COMPARISON OF THE EFFICIENCY OF SOME OF THE MOST USUAL DNA EXTRACTION METHODS FOR WOODY PLANTS IN DIFFERENT TISSUES OF *VITIS VINIFERA* L.

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Abstract

Aim: To compare different methods for extracting DNA from non-recalcitrant and recalcitrant tissues of *Vitis vinifera* woody plants and propose a modification of a previously published method to reduce the time and cost of extraction.

Methods and results: DNA was extracted from young and mature leaves as well as from stems and seeds using some of the most common methods of DNA isolation and two commercial kits. Another commercial kit, which does not require DNA extraction prior to PCR, was also used. Only two methods provided adequate results in all tissues. Other methods were only applicable to some tissues and some did not yield any functional DNA in any tissue. A modification of the method reported by Marsal *et al*. (2011) is proposed to reduce handling time and cost.

Conclusion: All of the methods studied here use a surfactant to improve the extractions. For DNA extraction from recalcitrant tissues to be optimal, it is best to use a combination of dodecyltrimethylammonium bromide (DTAB) and cetyltrimethylammonium bromide (CTAB). The changes made to the protocol reported by Marsal *et al*. (2011) enable functional DNA to be obtained from leaves in only 90 minutes and at very low cost (17 €/8 samples). However, this method cannot adequately isolate DNA from recalcitrant tissues (stems and seeds) and so, for this type of sample, we would recommend using the original method.

Significance and impact of the study: Nowadays, handling time and cost are key factors in selecting the most suitable DNA extraction method. This study compares not only the effectiveness of the various methods but also the handling time and cost. It also proposes a modification of the fastest and most economic DNA extraction method for leaves so that handling time and processing cost will be reduced even further.

Key words: *Vitis vinifera*, DNA extraction, leaf, stem, seed

Résumé

Objectif : Évaluation comparative de différentes méthodes d'extraction d'ADN à partir de tissus non récalcitrants et récalcitrants de plants ligneux de l'espèce *Vitis vinifera* et optimisation de la méthode précédemment publiée afin de réduire le temps de traitement pour en réduire son coût.

Méthodes et résultats : Des extractions d'ADN ont été réalisées à partir de feuilles jeunes et adultes, de sarments et de pépins de raisins en utilisant les méthodes les plus courantes ainsi que deux kits commerciaux. Un autre kit commercial, qui ne nécessite pas l'extraction d'ADN avant la PCR, a également été utilisé. Seulement deux méthodes ont donné des résultats satisfaisants pour tous les tissus. D'autres méthodes étaient uniquement applicables à certains tissus et quelques-unes n'ont pas permis d'obtenir de l'ADN fonctionnel. Une modification de la méthode de Marsal *et al*. (2011) est aussi proposée pour en réduire le temps de manipulation et le coût.

Conclusion : Toutes les méthodes étudiées utilisent certains tensio-actifs pour améliorer les extractions. Pour une extraction optimale d'ADN à partir de tissus récalcitrants, il est préférable d'utiliser une combinaison de DTAB (bromure de dodecyltriméthylammonium) et de CTAB (bromure de cetyltriméthylammonium). L'optimisation du protocole de Marsal *et al*. (2011) permet d'obtenir l'ADN fonctionnel de feuilles en seulement 90 minutes à un coût très bas (17 €/8 échantillons). Cependant, cette méthode ne permet pas une extraction suffisante d'ADN à partir de tissus récalcitrants (sarments et pépins) de sorte que la méthode sans modifications est recommandée pour ce type d'échantillons.

Signification et impact de l'étude : Actuellement, le temps de traitement et le coût sont des facteurs clés dans le choix de la méthode d'extraction d'ADN. Cette étude fournit non seulement une comparaison de l'efficacité des différentes méthodes existantes mais aussi une comparaison des temps de traitement et des coûts. Ainsi, ce travail nous permet de proposer une méthode plus rapide et moins onéreuse pour l'extraction de l'ADN de feuilles de vigne.

Mots clés : *Vitis vinifera*, extraction d'ADN, feuille, sarment, pépin

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INTRODUCTION

Most of the methodologies for analyzing DNA in plant material start with an extraction and purification step, which is key to the success of all the following steps. Consequently, the efficacy of DNA extraction must be a criterion for selecting the most appropriate procedure.

