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Preliminary study on rice straw degradation using microbial inoculant under shake flask condition

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Abundance and unmanaged agriculture residues lead to unutilized resource waste and environmental pollution. Application of microbial technology to manage agriculture waste could produce value-added product. A preliminary study on biodegradation of rice straw using different potential microorganisms was tested under controlled environment. Three different inoculant cultures were used to observe their efficiency in rice straw degradation. Combination cultures microorganisms coding AMB1 shows the potential degrading activity, which reduces the hemicellulose of rice straw by 50% from the raw material. The highest cellulase activity at 1.5 U/mL was also observed in rice straw treatment with AMB1 than single inoculant fungi and commercial microbial product. Overall, the results suggested that the biodegradation of rice straw could be improved by using combination culture. The ability of these cultures to enhance biodegradation shows potential to fasten the decomposing period and may be used to manage agriculture waste.

Key words: Cellulase, lignin, agriculture waste, biodegradation.

INTRODUCTION

Paddy is planted as an annual crop, thus producing two times of waste for the same area in a year. The ratio of the crop product to residue for paddy rice straw produce is 1: 1.4 (IPCC, 1996). In 2014, an amount of 2.98 million tonnes of rice straw was produced in Malaysia. Generally, on-field burning and incorporation of rice straw was practiced by farmers to manage this waste. Incomplete combustion of rice straw due to burning causes disturbance to air quality in the environment, losses of soil nutrient which will impact soil fertility and release of greenhouse gases (GHG) (Rosmiza et al., 2014). During rainy season, farmers practiced incorporation of rice straw to field to manage the abundance of this waste. The impact contributes to the production of methane (CH_4) during planting season due to the availability of organic matter in soil (Nishiwaki et al., 2015). Therefore, an efficient approach of managing rice straw which is environmental friendly with no impact to human health should be considered.

Rice straw consists of cellulose, hemicellulose and lignin with high silica content (Rashad, 2013). One of the approaches for eliminating this waste is by decomposition process of rice straw to produced improved product such as compost for the use in agriculture. On-field decomposition of rice straw occurs naturally, however the process is slow. The short period of gap between

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paddy planting seasons are insufficient for the decomposition to fully occur, thus led to the production of methane on flooded field during planting season. This decomposition of organic matter could be accelerated by microbial technology (Fang et al., 2012).

Decomposition involves microbial process where the substrate is broken down into more stabilize product (Mohammad et al., 2013). Different microorganisms may have different roles in the decomposition process. As an example, several fungi take place in degrading lignocelluloses meanwhile some bacteria act as cellulose degrading microorganisms (Singh and Nain, 2014). The celluloses and hemicelluloses contained in rice straw could be hydrolyzed by certain microorganisms using solid state as well as submerged fermentation to produce value added products (Thomas et al., 2016). Microorganisms have the ability to produce enzymes and metabolites which enhance the decomposition process of organic waste and increase humus quality in soil (Barker et al., 2006). This characteristic is useful in the process of managing rice straw waste. Besides, technology using microorganism for managing waste could be one of the alternatives in optimizing the used of available biomass besides being much more environmentally friendly. Therefore, the aim of this work was to identify and determine the microbial inoculant tested which led to fasten decomposition of rice straw.

MATERIALS AND METHODS

Microbial inoculant

Microorganisms used in this experiment were isolated from soil. Inoculants from lactic acid bacteria and cellulase degrader group were made as a mixed culture coded as AMB1. *Trichoderma* species inoculant used was obtained from MARDI Microbial Culture Collection (MMCC). The inoculants were grown on nutrient broth (NB) for AMB1 and potato dextrose broth (PDB) for *Trichoderma* spp. Commercial microbial product was used and obtained from the market which is known as Biodecomposer.

Rice straw

Rice straw used in the study was acquired from Tanjung Karang, Selangor, Malaysia; coordinates at 3.4627° N, 101.2208° E. Paddy planted in Selangor is located under the authority of Integrated Agriculture Development Area (IADA) in Barat Laut Selangor, Peninsular, Malaysia. IADA is one of the authorities which cover the granary area in Selangor. Granary areas refer to major irrigation schemes (areas greater than 4,000 ha) and are recognized by the government in the National Agricultural Policy (NAP) as the main paddy producing areas. Rice management cultivation applied in this area is under continuous flooding with two planting seasons per year.

Decomposition of rice straw using shake flask study

About 200 g of rice straw was added in sterile conical flask. AMB1 and *Trichoderma* spp. were added on flask containing rice straw, respectively. Commercial microbial product was added with rice straw following its usage instruction. Flask containing rice straw with water served as control. The simplified, treatment of shake flask study is as follow: T1 (Control); T2 (Rice straw treated with AMB1); T3 (Rice straw treated with *Trichoderma* spp.) and T4 (Rice straw treated with commercial microbial product). Each treatment was prepared in triplicate. Samples were collected at days 0, 2, 4, 8, 15, 20, 25, and 30 for physical and microbial analysis.

Microbial growth

Enumeration of each microorganism analysis was done for all treatment. Total aerobic culturable microorganisms were determined using dilution plate count technique. An initial of 5 g samples were diluted with 45 mL sterile phosphate sodium buffers and were preceded with serial dilutions till 10^5. About 1 ml of diluents were taken from dilution factors of $1:10^3$, $1:10^4$ and $1:10^4$ and transferred into the required media. Cultures showing between 30 and 200 colonies were counted using colony counter (Rebollido et al., 2008).

