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Optimization of DNA Extraction from Seeds of *Sorghum sudanense* (Piper) Stapf.

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Abstract

Sudan Grass (*Sorghum sudanense* (Piper) Stapf.) is a fine annual forage of Gramineae, Sorghum Genus that thrives in warm natural environment, with strong resistance against drought and barren soil, high yield, strong regeneration capability. DNA of the grass seeds was extracted by optimized Moller's method. The extraction of DNA is a premise for identifying *Sorghum sudanense* from malignant weed seeds such as *Sorghum halepense* by molecular biological methods. The effects of NH4Ac, different seed pretreatments, the ratio of seed powder to TES extraction solution volume (solid-liquid ratio), pyrolysis time, PVP (polyvinyl pyrrolidone) on the DNA extraction were studied. Based on results above, an optimized method for DNA extraction from *Sorghum sudanense* seeds was established. The results showed that NH4Ac had no effect on DNA extraction from *Sorghum sudanense* seeds. Soaking seeds in warm water 20 min before grinding, 1:10 for solid-liquid ratio, pyrolysing for 60 min and 1.5% (w/v) PVP in extraction solution could promote the quantity and purity of DNA, respectively.

Keywords: *Sorghum sudanense*, DNA extraction, Moller's method, optimization

Introduction

Sudan Grass (Sorghum sudanense (Piper) Stapf.) Sudan Grass can be used in graze, green chopped forage, green hay or silage, with good taste to stocks and perfect forage in pond fish farming (Bishnoi et al., 1993; Watanabe and Kasuga, 2000). At present, in China most of the Sudan Grass seeds are imported. The malignant weed seeds such as seeds of *Sorghum halepense* (Linn.) Per, one of the ten hardness weeds in the world, and ranked as the second most dangerous species according to the Chinese Import Act can be entrained in the imported seeds easily. They bear similarity to those of Sudan Grass and the seeds may be deformed and abrased during storage, loading and unloading. It is very difficult to distinguish them from each other morphologically (Feng et al., 2006). Cytogenetics method was used by Garber (1950) to classify different species in Sorghum Genus based on chromosome number, morphological features and chromosome pairing during meiosis. However, the number of chromosome varied significantly in different species of Sorghum Genus, the basic chromosome number whether n = 10 or n = 5 is still in dispute, so the taxonomic study of some species in Sorghum Genus with polyploid complex has not been used widely. With the extraction of DNA as the premise, molecular biological methods for identifying Sorghum sudanense from malignant weed seeds such as *Sorghum halepense* (Linn.) Per were studied (Chen et al., 2008), and the premise of the method was the extraction of enough DNA with satisfactory quality.

Four methods including CTAB, SDS, Moller's method (Moller *et al.*, 1992) and TaKaRa DNA extraction kit were used by Guo *et al.* (2005) to extract the seed DNA from seven related species of Sorghum Genus. The results showed that Moller's method was the best one for extraction of the genomic DNA in seven closely related species. However, there are differences in structure and composition in different plants or different parts of the same plant (Wang *et al.*, 2002), *e.g.*, some with a high degree of tissue lignification, and some with thick cell wall, while other cells contain more phenols, it is hard to find a general DNA extraction method which can be used for different plants. So we tried to improve Moller's method by modifying some aspects of procedures with an attempt to establish a more specific DNA extraction method for the *Sorghum sudanense* seeds.

Materials and methods

Plant materials

Sorghum sudanense seeds were obtained from Technical Center of Plant and Animal Quarantine, Shanghai Enter-Exit Inspection and Quarantine Bureau.

Extraction of DNA from Sorghum sudanense seeds (Modified from Moller's method)

1. About 30 - 60 mg seed was grinded in liquid nitrogen, and then put into a microtube containing 500μL TES (100 mM Tris, pH 8.0, 10 mM EDTA, 2% SDS); 50 - 100 μg Proteinase K was added. The sample was extensively mixed and incubated for 30 min up to 1 h at 55°C - 60°C. During the incubation, the sample was mixed occasionally and gently.

2. Salt concentration was adjusted to 1.4 M with 5 M NaCl, 1/10 volume 10% CTAB (cetyltrimethylammoniumbromide) was added, incubating for 10 min at 65°C.

3. Equal volume SEVAG (chloroform: isoamylalcohol, 24:1, v/v) was added and mixed gently, incubated at 0°C for 30 min, and centrifuged at 4°C, 13000g for 10 min.

4. Supernatant was transferred to a 1.5 mL tube, 225 μL 5 M NH_4Ac was added and mixed gently; placed on ice for approx. 30 min (the longer the better); centrifuged at 4°C, 13000g for 10 min.

