Assessment of DNA extraction methods for detection of arbuscular mycorrhizal fungi in plant roots by nested-PCR

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ABSTRACT. DNA extraction methods were evaluated for the yield and purity of DNA recovered from mycorrhized roots and whether the recovered DNA is suitable for amplification of arbuscular mycorrhizal (AM) fungal SSU rDNA. The DNeasy Plant Mini Kit and three extraction buffers were used alone or in combination with either polyvinylpyrrolidone (PVP), polyvinylpolypyrrolidone (PVPP) and/or activated charcoal (AC). Among the extraction methods tested, those based on the CTAB buffers yielded more DNA than those based on the TE buffer and the DNeasy Plant Mini Kit. Moreover, the use of AC alone or in combination with PVPP reduced DNA yield, while it significantly improved the purity of recovered DNA, whatever the extraction buffer. On the other hand, the success of nested-PCR amplification was negatively correlated with the amount of template DNA and positively correlated with the purity of recovered DNA. Three methods based on the TE buffer, two on the CTAB-βM buffer and one on the DNeasy Plant Mini Kit produced high-quality DNA in terms of purity and PCR performance. However, the TE buffer-based methods are less time consuming than the CTAB-βM buffer-based methods, and cheaper than the method based on the DNeasy Plant Mini Kit.

Keywords: activated charcoal, TE buffer, DNA quality.

Avaliação de métodos de extração de ADN para detecção de fungos micorrízicos arbusculares em raízes de plantas por nested-PCR

RESUMO. Diferentes métodos de extração de ADN, a partir de amostras de raízes, foram testados e o ADN obtido foi avaliado para a amplificação de genes ribossomais de fungos micorrízicos arbusculares (AM). Três tampões de extração foram utilizados isoladamente ou em combinação com polivinilpirrolidona (PVP), polivinipolipirrolidona (PVPP) e/ou carvão ativado (CA), além do DNeasy Plant Mini Kit. Entre os métodos de extração testados, aqueles com base no tampão CTAB renderam mais ADN do que os baseados no tampão TE e o DNeasy Plant Mini Kit. A utilização de CA ou PVPP nos diferentes tampões reduziram o rendimento de ADN, contudo, melhoraram significativamente a pureza do ADN recuperado, independentemente do tampão de extração. Por outro lado, o sucesso da amplificação por Nested-PCR foi negativamente correlacionado com a quantidade de DNA molde, e positivamente correlacionada com a pureza do ADN. Três métodos baseados no tampão TE, dois no tampão CTAB-βM e o DNeasy Plant Mini Kit produziram ADN de alta qualidade, em termos de pureza e rendimento da PCR. No entanto, os métodos baseados em tampão TE demandam menos tempo do que os métodos baseados em tampão CTAB-βM e são mais baratos do que o uso do DNeasy Plant Mini Kit.

Palavras-chave: carvão ativado, tampão TE, qualidade do DNA.

Introduction

Arbuscular mycorrhizal (AM) fungi belonging to the Glomeromycota (SCHÜSSLER et al., 2001) form obligate symbiosis with the majority of plants, resulting in the establishment of mixed organs called endomycorrhizae, which include fungal arbuscules, hyphae and vesicles (in 80% of AM fungi) within root cortex cells. The endomycorrhizae provide mineral nutrients and water to the plant which, as a reward, provides carbohydrates to its fungal partner (SMITH; READ, 2008). Thus, AM fungi are considered key to plant diversity and productivity (VAN DER HEIJDEN et al., 1998).

Staining and microscopy procedures have been used to monitor AM fungi in plant roots, according to morphological characters of their intraradical mycelia (SANDERS, 2004), however many of those characters are unstable during root colonization (JEFFRIES et al., 2003). To overcome the disadvantages of these procedures, molecular

techniques based on polymerase chain reaction (PCR) amplification have been successfully used to detected AM fungi in plant roots (REDECKER, 2002; VAN TUINEN et al., 1998). However, the sensitivity and efficiency of these molecular techniques depend on the quality of the recovered DNA.

