broomrape races is of a great importance for creation of sunflower hybrids resistant to this parasite. Nuclear DNA analysis represents an important tool for phylogenetic and diversity studies of parasitic flowering plants, and has already been used for more precise determination of broomrape variability (Gagne et al., 1998; 2000; Ciuca et al., 2004; Paran et al., 1997; Roman et al., 2003; Atanasova et al., 2005).

In molecular diversity studies, it is necessary to isolate sufficient quantity of good quality DNA. So far, DNA isolation in broomrape has been performed from fresh floral buds (Roman et al., 2003; Gagne et al., 1998; Gagne et al., 2000; Ciuca et al., 2004) and seeds (Joel et al., 1996; Guchetl et al., 2011). Benharrat et al. (2002) used frozen shoot and inflorescence tissue grounded in liquid nitrogen, while Paran et al. (1997) isolated DNA from fresh flowers. To our knowledge, there are no reports on DNA isolation from dry tissue of mature *Orobanche* plants. Since plants contain high amounts of many different substances, it is unlikely that just one DNA isolation protocol is suitable for all plants (Loomis, 1974). CTAB (cetyl trimethyl-ammonium bromide) based isolation methods are widely used for plant DNA isolation. Another method used for DNA

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isolation is based on use of detergent SDS (sodium dodecyl sulphate). In the past decade, commercially available DNA isolation kits are increasingly used, especially when DNA should be isolated in a limited amount of time. Benharrat et al. (2002) used Nucleon Phytopure DNA extraction kit (Amersham, Buchinghamshire, UK) for DNA isolation from broomrape shoots and inflorescence. Most commonly used protocols for DNA extraction from different broomrape tissue samples were differently modified CTAB based protocols. For DNA isolation from broomrape floral buds protocols reported by Doyle and Doyle (1991), Lassner et al. (1989) modified by Torres et al. (1993) and Rogers and Bendich (1985) were used, while for DNA isolation from seeds protocol reported by Fulton et al. (1995) with modification by Joel et al. (1996) was used (Atanasova et al., 2005). As collecting and transporting fresh broomrape tissue and seed samples for molecular analyses can be problematic, we tested the possibility of DNA isolation from dry tissue of mature broomrape plants, with the aim to find optimal method for sample storage and isolation of sufficient quantity of DNA for multiple PCR reactions needed for diversity studies.

MATERIAL AND METHODS

Broomrape sampling and storage

Mid parts of dry broomrape stalks were collected from three susceptible sunflower plants of the same hybrid (genotype) in the field near Subotica, Serbia. After collection, dry stalks were stored in four different ways (Table 1).

Mid parts of fresh (young) stalks were collected in the greenhouse, from the pots sown with susceptible sunflower inbred line and infested with broomrape. Stalks were immediately put in liquid nitrogen, and kept at -70^{o}C until DNA isolation.

Table 1. Storage of dry broomrape samples

Sample	Storage of materials
1	Broomrape dry stalks stored at -70^{o}C
2	Broomrape dry stalks stored in exsiccator
3	Broomrape dry stalks stored in a dry and ventilated place
4	Broomrape dry stalks kept for seven days in a dry and ventilated place, then stored at -70^{o}C
5	Fresh stalks stored at -70°C

DNA isolation protocols

Both in fresh and different dry samples, DNA were isolated from the bulks of three stems. Before DNA isolation, samples were mechanically homogenized in pre-cooled porcelain mortar with liquid nitrogen. Unless differently noted, in all tested protocols 50-100 mg of sample was used for DNA isolation. DNA was isolated using ten different protocols (Table 2).

Table 2. Protocols used for DNA isolation from fresh and dry broomrape stems

Protocol	Name	Modification	Reference/Producer
1	Modified CTAB protocol	None.	Permingeat et al., 1998
2	Genomic DNA Purification Kit	The sample size was 80 mg of grounded tissue. LB* increased from 400 µl to 600 µl. Centrifugation time increased from 2 min to 20 min. DNA precipitation with cold ethanol extended for 10 min.	Fermentas
3	Modified protocol 2	Sample size increased to 100 mg. LB and chloroform increased to 850 µl. Incubation time with LB prolonged for 5 min.	
4	Modified protocol 1	RB* (Deshmukh et al., 2007) with 0.05% of activated charcoal (Krizman at al., 2006) added before EB*. NaCl concentration in EB increased from 1.4 M to 2 M.	

