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Multiphase modification of Tissue Lyser Technique based Genomic DNA Isolation Protocol in Finger Millet (*Eleusine coracana* L)

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ABSTRACT: The semi-cereal crop Finger millet (*Eleusine coracana* (L) Gaertn) is presently gaining identity for its nutraceutical and climate resilient sustainable nature but research acceleration is being limited by non-application of high-throughput molecular biology protocols. 210 finger millet genotypes were taken by deploying time saving tissue lyser based DNA isolation technique for further molecular studies. Due to new crop biology *viz*., C_4 carbon fixation pathway and presence of complex biochemical compounds like phenols; and non-parallelism with other model plant species' a new crop specific DNA isolation protocol termed "easy step" was optimized. Utilizing standard reagents and chemicals of CTAB method of DNA isolation for plants, different aspect of DNA isolation viz. contamination with phenols, proper disintegration and high efficiency purification were optimized. Different combination, tissue lyser oscillation frequency and duration, along with other purification steps were optimized for high throughput and high-quality DNA isolation for Finger millet. The major challenge considered prior to study was limited protocol availability in the crop species with sophisticated biotechnological tools. We suggest from the study that taking 10-15 days old seedling using comb-stand method, addition of 1 percent (v/v) of Polyvinylpyrrolidone (PVP) and some standardized refrigerated microcentrifuge protocols for finger millet.

Keywords: Phenolic compounds, PVP, Tissue lyser, C_4 crop, Finger millet.

INTRODUCTION

Finger millet (*Eleusine coracana* (L.) is natural allotetraploid crop, comprised of two distinct genomes, (2n = 4X = 36, AABB), member of Family Poaceae and genus Eleusine. The genome size of finger millet is 1,593 Mb, and cliestogamous flower biology makes it naturally autogamous crop (Goron and Raizada, 2015). It is an annual (100-120 day's life cycle), herbaceous cereal grown and consumed largely by poor-middle income people of Asian and African continents. In Present day of health conscious or life style disease prone era, this crop is being recognised as "Nutraceutical", due to rich in protein, mineral nutrient as compared to other staple food sources namely rice, wheat, maize, sorghum etc. (Gupta *et al*., 2017; Sharma *et al*., 2017). The cultivated species of finger millet (*Eleusine coracana*; wild sp. *Eleusine africana*) is exceptionally rich in calcium (Ca^{++}) content, about 0.34% in whole seed, compare to 0.01-0.06% in other common cereals (Kumar *et al*., 2016). Additionally, its seeds are rich source of iron, zink, dietary fiber and other essential amino acids like leucine ($C_6H_{13}NO_2$), methionine ($C_5H_{11}NO_2S$), phenylalanine ($C_9H_{11}NO_2$), pytates ($C_6H_{18}O_{24}P_6$) and trypsin inhibitory factors and most importantly gluten free (Sood *et al*., 2016; Ceasar*et al*., 2018). The millet grains and seeds have long term storage property, even up to 5 years without insect damage, transforming it as valuable crop in drought-prone areas of south Asian and African countries (Latha *et al*., 2005). Apart from nutritional value, this crop is gaining world-wide popularity for its "marginal input-based production ecology" i.e., it can be grown of less fertile, eroded land where other fertilizer responsive crop *viz*., maize, rice etc can't be grown or give vary less productivity. Moreover, it requires vary less water to complete life cycle, hence, promising crop for present and future water stress prone areas.

Extraction of large quantity and high-quality DNA is often a limiting factor in genetic analysis of plant traits important to agriculture (Xin and Chen, 2012; Tiwari *et al*., 2017). Despite of nutritional, economical and agricultural significance of Finger millet, it has been addressed limitedly by modern day physiologists and molecular biologists. In current decade, however, some progress has been achieved for molecular marker assisted selection, QTL mapping, genome sequencing etc (Chelpuri *et al.*, 2019; Krishna *et al*., 2020; Wambi

et al., 2021). But was, mostly, restricted to limited laboratory and done by traditionally equipped techniques which are possible for small number of genotypes only. In this context, we optimised a "easy step" Tissue lyser based high throughput and high-quality DNA isolation technique. Genomic DNA of 210 genotypes was extracted using this approach, which was practically very difficult and time consuming with traditional Mortar-pestle based tissue homogenization. Additionally, optimization for preventing contamination from many phenolic compounds present in Finger millet which may inhibit downstream applications was carried out.