To be more specific, an efficient extraction and purification protocol must 1) provide pure, intact and functional DNA, 2) give a good yield, 3) be fast and simple, 4) be economic and 5) be reliable (Reddy, 2009). Several methods for the extraction and purification of DNA from woody plants that are theoretically appropriate for *Vitis vinifera* have been described in the literature. Some commercial kits allow faster DNA extraction compared to conventional methods, but they have the drawback of being expensive (Bashalkhanov and Rajora, 2008; Demeke and Jenkins, 2010). Other recent methodologies (Manen *et al*., 2005) or kits, which do not require the grinding of samples, are also becoming available. These «non-grinding» methods may even automate the process (Bashalkhanov and Rajora, 2008; Ki *et al*., 2007) but are more expensive (Demeke and Jenkins, 2010). This wide range of options makes it difficult to choose the most appropriate protocol for a given application.

Moreover, most of these methods are usually used in tissues from which it is easy to extract DNA such as young leaves (Doyle and Doyle, 1987; Thomas *et al*., 1993; Lodhi *et al*., 1994; Steenkamp *et al*., 1994; Hanania *et al*., 2004; Marsal *et al*., 2011) and very few are appropriate for more complicated tissues such as stems (Aljanabi *et al*., 1999; Khanuja *et al*., 1999; Wolf *et al*., 2001; Barzegari *et al*., 2010; Marsal *et al*., 2011) or seeds (Kang *et al*., 1998; Sharma *et al*., 2002; Kamiya and Kiguchi, 2003; Manen *et al*., 2003; Marsal *et al*., 2011). For this reason, stems and seeds are usually regarded as recalcitrant tissues for DNA extraction and purification. While leaves and other non-recalcitrant tissues have the obvious advantage of being easy to handle in the lab, they also have two drawbacks: first, the obvious dependence on the biological cycle, which prevents any DNA extraction during periods of dormancy (winter) and, second, the fragility of the tissues, which requires careful refrigeration during transport. In contrast, recalcitrant tissues (stems or seeds) have the disadvantage of being more «resistant» to DNA extraction but the advantages of being available year-round and easier to transport (Manen *et al*., 2005; Ki *et al*., 2007; Bashalkhanov and Rajora, 2008). In recent years, various methods for extracting DNA from seeds (Kang *et al*., 1998; Sharma *et al*., 2002; Kamiya and Kiguchi, 2003) and woody samples (Aljanabi *et al*., 1999; Khanuja *et al*., 1999; Barzegari *et al*., 2010) have been proposed for different plant species. Nevertheless, to our knowledge, only two have been specifically described for seeds (Manen *et al*., 2003; Marsal *et al*., 2011) and stems (Wolf *et al*., 2001; Marsal *et al*., 2011) in *Vitis vinifera*.

Extraction of high-quality DNA from the various organs of woody plants, such as grapevine, is particularly complicated because of the high concentrations of polysaccharides, polyphenols and other secondary metabolites that hinder the purification process. Some authors have also shown that the use of different DNA extraction protocols may influence the results of subsequent analyses (Jones *et al*., 1997; Boiteux *et al*., 1999). Specifically, the method may involve the presence of problematic contaminants, which consequently, may affect the quality of the purified DNA. The presence of phenolic compounds and polysaccharides, even at relatively low concentrations, can seriously inhibit the enzymatic activities of restriction endonucleases, polymerases and ligases (Barzegari *et al*., 2010; Do and Adams, 1991; Fang *et al*., 1992; Kim and Hamada, 2005), which may limit the success of further analysis. In addition, some of the reagents commonly used in most of these protocols can also affect the effectiveness of subsequent steps. In particular, it has been reported that some detergents (cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulphate (SDS), sarkosyl, etc.), antibiotics, enzymes (proteinase K), polysaccharides, lipids, proteins (casein), and other organic (polyphenols) and inorganic (sodium acetate, sodium chloride) compounds can affect or even inhibit the PCR reaction (Demeke and Jenkins, 2010; Rossen *et al*., 1992; Wilson, 1997; Peist *et al*., 2001; Rådström *et al*., 2008; Roux *et al*., 2009).

It is therefore obvious that the suitability of the DNA extraction method and the optimization of the PCR process are key strategies for guaranteeing a reliable DNA template for molecular analysis such as gene expression (Demeke and Jenkins, 2010) and organism identification using Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995; Cervera *et al*., 1998; Cervera *et al*., 2000) and Simple Sequence Repeats (SSR) (Thomas and Scott, 1993; Thomas *et al*., 1993; Bowers *et al*., 1996; This *et al*., 2004).

In this context, the choice of the method should depend on the tissue selected and downstream applications. Moreover, the simplicity, speed and cost of the procedure must also be taken into account. For

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these reasons, the aim of this study was to compare some of the most usual DNA extraction methods in different tissues of *Vitis vinifera* in order to make it easier for researchers to make decisions. To this end, we compared nine laboratory protocols (Dellaporta *et al*., 1983; Doyle and Doyle, 1987; Bowers *et al*., 1993; Thomas *et al*., 1993; Lodhi *et al*., 1994; Steenkamp *et al*., 1994; Wolf *et al*., 2001; Manen *et al*., 2003; Marsal *et al*., 2011), two extraction kits (DNeasy Plant Mini Kit and MasterPure™ Plant Leaf DNA Purification Kit) and one «non-grinding» kit (Phire Plant Direct PCR Kit) in three organs (leaves, stems and seeds) from the woody plant *Vitis vinifera*. We also modified a previously reported method (Marsal *et al*., 2011) in order to improve its simplicity, speed and cost.

