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Comparative Study of High Quality Genomic DNA Extraction Protocols in Rice and Tomato Crops

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A B S T R A C T

This study presents a rapid efficient DNA extraction protocol for rice and tomato crops. Five published protocols (Doyle & Doyle CTAB Procedure, Urea Method, Salt extraction method, IRRI Method, Dellaporta DNA Extraction method) were comparatively evaluated in rice and tomato crops for DNA purity and yield. The analysis of variance revealed that the yield was significantly different ($P < 0.01$) for all protocols in both crops, whereas there was no significant observation was observed for purity. The multiple Duncan test revealed that, Doyle & Doyle CTAB Procedure was best for rice crop (2481 ng/µl), whereas urea method was best for tomato crop (2685 ng/µl). Here Proposed that urea method, Doyle & Doyle CTAB Procedure is best method for good quality DNA and higher yield in tomato and rice crop respectively. Hence this method of extraction would be ideal for genotyping of large populations of rice and tomato genotypes.

Introduction

Biotechnology has emerged the most important new resource for achieving the global food production through release of new variety early. The emergence of plant transformation and molecular marker analyses in genome studies has greatly enhanced the speed and efficacy of crop improvement and breeding programmes. A prerequisite for taking advantage of these methods is the ability to isolate genomic DNA of superior quality and quantity for analyzing through Polymerase Chain Reaction (PCR), restriction enzyme digestion and subsequent Southern blot hybridization. The quality and quantity of DNA required depend upon the objective.

For marker assisted selection the amount of DNA needed is small and need not be stored for a long duration where as in QTL mapping or population studies of RILS, F_2 population required large amount of DNA (McCarthy *et al.,* 2002; Guo-Liang *et al.,* 1993) and also stored for long duration. However, to obtain accurate result, it is necessary to isolate good quality DNA that is relatively free from the many contaminants found in plant cells (Jobes *et al.,* 1995).

DNA extraction is used in the genetic modification of plants. Many agricultural companies use genetic extraction to create

DNA that they then modify to make a particular genetic strain of a crop that is resistant to various chemicals or pests. The DNA sequencing reliability entirely depends upon on purity and yield of DNA. Among the cereals and vegetables rice and tomato are very important crops respectively. Nowadays, crop improvement works are going on these crops such as cold tolerance in tomato and aerobic rice in case of rice. For crop improvement programme, the genomic DNA should be pure and high yield. A vital protocol of genomic DNA isolation helps in molecular characterization in these crops. Although Several protocols are available for genomic DNA isolation such as Dellaporta *et al.,* 1983; Chang *et al.,* 1993; Sperisen *et al.,* 2000; Sharma *et al.,* 2003; Kang and Yang, 2004; CTAB method (Doyle and Doyle, 1987) and its modified protocol (Huang *et al.,* 2000; Rogers and Bendich, 1985), procedures etc. But all these protocols don't give promising result in all cases. CTAB methods also have some demerits of obscuring absorbance readings at 260 nm due to the interference of residual CTAB in the final DNA solution (Doyle & Doyle, 1990). Liquid nitrogen and proteinase k are needed for CTAB protocols that are very expansive.

The choice of a method depends on many factors; the required quantity and molecular weight of the DNA, the purity required for downstream applications, time and expense. Since size, content and organization of genome and contents of metabolites of different plant systems vary from each other to a great extent, a single DNA isolation protocol is not likely to be applicable to all plant systems (Loomis, 1974). In recent decades, a well-known use for genetic extraction is germplasms characterization, genetic diagnostics, characterization of transformants, study of genome organization, phylogenetic analysis, marker-assisted selection, mapping Quantitative Trait Loci (QTL), DNA sequencing etc. (Gupta *et al.,* 1999). For these works need pure and high yield of DNA in limited resources.

Keeping in view the above point, the present experiment was conducted to identify the best DNA isolation method suited for isolation of reasonably pure DNA in sufficient amount from rice and tomato leaves that can be stored for a longer duration and lasting for several PCR reactions.