Physical and enzymatic analysis

pH was analyzed using pH Eutech Instrument pH2700. Enzyme activity assay was done to test degradation of cellulose and reducing sugar assay. Endoglucasnase activity (CMCase) was determined using carboxymethyl cellulose solution in citrate buffer. Reducing sugar assay was analyzed using 3,5-di-nitorcyalicylic acid (DNS) method (Miller, 1959).

Cellulose, hemicellulose and lignin analysis

Cellulose, hemicellulose and lignin content from rice straw was analyzed on day 30 using neutral detergent fibre (NDF) and acid detergent fibre (ADF) method (Sarkar et al., 2012).

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) using SAS software.

RESULTS AND DISCUSSION

Microbial growth

Degradation of rice straw was observed by submerged fermentation with different microbial inoculant. Cell density of microbial growth for each treatment is as shown in Figure 1. Higher growth was observed in AMB1 with the range of 3.6×10^8 to 1×10^9 CFU/mL. The density of AMB1 was maintained throughout the degradation process and started to drop at day 30. This indicated that AMB1 are in a favorable environment for its growth. The same trend was observed on commercial microbial product where cell density was increased during day 2 and maintained till day 20. A drop of microbial population of commercial microbial product was also observed at day 30. Therefore, this might be linked to the reduction of microbial population since low

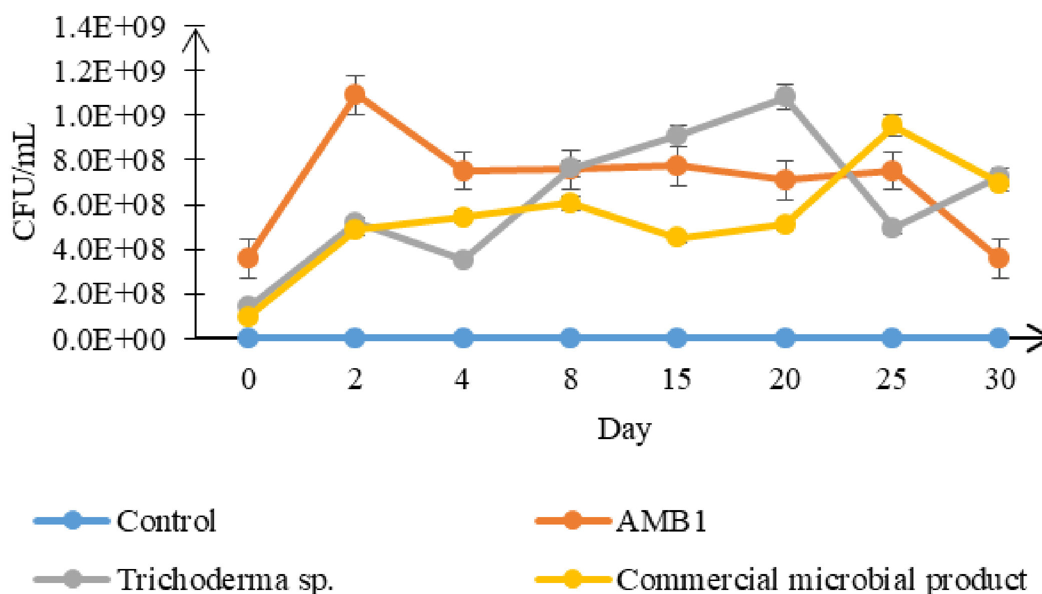

Figure 1. Population number of microorganisms during shake flask study.

substrates were available in the shake flask. This finding was similar to Jeude et al., (2006) where the study observed a constant of cell dry weight and a reduction of respiration activity after all carbon sources were exhausted. In contrast to other treatment, rice straw inoculated with *Trichoderma* spp. showed an increase of population number starting from day 4 to day 20 from 3.5 × 10^8 to 1.0^8 × 10^9. This may be explained by their slow growth characteristic which required being adapted in a new environment with rice straw as substrate. Approaching day 30 of the degradation process, only small pieces of rice straw were present and are compact with each other. The colour of the rice straw changed from light yellow to black indicating degradation process occurs.

pH

The pH range varied by microbial inoculant used. In early degradation, all treatments except rice straw inoculated with AMB1 were at pH 6 (Figure 2). In contrast, rice straw treated with AMB1 shows much lower pH value at the range of 3.2 to 4.2 on early degradation and throughout the fermentation time. This is due to the condition of the microbial culture itself since one of the components of AMB1 was lactic acid bacteria. Along the fermentation period, treatment of rice straw inoculated with commercial microbial product and control treatment are in the range of 5.9 to 8.2. This is a similar characteristic for rice straw degradation as reported by Zhao et al., (2014). Treatment with *Trichoderma* spp. shows a lower value of pH from day 2 to day 15 with the range of pH 4.3 to 4.5. However, the *Trichoderma* spp., tend to overcome the acidification condition during day 20 with pH at 6.5. *Trichoderma* spp. are known to grow and adapt well at variation of pH 8, thus the drop of pH did not interfere with the fungi growth (Belal, 2013).

Cellulase and sugar reducing activity

Cellulase is one type of enzymes which is involved significantly in degradation process of organic material. Cellulase activities were analyzed throughout the degradation period in order to understand the reaction of each inoculum towards the rice straw degradation. Higher amount of cellulase activity was observed in treatment AMB1 as compared to other treatments (Figure 3).