5. Supernatant was transferred to a fresh tube; 3μL RNase (10mg/mL) was added and the sample was incubated at 37°C for 30 min to remove RNA; centrifuged at 4°C, 13000g for 10 min.

6. Supernatant was transferred to a 1.5 mL tube; 0.55 volume isopropanol was added to precipitate DNA; sample was placed on ice for approx. 15-30 min; centrifuged at 4°C, 13000g for 10 min.

7. Supernatant was removed and pellets were washed twice with cold 70% ethanol, dried in ambient and dissolved in about 50 μL ddH_2O.

Optimization of DNA extraction from Sorghum sudanense seeds

Based on the procedure in 2.2.1, following modifications were done respectively: (1) without NH4Ac; (2) seed pretreatments by soaking seeds in warm water for 20 min, and with seed coat removed or intact; (3) set a set of serial ratio of seed powder to extraction solution: 1:5, 1:10, 1:20 and 1:30; (4) set a different pyrolysis time from 30 to 120 min; and (5) adjust the concentration of PVP in extraction solution to 1.0%, 1.5%, 3.0% and 4.0%, respectively.

Detection of the concentration and purity of DNA from the seeds of Sorghum sudanense

Concentration, purity (A_{260}/A_{280} ratio) and absorbance ratio at 260 nm to 230 nm (A_{260}/A_{230} ratio) were measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA).

DNA quality evaluation by agarose gel electrophoresis of PCR products

The genomic DNAs extracted from different pretreated seeds are used to amplify using two primers (Shanghai Sangon). The forward primer is D16: 5'-GTCACCGACGCATTCTTCA-3'; the reverse primer is D18: 5'-ATTCTTCCCGTTG CACTCG-3' (Chen *et al*., 2008). The amplification was performed in a FGENO2TD PCR Thermocycler (TECHNE, America) with initial condition at 94°C for 3 min, followed by 94°C for 45 s, 55°C for 45 s, 72°C for 30 s, 35 cycles, and at 72°C for 5 min. Reactions system consisted of 25 μL 1×PCR buffer (TIANGEN, China), 250 μmol L^{-1} dNTP, 200 μmol L^{-1} primer, 0.5 μL DNA templates obtained above seperately, 1 U Taq polymerase (TIANGEN, China). The amplified products were separated on 1% agarose gel in 1×TAE buffer with 120 V in voltage for 24min and visualized on UV transparent board by ethidium bromide staining.

Results and discussion

The effects of NH_4Ac on the extraction of DNA

Some parameters of DNAs extracted with and without addition of NH4Ac were listed in Tab. 1. Extraction ratio with NH4Ac was 12.9 μg/g, just 13.6% of control (without NH4Ac). Besides, the purity of DNA extracted with NH4Ac was also decreased to some degree compared with the control. Hence, it suggested that omitting NH4Ac from DNA extraction procedure could yield an improved performance for DNA extraction from *Sorghum sudanense* seeds.

Generally, the purpose of adding NH_4Ac in DNA extraction process is to reduce the redundant impurities (such as dNTP and polysaccharide) which can co-precipitate with nucleic acid. Okayam *et al.* (1982) pointed out that with the presence of 2 mol / L NH_4Ac, precipitating DNA twice, more than 99% of the dNTP can be removed from the DNA products. There was no dNTP pollution in this experiment, but the polysaccharide in the Sorghum Genus seeds was at a high level (Tan, 2007). Although the function of NH_4Ac is to improve the purity of DNA, the results of our experiment showed that NH_4Ac had little improvement on the purity of DNA, causing extraction ratio to drop significantly. One possible reason for this phenomenon was that adding NH_4Ac could only remove partial precipitation of protein and polysaccharide during DNA extraction. Since the effect of NH_4Ac on removing polysaccharides is not ideal, adding NH_4Ac is not suiTab. for plant DNA purification with higher content of polysaccharide. Also, the NH_4Ac addition makes the experiment process so complicate that may lead to loss and damage of DNA. This result was consistent with that of Liu *et al.* (2005).