MATERIALS AND METHODS

The present laboratory pre-experiment was conducted at Nuclear, Agricultural and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai, India. 210 genotypes of finger millet were chosen for study and grown in growth chamber with standard conditions. The seeds were sown in paper folds and two genotypes were taken in each row the fleshy leaf was cut for isolation after 10-15 days (discussed in details later). Qiagen Make "Tissue Lyser II" model was used for homogenization of leaf tissue using stainless tungsten carbidebeads of 5mm diameter. Refrigerated centrifuge "Eppendorf make model 5430R" with 2.0 – 1.5 ml centrifuge tube rotor was used for centrifugal purification and separation steps. Other standard chemicals used were liquid nitrogen, CTAB (Cetyl Trimethyl Ammonium Bromide), choloroform-isoamyl alcohol (CI), isopropenol, 75% alcohol and $T_{10}E_1$ buffer.

RESULTS AND DISCUSSION

A. Comb-stand method for seedling raise

After cleaning of com-stand apparatus, it was washed twice with stage II water followed by wiping with 70 percent alcohol. Whatman filter paper was cut at three equal parts (i.e., 15.3cm) than it was folded at every 5cm length and two 1cm length. The 1cm length formed notch for seed placing. The folding apparatus was kept on tray and started placing seed. Since in finger millet seeds are very small, two varieties of seeds were placed at a time (almost 20 seeds of each). Water added in tray and due care was taken that lower portion of paper-folds must be in contact to water so that water could reach to emerging seedlings through capillary action. The assembly was kept in dark for one day than transferred to growth chamber, supplied with routine light conditions. Liquid fertilizer or any kinds of nutrient application were not given. Cutting of leaf samples could have been done between 10-15 days, but after 12-13 days we observed yellowing of seedlings, which might be due to small sized endosperm of the crop. Hence, we finished the cutting of fleshy leaves within 10-12 days of sowing. Using this method, comparatively higher number of genotypes could be accommodated in a single sowing. In addition, by utilizing 10-15 days old seedling sufficient leaf tissue is available for DNA isolation in high amount and even repeat of some failed or accidently lost samples.

B. Addition of Polyvinylpyrrolidone PVP- $(C_6H_9NO)_n$

Additional to micro and macronutrients, Finger millet is good source of phytochemicals namely phenolic compounds which helps lowering down the probability of chronic diseases like diabetes, cancer and other cardiovasculardiseases (Chandrasekara and Shahidi, 2011a). The polyphenols consist of hydroxybenzoic (protocatechuic, *p*-hydroxybenzoic) acids, hydroxycinnamic (*p*-coumaric, ferulic, syringic) acids, flavonoids (quercetin, apigenin, catechin, epicatechin), and pro-anthocyanidins (Devi *et al*., 2014). But these compounds negatively affect the DNA isolation quality and quantity due to acidic phenol resulting in the denaturation of the DNA. Hence vary small quantity of DNA could be isolated in earlier phase of extraction (with only CTAB buffer). Additionally, we noticed that DNA pellet so isolated, was highly pigmented in colour which is an indication of poor quality. Therefore, we added 1 percent (v/v) of Polyvinylpyrrolidone (PVP) complemented CTAB extraction buffer yielded good amount of DNA pellet was obtained, and quality of purified DNA was also improved (Table 1). Previously efficiency of CTABDNA precipitation and pellet re-suspension was modified and improved by bringing down the NaCl concentration in extraction buffer with CTAB dilution buffer by Xin and Chan (2012). Most CTAB DNA extraction methods reported in literature (Mace *et al*., 2003; Flagel *et al*., 2005; Reynolds and Williams, 2004; Michiels *et al*., 2003) are variations of the method reported by Doyle and Doyle (1987), in which DNA in CTAB extraction buffer is precipitated by adding 0.5 volume isopropanol.