MATERIALS AND METHODS

1. Plant materials

Young and mature leaves, mature seeds and lignified stems were collected from Cabernet Sauvignon cultivar plants at the experimental vineyard of the Rovira i Virgili University in Constantí (A.O.C. Tarragona, Spain). Young leaves were collected in May and mature leaves in mid-July. The mature stems were collected at the end of August and the mature seeds on the day of harvest (21 September). The leaves were immediately washed and frozen in liquid nitrogen at the vineyard, whereas the seeds and stems were processed in the laboratory. The seeds were directly extracted from the grapes and the stems were obtained by cutting at about 25 cm from the shoot tip. The seeds were used directly while the stems were first bored to remove the crust. The seeds and bored stems were then washed with deionized water. All the samples were kept at -20°C until analysis.

2. Reagents and solutions

Reagents were of high purity and suitable for use in molecular biology (Sigma, St. Louis, MO). For each DNA extraction method, solutions were prepared as described in the literature. In the particular case of the proposed modified method, the following solutions were used. The lysis-extraction buffer (LE) consisted of 50 mM Tris-HCl pH 8.6, 100 mM boric acid, 0.75 M NaCl, 22.6 mM ethylenediaminetetraacetic acid (EDTA), 4% DTAB, and 2% (w/v) polyvinylpyrrolidone (PVP) (added just before use). The precipitation buffer (P) consisted of 1 M Tris-HCl pH 8.6, 200 mM boric acid, 1.5 M NaCl, 45.2 mM EDTA, and 1% CTAB. Both LE and P solutions were sterilized by autoclaving and stored at room temperature. The TE buffer consisted of 10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.5. This solution was sterilized by autoclaving and stored at 4°C.

3. DNA extraction

a. DNA extraction methods

DNA was extracted in accordance with each of the following published protocols: Dellaporta *et al*. (1983), Doyle and Doyle (1987), Bowers *et al*. (1993), Thomas *et al*. (1993), Lodhi *et al*. (1994), Steenkamp *et al*. (1994), Wolf *et al*. (2001), Manen *et al*. (2003), and Marsal *et al*. (2011). The following kits were used: DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), MasterPure™ Plant Leaf DNA Purification Kit (Epicentre Biotechnologies, Madison, USA), and Phire Plant Direct PCR Kit (Finnzymes, Espoo, Finland).

b. Optimized method

In an attempt to develop a faster method for extracting DNA, the original procedure of Marsal *et al*. (2011) was modified. Specifically, all the solutions were prepared in accordance with the original procedure, with the only exception of the LE buffer, which contained 2% (w/v) PVP. This compound was added just before use to improve the removal of polyphenols (Lodhi *et al*., 1994). The following specific changes were also made to the original method (Figure 1, modifications in bold type). After the cells had been disrupted, the sample was incubated for 10 min (instead of 20 min). The initial «mixing by inversion» step lasted for 1 min (instead of 10 min). Then, the sample was centrifuged for 5 min (instead of 25 min). Incubation with isopropanol took place at -20ºC for 15 min (instead of 30 min). Finally, the last centrifugation step lasted 5 min instead of 10 min.

A complementary study was carried out to determine the effect of conservation time after freezing (immediately after freezing, 1 day and more 30 days) on the extracted DNA yield. This study was performed in young and mature leaves using the original method developed by Marsal *et al.* (2011) and the proposed modified method.

4. Estimation of DNA purity and integrity

The purity of the isolated DNA samples was determined by spectrophotometric analysis (A260/A280) and the yield of the extraction process was measured using the A260 in accordance with a previously reported method (Surzycki, 2000). One µg of DNA sample was used to determine DNA integrity by means of agarose (Gibco BRL, Life-Technologies, Invitrogen, Carlsbad, CA) (2%) gel electrophoresis

