Optimization of DNA isolation and PCR protocol for RAPD analysis of banana / plantain (*Musa* spp.)

Das B.K.[1*], Jena R. C.[2] and Samal K.C.[3]
[1]Depertment of Horticulture,OUAT,BBSR,Orissa, Ph. 9437230926, Email. dr_bkd@yahoo.co.in
[2]Biotechnology cum Commercial Tissue Culture Centre, Orissa University of Agriculture and Technology, Bhubaneswar-751003, Orissa, Ph.9439203191, Email. jena_ramchandra@yahoo.co.in
[3]Department of biotechnology,OUAT,BBSR,Orissa, Ph.9437229140, Email. samalkc@rediffmail.com

Abstract- Genetic analysis of plants relies on high yields of pure DNA samples. Here we present the optimization of DNA isolation and Polymerase chain reaction (PCR) conditions for Random Amplified Polymorphic DNA (RAPD) analysis of banana/plantain. The leaf of banana contains high level of polysaccharides, poly phenols and secondary metabolites. The extracted DNA from these cultivars when subjected to PCR is often problematic, especially when mature tissues are used for DNA extraction. In order to overcome these problems a protocol has been developed, availing on a high salt concentration and on the combination of Polyvinyl pyrrolidone (PVP) and Cetyl trimethyl ammonium bromide (CTAB) in the extraction buffer, in order to prevent the solubilization of polysaccharides and polyphenols during the DNA extraction method. It also involves successive long term chloroform: Isoamylalcohol extractions, an long term RNAse treatment with all steps carried out at Room temperature (RT). Using this method, DNA was extracted from different banana species including young leaves, old leaves, frosted old leaves and withered old leaves. The yield of DNA ranged from 1-2 µg / µl per gram of the leaf sample / tissue and the purity ratio was between 1.6-1.7 indicating minimal levels of contaminating metabolites. The technique is ideal for isolation of DNA from different plant species / cultivars and the isolated DNA were used for RAPD analysis. The optimization of RAPD protocol was based on the use of 50 ng of template DNA, higher concentration of $MgCl_2$ (3 mM) and lower concentration of primer (0.6µM), Taq DNA polymerase (1.5 units) and an annealing temperature of 35°C, which resulted, optimal amplification. In all PCR reactions Reproducible amplifiable products were observed. Thus the results indicate that the optimized protocol for DNA isolation and PCR was applicable to plant species belonging to different genera and this process is suitable for further work on diversity analysis. Furthermore, here we used suitable DNA isolation protocol for RAPD analysis to study the genetic variation in the future in *Musaceae* species grown in Orissa.
Key Words: Banana, *Musa*, PCR, DNA isolation, RAPD

Introduction

The center of origin of banana (*Musa* spp.) is Asia (Primarily southern and southeastern). The plant Banana and plantains (*Musa* spp.), the forth most important fruit crop in the world, are vegetatively propagated crops with great economic important in tropical and sub tropical countries. Banana is an important member of the kingdom plantae, family Musaceae and belongs to genus *Musa* order Zingiberales. The estimated current world production of banana is 97.5 million tones per year, covering 10 million ha.. India is the largest producer of banana in the world contributing 19.71% to the total global production of banana, with a total production of 19.19 million tones from an area of 0.565 million ha. [1] and 3rd in area among fruit crops. It account for 13% of the total area. It seems that it is one of the earliest fruit grown by man kind at then dawn of civilization. Banana (Musa spp.), is the 2nd most important fruit crop in India next to mango. Its year round availability, affordability, varietals range, test, nutritive and medicinal value makes it the favorite fruit amongst all classes of people. Orissa, Karnatakka, Kerala, Madhya Pradesh, Maharashtra and west Bengal are major banana growing states in India [1]. Many plants are found in the genus; those that bear edible fruit are the most significant. In addition to fruit plantains and bananas provide many cultivars with medicines, beverages, fibers, edible floral parts, dyes, fuel, steam for cooking, cordage etc. Banana is the rich source of carbohydrates and vitamins, particularly vitamins B; it is also a good source of potassium, phosphorus, calcium and magnesium. The fruit is easy to digest free from fat and cholesterol. Banana powder is used as the fist baby food. It helps in reducing risk of heart disease when used regularly and is recommended for patients suffering from high blood pressure, arthritis, ulcer, gastroenteritis, and kidney disorders. Beside these it has great medicinal value. The multi purpose use has attracted global attention which prompted us to conduct preliminary studies on genetic diversity in *Musa* spp. Isolating high quality DNA is essential for molecular research. Banana leaf contains exceptionally high amounts of polysaccharides, polyphenols and secondary metabolites such as alkaloids, flavonoids and phenols, and terpenes which interfere with DNA isolation procedure. The problem encountered due to these compounds include co isolation of highly viscous polysaccharides degradation of DNA due to endonuclease, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with enzymatic reactions. Moreover, the contaminating RNA that precipitates along with DNA causes suppression of PCR amplification and other many problems [2, 3] interference with DNA amplification involving random primers e.g. RAPD analysis [3] and improper priming of DNA templates during thermal cycle sequencing. By using one DNA isolation protocol the optimal DNA yield may not possible from different plant taxa. For example, some closely related species of the same