The highest value was observed at day 3 with 1.5 U/ml. Trend of cellulase activity in AMB1 starts dropping at day 8 when approaching 0.2 U/ml by day 30. The increase of abundance of AMB1 during early degradation promotes higher cellulase activity since more cells are available to hydrolyze cellulose composition in rice straw. This indicates that higher decomposition activity occurs in treatment with AMB1. This suggests that mixed culture of microorganisms used could improve the decomposition process of organic materials. Several researchers had also reported that rice straw decomposition process could be accelerated by using mixed culture of microbial inoculant (Zhao et al., 2014; Mohamed et al., 2016).

Rice straw inoculated with commercial microbial product shows the second highest inoculant to produce cellulase activity in the shake flask study. The range of cellulase activity for this microbial inoculant are at 0.2 to

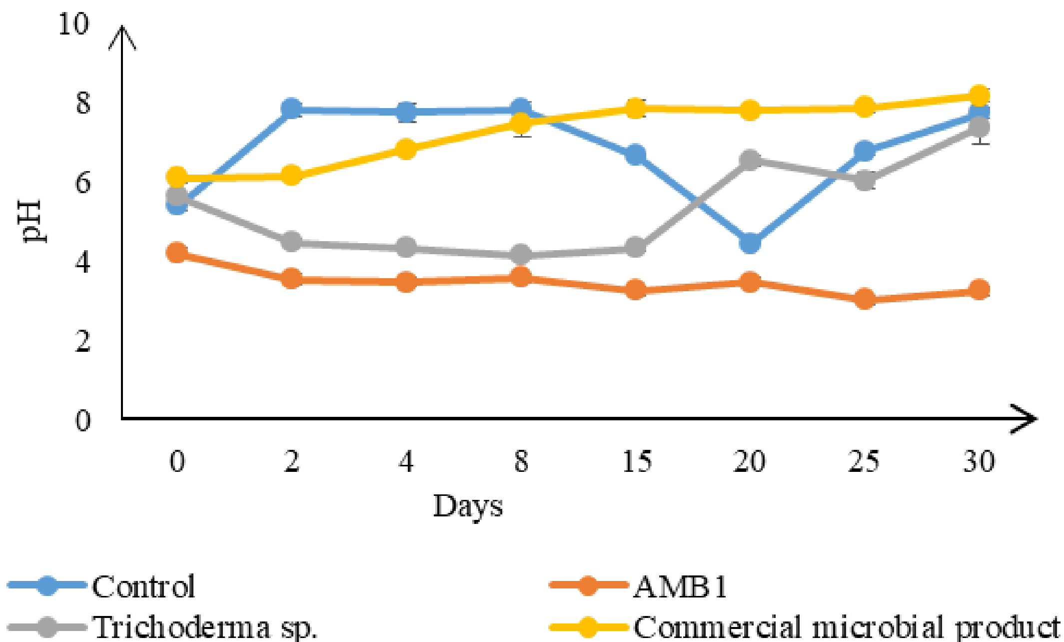

Figure 2. pH of rice straw during degradation proses.

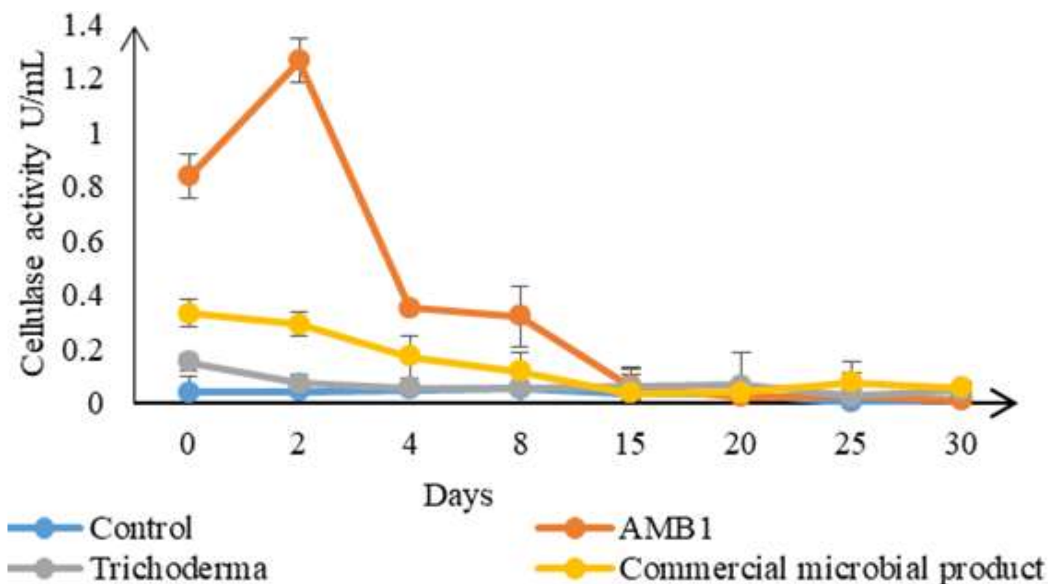

Figure 3. Cellulase activity of microbial inoculant.

0.6 U/ml. Lower cellulase activity was shown in *Trichoderma* spp. but an increase in trend is observed from days 8 to 30. This result is in line with the microbial growth where an increase of microbial population is observed at day 8 (Figure 1). Therefore, it is believed that the slow growth of *Trichoderma* spp. in shake flask condition is the cause of this condition. Previous research by Goyal and Sindhu (2011) observed that degradation of paddy straw with fungal cultures shows an increase and higher value of cellulase activity after day 30 of degradation process. Hence, a much longer period of degradation are needed to study and observed effects of fungal cultures on rice straw degradation. Control treatment showed the lowest activity which is expected since no inoculant were present to boost the degradation process.