Table 1: Effect of Addition of Polyvinylpyrrolidone in CTAB protocol.

Starting material of leaf (~300 mg)	Only CTAB buffer	CTAB buffer with 1% PVP
Total DNA yield (A260)	0.024	0.071
Typical concentration	238	712
A260/280 ratio	2.50	1.17
A260/230 ratio	0.068	0.086

C. Tissue lyser based approach for rapid isolation

Functioning principle. Tissue disruption and homogenization are the two bases of working of tissue lyser which is achieve through the beating and grinding effect of beads on the sample material as they are shaken together in 2ml sample tubes. Proper disruption and homogenization leaf sample is basic requirement for DNA isolation procedure. *Disruption*: Disruption is important to release the nucleic acids from the sample material. Complete disruption of cell walls (both primary and secondary) and plasma membranes of cells, including organelles membranes is absolute requirement for releasing entire nucleic acid. Samples of different crop species, varieties even genotypes have at least small variation for complete disruption and require optimization of homogenization protocol. Incomplete or improper disruption results in reduction in extracted DNA yields significantly. *Homogenization*: Homogenization reduces the viscosity of cell lysates formed by disruption. This step shears carbohydrates to create a homogeneous lysate otherwise inefficient binding of nucleic acids to apparatus silica membranes and magnetic particles and eventually reduction of DNA yields.

Procedure optimization for lysing/crushing. Lysing or crushing of tissue depends upon the nature of crop plant material, sample type (leaf, stem, seed etc) and age of seedling. In our optimization procedure we adopted five sets of crushing repeats with different combination of Frequency (1/seconds) and crushing time (Table 2). In finger millet, due to C_4 kind of fleshy leaf anatomy, the beads in the tube were striking and punched back to other end, and this was hampering the proper crushing of tissues. And when frequency was increased to 30/seconds, the tube started breaking; even at 25/seconds it got damaged. The frequency was set to 20/seconds and then the time was increased @ 30 seconds. Again at 90 seconds (at interval of 30), no proper grinding could be achieved. After 120 seconds (at interval of 30) small DNA pellets started appearing, and that was improved again to 150 seconds (at interval of 30), fair amount of DNA.In addition, some reports suggested chloroform extraction, which removes polysaccharides, lipids, and other nonpolar substances from aqueous phase, resulting in cleaner DNA (Richards *et al*., 1994).

D. Combination of Refrigerated centrifuge operational setup

Refrigerated centrifuge was deployed three times in the process of DNA isolation. Firstly, after addition of chloroform isoamyl alcohol and shaking at gel rocker for 15 minutes; at this stage 10000RPM spin was taken for 15 min at 4°C (Table 3). Some protocol opts for higher rpm, but to minimize the DNA shearing due to centrifugation slightly lower rpm was followed. At 2nd stage i.e., after addition chilled isopropanol and storage at -20°C for 30 minutes; we started with the combination of 4°C + 12000RPM + 15 min (I), Further we calibrated the combination to 4^0C + 10000RPM + 15 min (I), which yielded us the good quality pellet with reduced shearing of high molecular weight DNA. For the sake of saving resource i.e., 15 min of time; we again experimented with new reduced time combination i.e., 4°C + 10000RPM + 10 min.

Table 2: Combination of frequency and crushing time with tissue lyser for finger millet.

	Frequency (1/Seconds)	Time (Seconds)	Remarks	DNA quality
01	30	30	Fibrous tissue remained Breakage of 1.5mlsized tubes	No isolation
02	25	30 + 30	Fibrous tissue remained Damage of 1.5mlsized tubes	No isolation
03	20	30+30+20	No proper grinding of tissue	No isolation
04	20	30+30+30+30	Tissue almost grinded	Small DNA pallet appears
05	20	30+30+30+30+30	Tissue well grinded	Fair amount of DNA isolated

Table 3: Adjustment of Refrigerated centrifuge for finger millet.