Tab. 1 The effects of NH_4Ac on yield and purity of DNA extracted from *Sorghum sudanense* seeds

	Concentration (ng/μL)	Ratio (A_{260}/A_{280})	DNA Extraction Ratio (μg/g) #
Control	190.2±0.50	1.77	95.1
NH_4Ac	25.7±0.80	1.68	12.9

Note: #DNA Extraction Ratio (μg/g) = DNA concentration × Volume / Amount of seed power (g)

The effects of different seed pretreatments on DNA extraction

The effects of seed pretreatment on DNA extraction were shown in Tab. 2. By soaking seeds in warm water for 20 min and left seed coats not removed before grinding, the DNA extraction ratio was 116.1 μg/g, highest in three groups, 25.4% more than the control, and 3.7 times more than the group soaked in warm water for 20 min with seed coats removed. It indicated that soaking dry seeds of *Sorghum sudanense* in warm water for 20 min promoted DNA extraction efficiency. The DNA samples from three groups were amplified by primers D16 / D18, complementary to Chi-B partial sequence and producing about 594 bp amplified fragment (Fig. 1). According to the brightness of the amplified fragments, the effects of different pretreatments on DNA extraction could be determined. By using the same volume of DNA template, the picture of PCR products after agarose gel electrophoresis showed that the DNA extracted after soaking in warm water for 20 min with seed coats not removed gave the brightest amplified products, which indicated that it could be used for further research.

Wu *et al.* (2006) reported that softening dry maize seeds by soaking in water before DNA extraction could promote DNA quality so much that they can be used as template of molecular marker SSR. Our study showed that soaking in warm water could elevate the *Sorghum sudanense* seed DNA too. It could be due to the *Sorghum* Genus episperm, such as Sorghum, which is rich in anthocyanins and other polyphenols (Liu *et al.*, 2003). Besides, some substances of the sorghum seed clothing (Zhao *et al.*, 1997) may deteriorate the DNA extraction and soaking could alleviate this effect, so that DNA extraction ratios are improved. In order to rule out the interference of coat clothing, skin pigments and phenolic substances completely, the seed coat-peeled group was established in our experiment. If the seed coats were removed before grinding, however, the DNA extraction ratio decreased significantly. May be the embryo with higher level of DNA was abscised when seed coats were removed.

The effects of solid-liquid ratio on DNA extraction

Different ratios of seeds powder to TES extraction solution volume (i.e. solid-liquid ratio) exerted effects on DNA extraction ratio and DNA purity (Tab. 3).The results shown that the ratio of 1:10 produced highest extraction ratio (113.6μg/g), 14.6 times when the solid-liquid ratio was 1:5. When the solid-liquid ratio were 1:20 and 1:30, DNA extraction ratios decreased by 41.9% and 44.0% respectively. These results showed that when the solid-liquid ratio decreased below 1:10, extraction ratio dropped dramatically as extraction solution volume increased. The ratio of 1:10 also led to highest purity of DNA. Therefore, the solid-liquid ratio 1:10 was optimum for DNA extraction from *Sorghum sudanense* seeds.

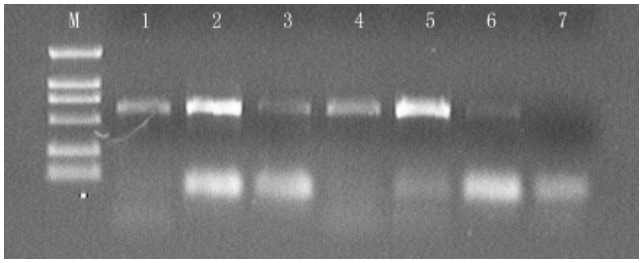

Fig. 1. The effects of different seed pretreatments on the *Sorghum sudanense* seed DNA extraction

M: Marker (DL 2000); 1, 4: control; 2, 5: soaked in warm water for 20 min with seed coats left; 3, 6: soaked in warm water for 20 min with seed coats removed; 7: negative control (no template); 4-5: half amount of DNA template of 1-3.

Cell lysing in TES was extremely important to the DNA release from the seed powder. If the extraction solution was not enough, the seed powder was more prone to adhere to the tube wall, resulting to incomplete extraction. The present study showed that when the solid-liquid ratio was 1:5, the extraction ratio was significantly low. However, when the amount of extraction solution rose to a certain degree, the seed powder was completely immersed, but it also led to the dilution of some active ingredients, such as proteinase K and RNase, thus weakening their effects, which can explain the reason why effectiveness of DNA extraction at solid-liquid ratio 1:20 and 1:30 was worse than 1:10.

The effects of pyrolysis time on DNA extraction

Different pyrolysis time exhibited impacts on *Sorghum sudanense* seed DNA extraction (Tab. 4). DNA extraction ratio was the highest in 60 min group, being 126.7 μg/g, 1.1 times, 7.1 times and 4.1 times as much as that in 30 min, in 90 min and in 120 min respectively. Meanwhile, its DNA purity was higher than those of other three groups. So, 60 min was chosen as the best pyrolysis time for *Sorghum sudanense* seed DNA extraction.