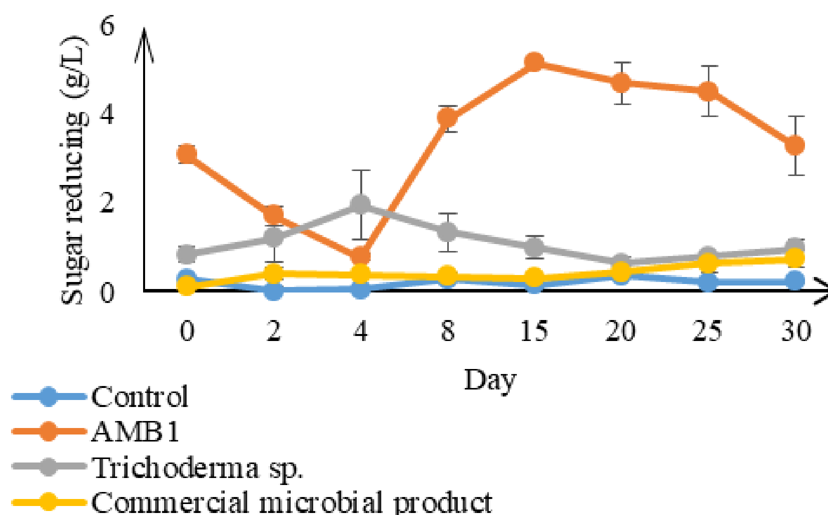

Figure 4. Reducing sugar.

Table 1. Composition of rice straw after treatment with microbial inoculant.

Treatment	Lignin	Hemicellulose	Cellulose
Control	8.89 ± 1.09[a]	26.11 ± 7.15[a]	53.14 ± 9.04[a]
Trichoderma spp.	8.59 ± 0.43[a]	18.06 ± 11.36[ab]	47.30 ± 1.18[ab]
AMB1	4.97 ± 0.50[b]	12.24 ± 1.96[b]	25.98 ± 1.01[c]
Commercial microbial product	8.48 ± 1.09[a]	19.94 ± 0.72[ab]	43.13 ± 7.19[b]

*Data shown are mean ± SD value with difference letters indicate significant difference at P<0.05.

The reducing sugar that accumulates during rice straw fermentation period with different inoculant shows significantly difference of treatment with AMB1 from other treatment (Figure 4).

The highest amount was obtained in treatment with AMB1 at 5 g/L during day 15. Reducing sugar was present after degradation of cellulose (Prasertsung et al., 2017). Treatment with *Trichoderma* also shows the formation of reducing sugar with the highest amount of 1.9 g/L at day 4. The presents of this reducing sugar indicate that enzyme activity of microorganisms for biomass breakdown is present which converts carbohydrate to further reducing sugar (Peng et al., 2015).

Cellulose, hemicellulose and lignin of rice straw

During degradation period, the composition of rice straw undergoes changes to much stable fraction. The amount of lignin, hemicellulose and cellulose composition in treatment with microorganisms' inoculant are much lesser than the control rice straw (Table 1).

Rice straw treated with AMB1 showed significantly lower amount of lignin at 4.97%, hemicellulose at 12.24% and cellulose at 25.98% indicating a rice straw hydrolysis. Significant differences were observed in AMB1 treatment for lignin, hemicellulose and cellulose. This is similar to the finding by Phutela et al. (2011) where the decrease in composition of rice straw occurrence indicated the results of breakdown or hydrolysis of complex sugar into fermentable sugars. Furthermore, it was parallel to the results of reducing sugar where AMB1 produced more amount of reducing sugar than other treatment (Figure 4). Composition of rice straw for treatment with *Trichoderma* spp. and commercial microbial product falls under the same grouping, which indicated that both treatments were at par for rice straw degradation. Both of these microbial inoculants, therefore, still show potential characteristic as rice straw degrader.

Conclusion

The present study suggests that usage of microbial input could aid in decomposition process of rice straw. A combination of microorganisms coded as AMB1 used has shown a performance of an efficient biodegradation of lignocellulosic material compared to single inoculum. Higher level of cellulase activity was observed in AMB1 as compared to other treatment which could lead to a benchmark strain for degradation of biomass. The

reduction of lignin, hemicellulose, and cellulose percentage in rice straw treated with AMB1 showed the potential degrading characteristics of this inoculant towards rice straw. However, *Trichoderma* spp. Also show a figure of strong degradation process towards the end of experimental period. Therefore, further studies are required to determine the maximum potential degradation by these microorganisms and their ability to degrade waste biomass on field and under exact environmental condition.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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DNA extraction from silica gel-preserved common bean (*Phaseolus vulgaris* L.) leaves