Indeed, AM fungal DNA is often extracted with potential inhibitors of PCR, such as polysaccharides, polyphenols, proteins and other plant and fungal secondary metabolites (WILSON, 1997). Therefore, the use of an extraction method able to provide good-quality DNA is required for accurate monitoring of AM fungi in roots. In this respect, several commercial extraction kits such as DNeasy Plant Mini Kit (QIAGEN) have been successfully used (ALGUACIL et al., 2009; DIÉDHIOU et al., 2010; WOLFE et al., 2007), but they are often expensive for large scale studies, particularly in developing countries.

Alternatively, numerous extraction methods combining detergents, reduction agents, and other additives have been used to recover from mycorrhized roots DNA suitable for PCR amplification. For instance, cetyl trimethylammonium bromide (CTAB) buffers including a reduction agent such as β-mercaptoethanol have been tested alone or in combination with either polyvinylpyrrolidone (PVP), or polyvinylpolypyrrolidone (PVPP) (STADDON et al., 2004; TURRINI et al., 2008). A Tris-HCl–EDTA (TE) buffer and activated charcoal (AC) have been also used to recover from mycorrhized roots DNA suitable for specific amplification of AM fungal DNA (FARMER et al., 2007).

The purpose of this study is to evaluate different DNA extraction methods based on CTAB and TE buffers and the DNeasy Plant Mini Kit for the yield and purity of DNA recovered from mycorrhized roots, and whether the recovered DNA is suitable for amplification of AM fungal SSU rDNA.

Material and methods

Root sampling and measurement of AM colonization

Roots of four month-old seedlings of *Mucuna pruriens,* a leguminous species which forms nitrogen-fixing and AM symbioses, were collected from the Embrapa experimental field of food crops (Fazendinha) in Seropédica, Rio de Janeiro State, Brazil. Root samples were kept in cool conditions (4°C) until arrival at the laboratory. They were then gently separated from surrounding soil and washed in a sieve (0.5 mm diameter) with tap water. Before DNA extraction, a subsample of lateral roots of 6 individual plants was stained according to Ishii and Loynachan (2004) to determine AM colonisation by the gridline intersection method. All stained root fragments were colonised, and the mean AM colonization rate was 23%.

Extraction buffers (CTAB and TE) and amendments

Two CTAB buffers and one TE buffer were prepared as follows:

The CTAB-β-mercaptoethanol (CTAB-βM) buffer was composed of 2% CTAB, 1.4 M NaCl, 20 mm EDTA (pH 8), 100 mm Tris-HCl (pH 8) and 1% β-mercaptoethanol;

The CTAB-dithiothreitol (CTAB-DTT) buffer was composed of 0.2% CTAB, 0.1 M NaCl, 50 mm EDTA, 0.2 M sodium phosphate (pH 8) and 1 mm DTT;

The TE buffer was composed of 10 mm Tris-HCl (pH 8) and 1 mm EDTA (pH 8).

Each extraction buffer was divided into three aliquots, and then the first aliquot was amended with 2% PVP, the second with 2% PVPP, while the third was not amended.

DNA extraction

Lateral mycorrhized roots were placed in a mortar and ground using a pestle in the presence of liquid nitrogen. To avoid the potential bias related to heterogeneity in mycorrhizal colonization, the ground root material (GRM) was well homogenized and aliquots of 50 mg were transferred to 1.5 mL microtubes. Before the addition of extraction buffers, the microtubes were randomly separated into two sets. For the first set, each aliquot of 50 mg GRM was amended with 10 mg of AC powder. The GRM and AC were then well mixed. For the second set, the aliquots did not receive AC.

DNA extraction with the CTAB-βM and CTAB-DTT buffers

For each extraction buffer, a volume of 500 μL of a buffer aliquot (amended or not with either 2% PVP or 2% PVPP) and 5 μL of proteinase K (20 mg mL^{-1}) were added to a 50 mg GRM amended or not with 10 mg AC. Afterward the samples were incubated at 65°C for 60 min., and centrifuged at 16,100 g for 10 min. The supernatant was subsequently transferred to 1.5 mL microtubes.