Stage of Centrifuge		Combination of Temperature, frequency and time		
		I	II	III
A.	After addition of CI and Shaking	4°C 10000 RPM 15 Min	-	-
B.	After addition of isopropanol	4°C 12000 RPM 15 Min	4°C 10000 RPM 15 Min	4°C 10000 RPM 10 Min
C.	After addition of 75% ethanol	4°C 10000 RPM 5 Min	4°C 10000 RPM 10 Min	-

At this level, the results were same as previous one, therefore we fixed this combination for 2nd stage of centrifugation. In last stage of centrifuge viz., after addition of 75% ethanol, we centrifuged at 4°C + 10000RPM + 5 min and we got the good-sized pellet. However, it was noticed that, at the time of ethanol decant the pallet was dropping down. In next isolation we enhanced the time of spinning (centrifuge) at 4°C + 10000RPM + 10 min, and obtained DNA pellet stick to opposite side of tube for longer time and easy to handle for further steps and minimizing loss of pellet.

Detailed protocol followed

- 0.3g of leaf sample was cut from fresh, healthy and disease-free seedlings
- Placed in liquid nitrogen by wrapping with aluminium foil
- When all samples were ready, put in 2ml tube and finally place in tissue lyser plates
- Run the tissue lyser as per standardized procedure discussed earlier
- After all the tissue crushed thoroughly, CTAB buffer was added at the rate of 800µl in each tube and left it for incubation at 60°C for 30 minutes
- This incubation can be done in water bath or any locally available box, by filling almost boiled water, measured its temperature and put the tissue lyser plate by removing its metal portion. Due care taken that water level must not reach at the level of tube cap, otherwise it would contaminate the crushed sample. Importantly, CTAB prepared always afresh by adding 1 percent β mercaptoethanol and 1 percent PVP.
- Equal amount of Chloroform isoamyl alcohol (CI) i.e., 800µl was added. CI at the rate of 24:1 had also to be prepared afresh as per samples to be isolated
- The tubes were left for shaking in gel rocker for 15 minutes
- Placed in refrigerated micro centrifuge for spun @ 4°C + 10000RPM + 15 min
- Decanted the supernatant into new tube, preferred 1.5ml tube because in later stage it's easier to handle this size tube.
- Addition of 1000µl chilled isopropanol and left at -20°C for 30 minutes.
- After stipulated time frame, the tubes were spun for 15 min at 10000 RPM and 4°C temperature.
- Carefully decanted the solution and kept the pallet and, further, added 1000µl of 75 percent ethanol and again spun @ 4°C + 10000RPM + 10 min.
- The ethanol was decanted and 200µl $T_{10}E_1$ buffer was added.

CONCLUSION

Involvement of substantial amount of time and laboratory resources persuaded us to summarize and recompile the work as manuscript. Although, initially the report can be taken casually due to well standardised in other crop species namely rice, wheat, maize, oilseeds, pulses etc.; but in Finger millet the sophisticated molecular biology tools are yet to be explored with fine tune. We suggest from the study that taking 10-15 days old seedling using comb-stand method, addition of 1 percent (v/v) of Polyvinylpyrrolidone (PVP) complemented CTAB extraction buffer, using tissue lyser for crushing of leaf samples for 150 seconds at the interval of every 30 seconds and above discussed combination of temperature, spinning and timing for isolation of quality DNA in this limited explored crop species. The high quality and rapid DNA isolate will enrich the way of *E. coracana* towards high throughput techniques like next generation sequencing (Nguyen *et al*., 2019). Through combining the efforts of molecular genetics, sequencing technologies, and bioinformatics, the modified technique will also render a rapid and precise way to distinguish earlier known, classify biological species and to retrieve sequence about them (Naeem *et al*., 2019).

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