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Extraction of non-degraded and contaminant-free DNA from field specimen requires collection under liquid nitrogen which is not readily available in resource constrained laboratories in low and middle income countries (LMICs). A method of extracting DNA from silica gel-preserved common bean (*Proteus vulgaris* L.) leaves is presented. The method, which does not involve the use of phenol, chloroform or isoamyl alcohol also obviates the need for low temperature incubation during the DNA extraction steps and the grinding of desiccated leaf tissue in liquid nitrogen. It relies on inactivating proteins using SDS and proteinase K along with precipitation of polysaccharides using a high salt solution (0.8 M NaCl). DNA is further purified by exploiting its insolubility in aqueous media. High quality pure DNA (mean concentration 2.84 ± 0.013 µg/g of dry leaf tissue) with mean DNA purity values of 2.1 ± 0.1 was extracted. The DNA was also found to be free of protein and polysaccharide contamination. This method enables DNA amplification using molecular markers routinely used in molecular biology laboratories like random amplified polymorphic (RAPD) markers, inter simple sequence repeat (ISSR) markers, sequence-characterized amplified region (SCAR) markers and simple sequence repeat (SSR) markers. The findings of this study show that it is possible to obtain high quality DNA from leaf tissue preserved in silica gel. The method used in this research will be invaluable to resource constrained laboratories in low and middle income countries (LMICs) that cannot afford to buy or access liquid nitrogen in order to extract high quality DNA and for research groups undertaking field surveys that require several days or weeks off station without laboratory freezers to maintain the integrity of the tissues which is crucial for obtaining high quality DNA.

Key words: Random amplified polymorphic (RAPD), inter simple sequence repeat (ISSR), simple sequence repeat (SSR), sequence-characterized amplified region (SCAR), deoxyribonucleic acid (DNA), low and middle income countries (LMICs).

INTRODUCTION

Good quality of DNA is a pre-requisite for most applications in a molecular biology laboratory. A number

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of DNA mini prep protocols have been developed and some of them (Agbagwa et al., 2012; Sahu et al., 2012; Aubakirova et al., 2014; Abdel-Latif and Osman, 2017; Shu et al., 2018) rely on modification of earlier sodium dodecylsulphate (SDS) or cetyltrimethyl ammonium bromide (CTAB) protocols (Rogers and Bendich, 1985; Edwards et al., 1991). Others are fast methods and rely on release of DNA from plant tissue using sodium hydroxide (Wang et al., 1993), by boiling (Thompson and Henry, 1995) or microwave treatment (Saini et al., 1999).

Extraction of DNA from plant tissues using in-house DNA extraction buffer formulations generally involves three stages; breaking the cell wall to release cellular constituents by grinding tissue in dry ice, liquid nitrogen or fine sand using a pestle and mortar or grinder; disrupting the cell membrane to release DNA into the extraction buffer; and use of detergents like SDS or CTAB to solubilize cell wall components and protect the DNA from nucleases using a chelating-like ethylenediaminetetracetic acid (EDTA). EDTA deprives the nucleases of magnesium ions thereby rendering them inactive.

Separation and denaturation of proteins from DNA is achieved by either emulsifying the buffer-tissue mixture with chloroform and/or phenol or by incorporating reducing agents like β- mercaptoethanol, dithiothreitol or sodium sulphite in the DNA extraction buffer (Baranwal et al., 2003; Abu-Romman, 2011; Das et al., 2013). Additional steps to minimize DNA degradation include minimizing turbulence by gently pipetting the DNA solution and exposure time between pulverization and contact with the DNA extraction buffer. However, despite all these safeguards, producing high quality DNA devoid of degradation remains a big challenge.

To mitigate the effects of degraded DNA, DNA extraction kits have been developed to facilitate the rapid extraction of very highly pure DNA. Such kits use a membrane within a column that binds DNA. Commonly used kits include DNeasy plant mini and maxi kits, Wizard genomic DNA purification kit and GENEspin (Tamari and Hinkley, 2016). The disadvantage of such kits is that they are not economically feasible in resource constrained laboratories or laboratories that process a huge volume of samples. In addition, the experimenters have limited options modifying the standard protocols. However, the kits are easy to use, contain all reagents for DNA extraction, require minimal laboratory equipment (usually a centrifuge and micropipettes) and use very few consumables (only pipette tips and micro centrifuge tubes). Another alternative to the DNA extraction kits is the extraction of DNA from leaf tissue immobilized on a paper matrix. A number of proprietary methods of extracting DNA from leaf tissue prepared this way include; IsoCode card (Schelidher and Schuell, Dassel, Germany), generation capture system (Biozym diagnostika GmbH, Hessisch-Oldendorf, Germany) and FTA cards (Whatman, Kent, UK). FTA methodology relies on spotting tissue onto the cards and cutting small discs (1.5 to 2 mm) using a tissue biopsy punch followed by washing of the discs using a proprietary reagent and isopropanol. The DNA remains are impregnated onto the disc which is then used for polymerase chain reaction (PCR) analysis. The method has been successfully used for human forensic studies (Zhong et al., 2001), wildlife DNA (Smith and Burgoyne, 2004), PCR based genotyping and plant diagnostics (Drescher and Graner, 2002).

Extraction of high quality DNA in the laboratory using in-house DNA extraction buffer formulations requires either freshly picked specimen collected under liquid nitrogen for immediate use or specimen collected under liquid nitrogen and stored at -80°C for later use. Both options are impractical because most field surveys are conducted hundreds of kilometers off station and therefore maintaining the integrity of the tissue to obtain high quality DNA is usually hard. The objective of this study was to develop a safe, inexpensive and robust protocol for extracting high quality DNA from silica gel dried common bean leaf specimen using equipment that is expected to be available in a basic molecular biology laboratory. In addition, the utility of the DNA obtained using common molecular marker systems- simple sequence repeat (SSR) markers, inter simple sequence repeat markers (ISSR), sequence characterized amplified region (SCAR) markers and random amplified polymorphic DNA (RAPD) markers is also demonstrated.