The purification of crude DNA was performed by adding 500 μL of phenol:chloroform:isoamyl alcohol (25:24:1), and centrifuging at 16,100 g for 10 min. The supernatant was transferred to 1.5 mL microtube. A volume of 500 μL chloroform:isoamyl

alcohol (24:1) was added. The solutions were mixed by inversion, and centrifuged at 16100 g for 10 min. The supernatant was transferred to 1.5 mL microtube, and 1 mL of isopropanol was added. The solutions were gently mixed, incubated at -20°C for 60 min., and then centrifuged at 16,100 g for 30 min. The DNA pellet was washed twice with 150 μL of 70% ethanol, and dried at room temperature. DNA was dissolved with 100 μL of sterile water.

DNA extraction with the TE buffer

A volume of 500 μL of a TE buffer aliquot amended or not with either 2 PVP or 2% PVPP, were added to a 50 mg GRM with or without 10 mg AC. Afterward the samples were incubated at 100°C for 10 min., and centrifuged at 16,100 g for 10 min. The supernatant was subsequently transferred to 1.5 mL microtubes. Then, the purification of crude DNA was performed as described above.

DNA extraction with the DNeasy Plant Mini Kit

DNA was extracted from 50 mg aliquots of GRM without AC, according to the manufacturer recommendations (QIAGEN Biotecnologia Brasil Ltda. - São Paulo State, Brazil). Six replicates were performed for each DNA extraction method.

DNA amplification

DNA recovered from each aliquot of GRM was taken to amplify a fragment of SSU rDNA of AM fungi. A nested-PCR was performed with the primers NS1 (5' -GTAGTCATATGCTTGTCTC- 3') and NS4 (5' –CTTCCGTCAATTCCTTTAAG- 3') (WHITE et al., 1990) for the first-round PCR, and the AM fungal specific primer AM1 (5'-GTTTCCCGTAAGGCGCCGAA- 3'; (HELGASON et al., 1998), in combination with the universal primer NS31 (5' -TTGGAGGGCAAGTCTGGTGCC- 3'; (SIMON et al., 1992) for the second-round PCR.

The first-round PCR was made with 15 μL reaction mixture, containing 0.5 μM each primer, 0.2 mm each dNTP, 2.5 mm $MgCl_2$, 0.18 μg mL^{-1} bovine serum albumin (BSA), 0.75% formamide, 0.75 U *Taq* DNA polymerase, 1X reaction buffer (Invitrogen, São Paulo State, Brazil), and 2 μL template DNA (1:20 diluted DNA sample). PCR amplification was performed as follows: initial denaturation at 95°C for 3 min. followed by 34 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 2 min., and final extension at 72°C for 10 min.

The second-round PCR was made with 20 μL reaction mixture, containing 0.2 μM each primer, 0.2 mm each dNTP, 2.5 mm $MgCl_2$, 0.135 μg μL^{-1} BSA, 0.5 U *Taq* DNA polymerase, 1X reaction buffer, and 3 μL template DNA (1:100 diluted first-round PCR product). PCR amplification was performed as follows: initial denaturation at 94°C for 3 min. followed by 34 cycles of denaturation at 94°C for 1 min., annealing at 59°C for 1 min., and extension at 72°C for 2 min., and final extension at 72°C for 5 min. Amplicons were visualized after electrophoretic migration on 1% agarose gels.

DNA measurement and statistical analyses

Three buffers (CTAB-βM buffer, CTAB-DTT buffer and TE buffer) amended or not with either PVP or PVPP and a DNeasy Plant Mini Kit were used to extract DNA from GRM amended or not with AC. Hence, 18 DNA extraction methods were obtained. For each DNA extraction method, a volume of 2 μL DNA was taken from each replicate to measure DNA concentration at 260 nm and determine DNA purity by the A_{260}/A_{280} ratio using a NanoDrop Spectrophotometer (ND-1000, Thermo Fisher Scientific Wilmington, DE, USA). The raw data of DNA yield (ng mg^{-1} fresh weight of GRM) and DNA purity (A_{260}/A_{280} ratio) were log$_{10}$-transformed before further statistical analyses. Comparisons of extraction methods were made using a one-way analysis of variance (ANOVA) with Tukey post-hoc tests for pairwise comparisons ($p < 0.05$). The effects of extraction buffer and amendment on the yield and purity of the recovered DNA were determined using a two-way unbalanced ANOVA with interactions. The DNeasy Plant Mini Kit was excluded from this analysis.