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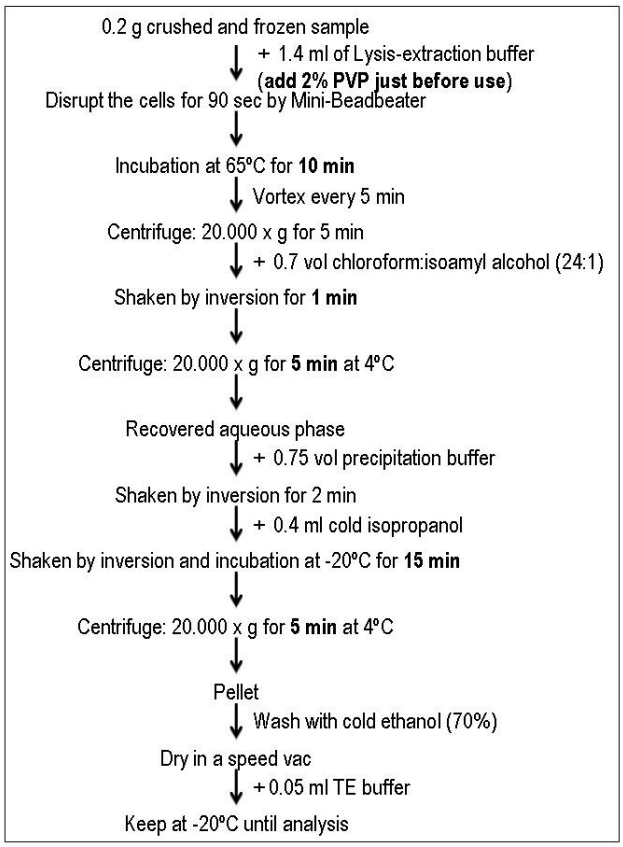


Figure 1 - Flux diagram of the proposed modified method. The modifications from Marsal *et al.* (2011) are shown in bold type.

after staining with ethidium bromide (Sigma, St Louis, MO). The band was observed under UV light.

5. Microsatellite technique: PCR and allele length measurement

The grapevine gene pool was genotyped at the following six SSR loci: VVS2 (Thomas and Scott, 1993); VVMD5 and VVMD7 (Bowers *et al*., 1996); VVMD27 (Bowers *et al*., 1999); and VrZAG62 and VrZAG79 (Sefc *et al*., 1999). The microsatellite amplifications were made by PCR analysis using a MyCycler thermocycler (Bio-Rad Laboratories, Hercules, CA), with the only exception of the Phire Plant Direct PCR kit, for which we used the thermocycler advised by the manufacturer (Piko Thermal Cycler, Finnzymes). PCR was performed with 50 ng of DNA and 1 µM of each primer labelled with a fluorescent dye in the upper primer (6-FAM, HEX, or NED) using the AmpliTaq DNA Polymerase kit (Applied Biosystems, Foster City, CA) for all DNA extraction protocols. except the three commercial kits, for which we used the supplied DNA Polymerase. The SSR were divided into three groups according to Marsal *et al*. (2011). Amplification products were mixed with 20 µL of deionized formamide and 0.5 µL of DNA size standard (GeneScan 500-ROX, Applied Biosystems, Foster City, CA), and denatured at 95ºC for 5 min. The amplified fragments were separated by capillary electrophoresis and measured with an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA).

6. Statistical study

All purity indices and yield values are expressed as the arithmetic average ± standard deviation of five replicates. Statistical comparisons between values were established with one-factor ANOVA and Scheffe's test using SPSS software ($p < 0.05$).

RESULTS AND DISCUSSION

1. General characteristics of the methods studied

Several protocols have been drawn up in an attempt to improve DNA extraction from *Vitis* species and/or other woody plants (Dellaporta *et al*., 1983; Lodhi *et al*., 1994; Aljanabi *et al*., 1999; Hanania *et al*., 2004).

All these protocols use detergents such as SDS, CTAB, DTAB and sarkosyl in the extraction buffer (Zain *et al*., 2008; Marsal *et al*., 2011).

Anionic detergents (SDS and sarkosyl) are used to selectively solubilize membrane proteins, whereas cationic detergents (CTAB and DTAB) are used to enhance the solubility of membrane- and lipid-associated proteins and/or proteins with hydrophobic domains. All these detergents disrupt the cell membrane, thereby releasing the cell contents (Surzycki, 2000; Palomera-Avalos *et al*., 2008). However, the use of the cationic detergents DTAB and CTAB improves solubilization efficiency in plant tissues (Manen *et al*., 2003; Marsal *et al*., 2011).

It has been reported that sarkosyl and CTAB give better results than SDS in the particular case of recalcitrant tissues (Zain *et al*., 2008). Nevertheless, the combination of DTAB and CTAB is probably the best option (Armaleo and Clerc, 1995; Manen *et al*., 2003; Marsal *et al*., 2011).

Here DNA was extracted using ten of the most common procedures for DNA isolation (including the modified procedure) and two commercial kits. Another commercial kit that does not require DNA isolation prior to PCR was also used. Table 1 summarizes the main characteristics of all these methods.