MATERIALS AND METHODS

Bean seed was sown into sterilized soil in 5-L plastic pots in the screenhouse at Kawanda, Wakiso district of Uganda. A trifoliate leaf was picked from one of the three plants 10 days after sowing, weighed, wrapped in aluminum foil and put in a sealable plastic bag containing 10 g silica gel. The plastic bag was then put in a Secador desiccator (LabSource, Northlake, IL), stored for seven days and re-weighed. Just before DNA extraction, a DNA extraction buffer containing 0.2 M Tris-HCl, 0.8 M NaCl, 1% SDS, 20 mM EDTA and 60 µg/mL Proteinase K was prepared.

DNA extraction began by grinding the dry leaf in 700 µL of the buffer using a pestle and mortar. The solution was then incubated in a water bath at 65°C for 1 h. After incubation, the mixture was centrifuged at 13200 rpm for 10 min and 300 µL was transferred to a fresh 1.5 mL tube. Thereafter, 150 µL of 5 M NaCl and 900 µL of room temperature absolute ethanol was then added to the supernatant and the mixture was left at room temperature for 2 h to precipitate the DNA. The mixture was further centrifuged at 13200 rpm for 10 min and the supernatant discarded. The resultant pellet was washed twice with 70% ethanol at room temperature. The pellet was then dried at room temperature for 20 min and dissolved in 70 µL of 1 X TE buffer (10 mM Tris-HC1 [pH 8], 1 mM EDTA). The DNA solution was then treated with RNase A at a final concentration of 50 µg/mL for 2 h, quantified using the DQ300 fluorometer and immediately used for molecular assays. DNA quality and purity were further assessed by comparing the ratio of the absorbance at 260 nm to absorbance 280 nm (A260/280) and the ratio of absorbance at 260 nm to absorbance 230 nm (A260/230) respectively, using a NanoDrop 2000c spectrophotometer (Thermo Fisher, Wilmington, DE). Prior to quantification of the DNA, a standard curve (Figure 1) was generated from various concentrations (100-500 ng/µL) of a

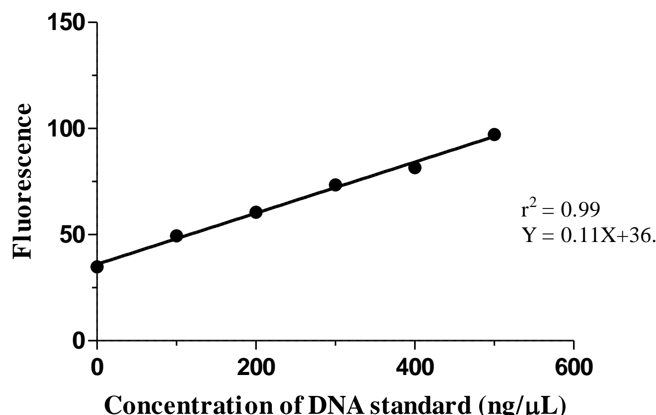

Figure 1. Standard curve from various concentrations of the DNA standard. Points are mean values of duplicate readings of the standard.

Table 1. Names and sequences of molecular markers used to amplify DNA from silica-gel dried common bean leaves.

Marker Name	Marker sequence (5'- 3')
SW13	F CACAGCGACATTAATTTTCCTTTC R CACAGCGACAGGAGGAGCTTATTA
OPV12*	ACCCCCCACT
BMd 502	F ATTCTCAGGCAGGAAACATA R ACGACCCACAATCACTTAAA
RAMS 4*	GAG(ACA)5

F and R denote forward and reverse primer respectively. Marker systems denoted by * use only 1 primer.

known DNA standard (calf thymus DNA) using the DyNAquant DQ 300 fluorometer (Hoefer, Holliston, MA) with the Hoechst 33258 stain (Thermo Fisher, Wilmington, DE) as the fluorescent dye. The standard curve was plotted using the GraphPad prism software v.5.00 (GraphPad Software, La Jolla California USA, www.graphpad.com).

PCR amplification

All PCR amplifications were carried out in 20 µL reactions consisting of 100 ng of DNA, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1 × PCR buffer, 0.5 U *Taq* DNA polymerase (Bioneer, Daejeon, Korea) and 0.5 µM of primers.

For the SSR marker BMd 502 (Blair et al., 2009), the reaction mixture was amplified using an initial denaturation at 95°C for 5 min and 36 cycles of a touchdown profile each consisting of a denaturation step at 95°C for 20 s, an annealing step at 0.5 to 70°C each cycle for 20 s and an extension step at 72°C for 30 s. Thereafter, a final extension step was performed at 72°C for 5 min and the reaction was stored at 8°C.

The RAPD marker OPV 12 (Operon RAPD 10mer kit, Cosmogenetech, Seongdong-gu, Korea) and the ISSR marker RAMS 4 (Hantula et al., 1996) both used a similar thermal cycling profile consisting of an initial denaturation at 95°C for 5 min and 40 cycles each consisting of a denaturation step at 95°C for 20 s, an annealing step at 45°C for 40 s and an extension step at 72°C for 60 s with a final extension step at 72°C for 10 min and storage at 8°C.