Continued table 2

5	CTAB based DNA isolation protocol	Centrifugation time up to 15 min.	Somma, 2006
6	DNeasy® Plant Mini Kit	Sample size was 0.02 g of grounded tissue.	Quiagen
7	CTAB based DNA isolation protocol	500 µl of CTAB buffer was used and samples incubated at 65°C for 20 min. Centrifugation time up to 5 minutes. At precipitation step, samples were kept for 20 min at -20°C. RNAse treatment was omitted.	Rogers and Bendich, 1985
8	SDS based DNA isolation protocol	None.	Jobes et al., 1995
9	CTAB based DNA isolation protocol	Grounded sample quantity doubled (50 mg).	Shepherd and McLay, 2011
10	Modified protocol 9	Chloroform/isoamyl alcohol (24:1) was added to the sample. Cold isopropanol was used for DNA precipitation, and the samples were kept for 20 min at -20°C.	

*RB - rinsing buffer; EB - extraction buffer; LB - lysis buffer.

DNA quantification

The amount and purity of the isolated DNA was determined spectrophotometrically on Spectrofotometer Ultrospec 2000 (Pharmacia Biotech) at absorbances of 260 nm, 230 nm and 280 nm. A_{260}/A_{280}, A_{260}/A_{230} ratios were calculated.

Visualization of the isolated DNA was performed on 0.7% agarose gel. Isolated DNA was compared with various amounts of standards (λ DNA).

PCR amplification

Amplification of isolated DNA was performed with two primers (Table 3). The first primer, 26S, was created in the Laboratory for Plant Molecular Biology of Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia. It is designed according to conserved region that encodes 26S rRNA, which is present in all plant species (Jovanović et al., 2011). Second primer used, was RAPD (Random Amplified Polymorphic DNA) primer, UBC 358, created at University of British Columbia, Vancouver.

PCR mix in final volume of 25 µl contained 1x Taq buffer, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 2.5 µg BSA, 0.2 µM primer mix, 1 U Taq polymerase or *Dream Taq* polymerase, and approximately 25 ng of isolated DNA. PCR amplification was performed according to Nagl et al. (2011) in Mastercycler Gradient, Eppendorf.

Table 3. Sequences of used primers

Primer name	Sequence 5'-3'
26S F	TTCCCAAACAACCCGACTC
26S R	GCCGTCCGAATTGTAGTCTG
UBC 358	GGTCAGGCCC

PCR products were separated on 2% agarose gels containing 10 mg/ml ethidium bromide in TBE buffer. Gels were visualized under UV light, by use of BIO-Print system (Vilber Lourmat, Marne la Velee, France).

RESULTS AND DISCUSSION

In this paper we propose the use of dry broomrape stalks for isolation of sufficient quantity of DNA for extensive molecular research. There are several advantages of the use of mature stalk material. It is easy to collect dry stalks in the field and store them, it is easy to transport them within the country, but also between countries, as there is no danger of uncontrolled spread of the parasite, and finally, a sufficient quantity of plant material necessary for DNA isolation could be collected from one site.

The effect of sample storage on DNA yield and quality

In order to find the optimal way of sample storage, we stored dry stalks in four different ways (Table 1) and compared the

yield and quality of isolated DNA among variants and with the results obtained with fresh tissue (Table 4). A_{260}/A_{280} and A_{260}/A_{230} ratios of dry samples indicated that all isolated DNAs were contaminated with proteins and polysaccharides. A_{260}/A_{280} ratio was mostly near optimal value (1.8-2.0) in DNA isolated from fresh samples (Table 4).

This ratio was generally lower in dry samples, in which the lowest values were determined in DNA from sample 4, regardless of the isolation method used, and the highest in DNA from samples 2 and 3. Low A_{260}/A_{280} ratio in DNA from dry samples indicates that protein contamination could affect DNA quality in dry samples. A_{260}/A_{230} ratio was not near optimal value (higher than 1.8) indicating that there was contamination with polysaccharides in DNA isolated from both fresh and dry samples.

