Optimization of a Rapid DNA Extraction Protocol in Rice Focusing on Age of Plant and EDTA Concentration

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***Abstract*—An increase in the world population causes the need to improve the crop and food productivity. It is generally suggested that an extensive attention should be paid to plant breeding efforts to address the issues of food security. Marker Assisted Selection (MAS) is referred to as the use of DNA markers for the selection of desirable traits in segregating/breeding populations. DNA based breeding selection is much more reliable than traditional breeding methods. Breeding populations used to select the desirable traits called segregating. Yet segregating populations need huge financial as well as leisure requirement. However DNA extraction in segregating populations has been always being a limiting factor either because of quantity or quality. The present study was conducted to improve the DNA quality and quantity of rice by investigating the effects of the age of the seedlings and EDTA buffer concentration in order to apply more reliable downstream processes and subsiquantly to gain better results. As a result, Four weeks old seedlings gave the best quantity of DNA (118.9ng/µl). The concentrations of EDTA in the first step buffer (2mM) and second step buffer (0.1mM) were the best for DNA extraction.**

***Index Terms*—rice breeding, age effect, EDTA concentration, marker assisted selection, DNA extraction.**

I. Introduction

Rice has become an important food for the people all over the world. Researchers are trying to improve rice systems in order to increase the crop production because world's population is rapidly growing. Conventional breeding is not prevalent anymore. Therefore, scientists have started to use breeding systems based on molecular studies. The core of many applications in breeding programs is the capability to recognize individual plants and characterize the plant genotypes individually.

Another major goal of breeding programs is investigation of genetic diversity and relationships among breeding lines to facilitate parent selection in hybrid rice breeding programs [1]. Therefore it is necessary to identify plant populations and individual plants by molecular studies [2]. The most important raw material for molecular studies and breeding systems is high quality DNA. Consequently, it is essential to use a suitable DNA extraction protocol.

DNA extraction is the most important step in molecular studies and is the main key to acquire results with impressive resolutions in gel-based systems. Although many protocols and kits have been introduced during past decades, there are still some difficulties with these protocols such as using rare and dangerous chemicals like liquid nitrogen and β-mercaptoethanol and also triggering special equipments like zirconium balls and steel beads which may not be found in all laboratories. Furthermore, there are some hardships with procedures like altering heat shock at -80 ℃ and 60 ℃. In addition, contamination with high polysaccharide concentration, which is an inhibitor for the enzymes in the case of downstream process such as Polymerase Chain Reaction (PCR), is a big problem with many DNA extraction protocols [3]. Besides, extraction kits are too expensive. All mentioned difficulties are prevented in our method to have an easy, fast and cost-effective procedure.

A DNA extraction method which is efficient and rapid is essential when a large number of samples should be analyzed rapidly. For example, in marker assisted selection and breeding, it is a big deal [4]. In other words, the absence of good-quality DNA would be a limiting factor to the success of polymerase chain reaction (PCR)-based downstream applications such as random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), sequence-related amplified polymorphism (SRAP), and amplified fragment length polymorphism (AFLP) [4].

In this study, DNA was extracted from seedlings of different ages to detect if the age of the seedlings has any effect on quality and quantity of extracted DNA. TE buffer with different concentrations of EDTA was applied in the DNA extraction protocol. This protocol only requires TE buffer, micro tube and disposable pipette tip.

Manuscript received May 12, 2013; revised July 11, 2013.

doi: 10.12720/jomb.2.3.218-223

II. Matherial and Method

The core of this study is focusing on laboratory researches in order to optimize a DNA extraction method which is able to extract high quality and quantity DNA from small amounts of fresh rice leaves.

A. Plant Cultivation

Pokkali rice was used as the source for DNA extraction. 200 Pokkali seeds were soaked in distilled water in Petri dishes for one week. After germination they transferred into plastic seedling boxes. Samples were taken (0.02g) when the seedlings were 2, 3 and 4 weeks old.

B. DNA Extraction Protocol

The optimized DNA extraction protocol was carried out based on an original method described by Ikeda *et al.* (2001) because this method is (among exist protocols) simple, low cost and effective. The leaves were vigorously rinsed in distilled water to remove particles on leaf surface. 0.02 g of fresh tissue was weight out. The tissue was chopped into very small pieces helping sharp scissors (scissors were dipped in absolute ethanol before use). Pieces were placed in a 1.5 ml micro tube. 200 µl of first TE buffer was added and the leaves were ground by a blue pipette tip (0.1-1 ml tip). Tubes were placed in boiling water (100 ˚C) for 20 minutes to insert and wash out potentially existing DNase and take out the DNA from the cell nucleus. 800 µl of second TE buffer was added, mixed by vortex for 20-30 seconds and centrifuged at 14,000 rpm at room temperature for 3 minutes. Finally, the supernatant which contains the genomic DNA was transferred to a new 1.5 ml tube, and it was stored at -20 ˚C for PCR amplification and other molecular experiments.