Principal Component Analysis (PCA) was performed to (i) determine the relationship between the PCR amplification success, purity of the recovered DNA and amount of the template DNA submitted to nested-PCR, (ii) and effectively visualize the differences of extraction methods in DNA recovery and PCR performance. To perform this analysis, amplification success codes were assigned to each DNA submitted to nested-PCR. The success codes were: 2, for successful amplification for both first-round and second-round PCR; 1, for successful amplification for the second-round PCR; and 0, for unsuccessful amplification for both first-round and second-round PCR. All statistical analyses were performed by using the XLSTAT software (Addinsoft, Paris, France).

Results

Among the 18 DNA extraction methods, 6 (CTAB-βM-AC, CTAB-βM/PVP-AC, CTAB-βM/PVPP-AC, CTAB-βM, CTAB-βM/PVP and CTAB-βM/PVPP) were based on the CTAB-βM buffer, 6 (CTAB-DTT-AC, CTAB-DTT/PVP-AC, CTAB-DTT/PVPP-AC, CTAB-DTT, CTAB-DTT/PVP and CTAB-DTT/PVPP) on the CTAB-DTT buffer, 5 (TE-AC, TE/PVP-AC, TE/PVPP-AC, TE/PVP and TE/PVPP) on the TE buffer and one on the DNeasy Plant Mini Kit. For each DNA extraction method, the yield and purity of the recovered DNA are shown in Table 1.

Table 1. Experimental design for comparison of DNA extraction methods, and yield (ng mg^{-1} fresh weight of GRM) and purity (A_{260}/A_{280} ratio) of recovered DNA. Values represent means ± standard errors (n = 6).

Extraction buffer	Amendment	DNA yield (ng mg^{-1} GRM)	DNA purity (A_{260}/A_{280})
CTAB-βM		894.51 ± 78.42	1.65 ± 0.02
	PVP	1061.07 ± 153.46	1.59 ± 0.02
	PVPP	906.01 ± 61.78	1.68 ± 0.02
	AC	730.89 ± 132.29	1.80 ± 0.05
	PVP + AC	1329.78 ± 163.28	1.64 ± 0.02
	PVPP + AC	789.18 ± 171.37	1.75 ± 0.03
CTAB-DTT		1272.54 ± 175.61	1.57 ± 0.01
	PVP	726.87 ± 49.08	1.61 ± 0.02
	PVPP	1185.29 ± 292.02	1.62 ± 0.02
	AC	552.84 ± 65.31	1.68 ± 0.02
	PVP + AC	832.46 ± 88.40	1.54 ± 0.02
	PVPP + AC	625.94 ± 66.62	1.68 ± 0.03
TE	PVP	117.92 ± 19.15	1.51 ± 0.02
	PVPP	403.71 ± 68.43	1.70 ± 0.03
	AC	27.28 ± 8.42	1.74 ± 0.04
	PVP + AC	151.67 ± 32.79	1.83 ± 0.02
	PVPP + AC	34.94 ± 10.54	2.02 ± 0.05
DNeasy Pl. Mi. Kit		80.55 ± 8.51	1.64 ± 0.02

Comparison of DNA yield

The yield of DNA (mean 951.91 ± 60.58 ng mg^{-1}) obtained by the methods based on the CTAB-βM buffer was comparable to that (mean 865.99 ± 72.98 ng mg^{-1}) obtained by the methods based on the CTAB-DTT buffer, and was higher than that obtained by the methods based on the TE buffer (mean 147.10 ± 29.38 ng mg^{-1}) and the DNeasy Plant Mini Kit (mean 80.55 ± 8.51 ng mg^{-1}).

A two-way ANOVA revealed significant effects of extraction buffer, amendment and their interaction. The highest impact on the model explaining over 89% of the variability of DNA yield was achieved with extraction buffer (Table 2). Subsequently, when the methods based on the CTAB-βM buffer were considered all together, a one-way ANOVA revealed that the methods using the different amendments (PVP, PVPP, AC, PVP, and AC or PVPP and AC) did not significantly affect the yield of DNA when compared to the method without amendment.

Nevertheless, the method using PVP and AC produced DNA of higher yield than the method using AC alone (Figure 1). For the method based on the CTAB-DTT buffer, the statistical analysis showed that the method using AC alone and that using PVPP and AC resulted in a significant loss of DNA. For the methods based on the TE buffer, the yield of recovered DNA was highest with the method using PVPP alone and lowest with the method using AC alone and that using PVPP and AC (Figure 1).