The lowest A_{260}/A_{230} ratio was observed in sample 4, and the highest in samples 2 and 3, respectively. Decreased quality of DNA isolated from dry samples in comparison to the fresh ones has been reported by other authors, as well (Drabkova et al, 2002; Ribeiro and Lovato, 2007). Usually, DNA isolation from mature plant tissues is avoided because of presence of polysaccharides, polyphenols and other secondary metabolites (Dabo et al., 1993).

In general, fresh samples provided higher DNA yield in comparison to dry samples (Table 4). In dry samples, the highest DNA yield was obtained from sample 4, but the DNA quality was low.

Table 4. Yield and purity of isolated DNA from dry and fresh broomrape samples extracted by different protocols

Protocol used	Sample number	µg/µl	A_{260}/A_{280} ratio	A_{260}/A_{230} ratio	Protocol used	Sample number	µg/µl	A_{260}/A_{280} ratio	A_{260}/A_{230} ratio
Protocol 1	1	0.404	1.45	1.13	Protocol 6	1	0.136	1.23	0.90
	2	0.192	1.46	1.19		2	0.055	1.38	0.92
	3	0.260	1.56	1.15		3	0.076	1.25	0.88
	4	0.172	1.55	1.42		4	0.045	1.13	0.69
	5	0.591	1.89	1.19		5	0.081	1.23	0.84
Protocol 2	1	0.005	-*	-*	Protocol 7	1	0.056	1.11	0.71
	2	0.136	1.69	1.23		2	0.024	1.20	1.46
	3	0.217	1.65	1.16		3	0.036	1.00	0.56
	4	0.162	1.22	0.79		4	0.069	1.03	0.83
	5	0.237	1.88	1.21		5	0.156	1.92	2.03
Protocol 3	1	0.040	1.14	0.89	Protocol 8	1	0.030	1.50	0.83
	2	0.076	1.50	1.50		2	0.061	1.50	0.11
	3	0.045	1.00	0.90		3	0.057	1.55	0.74
	4	0.076	1.00	1.00		4	0.166	1.19	0.84
	5	0.101	1.82	1.25		5	0.149	2.11	1.85
Protocol 4	1	0.167	1.32	1.10	Protocol 9	1	0.254	1.26	0.64
	2	0.152	1.43	1.30		2	0.242	1.43	0.89
	3	0.126	1.25	1.19		3	0.507	1.48	1.04
	4	0.055	1.50	1.00		4	0.537	1.22	0.82
	5	0.288	1.54	1.12		5	0.121	1.67	0.95
Protocol 5	1	0.010	-*	-*	Protocol 10	1	0.349	1.12	0.72
	2	0.010	-*	-*		2	0.176	1.36	0.76
	3	0.061	-*	-*		3	0.160	1.79	1.18
	4	0.040	-*	-*		4	0.547	1.11	0.72
	5	0.015	-*	-*		5	0.089	1.76	0.98

*Due to extremely low absorbance values, it was impossible to determine absorbance ratio.

Ribeiro and Lovato (2007) found that decrease in DNA quality isolated from dry herbarium samples is a result of collecting conditions and sample preservation. Best quality of isolated DNA in dry samples was obtained from samples 2 (stored in exsiccator) and 3 (stored in ventilated place). Air-drying is considered to be a better way of preservation of plant tissues than storage in silica gel or anhydrous $CaSO_4$ (Taylor and Swann, 1994). Therefore, the storage in a dry and ventilated place could be recommended as an efficient and simple method for preservation of dry broomrape samples, with no need for special equipment (exsiccator) and sufficient yield and quality of isolated DNA.