Pyrolysis time in a water bath is also another extremely important factor in the DNA extraction process. Sun *et al.*

Tab. 2. The effects of different seed pretreatments on the *Sorghum sudanense* seed DNA extraction

Variant	Concentration（ng/μL）	Ratio (A260/A280)	Extraction ratios of DNA（μg/g）
Control	185.2±0.40	1.78	92.6
Soaked with seed coats left	232.2±0.70	1.77	116.1
Soaked with seed coats removed	49.8±0.20	1.69	24.9

Tab. 3. The effects of solid-liquid ratio on the extraction of DNA from *Sorghum sudanense* seeds

Solid-liquid ratio	Concentration (ng/μL)	Ratio (A_{260}/A_{280})	Extraction ratios of DNA (μg/g)
1:5	15.5±0.02	1.78	7.8
1:10	227.2±0.06	1.82	113.6
1:20	132.0±0.40	1.58	66.0
1:30	127.2±0.30	1.63	63.6

(2007) found that the best time period in the water bath was 30 min to 40 min when the *Myrica rubra* leaf genomic DNA was isolated by CTAB. DNA extraction ratio would decrease when time exceeds 50 min. As longer water bath time period would decompose genomic DNA or increase other impurities, *i.e.* the effect wouldn't be better for longer time period. Our study confirmed the above point, it shared same tendency but different in time period. The optimum time for pyrolysis in a water bath was 60 min when genomic DNA was isolated from *Sorghum sudanense* seeds

Tab. 4. The effects of pyrolysis time on the extraction of DNA from the seeds of *Sorghum sudanense*

pyrolysis time	Concentration (ng/μL)	Ratio (A_{260}/A_{280})	Extraction ratios of DNA (μg/g)
30 min	220.5±0.03	1.62	110.3
60 min	253.3±0.08	1.80	126.7
90 min	35.5±0.50	1.40	17.8
120 min	61.8±0.30	1.72	30.9

with the modified Moller's method, and DNA extraction ratios decreased significantly after 60 min. Perhaps, this is determined by the differences of the experimental materials. Generally, leaf cells are easier to crack than seed cells, and it is easier for DNA to release from leaves than from seeds, therefore *Sorghum sudanense* seeds need longer pyrolysis time.

The effects of PVP on DNA extraction

The highest extraction ratio existed with 1.5% PVP group, being at approximately the same level of the control group (Tab. 5). In groups 1.0%, 3.0% and 4.0%, the DNA extraction ratios decreased by 32.2%, 49.8% and 90.2%, respectively, compared with control group. Besides, the DNA purity in 1.5% group was highest; the ratio of A_{260}/A_{280} was 1.82, close to the standard absorbance ratio 1.80 and was accompanied by the highest absorbance ratio of A_{260}/A_{230}. Therefore, 1.5% PVP had the best contribution to the extraction.

The *Sorghum sudanense* seeds generally contain tannin, pigment and some phenolics, so the DNA precipitation pellets were slightly yellow-brown. Polyphenol binder PVP can prevent polyphenols from being oxidize to form quinones effectively, and also PVP can be used as a clarifying agent to adsorb impurities such as polysaccharides (Cui and Lin, 2008). After 1.5% PVP (m/v) was added, the color of the genomic DNA extracted this way became bright and white. Although the addition of 1.5% PVP had little impact on the extraction ratio, the changes in ratios of A_{260}/A_{230} and A_{260}/A_{280} showed clearly that the pollution of polyphenols was reduced significantly.

Conclusions

DNA extraction system of Sudan Grass (*Sorghum sudanense* (Piper) Stapf.) seeds was established by optimizing Moller's method.

Omitting NH_4Ac from DNA extraction procedure could yield an improved performance for DNA extraction.

Soaking seeds in warm water 20 min before grinding promoted DNA extraction efficiency.

Solid-liquid ratio (1:10), pyrolysing for 60 min and 1.5% (w/v) PVP in extraction solution could, also, promote the quantity and purity of DNA, respectively.

DNA extraction method established for the *Sorghum sudanense* seeds could give reference for the DNA extraction from other sorghum genus seeds (such as *Sorghum*

Tab. 5. The PVP effects on the *Sorghum sudanense* seed DNA extraction

PVP (%)	Concentration (ng/μL)	Ratio (A_{260}/A_{280})	Extraction ratios of DNA (μg/g)	A_{260}/A_{230}
0.0 (control)	221.4±0.30	1.74	110.7	0.51
1.0	150.0±0.50	1.49	75.0	0.69
1.5	238.3±0.10	1.82	119.2	1.29
3.0	111.2±0.40	1.45	55.6	0.65
4.0	21.7±0.60	1.19	10.9	0.36

halepense seeds) and also could be helpful for the detection of *Sorghum halepense* by molecular biology methods.

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