Materials and Methods

Collection of plant sample

The plants used for genomic DNA extraction were grown in a greenhouse. The leaf sample was collected from rice variety-IR 64 and tomato variety Arka Alok. Rinsed the leaf sample in running tap water then dried and stored for 24 hrs in a deep freezer a (-20° C). In all method DNA was extracted without using liquid nitrogen.

DNA extraction

The DNA was extracted from healthy fresh young leaf tissue of rice and tomato variety with four replications by following five published protocols viz: A. Doyle & Doyle CTAB Procedure (Doyle & Doyle, 1987) B. Urea Method (Nalini *et al.,* 2003) C. Salt extraction method (Aljanabiand Martinez, 1997) D. IRRI Method, (Zheng *et al.,* 1995) E. Dellaporta DNA Extraction method, (Dellaporta *et al.,* 1983). The DNA was extracted by all five methods in both crops with four replications.

Doyle & Doyle CTAB procedure (Doyle & Doyle, 1987)

5.0 -7.5 ml of CTAB isolation buffer (2 % cetyltrimethyl ammonium bromide [CTAB], 1.4 M sodium chloride [NaCl], 0.2 % 2-

mercaptoethanol, 20 mM ethylenediamine tetra acetic acid [EDTA], 100 mM Tris-HCl, pH 8.0) was preheated in a 30 ml glass centrifuge tube to 60 ^{o}C in a water bath. Fresh leaf tissues were collected 0.5 g and grinded in CTAB isolation buffer in a preheated mortar. Samples were then incubated at 60°C for 30 min. with occasional gentle swirling. Then it was extracted with chloroform-isoamyl alcohol (24:1), by mixing gently followed by centrifugation at 6,000 x g for 10 min. at room temperature. Aqueous phase was removed with wide bored pipette and transferred to clean glass centrifuge tube in which 2/3 volumes cold isopropanol was then added to precipitate nucleic acids for an hour. After precipitation of nucleic acid, the supernatant carefully removed and washed with 70 % ethanol gently followed by allow it to air dry briefly at room temperature. Finally nucleic acid pellet was re-suspended in 100 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).

Urea Method (Nalini, *et al.,* 2003)

The 0.5 g of leaf tissue was grinded in mortar after adding 2 ml of extraction buffer. The extraction buffer (pH 8.0) contains 100 mM Tris, 20 mM EDTA, 0.5 M NaCl, 7 M Urea, 0.1 % β-mercaptoethanol and 2 % Sodium dodecyl sulfate [SDS]. The homogenates were transferred to a 2 ml-microfuge tubes. An equal volume of phenol: chloroform: Isoamlyalcohol (25:24:1) was added to the tubes and mixed well.The tubes were centrifuged at room temperature for 15 min. at 15,000 rpm. The upper aqueous phase was collected in a new tube and an equal volume of chloroform: Isoamlyalcohol (24:1) was added and mixed. The upper aqueous phase obtained after centrifuging at room temperature for 10 min. at 15,000 rpm was transferred to a new tube. The DNA was precipitated from the solution by adding 0.1 volume of 3 M Sodium acetate pH 7.0 and 0.7 volume of Isopropanol. After 15 min. of incubation at room temperature the tubes were centrifuged at 4ºC for 15 min. at 15,000 rpm. The DNA pellet was washed with 70 % ethanol and then very briefly with 100 % ethanol and air dried. The DNA was dissolved in 100 µl TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM).