TABLE I. Nine Applyed TE Buffer Treatments for DNA Extraction Method

Treatment Number	First TE Buffer	Second TE Buffer
1	TE buffer 1-1	TE buffer 2-1
2	TE buffer 1-1	TE buffer 2-1
3	TE buffer 1-1	TE buffer 2-1
4	TE buffer 1-2	TE buffer 2-2
5	TE buffer 1-2	TE buffer 2-2
6	TE buffer 1-2	TE buffer 2-2
7	TE buffer 1-3	TE buffer 2-3
8	TE buffer 1-3	TE buffer 1-1
9	TE buffer 1-3	TE buffer 2-3

To optimize the buffer composition, two groups including six TE buffers were used containing different EDTA concentrations. First group of TE buffers were TE buffer 1-1: 10 mM Tris-HCl, 1 mM EDTA pH=8, TE buffer 1-2: 10 mM Tris-HCl, 2 mM EDTA pH=8, TE buffer 1-3: 10 mM Tris-HCl, 0.5 mM EDTA pH=8 and. Second group of TE buffers were TE buffer 2-1: 10 mM Tris-HCl, 0.1 mM EDTA pH=8, TE buffer 2-2: 10 mM Tris-HCl, 0.2 mM EDTA pH=8 and TE buffer 2-3: 10 mM Tris-HCl, 0.3 mM EDTA pH=8. TE buffer treatments in each experiment are listed in Table II. All treatment applications are listed in Table I.

TABLE II. Applications of Treatments for DNA Extraction Protocol Including Age of the Seedlings and TE Buffer Concentrations (Description of Numbers 1-9 for TE Buffer Is Listed Is Table 1).

Age	TE Buffer
Week 2	1 to 9
Week 3	1 to 9
Week 4	1 to 9

C. DNA Quantification

DNA concentration was quantified using a Nano Drop Spectrophotometer and some statistical analysis was performed to investigate the optimum age and EDTA concentration among all the treatments. The quality of genomic DNA was determined in ratio absorbance of A_{260}/A_{280} using NanoDrop-1000 Spectrophotometer.

D. Determining DNA Concentration and Quality

The concentration and quality of extracted rice DNA were confirmed by using 0.8% (w/v) agarose gel.

E. PCR Amplification and SSR Analysis

After optimization of PCR condition, PCR of amplifi--cation of extracted genomic DNA was carried out. The final reaction volume was 25 µl, containing 2 µl template DNA, 2.5 µl 10x PCR buffer, 0.625 µl 10 mM dNTPs, 0.625 µl 100 mM MgCl2, 1.0 µl 10 µM primers, 0.8 units of BIORON Taq DNA polymerase and suitable amount of sterile deionized water. For PCR amplification, RM 171 was used which is a universal primer and the sequence is located on chromosome 10 of rice. The PCR reaction was performed in a MJ Gradiant Thermocycler (Bio-Rad Laboratories). The reaction mixtures underwent initial denaturation at 95 ℃ for 5 min followed by 35 cycles; 1 min at 94 ℃, 1 min at 57 ℃ and 1 min 72 ℃. A final extension was also performed at 72 ℃ for 7 min. Then a mixture consists of 4 µl PCR product and 2 µl loading dye were loaded onto a 2.5% agarose gel, electrophoresed at 80V for 40 min, and visualized using ethidium bromide and gel viewer.

F. Electrophoresis and Staining Procedure

PCR products were separated on 2.5% (w/v) agarose gel electrophoresis for 45 minutes and 80 volts and stained with ethidium bromide solution. 0.05% ethidium bromide (1 µg/ml) for 5 min was used for staining. The bands were visualized under UV under Gel Documentation system (gel viewer machine).

III. Results and Discussion

A very simple rice DNA extraction protocol was optimized in terms of age of the seedlings and EDTA

concentration used in TE buffer. To find the optimum age and EDTA concentration qualitative and quantitative analysis were performed and discussed.