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Table 1 - Main characteristics of the methods studied

Authors/Company	Starting weight sample (mg)	Extraction System	Use of RNAse	Volume Tubes
Dellaporta *et al.* (1983)	750	SDS lysis	no	high
Doyle and Doyle (1987)	750	CTAB lysis	yes	high
Bowers *et al*. (1993)	3000	CTAB lysis	yes	high
Thomas *et al. (*1993)	2000	Sarkosyl lysis	yes	high
Lodhi *et al.* (1994)	500	CTAB lysis	yes	high
Steenkamp *et al.* (1994)	1500	CTAB lysis	yes	high
Wolf *et al.* (2001)	500	CTAB-Urea lysis	no	low
Manen *et al*. (2003)	200	DTAB lysis	no	low
Marsal *et al.* (2011)	200	DTAB lysis	no	low
Modified method of Marsal *et al.* (2011)	200	DTAB lysis	no	low
DNeasy Plant Mini Kit	100	Silica membrane binding	yes	low
MasterPure Plant Kit	35	*	no	low
Phire Plant. Direct PCR Kit	#	#	no	low

(*) Data not provided by the company. (#) Not required.

The methods reported by Dellaporta *et al*. (1983), Doyle and Doyle (1987), Bowers *et al*. (1993), Thomas *et al*. (1993), Lodhi *et al*. (1994), Steenkamp *et al*. (1994) and Wolf *et al*. (2001) have the drawback of needing high amounts of starting sample (≥ 500 mg). In contrast, the methods described by Manen *et al*. (2003), Marsal *et al*. (2011), and the two standard commercial kits (DNeasy Plant mini Kit and MasterPure Plant Kit) have the advantage of requiring low amounts of starting sample (≤ 200 mg). Moreover, the methods described by Dellaporta *et al*. (1983), Doyle and Doyle (1987), Bowers *et al*. (1993), Thomas *et al*. (1993), Lodhi *et al*. (1994) and Steenkamp *et al*. (1994) also need high volumes of all solutions. In contrast, the methods reported by Wolf *et al*. (2001), Manen *et al*. (2003), Marsal *et al*. (2011), and both standard commercial kits only require small volumes of all solutions. Consequently, they have the advantage that work can be done in Eppendorf tubes.

Some of these methods also have the disadvantage of needing an incubation process in the presence of RNAse (Doyle and Doyle, 1987; Bowers *et al*., 1993; Thomas *et al*., 1993; Lodhi *et al*., 1994; Steenkamp *et al*., 1994; DNeasy Plant Mini Kit), whereas the other methods do not.

All the methods tested also have different extraction buffer composition, especially in the surfactants used. To be more specific, one method uses SDS (Dellaporta *et al*., 1983), one method uses sarkosyl (Thomas *et al*., 1993), four methods use CTAB (Doyle and Doyle, 1987; Bowers *et al*., 1993; Lodhi *et al*., 1994; Steenkamp *et al*., 1994), one method uses a combination of CTAB and urea (Wolf *et al*., 2001) and two methods use DTAB (Manen *et al*., 2003; Marsal *et al*., 2011). In the particular case of the standard commercial kits, the MasterPure Plant Kit provides no information about the composition of the extraction buffer and the DNeasy Plant Mini Kit uses a silica membrane binding system for DNA extraction. Neither of them provides any information about any of the solvents used. Finally, the Phire Plant Direct PCR Kit does not need DNA extraction.

Nowadays, the cost of a lab process is one of the main criteria for choosing the most suitable analysis method. It is obvious, then, that the time and cost of processing are key factors. From this point of view, working with high amounts of starting sample is more expensive because higher quantities of reagents are required. Moreover, higher sampling volumes require larger tubes, which also increases the final cost of the process. In contrast, the possibility of working with Eppendorf tubes notably reduces the cost. Moreover, using RNAse is also more expensive because of its high price and the longer processing time.

2. Yield and quality of extracted DNA

It is difficult to extract nucleic acids (DNA and RNA) from *Vitis vinifera* and other woody plants because of the presence of high concentrations of compounds such as polyphenols, polysaccharides and proteins (Lodhi *et al*., 1994; Hanania *et al*., 2004; Tattersall *et al*., 2005; Fort *et al*., 2008). DNA extraction from mature leaves is considered to be more complicated than from young leaves because of the higher presence of these compounds (Marsal *et al*., 2011). Other tissues such as seeds and stems also contain

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Table 2 - Comparison of results obtained by the different methods studied