Salt Extraction Method (Aljanabiand Martinez, 1997)

The fresh 0.5 g tissue was homogenized in 400 µl of sterile salt homogenizing buffer (0.4 M NaCl 10 mM Tris-HCl pH 8.0 and 2 mM EDTA pH 8.0), using a Polytron Tissue Homogenizer, for 10-15 sec. Then 40 µl of 20 % SDS (2 % final concentration) and 8 µl of 20 mg/ml protenase K (400 µg/ml final concentration) were added and mixed well. The samples were incubated at 55-65 ^{0}C for at least 1 hr. or overnight, after which 300 µl of 6 M NaCl (NaCl saturated H_2O) was added to each sample. Samples were vortexed for 30 s at maximum speed, and tubes spun down for 30 min at 10000 g. The supernatant was taken and transferred to fresh tubes. An equal volume of isopropanol was added to each sample, mixed well, and samples were incubated at –20 ^{0}C for 1 hr. Samples were then centrifuged for 20 min, 4 ^{0}C, at 10 000 g. The pellet was washed with 70 % ethanol, dried and finally resuspended in 100 µl TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM).

IRRI Method (Zheng *et al.,* 1995)

The 0.5 g Leaf tissue was grinded in 200 µl extraction buff er [Tris-HCl 50 mM (pH 8), EDTA 25 mM, NaCl 300 mM, SDS 1%]. After grinding, 300 µl of extraction buff er was added and transferred to 1.5 ml tube containing another 500 µl. In order to remove proteins, 500 µl chloroform/isoamylalcohol (24:1) was added to the tube and mixed by inversion. The samples were then centrifuged for 1 min at 15115 g. Supernatant (750 µl)

was removed and 750 µl of 100 % ethanol was added to precipitate the DNA. After mixing by gentle inversion, the samples were centrifuged for 3 min. at >13000 rpm. The supernatant was then discarded and washed with 70 % ethanol. The pellet was then dried by leaving on the on bench at room temperature for at least 20 min (for the evaporation of residual ethanol). Finally, the DNA was re-suspended in 100 µl of TE Buff er (10 mM TrisHCl, 1 mM EDTA pH 8.0) and stored at - 20^0C.

Dellaporta DNA extraction method (Dellaporta, *et al.,* 1983)

Weigh 0.5 g of tissue, freeze in -80^0c and grind to fine powder in a mortar and pestle. Transfer the fine powder into a 30 ml Oak Ridge Tube and add 15 ml extraction buffer. Add 1 ml of 20 % SDS to each tube. Make sure libs are screwed on tightly.

Mix thoroughly by vigorous shaking, and then incubate tubes in a 65°C water bath for 10 min. Add 5 ml of 5 M Potassium acetate [KOAC]. Mix thoroughly by vigorous shaking, then incubate tubes at 0°C (on ice) for 20 min. Spin tubes at 13,000 rpm for 20 min. in a Sorval centrifuge with the SA-600 rotor(~25,000 x g) Pour supernatant through Miracloth (cheesecloth) into a 30 ml Oak Ridge Tube containing 10 mM isopropanol invert 20 times to mix well and incubate at -20 °C for 30 min. Spin tubes at 12,000 rpm for 15 min. in a Sorval centrifuge with the SA-600 rotor(~20,000 x g).

DNA quantification

The amount of genomic DNA was quantified by ND-1000 spectrophotometer (Nano Drop inc. 2007). The purity of genomic DNA of rice and tomato was measured by obtaining the absorbance ratio A260/280 using the Nanodrop spectrophotometer.

DNA purification

The genomic DNA was treated with 10 µl of (10 U/µl) RNase A. Incubate at 37° C for 10 min. after that sample was inactivated by heat treatment at 70^0 C for 15 min. and kept sample on ice to cool. For ethanol precipitation added 1/10 volume of 3 M Sodium Acetate and 2.5 volumes of 100 % ethanol mixed the sample and Placed at-20^0 C overnight. The sample centrifuged at 4^0 C for 20 min. washed the pellet with 70 % ethanol (cold). Spin sample at 13,000 rpm at 4^0 C for 3-5 min. The pellet dried and resuspend with 100 ul of TE buffer.

Agarose gel electrophoresis

To evaluate the quality and intactness of the extracted DNA, gel electrophoresis was used. The extracted DNA of both crop tomato and rice 500 ng/ µl (5 µl) was loaded on 0.8 % agarose gel (Genei, Bangalore), which contained ethidium bromide for DNA staining. For image acquisitions, gels were visualized under UV light and documented by using the gel documentation system (Alpha Innotech, Fluor ChemR FC2).