The concentration of DNA in each sample was checked by spectrophotometry. A Nano Drop Spectrophotometer is suitable for this purpose. Table III shows the three highest quantified amounts and their related treatments. The maximum average of DNA quantity for the present method was 115.7ng/µ.l when the DNA was extracted by TE buffer 1 from 4 weeks seedlings. The DNA quantity for Ahmed *et al.* [5] and Sun *et al.* [6] was 20-30 µg/cm² and 30 µg respectively. The concentration of extracted DNA in some previous methods such as Xu et al. [4], Ahmed et al. [5] and Sun et al. [6] was 5-20 ng/cm², 30 µg and 20-30 µg/cm² respectively. However, the use of rare materilas such as zirconium ball (Xu et al. [4]) and steal bead (Sun et al. [6] and also dangerous material like ether (Ahmed et al. [5]) was observed in these methods. Based on presented data in Table III, statistical analysis was run to survey if there is any significant interaction between independent factors (age, and EDTA concentration).

TABLE III. MAXIMUM DNA CONCENTRATIONS (NG/ML) EXTRACTED FROM 2, 3 AND 4 WEEKS AND THEIR RELATED TE BUFFER.

Age	DNA (ng/µl)	TE buffer	DNA (ng/µl)	TE buffer	DNA (ng/µl)	TE buffer
Week 2	78.6	1	71.3	3	70.4	4
Week 3	117.9	1	101.1	3	98.3	4
Week 4	121.7	1	117.5	4	108.2	3

Table IV shows the results for Test of Between-Subjects Effect [7] which expresses significant mean differences between groups for two independent variables; age, and EDTA concentration and their interaction (age * EDTA). If $P < 0.05$ means that there is a significant interaction between the independent factors. For all factors and their interactions P value was less than 0.05 meaning that the age of the seedling and EDTA concentration have a significant effect on the quality of the extracted DNA. To find the optimum age and EDTA concentration, Turkey HSD test was performed. For more information about this test, please refer to Ref [8].

TABLE IV. SIGNIFICANT MEAN DIFFERENCES BETWEEN GROUPS (DEPENDENT VARIABLE: DNA QUANTITY)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Age	46539.998	2	23269.999	1022.413	0
EDTA	25771.508	8	3221.438	141.54	0
Age* EDTA	4946.945	16	309.184	13.585	0

The Turkey post hoc analysis will illustrate where the statistical differences are located. Multiple comparison table (refer to Appendix A) reports the mean differences between the different ages and EDTA concentrations. It can be reported that the quantities of extracted DNA from seedlings in age 3 and 4 weeks old are approximately equal and moreover their quantity mean is higher than that of seedlings which are examined in week 2. The effect of different EDTA concentrations on the quantity of extracted genomic DNA was observed as well. TE buffer 1 was the most effective reagent among all nine buffers followed by TE buffer 3 and 4. Based on this data, the most proper TE buffers for extracting genomic DNA from rice leaves would be TE buffer 1, 3 and 4. However, the most effective one was TE buffer 1. In the next step linear models were performed to create a clear comparison between factors and their treatments.

Fig. 1 shows the interactions between age and EDTA concentration. The maximum estimated means of DNA quality can be seen in age 2 (week 3) and TE buffer 1 ($EDTA_1$ = 1 mM, $EDTA_2$ = 0.1 mM). The means grow sharply for TE buffer 1 ($EDTA_1$ = 1 mM, $EDTA_2$ = 0.1 mM), TE buffer 3 ($EDTA_1$ = 1 mM, $EDTA_2$ = 0.3 mM) and TE buffer 4 ($EDTA_1$ = 2 mM, $EDTA_2$ = 0.1 mM). The means show a considerable growth from age 1 (week 1) to age 2 (week 3). Based on quantitative analysis, it can be said that using this method, the optimum age was age 3 (week 4) and the optimum EDTA concentration was that mentioned volume in TE buffer 1. To check the quality of extracted DNA gel-based analysis was run.

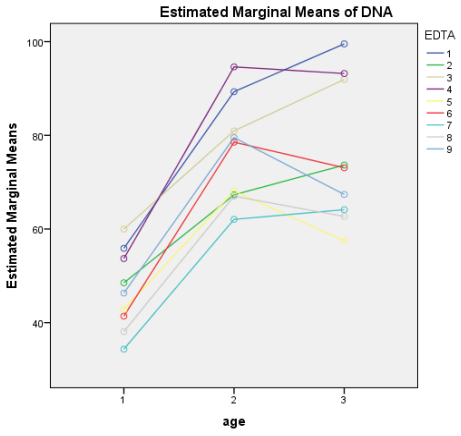


Figure 1. Estimated marginal means of DNA quality based on the effect of age and EDTA