genus require different isolation protocols. So we have to standardize an efficient protocol for DNA isolation and optimization of the PCR condition. Various protocols for DNA extraction have been successfully applied to many plant species [4-9] We have tested previously mentioned DNA isolation protocols in some fruit crops that contain high polysaccharide level such as different banana (*Musa species*), lichi (*lichi chinensis* S.) guava (*psidium guajava* L.).But these methods resulted in DNA with lot of impurities and not very suitable for RAPD analysis. Therefore, we report here a total genomic DNA isolation protocol derived from a method originally developed for other plants [9]. Modification were made to minimize phenols, polysaccharide co-isolation and to simplify the procedure for processing of large number of DNA samples, Using polyvinylpyrolidone (PVP) and high salt concentration; [10, 11] DNA extraction from plant is preferentially performed from young tissues due to the lower content of polysaccharides, Polyphenols and other secondary metabolites which co-precipitate with DNA in the extraction procedure, inhibit DNA digestion and PCR [12] , presumably by irreversible interactions with DNA [13]. The use of primers, Taq polymerase, quantity of DNA, DNTPs, and the reaction volume to standardize the protocol for RAPD is proved to be inexpensive. Thus the protocol derived for both RAPDs and genomic DNA isolation, is genus independent, efficient, inexpensive, simple, and yields pure DNA amplifiable by PCR as indicated by the results of the RAPD technique. The DNA which is isolated by the present protocol is suitable for further downstream applications.

Materials and Methods

Plant Material

The 12 banana (*Musa* spp.) cv. Pata kapoora, Chepta bantala, Grandnine, Singapoori, Robusta, Champa, Banua bantala, Batisia bantala, Amruta pani, Paunsia bantala, Katia, Gaja bantala etc were used in this research. The leaf samples of 12 varieties were collected from various places in the state of Orissa. One gram of young leaves was harvested fresh for DNA isolation.

Reagents/Chemicals used

An extraction buffer consisting of 3 % CTAB (w/v), NaCl (2 M), Tris HCl pH 8.0 (100 mM) and EDTA pH 8.0 (20 mM), PVP (1.5 %) , β-mercaptoethanol 2.5 % (V/v). was prepared. Ribonuclease A (10 mg/ml), chloroform: Isoamylalcohol (24:1) v/v/v), Ethanol (70 %, 100 %), Sodium acetate (3M) solution (pH 8.0), and TE buffer (Tris HCl, 10 Mm,pH 8.0 , 1mM EDTA; pH 8.0) were the additional solutions required.

DNA isolation protocol

a. Freshly harvested young and tender or old leaf samples (1 g) were ground in liquid Nitrogen using a pre-chilled mortar and pestle.
b. The ground powder was quickly transferred into a clear autoclaved 50 ml centrifuge tube and then 10 ml of pre-warmed (60^{o}C) extraction buffer was added and shaken gently to form slurry.
c. The tubes were incubated at 60^{o}C in circulating water bath for one hour with intermittent shaking for every 10 minutes with occasional inversion and cold to normal temperature.
d. An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed properly by inverting the tubes 20-25 times to form an emulsion and centrifuged at 12000 rpm for 20 minutes at RT to separate the phases (long term mixing of samples in chloroform: isomyl alcohol approximately for 30 minutes, will help in removal of pigments and formation of brownish colour in DNA sample can be omitted).
e. The supernatant was carefully decanted and transferred to a new tube and the second chloroform: isoamyl alcohol (24:1) extraction performed for the cloudy nature of aqueous phase due to presence of PVP.
f. Again the supernatant was carefully decanted and transferred to a new tube and was precipitated with two volume of pre chilled (-20^{o}C) 95 % ethanol and sodium acetate (final concentration 0.3 M), and gently mixed by inverting up and down (10 minutes) to produce fibrous DNA and incubated at -20^{o}C for a minimum of one hour.
g. The samples were centrifuged at speed 10,000 rpm for 15 minutes. Pour off the supernatant and the pellet was washed twice and thrice with 70 % ethanol. Decanted the supernatant and air dried DNA pellet at RT until the whitish pellet turned to transparent and resuspended in 300 µl of TE Buffer and 6µl of RNAase (10 µg/µl) was added incubated at 37^{o}C for two hour (RNAase treatment helped achieving in proper genomic DNA). To this 600 µl of Ice chilled ethanol and 10 ml of 3M sodium acetate was added and incubated at -20^{o}C for one hour to re-precipitate DNA.
h. The solution was centrifuged at 10,000 rpm for 15 minutes; DNA pellet was dried at 37^{o}C and resuspended in 300 µl of Tris–EDTA (TE) buffer. All the centrifugation steps were carried out at RT to avoid precipitation with (CTAB, DNA degradation and to obtain good quality of DNA.