Statistical analysis

The mean and variance of yield and purity of genomic DNA mentioned above were subjected to statistical analysis by R software 3.0 (online free version).

Results and Discussion

The result of analysis of variance revealed that significant differences were observed between the mean DNA yields of rice and tomato using the five methods (Table 1). There was no significant variance observed for DNA purity of both crops using all methods. It is due to the genomic DNA was treated with Rnase A enzyme so RNA

contamination was eliminated. The result of Duncan's Multiple Range Test state that all five method of the mean yield of DNA were significantly different in both crop rice and tomato (Table 2). In rice, the genomic DNA yield was maximum observed by Doyle & Doyle CTAB Procedure, (2481 ng/µl) followed by Urea Method (1928 ng/µl), Dellaporta DNA Extraction method (1235 ng/µl) whereas, in tomato, the maximum was observed by Urea Method (2685 ng/µl) followed by Dellaporta DNA Extraction method (1979 ng/µl) and Doyle & Doyle CTAB Procedure (1419 ng/µl) (Table 3).

The amount of DNA was obtained in rice 2481 ng/µl and in tomato 1419 ng/µl by Doyle & Doyle CTAB Procedure. It indicates that this method is good for high amount of DNA isolation in both crops. CTAB is an ionic detergent, which form an insoluble complex with nucleic acids in a low salt environment. Under these conditions, polysaccharides, phenolic compounds and other contaminants remain in supernatant and can be washed away. The DNA complex is solubilised by raising salt concentration resulting a good yield and purity.

The yield and purity of genomic DNA were compared in both of the crops for all the methods. By Doyle & Doyle CTAB Procedure, amount of DNA was maximum in rice crop where as in tomato third most. The amount of DNA was low and purity also poorer in both crops rice and tomato crops by IRRI Procedure therefore this method is not suitable for longer storage and genotyping of the crops.

By urea method, the amount of DNA was maximum in tomato sample (2685 ng/µl) and second most in rice sample (1928 ng/µl). This amount of DNA yield was better than recent studies (Laxmi prasanna *et al.,* 2013; Healey *et al.,* 2014; Rawat *et al.,* 2016; Sajib *et al.,* 2017). Urea is a chaotropic agent, and its role is to remove DNA from the histones and denature other proteins and promote more stability to the system. Sodium dodecyl sulphate (SDS) is a strong anionic detergent that can solubilize the proteins and lipids that form the membranes. This helps to rupture cell membrane and nuclear envelop and expose the chromosomes that contain the DNA. In addition to remove the membrane barriers, SDS helps release the DNA from histones and other DNA binding proteins by denaturing them. Hence in urea method, the urea along with SDS helps to get a higher concentration of DNA. Nalini *et al.,* (2003), DNA was obtained on an average 200 µg of from the 0.5 g of leaf tissue of wheat plants. For PCR reaction 100 ng is required for one reaction so this amount of DNA is sufficient for 2000 reactions. In present study, the amount of DNA was obtained 2685 ng/µl from 0.5 g of leaf tissue of tomato and total volume maintained at 100 µl which is equivalent to 2685 PCR reactions. The result showed that the DNA isolated by urea method provides high quality pure DNA and high yield which is suitable for genotyping.

The purity of DNA extracted by this method was measured by ND-1000 spectrophotometer and running DNA on 0.8 % agarose gel. The purity of DNA was estimated by the ratio of absorbances at 260 nm (A260) and 280 nm (A280), A260/A280. The purity of DNA is considered to be best if it ranges between 1.8-2.0. In this study the average range was observed between 1.8-2.0 in both crops (Table 3 and 4). It indicates that there is no much contamination in all methods. Pure DNA was obtained from these protocols could be as a result of the RNase A treatment. It was reported that the absence of RNA are evidence of a good quality genomic DNA (Sambrook, 2001). The A260/A230 values for pure nucleic acid are often higher than the respective A260/A280 values.