Table 2. ANOVAs for effects of extraction buffer, amendment and their interactions on DNA yield and DNA purity.

Dependent Variable: DNA yield					
Source	df	SS	MS	F	p > F
Buffer	2	22.52	10.26	226.07	< 0.0001
Amendment	5	4.14	0.83	18.23	< 0.0001
Buffer x Amendment	9	3.20	0.36	7.85	< 0.0001
Error	85	3.86	0.05		
Corrected total	101	33.73			
CV	0.72%				
Dependent Variable: DNA purity					
Source	df	SS	MS	F	p > F
Buffer	2	0.02	0.01	25.80	< 0.0001
Amendment	5	0.04	0.01	25.70	< 0.0001
Buffer x Amendment	9	0.03	0.00	10.11	< 0.0001
Error	85	0.03	0.00		
Corrected total	101	0.12			
CV	0.08%				

Comparison of DNA purity

The A_{260}/A_{280} ratio of DNA (mean 1.76 ± 0.03) obtained by the methods based on the TE buffer was comparable to that obtained by the methods based on the CTAB-βM buffer (mean 1.69 ± 0.02), and the DNeasy Plant Mini Kit (mean 1.64 ± 0.02), and was higher than that obtained by the methods based on the CTAB-DTT buffer (mean 1.62 ± 0.01).

A two-way ANOVA revealed significant effects of extraction buffer, amendment and their interaction (Table 2). The model explained over 77% of the variability in DNA purity. When the methods based on the same extraction buffer were considered all together, significant differences in