The effect of isolation method on DNA yield and quality

Ten different protocols were used for DNA isolation from dry and fresh broomrape samples. Analysis of yield of isolated DNA showed that in average the highest yield was obtained with protocol of Shepherd and McLay (2011), and the lowest with protocol of Somma (2006) (Table 4). As A_{260}/A_{280} and A_{260}/A_{230} ratios of DNA isolated from dry samples showed that most of the isolated DNA was contaminated, several attempts were made in order improve DNA quality. Protocol 1 was modified (protocol 4) and the number of rinsing steps increased to four, which lead to discolouring of the DNA pellet. However, A_{260}/A_{280} and A_{260}/A_{230} ratios were not improved in comparison to protocol 1. This is in contrast to results of Deshmukh et al. (2007), where rinsing buffer successfully removed contaminations from dry and fresh *Terminalia arjuna* L. leaf samples. Furthermore, despite the increase of NaCl in extraction buffer (2 M NaCl), absorbance ratio showed that polysaccharides were still not eliminated. In previously published protocols, polysaccharides were effectively eliminated by increasing NaCl concentration in extraction buffer (Varma et al., 2007).

As precipitation step is very important for DNA recovery, protocols with different precipitation steps were used. Ribeiro and Lovato (2007) found that longer precipitation time was very important step in isolation of DNA from herbarium specimens. In contrast to this results, DNA yield of dry broomrape samples was not significantly increased in protocol with long (overnight) precipitation step (protocol 8) in comparison to other protocols used. Shepherd and McLay (2011) found that precipitation of DNA with room-temperature isopropanol lead to slight decrease in polysaccharide contamination, in comparison to use of ice-cold isopropanol, which was not the case in our experiment (Table 4). However, the highest yield of DNA from dry samples with satisfactory quality parameters was obtained with the protocol of the same authors (protocol 9).

PCR amplification

In order to determine whether isolated DNA could be used for analysis with molecular markers, PCR amplification with two types of primers was performed. Primer specific for plant 26S rRNA region in genomic DNA amplified one band of the same length in most of the samples tested (Figure 1). In some cases DNA isolated from dry tissue did not amplify, especially when protocol 9 was used for DNA isolation (Figure 1c and 1d), probably due DNA degradation.

Amplification of bands with RAPD primer, UBC358, occurred in less samples than with primer 26S (Figures 2 and 3). Drabkova et al. (2002) found that most effective amplification of DNA isolated from herbarium specimens was when the product size was small, approximately 300 bp, while the amplification effectiveness was lowered when product size excided 400 bp. This could be an explanation of why amplification with primer 26S was more successful than with UBC358.

Although, protocol 1 was the most effective when it came to removing polysaccharides from DNA samples (the highest value of A_{260}/A_{230} ratio), it seems that this was not essential for successful PCR amplification, since no bands were amplified in any of the DNA samples (Figure 2a). None of DNAs isolated by protocol 9 produced any

bands, while in protocols 4, as well as 8 and 10, only two and one DNA sample, respectively, had amplify with UBC 358 (Figure 2). Primer UBC 358 amplified bands with most of the DNA samples extracted by Genomic DNA Purification Kit (Fermentas) (protocols 2 and 3) (Figure 2a) and by protocol 8 (Figure 2b).