Method	Organ	$A_{260/280}$	Yield (µg/g tissue)	SSR					
				VVS2	VVMD5	VVMD7	VVMD27	VrZAG62	VrZAG79
Dellaporta *et al.* (1983)	young leaf	0.7±0.1	97±10	-	-	-	-	-	-
	mature leaf	0.9±0.1	52±09	-	-	-	-	-	-
	wood	0.5±0.05	25±04	-	-	-	-	-	-
	seed	-	-	-	-	-	-	-	-
Doyle and Doyle (1987)	young leaf	1.3±0.1	264±18	-	-	-	-	-	-
	mature leaf	1.2±0.1	198±19	-	-	-	-	-	-
	wood	0.9±0.05	53±06	-	-	-	-	-	-
	seed	-	-	-	-	-	-	-	-
Bowers *et al.* (1993)	young leaf	1.8±0.1	43±05	+	+	+	+	+	+
	mature leaf	1.4±0.1	7±01	-	-	-	-	-	-
	wood	-	-	-	-	-	-	-	-
	seed	-	-	-	-	-	-	-	-
Thomas *et al.* (1993)	young leaf	1.8±0.1	129±09	+	+	+	+	+	+
	mature leaf	1.6±0.1	39±05	+	-	-	-	+	+
	wood	-	-	-	-	-	-	-	-
	seed	-	-	-	-	-	-	-	-
Lodhi *et al.* (1994)	young leaf	1.9±0.1	526±21	+	+	+	+	+	+
	mature leaf	1.7±0.1	349±27	+	+	+	+	+	+
	wood	1.3±0.05	131±16	-	-	-	-	-	-
	seed	0,8±0.05	39±15	-	-	-	-	-	-
Steenkamp *et al.* (1994)	young leaf	1.8±0.1	172±15	+	+	+	+	+	+
	mature leaf	1.4±0.1	27±03	-	-	-	-	-	-
	wood	-	-	-	-	-	-	-	-
	seed	-	-	-	-	-	-	-	-
Wolf *et al.* (2001)	young leaf	2.0±0.05	488±39	+	+	+	+	+	+
	mature leaf	1.9±0.1	421±41	+	+	+	+	+	+
	wood	1.9±0.1	220±12	+	+	+	+	+	+
	seed	1,1±0.1	48±03	-	-	-	-	-	-
Manen *et al*. (2003)	young leaf	1.8±0.1	411±31	+	+	+	+	+	+
	mature leaf	1.8±0.1	332±39	+	+	+	+	+	+
	wood	1.9±0.1	297±19	+	+	+	+	+	+
	seed	1.9±0.1	81±10	+	+	+	+	+	+
Marsal *et al.* (2011)	young leaf	2.0±0.05	547±39	+	+	+	+	+	+
	mature leaf	1.9±0.1	419±41	+	+	+	+	+	+
	wood	1.8±0.1	403±24	+	+	+	+	+	+
	seed	1.8±0.1	108±24	+	+	+	+	+	+
Modified method of Marsal *et al*. (2011)	young leaf	2.0±0.1	560 ± 32	+	+	+	+	+	+
	mature leaf	1.9±0.2	330± 9	+	+	+	+	+	+
	wood	1.3±0.1	160±24	-	-	-	-	-	-
	seed	1.1±0.1	60±11	-	-	-	-	-	-
DNeasy Plant Mini Kit	young leaf	1.9±0.1	405±51	+	+	+	+	+	+
	mature leaf	2.0±0.05	351±29	+	+	+	+	+	+
	wood	1.1±0.05	123±17	-	-	-	-	-	-
	seed	-	-	-	-	-	-	-	-
MasterPure Plant Kit	young leaf	1.5±0.1	25±03	-	-	-	-	-	-
	mature leaf	1.2±0.1	13±01	-	-	-	-	-	-
	wood	-	-	-	-	-	-	-	-
	seed	-	-	-	-	-	-	-	-
Phire Plant Direct PCR Kit	young leaf	#	#	-	-	-	-	-	-
	mature leaf	#	#	-	-	-	-	-	-
	wood	#	#	-	-	-	-	-	-
	seed	#	#	-	-	-	-	-	-

(+) positive answer; (-) negative answer; (#) not done.

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high amounts of these compounds. In addition, seeds and stems are difficult to grind, which complicates the extraction process even more. These are some of the reasons why seeds and stems are often perceived as recalcitrant tissues (Manen *et al*., 2003; Marsal *et al*., 2011). Table 2, which shows the DNA purity indices and yields, confirms this fact.