Quantification of extracted DNA and purity checking

The yield of DNA per gram of leaf tissue isolated was measured using a UV spectrophotometer at 260 nm. The purity of DNA was determined by estimating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the samples on 0.8 %. Agarose gel depending on the intensities of band when compared with lambda DNA marker (used to determine the concentration). The nucleic acid concentration was estimated following [14].

Optimization of RAPD reaction

For the optimization of RAPD reaction using DNA extracted from 12 different banana varieties, Oligonucleotide primers from RPI-C series (Bangalore Genei, Bangalore ,India) and also OPC series (Operon, Technologies Inc. Almeda CA, USA) were used for amplification to standardize the PCR conditions. The reactions were carried out in a DNA thermocycler (Bio Rad). Reactions without DNA were used as negative controls. Each 25 µl reaction volume contained about 1 x PCR Buffer (10mM Tris-HCL pH 8.3;50mM KCL), 3mM $MgCl_2$, 200 µM dNTP mix, 1.5U of Taq DNA polymerase(Genei, Bangalore) 0.6µM of single primer (Genei, Bangalore and Operon Technologies Inc. Almeda CA, USA), 50 ng of temptate DNA. The thermocycler was programmed for an initial denaturation step of 2 min at 94 ^oC, followed by 36 cycles of 1 min at 94^oC, 1min at 35^oC, extension was carried out at 72^oC for 3 minutes and final extension at 72^oC for 5 minutes and at last the hold temperature was of 4^oC. PCR products were electrophoresed on 1.5% (W/V) agarose gels, in 1 x Tris Borate-EDTA (TBE) Buffer at 70 V for Five hour and then stained with elthidium bromide (1.0 µg/ml). Gels with amplification fragments were visualized and photographed under UV light. Medium range DNA Ruller was used as molecular marker (Bangalore Genei, Bangalore, India) to know the size of the fragments. For each experiment the reproducibility of the amplification products was tested twice using similar reaction conditions at different times.

Results and discussion

At present several banana varieties have many synonyms in different regions which make identification difficult. Differentiation of cultivars through morphological features is inefficient and inaccurate. Several methods have been used to investigate the genetic variability present in *Musa* germplasm. Morpho-texonomic cherecters were the first developed and optimized for bananas and 119 descriptors were defined to cherecterize Musa germplasm [15]. Biochemical markers such as Isoenzyme and protein patterns though minimally influenced by the environment offer limited Polymorphism and often do not allow discrimination between closely related genotypes. Many of these drawbacks of morphological and Bio-chemical markers can be over come through direct identification of genotypes with DNA based genetic markers. Recently reliable DNA based genetic markers have been developed and introduced for Banana varieties identification .Unlike protein markers, DNA markers segregate as single gene and they are not affected by the environment. Many researches on DNA based molecular markers is in progress in various research institutions all over the world like RAPD, ISSR, AFLP, and Micro satellite etc. A simple, reliable, unambiguous, fast and cost effective determination of genetic diversity in plant varieties is essential for the proper varietals identification, classification, and conservation, finally helpful for plant improvement. The RAPD (Random Amplified Polymorphic DNA) method has advantages over other kinds of DNA-based genetic markers, it is relatively quick, easy to perform, comparatively cheap, highly informative, need no information of template DNA sequence and synthesis of specific markers more amiable to automation then conventional techniques and nanogram quantities of DNA are required.

DNA extraction was standardized by modifying some of the steps in original CTAB DNA isolation protocol [9]. Different stages of leaves i.e. tender, young, old are used for DNA isolation. Samples frozen in liquid nitrogen and preserved at -20^oC for several weeks can also be used. The procedure Presented here resulted in extracting, high quality, low polysaccharide genomic DNA from 12 different banana varieties belonging to genus *Musa*. Tannins, terpenes and resins considered as secondary metabolites are also difficult to separate from DNA [7]. Certain polysaccharides are known to inhibit RAPD reactions. They distort the results in many analytical applications and therefore, lead to wrong interpretations [16]. Polysaccharide co-precipitations avoided by adding a selective precipitant of nucleic acids, i.e., centyl trimethyl ammonium bromide (CTAB) to keep polysaccharides in solution through SDS [17].The presence of polyphenols , which are powerful oxidizing agents present in many plant species, can reduce the yield and purity by binding covalently with the extracted DNA making it useless of most research applications [3, 18-20].The light brown DNA Obtained in some cases is due to oxidation of phenolic compounds[21].Mixing of PVP along with CTAB may bind to the polyphenolic compounds by forming a complex with hydrogen bonds and may help in removal of impurities to some extent. The removal of chlorophyll and other colouring substances such as pigments, dyes, etc. occured by the help of Long term chloroform: isoamyl alcohol treatment. Many DNA isolation procedures also yield large amounts of RNA, especially 18S and 25S rRNA [9]. The yield of PCR reduction can be possible by large amounts of RNA in the sample. A