DNA purity were detected, whatever the extraction buffer. For instance, for the methods based on the CTAB-βM buffer, a one-way ANOVA revealed that the method using AC alone significantly improved the purity of recovered DNA (Figure 1). For the methods based on the CTAB-DTT buffer, the purity of recovered DNA was highest with the method using AC alone and that using PVPP and AC, intermediate with the method using PVP alone and that using PVPP alone, and lowest with the method using PVP and AC and that without any amendment. For the methods based on the TE buffer, the A_{260}/A_{280} ratio of recovered DNA was highest with the method using PVPP and AC, and lowest with the method using PVP alone (Figure 1).
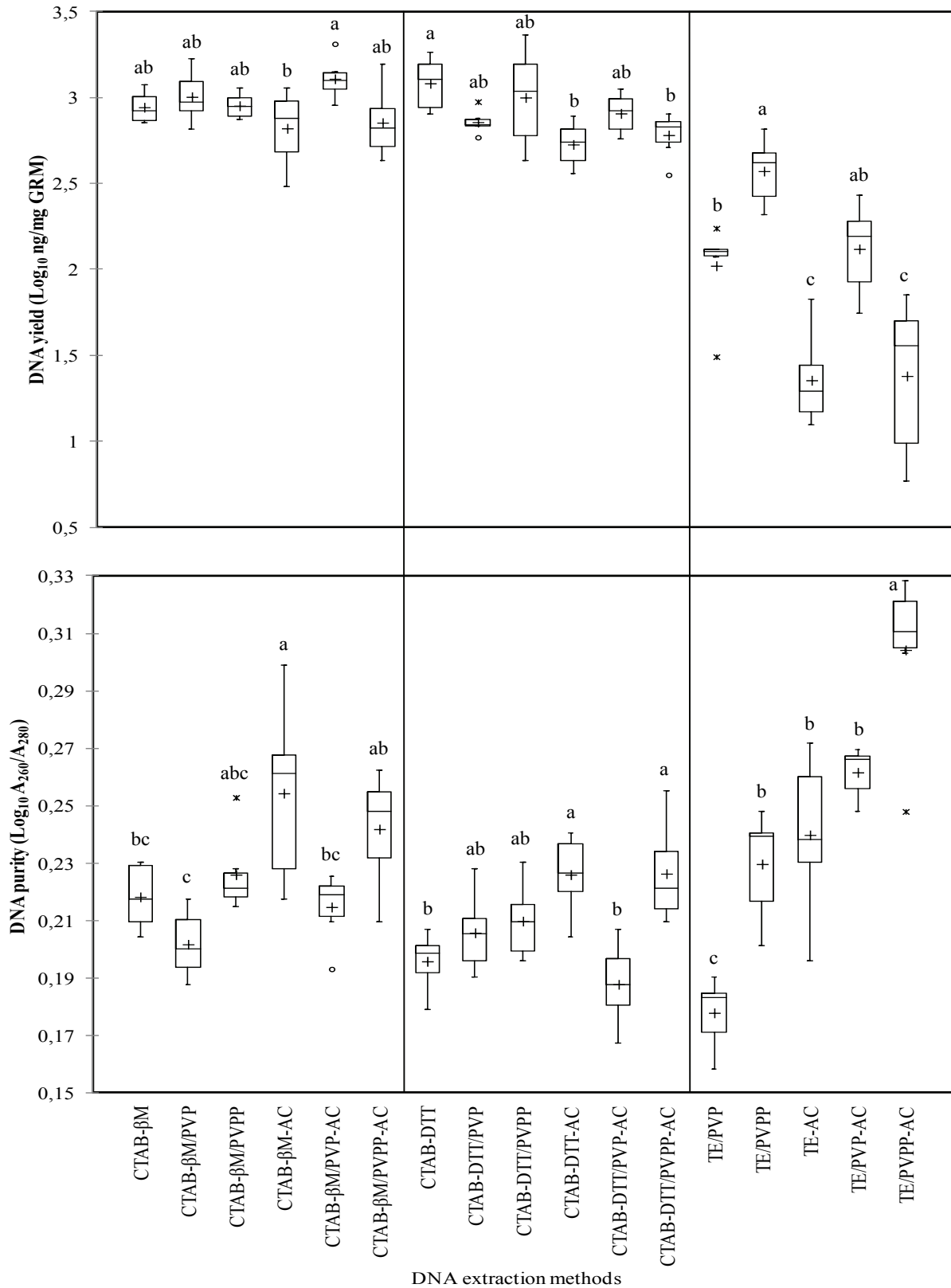
Figure 1. Comparison of DNA yield and purity. For each method, the box indicates the range between the 25th to the 75th percentile; the line in the box indicates the median value; plus sign indicates mean value; upper and lower boundaries of whiskers indicate maximum and minimum values and circles above the whiskers indicate outliers. Letters indicate significant (p < 0.05) differences, by Tukey post-hoc test, between methods using the same extraction buffer (within each rectangle box).