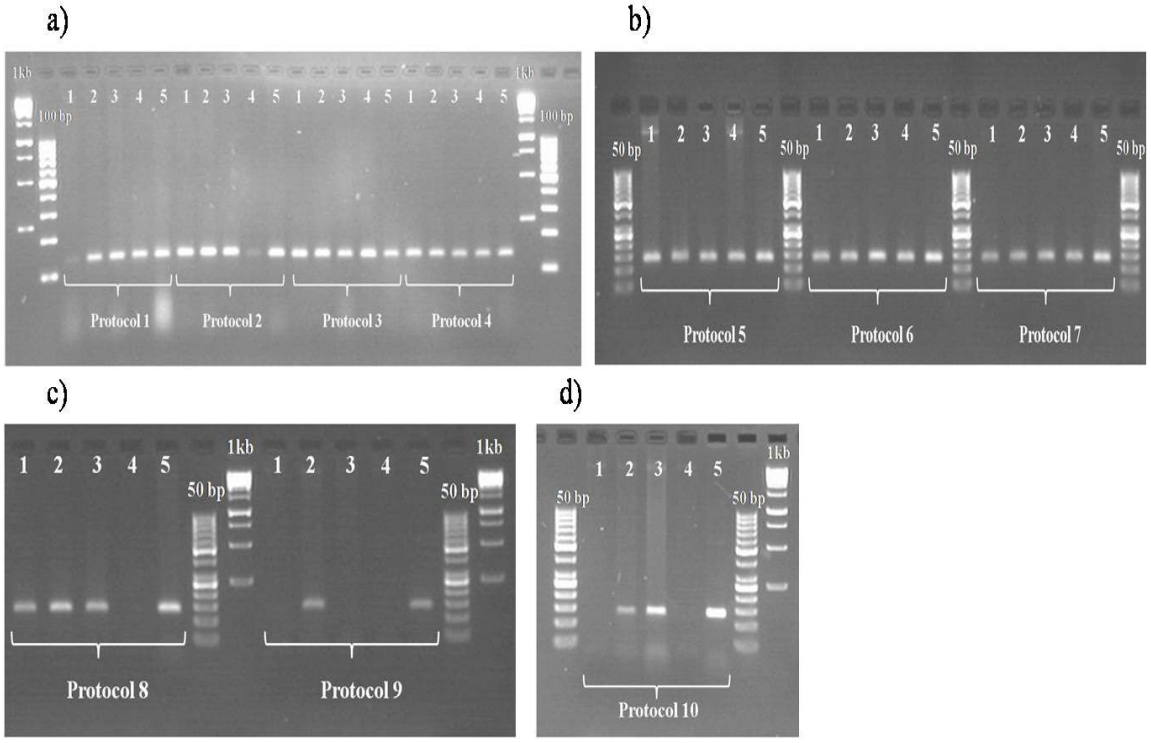

Figure 1. Amplification of conserved plant DNA region using primer 26S:
a) Protocols 1-4; b) Protocols 5-7; c) Protocols 8, 9;
d) Protocol 10; 1-5 - DNA samples as in table 1.