The SCAR marker SW13 (Melotto et al., 1996) was amplified with the following thermal profile; initial denaturation at 95°C for 5 min and 30 cycles each consisting of a denaturation at 94°C for 10 s, annealing at 50°C for 20 s and extension at 72°C for 40 s. Thereafter, there was a final extension at 72°C for 5 min and finally, the reaction was stored at 8°C. The marker names, types and sequences used are shown in Table 1.

Electrophoresis, staining, visualization and gel documentation

The amplicons obtained with the BMd 502 marker were resolved on acrylamide gel using the horizontal polyacrylamide gel electrophoresis (hPAGE) set (Cleaver Scientific, Warwickshire, UK). Briefly, 50 mL of a 6% denaturing polyacrylamide gel consisting of

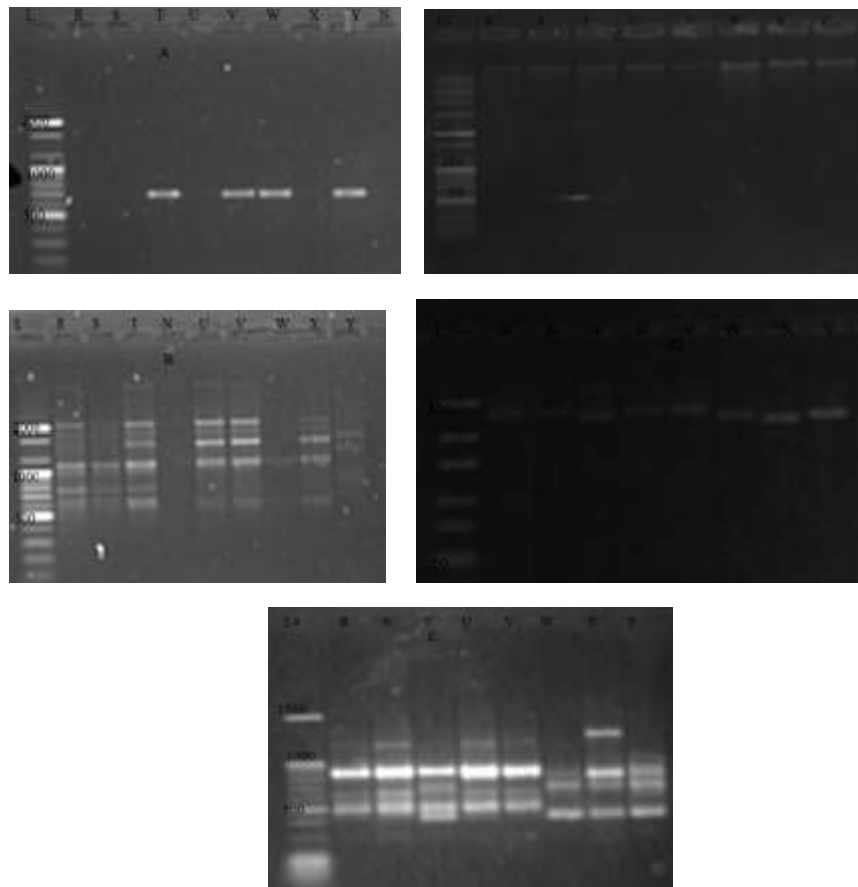

Figure 2. Amplicons were obtained using the SW13 (A), RAMS 4 (B), BMd 502 (D) and OPV 12 (E) markers. C shows genomic DNA obtained using our method. L is the 100 bp DNA ladder (Bioneer, Daejeon, Korea), L2, L3 and L4 are the GeneRuler 1 Kb plus DNA ladder, O'GeneRuler ultra-low range DNA ladder and the 100 bp DNA ladders (Thermo Fisher Scientific, Waltham, MA) respectively. N is the non-template control.

21 g urea, 1 mL 50 X TAE, 7.5 mL of 40% acrylamide-bis acrylamide (19:1 acrylamide: bis-acrylamide) mix, 100 µL TEMED and 500 µL of 10% APS was made. The solution was then cast onto a hPAGE gel casting unit and a quarter plate overlaid. The mixture was left at room temperature for 30 min to allow complete polymerization of the acrylamide gel mixture. Electrophoresis was performed at 100 V in 1 X TAE (0.04 M Tris–Acetate, 0.0001 M EDTA) for 2 h after which the gel was put for staining in 0.5 µg/mL ethidium bromide for 40 min. Finally, the image was captured using the G: BOX gel documentation system (Syngene, Fredrick, MD).

Amplicons of the RAMS 4, SW13 and OPV12 markers were resolved on 1.5% agarose gel in 1 X TAE at 100 V for 1 h. The staining procedures and image documentation were like for the SSR marker.

RESULTS

A clear, non-viscous DNA solution was obtained after dissolving the DNA pellet in the TE buffer. The DNA also showed minimum degradation and it was amplified by all the molecular markers assayed (Figure 2). The readings were derived from the standard curve (Figure 1) which showed high accuracy. The standard curve had a high value of r^2 (0.99).

The average mass of the fresh and dry leaves was 2.09 and 2.01 g respectively representing a 4% change in mass of tissue (Table 2). The mean amount of DNA per gram of dry tissue was 2.84 µg. The DNA obtained was also pure with a mean value of the ratio of the absorbance at 260 nm to absorbance 280 nm (A260/280) of 2.1. The mean value of the ratio of absorbance at 260 nm to absorbance 230 nm (A260/230) indicates that the protein concentration was 2.3. These values are summarized in Table 3.