prolonged 2 hour RNase treatment degraded RNA into small ribbon nucleosides that do not contaminate DNA preparation and yielded RNA Free pure DNA. To avoid the DNA degradation and precipitation for some extent all the steps were carried out at RT .Additional centrifugation steps, modified speed and time removed large amounts of precipitates like protein and polysaccharides .we found these modified steps necessary to standardize and increase the quality and quantity of genomic DNA. The degree of purity and quantity varies between applications [3]. Isolation of good quality DNA suitable for analysis from semi-processed or processed botanicals is also a challenge [22].The isolated DNA was of high quality as it showed a reading in between 1.6 to 1.7 after calculating the ratio of absorbance 260/280 nm Fig.(1). The DNA yield found ranged from 1 to 2 ng /µl. DNA isolated by this method yielded strong and reliable amplification products showing its compatibility for RAPD-PCR using random decamer primers Fig. (2). The amplified fragments size ranged in between to 200 bp to 3500 bp. For RAPDs almost all the tested parameters like the primer, Taq polymerase, dNTPs, magnesium chloride, concentration of template DNA and temperature and time intervals during denaturation , annealing and elongation were optimized which also had an effect on amplification, reproducibility and banding patterns. The described conditions in the present work, modified for use in RAPD analysis, consistently amplified DNA fragments of different Banana varieties belonging to genus *Musa*, which are highly recalcitrant. The optimized DNA isolation and RAPD technique may serve as an efficient tool for further genetic studies. We have recently performed this protocol for genomic DNA isolation from withered old leaves and young leaves of other fruit crops. These samples included lichi (*lichi chinensis* S.) and guava (*psidium guajava* L.). Results prove the reproducibility, reliability and practicality of this protocol. Thus we concluded that not only the present protocol describes a reliable, simple, and consistent DNA isolation method for *Musa spp*. but also applicable for other common fruit crops like lichi, guava etc.

Abbreviations

RAPD- Random Amplified Polymorphic DNA
PCR- Polymerase chain reaction
PVP- Polyvinyl pyrrolidone
CTAB- Cetyl trimethyl ammonium bromide
RT- Room temperature
Taq- Thermus aquaticus
PVP- Polyvinylpyrolidone
TE- Tris–EDTA
TBE- Tris borate-EDTA

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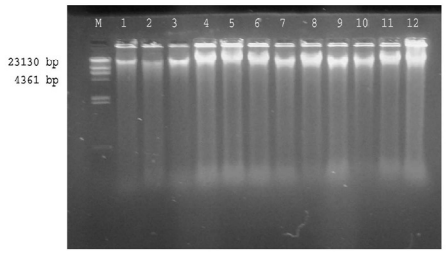

Fig. (1) : Genomic DNA were loaded in 0.8% agarose gel and separated by electrophoresis for 90 min at 100 volt.
Lane M : 1 Kb ladder size stander marker
Lane 1-12 : Genomic DNA isolated from different Banana cultivars. The numbers on the top of the lanes correspond the genotypes of Banana as given in Figure 2

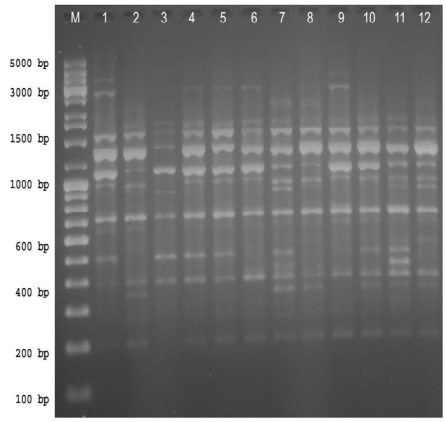

Fig. (2): RAPD-PCR amplification product of 12 different banana varieties viz. 1. Pata kapoora, 2.Chepta bantala, 3.Grand nino 4.Singapoori 5.Rabasta 6.Champa 7.Banua bantala 8.Batisia bantala 9.Amruta pani 10.Paunsia bantala 11.Katia 12.Gaja bantala. by using Genei, Bangalore primer RPI- C Series, RPI-4 (5'-ACCGCCTATG-3') indicated in Figure 2.
The amplification product were fractioned in a 1.5% agaroge gel.
Lane M : Medium Range DNA Ruler