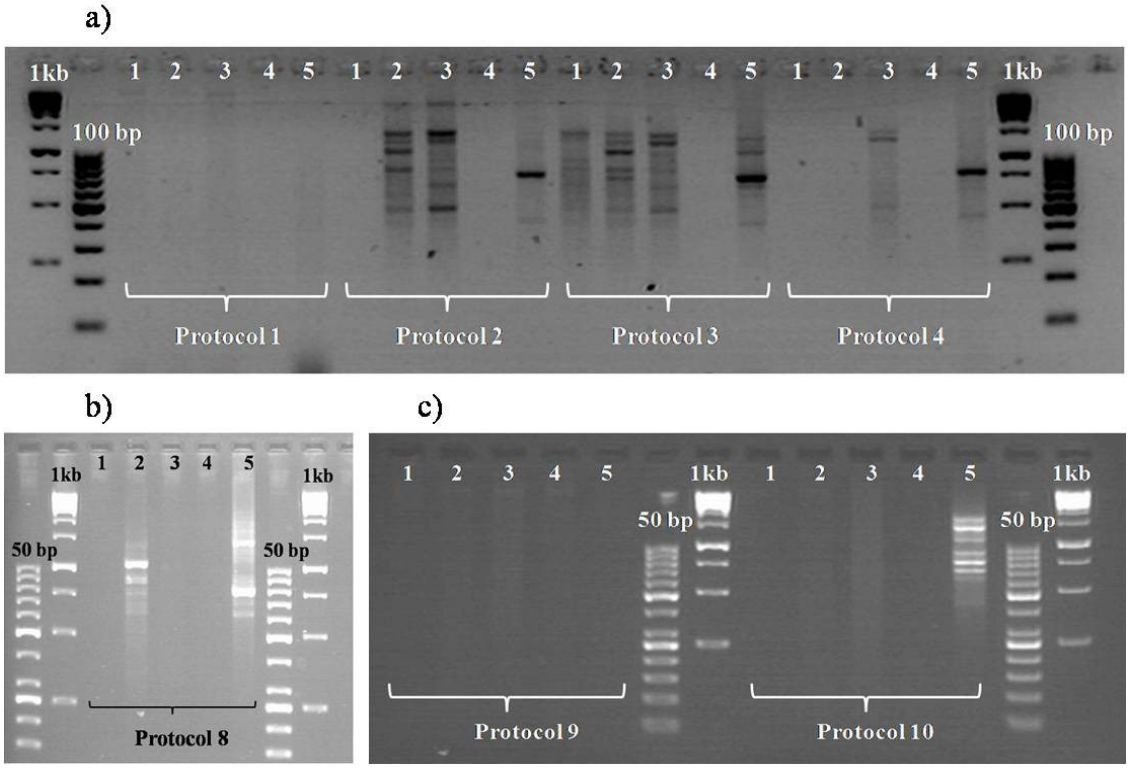

Figure 2. Amplification with primer UBC 368 of DNAs isolated with:
a) Protocols 1-4; b) Protocol 8; c) Protocols 9-10; 1-5 - DNA samples as in table 1.

The best amplification with UBC358 was obtained with DNA isolated by protocols 5, 6 and 7 (Figure 3). Although it has enabled isolation of DNA of good PCR quality, protocol 5 (Somma, 2006) is labour intensive, and the yield of DNA is low. In protocol 6, DNA was isolated with DNeasy® Plant Mini Kit (Qiagen), which has already been used for isolation of DNA from frozen (Delavault et al., 2002) and fresh tissue (Stoyanov et al., 2012), as well as from seeds (Dongo et al., 2012) of different *Orobanche* species As in work of Gagne et al. (1998), who used this method for DNA isolation from broomrape flower buds, in our work slightly modified protocol by Rogers and Bendich (1985) (protocol 7) was also found to be suitable for DNA isolation from dry broomrape stems, as sufficient quantity and quality of DNA for RAPD analysis was isolated from dry broomrape samples.

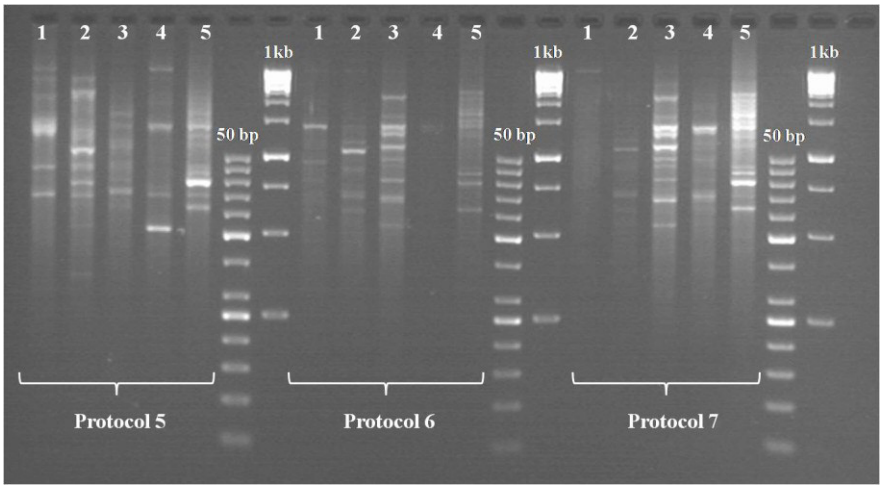

Figure 3. Amplification with primer UBC 368 of DNA isolated with protocols 5-7; 1-5 - DNA samples as in table 1.

CONCLUSIONS

In our work we found that dry mature broomrape stalks could be used for DNA isolation in broomrape diversity studies. DNA yield and quality depended on the way of sample storage, as well as on the method of isolation used. The best results regarding DNA yield and quality were obtained with dry samples stored at room temperature in ventilated space. DNA suitable for RAPD analysis was isolated with three of ten tested methods. As protocol of Somma (2006) is labour intensive and produced the lowest DNA yield, only DNA isolation with DNeasy® Plant Mini Kit, Qiagen and protocol by Rogers and Bendich (1985) could be recommended for future studies.

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REFERENCES

Atanasova, R., Batchvarova, R., Todorovska, E., Atanassov, A., 2005. *Molecular study of broomrape (Orobanche spp.) by RAPD analysis*. Biotechnol. & Biotechnol. Eq., 19(3): 51-60.