DISCUSSION

The objective of this study was to develop a DNA extraction protocol for the recovery of high quality DNA for the DNA concentration were very accurate since they from field specimen. A moderate amount of DNA was

Table 2. Mass and percentage change in mass of a single leaf trifoliate from eight common bean lines.

Sample ID	Fresh mass (g)	Dry mass (g)	% change in mass
X	2.27	2.12	0.066
U	1.98	1.94	0.020
R	2.13	2.04	0.042
V	2.07	2.02	0.024
S	2.12	2.08	0.019
Y	2.15	2.06	0.042
T	2.08	1.92	0.077
W	1.97	1.91	0.030
Mean	2.09(0.098)	2.01(0.078)	0.04(0.02)

Values in parentheses are standard error of the mean of the observations.

Table 3. Fluorescence values, DNA yield and DNA purity parameters of a single leaf trifoliate from eight common bean lines.

Sample ID	Reading 1	Reading 2	Mean readings	DNA yield (µg)	$A_{260/A280}$	$A_{260/A230}$
R	40.45	42.52	40.56	2.83	2.13	2.16
S	43.08	43.3	40.75	2.85	2.19	2.2
T	44.38	41.91	40.74	2.85	2.03	2.34
U	46.19	46.83	41.11	2.87	2.09	2.49
V	42.79	41.64	40.64	2.84	1.98	2.34
W	44.65	44.94	40.92	2.86	2.07	2.39
X	42.06	39.71	40.49	2.83	2.31	2.13
Y	42.73	43.38	40.73	2.85	2.1	2.54
Mean				2.84(0.014)	2.11(0.101)	2.32(0.15)

Reading denotes fluorescence values obtained with the fluorometer. DNA concentration was determined by substituting the values of the mean readings of fluorescence into the equation of the standard curve Y=0.11X+36 and multiplying the value by the volume of TE buffer which was used to dissolve the pellet (70 µl). Value in brackets is the standard deviation.

extracted. The findings here are lower than those of Agbagwa et al. (2012) who obtained up to 10 µg of DNA per gram of tissue of *Cajanus* species. The reason for the difference in the amount of DNA recovered could be the method of quantification. The study by Agbagwa et al. (2012) used spectrophotometric methods to quantify the DNA while the present study used fluorescence. Spectrophotometric (absorbance) methods overestimate the amount of DNA by quantifying single stranded DNA and RNA alongside double stranded (ds) DNA (O'Neill et al., 2011; Sironen et al., 2011) but fluorescent dyes bind only to dsDNA. The Hoechst 33258 dye used in our study is a class of non-intercalating benzimidazole fluorescent dyes that preferentially binds to A/T-rich regions in the minor groove of dsDNA (Weisblum and Haenssler, 1974). Therefore, the amount of DNA obtained with our method will vary according to the proportion of A+T content of the genomic DNA of various plant species.

The DNA solution was clear and not viscous. These observations are indicative of the quality of the DNA and they are supported by our values of the measures for DNA purity and absence of DNA contaminants being within the ranges stipulated for highly pure DNA ($A_{260/280}$ = 1.7-2, $A_{260/230}$ >2, respectively) (Thermo Scientific, 2010; Green and Sambrook, 2018). The clarity of the DNA is suggestive of absence of contaminating secondary metabolites especially polyphenolic compounds like tannins and terpenoids. These compounds are rapidly oxidized when released from tissue and bind to the phosphate backbone of DNA imparting a brown-red color to the DNA (Sahu et al., 2012). A non-viscous DNA solution is indicative of minimum contamination of DNA with polysaccharides. It has been reported that the chemical properties of polysaccharides make them co-precipitate with genomic DNA giving DNA solutions a viscous appearance (Porebski et al., 1997). Two reasons were advanced for the low levels of contaminants. The age of the tissue used and use of high concentration of NaCl in the extraction buffer and in the precipitation of DNA (instead of sodium, potassium or ammonium acetate). Young leaf tissues contain low levels of secondary metabolites like polysaccharides and

polyphenols while NaCl not only minimizes the formation of polysaccharides but also prevents them from co-precipitating with the DNA (Carrier et al., 2011; Sahu et al., 2012).

The DNA obtained showed minimal degradation. It is believed this was due to the activity of DNases during the maceration with sand. Labuza (1970) reviewed the properties of water under food preservation conditions and concluded that the water in food does not leak out unless there is damage to the membranes. Based on this observation, we want to think that drying under silica gel is somewhat stringent resulting in the lysis of the membranes. DNases then gain access to the DNA causing its degradation. Macerating the tissue in liquid nitrogen can reduce degradation.

This research has demonstrated an inexpensive, reliable and scalable method that recovers good quality DNA from field specimen without prior preservation in liquid nitrogen. The DNA was also amplified using common molecular marker systems. The method used in this study will find particular utility in resource constrained laboratories in low and middle income countries (LMICs) that are keen on using marker assisted selection (MAS) in their breeding activities.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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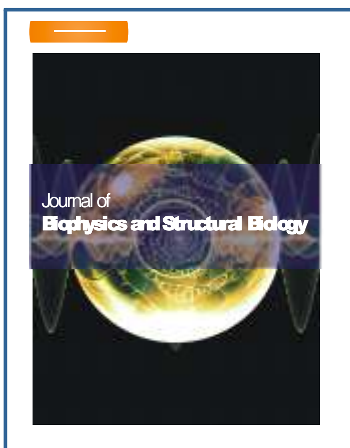

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