DNA yield from young leaves was higher than from mature leaves, seeds and stems in most of the methods compared. The methods using DTAB in the extraction buffer (Manen *et al*., 2003; Marsal *et al*., 2011) produced the best yields with adequate purity indices (A260/280 = 1.8-2.0) in all the tissues studied. The method reported by Wolf *et al*. (2001) also gave good results in both young and mature leaves, and in stems but not in seeds. The methods described by Lodhi *et al*. (1994) and the proposed modified method of Marsal *et al*. (2011) also provided good results in young and mature leaves but not in recalcitrant tissues (seeds and stems). Other methods (Bowers *et al*., 1993; Thomas *et al*., 1993; Steenkamp *et al*., 1994) only provided adequate results in young leaves. The remaining protocols all gave inadequate purity indices, although some gave adequate yield.

The DNeasy Plant Mini Kit gave acceptable results in young and mature leaves but not in recalcitrant tissues. In contrast, the MasterPure Plant Kit gave very low yields and purity indices in all the tissues.

Table 2 also shows the results obtained by microsatellite analysis. These results are very clear and confirm that the results of the microsatellite analysis were positive only when the DNA purity indices were acceptable. However, when the DNA quality was inadequate, the amplifications were negative. Therefore, DNA extraction needs to be efficient if these techniques are to be used in cultivar identification because the presence of contaminants probably interferes with DNA isolation and/or PCR analysis (Lodhi *et al*., 1994; Steenkamp *et al*., 1994; Marsal *et al*., 2011). In all, these results show that only the combination of DTAB and CTAB provides good DNA yields and satisfactory results in subsequent microsatellite analysis in all tested tissues, with the only exception of the proposed modified method of Marsal *et al*. (2011). This method is only applicable to leaves but has the advantage of considerably reducing the processing time.

3. Economic comparison of the different protocols

As has been mentioned above, nowadays economic aspects are fundamental in the choice of a procedure. Table 3 compares the performance (processing time and number of samples per day) and the estimated cost of each of the procedures studied. Cost was estimated based on the following factors: the cost of the reagents and the cost of the processing time (8 euros/work hour according to Tattersall *et al*. (2005)). For purposes of simplification, these data were considered only for DNA extraction in young leaves. Naturally, the methods that did not yield any functional DNA were not considered.

Table 3 - Main economic traits: Final cost for eight samples, cost per mg DNA extracted and estimation of number of samples processed per day

Protocols or commercial kits	Cost of reagents (€)		Cost handling time per 8 samples (€)		Final cost per 8 samples	Final cost per mg (€) extracted per 8 samples (+)	Estimated DNA samples per day (º)
	A sample	8 samples	Estimated extraction time (h)	Cost hands-on-time (^)	Reagents and handling time	Cost per mg DNA	
Bowers *et al*. (1993)	20.38	163.02	8.00	64.00	227.02	659.94	8
Thomas *et al. (*1993)	3.17	25.37	3.00	24.00	49.37	47.84	36
Lodhi *et al.* (1994)	3.95	31.63	3.80	30.40	62.03	14..74	24
Steenkamp *et al.* (1994)	4.00	32.02	3.30	26.40	58.42	42.46	24
Wolf *et al.* (2001)	0.50	4.01	3.00	24.00	28.01	7.17	36
Manen *et al*. (2003)	0.29	2.36	3.50	28.00	30.36	9.23	24
Marsal *et al.* (2011)	0.63	5.08	2.50	20.00	25.08	5.73	48
Modified method of Marsal *et al.* (2011)	0.63	5.08	1.50	12.00	17.08	3.81	96
DNeasy Plant Mini Kit	4.10	32.80	2.50	20.00	52.80	16.30	48

(^) 8€/h (Tatersall et al. (2005)). (+) Samples: young leaves. (º) Calculated on the basis of the column of estimated extraction time

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Table 4 - Influence of conservation time after freezing on DNA yield

Method	Age of the leaf	DNA yield (ng/g)		
		Freezing time (days)		
		Immediately processed	1	> 30
Marsal *et al*. (2011)	Young	547.1 ± 39 A, a	248.7 ± 31.2 B, a	220.9 ± 15.4 B, a
	Mature	419.6 ± 41.3 A, b	191.1 ± 2.49 B, b	126.6 ± 9.6 C, b
Modified method of Marsal *et al.* (2011)	Young	560.9 ± 32.6 A, a	227.5 ± 8.9 B, a	219.4 ± 15.6 B, a
	Mature	330.3 ± 9.2 A, b	110.0 ± 11.3 B, b	93.0 ± 9.4 B, b

Statistical analysis: One-factor ANOVA and Scheffe's test (both p = 0.05). Different letters indicate statistical differences. Lower-case letters are used to compare the DNA yield from leaves of different ages, whereas capital letters are used to compare yields with respect to freezing time.