Amplification of AM fungal SSU rDNA

A total of 108 DNA samples obtained by the 18 extraction methods, each being performed with 6 replicates, were submitted to nested-PCR. The nested-PCR amplification success rate was 84% (91/108) (Figure 2).

The methods based on the DNeasy Plant Mini Kit (6 successful nested-PCRs out of 6) and the TE buffer (30 out of 30) were the most successful, followed by the CTAB-βM buffer-based methods (30 out of 36), and the CTAB-DTT buffer-based methods (25 out of 36). Considering the methods based on the same CTAB buffer, those using AC alone or in combination with PVPP almost had the best PCR performances (Figure 2).

The PCA results revealed that the DNA amplification success was negatively correlated (r = -0.74, p < 0.05) with the amount of template DNA, and positively correlated (r = 0.73, p < 0.05) with the purity of recovered DNA. A negative correlation (r = -0.50, p < 0.05) was also revealed between the amount and purity of recovered DNA. PCA explaining 93.91% of the variability in the first two factors discriminated DNA extraction methods (Figure 3).
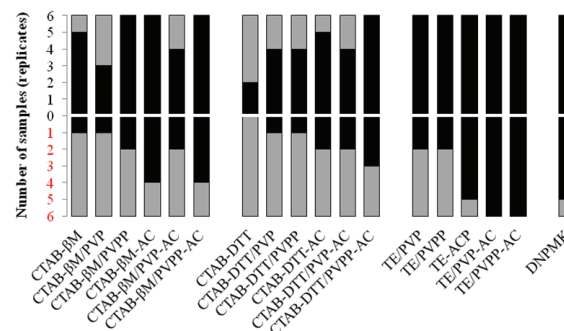
Figure 2. DNA samples submitted to nested-PCR and amplification results for the first-round PCR (below the zero line of the histogram) and the second-round PCR (above the zero line of the histogram). The black color indicates the successfully amplified DNA samples, and the grey color indicates the unamplified DNA samples.
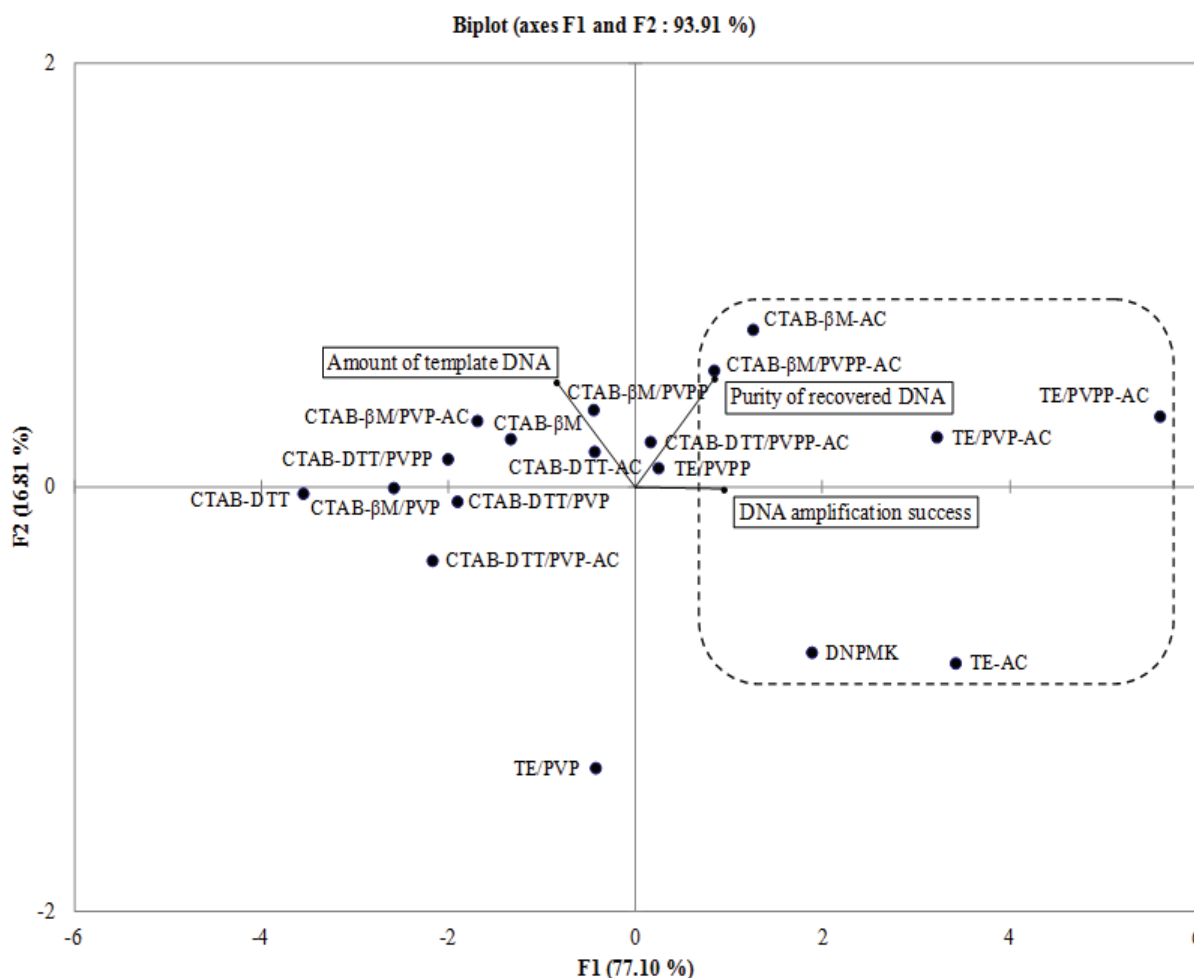
Figure 3. Projection of the two largest factors in Principal Component Analysis (PCA). The axes F1 and F2 represent 77.10% and 16.81% of the total variability, respectively. The dashed box includes the methods which produced high-quality DNA in terms of purity and PCR performance.