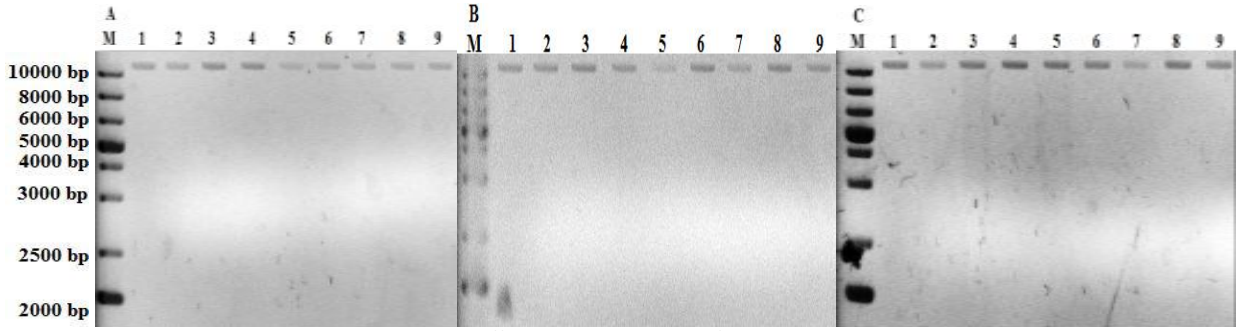
Prepared DNA samples were subjected to gel electrophoresis. All the genomic DNA samples produced clear bands with varying intensities (Fig. 2). Based on the gel results and quantitative results, 9 highest DNA samples in terms of quality and quantity were chosen (Table III and Fig. 3) and PCR-based amplification of SSR fragments of extracted genomic DNA was carried out. According to TE buffer treatment, DNAs treated with TE buffer 1, 3 and 4 possess the best quality and quantity. PCR results are shown in Fig. 3. It indicates that

the extracted DNA using this method is stable, in good quality and suitable for diverse molecular studies.

The present DNA extraction protocol only utilizes two TE buffers for DNA extraction. TE buffer contained EDTA and Tris-HCl. The first TE buffer was added for easy grinding of the leaves [9]. EDTA protects DNA against the action of the nucleus by binding to Mg ions which are cofactors for nucleases.

EDTA is often included in extraction buffers. The pH stability of the Tris-HCl buffer is important for inactivating inhibitors in plant material. Ref. [10] examined DNA extraction buffers with different components for consistency of PCR amplification. The extraction buffer contained 20mM EDTA, 100mM Tris-HCl and 28.6mM mercaptoethanol. PCR results for this treatment was reproducible and reliable. However this method utilizes mercaptoethanol which is a dangerous material. Therefore, the present method in this research is safer than that of Kawata *et al.* [10].

Comparability Groups	Mean Differences
TE buffer 1 ($EDTA_1$ = 1 mM. $EDTA_2$ = 0.1) is higher than TE buffer 2 ($EDTA_1$ = 1 mM. $EDTA_2$ = 0.2)	18.41 ng/µl
TE buffer 1 ($EDTA_1$ = 1 mM. $EDTA_2$ = 0.1) is higher than TE buffer 5 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.2).	25.42 ng/µl
TE buffer 1 ($EDTA_1$ = 1 mM. $EDTA_2$ = 0.1) is higher than TE buffer 6 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.3)	17.23 ng/µl
TE buffer 1 ($EDTA_1$ = 1 mM. $EDTA_2$ = 0.1) is higher than TE buffer 7 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.1)	28.06 ng/µl
TE buffer 1 ($EDTA_1$ = 1 mM. $EDTA_2$ = 0.1) is higher than TE buffer 8 ($EDTA_1$ = 1 mM. $EDTA_2$ = 0.2)	25.64 ng/µl
TE buffer 1 ($EDTA_1$ = 1 mM. $EDTA_2$ = 0.1) is higher than TE buffer 9 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.3)	18.41 ng/µl
TE buffer 2 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.1) is higher than TE buffer 5 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.2).	7.01 ng/µl
TE buffer 2 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.1) is higher than TE buffer 7 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.1)	9.65 higher
TE buffer 2 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.1) is higher than TE buffer 8 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.2)	7.32 ng/µl
TE buffer 3 ($EDTA_1$ = 1 mM. $EDTA_2$ = 0.3) is higher than TE buffer 2 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.1)	14.46 ng/µl
TE buffer 3 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.1) is higher than TE buffer 3 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.1)	21.47 ng/µl
TE buffer 3 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.1) is higher TE buffer 6 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.3)	13.28 ng/µl
TE buffer 3 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.1) is higher than TE buffer 7 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.1)	24.11 ng/µl
TE buffer 3 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.1) is higher than TE buffer 8 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.2)	21.69 ng/µl
TE buffer 3 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.1) is higher than TE buffer 9 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.3)	13.21 ng/µl
TE buffer 4 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.1). is higher than TE buffer 2 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.1)	17.34 ng/µl
TE buffer 4 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.1) is higher than TE buffer 9 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.3)	16.16 ng/µl
TE buffer 4 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.1) is higher than TE buffer 8 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.2)	24.57 ng/µl
TE buffer 4 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.1) is higher than TE buffer 5 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.2)	24.35 ng/µl
TE buffer 4 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.1) is higher than TE buffer 6 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.1)	26.99 ng/µl
TE buffer 6 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.3) is higher than TE buffer 5 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.2)	8.19 ng/µl
TE buffer 6 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.3) is higher than TE buffer 7 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.1)	10.83 ng/µl
TE buffer 6 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.3) is higher than TE buffer 8 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.2)	8.41 ng/µl
TE buffer 9 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.3) is higher than TE buffer 5 ($EDTA_1$ = 2 mM. $EDTA_2$ = 0.2)	8.26 ng/µl
TE buffer 9 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.3) is higher than TE buffer 7 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.1)	10.90 ng/µl
TE buffer 9 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.3) is higher than TE buffer 8 ($EDTA_1$ = 0.5 mM. $EDTA_2$ = 0.2)	8.47 ng/µl