Benharrat, H., Veronesi, C., Theodeet, C., Thalouarn, P., 2002. *Orobanche species and population discrimination using intersimple sequence repeat (ISSR)*. Weed Res., 42: 470-475. DOI: 10.1046/j.1365-3180.2002.00305.x

Ciuca, M., Păcureanu, M., Iuoras, M., 2004. *RAPD markers for polymorphism identification in parasitic weed Orobanche cumana Wallr*. Rom. Agric. Res., 21: 29-32.

Dabo, S.M., Mitchell, E.D., Melcher, U., 1993. *A method for the isolation of nuclear DNA from cotton (Gossypium) leaves*. Anal. Biochem., 210(1): 34-38. DOI: 10.1006/abio.1993.1146

Delavault, P., Simier, P., Thoiron, S., Veronesi, C., Fer, A., Thalouarn, P., 2002. *Isolation of mannose 6-phosphate reductase cDNA, changes in enzyme activity and mannitol content in broomrape (Orobanche ramosa) parasitic on tomato roots*.

Physiol. Plantarum, 115: 48-55. DOI: 10.1034/j.1399-3054.2002.1150105.x.

Deshmukh, V.P., Thakare, P.V, Chaudhari, U.S., Gawande, P.A., 2007. *A simple method for isolation of genomic DNA from fresh and dry leaves of Terminalia arjuna (Roxb.) Wight and Arnot*. Electronic J. Biotechnol., 10(3): 468-472. DOI: 10.2225/vol10-issue3-fulltext-5.

Dongo, A., Leflon, M., Simier, P., Delavault, P., 2012. *Development of a high-throughput real-time quantitative PCR method to detect and quantify contaminating seeds of Phelipanche ramosa and Orobanche cumana in crop seed lots*. Weed Res., 52: 34-41. DOI: 10.1111/j.1365-3180.2011.00891.x.

Doyle, J.J., Doyle, J.L., 1987. *A rapid DNA isolation procedure from small quantities of fresh leaf tissue*. Phytochem. Bull., 19: 11-15.

Drabkova, L., Kirschner, J., Vlcek., C., 2002. *Comparison of seven DNA extraction and amplification protocols in historical herbarium specimens of Juncaceae*. Plant Mol. Biol. Rep., 20: 161-175. DOI: 10.1007/BF02799431.

Fulton, T.M., Chunwongse, J., Tanksley, S.D., 1995. *Microprep protocol for extraction of DNA from tomato and other herbaceous plants*. Plant Mol. Biol. Rep., 13: 207-209. DOI: 10.1007/BF02670897.

Gagne, G., Roeckel-Drevet, P., Grezes-Besset, B., Shindrova, P., Ivanov, P., Grand-Ravel, C., Vear, F., Tourvieille de Labrouhe, D., Charmet, G., Nicolas, P., 1998. *Study of the variability and evolution of Orobanche cumana populations infesting sunflower in different European countries*. Theor. Appl. Genet., 96: 1216-1222. DOI: 10.1007/s001220050859.

Gagne, G., Roeckel-Drevet, P., Grezes-Besset, B., Shindrova, P., Ivanov, P., Grand-Ravel, C., Vear, F., Charmet, G., Nicolas, P., 2000. *Amplified Fragment Length Polymorphism (AFLP) as Suitable Markers to Study Orobanche cumana Genetic Diversity*. J. Phytopathology, 148: 457-459. DOI: 10.1046/j.1439-0434.2000.00528.x.

Guchetl, S.Z., Tchelustnikova, T.A., Ramazanova, S.A., 2001. *Interpopulation variability of Orobanche cumana Wallr. affecting sunflower in south regions of Russia revealed by molecular-genetic markers*. Oil Crops Scientific and Technical Bulletin of VNIIMK, 1(146-147): 119-122.

Jobes, D.V., Hurley, D.L., Thien, L.B., 1995. *Plant DNA isolation: a method to efficiently remove polyphenolics, polysaccharides, and RNA*. Taxon, 44: 379-386.

Joel, D.M., Portnoy, V., Katzir, N., 1996. *Identification of single tiny seeds of Orobanche using RAPD analysis*. Plant Mol. Biol. Rep., 14: 243-248. DOI: 10.1007/BF02671659.