Fig.1 Agarose gel profile of Genomic DNA of all five methods with four replications in rice crop

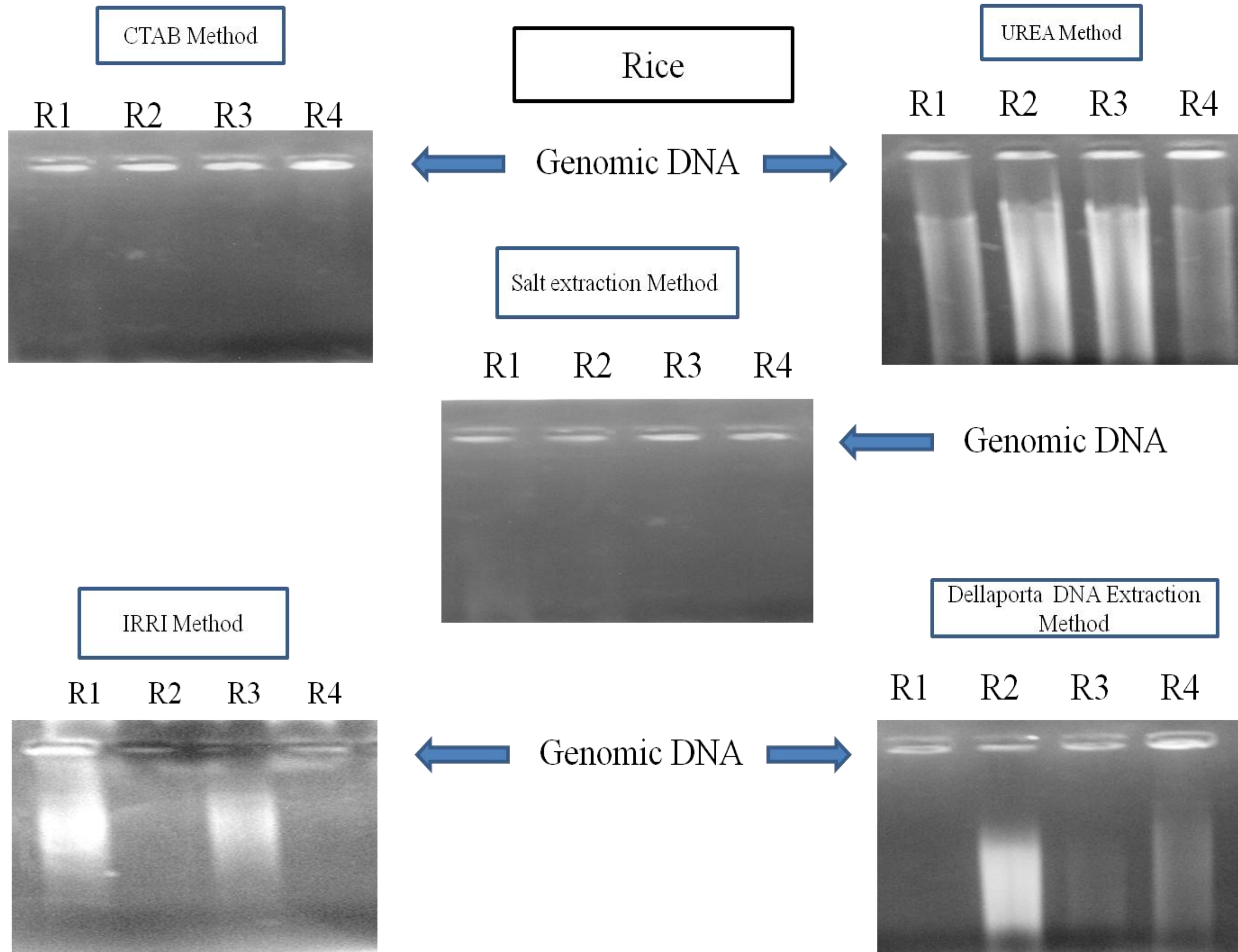

Fig.2 Agarose gel profile of Genomic DNA of all five methods with four replications in tomato crop