Therefore, six methods of which three were based on the TE buffer (TE/PVPP-AC, TE/PVP-AC and TE-AC), two on the CTAB-βM buffer (CTAB-βM/PVPP-AC and CTAB-βM-AC) and one on the DNeasy Plant Mini Kit were relatively far away from the others on the positive side of F1 axis (explaining 77.10% of the variability). The DNA samples obtained by these extraction methods showed high quality in terms of purity and PCR performance (Table 1, Figure 2).

Discussion

Although the staining and microscopy procedures are still used to reveal AM fungi in plant roots (VAN TUINEN et al., 1998), in recent years, considerable attention has been given to molecular techniques based on PCR amplification. However, it has been shown that the sensitivity and efficiency of these molecular techniques depend on the quality of the recovered DNA (VROH BI et al., 1996; WILSON, 1997). In this respect, we tested 18 DNA extraction methods for the yield and purity of DNA recovered from mycorrhized roots, and whether the recovered DNA is suitable for amplification of AM fungal SSU rDNA.

Our results showed that the methods based on the CTAB buffers yielded more DNA than the methods based on the TE buffer and the DNeasy Plant Mini Kit. This high sensitivity of the methods based on the CTAB buffers in assessing the total DNA may be related to the incubation procedure (e.g. 60 min. at 65°C for the CTAB buffers-based methods *versus* 10 min. at 100°C for the TE buffer-based methods), and/or the extraction buffer components, particularly CTAB and NaCl. Indeed, CTAB is known to form an insoluble complex with nucleic acids, and NaCl to break down the formed complex and thereby releases large amounts of nucleic acids (BROWN, 2010). Moreover, the use of AC alone or in combination with PVPP reduced DNA yield, while it significantly improved the purity of recovered DNA, whatever the extraction buffer. A possible explanation for this result is the high adsorption capacity of AC due to its large surface area and pore volume (BAKER et al., 1992). AC has thus been successfully used to remove humic acids, polysaccharide and polyphenolic compounds and other impurities, which are often co-extracted with DNA (DESAI; MADAMWAR, 2006; VERMA; SATYANARAYANA, 2011; VROH BI et al., 1996).

On the other hand, unlike the DNA samples obtained by some extraction methods based on the CTAB buffers, those obtained by the methods based on the TE buffer and the DNeasy Plant Mini Kit were consistently amplified. This suggests that the TE buffer-based methods can be reliable methods for recovering DNA suitable for detection of AM fungi in roots (FARMER et al., 2007; IKEDA et al., 2001). In addition, the nested-PCR success was negatively correlated with the amount of template DNA, and positively correlated with the purity of recovered DNA, suggesting that a relatively low quantity and high purity of DNA is required for successful amplification of AM fungal SSU rDNA. Indeed, since the DNA samples recovered from AM roots were composed of a mixed plant–fungal DNA and other impurities, the use of a large amount of template DNA leads to increase the amount of the non-target DNA and impurities which may inhibit the amplification of the target AM fungal SSU rDNA (WILSON, 1997). Nevertheless, dilutions of DNA samples can contribute to improve nested-PCR success.

Six methods of which three were based on the TE buffer (TE/PVPP-AC, TE/PVP-AC and TE-AC), two on the CTAB-βM buffer (CTAB-βM/PVPP-AC and CTAB-βM-AC) and one on the DNeasy Plant Mini Kit produced high-quality DNA in terms of purity and PCR performance. However, the TE buffer-based methods are less time consuming than the CTAB-βM buffer-based methods, and cheaper than the method based on the DNeasy Plant Mini Kit. In this respect, the performance of the TE buffer-based methods should be tested on other types of samples to confirm that they could provide useful alternatives to the use of commercial DNA extraction kits.

Conclusion

Our work revealed that the choice of the type of extraction buffer and amendment has significant implications for DNA recovery and PCR success. Hence, the TE buffer-based methods are not only simple and reliable for DNA isolation, but are also less time consuming and cheaper than the other methods. Finally, we anticipate that the TE buffer-based methods, particularly those using AC can be used for the isolation of genomic DNA from roots and other plant organs such as leaves of *M. pruriens* and other plants species, including important crops.

Acknowledgements

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