Jovanović, Z., Stanisavljević, N., Nikolić, A., Medović, A., Mikić, A., Radović, S., Đorđević, V., 2011. *Extraction of the ancient DNA from charred seeds from the site of Hissar in South Serbia*. Ratar. Povrt. / Field Veg. Crop Res., 48: 227-232.

Krizman, M., Jakse, J., Baricevic, D., Javornik, B., Prosek, M., 2006. *Robust CTAB-activated charcoal protocol for plant DNA extraction*. Acta Agriculturae Slovenica, 87(2): 427-433.

Lassner, M.W., Peterson, P., Yoder, J.I., 1989. *Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny*. Plant Mol. Biol. Rep., 7: 116-128. DOI: 10.1007/BF02669627.

Loomis, W.D., 1974. *Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles*. Methods Enzymol., 31: 528-544. DOI: 10.1016/0076-6879(74)31057-9.

Nagl, N., Taski-Ajdukovic, K., Barac, G., Baburski, A., Seccareccia, I., Milic, D., Katic, S., 2011. *Estimation of the genetic diversity in tetraploid alfalfa populations based on RAPD markers for breeding purposes*. Int. J. Mol. Sci., 12: 5449-5460. DOI: 10.3390/ijms12085449.

Paran, I., Gidoni, D., Jacobsohn, R., 1997. *Variation between and within broomrape (Orobanche) species revealed by RAPD markers*. Heredity, 78: 68-74. DOI: 10.1038/hdy.1997.8.

Permingeat, H.R., Romagnoli, M.V., Vallehos, R.H., 1998. *A simple method for isolating high yield and quality DNA from cotton (Gossypium hirsutum L.) leaves*. Plant Mol. Biol. Rep., 16: 89. DOI: 10.1023/A:1007466522028.

Ribeiro, R.A., Lovato, M.B., 2007. *Comparative analysis of different DNA extraction protocols in fresh and herbarium specimens of the genus Dalbergia*. GMR, 6(1): 173-187.

Rogers, S.O., Bendich, A.J., 1985. *Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues*. Plant Mol. Biol., 5: 69-76. DOI: 10.1007/BF00020088.

Roman, B., Alfaro, C., Torres, A.M., Moreno, M.T., Satovic, Z., Pujadas, A., Rubiales, D., 2003. *Genetic relationships among Orobanche species as revealed by RAPD analysis*. Ann. Bot., 91: 637-642. DOI: 10.1093/aob/mcg060.

Shepherd, L.D., McLay, T.G.B., 2011. *Two micro-scale protocols for the isolation of DNA from polysaccharide-rich plant tissue.* J. Plant Res., 124: 311-314. DOI: 10.1007/s10265-010-0379-5.

Somma, M., 2006. *Extraction and purification of DNA* (Session 4). In: Querci, M., Jermini, M., Van den Eede, G. (eds), The Analysis of Food Samples for the Presence of Genetically Modified Organisms. Luxembourg: Office for Official Publications of the European Communities, 15-16.

Stoyanov, K., Gevezova, M., Denev, I., 2012. *Identification of ISSR markers for studying the biodiversity of Bulgarian representatives of genus Orobanche subsection Minores*. Biotechnol. & Biotechnol. Eq., 26(1): 2743-2749. DOI: 10.5504/bbeq.2011.0139.

Taylor, J.W., Swann, E.C., 1994. *Dried samples: soft tissues, DNA from herbarium specimens*. In: Herrmann, B., Hummel, S. (eds), Ancient

DNA: Recovery and Analysis of Genetic Material from Paleontological, Archaeological, Museum, Medical, and Forensic Specimens. Springer Verlag: 166-181.

Torres, A.M., Weeden, N.F., Martin, A., 1993. *Linkage among isozyme, RFLP and RAPD markers in Vicia faba*. Theor. Appl. Genet., 85: 937-945. DOI: 10.1007/BF00215032.

Varma, A., Padh, P., Shrivastava, N., 2007. *Plant genomic DNA isolation: An art or a science*. Biotechnol. J., 2: 386-392. DOI: 10.1002/biot.200600195.