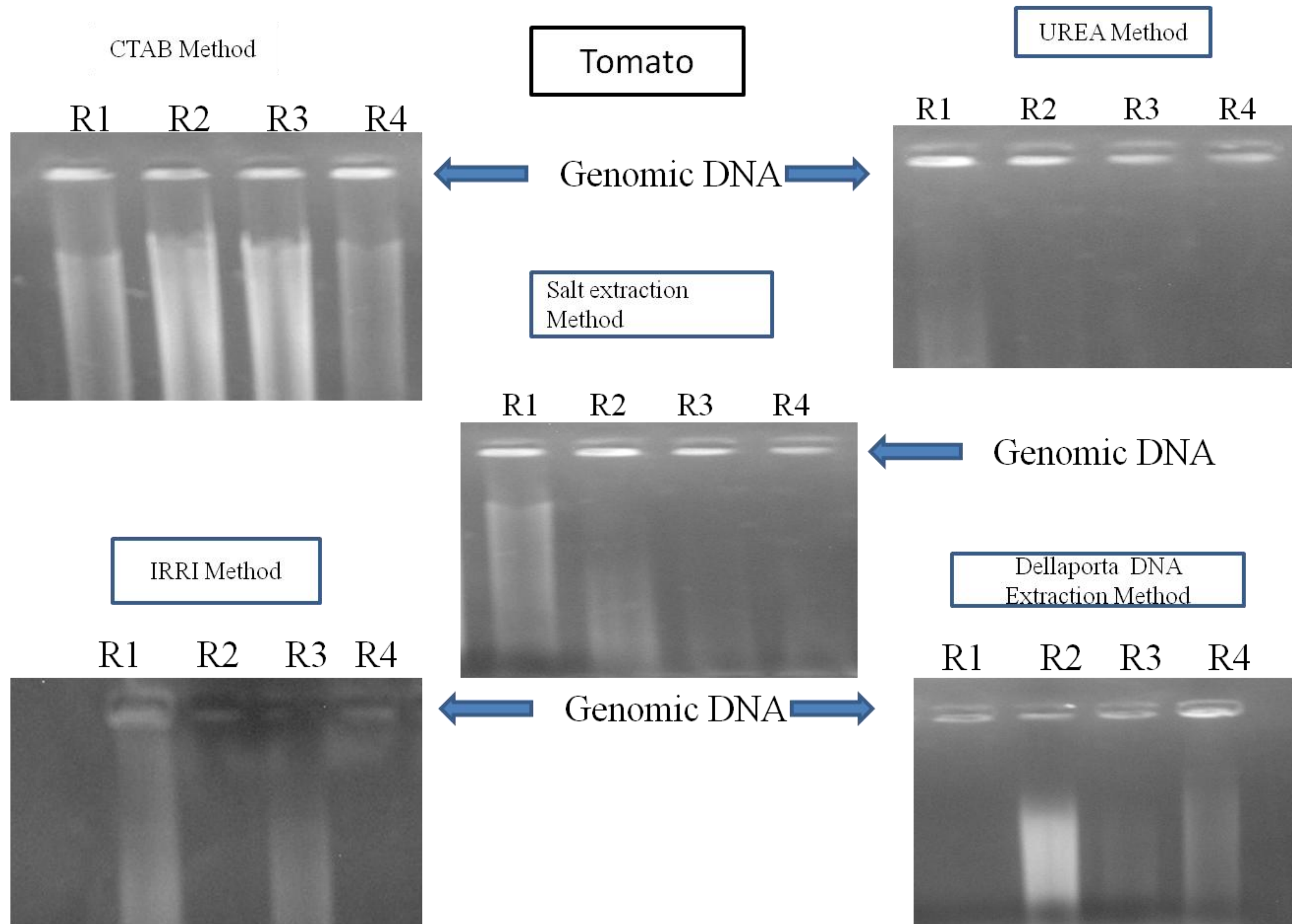

Table.1 Analysis of variance for amount of genomic DNA of rice and tomato

Source of variation		Degrees of freedom	Mean sum of squares	
Sl. No.			Rice	Tomato
1	Treatments	4	2749805.20*	3279977.20*
2	Error	15	9413.13*	6628.40*
3	Total	19		
4	CD (0.01)		202.17	169.65
5	Coefficient of Variation		7.011	5.55