One of the most important aspects in this economic comparison was the cost of the reagents. It varied widely among methods, ranging between 0.29 €/sample for the cheapest method (Manen *et al*., 2003) and 20.38 €/sample for the most expensive one (Bowers *et al*., 1993). In total, four of these methods can be regarded as relatively economic (Manen *et al*., 2003; Wolf *et al*., 2001; Marsal *et al*., 2011 and its modification) with a cost between 0.29 and 0.63 €/sample. The other methods (Thomas *et al*., 1993; Lodhi *et al*., 1994; Steenkamp *et al*., 1994) are considerably more expensive with costs ranging between 3.17 and 4.00 €/sample. These wide differences are affected by many factors, among which the handling volume and the use of RNase are probably the most important. The higher the handling volume, the more reagents are required. Costs are particularly high for those methods that use expensive reagents such as RNase and high volumes.

Another aspect to be borne in mind is the handling time for each method, which also varied considerably: from 1 hour and 30 minutes for the fastest method (modified method of Marsal *et al*., 2011) to 8 hours for the slowest (Bowers *et al*., 1993), with the other methods ranging between 2 hours and 30 minutes and almost 4 hours. These handling times determine the number of samples that can be processed by one technician in a day, which is also a criterion that has to be assessed. In batteries of eight samples, the method that performed best was the modified method of Marsal *et al*. (2011), which can be used to process 96 samples a day. In contrast, the method that performed worst was the one reported by Bowers *et al*. (1993), which only processes eight samples a day.

Taking both the cost of the reagents and the cost of the handling time into account, the final cost of the different protocols varies considerably. The cheapest is the modified method of Marsal *et al*. (2011), which has an overall cost of 17€ for eight samples, and the most expensive is the protocol reported by Bowers *et al*. (1993), with a cost of 227€ for eight samples. Once again, some of the methods are economic (Marsal *et al*., 2011 and its modification; Wolf *et al*., 2001; Manen *et al*., 2003) with costs below 31€ for eight samples. On the other hand, the other methods (Thomas *et al*., 1993; Steenkamp *et al*., 1994; Lodhi *et al*., 1994; Bowers *et al*., 1993) are much more expensive.

Commercial kits are usually faster than standard protocols. In theory, this is the advantage that compensates for their higher price. As has been mentioned above, only one of the two commercial kits successfully extracted DNA from young leaves (DNeasy Plant Mini Kit). The estimated extraction time of this commercial kit is around 2 hours and 30 minutes, which means that 48 samples can be processed per day. This extraction time is shorter than for the other methods with the exception of the method reported by Marsal *et al*. (2011) and its proposed modification. The estimated cost of this commercial kit is 52.80€ for 8 samples. Although this cost is reasonable, it is more expensive than the methods regarded as being economic (Marsal *et al*., 2011; Wolf *et al*., 2001; Manen *et al*., 2003) and nearly three times more expensive than the most economic one (modified method of Marsal *et al*., 2011).

Taking all these data into account, the proposed modification of the protocol reported by Marsal *et al*. (2011) provides the best performances as far as cost and processing time are concerned. However, this method only works well for leaves and therefore should not be considered for other tissues.

4. Influence of the time of conservation after freezing on DNA yield

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Table 4 shows the results obtained by applying Marsal *et al*.'s method (2011) and its proposed modification to three different samples of young and mature leaves: leaves that were processed immediately, and leaves that had been frozen for 1 day or more than 30 days at -20ºC. In general terms, the results indicate that freezing led to significant decreases in DNA yield after only 24 hours of storage at -20ºC. These decreases seemed to be greater for mature leaves. However, in all cases, the DNA extraction yield was high enough to be used for further DNA analysis. Moreover, no significant differences were found between samples processed 24 hours and more than 30 days after sampling. This data confirms that it is possible to sample leaves some days before DNA extraction as long as the samples are frozen and stored appropriately.

CONCLUSIONS

The comparison of several protocols for extracting DNA from different *Vitis vinifera* tissues indicates that very few of them provide acceptable results in young and mature leaves, stems and seeds (Manen *et al*., 2003; Marsal *et al*., 2011). One method (Wolf *et al*., 2001) can be used in young and mature leaves and stems but not in seeds. The others can be used in young and mature leaves (Lodhi *et al*., 1994; DNeasy Plant Mini Kit) or in young leaves only (Bowers *et al*., 1993; Thomas *et al*., 1993; Steenkamp *et al*., 1994). The other methods studied do not provide acceptable results in any *Vitis vinifera* tissue.

Some of the methods for leaf DNA extraction are very time-consuming and/or expensive (Thomas *et al*., 1993; Steenkamp *et al*., 1994; Lodhi *et al*., 1994; Bowers *et al*., 1993). The other protocols can be regarded as adequate.

This article proposes some modifications to the method reported by Marsal *et al*. (2011). This modified method, which is very fast and economic, allows the processing of about 96 samples per day at a really low cost, however, it is only applicable to leaves.

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