A= week 2, B= week 3 and C= week 4. 1-9: different reagent treatments for DNA extraction method, M= 1kbp DNA ladder

Figure 2. Extracted DNA samples on 0.8% gel agarose.

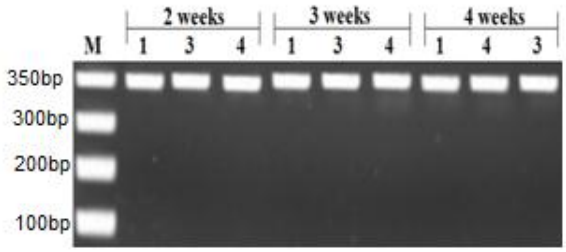


Figure 3. PCR amplification uses SSR marker. Lanes 1, 3 and 4 refer to TE buffer treatments 1, 3 and 4. M= 100bp DNA ladder.

The present method has several advantages. It is safe, low cost, fast and reliable for molecular analysis of large samples. Investigating the effect of the age of the seedling on the quality and quantity of extracted DNA increases the potential of this method with the best age of 4 weeks. It can be said that this method is time saving because samples can be collected at the seedling stage (after four weeks). Similarly, most of previous reports use samples from the seedling stage for DNA extraction (Takakura and Nishito [11], Sun *et al* [6], Xu *et al.*[4] Ahmadikhah [12], Ahmed *et al.*[5], Kawata *et al.* [10], Chen *et al.* [13].

However, the present protocol has more advantages compared to the others. Ref. [6] and ref. [4] which took the samples when the seedling one week. Zirconium Balls (Xu *et al.* [4]) and Steel Bead (Sun *et al.* [6]) were used in these methods. These objects cannot be found in every laboratory easily. In another protocol presented by Chen *et al.* [13], samples were collected after 6 days. But this method is introduced for etiolated seedlings. Whether or not, this method is more simple, time saving and safer compare with others. They use hazardous reagents such as 2-mercaptoethanol (Sun *et al.* [6]), SDS and chloroform (Xu *et al.* [4]). Sample collection in Ahmadikhah [12] method was similar seedling age as the present method (14 days). Nevertheless, the use of chloroform, which is a very dangerous material, is necessary in Ahmadikhah method.

The present DNA extraction protocol is very simple and safe without the need to use expensive or rare materials and laboratory apparatus. The amount of DNA produced was sufficient and the quality was acceptable for PCR amplification as shown in Fig. 3.

IV. Conclusion

DNA was extracted from Pokkali rice through a protocol introduced by Ikeda *et al.* [9]. In order to optimize this protocol two factors were investigated. These two factors were the age of the seedlings and EDTA concentration. The DNA extraction protocol was performed with different treatment of each factor. The results illustrated that these factors possess a significant impact on the DNA quantity. The effect reagents (TE buffer) were more considerable among these two. In addition, the effect of the age of the seedlings was obvious. Based on the results the optimized conditions are: TE buffer 1 for EDTA reagents and 4 weeks for age of the seedlings.

Appendix A

The list of significant means differences in EDTA concentration. (Measured parameter is DNA concentration)

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