*significance at level 1%

Table.2 Amount of genomic DNA of rice and tomato in replicated data

Methods	R1(ng/µl)		R2(ng/µl)		R3(ng/µl)		R4(ng/µl)		Average (ng/µl)	
	Rice	Tomato	Rice	Tomato	Rice	Tomato	Rice	Tomato	Rice	Tomato
Doyle & Doyle CTAB Procedure	2410	1521	2600	1390	2690	1460	2224	1305	2481 a	1419 abc
Urea Method	1960	2710	1983	2640	1900	2790	1869	2600	1928 ab	2685a
Salt extraction method	860	750	890	900	880	790	878	960	877 cd	850bc
IRRI Method	395	450	398	360	405	415	396	351	398.5 d	394c
Dellaporta DNA Extraction	1190	2015	1250	1960	1231	2060	1269	1881	1235 bc	1979ab

Values followed by the same letters are not significantly different

Table.3 Evaluation of tomato genomic DNA samples extracted using five protocols according to DNA purity and yield (ng/ul)

Sl. No.	Methods	Avg. DNA conc. (ng/ul)	Avg. Purity of DNA obtained(nm)		RNase A Treatment
			260/280	260/230	
1.	Doyle & Doyle CTAB Procedure	1419.37	1.89	1.99	Yes
2.	Urea Method	2685.22	1.83	2.00	Yes
3.	Salt extraction method	850.92	1.69	1.99	Yes
4.	IRRI Method	394.2	1.76	1.05	Yes
5.	Dellaporta DNA Extraction	1979.57	1.86	1.98	Yes

Table.4 Evaluation of rice genomic DNA samples extracted using five protocols according to DNA purity and yield (ng/ul)

Sl. No.	Methods	Avg. DNA conc. (ng/ul)	Avg. Purity of DNA obtained(nm)		RNase A Treatment
			260/280	260/230	
1.	Doyle & Doyle CTAB Procedure	2481.36	1.85	2.2	Yes
2.	Urea Method	1928.06	2.00	2.01	Yes
3.	Salt extraction method	877.80	1.83	1.99	Yes
4.	IRRI Method	394.2	1.77	1.50	Yes
5.	Dellaporta DNA Extraction	1235.6	1.79	1.91	Yes

Expected A260/A230 values are commonly in the range of 2.0-2.2. In present study the average range was observed between 2.0-2.2 except by IRRI method in both crops rice and tomato (Table 3 and 4).

The integrity of the extracted DNA was also analyzed by agarose gel electrophoresis. Both crop of genomic DNA were electrophoresed with four replications and visualized in gel documentation system. Intact DNA was observed in all samples. This clearly demonstrated that the isolated total DNA from isolates was of a high quality and could be used in downstream applications. The DNA band was visible in all samples except in IRRI method where the amount of DNA was low (394.2 ng/μl) in both crops rice and tomato (Figs. 1 & 2). The good quality of DNA extracted in our study is comparable to various other studies where it was reported that good quality DNA can be isolated without using liquid nitrogen (Chandra and Tewari, 2009; Sharma *et al.,* 2010; Ferdous *et al.,* 2012; Ambawat *et al.,* 2017). The genomic DNA was isolated by five methods in both crops rice and tomato. The amount of DNA quantified and identified the urea method is best for tomato and Doyle & Doyle CTAB Method is best rice crop. For high amount of DNA, the urea method can be used in tomato crops and Doyle & Doyle CTAB Method can be used in rice crop for further genotyping of crops.

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